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RESEARCH ARTICLE

Immunological resilience of a temperate catshark to a simulated marine heatwave

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ABSTRACT

Marine heatwaves (MHWs) have recently been proposed to be more relevant in driving population changes than the continuous increase in average temperatures associated with climate change. The causal processes underpinning MHW effects in sharks are unclear but may be linked to changes in fitness caused by physiological trade-offs that influence the immune response. Considering the scarcity of data about the immune response of sharks under anomalous warming events, the present study analyzed several fitness indices and characterized the immune response (in the blood, epigonal organ, liver, spleen and intestine) of temperate adult small-spotted catsharks (*Scyliorhinus canicula*) after a 30 day exposure to a category II MHW. The results indicated that adult small-spotted catsharks have developed coping strategies for MHWs. Specifically, among the 35 parameters investigated, only the gonad-to-body ratio (GBR) and plasma glucose concentration showed significant increases. In contrast, gene expression of *igm* and tumor necrosis factor receptor (*tnfr*) in blood cells, and *tnfr* in the epigonal organ, as well as the number of monocytes, all significantly decreased. Although a decline in immune function in small-spotted catsharks was revealed following MHW exposure, energy mobilization restored homeostasis and indicated a shift in energy allocation towards reproduction. Group resilience may be due to the variable tolerance of individuals, the phenotypic plasticity of cellular immunity, thermal imprinting and/or metabolic capacity of the individuals.

KEY WORDS: Climate change, Fitness, Immune response, Marine heatwave, *Scyliorhinus canicula*

INTRODUCTION

The effects of climate change on the global ocean are real. The ocean absorption of some 93% of potential atmospheric heating caused by anthropogenic activity has resulted in ocean warming (IPCC, 2023; Pryia, et al., 2023; Venegas et al., 2023). Extreme weather events, such as marine heatwaves (MHWs), are generally defined as severe environmental events (Herring et al., 2015). MHWs are anomalously warm water events defined as periods when seawater temperatures exceed a seasonally varying threshold (usually the 90th percentile) for at least five consecutive days, potentially lasting several months (Hobday et al., 2016). MHWs are categorized by severity (category I to IV) based on how much temperatures exceed the local climatological threshold (Hobday et al., 2018), and are projected to increase in frequency, duration, spatial extent and intensity. This means the upper temperature threshold of organisms may be exceeded more frequently and by greater magnitude and may push a system to near or beyond the ends of its normally observed range (IPCC, 2014; Frölicher et al., 2018; Hobday et al., 2018; IPCC, 2023; Oliver et al., 2018). Elevated temperatures increase basal metabolic rates, and the energy demand can exceed the metabolic capacity of the species (Lemoine and Burkepile, 2012), which can have severe consequences for individual performance (Smith et al., 2023). The negative consequences of MHWs vary from sublethal effects on core physiological processes to mortality (Smith et al., 2023), with metabolism, reproduction, growth or immune function profoundly affected (Pegado et al., 2020b; Santos et al., 2021; Shanks et al., 2020; Trégarot et al., 2024). In response to these extreme events, marine ecosystem changes also include shifts in species' geographical distribution, and population collapse that leads to changes in food availability and food web structure (IPCC, 2023). Examples of MHW impacts include the mass mortality of oysters (Oliver et al., 2017), accelerated mortality rates of the keystone predator sunflower sea star (Tracy et al., 2019), mass mortalities of more than 40 species of coral reef fishes (Genin et al., 2020) and farmed Atlantic and coho salmon and trout (Trainer et al., 2020), temporary and semipermanent range shifts of tropical fish species (e.g. Cavole et al., 2016; Lenanton et al., 2017), algal blooms or seagrass loss affecting the abundance of sharks, sea snakes, dolphins, turtles or penguins (e.g. Nowicki et al., 2019). By contrast, these strong environmental perturbations can be major drivers of evolutionary change, shaping phenotypes and life histories and creating high selection pressure and the potential resilience of some populations (Cohen et al., 2012; Grant et al., 2017; Palumbi et al., 2019; Pörtner and Farrell, 2008; Ricklefs and Wikelski, 2002; Smith et al., 2023).

Most sharks are apex predators, occupying high trophic levels in marine ecosystems, and exerting significant top-down control over community structure and function (Roff et al., 2016). Mesopredatory sharks, owing to their smaller size, are vulnerable to predation by apex predators (e.g. Mourier et al., 2013) and this explains the inter-connectivity of sharks seen in food web models (Bascompte et al., 2005). The decline or loss of mesopredatory sharks can trigger an inverse cascade and cause apex shark populations to decrease. Additionally, when large-bodied sharks are lost, there is mesopredator release (Heupel et al., 2014), allowing the generation of new apex predators (Roff et al.,

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2016). Therefore, the small-spotted catshark (*Scyliorhinus canicula*), as a mesopredator, has high trophic plasticity and is important in shaping the structure and function of ecosystems (Barría et al., 2018). Furthermore, the small-spotted catshark is demersal and its site fidelity (Kousteni et al., 2015) may potentially limit its ability to undertake large-scale migrations to cooler thermal conditions (Vilmar and Di Santo, 2022) under ocean warming.

Since the 1970s, the global abundance of oceanic sharks has declined by 71%, mainly driven by excessive fishing pressure and habitat degradation (Dulvy et al., 2017; Pacoureau et al., 2021). Despite their resilient evolutionary history, the classic k-strategy of long generation times, low fecundity and low intrinsic population growth rates make sharks inherently susceptible to environmental change because of their reduced potential for thermal transgenerational adaptations (Cortés, 2002; Dulvy et al., 2017; García et al., 2008; Pimiento et al., 2017; Rosa et al., 2017; Santos et al., 2021).

The immune system, which is costly in energy and nutrients (Lochmiller and Deerenberg, 2000) has been proposed as a mediator of long-term trade-offs between other metabolically expensive processes, such as reproduction and functions that are necessary for survival (e.g. swimming, respiration, predatory or anti-predatory behavior) (Sheldon and Verhulst, 1996), and may be the most important determinant of lifetime reproductive success and fitness for many species (Lochmiller and Deerenberg, 2000). Likewise, immune responses are an important component of survival because of their role in protection from pathogens (Downs and Stewart, 2014). From this perspective, the immune system and its effective and appropriate response to pathogens are a focus of natural selection (Ardia et al., 2011). In addition to the mechanistic link between disease dynamics and the consequences for host populations, the immune response is also important for understanding individual heterogeneity (Downs and Stewart, 2014; Lochmiller, 1996). From the perspective of physiological trade-offs, disturbances caused by environmental change may lead to a compromised immune response in aquatic organisms if they reallocate resources to competing physiological systems, which may render them vulnerable to infection and disease (Barange et al., 2018; Dittmar et al., 2014).

Interactions between the immune response and ocean warming have mainly been studied in teleost fishes. The diversity of parameters studied includes: (1) changes in expression of antiviral and inflammatory genes such as pro-inflammatory mediators and receptors, toll-like receptors, cytokines, immunoglobulins, histocompatibility complex, complement, heat shock proteins and iron regulation; and (2) quantification of osmolality, plasma ions, levels of lysozyme, cortisol, complement activity, the respiratory burst, proliferation, phagocytic activiy or hematopoietic factors in fish such as grouper, three-spined stickleback, medaka, Atlantic cod, zebrafish, sturgeon, turbot and Atlantic halibut (e.g. Bowden et al., 2004; Cataldi et al., 1998; Cheng et al., 2009; Dios et al., 2010; Dittmar et al., 2014; Huang et al., 2011, 2015; Pérez-Casanova et al., 2008; Prophete et al., 2006). The response of fish species, however, is not uniform (Campbell et al., 2021), with those having narrow thermal niches being more negatively affected than those with broader thermal tolerance (Smith et al., 2023). Ocean warming also impacts populations differently, based on population disturbance history and event characteristics (Smith et al., 2023). Furthermore, the immune response of teleosts depends on species and the experimental temperature range (Dittmar et al., 2014). This means that the unique thermal tolerance, adaptive capacity and ecological circumstances of different species influence how ocean warming affects immune function and limits the universality of findings. Chondrichthyan fishes are much less studied, and so far, the effects of climate change on their physiology have focused on elasmobranch survival, development and growth, behavior, metabolic performance, feeding and digestion, and oxidative stress (Crear et al., 2019; Gervais et al., 2016; Hume, 2019; Rosa et al., 2014, 2016a,b). Relatively few data exist about elasmobranch immunity or the immune response under climate change and those that do are limited to warming and blood cell quantification, microhematocrit, blood lactate, hemoglobin quantification and antioxidant enzymes (Pegado et al., 2020b; Santos et al., 2021). The knowledge gaps in stress-related research on sharks, particularly immune competence that can determine survival is a research priority.

The physiological state of a fish with disturbed homeostasis can be captured by recording indicative signs and measurable characteristics of the response to environmental challenges (Chrousos, 2009). Blood parameters are essential indicators of physiological stress (Cataldi et al., 1998; Seibel et al., 2021; Shahjahan et al., 2019) and may directly reflect changes in water parameters in poikilothermic organisms (Sadiqul et al., 2016; Wilson and Taylor, 1993). However, there is a scarcity of high-throughput approaches using fish blood as a test material for fish physiology studies (Seibel et al., 2021). Even though, peripheral blood is an accessible, non-lethal source of cells and provides substantial information about whole animal homeostasis and an integrated picture of the immune system under different environmental conditions (Seibel et al., 2021). Considering the lack of information about the immune response in sharks under warming conditions, the present study evaluated this research top in the temperate small-spotted catshark (*Scyliorhinus canicula*) submitted to a MHW for 30 days. *Scyliorhinus canicula* is considered to be the most abundant species of catshark in European inshore waters (Ellis and Shackley, 1997). The phylogenetic position of *S. canicula* among chondrichthyans confirms this species as an informative and interesting representative of the chondrichthyan clade (Coolen et al., 2008) and holds the important distinction of being one of the most evolutionarily basal members of the Carcharhiniformes, a group that has become the most dominant group of sharks alive today (Coolen et al., 2008). Parameters considered were blood cell characteristics and quantification, plasma biochemistry, immunoassays with plasma and leukocytes from the peripheral circulation, and variations in the expression of key genes of the innate and adaptative immune response in the blood and lymphoid tissue, intestine, spleen, liver and epigonal organ.

MATERIALS AND METHODS

Ethics statement

The capture of wild *S. canicula* adults did not require a license from the responsible Portuguese institution - ICNF (Instituto da Conservação da Natureza e das Floresta/Institute for the Conservation of Nature and Forests), as this species is not included in the annexes of the Habitats Directive (Portuguese Decreto-Lei 140/99, de 24 de Abril) nor the Bonn Convention (Convention on the Conservation of Migratory Species of Wild Fauna).

All procedures involving animals were reviewed and approved by the Ethical Committee of the Faculty of Sciences of Lisbon University (ORBEA, Statement 2/2021) and conducted according to the requirements of the European Parliament's Directive 2010/63.

Animal collection and acclimation

Seventeen adult male small-spotted catsharks (mass=603.59±81.31 g; total length=57.74±3.17 cm; mean±s.d.) were caught by fishermen using traps aboard trawlers at Figueira da Foz (Portugal) in February 2021. After collection, the sharks were transferred, under controlled

water and thermal conditions, to the facilities of the Laboratório Marítimo da Guia (LMG - Cascais, Portugal), where they were randomly distributed between six tanks (three males per 600 liter tank per replicate). The animals were kept in a 1080 liter semi-closed system, maintaining conditions similar to those of their natural habitat (16°C and P_{CO_2} ~400 µatm; Pegado et al., 2020a; Varela et al., 2023). Sharks were fed daily at 4–6% total tank wet fish weight week^{-1} with fish and squid (Varela et al., 2023).

