UNIVERSIDADE DE LISBOA
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Effects of climate change in the early ontogeny of temperate and tropical sharks

"Documento Definitivo"

Doutoramento em Ciências do Mar

Maria Rita de Carvalho Godinho Macedo Pegado

Tese orientada por:
Professor Doutor Rui Afonso Bairrão da Rosa

Documento especialmente elaborado para a obtenção do grau de doutor

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"Cada um que passa na nossa vida, passa sozinho, mas não vai só, nem nos deixa sós;

deixa um pouco de si e leva um pouco de nós"

Antoine de Saint-Exupéry

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List of abbreviations and units

Abbreviations

8-OHdG	8-hydroxy-2'-deoxyguanosine
abs	Absorbance
AChE	Acetylcholinesterase
A_T	Total alkalinity
ATP	Adenosine triphosphate
bs	Brain stem
BSA	Bovine serum albumin
ca.	Circa
$CaCO_3$	Calcium carbonate ions
CAT	Catalase
cer	Cerebellum
CO_2	Carbon dioxide
die	Diencephalon
e.g.	For example
ELISA	Enzyme-linked immunosorbent assay
ENA	Erythrocytes with nuclear anomalies
g	Relative centrifugal force
GLM	Generalized linear models
GPx	Glutathione peroxidase
GR	Specific growth rate
H^+	Hydrogen ions
[Hb]	Concentration of hemoglobin
H_2O_2	Hydrogen peroxide

$HO^{\bullet}$	Hydroxyl radical
HSP	Heat shock protein
IPCC	Intergovernmental panel on climate change
K	Fulton condition
LPO	Lipid peroxidation
MCHC	Mean corpuscular hemoglobin concentration
MDA	Malondialdehyde
MS-222	Tricaine methanesulfonate
n	Number of individuals
$O_2^{\bullet}$	Superoxide ion
O_2	Oxygen
OA	Ocean acidification
opt	Optic lobes
olf	Olfatory lobes
OAW	Ocean acidification and warming
p-value	Probability of statistical test
PBS	Phosphate buffered saline
pCO_2	Carbon dioxide partial pressure
pH	Power of hydrogen
ppm	Part per million
RAS	Recirculating aquaculture system
ROS	Reactive oxygen species
SD	Standard deviation
SGR	Somatic growth rate
SOD	Superoxide dismutase

spc Spinal cord

SST Sea surface temperature

tel Telencephalon

Ub Ubiquitin

XOD Xanthine oxidase

Units

° C Degrees celsius

% Percentage

± Plus – minus

~ Approximately

Ω Saturation State

Δ Delta

BL Body length

cm Centimeters

h Hour

kg Kilogram

L Litter

m Meter

mL Mililiter

M Molar

min Minutes

mg Milligram

mL Milliliter

mM Millimolar

nm Nanometers

nmol Nanomole

s Seconds

W Weight

w/v Weight per volume

µatm Micro atmosphere

µL Microliter

µm Micrometer

µM Micromolar

µm Micromole

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25th and 75th percentiles and triangles represent the mean value. Lower-case letters indicate statistical differences between tissues. More statistical information is present in Supplemental Table S1.

Chapter 3. Impact of a simulated marine heatwave in the hematological profile of a temperate shark (*Scyliorhinus canicula*)

Figure 1. Category II MHW reconstructed from a dataset of local sea surface temperatures for the last 30 years (Cascais, Portugal; acquired from NOAA (Daily Optimum Interpolation SST, version 2; Banzon et al., 2016). Temperatures above threshold (90th percentile in relation to the long-term climatology) represented as yellow (Category I) and orange (threshold 2x; Category II) (Hobday et al, 2016).

Figure 2. Impact of a simulated category II MHW (+3 °C) on the proportions of: A) erythrocytes, B) leukocytes, C) thrombocytes and D) erythrocytes with micronucleus from blood smears of small-spotted catsharks (*Scyliorhinus canicula*, n = 12 per treatment). Black triangles represent the mean, bold horizontal lines represent the median, boundaries represent 25th and 75th percentiles and whiskers represent the lowest and highest values. Lower case letters indicate significant statistical difference between treatments. Additional statistical information in Supplemental table S1.

Figure 3. Impact of a simulated category II MHW (+3 °C) on: A) nucleus to cytoplasmic ratio, B) heart to body ratio and C) spleen to body ratio of small-spotted catsharks (*Scyliorhinus canicula*, n = 12 per treatment). Black triangles represent the mean, bold horizontal lines represent the median, boundaries represent 25th and 75th percentiles and whiskers represent the lowest and highest values. Lower case letters indicate significant statistical difference between treatments. Additional statistical information in Supplemental table S1.

Figure 4. Impact of a simulated category II MHW (+3 °C) on the ventilation rates (breaths per minute) of small-spotted catsharks (*Scyliorhinus canicula*, n = 12 per treatment): T0 – 9 days before the treatment started; T1 - the first day of treatment; and T15 - after 15 days of treatment. Triangles represent the mean and boundaries represent 95% confidence intervals. Lower case letters indicate significant statistical differences. Additional statistical information in Supplemental table S1.

Chapter 4. Lack of oxidative damage on temperate juvenile catsharks after a long-term ocean acidification exposure

Figure 1. Effects of high CO_2 levels (ΔpCO_2 ~ 500 µatm) on the levels of: i) SOD (superoxide dismutase), ii) CAT (catalase) and iii) GPx (glutathione peroxidase) in the gills, liver and muscle of small-spotted catsharks (*Scyliorhinus canicula*, n = 18 per treatment). Bold horizontal line within the box represents the median, boundaries represent 25th and 75th percentiles and the triangle represents the mean value. Different lower-case letters represent statistical differences between tissues, while asterisks (*) indicate significant statistical differences within the tissues. Additional statistical information present in Supplemental table S1.

Figure 2. Effects of high CO_2 levels (ΔpCO_2 ~ 500 µatm) on the levels of: i) HSP (heat shock proteins) and ii) Ub (ubiquitin) in the gills, liver and muscle of small-spotted catsharks (*Scyliorhinus canicula*, n = 18 per treatment). Bold horizontal line within the box represents the median, boundaries represent 25th and 75th percentiles and the triangle represents the mean value. Different lower-case letters represent statistical differences between tissues. Additional statistical information present in Supplemental table S1.

Figure 3. Effects of high CO_2 levels (ΔpCO_2 ~ 500 µatm) on the levels of: i) MDA (malondialdehyde production due to lipid peroxidation) and ii) 8-OHdG (8-hydroxy-2′-deoxyguanosine due to DNA damage) in the gills, liver and muscle of small-spotted catsharks (*Scyliorhinus canicula*, n = 18 per treatment). Bold horizontal line within the box represents the median, boundaries represent 25th and 75th percentiles and the triangle represents the mean value. Different lower-case letters represent statistical differences between tissues. Additional statistical information present in Supplemental table S1.

Chapter 5. Effects of elevated carbon dioxide on the hematological parameters of a temperate catshark

Figure 1. Effect of high CO_2 (ΔpCO_2 ~ 500 µatm) on the estimate of the proportion of: A) erythrocytes, B) leukocytes and C) thrombocytes from blood samples of small-spotted catsharks (*Scyliorhinus canicula*, n = 18). Horizontal lines represent the median, the whiskers represent the lowest and highest values of the results, and boundaries represent the 25th and 75th percentiles. Additional statistical information in Supplemental table S1.

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response - HSP and ubiquitin - Ub), and oxidative damage in lipids (lipid peroxidation – LPO) and DNA (DNA damage, 8-OHdG). Std Error – Standard Error.

Chapter 5. Effects of elevated carbon dioxide on the hematological parameters of a temperate catshark

Table 1. Seawater physicochemical parameters for the experimental conditions. Salinity, pH, temperature and total alkalinity (A_T) were used to calculate the carbonate system parameters [pCO_2 (carbon dioxide partial pressure), HCO_3 (bicarbonate concentration) and Ω Arg (aragonite saturation state)]. Values indicate the mean ± SD.

Supplemental table S1. Statistical analysis of *S. canicula*'s normal blood cells (leukocytes, thrombocytes and erythrocytes), erythrocytes with nuclear anomalies (ENAs) and ratios (nucleus to cytoplasmic, spleen and heart to body ratios). Std Error – Standard Error.

Chapter 6. Reduced impact of ocean acidification on growth and swimming performance of newly hatched tropical sharks (*Chiloscyllium plagiosum*)

Table 1. Seawater carbonate chemistry data of the experiments with embryos and juveniles of whitespotted bamboo shark (*Chiloscyllium plagiosum*). 1. Embryos at control conditions; 2. Juveniles at control conditions; 3. Juveniles at future oceanic conditions. Carbon dioxide partial pressure (pCO_2) was calculated with CO2SYS using salinity, temperature, pH, and total alkalinity (A_T). Values are mean ± SD (standard deviation).

Supplemental table S1. Results from the statistical analysis (* - $p < 0.05$).

List of papers

As author of the present dissertation, I hereby announce that I contributed for the conception of each chapter here presented. I was responsible for rearing the animals, performing blood collection and biochemical analysis, analyzing the data and writing each manuscript. The present dissertation is composed of five scientific papers from Chapter 2 to 6, of which three are published, and another is submitted.

Chapter 2

Pegado MR, Santos CP, Raffoul D, Konieczna M, Sampaio E, Temporão A, Diniz M & Rosa R. Effects of a simulated marine heatwave on the oxidative stress response of juvenile temperate catsharks. (*in prep*).

Chapter 3

Pegado MR, Santos CP, Raffoul D, Konieczna M, Sampaio E, Diniz M & Rosa R. 2020. Impact of a simulated marine heatwave in the hematological profile of a temperate shark (*Scyliorhinus canicula*). Ecological Indicators. 114:106327. DOI: 10.1016/j.ecolind.2020.106327.

Chapter 4

Pegado MR, Santos CP, Pimentel M, Cyrne R, Sampaio E, Temporão A, Röckner J, Diniz M & Rosa R. Lack of oxidative damage on temperate juvenile catsharks after a long-term ocean acidification exposure. Marine Biology. (*Submitted*).

Chapter 5

Pegado MR, Santos CP, Pimentel M, Cyrne R, Paulo M, Maulvaut AL, Raffoul D, Diniz M, Bispo R & Rosa R. 2019. Effects of elevated carbon dioxide on the hematological parameters of a temperate catshark. Journal of Experimental Zoology Part A: Ecological and Integrative Physiology. 333(2):126-132. DOI: 10.1002/jez.2333.

Chapter 6

Pegado MR, Santos C, Couto A, Pinto E, Lopes AR, Diniz M & Rosa R. 2018. Reduced impact of ocean acidification on growth and swimming performance of newly hatched tropical sharks (*Chiloscyllium plagiosum*). Marine and Freshwater Behaviour and Physiology. 51(6):347-357. DOI: 10.1080/10236244.2019.1590120.

Abstract

Anthropogenic activities release carbon dioxide into the atmosphere, which is leading to physical and chemical changes on our once-pristine oceans. The atmospheric levels of carbon dioxide are rising at unprecedented rates, resulting in ocean warming (including marine heatwaves) and acidification. Alongside, shark populations are dangerously declining and, as key elements of marine ecosystems, it is of extreme relevance to determine their vulnerability to the potential risks to climate change. Hence, the purpose of the present dissertation was to investigate the impacts that marine heatwaves and ocean acidification may have on the ecophysiology of juvenile temperate (*Scyliorhinus canicula*) and tropical (*Chiloscyllium plagiosum*) benthic sharks, including oxidative stress response, hematological parameters, growth and swimming performance. The findings suggest that the temperate species is more sensitive to warming than to acidification, as exposure to a marine heatwave altered normal blood cells counts and increased ventilation rates, while ocean acidification did not elicit significant alterations. Moreover, ocean acidification only affected the tropical species, as a short-term exposure was enough to reduce growth rates and time spent swimming and even to reduce acetylcholinesterase's activity in the brain. The observed effects may indicate an overall resilience of sharks towards these physical and chemical changes in the ocean. Nonetheless, these sublethal effects may reduce sharks' fitness and sustainability at long-term, with potential cascading effects at ecosystem level.

Keywords: Marine heatwaves, ocean acidification, sharks, physiology, hematology, oxidative stress.

Resumo

A atividade antropogénica está a libertar dióxido de carbono para a atmosfera, que se traduz em alterações físicas e químicas dos nossos oceanos outrora pristinos. Os níveis de dióxido de carbono estão a aumentar a uma velocidade sem precedentes o que resulta no aumento de temperatura (incluindo um aumento de ondas de calor marinhas) e acidificação dos oceanos. Simultaneamente as populações de tubarões estão a diminuir e, como elementos essenciais dos ecossistemas marinhos, é imperativo determinar a sua vulnerabilidade aos potenciais riscos que um oceano em mudança acarreta. Assim, o objetivo da presente dissertação foi investigar os impactos que uma onda de calor marinha e a acidificação dos oceanos poderão ter na ecofisiologia de tubarões temperados (*Scyliorhinus canicula*) e tropicais (*Chiloscyllium plagiosum*) juvenis bentónicos, incluindo respostas de stress oxidativo, parâmetros hematológicos, crescimento e performance natatória. Os resultados sugerem que a espécie temperada estudada é mais sensível ao aquecimento que à acidificação, uma vez que a exposição a uma onda de calor marinha alterou a contagem de células sanguíneas e aumentou a taxa de ventilação enquanto a acidificação não surtiu efeitos significativos. Além disso, a acidificação só afetou a espécie tropical estudada, uma vez que uma curta exposição foi suficiente para reduzir a taxa de crescimento, o tempo de natação e a atividade da acetilcolinesterase no cérebro. Os impactos observados parecem indicar alguma resiliência às alterações climáticas. Contudo, até estes efeitos subletais podem reduzir o estado físico e a sustentabilidade destes tubarões a longo curso, com potenciais efeitos cascata ao longo do ecossistema.

Palavras chave: Ondas de calor no mar, acidificação dos oceanos, tubarões, fisiologia, hematologia, stress oxidativo.

Resumo alargado

As atividades antropogénicas, como a queima de combustíveis fósseis e a desflorestação, estão a aumentar a concentração de dióxido de carbono (CO_2) na atmosfera. Ao longo de milhares de anos os níveis de CO_2 mantiveram-se estáveis, não ultrapassando as 310 partes por milhão (ppm), mas atualmente já ultrapassaram as 400 ppm. Se estas emissões não forem reduzidas, as projeções científicas indicam que no final deste século irão atingir 1000 ppm. Deste aumento sem precedentes advêm uma série de consequências. O CO_2 existente na atmosfera absorve parte da radiação solar sob a forma de calor, e o aumento da sua concentração leva a um aumento da temperatura média do planeta. Uma grande parte deste calor e do CO_2 adicionais têm sido absorvidos pelos oceanos, caso contrário a temperatura à superfície do nosso planeta seria ainda mais elevada. Contudo, a absorção de calor e de CO_2 faz com que também o oceano fique mais quente e, além disso, mais ácido. Ao ser absorvido parte do CO_2 reage com a água do mar para formar ácido carbónico, num fenómeno designado de acidificação dos oceanos. Desde o início da revolução industrial o pH diminuiu cerca de 0.1 unidades e a temperatura média da superfície dos oceanos subiu cerca de 1 °C. Num cenário de emissões continuas como se verificou até à atualidade é possível que o pH diminua mais 0.3 unidades e a temperatura da superfície marinha suba mais 4.8 °C até ao fim do século. Face a estas projeções, torna-se imperativo compreender o impacto que estas alterações físicas e químicas possam ter na vida marinha.

Começando pelo nível molecular, estas alterações do meio representam um fator de stress para os organismos marinhos, o que pode despoletar o aumento de espécies reativas de oxigénio (ROS). As ROS têm funções importantes de sinalização celular e de destruição de organismos patogénicos, e são naturalmente produzidas pelos organismos, especialmente durante processos metabólicos como a respiração celular. No entanto, em grandes quantidades podem tornar-se altamente tóxicos para as células. Basicamente, durante o processo de produção de energia, uma pequena porção do oxigénio utilizado pelas mitocôndrias é parcialmente reduzido originando o ião superóxido ($O_2^{\bullet}$), o qual através de várias reações químicas pode originar peróxido de hidrogénio (H_2O_2) e o radical hidroxilo ($HO^{\bullet}$). Estes três radicais livres

representam os principais ROS capazes de causar dano oxidativo em lípidos, proteínas e até no DNA dos organismos marinhos. Para combater o efeito nocivo das ROS, os organismos contam com um conjunto de enzimas antioxidantes. Entre elas a superóxido dismutase, que converte $O_2^{\bullet}$ em H_2O_2, bem como a catalase e glutationa peroxidase que transformam H_2O_2 em H_2O. Simultaneamente, para recuperar proteínas danificadas pela ação das ROS ou lidar com o stress térmico, o qual também danifica as proteínas, os organismos produzem proteínas de choque térmico que atuam na recuperação da conformação proteica. Se o dano for além da recuperação, o organismo produz ubiquitinas que marcam as proteínas irrecuperáveis para estas serem removidas. Tanto o aumento de temperatura como a diminuição do pH dos oceanos podem levar, não só ao aumento das ROS, como a diminuição da capacidade enzimática de defesa contra elas e em último caso aumentar o dano oxidativo. É também importante referir que, quando um organismo marinho é exposto a um ambiente mais ácido, há uma disrupção no equilíbrio ácido-base. Para repor este equilíbrio, os animais acumulam bicarbonatos (HCO_3^-) e libertam iões hidrogénio (H^+) para o meio ambiente. Este processo requer energia que poderá afetar outros processos fisiológicos.

A nível celular, estas alterações nos oceanos podem afetar o metabolismo basal ou até diminuir a resposta imunitária dos organismos. O aumento de temperatura reduz a saturação de oxigénio disponível no meio e, além disso, tanto o aumento de temperatura como a diminuição do pH podem alterar a conformação das proteínas responsáveis pelo transporte de oxigénio, comprometendo o seu fornecimento aos tecidos. A redistribuição energética necessária, tanto para a manutenção do metabolismo basal como para o equilíbrio ácido-base, pode reduzir a capacidade do sistema imunitário.

Por último, as alterações fisiológicas observadas poderão manifestar-se ao nível do organismo. A energia necessária para combater os efeitos do aumento de temperatura e da acidificação não poderá ser utilizada para outros processos, como é o caso do crescimento ou alterações comportamentais. O aumento da temperatura poderá levar à redistribuição geográfica das espécies enquanto as alterações químicas causadas pela acidificação poderão alterar funções neurológicas, traduzindo-se em comportamentos anormais ou de risco.

Os tubarões são elementos importantes dos ecossistemas marinhos. Alguns estudos científicos apontam para que, tanto o aumento de temperatura como a acidificação dos oceanos, possam ter um impacto na fisiologia dos tubarões. Contudo, não existe muita informação disponível sobre as respostas dos tubarões ao stress e, sobretudo, aos possíveis impactos das alterações climáticas. Neste contexto, a presente dissertação foi dividida em duas partes: Parte I – com o objetivo de estudar os efeitos de uma onda de calor marinha, causada pelo aumento de temperatura, na resposta ao stress oxidativo, nos parâmetros hematológicos e na taxa de ventilação de uma espécie temperada (*Scyliorhinus canicula*; capítulos dois e três, respetivamente) e Parte II – de forma a estudar os impactos da acidificação dos oceanos na resposta ao stress oxidativo e nos parâmetros hematológicos da mesma espécie temperada (*Scyliorhinus canicula*; capítulos quatro e cinco, respetivamente) e nas taxas de crescimento, performance natatória e no sistema colinérgico de um tubarão tropical (*Chiloscyllium plagiosum*; capítulo seis).

De acordo com os resultados, tanto a onda de calor como acidificação não causaram alterações nas respostas enzimáticas ao stress oxidativo, nem causaram dano oxidativo na espécie temperada (*Scyliorhinus canicula*). Contudo, houve um aumento da atividade da catalase no músculo de tubarões expostos a acidificação. Tal pode ter ocorrido uma vez que, quando um organismo é exposto a acidificação, existem mais iões hidrogénio (H^+) disponíveis o que podem levar ao aumento de peróxido de hidrogénio (H_2O_2). Quando tal acontece, a catalase é a principal enzima antioxidante que promove a transformação deste ROS em água (H_2O) e oxigénio (O_2). De qualquer forma, este aumento serviu para neutralizar as ROS produzidas, e que não resultou em dano oxidativo.

Ao analisar o sangue, a acidificação não teve qualquer efeito, enquanto a onda de calor provocou várias alterações. O aumento da temperatura por si só já leva a uma diminuição de oxigénio disponível e por isso há maior necessidade deste por parte do organismo. Em reposta, o aumento de temperatura levou a um aumento da taxa de ventilações e de eritrócitos, e para lidar com estas limitações de oxigénio na espécie temperada (*Scyliorhinus canicula*). Além disso, a onda de calor levou também a um aumento de leucócitos e trombócitos possivelmente para lidar com um possível aumento de agentes patogénicos.

Por último, a acidificação dos oceanos promoveu várias alterações na fisiologia da espécie tropical (*Chiloscyllium plagiosum*), nomeadamente, provocou uma diminuição na taxa de crescimento somático, uma diminuição da percentagem de tempo passado a nadar e a diminuição da atividade de acetilcolinesterase em duas macro áreas do cérebro. A diminuição da natação aleada à diminuição do crescimento fazem com que este tubarão juvenil esteja mais propenso a ser predado e/ou a não ter tanto sucesso como predador.

Assim sendo, a presente dissertação indica que os tubarões podem ser tolerantes ao oceano em mudança até certo ponto. A temperatura parece ter um efeito mais negativo que a acidificação na espécie temperada (*Scyliorhinus canicula*). Além disso, a espécie tropical (*Chiloscyllium plagiosum*) parece ser suscetível à acidificação. Assim, apesar das alterações observadas não serem letais podem ainda assim ter um impacto negativo nestas espécies. Uma vez que os efeitos das alterações climáticas se estão a intensificar, é importante e necessário tomar medidas para combater e mitigar estas alterações de forma a proteger as espécies marinhas, e sobretudo os tubarões.

“We need to respect the oceans and take care of them as if our lives depended on it.

Because they do.”

Sylvia Earle

1 General Introduction

1.1. Carbon dioxide

Anthropogenic activities, such as combustion of fossil fuels and deforestation, are contributing to the increase of carbon dioxide (CO_2) in the atmosphere, and changing the physical and chemical properties of our once-pristine oceans (Doney & Schimel, 2007; Hartmann et al., 2013; Pörtner et al., 2014). Over the past 400.000 years, CO_2 levels have steadily oscillated between 180 and 310 parts per million (ppm) (Petit et al., 1999), however, as a result of this activity, within the last two centuries levels have surpassed 400 ppm (Fischer et. al, 2018; NOAA, 2020; Fig. 1).

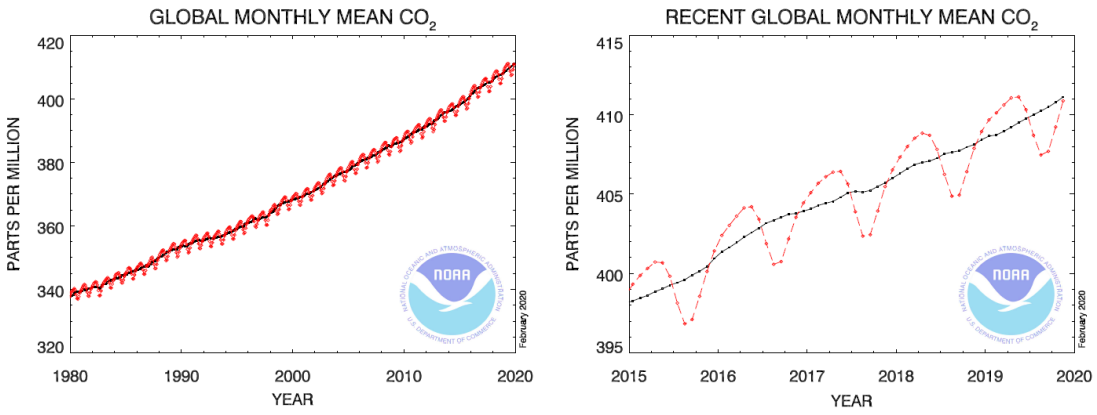

Figure 1. Monthly measurements of global mean carbon dioxide over marine surface areas from 1980 to the present day (left), and from 2015 to the present day (right). Data measured at globally distributed sampling sites. The red line represents monthly mean values and black line represents the same after rectification for mean seasonal cycle (Conway, 1994; NOAA, 2020).

To predict the consequences of these emissions, the Intragovernmental Panel on Climate Change (IPCC) created four Representative Concentration Pathways (RCPs) that project different impacts regarding anthropogenic radiative forcing (W m^{-2}, in relation to 1750) by the end of this century. These include: RCP2.6, RCP4.5, RCP6.0, and RCP8.5. The best-case scenario is given by the RCP2.6, which represents a

reduction of greenhouse gas emissions through a severe mitigation plan that limits global warming to less than 2 °C by 2100 (Allen et al., 2018). While the worst-case scenario is given by the RCP8.5, which represents a business-as-usual scenario of high greenhouse gas emissions without beneficial mitigation policies, heading to an increase of greenhouse gas concentrations in the atmosphere (Riahi et al., 2011). The other two RCPs fall between these extreme scenarios. If anthropogenic emissions continue to grow as in the present day, they will follow the RCP8.5 trajectory (Le Quéré et al., 2018), and CO_2 levels are expected to reach around 1000 ppm by the end of the century (IPCC, 2019). The main consequence regarding CO_2 increase is the slow timescale of the carbon cycle (Lenton & Britton, 2006), which dictates that approximately 35% of CO_2 that has been emitted to the atmosphere is likely to remain there even after a thousand years (IPCC, 2013). Moreover, the CO_2 accumulated absorbs long-wavelength radiation, trapping additional heat in the atmosphere which results in higher temperatures around the globe (Raghuvanshi, Chandra & Raghav, 2006).

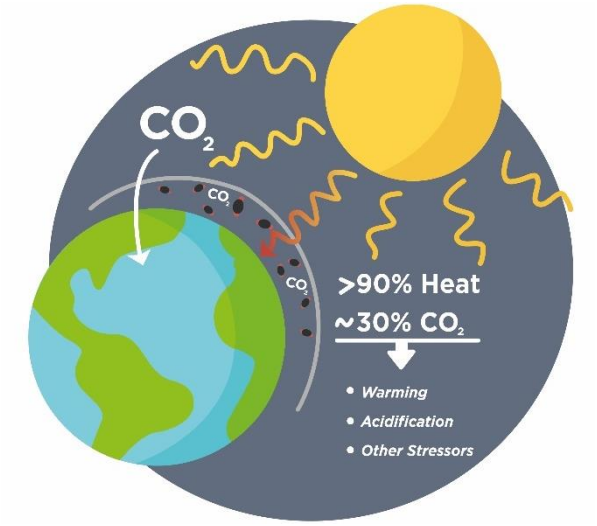

Figure 2. Schematic representation of CO_2 (carbon dioxide) effects on the ocean.

The oceans are extremely important to the health of our planet, as they cover around 70% of the surface, host a large percentage of biodiversity and regulate the climate (Hoegh-Guldberg & Bruno, 2010). Without the oceans, the impacts of anthropogenic emissions would be much more severe (Barnett, Pierce & Schnur, 2001). The large extent of sea surface area allows the exchange of important gases between the atmosphere and the ocean, including CO_2 (Bigg et al., 2003; Fig. 2). As a result, the ocean has absorbed 93%

of excess heat present in the atmosphere and approximately 30% of the CO_2 that has been released (IPCC, 2014). Although the ocean has contributed to soften an otherwise drastic climate change (Barnett, Pierce & Schnur, 2001), this unprecedented rate of CO_2 release will have irreversible climatic consequences, some of which are already taking place. Since pre-industrial times the absorption of heat has been increasing global sea surface temperature, leading to ocean warming (Wolf-Gladrow, 2018) and the absorption of CO_2 has been decreasing seawater pH, leading to ocean acidification (IPCC, 2014). As the rate of these climate changes are unprecedented, the negative effects that these changes may have on marine life and ecosystems is likely to be unprecedented as well.

1.2. Marine heatwaves

The average sea surface temperature (SST) around the globe has already increased about 1 °C since the Industrial Revolution (IPCC, 2014). According to scientific projections, in a business-as-usual scenario, the mean global sea surface temperature will further rise between 1.64–3.51 °C until the end of the century (Allen et al., 2018). Due to their heat capacity and thermal inertia, the oceans are slowly absorbing this extra heat (Barnett, Pierce & Schnur, 2001), which is also increasing the frequency of extreme sudden warming events designated as marine heatwaves (MHW). MHW have been designated as periods of at least five consecutive days in which SST is above the 90th percentile in relation to the long-term local climatology (Hobday et al., 2016). According to these authors, they can be divided into four categories, as multiples of the difference between the normally-occurring climatology and the 90th percentile as: moderate (Category I, 2×), strong (Category II, 3×), severe (Category III, 4×), and extreme (Category IV, >4×), as represented in the scheme (Hobday et al. 2018; Fig. 3).

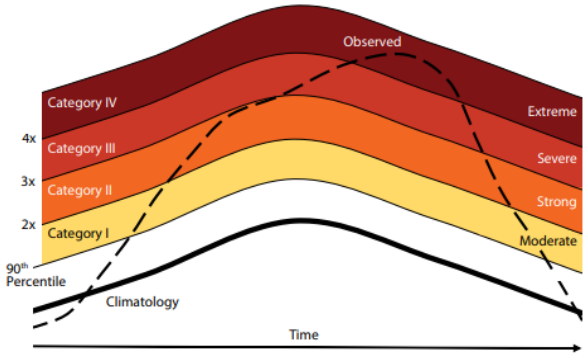

Figure 3. Schematic categorization of marine heatwaves (MHW). Dashed line indicates observed temperature through time, bold line represents long-term regional climatology, and thin line indicates the 90th percentile difference. Multiples of the 90th percentile (twice, three times, etc.) from the climatology value describe each category (Hobday et al., 2018).

Furthermore, when analyzing MHW, two metrics can be considered: duration, i.e. amount of time between the begin and ending dates, and maximum intensity, i.e. the peak of anomalous temperature (Herring et al., 2018). There is evidence to suggest that MHW duration and frequency are increasing (Oliver et al., 2018), and are expected to further intensify in the future (Frölicher, Fischer & Gruber, 2018). While the trends of global SST are well known, the mechanistic understanding of the processes leading up to MHW are currently missing (Frölicher & Laufkötter, 2018). In comparison, there is a better understanding regarding MHW-elicited direct impacts on biological systems (Frölicher, Fischer & Gruber, 2018). These sudden warming events are restructuring marine communities, not only due to massive mortality of numerous marine animals (Garrabou et al., 2009; Oliver et al., 2017) and loss of kelp forest (Wernberg et al., 2016), but also by changing the geographical distribution of several species (Poloczanska et al., 2013; Pinsky et al., 2013; Frölicher, Fischer & Gruber, 2018). Organisms generally suffer stronger impacts by extreme changes in the environment, compared to slower, gradual alterations of the natural conditions (Gaines & Denny, 1993). Thus far, MHWs have caused severe impacts on key ecosystems, such as the Great Barrier Reef, as an extensive area of over 2300 km of coral assemblage suffered mass mortality due to bleaching (Hughes et al., 2017). As they intensify, these warming events are breaking new records. The longest MHW that was ever recorded lasted 3 years, and it was designated as “the Blob” (Cavole et al., 2016; Di Lorenzo & Mantua, 2016). This heatwave, began in 2013 and lasted until 2016, in the northeastern Pacific (Cavole et al., 2016; Di Lorenzo & Mantua, 2016), causing unprecedented rates of harmful algal bloom growth, massive deaths of marine mammals and sea birds and shifts of several marine warm water animals (Cavole et al., 2016). Warmer years lead to warmer sea surface temperature anomalies that can also extend to deeper waters (Zador et al., 2018), having already reached depths of 300 m (Walsh et al., 2018). This continuous intensification of MHWs may enhance the risk of acute and long-term repercussions on many marine organisms (Frölicher, Fischer & Gruber, 2018). Given the probable intensification of these extreme warming events as a result of anthropic climate change and the potential for deep ecological impacts, understanding the full extent of the impacts that MHW may have on marine life is a pressing issue.

1.3. Ocean acidification

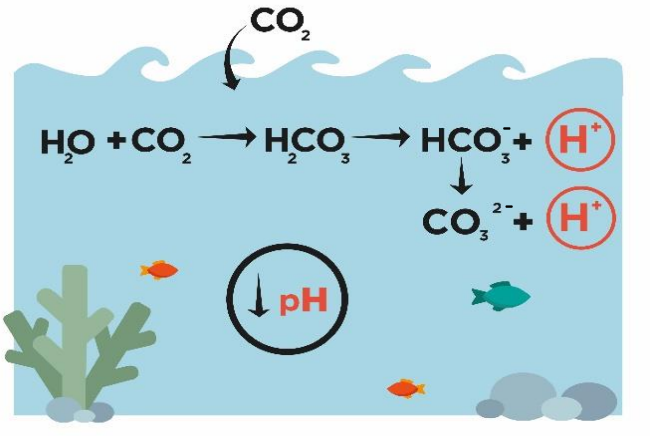

Figure 4. Scheme of the chemical reactions that drive ocean acidification (CO_2 – carbon dioxide, H_2O – water, H_2CO_3 – carbonic acid, HCO_3^- – bicarbonate ion, CO_3^{2-} – carbonate ion, H^+ – hydrogen ions).

The ocean has absorbed around 30% of CO_2 in the atmosphere, altering the ocean's carbonate chemistry and decreasing the pH (IPCC, 2014). As it is absorbed, CO_2 reacts with seawater to form carbonic acid (H_2CO_3) which rapidly dissociates into carbonates (CO_3^{2-}) and hydrogen ions (H^+) (Zeebe & Wolf-Gladrow, 2001; Fig. 4). This increase of H^+ contributes to a decrease in the seawater pH which leads to ocean acidification (OA). Since pre-industrial times, surface seawater pH has decreased by approximately 0.1 units (Doney et al., 2009) from 8.21 to 8.10 (Raven et al., 2005). It should be emphasized that pH is measured on a logarithmic scale, and even a small change as 0.1 units corresponds to a 30% increase of H^+ ions (Wolf-Gladrow, 2018). Therefore, the amount of CO_2 that is absorbed has an enormous impact on the resultant ocean's pH (Raven et al., 2005). In a business-as-usual scenario, if atmospheric CO_2 levels continue to rise, the pH is expected to further decrease around 0.3 units (Allen et al., 2018).

