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IMMUNITY AND TEMPERATE CORAL CRISIS: A NOVEL APPROACH TO DISCOVER IMPACTS AND RESILIENCE UNDER CLIMATE CHANGE SCENARIOS

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"Everything you can imagine, nature has already created"

Albert Einstein

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List of papers

Papers directly connected with the thesis

Bisanti, L., La Corte, C., Dara, M., Bertini, F., Parisi, M.G., Chemello, R., Cammarata, M., & Parrinello, D. (2024). Global warming-related response after bacterial challenge in *Astroides calycularis*, a Mediterranean thermophilic coral. Scientific Reports, 14(1), 8495. https://doi.org/10.1038/s41598-024-58652-0

Bisanti, L., La Corte, C., Dara, M., Bertini, F., Parrinello, D., Chemello, R., Cammarata, M., & Parisi, M. G. (2024). How does warmer seawater change the sensitivity of a Mediterranean thermophilic coral after immune-stimulation? Coral Reefs, 43(1), 137-150. https://doi.org/10.1007/s00338-023-02454-9

Other relevant papers

Bisanti, L., de Sabata, E., Visconti, G., & Chemello, R. (2022). Towards a local mass mortality of the Mediterranean orange coral *Astroides calycularis* (Pallas, 1766) in the Pelagie Islands Marine Protected Area (Italy). Aquatic Conservation: Marine and Freshwater Ecosystems, 32(3), 551-557. https://doi.org/10.1002/aqc.3772

Bisanti, L., Visconti, G., Scotti, G., & Chemello, R. (2022). Signals of loss: Local collapse of neglected vermetid reefs in the western Mediterranean Sea. Marine Pollution Bulletin, 185, 114383. https://doi.org/10.1016/j.marpolbul.2022.114383

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La Corte, C., Dara, M., Bertini, F., **Bisanti, L.**, Cammarata, M., & Parisi, M.G. (2024). Sea anemones, methylmercury, and bacterial infection: A closer look at multiple

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Bisanti, L., La Corte, C., Dara, M., Bertini, F., Vizioli, J., Parisi, M.G., Cammarata, M., & Parrinello, D. (2024). The Interplay of TLR-NFκB Signalling Pathway and Functional Immune-Related Enzymes in the Inflammatory Response of *Ciona robusta*. Animals, 14(15), 2169. https://doi.org/10.3390/ani14152169

Summary

Consequences of anthropogenic carbon emissions are warming warming seawater worldwide, pushing the limits of tolerance for corals and driving a decline in biodiversity. The Mediterranean Sea is a climate change hotspot where temperature impacts are very pronounced and have been well-documented across the region over the last 30 years. To determine the influence of warmer conditions on the immune activities of temperate corals, several experimental designs were implemented in both the laboratory and the field, using a wide variety of functional and histological techniques as well as antibodies towards specific molecular targets. The findings of experiments carried out in the laboratory confirmed the regulation of the TLR-NF-kB pathway in colonies of *Astroides calycularis* under bacterial challenge at control temperature, as well as the interplay of HSP70 in coral responses. Under warmer conditions plus pathogen elicitation, TLR4-NF-kB activation was almost completely suppressed, while an upregulation of HSP70 appeared in both treatments at elevated temperature. Results from laboratory and field experiments revealed that several immune-related enzymes (phenoloxidase, glutathione peroxidase, lysozyme, alkaline phosphatase, and esterase) are involved in the responses of *A. calycularis*, *Cladocora caespitosa* and *Balanophyllia europaea* corals to pathogen elicitation and physical injury. However, all species considered showed a strong response alteration (in almost all immune parameters) with thermal stress, indicating a general trend of enhanced effects on constitutive activities but a suppressive impact on injury and/or bacterial challenge. Microbiological analyses from *in situ* experiments also revealed a shift in the coral-associated bacterial community during the summer period towards pathogen taxa after injuries. These species have the ability to acquire and maintain warming tolerance through stress memory. This is a phenomenon wherein a transient "priming" stimulus leads to a modified and often enhanced response to a subsequent "triggering" stress exposure. The results on *C. caespitosa* indicated a durable thermal-protective effect in temperate corals due to stress memory, which significantly improved immune tolerance and a bleaching resistance for next 75 days after the priming exposure. Additionally, a significant relationship was found between genomic methylation levels and accumulated thermal stress in corals, preliminarily suggesting an epigenetic regulation dynamic of coral stress tolerance in response to climate change. Such an approach

was useful for discovering the impacts of climate change and its effects on the survival chances of temperate corals, combining new immunological information with physiological natural history to better understand what drives the health and resistance of endangered corals.

Keywords: seawater warming; temperate coral; innate immunity; stress memory; DNA methylation

General introduction

Anthropogenic climate-change drivers (e.g., rising seawater temperatures, increased nutrient loads, and increased ocean acidification) are the greatest selective pressures on corals worldwide (Davies et al., 2015; Hughes et al., 2018; Nolan et al., 2018). A healthy coral is generally host to endosymbiotic algae (e.g., *Symbiodinium*), bacteria, fungi, viruses and archaea; however, recurrent and prolonged anthropogenic pressures can compromise the health-state of these organisms, increasing bleaching and disease (Miller et al., 2006; Muller et al., 2008; Miller et al., 2009; Hughes et al., 2017). Immune systems promote homeostasis and orchestrate relationships between hosts, mutualists, commensals, and pathogens of multipartite corals. Thus, immunity underlies the health and survival of these organisms, resisting disruptions and re-establishing homeostasis in the face of biotic and abiotic perturbations.