The tank water was continuously renewed with a daily substitution rate of ~10% supplied through a drip system, with filtered (0.35 µm, Harmsco, USA) and UV-irradiated seawater (Vecton 300, TMC Iberia, Portugal). The water quality in the circuit was maintained using biological filtration (Ouriço® bio balls, Fernando Ribeiro, Portugal), sand filters (model FSBF 1500, TMC Iberia, Portugal), and protein skimmers (Schuran, Germany). A photoperiod of 12 h:12 h (light:dark cycle) was provided by artificial illumination.

The water temperature in the semi-closed system was regulated automatically and independently in each tank with temperature controllers (T controller twin, Aquamedic, Germany) connected to chillers (Hailea, China) and thermostats (V^2 Therm 100, TMC Iberia, Portugal), and hysteresis was set to ±0.2°C. Tanks were aerated using air stones and CO_2-filtered air (using soda lime, Sigma-Aldrich). Temperature, pH, and dissolved oxygen were monitored daily using a handheld WTW Multi 3510 IDS SET 4 with a pH-electrode SenTix® 940 (WTW, Germany) and a dissolved oxygen sensor FDO® 925 (WTW, Germany), and salinity was verified using a water salinity tester Hanna HI98319 (Hanna Instruments Portugal, Lda). Ammonia (NH_3/NH_4^+) and nitrite (NO_2^-) were monitored regularly using colorimetric test kits and were below detectable levels (Tropic Marin, USA).

Specimens were acclimated to the experimental facility for a period of 90 days, and then randomly divided into two treatment groups, each one with 3 replicates: (1) control [C1, C2 and C3; temperature=16.3±0.3°C, pH=7.980±0.049 pH units, salinity=34.5 ±0.7 g l^{-1}, dissolved oxygen=9.60±0.07 mg l^{-1} (mean±s.d.); n=8] and (2) a simulated scenario of a category II marine heatwave [W1, W2, and W3; temperature=19.0±0.7°C, pH=7.981±0.058 pH units, salinity=34.6±1.1 g l^{-1}, dissolved oxygen=9.13±0.10 mg l^{-1} (mean±s.d.); n=9] for 30 days.

Concurrent with the random distribution of the animals between tanks they were weighed on a Scout™ scale (Ohaus, Switzerland) and photographed dorsally to facilitate individual identification using their pigmentation patterns. The individual daily health status was checked by confirmation of food intake, behavior during feeding, abnormal behavior (e.g. aggression, lethargy) and their swimming pattern throughout the experimental period. Lateral images were used to determine the fish length using ImageJ software.

Experimental design

This study followed the 3Rs framework, prioritizing the humane use of the animals. The authors have solid experience in maintaining this species under laboratory conditions (e.g. Pegado et al., 2020a,b; Varela et al., 2023), and the experimental protocols were refined. However, owing to the absence of effect size data from previous studies, we lacked a realistic idea of the expected effect size and variability. Consequently, the sample size could not be pre-determined. Therefore, we opted for a minimum of three animals per replicate to minimize bias. This decision was also influenced by the relatively large size of small-spotted catsharks, which restricted the number of animals per replicate to ensure optimal husbandry conditions while using three technical replicates to mitigate experimental errors. As small-spotted catsharks show distinct sexual segregation in both habitat use and activity profile (Sims et al., 2001), we decided to opt for one sex, the one that was captured in greater quantity.

Recent rapid warming trends across much of the global ocean have increased the likelihood of thresholds being exceeded relative to a fixed climatology and, as such, the occurrence and frequency of MHWs (Smith et al., 2023). Moreover, there is a 24% increased trend in the area of the ocean where category II MHWs occur (Hobday et al., 2018). Accordingly, the experimental MHW followed the definitions of Hobday et al. (2016, 2018) and the global trend of MHWs severity. A 30 year dataset for seawater surface temperature (Daily Optimum Interpolation SST version 2) (Banzon et al., 2016) in the region of Cascais (Portugal; local climatology) was acquired from the National Oceanic and Atmospheric Administration (NOAA). The R package 'heatwaveR' was used to reconstruct a category II marine heatwave (MHW II; threshold 2×) and animals were exposed to it across 30 days. Between day one and day seven, the temperature increased by 0.5°C per day, from 16.0°C (local climatology for the first 6 months of the year) to 19.0°C (2× the threshold of local climatology for the first 6 months of the year). From day seven until day 30, the temperature was maintained at 19.0±0.7°C.

Biometry and sample collection

A total of 17 sharks (control, n=8; MHW, n=9) were collected after 30 days of exposure to the experimental challenge, euthanized by immersion in seawater containing 300 mg l^{-1} ethyl 3-aminobenzoate methanesulfonate (Tricaine MS-222; E10521, Sigma-Aldrich) buffered with 300 mg l^{-1} sodium bicarbonate (S5761, Sigma-Aldrich) for at least 15 min. After visual confirmation of complete cessation of body and gill slit movements (between 15 and 30 min), sharks were weighed on a Scout™ scale, and the total length was measured (from the tip of the snout to the tip of the tail). Length and weight were subsequently used to calculate Fulton's condition factor (K), which is the total mass (g) divided by total length (cm) cubed ×100 (Ricker, 1975).

Peripheral blood was collected by puncture of the caudal vein using a sterile syringe (2 ml) with a 27 gauge, 2.5 cm needle and immediately transferred to a microcentrifuge tube. Both syringes/needles and the microcentrifuge tubes were previously washed with 1000 units ml^{-1} heparin (H0878-100KU, Sigma-Aldrich) in elasmobranch-modified phosphate-buffered saline (E-PBS), according to Smith et al. (2004), to avoid blood clotting and cell deformation. Plasma was collected by centrifugation at 10,000 ***g*** for 10 min (Sorvall™ Legend™ Micro 21R Microcentrifuge, Thermo Scientific) and immediately stored at −80°C for immunoassays and measurement of biochemical parameters. The blood cell pellet was diluted in 1 ml RNAlater solution (466.6 g l^{-1} ammonium sulphate, 7.82 g l^{-1} sodium citrate, 2.4 g l^{-1} EDTA in sterile MilliQ water with pH adjusted to 5.2) and stored at −20°C until RNA extraction.

Sharks were dissected, and the liver, spleen, and testis were removed and weighed on a Sartorius balance (CPA225D, Germany) to calculate liver, spleen and gonad-to-body ratios (LBR, SBR and GBR, respectively), as a percentage of the total fish mass (Pegado et al., 2020b). A portion of the anterior spiral intestine (the first third contiguous to the proximal intestine), spleen, liver and epigonal organ were removed and immediately placed in dry ice and stored at −80°C until RNA extraction.

Manual blood cell analysis

The hematocrit (Ht), or packed cell volume, was examined using the microhematocrit method. Approximately 40 µl of whole blood was taken up by a microhematocrit capillary (BR749321 BRAND®,

Sigma Aldrich). The capillary was sealed with wax (TX82.1 Marienfeld, betaLab, Portugal) and centrifuged at 2000 rpm for 7 min in a Hettich Universal 320/320R centrifuge (Germany). The hematocrit value was determined by measuring the percentage of erythrocytes in the total blood volume using a ruler and was expressed as a percentage of total blood volume. Absolute counts of blood cells (erythrocytes and leukocytes) were determined by diluting whole blood (1:50) in E-PBS and counting cells in an improved Neubauer hemacytometer (717805 Brand™, ThermoFisher Scientific) (Smith et al., 2004).

Three blood smears per animal were prepared on pre-washed glass microscopy slides (D100004, Deltalab), allowed to air-dry, and subsequently stained following the manufacturer's guidelines with a Giemsa's azur Eosin–Methylene Blue staining solution (1.09204, Sigma-Aldrich) buffered at pH 7.4. Stained blood smears were mounted in DPX (1.00579, Sigma-Aldrich) and a glass coverslip and stored in the dark. The blood smears were photographed at a magnification of 40× in a Leica DM1000 microscope equipped with a Leica DGC4590 monochrome camera. Between 1500 and 1700 cells for each animal, selected from the three smears prepared, were examined, and counted by a single observer using ImageJ software. Erythrocytes (E), leukocytes (Lk) and thrombocytes (Tb) were identified based on the cell cytology reported in Parish et al. (1986). Leukocytes were divided into lymphocytes (Lph), granulocytes (GR), and monocytes (Mn). Granulocytes were subdivided into 3 types: (1) neutrophils [Nt; termed granulocytes type III in Parish et al. (1986), and neutrophils in Arnold (2005)]; (2) eosinophils type I [EI; termed granulocytes type I in Parish et al. (1986) and coarse eosinophilic granulocytes in Arnold (2005)]; and (3) eosinophils type II [EII; termed granulocytes type II in Parish et al. (1986) and fine eosinophilic granulocytes in Arnold (2005)]. Erythroblasts (Et), which included basophilic erythroblasts and orthochromatophilic erythroblasts, were identified according to Shahjahan et al. (2020) and Smith et al. (2004). Lastly, the number of erythrocyte nuclear abnormalities (ENAs) was counted and cellular abnormalities (ECAs) were observed using an optical microscope and classified in accordance with Carrasco et al. (1990) and Shahjahan et al. (2020).

Leukocytes were separated with a density gradient using Percoll (P4937, Sigma-Aldrich) diluted to 1.077 g ml^{-1} [adapted from Guardiola et al. (2017) and Samaï et al. (2018)]. Briefly, whole blood diluted 1:20 in E-PBS was overlayed on Percoll (1:30) and centrifuged at 100 ***g*** for 10 min and the supernatant collected, diluted (1:30 in E-PBS) and centrifuged at 100 ***g*** for 10 min. The pellet (leukocytes) was resuspended 1:2 in E-PBS and stored on ice until further analysis. Leukocyte viability (Lk_VB) was assessed by diluting the isolated leukocytes 1:10 in 0.2% E-Trypan Blue (Trypan Blue 15250061, ThermoFisher diluted in E-PBS) and live cells counted in an improved Neubauer hemacytometer (717805 Brand™, Thermo Fisher Scientific) (Smith et al., 2004).

Flow cytometry – blood cell analysis

Two types of samples were run on an LSRFortessa cytometer (BD Biosciences) and analyzed with FlowJo software (TreeStar): (1) whole blood (WB) and (2) the Percoll fraction (PF). The subpopulations were analyzed through the scatter and fluorescent characteristics of the cells (adapted from Inoue et al., 2002; Konomi et al., 2014; Pierrard et al., 2012; Priya et al., 2017; Yakhine-Diop et al., 2019; Zheng et al., 2017). Erythrocytes were distinguished from leukocytes and thrombocytes using 3,3-dihexyloxacarbocyanine iodide staining [DiOC6(3), 318426, Sigma-Aldrich; 405 nm Laser], which was optimal after 10 min staining and was stable for at least 1 h at 4°C. Necrotic (N) and apoptotic (A) cells were quantified using Propidium Iodide (PI; Acros Organics, 440300250; 488 nm Laser) and Vybrant® DyeCycle™ Violet (Invitrogen, V35003; 405 nm laser), respectively. Gating criteria and fluorescent staining previously optimized for shark blood cell samples were used with a flow cytometer sorter BD FACSAria Ilu (BD Biosciences; nozzle=100 µm, 20°C, 300 rpm) and the output analyzed with FlowJo software (TreeStar).

For whole blood samples (WB), 1910 µl of E-PBS were added to 50 µl of whole blood, followed by 1 µl of Violet stain, as above. The suspension was gently mixed and incubated at RT protected from light for 20 min and then 40 µl of DiOC6(3) was added and incubated for a further 10 min at RT in the dark. For leukocyte suspensions (PF), 1900 µl of E-PBS was added to 50 µl of a single-cell suspension, followed by 1 µl of violet stain, mixed well and incubated at RT for 30 min, protected from light. Then, 95 µl of 10 µg ml^{-1} PI staining solution was added, mixed gently, and the cells incubated for 1 min in the dark. A minimum of 250,000 and 9000 events were analyzed for whole blood and Percoll fractions, respectively. The contribution of different leukocyte subpopulations determined using the flow cytometer was established by relating the results to the total blood cell quantification using a hemacytometer.