These chemical changes are expected to affect marine life in the near future (Seibel & Fabry, 2003; Fabry et al. 2008; Wittmann & Pörtnerz, 2013). Vulnerability to OA differs between species. Some are more sensitive and suffer high mortality rates (Dupont et al., 2008), especially during early life stages (reviewed in Pörtner, Langenbuch & Michaelidis, 2005), while others present sublethal (Dupont, Dorey & Thorndyke, 2010) or even positive effects (Dupont, Lundve & Thorndyke, 2010; Kroeker et al., 2013). Nonetheless, even the sublethal effects may come with costs (Esbaugh et al., 2016). Exposure to OA dirupts acid-base equilibrium and, to compensate, marine organisms accumulate HCO_3^- while excreting H^+ to the environment. If compensation of this imbalance cannot be achieved some species may suppress their

metabolism (Reipschläger & Pörtner, 1996; Guppy & Withers, 1999), which may lead to physiological impairments, such as reduced growth rates (Fabry et al., 2008) and/or altered behaviours (Pörtner, Langenbuch & Reipschläger, 2004). As CO_2 emissions continue to increase it is imperative to measure the biological responses of marine life to these chemical changes.

1.4. Biological responses of marine life to climate change

Knowledge regarding the responses of marine life to climate change is a sheer drop in the ocean when compared to terrestrial ecosystems (Parmesan & Yohe, 2003), as their attribution and detection imposes higher challenges for scientists (Richardson et al., 2012). However, increasing evidence suggests that marine life could respond just as fast, if not faster, than terrestrial (Richardson & Poloczanska, 2008; Sorte, Williams & Carlton, 2010). Hence, in a changing ocean, marine species may be directly affected at different organizational levels, e.g. from molecular to community levels (Pörtner & Farrell, 2008; Tuomainen & Candolin, 2011).

1.4.1. Molecular level (e.g. GABA and reactive oxygen species)

The chemical changes that drive OA can impact marine life, starting at the molecular level. For instance, it has been theorized that exposure to a lower pH can alter behaviours due to ionic changes in the $GABA_A$ receptor's membrane (Nilsson et al., 2012; Lai, Jutfelt & Nilsson, 2015). Gamma-aminobutyric acid (GABA) is an inhibitory neurotransmitter (Jessen et al., 1979), and the imbalanced gradients in the neuronal membrane experienced under high CO_2 levels may origin a shift in neuronal circuits from inhibitory to excitatory, leading to abnormal behaviours (Nilsson et al., 2012). In normal conditions, $GABA_A$ receptors have a specific conductance for HCO_3^- and Cl^-, and when activated by GABA an inflow of Cl^- reduces neural excitability through hyperpolarizaton (Nilsson et al., 2012). However, when CO_2 concentrations are elevated, a new gradient of HCO_3^- and Cl^- is established across the neural membrane, and the activation $GABA_A$ receptor can result in an outflow of these anions which depolarizes the membrane (Nilsson et al., 2012; Heuer and Grosell, 2014; Fig. 5). Ultimately, this process can pottentially cause changes in behaviour,

olfactory preference, vision, and even learning (Nilsson et al., 2012; Chivers et al., 2014; Lai et al., 2015). In this way, OA can alter a great variety of behaviours that are connected to cognition and physiology, as activity levels, lateralization and swimming behaviour (Nagelkerken & Munday, 2016).

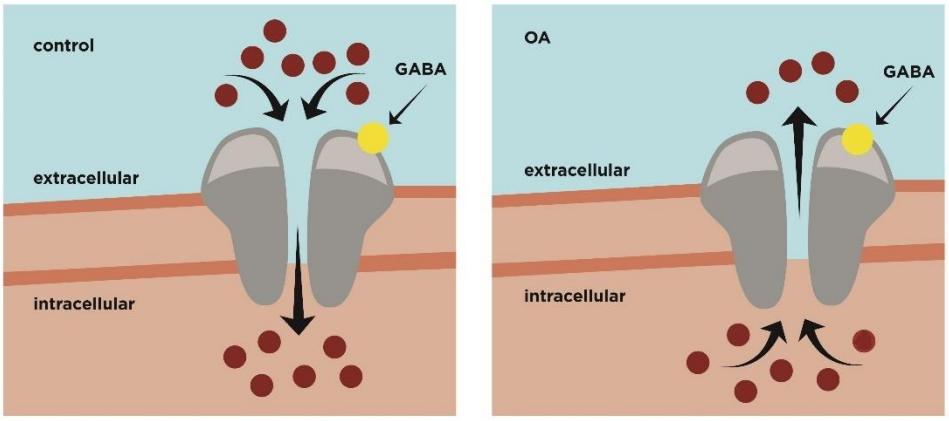

Figure 5. Scheme of $GABA_A$ receptor function alteration during OA exposure. Red dots represent the flow of Cl^- ions in the neuronal membrane.

Moreover, at a molecular level, the stress caused by changes in the environment such as warming or acidification can lead to the overproduction of reactive oxygen species (ROS) (Lesser, 2006). ROS are oxygen-containing molecules (Lambert & Brand, 2009) that result from the partial reduction of oxygen (O_2), such as $O_2^{\bullet}$, H_2O_2 and $HO^{\bullet}$ (Wilhelm Filho, 2007). ROS are normally generated as a by-product of normal cell metabolism in the mitochondria (Le Bras et al., 2005). During ATP production, around 1-3% of O_2 can be partially reduced to superoxide anion ($O_2^{\bullet}$) (Birben et al., 2012; Fig. 6), which can either be further reduced to H_2O_2, by the catalytic action of superoxide dismutase (SOD), or react with reactive nitrogen species, to produce $HO^{\bullet}$ (Bresciani, da Cruz & González-Gallego, 2015), the most harmful ROS (Boveris, 1977). Alternatively, H_2O_2 can be reduced to $HO^{\bullet}$ in successive reactions designated as Haber–Weiss (Cu^{2+}) or Fenton (Fe^{2+}) reactions (Hippeli & Elstner, 1997).

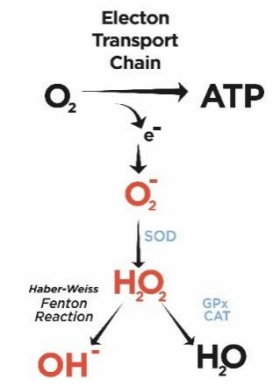

Figure 6. Schematic representation of ROS production ($O_2^{\bullet}$, H_2O_2 and $HO^{\bullet}$), represented in red. Neutralized by antioxidant enzyme defenses (SOD, CAT and GPx), represented in blue.

Excessive ROS production can lead to oxidative stress (Le Bras et al., 2005), which can lead to oxidative damage in the lipids, proteins or in the DNA (Abele & Puntarulo, 2004; Lesser, 2006). Cell membranes contain polyunsaturated fatty acids, which are highly susceptible to peroxidation (Niki et al., 2005). ROS can trigger lipid peroxidation (LPO) through a series of chain reactions (Birben et al., 2012) that consist in three points: initiation, propagation, and termination (Niki et al., 2005). Briefly, the initiation phase starts when a ROS (usually $HO^{\bullet}$) removes one hydrogen atom from a polyunsaturated fatty acid, which produces a lipid radical ($L^{\bullet}$) that reacts with O_2 to form a lipid peroxyl radical ($LOO^{\bullet}$); the latter quickly abstracts a hydrogen atom from another lipid, thus producing a lipid hydroperoxide and a new lipid radical that propagates the reaction (Regoli & Giuliani, 2014). The reaction terminates in the formation of aldehydes and malondialdehydes (MDA), which disrupt cell membranes integrity (Birben et al., 2012; Fig. 7). Injury to mitochondria through LPO can lead to further ROS formation, intensifying oxidative damage (Green & Reed, 1998).

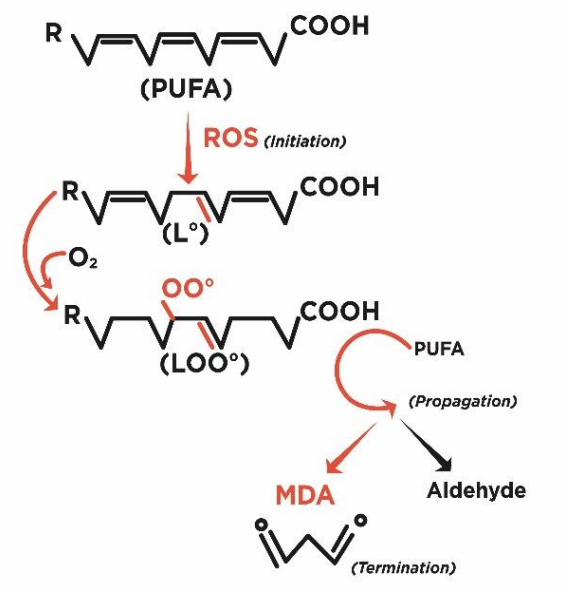

Figure 7. Schematic representation of LPO and consequence formation of MDA. Cytotoxic elements are represented in red.

Exposure to ROS can also lead to a series of reversible and irreversible changes on the side-chains of amino acids in a protein (Ghezzi & Bonetto, 2003) and change its structure or function. The side-chains of amino acids can be irreversibly altered via carbonylation leading to protein aggregation or degradation (Levine et al., 2000). ROS can also cause DNA damage in numerous ways, through the degradation of bases, alterations of sugar-bound, mutations, translocations or deletions and even single- or double-strand DNA breaks. Formation of 8-hydroxy-2'-deoxyguanosine (8-OHdG) is the best-known biomarker for DNA damage occurring due to oxidative stress (Birben et al., 2012; Fig. 8).

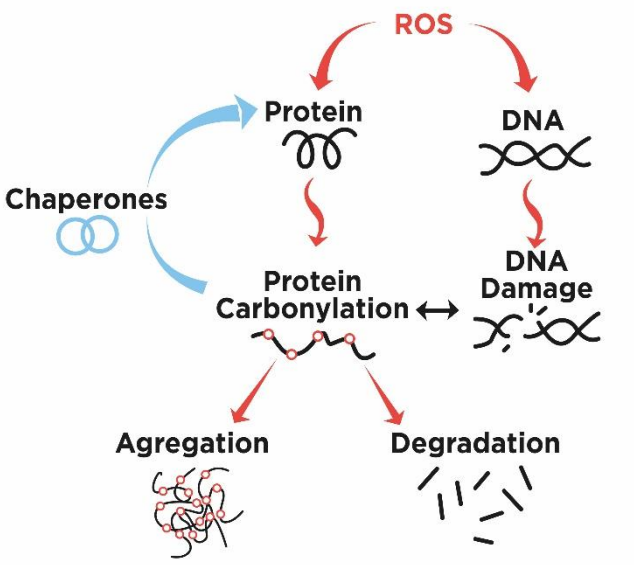

Figure 8. Schematic representation of protein and DNA damage caused by ROS. Cytotoxic elements are represented in red.

The potential defense against this oxidative damage can be measured through the activity of antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), that are able to neutralize ROS (Scandalios, 2005). The antioxidant machinery neutralizes ROS through a direct reaction, in which antioxidants are momentarily oxidized, and then are reconverted to their active form by specific reductases (Regoli & Giuliani, 2014). The first enzyme that participates in the antioxidant defense is SOD (Bandyopadhyay, Das & Banerjee, 1999) that converts $O_2^{\bullet}$ to H_2O_2, followed by CAT that further oxidizes H_2O_2 to water and O_2 (Giorgio et al., 2007), or GPx that oxidizes H_2O_2 to water and oxidized glutathione, thus protecting the cell from oxidative damage (Deisseroth & Dounce, 1970). Marine organisms also count with non-enzymatic defenses from an endogenous and/or dietary origin which include ascorbate, carotenoids, glutathione reductase, vitamin E, polyphenols, bilirubin and uric acid (Hermes-Lima & Zenteno-Savín, 2002). In the case of elasmobranchs, besides its main function to sustain plasma osmolality, urea has been suggested to also act as a supplement to their non-enzymatic antioxidant machinery to neutralize ROS (Rudneva et al., 2014) and due to its lower molecular weight can pass through membranes and has a greater mobility than enzymatic antioxidants (Wang et al., 1999). Trimethylamine N-oxide (TMAO) can be

accumulated to counteract the toxic effects of urea in elasmobranchs (Seibel & Walsh, 2002) but also in other marine groups to stabilize proteins (Samerotte et al., 2007; Bockus & Seibel, 2018) and prevent protein degradation (Ufnal, Zadlo & Ostaszewski, 2015). In the same manner, heat shock proteins (HSP) function as chaperones to refold proteins, and thus are considered as good indicators of sub-lethal protein damage (Sanders, 1993; Feder & Hofmann, 1999). However, when proteins are too damaged by oxidation, ubiquitin (Ub) targets them and promotes their degradation (Wilkinson, 2000). In normal conditions, these defense mechanisms counterbalance excessive ROS formation and avoid oxidative damage (Lushchak, 2011). However, in a highly stressful environment such as elevated temperatures or lower pH, ROS can be overproduced (Lesser, 2006) while antioxidant enzyme machinery can be affected, which can lead to oxidative damage. Both temperature and pH can affect enzymatic activity or function; high temperatures destabilize the protein's structural integrity which hinders its normal function while changes in pH display differences in ionic environments which may limit enzymatic activity (Banerjee et al., 2015). Exposure to warmer temperatures can cause an increase of antioxidant activity to protect against oxidative damage (Klein et al., 2017), but, as temperature can affect the structural integrity of the enzymatic antioxidants, these defenses are usually inefficient and this exposure mostly results in oxidative damage (Rosa et al., 2016; Sampaio et al., 2018; Pimentel et al., 2015; Madeira et al., 2013; Madeira, Vinagre & Diniz, 2016; Lopes et al., 2018). OA exposure can lead to upregulating genes and important proteins responsible for counterbalance the impacts of oxidative stress, however, the limiting enzymatic activity experienced in a lower pH environment, can still lead to oxidative damage (Tomanek et al., 2011; Lopes et al., 2018).

It is worth mentioning that while excessive formation of ROS leads to oxidative stress (Le Bras et al., 2005), a small increase within the cells act as an important second messenger for several intracellular signaling pathways (Le Bras et al., 2005) essential for cell function, or as cellular response after the successful recognition of a pathogen (Simon, Haj-Yehia & Levi-Schaffer, 2000; Torres, Jones & Dangl, 2006). The release of reactive oxygen metabolites can be mediated by blood cells responsible for the immune response,

including phagocytosis of unfamiliar or diseased cells (Simon, Haj-Yehia & Levi-Schaffer 2000; Torres, Jones & Dangl, 2006).

1.4.2. Cellular level (e.g. hematological parameters)

At cellular level, the ability to cope with a dynamic abiotic environment depends on the adjustment of internal components, including blood cells, and processes, such as acid-base regulation, to maintain homeostasis (Shultz et al., 2014). Blood parameters have been considered essential indicators of physiological stress in fish due to any internal or external changes that affect homeostasis (Sharmin et al., 2015; Salam et al., 2015). Basically, blood is a complex combination of cells immersed in plasma, that enables several vital functions. These cells consist of erythrocytes that transport oxygen and carbon dioxide from and to the gills and tissues, leukocytes that protect the body against pathogens, and thrombocytes that can form clots to protect the body from excessive blood loss following an injury (Walsh & Luer, 2004; Fig. 9).

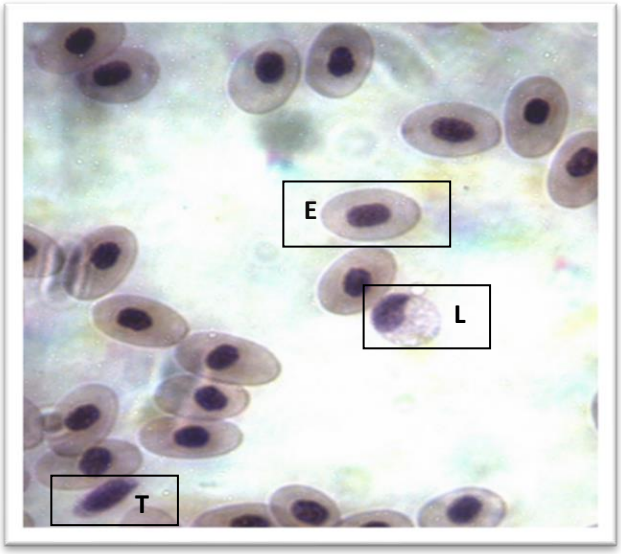

Figure 9. Blood cell components. (E) Erythrocytes, (L) Leukocytes, and (T) Thrombocytes.

Elevated temperatures can impact the hematological parameters of marine organisms (Mariana & Badr, 2019). Warmer waters reduce oxygen availability (Schmidtko, Stramma & Visbeck, 2017) which compromises oxygen transport by erythrocytes (Madeira et al., 2016) and to cope, organisms have to increase their respiration rates (Coma, 2002) as, simultaneously, more oxygen is required to produce energy (Strobel et al., 2013). An organism that has to endure such a response will certainly reduce energy storage over time (Mackenzie et al., 2014). Elevated temperatures may change the conformation of mitochondrial membrane and transmembrane proteins, altering protons permeability and ultimately compromizing

energy production by mitochondrias (Strobel et al., 2013). These metabolic changes may lead to several detrimental consequences, such as reduced ability to build an effective immunological response (Vezzulli et al., 2010) particularly if parallel with shifts in parasite loads and pathology. In fact, it is know that higher temperatures contribute to the increase of disease transmission, pathogen survival and development (Harvell et al., 2002; Lafferty, Porter & Ford, 2004; Ford, 1996).

Lower pH values in the environment can disturb the acid-base equilibrium in body-fluids, including the blood (Pörtner, 2008; Melzner et al., 2009). Hence, to cope with this disturbance, many marine organisms can retain HCO_3^- in the blood plasma (Heuer & Grosell, 2014) and actively transport ions through tissues (Claiborne & Evans, 1992) followed by a release of H^+ to the ambient water, which raises the pH in the blood plasma back to control levels, and confers these marine organisms an advantage to OA (Heuer & Grosell, 2014). However, this expensive coping mechanism requires energy redistribution (Reipschläger & Pörtner, 1996; Guppy & Withers, 1999) which may lead to immunity depression as an indirect consequence (Liu et al., 2016). On the other hand, the changes in the internal pH caused by OA may directly affect enzymatic activity and consequently may affect the immunity of marine organisms, since several molecules related to the immune response are sensitive to the pH (Liu et al., 2016). Simultaneously, exposure to OA may affect oxygen supply, due to a disruption of oxygen transport in the blood through oxygen binding proteins (Wittmann & Pörtner, 2013; Pörtner, Langenbuch, & Reipschläger, 2004) and decrease heart rate (Kent & Peirce, 1978; Perry et al., 1999).

A stressful set of conditions can also lead to the formation of erythrocytes with nuclear abnormalities (ENAs; Eiras, 1983; Takashima & Hibiya, 1995; Clauss et al., 2008; Fig. 10). For instance, ENAs can be formed due to increased products of lipid peroxidation in erythrocytes of fish exposed to elevated temperatures (Bai et al., 2014; Ghaffar et al., 2015). Micronuclei are formed during the telophase of the cell cycle division as either chromosome fragments or whole chromosomes which are encapsulated in a nuclear envelope and adopt the features of an interphase nucleus that is significantly reduced in size (Al-Sabti & Metcalfe, 1995).

The precise pathways leading to the formation of other nuclear abnormalities of erythrocyte cells are less understood, but these consist of segmented or blebbed nucleus (Braham et al., 2017).

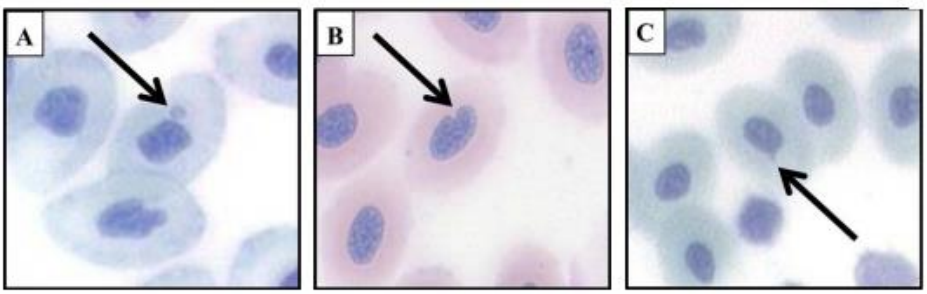

Figure 10. Erythrocytes with nuclear abnormalities. (A) Micronuclei, (B) Segmented nucleus, and (C) Blebbed nucleus. Images were captured at 600x magnification (Braham et al. 2017).

Regardless, even a possible adaptation to climate change conditions may come with costs (Esbaugh et al., 2016), as the disturbance of all these internal components can be reflected in physiological changes of the whole organism (Somero, 2010).

1.4.3. Organismal level (metabolism and behavior)

Temperature is considered as the most piercing climate-related impact on biological function (Johnston & Bennett, 1996, Kaslbeek et al., 2012). Most biological processes are sensitive to temperature, e.g. marine organisms function optimally within a slim thermal window and outsite this window their performance declines sharply (Kearney & Porter, 2009). To explain the connection between organism performance and environmental temperature the concept of oxygen- and capacity-limited thermal tolerance (OCLTT) was established (Pörtner, 2001; Pörtner

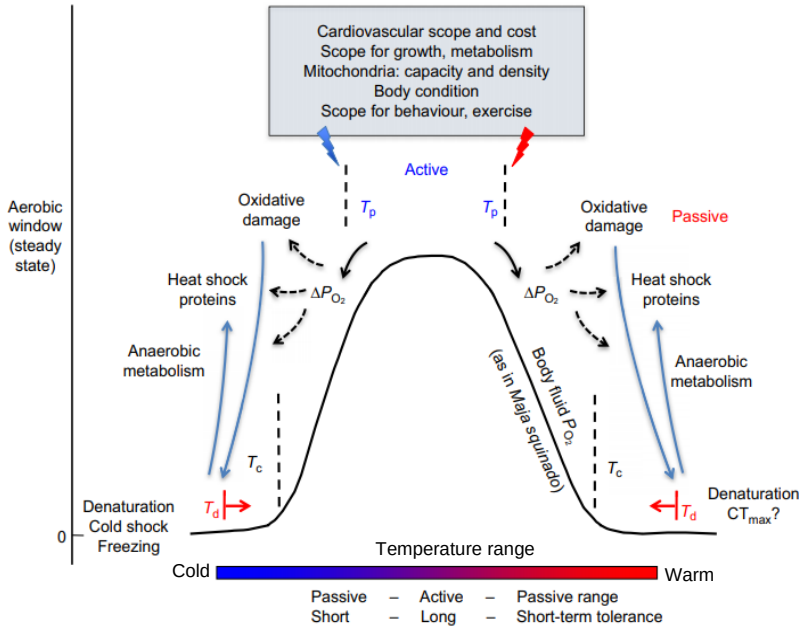

Figure 11. Conceptual model for the oxygen- and capacity-limited thermal tolerance hypothesis (adapted from: Pörtner HO et al., 2017).

et al., 2009, 2017; Fig. 11). OCLTT suggests that there is an optimal thermal range of performance close to the pejus temperature (Tp, "pejus" means getting worse), above which there is insufficient oxygen supply to the tissues, resulting in a gradual reduction of the organism's performance. Beyond a critical temperature (Tc), the organism can no longer sustain an aerobic budget which results in the onset of anaerobiosis and ultimately, its death (Td). To determine the impacts of temperature on the aerobic power scope, several traits that are associated to oxygen uptake and its distribution can be measured as proxy, including the blood oxygen content, cardiac function and ventilation rates, which ideally should be combined with performance indicators for the whole animal such as growth or swimming performance (Eymann et al., 2020). The exposure to elevated temperature promotes an acceleration of metabolic demands (Moran & Woods, 2007) and the reallocation of energy to support these additional demands is taken away from non-vital processes. First of all, it affects growth (Kennedy, Newell & Shumway, 1996, Katersky & Carter, 2007; Wang & Overgaard, 2007), but also reproductive output, timing of reproduction (Munday et al., 2008), and physical function (Peck, Webb & Bailey, 2004). Furthermore, additional drivers such as OA may also influence these responses to elevated temperatures.

Ocean warming and acidification may influence marine organisms' behaviour through several ways. Elevated temperatures can alter the range size and species distribution while acidification can modify population size and change species dynamics, thus altering species interactions behaviours (Edwards & Richardson, 2004; Harley, 2011; Tunney et al., 2014; Nagelkerken et al., 2015). These stressors may even directly influence physiological processes that underpin behaviour, such as higher metabolic demands during warming (Portner et al., 2004) or neurological shifts during exposure to OA (Nilsson et al. 2012). The neurological changes caused by OA can lead to riskier behaviours and increase predation, mainly due to impairments in the vision, olfaction, audition (Nagelkerken & Munday, 2016) and lateralization. Laterality is a key process for simple locomotory responses, especially during escape from predators (Nepomnyashchikh & Izvekov, 2007) and in fact, both warming and OA may affect brain lateralization by affecting cerebral

processes responsible for right/left choice (Domenici et al., 2014), with further consequences in complex cognitive functions (Nepomnyashchikh & Izvekov, 2007).

1.5. Sharks and climate change

As apex predators, sharks are vital for maintaining a healthy ecosystem and the structure of food webs (Torres et al., 2014). They are classified as cartilaginous fish, as their skeleton is made of calcified cartilage, and have been considered as the most ancient living jawed vertebrates (Bird et al., 2002). Sharks have been roaming our oceans over the past 400 million years, having survived through five mass extinctions (Kriwet et al., 2008) and successfully coped with high fluctuations of CO_2 levels in the environment. Throughout time, sharks faced prolonged periods of elevated CO_2 levels, that even exceeded 4000 ppm, and thrived (Royer et al., 2004; Clack, 2007; Fig. 12). Even though they have successfully evolved through different conditions and marine habitats, the ability to adapt to a fast-changing ocean may be limited (Field et al., 2009; Chin et al., 2010) due to their k-life strategy. Sharks have long lifespans characterized by late maturity age and few offspring (Compagno, 1990), and as their populations are in deep decline (> 90%) (Musick et al., 2000) they are probably at higher risk from climate change (Cortés 2002; García, Lucifora & Myers 2008).

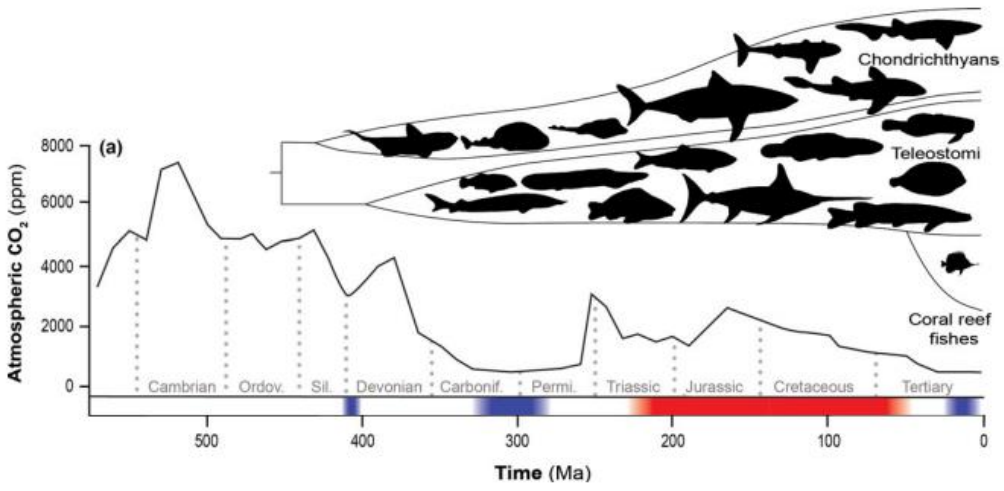

Figure 12. Schematic representation of the evolutionary timeline of Chondrichthyans and Teleostomi, with respective CO_2 levels in the atmosphere in parts per million (ppm). Warmer periods are represented in red and colder periods in blue (Rummer & Munday 2017).

The decline of top predators can have cascading effects throughout ocean ecosystems (Stevens et al., 2000; Myers et al., 2007) thus assessing the impacts of yet another potential threat is extremely important. As both warming and OA may pose a considerable threat to marine life, we are still far from fully understanding how these environmental changes will impact shark species. Indeed, the scientific community is unraveling the effects that climate change may have on sharks. In an OA scenario, epaulette shark embryos (*Hemiscyllium ocellatum*) showed no impacts on growth rate, yolk usage, tail oscillations, gill movements and survival rates (Johnson et al., 2016), and juveniles also did not show impacts on foraging behaviour (Heinrich et al., 2015) nor on metabolic performance (Heinrich et al., 2014). This species appears to be incredibly tolerant to OA as it also exhibits exceptionally high tolerance to severe hypoxia (Heinrich et al., 2014). Adult small-spotted catsharks (*Scyliorhinus canicula*) showed no alterations in growth, but experienced changes in blood chemistry and behaviour, consisting of swimming pattern shifts and increased lateralization (Green & Jutfelt, 2014). High CO_2 levels led to DNA damage in the liver of newly hatched whitespotted bamboo sharks (*Chiloscyllium plagiosum*) (Lopes et al., 2018). Lastly, OA reduced the ability of smooth dogfish (*Mustelus canis*) to properly respond to an olfactory cue (Dixson et al., 2015), and the puffadder shyshark (*Haploblepharus edwardsii*) showed dermal denticle corrosion following chronic exposure to OA (Dziergwa et al., 2019). Warming alone accelerated the embryogenesis process of small-spotted catshark embryos, increased growth rates and yolk-consumption of embryos and increased Fulton's condition factor of hatchlings (Musa et al., 2020). It also decreased embryonic development time (Pistevos et al., 2015) and altered Port Jackson sharks (*Heterodontus portusjacksoni*) absolute lateralization, as sharks at warmer waters were significantly biased to the right comparatively to the ones reared at control conditions (Vila Pouca et al., 2018). Elevated temperatures increased mortality rate and learning abilities of these tropical sharks (Vila Pouca et al., 2019). A biochronological analysis of Port Jackson shark growth for 15 years showed significant year-to-year deviations in growth negatively correlated with mean SST (Izzo & Gillanders, 2020). Moreover, the combination of both stressors had negative effects on Port Jackson sharks; it increased their energetic demands, while reducing their metabolic efficiency and their ability to detect

food through olfaction, which led to a substantial decrease in growth rates (Pistevos et al., 2015). Elevated temperatures also increased their swimming activity while feeding which increased motivational drive to locate prey, independently of CO_2 levels. However, high CO_2 canceled chemical and visual responses that enable successful predation. Thus, the interactive effects of OA and warming appear to reduce Port Jackson shark's potential to efficiently hunt which in combination with the increased energetic demand, suggest this species will need energetic trade-offs to sustain themselves in a changing ocean (Pistevos et al., 2017). Exposure to OA and warming (OAW) affected bamboo sharks' routine metabolic rates during embryogenesis and after hatching. Both stressors also negatively affected juveniles' Fulton condition, survival (up to 44%) and ventilation rates (Rosa et al., 2014). Regarding their brains, OAW decreased aerobic potential while increased peroxidative damage and cholinergic neurotransmission (Rosa et al., 2016a). Lastly, regarding their digestive enzymes, pancreatic trypsin levels and alkaline phosphatase activity increased under warming and decreased under OA (Rosa et al., 2016b). Hence, the impacts of OA appear to be species-specific, while warming seems to have an overall negative influence on sharks.

1.5.1. Biology and ecology of the studied species

1.5.1.1. Temperate small-spotted catsharks (*Scyliorhinus canicula*)

The small-spotted catshark, or lesser spotted dogfish (*Scyliorhinus canicula*; Linnaeus, 1758; Fig. 13), is an abundant oviparous and small bottom-living species that can be found in the north-eastern Atlantic Ocean, along the coasts of Europe, and in the Mediterranean and Adriatic Seas (Halit & Taşkavak, 2006, Weigmann, 2016).

Figure 13. Small-spotted catshark.

Their eggs are laid in pairs, covered with a protective case and can generally be found attached to macroalgae or other solid surfaces in the subtidal (Ellis & Shackley, 1997). They perform diel vertical migrations to enhance energetic benefits through the exchange of their thermal resources on

expended energy and foraging opportunities (Sims et al., 2005). These catsharks are usually in waters shallower than 450 m (Rodríguez-Cabello et al., 2007), but can be found in deeper waters, at 800 m of depth (Soto & Mincarone, 2004). This species is assessed as least concern in the IUCN red list (Ellis et al., 2009).

1.5.1.2. Tropical whitespotted bamboo shark (*Chiloscyllium plagiosum*)

Figure 14. Whitespotted bamboo shark. From Florida Museum of Natural History, by Doug Perrine.

Whitespotted bamboo shark (*Chiloscyllium plagiosum*, Bennet; Fig. 14) is a relatively small, oviparous, nocturnal and bottom dwelling species (Compagno, 2001), distributed throughout the western and eastern Indian Ocean and the south-western and north-western Pacific Ocean (Weigmann, 2016). This species has a bathymetric range between 0–50 m of depth (Weigmann, 2016). Although in the last decades they were considered as common (Compagno, 2001) these sharks are now assessed as near threatened in the IUCN red list (Kyne & Burgess, 2006).

1.6. General objectives

Sharks are essential elements for the health of marine ecosystems and understanding their vulnerability to a changing ocean is imperative. Hence, the present dissertation aims to unravel the impacts that key climate change-related stressors, namely marine heatwaves (Part I) and ocean acidification (Part II), may have on the ecophysiology of juvenile temperate and tropical sharks. More specifically, in Part I:

Chapter 2: The purpose of this chapter was to investigate the effects of a 15-day long category II marine heatwave (Δ3 °C) on the oxidative stress response of juvenile small-spotted catsharks (*Scyliorhinus canicula*) by quantifying oxidative stress biomarkers in three different tissues (gills, liver and muscle). These biomarkers include superoxide dismutase, catalase, glutathione

peroxidase, heat shock proteins, ubiquitin, and we also quantified oxidative damage, i.e. production of malondialdehyde (MDA) due to lipid peroxidation, and DNA damage.

Chapter 3: The aim of this chapter was to study the impact of a 15-day category II marine heatwave (Δ3 °C) on different physiological parameters of juvenile small-spotted catshark (*Scyliorhinus canicula*). We analyzed erythrocytes, leukocytes, thrombocytes, and erythrocytes with nuclear abnormalities proportions, and calculated erythrocyte nucleus to cytoplasm ratio. To assess potential impacts on the cardiac and hematopoietic systems we calculated the heart and spleen to body ratios. Lastly, we measured their ventilation rates.

