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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) hematological parameters (total erythrocytes, leukocytes, thrombocytes, erythrocyte nuclear abnormalities (ENAs) counts, and nucleus to cytoplasmic ratio), ii) heart and spleen to body ratios, and iii) ventilation rates of juvenile catsharks (*Scyliorhinus canicula*). We found that MHW exposure led to significant changes in normal blood cell counts, by lowering erythrocyte counts and nucleus to cytoplasm ratio, and increasing leukocyte and thrombocyte counts. Moreover, ventilation rates increased consistently over the course of the MHW. 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.

1. 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 2300 km (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) can result in constraints on hematological and cardio-respiratory systems (Mariana & Badr 2019).

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Specifically, as the temperature rises there is an increase of respiration rates (Coma 2002) that can compromise oxygen transport by red blood cells (Madeira et al. 2016) and affect 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 can increase cardiac output in fish (Farrell 2009), which is allied to a decrease in cardiac mass (Gamperl & Farrell 2004). Contrarily, at colder waters, fish can enlarge their hearts to maintain cardiac output (Driedzic et al., 1996; Gamperl & Farrell 2004), even during a short-term exposure of 4 weeks (Graham & Farrell 1989). Thermal stress has also implications over the metabolism (Pörtner & Knust 2007), through changes in energy allocation, alongside a reduction in animal's immunity by increased bacterial pathogenicity and infections (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., 2008). 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 and Farrell, 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., 2018) MHW (Δ3 °C, 15 days) on different physiological parameters of juvenile catsharks (*Scyliorhinus canicula*), including: i) hematological parameters (total erythrocytes, leukocytes, thrombocytes, erythrocyte with nuclear abnormalities counts, and erythrocyte's nucleus to cytoplasm ratio), ii) heart and spleen to body ratios and iii) ventilation rates.

2. Materials and methods

2.1. 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).

2.2. 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 at least 4 weeks 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 around 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) 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 12:12 h was kept throughout the experiment.

2.3. 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 (Schlegel and Smit, 2018), 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 et al. (2018) (See Fig. 1).

2.4. Ventilation rates

Sharks were individually observed for one minute and the number of breaths (i.e. gill movements) 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 °C; and iii) after 15 days of treatment. These observations were made before feeding to exclude any possible bias on the respiration.

2.5. 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 24 h, 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). An average of 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

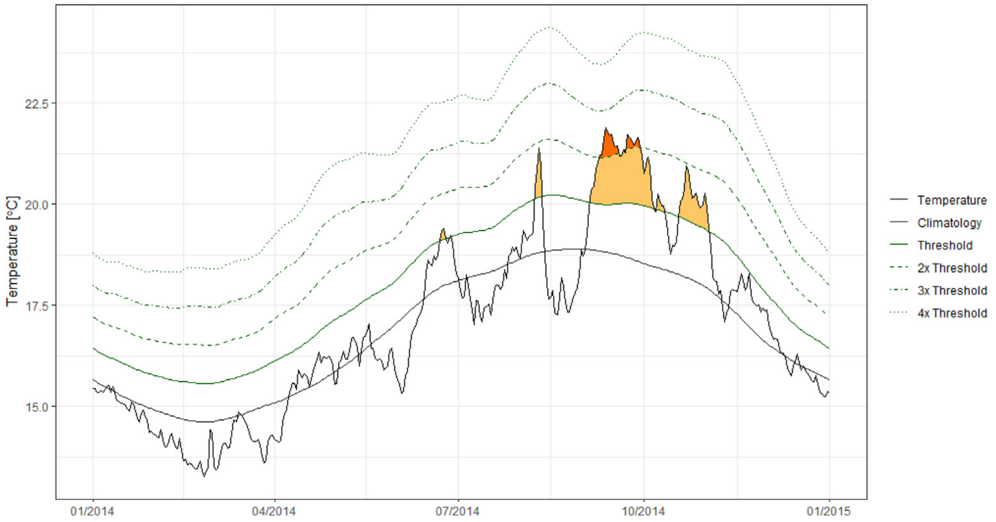

Fig. 1. Category II MHW reconstructed from a dataset of local sea surface temperatures for the last 30 years (Cascais, Portugal; acquired from NOAA (Daily Optimum Interpolation SST, version 2)). Temperatures above threshold (90th percentile in relation to the long-term climatology) represented as yellow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

Table 2
Values of the studied parameters.