Biochemistry of leukocytes (Percoll fraction)

Respiratory burst activity measurements were performed for the detection of reactive oxygen species (ROS) in a Synergy HTX microplate reader (BioTek Instruments Inc., USA) and data were collected using Gen5 software (BioTek Instruments Inc., USA). Following the manufacturer's instructions and Yazdani et al. (2015), samples (3 technical replicates per animal) were diluted in DRH123 probe (KP06004-250, Bioquochem) to a concentration of 1×10^6 leukocytes ml^{-1}, incubated in the dark for 30 min at RT, then centrifuged 500 ***g*** at RT for 10 min. The supernatant was removed, and the pellet was resuspended in PBS to give 1×10^5 cells ml^{-1}. The fluorescence was measured immediately 10 times (excitation: 485/20, emission: 528/20). ROS were expressed as FU (fluorescence units).

The leukocyte suspension was diluted 1:10 in 1000 units ml^{-1} heparin (H0878-100KU, Sigma-Aldrich) prepared with E-PBS (Smith et al., 2004) to avoid cells clotting in cytotoxicity and proliferation assays. Cytotoxicity was assessed using a Cytotoxicity LDH Assay Kit-WST (91963, Sigma-Aldrich), according to the manufacturer's instructions. The cell concentration optimized for this assay was 250,000 cells ml^{-1}. For each replicate, 1:10 lysis reagent was added, followed by incubation at 37°C for 30 min. Then, 1:1 of reagent solution was added to each well and the plate was incubated at RT protected from light for 20 min. To stop the reaction, stop solution (1:2 dilution) was added, and the absorbance was measured at 490 nm in a Multiskan SkyHigh Microplate Spectrophotometer (ThermoFisher Scientific, USA). To determine cytotoxicity, lactate dehydrogenase (LDH) activity was measured in the cells (after lysis; Cyt_Cl) and in the supernatant (released spontaneously; Cyt_Sup).

Proliferation (Prol) was assessed using a Calbiochem® Rapid Cell Proliferation Kit (QIA-127, Thermo Fisher Scientific, USA), following the manufacturer's instructions. Samples of 250,000 cells ml^{-1}, in triplicate, were transferred to a flat bottomed plate and incubated for 30 min at RT to allow cell adhesion. 1:10 of WST-1/EMS mixture was added to each well and incubated for 2 h at 37°C. Absorbance was measured at 450 nm at 37°C (after gently shaking) in a Multiskan SkyHigh Microplate Spectrophotometer (ThermoFisher Scientific, USA). Absorbance was proportional to formazan formation, the cleavage product of WST-1 formed by mitochondrial dehydrogenases and is proportional to cell number.

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Lysozyme and total anti-protease activity in plasma

Lysozyme activity (Lys) was measured using a turbidimetric assay (Ellis, 1990a) with plasma samples diluted 1:2. Lysozyme was expressed as U mg^{-1} protein and determined via a standard curve using hen egg white lysozyme (HEWL, Sigma-Aldrich). One unit of lysozyme activity gave a reduction in absorbance of 0.001 per minute. Total anti-protease activity (Aprot) was determined using a spectrophotometric method (Ellis, 1990b) modified by Hanif et al. (2004). The total anti-protease activity was expressed based on the percentage of trypsin activity and normalized using the protein content of the plasma (% of inhibition mg^{-1} protein). All measurements were carried out on a microplate reader BioTek Synergy 4 (BioTek Instruments, Inc., USA) at 450 nm.

Plasma biochemistry

Clinical chemistry parameters were obtained from plasma using automated analyzers with specific cartridges for each analyzer, according to the manufacturer's instructions. Urea (U; mg dl^{-1}), glucose (Glu; mg dl^{-1}), total protein (TP; g dl^{-1}), triglycerides (Tg; mg dl^{-1}), total cholesterol (TC; mg dl^{-1}), and calcium (Ca; mg dl^{-1}), were measured in SPOTCHEM™ EZ SP 4430 (Arkray, Japan), sodium (Na; mmol l^{-1}), chloride (Cl; mmol l^{-1}), and potassium (K; mmol l^{-1}) were measured in IDEXX VetStat (IDEXX Laboratories, Westbrook, USA). Total protein (TP_BD; mg ml^{-1}) was determined using the Bradford method (Bradford, 1976) with a Quick Start™ Bradford Protein Assay (5000202, Bio-Rad, USA) and using bovine serum albumin (BSA) as the standard. Plasma was diluted (1:40 or 1:80) with the assay dye reagent and measured at 595 nm using a microplate reader BioTek Synergy 4 (BioTek Instruments, Inc., USA).

Analysis of gene expression by quantitative real-time PCR (qPCR)

RNA was extracted from up to 15 mg of the spleen, 20 mg of the epigonal organ, 25 mg of intestine and liver and 300 µl of blood cells (blood pellet after centrifugation of whole blood at 10,000 ***g*** for 10 min) diluted in RNAlater, using an E.Z.N.A.® Total RNA Kit I Protocol (SKU: R6834-02, Omega Bio-tek, Inc.) with 2-mercaptoethanol (A1108,0100, PanReac), following the manufacturer's protocol after previous optimizations. Tissues were lysed in a Retsch MM400 (Scansci) using 3 cycles of 30 s per cycle. RNA was eluted in 30 µl (liver, epigonal organ, intestine, and blood) or 40 µl (spleen) nuclease-free water. To eliminate genomic DNA contamination of extracted total RNA, it was treated using a Precision™ DNase kit (Primerdesign), following the manufacturer's protocol. The purity and concentration of RNA were assessed with a NanoDrop ONE^{c} (Thermo Scientific). RNA integrity was checked by agarose gel electrophoresis by loading 6 µl of 150 ng $µl^{-1}$ total RNA on 0.8% Seakem® LE Agarose (50004, Lonza) and run in a PowerPac Basic tray (BioRad).

Synthesis of cDNA was performed using 39 ng $µl^{-1}$ of total RNA, which was denatured at 65°C for 5 min, before adding 7 µl of a mix containing 10 mmol l^{-1} dNTPs, 100 µmol l^{-1} (200 ng) random hexamers (Jena Biosciences), 40 U $µl^{-1}$ Ribolock™ RNase inhibitor (ThermoFisher) and 200 U $µl^{-1}$ RevertAid™ reverse transcriptase (ThermoFisher). Conditions for synthesis of cDNA were as follows, 20°C for 10 min, then 42°C for 50 min, before heat inactivation of the enzyme at 72°C for 5 min. PCR reactions were carried out in a 25 µl final reaction volume containing 2 µl total cDNA 1:10, 10 mmol l^{-1} dNTPs, DreamTaq (VWR), 10 mmol l^{-1} RT4 primers (Table 1) and 10×reaction buffer, and incubating at 95°C for 5 min, followed by 27

Table 1. Primer sequences, amplicon length, primer annealing temperature (T_a), qPCR efficiency and R^2 for all small-spotted catshark (*Scyliorhinus canicula*) genes amplified

Gene	Accession no.	Symbol	Primer	Sequence (5′–3′)	Amplicon (bp)	T_a (°C)	Efficiency (%)	R^2
Large ribosomal subunit protein 13	XM_038806482	*RPL13*	F	GCTCCAAGTTAATCATCTTCCCA	172	60	102.6	0.995
			R	GCCTTGAAATTCTTCTCATCCTC				
Large ribosomal subunit protein 29	XM_038776343	*RPS29*	F	CATCAGCAGCTTTACTGGTCTCATC	150	60	97.2	0.997
			R	GAAGCCGATGTCTTTAGCGTATTG				
Perforin 1-like	XM_038780370.1	*perf*	F	GATGGGTTTCGGCACCAGTA	121	60	91	0.998
			R	GGAGAATTGGGCGGACAAGA				
Melanotransferrin-like	XM_038815982.1	*meltf*	F	AGGGTGGAATGCTCCTGTTG	159	60	93.8	0.999
			R	CCGACACACAACTGGCAAAG				
Ferrochelatase	XM_038790571.1	*fch*	F	CTGGGGCAGACTGAGTCAGA	137	60	92.2	0.998
			R	GACAGCCTCAGCAACGCCAT				
Ferritin heavy chain b-like+oocyte isoform	XM_038783372.1, XM_038783766.1	*fer*	F	GATGTTGCCCTGCGTCACT	163	60	95.3	0.995
			R	CCTCCAGACCATTGCTCCAC				
Immunoglobulin heavy chain c region-like	JX556041.1, JX556021.1, JX556022.1	*ig1*	F	GCCACTCTGATGTGCGAAGT	168	60	92.4	0.998
			R	ATCCCATTCTTCCACGGTGC				
Immunoglobulin M heavy chain	JX555996.1	*igm*	F	GCTGTTTGGCGATGGACTACT	155	60	88	0.997
			R	CTCTGACTCGGTGATGGTTA				
Interferon regulatory factor 2	XM_038805685.1	*irf2*	F	CCCGCCTATCTGTCAGCATC	132	60	100.7	0.999
			R	CCCATCAGTTCTTGCCCCTG				
tnfrsf1a-associated via death domain (tradd)	XM_038806934.1	*tnfr*	F	GCGTGGCTGAGGATGGATTC	88	60	108.7	0.979
			R	GGGCCGATAGATCCGTCTTG				
Lysozyme G-like1	XM_038775695.1	*lys*	F	ATTAAGTGGAGGGTGGAGCG	184	60	91.8	0.999
			R	TGCCAGTCGAATGTCCTTTAGC				
T-cell receptor beta, B constant region (TCRbB)	KY434203	*tcrb*	F	CGTCAATGGCGAAGAAATGC	194	60	89.6	1.000
			R	CATGTTGCGTGCTCTTGGTG				
T-cell receptor delta	KY434205.1	*tcrd*	F	GGCATCAGACTTCTACCCCAAG	222	60	93.3	0.995
			R	GGAACAACGGTTACCCAGGTG				
Ribosomal protein S18	–	*RT4*	F	TGACGGAAGGGCACCACCA-G	For genomic contamination control of cDNA			
			R	AATCGCTCCACCAACTAAGAACGG				

cycles of 95°C for 20 s, and then 60°C for 20 s, 72°C for 20 s, with a final cycle of 72°C for 5 min. The cDNA synthesis and PCR reactions were carried out in a T100™ thermal cycler (Biorad). Genomic contamination of total RNA was monitored before cDNA synthesis using it directly in PCR and amplifying ribosomal protein 18S and then analyzing 10 µl of the reaction products by agarose gel electrophoresis (as outlined above). Samples contaminated with genomic DNA were retreated with DNase (as outlined above).

Quantitative PCR (qPCR) was used to analyze the mRNA expression of candidate indicator genes of the innate and acquired immune response in cDNA prepared from control and the heatwave-challenged sharks. To monitor how this environmental stressor modulated the activity of the immune response, ferritin heavy chain b (*fer*), melanotransferrin (*meltf*), ferrochelatase (*fch*), lysozyme (*lys*), T-cell receptors beta and delta (*tcrb* and *tcrd*, respectively), tumor necrosis factor receptor SF1A associated via death domain (*tnfr*), perforin 1 (*perf1*), immunoglobulin IgNAR transmembrane and the secretory heavy chain constant region (*ig1*), immunoglobulin M secretory heavy chain (*igm*) and interferon regulatory factor 2 (*irf2*) were measured in cDNA of blood cells, epigonal organ, intestine, spleen and the liver.