And, in Part II:

Chapter 4: The aim of this chapter was to investigate the oxidative stress-related responses of juvenile small-spotted catsharks (*Scyliorhinus canicula*) exposed to ocean acidification. We performed a long-term acclimation to the expected conditions (pCO_2 ~ 900 µatm) for 9 months, and measured changes in three different tissues (gills, liver and muscle) in the primary antioxidant defenses (superoxide dismutase, catalase and glutathione peroxidase), in protein repair mechanisms (through the quantification of heat shock proteins and ubiquitin), and oxidative damage (in lipids and DNA).

Chapter 5: The purpose of this chapter was to investigate the effects of ocean acidification on the hematological parameters of juvenile small-spotted catsharks (*Scyliorhinus canicula*). Following a long-term acclimation of 9 months to increased CO_2 levels (pCO_2 ~ 900 µatm), we determined leukocyte, erythrocyte, and thrombocyte proportions; formation of erythrocyte with nuclear abnormalities and calculated erythrocyte nucleus to cytoplasmic ratio. Additionally, heart and spleen to body ratios were assessed to evaluate potential effects over the cardiac and hematopoietic conditions.

Chapter 6: The aim of the present chapter was to understand how ocean acidification may influence the early stages of a tropical shark, the whitespotted bamboo shark (*Chiloscyllium plagiosum*). After 45 days of exposure to high CO_2 levels (pCO_2 ~ 900 µatm), we assessed sharks growth rates and swimming performance. Moreover, since the cholinergic system has been linked to neuromuscular function (Soreq and Seidman, 2001), shark brains were dissected in seven micro areas: telencephalon, cerebellum, optic lobes, olfactory bulbs, diencephalon, brainstem and spinal cord, to determine acetylcholinesterase's (AChE) activity levels.

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Part One

Marine Heatwaves

2 Effects of a simulated marine heatwave on the oxidative stress response of juvenile temperate catsharks

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Abstract

Marine heatwaves (MHW) are becoming more frequent and intense, as so, understanding how species may be affected becomes imperative. Extreme temperatures can increase reactive oxygen species (ROS) and potentially lead to oxidative damage, which is buffered by antioxidant defense mechanisms. Thus, to unravel the effects of a 15-day MHW (category II, Δ 3 °C) on the oxidative stress response of juvenile small-spotted catsharks (*Scyliorhinus canicula*), we performed several biochemical analyses. We measured enzymatic levels of the antioxidant defense mechanism (superoxide dismutase - SOD, catalase - CAT and glutathione peroxidase - GPx), quantified heat shock proteins (HSP) and ubiquitin (Ub) levels, and lastly evaluated markers of oxidative damage (lipid peroxidation - LPO and DNA damage). According to our results, MHW did not evoke detectable antioxidant and heat shock responses nor led to oxidative damage. These sharks display diel vertical migrations as adults and experience abrupt temperature changes, which may have conferred this species a resistance to sudden thermal fluctuations in their environment, enabling them to tolerate the sudden warming events that are currently occurring. Although oxidative stress is a trending approach used to estimate the metabolic status of an organism, we did not assess other metabolic parameters of these sharks. Future research should, therefore, measure this species' metabolic rates to fully understand the extent of MHW at organism-level.

Keywords: marine heatwave, reactive oxygen species, oxidative stress, elasmobranch, *Scyliorhinus canicula*

Introduction

Climate is markedly changing, triggered by excessive human activities (Feely et al. 2004). Anthropogenic emissions of greenhouse gases are inducing physicochemical changes that disturb the natural equilibrium of entire ecosystems (IPCC 2014; Sampaio & Rosa 2019), as the buildup of carbon dioxide in the atmosphere increases the temperature of the planet's surface (Stocker et al. 2013). In the oceans, this warming led to an increase of approximately 1 °C in average sea surface temperature since the Industrial Revolution (Allen et al. 2018), and if emissions continue to rise may even reach 4 °C by the end of this century (Collins et al. 2013). This warming is also intensifying marine heatwaves (MHW), which are expected to increase in frequency and magnitude (Oliver et al. 2018). Contrarily to global warming that is driven by annual increase of the mean temperature, MHW are discrete events of extreme thermal stress (Pansch et al. 2018) that occur in a specific location (Hobday et al. 2016). Thus, while chronic increase of temperature allows time for adaptation, a sudden warming event exposes an organism to rapid adverse conditions (South & Dick 2017).

Extreme temperatures such as those experienced during MHW can result in considerable changes and impacts on marine life and organism physiology (Randall & Szmant 2009; Hemmer-Brepson et al. 2014; Jeffries et al. 2014). Elevated temperatures can induce the formation of reactive oxygen species (ROS) due to increased respiration rates (Abele et al. 2002; Halliwell & Gutteridge 2015). ROS are mostly produced in the mitochondria (Boveris & Chance 1973; Halliwell 2006) during ATP production. ATP is produced through a series of complex reactions, and a small percentage of the O_2 used in these reactions can be partially reduced to superoxide anion ($O_2^{\bullet}$) (Chance et al. 1979). The superoxide anion is then converted into H_2O_2 by the catalytic action of mitochondrial superoxide dismutase (Mn-SOD), which can eventually produce high amounts of the extremely toxic hydroxyl radical ($HO^{\bullet}$) (Boveris 1977). The denomination of ROS has been commonly applied to these three species (i.e. $O_2^{\bullet}$, H_2O_2 and $HO^{\bullet}$) that result from the partial reduction of O_2 (Wilhelm Filho 2007). When the production of ROS is not buffered, organisms experience oxidative stress (Blier 2014), which can ultimately lead to oxidative damage through lipid peroxidation (LPO), protein

carbonylation or DNA degradation (Abele & Puntarulo 2004; Lesser 2006). The potential for an organism to be protected against oxidative damage can be measured by the activity of antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), which in simple redox reactions are able to detoxify ROS (Scandalios 2005). For instance, SOD converts $O_2^{\bullet}$ into H_2O_2, that in turn is oxidized to water by CAT and GPx. In this way, different ROS are buffered to control redox balance inside cells, thus avoiding extreme oxidation in cellular components (Giorgio et al. 2007). Simultaneously, to mitigate heat stress, organisms produce heat shock proteins (HSP) and ubiquitin (Ub), which also quench ROS production and limit oxidative damage to proteins (Lesser 2006).

As ectotherms, small-spotted catsharks can be particularly affected by changes in environmental temperature. Especially early stages which appear to be more vulnerable to higher temperatures (Thomason et al. 1996; South & Dick 2017). Here, we investigated the effects of a 15-day long category II MHW (Δ3 °C) on the oxidative stress response of juvenile small-spotted catsharks (*Scyliorhinus canicula*) by quantifying oxidative stress biomarkers in three different tissues (i.e gills, liver and muscle). These biomarkers include superoxide dismutase (SOD), catalase (CAT), glutathione-S.transferase (GPx), heat shock proteins (HSP), ubiquitin (Ub), and we also quantified oxidative damage, i.e. production of malondialdehyde (MDA) due to lipid peroxidation (LPO), and DNA damage.

Materials and methods

Ethics statement

The Experiments followed the requirements of the European Parliament (Directive 2010/63/EU) and the Council of 22 September 2010 concerning animal safety for scientific research. The experimental procedures were revised and approved by Animal Welfare Body of FCUL (Statement 5/2016), animal ethics committee (ORBEA), and National Veterinary Medicines Directorate (DGAV).

Animal collection and acclimation

Juvenile sharks (n = 24) were acquired from a public aquarium (Aquário Vasco da Gama, Algés, Portugal). Upon arrival to the facilities of Laboratório Marítimo da Guia they were placed in 600L tanks with semi-open systems, and a baseline of 18 °C was used (i.e. before MHW). After an acclimation period of 3 months, sharks were divided in two conditions for 15 days: control (18 °C; n = 12) and a simulated category II MHW (21 °C; n = 12; more information in Table 1).

Table 1. Seawater parameter values (temperature, pH and salinity).

	Control	Marine Heatwave
Temperature (° C)	18.1 ± 0.4	21.2 ± 0.3
pH	8.05 ± 0.04	8.08 ± 0.04
Salinity	35 ± 0.7	35 ± 0.7

To simulate a MHW, water temperature was gradually raised for six consecutive days. New seawater was constantly dripping to the tanks, previously filtered (1 µm; Harmsco, USA) and UV-sterilized (Vecton 300, TMC Iberia, Portugal). Temperature was maintained and automatically adjusted by chillers (Hailea chillers, China) and thermostats (V^2 Therm 100, TMC Iberia, Portugal). Temperature (WTW, Multi 3510 IDS SET4, Germany), pH (VWR pHenomenal, Germany) and salinity (V2 TMC, Iberia, Portugal) were monitored daily, while ammonia, nitrites and nitrates (Tropic Marin, Germany) were registered twice a week. Water quality was improved with protein skimmers (Schuran, Jülich, Germany) and a biological filter (Ouriço® bioballs,

Fernando Ribeiro, Portugal) previously matured with nitrifying bacteria. Throughout the experiment sharks were fed *ad libitum*, with squid (Sims 1994) and a photoperiod of 12:12h was maintained.

Experimental design

Data collection of the last 30 years of sea surface temperatures (SST) in Cascais (Portugal) was obtained from NOAA (Daily Optimum Interpolation SST version 2; (Banzon et al. 2016)). The heatwaveR (R package) was used to determine the average duration (14 days) and a peak of temperature registered in the zone (21 °C; that corresponds to a category II MHW when the climatology is at control temperatures ~18 °C). This package applies the MHWs definition by Hobday (2016).

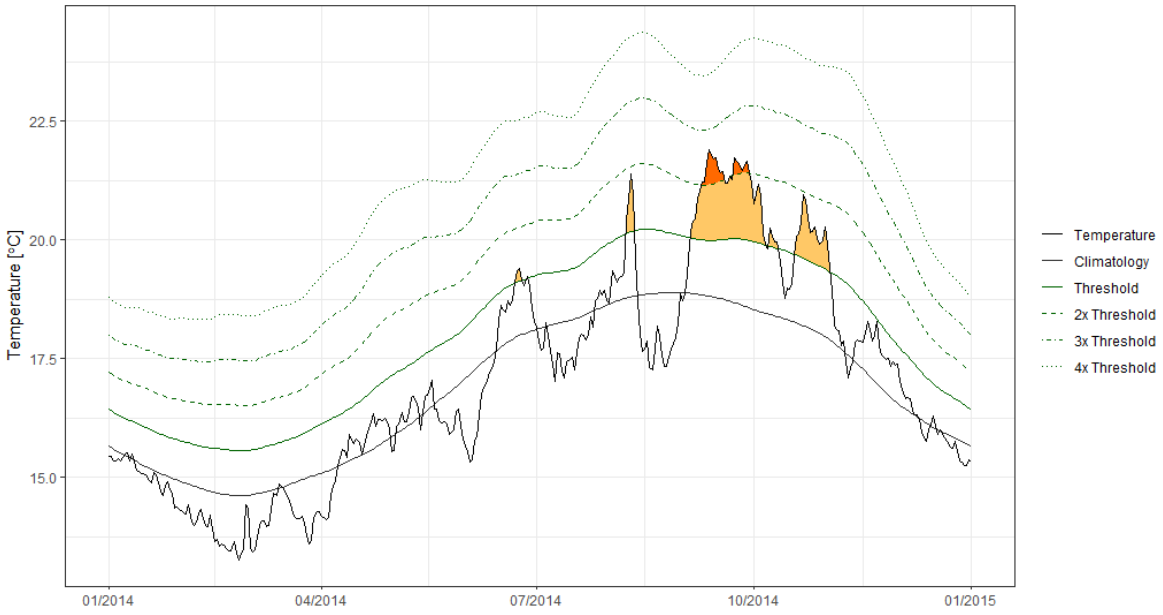

Figure 1. Reconstruction of a category II MHW from a dataset of the last 30 years of the local sea surface temperatures (Cascais, Portugal). Temperatures above the threshold (given by the 90th percentile regarding the long-term climatology) are represented as yellow and orange respectively as category I and II, according to Hobday (2018).

Biochemical analyses

1. Preparation of tissue extracts

Each shark was euthanized with an overdose of tricaine solution (MS222, buffered with sodium bicarbonate at 1:1 ratio). Tissue samples (n = 12 per treatment) of gills, liver and muscle were homogenized in 1.0 mL of phosphate-buffered saline solution (PBS: NaCl, KH_2PO_4, Na_2HPO_4 and miliQ H_2O; pH 7.4) and grinded using an Ultra-Turrax (Staufen, Germany). After, homogenates were centrifuged (14,000×g for 20 min at 4 °C), the supernatant fraction (~1.0 mL) was transferred to a new Eppendorf and stored until further analyses at - 80 °C. Each sample was run in duplicate to quantify the total protein content by following Bradford assay (1976). Total protein contents were used to normalize the biomarkers results.

2. Antioxidant enzyme activities

2.1. Superoxide dismutase activity (SOD)

Superoxide dismutase (SOD) as percentage of inhibition was quantified following McCord and Fridovich (1969), after optimizing the method for 96-well microplates. Briefly, 200 µL of buffer solution (50 mM K_3PO_4, Sigma-Aldrich, Germany; pH ~8.0) was placed in each well of a 96-well microplate (Greiner Bio-one, Austria). After which, 10 µL of each was added: EDTA (3 mM; Riedel-de Haën, Germany), NBT (0.75 mM; Sigma-Aldrich, Germany), xanthine (3 mM; Sigma, Germany) and then the sample. Following, 10 µL of xanthine oxidase (100 mU; XOD, Sigma-Aldrich, Germany) was added to start the inhibition reaction. Lastly, the absorbance was read with a microplate reader (Biotek Synergy HTX multi-mode reader, USA) at 560 nm every minute during the first 5 minutes, and then at 10 and 15 minutes. SOD activity was normalized with total protein content and determined as percentage of inhibition (% inhibition/min/mg protein).

2.2. Catalase activity (CAT)

Catalase (CAT) activity was measured after adapting a method from Johansson and Borg (1988) to 96-well microplate. Initially, 100 µL of potassium phosphate (100 mM K_3PO_4; Sigma-Aldrich, Germany; pH 7.0) was

added to each well. Then, 30 µL of methanol (CH_3OH) and 20 µL of each sample or standard were placed in each well of a 96-well microplate. To initiate the reaction 20 µL of hydrogen peroxide (0.035 M, Sigma-Aldrich, Germany) was added to the 96-well microplate and incubated at room temperature for 20 min. Afterwards, 30 µL of Purpald (34.2 mM in 0.5 M HCl, Aldrich, Germany) and 30 µL of potassium hydroxide (10 M KOH, Chem-Lab, Belgium) were added to each well. Afterwards the microplate was covered and incubated at room temperature in a shaker for 10 min. Following the incubation period, 10 µL of potassium periodate (65.2 mM in 0.5 M KOH; Chem-lab, Belgium) was added to each well and the 96-well microplate was shaken again for 5 min. To determine the concentrations of formaldehyde in the samples, a standard curve was built ranging from 0 to 75 µM of formaldehyde (Sigma, Germany). The enzymatic activity was read at 540 nm using a microplate reader (Biotek Synergy HTX multi-mode reader, USA). To calculate CAT activity, the amount of enzyme that would produce 1.0 nmol of formaldehyde for each minute at 25 °C was considered as one unit of CAT, and the results were expressed in total protein content (nmol/min/mg protein).

2.3. Glutathione peroxidase activity (GPx)

The activity of glutathione peroxidase (GPx) was quantified following a method adapted from Lawrence and Burk (1976) method. First, 140 µL of assay buffer was added to two wells for negative control and the remaining wells were filled with 120 µL of assay buffer and 20 µL of each sample. The assay buffer is composed of EDTA (5 mM, Riedel-de Haën, Germany; pH 7.6) and potassium phosphate buffer (50 mM, Sigma-Aldrich, Germany; pH 7.4). Subsequently, 50 µL of co-substrate mixture [nicotinamide adenine dinucleotide phosphate (1 mM, NADPH, Sigma-Aldrich, Germany), sodium azide (4 mM, Sigma-Aldrich, Germany), reduced glutathione (4 mM, GSH, Sigma, Germany) and glutathione reductase (4 U/mL, GSSG-reductase, Sigma, Germany)] was added to each well. Finally, 20 µL of hydroperoxide cumene (15 mM, Sigma-Aldrich, Germany) were added to the 96-well microplate, and the absorbance was read for 6 min at

340 nm in a microplate reader (Biotek Synergy HTX multi-mode reader, USA). GPx activity was quantified using a coefficient of extinction of 3.73 mM^{-1} cm^{-1}. The results were expressed as nmol/min/mg protein.

3. Protein repair and removal mechanisms

3.1. Heat shock response

Content of Heat Shock Protein (HSP_{70}) was measured by Enzyme-linked Immunosorbent Assay (ELISA) by adapting a method from Njemini et al. (2005), also described by Maulvault et al (2019). Firstly, 50 µL of diluted sample (1:100) was added to each well of a 96-well microplate (Greiner Bio-one Microlon®600, Austria) and incubated at 4 °C overnight. Following 24 h, the microplates were washed three times with 0.05% PBS-Tween-20 solution (Sigma-Aldrich, Germany). Afterwards, 100 µL of blocking solution (1% bovine serum albumin (BSA, Sigma-Aldrich, USA) in PBS) was added to the 96-well microplate which was incubated (Labnet, USA) for 90 min at 37 °C. Each microplate was washed in the same manner and then 50 µL of a primary antibody solution (1 µg/mL of anti-HSP_{70}/HSC_{70} in 1% BSA solution; OriGene, USA) was added to each well of the microplate followed by an incubation at 4 °C overnight. After the incubation period, the microplates were once more washed to remove non-linked antibodies, three times. Then, 50 µL of a second antibody (anti-mouse IgG Fc alkaline phosphatase conjugated, Sigma-Aldrich, Germany; diluted to 1 µg/mL in 1% BSA solution) was added to each well and incubated at 37 °C for 90 min. Following another washing procedure, 100 µL of alkaline-phosphatase substrate [$MgCl_2$ (50 mM, Sigma-Aldrich, Germany), PnPP (27 mM; 4-nitrophenyl phosphate disodium salt hexahydrate (Sigma, Germany); pH 8.5), Tris-HCl (100 mM; Sigma-Aldrich) and NaCl (100 mM; Panreac, Spain)] was added to each well and the microplate incubated at room temperature for 30 min. Lastly, each well was filled with 50 µL of a stop solution (3 M NaOH; Panreac, Spain) and the absorbance was read at 405 nm in a microplate reader (Biotek Synergy HTX multi-mode reader, USA). The content of HSP_{70}/HSC_{70} in the samples was calculated using a standard curve of purified active HSP_{70} protein (OriGene Technology, USA), through serial dilutions to obtain a range from 0 to 2 µg/mL. The results are expressed as µg/mg of total protein.

3.2. Ubiquitin Content (Ub)

The content of ubiquitin (Ub) was measured by indirect ELISA as previously described by Lopes et al. (2018). Briefy, 10 µL of sample was added to 40 µL PBS in a 96-well microplate (Greiner Bio-one Microlon®600, Austria) and incubated at 4 °C overnight. After 24 h, each microplate was washed with PBS-Tween-20 solution (0.05%, Sigma-Aldrich, Germany) three times. Then 100 µL of blocking solution consisting of bovine serum albumin (1%) in PBS (Phosphate buffer saline solution) (Sigma-Aldrich, USA) was added and incubated (Labnet, USA) for 90 min at 37 °C. Next, the same washing procedure was done as described before, followed by adding 50 µL of a primary antibody solution (Ub-.P4D1, Sc-8017, mouse monoclonal antibody, Santa Cruz Biotechnology, USA); diluted to 1 µg/mL in 1% BSA in PBS) added to the microplates wells and incubated at 4 °C overnight. Afterwards, the microplates were washed again to remove non-linked antibodies. Then, 50 µL of a secondary antibody (antimouse IgG, fc-specific alkaline phosphatase conjugate, Sigma-Aldrich, Germany) was added to the 96-well microplates and incubated for 90 min at 37 °C. After the incubation period, the microplates were washed with PBS-Tween20 (3X) and 100 µL of alkaline-phosphatase substrate [Tris-HCl (100 mM, Sigma-Aldrich), NaCl (100 mM, Panreac, Spain), PnPP (27 mM, 4-nitrophenyl phosphate disodium salt hexahydrate, Sigma, Germany) and $MgCl_2$ (50 mM ,Sigma-Aldrich, Germany); pH 8.5] was added to the microplates and incubated for 30 min at room temperature. Finally, 50 µL of stop solution (3 M NaOH, Panreac, Spain) was added to the microplate wells and the absorbance was read at 450 nm in a microplate reader (Biotek Synergy HTX multi-mode reader, USA). The content of total Ubiquitin was quantified using a standard curve, which was built using sequential dilutions of a purified active ubiquitin protein (Santa Cruz Biotechnology, USA), ranging from 0.8 to 0.0125 µg/µL. The results were given as µg/mg total protein, in relation to total protein content of the samples.

4. Lipid peroxidation and DNA damage

To determine lipid peroxidation (LPO), production of malondialdehyde (MDA) was quantified based on thiobarbituric acid reactive substance (TBARS) assay (Uchiyama and Mihara 1978). First, 5 µL of standard or

sample was added to 1.0 mL microtubes, then 45 µL of monobasic sodium phosphate buffer (50 mM, Sigma-Aldrich, Germany), 12.5 µL of sodium dodecyl sulfate (8.1% SDS, Merck, Germany), 93.5 µL of thio barbituric acid (TBA 1%, Sigma-Aldrich, Germany), 93.5 µL of trichloroacetic acid (20% TCA, Panreac, Spain; pH 3.5), and 50.5 µL of water (Milli-Q ultrapure). Subsequently, the mixtures were centrifuged (2,000×g) and incubated in boiling water (100 °C) for 10 min. After that, microtubes were placed on ice to lower the temperature for a few minutes, 62.5 µL of water (Milli-Q ultrapure) was added and the mixture was again centrifuged (2,000×g) for 1 min. 150 µL of duplicate supernatant fraction of the sample was added to 96-well microplate (Greiner Bio-one, Austria), and lastly the absorbance was read in a microplate reader (Biotek Synergy HTX multi-mode reader, USA) at 530 nm. The concentration of MDA was measured using a calibration curve ranging from 0 to 0.1 µM TBARS, using MDA as a standard (Sigma-Aldrich, Germany). The results are presented as nmol/mg of total protein.

The DNA damage, such as strand break or DNA base mismatch, was determined through the measure of 8-hydroxy-2′-deoxyguanosine (8-OHdG). 8-OHdG was quantified by an ELISA method, according to Shen et al. (2007). First, 100 µL of each sample was added into a 96-well microplate and incubated at 4 °C overnight. Then, microplates were washed with PBS-Tween 20 (0.05%) three times and incubated for 90 min at room temperature after adding 200 µL of blocking solution (BSA). After new washing procedure, microplates were incubated again at 4 °C overnight after adding a primary antibody (anti-8-OHdG, Sigma-Aldrich, Germany) to each well of the 96-well microplate. After the incubation period, the microplates were washed again with PBS-Tween 20 (0.05%) three times to remove non-linked antibodies and incubated for 90 min at 37 °C with a secondary antibody (anti-mouse IgG fc-specific, alkaline phosphatase-conjugated, Sigma-Aldrich, USA). After a final washing procedure (3X), substrate (SIGMA FAST™ p-Nitrophenyl Phosphate Tablets, Sigma-Aldrich, USA) was added to the microplates which were then incubated for 30 min at room temperature. Finally, 100 µL of NaOH (3 M) were added to stop the reaction and the absorbance was read (Biotek Synergy HTX multi-mode reader) at 405 nm. Given the absence of 8-OHdG standard, results are presented as absorbance standardized with total protein (mg).

Statistical analyses

To perform the statistical analyses, generalized linear mixed-effects models (GLMM) with either Gaussian distribution (identity link function) or Gamma (log link function) were used to infer statistical differences between the tissues analyzed and between MHW treatments. The tissues (gills, liver and muscle) were first used as explanatory variables for each one of the dependent variables (antioxidant machinery: SOD, CAT, GPx; protein repair and removal: HSP and Ub; oxidative damage: LPO and DNA). Models included tissues and treatments as fixed effects and replicates as random effects, to account for eventual non independence between observations of the same treatment. Subsequently, in each tissue, treatments (control and MHW) were used as explanatory variables to identify statistical differences between treatments. Models included the treatments as fixed effects and replicates as random effects. All residuals were verified for possible influential observations and departures from the models' assumptions (homoscedasticity and distribution). No outliers or significant deviations from the models' assumptions were found. Statistical analysis was performed in R, and a *p*-value inferior to 0.05 was viewed as statistically significant.

Results

Concerning the primary antioxidant defense, SOD activity was significantly higher in the muscle than the liver ($p < 0.001$, Table S1; Fig. 2A). The activity of CAT was significantly different between all tissues ($p < 0.001$, Table S1; Fig. 2B), presenting higher levels in the liver, than the gills and the muscle. GPx activity was significantly higher in the liver than the remaining tissues ($p < 0.001$, Table S1; Fig. 2C). In each tissue, SOD, CAT and GPx activities were not significantly different between treatments ($p > 0.05$, Table S1; Fig. 2).

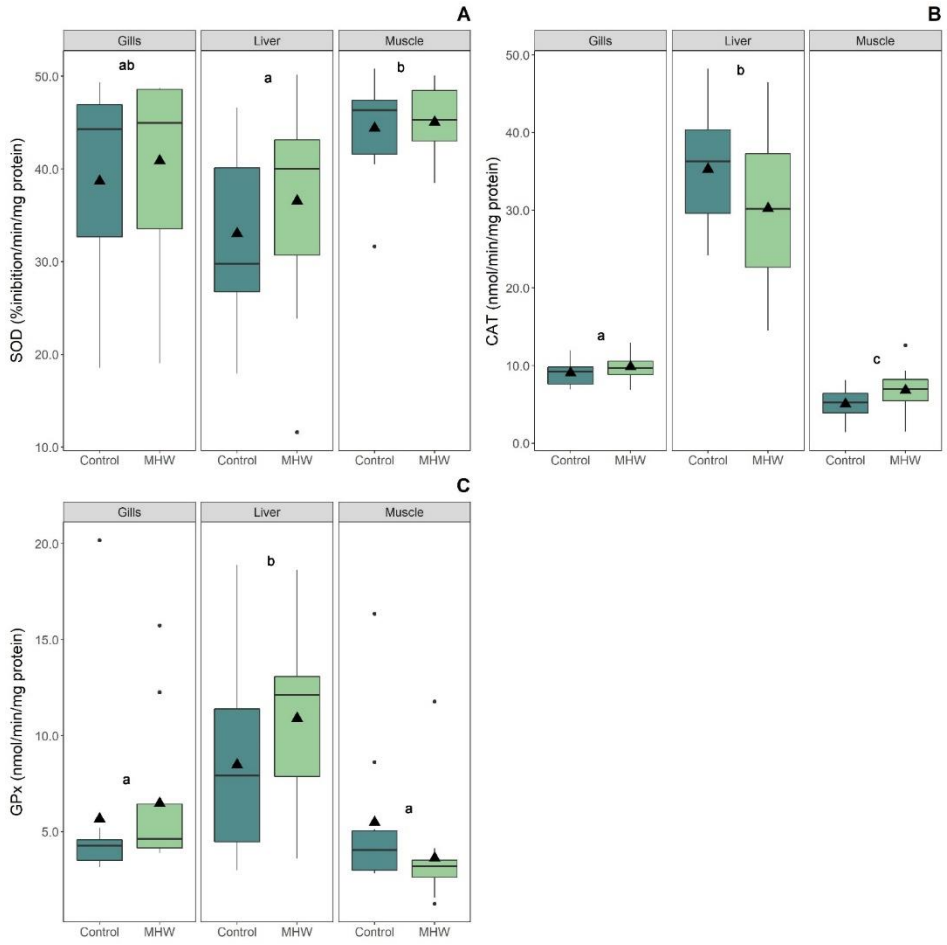

Figure 2. Effects of a Category II MHW (Δ +3 °C) on the enzymatic antioxidant defense: A) SOD (superoxide dismutase), B) CAT (catalase) and C) GPx (glutathione peroxidase) in three different tissues (gills, liver and muscle) of small-spotted catsharks (*Scyliorhinus canicula*, n = 12 per treatment). Middle horizontal lines represent the median, boundary lines represent 25th and 75th percentiles and triangles represent the mean value. Lower-case letters indicate statistical differences between tissues. More statistical information is present in Supplemental Table S1.

Regarding protein repair and removal enzymes, both HSP and Ub levels were significantly higher in the muscle than the gills (respectively, $p < 0.001$, Table S1; Fig. 3A, and $p < 0.05$, Table S1, Fig. 3B) and HSP levels in the muscle were also higher than the liver ($p < 0.001$, Table S1; Fig. 3A). While in each tissue, both HSP and total Ub levels were not significantly different between treatments ($p > 0.05$, Table S1; Fig. 3).

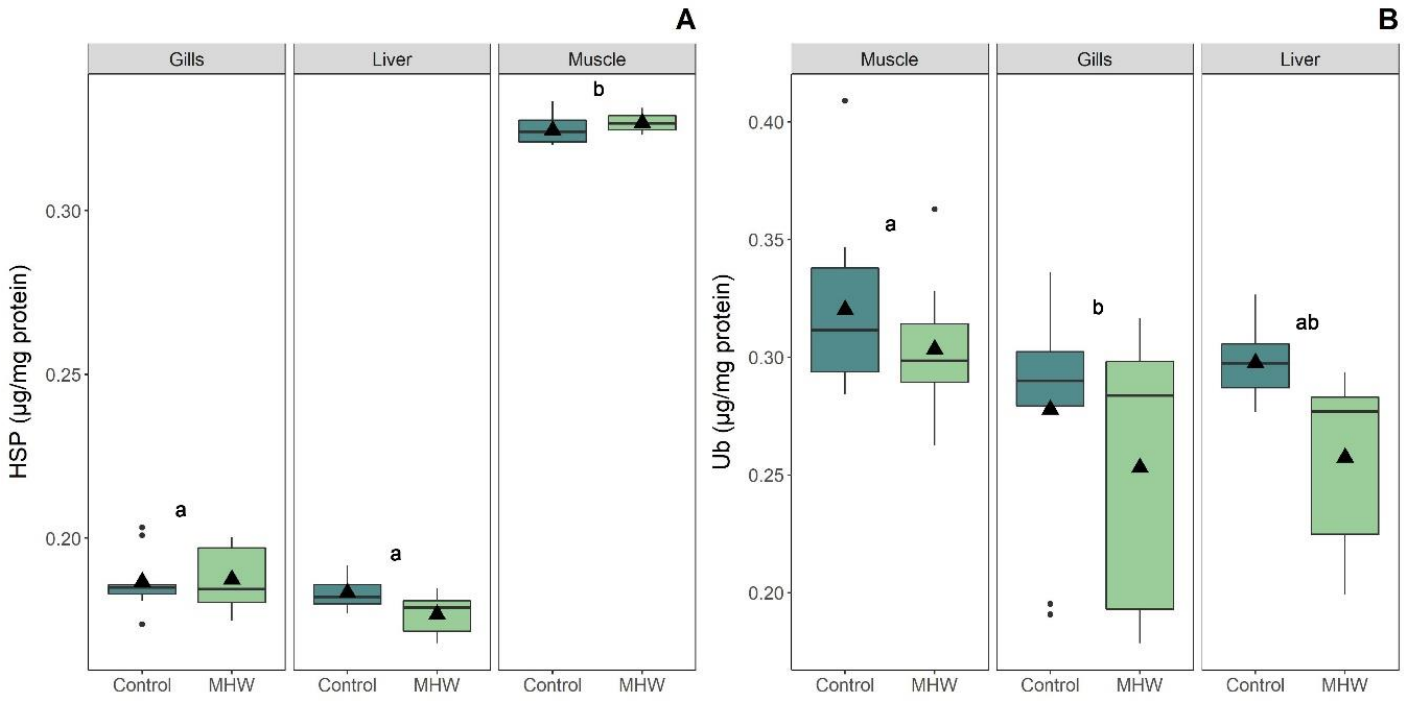

Figure 3. Effects of a Category II MHW (Δ +3 °C) on protein repair and removal: A) HSP (heat shock protein), B) Ub (ubiquitin) levels, in three different tissues (gills, liver and muscle) of small-spotted catsharks (*Scyliorhinus canicula*, n = 12 per treatment). Middle horizontal lines represent the median, boundary lines represent 25th and 75th percentiles and triangles represent the mean value. Lower-case letters indicate statistical differences between tissues. More statistical information is present in Supplemental Table S1.

Lastly, both LPO and DNA damage were significantly higher in the liver compared to the gills (respectively, $p < 0.05$, Table S1; Fig. 4A and $p < 0.001$, Table S1; Fig. 4B) and DNA damage was also significantly higher in the muscle and liver than the gills ($p < 0.001$, Table S1; Fig. 4B) with no further significant differences among other tissues or between treatments ($p > 0.05$, Table S1; Fig. 4). In short, a marine heatwave scenario did not elicit any significant changes in the antioxidative response of small-spotted catsharks (*Scyliorhinus canicula*).