Coral immune system

Corals possess a complex innate immune system that is capable of self-non-self-recognition, initiating signalling responses, and mounting downstream responses to regulate coral health (Palmer and Traylor-Knowles, 2012; Bosch, 2013; Traylor-Knowles and Connelly, 2017; Parisi et al., 2020) (Figure 1). The coral immune system consists of different types of receptors and signalling pathways, leading to many different effector responses. These pattern recognition receptors (PRRs) include scavenger receptors, toll-like receptors (TLRs), interleukin receptors (IL-Rs), lectins, complement receptors, and tumour necrosis factor receptors (TNFRs). TLRs are a superfamily of PRRs including pro-inflammatory interleukin-1 (IL-1R) receptors that bind microbe-associated molecular patterns (MAMPs) via extracellular leucine-rich repeat domains (LRRs) and transduce signals via intracellular toll-interleukin receptor 1 (TIR) domains (Schwarz et al., 2008; Palmer and Traylor-Knowles, 2012; Libro et al., 2013). Lectins are another PRR class represented in corals (e.g., c-type lectins, rhamnose-binding lectins, and tachylectins) that possess extracellular LRRs to bind oligosaccharides and other MAMPs (Kvennefors et al., 2008; Zhou et al., 2017). NOD-like receptors (NLRs) and RIG-I-like receptors (RLRs) are cytoplasmic PRRs that bind bacterial PAMPs and viral DNA, their functional characterization in corals is still incomplete (Shinzato et al., 2011; Hamada et al., 2013; Anderson et al., 2016). The TNFR gene family is involved in the maintenance, activation and modulation of the immune

system, as well as apoptosis (MacEwan, 2002; Gaur and Aggarwal, 2003). Remarkably, corals possess a great diversity of these genes: the highest level of diversity of all known multicellular organisms (Quistad and Traylor-Knowles, 2016).

Numerous immune signalling pathways converge in the activation of nuclear factor kappa B (NFkB), a key factor regulating immune gene transcription (Palmer and Traylor-Knowles, 2012; Traylor-Knowles and Connelly, 2017; Parisi et al., 2020). Corals possess TLRs and NLRs with TIR domains, which can interact with genetic homologues of myeloid differentiation primary response protein 88 (MyD88), IL-1R-associated kinase (IRAK), TNF receptor-associated factor 6 (TRAF) and IkB kinase (Ikk) that cleave NFkB inhibitors (IkBs) and allow NFkB protein dimers to translocate into the nucleus and enhance the expression of inflammatory cytokines (Schwarz et al., 2008; Oliver et al., 2017). In the complement cascade, homologous of C3 protein, factor B (Bf) and mannose-associated serine protease (MASP) that are activated by lectin PRRs have been identified in corals (Poole et al., 2014; Traylor-Knowles and Connelly, 2017).

Upon activation by MAMP sensing, the coral phenoloxidase (PO) cascade employs multiple POs to hydroxylate monophenol and diphenol substrates into polymeric melanin deposits that produce cytotoxic defences and create barriers to infection (Mydlarz and Palmer, 2011; Palmer et al., 2011; van de Water et al., 2015). Reactive oxygen species (ROS) are free radicals derived from oxygen or nitrogen, involved in cellular reduction-oxidation (redox) reactions and counterbalanced by antioxidant compounds. At low doses, ROS act as signalling molecules (e.g., in immune response and apoptosis), while at higher doses they produce oxidative stress that is harmful to cellular components, such as nucleic acids, proteins, and lipids (Evans and Halliwell, 1999). During respiratory bursts in corals, hydrogen peroxide (H_2O_2) can be used to destroy pathogens or as a by-product of melanin and oxidase enzyme formation (Mydlarz and Jacobs, 2006). To prevent oxidative stress, the cell uses various enzymatic and non-enzymatic antioxidants as nitrogen (Evans and Halliwell, 1999; Parisi et al., 2020). Enzyme-based antioxidants are scavenging molecules stable enough to donate an electron to ROS, thus reducing its capacity to damage. Corals possess superoxide dismutase (SOD), peroxidase (PXD), and catalase (CAT) and can express these antioxidants to spatially regulate oxidative cytotoxicity (Csaszar et al., 2009;

Krueger et al., 2015; Traylor-Knowles and Connelly, 2017). Fluorescent proteins (FPs) and non-fluorescent chromoproteins have also been shown to scavenge ROS, suggesting a more important role in mitigating cellular stress (Dove et al., 2006; Palmer et al., 2009). Antimicrobial peptides (AMPs) may also be critical for directed microbial killing in corals; however, the only AMP that has been characterized to date is damicornin from *Pocillopora damicoris* (Vidal-Dupiol et al., 2011).

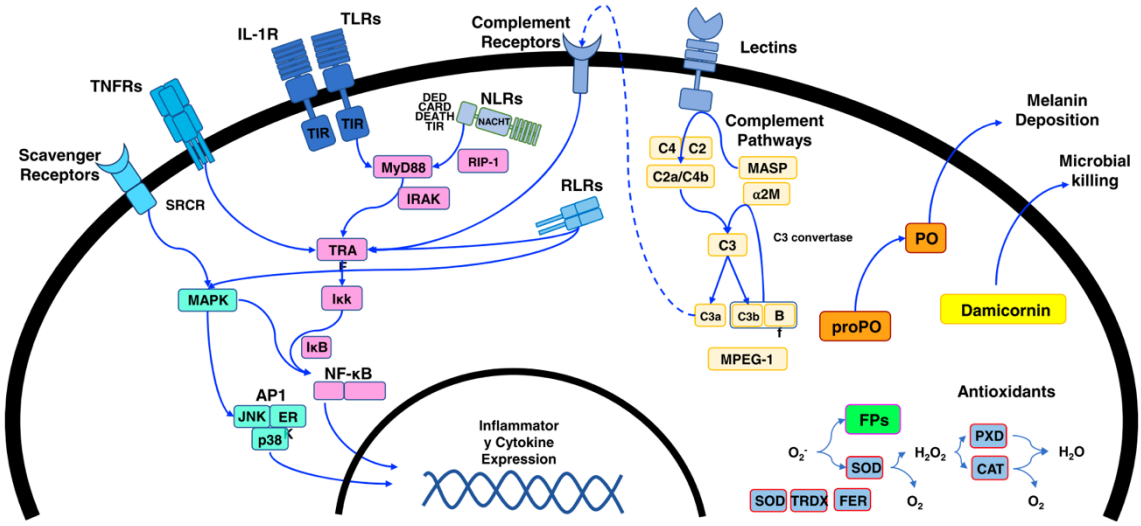

Figure 1 *The diversity of pattern recognition receptors (PRRs), intracellular signalling pathways, and effector mechanisms that exist within the coral innate immune system (from Traylor-Knowles and Connelly, 2017).*

