Alexandro Autiero

Physiological response of *Palaemon elegans* (Rathke, 1836) to anthropogenic stressors: synergistic impacts of heatwaves and UV filters from sunscreens

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Mestrado em Biologia Marinha

Supervisors:
Alicia Herrera Ulibarri

Co-supervisor

Catarina Vinagre

UNIVERSIDADE DO ALGARVE

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Abstract

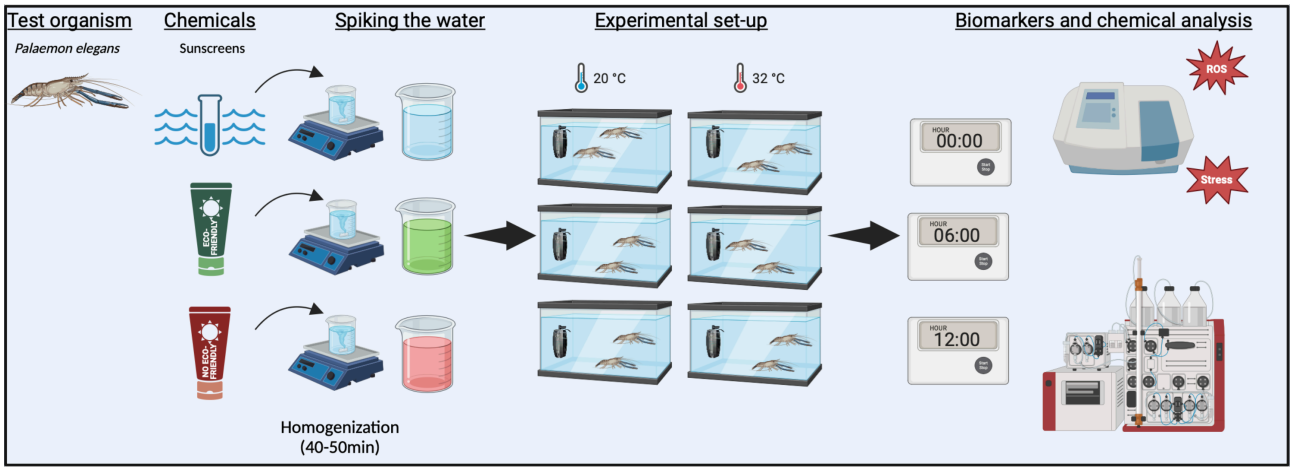
Heat waves are prolonged periods of unusually high temperatures representing a threat to marine organisms. Their intensification raised concerns about the impacts on aquatic organisms and ecosystem dynamics. On the other hand, the rapid escalation of tourism particularly in tropical and subtropical regions and the greater awareness of photodamage caused by solar UV radiation increased the use of sunscreens. Indeed, UV filters found in sunscreen have recently been identified as emerging pollutants in coastal waters due to their stability in the environment and the potential to accumulate in marine organisms. Exposure to these components has been proven toxic to many aquatic species. UV filters cause substantial levels of reactive oxygen species (ROS).

This study analyses the effects of extreme weather temperatures with two different sunscreens on *Palaemon elegans* (Rathke, 1836). Two temperatures (20°C, and 32°C) and two different sunscreens (one labelled as eco-friendly and the other not) were tested in a full factorial experiment for 12 hours. Shrimps had three sampling times: 1, 6 and 12 hours of exposure. At each of sampling time, the combined effect of temperatures and sunscreens was analysed in hepatopancreas and muscle. Cellular biomarkers associated with oxidative stress such as GST – Glutathione-S-Transferase, SOD – Superoxide Dismutase, with oxidative damage, LPO – Lipid peroxidation, and metabolic biomarkers such as COX – Cytochrome c oxidase, ETS – Electron transport system, were used as indicators of the shrimp's physiological response.

Analysis of metabolic biomarkers in the muscle showed that while ETS exhibited a higher metabolic demand at elevated temperatures, decreased levels of COX indicated mitochondrial dysfunction caused by oxidative stress at high temperatures, further enhanced by exposure to non-ecofriendly sunscreen. These effects were increased in the non-eco-friendly sunscreen. LPO activity showed oxidative stress in organisms exposed to elevated temperatures and treatments with non-ecofriendly sunscreen. Whereas oxidative stress biomarkers such as GST and SOD showed that these antioxidant defences were saturated due to ROS accumulation related to high temperature and chemical pollutants.

Keywords: Heatwaves, oxidative stress, UV sunscreens filters, biomarkers, environmental stressors, *P.elegans*

Graphical Abstract



Resumo

As ondas de calor são períodos prolongados caracterizados por temperaturas muito elevadas que representam uma grave ameaça ambiental. Tem, de facto, um impacto significativo sobretudo para os organismos marinhos. Devido às graves alterações antropogénicas no meio ambiente, a frequência, duração e intensidade das ondas de calor aumentaram a nível global, transformando-se em riscos relevantes para os ecossistemas aquáticos. As altas temperaturas, acima do normal, têm vindo a afetar severamente os organismos marinhos, influindo tanto nos processos metabólicos dos organismos, como também a nível abiótico provocando uma redução da solubilidade do oxigénio na água levando a stress fisiológico. Este processo poder ter consequências letais para o ecossistema marinho podendo levar à morte dos organismos ou a mudanças nas dinâmicas populacionais. Os impactos que as ondas de calor podem ter sobre os invertebrados marinhos, como os crustáceos são motivo particular de preocupação. De facto, estes organismos marinhos desempenham papeis cruciais no funcionamento da cadeia trófica e, em geral, no funcionamento dos ecossistemas costeiros. Portanto, compreender os efeitos combinados do stress térmico e de outros poluentes ambientais é fundamental para prever as possíveis consequências para a vida marinha e desenvolver estratégias para a conservação desses ecossistemas.

O incremento da população humana mundial e o rápido crescimento do turismo de massa, especialmente em regiões tropicais e subtropicais, levou a um aumento da pressão sobre os ecossistemas marinhos locais. Além disso, as novas descobertas científicas em relação à prevenção de tumores da pele, levou a uma maior preocupação em relação à exposição solar e uso constante de protetores solares. Desta forma, a crescente conscientização dos efeitos nocivos da radiação solar ultravioleta (UV), sendo causa entre as mais comuns do fotoenvelhecimento e dos melanomas, contribuiu para um aumento significativo na aplicação de cremes solares. No entanto, a maioria dos protetores solares contêm diferentes filtros UV, os quais podem ser tanto físicos, como químicos, que refletem ou absorvem a radiação UV. Esses filtros, embora benéficos (em parte) para o ser humano, têm recentemente sido identificados como poluentes emergentes em águas costeiras. Isto deve-se tanto à capacidade dos filtros UV de estar quimicamente estáveis na água, persistindo por largos períodos no ambiente marinho e podendo acumular-se nos organismos aquáticos, representando uma ameaça significativa para a vida marinha.

Estudos anteriores demonstraram que os filtros UV podem ser tóxicos para uma ampla variedade de espécies marinhas. A toxicidade dos filtros UV, absorvida pelos organismos em directo contacto na água, são causa principal de atraso no desenvolvimento, decrescimento na taxa de reprodução e principalmente levando à geração de espécies reativas de oxigénio (ROS). Isto leva a um incremento da atividade das enzimas antioxidantes, e se não é suficiente, leva a vários danos celulares e a

alterações dos processos fisiológicos. Além disso, os filtros UV podem interagir com os micronutrientes inorgânicos em habitats costeiros, alterando a dinâmica de nutrientes e afetando ainda mais os ecossistemas marinhos locais. Apesar da crescente preocupação, ainda há algumas lacunas sobre os impactos fisiológicos específicos desses poluentes em espécies marinhas, em particular em condições de temperaturas elevadas associadas às ondas de calor.

O estudo conduzido neste trabalho pretende analisar e compreender os efeitos combinados das ondas de calor marinhas em associação à exposição a diferentes tipos de filtros UV, contidos em protetores solares, no camarão *Palaemon elegans* (Rathke, 1836). O foco nesta espécie, amplamente distribuída em habitats costeiros rochosos das Ilhas Canárias (Espanha), e em particular no norte da ilha de Gran Canaria, deve-se ao facto de que este organismo representa um componente essencial da cadeia trófica marinha e devido a sua ampla distribuição. Este estudo tem como objetivo elucidar o impacto combinado do stress térmico e da poluição química na saúde fisiológica deste organismo marinho.

O desenho experimental tem em consideração duas temperaturas diferentes: uma de 20°C, que representa uma temperatura média das poças de maré naturais durante a baixa maré, e outra de 32°C, que representa condições extremas que se verificam durante as ondas de calor, sempre em baixa maré. Além dessas condições, duas distintas formulações de protetor solar (uma rotulada como ecológica e não prejudicial para o meio ambiente e outra não) foram testadas, em associação às diferentes temperaturas.

O estudo foi feito durante um período total de 12 horas em que as amostras foram recolhidas após 30 minutos de exposição ao creme solar e às temperaturas (sendo este o T1); passadas 6 horas (T6) e depois de 12 horas (T12). Além destas, foram recolhidas no dia anterior, amostras representando o T0, à temperatura de 20°C.

Os efeitos combinados da temperatura e da exposição aos protetores solar foram avaliados analisando biomarcadores de stress oxidativo tanto no tecido muscular (sem quitina) como no hepatopâncreas dos camarões. Os biomarcadores analisados foram Citocromo c oxidase (COX), Sistema de Transporte de Elétrões (ETS), Glutationa S-Transferase (GST), Superóxido Dismutase (SOD) e Peroxidação Lipídica (LPO). Esses biomarcadores foram escolhidos para fornecer um quadro abrangente das respostas fisiológicas face a um possível stress oxidativo nos camarões. COX e ETS são biomarcadores envolvidos na respiração celular e no metabolismo energético. GST e SOD desempenham papéis críticos na desintoxicação dos radicais livres e na proteção contra danos oxidativos. Por fim, a LPO serve como um indicador de danos oxidativos às membranas celulares.

Para quantificar a incorporação e a acumulação de componentes dos filtros UV nos tecidos dos camarões ao longo do período de exposição, utilizou-se a cromatografia líquida de alta eficiência (HPLC). Essa técnica analítica permitiu a medição precisa das concentrações de filtros UV nos

camarões, fornecendo importantes informações sobre se o período de exposição de 12 horas é suficiente para observar acumulação, e de como esta pode variar ao largo do tempo de acordo com as diferentes temperaturas. Além disso, os dados gerados por este estudo podem ajudar a perceber se os protetores solares apresentados como ecológicos diferem das formulações convencionais em termos de seu impacto nos organismos marinhos e em condições ambientais variáveis.

Das anaálises de biomarcadores feitas no tecido muscular do organismo, resulta que, as elevadas temperaturas aumentaram a demanda metabólica a nível celular, justificada face um aumento da atividade da ETS. Contrariamente, um decrescimento da atividade da COX indica que, as elevadas temperaturas, causaram uma disfunção mitocondrial devido a stresse oxidativo. Estes efeitos foram amplificados no tratamento com protetor solar não ecológico. A atividade da LPO indicou um aumento de stresse oxidativo em organismos em condições de elevada temperatura, e associados ao protetor solar não ecológico. Contrariamente, GST e SOD indicaram que estas defesas contra radicais livres já não estão reagindo adequadamente, possivelmente devido a elevada acumulação de espécies reativas de oxigénio.

A análise feita por HPLC revelou que os filtros UV de ambos os protetores solares foram rapidamente absorvidos e acumulados nos camarões. Foi observado que as concentrações de filtros UV nos tecidos foram maiores após 12 horas de exposição, mesmo na temperatura mais baixa, indicando que há bioacumulação e que pode ocorrer rapidamente, podendo levar a efeitos prejudiciais de longo prazo nos organismos marinhos, se estas forem repetidas no tempo. Isto pode permitir também que os contaminantes químicos passem na cadeia trófica podendo bioacumular-se em outros organismos marinos. Esses resultados ressaltam a importância de avaliar concretamente o impacto ambiental dos filtros UV e de reconsiderar seu uso em produtos de consumo, especialmente em regiões propensas a ondas de calor e de turismo massivo.

Este estudo fornece novas perspetivas sobre a interação entre os fatores de stress ambientais, como ondas de calor, e novos poluentes, como os filtros UV. Através o uso de biomarcadores de estres oxidativo e metabólicos foi possível evidenciar que a combinação desses stressores antropogénicos induzem efeitos adversos. Estes são visíveis numa variação dos valores de biomarcadores metabólicos como ETS e COX, numa saturação dos biomarcadores de stresse oxidativo como GST e SOD, e em um aumento dos níveis de LPO. As evidências sugerem que organismos marinhos, como *Palaemon elegans*, são particularmente vulneráveis aos efeitos combinados de temperaturas elevadas e exposição química. E que as respostas face ao stress oxidativo observadas ressaltam que os impactos acumulados na saúde e na sobrevivência desses organismos em cenários climáticos futuros (inclusivamente mais críticos) pode piorar, comprometendo o ecossistema, e pudendo alcançar largas distâncias através a propagação dos poluentes químicos através a cadeia trófica. Além disso, a rápida

bioacumulação de filtros UV no tecido do organismo, após apenas 6 horas de exposição, levanta grande preocupação sobre as possíveis consequências de longo prazo nas cadeias alimentares marinhas e sobretudo na estabilidade dos ecossistemas.

As descobertas deste estudo têm importantes implicações para a conservação e gestão marinha e sobretudo sobre os efeitos antropogénicos em organismos marinhos. A evidência de rápida bioacumulação e de stress oxidativo indica que se necessita de nova regulação e que os produtos usados sejam minuciosamente testados para abordar a ameaça emergente dos filtros UV. Além disso, há grande necessidade de maior conscientização pública sobre os potenciais impactos ambientais dos protetores solares e há que desenvolver alternativas verdadeiramente ecológicas e a preço acessível (sendo esta a maior causa de estes produtos não serem comprados). Políticas que promovam o uso dessas alternativas mais saudáveis tanto para o meio ambiente como também para o ser humano, especialmente em áreas com alta densidade turística, poderiam ajudar a mitigar os riscos apresentados por esses poluentes.

Ao compreender as respostas fisiológicas do *Palaemon elegans* face ao stress térmico em combinação com a exposição aos filtros UV, este estudo pretende contribuir para uma maior compreensão de como as alterações climáticas interferem nos ecossistemas marinhos. Os resultados ressaltam a vulnerabilidade dos organismos marinhos a múltiplos fatores de stress e destacam a necessidade de medidas urgentes proativas para proteger a biodiversidade marinha.

Palavras-chave: Ondas de calor, filtros UV, protetores solares, organismos marinhos, stress oxidativo, bioacumulação, *Palaemon elegans*, fatores de stress ambiental, conservação marinha.

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Many people also supported this study and the journey leading to it, which began back in 2015 when I had the opportunity to study in Faro—a completely different experience. There, a dream of mine came true: to live right next to the ocean. With that came the realization of something I had never acknowledged before—that I simply love the ocean and that one can also study it. For this, I want to thank the professor who gave me that opportunity at the time: thank you, Barbara Gori.

After wandering around for a while, I finally found my aspiration, and Porto was the starting point of this new chapter. It was there where I first immersed myself in studying Marine Biology. For this, I really want to thank Professor Alexandre da Cunha, who at the time was on the selection committee and who gave me the opportunity to be admitted to the course. The time in Porto was wonderful, and I was lucky enough to find my chosen family. I want to thank Fede and Kate, Pedro, Lili, Bia, and her whole family.

Then it was time to move again, this time to the Canary Islands for an Erasmus exchange. This small place in the world gave me so much and filled my heart with people I will cherish for the rest of my life. Thank you, Alvarito y Clarita, Lucky, and Vic. I am pretty sure that without you, the whole experience would not have been the same.

Again, time to move. Time to close the circle. Time to go back to where it all started—back to Faro to begin the academic path that, with this thesis, I am closing right now. I want to thank again Professor Alexandre Lobo da Cunha, Professor Maria Salomé Gomes and Professor Carilina Lemos who helped me get to Faro.

This new path took me back to an old one—back to the Canary Islands, and the EOMAR research group to write this thesis. For this, I want to thank the whole EOMAR research lab for the opportunity they gave me. I want to thank everyone who helped me and who took part of their time for the success of this work and for believing in what I was doing. A special thanks to Ico Martinez and her infinite patience in the lab and not only, to Alicia Herrera, who believed and helped me throughout this journey, and to Sarah Montesdeoca Esponda who immediately showed interest in this project and made herself available. Thanks also to Eva and Margaux for sharing with me their knowledge and tips in liquid chromatography. I want to give a special thanks also to Catarina Vinagre, who supported me and gave me the opportunity to present this work in Valencia at ISMS 24.

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obstacles of life and for his wisdom. A really special thank you goes to Vic, for your patience, for encouraging me when I was at my lowest, and for believing in me and my passion. You had a special role in the success of this work and of many others yet to come. We were flatmates and friends; now we are vanmates and partners. I want to dedicate this work also to you and to the many future adventures that will come.