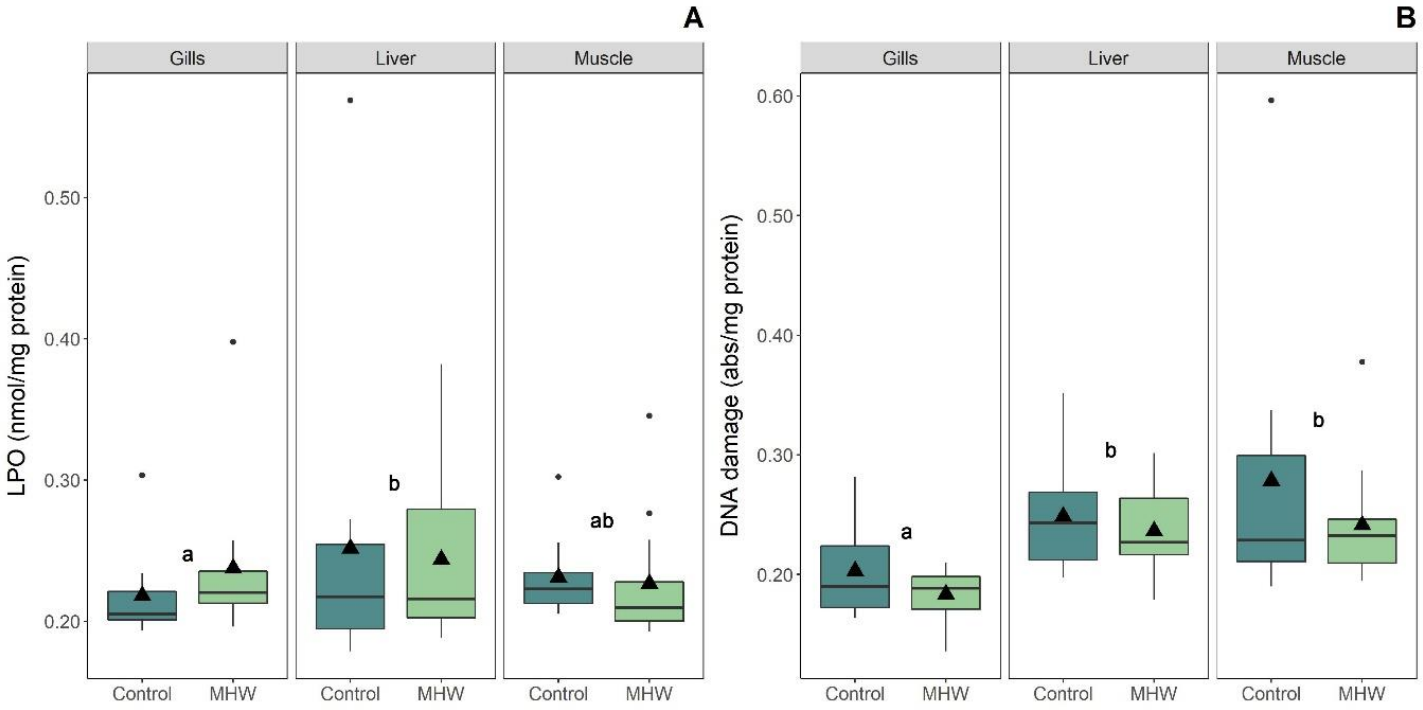

Figure 4. Effects of a Category II MHW (Δ +3 °C) on oxidative damage: A) LPO (lipid peroxidation), B) DNA (DNA damage) in three different tissues (gills, liver and muscle) of small-spotted catsharks (*Scyliorhinus canicula*, n = 12 per treatment). Middle horizontal lines represent the median, boundary lines represent 25th and 75th percentiles and triangles represent the mean value. Lower-case letters indicate statistical differences between tissues. More statistical information is present in Supplemental Table S1.

Discussion

In the present work, a realistic category II MHW (Δ3 °C, T = 21 °C) did not elicit detectable antioxidant and heat shock defenses, nor led to oxidative damage in small-spotted catsharks. As adults, in the North Atlantic, they perform diel vertical migrations between deeper cold waters and hotter shallow waters, crossing thermoclines in which temperatures can fluctuate around 1 to 8 °C (Sims et al. 2006). Therefore, it is possible that these diel changes between temperatures may have conferred these species an increased resistance to abrupt thermal fluctuations in their environment, enabling them to endure the sudden warming events that are occurring, without significant disruption of physiological homeostasis. While exposure to acute thermal stress can immediately promote a cellular response, such as HSP synthesis, to prevent irreversible protein aggregation caused by warming (Feder & Hofmann 1999), heat shock response can differ between organisms based on their thermal history (Tomanek 2008), which may explain why HSP levels remained stable during the experimental MHW.

Exposure to MHW has been found to alter this species' blood cell counts (including a decrease of erythrocyte's nucleus to cytoplasmic ratio) and increased ventilation rates (Pegado et al., 2020; see next Chapter). Generally, increased ventilation rates intensify oxygen uptake which can promote additional ROS formation and increase the potential for oxidative damage (Abele et al. 2002; Halliwell & Gutteridge 2015). Since we did not measure ROS formation directly, the lack of both antioxidant response and oxidative damage, can mean: i) either ROS formation was not intensified, or ii) that it was reduced via alternative mechanisms. For instance, the reduction in the nucleus to cytoplasmic ratio can promote faster oxygen exchange (Lay & Baldwin 1999) which could have avoided excessive production of ROS in the first place. However, if additional ROS production is occurring, the lack of enzymatic antioxidant response, such as SOD, CAT, and GPx increase, may suggest that non-enzymatic antioxidant mechanisms could be acting as primary line of defense. These non-enzymatic antioxidants include urea, ascorbic acid and α-tocopherol, which play an important role in cartilaginous fish's protection against oxidative stress (Rudneva 1997). Bony fish are also equipped with a non-enzymatic antioxidant defense, that differs from elasmobranchs as it does not

include urea (Guerriero et al. 2002), and when exposed to elevated temperatures they generally reveal oxidative stress and damage (Madeira et al. 2013, 2016; Madeira, Vinagre, and Diniz 2016; Sampaio et al. 2018; Pimentel et al. 2015). It has been argued that sharks may have more efficient antioxidant mechanisms (Buffenstein et al. 2008) conferring them a possible evolutionary advantage to cope with oxidative stress. However, a tropical shark species exposed to warming showed increased lipid damage (LPO) in the muscle and brain (Rosa et al. 2016b). Tropical species inhabit stable environments, with narrower thermal intervals, and may already live closer to their thermal tolerance capacity, which makes them more susceptible to rising temperatures (Beitinger et al. 2000; Stillman 2003). Tropical sharks exposed to elevated temperatures were also impaired in their hunting abilities (Pistevos et al. 2015), digestive capacity (Rosa et al. 2016a) and reduced aerobic potential (Rosa et al. 2016b).

As the deep ocean (700–2000 m) is also warming and these sudden warming events are expected to intensify even at these depths (Cheng et al. 2015), temperate sharks also face thermal challenges. Alongside the warming trend, deoxygenation and acidification are simultaneously occurring in the ocean since they are all caused by the same process (Gruber 2011). Considering this, further studies should evaluate the effects of these multi-stressors over the oxidative stress response of these temperate sharks, to better understand the limitations that this species may endure.

Although oxidative stress is a trending approach used to estimate the metabolic status of an organism (Blier 2014) we did not assess other metabolic parameters of these sharks. It would be therefore interesting to measure this species' metabolic rates to fully understand the extent of MHW effects at organism-level. Providing a holistic overview of the potential of shark physiology to overcome future abiotic challenges is key to understand how these charismatic animals will fare in the oceans of tomorrow and to help decision-makers to effectively incorporate the necessary measures in the oceans of today.

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3 Impact of a simulated marine heatwave in the hematological profile of a temperate shark (*Scyliorhinus canicula*)

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Abstract

As Earth's temperature continues to rise, sudden warming events, designated as marine heatwaves (MHWs), are becoming more frequent and longer. This phenomenon is already shown to significantly impact marine ecosystems and respective fauna. While experimental acclimation to higher temperatures is known to affect predatory behavior, metabolism and overall fitness of sharks, the effects of short-term exposure to high temperatures on sharks' physiology has yet to be investigated in a MHW context. Thus, the aim of our work was to study the impact of a category II MHW (Δ3 °C, 15 days) on: i) ventilation rates ii) hematological parameters (erythrocytes, leukocytes, thrombocytes, erythrocyte with nuclear abnormalities (ENAs), and nucleus to cytoplasmic ratio), and iii) heart and spleen to body ratios of juvenile catsharks (*Scyliorhinus canicula*). We found a significant increase of ventilation rates over the course of the MHW. Moreover, exposure to this sudden warming event significantly changed normal blood cell proportions, by increasing leukocytes and thrombocytes while lowering erythrocytes, and decreased nucleus to cytoplasm ratio. However, there were no changes regarding the presence of ENA, as well as spleen and heart to body ratios. Our findings indicate limited capabilities for coping with sudden warming events, suggesting potential disruption in shark physiological homeostasis as the frequency, duration and intensity of MHWs are expected to be strengthened.

Keywords: marine heatwaves, blood, juveniles, elasmobranch, sharks, *Scyliorhinus canicula*.

Introduction

Marine heat waves (MHW) are sudden warming events that occur in the ocean, caused by a conjunction of oceanographic and atmospheric processes (Hobday et al. 2016). In particular, small and large-scale atmospheric forcing, oceanic forcing, or the combination of both can contribute to extreme temperatures at the surface of the ocean, causing a MHW (Frölicher & Laufkötter 2018). Since the last century, MHWs frequency and duration has increased by 54% (Oliver et al. 2018) as consequence of rising greenhouse gases emissions (Meehl & Tebaldi 2004). Concurrently, these warming events are expected to continue increasing in the future, both in intensity and in frequency (Frölicher et al. 2018; Oliver et al. 2019). MHWs have already cause major impacts in key ecosystems, e.g. the Great Barrier Reef, where coral assemblages suffered massive mortality over 2300km (Hughes et al. 2017), which may impact reef fish diversity and abundance (Munday et al. 2008). In fact, starting from 2013, in the northeastern Pacific, the longest MHW ever recorded (known as "the Blob") lasted for an unprecedented 3 years (Cavole et al. 2016; Di Lorenzo & Mantua 2016). As a consequence, MHWs are reshaping marine communities, through changes in the distribution of keystone species (Pinsky et al. 2013; Poloczanska et al. 2013; Frölicher et al. 2018), the loss of kelp forests (Wernberg et al. 2016), severe coral senescence due to bleaching (Hughes et al. 2017), and mass mortality of several marine animals due to heat stress (Garrabou et al. 2009; Oliver et al. 2017; Smale et al. 2019).

Animals exposed to warmer, but sublethal temperatures, face thermal stress (Mariana & Badr 2019) and a decline in oxygen availability (Schmidtko et al. 2017) which results in constraints on hematological and cardio-respiratory systems (Mariana & Badr 2019). Moreover, as temperature rises, heme-binding proteins can be down-regulated, compromising oxygen transport by red blood cells (Madeira et al. 2016) and altering the heart rate (Carlson et al. 2004). A basic parameter to determine functional efficiency of an animal's cardiac condition is the relationship between heart and body weight (Ostadal & Dhalla 2012). Elevated temperatures increase cardiac output in fish (Farrell 2009), allied to a decrease in cardiac mass (Gamperl & Farrell 2004). Contrarily, at colder waters, fish can enlarge their hearts to maintain cardiac output (Driedzic

1996; Gamperl & Farrell 2004), even during a short-term exposure of 4 weeks (Graham & Farrell 1989). Energy allocation that occurs to sustain the metabolism during thermal stress (Pörtner & Knust 2007) may compromise the immune system while simultaneously there is an increase in bacterial pathogenicity (Vezzulli et al. 2010). These infections can be responsible for changes in the biometric characteristics of fish (Tavares-Dias et al. 2000) such as increased spleen size (Hadidi et al. 2009). The spleen plays an important part during a physiological response to stress and in a few short minutes can change in size (Pearson & Stevens 1991). A quantifiable immune parameter is the spleno-somatic index which can be measured as the spleen to body ratio (Lefebvre et al. 2004). Thus, identifying species' thermal window of performance is essential to define their vulnerability to warming (Pörtner 2008).

Top and mesopredator sharks are known to play a key role in ecosystems (Terborgh & Estes 2013; Wallach et al. 2015), with recent studies showing that increased temperatures (not within MWH contexts) can elicit negative effects on sharks. For instance, warming has been shown to significantly change digestive abilities (Rosa et al. 2016), reduce the duration embryonic development (Rosa et al. 2014; Pistevos et al. 2015), and increase ventilation rates and mortality (Rosa et al. 2014). However, to our knowledge, there is no experimental data on the effects of ecologically realistic MHWs on shark physiology, which depend on external temperature to maintain vital processes. Since early stages are expected to be more vulnerable to sudden changes in the environment (Pörtner & Farrell, 2008), the aim of our work was to study, for the first time, the impact of a category II (see MHW category definitions in Hobday et al, 2016) MHW (Δ3 °C, 15 days) on different physiological parameters of juvenile catsharks (*Scyliorhinus canicula*), including: i) hematological parameters (erythrocytes, leukocytes, thrombocytes, erythrocyte with nuclear abnormalities, and erythrocyte's nucleus to cytoplasm ratio), ii) heart and spleen to body ratios and iii) ventilation rates.

Materials and methods

Ethics statement

During this work, all procedures followed the requirements of the European Parliament (Directive 2010/63/EU) and the Council of 22 September 2010 on animal protection used for science. Experimental procedures were also reviewed and approved by the animal ethics committee ORBEA, the Animal Welfare Body of FCUL (Statement 5/2016) and the National Veterinary Medicines Directorate (DGAV).

Animal collection and acclimation

Small-spotted catshark juveniles (n = 24) around 15.8 cm (± 1.7) were brought to our aquaculture facilities from a public aquarium (Aquário Vasco da Gama, Algés, Portugal) in September 2018. They were all placed in one 600L semi-open system and fed *ad libitum*, with fish or squid. After 3 months of acclimation at control temperature conditions (18 °C), sharks were divided in three 600L semi-open systems (replicates) per treatment: control (18 °C; n = 12) and a simulated scenario of a category II MHW (21 °C; n = 12) for 15 days (for more information see Table 1). Sharks were also divided per sex: 7 females and 5 males per treatment.

Table 1. Seawater parameters during the experiment (temperature, pH and salinity).

	Control	MHW
Temperature (° C)	18.1 ± 0.4	21.2 ± 0.3
pH	8.05 ± 0.04	8.08 ± 0.04
Salinity	35 ± 0.7	35 ± 0.7

In the MHW treatment, the water temperature was increased 0.5 °C per day. Water was constantly renewed with a drip-system, which constantly supplied UV-sterilized (Vecton 300, TMC Iberia, Portugal) and filtered (1 µm; Harmsco, USA) seawater. Water temperature was controlled and adjusted automatically with thermostats (V^2 Therm 100, TMC Iberia, Portugal) and chillers (Hailea chillers, China). Seawater parameters such as salinity (V2 TMC, Iberia, Portugal), pH (VWR pHenomenal, Germany) and temperature (WTW, Multi 3510 IDS SET4, Germany) were monitored daily, while ammonia, nitrites and nitrates (Tropic Marin,

Germany) were measured twice a week. Water quality was further ensured with protein skimmers (Schuran, Jülich, Germany) and biological filter (Ouriço® bioballs, Fernando Ribeiro, Portugal) matured with nitrifying bacteria. A photoperiod of 12:12h was kept throughout the experiment.

Experimental design

A 30 years dataset for seawater surface temperature in the region of Cascais (Portugal) was acquired from NOAA (Daily Optimum Interpolation SST version 2; (Banzon et al. 2016)). The R package, heatwaveR, was used to determine the average duration (14 days) and the maximum temperature registered (21 °C; corresponding to a category II MHW when the climatology was ~18 °C). This package applies the MHWs definition by Hobday (2016).

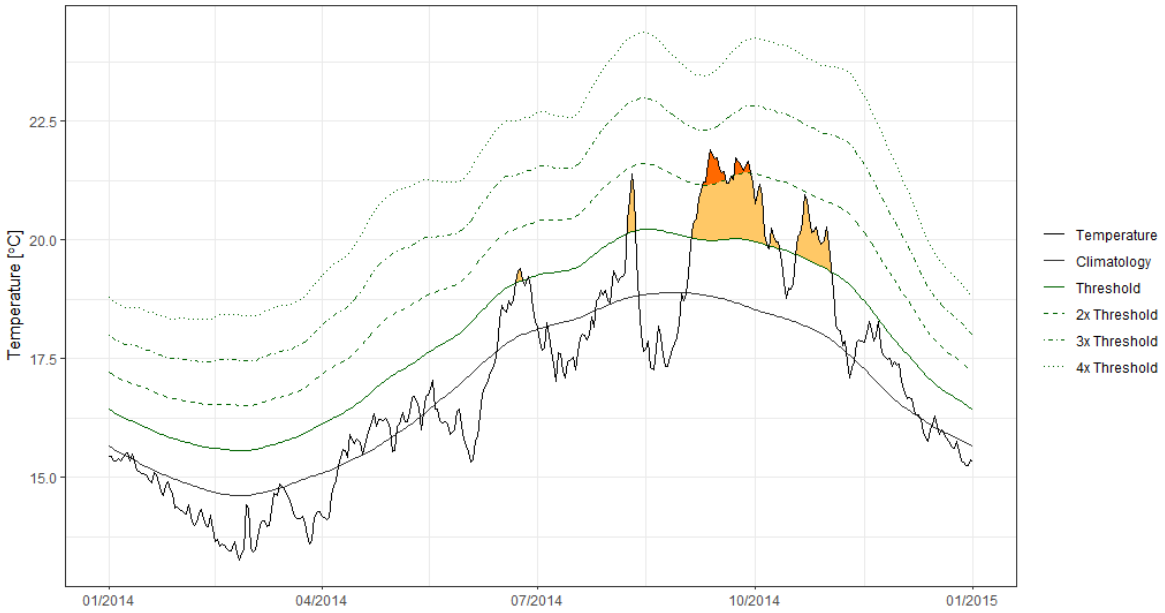

Figure 1. Category II MHW reconstructed from a dataset of local sea surface temperatures for the last 30 years (Cascais, Portugal; acquired from NOAA (Daily Optimum Interpolation SST, version 2)). Temperatures above threshold (90th percentile in relation to the long-term climatology) represented as yellow (Category I) and orange (threshold 2x; Category II) (Hobday et al, 2018).

Ventilation rates

Sharks were individually observed for one minute and the number of breaths (i.e. opening of the gills) was registered. Each observation was repeated 3 times to obtain a mean value of the ventilation rate per shark.

Ventilation rates were measured: i) 9 days before the experiment started; ii) the first day with +3 ºC; and iii) after 15 days of treatment. These observations were made before feeding to exclude any possible bias on the respiration.

Sample preparation and hematological parameters

Each shark was collected from the respective treatment (n = 24) and euthanized with an overdose of MS222 solution (buffered with addition of sodium bicarbonate at 1:1 ratio). Afterwards, sharks were weighted, and blood was collected from the caudal vein using a heparinized syringe. Blood smears of each individual were prepared, by placing a drop of blood in a microscope glass slide. Then blood was spread by capillary action using a second slide as the spreader slide, being subsequently allowed to air-dry and fixed for one minute in methanol. Following 24h, the fixed blood smears were stained with Hemacolor staining reagent (Hemacolor® Rapid, Sigma-Aldrich) and counter-stained with safranin (Sigma-Aldrich). Staining allowed the count of normal blood cells (erythrocytes, leukocytes and thrombocytes) and the identification of erythrocytes nuclear abnormalities (ENAs) through optical microscopy. After staining, the glass slides were mounted with a drop of DPX (BDH, Poole, England) and Xylene (mixture of isomers ≥ 98.5%, AnalaR NORMAPUR® ACS). At least 500 cells per animal was counted under the microscope (40× magnification (pixel 0.14 µm, DFC 320), Leica DM LB2 microscope), classified as normal blood cells according to Arnold (2005) and ENAs according to Carrola et al. (2014) classification. An average of 100 erythrocytes per shark was measured using ImageJ software, to determine the nucleus to cytoplasmic ratio (NCR) according to Doughty (2012), with the following formula: NCR = nucleus area / (cell area – nucleus area). All cell counts and measurements were performed by an investigator blind to the treatment.

After blood collection, sharks were dissected, the heart and spleen were removed and weighed to calculate heart and spleen to body ratios, according to the formula:

$$\mathbf{HBR} = \frac{\text{Heart weight (g)}}{\text{Body weight (g)}} \text{ x } 100 \qquad \mathbf{SBR} = \frac{\text{Spleen weight (g)}}{\text{Body weight (g)}} \text{ x } 100$$

Statistical analyses

Generalized linear mixed-effects models (GLMM) were used to analyse the data. To model normal blood cells (i.e. erythrocytes, leucocytes, and thrombocytes) and ENAs proportions the binomial distributional family (logit link function) was used. To model continuous quantities as HBR and SBR, the Gaussian distribution (identity link function) was used instead, while for NCR we used the Gamma distribution (log link function). These models included the MHW level as a fixed effect and a random effect to account for potential non-independency between observations within the same tank (i.e. replicates). To model the ventilation rates the Gaussian distribution (identity link function) was used, with individual identity as a random effect, time of the measurement and treatment as covariates. All model residuals were examined for departures from the models' assumptions (i.e. residuals distribution and homoscedasticity) and for possible influence from observations. No significant deviations from the models' assumptions or outliers were found. Statistical analyses were performed in R, using *lme4* and *nlme* packages (Pinheiro et al. 2012; Bates et al. 2014).

Results

Under the control conditions, the average hematological composition of sharks comprised of erythrocytes (747 ± 202 cells), leukocytes (39 ± 29 cells) and thrombocytes (6 ± 8 cells, Table 2).

Table 2. Values of the studied parameters: Ery – Erythrocytes, Leu – Leukocytes, Thr – Thrombocytes, Mic – Erythrocytes with micronucleus, NCR – Erythrocyte's nucleus to cytoplasmic ratio, HBR – Heart to body ratio and SBR – Spleen to body ratio.

	Ery	Leu	Thr	Mic	NCR	HBR	SBR
Control	747 ± 202	39 ± 29	6 ± 8	1 ± 2	0.28 ± 0.13	0.11 ± 0.02	0.22 ± 0.08
MWH	642 ± 235	72 ± 45	12 ± 6	1 ± 0	0.20 ± 0.06	0.13 ± 0.03	0.21 ± 0.06

Exposure to a category II MHW significantly altered normal blood cell proportions. More specifically, the proportion of erythrocyte cells was significantly decreased (MHW: 642 ± 235, Table 2; $p < 0.05$, Table S1; Fig. 2A), while the proportion of leukocytes (MHW: 72 ± 45, Table 2; $p < 0.05$, Table S1; Fig. 2B) and thrombocytes (MHW: 12 ± 6, Table 2; $p < 0.05$, Table S1; Fig. 2C) increased. Concerning the presence of ENAs, none of the individuals displayed erythrocytes with segmented and blebbed nucleus, and there was no significant difference between treatments regarding micronucleus (control: 1 ± 2, MHW: 1 ± 0, Table 2; $p > 0.05$, Table S1; Fig. 2D). Nucleus to cytoplasm ratio (NCR) was significantly lower (control: 0.28 ± 0.13, MHW: 0.20 ± 0.06, Table 2; $p < 0.05$, Table S1; Fig. 3A) in the MHW treatment, while the other ratios were not significantly different between treatments; i.e. heart (g/g) (control: 0.11 ± 0.02, MHW: 0.13 ± 0.03, Table 2; $p > 0.05$, Table S1; Fig. 3B) and spleen to body ratio (g/g) (control: 0.22 ± 0.08, MHW: 0.21 ± 0.06, Table 2; $p > 0.05$, Table S1; Fig. 3C). However, ventilation rates significantly increased through time in sharks from the MHW treatment, and between treatments (control (T0): 58 ± 13, MHW (T0): 53 ± 6, control (T1): 59 ± 10, MHW (T1): 67 ± 7, control (T15): 53 ± 7; MHW (T15): 66 ± 11; $p < 0.01$, Table S1; Fig. 4). More specifically, sharks exposed to a MHW significantly increased their ventilations from T0 to T1 ($p < 0.001$,

Table S1; Fig. 4) and T15 ($p < 0.001$, Table S1; Fig. 4), and also increased between treatments, from control T1 to MHW T15 ($p < 0.01$, Table S1; Fig. 4) and from control T15 to MHW T15 ($p < 0.01$, Table S1; Fig. 3).

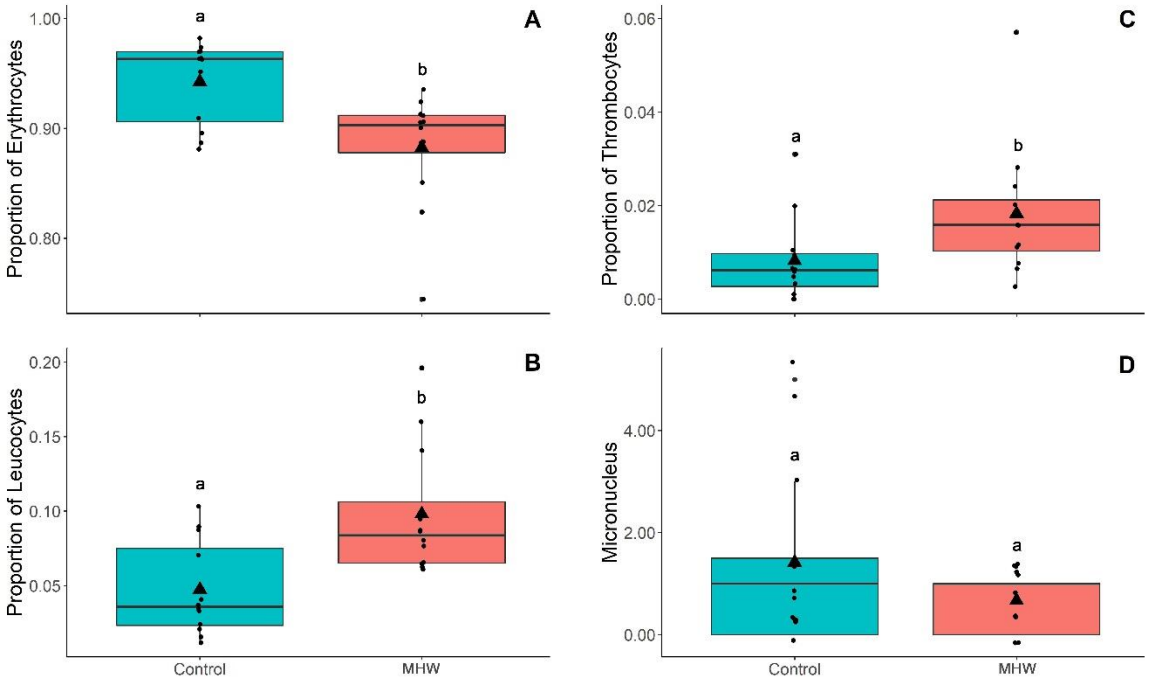

Figure 2. Impact of a simulated category II MHW (+3 °C) on the proportions of: A) erythrocytes, B) leukocytes, C) thrombocytes and D) erythrocytes with micronucleus from blood smears of small-spotted catsharks (*Scyliorhinus canicula*, n = 12 per treatment). Black triangles represent the mean, bold horizontal lines represent the median, boundaries represent 25th and 75th percentiles and whiskers represent the lowest and highest values. Lower case letters indicate significant statistical difference between treatments. Additional statistical information in Supplemental Table S1.

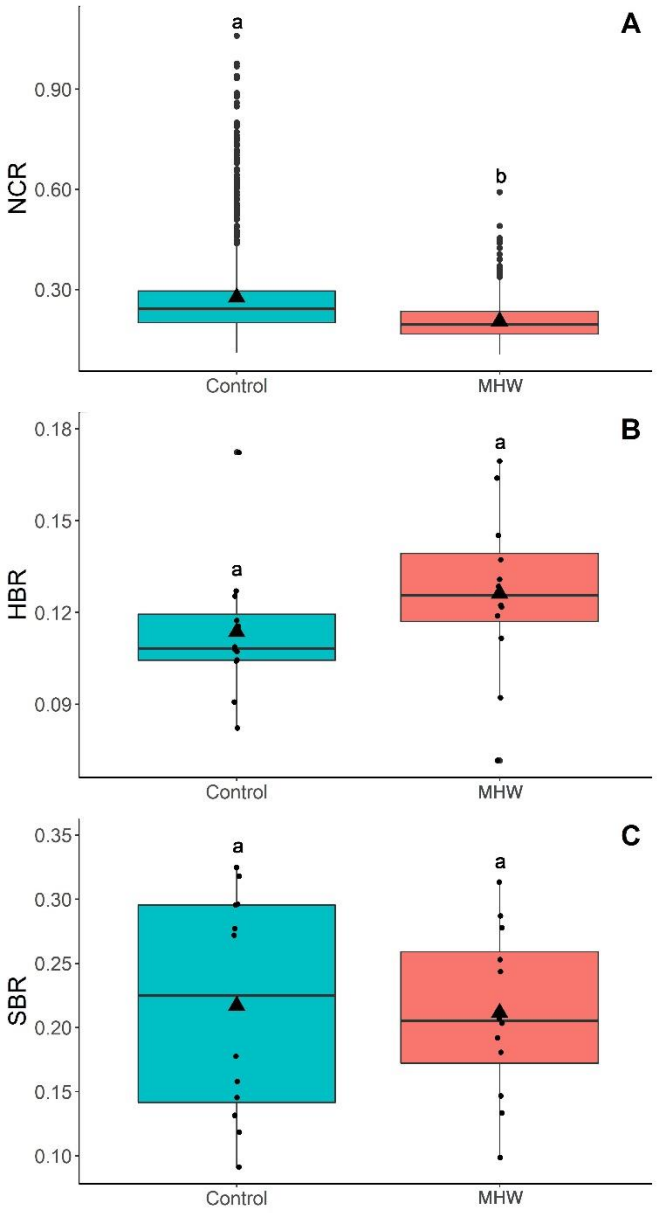

Figure 3. Impact of a simulated category II MHW (+3 °C) on: A) nucleus to cytoplasmic ratio, B) heart to body ratio and C) spleen to body ratio of small-spotted catsharks (*Scyliorhinus canicula*, n = 12 per treatment). Black triangles represent the mean, bold horizontal lines represent the median, boundaries represent 25th and 75th percentiles and whiskers represent the lowest and highest values. Lower case letters indicate significant statistical difference between treatments. Additional statistical information in Supplemental Table S1.

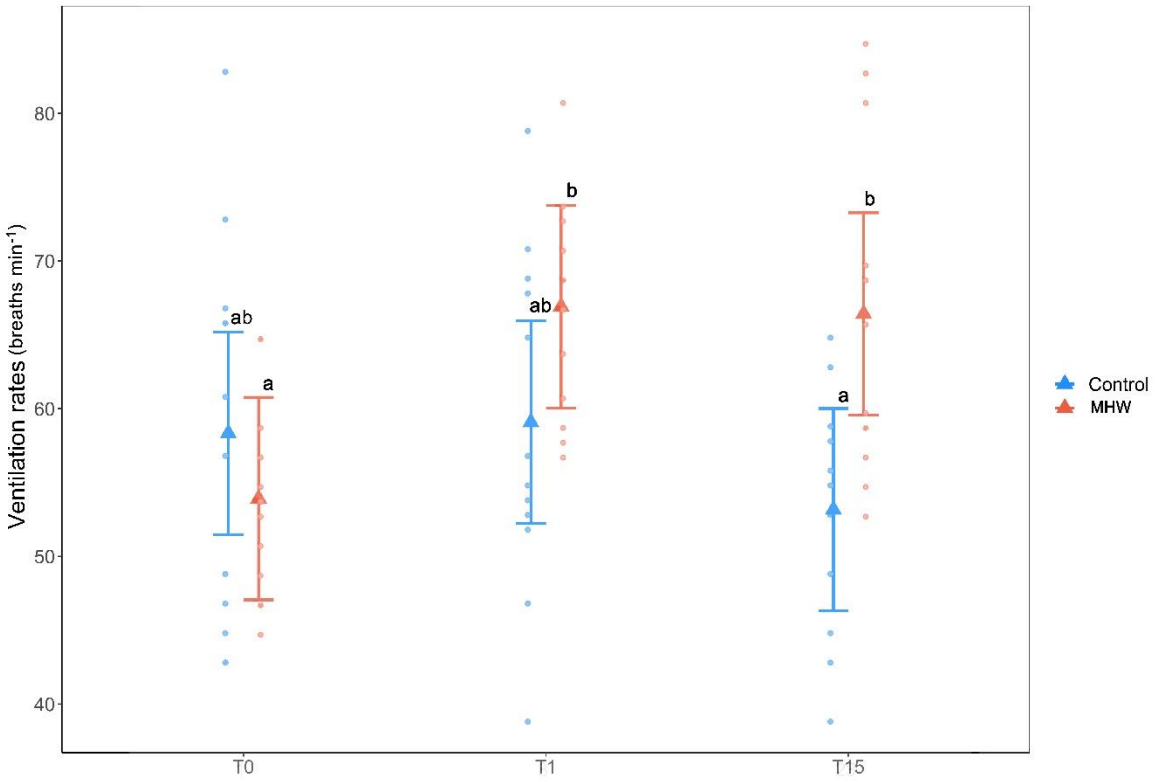

Figure 4. Impact of a simulated category II MHW (+3 °C) on the ventilation rates (breaths per minute) of small-spotted catsharks (*Scyliorhinus canicula*, n = 12 per treatment): T0 – 9 days before the treatment started; T1 - the first day of treatment; and T15 - after 15 days of treatment. Triangles represent the mean and boundaries represent 95% confidence intervals. Lower case letters indicate significant statistical differences. Additional statistical information in Supplemental Table S1.

Discussion

In the present study, we found that a category II MHW has the potential to alter blood cell proportions, increase all blood cell counts (data not shown) and ventilation rates of juvenile small-spotted catsharks. In a warmer environment, animals are simultaneously tackled with lower dissolved oxygen levels and a higher oxygen demand, which leads to an increased effort for animals to obtain proper oxygen supply (Pörtner 2006). Indeed, as a response to this demand, we observed a reduction in nucleus to cytoplasm ratio (NCR) of erythrocytes that indicates the formation of new cells, and consequently leading to an increase of erythrocyte counts. The opposite result was found in a tropical shark species (*Heterodontus francisci*) exposed to a temperature increase of 10 °C (Neale et al. 1977). The authors report that erythrocyte counts decreased while mean corpuscular volume and size increased. In this case, as elevated temperatures can affect the structure of cell membranes (Farkas et al. 2001), a rise of 10 °C may have increased apoptosis, resulting in a lower count. Moreover, to effectively deal with oxygen limitations, catsharks increased ventilation rates through buccal pumping, as it intensifies waterflow through the gills (Houston 1980). This behavior was also observed in the wild, in a survey performed during a MHW along the Western Australian coast, where wobbegong sharks were observed laying on the bottom of the ocean venting their gills more than normal (Pearce et al. 2011).

Changes in environmental temperature can have severe effects over the immune system (Magnadottir 2010). Here, we detected an increase of leukocytes, which represents the first line of defense in an immune response (Opdenakker et al. 1998). Fish exposed to elevated temperatures tend to develop bacterial illnesses (Zaragoza et al. 2008), hence, this increase may indicate the protection against pathogens that are invading the body (Opdenakker et al. 1998). Similarly, there was an increase of thrombocytes. In elasmobranch, these blood cells can engulf latex beads, suggesting that they too play a role in immune responses (Carrier et al. 2012); thus, the observed thrombocyte increase may serve as another protective barrier against pathogens. On the other hand, thrombocytes are also responsible for coagulation, which can be increased by stress (Ruis & Bayne 1997). Thus, alternatively or complementarily, the increase of

thrombocytes could be beneficial to prepare the animal for possible stress-related impairments. It is worth noting however, that long-term exposure to heat has been shown to permanently change clotting time, which may implicate severe hemostatic problems (Tavares-Dias & Oliveira 2009). Furthermore, warming conditions stimulate the respiratory frequency (Miklos et al. 2003), which increases heart rate (Butler & Taylor 1975) and blood pressure, which in turn can be correlated to heart growth. Changes in spleen size could also indicate differences in blood storage since sharks' spleen has several functions, including the production and storage of blood cells, and the removal of damaged or aged blood cells (Fänge & Nilsson 1985). Yet, no significant results were observed regarding SBR and HBR, most probably due to the short time of the MHW exposure.