Despite the fact that anthropogenic stressors are clearly understood to disrupt coral health, much remains to be discovered about the cellular and molecular immune mechanisms underlying the responses, with the type, magnitude and timing variation of stressors further complicating our understanding of their effects. To be effective, the complexities of coral holobiont health must be fully explored and embraced. This will require understanding the role of immunity in the maintenance and deterioration of homeostasis, including mutualisms, under the challenges of environmental changes.

Effect of thermal stress on coral immunity

Global sea-warming is the major component of marine climate change that affects corals worldwide. Many heat stress studies have utilized genomic techniques and have discovered a number of previously unknown immune genes involved in coral responses (e.g., DeSalvo

et al., 2008; Shinzato et al., 2011; Palumbi et al., 2014). Other research has instead focused on the symbiotic relationship between corals and their algal symbionts, and have specifically targeted candidate immune genes that aid in the recognition of potential symbionts or which are involved in the disruption of symbiosis (e.g., Vidal-Dupiol et al., 2009; Poole et al., 2016). For example, in *Pocillopora damicornis*, following exposure to heat stress, a mannose-binding c-type lectin was found to be downregulated under warmer conditions, suggesting it may have a role in maintaining a healthy symbiotic state and that heat stress disrupts this relationship (Vidal-Dupiol et al., 2009). This pattern of downregulation has been found in other heat-stressed corals, including *Acropora hyacinthus* adults and *Acropora millepora* larvae (Rodriguez-Lanetty et al., 2009; Barshis et al., 2013), implying that lectins are critical components in maintaining healthy symbiotic relationships for many different coral species. Another key response factor to heat stress conditions are TNFRs. Coral colonies exposed to a fluctuating temperature treatment for 72 h were observed to have a single upregulated TNFR, with a threefold increase in response to heat stress (Barshis et al., 2013). In a field experiment, several TNFRs had increased transcript numbers in cross-transplanted corals originating from a warmer and more variable environment (Palumbi et al., 2014). Under thermal stress, the NOD-like receptor family CARD domain containing 3 (NLRC3) has been shown to suppress the immune system of *Acropora aculeus* via the negative regulation of the NFkB signalling pathway, which the authors suggest causes bleaching (Zhou et al., 2017). Evidence from transcriptomic studies has also highlighted that the complement system plays a key role during heat stress responses (van de Water et al., 2015). In a bleaching experiment on the tropical species *Orbicella faveolata*, mannose-binding lectin associated serine protease-1 (MASP1) was downregulated in the bleached specimens (Pinzon et al., 2015). However, in a different study on *Pocillopora damicornis*, components of the complement system were upregulated in response to heat stress (Vidal-Dupiol et al., 2014).

The importance of certain enzymatic pathways of innate immunity has been shown in coral responses to warming conditions, with many studies focusing on antioxidant responses and the melanin pathway (e.g., Mydlarz et al., 2010; Palmer et al., 2011). Many studies have shown that the main driver of coral bleaching is the production of ROS caused by the deterioration of photosystem II (PSII) in the thylakoid membrane of *Symbiodinium*

chloroplasts (Tchernov et al., 2004; Bieri et al., 2016). At least five SODs and as many CAT homologues have been identified in the transcriptomes of the genus *Acropora*, and have been shown to be upregulated when exposed to thermal stress conditions (Seneca et al., 2010; Louis et al., 2017). Furthermore, ferritin, an iron-binding antioxidant that scavenges active oxygen intermediates, was found to be upregulated in coral responses to heat stress (Csaszar et al., 2009). Another enzymatic mediator studied in the context of coral immune response is the PO cascade (Mydlarz and Palmer, 2011; Palmer et al., 2011; van de Water et al., 2015). This rapidly inducible proteolytic cascade results in the deposition of pigmented cytotoxic melanin along the front of a wound margin, clot, or near the infection areas (Cerenius et al., 2008). In corals, strong PO enzymatic activity has generally been correlated with lower susceptibility to bleaching and disease (Mydlarz and Harvell, 2007; Palmer et al., 2010; Mydlarz and Palmer, 2011; Palmer et al., 2011). PO enzymatic factors are activated in response to thermal stress and bacterial challenge (Palmer et al., 2010), and it appears that different coral species can modify this system for their immune needs (Palmer et al., 2010; Palmer et al., 2011). AMPs have also been studied in thermal-stressed corals (Banin et al., 2001; Vidal-Dupiol et al., 2011; Vidal-Dupiol et al., 2014). These peptides are small protein products (4-15 kDa) of single genes, capable of killing both gram-negative and gram-positive bacteria and are involved in the immunomodulation of the innate immune system (Zasloff, 2002; Krediet, et al., 2013; Destoumieux-Garzon et al., 2016). Damicornin, along with two less-described AMPs (mytimacin and LBP-BPI), were repressed when a vibrio (*Vibrio coralliilyticus*) was in its virulent state activated by higher temperatures (Vidal-Dupiol et al., 2011; Vidal-Dupiol et al., 2014). In contrast, these AMPs were activated in response to the non-virulent state of this vibrio species (Vidal-Dupiol et al., 2011; Vidal-Dupiol et al., 2014). The warmer condition-dependent activation of *V. corallyticus* and subsequent repression of these AMPs is compelling evidence that increasing temperatures will exacerbate the likelihood of immune suppression and pathogen activation. A summary overview of heat stress effects on the coral innate immune system is provided in Figure 2.