To design primers for the amplification of gene transcripts the FASTA sequences of the candidate and reference genes were retrieved from the Nucleotide database from NCBI, where the genome of *Scyliorhinus canicula* is available, and primers were designed using Primer3 and the BLAST tool from NCBI. qPCR reactions were performed in duplicate for a 6 µl final volume, containing a specific concentration of cDNA for each tissue (liver, 500 ng μl^{-1}; spleen and intestine, 250 ng μl^{-1}; epigonal organ and blood, 100 ng μl^{-1}), 10 µmol l^{-1} of each primer (Table 1) and SsoFast EvaGreen supermix (Bio-Rad Laboratories). The samples and the reaction mix were loaded into 384 plates using a pipetting robot CyBio® FeliX CHOICE™ (AnalytikJena, Germany).

qPCR reactions were carried out in a StepOnePlus qPCR thermocycler and data were analyzed with Bio-Rad CFX Maestro 1.0 software (Bio-Rad Laboratories, USA). Thermocycling conditions were 95°C for 1 min, 39 cycles of 95°C for 5 s and 60°C for 10 s, followed by a final melt curve between 60 and 95°C, which gave single product/dissociation curves in all reactions. Control reactions included a no-template control and a cDNA synthesis control (reverse transcriptase omitted from the reaction). Quantification of the transcripts was performed using the relative standard curve method (Vieira et al., 2012), generated through serial dilutions of specific qPCR products for each gene. All amplicons were sequenced to confirm the specificity of the qPCR primers. Two candidate reference genes, ribosomal protein L13 (RPL13) and ribosomal protein S29 (RPS29) were analyzed using the CFX Maestro Reference Gene Selection Tool (Bioradiations, 2020) and the GeNorm algorithm (Vandesompele et al., 2002). Both candidate reference genes were stable in all tissues across all treatments. The qPCR results were normalized by dividing the detected copy number of the target gene by the geometric mean of the reference genes in each cDNA sample.

Statistical analyses

All statistical analyses were performed using R Studio software (version 2022.02.2+485; r-project.org). Generalized linear mixed models using template model builder ('glmmTMB' function; https://CRAN.R-project.org/package=glmmTMB) were used to infer the effects of the treatment on the parameters analyzed. Treatments were set as a two-level factor (Control and MHW), as well as replicates (C1-3, MHW1-3). The model residuals were plotted ('check_model' function) to check assumptions of normality, homoscedasticity, and independence among residuals. The adequacy of the statistical model of each variable involved, ensuring that the model was a good fit for the data, was assessed by verifying that all model assumptions were met. The final model was chosen whenever all assumptions were met.

Type II Wald Chi-squared tests ('Anova' function) were performed on the models to evaluate the effect of the treatments and replicates on the response variables. The effect size was determined using the package 'effsize' (https://CRAN.R-project.org/package=effsize; 'cohen.d' function) and the statistical power through 'pwr.anova.test' function. Statistical significance was accepted at α=0.05. No significant differences were detected between replicates. When very small eigenvalues were detected (ENAs proportion with binucleated cells in blood smears and *meltf* in spleen), values were multiplied by 1000. No significant differences were detected in the weight and length of the sharks, which were randomly distributed between treatments. Parameters were plotted using the 'ggplot' package (https://CRAN.R-project.org/package=ggplot). Correlations were performed using the 'corrplot' package (https://CRAN.R-project.org/package=corrplot). Additional statistical information, such as the *P*-value, effect size with confidence intervals, and statistical power is provided in Table S2.

RESULTS

Morphometric indices

The gonad-to-body ratio (GBR) indicated that males exposed to the MHW allocated a greater proportion of body mass and energy expenditure to testicular tissue (P<0.0001; Table S2) compared with the spleen and liver (Fig. 1).

Blood smear analysis

The cellular immune system response was characterized by the relative frequency of each type of blood cell in the peripheral circulation. Erythrocytes (E), thrombocytes (Tb), lymphocytes (Lph) and monocytes (Mn) were easily identified. Erythroblasts (Et) were identified as polychromatic nucleated cells, with a less condensed nucleus, and were smaller and less elongated than the mature erythrocytes [according to Shahjahan et al. (2020) and Smith et al. (2004)]. The cytoplasm of erythroblasts was less acidophilic compared to the mature red blood cells. Eosinophil type I (EOI) was characterized by the presence of light pink eosinophilic granules in the cytoplasm and an irregular nucleus but were rarely polymorphic. Eosinophil type II (EOII) stained brighter and pinker than EOI and were polymorphonuclear. Neutrophils (Nt) appeared round with lightly stained granules and an eccentric nucleus with a dumb-bell shape [according to Arnold (2005) and Parish et al. (1986); Fig. S1].

Monocytes (Mn) were significantly lower in the MHW treatment (P<0.05; Table S2) compared with the control. Number of thrombocytes (Tb), lymphocytes (Lph), eosinophils (EO), neutrophils (Nt), erythroblasts (Et) and erythrocytes (E), as well as the hematocrit (Ht) and the neutrophil-to-lymphocyte ratio (NLR) did not differ significantly between treatments (Fig. 2; Table S2).

Absolute blood cell counts

Absolute blood cell counts were determined to establish baseline values for small-spotted catshark male adults (Table 2).

Morphological changes of erythrocytes

Two different types of erythrocyte nuclear abnormalities (ENAs) were observed and quantified: (1) notched nucleus (cells that did not contain nuclear material) and (2) binucleated (cells with two nuclei)

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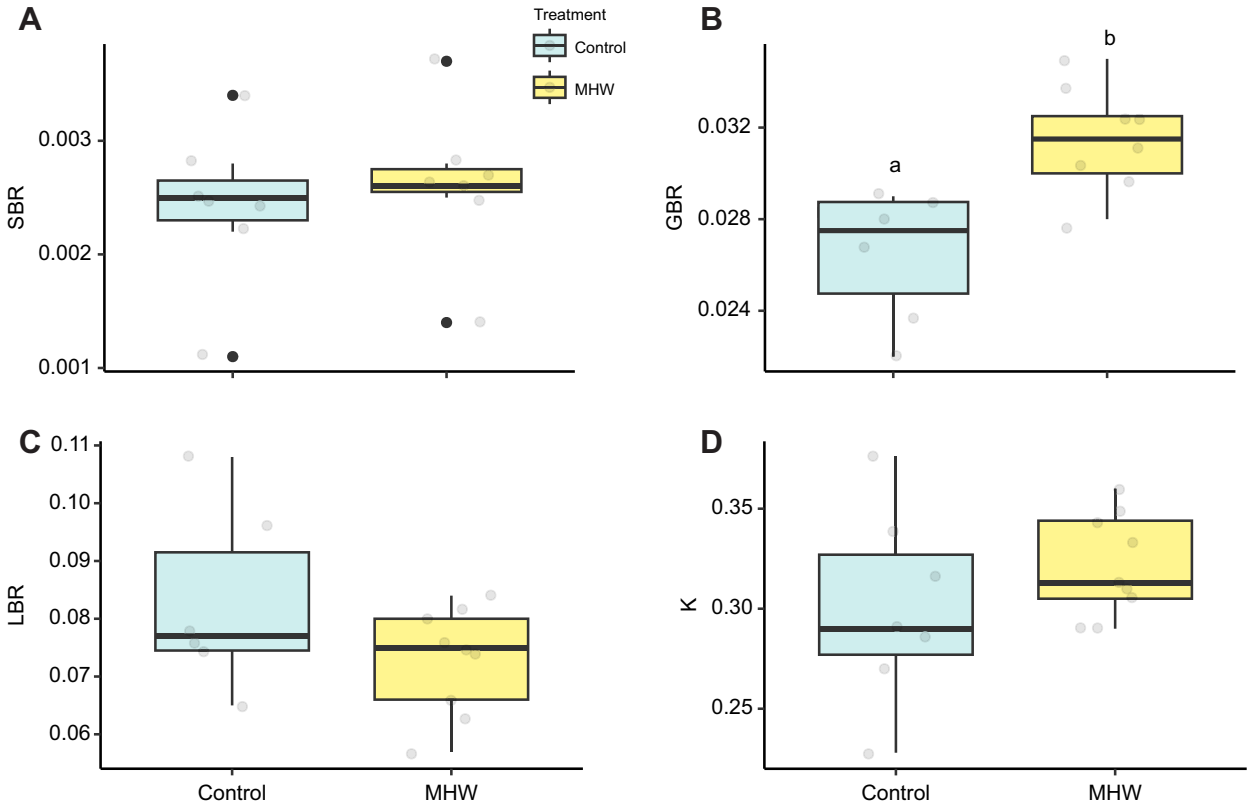

Fig. 1. Impact of a marine heatwave (MHW) on the small-spotted catshark (*Scyliorhinus canicula*) after 30 days of exposure. Effects of MHW on the (A) spleen-to-body ratio (SBR), (B) gonad-to-body ratio (GBR), (C) liver-to-body ratio (LBR) and (D) Fulton's condition factor (K) (control, n=8; MHW, n=9). SBR, GRB and LBR scale is a proportion. Values with different letters are significantly different (P<0.05). Horizontal lines represent the median, the whiskers represent the lowest and highest values of the results, and the boundaries represent the 25th and 75th percentiles. Black dots represent outliers and grey dots represent individual data points. Additional statistical information is provided in Table S2.

(Fig. S1a,b). The frequency of both ENAs did not differ significantly between blood from the control and MHW sharks (P>0.05; Table S2). Cells with notched nuclei were observed in two replicates of the control group and in all replicates of the MHW group and binucleated cells were observed in one replicate from the control and in two MHW replicates. The erythrocyte cellular abnormalities (ECAs) observed were twins (two cells joined at the cell surface), teardrops, elongated, echinocytic shapes and other abnormal shapes (Fig. S1c). ECAs were observed in both treatments across the three replicates.

Cellular characterization

Whole blood

3,3-dihexyloxacarbocyanine iodide staining [DiOC6(3)] distinguished erythrocytes (E) from leukocytes and thrombocytes (Lk and Tb) (Fig. 3A, right panel); whole blood samples were treated since these did not differ between treatments (Fig. 3A; P>0.05; Table S2). The level of apoptosis was similar in both treatments (Fig. 3B; P>0.05; Table S2). Gating of cells, after erythrocyte removal, was performed considering their forward scatter-height (FSC-H) versus side scatter-height (SSC-H) properties. Population 1 (P1) had smallest, least granular cells and population 4 (P4) had the largest, most granular cells (Fig. 3C). Subpopulation identifications were performed considering: (1) FSC-H versus SSC-H properties, (2) characterization of blood cells from *S. canicula* blood smears, (3) previous identification of subpopulations after sorting of whole blood using a flow cytometer. Therefore, P1 and P2 may be identified as thrombocytes and lymphocytes, P3 as eosinophils, P4 as neutrophils and P5 as monocytes. Subpopulations did not differ significantly between treatments (Fig. 3C, bottom right panel; P>0.05; Table S2).

Leukocyte suspension (Percoll fraction)

Flow cytometry-specific staining revealed that the proportion of necrotic and apoptotic leukocytes, and leukocyte death (manual Trypan Blue method) had similar means values between treatments (Fig. 4A; P>0.05; Table S2). When comparing apoptosis levels in the leukocytes from whole blood (Fig. 3B) and the leukocytes from the Percoll fraction (Fig. 4A), although not significantly different (P>0.05; Table S2), it seems that Percoll may induce some cell death. Considering these data, we propose whole blood samples be used as the population gating since quantification was more informative and may be more accurate.

Three subpopulations of leukocytes were identified using FSC-H versus SSC-H properties. Population 1 (P1) had smaller and less granular cells, P2 had cells with higher granularity than P1 and P3 had larger cells than P1 with a similar granularity to P2 (Fig. 4B, bottom panel). Subpopulation identification was performed considering the FSC-H versus SSC-H properties and previous identifications of subpopulations after sorting whole blood using flow cytometry. Therefore, P1 may contain lymphocytes, thrombocytes and monocytes, which correspond to P1, P2 and P5 in whole blood (Fig. 3C) and were preferentially kept after Percoll treatment. P2 contains eosinophils which corresponds to P3 in whole blood (Fig. 3C) and P3 to neutrophils which corresponds to P4 in whole blood (Fig. 3C).