Depth range of small-spotted catsharks varies from 10 to 780 m, allowing them to avoid warmer temperatures, however, juveniles are generally found in shelves, i.e. above ~ 200 m (Ayas & Çiftçi 2018). Since MHW are intensifying and heat is expected to expand in depth as well, these juvenile sharks may not be able to avoid warmer temperatures. Indeed, recent anomalous temperatures have already reached a depth of 300 m and are expected to expand even deeper to 1000 m (Walsh et al. 2018). Hence, this species may face the tested warming conditions in their natural environment, resulting in changes in their hematological profile. Nonetheless, there is a possibility for recovery to a normal hematological and respiratory state as MHWs are occurring for relatively short periods of time, and to further understand these impacts successive blood collection should test this possibility. On the other hand, MHWs are expected to become longer and more frequent, and prolonged thermal stress may lead to impact exacerbation and override hematological recover. Thus, future research should also analyze the impacts of longer or even repeated sudden warming events on the hematological parameters of sharks. Understanding the impacts that drastic climatic events may impose on marine life is essential to, not only facilitate policymakers' decisions to protect vulnerable species, but also to stimulate further research in this field.

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Part Two

Ocean acidification

4 Lack of oxidative damage on temperate juvenile catsharks after a long-term ocean acidification exposure

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Abstract

Ocean acidification is a consequence of chemical changes driven mainly by a continuous uptake of carbon dioxide, resulting in pH decrease. This phenomenon represents an additional threat to marine life, with expected effects ranging from changes in behavioral responses and calcification rates to the potential promotion of oxidative stress. In order to unravel the impacts of ocean acidification on the antioxidant system of sharks, we performed a long-term exposure (9 months, since early embryogenesis) to high CO_2 conditions (pCO_2 ~ 900 µatm) on a temperate shark (*Scyliorhinus canicula*). The following biomarkers were measured: enzymatic antioxidant defense (superoxide dismutase, catalase and glutathione peroxidase), protein repair and removal (heat shock proteins and ubiquitin), and oxidative damage on lipids (malondialdehyde) and DNA (8-hydroxy-2'-deoxyguanosine). Changes in the antioxidant enzyme defense were restricted to an increase in catalase activity in the muscle, an enzyme that plays a major role in oxidative stress mitigation. On the other hand, no evidence of oxidative damage was found, indicating that the observed increase in catalase activity may be enough to neutralize the effects of potentially higher reactive oxygen species. These results further indicate that these sharks' antioxidant system can successfully cope with the levels of carbon dioxide projected for the end of the century. Nonetheless, the interaction between ocean acidification and the rise in temperature expected to occur in a near future may disturb their antioxidant capacity, requiring further investigation.

Keywords: ocean acidification, oxidative stress, reactive oxygen species, elasmobranch, *Scyliorhinus canicula*, early ontogeny

Introduction

Part of the carbon dioxide (CO_2) released into the atmosphere has been absorbed by our oceans, leading to a phenomenon commonly designated as ocean acidification (OA) (Doney et al. 2009; Gattuso and Hansson 2011; IPCC 2014). Dissolved CO_2 reacts with seawater to form carbonic acid, which rapidly dissociates into carbonate ions and H^+, reducing seawater pH (Caldeira and Wickett 2003; Orr et al. 2005). Thus far, the pH has already dropped 0.1 units (Haugan and Drange 1996) and projections for the end of this century suggest a further decrease of 0.3 units (IPCC 2014) potentially threatening marine life (Hendriks et al. 2010; Kroeker et al. 2010; Wittmann and Pörtner 2013). Studies on this topic indicate that responses to OA are species-specific (Ries et al. 2009; Kroeker et al. 2010) and effects on fitness-related parameters oscillate from negative to positive (Dupont et al. 2013). Understanding this response variability is currently a challenge in OA research. In addition, most OA studies are performed over short-term exposures of high CO_2 levels, therefore disregarding potential acclimation to the new abiotic conditions, which may over or underestimate the real effects of chronic exposure to OA (Dupont et al. 2013). Thus, studies featuring prolonged exposure to high CO_2 levels are necessary as a more adequate proxy of the future impacts of OA. Fish exposed to OA can compensate the consequential acid-base disturbance, by adjusting the levels of HCO_3^- and H^+ in the blood plasma (Heuer and Grosell 2014). However, this ion imbalance facilitates redox reactions that can form reactive oxygen species (ROS) (reviewed in Dean 2010). ROS are produced through continuous reduction of O_2, such as O_2^-, H_2O_2 and HO^-, and are highly reactive with other molecules (Cadenas 1989; Halliwell and Gutteridge 2015). Overproduction and accumulation of ROS beyond the organism coping capacity creates an oxidative environment within the cells (i.e. oxidative stress) that triggers the oxidation of important biomolecules, such as lipids, proteins, and DNA (Ighodaro and Akinloye 2018). For instance, ROS can disrupt the normal morphology and function of phospholipidic membranes through lipid peroxidation (LPO), and the products of this degradation include the formation of malondialdehyde (MDA), which is highly cytotoxic (Lesser 2012). DNA damage can also occur, including the formation of 8-hydroxy-

2'-deoxyguanosine (8-OHdG), which is the most commonly used biomarker for damage in the DNA associated with changes in protein expression (Birben et al. 2012).

Antioxidant enzymes act as a primary defense mechanism to avoid oxidative stress, in which the enzyme superoxide dismutase (SOD) converts O_2^- to H_2O_2 while both catalase (CAT) and glutathione peroxidase (GPx) remove H_2O_2, avoiding its build-up in cells and tissues (Lesser 2012; Halliwell and Gutteridge 2015; Ighodaro and Akinloye 2018). Under stressful environmental conditions, organisms can also produce heat shock proteins (HSPs) (Parsell and Lindquist 1993) that refold and remove any damaged or denatured protein (Wang et al. 2004). When protection by HSPs fails, ubiquitin (Ub) acts as the last defense mechanism, targeting irreversibly damaged proteins and permanently removing them (Bond et al. 1988; Hanna et al. 2007; Lopes et al. 2018). The balance between cellular damage and its repairing mechanisms regulates the fate of the cell (Lesser 2006). In this context, both oxidative degradation products and antioxidant enzymes are widely used biomarkers to estimate oxidative stress, conveying information regarding the antioxidant state of an organism (Valavanidis et al. 2006).

Cellular redox balance disturbances and subsequent ROS formation are a frequent consequence of OA exposure across several taxa of marine organisms (Lesser 2006; Lesser 2012; Rosa et al. 2012; Sokolova et al. 2012; Matoo et al. 2013; Matozzo et al. 2013; Pimentel et al. 2015), however, responses can vary according to species, taxonomic group, life-strategy and ontogeny (Doney et al. 2012; Gibson et al. 2011). Indeed, while previous studies indicate that sharks display a certain resistance to oxidative stress under simulated OA, effects have only been addressed in two closely related tropical species (Rosa et al. 2016; Lopes et al. 2018). In this context, comprehending if other sharks (e.g. temperate sharks) can cope with the expected OA conditions would yield useful information regarding the future of shark populations. Hence, as there is growing evidence that climate change may impact shark physiology (Green and Jutfelt 2014; Heinrich et al. 2014; Rosa et al. 2014; Dixson et al. 2015; Pistevos et al. 2015; Johnson et al. 2016; Rosa et al. 2017; Pegado et al. 2019), the aim of our study was to investigate the oxidative stress-related responses of temperate catsharks (*Scyliorhinus canicula*) exposed to OA (pCO_2 ~ 900 µatm) over their early

developmental stages. We performed a long-term acclimation to the expected future OA conditions for 9 months (4 months during the whole embryogenesis plus 5 months after hatching), and measured changes in the primary antioxidant defenses (SOD, CAT and GPx), in protein repair and elimination mechanisms (HSP and Ub), and oxidative damage (MDA and 8-OHdG).

Materials and methods

Animal collection and acclimation

Adults small-spotted catsharks (*Scyliorhinus canicula*) were captured in June 2017 at Figueira da Foz (Portugal) and transported to Laboratório Marítimo da Guia facilities (Cascais, Portugal). Sharks were placed in 600 L semi-closed systems at control conditions (18 °C and pCO_2 ~ 400 µatm) and fed *ad libitum* with squid and fish (Sims 1994). Water quality ensured with protein skimmers (Schuran, Jülich, Germany) and biological filtration (Ouriço® bioballs, Fernando Ribeiro, Portugal), and temperature was maintained with chillers (Hailea Chillers, China) in a range of ± 1 °C.

Egg deposition was monitored daily. Females normally laid a pair of eggs, that would be collected and randomly assigned to one of two treatments: control (~ 400 µatm CO_2) or a simulated scenario of high CO_2 (ΔpH = - 0.3 pH units; ~ 900 µatm CO_2; according to IPCC's RCP scenario 8.5 (IPCC 2014)). In each treatment, eggs were reared in 50 L tanks (3 replicates per treatment) during the whole embryogenesis (around 4 months) plus 5 months after hatching, for a total of 9 months. The eggs were suspended ~5 cm below the surface. A drip system would then provide each tank a constant flow of filtered (1 µm; Harmsco, USA) and UV-sterilized (Vecton 300, TMC Iberia, Portugal) seawater via a mixing tank, in which the pH values were regulated. Seawater pH was regulated in the mixing tank with an automated controlling system (Profilux 3.1, GHL, Germany) linked to individual probes (GHL, Germany), and monitored every two seconds. Upregulation of the pH was done with soda lime filtered air and down-regulation was done via solenoid valves (Etopi, Portugal) with CO_2 injection (certified by Air Liquid, Portugal) and hysteresis was maintained around ± 0.05 units of pH. Experimental tanks were immersed in a water bath and the temperature was controlled with a chiller (Hailea Chillers, China). Water parameters were manually measured daily: pH with a portable pH probe (VWR pHenomenal, Germany), temperature with a thermometer (TFX 430, WTW GmbH, Germany) and salinity with a refractometer (V2, TMC Iberia, Portugal); for additional information see Table I. A photoperiod of 12 h light and 12 h dark was kept during the entire experiment. Ammonia,

nitrites and nitrates were monitored (Tropic Marin, Germany) weekly and kept under detectable levels. Seawater carbonate system speciation (Supplementary Table I) was also measured weekly, and calculated from pH measurements and total alkalinity (Sarazin et al. 1999). CO2SYS software (Lewis and Wallace 1998) was used to calculate total dissolved inorganic carbon (DIC), bicarbonate, aragonite saturation and pCO_2 levels, adjusted by Dickson and Millero (1987), using the dissociation constants from Mehrbach et al. (1973). After the embryonic period, sharks were fed every other two days to satiation, with squid (Sims 1994).

Table 1. Seawater parameters measured during the experiment. Values indicate the mean ± SD (standard deviation). Temperature, pH, salinity and total alkalinity (A_T) were measured and used to calculate the values from the carbonate system parameters [i.e. pCO_2 (carbon dioxide partial pressure), HCO_3 (bicarbonate concentration) and Ω Arg (aragonite saturation state)].

		Control	High CO_2
Measured			
	Temperature (° C)	18 ± 0.8	18 ± 0.7
	pH (NBS scale)	8.04 ± 0.07	7.75 ± 0.09
	Salinity	36 ± 0.7	36 ± 0.7
	A_T (µmol kg^{-1} SW)	2489.1± 214.3	2481.3± 189.4
Calculated			
	pCO_2 (µatm)	442.9 ± 65.0	915.5 ± 111.3
	HCO_3 (mmol kg^{-1} SW)	2026.4 ± 175.7	2215.7 ± 169.0
	Ω Arg	2.9 ± 0.4	1.7 ± 0.2

Biochemical analyses

1. Preparation of tissue extracts

Samples of gills, muscle and liver (n = 18 individuals per treatment) were kept on ice throughout the homogenization process, to maintain protein integrity and loss of enzyme activity. Samples were homogenized in 1.0 mL phosphate-buffered saline solution (PBS, pH 7.4: NaCl, Na_2HPO_4, KH_2PO_4 and miliQ

H_2O) using Ultra-Turrax (Staufen, Germany). Homogenates were subsequently centrifuged (14,000×g for 20 min at 4 °C) and the fraction of supernatant (~1.0 mL) was transferred to new Eppendorfs and frozen (at -80 °C) until further analyses. The determination of total protein content was performed according to Bradford (1976). Each sample was run in duplicate (technical replicates), and then results were used for normalizing biomarkers results.

2. Antioxidant enzyme activities

2.1 Superoxide Dismutase Activity (SOD)

Superoxide dismutase (SOD) inhibition was measured according to McCord and Fridovich (1969). In brief, 96-well microplate (Greiner Bio-one, Austria) wells were filled with 200 µL buffer solution (pH ~8.0, 0.5 mM K_3PO_4, Sigma-Aldrich, Germany) followed by adding 10 µL of each: 3 mM EDTA (Riedel-de Haën, Germany), 3 mM xanthine (Sigma, Germany), 0.75 mM NBT (Sigma-Aldrich, Germany) and then the sample. After which, the reaction was initiated by adding 10 µL of 100 mU xanthine oxidase (XOD, Sigma-Aldrich, Germany). The absorbance was read at 560 nm with a microplate reader (Biotek Synergy HTX multi-mode reader, USA) every minute for 5 min, and then at 10 and 15 min. SOD activity was normalized to total protein content and expressed in percentage of inhibition per milligram of total protein.

2.2 Catalase Activity (CAT)

Catalase (CAT) activity was measured using an adapted version of a method described by Johansson and Borg (1988). Each well of a 96-well microplate (Greiner Bio one, Austria) was filled with 100 µL of 100 mM potassium phosphate (K_3PO_4), 30 µL of methanol (CH_3OH) and 20 µL of each sample. A standard curve was built to determine the formaldehyde produced in samples, based on a calibration curve with range from 0 to 75 µM of formaldehyde (Sigma, Germany) and 100 µL of 25 mM potassium phosphate (Sigma-Aldrich, Germany; pH 7.0), 1 mM EDTA (Riedel-de Haën, Germany), 0.1% BSA (Nzytech, Portugal). The reaction was initiated with the addition of 20 µL of hydrogen peroxide (0.035 M, Sigma-Aldrich, Germany) followed by

incubation for 20 min at room temperature. Subsequently, 30 µL of potassium hydroxide (10 M KOH, Chem-Lab, Belgium) and 30 µL of Purpald (34.2 mM in 0.5 M HCl, Aldrich, Germany) was added to each well, the microplate was shaken and incubated for another 10 min at room temperature. Afterwards, 10 µL of potassium periodate (65.2 mM in 0.5 M KOH; Chem-lab, Belgium) was added to each well, the microplate was placed in a shaker and incubated for 5 min. Then, the enzymatic activity was measured spectrophotometrically at 540 nm with a microplate reader (Biotek Synergy HTX multi-mode reader, USA). Catalase activity was quantified by considering one unit of catalase as the amount of enzyme that would produce 1.0 nmol of formaldehyde per minute, at 25 ºC. Results were expressed relative to the total protein content (nmol/min/mg total protein).

2.3 Glutathione Peroxidase Activity (GPx)

Glutathione peroxidase activity (GPx) was determined following Lawrence and Burk (1976), adapted to 96-well microplates (Greiner Bio one, Austria). Briefly, 20 µL of each sample was added to 120 µL of assay buffer and the remaining wells were filled with 140 µL of assay buffer for the negative control. The assay buffer contained potassium phosphate buffer (50 mM, pH 7.4, Sigma-Aldrich, Germany) and EDTA (5 mM, pH 7.6; Riedel-de Haën, Germany). After, 50 µL of the co-substrate mixture [sodium azide (4 mM, Sigma-Aldrich, Germany), nicotinamide adenine dinucleotide phosphate (1 mM, NADPH, Sigma-Aldrich, Germany), glutathione reductase (4 U/mL, GSSG-reductase, Sigma, Germany) and reduced glutathione (4 mM, GSH, Sigma, Germany)] was added. Following, 20 µL of hydroperoxide cumene was added (15 mM, Sigma-Aldrich, Germany), and the absorbance was read at 340 nm for 6 min (Bio-Rad, Benchmark, USA). The GPx activity was determined with the β-NADPH coefficient extinction (3.73 mM^{-1} cm^{-1}, adapted for the solution path length in a microplate well, CGP1, Sigma Technical Bulletin; Equation 11), and the results were given in nmol/min/mg.protein.

3. Protein repair and elimination

3.1 Heat Shock Response (HSP_{70}/HSC_{70} content)

Heat Shock Protein (HSP_{70}) content was quantified through Enzyme-linked Immunosorbent Assay (ELISA) according to Njemini et al. (2005). Briefly, 10 µL of each sample was diluted in 990 µL of PBS. Afterwards, 50 µL of each diluted sample was added to 96-well microplates (Greiner Bio-one Microlon, Austria) and incubated overnight at 4 °C. This step allows the antigen to bind to the plate wells (antigen coating). After 24 h, each microplate was washed three times with 0.05% PBS-Tween-20 solution (Sigma-Aldrich, Germany). Subsequently, 100 µL of blocking solution (1% BSA, bovine serum albumin (BSA, Sigma-Aldrich, USA) in PBS) was added to each well and incubated for 90 min at 37 °C (Labnet, USA). Microplates were then washed in the same manner and 50 µL of primary antibody solution (1 µg/mL in 1% BSA solution: anti-HSP_{70}/HSC_{70}, OriGene, USA) was added to each well and incubated overnight at 4 °C, allowing the antibody to bind specifically to the antigen (protein capture). Then, microplates were washed three times to remove non-linked antibodies and a second antibody conjugated to alkaline phosphatase (anti-mouse IgG Fc specific - alkaline phosphatase, Sigma-Aldrich, Germany; diluted to 1 µg/mL in 1% BSA solution) was added (50 µL) to each well and incubated for 90 min at 37 °C, binding to the primary antibody. After another washing step, 100 µL of alkaline-phosphatase substrate [100 mM NaCl (Panreac, Spain), 50 mM $MgCl_2$ (Sigma-Aldrich, Germany), 100 mM Tris-HCl (Sigma-Aldrich) and 27 mM PnPP (pH 8.5, 4-nitrophenyl phosphate disodium salt hexahydrate (Sigma, Germany)] was added to each well and incubated at room temperature for 30 min. Finally, 50 µL of stop solution (3 M NaOH, Panreac, Spain) was added to each well and the absorbance was read at 405 nm using a microplate reader (Biotek Synergy HTX multi-mode reader, USA). HSP_{70}/HSC_{70} sample contents were calculated from the standard curve of purified HSP_{70} active protein (OriGene Technology, USA) using a series of dilutions that ranged from 0 to 2 µg/mL. Results were expressed as µg/mg total protein, in relation to the total protein content of the samples.

3.2 Ubiquitin Content (Ub)

Ubiquitin (Ub) content was assessed through an indirect ELISA according to Njemini et al. (2005). 10 µL of each sample plus 40 µL PBS was added to 96-well microplate (Greiner Bio-one, Austria) and incubated overnight at 4 °C. After 24 h, microplates were washed three times with 0.05% PBS-Tween-20 solution (Sigma-Aldrich, Germany) and 100 µL of blocking solution (1% BSA, bovine serum albumin (BSA, Sigma-Aldrich, USA) in PBS) was added to each well and left to incubate for 90 min at 37 °C (Labnet, USA). Microplates were then washed in the same manner and 50 µL of primary antibody solution ((Ub (P4D1) Sc-8017, mouse monoclonal IgG, Santa Cruz Biotechnology, USA; diluted to 1 µg/mL in 1% BSA in PBS solution) was added to each well and incubated overnight at 4 °C. After another 24h, microplates were again washed three times to remove non-linked antibodies. A secondary antibody (antimouse IgG, fc-specific, alkaline phosphatase conjugate, Sigma-Aldrich, Germany) was added (50 µL) to each well and incubated for 90 min at 37 °C. After another washing procedure, 100 µL of alkaline-phosphatase substrate [NaCl (100 mM, Panreac, Spain), Tris-HCl (100 mM, Sigma-Aldrich), $MgCl_2$ (50 mM ,Sigma-Aldrich, Germany) and PnPP (27 mM, 4-nitrophenyl phosphate disodium salt hexahydrate (Sigma, Germany): pH 8.5] was added to each well and incubated at room temperature for 30 min. Finally, 50 µL of 3 M stop solution (NaOH, Panreac, Spain) was added to each well and the absorbance at 450 nm was read in a microplate reader (Biotek Synergy HTX multi-mode reader, USA). Ubiquitin content was determined from a standard curve, prepared using sequential dilutions of purified ubiquitin active protein (Santa Cruz Biotechnology, USA), which ranged from 0.8 to 0.0125 µg/µL. Results were expressed as µg/mg total protein, relating them to the total protein content of the samples.

4. Lipid peroxidation and DNA damage

Lipid peroxidation (LPO) was estimated through the quantification of malondialdehyde (MDA), which is a specific end-product of the process of lipid oxidative degradation, based on the thiobarbituric acid reactive substance (TBARS) assay (Uchiyama and Mihara, 1978). Briefly, 5 µL of sample or standard was added to a

1-mL microtube, followed by 45 µL of 50 mM monobasic sodium phosphate buffer (Sigma-Aldrich, Germany), 12.5 µL of sodium dodecyl sulfate (SDS 8.1%, Merck, Germany), 93.5 µL of trichloroacetic acid (TCA 20%, pH 3.5; Panreac, Spain), 93.5 µL of thio barbituric acid (TBA 1%, Sigma-Aldrich, Germany), and 50.5 µL of Milli-Q ultrapure water. Then, the mixture was centrifuged (2000×g) and incubated for 10 min in boiling water (100 °C). The resulting mixture was placed on ice for a few minutes to lower the temperature, 62.5 µL of Milli-Q ultrapure was added and the microtubes were again centrifuged (2000×g) for 1 min. Duplicates of 150 µL of the supernatant fraction of each sample were added to 96-well microplate (Greiner Bio-one, Austria), and absorbance was read at 530 nm in a microplate reader (Biotek Synergy HTX multi-mode reader, USA). MDA concentrations were quantified from a calibration curve that ranged from 0 to 0.1 µM TBARS, performed with MDA (Sigma-Aldrich, Germany), to calculate lipid peroxides formed in each sample. Results were expressed in µM/mg total protein, relating them to total protein content.

To determine potential DNA damage, 8-hydroxy-2'-deoxyguanosine (8-OHdG) was quantified, as it is widely used as a biomarker for oxidative DNA damage and can lead to DNA base mismatch or strand break. 8-OHdG was measured through an ELISA method, following Shen et al. (2007). Briefly, 100 µL of each sample was added to each well of a 96-well microplate and incubated overnight at 4 °C. After 24 h of incubation, plates were washed (4×) with PBS-Tween 20 (0.05%) and re-incubated with a blocking solution (200 µL of BSA) at room temperature for 90 min. After another washing procedure (4×), microplates were again incubated overnight at 4 °C with primary antibody (anti-8-OHdG, Sigma-Aldrich, Germany). On the following day, microplates were washed (4×) with PBS-Tween 20 (0.05%) to eliminate non-linked antibodies and then were incubated at 37 °C for 90 min with the secondary antibody (alkaline phosphatase-conjugated anti-mouse IgG fc-specific, Sigma-Aldrich, USA). After a final wash procedure, microplates were incubated with the substrate (SIGMA FAST™ p-Nitrophenyl Phosphate Tablets, Sigma-Aldrich, USA) for 30 min at room temperature. The reaction was stopped with 100 µL of NaOH (3 M), and the absorbance was read at 405 nm (Biotek Synergy HTX multi-mode reader). Given the absence of a standard for 8-OHdG, the results are presented as absorbance normalized with the total protein.

Statistical analyses

Statistical analyses were performed with generalized linear mixed-effects models (GLMM) using Gamma (log link function) or Gaussian distribution (identity link function) to infer the statistical difference between treatments and between the tissues analyzed. Firstly, the tissues (muscle, gills and liver) were used as explanatory variables for each dependent variable (antioxidant capacity: SOD, CAT, GPx; protein repair and removal: HSP and Ub; oxidative damage: DNA and LPO). Models included CO_2 treatment and tissues as fixed effects and the replicates as random effect, to account for possible non independence between observations from the same treatment. Afterwards, CO_2 levels (control and high CO_2) were used as explanatory variables to identify the effect of CO_2 in each type of tissue. Models included CO_2 treatment as a fixed effect and the replicates as a random effect. All residuals were checked for potential influential observations and departures from models' assumptions (distribution and homoscedasticity). No significant deviations or outliers from models' assumptions were found. The statistical analysis was performed in R (Version 1.1.453 – © 2009-2018 RStudio, Inc.), and a *p*-value inferior to 0.05 was considered statistically significant.

Results

Regarding the tissue-specific patterns of primary antioxidant defense, SOD activity was significantly lower in the muscle than the gills ($p < 0.001$, Supplemental table 1; Fig. 1A) and the liver ($p < 0.001$, Supplemental table 1; Fig. 1A), while CAT and GPx activities were significantly different between all tissues (both $p < 0.001$, Supplemental table 1; Fig. 1B, C), with higher levels in the liver, followed by the gills and lastly the muscle. Regarding the effects of CO_2 treatment, high CO_2 increased CAT activity in the muscle ($p < 0.05$, Supplemental table 1; Fig. 1B) with no alteration in the other tissues ($p > 0.05$, Supplemental table 1; Fig. 1B), SOD and GPx activities remained unaltered in all tissues ($p > 0.05$, Supplemental table 1; Fig. 1C). Concerning protein repair and elimination mechanisms, both HSP and Ub levels were significantly different between tissues and both were higher in the muscle, then the liver and lastly the gills (both $p < 0.001$, Supplemental table 1; Fig. 2A, B). Furthermore, there were no significant differences between CO_2 treatments across all tissues ($p > 0.05$, Supplemental table 1; Fig. 2A, B). MDA levels were only significantly higher in the gills compared to the liver ($p < 0.05$, Supplemental table 1; Fig. 3A), with no differences between other tissues or between CO_2 treatments ($p > 0.05$, Supplemental table 1; Fig. 3A). Lastly, regarding DNA damage there were neither significant differences between tissues ($p > 0.05$, Supplemental table 1; Fig. 3B), nor between CO_2 treatments ($p > 0.05$, Supplemental table 1; Fig. 3B).

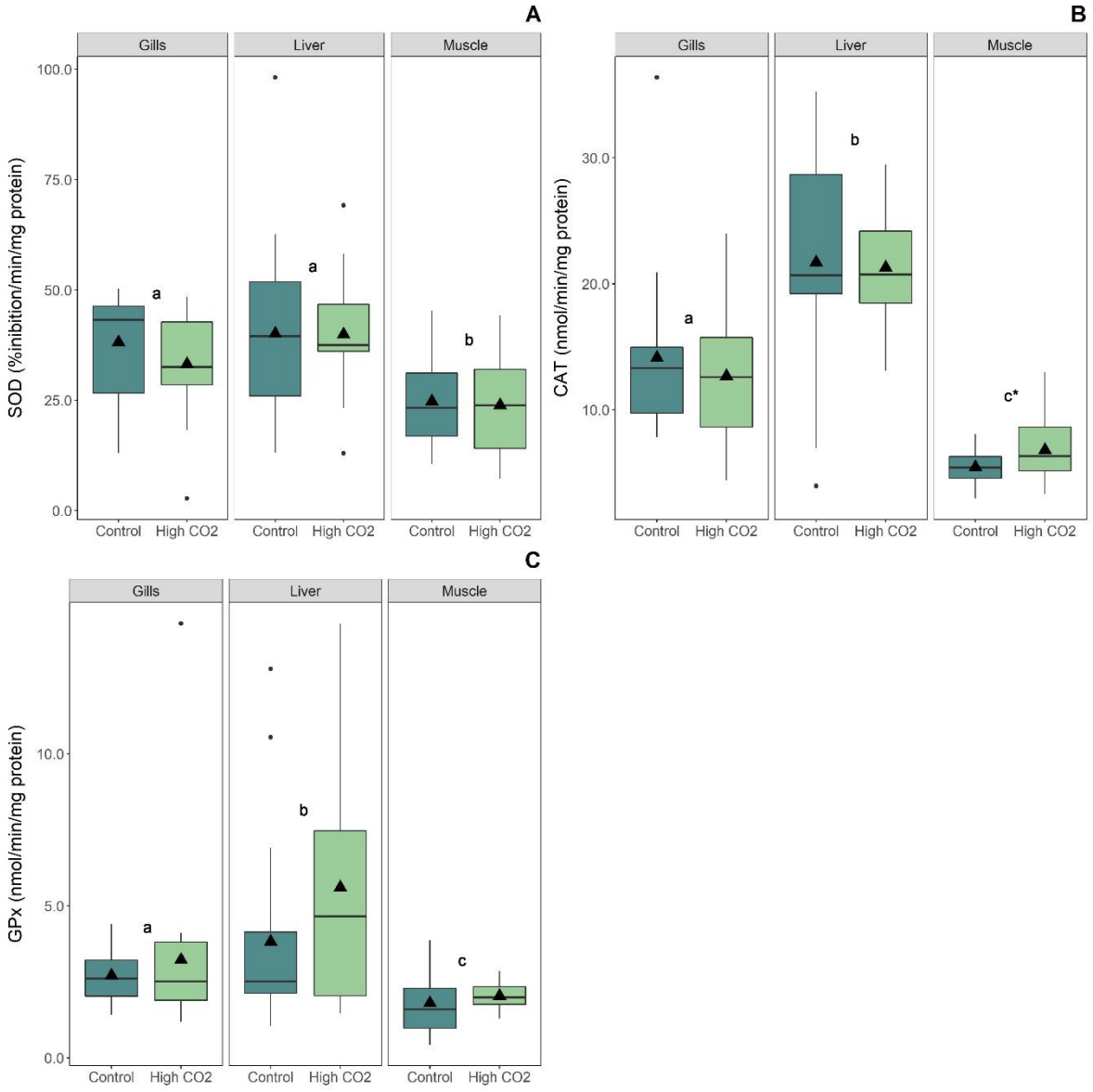

Figure 1. Effects of high CO_2 levels ($\Delta\ pCO_2$ ~ 500 µatm) on the levels of: i) SOD (superoxide dismutase), ii) CAT (catalase) and iii) GPx (glutathione peroxidase) in the gills, liver and muscle of small-spotted catsharks (*Scyliorhinus canicula*, n = 18 per treatment). Bold horizontal line within the box represents the median, boundaries represent 25th and 75th percentiles and the triangle represents the mean value. Different lower-case letters represent statistical differences between tissues, while asterisks (*) indicate significant statistical differences within the tissues. Additional statistical information present in Supplemental Table S1.

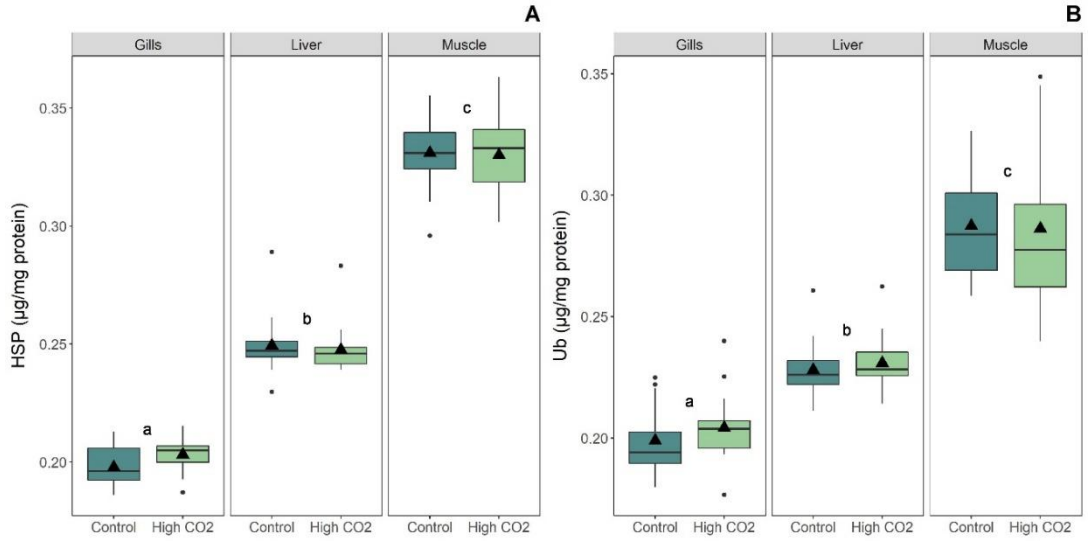

Figure 2. Effects of high CO_2 levels (Δ pCO_2 ~ 500 µatm) on the levels of: i) HSP (heat shock proteins) and ii) Ub (ubiquitin) in the gills, liver and muscle of small-spotted catsharks (*Scyliorhinus canicula*, n = 18 per treatment). Bold horizontal line within the box represents the median, boundaries represent 25th and 75th percentiles and the triangle represents the mean value. Different lower-case letters represent statistical differences between tissues. Additional statistical information present in Supplemental Table S1.

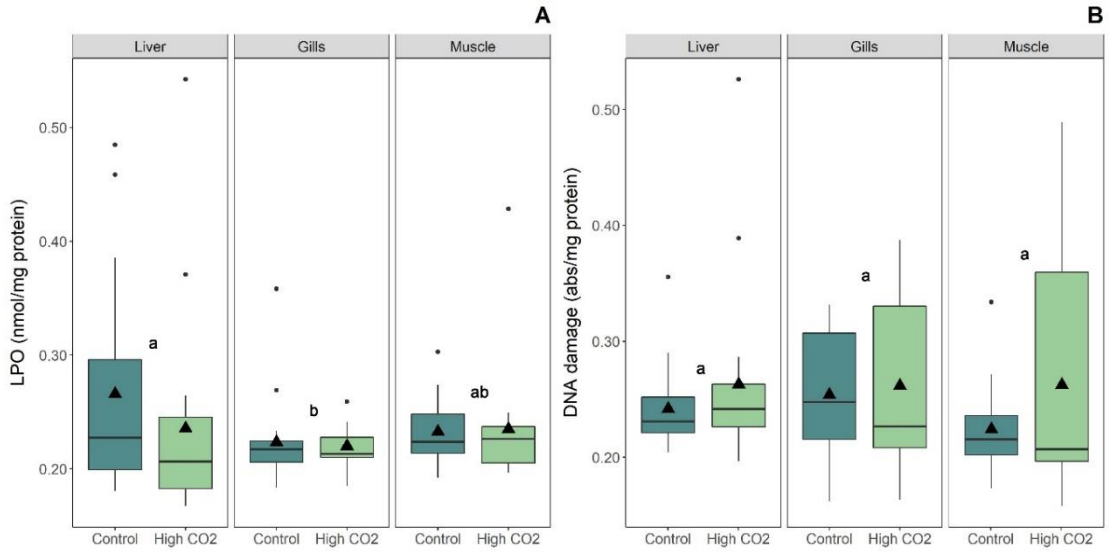

Figure 3. Effects of high CO_2 levels (Δ pCO_2 ~ 500 µatm) on the levels of: i) MDA (malondialdehyde production due to lipid peroxidation) and ii) 8-OHdG (8-hydroxy-2′-deoxyguanosine due to DNA damage) in the gills, liver and muscle of small-spotted catsharks (*Scyliorhinus canicula*, n = 18 per treatment). Bold horizontal line within the box represents the median, boundaries represent 25th and 75th percentiles and the triangle represents the mean value. Different lower-case letters represent statistical differences between tissues. Additional statistical information present in Supplemental Table S1.