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As a song from Stereophonics says, "Life is not easy, but it's the one we all got." I hope this is only the start of this amazing path!

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List of abbreviation

- ABS – Absorbance
- ACN – Acetonitrile
- BEH – Bridged Ethyl Hybrid
- BMDBM – Butyl Methoxy Dibenzoyl Methane
- BP-3 – Benzophenone-3
- BF – Buffer
- BHT – Butylated hydroxytoluene
- BSA – Bovine serum albumin
- CDNB – Chloro Di Nitro Benzene
- COX – Cytochrome c oxidase
- CYT-C – Cytochrome c
- CTMax – Critical Temperature maximum
- DTT – Dithiothreitol
- ECA – European Chemical Agency
- EDTA – Ethylenediaminetetraacetic acid
- ETC – Electron Transport Chain
- EHS – Ethylhexyl Salicylate
- ESI – Electrospray Ionization
- ETS – Electron Transport System
- FADH2 – Flavin Adenine Dinucleotide
- GST – Glutathione-*S*-transferase
- HEX – Hexane
- HPLC-MS – High-Performance Liquid Chromatography-Mass Spectrophotometry
- INT– p-Iodonitrotetrazolium violet (Iodonitrotetrazolium chloride)
- IS – Internal standard
- MCEs – Marine and coastal ecosystems
- MHWs – Marine heatwaves
- NADH – Nicotinamide Adenine Dinucleotide
- NADPH – Nicotinamide Adenine Dinucleotide Phosphate
- NBT – Nitroblue Tetrazolium
- OC – Octocrylene

- PET - Polyethylene Terephthalate
- PMS – Phenazine Methosulfate
- PVP – Polyvinylpyrrolidone
- ROS – Reactive Oxygen Species
- SPA – Solid Phase Extraction
- TBA – Thiobarbituric Acid
- TBARS – Thiobarbituric Acid Reactive Substances
- TCA – Trichloroacetic Acid
- SOD – Superoxide Dismutase
- UV – Ultraviolet
- WR – Working Reagent

State-of-the-art introductory section

Globally, marine and coastal ecosystems have been increasingly suffering from a higher anthropogenic pressure (Rocha et al., 2015; Selim et al., 2016), threatening biodiversity, disrupting its ecological balance and leading to species losses (Worm et al., 2006). Multiple anthropogenic stressors interact daily with aquatic ecosystems creating difficult challenges for conservationists. This is happening more directly with chemicals and pollutant release (Cadena-Aizaga et al., 2022; Madhav et al., 2020), marine litter (Camacho et al., 2019; Herrera, Rivera, et al., 2022; Reinold et al., 2020; Ugwu et al., 2021), fisheries (Pusceddu et al., 2014; Thrush & Dayton, 2002), and indirectly through marine heatwaves (de Luzinais et al., 2024; Fragkopoulou et al., 2023) and eutrophication (Malone & Newton, 2020; Maúre et al., 2021; V. H. Smith, 2003). Even though it is difficult to assess the specific weight of each stressor, it is crucial to consider that the worst effect they can have is when they pass from the environment to animal organisms leading to the phenomenon of bioaccumulation and biomagnification, which increases the toxicity of pollutants (Cruz & Barceló, 2015; Herrera, Acosta-Dacal, et al., 2022; Lozano et al., 2020).

In Europe have been established precise criteria to protect and monitor the aquatic environment. Those legislations, such as the Water Framework (WFD; 2000/60/EC) and the Marine Strategy Framework Directive (MSFD; 2008/56/EC) have the objective of restoring the ecological and environmental good status of estuarine, coastal and offshore waters. While the WFD aims to protect and restore waters by promoting sustainable use and reducing pollutants, the MSFD promotes the use of biological elements such as phytoplankton and benthic invertebrate fauna to preserve the health and to have an overview of the quality status (EC–European Commission, 2003). This legislation has been implemented with a series of multimetric indexes to assess a more accurate overview to separate and target specific areas that need specific attention.

To specifically assess the impact of these stressors, biomarkers and bioindicators based on determined parameters have become essential tools for providing early signs of environmental stress (Samanta et al., 2018; Sanchez & Porcher, 2009). Originally, biomarkers were defined as “a xenobiotically-induced variation in cellular or biochemical components or processes, structures or functions that is measurable in a biological system or sample” (Shugart et al., 1992) and were mainly used in pharmacology and medical toxicology. Later on, McCarty et al., (2002) modified the concept of biomarkers, adding “A biomarker is an anthropogenically-induced variation in biochemical, physiological, or ecological components or processes, structures, or functions that are measurable in a biological sample or system”. According to this, to understanding the effect or detecting pollutants dispersion in the water, using biomarkers for aquatic organisms can be considered as an effect tool

(Hagger et al., 2008). Fossi and Marsili (1997), define a biomarker as an effective tool for environmental assessment if it is reliable, easy to sample, affordable and sensitive to pollutants. Even though baseline data is essential to understand the relationship between natural variability and contaminant-induced stress clearly, it is firstly necessary to establish the relationship between biomarker response and pollutant exposure (Lomartire et al., 2021) beforehand.

Biomonitoring tools such as bioindicators and biomarkers are the main components of the multi-level biomarker approach, which includes biochemical, physiological and ecological responses from aquatic organisms to understand and detect environmental pollutants (Martinez-Haro et al., 2015). Biomarkers are classified into four classes: biochemical (enzymes, hormones); physiological (growth, reproduction, energetics), toxicological (behaviour, lethality, teratogenicity, mutagenicity, carcinogenicity) and ecological/community (additions, deletions, alterations in ecosystem/community structures and relationships) (McCarty & Munkittrick, 1996). Because genetic changes precede a cellular response, early detection via molecular tools provides insights before it manifests in the organism (Lam, 2009). This multi-level biomarker approach can provide a wider and more precise understanding of the organism's health (Samanta et al., 2018).

The WHO (World Health Organization) defines different categories of biomarkers: a biomarker of exposure is "an exogenous substance or its metabolite or the product of an interaction between a xenobiotic agent and some target molecule or cell that is measured in a compartment within an organism"; a biomarker of effect is considered "a measurable biochemical, physiological, behavioural or other alteration within an organism that, depending on the magnitude, can be recognized as associated with an established or possible health impairment or disease" (World Health Organization, 1993).

For instance, oxidative stress biomarkers are widely used bioindicators in marine organisms (Valavanidis et al., 2006). Those give interesting insight and are useful tools for monitoring ecosystems and organisms' health (Chahouri et al., 2023) , even if, in many cases, they might be very species-specific and vary according to organisms and stressors (Madeira et al., 2013), making it hard to establish precise criteria and patterns. That is why, generally, scientists remain divided on the use of biomarkers at different biological levels due to their varying predictive capacities. In fact, while high-level biomarkers (genetic expression) are more ecologically relevant, they are slow to respond and hard to detect; low-level biomarkers offer early warnings about contaminants, but they are limited in predicting broader biological effects, responding differently according to the general stressors (Armon & Hänninen, 2015; Lam, 2009; Wu et al., 2005). Because of different environmental factors and physiological-specific conditions, it is fundamental to establish a clear dose-response relationship for each contaminant or environmental stressor (McCarty & Munkittrick, 1996) and validate the

respective biomarker for the specific moment and species, considering that some can also exhibit bioremediation and adaptability, leading to underestimated biomarker levels (Ching et al., 2001; Nacci et al., 2010).

Biomarkers have largely been used by different scientists to understand the effects of marine heat waves on marine and coastal organisms (Madeira et al., 2012, 2015, 2016; Vinagre et al., 2012, 2014, 2018). Because of global changes due to anthropogenic stressors, one of the consequences is the rising of the average ocean temperatures (Venegas et al., 2023), together with periods of relatively higher than the average ocean temperature leading to marine heat waves, which have important consequences on biodiversity and marine and coastal ecosystems (K. E. Smith et al., 2024). Heatwaves driven by global climate change have doubled their frequency and become longer, more intense and more extensive (Oliver et al., 2018) and have been shown to cause also mass mortality events in different marine species (Garrabou et al., 2022; Hughes et al., 2017), altering their distribution and ecological interaction (Fragkopoulou et al., 2023). Different studies have reported that marine heatwaves do have a physiological interaction with aquatic organisms leading to reduced reproductive success (Wild et al., 2019) and inducing physiological stress on marine invertebrates assessed with oxidative stress biomarkers (Vinagre et al., 2014), showing that the oxidative stress response increases with temperature. However, this is a very different and species-specific reaction where also other environmental constrains have to be taken into consideration. (Vinagre et al., 2014).

Beyond the thermal stress, marine organisms are increasingly exposed to chemical pollutants such as UV filters from sunscreens, accumulating in coastal water, which originate mainly from wastewater plants (Cadena-Aizaga et al., 2022). However, another major driver of the presence of these pollutants is the rising concerns over the harmful effects of UV rays on human health enhancing the use of sun protectors (Tang et al., 2024) associated with the increasing pressure due to tourism (Sánchez-Quiles & Tovar-Sánchez, 2015). The collateral effects of UV filters contained in sunscreen have been largely studied on different marine organisms and ecosystems. The amount of UV filters present in sunscreen lotions is regulated by the European Chemical Agency (ECHA) and by a specific 'REGULATION (EC) No 1223/2009 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 30 November 2009 on cosmetic products' which established specific criteria and maximum concentration allowed. Despite this, while UV filters like oxybenzone (BP3) and octinoxate have been recognised as harmful to coral reef ecosystems, being banned in Hawaii (Levine, 2020), in Europe they are present in sunscreens. Researches indicate UV filters can bioaccumulate in coastal organisms (Wang et al., 2022). Consequently, these contaminants are not only present within the coastal food webs, but they have the potential to be transported across marine ecosystems along the

food web and trophic transfer, spreading beyond coastal zones into the open ocean (Cadena-Aizaga et al., 2022; Tovar-Sánchez et al., 2013).

To date, no scientific research has specifically addressed the combined effects of MHW and UV filters from sunscreens on marine organisms. While on the one hand, there is a great amount of research on each factor, the synergistic impact of these two stressors remains unexplored. Because of the increasing occurrence and duration of marine heatwaves and the rising and widespread presence of UV filters in coastal water, this gap in research is fundamental for understanding the potential threat to marine ecosystems.

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1. Introduction

Marine and coastal ecosystems (MCEs) are fundamental to sustaining life on Earth (Lopez-Rivas & Cardenas, 2024) but are increasingly threatened by different anthropogenic pressures (Halpern et al., 2007), which put a strain on these fragile environments. Human activities have been directly and indirectly involved in the decline of marine ecosystems at global, regional, and local scales (Korpinen et al., 2021). Climate change and human development have accelerated the degradation of coral reefs (Good & Bahr, 2021), increased pollution levels (Bowler et al., 2020), and disrupted marine food webs, leading to significant ecological imbalances (Gomes et al., 2024). The complex interaction between climate-driving forces, such as rising sea temperatures, leads to more frequent and lethal marine heatwaves (Smale et al., 2019). This, in association with the continually increasing concentration of chemical pollutants, such as UV filters present in sunscreens (Tovar-Sánchez et al., 2013), remains a concerning stressor that increasingly undermines the resilience, health, and biodiversity of MCEs (Chapman, 2017; Simeoni et al., 2023). This will consequently increase the overall vulnerability, making these natural environments more susceptible to further environmental changes and challenges (Berrouet et al., 2018).

Over the past few decades, significant warming of the biosphere has resulted in broad impacts on the integrity of ecosystems and the sustainability of natural resources (Cardinale et al., 2012). Projections of heatwaves indicate that they are expected to occur more frequently and with greater intensity (Wedler et al., 2023), representing a threat to global biodiversity. These events occur both in the atmosphere and in the ocean. Marine heatwaves (MHWs) are caused by a variety of processes occurring at different spatial and temporal scales, from localized air-sea heat flux (atmospheric processes) to large-scale climate drivers such as the El Niño Southern Oscillation (oceanographic processes) (Hobday et al., 2016). Regional case studies have demonstrated that MHWs can significantly alter ecosystems, leading to widespread mortality, species distribution shifts, and community structure changes (Smale et al., 2019). MHWs are generally defined as prolonged periods of anomalously warm water lasting for at least five or more consecutive days with temperatures warmer than the 90th percentile based on a 30-year historical baseline period (Hobday et al., 2016). Those extreme events negatively impact marine ecosystems and fisheries (Cavole et al., 2016; Oliver et al., 2017). Therefore, it is vital to investigate MHWs deeply and to understand the impact they have on marine ecosystems fully.

Parallel to the climatic challenges, human activities have added another layer of stress on marine ecosystems. The rapid expansion of tourism, particularly in tropical and subtropical regions, has led to an increased awareness of the harmful effects of solar ultraviolet (UV) radiation (Thallinger et al.,

2023). To mitigate the risks of sunburn, photoaging, and skin cancer due to UVA (400-320nm), UVB (320-280nm), and UVC (280-100nm) radiations, the use of sunscreens has become widespread (Henderson et al., 2022; Sander et al., 2020; Silva et al., 2018). While sunscreens play a crucial role in protecting human health (Fig. 1.1), they have inadvertently become a source of pollution in marine environments (Tovar-Sánchez et al., 2013). The active components in sunscreens, the UV filters, are either physical filters like titanium dioxide and zinc oxide or organic molecules absorbing light in the UV range. These are now recognized as emerging pollutants due to their persistence and accumulation in coastal waters (Cadena-Aizaga et al., 2022; Chatzigianni et al., 2022).

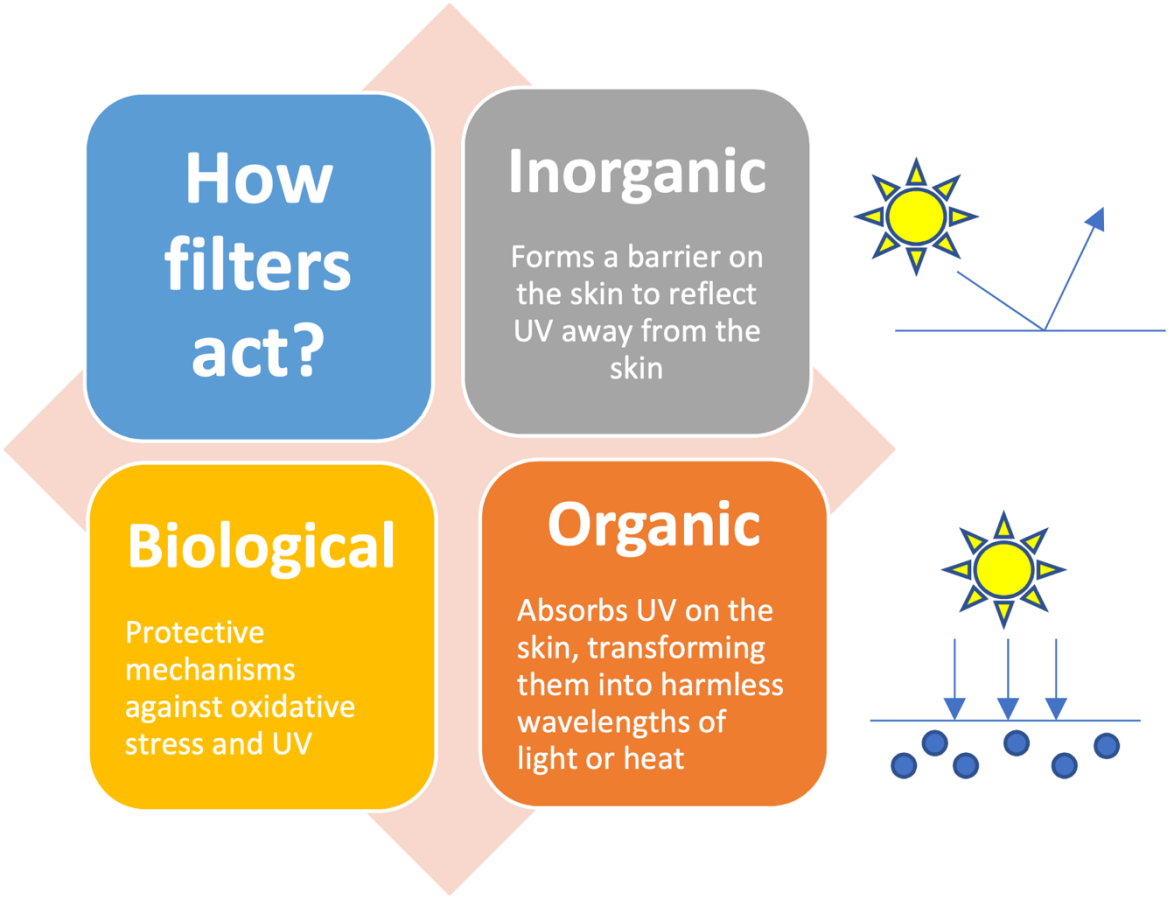

Fig. 1.1 *Categories of filters and their mechanisms of action (Santander Ballestín & Luesma Bartolomé, 2023)*

UV filters such as oxybenzone, octinoxate, and others are known to leach into marine environments through various pathways as discharges of lakes and rivers (Balmer et al., 2005), recreational activities and wastewater discharge (Fent et al., 2010; Plagellat et al., 2006; Rodil et al., 2008), especially in Canary Island (Montesdeoca-Esponda et al., 2021). Moreover, the accelerated growth of coastal tourism, coupled with the widespread application of sunscreen, constitutes an important pathway for introducing UV filters into the marine environment (Fig. 1.2) as showed by Sánchez-Quiles & Tovar-Sánchez, (2015). These compounds have been detected in significant concentrations in coastal areas (Sánchez Rodríguez et al., 2015; Sharifan et al., 2016), in particular in primary marine

consumers (Isabel Cadena-Aizaga et al., 2022), posing a toxic threat to marine life (Bachelot et al., 2012). Researchers have shown that UV filters can induce endocrine disruption both in mammals (Ma et al., 2023; Schlumpf et al., 2008) and fish (Kinnberg et al., 2015) , neurotoxicity (Araújo et al., 2018) , oxidative stress (Nataraj et al., 2020), absorption and bioaccumulation (Rodil et al., 2019). The generation and increase of reactive oxygen species (ROS) which leads to several oxidative damages (Huang et al., 2020; Sureda et al., 2018) and the release of inorganic macromolecules by these filters can further disrupt the ecological balance of coastal habitats.