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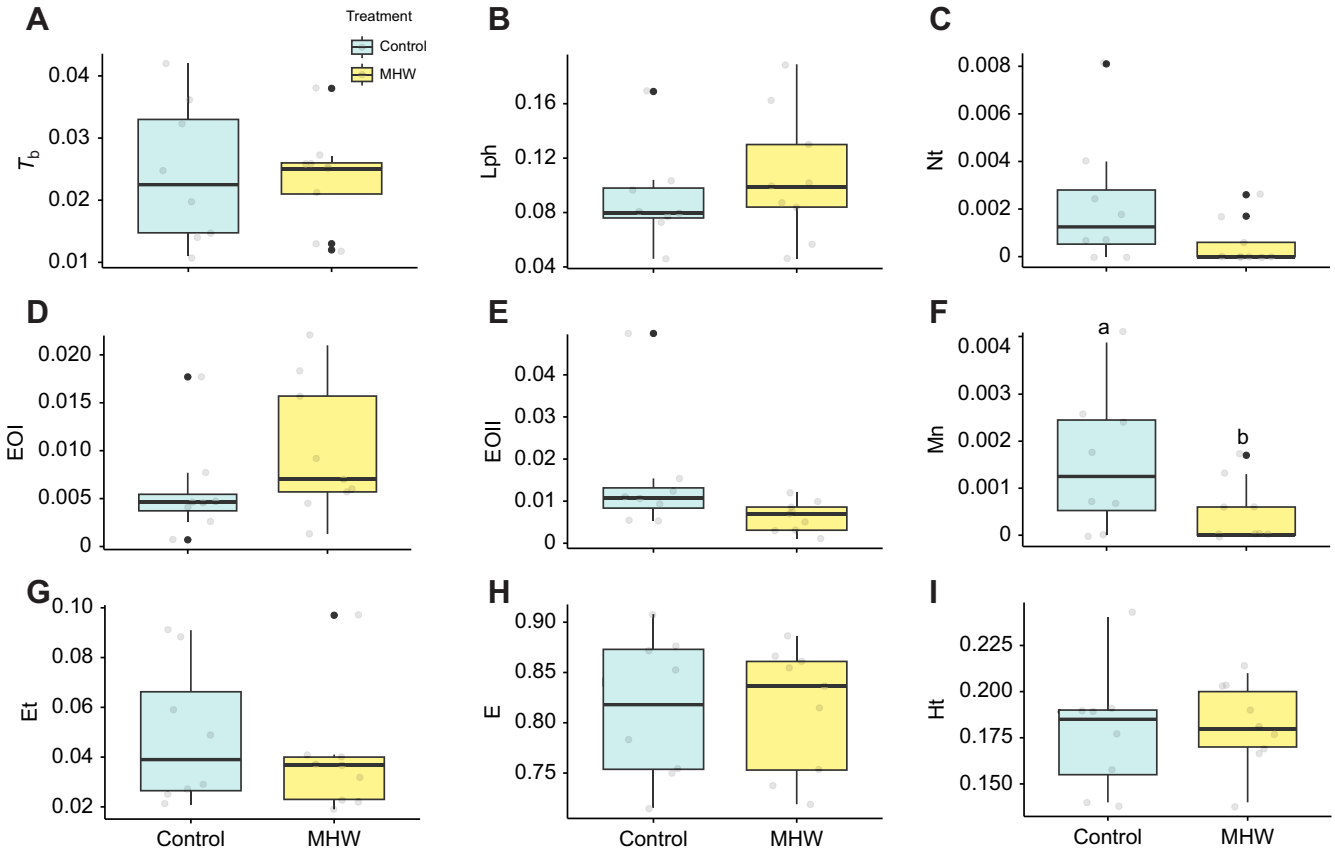

Fig. 2. Proportion of different cell types relative to total blood cells in whole blood (manual counting) and the hematocrit of small-spotted catsharks (*Scyliorhinus canicula*) after 30 days of exposure to a MHW. A mean of 1600 cells was observed per animal. (A) Thrombocytes (Tb). (B) Lymphocytes (Lph). (C) Eosinophils type I (EOI). (D) Eosinophils type II (EOII). (E) Neutrophils (Nt). (F) Monocytes (Mn). (G) Erythroblasts (Et). (H) Erythrocytes (E). (I) Hematocrit (Ht; proportion of red blood cells). Values with different letters are significantly different (P<0.05). Additional statistical information is provided in Table S2. MHW, marine heatwave; control, n=8; MHW, n=9.

The gated subpopulations of leukocytes were not significantly different between treatments (Fig. 4B; P>0.05; Table S2). The percentage of the subpopulations after the density gradient may differ from whole blood percentages because Percoll separates cells by density and during this process, larger cells may be selectively lost. As a result of the Percoll treatment, it was not possible to compare P2 and P3 between treatments in the Percoll fraction.

Plasma biochemistry

Fig. 5 shows that the MHW elicited a significant increase in the concentration of plasma glucose (P<0.05; Table S2) after 30 days of exposure. Triglycerides (Tg), total protein (TP), calcium (Ca), total cholesterol (TC), urea (U) and potassium (K) were not affected by the warming event (Fig. 5). Owing to the volume of the available sample, it was not possible to run the monovalent ions chloride and sodium.

Table 2. Absolute counts (cells ml^{-1}; mean±s.d.) in blood smears of small-spotted catsharks (*Scyliorhinus canicula*) for the control treatment (n=8)

Cell type	Absolute count
Erythroblasts	39,996±22,974
Erythrocytes	674,483±270,398
Thrombocytes	19,029±8881
Lymphocytes	71,086±30,952
Eosinophils type I	5582±3570
Eosinophils type II	7798±3750
Neutrophils	2025±2433
Monocytes	1366±1157

Cell activity assays

Cell activity assays performed with the Percoll cell fraction (leukocytes) such as respiratory burst activity detected by measurement of reactive oxygen species (ROS), proliferation measured through formazan formation, cell cytotoxicity through lactate dehydrogenase activity, and lysozyme and total anti-protease activities measured in plasma were not significantly impacted by the MHW treatment (Fig. 6; P>0.05; Table S2).

Quantitative qPCR

The warming challenge had significant effects on the expression of tumor necrosis factor receptor SF1A associated via death domain (*tnfr*), which was downregulated in blood cells and the epigonal organ (P<0.05; Table S2). The expression of immunoglobulin M heavy chain (*igm*) in the blood was likewise significantly decreased in the MHW treatment (P<0.05; Table S2). However, the effect size as well as the statistical power of the *tnfr* and *igm* genes in blood cells was very low (Table S2). The expression levels of the remaining genes did not exhibit significant changes between treatments (Table S2). Considering the significant gene expression variations in each tissue analyzed, the immune-related genes in the blood cells and epigonal organ were downregulated, while the levels

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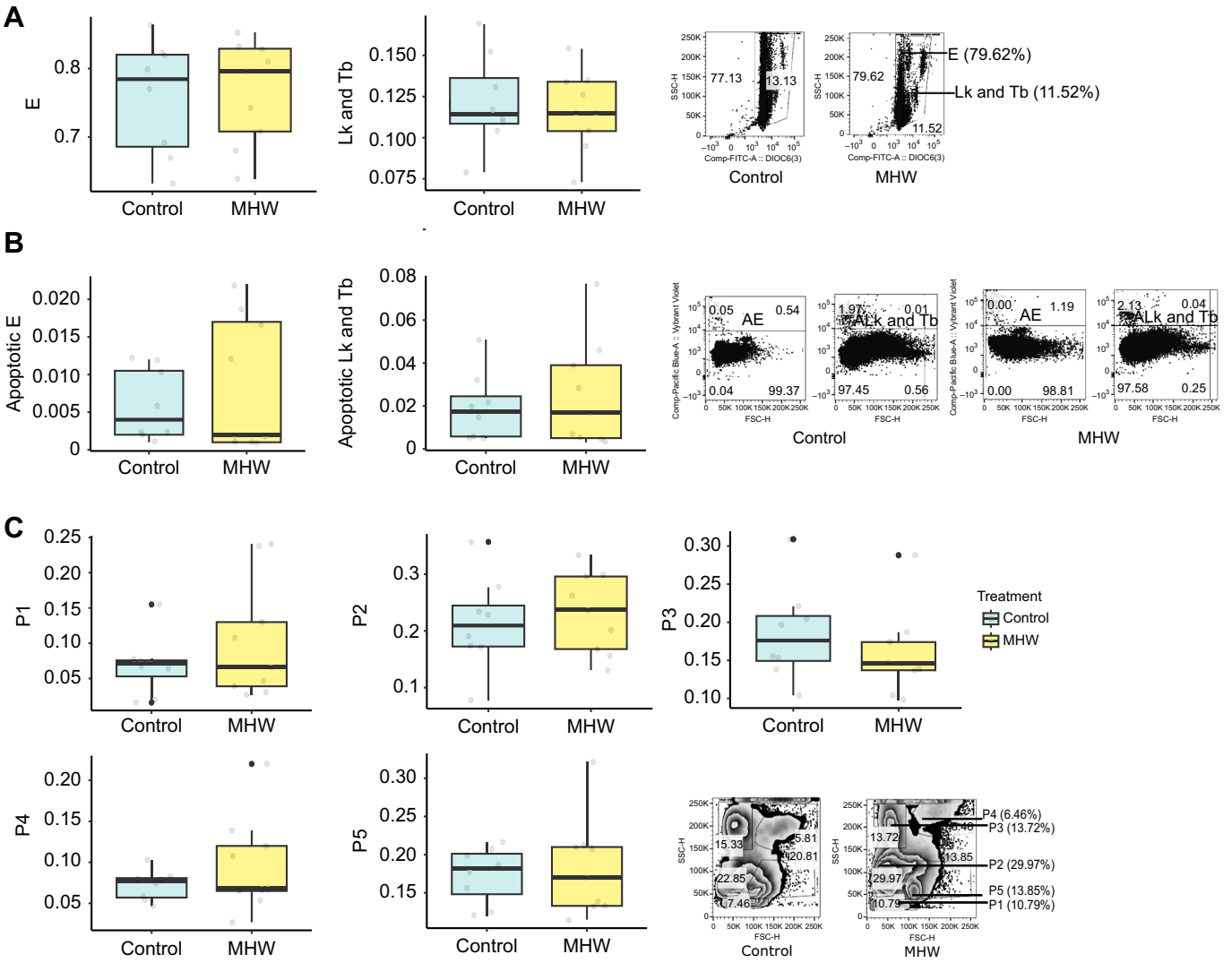

Fig. 3. Flow cytometry results in whole blood of small-spotted catsharks (*Scyliorhinus canicula*) after 30 days of exposure to a MHW. (A) Proportion of erythrocytes and leukocytes plus thrombocytes (Lk and Tb) stained with DiOC6(3). Representative flow cytometry plots of population gating are shown on the right. (B) Proportion of apoptotic erythrocytes (AE) and apoptotic leukocytes plus thrombocytes (Alk&Tb) stained with Vybrant® DyeCycle™ Violet. Representative flow cytometry plots of apoptotic cell gatings are shown on the right. (C) Proportion of subpopulations of leukocytes plus thrombocytes gated after DiOC6(3) staining. Representative flow cytometry plot of 5 subpopulations of leukocytes plus thrombocytes (P1–P5) are shown on the bottom right. Subpopulations were divided by their forward scatter-height (FSC-H) versus side scatter-height (SSC-H) properties. No significant differences were detected in cell populations between treatment groups (P>0.05). Additional statistical information is provided in Table S2. Control, n=8; MHW, n=9.

of expression of the genes remained unchanged in the liver, intestine and spleen (Fig. 7).