Discussion

Oxidative stress can be exacerbated at lower pH values (Tomanek et al. 2011) and, to prevent it, some marine organisms increase antioxidant enzyme activity and heat shock response (Matoo et al. 2013; Pimentel et al. 2015; Wang et al. 2016). In our study, albeit there was a significant increase of catalase (CAT) activity in the muscle, a long-term acclimation to OA did not elicit oxidative damage in small-spotted catsharks. Exposure to OA conditions can increase the amount of H^+ ions in extracellular fluids, which can interact with the superoxide ion (O_2^-) or H_2O to form peroxide hydrogen (H_2O_2) (Tiedke et al. 2013). CAT is generally the primary enzyme in the elimination of H_2O_2 (López-Cruz et al. 2010) converting it back to H_2O and O_2 (Fridovich 1983), suggesting an underlying increase of this ROS. Regarding the rest of the studied antioxidant enzymes, no significant changes were observed. Moreover, there was no evidence of oxidative damage (i.e. lipid peroxidation or DNA damage), which further indicates that this species was able to avoid oxidative stress that could be elicited by the tested CO_2 levels, through an increase in CAT activity. It is worth mentioning that, apart from CAT, other key antioxidant enzymes, which act as electron donors, are also present in cells and could be acting to neutralize excess H_2O_2, such as thioredoxins and peroxiredoxins (Andreyev et al. 2005; Hanschmann et al. 2013). Nonetheless, higher levels of oxidative stress could have preceded physiological acclimation since all endpoints were evaluated following a prolonged exposure (i.e. 9 months).

To our knowledge, only two other studies analyzed shark's oxidative response to OA, both focusing on closely related tropical species (Rosa et al. 2016; Lopes et al. 2018), which could present higher aerobic demands (Baldwin and Wells 1990) that could lead to increased ROS production (Halliwell and Gutteridge 2015). However, both studies provided little evidence of cellular damage. The first, a prolonged exposure (i.e. whole embryogenesis plus 30 days, pCO_2 ~ 1500 µatm) of bamboo sharks (*Chiloscyllium punctatum*) found no evidence of oxidative damage as a result of OA-exposure (Rosa et al. 2016), while the second, a short-term exposure (i.e. 50 days, pCO_2 ~ 900 µatm) of another species of bamboo sharks (*Chiloscyllium*

plagiosum) presented changes on the antioxidant system and increased DNA damage in the liver, which is a main detoxifying center (Lopes et al. 2018). Since a short-term exposure to OA may not be enough to allow for acclimation to new abiotic conditions and may overestimate the impacts caused by chronic exposure, the existing literature suggests that sharks' antioxidant system will be able to prevent oxidative damage under chronic OA conditions.

Based on currently available research, sharks appear to be quite resilience to OA-induced oxidative stress, particularly in comparison to invertebrates (Tomanek et al. 2011; Lesser 2012), mollusks (Sokolova et al. 2012) and teleost fishes (Lesser 2006; Pimentel et al. 2015). However, studies have so far been restricted to small oviparous and relatively sedentary shark species, thus precluding broad generalization. Moreover, further research is needed to unravel the mechanisms that may confer sharks this resilience. Unlike teleost fish, the unique osmoregulation mechanisms of elasmobranchs rely mainly on urea and trimethylamine N-oxide (TMAO) which may have important roles in the stress response. For instance, urea possesses ROS scavenging properties, with protective action against oxidative stress, including the ability to protect whole organs from oxidative damage (Wang et al. 1999). On the other hand, TMAO may function as a chaperone in a stressful environment, as its main function is to counteract the highly toxic effects of urea (Seibel & Walsh, 2002) by stabilizing proteins (Samerotte et al., 2007; Bockus and Seibel, 2018) and preventing protein degradation (Ufnal, Zadlo and Ostaszewski, 2015). Shark cartilage also appears to have a scavenging role against ROS (Gomes et al. 1996; Felzenszwalb et al. 1998) protecting cells from the negative effects caused by H_2O_2 (Gomes et al. 1996).

Physiological compensation at organismal level is established primarily through molecular and biochemical changes (Hochachka and Somero 2002). The present study suggests that near-future CO_2 levels represent a low oxidative risk to small-spotted catsharks, although there may be a potential energetic cost due to the upregulation of enzymatic ROS scavenging machinery, i.e. increased CAT activity. Moreover, oxidative stress levels can change dramatically when paralleled with major variations in metabolism, such as increased temperature (Tomanek 2011). In these situations, the energy necessary to keep the antioxidant defenses

upregulated or to activate repair systems, may be allocated to other physiological traits, potentially resulting in oxidative damage (Pamplona and Costantini 2011). Indeed, some studies where exposure to OA did not induce oxidative stress, reported an interactive effect associated with increased temperature (Matoo et al. 2013; Maulvault et al. 2018). This effect has also been registered in tropical sharks, with an interactive effect of OA with warmer temperatures in the induction of oxidative stress and associated antioxidant responses (Rosa et al. 2016). This suggests that antioxidant defenses may not be enough in a multi-stressor context. Ocean acidification is expected to take place side-by-side with ocean warming and deoxygenation, among other climate-related stressors. Hence, future research on the subject should investigate the cumulative effects of the combined multi-stressors expected to occur in the near future.

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5 Effects of elevated carbon dioxide on the hematological parameters of a temperate catshark

Running title: Shark hematology under high CO_2

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Abstract

Atmospheric CO_2 levels have been rising due to an increase in anthropic activities and its implications over marine ecosystems are unprecedented. The present study focused on the effects of ocean acidification (OA) on key hematological parameters of the juvenile small-spotted catsharks (*Scyliorhinus canicula*). Eggs were reared throughout the entire embryogenesis (~4 months) plus 5 additional months, in two experimental treatments (control: pCO_2 ~ 400 µatm; and high CO_2: pCO_2 ~ 900 µatm, Δ - 0.3 pH units). After blood collection, the following hematological parameters were evaluated: i) normal blood cells (erythrocytes, leukocytes and thrombocytes), ii) presence of erythrocytes with nuclear abnormalities (ENAs) and iii) erythrocyte nucleus to cytoplasmic ratio. Concomitantly, to determine the cardiac and hematopoietic conditions, the spleen and heart to body ratios were also assessed. The present findings indicate that the measured variables may not be affected by elevated pCO_2 in this temperate species, as no significant differences were observed between treatments across all the endpoints tested. Nonetheless, it is worth mentioning a decreasing trend observed in number of thrombocytes associated with OA, which should foster further investigation, regarding other aspects of their coagulation response. Along with OA, other stressors are expected to impact marine life, such as warming and hypoxia. Thus, future research should aim to investigate the cumulative effect of these stressors on hematological parameters in sharks.

Keywords: ocean acidification, hematology, early stages, elasmobranch, *Scyliorhinus canicula,* shark

Introduction

Anthropogenic activities, such as fishing (Musick et al. 2000) and habitat destruction (Chin et al. 2010), have been exerting pressure on shark populations for the last decades (Baum et al. 2003). Ocean acidification (OA) may represent an additional anthropogenic threat, as it constitutes an underway process (IPCC 2013) and is expected to intensify alongside carbon dioxide (CO_2) emissions during the current century (Caldeira and Wickett 2003; IPCC 2014). OA results from the accumulation of atmospheric CO_2 by the oceans and leads to changes in the seawater carbonate system along with a reduction of the pH (IPCC 2013). The potential impact of OA on marine life is an increasingly concerning topic, since many marine organisms have been documented to be affected by these chemical changes (Dupont, Dorey, and Thorndyke 2010; Kroeker et al. 2010; Hendriks, Duarte, and Álvarez 2010).

The decrease of seawater pH can disrupt the acid–base balance of extracellular body fluids, e.g. blood (Pörtner 2008; Melzner et al. 2009). Highly energetic organisms, such as sharks, can compensate this disturbance by active ion transport in most tissues (Claiborne and Evans 1992), and by retaining HCO_3^- in the blood plasma (Heuer and Grosell 2014). Through HCO_3^- accumulation, the pH in the blood plasma returns to control levels, which confers them an advantage to OA exposure (Heuer and Grosell 2014). However, phenotypic plasticity may come with a cost over other physiological traits (Esbaugh et al. 2016). The ability to compensate for an acute acid–base disturbance may not translate to health over long-term exposure (Ishimatsu, Hayashi, and Kikkawa 2008; Pörtner 2008), as it can actually have repercussions on fundamental processes. For instance, long-term exposure to high CO_2 may inhibit the adequate transport of oxygen through the blood (Wittmann and Pörtner 2013; Pörtner, Langenbuch, and Reipschläger 2004), decrease heart rate [bradycardia; (Kent and Peirce 1978; Perry et al. 1999)] or cardiac output, i.e. blood volume pumped per unit of time (Perry and Gilmour 2002), and also result in a weaker immune system (de Souza et al. 2014).

Recently, the scientific community started to investigate the effects that OA may have on sharks' physiology and behaviour (Green and Jutfelt 2014; Heinrich et al. 2014; Rosa et al. 2014; Dixson et al. 2015; Heinrich

et al. 2015; Pistevos et al. 2015; Johnson et al. 2016; Rosa et al. 2016a; Rosa et al. 2016b; Pistevos et al. 2017; Rosa et al. 2017; Lopes et al. 2018). Two studies have so far indicated the presence of compensation mechanisms in sharks exposed to OA. Exposure to higher CO_2 in the environment increased HCO_3^- and Na^+ levels in the blood plasma of small-spotted catshark adults (Green and Jutfelt 2014). A similar acid-base compensation in the blood plasma was observed in the epaulette shark (*Hemiscyllium ocellatum*) along with an increase in [Hb] (hemoglobin concentration) and MCHC (mean cell hemoglobin concentration) (Heinrich et al. 2014). As sharks rely on their environment to maintain vital processes (Seamone and Syme 2015), exposure to high CO_2 levels have also shown a decrease of digestive enzymatic activities (Rosa et al. 2016a) and reduced ability to hunt (Pistevos et al. 2015), which could lead to less nutrient uptake. As erythrocyte nuclear abnormalities (ENAs) formation can occur due to nutritional deficiencies (Eiras 1983; Takashima and Hibiya 1995; Clauss et al. 2008) we hypothesized that future OA may change normal blood cells count and lead to chromosomal damage measured as erythrocytic nuclear abnormalities. Nonetheless, no study has so far addressed potential changes in the actual cellular components of the blood. Hence, the purpose of this study was to investigate, for the first time, the effects of increased CO_2 levels (pCO_2 ~ 900 µatm) on the hematological parameters (erythrocytes, leukocytes and thrombocytes; presence of erythrocyte nuclear abnormalities and nucleus to cytoplasmic ratio) of early-stage small-spotted catsharks (*Scyliorhinus canicula*). Additionally, heart and spleen to body ratios were assessed to evaluate potential OA effects over the cardiac and hematopoietic conditions.

Materials and methods

Ethics statement

During this research, all experimental procedures followed the requirements of Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on animal protection used for scientific purposes. They were also approved and reviewed by the Animal Welfare Body of Faculdade de Ciências da Universidade de Lisboa (Statement 5/2016), animal ethics committee ORBEA and the National Veterinary Medicines Directorate (DGAV).

Animal collection and acclimation

Adult small-spotted catsharks (n = 14, 9 females and 5 males) were caught by fishermen at Figueira da Foz (Portugal) in June of 2017. After collection they were transferred to the facilities of Laboratório Marítimo da Guia (Cascais, Portugal). Adults (3 females and 1 or 2 males per tank) were kept in 600 L semi-closed systems, at conditions similar to the natural environment (18 °C and pCO_2 ~ 400 µatm) and fed *ad libitum* with fish or squid. Water quality was improved by biological filtration (Ouriço® bioballs, Fernando Ribeiro, Portugal) and a protein skimmer (Schuran, Jülich, Germany). Water temperature was adjusted automatically with a chiller (Hailea Chillers, China) and hysteresis was kept around ± 1 °C. Egg deposition was checked every day, however most of the time it was not possible to determine their origin since multiple females were kept together. Generally, females laid a pair of eggs that would be collected and divided in two treatments: control (~400 µatm CO_2; n = 18) or a simulated scenario of high CO_2 (~900 µatm CO_2, ΔpH = -0.3 pH units; n = 18; IPCC's RCP scenario 8.5 (IPCC 2014)). Unless there were multiple eggs or only one egg was laid, in which case they were randomly assigned to a treatment. The eggs were placed in a 50 L aquarium (3 aquaria per treatment) for the whole embryogenesis (~4 months) plus, at least 5 more months after hatching, for a total of approximately 9 months. Eggs were suspended 5 cm below the surface, to ensure good aeration. A dripping system provided each aquarium with a constant flow of UV-sterilized (Vecton 300, TMC Iberia, Portugal) and filtered (1 µm; Harmsco, USA) seawater (~500% daily water change)

via a mixing tank, in which pH levels were regulated using an automated controlling system (Profilux 3.1, GHL, Germany) connected to individual probes (GHL, Germany). Proper seawater pH was guaranteed by upregulating the pH with soda lime filtered air or down-regulating with CO_2 injection (certified by Air Liquid, Portugal) via solenoid valves (Etopi, Portugal) monitored every two seconds and hysteresis kept the values around ± 0.05. The water temperature was maintained by a water bath controlled by a chiller (Hailea Chillers, China). Seawater parameters were manually measured daily, namely pH, using a portable pH probe (VWR pHenomenal, Germany), temperature, with a thermometer (SFT-75 Sanitas Thermometer, Germany) and salinity, with a refractometer (V2, TMC Iberia, Portugal); for further information see Table 1.

Table 1. Seawater physicochemical parameters for the experimental conditions. Salinity, pH, temperature and total alkalinity (A_T) were used to calculate the carbonate system parameters [pCO_2 (carbon dioxide partial pressure), HCO_3 (bicarbonate concentration) and Ω Arg (aragonite saturation state)]. Values indicate the mean ± SD.

		Control	High CO_2
Measured			
	Temperature (° C)	18 ± 0.8	18 ± 0.7
	pH (NBS scale)	8.04 ± 0.07	7.75 ± 0.09
	Salinity	36 ± 0.7	36 ± 0.7
	A_T (µmol kg^{-1} SW)	2489.1± 214.3	2481.3± 189.4
Calculated			
	pCO_2 (µatm)	442.9 ± 65.0	915.5 ± 111.3
	HCO_3 (mmol kg^{-1} SW)	2026.4 ± 175.7	2215.7 ± 169.0
	Ω Arg	2.9 ± 0.4	1.7 ± 0.2

Ammonia, nitrites and nitrates were monitored weekly (Tropic Marin, Germany), along with seawater carbonate system speciation (see Table 1), which was calculated from total alkalinity and pH measurements (Sarazin, Michard, and Prevot 1999). Total dissolved inorganic carbon (DIC), aragonite saturation, bicarbonate and pCO_2 levels were calculated using the CO2SYS software (Lewis and Wallace 1998), adjusted by Dickson and Millero (1987) with dissociation constants from Mehrbach et al. (1973). A photoperiod of

12 h light and 12 h dark was maintained for the entire experiment. After hatching, juveniles were fed to satiation every other two days with fish and squid.

Sample preparation and hematological parameters

After the experimental period, a total of 18 sharks were collected from each treatment (n = 36), euthanized by immersion in an overdosed MS222 solution (2000 mg L^{-1}, Sigma-Aldrich, USA) buffered with sodium bicarbonate (1 g of $NaHCO_3$ to 1 g of MS222 to 1 L of seawater) for 10 min. Euthanized sharks were weighted, and peripheral blood was collected by puncture of the caudal vein using a syringe (2 mL) washed with potassium EDTA (10% w/v in distilled water) to avoid blood clotting. A blood smear was prepared on preclean glass microscopy slides, allowed to air-dry and subsequently fixed in methanol (100%). Following fixation, blood smears were stained with Hemacolor staining reagent (Hemacolor® Rapid blood smear staining, Sigma-Aldrich) and counter-stained with safranin. Following staining, microscope glass slides were mounted using DPX (BDH, Poole, England) and stored. Subsequently, samples were analyzed using a Leica microscope (model DMLB equipped with a DFC480 digital camera) for the total count of normal blood cells (erythrocytes, leukocytes and thrombocytes, according to Arnold (2005)). The presence of erythrocytes nuclear abnormalities (ENAs: i) erythrocytes with segmented nucleus, ii) erythrocytes with blebbed nucleus and iii) erythrocytes with micronucleus) were also counted through optical microscopy. An average of at least 700 cells per slide were examined following the ENAs and micronuclei classification in agreement with Carrola et al. (2014). Additionally, a total of 100 erythrocytes per shark were measured using ImageJ software, to calculate nucleus to cytoplasmic ratio (NCR) according to Doughty (2012), i.e. NCR = nucleus area / (cell area – nucleus area). All cell counts were performed by an observer that was blind to the treatment. Sharks were dissected, their heart and spleen were collected and weighted to determine both heart and spleen to body ratios, using the following formulas:

$$\mathbf{HBR} = \frac{\text{Heart weight (g)}}{\text{Body weight (g)}} \text{ x } 100 \qquad \mathbf{SBR} = \frac{\text{Spleen weight (g)}}{\text{Body weight (g)}} \text{ x } 100$$

Statistical analyses

Data were analyzed using generalized linear mixed-effects models (GLMM). The binomial distributional family (logit link function) was used to model normal blood cells and ENAs proportions. The Gaussian distribution (identity link function) was used to model continuous quantities as HBR and SBR, while the Gamma distribution (log link function) was used to model NCR. The models included the CO_2 level as a fixed effect, and replicates as random effect to account for possible non independency between observations within the same aquariums. Model residuals were checked for departures from models' assumptions (residuals distribution and homoscedasticity) and for potential influent observations. No significant deviations from models' assumptions or outliers were found. All statistical analyses were implemented in R, using *lme4* and *nlme* packages (Bates et al. 2015, Pinheiro et. al 2018). A p value < 0.05 was considered statistically significant.

Results

Exposure to elevated CO_2 levels did not significantly affect total erythrocytes (p = 0.428, Supplemental Table S1; Fig. 1A) and total leukocytes proportions (p = 0.631, Supplemental Table S1; Fig. 1.B). Although not significant, the proportion of thrombocytes was lower in sharks exposed to increased CO_2 (p = 0.057, Supplemental Table S1; Fig. 1.C). Moreover, the proportion of erythrocytes with nuclear abnormalities, particularly (i) segmented nucleus (p = 0.124, Supplemental Table S1; Fig. 2.A), (ii) blebbed nucleus (p = 0.675, Supplemental Table S1; 2.B), and (iii) micronucleus formation (p = 1.00, Supplemental Table S1; 2.C), did not significantly vary between treatments. Similarly, there were no significant differences in the ratios analyzed, namely in (i) cytoplasm to nucleus ratio (p = 0.090, Supplemental Table S1; Fig. 3.A), (ii) spleen to body ratio (p = 0.999, Supplemental Table S1; 3.B), and (iv) heart to body ratio (p = 0.394, Supplemental Table S1; 3.C).

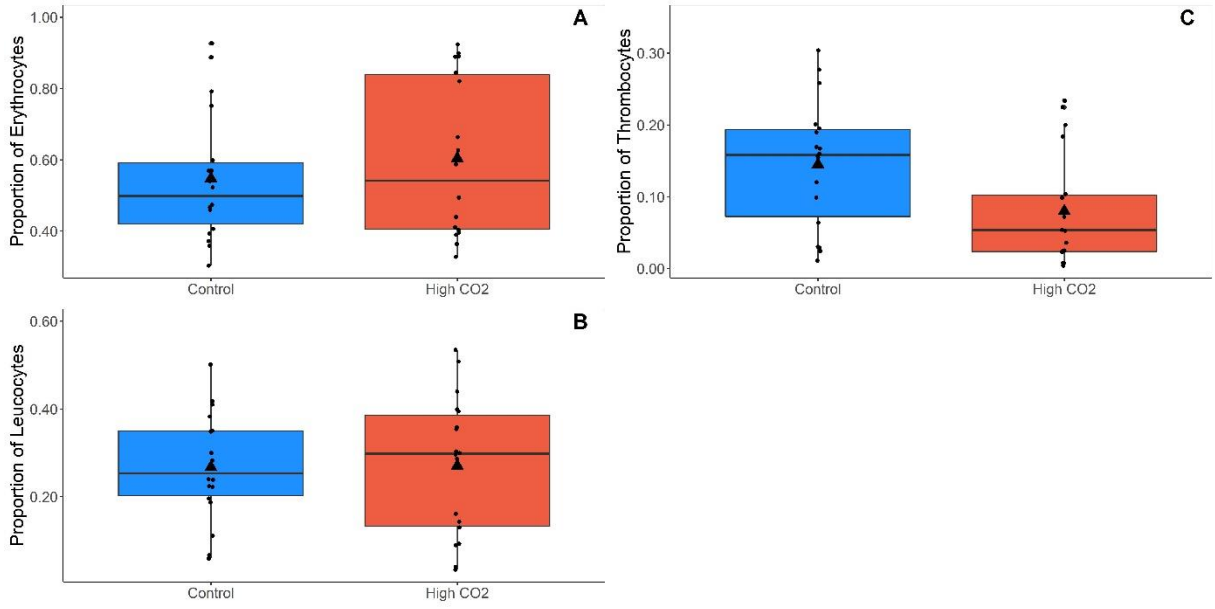

Figure 1. Effect of high CO_2 ($\Delta\ pCO_2$ ~ 500 µatm) on the estimate of the proportion of: A) erythrocytes, B) leukocytes and C) thrombocytes from blood samples of small-spotted catsharks (*Scyliorhinus canicula*, n = 18). Horizontal lines represent the median, the whiskers represent the lowest and highest values of the results, and boundaries represent the 25th and 75th percentiles. Triangles represent the mean. Additional statistical information in Supplemental Table S1.

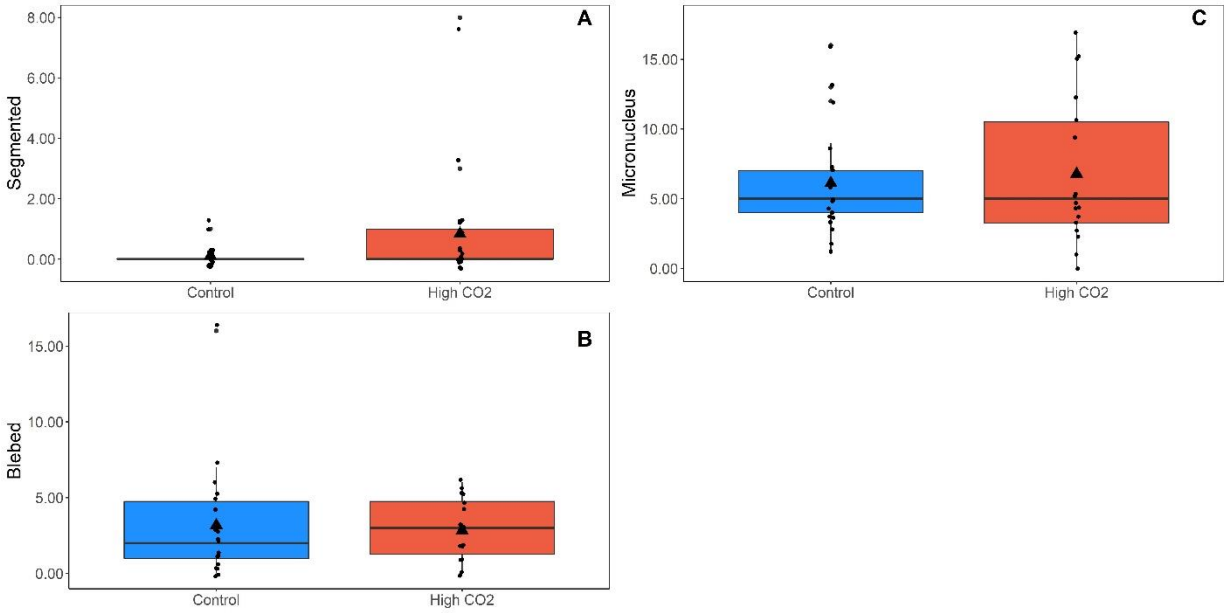

Figure 2. Effect of high CO_2 ($\Delta\ pCO_2$ ~ 500 µatm) on the estimate of the proportion of erythrocytes with nuclear anomalies: A) segmented nucleus, B) blebbed nucleus and C) micronucleus from blood samples of small-spotted catsharks (*Scyliorhinus canicula*, n = 18). Horizontal lines represent the median, the whiskers represent the lowest and highest values of the results, and boundaries represent the 25th and 75th percentiles. Triangles represent the mean. Additional statistical information in Supplemental Table S1.

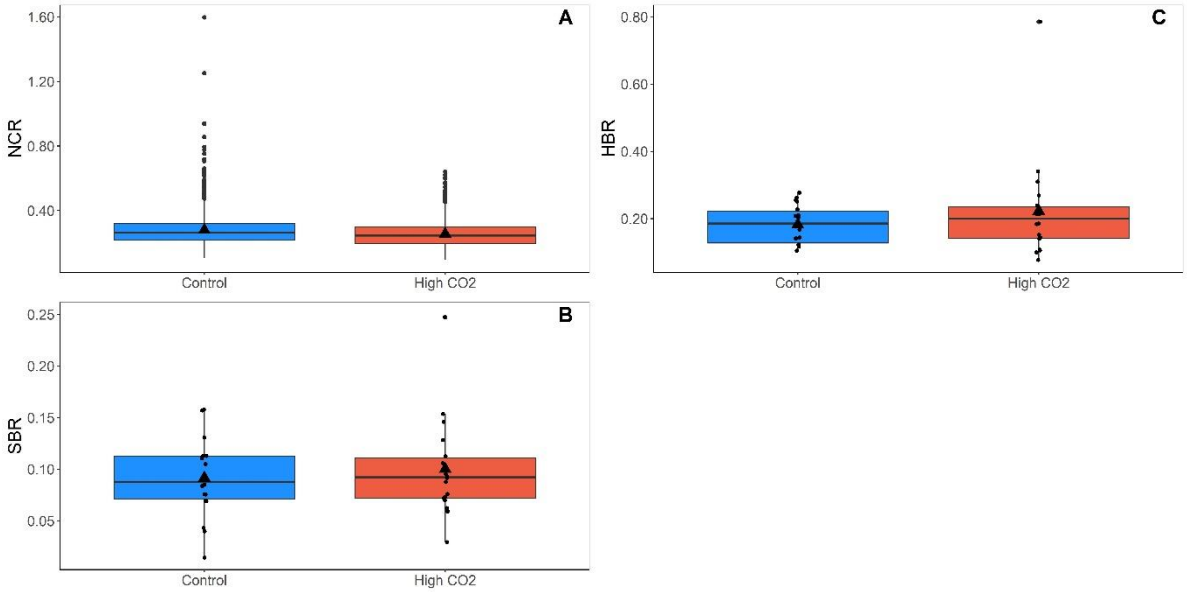

Figure 3. Effect of high CO_2 ($\Delta\ pCO_2$ ~ 500 µatm) on: A) nucleus to cytoplasmic ratio, B) spleen to body ratio and C) heart to body ratio of small-spotted catshark (*Scyliorhinus canicula*, n = 18). Horizontal lines represent the median, the whiskers represent the lowest and highest values of the results, and boundaries represent the 25th and 75th percentiles. Triangles represent the mean. Additional statistical information in Supplemental Table S1.

Discussion

Anthropic activities are dramatically increasing shark's vulnerability worldwide, mostly due to overfishing and habitat degradation (Baum et al. 2003), but little is known on how OA may affect sharks' physiology, including hematological parameters. Exposure to OA did not elicit significant alterations on this species' hematological parameters nor on the studied ratios, which may suggest a relative resilience of this species towards elevated pCO_2. Indeed, adult small-spotted catsharks have also been shown to compensate pH imbalance through elevation of HCO_3^- concentrations in their blood plasma with no changes in other physiological traits (Green and Jutfelt 2014). Sharks' sophisticated acid excretion processes (Wood, Pärt, and Wright 1995) and the fact that their hemoglobin has a higher buffering capacity than most teleost (Berenbrink et al. 2005) may confer them a certain degree of resilience to the acid–base disturbances caused by OA. Actually, it has been hypothesized that oviparous shark species may have developed successful mechanisms to cope with the higher CO_2 levels inside the egg capsules (Rosa, Rummer and Munday 2017), thus further research should focus on the effects of high CO_2 over species from other reproductive guilds.

While the present findings align with other studies, that showed that several shark species appear to be somewhat resilient to OA (Heinrich et al. 2015; Johnson et al. 2016; Rosa et al. 2017; Lopes et al. 2018; Pegado et al. 2019), there was striking, albeit not statistically significant, reduction in the proportion of thrombocytes. Thus, the present study suggests that a long-term exposure to high CO_2 translated in a tendency for a lower thrombocyte count. This is worth mentioning as future studies should further analyze the extent of this reduction along with coagulation factors, which appears to be a precise indicator of a fish's physiological state (Tavares-Dias and Oliveira 2009). Thrombocytes are essentially responsible for blood clotting, controlling loss of fluids from open wounds (Clauss, Dove and Arnold 2008), and may also have an immune function [e.g. phagocytosis; (Carrier, Musick and Heithaus 2012)]. In this context, further research is necessary to better understand the effects of OA over shark's hemostasis. Further research

should also consider the concomitant effects of OA with ocean warming, ocean deoxygenation and water contaminants on shark's hematology.

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RAPID COMMUNICATION

6 Reduced impact of ocean acidification on growth and swimming performance of newly hatched tropical sharks (*Chiloscyllium plagiosum*)

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Abstract

Sharks have been facing unprecedented pressure over the last decades, and ocean acidification may represent an additional threat, particularly during their most susceptible life stages. Hence, the present study aimed to investigate the effects of ocean acidification (control pCO_2 ~ 400 µatm; high pCO_2 ~ 900 µatm) on the growth, swimming performance and cholinergic system of juvenile whitespotted bamboo sharks (*Chiloscyllium plagiosum*). After 45 days of exposure, we observed that high CO_2 did not affect most of the endpoints studied. However, somatic growth rate and the percentage of time that sharks spent swimming was significantly reduced under high CO_2 conditions. Moreover, AChE activity decreased in two of the seven brain macroareas analyzed, the telencephalon and optic lobes. As this near-threatened shark species showed small sub-lethal effects to high CO_2 levels, we argue that within a longer timeframe they can potentially reduce individual performance with cascading consequences to shark population dynamics.

Keywords: elasmobranch; climate change; AChE; behaviour; juveniles

Introduction

The increasing emissions of carbon dioxide (CO_2) observed over the past 200 years have been disrupting the carbonate system of the oceans (Gattuso and Lavigne 2009). The CO_2 partial pressure (pCO_2) in the seawater has risen along with atmospheric values, due to the constant absorption of CO_2 by the ocean (Sabine et al. 2004) – leading to ocean acidification (OA). This has already resulted in a reduction of 0.1 units on the seawater pH (Haugan and Drange 1996) and is expected to further decrease by up to 0.32 units by the end of the century, as pCO_2 levels surpass 900 ppm – IPCC RCP scenario 8.5 (IPCC 2014). Over the past decade, it has become clear that these unprecedented rates of physicochemical change can negatively impact key biological traits of marine organisms, including calcification rates (Kleypas et al. 2005; Orr et al. 2005; Feely et al. 2009), survival (Seibel and Fabry 2003; Kroeker et al. 2010), growth (Seibel and Fabry 2003; Kroeker et al. 2010), reproduction (Kroeker et al. 2010), physiology (Pörtner et al. 2004; Heuer and Grosell 2014) and behaviour (Nilsson et al. 2012; Jutfelt et al. 2013; Munday et al. 2013; Green and Jutfelt 2014). Sharks have been roaming our oceans for 400 million years (Hashimoto et al. 1992) and, most as apex predators, represent a key element in structure and function of marine ecosystems (Heithaus et al. 2008). Having survived several mass extinctions (Raup and Sepkoski 1982; Hashimoto et al. 1992), and despite their long evolutionary history, their populations have been declining worldwide by ≥ 90% (Baum et al. 2003) due to anthropogenic pressure, such as overexploitation (Musick et al. 2000) and habitat degradation (Chin et al. 2010; DiBattista et al. 2011). Moreover, their k-strategy and long lifecycle (Carrier et al. 2012) limits their adaptive potential to a rapidly changing environment (Field et al. 2009; Chin et al. 2010). Nonetheless, the direct impacts that OA may have on sharks have only recently started to be empirically addressed (see review in Rosa et al. (2017)). Sharks exposed to elevated CO_2 showed impairments at different levels of biological organization, from molecular to organismal levels (Green and Jutfelt 2014; Rosa et al. 2014, 2016a, 2016b; Dixson et al. 2015; Heinrich et al. 2015; Pistevos et al. 2015; Johnson et al. 2016). Among the studied behavioural responses, significant OA effects were detected in odour tracking (Dixson et al. 2015), hunting (Pistevos et al. 2015) and swimming abilities (Green and Jutfelt

2014) of certain shark species. Most of these high CO_2-related effects were attributed to CO_2 interference in neurologic processes (Nilsson et al. 2012). Within this context, the aim of the present study was to understand how the increased levels of CO_2 predicted for the near future (pCO_2 ~ 900 µatm) may influence the early ontogeny of a tropical benthic shark, the whitespotted bamboo shark (*Chiloscyllium plagiosum*). This species is a tropical benthic predator that inhabits complex reef environments (Compagno 1984) of the Indo-Pacific region (Chen and Liu 2006). After 45 days of exposure to high CO_2 levels, the newly hatched juveniles were assessed regarding growth (namely Fulton condition, specific and somatic growth rates) and swimming performance (namely maximum reached velocity, percentage of time spent swimming, number of bursts, and pre and post-swimming ventilation rates). Moreover, and because the cholinergic system has been linked to neuromuscular function (Soreq and Seidman 2001), shark brains were macrodissected in seven areas: telencephalon, cerebellum, optic lobes, olfactory bulbs, diencephalon, brainstem and spinal cord, to determine acetylcholinesterase's (AChE) activity levels.