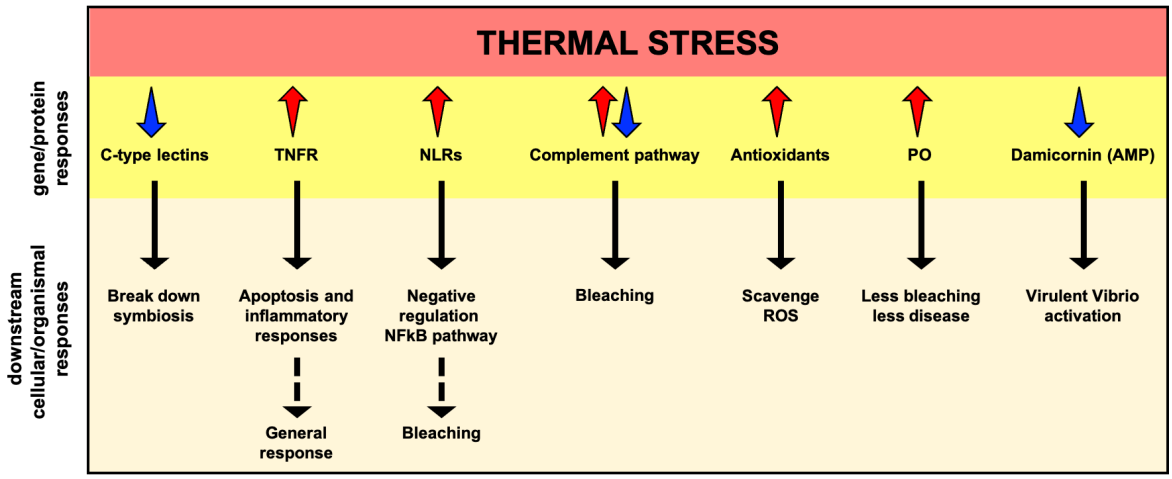

Figure 2 *Summary overview of coral immune system responses to thermal stress. The yellow box represents the gene or protein response, and the brownish box represents the downstream cellular or organismal response already known. Red arrows indicate upregulation and blue arrows represent downregulation (adapted from Traylor-Knowles and Connelly, 2017).*

Coral stress memory

A comprehensive understanding of the response mechanisms to warming seawater must also consider the ability of coral organisms to cope with environmental variation through adaptive processes, in order to more accurately predict the impacts of continuous global change (Pandolfi, 2015). In addition to genetic adaptation (a dynamic evolutionary process that adapts and maintains the phenotypes of organisms in their environment, improving their evolutionary fitness through natural selection) across generations, corals can respond more rapidly to environmental changes through non-genetic mechanisms involving changes in phenotype without changes in the underlying DNA sequences (van Oppen et al., 2015). Priming (or stress memory) is one of these mechanisms, characterized by the temporal separation between a "priming" stimulus and a subsequent "triggering" stimulus that elicits distinct responses compared to naïve specimens (Hilker et al., 2016). In contrast to acclimation (a phenotypic response to a prolonged change in environmental conditions in a laboratory setting) and acclimatization (a coordinated phenotypic response to stressors under natural conditions), which include environmental-dependent phenotypic plasticity (Rivest et al., 2017), stress memory requires the retention of information from the priming cue to modify the response to subsequent stress exposure (Hilker et al., 2016; Hackerott et al., 2021). The process of developing memories of abiotic stressors (i.e., environmental

memory) is a strategy that resists drivers of global climate change without relocation, which is of fundamental importance for long-lived, sessile marine organisms such as corals (Putnam et al., 2017). Indeed, physiological interventions dependent upon environmental memory have been identified as strategies that increase coral resilience worldwide (Hackerott et al., 2021).

The "primed" state represents a set of modified responses to a secondary triggering stimulus, which may be characterized by a faster, stronger, or more sensitive response compared to naïve (un-primed) specimens (Hilker et al., 2016; Lämke and Bäurle, 2017). Such primed responses can be achieved through changes at the transcriptional, translational and post-translational levels, with some of these molecular modifications persisting between priming and activating stimuli (Figure 3) (Crisp et al., 2016; Hackerott et al., 2021). For example, the thermotolerant western lobes of the coral *Goniastrea aspera* showed higher concentrations of antioxidants and heat shock proteins before and during a thermal challenge (Brown et al., 2002). The increase in these biomarkers suggests that coral environmental memory is mediated by preparatory defences sustained between the priming and the triggering stimulus, as well as by a stronger response to secondary stress exposure (Hackerott et al., 2021). However, the molecular mechanisms underlying such enhanced responses are not well understood. In the few studies that have assessed coral gene expression during priming and acclimation, increased thermal tolerance has been associated with dampened transcriptomic responses to thermal stress (Bay and Palumbi, 2015; Ainsworth et al., 2016). In cases of long-term acclimation to variable thermal regimes, such an attenuated response could be due to a constitutive upregulation of genes that respond to stress more quickly following a triggering stimulus (Barshis et al., 2013; Palumbi et al., 2014). Mechanistic explanations for the reduced transcriptional stress responses in pre-exposed corals without transcriptional frontloading are lacking, especially since such a model apparently contradicts the enhanced response expected for primed colonies (Hilker et al., 2016; Lämke and Bäurle, 2017). However, a recent short-term exposure study highlighted improved thermal tolerance correlated with substantial changes in gene expression of stressed colonies, involving the innate immune response, downregulation of DNA replication and protein ubiquination (Drury et al., 2022).