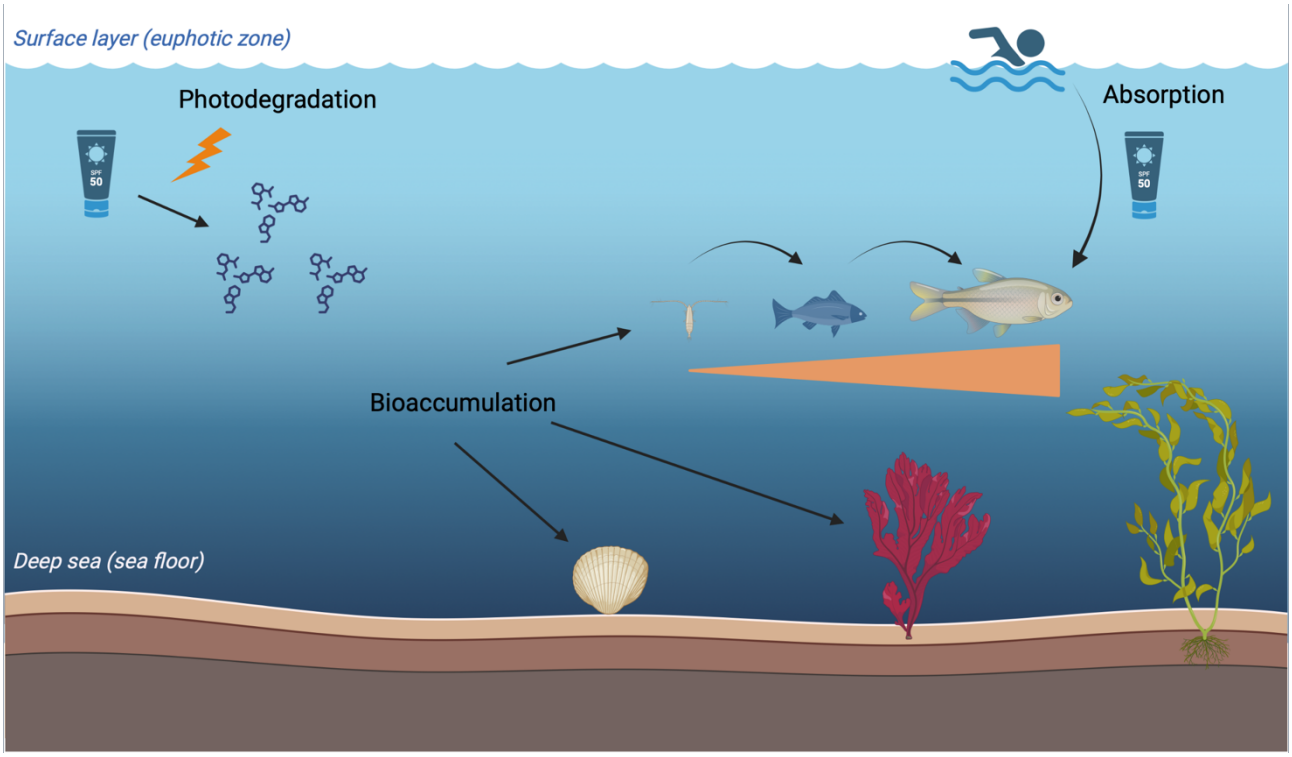

Fig. 1.2 *Source and faith of UV filters in the marine environment*

A specific type of MCE that is particularly subject to multiple anthropogenic stressors are the rocky intertidal pools. This ecosystem represents a unique coastal habitat found in the intertidal zone, the area between the high and low tide. These pools are formed in depressions on rocky shores where water is trapped during low tide and are highly variable in surface area and volume. Tidal pools serve as a fundamental microhabitat for different marine organisms, providing shelter and nursery ground to small fish, invertebrates, and cephalopods, as well as to different species of algae (Amara & Paul, 2003; Vinagre et al., 2015).

As a type of MCE, rocky intertidal pools are highly exposed to different anthropogenic pressures since these pools are often located in coastal areas where human activities are concentrated. On the one hand, the limited water volume and frequent isolation from the open ocean during low tide make these pools particularly vulnerable to accumulating pollutants such as sunscreens. On the other hand, due to global warming especially in sub-tropical and tropical areas where temperatures are more

thermally stable, marine organisms have a higher sensitivity to projected higher temperatures (Donelson, 2015). This is because they already live at their thermal limit (Donelson, 2015; Vinagre et al., 2019). Furthermore, due to their shallow waters, rocky intertidal pools can become ecological traps during summer ebb tides and heatwaves (Vinagre et al., 2018). The synergistic combination of these two anthropogenic stressors on rocky pool populations may result in important population loss. This scenario is real in the north of Gran Canaria's coastline, which is abundantly rich in rocky shore ecosystems. The unique wildlife in the Canary Islands has been recognised worldwide, counting many endemic species both in the terrestrial and marine realms. However, the biodiversity of this archipelago is under severe threat due to escalating environmental challenges. One of the most significant stressors in the marine ecosystem is the pressure from growing coastal populations and tourism (Ley Bosch et al., 2024) together with a consistent increase in air temperatures of 0.09 $\pm$ 0.04°C that the Canary Islands have experienced (Martín et al.,2012). Nevertheless, no ecological studies have yet been carried out on the effects of the rising air temperatures that affect intertidal and supralittoral marine communities (Riera et al., 2014).

Biomarkers related to oxidative stress have largely been measured to understand the impact of a heatwave (Madeira et al., 2015) on *Palaemon elegans'* physiology and how UV filters induce oxidative stress in bivalves (Bordalo et al., 2020; Falfushynska, 2021). Several studies confirm that the effects of anthropogenic pollutants, like microplastic ingestion (Herrera et al., 2019) increase ROS production, leading to oxidative stress by altering the antioxidant defense system in marine organisms (Solomando et al., 2020). Increasing concerns due to the impact of heatwaves (Vinagre et al., 2014) and UV filters (Bordalo et al., 2020; Sureda et al., 2018) on marine organisms have highlighted the need for further investigation into their combined effects.

The study aimed to assess *Palaemon elegans'* (Rathke, 1836) *physiological reactions* under the combined impact of extreme temperatures and sunscreens. Researchers tested two temperatures (20 and 32°C) and two sunscreens (one eco-friendly) in a 12-hour full factorial experiment. Biomarkers linked to oxidative stress, including Cytochrome c oxidase (COX), Glutathione-S-Transferase (GST), Superoxide Dismutase (SOD), Lipid peroxidation (LPO), and metabolic marker Electron Transport System (ETS), were measured to evaluate the shrimp's response. Additionally, the absorption of UV filters—Benzophenone-3 (BP-3), Octocrylene (OC), Butyl Methoxydibenzoylmethane (BMDBM), and Ethylhexyl Salicylate (EHS)—was investigated using high-performance liquid chromatography to determine if 12 hours is enough to see significant effects.

2. Materials and Methods

Animal collection and husbandry

Prawns were collected by hand net (Fig. 2.1) during low tide from rocky pools from different natural rockpools in Gran Canaria (Canary Island, Spain) between March and April 2024. Shrimps were sampled from several rockpools along Gran Canaria's coast. La Laja beach, (Las Palmas de Gran Canaria, 28°03′28″N 15°25′07″O); Cueva El Camello, (Arucas, 28°15'49″N -15°53'41″O); Los Charcones de Bañaderos (28°08′47″N 15°32′00″O); Piscinas Natural El Altillo (Moya, 28.14°59'95″N 15°56'56″O); Charco Natural de San Lorenzo (Moya, 28°14'45″N 15°57'74″O) and from Piscina Natural El Agujero (Gáldar, 28°16'16″N 15°66'06″O). They were transported to the laboratory in plastic tanks at a controlled temperature. Once in the laboratory, they were allowed to recover and kept for 14 days in quarantine and acclimatization in 80-litre tanks with aerated filtered saltwater flow with bacteria filters (bio balls). Prawns were kept at a controlled room temperature of 20°C, in direct contact with natural light and fed three times a week *ad libitum* with cultured *Artemia franciscana*. Tank parameters (temperature, dissolved oxygen, ammonia, nitrites and nitrates) were measured twice a week.

Fig. 2.1 *Hand net used to catch prawns*

Experimental idea

This experiment aims to investigate whether the synergistic effects of elevated temperature, simulating a heatwave, and UV filters from two distinct sunscreen formulations can adversely affect the physiological health of *P.elegans* by inducing oxidative stress. To achieve this, two temperature regimes were employed: 20°C as the control and 32°C to mimic heatwave conditions, bringing the organism close to its CTMax (Madeira et al. 2012; Vinagre et al., 2016). Additionally, two sunscreen

formulations were tested: one marketed as eco-friendly and the other as a conventional, non-eco-friendly product.

Experimental preparation

The day before the experiment a 12-tank system aquarium was filled with filtered sea water. Each tank was provided with an air-flow pump giving a constant oxygen flux to each tank. A total of 30 organisms (360 in total) were randomly transferred from the quarantine tank to each tank using a hand net. Tanks that corresponded to the heatwave treatment (32°C) were equipped with a thermostat, which was turned on at 8:00 pm of the night before the experiment (based on previous experiments performed to understand how long it takes to the water to increase the temperature from 20°C to 32°C). The remaining organisms (n=8) were used as T0, to understand the condition of the prawn without any stress given by the presence of the sunscreen and a higher temperature.

All the material used for sampling, euthanasia, and storing the prawns was prepared the day before. Three different hand nets were properly marked according to the treatment they were used for. Letter C was used for the control; letter G was used for the European eco-friendly sunscreen (named good for the experiments), and letter B was used for the American sunscreen containing BP-3 (named bad for the experiments). The main ingredients (i.e., UV filters), their sun protection factor (SPF), and the format (i.e., application type) of the sunscreens are described in Table 2.1. Three different workstations were designated to prevent contamination among the treatments C, G, and B, respectively and to remove the heads and exoskeletons of the prawns.

Table 2.1 List of ingredients present in the two selected sunscreens: SPF (sun protection factor), format (application type), and UV filters included in their composition. In bold UV filters which have been analysed with high-performance liquid chromatography.

SUNSCREEN	SPF	FORMAT	UV FILTERS (CHEMICALS)
G, ECOFRIENDLY LABELLED SUNSCREEN (EUROPEAN)	30	Lotion	**Ethylhexyl Salicylate,** Bis-ethylhexyloxyphenol methoxyphenyl triazine, **Butyl methoxydibenzoylmethane.**
B, NOT ECOFRIENDLY SUNSCREEN (AMERICAN)	15	Lotion	**Ethylhexyl Salicylate**, **Butyl methoxydibenzoylmethane, Octocrylene, Benzophenone – 3,** Polysilicone – 15.

Experimental setup

Collection of shrimps and experimental setup

The experiment took place at the EOMAR research group laboratory. Before the start of the experiment, sunscreen solutions were prepared for each tank. A final concentration of 60 mg/L of sunscreen (Sendra et al., 2017; Sureda et al., 2018) was established for each tank. To simulate the introduction of sunscreens into the environment, the sunscreens were thoroughly homogenised in filtered seawater, achieving dilution in the aqueous medium through continuous agitation (Araújo et al., 2020) with a magnetic stirrer for 50 minutes. The established amount of sunscreen was weighted on a Petri glass dish using an automated precision balance (Precisa LT120A). Once the desired amount of sunscreen was on the dish a metal stick was used to mix it with the filtered seawater previously poured in a glass beaker. During homogenization, the glass beaker was entirely covered with aluminium foil to avoid any sunscreen degradation (Romanhole et al., 2016) and contamination due to sprinkling. Shrimps were kept in starvation for 24 hours before the start of the experiment.

Once all the prepared solutions were introduced into the tanks, the initial sampling (T1) was conducted after 30 minutes. Subsequent samplings were carried out at six-hour intervals, with the second sampling (T6) occurring six hours after T1, and the third sampling (T12) taking place six hours following T2. Prawns were collected using a dedicated hand net that was exclusively reserved for each sunscreen treatment and put in small beakers containing ice used to sedate the organisms (Saborowski et al., 2022) and covered with a net (to prevent prawns from jumping out of it). Eight prawns for each tank were sampled at each time: five individuals were used for biomarkers and three for liquid chromatography analysis.

Samples preparation for biomarkers

Once the shrimp were sedated, the full body length was measured with a ruler and weighed using a precision balance (Precisa LT120A). They were then stored individually in previously marked plastic vials and placed on ice. Depending on the sunscreen treatment (C, G, and B), shrimps were handled by different individuals.

Both the midgut gland and the muscle were extracted on a Petri dish placed on ice and stored in a 2 mL vial, which was immediately frozen in dry ice. Both midgut and muscle were then stored at -80°C until the analysis. Between each time of the experiment (T1, T6, and T12), all the metal sticks used, surgical scalpel, and Petri dish were cleaned with ethanol 96%.

Biomarkers analysis

For biomarker analysis regarding the muscle, five prawns for each treatment were singularly homogenized for 2 minutes in a Potter-Elvehiem type Teflon glass tissue grinder rotating at 2600rpm (70%), in 1 mL of 0.1 M sodium potassium phosphate buffer (0.1M Na_2HPO_4, 0.1mM KH_2PO_4, 75µM $MgSO_4$, $7H_2O$, 1.5% (Polyvinylpyrrolidone) PVP and 0.2% Triton X-100) at pH 8.5 (Owens & King, 1975). Samples were kept at 0-4°C in an ice bath throughout the process. Crude homogenates were centrifuged for 10 minutes at 4000 rpm, 0ºC, and the supernatant fluid was used for measurements. A pool of five hepatopancreases was pooled and subjected to sonication for 45 seconds at 65% of the maximum amplitude (127.4 µm) using an ultrasonic probe (Vibracell VXC 130). The sonication process aimed to efficiently disrupt the tissues, ensuring uniform homogenization for subsequent analysis. This method was chosen to optimize tissue breakdown while minimizing the risk of overheating or damage to sensitive cellular components. According to the total biomass, between 1.2 mL and 0.9 mL of 0.1 M sodium potassium phosphate buffer (0.1M Na_2HPO_4, 0.1mM KH_2PO_4, 75µM $MgSO_4$, $7H_2O$, 1.5% (Polyvinylpyrrolidone) PVP and 0.2% Triton X-100) at pH 8.5 (Owens & King, 1975). All biomarker analyses were conducted using a FLUOstar Omega microplate reader from BMG Labtech. The enzymatic activities (ETS, COX, SOD, GST) were measured at controlled temperatures of 20°C and 32°C.

Cytochrome c oxidase

The experimental procedure followed the methodology outlined by Gauthier et al. (2008), with slight modifications. Initially, a DTT (dithiothreitol) solution was prepared at a concentration of 100 mM by diluting it in BF_{COX} buffer [BF_{COX} = 0.1M Na_2HPO_4, 0.1mM KH_2PO_4, pH 7.1]. Subsequently, a reduced cytochrome c (Cyt-C) solution was prepared by diluting a specified quantity of oxidized Cyt-C (Sigma, ref. C3506) and 77.15 µL of the previously prepared DTT solution in BF_{COX}. The final concentrations achieved in this solution were 220 mM for Cyt-C and 0.5 mM for DTT, with the total volume adjusted based on the number of samples to be analyzed. This solution was gently agitated for 15 minutes, during which the colour transition from orange/red to pink/red indicated the reduction of Cyt-C.