DISCUSSION

Little information is available about the effects of MHWs on adult shark immunology and how this environmental stressor might contribute to shark population declines. The present study addressed this knowledge gap, taking a mechanistic approach that provides a comprehensive understanding of the physiological response of small-spotted catsharks to MHWs. Out of the 45 parameters analyzed, six significantly changed between the two treatments. MHW-treated sharks had increased gonad-to-body ratio (GBR) and glucose levels, but had reduced levels of monocytes in peripheral blood, *igm* gene expression in blood cells and *tnfr* gene expression in blood cells and in the epigonal organ (Fig. 8). As demonstrated in Fig. 8, energy was reallocated by increasing glucose levels and enhancing reproductive performance to the detriment of immune-related factors. This re-budgeting revealed the resilience of small-spotted catsharks under the MHW conditions, with these mechanisms working to restore homeostasis and prevent mortality. These findings suggest that the applied MHW may have triggered adaptive phenotypic mechanisms such as allostasis, an adaptive process that actively maintains stability through change (Korte et al., 2005), allowing the sharks to cope with the applied MHW. However, the thermal stress may have compromised immunocompetence, which is the ability to resist or tolerate infection (Råberg et al., 2009; Simms, 2000). If allostasis becomes significantly impaired by prolonged or frequent stress, it could overwhelm the catsharks' adaptive mechanisms and, when combined with acute immune response impairments (Tort, 2011), pose long-term risks to this species. The absence of statistically significant differences in the other measured parameters may be attributed to the high variability in responses among individuals, which is typical of wild, genetically diverse animals (Downs and

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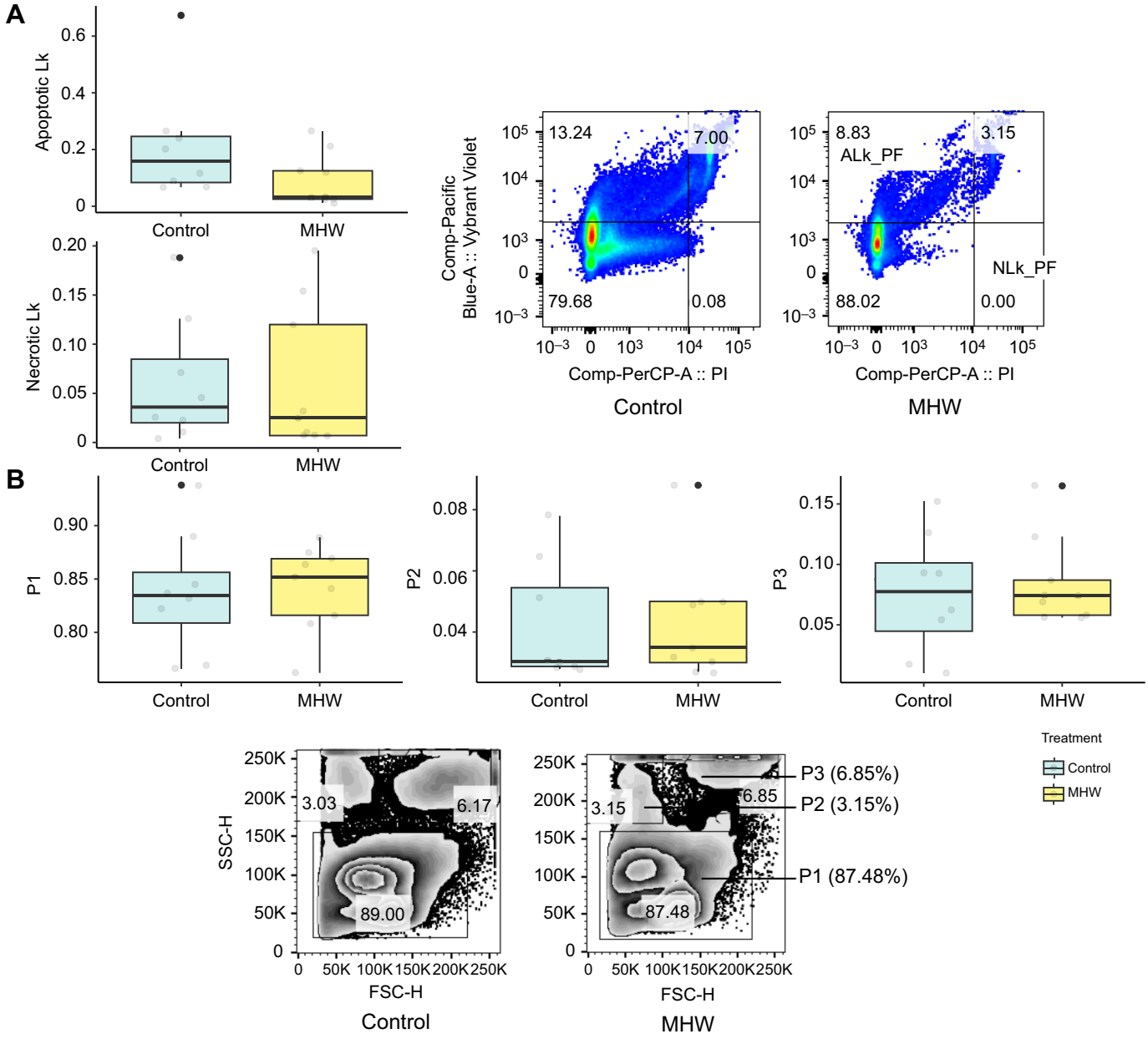

Fig. 4. Flow cytometry results for the Percoll fraction of small-spotted catsharks (*Scyliorhinus canicula*) after 30 days of exposure to a MHW. (A) Proportion of apoptotic leukocytes stained with Vybrant® DyeCycle™ Violet and necrotic leukocytes stained with Propidium iodide (PI). Representative flow cytometry plot gating showing percentage of apoptotic (ALk_PF) and necrotic leukocytes (NLk_PF) are shown on the right. (B) Proportion of subpopulations of leukocytes gated after DiOC6(3) staining in populations 1–3 (P1–P3). Representative flow cytometry plot gating showing percentage of P1–P3 (bottom); subpopulations were divided by their FSC-H versus SSC-H properties. No significant differences were detected (P>0.05) between the blood from control and MHW treatments. Additional statistical information is provided in Table S2. Control, n=8; MHW, n=9.

Stewart, 2014). The adult sharks used in this experiment likely experienced distinct life histories prior to capture and have different genotypes, which could account for the high variability in their responses to the MHW exposure.

Our study involved adult male sharks and given that sex-based differences may influence reproductive decisions – often shaped by energetic costs associated with physiological maintenance, growth and survival, which includes immune responses (Hammerschlag et al., 2018) – this aspect requires further discussion. There are few studies on how sex affects the immune response in fishes (Campbell et al., 2021). Moreover, their sex-based immune response has been indirectly identified during the spawning season when there is a decline in immune function, which is speculated to arise from inhibition to offset the increased energetic costs of reproduction (Campbell et al., 2021). However, in some species of teleost fish, both males and females experience reduced immune competence with decreases in immune cells and markers across various organs, in other species, a decline in innate immune markers only occurs in females (e.g. Ghobeishavi et al., 2016; Hou et al., 1999; Kortet et al., 2003; Pickering and Pottinger, 1987). Because the spawning period in fish is related to specific seasons, the immune response may also have a seasonal pattern (Campbell et al., 2021). Animals adopt various life-history strategies and resource utilization tactics to meet the high energy demands of reproduction (Hammerschlag

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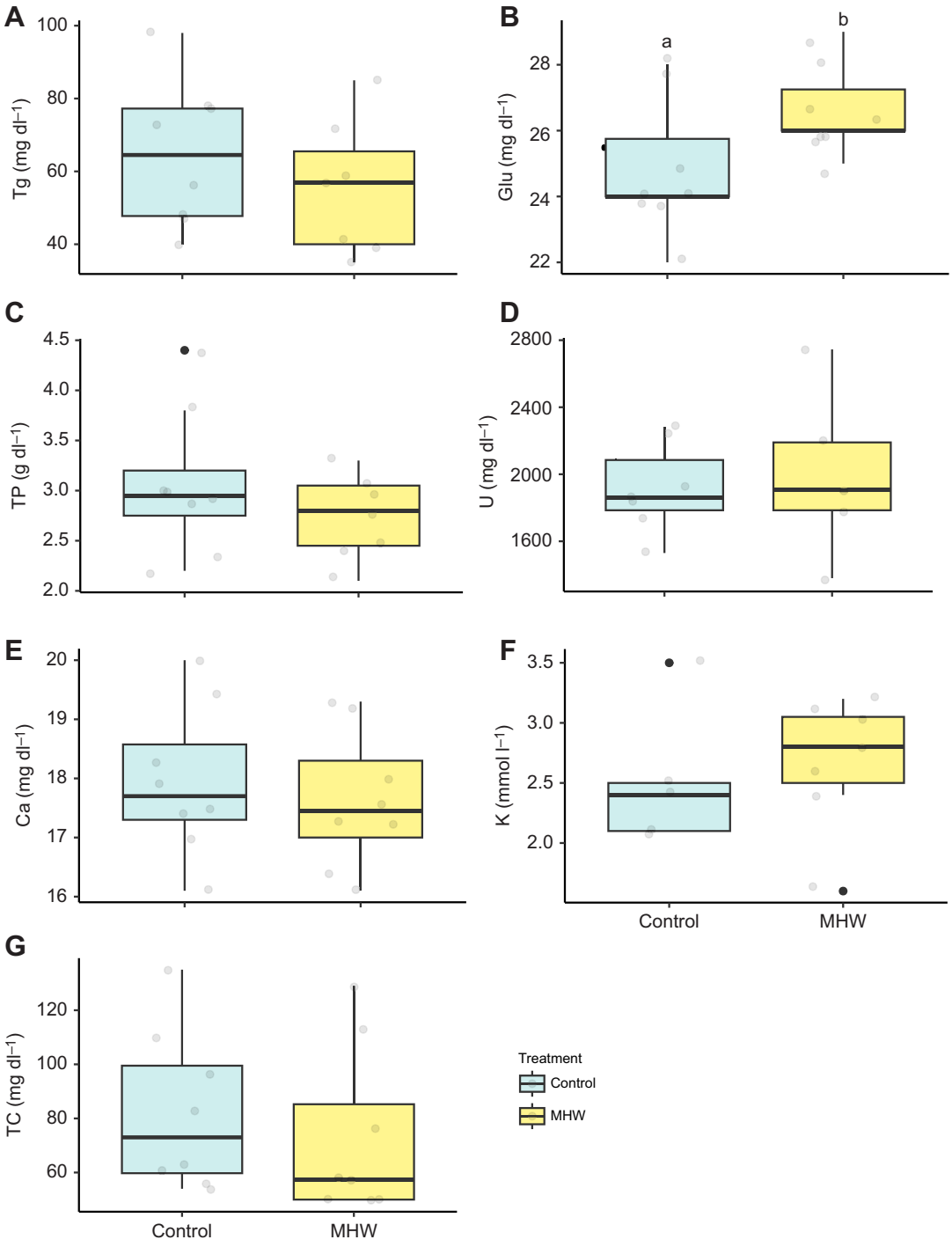

Fig. 5. The impact of MHW on plasma biochemistry in small-spotted catsharks (*Scyliorhinus canicula*) after 30 days of exposure. (A) plasma triglycerides (Tg; mg dl^{-1}). (B) Glucose (Glu; mg dl^{-1}). (C) Total protein (TP; g dl^{-1}). (D) Urea (U; mg dl^{-1}). (E) Calcium (Ca; mg dl^{-1}). (F) Potassium (K; mmol l^{-1}). (G) Total cholesterol (TC; mg dl^{-1}). Values with different letters in superscript are significantly different (P<0.05) between the control and MHW sharks. Additional statistical information is provided in Table S2. Control, n=8; MHW, n=9.

et al., 2018); these involve specific immune hypothalamic–pituitary–gonadal axis interactions and limiting demands are placed on the immune system (Campbell et al., 2021). However, comparable information on the relationship between the reproductive and energetic states of many shark species is lacking (Hammerschlag et al., 2018). Researchers should consider oviparous species such as small-spotted catsharks (Coolen et al., 2008) and viviparous shark species since the energetic investment in reproduction may differ. Furthermore, seasonal changes in the gonadosomatic index are only significant for female small-spotted catsharks (Kousteni et al., 2010) and their polyandric reproductive strategy (Griffiths et al., 2012) may require higher energetic demands for females. More studies are needed on both sexes of *S. canicula* to establish the cost of physiological traits and how they impact metabolically costly mechanisms, such as their immune response under climate change stressors.