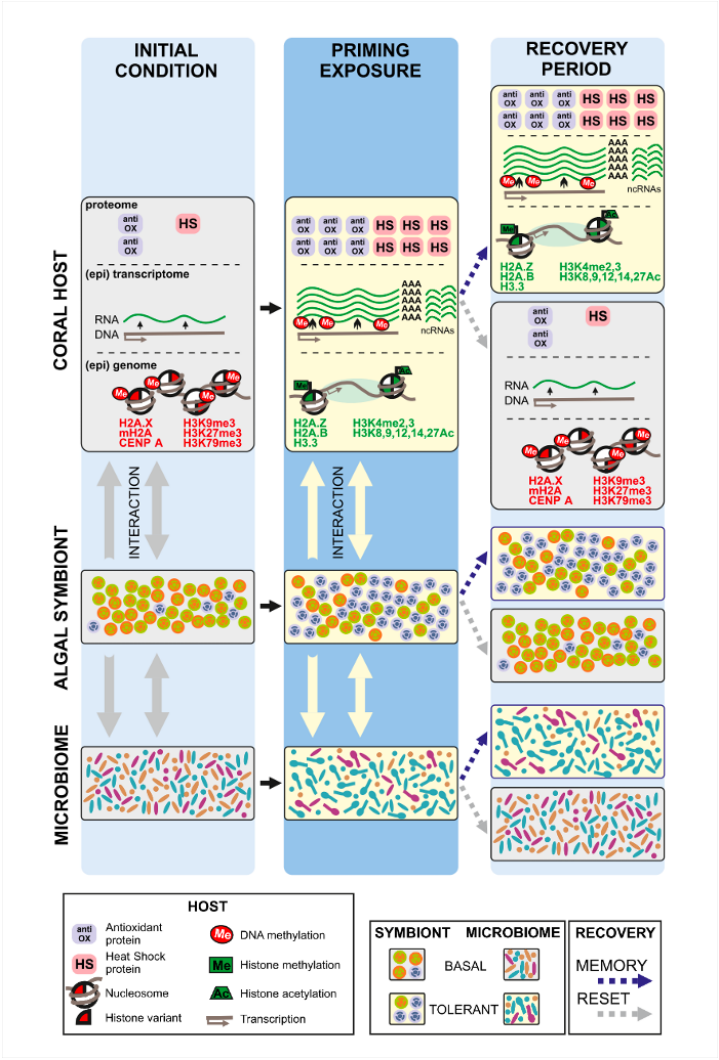

Figure 3 *Possible mechanisms underlying coral environmental memory. An exposure to priming stress can elicit a variety of cellular changes within the host coral, shifts of symbiont communities and/or algal microbiomes towards more tolerant assemblages. With the return to environmental conditions, these changes can be restored (i.e., memory loss) or maintained in the absence of the priming stimulus (i.e., memory) to modify the primed organism's response to a subsequent stimulus. Within corals, cellular responses may include preparatory defences sustained during recovery (e.g., persistence of antioxidants or heat shock proteins, and/or constitutive upregulation of defence-related genes) and/or epigenetic modifications (e.g., post-translational histone modifications or variants, DNA methylation) that facilitate the increased reactivation of stress-responsive genes following subsequent stress exposure (from Hackerott et al., 2017).*

An enhanced transcriptional response to secondary thermal stress exposure may also be mediated by cellular mechanisms that alter the activation kinetics of gene expression (Lämke and Bäurle, 2017; Bäurle and Trindade, 2020). Epigenetic mechanisms (mechanisms capable of regulating gene expression through alternative states of activity in the context of the same DNA sequence) act between environmental conditions and genome functions,

dynamically altering and, in some cases, perpetuating states of gene activities (Cavalli and Heard, 2019). As a carrier of environmental messages to the genome, epigenetic regulation likely plays a key role in the creation and maintenance of environmental memory, within the boundaries established by the organism's genome sequence (Adrian-Kalchhauser et al., 2020). These epigenetic mechanisms include post-translational histone modifications (PTMs) (Lämke et al., 2016; Fabrizio et al., 2019), reduced nucleosome occupancy (Brzezinka et al., 2016), DNA methylation (Wibowo et al., 2016), as well as genetic regulation by small RNAs (Stief et al., 2014). Recently, transcriptional memory response in corals has been associated with epigenetic modifications that influence the accessibility of stress-responsive genes, revealing changes in DNA methylation and histone PTMs (Putnam et al., 2016; Dixon et al., 2018; Liew et al., 2018). In particular, DNA methylation could play a key role in reducing spurious transcription in corals (Liew et al., 2018) and in the anemone *Aiptasia pallida* (Li et al., 2018), which could be critical in regulating more efficient transcriptional responses in primed individuals. Seasonal patterns of DNA methylation have also been reported in *Acropora cervicornis* (Rodríguez-Casariego et al., 2020), suggesting the role of epigenetic modifications in mediating seasonal acclimation in corals (Jurriaans and Hoogenboom, 2020). Overall, current studies support that epigenetic changes are environmentally driven and may be inherited in corals (Liew et al., 2020; Dimond and Roberts, 2020).

Here, the study of several Mediterranean coral species has enabled a better understanding of immune dynamics in a global sea-warming scenario, acquiring new basic knowledge about the physiological mechanisms related to thermal stress-responses in ecologically relevant organisms. Additionally, corals' ability to acquire and maintain enhanced stress tolerance across transient exposures to thermal-stress conditions (i.e., coral priming) was explored, filling in some gaps regarding stress hardening mechanisms that could increase coral resilience. This research provides an attractive cross-cutting path of exploration from immunology to ecology, which aims to better estimate the trajectories of marine organisms threatened by global climate change, as well as favour future applications for designing new conservation and restoration approaches.