The subsequent step involved preparing the MIX solution (MS) by diluting 2.22 mL of the reduced Cyt-C solution with 17.78 mL of BF_{COX}. The total volume of the MS was adjusted according to the sample size. For the assay, 20 µL of the sample was combined with 180 µL of the MS in a 96-well microplate. The decrease in absorbance at 550nm was then measured over a 6-minute interval. Cytochrome c oxidase (COX) activity was quantified as the oxidation of 1 µmol of ferrocytochrome c per minute, calculated based on the decrease in absorbance at 550nm, corresponding to the

oxidation of cytochrome c. The reaction involved can be represented as 4 Cyt c_red + O_2 + 4 H^+ → 4 Cyt c_ox + 2 H_2O, with an extinction coefficient of 21.84mM^{-1} cm^{-1} (Berry & Trumpower, 1987).

Electron transport system

ETS (electron transport system) activity was measured following the methodologies described by Packard & Christensen (2004) and Owens & King (1975). The procedure consists of mixing 40 µL of sample with 120 µL of buffer as substrate (1.7mM NADH and 0.25mM NADPH dissolved in a buffer made of 0.1M of Na_2HPO_4, 0.1mM of KH_2PO_4 and 0.2% of Triton x100), with 40µl of a 4% INT solution (p-iodonitrotetrazolium violet). The INT reduction to a dark red solution is kinetically followed for 8 minutes at 490nm. In the control, without substrate, the sample was measured as a standard for INT enzymatic reduction (Maldonado et al.,2012). To calculate ETS activity, it was used a molar coefficient (Ɛ) of 15.27mM^{-1} cm^{-1} (the Ɛ was previously determined from a standardized INT experiment, according to Maldonado et al., (2012). Stoichiometrically, 2 µmol of formazan produced during INT reduction is equivalent to 1 µmol of O_2 consumed if all electrons are transferred to molecular oxygen. Therefore, ETS activity is quantified in formazan µmol based on the kinetic slope (Eq. 1). The consumed oxygen is converted into µmol O_2 units. Equation 1 defines ETS activity as $[(S_s - B_s)\ V_c] / (V_{ext}\ \varepsilon)$, where S_s and B_s represent slopes (S_s from the enriched substrate minus the blank substrate; B_s from the sample without the substrate minus the zero-reactive substrate in the blank). V_c is the total volume of the reaction mixture in the cuvette, V_{ext} denotes the homogenized extract, and ε was defined earlier.

Glutathione-S-Transferase

GST activity (glutathione-*S*-transferase) was calculated at 340 nm for 5 minutes, using the methodology described by Frasco & Guilhermino (2012) and by Habig et al. (1974). 100 µL of the sample, and 200 µL of WR_{GST}, (the WR_{GST} is composed of 4.95 mL of phosphate buffer (0.1M, pH6.5); 0.9 mL of GSH solution 10 mM (GSH was dissolved in the BF_{GST}) and finally of 0.15 mL CDNB solution (60 mM dissolved in ethanol)). The equation (Eq.2) is the following: $[GST\ activity\ (\mu mol \bullet ml^{-1} \bullet min^{-1}) = ((GSTslope_{340} - RBslope_{340}) * VR) / (Vext * (\mathcal{E} * f))]$. $GSTslope_{340}$ and $RBslope_{340}$ are the slopes for the sample and reagent blank respectively; the VR is reaction volume (0.3ml); the V_{ext} is the homogenate subsample volume (calculated in microliters of sample in the reaction; in this case, 100 µL), Ɛ = extinction coefficient (9.6 $mM^{-1}cm^{-1}$) and finally *f* is pathlength correction.

Superoxide-Dismutase

SOD (Superoxide-Dismutase) activity was measured following a kinetic assay performed for 3 minutes at 560nm, using the methodology described by Ewing & Janero (1995) as follows. SOD activity was quantified using the nitro blue tetrazolium (NBT) reduction assay in which 25 µL of the

homogenized sample was mixed with 200 µL of working reagent (WR) containing 50 mM of sodium carbonate buffer, 0.1 mM EDTA, =.1mM NBT and 0.1 NADH. The reaction was initiated by adding 25 µL of 0.1 nM phenazine methosulfate (PMS) solution. After incubating at room temperature, the absorbance was measured at 560nm. A negative control (NBTmax) was prepared by substituting the sample with 25 µL of homogenization buffer. SOD activity was calculated based on the inhibition of NBT reduction. For the calculations, the methodology used was described by Zhang et al. (2016) as follows: $[(NBT_{max} - SOD)/(SOD)]*df$. The SOD activity in the total sample (U) = $[(NBT_{max} - SOD)/(SOD)*df*(V_{homog}/V_{ext})$. For NBT_{MAX} 25 µL of the homogenization buffer was pipeted into their respective well; 200 µL of the WR_{SOD} into each well and 25 µL of PMS into each well.

Lipid peroxidation oxidation

Based on (Barboza et al., 2018; Bird & Draper, 1984; Ohkawa et al., 1979), with slight modifications, the lipid peroxidation was determined by quantifying the concentration of the thiobarbituric acid reactive substances (TBARS). First, 250 µL of fresh homogenate was mixed with 4 µL BHT 4% to obtain a BHT-homogenate mix, and stored at -80ºC until analysis, to avoid artefactual lipid oxidation. For the analysis, the assay was done by mixing 60 µL of that BHT-homogenate mix with 300 µL of TCA 12%. After that, vortex, and add 240 µL of a TRIS-DTPA solution (TRIS-HCl 60 mM plus DTPA 0.1mM dissolved in ultrapure water at pH 7.4) and 300 µL of TBA 0.73%. That mix was incubated for 1h at 100ºC in a water bath, and after being cooled to room temperature, centrifuged at 11500rpm for 5min. Then, read the absorbance at 535 nm. TBARS concentration was determined according to equation 3, described below. TBARS concentration ($\mu mol \cdot ml^{-1}$) = $(ABS_{TBARS} - ABS_{blk})*V_R)/ (V_{ext}* Ɛ)$. ABS_{TBARS} indicates the optical density of the sample reaction, while ABS_{blk} is the reagent blank optical density. V_R and V_{ext} are the volumes, in mL, of the reaction (0.9 mL) and the sample in the reaction (0.06mL) respectively, and Ɛ represents the extinction coefficient of $156 mM^{-1} \cdot cm^{-1}$.

Proteins

Protein concentrations were determined using the Pierce™BCA Protein Assay Kit (Thermo Scientific, ref. 23225) adapted for microplate analysis with a plate reader. Fresh bovine serum albumin (BSA), from 0 to 2000 µg/mL µL was prepared as a homogenization buffer on the day of the assay. 25 µL from each sample and BSA standard were pipetted into a 96-well microplate. The working reagent (WRBCA) was prepared by mixing 50 parts of BCA Reagent A (Pierce™ BCA Reagent B, ref 23223) with 1 part of BCA Reagent B (Pierce™ BCA Reagent B, ref 23224). Then, 200 µL of the WRBCA was added to each well. The microplate was incubated at 37°C for 30 minutes. Following the incubation, the absorbance was measured at 562 nm using a microplate reader. Protein

concentrations in the samples were calculated by comparing their absorbance values to the standard curve generated from the BSA standards.

UV filters analysis

Reagents and consumables

The target compounds were obtained from Sigma-Aldrich (Madrid, Spain). The solvents used as mobile phases (mass spectrometry grade water and methanol (MeOH), as well as those used as extractants (MeOH and acetonitrile (ACN), all of them with the minimum assay of 99.9%, were obtained from Panreac Quimica (Barcelona, Spain). The 0.2 µm syringe polyethylene terephthalate (PET) filters were supplied by Macherey-Nagel (Dueren, Germany). SPE 500 mg Sep-pak C18 cartridges from Waters (Madrid, Spain) were used.

Pre-treatment and extraction procedure from water analysis

Once shrimps were sedated, the full body length was measured with a ruler and weighed using a precision balance (Precisa LT120A). After that, they were gently washed with distilled water and then they were stored in aluminium boxes, covered with aluminium foil, and stored at -80 °C until further analysis.

At T0 and T12 samples of water according to each treatment at both temperatures were taken with one litre of glass jars. Those were previously fully covered with aluminium foil to avoid any UV filter degradation due to direct light exposure. Sampled water was acidified with formic acid to pH = 3 to inhibit any microbial activity and stored at 4 °C until analysis.

Sample extraction was done using solid phase extraction SPE according to the procedure published by Cadena-Aizaga et al., (2022). C18 cartridges were conditioned before each extraction with 5 mL of MeOH and 5 mL of Milli-Q water. 700 mL of water was passed through a cartridge, followed by a clean-up step using 5 mL of Milli-Q water. Then the cartridges were dried for 1 min and the retained compounds were eluted with 5 mL of MeOH: ACN (1:1, v/v). Under these conditions, a preconcentration factor of 140 times is achieved.

Pre-treatment and extraction procedure from *P.elegans* samples

All samples were freeze-dried for 24 hours in small glass vials. After this process, a porcelain mortar with a pestle was used to homogenise and grind the samples into powder.

For the extraction of target compounds, a Microwave-assisted extraction (MAE) method from Guazé et al. (pending publication) was adapted. Briefly, 50 mg of the dried powder was weighed with a precision balance, Precisa (LT120A), and placed into microwave digestion vessels. The microwave oven used for extraction was a TITANMPS with 16 vessels (230 V, 50–60 Hz, 40 bar) which were purchased from Perkin Elmer (Madrid, Spain). 7 mL of Hexane (HEX) was used as an extractant

solvent. MAE equipment was then used to heat solvents at 68 °C in contact with samples for 15 minutes. After allowing samples to reach room temperature, the solvent was evaporated with a nitrogen stream to reconstitute in a solvent compatible with the detection system. Moreover, this step enables sample concentration, by using a lower volume (1 mL of MeOH). Then, samples were sonicated for 30 minutes and filtered through a 0.2 μm syringe into chromatographic glass vials.

Liquid chromatography determination

The selected organic UV filters were determined in an ACQUITY UHPLC system equipped with a binary solvent manager, a thermostated autosampler and a tandem triple quadrupole mass spectrometer detector (MS/MS) with electrospray ionisation (ESI). All the components were controlled by the MassLynx Mass Spectrometry software (Waters Chromatography, Barcelona, Spain). The ESI parameters were fixed as follows: capillary voltage at 4 kV, 15 V of cone voltage, 120 and 450 °C for source and desolvation temperature, respectively, and 500 L h^{-1} of desolvation gas flow. Nitrogen was used for desolvation and argon for collision. Detailed information about the fragmentation of target analytes is shown in Table 2.2.

As BEH (Bridged Ethyl Hybrid) C18 column (50 × 2.1 mm, 1.7 μm particle size) was used for the chromatographic separation. The mobile phase consists of MeOH (A) and water (B) of LC-MS grade with 0.1% (v/v) formic acid at a flow rate of 0.3 mL min^{-1}. The following gradient was employed; starting with 25% A:75% B, which was left for 3 min and then lowered to 0% of A in 2 min and held for 1 min. Finally, A was increased to 25% for 1 min and held for 1 min for the next injection. The injected extract volume was 10 μL. Quantification was performed by an internal standard (IS) method using deuterated compound BP-d10 at 200ng/g.

Table 2.2 Main characteristics of the target compounds and mass spectrometer conditions for their determination.

COMPOUND	CAS NUMBER	LOG K_{OW}*	PRECURSOR ION (M/Z)	CONE VOLTAGE (V)	QUANTIFICATION ION (M/Z)	COLLISION POTENTIAL (V)	CONFIRMATION ION (M/Z)	COLLISION POTENTIAL (V)
BENZOPHENONE-3 (BP-3)	131-57-7	3.6	229.0	32	151.0	20	105.0	25
2-ETHYLHEXYL SALICYLATE (EHS)	118-60-5	5.7	251.1	15	139.1	10	138.1	10
BUTYL METHOXYDI-BENZOYLMETHANE (BMDBM)	70356-09-1	4.8	311.2	30	161.2	23	135.1	23
OCTOCRYLENE (OC)	6197-30-4	7.1	362.4	28	250.0	12	332.0	20

*obtained from PubChem

3. Results

Statistical analysis

Statistical analysis were performed using R software (version 4.4.1, R Core Team, 2024). For differences between two independent groups, the Mann-Whitney U test was employed to analyse the biomarkers in the muscle and the hepatopancreas, and to understand if there is accumulation in the organisms (p-value < 0.05), as it is a non-parametric method convenient for data that do not follow a normal distribution. Additionally, the Jonckheere-Terapista trend test was used to assess the presence of an ordered trend across different groups according to temperature exposure (p-value < 0.05). It provides a non-parametric approach to detecting monotonic trends. Graphics were performed with R studio, using the package ggplot2.

Biomarkers analysis

Muscle tissue

COX, ETS, GST, LPO, and SOD levels were analysed in the homogenised muscle tissue from both treatment groups (UV filters good and UV filters bad) across the established temperatures (20 and 32°C) at the specified time points (T1, T6, and T12).

In COX activity, (shown in Table 3.1), the highest means values were found in T1 and T6, in the treatment with 'UV filters bad' at 20°C (respectively of 7.3 ± 2.9 nmol/min/mg proteins and 6.8 ± 3.6nmol/min/mg proteins respectiveley).

Table 3.1 Values of COX in nmol/min/mg proteins, present in the homogenized muscle tissue of *P.elegans* (each treatment shows mean, standard deviation, median, minimum and maximum values for the specified time points sampling with the control, T1 was measured 30 minutes after the start of the experiment; T6 after 6 hours and T12 after 12 hours). Treatments are the control (without any sunscreen), UV filters good (applied for the treatments with the eco-friendly sunscreen), and UV filters bad (for the not labelled eco-friendly sunscreen).

COX activity, nmol/min/mg prot

Mean, sd, median, minimum and maximum values by treatment

time	temperature	treatment	mean	sd	median	min	max
T0	20	control	3.5	1.3	3.4	1.6	6.17
T1	20	control	3.8	1.7	3.9	0.8	6.52
T1	20	UV filters good	5.6	1.2	5.8	4.2	8.17
T1	20	UV filters bad	7.3	2.9	6.4	4.0	12.52
T1	32	control	3.3	1.7	3.5	0.1	5.37
T1	32	UV filters good	6.3	4.2	6.1	1.1	14.15
T1	32	UV filters bad	3.1	2.2	2.8	0.1	6.38
T6	20	control	5.9	2.8	5.0	3.1	10.65
T6	20	UV filters good	4.5	1.5	4.2	3.1	7.73
T6	20	UV filters bad	4.1	2.2	4.5	0.6	6.34
T6	32	control	4.8	2.5	4.2	2.6	10.84
T6	32	UV filters good	3.1	1.7	3.3	0.1	5.55
T6	32	UV filters bad	5.8	1.9	6.3	3.2	8.74
T12	20	control	5.6	1.4	6.1	2.8	7.16
T12	20	UV filters good	6.2	1.4	6.6	3.7	8.03
T12	20	UV filters bad	6.8	3.6	6.6	0.5	14.75
T12	32	control	4.3	4.0	3.3	1.1	15.00
T12	32	UV filters good	3.3	2.6	2.9	0.2	6.85
T12	32	UV filters bad	2.0	1.9	1.6	0.1	5.53

A significant difference was found in T1 (Fig.3.1), within the ‘bad UV filter treatment’ group. Specifically, the comparison between the 20°C and 32°C exposures revealed a significant difference ($W = 88$, p-value = 0.002879) indicating a pronounced effect of temperature under this treatment.

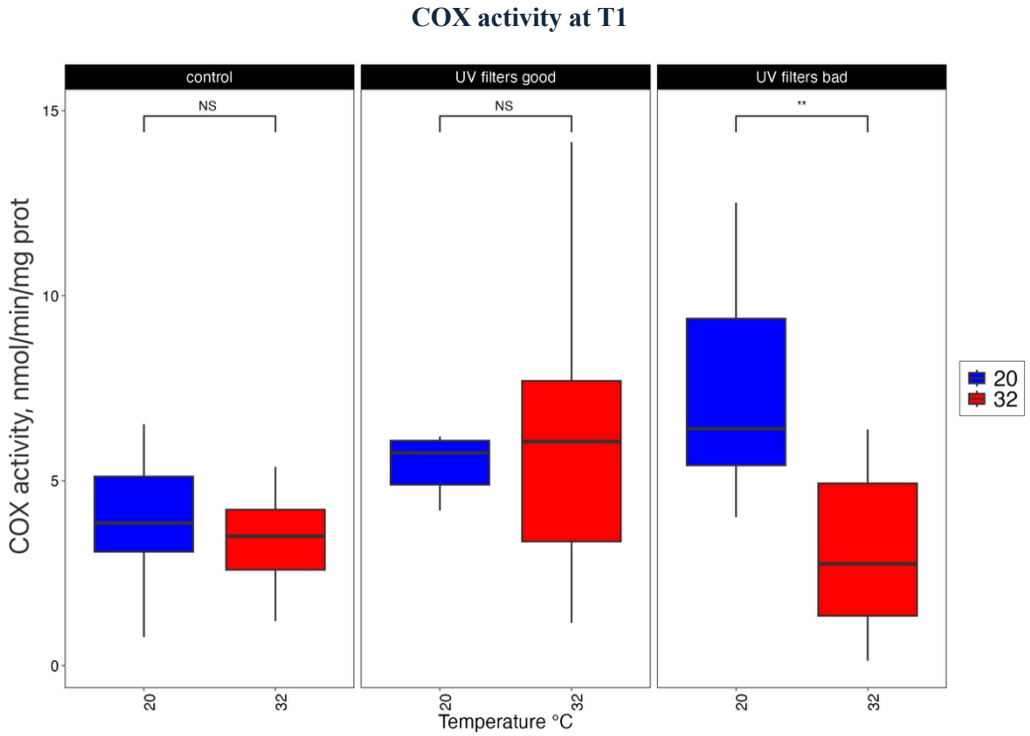

Fig. 3.1 COX values, at T1, stratified by treatment conditions and corresponding temperature exposures. The boxplot illustrates that at T1, there is a significant difference in COX levels between groups exposed to 20°C and 32°C under the bad UV filter treatment.