Materials and methods

Ethics statement

The experimental procedures implemented for this work were in accordance with the requirements of Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. They were also reviewed and approved by the animal ethics committee ORBEA ± Animal Welfare Body of FCUL (Statement 5/2016) and by the National Veterinary Medicines Directorate (DGAV).

Animal collection and acclimation

Whitespotted bamboo shark eggs were hand-collected by local fishermen near Lungsod Ng Cebu (Philippines: 10°11′N 123°58′E), during June–July 2016, and transported through a certified commercial supplier (TMC – Iberia). Upon arrival to the experimental aquaculture facilities at Laboratório Marítimo da Guia (Cascais, Portugal), the eggs were suspended in a recirculating aquaculture system (RAS; 200 L) under similar conditions to their natural environment (26 °C and pCO_2 ~ 400 µatm). Once hatched, each shark was transferred to an individual 50 L opaque tank coupled into a RAS and randomly allocated to either control conditions (~390 µatm CO_2; n = 5) or a simulated scenario of high CO_2 (~890 µatm CO_2, Δ – 0.3 pH units; n = 5; IPCC's RCP scenario 8.5) for 45 days (IPCC 2014). A drip-system fed each RAS, ensuring water quality and adequate carbonate speciation through a daily 50% water change with new filtered (0.35 µm; Harmsco, USA) and UV-sterilized (Vecton 600, TMC Iberia, Portugal) natural seawater. Water quality was further assured by protein skimmers (Schuran, Jülich, Germany) and biological filtration (Ouriço®, Fernando Ribeiro Lda, Portugal). Seawater pH values were monitored every 2 s and adjusted through an automated controlling system (Profilux 3.1, GHL, Germany) connected to individual probes (GHL, Germany). Required seawater pH values were achieved through pH downregulation, by injection of a certified CO_2 gas mixture (≥ 99,7%, Air Liquid, Portugal) via solenoid valves (Etopi, Portugal), or upregulation, using soda lime filtered air. Hysteresis maintained pH levels at ± 0.05 margins. Temperature was automatically adjusted with

heaters. Additionally, seawater parameters, namely pH (NBS scale), temperature, and salinity, were manually measured on a daily basis with a portable pH probe (SevenGo pro SG8, Mettler Toledo), a thermometer (TFX 430, WTW GmbH, Germany), and a refractometer (V2, TMC-Iberia, Portugal), respectively; the set-points of each RAS were adjusted as required. Ammonia, nitrites and nitrates were also monitored daily (Profi Test, Salifert, Holland). Seawater carbonate system speciation (Table 1) was calculated every week from total alkalinity (A_T) and pH measurements (Sarazin et al. 1999). Total dissolved inorganic carbon, bicarbonate, aragonite saturation and pCO_2 levels were calculated using the CO2SYS software (Lewis et al. 1998), with dissociation constants from Mehrbach et al. (1973) as adjusted by Dickson and Millero (1987). The photoperiod was kept as 12 h light: 12 h dark throughout the duration of the experiment; opaque shelters (PVC tubes) were available in each tank. Feeding took place every other day, in the late afternoon, alternating between shrimp, kingfish and squid.

Table 1. Seawater carbonate chemistry data of the experiments with embryos and juveniles of whitespotted bamboo shark (*Chiloscyllium plagiosum*). 1. Embryos at control conditions; 2. Juveniles at control conditions; 3. Juveniles at future oceanic conditions. Carbon dioxide partial pressure (pCO_2) was calculated with CO2SYS using salinity, temperature, pH, and total alkalinity (A_T). Values are mean ± SD (standard deviation).

	Temperature (°C)	Salinity	pH (total scale)	A_T (µmol kg^{-1} SW)	pCO_2 (µatm)
Embryos	25,7 ± 0,42	36 ± 0,57	8,145 ± 0,09	2251,06 ± 407,16	330,43 ± 72,93
Juveniles					
Control	26,0 ± 0,11	35 ± 0,02	8,003 ± 0,11	2269,53 ± 163,6	411,44 ± 35,81
High CO_2	26,1 ± 0,14	35 ± 0,01	7,730 ± 0,01	2323,71 ± 68,81	891,70 ± 17,83

Biometric assessment

Each animal was weighted (W) and measured (body length – BL) after hatching and following the 45 days of exposure to the experimental conditions. The Fulton condition (K) was calculated using the following formula: $K = (W/BL^3) \times 100$. Specific Growth Rate (GR), i.e. percentage of body weight per day, was calculated using the following formula: $GR = 100 (\ln W_f - \ln W_i)/t$, where W_f and W_i are the final and initial body weights (g), respectively, and t the time in days. Finally, Somatic Growth Rate (SGR), i.e. percentage of body

length per day, was determined according to the formula: SGR = 100 (Ln BL_f − Ln BL_i)/t, where BL_f and BL_i are the final and initial body lengths (g), respectively, and t the time in days.

Swimming performance

Following the biometric assessment at 45 days post-hatching, each shark was transferred to a swimming tunnel (swim chamber: 30 × 7.5 × 7.5 cm; Loligo Systems, Denmark) previously filled with water at the corresponding treatment conditions and immersed in a temperature-controlled bath. The shark could roam freely at a basal flux velocity of 0.06 $m.s^{-1}$ (ca. 0.5 body length per second) and during the first hour the percentage of time the animal spend swimming was recorded (Legria HF R56, Canon). Additionally, the number of resting ventilations were counted at three moments. This process was repeated twice, to perform two separate tests:

1. Maximum velocity and bursts

Water flow velocity was increased at a rate of 1 cm min^{-1} until the shark could no longer swim and was considered exhausted. Whenever a shark stopped swimming, it was encouraged to restart through a reversal of the water flux. This flux reversal would last a few seconds to determine if the shark was resting or in fact exhausted. In the later scenario, the animal would be unable to resume swimming and, therefore, the test would be concluded by returning to the basal flux velocity. The number of post-swimming ventilations was also registered at the end of the test, using the same methodology as above. The tests were recorded with a camera (Legria HF R56, Canon), the maximum velocity at which each shark was able to swim, and the number of bursts was registered from video analysis. A burst was considered as the impulse given to increase swimming velocity.

2. Chase protocol

A chase protocol was performed to determine ventilation in distress (Reidy et al. 2000). The animal was removed from the swimming tunnel and chased in a bucket by hand until it could no longer swim and was considered exhausted. The number of post-chase ventilations was again registered at the end of the test, using the same methodology as above. A significant water change (> 95%) took place between all trials.

Acetylcholinesterase (AChE) activity

Five days after the swimming performance test, sharks were sacrificed by immersion in MS222 (1000 mg/L, Sigma–Aldrich, USA; (Smith et al. 2004)) and their brains were dissected into seven macroareas: telencephalon (tel), diencephalon (die), cerebellum (cer), optic lobes (opt), olfactory bulbs (olf), brain stem (bs) and spinal cord (spc). Each of the seven brain macroareas (5 per treatment) were individually weighted and homogenized with a Teflon grinder, in 0.1 mL of phosphate buffered saline solution (PBS: 0.14 M NaCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4 and 2.7 mM KCl, pH 7.4). Homogenates were centrifuged at 10000 × g for 15 min at 4 °C and frozen at −80 °C until further analyses. AChE activity was determined through the adaptation of the method described by Ellman et al. (1961) to 96-well microplates (Dizer et al. 2001). Briefly, 50 µL of each sample and 250 µL of a mix solution (50 mM of sodium phosphate, 75 mM acetylthiocholine iodide and 1 mM of 5,5′-dithio-bis-2-nitrobenzoic acid) were added to a 96-well microplate. Afterwards, the absorbance was read every minute for 10 min, at 415 nm in a microplate reader (Bio-Rad, Benchmark, USA). AChE activity in each macroarea was standardized to total protein concentration (nmol min^{-1} mg^{-1} protein). Total protein content was measured spectrophotometrically through the method described by Bradford (1976).

Statistical analyses

Generalized linear models (GLM) were conducted to detect significant differences between control and high CO_2 treatments. Length was used as a covariate for swimming related statistical analysis, and pre-ventilation rates were used as a covariate of post-ventilation rates. All data analyzed followed a normal distribution, except the percentage of time spent swimming and the number of bursts, which followed a Gamma and Poisson distributions, respectively. The assumptions of normality, homogeneity of variances and absence of outliers were confirmed from residuals. Statistical analyses were performed for a significance level of 0.05, using R (Version 1.1.453 – © 2009–2018 RStudio, Inc.).

Results

All shark individuals survived the exposure period, and high CO_2 did not affect Fulton condition index (K; $p > 0.05$, Table S1; Figure 1A) neither specific growth rate (% body weight per day; $p > 0.05$, Table S1; Figure 1B). However, somatic growth rate (% body length per day; $p < 0.05$, Table S1, Figure 1C) was significantly reduced under high CO_2 exposure. Concerning the swimming performance, both maximum velocity and number of bursts were not significantly different between treatments (both $p > 0.05$, Table S1; Figure 1D, F). However, the percentage of time spent swimming was significantly lower in animals exposed to high CO_2 conditions ($p < 0.05$, Table S1; Figure 1E). The ventilation rates (for both tests) showed no differences between treatments ($p > 0.05$, Table S1; Figure 1G, H). AChE activity only decreased significantly in two brain macroareas, the telencephalon and optic lobes, under high CO_2 levels ($p < 0.05$, Table S1; Figure 2).

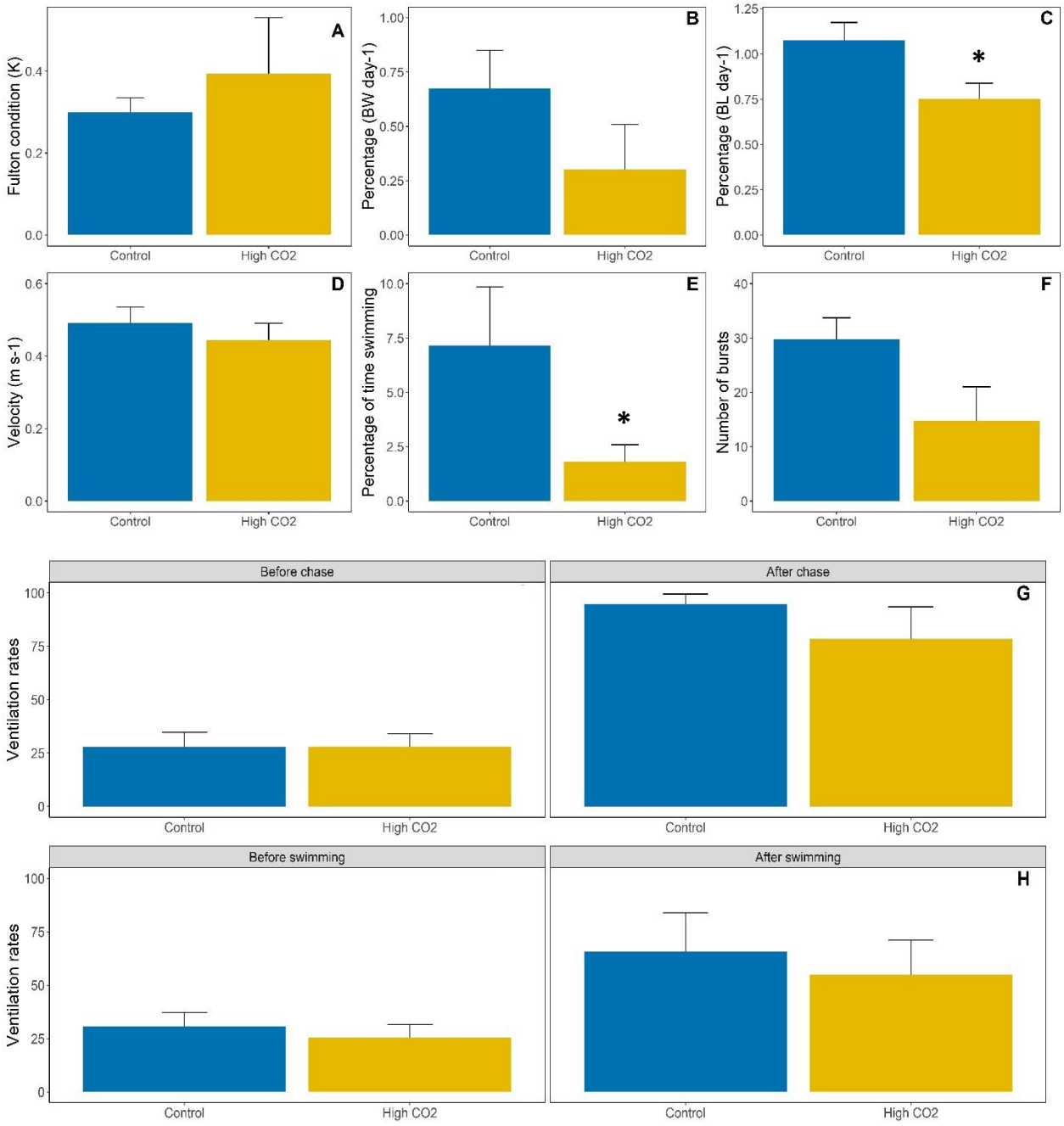

Figure 1. Effect of high CO_2 ($\Delta\ pCO_2$ ~ 500 µatm) on: (A) Fulton condition index (K), (B) specific growth rate (% body weight per day), (C) somatic growth rate (% body length per day), (D) maximum reached velocity (m s^{-1}), (E) percentage of time swimming (%), (F) number of bursts, (G) pre and post-chase ventilation rates (breaths min^{-1}) and (H) pre and post-swimming ventilation rates of juvenile whitespotted bamboo shark (*Chiloscyllium plagiosum*, n = 5). Values represent the mean (+ SD). Asterisks represent significant differences between treatments. For more statistical information see Supplemental Table S1.

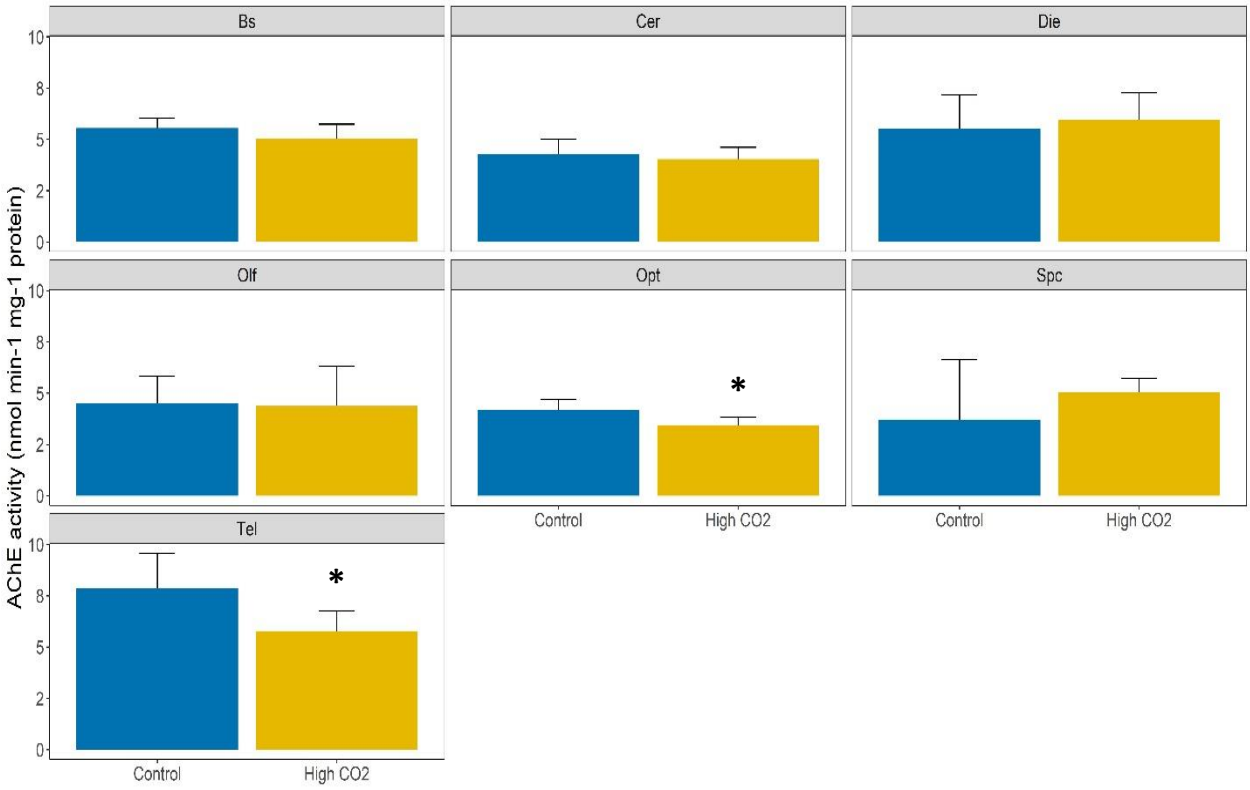

Figure 2. Impact of high CO_2 (Δ pCO_2 ~ 500 µatm) on acetylcholinesterase's (AChE) activity on seven different macroareas of juvenile whitespotted bamboo shark (*Chiloscyllium plagiosum*, n = 5). Brain areas: Bs – brainstem, Cer – cerebellum, Die – diencephalon, Olf – olfactive lobes, Opt – optic lobes, Spc – spinal cord, Tel – telencephalon. Values represent the mean (+ SD). Asterisks represent significant differences between treatments. For more statistical information see Supplemental Table S1.

Discussion

Most sharks rely on their swimming abilities to forage for food, refuge and eventually mate, as well as to escape larger predators, particularly as juveniles. In the present work, most of the studied (growth and swimming-related) response variables were not affected by environmental hypercapnia. In fact, only swimming duration and somatic growth rate were significantly reduced under high CO_2 conditions. We argue that such findings may be associated with energetic trade-offs as energetic demands for vital processes are prioritized under non-optimal conditions (Pörtner 2008; Heuer and Grosell 2014). Both growth (Post and Parkinson 2001) and swimming are highly energy-demanding ordeals (Lauder and Di Santo 2015) and the energy they require may be allocated to a continuous acid–base regulation due to exposure to high CO_2 (Pörtner et al. 2004). This chemical compensation is highly important to avoid metabolic depression (Pörtner et al. 2004; Fabry et al. 2008), although there is no evidence of metabolic compensation in sharks (Chapman and Renshaw 2009; Rosa et al. 2014; Renshaw et al. 2012). On the other hand, one may argue that diminished willingness to swim may be related to the decrease of acetylcholinesterase's (AChE) activity (Marigoudar et al. 2009), especially in the telencephalon, which activity has been linked to motivation (Flood et al. 1976). Additionally, AChE is mainly responsible for regulating the concentration of the neurotransmitter acetylcholine in synapses (Soreq and Seidman 2001) and its activity has also been linked to enhance of dopamine release (Holmes et al. 1997), a key component of motivation and reward (Berridge and Robinson 1998). Reduced AChE activity, particularly in the telencephalon (Riedel 1998), may affect an animal's behaviour since it generates an accumulation of acetylcholine that results in prolonged excitatory cholinergic postsynaptic potentials (Marigoudar et al. 2009; Bonansea et al. 2016). Considering that AChE is widely implicated in several processes (e.g. learning, memory) (Fibiger 1991), which take place specially in the telencephalon (de Bruin 1980), further consequences over the cholinergic system should also be investigated. Furthermore, a closely related shark (*C. punctatum*) exposed to high CO_2 showed no changes in AChE activity levels in the whole brain (Rosa et al. 2016b). This difference may result from an undifferentiated brain analysis instead of the approach used in our experiment. Moreover, according to

cytochemical data, AChE expression is spatiotemporally regulated during early stages of embryogenesis (Fitzpatrick-McElligott and Stent 1981), and in contrary to the present study, *C. punctatum* were exposed to much higher levels of CO_2 (pCO_2 ~ 1400 ppm) during the whole embryogenesis.

Changes in swimming behaviour have been previously reported in temperate adult catsharks. Green and Jutfelt (2014) reported a decreased number of swimming events per hour for *Scyliorhinus canicula*. Nonetheless, this is the first time a similar result is described in a tropical species and at much early developmental stages. Hence, the OA-associated changes in swimming behaviour appear to extend beyond species, life-stage and climatic region. Moreover, despite ecomorphological similarities between these species, they represent distinct branches of the elasmobranch phylogenetic tree (Da Cunha et al. 2017). Nonetheless, recently hatched sharks exposed to high CO_2 concentrations did not exhibit significant changes in their Fulton condition index, specific growth rate, maximum velocity, ventilation rates and AChE activity in the majority of the macroareas. Low statistical power may conceal some OA-induced effects, and therefore we emphasize the need for further investigation. As hypothesized by Heinrich et al. (2014) the epaulette shark (*Hemiscyllium ocellatum*) may have developed physiological features that enable them to tolerate steep chemical changes (Heinrich et al. 2014) and this type of physiological adaptation is ubiquitous to reef fishes (Nilsson and Östlund-Nilsson 2004). Since whitespotted bamboo sharks are a closely related species that can be found in coral reefs (Compagno 1984) our results also suggest a certain degree of resilience to OA, which is aligned with our previous oxidative stress-related findings (Lopes et al. 2018). Regardless, this species is already accessed as near threatened in the IUCN Red List (Kyne and Burgess 2006) and even the sub-lethal effects, as the ones here observed, have the potential to reduce individual performance with consequences over population dynamics. Removal of top predators, such as sharks, can exacerbate stress on the food web with cascading down effects, disturbing the equilibrium of prey populations (Myers et al. 2007). Moreover, we reinforce the importance of multi-stressor studies, since the effects of OA may be intensified when associated with elevated temperatures expected to occur in the future.

Disclosure statement

No potential conflict of interest was reported by the authors.

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7 General discussion and final remarks

The purpose of the present dissertation was to contribute to a deeper understanding of how ocean warming and acidification (OA) may affect important physiological traits of temperate (*Scyliorhinus canicula*) and tropical (*Chiloscyllium plagiosum*) shark species, from the molecular to organismal levels (Fig. 1). It is well known that vulnerability to climate change is species-specific, so providing information on the biological responses of shark early life stages (the most vulnerable ontogenetic stages) to climate change from two distinct climatic regions (tropical and temperate) is of paramount importance.

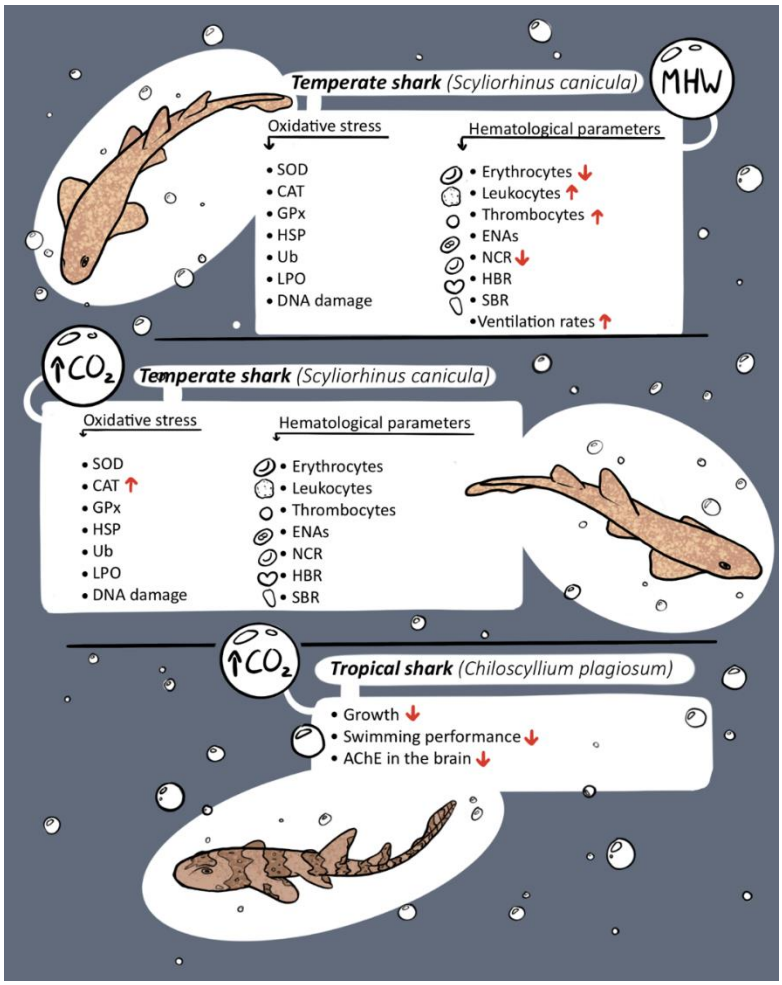

Figure 1. Summary of the main findings of the present dissertation.

7.1. Impacts of climate change across climate regions

Responses of marine species to climate change show substantial variability across climate regions (Poloczanska et al., 2013). As temperature increases, the broadest thermal windows are generally found in species from temperate latitudes that face strong seasonality (Poloczanska et al., 2016). Contrarily, tropical species seem to have relatively slim thermal windows, especially the ones that inhabit the warmest waters and are therefore near their physiological thermal tolerance limits (Storch et al., 2014). Here, the temperate species was able to acclimate to a marine heatwave (Δ 3°C), displaying significant changes in the hematological parameters (Fig. 1, Chapter 3) with no evidence of oxidative damage (Fig. 1, Chapter 2). Moreover, in an ocean warming scenario (Δ 5°C) elevated temperatures accelerated the process of embryogenesis and growth rates (Musa et al. 2020), as expected since temperature is a key factor for embryonic development of ectotherms (Wheeler et al., 2020), with no further effects on survival (Musa et al. 2020). Hence, these findings suggest that temperate small-spotted catsharks are somewhat tolerant to warming. Regardless, elevated temperatures can still drive temperate species to migrate towards the cooler waters of the poles. However, avoiding physiological issues prompted by warming may lead to exposure to higher CO_2 levels. In the poles, cooler waters absorb more CO_2 from the atmosphere, which is lowering seawater pH further than in other regions (Orr et al. 2005; Steinacher et al. 2009).

Although changes in temperature seem to have a greater effect in animal physiology than shifts in CO_2 or pH (Turley & Findlay 2016), species' responses to OA are still complex to predict and are probably influenced by each species' ecological traits (Poloczanska et al., 2016). For instance, temperate small-spotted catsharks are extremely common throughout the northeast Atlantic coast, from Southern Norway to Senegal, including in the Adriatic and Mediterranean Seas (Rodríguez-Cabello, Sánchez & Olaso 2007), and can be found at a maximum depth of 800 m (Weigmann, 2016). These ecological traits may confer this species higher tolerance to OA, since the reduction of pH did not affect specific and somatic growth rates, Fulton condition, dermal denticles' structure (Green & Jutfelt 2014), hematological profile (Fig. 1, Chapter 5) and

oxidative damage (Fig. 1, Chapter 4). OA appears to only affect behavioural traits of this species, as it reduced the number of swimming events and increased lateralization (Green & Jutfelt, 2014).

The whitespotted bamboo shark is a tropical bottom-dwelling fish that can be found from Madagascar to the Philippines, in the Indo-West Pacific ocean (Compagno 2001), on coral reefs, in tidal pools and rock crevices (Last et al., 2010), and has a smaller depth range from 0-50 m (Weigmann, 2016). Due to ocean warming, tropical species may also have to migrate towards cooler waters and endure an environment with more CO_2 (Orr et al. 2005; Steinacher et al. 2009). As whitespotted bamboo sharks live in a more stable environment, it appears that the shifts in pH predicted for the future may affect this species. Indeed, exposure to OA affected numerous physiological traits, elicited DNA damage in the liver (Lopes et al. 2018), decreased somatic growth rate, the percentage of time spent swimming and AChE activity in the brain of this tropical shark (Fig. 1, Chapter 6).

7.2. Impacts at molecular and cellular levels

7.2.1. Oxidative stress response

Exposure to elevated temperatures (Lesser, 2006) and lower pH levels can increase oxidative stress in marine organisms (Tomanek et al., 2011). Warming prompts the increase of mitochondrial oxygen turnover followed by progressive hypoxia in body fluids which, consequently, enhances oxidative stress (Abele et al., 2001). Similarly, increase of CO_2 levels can affect mitochondrial function directly and aggravate oxidative stress (Murphy, 2009; Tomanek et al., 2011). To prevent this, organisms rely on natural defense mechanisms, comprised by antioxidant enzymatic activity and a heat shock response (Wang et al. 2016; Pimentel et al. 2015; Matoo et al. 2013). In the present dissertation, small spotted catsharks exposed to a 15-day MHW (21 °C, corresponding to a category II; Chapter 2) and a long-term acclimation to OA (pCO_2 ~900 µatm; Chapter 4) did not show an overall antioxidant and heat shock responses, nor showed oxidative damage in any of the studied tissues (i.e. gills, liver and muscle). However, exposure to OA led to a significant increase of CAT activity in the muscle, displaying tissue-specific differences in activity (Chapter 4). One of

the negative impacts of OA is an increase of H^+ ions, which can directly interact with H_2O or the superoxide ion (O_2^-) to form peroxide hydrogen (H_2O_2) leading to an increased toxicity level (Tiedke et al. 2013). CAT is responsible for converting H_2O_2 back to H_2O and O_2 (Fridovich, 1983) and at higher levels of H_2O_2 this enzyme becomes more important than other enzymes to eliminate this ROS (López-Cruz et al., 2010). Accordingly, it appears that this increased CAT activity in the muscle sufficed to neutralize higher ROS production.

Moreover, even the short-term acclimation to elevated temperatures should, in theory, have increased antioxidant enzyme activity, and especially heat shock proteins. Similar findings were reported in teleost fish where warmer temperatures did not produce any alterations in antioxidant enzyme activities (Grim et al., 2010; Mueller et al., 2012; Machado et al., 2014). However, heat shock proteins are among the most eminent proteins induced by elevated temperatures and were thus expected to increase. Yet, sharks are also equipped with trimethylamine N-oxide (TMAO), that besides counteracting the toxic effects of urea (Seibel & Walsh 2002), also function as chaperones to refold damaged protein and may enhance these shark's thermal tolerance.

In elasmobranchs, a short-term exposure of 50 days (pCO_2 ~ 900 µatm) elicited increased DNA damage in the liver of a tropical shark's (*Chiloscyllium plagiosum*) (Lopes et al., 2018). This tissue-specific damage can occur as the liver is considered the most active tissue metabolically (Van der Oost et al., 2003), where many reactions occur and numerous sources of ROS exist (Regoli & Giuliani, 2014). A prolonged exposure during the whole embryogenesis plus 30 days (pCO_2 ~ 1500 µatm) of another tropical shark (*Chiloscyllium punctatum*) did not elicit any oxidative stress response or damage. However, the combination of warming and OA increased antioxidant response, mainly in the brain and increased lipid peroxidation in the muscle and brain (Rosa et al., 2016). This increased susceptibility of tropical sharks species may arise from their higher demand for oxygen uptake (Baldwin & Wells, 1990), which is the main source of ROS production (Halliwell & Gutteridge, 2015).

Since physiological compensation at the organism level is ultimately established through changes at the molecular and biochemical levels (Hochachka & Somero 2002), the present findings suggest that small-spotted catsharks will be able to cope with sudden warming events that are occurring in the ocean and the high CO_2 levels expected to occur in the future.

7.2.2. Acetylcholinesterase activity

Acetylcholinesterase (AChE) is an enzyme responsible for degrading and regulating the levels of acetylcholine, which is the primary neurotransmitter in the neuromuscular and sensory systems of fish (Kirby et al., 2000). The activity of these two components is essential for muscular function and normal behaviour (Payne et al., 1996). However, as an enzyme, AChE activity has an optimum pH range outside of which its activity decreases (Reiner & Aldridge, 1967) and from this reduction can result a buildup of acetylcholine responsible for abnormal behaviours (Kirby et al., 2000). In the present dissertation, exposure to OA reduced AChE activity in whitespotted bamboo sharks' brain (Chapter 6), indeed, high levels of CO_2 could lead to stronger AChE inhibition (Sampaio et al. 2016). This result could also be related to increased oxidative stress found in this tropical shark's liver (Lopes et al., 2018), since oxidative stress can be responsible for inactivating AChE (Weiner et al. 1994). A different result was shown in a closely related shark (*Chiloscyllium punctatum*), as there were no changes of AChE levels in the whole brain (Rosa et al. 2016). This contradictory output could have resulted from the undifferentiated brain analysis, the higher levels of CO_2 used and the longer time of exposure. Regardless, AChE activity was particularly reduced in the telencephalon of whitespotted bamboo sharks (Chapter 6), which is considered as a high order coordinating center in elasmobranchs, responsible for influence motor behaviour and multisensorial input (Smeets, 1990). Hence, changes in the cholinergic system due to OA exposure could not only be affecting sharks' behaviour but also their ability to properly respond to sensory cues. However, this mechanism has not been studied in fish. Behavioural changes associated to OA exposure have been linked to changes of $GABA_A$ receptor from inhibitory to excitatory in teleost fish (Nilsson et al., 2012), which has been found to

potentiate behavioural and sensorial impairments, such as lateralization and loss of olfaction (Domenici et al. 2012). In fact, exposure to OA showed increased lateralization in adult small-spotted catsharks (Green & Jutfelt 2014) and reduced the ability of smooth dogfish to follow an olfactory cue (Dixson et al., 2015). In any case, the underlying mechanisms responsible for behavioural changes in sharks have not yet been studied.

7.2.3. Hematological changes

Both ocean warming and acidification may elicit hematological changes in marine organisms. Elevated temperatures can affect the blood in several ways; warming can disturb the normal structure of cell membranes (Farkas et al., 2001), it can lower oxygen dissolved in seawater while increasing its requirement to sustain the metabolism (Pörtner, 2006), and severely affect the immune system (Magnadottir, 2010). On the other hand, to compensate pH imbalance caused by OA, marine animals can accumulate HCO_3^- in the blood plasma (Green & Jutfelt 2014). The reduction of pH may also reduce effective oxygen transport and an efficient acid-base regulation under high CO_2 can elicit changes on blood cells (Pörtner et al., 2011), such as a weakened immune system (Liu et al., 2016). In the present dissertation, exposure to a 15-day MHW (21°C, corresponding to a category II; Chapter 3) altered small-spotted catshark blood cell proportions (including a decrease of erythrocyte's nucleus to cytoplasmic ratio) and increased ventilation rates while a long-term acclimation to OA (pCO_2 ~900 µatm; Chapter 5) did not elicit any blood-related changes, which suggests a certain resilience of this species towards OA.