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Chapter 1

Global warming-related response after bacterial challenge in *Astroides calycularis*, a Mediterranean thermophilic coral

Abstract

A worldwide increase in the prevalence of coral diseases and mortality has been linked to ocean warming due to changes in coral-associated bacterial communities, pathogen virulence, and immune system function. In the Mediterranean basin, the worrying upward temperature trend has already caused recurrent mass mortality events in recent decades. To evaluate how elevated seawater temperatures affect the immune response of a thermophilic coral species, colonies of *Astroides calycularis* were exposed to environmental (23°C) or elevated (28°C) temperatures, and subsequently challenged with bacterial lipopolysaccharides (LPS). Using immunolabeling with specific antibodies, we detected the production of Toll-like receptor 4 (TLR4) and nuclear factor kappa B (NF-kB), molecules involved in coral immune responses, and heat shock protein 70 (HSP70) activity, involved in general responses to thermal stress. A histological approach allowed us to characterize the tissue sites of activation (epithelium and/or gastroderm) under different experimental conditions. The activity patterns of the examined markers after 6 hours of LPS stimulation revealed an up-modulation at environmental temperature. Under warmer conditions plus LPS-challenge, TLR4-NF-kB activation was almost completely suppressed, while constituent elevated values were recorded under thermal stress only. An HSP70 up-regulation appeared in both treatments at elevated temperature, with a significantly higher activation in LPS-challenge colonies. Such an approach is useful for further understanding the molecular pathogen-defense mechanisms in corals in order to disentangle the complex interactive effects on the health of these ecologically relevant organisms related to global climate change.

Keywords: thermal stress, Mediterranean Sea, innate immunity, LPS, temperate coral

Introduction

Direct and indirect impacts of global warming are proving detrimental effects to the health of marine species and are the primary cause of coral death worldwide (e.g., van de Water et al., 2016; Hughes et al., 2018; Beavers et al., 2023), as well as affecting the Mediterranean (Garrabou et al., 2019; Garrabou et al., 2022). The coral holobiont is an obligate association between the coral animal and a plethora of mutualists (i.e., an appropriate commensal microbiota), including dinoflagellate endosymbionts of zooxanthellate species (Palmer et al., 2018); however, this association is delicately balanced, and coral species worldwide are increasingly threatened by warming seawater and temperature-driven disease outbreaks (Harvell et al., 2002; Hughes et al., 2003; Ward and Lafferty, 2004; Beavers et al., 2023). Indeed, the risk of climate anomalies in the Mediterranean basin has increased sharply over the last few decades, with warming temperatures exceeding the range of normal fluctuations historically experienced by marine organisms (Lejeusne et al., 2010; Darmaraki et al., 2019; Garrabou et al., 2022). This trend has boosted well-documented disease outbreaks and, consequently, mortality events that have affected several anthozoan species across varying geographic extents (Bally and Garrabou 2007; Vezzulli et al., 2010; Rubio-Portillo et al., 2016; Garrabou et al., 2019). Despite recent advances, much remains to be understood about the molecular processes that underpin coral immune responses to pathogens. Consequently, it is crucial to determine the immune mechanisms involved and how they may be influenced by environmental signals (e.g., temperature).

From an anatomical point of view, corals have a simple body structure consisting of only two true layers of tissue, and no organs. Depending on the stage of development - developing polyp or adult coral - these tissues are called ectoderm/epithelium and endoderm/gastroderm, respectively, interlined by the mesoglea (an amorphous and practically acellular extracellular matrix) (Kvennefors et al., 2010; Parisi et al., 2020). In zooxanthellate species, *Symbiodinium* spp. (coral-dinoflagellate symbiont) reside mainly in the gastrodermal areas between the gut and the external epithelium (barrier to the surrounding environment) in the oral end, and more rarely in the gastrodermal areas between the intestine and the skeleton of the organism. Instead, nematocysts are present only in the epithelium of the oral end, but not in the calicoblastic epithelium (Parisi et al., 2020). The majority of host corals have only

species-specific associations with specific bacterial phylotypes (including *Symbiodinium* spp.) which populate the different habitats of coral anatomical compartments, such as the surface mucus, tissues, and skeleton (Apprill et al., 2016; Biagi et al., 2020). This suggests the presence of a well-developed self/non-self-recognition system in which appropriate strains multiply by establishing a stable symbiosis, while unsuitable strains are actively removed through cellular processes (Weis et al., 2008; Dunn and Weis, 2009; Palmer et al., 2018).

Corals and other cnidarians contain a surprisingly high degree of genetic complexity (Kortschak et al., 2003; Technau et al., 2005; Miller et al., 2007; Putnam et al., 2007), with homologs of many proteins involved in vertebrate immunity being described for these organisms in the last two decades (e.g., Miller et al., 2007; Traylor-Knowles and Connelly 2017; Williams et al., 2018; Parisi et al. 2020). The primary function of the anthozoan innate immune system is to recognize specific patterns of non-self-entities (Janeway and Medzhitov, 2002; Medzhitov and Janeway, 2002). These patterns are called pathogen-associated molecular patterns (PAMPs), or microbe-associated molecular patterns (MAMPs), such as lipopolysaccharide (LPS), peptidoglycan, and mannan components of the microbial cell wall. These and other PAMPs are recognized by proteins called pattern recognition receptors (PRRs), which are molecules that include both membrane-bound proteins, such as Toll-like receptors (TLRs), and soluble proteins, such as lectins (Palmer and Traylor- Knowles, 2012; van de Water et al., 2016; Traylor-Knowles and Connelly 2017; Parisi et al. 2020). Corals possess TLRs and nucleotide oligomerization domain (NOD)-like receptors (NLRs) with intracellular Toll/interleukin-1 receptor (TIR) domains which can interact with genetic homologs of myeloid differentiation primary response protein 88 (MyD88), IL-1R-associated kinase (IRAK), receptor-associated factor 6 of TNF (TRAF), and IkB kinase (Ikk). These in turn can cleave nuclear factor kappa B (NF-kB) inhibitors and allow NF-kB protein dimers to translocate into the nucleus, enhancing the expression of inflammatory cytokines, the production of antimicrobial peptides (AMPs), cell survival, and apoptosis (Palmer and Traylor- Knowles, 2012; Traylor-Knowles and Connelly, 2017; van de Water et al., 2018; Parisi et al., 2020). For example, the innate immune pathway from TLR to NF-kB in the tropical species *Orbicella faveolata* was recently characterized. Compared to human TLRs, the intracellular TIR domain is very

similar to TLR4, and treatment of *O. faveolata* tissue with lipopolysaccharides (LPS; a common ligand for mammalian TLR4) led to changes in gene expression consistent with the mobilization of the NF-kB pathway (Williams et al., 2018).