At T12 (Fig.3.2) significant differences were observed between the 20°C and the 32°C conditions for the control group (W = 89, p-value = 0.002089), as well as for the 'good UV filter' treatment (W = 82, p-value = 0.01469*)* and the 'bad UV filters' treatment (W = 76, p-value = 0.01013), indicating that mainly temperature (32°C) had a significant effect across all the treatments.

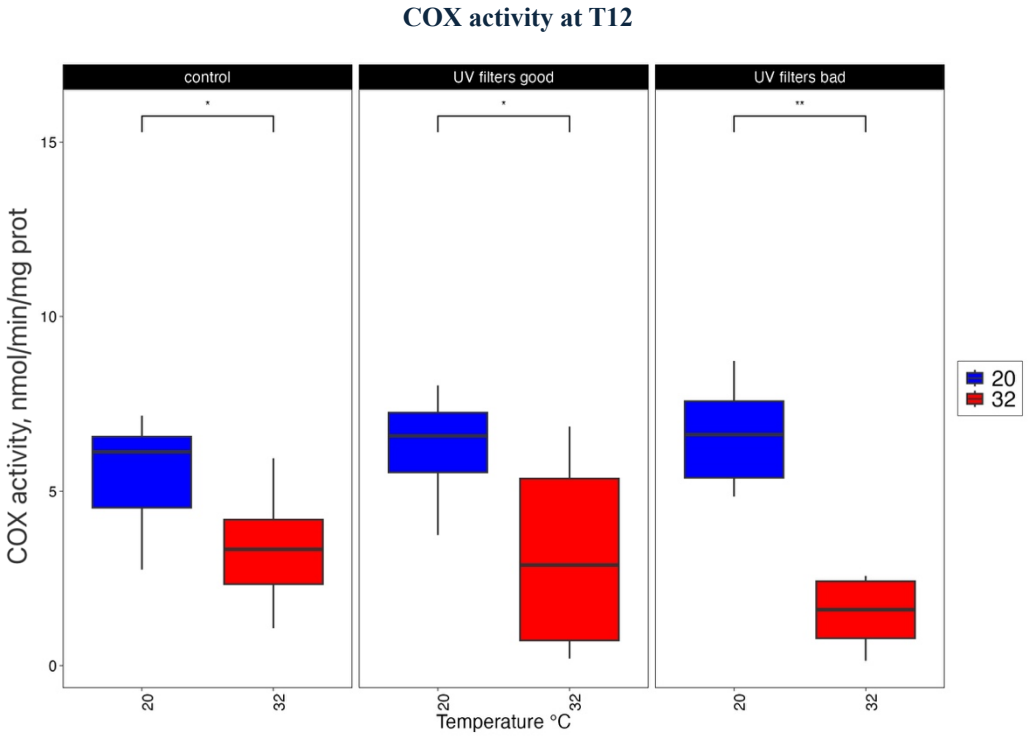

Fig. 3.2 COX values, at T12 stratified by treatment conditions and corresponding temperature exposures. The boxplot illustrates that at T1, there is a significant difference in COX levels between groups exposed to 20°C and 32°C under the bad UV filter treatment.

In ETS activity (shown in Table 3.2), the highest means values were found in T1 at 32°C for the control (6.1 ± 2.8µl O_2/min/mg protein) and for the bad UV filter, treatment (6.1 ± 4.0µl O_2/min/mg protein) and in T6 at 32°C for the bad UV filter treatment (7.6 ± 10.4µl O_2/min/mg protein).

Table 3.2 Values of ETS µl O_2/min/mg protein, present in the homogenized muscle tissue of *P.elegans* (each treatment shows mean, standard deviation, median, minimum and maximum values for the specified time points sampling with the control, T1 was measured 30 minutes after the start of the experiment; T6 after 6 hours and T12 after 12 hours). Treatments are the control (without any sunscreen), UV filters good (applied for the treatments with the eco-friendly sunscreen), and UV filters bad (for the not labelled eco-friendly sunscreen).

ETS µl O2/min/mg protein

Mean, sd, median, minimum and maximum values by treatment

time	temperature	treatment	mean	sd	median	min	max
T0	20	control	1.9	0.4	2.0	1.2	2.401589
T1	20	control	3.2	1.7	2.7	1.4	7.225243
T1	20	UV filters good	3.9	1.4	4.0	1.3	6.232700
T1	20	UV filters bad	5.5	2.0	5.9	2.6	8.146830
T1	32	control	6.1	2.8	5.6	2.9	11.177851
T1	32	UV filters good	5.2	2.6	5.9	0.8	8.780117
T1	32	UV filters bad	6.1	4.0	5.4	2.2	15.545681
T6	20	control	5.4	1.2	5.3	4.1	7.780880
T6	20	UV filters good	4.7	2.9	5.8	0.6	8.484752
T6	20	UV filters bad	3.9	2.9	3.6	0.8	8.355030
T6	32	control	5.4	1.6	5.4	2.9	8.055298
T6	32	UV filters good	4.8	1.4	4.3	3.2	6.845849
T6	32	UV filters bad	7.6	10.4	5.2	−1.2	36.248683
T12	20	control	1.5	0.7	1.3	0.3	2.858734
T12	20	UV filters good	2.1	1.2	1.9	0.6	4.122987
T12	20	UV filters bad	2.0	0.7	2.1	1.0	3.017350
T12	32	control	4.1	1.2	3.7	2.7	6.111257
T12	32	UV filters good	3.9	1.3	4.0	1.9	6.077430
T12	32	UV filters bad	4.3	3.1	3.9	0.2	11.063280

At T12 (Fig.3.3), statistical differences were detected across all three treatments between the 20°C and the 32°C exposures. Significant differences were found in the control group (W = 1, p-value = 2.165e-05), the 'good UV filter' treatment (W = 14, p-value = 0.01013) and the 'bad UV filter' treatment (W = 23, p-value = 0.04326). Indicating that temperature had a greater impact on each treatment for the 32°C condition after 12 hours of exposure.

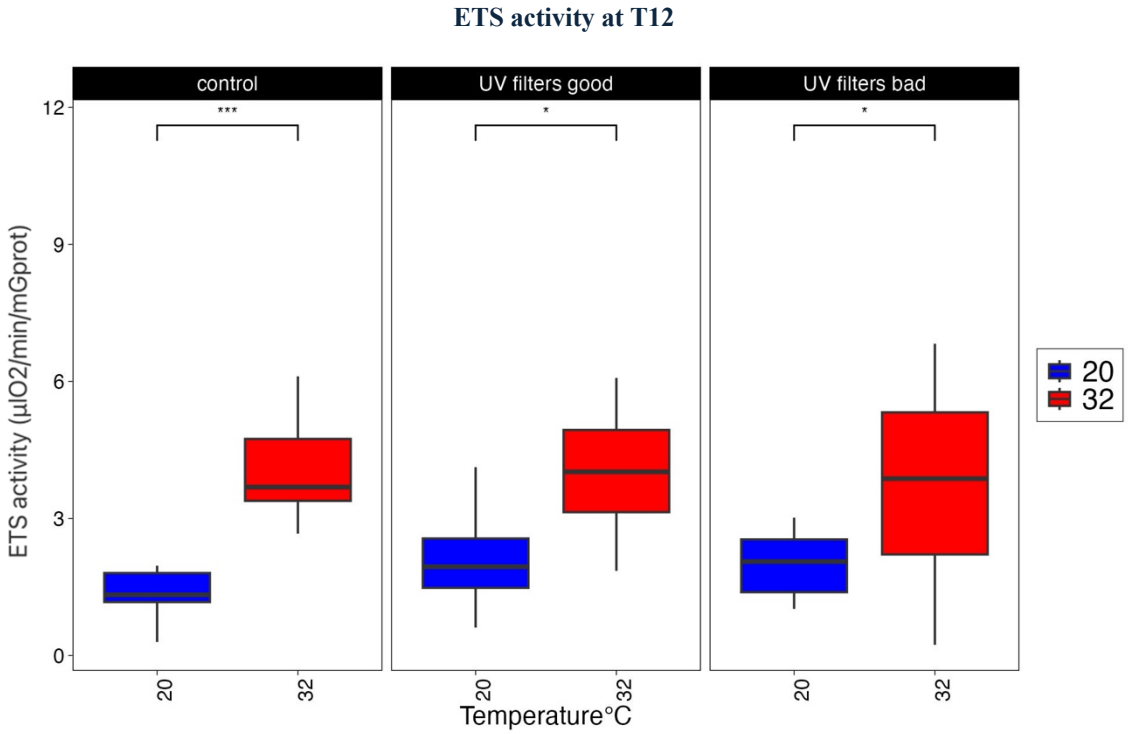

Fig. 3.3 ETS values, at T12 stratified by treatment conditions and corresponding temperature exposures. The boxplot illustrates that at T12, there is a significant difference in ETS levels between groups exposed to 20°C and 32°C for the control, the good and bad UV filter treatment.

In GST activity (shown in Table 3.3), the highest means were found in T6 for the UV filter bad treatment at 32 °C (5.4 ± 10.0nmol/min/mg protein) and at 20°C (5.0 ± 4.4nmol/min/mg protein), and for the control in T12 at 20°C (5.1 ± 2.1 nmol/min/mg protein). In this case, a statistical difference was found at T12 (Fig.7), between the 20°C and 32°C conditions for the control group (W = 80, p-value = 0.02323).

Table 3.3 Values of GST nmol/min/mg protein, present in the homogenized muscle tissue of *P.elegans* (each treatment shows mean, standard deviation, median, minimum and maximum values for the specified time points sampling with the control, T1 was measured 30 minutes after the start of the experiment; T6 after 6 hours and T12 after 12 hours). Treatments are the control (without any sunscreen), UV filters good (applied for the treatments with the eco-friendly sunscreen), and UV filters bad (for the not labelled eco-friendly sunscreen).

GST nmol/min/mg protein

Mean, sd, median, minimum and maximum values by treatment

time	temperature	treatment	mean	sd	median	min	max
T0	20	control	2.0	0.9	2.2	0.0	3.209659
T1	20	control	3.5	2.9	2.6	0.7	10.300732
T1	20	UV filters good	3.4	1.6	3.0	1.9	7.590044
T1	20	UV filters bad	3.5	3.2	2.6	0.3	11.607219
T1	32	control	2.6	0.9	2.6	1.4	3.950775
T1	32	UV filters good	3.2	2.9	2.5	−0.3	10.156032
T1	32	UV filters bad	4.4	5.9	2.1	0.3	20.432611
T6	20	control	3.0	2.7	2.3	1.0	10.113675
T6	20	UV filters good	4.9	4.5	3.8	1.2	17.306802
T6	20	UV filters bad	5.0	4.4	4.5	1.2	16.257560
T6	32	control	3.6	0.9	3.8	1.8	4.681333
T6	32	UV filters good	2.3	1.5	2.2	−0.5	5.264340
T6	32	UV filters bad	5.4	10.0	2.2	0.8	33.425659
T12	20	control	5.1	2.1	5.3	1.1	8.267923
T12	20	UV filters good	3.1	2.2	2.3	1.3	8.997749
T12	20	UV filters bad	2.7	1.3	2.6	1.0	5.135043
T12	32	control	3.0	2.8	2.4	0.6	10.172502
T12	32	UV filters good	3.0	1.6	3.2	0.0	5.024879
T12	32	UV filters bad	2.0	1.4	1.5	0.4	4.603320

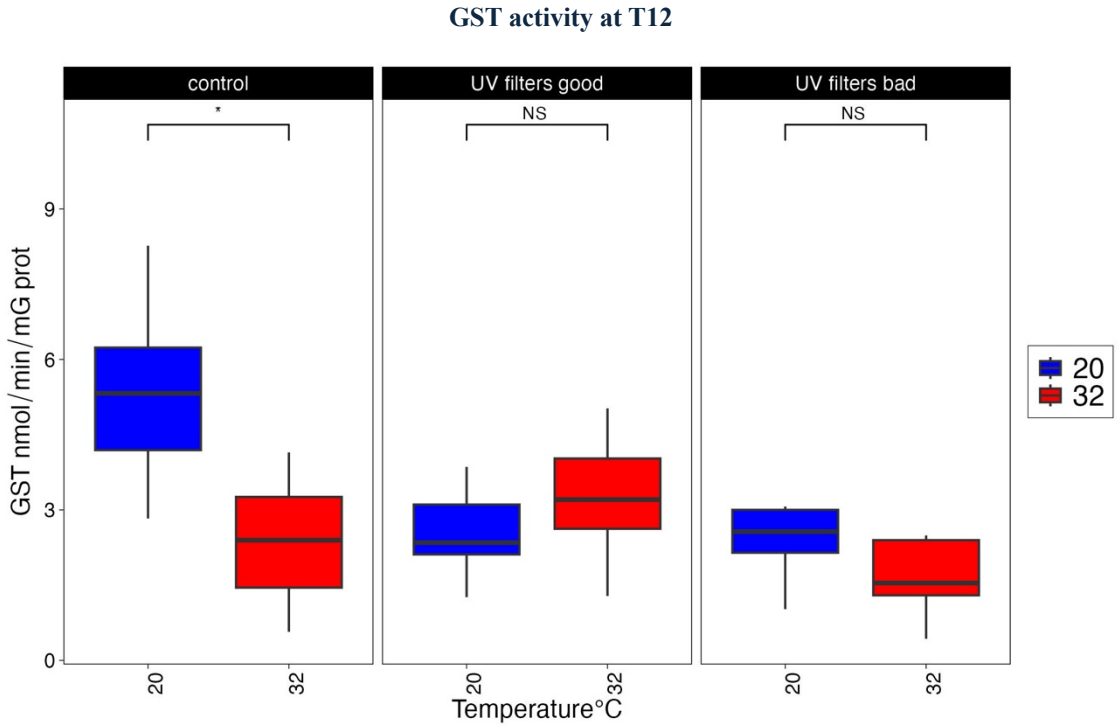

Fig. 3.4 GST values nmol/min/mg protein, at T12 stratified by treatment conditions and corresponding temperature exposures. The boxplot illustrates that at T12, there is a significant difference in GST levels between groups exposed to 20°C and 32°C for the control treatment.

As shown in Table 3.4, the mean of LPO activity was generally low. The highest values were observed in T1 (0.40 nmolTBARS·mgprot^{-1}) and T6 (1.11 nmolTBARS·mgprot^{-1}) both at 32°C for the 'UV filter bad' treatment.

Table 3.4 Values of LPO nmolTBARS·mgprot^{-1}, present in the homogenized muscle tissue of *P.elegans* (mean, standard deviation, median, minimum and maximum values are shown by each treatment for the specified time points sampling with the control, T1 was measured 30 minutes after the start of the experiment; T6 after 6 hours and T12 after 12 hours). Treatments are the control (without any sunscreen), UV filters good (applied for the treatments with the eco-friendly sunscreen), and UV filters bad (for the not labelled eco-friendly sunscreen).

LPO nmolTBARS·mgprot^{-1}

Mean, sd, median, minimum and maximum values by treatment

time	temperature	treatment	mean	sd	median	min	max
T0	20	control	0.089	0.045	0.075	0.022	0.1585060
T1	20	control	0.069	0.038	0.068	0.010	0.1182198
T1	20	UV filters good	0.136	0.076	0.104	0.075	0.3022759
T1	20	UV filters bad	0.161	0.042	0.141	0.112	0.2288751
T1	32	control	0.128	0.036	0.137	0.044	0.1616285
T1	32	UV filters good	0.138	0.054	0.125	0.086	0.2758383
T1	32	UV filters bad	0.175	0.096	0.155	0.068	0.4097541
T6	20	control	0.192	0.072	0.171	0.098	0.3375310
T6	20	UV filters good	0.183	0.060	0.172	0.108	0.2858560
T6	20	UV filters bad	0.194	0.073	0.177	0.099	0.2979922
T6	32	control	0.115	0.034	0.107	0.074	0.1783087
T6	32	UV filters good	0.164	0.080	0.156	0.078	0.3408836
T6	32	UV filters bad	0.223	0.318	0.121	0.067	1.1189828
T12	20	control	0.157	0.106	0.122	0.055	0.3674692
T12	20	UV filters good	0.147	0.032	0.147	0.107	0.2079342
T12	20	UV filters bad	0.114	0.032	0.112	0.052	0.1541859
T12	32	control	0.183	0.087	0.154	0.069	0.3765107
T12	32	UV filters good	0.175	0.052	0.176	0.118	0.2718836
T12	32	UV filters bad	0.165	0.076	0.151	0.093	0.3572991

Lipid peroxidation (LPO) showed a statistically significant decrease in the control group at T6, when comparing the 20°C and 32°C temperature conditions (W = 88, p-value = 0.002879) (Fig.3.5). However, in both the 'good UV filter' and the 'bad UV filter' treatments, no significant differences were observed between the two treatments. Indicating that temperature has a pronounced effect in the control, whereas no similar response in the UV filter treatment groups.