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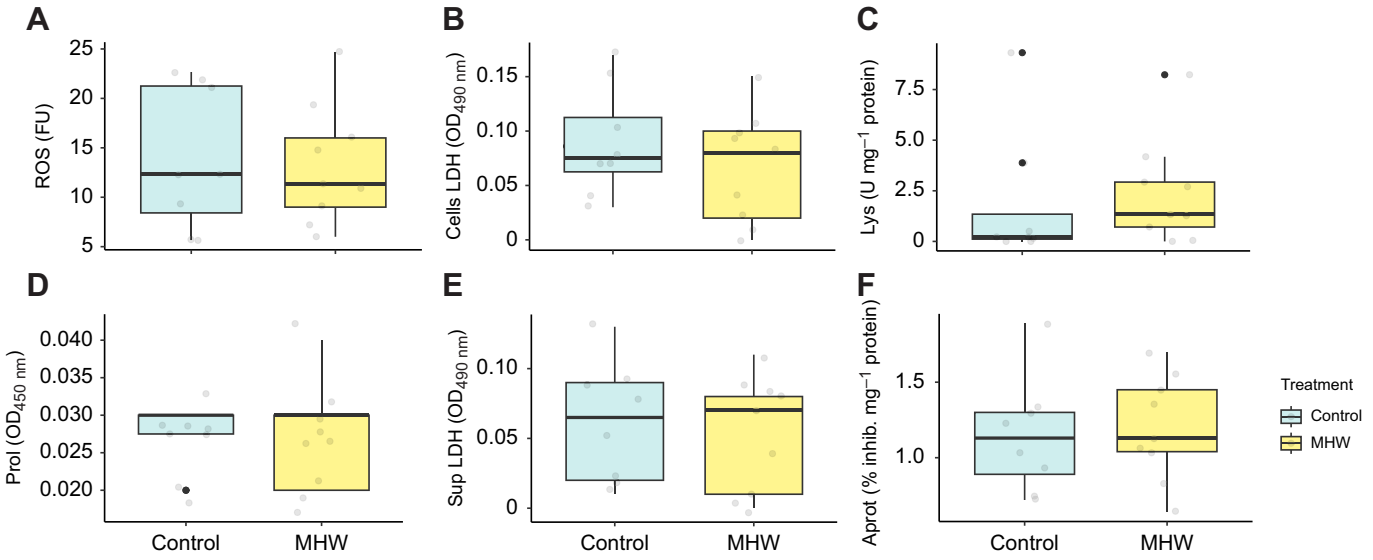

Fig. 6. Impact of a MHW on leukocyte activity in small-spotted catsharks (*Scyliorhinus canicula*) after 30 days of exposure to a MHW. (A) Reactive oxygen species (ROS; fluorescence units, FU), (D) proliferation (Prol) measured through formazan formation at 450 nm, (B,E) cytotoxicity measured by cell (Cells) and supernatant (Sup) lactate dehydrogenase (LDH) activity detected at 490 nm; in plasma: (C) lysozyme activity (Lys), (F) total anti-protease activity (Aprot), both normalized to the protein content of the plasma. No significant differences were detected (P>0.05). Additional statistical information is provided in Table S2. Control, n=8; MHW, n=9.

Morphometric indices clarify the nutritional status and relative health of fish. Fulton's condition factor makes the assumption that a heavier fish at a given length has greater energy reserves and, consequently, is in better condition (Bolger and Connolly, 1989). Gonad, liver and spleen ratios (GBR, LBR and SBR, respectively) can describe life history traits, fat accumulation and the response of fish to environmental effects (Brown and Murphy, 2004). Of the four morphometric indices, only GBR was significantly different and was higher in *S. canicula* after the 30 day MHW (Fig. 1). Additionally, GBR was strongly and negatively correlated with LBR (Fig. S2). This result may indicate that the sharks exposed to the MHW allocated energetic resources to reproduction and less to immune function, which was impaired (Fig. 8). This echoes what occurs in the Pacific salmon where energy was diverted away from several processes, including the immune response, to ensure the fish could spawn (Campbell et al., 2021). Schreck (2010) also reported that in some teleosts low levels of stress can positively influence reproductive processes. Thus, an increased GBR in the MHW-treated sharks may be a response to the MHW exposure. Furthermore, although it has been reported that only small-spotted catshark females have a seasonal change in the gonadosomatic index (Kousteni et al., 2010), the GBR increased in shark males during the breeding season (June and July; Ellis and Shackley, 1997) but only in the MHW treatment, further supporting the conclusion that increased GBR may be a response to MHW exposure. Conversely, the ability of fish to resist pathogens and cope with stress depends mostly on their nutritional status (Martin and Król, 2017) and the metabolic trade-offs of the sharks in this study could have been influenced by the food consumption rate. However, these sharks were fed daily at adequate levels for benthic sharks (Smith et al., 2004; Varela et al., 2023) and feeding sessions were monitored to ensure all the sharks were consistently satiated. On the other hand, all organisms have an optimal temperature range for growth, reproduction and ultimately, their survival (Angilletta, 2009), with immune function being a key component of survival. Spawning adults, such as the sharks in this study, have narrower thermal tolerance ranges than other developmental stages, making them particularly vulnerable to climate warming (Clarke, 2017; Dahlke et al., 2020). Metabolically expensive processes such as growth, organ development and gamete production can modify their highest acute temperature tolerance, known as the critical thermal maximum (CT_{max}) (Dahlke et al., 2020). Since spawners have a lower CT_{max} (Dahlke et al., 2020), the small-spotted catsharks in this experiment may have reduced their capacity to resist ocean warming. This energetic trade-off in thermal adaptation probably accounts for the significant decline in the immune-related parameters discussed below.

The secondary response, readily measurable in the peripheral circulation, can influence fitness and the immune response, leading to sublethal tertiary organismal and population-level responses (Skomal and Mandelman, 2012). Secondary responses to stress were assessed through blood cell quantification, plasma biochemistry and the use of immunological indicators (Skomal and Mandelman, 2012; Fig. 8).

The small-spotted catshark peripheral blood analysis showed a significant decrease in monocytes after the warming exposure (Fig. 2), which may be indicative that these long-lived phagocytic cells associated with defense against infections and bacteria (Davis et al., 2008) were migrating to inflammatory sites. Additionally, changes in leukocyte number may be indicative of a secondary stress response (Shahjahan et al., 2020), altering the immune function after the MHW (Fig. 8). The number of erythrocytes in the sharks did not differ between treatments, however, erythrocytic nuclear abnormalities (ENAs), although not significantly different, were more frequently observed in sharks from the warming treatment. In fact, it has previously been reported that adverse environmental conditions affect the shapes of circulating erythrocytes due to tubulin polymerization deficiency (Pegado et al., 2020b; Shahjahan et al., 2019).

The plasma biochemical parameters here quantified are proxies of health (AtallahBenson et al., 2020), and revealed these sharks experienced a secondary stress response after the MHW exposure (Fig. 8). Blood glucose, a vital indicator of stress is widely recognized as one of the most acceptable methods to assess the physiological status of shark species under stress (Falco et al., 2023; Schoen et al.,

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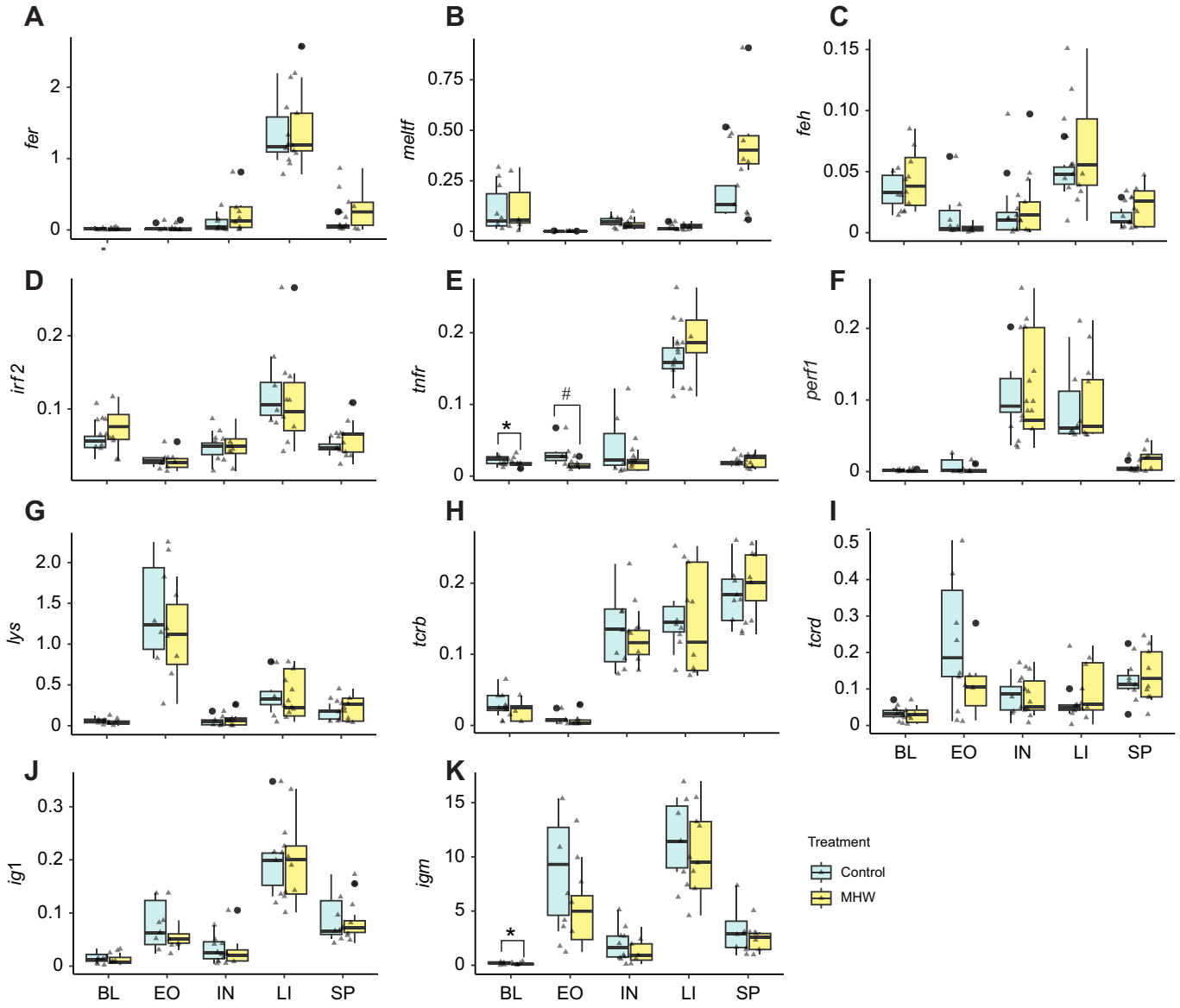

Fig. 7. Expression of immune-related genes in tissues of small-spotted catsharks (*Scyliorhinus canicula*) after 30 days of exposure to a MHW. (A) *fer*, ferritin heavy chain; (B) *meltf*, melanotransferrin; (C) *fch*, ferrochelatase; (D) *irf2*, interferon regulatory factor 2; (E) *tnfr*, tumor necrosis factor receptor SF1A-associated via death domain; (F) *perf1*, perforin 1; (G) *lys*, lysozyme; (H) *tcrb*, T-cell receptor beta; (I) *tcrd*, T-cell receptor delta; (J) *ig1*, immunoglobulin IgNAR transmembrane and secretory heavy chain constant region; (K) *igm*, immunoglobulin M secretory heavy chain. BL, blood; EO, epigonal organ; IN, intestine; LI, liver; SP, spleen. Genes are expressed as SQ mean, normalized by the geometric mean of the reference genes (RPL13 and PRS29). Symbols indicate significant differences between treatments (P<0.05). Additional statistical information is provided in Table S2. Control, n=8; MHW, n=9.