	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

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.

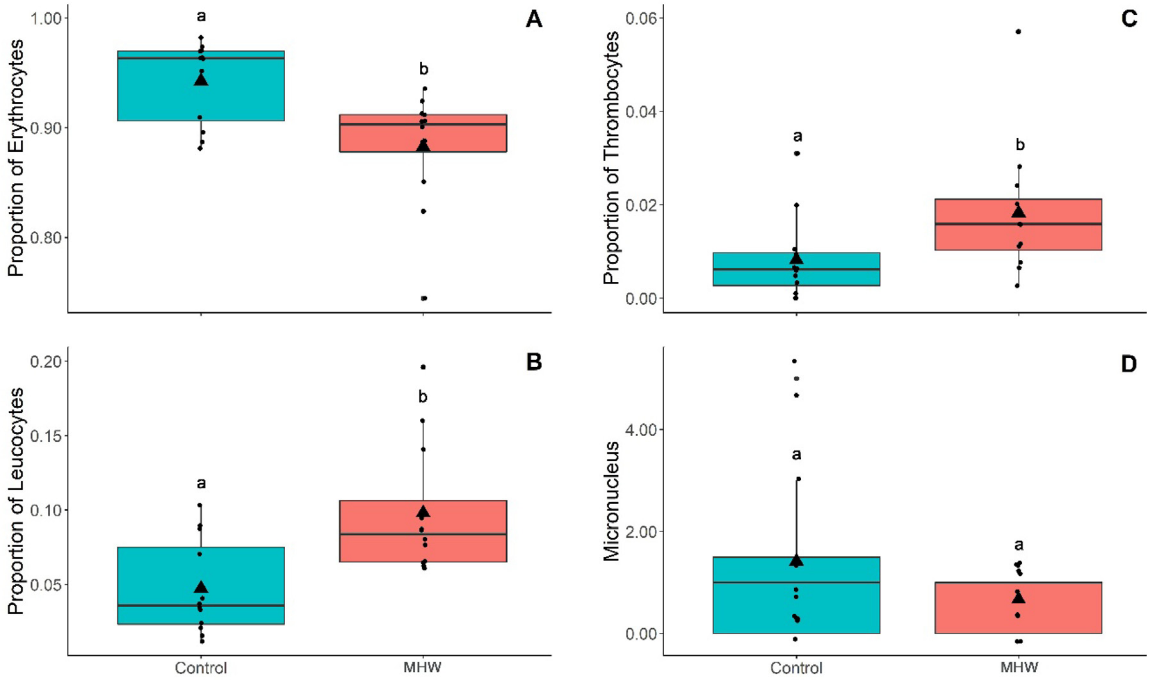

Fig. 2. Impact of a simulated category II MHW (+3 °C) on the proportions of: A) erythrocytes, B) leukocytes, C) thrombocytes and D) erythrocytes with micronucleus from blood smears of small-spotted catsharks (*Scyliorhinus canicula*, n = 12 per treatment). Black triangles represent the mean, bold horizontal 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.

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:

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

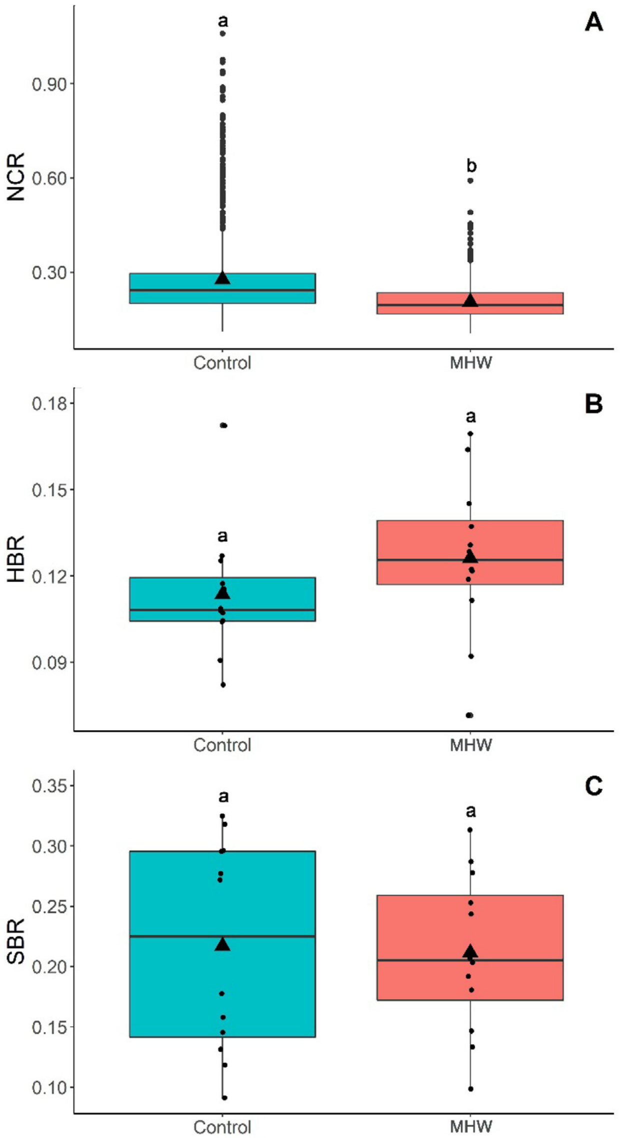

Fig. 3. Impact of a simulated category II MHW (+3 °C) on: A) nucleus to cytoplasmic ratio, B) heart to body ratio and C) spleen to body ratio of small-spotted catsharks (*Scyliorhinus canicula*, n = 12 per treatment). Black triangles represent the mean, bold horizontal lines represent the median, boundaries 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.

2.6. *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 (R Core Team, 2014), using *lme4* and *nlme* packages (Pinheiro et al. 2012; Bates et al., 2015).

3. Results

Under the control conditions, the average hematological composition of sharks comprised a total erythrocyte count of 747 ± 202 cells, a total leukocyte count of 39 ± 29 cells and a total thrombocyte count of 6 ± 8 cells (Table 2).

Exposure to a category II MHW significantly altered normal blood cell counts. More specifically, the number of erythrocyte cells was significantly decreased (MHW: 642 ± 235, Table 2; $p < 0.05$, Table S1; Fig. 2A), while the number 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).

4. Discussion

Small-spotted catsharks occupy open and coastal waters on corals or rocky bathyal bottoms (Ayas & Çiftçi 2018). Although their depth range varies from 10 to 780 m, juveniles are generally found in the shelves, i.e. above ~ 200 m (Ayas & Çiftçi 2018). Because MHW are intensifying, and heat is expected to expand in depth as well, these juvenile sharks may not be able to avoid warmer temperature conditions. For example, recent anomalous warmer temperatures have reached a depth of 300 m in the Pacific Ocean (Walsh et al. 2018). Here we show that a category II MHW has the potential to alter blood cell counts (erythrocytes, leukocytes and thrombocytes) and increase ventilation rates in juvenile small-spotted catsharks. As temperature can affect the structure of cell membrane (Farkas et al. 2001) the present data suggests that a 3 °C increase may cause erythrocyte, i.e. red blood cell (RBC), membranes to become more fragile and susceptible to apoptosis, which ultimately resulted in a lower RBC count. A similar reduction was observed in another shark species (*Heterodontus francisci*) exposed to warmer waters (Neale et al. 1977). This RBC reduction may impose a significant challenge as, 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). Accordingly, to cope with MHW-related challenges, small spotted catsharks registered reduced erythrocyte's nucleus to cytoplasm ratio (NCR) and increased ventilation rates. Erythrocyte's NCR appears to diminish as a response to the heat, since smaller erythrocytes could improve oxygen delivery by allowing a faster oxygen transfer rate (Lay & Baldwin 1999). It has been proposed