One group of proteins used as ubiquitous and putative markers of temperature-induced cellular stress in corals are the heat shock proteins (HSPs) (Downs et al., 2000; Chow et al., 2012; Olsen et al., 2013; Louis et al., 2017; Seveso et al., 2020). HSPs are differentiated by molecular weight into several major chaperone families (HSP40, HSP60, HSP70, HSP90, HSP100, and the small HSPs), with specific intracellular localization and function (Pockley 2003). As molecular chaperones, they support protein homeostasis by facilitating proper protein folding and translocation, and by aiding in the folding or degradation of proteins damaged by heat or other environmental stresses (Bozaykut et al., 2014; Balchin et al., 2016). The involvement of these proteins in the immune system has been widely reported in both vertebrates and invertebrates, increasing after exposure to biotic challenges such us during the development of infections and/or diseases (e.g., Prohaszka et al., 2002; Sung et al., 2008). For example, human HSP70 activates the TIR receptor signaling pathway during a highly inflammatory response (Prohaszka et al., 2002; Sung et al., 2008). In corals, the increased expression of HSP70 in tropical species colonies showing signs of disease indicates their potential involvement in the immune and stress responses to pathogenic challenges (Seveso et al., 2012; Brown et al., 2013).

The present study, using a manipulative aquarium-based experiment, aims to assess the impacts of warmer conditions on the activity of TLR4, NF-kB, and HSP70 markers under bacterial LPS challenge in an azooxanthellate coral species, *Astroides calycularis* (Pallas, 1766). This species is commonly found in the central-southern part of the Mediterranean Sea, covering vertical rocky reefs, overhangs, and caves below the intertidal fringe (Ingrosso et al., 2018). *A. calycularis* occupies both well-lit and sciaphilous habitats and is considered a tolerant thermophilic species, thriving at relatively high temperatures (Ingrosso et al., 2018). However, recent field observations indicate that this orange coral is impacted by seawater temperatures reaching and exceeding 28°C (Gambi et al., 2018; Bisanti et al., 2022). In this experiment, the use of bacterial LPS allowed us to avoid the well-documented difficulties of infecting coral with live strains (Lesser et al., 2007), while testing the

induction/suppression of immune pathways by PRRs (Kvennefors et al., 2010; Liu et al., 2011; Palmer et al., 2011).

Materials and methods

Coral collection and experimental design

As described in Figure 1, 54 comparably sized (3.5 ± 1.0 cm colony diameter) and visibly healthy adult colonies of *A. calycularis* were sampled in October 2022 from the upper littoral zone (~ 5 m depth) of Capo Zafferano Bay (38° 06′ 25″ N; 13° 32′ 10″ E; NW coast of Sicily, Italy). Each colony was carefully removed with the aid of a hammer and chisel and transported in a 1 µm filtered seawater tank to the laboratory. At the Marine Immunobiology Laboratory (Department of Earth and Marine Sciences, University of Palermo), corals were equally and randomly assigned among four large aquaria supplied with continuous flow-filtered seawater (1µm) at environmental temperature during the sampling periods (daily averages temperature ranged from 22.90 ± 0.17°C to 23.54 ± 0.01°C). Aquaria conditions were maintained with a 15:9 h photoperiod of daily light/dark cycles to match the natural photoperiod at the collection sites (metal halide lights, with levels maintained at 150-250 µmol $m^{-2}s\text{-}^{1}$), and in dimmed light conditions to mimic their natural sciaphilous environment (the tanks were under a canopy of 70% light-reducing shade-cloth). During the 20 days acclimatization period, the colonies were fed twice a week with a commercial preparation of plankton (Elos Coral Foods SvC) prior to examination (Franzellitti et al., 2018). After the acclimatization period, 3 randomly selected samples were fixed for the histological experiments (detailed in the histological analysis section) or snap frozen and stored at -30°C for tissue extraction (detailed in the Western blotting section) as pre-treatments controls. Two aquaria were then randomly selected for the elevated temperature treatment, in which temperature was increased by 1.0 to 1.5°C per day for 3 days and stabilized at 28°C (daily averages temperature ranged from 28.00 ± 0.17°C to 28.41 ± 0.19°C).