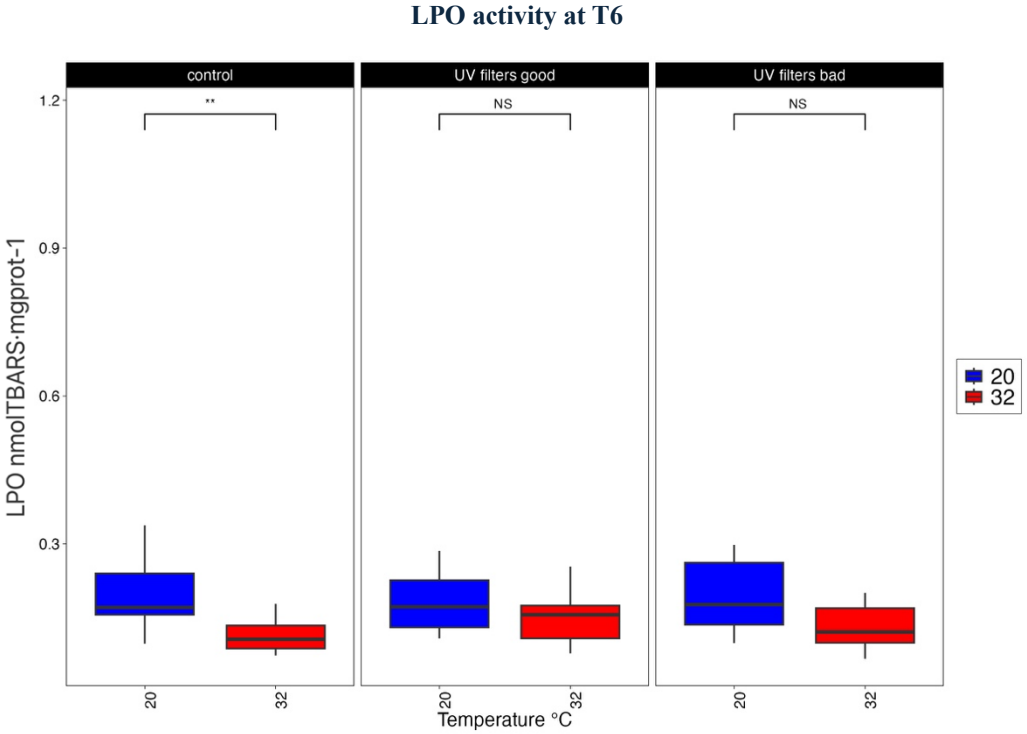

Fig. 3.5 LPO values nmolTBARS·mgprot^{-1}, at T6 stratified by treatment conditions and corresponding temperature exposures. The boxplot illustrates that at T6, there is a significant difference in LPO levels between groups exposed to 20°C and 32°C for the control treatment, with those being lower for the heatwave-exposed groups.

Iin T12 LPO values (Fig.3.6) showed a statistically significant increase in the 'bad UV filters' treatment when comparing the 20°C and 32°C temperature groups (W = 23, p-value = 0.04326). However, in both the 'good UV filter' and the 'bad UV filter' treatments, no significant differences were observed between the two treatments. This indicates that temperature, associated with the treatment of 'bad UV filters, ' had a noticeable effect on lipid peroxidation.

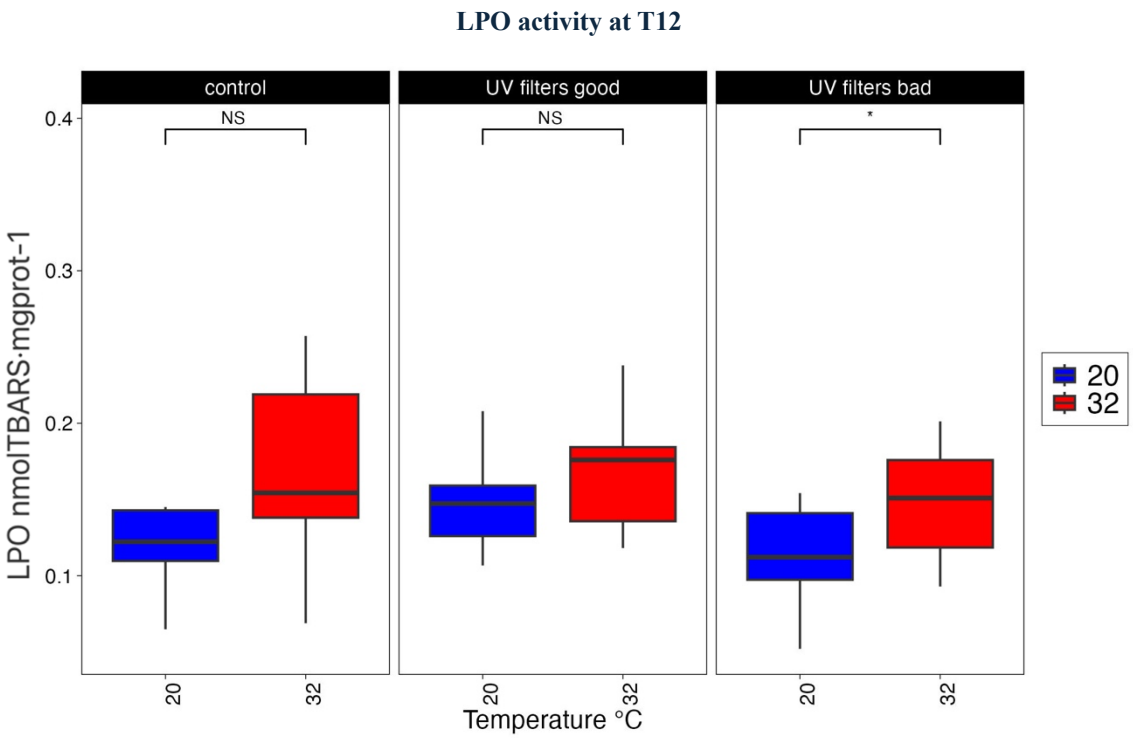

Fig. 3.6 LPO values nmolTBARS·mgprot^{-1}, at T12 stratified by treatment conditions and corresponding temperature exposures. The boxplot illustrates that at T12, there is a significant difference in LPO levels between groups exposed to 20°C and 32°C for the UV filters bad treatment, with those being higher for the heatwave-exposed groups.

Finally, in SOD activity (shown in Table 3.5), the highest means values were found in T6 at 20°C with the UV filters bad treatment (31.6 ± 33.1 µg/mg protein), and in T12 at 20°C in the control treatment (24.7 ± 13.3 µg/mg protein).

Table 3.5 SOD U/mg protein, present in the homogenized muscle tissue of *P.elegans* (mean, standard deviation, median, minimum and maximum values are shown by each treatment for the specified time points sampling with the control, T1 was measured 30 minutes after the start of the experiment; T6 after 6 hours and T12 after 12 hours). Treatments are the control (without any sunscreen), UV filters good (applied for the treatments with the eco-friendly sunscreen), and UV filters bad (for the not labelled eco-friendly sunscreen).

SOD activity

Mean, sd, median, minimum and maximum values by treatment

time	temperature	treatment	mean	sd	median	min	max
T0	20	control	85.7	29.6	85.4	35.9	126.42025
T1	20	control	36.3	50.0	13.0	7.2	154.55843
T1	20	UV filters good	12.9	6.9	12.7	5.2	25.87766
T1	20	UV filters bad	12.7	10.4	8.8	2.3	31.78357
T1	32	control	11.9	7.0	15.6	0.7	19.24914
T1	32	UV filters good	17.1	7.4	16.9	6.2	30.39611
T1	32	UV filters bad	22.8	20.3	16.4	3.5	66.36478
T6	20	control	19.0	32.5	6.5	5.5	99.09588
T6	20	UV filters good	9.4	9.8	4.2	0.3	29.03305
T6	20	UV filters bad	31.6	33.1	18.0	2.7	91.29265
T6	32	control	7.4	3.8	7.1	1.6	12.19213
T6	32	UV filters good	14.5	17.2	10.0	0.2	53.85786
T6	32	UV filters bad	13.2	12.5	9.6	2.6	47.03696
T12	20	control	24.7	13.3	20.2	4.4	46.24275
T12	20	UV filters good	16.7	13.3	12.2	3.2	43.42240
T12	20	UV filters bad	15.2	13.1	10.2	4.1	46.31990
T12	32	control	11.5	4.4	10.1	5.0	18.22237
T12	32	UV filters good	17.2	16.9	10.9	5.2	57.52608
T12	32	UV filters bad	11.9	9.5	8.6	2.8	28.01279

A significant difference shown in Fig. 3.7, was found in T12 in the control treatment between 20°C and 32°C temperature conditions (W = 77, p-value = 0.00762).

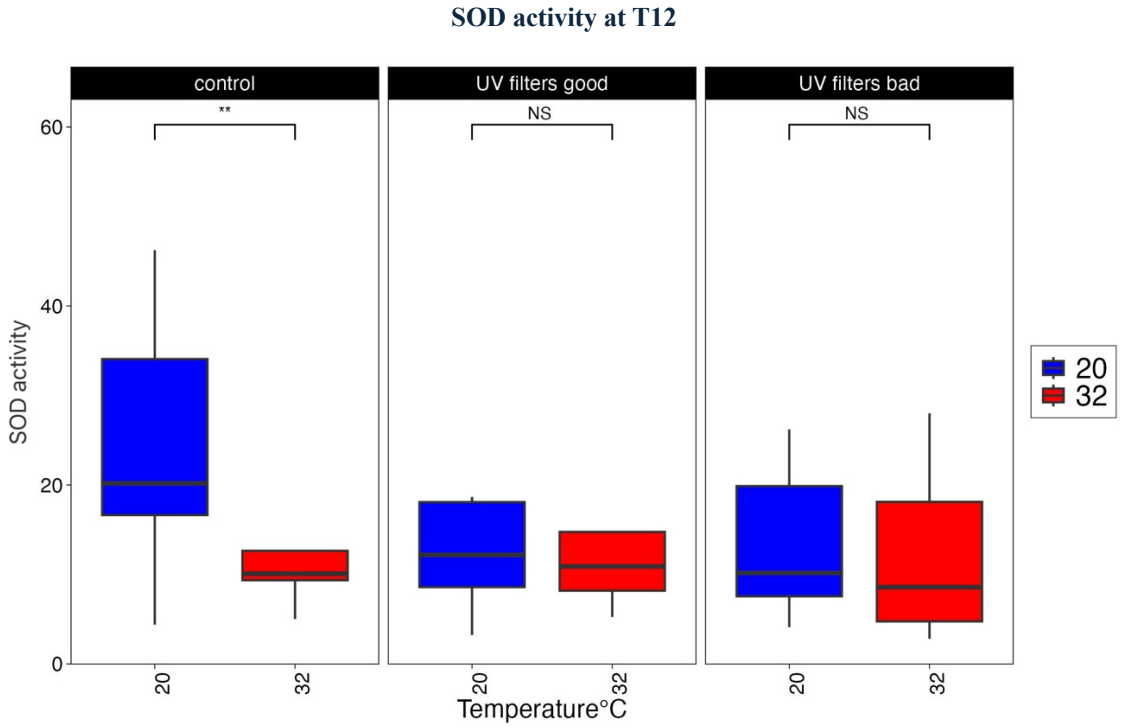

Fig. 3.7 SOD U/mg protein, at T12, stratified by treatment conditions and corresponding temperature exposures. The boxplot illustrates that at T12, there is a significant difference in SOD levels between groups exposed to 20°C and 32°C for the UV control treatment.

Hepatopancreas

Analysis of the hepatopancreas of *P.elegans* revealed no significant differences in the biomarkers examined across the different treatments (control, 'good UV filters' and 'bad UV filters') and within the temperature conditions (20°C and 32°C) at the sampling times (1, 6, 12) (p-value > 0.05 for all comparisons).

UV filters analysis

The stability and the accumulation of the studied UV filters – BP3, EHS, BMDBM and OC were analysed using UHPLC-MS/MS. The results of the Kruskal-Wallis test revealed no significant differences in the concentration of any of the UV filters (BP3, EHS, BMDBM and OC) across the different temperatures (20°C and 32°C) and time points (1, 6 and 12 hours) (p > 0.05). Additionally, the Wilcoxon rank-sum test was conducted to compare individual time points with temperature conditions. It confirmed that there were no statistically significant differences between the UV filter concentrations (p-value > 0.05)

The graphical analysis showed that the concentration of the UV filters in *P.elegans* generally tended to increase over time, particularly at higher temperatures (32°C). However, despite this trend, statistical analysis did not confirm these values.

Specifically:

BP3: An upward trend, not statistically significant (p-value > 0.05 for both Kruskal-Wallis and Wilcoxon-rank-sum tests) was observed in the 32°C group, shown in Figure 3.8. Respective values are shown in Table 3.6.

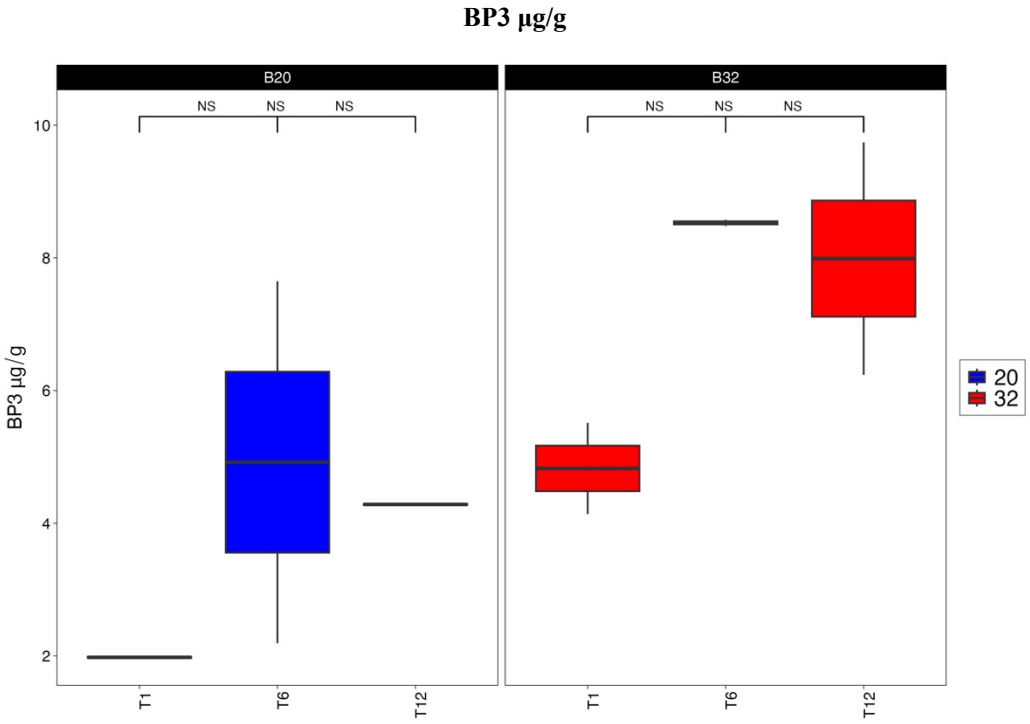

Fig. 3.8 BP3 µg/g detected with High-Performance Liquid Chromatography from a pool of three individuals of *P.elegans* previously dry frozen and homogenized for each temperature (20 and 32°C) and each sampling time (T1, T2 and T12).

Table 3.6 BP3 values, only presents in the non-ecofriendly sunscreen (mean, standard deviation, median, minimum and maximum values are shown at each sampling time point, for 20°C and 32°C groups).

BP3 µg/g

Mean, sd, median, minimum and maximum values in ng/g by treatment

time	temperature	mean	sd	median	min	max
T1	B20	2.0	0.0	2.0	2.0	2.0
T1	B32	4.8	1.0	4.8	4.1	5.5
T6	B20	4.9	3.9	4.9	2.2	7.6
T6	B32	8.5	0.1	8.5	8.5	8.6
T12	B20	4.3	0.0	4.3	4.3	4.3
T12	B32	8.0	2.5	8.0	6.2	9.7

EHS: The UV filter, contained in the eco-friendly sunscreen, showed a clearly increased concentration for the 32°C group during the exposure time, although not statistically significant (p-value > 0.05), as shown in Figure 3.9, respective values are shown in Table 3.7, with respective values shown in Table 3.7. On the other hand, the UV filter contained in the non-ecofriendly sunscreen, in Figure 3.10, shows a potential upward trend accumulation for both 20°C and 32°C groups, although not statistically significant (p-value > 0.05), respective values are shown in Table 3.8.