Exposure to warmer waters increased erythrocytes (Chapter 3) as a result of erythropoiesis, i.e. formation of new erythrocytes. Moreover, the increased number of erythrocytes with lower nucleus to cytoplasm ratio (NCR) also indicates that new and immature erythrocytes are being produced. Hence, to cope with the environmental changes caused by warming, sharks produced more and smaller erythrocytes and increased their ventilation rates, to allow a quicker oxygen transfer rate and improve oxygen delivery to the cells (Lay & Baldwin, 1999).

To further evaluate the stress caused by environmental changes, erythrocytes with nuclear abnormalities (ENAs) and differential leukocyte counts are important analytical factors (Ghaffar et al. 2015). ENAs can increase due to lipid damage in erythrocytes of fish that were exposed to elevated temperature (Bai et al., 2014; Ghaffar et al., 2015). In the present dissertation, there was no evidence of increased ENAs during exposure to elevated temperatures (Chapter 3), or high CO_2 levels (Chapter 5) which was expected since we did not find increased lipid peroxidation in any of the tissues analyzed (Chapter 2 and 4). However, to deal with increased pathogenesis potentiated by warmer temperatures (Zaragoza et al., 2008), small spotted catsharks raised leukocyte and thrombocyte cells (Chapter 3) as both are essential for protection against pathogens (Opdenakker et al. 1998; Carrier et al. 2012). Since small spotted catsharks perform diel vertical migrations in which temperatures can vary from 1 to 8 °C (Sims et al., 2006), the hematological changes here observed, associated with the lack of oxidative damage suggest that these diel changes between temperatures may have conferred them an increased resistance to the sudden warming events that are currently occurring without a substantial disruption of physiological homeostasis, and could even return to normal levels as temperatures returns to normal. Thus, it appears that both ocean warming as acidification will not have detrimental effects on these juvenile shark species.

It is worth mentioning that small-spotted catsharks exposed to warming conditions (Δ 5°C) during the entire embryogenesis showed significant impacts on important physiological traits. Elevated temperatures accelerated the embryogenesis process, increasing growth and specific growth rates, and yolk-consumption. This resulted in smaller newly hatched sharks, with reduced body length and mass while increased the Fulton's condition factor (Musa et al. 2020). In this extreme context, warming appears to have significant implications for the ecology of this juvenile shark. Nonetheless, the impacts that these two stressors combined may have on this species is still unknown.

7.3. Impacts at organismal level

7.3.1. Growth rates

Among the negative effects of CO_2 on animal physiology are the associated costs of maintaining acid-base regulation (Perry & Gilmour, 2006). Disturbances in the acid–base balance during OA exposure can result in metabolic depression and consequently reduced growth (Pörtner et al., 2004; Michaelidis et al., 2005, 2007). In the present dissertation, physiological compensation to high CO_2 exposure may come with energetic costs that reduced somatic growth rate of whitespotted bamboo sharks (Chapter 6). The effects of OA on growth rates can vary among different groups and life stages (Kroeker et al., 2010). For instance, in epaulette shark embryos, OA did not elicit changes on growth rates (Johnson et al., 2016). Similarly, juvenile Port Jackson sharks exposed to OA alone did not manifested alterations in growth. However, when exposed to OA in combination with warming their growth was reduced, and when held in mesocosms where these sharks had to hunt for their food, exposure to OA alone was enough to also reduced growth rates (Pistevos et al., 2015). Adults puffadder shy shark (Dziergwa et al., 2019) and small spotted catshark (Green & Jutfelt, 2014) showed no alterations on growth rates. As the effects on growth rate are indeed species-specific, OA may have profound effects on marine food webs, as impairment of efficient growth in predator populations may reduce its sustainability (Pistevos et al., 2015). The predator/prey relationship throughout marine ecosystems depends deeply on the predator's size (Barnes et al., 2010) and different vulnerability levels to increase CO_2 levels that leads to changes in predator's body size will have cascading effects to other species (Pistevos et al., 2015).

As predators, and specially as preys during their early life stages, whitespotted bamboo sharks can be at risk when exposed to high CO_2 levels (Chapter 6). Furthermore, although not significant, the Fulton condition was tendentially higher during OA exposure, and field studies have shown that there is a predatory selection over juveniles with slower growth rates and higher Fulton's condition factor (Hoey and McCormick, 2004). Although the impacts over the mechanisms responsible for growth in acidified waters are not yet known, a fast growth rate and a larger size would had benefited these newly hatched juveniles (Bergenius et al., 2002;

Meekan et al., 2003) as it would offer a competitive advantage and less time during which the individuals would be smaller and thus more vulnerable (Jones & McCormick, 2002). Hence, although the reduction in growth rates observed in whitespotted bamboo sharks is a sublethal effect (Chapter 6), it may still be detrimental and translate into a higher level of vulnerability to predators.

7.3.2. Swimming performance

As juveniles, sharks must rely on their swimming abilities to hunt, escape and hide from predators. Here, exposure of whitespotted bamboo sharks to OA did not alter most of the swimming-related response variables. However, the duration of swimming was significantly reduced (Chapter 6). Similarly to the reduction of somatic growth rate, the reduction of time spent swimming could be associated with the energetic trade-offs caused by exposure to OA. Swimming is extremely costly, as it makes up for a considerable part of the costs on the energy budget of a marine animal (Videler, 1993).

When sharks swim, their bodies undulate smoothly through the water as their skin has unique drag reducing properties. Shark's skin is composed by small bony dermal denticles embedded into the dermis (Kemp, 1999; Motta, 1977) to enhance swimming performance. Recently, exposure to OA evidenced denticle corrosion on the skin of puffadder shy sharks (*Haploblepharus edwardsii*), a tropical shark. The corrosion of these dermal structures can potentially undermine skin protection and hydrodynamics (Dziergwa et al., 2019), with possible consequences on swimming performance. When analyzing swimming performance, OA had no effect on the swimming speed or activity level of smooth dogfish (*Mustelus canis*) (Dixson et al., 2015) and Port Jackson sharks (*Heterodontus portujacksoni*) (Pistevos et al., 2017); while it decreased the number of swimming events per hour of adults small spotted catshark (*Scyliorhinus canicula*), a temperate shark (Green & Jutfelt, 2014). Hence, the OA-related changes on swimming behaviour appear to impact sharks differently across species, life-stages and climatic region.

The reduction of time that whitespotted bamboo sharks spent swimming (Chapter 6) may increase these sharks' susceptibility to predators and/or reduce their hunting success, which in turn could further reduce their growth rates in a positive feedback loop.

Lastly, this swimming reduction could also be due to behavioural impairments caused by the chemical changes driven by high CO_2 in the environment

7.4. Conclusions and future directions

The present dissertation has shown that sharks may be to some extent tolerant to a changing ocean. Nonetheless, temperature appears to have a greater impact over the hematological parameters of the studied temperate shark than OA. Exposure to increased temperatures elicited changes in normal blood cells count and increased ventilation rates, suggesting that temperature plays an important role over this species' vulnerability status. Moreover, the studied tropical species appears to be more vulnerable to OA than the temperate one, presenting sublethal impacts over important physiological traits. As OA is intensifying and sudden warming events in the oceans are becoming more frequent, sharks may be particularly vulnerable in the future. Hopefully, the insights of the present dissertation will instigate further research on this field and can promote effective management actions to mitigate and diminish the negative impacts of anthropogenic CO_2 emissions on marine ecosystems.

As the present dissertation sheds a light over some physiological implications that ocean warming and acidification may have on temperate and tropical sharks, there is still a limitation to these predictions. To fully understand the impacts of these two stressors, further investigation should study the effects of both warming and acidification combined. Moreover, as marine heatwaves solely elicited several hematological changes, further research should investigate the possibility for recovering as temperatures return to control levels. Lastly, since there where changes in AChE activity during OA exposure, future research should study the impacts that this stressor may have on shark's behaviour and the underlying mechanisms that caused such changes. The acid-base disturbances caused by OA could affect the $GABA_A$ receptors and other

neurobiological mediators such as glycine (Tresguerres & Hamilton et al. 2017) or cholinergic receptors. Identifying these mechanisms may also provide insight into their adaptive capacity to OA and allow more accurate predictions of the impacts to shark populations in more acidic oceans.

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Supplemental Material

Chapter 2

Table S1. Statistical analyses of juvenile small-spotted catshark (*Scyliorhinus canicula*) response to a marine heatwave on the: antioxidant machinery (superoxide dismutase - SOD, catalase - CAT and glutathione peroxidase - GPx), protein repair and removal (heat shock response - HSP and ubiquitin - Ub), and oxidative damage in lipids (lipid peroxidation - LPO) and DNA (DNA damage). Std Error – Standard Error.

Model: GLM (Gamma) Random effect: Replicates				Response variable: SOD
	Estimate	Std. Error	*t* value	*p* value
(Intercept)	3.649	0.080	45.359	**< 2 e-16 *** **
Marine heatwave	0.066	0.092	0.719	0.472
Gills - Liver	-0.141	0.074	- 1.900	0.058
Gills - Muscle	0.113	0.074	1.527	0.127
Muscle - Liver	0.254	0.072	3.522	**0.0004 *** **
Model: GLM (Gamma) Random effect: Replicates				Response variable: SOD (muscle)
	Estimate	Std. Error	*t* value	*p* value
(Intercept)	3.793	0.031	122.772	**< 2 e-16 *** **
Marine heatwave	0.014	0.043	0.333	0.739
Model: GLM (Gamma) Random effect: Replicates				Response variable: SOD (gills)
	Estimate	Std. Error	*t* value	*p* value
(Intercept)	3.639	0.117	31.019	**< 2 e-16 *** **
Marine heatwave	0.068	0.158	0.427	0.669
Model: GLM (Gamma) Random effect: Replicates				Response variable: SOD (liver)
	Estimate	Std. Error	*t* value	*p* value
(Intercept)	3.482	0.125	27.89	**< 2 e-16 *** **
Marine heatwave	0.106	0.173	0.61	0.542
Model: GLM (Gamma) Random effect: Replicates				Response variable: CAT
	Estimate	Std. Error	*t* value	*p* value
(Intercept)	2.203	0.095	23.071	**< 2 e-16 *** **
Marine heatwave	0.074	0.105	0.706	0.48
Gills - Liver	1.249	0.097	12.834	**< 2 e-16 *** **
Gills - Muscle	-0.47	0.097	- 4.840	**1.3 e-06 *** **
Muscle - Liver	1.719	0.097	17.703	**< 2 e-16 *** **

Model: GLM (Gaussian)				Response variable: CAT (muscle)
Random effect: Replicates				
	Estimate	Std. Error	*t* value	*p* value
(Intercept)	5.004	1.136	4.404	**0.0105 ***
Marine heatwave	1.808	1.596	1.133	0.319
Model: GLM (Gamma)				Response variable: CAT (gills)
Random effect: Replicates				
	Estimate	Std. Error	*t* value	*p* value
(Intercept)	2.19	0.076	28.708	**< 2 e-16 *** **
Marine heatwave	0.091	0.106	0.857	0.391
Model: GLM (Gaussian)				Response variable: CAT (liver)
Random effect: Replicates				
	Estimate	Std. Error	*t* value	*p* value
(Intercept)	35.286	2.662	13.254	**1.114 e-11 *** **
Marine heatwave	-5.058	3.686	-1.372	0.184
Model: GLM (Gamma)				Response variable: GPx
Random effect: Replicates				
	Estimate	Std. Error	*t* value	*p* value
(Intercept)	1.725	0.199	8.653	**< 2 e-16 *** **
Marine heatwave	0.015	0.244	0.062	0.951
Gills - Liver	0.541	0.157	3.438	**0.0005 *** **
Gills - Muscle	-0.275	0.158	- 1.745	0.081
Muscle - Liver	0.817	0.155	5.281	**1.29 e-07 *** **
Model: GLM (Gaussian)				Response variable: GPx (muscle)
Random effect: Replicates				
	Estimate	Std. Error	*t* value	*p* value
(Intercept)	5.551	1.278	4.342	**0.014 ***
Marine heatwave	-1.92	1.755	- 1.094	0.346
Model: GLM (Gamma)				Response variable: GPx (gills)
Random effect: Replicates				
	Estimate	Std. Error	*t* value	*p* value
(Intercept)	1.603	0.2717	5.904	**3.54 e-09 *** **
Marine heatwave	0.167	0.382	0.438	0.661
Model: GLM (Gamma)				Response variable: GPx (liver)
Random effect: Replicates				
	Estimate	Std. Error	*t* value	*p* value
(Intercept)	2.138	0.152	14.034	**< 2 e-16 *** **
Marine heatwave	0.252	0.211	1.193	0.233

Model: GLM (Gamma)				Response variable: HSP
Random effect: Replicates				
	Estimate	Std. Error	*t* value	*p* value
(Intercept)	-1.672	0.013	-130.883	**< 2 e-16 *** **
Marine heatwave	-0.008	0.016	-0.517	0.605
Gills - Liver	-0.039	0.009	-4.349	**1.37 e-05 *** **
Gills - Muscle	0.554	0.009	61.073	**< 2 e-16 *** **
Muscle - Liver	0.593	0.009	66.188	**< 2 e-16 *** **
Model: GLM (Gamma)				Response variable: HSP (muscle)
Random effect: Replicates				
	Estimate	Std. Error	*t* value	*p* value
(Intercept)	-1.125	0.004	-312.654	**< 2 e-16 *** **
Marine heatwave	0.007	0.005	1.362	0.173
Model: GLM (Gamma)				Response variable: HSP (gills)
Random effect: Replicates				
	Estimate	Std. Error	*t* value	*p* value
(Intercept)	-1.675	0.028	-59.588	**< 2 e-16 *** **
Marine heatwave	-0.0003	0.040	-0.008	0.994
Model: GLM (Gamma)				Response variable: HSP (liver)
Random effect: Replicates				
	Estimate	Std. Error	*t* value	*p* value
(Intercept)	-1.698	0.020	-84.819	**< 2 e-16 *** **
Marine heatwave	-0.035	0.028	-1.231	0.218
Model: GLM (Gamma)				Response variable: Ub
Random effect: Replicates				
	Estimate	Std. Error	*t* value	*p* value
(Intercept)	-1.284	0.043	-29.541	**< 2 e-16 *** **
Marine heatwave	-0.094	0.049	-1.905	0.057
Gills - Liver	0.045	0.042	1.088	0.277
Gills - Muscle	0.165	0.042	3.980	**6.9 e-05 *** **
Muscle - Liver	-0.12	0.041	-2.929	**0.003 ****
Model: GLM (Gamma)				Response variable: Ub (muscle)
Random effect: Replicates				
	Estimate	Std. Error	*t* value	*p* value
(Intercept)	-1.148	0.048	-23.538	**< 2 e-16 *** **
Marine heatwave	-0.046	0.069	-0.662	0.508
Model: GLM (Gamma)				Response variable: Ub (gills)
Random effect: Replicates				
	Estimate	Std. Error	*t* value	*p* value
(Intercept)	-1.297	0.101	-12.842	**< 2 e-16 *** **
Marine heatwave	-0.086	0.142	-0.608	0.544

Model: GLM (Gamma) Random effect: Replicates				Response variable: Ub (liver)
	Estimate	Std. Error	*t* value	*p* value
(Intercept)	-1.214	0.080	-15.112	**< 2 e-16 *** **
Marine heatwave	-0.154	0.116	-1.333	0.182
Model: GLM (Gamma) Random effect: Replicates				Response variable: LPO
	Estimate	Std. Error	*t* value	*p* value
(Intercept)	-1.489	0.029	- 52.084	**< 2 e-16 *** **
Marine heatwave	0.014	0.055	0.256	0.798
Gills - Liver	0.081	0.029	2.816	**0.005 ****
Gills - Muscle	0.007	0.028	0.244	0.807
Muscle - Liver	-0.074	0.073	- 1.021	0.307
Model: GLM (Gamma) Random effect: Replicates				Response variable: LPO (muscle)
	Estimate	Std. Error	*t* value	*p* value
(Intercept)	-1.466	0.024	- 60.948	**< 2 e-16 *** **
Marine heatwave	-0.024	0.026	- 0.917	0.359
Model: GLM (Gamma) Random effect: Replicates				Response variable: LPO (gills)
	Estimate	Std. Error	*t* value	*p* value
(Intercept)	-1.533	0.079	-19.326	**< 2 e-16 *** **
Marine heatwave	0.081	0.112	0.726	0.468
Model: GLM (Gamma) Random effect: Replicates				Response variable: LPO (liver)
	Estimate	Std. Error	*t* value	*p* value
(Intercept)	-1.409	0.121	-11.69	**< 2 e-16 *** **
Marine heatwave	-0.006	0.167	-0.04	0.968
Model: GLM (Gamma) Random effect: Replicates				Response variable: DNA
	Estimate	Std. Error	*t* value	*p* value
(Intercept)	-1.596	0.052	- 30.772	**< 2 e-16 *** **
Marine heatwave	-0.097	0.051	- 1.910	0.056
Gills - Liver	0.229	0.061	3.750	**0.0001 *** **
Gills - Muscle	0.292	0.062	4.708	**2.5 e-06 *** **
Muscle - Liver	0.063	0.061	1.043	0.297
Model: GLM (Gamma) Random effect: Replicates				Response variable: DNA (muscle)
	Estimate	Std. Error	*t* value	*p* value
(Intercept)	-1.281	0.961	- 13.33	**< 2 e-16 *** **
Marine heatwave	-0.143	0.131	- 1.087	0.277

Model: GLM (Gamma) Random effect: Replicates			Response variable: DNA (gills)	
	Estimate	Std. Error	*t* value	*p* value
(Intercept)	-1.595	0.05	-33.372	**< 2 e-16 *** **
Marine heatwave	-0.101	0.066	-1.526	0.127
Model: GLM (Gamma) Random effect: Replicates			Response variable: DNA (liver)	
	Estimate	Std. Error	*t* value	*p* value
(Intercept)	-1.393	0.051	-27.739	**< 2 e-16 *** **
Marine heatwave	-0.05	0.069	-0.729	0.466

Table S1. Statistical analysis of small-spotted catshark's normal blood cells (leukocytes, thrombocytes and erythrocytes), erythrocytes with micronucleus, ratios (nucleus to cytoplasmic, spleen and heart to body ratios) and ventilation rates. Std Error – Standard Error.

Model: GLM (Binomial) Random effect: Replicates			Response variable: Erythrocytes (proportion)	
	Estimate	Std. Error	*z* value	*p* value
(Intercept)	2.761	0.081	33.975	**< 2 e-16***
MHW	- 0.707	0.112	6.332	**2.42 e-10***
Model: GLM (Binomial) Random effect: Replicates			Response variable: Leukocytes (proportion)	
	Estimate	Std. Error	*z* value	*p* value
(Intercept)	- 2.964	0.085	- 34.807	**< 2 e-16***
MHW	0.731	0.117	6.261	**3.83 e-10***
Model: GLM (Binomial) Random effect: Replicates			Response variable: Thrombocytes (proportion)	
	Estimate	Std. Error	*z* value	*p* value
(Intercept)	- 4.785	0.178	- 26.903	**< 2 e-16***
MHW	0.648	0.239	2.718	**0.007***
Model: GLM (Binomial) Random effect: Replicates			Response variable: ENAs (Micronucleus)	
	Estimate	Std. Error	*z* value	*p* value
(Intercept)	0.693	0.612	1.132	0.258
MHW	0.916	0.908	1.009	0.313
Model: GLM (Gaussian) Random effect: Replicates			Response variable: Spleen to body ratio	
	Estimate	Std. Error	*t* value	*p* value
(Intercept)	0.217	0.029	7.359	0.000
MHW	- 0.006	0.042	- 0.138	0.897
Model: GLM (Gaussian) Random effect: Replicates			Response variable: Heart to body ratio	
	Estimate	Std. Error	*t* value	*p* value
(Intercept)	8.805	0.508	17.323	**< 2 e-16***
MHW	- 0.877	0.684	- 1.282	0.200

Model: GLM (Gamma)			Response variable: Nucleus to cytoplasmic ratio	
Random effect: Replicates				
	Estimate	Std. Error	*t* value	*p* value
(Intercept)	- 1.343	0.078	- 17.311	**< 2 e-16***
MHW	- 0.247	0.109	- 2.249	**0.025***
Model: GLMM (Gaussian)			Response variable: Ventilation rates	
Random effect: Replicates				
	numDF	denDF	*F* value	*p* value
(Intercept)	1	64	633.339	< 0.001
Treatment	1	64	6.2408	0.0151
Ventilations	2	64	3.1904	0.0478
Treatment: Ventilations	2	64	5.5214	0.0061
	Estimate	Std. Error	*z* value	*p* value
(Intercept)				
MHW (T0) – Control (T0)	4.417	4.053	- 1.090	0.87505
Control (T1) – Control (T0)	0.750	2.758	0.272	0.99977
MHW (T1) – Control (T0)	8.583	4.053	2.118	0.25860
Control (T15) – Control (T0)	- 5.167	2.758	- 1.874	0.39546
MHW (T15) – Control (T0)	8.083	4.053	1.994	0.32386
Control (T1) – MHW (T0)	5.167	4.053	1.275	0.78284
MHW (T1) – MHW (T0)	13.000	2.758	4.714	**< 0.001 *** **
Control (T15) – MHW (T0)	- 0.750	4.053	-0.185	0.99997
MHW (T15) – MHW (T0)	12.500	2.758	4.533	**< 0.001 *** **
MHW (T1) – Control (T1)	7.833	4.053	1.933	0.35935
Control (T15) – Control (T1)	-5.917	2.758	- 2.146	0.24498
MHW (T15) – Control (T1)	7.333	4.053	1.809	0.43611
Control (T15) – MHW (T1)	- 13.750	4.053	- 3.392	**0.00812 ** **
MHW (T15) – MHW (T1)	- 0.500	2.758	- 0.181	0.99997
MHW (T15) – Control (T15)	13.250	4.053	3.269	**0.01240 ***

Table S1. Statistical analysis of *Scyliorhinus canicula*'s antioxidant response (superoxide dismutase - SOD, catalase - CAT and glutathione peroxidase - GPx), oxidative stress response (heat shock response - HSP and ubiquitin - Ub), and oxidative damage in lipids (lipid peroxidation – LPO) and DNA (DNA damage, 8-OHdG). Std Error – Standard Error.

Model: GLM (Gamma) Random effect: Replicates			Response variable: SOD	
	Estimate	Std. Error	*t* value	*p* value
(Intercept)	3.602	0.087	41.267	**<2 e-16 *****
High CO_2	-0.061	0.088	-0.686	0.492
Gills - Liver	0.116	0.107	1.083	0.279
Gills - Muscle	-0.38	0.109	-3.483	**0.0005 *****
Muscle - Liver	0.496	0.109	4.517	**6.26 e-06 *****
Model: GLM (Gamma) Random effect: Replicates			Response variable: SOD (muscle)	
	Estimate	Std. Error	*t* value	*p* value
(Intercept)	3.207	0.124	25.79	**<2 e-16 *****
High CO_2	-0.035	0.175	-0.202	0.84
Model: GLM (Gamma) Random effect: Replicates			Response variable: SOD (gills)	
	Estimate	Std. Error	*t* value	*p* value
(Intercept)	3.641	0.101	36.239	**<2 e-16 *****
High CO_2	-0.139	0.14	-0.993	0.321
Model: GLM (Gamma) Random effect: Replicates			Response variable: SOD (liver)	
	Estimate	Std. Error	*t* value	*p* value
(Intercept)	3.641	0.141	26.024	**<2 e-16 *****
High CO_2	0.002	0.202	0.012	0.991
Model: GLM (Gaussian) Random effect: Replicates			Response variable: CAT	
	Estimate	Std. Error	*t* value	*p* value
(Intercept)	13.482	1.0345	13.032	**<2 e-16 *****
High CO_2	-0.182	1.027	-0.177	0.86
Gills - Liver	8.103	1.249	6.486	**3.29 e-09 *****
Gills - Muscle	-7.272	1.267	-5.739	**1.00 e-07 *****
Muscle - Liver	15.375	1.258	12.218	**<2 e-16 *****

Model: GLM (Gamma)			Response variable: CAT (muscle)	
Random effect: Replicates				
	Estimate	Std. Error	*t* value	*p* value
(Intercept)	1.693	0.07	24.028	**<2 e-16 *** **
High CO_2	0.224	0.099	2.247	**0.025 ***
Model: GLM (Gamma)			Response variable: CAT (gills)	
Random effect: Replicates				
	Estimate	Std. Error	*t* value	*p* value
(Intercept)	2.648	0.102	26.085	**<2 e-16 *** **
High CO_2	-0.109	0.141	-0.773	0.439

Model: GLM (Gaussian)			Response variable: CAT (liver)	
Random effect: Replicates				
	Estimate	Std. Error	*t* value	*p* value
(Intercept)	21.696	1.509	14.381	**5.14 e-16 *** **
High CO_2	-0.404	2.134	-0.189	0.851
Model: GLM (Gamma)			Response variable: GPx	
Random effect: Replicates				
	Estimate	Std. Error	*t* value	*p* value
(Intercept)	0.958	0.125	7.647	**2.05 e-14 *** **
High CO_2	0.238	0.141	1.687	0.092
Gills - Liver	0.45	0.131	3.436	**0.001 *** **
Gills - Muscle	-0.429	0.1339	-3.205	**0.001 *** **
Muscle - Liver	0.879	0.133	6.607	**3.92 e-11 *** **
Model: GLM (Gamma)			Response variable: GPx (muscle)	
Random effect: Replicates				
	Estimate	Std. Error	*t* value	*p* value
(Intercept)	0.569	0.131	4.338	**1.44 e-05 *** **
High CO_2	0.141	0.185	0.765	0.444
Model: GLM (Gamma)			Response variable: GPx (gills)	
Random effect: Replicates				
	Estimate	Std. Error	*t* value	*p* value
(Intercept)	0.9919	0.145	6.857	**7.02 e-12 *** **
High CO_2	0.159	0.203	0.785	0.433
Model: GLM (Gamma)			Response variable: GPx (liver)	
Random effect: Replicates				
	Estimate	Std. Error	*t* value	*p* value
(Intercept)	1.339	0.16	8.365	**<2 e-16 *** **
High CO_2	0.385	0.226	1.699	0.089

Model: GLM (Gaussian)				Response variable: HSP
Random effect: Replicates				
	Estimate	Std. Error	*t* value	*p* value
(Intercept)	0.2	2.81 e-03	9.179	**6.6 e-14 *****
High CO_2	7.88 e-04	3.21 e-03	0.395	0.818
Gills - Liver	4.80 e-02	2.81 e-03	97.01	**<2 e-16 *****
Gills - Muscle	0.13	4.01 e-03	97.18	**<2 e-16 *****
Muscle - Liver	0.082	0.003	97.069	**<2 e-16 *****
Model: GLM (Gamma)				Response variable: HSP (muscle)
Random effect: Replicates				
	Estimate	Std. Error	*t* value	*p* value
(Intercept)	-1.105	0.013	-84.675	**<2 e-16 *****
High CO_2	-0.003	0.018	-0.185	0.853
Model: GLM (Gamma)				Response variable: HSP (gills)
Random effect: Replicates				
	Estimate	Std. Error	*t* value	*p* value
(Intercept)	-1.62	0.019	-87.28	**<2 e-16 *****
High CO_2	0.026	0.026	0.979	0.327
Model: GLM (Gamma)				Response variable: HSP (liver)
Random effect: Replicates				
	Estimate	Std. Error	*t* value	*p* value
(Intercept)	-1.389	0.013	-105.504	**<2 e-16 *****
High CO_2	-0.007	0.018	-0.388	0.689
Model: GLM (Gamma)				Response variable: Ub
Random effect: Replicates				
	Estimate	Std. Error	*t* value	*p* value
(Intercept)	-1.607	0.018	-87.698	**<2 e-16 *****
High CO_2	0.011	0.022	0.516	0.606
Gills - Liver	0.129	0.016	7.934	**2.12 e-15 *****
Gills - Muscle	0.353	0.017	21.295	**<2 e-16 *****
Muscle - Liver	-0.223	0.016	-13.577	**<2 e-16 *****
Model: GLM (Gaussian)				Response variable: Ub (muscle)
Random effect: Replicates				
	Estimate	Std. Error	*t* value	*p* value
(Intercept)	0.287	0.007	32	**<2 e-16 *****
High CO_2	-0.001	0.009	32	0.91
Model: GLM (Gamma)				Response variable: Ub (gills)
Random effect: Replicates				
	Estimate	Std. Error	*t* value	*p* value
(Intercept)	-1.615	0.028	-57.45	**<2 e-16 *****
High CO_2	0.025	0.039	0.64	0.522

Model: GLM (Gamma)			Response variable: Ub (liver)	
Random effect: Replicates				
	Estimate	Std. Error	*t* value	*p* value
(Intercept)	-1.479	0.016	-89.816	**<2 e-16 *** **
High CO_2	0.013	0.023	0.586	0.57
Model: GLM (Gamma)			Response variable: LPO	
Random effect: Replicates				
	Estimate	Std. Error	*t* value	*p* value
(Intercept)	-1.479	0.085	-17.332	**<2 e-16 *** **
High CO_2	-0.053	0.114	-0.46	0.645
Gills - Liver	0.105	0.046	2.269	**0.023 ***
Gills - Muscle	0.046	0.047	0.975	0.329
Muscle - Liver	-0.059	0.047	-1.278	0.201

Model: GLM (Gamma)			Response variable: LPO (muscle)	
Random effect: Replicates				
	Estimate	Std. Error	*t* value	*p* value
(Intercept)	-1.458	0.059	-24.811	**<2 e-16 *** **
High CO_2	0.0005	0.083	0.006	0.995
Model: GLM (Gamma)			Response variable: LPO (gills)	
Random effect: Replicates				
	Estimate	Std. Error	*t* value	*p* value
(Intercept)	-1.503	0.049	-30.46	**<2 e-16 *** **
High CO_2	-0.0137	0.069	-0.196	0.844
Model: GLM (Gamma)			Response variable: LPO (liver)	
Random effect: Replicates				
	Estimate	Std. Error	*t* value	*p* value
(Intercept)	-1.349	0.151	-8.938	**<2 e-16 *** **
High CO_2	-0.122	0.213	-0.572	0.568
Model: GLM (Gamma)			Response variable: DNA Damage	
Random effect: Replicates				
	Estimate	Std. Error	*t* value	*p* value
(Intercept)	-1.425	0.078	-18.227	**<2 e-16 *** **
High CO_2	0.083	0.101	0.815	0.415
Gills - Liver	0.015	0.057	0.269	0.788
Gills - Muscle	-0.035	0.056	-0.633	0.527
Muscle - Liver	0.051	0.058	0.874	0.382
Model: GLM (Gamma)			Response variable: DNA Damage (muscle)	
Random effect: Replicates				
	Estimate	Std. Error	*t* value	*p* value
(Intercept)	-1.505	0.117	-12.823	**<2 e-16 *** **
High CO_2	0.159	0.166	0.954	0.34

Model: GLM (Gaussian) Random effect: Replicates			Response variable: DNA Damage (gills)	
	Estimate	Std. Error	*t* value	*p* value
(Intercept)	0.253	0.024	3.736	**0.0007 *****
High CO_2	0.054	0.035	3.742	0.884
Model: GLM (Gamma) Random effect: Replicates			Response variable: DNA Damage (liver)	
	Estimate	Std. Error	*t* value	*p* value
(Intercept)	-1.425	0.075	-19.115	**<2 e-16 *****
High CO_2	0.083	0.105	0.782	0.434

Table S1. Statistical analysis of *S. canicula*'s normal blood cells (leukocytes, thrombocytes and erythrocytes), erythrocytes with nuclear anomalies (ENAs) and ratios (nucleus to cytoplasmic, spleen and heart to body ratios). Std Error – Standard Error.

	Estimate	Std. Error	*t* value	*z* value	*p* value
Normal cells					
Leukocytes	0.184	0.383	-	0.480	0.631
Thrombocytes	0.726	0.382	-	1.902	0.057
Erythrocytes	-0.364	0.459	-	-0.792	0.428
ENAs					
Segmented nucleus	-1.386	0.901	-	-1.538	0.124
Blebbed nucleus	-0.357	0.849	-	-0.420	0.675
Micronucleus	3.363 e+01	1.582 e+07	-	0.000	1.000
Ratios					
Nucleus to cytoplasmic	-0.101	0.059	-1.695	-	0.090
Spleen to body	3.32 e-06	0.012	0.000	-	0.999
Heart to body	0.022	0.023	0.955	-	0.394

Table S1. Results from the statistical analysis (* - $p < 0.05$).

Fixed effects	Random effect		Estimate	SE	*t* value	*p* value
Fulton condition (K)	**(Intercept)**		0.394	0.045	8.807	2.16e-05
	Control		-0.094	0.063	-1.482	0.177
Specific growth rate	**(Intercept)**		0.300	0.193	1.559	0.158
	Control		0.375	0.272	1.375	0.206
Somatic growth rate	**(Intercept)**		0.752	0.093	8.106	3.97e-05
	Control		0.324	0.131	2.468	**0.038***
Maximum velocity	**(Intercept)**		0.732	0.598	1.223	0.261
	Control		0.071	0.083	0.860	0.418
	Length		-0.019	0.039	-0.482	0.644
Time swimming	**(Intercept)**		0.597	0.404	1.477	0.178
	Control		1.371	0.571	2.399	**0.043***
Bursts	**(Intercept)**		-3.618	1.483	-2.440	0.0147
	Control		0.262	0.169	1.554	0.120
	Length		0.416	0.096	4.322	**1.54e-05***
Ventilation rates (post) Swimming	**(Intercept)**		67.74	26.46	2.560	0.038
	Control		13.45	12.60	1.067	0.321
	Pre-VR		-0.509	0.991	-0.514	0.623
Ventilation rates (post) Chase	**(Intercept)**		1.42e-02	2.7e-03	5.152	0.001
	Control		-2.21e-03	1.1e-03	-2.002	0.085
	Pre-VR		-4.97e-05	9.3e-05	-0.538	0.607
AChE activity	**(Intercept)**	Tel	5.778	0.627	9.216	1.56e-05
	Control		2.073	0.887	2.339	**0.048***
	(Intercept)	Cer	4.033	0.2965	13.602	8.21e-07
	Control		0.247	0.419	0.589	0.572
	(Intercept)	Opt	3.437	0.211	16.294	2.03e-07
	Control		0.727	0.298	2.437	**0.041***
	(Intercept)	Olf	4.369	0.740	5.903	0.0004
	Control		0.145	1.047	0.138	0.894
	(Intercept)	Die	5.955	0.666	8.942	1.94e-05
	Control		-0.427	0.942	-0.453	0.663
	(Intercept)	Bs	5.029	0.299	16.831	6.4e-07
	Control		0.515	0.401	1.284	0.240
	(Intercept)	Spc	5.040	0.953	5.290	0.00074
	Control		-1.321	1.348	-0.980	0.356