2021). In this study, glucose levels were significantly higher in sharks submitted to the MHW (Fig. 5), although the measured immunological indicators in the sharks' plasma and leukocytes (plasma lysozyme, reactive oxygen species, proliferation, cell cytotoxicity and total anti-protease activity) were not significantly affected by the MHW (Fig. 6). Increased levels of plasma glucose in the small-spotted catshark are consistent with previous studies in elasmobranchs that showed a strong increase in glucose due to metabolite mobilization after thermal stress (Bouyoucos et al., 2018; Falco et al., 2023; Scarponi et al., 2021; Schoen et al., 2021). The effect of stress triggers a rapid mobilization of glucose into the bloodstream to meet increased energetic demands, aiding in the restoration of homeostasis during and after a stressful event (Schoen et al., 2021; Skomal and Mandelman, 2012). Confounding factors such as seasonal variations in glucose levels (Gutiérrez et al., 1988), seem unlikely to explain the significant rise in the sharks' glucose levels since it only occurred in the MHW treatments and not the control. Therefore, this study showed that the MHW-exposed *S. canicula* males underwent significant energy mobilization by increasing levels of glucose in the peripheral circulation. Additionally, all the sharks' plasma osmolytes were positively correlated after the MHW treatment (Fig. S2). These adjustments in metabolic allocation and osmoregulation after the MHW exposure may impact systemic health and the overall redistribution or suppression of immune function (Chrousos, 2009; Seibel et al., 2021; Wendelaar Bonga, 1997).

The MHW event significantly suppressed the expression of the sharks' immune-related genes *igm* and *tnfr* in blood cells and in the primary lymphoid organ of sharks, the epigonal organ (Smith et al., 2004) (Fig. 7). The reduction in expression of two core genes of the immune response in the MHW-exposed sharks suggest there was a

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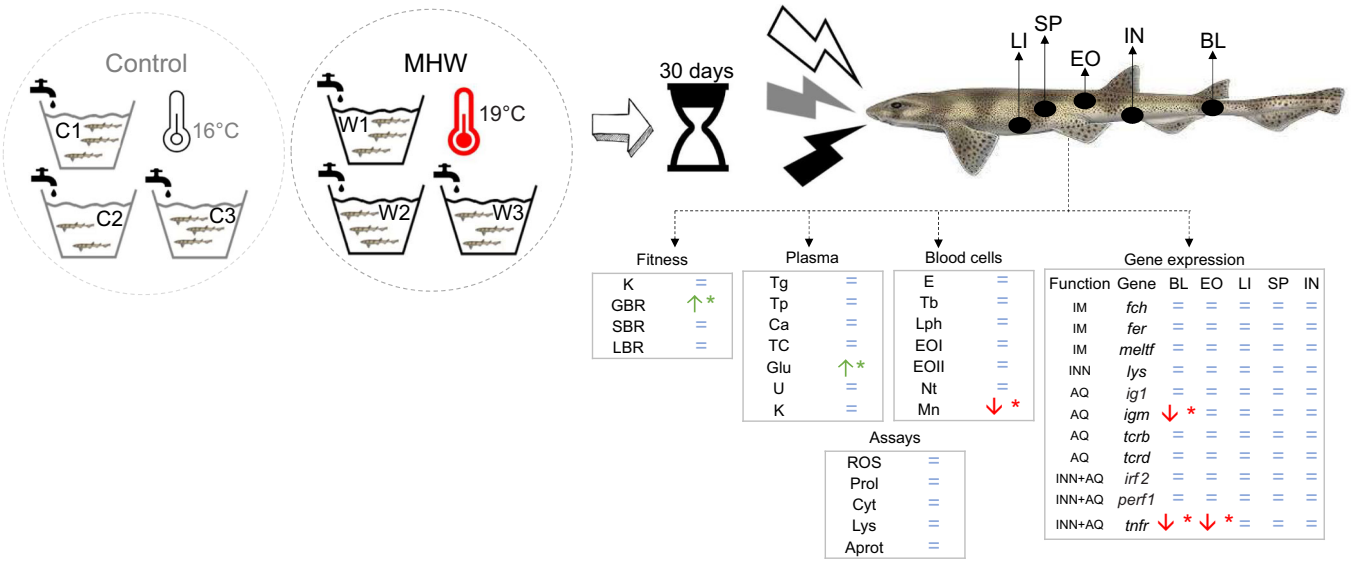

Fig. 8. GBR, *igm* and *tnfr* gene expression in blood cells and *tnfr* in the epigonal organ, monocyte number and plasma glucose were significantly modified in adult small-spotted catsharks. Temperature control was set at 16°C, with an increase of 3°C for the MHW for 30 days. Each treatment had three replicates. After 30 days of exposure, fitness indices (GBR, SBR, LBR, K) were calculated, and sharks were euthanized to collect blood (BL), and a portion of the epigonal organ (EO), liver (LI), spleen (SP) and intestine (IN). Immune assays [reactive oxygen species (ROS), proliferation (Prol), cytotoxicity (Cyt), lysozyme (Lys), total anti-protease (Aprot)], plasma biochemistry [triglycerides (Tg), total protein (Tp), calcium, total cholesterol (TC), glucose (Glu), urea (U) and potassium (K)] and blood cell quantification [erythrocytes (E), thrombocytes (Tb), lymphocytes (Lph), eosinophils (EO), neutrophils (Nt), monocytes (Mn)] were performed. Expression of genes related to iron metabolism (IM), innate immune response (INN) and acquired immune response (AQ) was quantified in the lymphoid organs and in the blood. GBR and Glu increased significantly with warming, while Mn, gene expression of *igm* in blood, and gene expression of *tnfr* in blood and the epigonal organ followed an opposite trend. ↓, decreased; ↑, increased; =, level maintained compared with control. *Parameter with significant differences between treatments (P<0.05).

decline in their adaptive immune capacity. This effect was measured after 30 days of treatment and the absence of significantly altered expression levels of the remaining nine genes (Figs 7 and 8) may be linked to their function. The liver was the organ where *irf2* was most highly expressed in *S. canicula* (Fig. 7), which contrasts with the elephant shark where the spleen had the highest and broadest expression of IRFs (including IRF2) (Du et al., 2018). In *S. canicula*, IgNAR (*ig1*) was predominantly expressed in the liver and *igm* in the epigonal organ and liver (Fig. 7), which contrasts with the finding of Li et al. (2013), who reported the highest transcript levels of immunoglobulins in the spleen, pancreas, epigonal and Leydig organs of the spiny dogfish (*Squalus acanthias*). Isotypes *igm* and *ig1* (IgNAR) seemed to respond differently and independently to environmental stress in *S. canicula* (Fig. 7). In fact, Rumfelt et al. (2002) reported no overlap between IgM- and IgNAR-secreting cells in nurse sharks, also indicating isotypic exclusion.

Overall, following exposure to the MHW, we observed non-lethal stress effects on the small-spotted catsharks, and the significant results indicate an increase in reproduction-related indices (GBR) and glucose levels, along with cellular stress responses through decreased immune-related gene expression (Fig. 8). The sharks' energy mobilization was aimed at facilitating the restoration of homeostasis by increasing glucose levels (Figs 5 and 8) and optimizing the reproductive performance by increasing the GBR (Figs 1 and 8). The lack of statistical differences in most of the parameters analyzed (Fig. 8) suggests that adult small-spotted catsharks were resilient to a MHW category II (plateau two times the threshold of the local climatology: >3°C compared with control temperature). This 30 day MHW is defined as an acute event that caused a chronic stress response, disturbing the sharks' individual physical integrity to some extent (Scheiner et al., 2020) (Fig. 8). Based on the results obtained, the applied MHW may have triggered adaptative phenotypic mechanisms in these sharks, enabling them to cope with an acute warming event. In addition, the phenotypic plasticity of cellular immune system components (Scheiner et al., 2020) and the majority of significantly unchanged parameters may be because the adult sharks had already been exposed to adverse environmental conditions in the wild and developed some plasticity (thermal imprinting). It has been reported that repeated exposure to low-intensity stressors can trigger an adaptive response over time and attenuate acute stress reactions (Schreck et al., 2016). This phenotypic plasticity, along with optimum physical conditions to survive and reproduce, can potentially keep pace with the predicted environmental changes and is one mechanism proposed to prevent the extinction of species (Scheiner et al., 2020). Microevolutionary changes in response to climate change remain largely unclear, as phenotypic adaptations can either delay or facilitate genetic change (Grazer and Martin, 2012). By increasing reproduction, offspring generation is enhanced to maintain species survival through legacy preservation, increasing the potential for adaptation, divergence, and speciation (Grazer and Martin, 2012). However, other parameters under warming should be evaluated, such as sperm viability and female general responses, including reproductive fitness and immune and endocrine response.

The allostasis that these sharks achieved after the exposure to a 30 day MHW may temporarily present a resilient state, but it can prove dysfunctional if the compensatory mechanisms or the energetic reserves are not sufficient for long-term stress events. Therefore, persistent stressors such as repeated MHWs and long-term exposure may overwhelm small-spotted catsharks' adaptive mechanisms, posing long-term risks. Recognized as a mesopredator because of its high trophic plasticity, the small-spotted catshark plays a crucial role in shaping the structure and function of ecosystems, as highlighted in a study by Barría et al. (2018). The potential decline in *S. canicula* populations due to the projected

increase in frequency, duration, spatial extent and intensity of MHWs could disrupt ecosystems from temperate regions from Norway to the south of Senegal and the Mediterranean Sea (Ellis and Shackley, 1997). Despite the presence of several genetic stocks across different populations (Kousteni et al., 2015), the loss of genetic diversity may increase the risk of extinction (Frankham, 2005) for this critical mesopredator in the coming decades. Moreover, polyandry and multiple paternity in *S. canicula* may alter the potential for adaptation within populations (Frankham, 2005); thus, this matter should also be investigated.

In further studies, a higher number of animals could result in a more homogenous response, diminishing individual variability inherent to the immune response. This would enhance study power and practical significance and prevent type II errors, thus possibly uncovering the catsharks' resilience and capacity to cope with additional stressors. Lastly, it is important to highlight that the present study optimized many techniques, developed tools for peripheral blood analysis in sharks, and revealed the utility and cost-effectiveness of the hemacytometer, which yielded similar results to those obtained using technical and costly flow cytometry methods (cf. Tables S2 and S3).

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: S.M., C.P.S., R.R., D.M.P.; Methodology: S.M., C.F., A.P.M., C.P.S., J.F., R.R., D.M.P.; Validation: S.M., C.F., A.P.M., J.F., R.R., D.M.P.; Formal analysis: S.M.; Investigation: S.M., C.F., A.P.M., C.P.S., J.F.; Resources: C.F., J.F., R.R., D.M.P.; Data curation: S.M.; Writing - original draft: S.M.; Writing - review & editing: S.M., C.F., A.P.M., J.F., R.R., D.M.P.; Visualization: S.M., R.R., D.M.P.; Supervision: R.R., D.M.P.; Project administration: R.R., D.M.P.; Funding acquisition: R.R., D.M.P.

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Data availability
All relevant data can be found within the article and its supplementary information.

ECR Spotlight
This article has an associated ECR Spotlight interview with Sandra Martins.

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