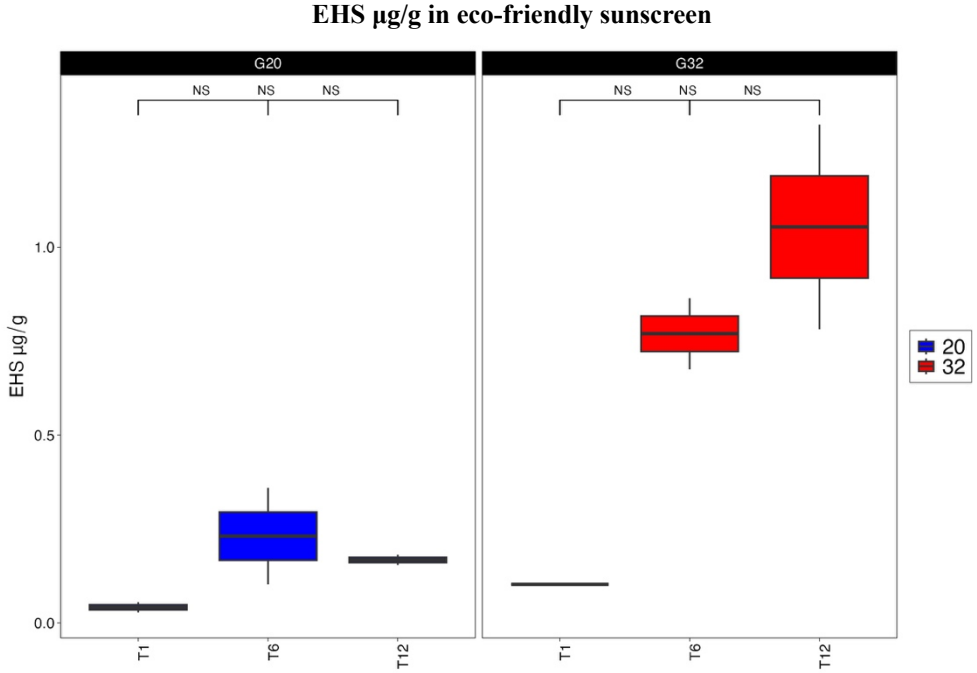

Fig. 3.9 EHS µg/g (contained in the eco-friendly sunscreen) detected with High-Performance Liquid Chromatography from a pool of three individuals of *P.elegans* previously dry frozen and homogenized for each temperature (20 and 32°C) and each sampling time (T1, T2 and T12).

Table 3.7 EHS µg/g values contained in the eco-friendly sunscreen (mean, standard deviation, median, minimum and maximum values are shown at each sampling time point, for 20°C and 32°C groups).

EHS µg/g (good)

Mean, sd, median, minimum and maximum values in ng/g by treatment

time	temperature	mean	sd	median	min	max
T1	G20	0.04	0.02	0.04	0.03	0.06
T1	G32	0.10	NA	0.10	0.10	0.10
T6	G20	0.23	0.18	0.23	0.10	0.36
T6	G32	0.77	0.13	0.77	0.67	0.86
T12	G20	0.17	0.02	0.17	0.15	0.18
T12	G32	1.05	0.39	1.05	0.78	1.33

EHS µg/g in non-ecofriendly sunscreen

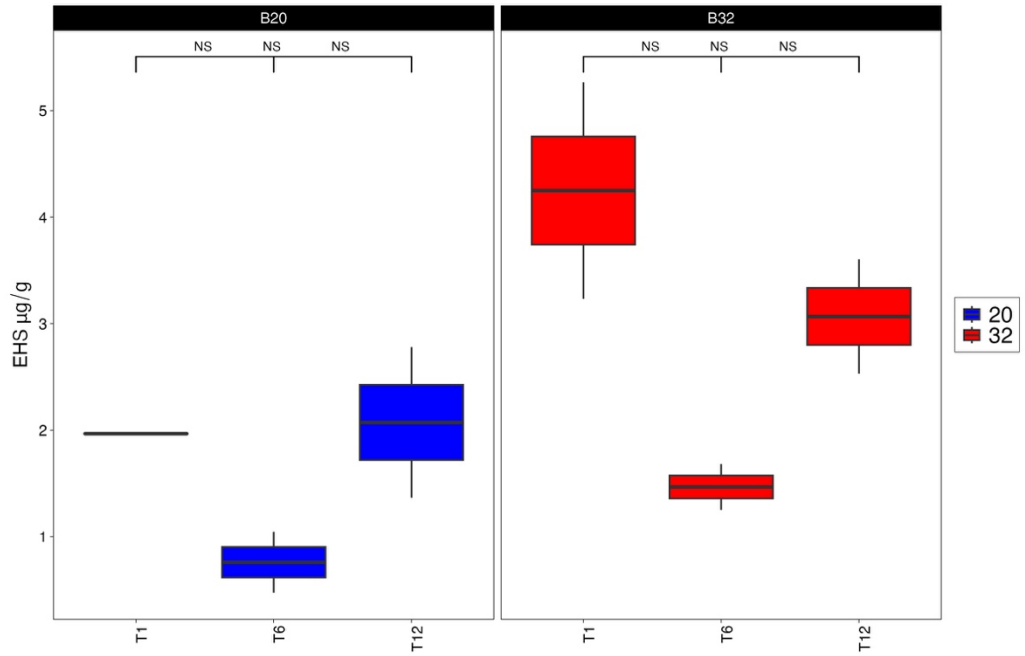

Fig. 3.10 EHS µg/g (contained in the **non**-eco-friendly sunscreen) detected with High-Performance Liquid Chromatography from a pool of three individuals of *P.elegans* previously dry frozen and homogenized for each temperature (20 and 32°C) and each sampling time (T1, T2 and T12).

Table 3.8 EHS values contained in the non-eco-friendly sunscreen (mean, standard deviation, median, minimum and maximum values are shown at each sampling time point, for 20°C and 32°C groups).

EHS µg/g (bad)

Mean, sd, median, minimum and maximum values in ng/g by treatment

time	temperature	mean	sd	median	min	max
T1	B20	1.97	0.01	1.97	1.96	1.97
T1	B32	4.25	1.44	4.25	3.23	5.27
T6	B20	0.76	0.40	0.76	0.47	1.05
T6	B32	1.47	0.30	1.47	1.25	1.68
T12	B20	2.07	1.00	2.07	1.37	2.78
T12	B32	3.07	0.76	3.07	2.53	3.60

BMDBM: Figure 3.11 shows an upward concentration trend for the UV filter contained in the eco-friendly sunscreen, more evident in the 32°C groups, but no statistically significant differences were found (p-value > 0.05), respective values showed in Table 3.9. On the other hand, for the UV filter contained in the non-ecofriendly sunscreen, shown in Figure 3.12, an upward trend seems to stabilize at T6 in both temperature groups (20 and 32°C), showing then a decrease at T12. Also in this case,

no statistically significant differences were found (p-value > 0.05) among groups and times. Respective values are shown in Table 3.10.

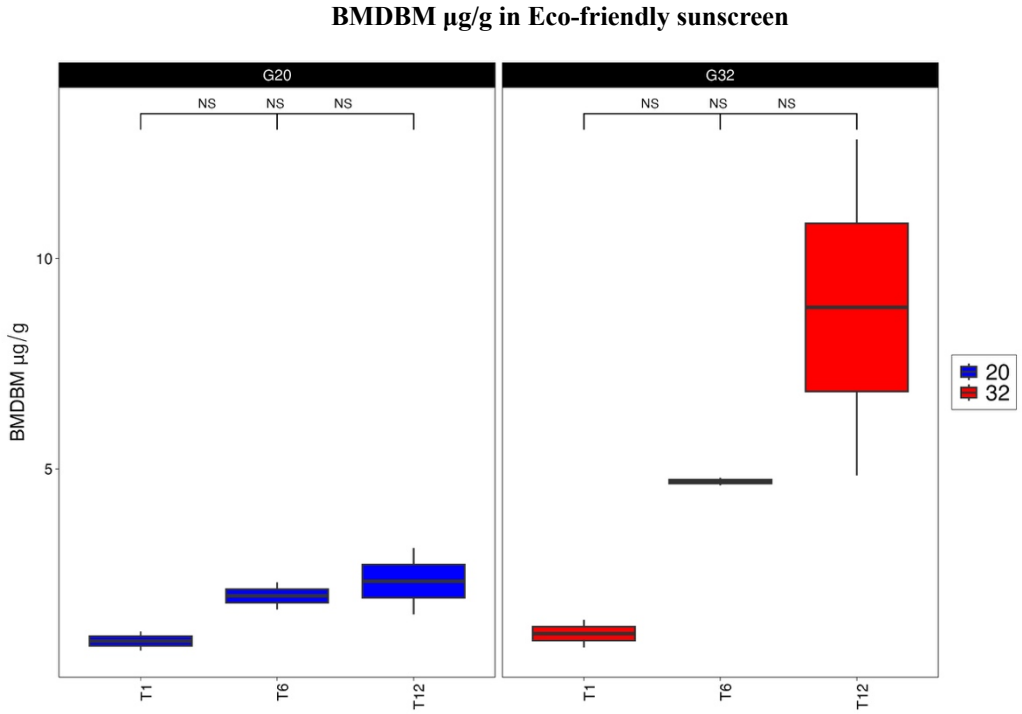

Fig. 3.11 BMDBM µg/g (contained in the eco-friendly sunscreen) detected with High-Performance Liquid Chromatography from a pool of three individuals of *P.elegans* previously dry frozen and homogenized for each temperature (20 and 32°C) and each sampling time (T1, T2 and T12).

Table 3.9 BMDBM µg/g contained in the eco-friendly sunscreen (mean, standard deviation, median, minimum and maximum values are shown at each sampling time point, for 20°C and 32°C groups).

BMDBM µg/g (good)

Mean, sd, median, minimum and maximum values in ng/g by treatment

time	temperature	mean	sd	median	min	max
T1	G20	0.91	0.32	0.91	0.68	1.14
T1	G32	1.08	0.47	1.08	0.75	1.41
T6	G20	1.98	0.46	1.98	1.66	2.30
T6	G32	4.70	0.13	4.70	4.61	4.79
T12	G20	2.33	1.12	2.33	1.54	3.12
T12	G32	8.84	5.65	8.84	4.84	12.83

BMDBM µg/g in non-eco-friendly sunscreen

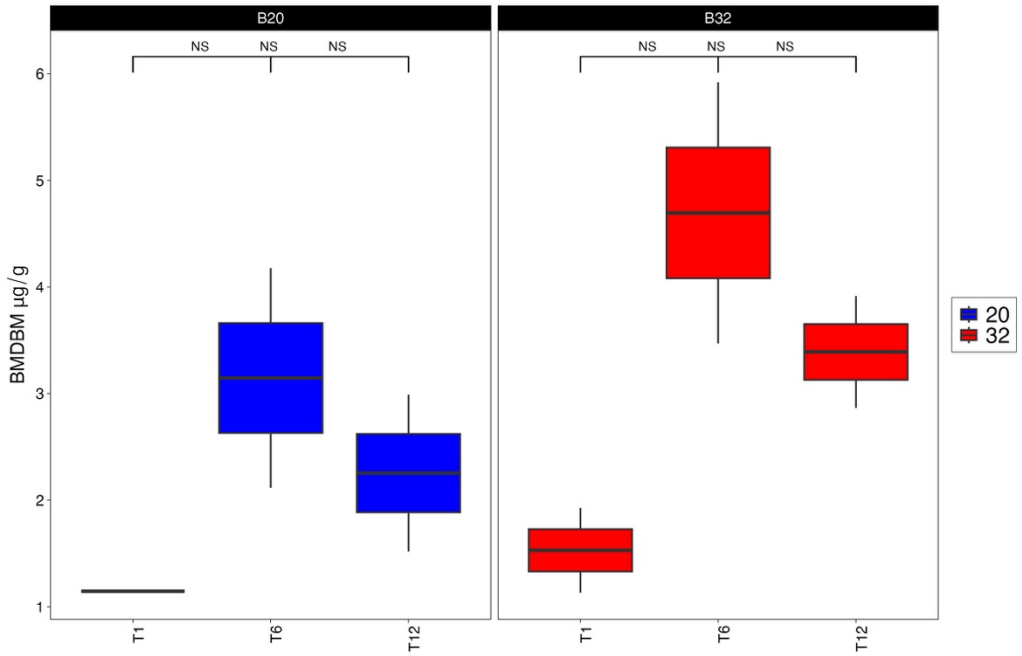

Fig. 3.12 BMDBM µg/g (contained in the **non**-eco-friendly sunscreen) detected with High-Performance Liquid Chromatography from a pool of three individuals of *P.elegans* previously dry frozen and homogenized for each temperature (20 and 32°C) and each sampling time (T1, T2 and T12).

Table 3.10 *BMDBM* µg/g contained in the non-eco-friendly sunscreen (mean, standard deviation, median, minimum and maximum values are shown at each sampling time point, for 20°C and 32°C groups).

BMDBM µg/g (bad)

Mean, sd, median, minimum and maximum values in ng/g by treatment

time	temperature	mean	sd	median	min	max
T1	B20	1.15	0.00	1.15	1.15	1.15
T1	B32	1.53	0.56	1.53	1.13	1.93
T6	B20	3.15	1.46	3.15	2.12	4.18
T6	B32	4.69	1.73	4.69	3.47	5.92
T12	B20	2.25	1.04	2.25	1.52	2.99
T12	B32	3.39	0.74	3.39	2.87	3.91

Octocrylene: An upward trend in the concentration of Octocrylene shown in Fig. 3.13, contained in the non-ecofriendly sunscreen, is evident along the exposure time for both temperature groups (20 and 32°C). Indeed, no statistical difference was found across treatments and across exposure times (p-value > 0.05), respective values are shown in Table 3.11.

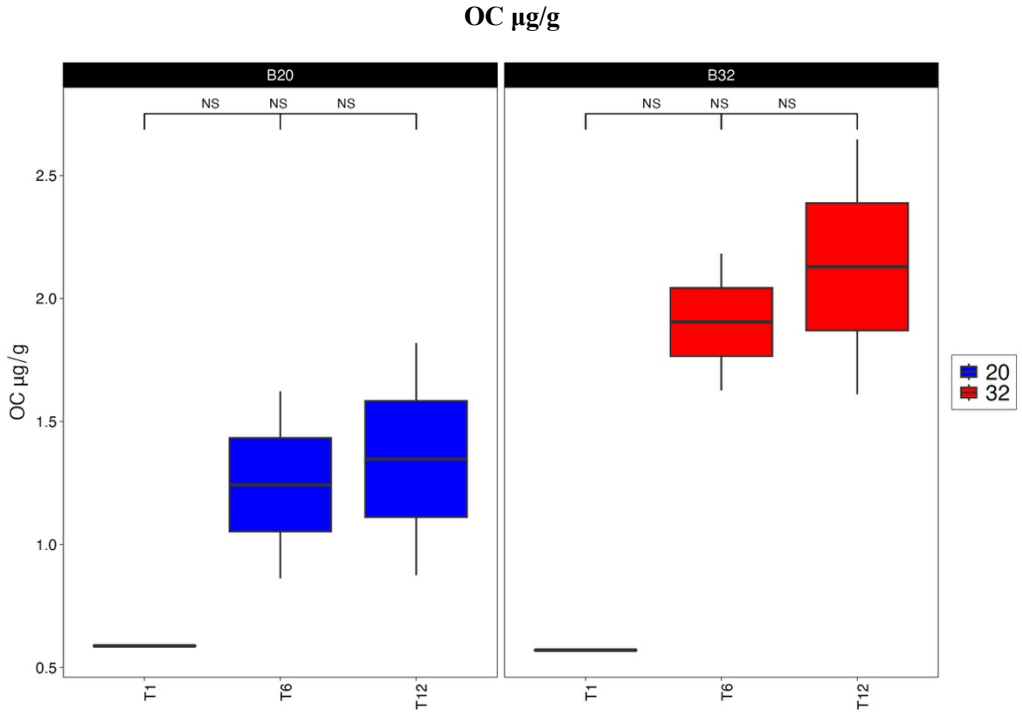

Fig. 3.13 Octocrylene µg/g (contained in the **non**-eco-friendly sunscreen) detected with High-Performance Liquid Chromatography from a pool of three individuals of *P.elegans* previously dry frozen and homogenized for each temperature (20 and 32°C) and each sampling time (T1, T2 and T12).

Table 3.11 Octocrylene values contained only in the non-eco-friendly sunscreen (mean, standard deviation, median, minimum and maximum values are shown at each sampling time point, for 20°C and 32°C groups).