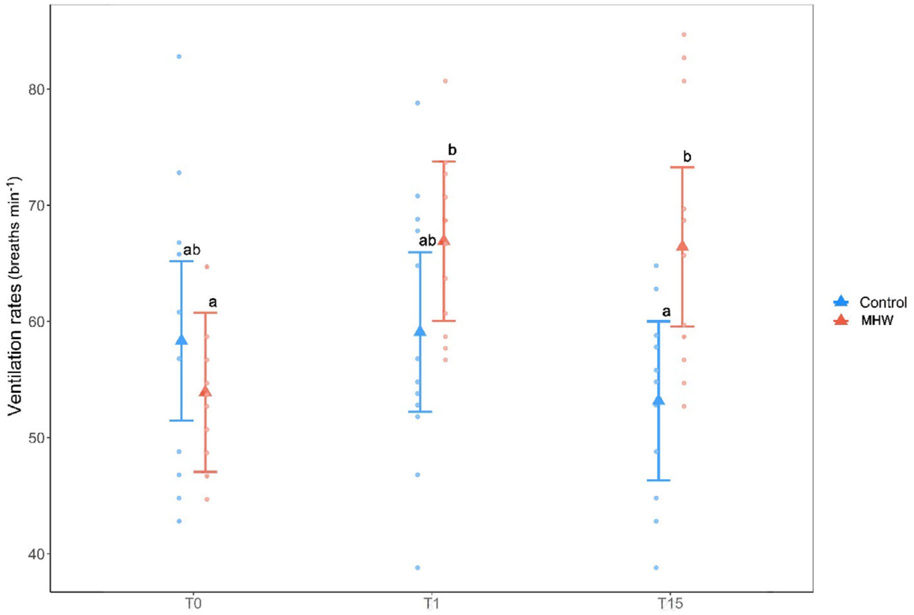

Fig. 4. Impact of a simulated category II MHW (+3 °C) on the ventilation rates (breaths per minute) of small-spotted catsharks (*Scyliorhinus canicula*, n = 12 per treatment): T0 – 9 days before the treatment started; T1 – the first day of treatment; and T15 – after 15 days of treatment. Triangles represent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

that animals with higher metabolism tend to have smaller cells (Szarski 1970). For instance, warm and active elasmobranchs tend to have RBC with a lower volume than temperate ones (Baldwin & Wells 1990). Simultaneously, sharks exposed to a MHW increased ventilation rates through buccal pumping, as rising ventilations offer another mean of intensifying waterflow over the gills, to effectively deal with the oxygen limitations (Houston 1980). This behavior was also verified in the wild, in a survey performed during a MHW along the Western Australian coast, where wobbegong sharks were observed laying on the ocean bottom venting their gills more than normal (Pearce et al. 2011).

Changes in environmental temperature can have severe effects over the immune system (Magnadottir 2010). In this experiment, we detected an increase of sharks' white blood cells (WBC) count, which can be designated as leukocytosis (Chabot-Richards & George, 2014). Fish exposed to elevated temperatures have a tendency to develop bacterial illnesses (Zaragoza et al. 2008), hence, leukocytosis is essential in the first line of defense, and may occur to maximize protection against pathogens that can arise (Opdenakker et al. 1998). Additionally, oxygen limitation can affect erythrocyte's integrity increasing the rate of erythrophagocytosis, i.e. phagocytosis of RBC (Pulsford et al. 1994), which would further explain the observed lower RBC count. Along with leukocytosis, there was also an increase of thrombocyte counts. Elasmobranch thrombocytes, specifically, can engulf latex beads, suggesting they also 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 complementary, 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). 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.

Summing up, our findings indicate an overall negative impact of a present-day category II MHW juvenile shark condition. Similar findings were previously observed in juvenile tropical sharks exposed to ocean warming expected to occur by the end of the century (Rosa et al. 2014). As MHWs occur for determined, relatively short periods, there is a possibility of recovery to a normal hematological and respiratory state. Since these physiological changes are non-detrimental, recovery should be tested by successive blood collection over 15-day periods. However, since MHWs are expected to become longer and more frequent, prolonged thermal stress may lead to impact exacerbation and override hematological recover. Thus, future research should further investigate the impacts of longer or even repeated sudden warming events on the hematological parameters of sharks. Understanding the impact that these 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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CRediT authorship contribution statement

Maria Rita Pegado: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing - original draft, Visualization, Project administration. **Catarina P. Santos:** Conceptualization, Methodology, Investigation, Data curation, Writing - review & editing, Project administration. **Dayanne Raffoul:** Investigation, Data curation, Writing - review & editing. **Marta Konieczna:** Investigation, Data curation. **Eduardo Sampaio:** Formal analysis, Writing - review & editing. **Ana Luísa Maulvault:** Writing - review & editing, Investigation. **Mário Diniz:** Writing - review & editing, Supervision. **Rui Rosa:** Conceptualization, Resources, Investigation, Writing - review & editing, Supervision, Funding acquisition, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecolind.2020.106327.

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