OC µg/g

Mean, sd, median, minimum and maximum values in ng/g by treatment

time	temperature	mean	sd	median	min	max
T1	B20	0.59	0.00	0.59	0.58	0.59
T1	B32	0.57	0.00	0.57	0.57	0.57
T6	B20	1.24	0.54	1.24	0.86	1.62
T6	B32	1.90	0.39	1.90	1.63	2.18
T12	B20	1.35	0.67	1.35	0.87	1.82
T12	B32	2.13	0.73	2.13	1.61	2.65

4. Discussion

Oxidative stress in the muscle

This work shows, for the first time, the synergistic interaction between UV filters contained in sunscreens with high-temperature stress in the physiology of *P.elegans,* a small crustacean inhabiting rocky tide pools. The antioxidant enzymes analysed respond differently to the increasing temperature and the presence or absence of UV filters. This reflects changes in the susceptibility to both thermal stress and chemical pollutants.

Oxidative stress biomarkers are useful tools for adverse effects that enable us to identify, monitor, and assess several threats to marine biodiversity (Benedetti et al., 2015). Because physiological activities normally generate reactive molecules and free radicals, these molecules can damage different structures such as lipids, DNA, or proteins, and eventually leading to irreversible damage (Félix et al., 2020a). When ROS production surpasses the removal, then oxidative stress occurs (Aranda-Rivera et al., 2022). Despite this, cellular defences help detoxify these molecules, reducing their harmful effects (Birben et al., 2012).

ETS activity represents an important metabolic biomarker since gives us important information on the metabolic demand of the cell, because this process is directly involved in energy production (Coen & Janssen, 1997). An increased stress, should increase ETS activity and, at the same time reduce the energy available that could potentially be utilized for somatic and reproductive growth (Lemos, 2021).

In this study, a significant increase in ETS activity was observed at T12 under across all three treatments (control, UV filters good and UV filters bad), for organisms exposed at 32°C (simulating the effects of a marine heatwave). The combination of elevated temperatures and the presence of UV filters led to significantly higher ETS activity compared to organisms maintained at lower temperatures. The higher activity of this metabolic biomarker suggests that the thermal stress might trigger metabolic adjustments, reflecting the organism's ability to face the energy demand under this condition, wich is directly linked to ATP production. In fact, the prolonged warmer environment, exceeding the duration of a normal tidal cycle, may upregulate the mitochondrial activity in response to an increased metabolic demand. This might be correlated to the fact that at the two previous sampling times (T1 and T6), no significant difference ($p > 0.05$) were found within the treatments and between the temperature groups. These results highlight that heat stress had can have a greater impact than UV filters, with temperature being the dominant factor on metabolic pathways after 12 hours. This might be a strategy to compensate for the potential damage caused by the elevated temperatures. Previous studies showed that assessing the ETS activity has been considered to be an accurate method for estimating metabolic activity in different marine organisms like *Hippoglossoides*

platessoides (Fabricious, 1780) (Smith & Chong, 1982) and the *Oryzias latipes* (Temminck and Schlegel, 1846), (G.-Toth et al., 1995). It was observed that organisms exposed to warmer waters tend to increase their metabolic rates to cope with heat-induced stress (Simčič et al., 2015), similar to our findings.

On the other hand, COX activity for organisms sampled at T1 show higher levels in the 'bad UV filter' treatment at 20°C than the ones at 32°C. Moreover, at T12, in all treatments, the 20°C group, showed higher levels of COX in comparison to those at 32°C. COX represents a transmembrane molecule, which is found in the mitochondria of eukaryotes and plays a fundamental role in producing energy via ATP generation, through the electron transport system (Antonini et al., 1970; Wikström & Sharma, 2018), being the major regulatory enzyme of the electron transport chain (Srinivasan & Avadhani, 2012). So far, different studies have been investigating the aerobic respiration in osmoregulatory organs of euryhaline teleost (*Chanos chanos, Oreochromis mossambicus* and *Oryzias dancena*) (Hu et al., 2018), the oxygen level fluctuation impacts on the mitochondrial function and the aerobic metabolism in estuarine and coastal benthic organisms (Ouillon et al., 2021). Hence our study included a multifactorial stressors assessment on a rocky tidal pool crustacean, *P.elegans,* with the objective of understanding, if higher water temperature associated with UV filters, might lead to metabolic changes and, consequently, increase the production or ROS, we have little literature to compare our data analysis to. However, studies indicate that COX dysfunction is highly related to increased mitochondrial reactive oxygen species production, affecting ATP production and promoting oxidative stress levels (Fornuskova et al., 2010; Galati et al., 2009). Under normal conditions, the COX uses oxygen, allowing the electron flow and ATP production. However, a MHW episode in a rocky tidal pool can cause a situation of low oxygen concentrations (Roman & Pierson, 2022), so that the oxygen flow might not be enough, leading to the upstream accumulation of electron-rich intermediaries (NADH, FADH2) in the electron transport chain (ETC), which enhance the production of ROS, causing oxidative damage (Srinivasan & Avadhani, 2012). According to this, our data show that already at T1, the ECT in the cells' mitochondria, under the synergistic effect of the UV filters associated with 32°C water temperature, is already accumulating ROS that may reduce the energy allocation. Moreover, at T12, data, indicate that the most critical stressor is the temperature (Fig.5). In fact, in all the 32°C groups, COX values were significantly lower than in the 20°C groups, highlighting a possible dysfunction of this enzyme at a 12-hour protected high temperature. As a secondary consequence of this, the ATP generation might be compromised as well due to a possible ROS accumulation in the ECT.

Regarding the GST, this biomarker plays a fundamental role in detoxifying and preventing lipid peroxidation (Sureda et al., 2018). The results of our study (Fig. 7), show that only for the control

treatment at 12 hours, the 20°C group has higher levels of GST than the one at 32°C. GST is widely used as a biomarker for assessing environmental conditions, particularly in evaluating how organisms respond to stressors like pollution and temperature fluctuations (Cheung et al., 2001; Gowland et al., 2002). GST activity in invertebrates has been shown to increase according to the concentration of xenobiotics present in the seawater, however, measuring this biomarker is often challenging and typically results in patterns that are difficult to interpret (Cunha et al., 2005). Moreover, it has already been shown that the temperature positively interacts with the GST activity, promoting higher activity of this enzyme (Booth et al., 2000). The reason of our findings might be due to the fact that the organism at 32°C has already exceeded the upper-temperature threshold limit, inducing the collapse of the antioxidant system. This theory is reinforced by the fact that GST levels for the 20°C group are low in both UV filters 'good' and 'bad' treatments, and there are no significant differences with the 32°C groups. In this case, the presence of the chemical compounds represented by the UV filters from one side, increased by the heat stress from the other, might trigger the antioxidant response. Despite these theories, it is necessary to affirm that the GST does not show predictable trends, as previously reported in other studies, where this biomarker produced highly variable and inconsistent results (Domingues et al., 2010).

Similar to the GST, also the superoxide dismutase (SOD) activity shows statistically significant values at T12, for the control treatment, with higher values in the 20°C group in comparison with the 32°C group. This biomarker found in cells' cytosol, increases as the oxidative stress accumulates in the organism, and it is a fundamental defence against ROS which inhibits their accumulation (Downs et al., 2001). Verlecar et al.,(2007) found a high SOD activity at 32°C than 26°C for *Perna viridis* digestive gland and gills. Comparable patterns of SOD variability were reported in gastropods where an increased temperature exposure resulted in higher SOD activity (De Oliveira & Greco, 2015). By contrast, in the present study higher SOD activity was found in the control group at 20°C, maybe due to the collapse of this antioxidant defence system, which would not be able to catalyse the oxidation of superoxides in the hydrogen peroxide (H_2O_2) (Ilijin et al., 2021). The high-temperature-related stress was beyond the tolerance range of *P.elegans*, therefore the SOD activity decreased at 32°C, as the high-temperature-related stress acting together with the chemical pollutants represented by the UV filters lead to low SOD levels.

Lipid peroxidation (LPO) is one of the most common biomarkers for free radical formation because unsaturated fatty acids, such as the ones found in cellular membranes are frequently a target point of free radicals (Félix et al., 2020a). This process occurs when a free radical removes a hydrogen atom from an unsaturated carbon, forming water and an unpaired electron, which reacts with oxygen, forming a peroxyl radical (Félix et al., 2020b). Our study showed that at T6, a higher LPO activity

for the 20°C group than for the 32°C group. This might indicate a potential limit in the organism's ability to counteract oxidative damage under the prolonged exposition (Abele et al.,2002; Kregel, 2002). Other studies showed similar results for LPO. For instance, Madeira et al., (2013) found a decrease in LPO activity when specimens were tested with a simulated summer heat wave. On the contrary, at T12 following prolonged exposure to both thermal and chemical stressors, for the 'bad UV treatment', LPO showed statistically significant higher values in the 32°C group, than in the 20°C group. These results align with previous studies conducted in the muscle of *Sparus aurata* by Madeira et al., (2016), and also by Vinagre et al., (2012) in *Dicentrarux labrax* assessing that when temperature increases, outside the species-specific thermal optimum, LPO activity increases as well. This has to be taken into consideration consciously because this response varies between species. Yet the synergistic effect of the thermal stressor associated with the UV filters contained in the sunscreen, after 12 hours, led to the production of a higher amount of ROS causing cell membrane damage and unregulated function (Lesser, 2006).

Oxidative stress in the hepatopancreas

Hepatopancreas data analysis is unclear and does not allow the formulation of any hypothesis. The statistical analysis performed showed no significant differences (p-value> 0.05) in the biomarkers across the different treatments and temperatures at any of the sampling times (1, 6, and 12 hours). These results might be due because of the small sample size obtained from the experiment, which did not allow to perform a solid statistical analysis. Moreover, the hepatopancreas is a very sensitive organ, and since our values were obtained from a pool of 5 shrimps that were dissected, this might have influenced and contaminated the outcome. In addition, due to this organ's high sensitivity, it needs to be carefully handed. The procedures adopted during the extraction might have inferred the final results.

Even though the hepatopancreas is the principal organ for the digestion and xenobiotic detoxification in aquatic invertebrates, it is highly responsive to environmental changes, making it difficult to establish oxidative stress reaction patterns (Webb, 2011). In fact, the organism might have already gone above the peak of the antioxidant response, resulting in a counter effect lowering the antioxidant pathways levels. Another point of view could be that the organisms might shift to prioritize another part to maintain homeostasis under the effect of several stressors, like in the muscle, for example.

The biomarkers selected for this study, and analysed in the muscle have suggested that there is a physiological response to the anthropogenic stressors to which *P.elegans* was exposed. The response that those invertebrates have is very species-specific, and there are many other variables to take into consideration. The hepatopancreas might be a very sensible organ which needs an appropriate workflow for the analysis that has to be optimised if considering the stressors applied in this study. It

is important to consider the complexity of biological responses are organisms can employ multiple defense mechanisms, some of which may not be captured by the specific biomarkers chosen.

UV filters accumulation

This study of the accumulation of UV filters in marine invertebrates populating coastal regions highly frequented by humans could be a proxy for identifying if these compounds can accumulate in the organism and eventually biomagnified along the food web. In this study, we mostly focused on assessing a potential direct uptake of the contaminants from the environment, intending to regulate and limitate those pollutants.

Due to circumstances beyond our control, in this experiment, we used a pool of 3 dry frozen, then triturated shrimps for the HPLC analysis. From this, we obtained a single replicate which was compared to another one (resulting in a total of 2 replicates for each treatment). The lack of more replicates limits the statistical analysis but still might be a key starting point for analysing and monitoring UV filters. Moreover, the methodology used for the extraction of the pollutants was successful but optimized for zooplankton and not for bigger crustacea such as *P.elegans.*

BP3 concentration was constantly detected at higher concentrations in the MHW simulated treatment, increasing from T1 to T6 than stabilizing at T12, (Fig. 11). This UV filter has been detected worldwide, from the USA (He et al., 2017) to China (Sang & Leung, 2016) and from the Antarctic (Emnet et al., 2015) to Spain (Isabel Cadena-Aizaga et al., 2022).

EHS concentration as well, was constantly detected at higher levels for the treatment at 32°C with a higher concentration in the treatment where the "non-labelled eco-friendly sunscreen". In the organisms exposed to the non-eco-friendly sunscreen, on average more than one ug/G of the EHS was found. Even if no statistical significance was found, the non-ecofriendly treatment presented a higher concentration of this UV filter.

BMDBM is the most common UV filter found in the Canary Islands, as reported by Cadena-Aizaga et al., (2022), and also the one with the highest concentration. In our study, this UV filter showed an upward trend in the eco-friendly treatment, but not in the non-ecofriendly treatment, which decreased from T6 to T12. This might be due to the fact that the organism might be able to process this filter differently according to the treatment. However, in both treatments, the organisms exposed at 32°C constantly had a higher concentration of the filters. This shows that the solubility is higher according to the temperature, and that can accumulate more easily in the organism during a MHW.

Finally, octocrylene, found only in the non-ecofriendly sunscreen, the concentration for both 20 and 32°C increased with time. This means that 12 hours might be enough for this UV filter to accumulate in the organism, with the possibility of biomagnifying along the food web. In fact, it has been reported that this compound is not easily biodegradable due to its low solubility and high Log K_{OW},

contributing to the tendency to accumulate (Pawlowski et al., 2019), and possibly to biomagnify along the food web.

5. Conclusion

This study provides insights into the synergistic effects of UV filters contained in sunscreen and marine heat waves on the physiology of the rocky tidal pool *Palaemon elegans.* Our findings show that biomarkers could be used as a proxy for the health of this organism, and also that these are very species-specific. Metabolism-related biomarkers, such as ETS and COX, and ROS-related biomarkers, like GST, SOD and LPO showed that they could be influenced by prolonged exposure to thermal stress, close to its species-specific CTMax. In fact, the thermal stress set for the MHW simulation, 32°C, represented the main driver of the physiological stress for the organism, even though when associated with the non-ecofriendly labelled sunscreen results were amplified. High ETS activity indicated an increased metabolic demand at a higher temperature, and low COX levels indicated that the ATP system production had been compromised, causing mitochondrial dysfunction together with a possible ROS accumulation. GST and SOD activities showed treatment-specific responses, indicating that prolonged heat stress might have overwhelmed the antioxidant system, leading to a decrease in the biomarkers values.

The HPLC confirmed that the UV filters found in the sunscreens accumulate in the organisms as early as 6 hours after the exposure, with higher levels after 12 hours. This was enhanced by higher temperatures, facilitating the uptake. Despite the unsuccess of the statistical analysis, influenced by the small sample size analysis, this study indicates the potential for chemical pollutants to accumulate in the organism after only a brief period of exposure. Moreover, it showed that the synergistic activity of UV filters and MHW represent a threat to this species. Further studies need to focus on the optimisation of the UV filter extraction from this species for the HPLC, and for the hepatopancreas analysis.

The two sunscreens do not seem to enhance a different oxidative response in the organisms or change at a metabolic level in general. This is probably because both filters similarly harm the organism. On the contrary, LPO activity shows higher levels in the non-ecofriendly sunscreen at 32°C. According to this, further analysis are necessary, testing individually the effect of the UV filters and identifying the specific component responsible for the damage.

For a sub-tropical region like the Canary Islands, the temperate temperatures throughout the whole year make this region a major truistic destination, this ends up increasing the pressure on the coastal ecosystem. One of the possible consequences of this is the increased use of solar protectors, and while many of those present UV filters that are regulated, others present UV filters that have been banned in certain countries, but that are still sold in the Canary Islands. The results shown in this study highlight the potential interaction between marine heatwaves and chemical pollutants as UV filters

contained in sunscreens, suggesting that rising ocean temperatures might exacerbate the negative effects of these pollutants on marine organisms. This justifies the need for further investigation into their combined impacts.

Personal Note

As demonstrated by this small study and numerous published scientific articles, higher temperatures are a real threat. The ocean is “boiling”, and as we might say in Italy, it is time to “put in the pasta”. Many researchers are striving to draw more attention to this issue. Marine and coastal ecosystems represent a beautiful and colourful but delicate and fragile environment rich in biodiversity and life. At the same time, are among the most vulnerable, threatened by humans, endangering the whole biota. Lowering temperatures might not be an easy task to deal with, but there are many things that we as humans can do. Despite this, we could promptly decide to spend more time looking at the products that we use, assuring that those really respect the environment and do not contain substances harmful to the ecosystem. The world that we are living in is not an easy one, but it is the only one we have. Everything and everyone is moving fast, too fast. We want everything, and we want it now. There is no time anymore that we humans can wait for, but then it is funny to realise how much time we lose on silly and superfluous things. We, people, do not have to stop trying to make this call. We have to keep trying to bring attention on issues like the one I dealt with in this study, with the hope that this message will spread throughout, inspiring people to drive meaningful changes. We know it is difficult. We do not have to stop. We do have to keep moving and stirring the pot. We must believe in what we do, in our colleagues and in this passion for the blue, that we all share!

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