Two small plastic tanks, holding ~ 5 l seawater and equipped with individual air-stones, were submerged within each of the four large aquaria, for a total of 8 small tanks. Three colonies were placed in each small tank (± 5 cm away from each other), and thus did not come into contact with each other. These tanks were randomly designated for one of four treatments: (1) environmental temperature (~ 23°C) and control (no-LPS); (2) environmental temperature with 5 µg ml^{-1} LPS, lyophilized from Escherichia coli (ATCC 25922 strain; Chrisope Technologies, Louisiana, USA) and dissolved in sterile filtered seawater (Palmer

et al., 2011); (3) elevated temperature (~ 28°C) and control (no-LPS); (4) elevated temperature with 5 µg ml^{-1} LPS. Corals were exposed to a constant flow (from the larger aquaria) of 20 µm-filtered seawater at either environmental or elevated temperature for 2 days prior to LPS treatment. Over the LPS exposure, the system was closed by stopping the water circulation in the small tanks (raising the rim slightly above the waterline of the larger aquaria), and the seawater was carefully exchanged with relative-temperature 20 µm sterile filtered seawater with LPS (5 µg ml^{-1}) or without LPS in the designated tanks. Through this method, the seawater within each small tank remained isolated, while temperatures continued to be maintained at the same values as in the large aquaria (i.e., ~ 23°C or ~ 28°C). The LPS treatment occurred over a period of 6 hours, keeping the water moving via air stones in each small tank which flowed vigorously during the entire exposure. Once the period of LPS exposure was over, colonies were sampled from all treatment groups and immediately fixed or stored at -30°C for histological experiments and tissue extraction, respectively. For the whole duration of the experiment, no mortality occurred, and all coral branches appeared healthy and without any visible lesions.

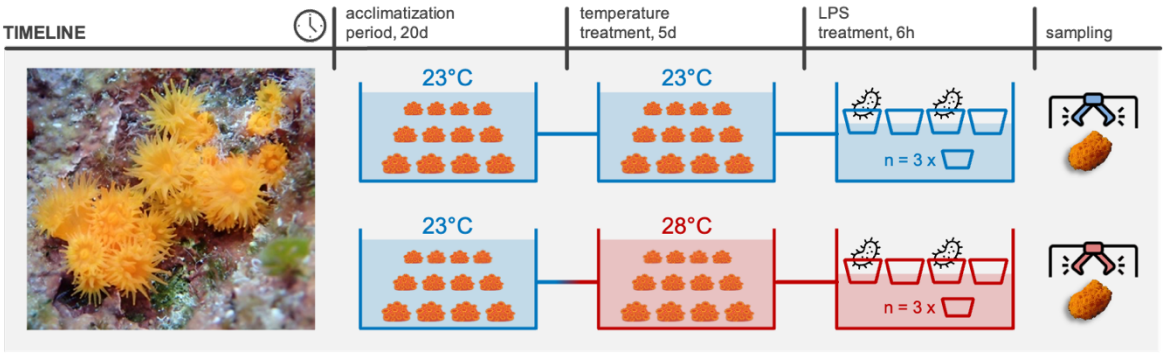

Figure 1 *Schematic drawing summarizing the experimental timeline. Experimental treatments were carried out in duplicate. Number of biological replicates, n*

Western blotting

Western blot analysis was performed to determine the antibody specificity against selected target proteins in samples of *A. calycularis* (Dara et al., 2021; Parisi et al., 2022; La Corte et al., 2023). Compatibility with the antibody-epitopes used was verified on the NCBI (National Center for Biotechnology Information) and UniProt databases, based on similarity between the protein sequences present in species phylogenetically close to the orange coral

and those used for antibody cultivation (human TLR4, NF-kB, HSP70) (Fig. S4; Table S1). Tissues were removed from frozen samples and subsequently homogenized into polycarbonate tubes with 500 µl TBS-buffer (NaCl 150 mM, Tris-HCl 10 mM, pH 7.4) containing a complete EDTA-free cocktail of protease inhibitors (12352204, Sigma-Aldrich) on ice; the resultant slurry was then centrifuged (36,200 x g for 20 min at 4°C). The supernatant was collected and sample absorbance was read at 595 nm (RAYTO RT-2100C) with TBS as blank, and a calibration curve defined through bovine serum albumin was used to obtain the protein concentration (Bradford, 1976). Coral extracts were adjusted to 0.5 mg/ml prior to the experiments. SDS-PAGE was carried out using a 4% (stacking) and a 10% (separating) polyacrylamide gel for 50 min at 190 V using a Bio-Rad mini gel kit (Laemmli, 1970). Coral extracts were run in parallel lines, following antibody suppliers' recommendations. Proteins separated by SDS-PAGE were electroblotted onto a nitrocellulose membrane. The gels were prepared in blotting buffer (20 mM Tris-HCl, 192 mM glycine, 20% methanol, pH 8.8), and a semi-dry blotting bath (Bio-Rad Laboratories) was used (0.8 mA cm^2 for 75 min). The filter membrane was soaked in blocking buffer (TBS containing 3% BSA and 1% Tween-20) and incubated overnight with the following appropriate primary antibodies diluted in blocking solution (TBS containing 0.1% BSA and 1% Tween-20): polyclonal anti-TLR4 produced in rabbit (SAB5700684, Sigma-Aldrich) (1:1000); polyclonal anti-NF-kB produced in rabbit (SAB4501989, Sigma-Aldrich) (1:1000); monoclonal, anti-HSP70 produced in mouse (SAB4200714, Sigma-Aldrich) (1:1000). Membranes were then washed with blocking buffer and incubated with the appropriate secondary antibody: goat anti-rabbit IgG-alkaline phosphatase (A3812, Sigma-Aldrich) or goat anti-mouse IgG-alkaline phosphatase (A3562, Sigma-Aldrich) (1:15000 in washing buffer, 0.1% BSA) for 1 hour. Antibody binding was detected by chromogen substrate BCIP/NBT (Sigma-Aldrich).

Histological analysis

For histological assessment, colonies were fixed in 4% paraformaldehyde suspended in PBS-buffer solution (NaCl 137mM, KH_2HPO_4 10mM, KH_2HPO_4 2mM, KCl 2.7 mM, pH 7.6) at 4°C. After decalcification in 10% EDTA and dehydration in ethanol, polyps were embedded in paraffin (Bio-Optica, Italy). Histological sections (7 µm thick) were cut with a

rotary automatic microtome (Leica Microsystems HM350S, Wetzlar, Germany), stained with Hematoxylin/Eosin (H/E) and Gomori trichrome to evaluate the morphological features of the samples. Slides were analyzed with a light microscope (Leica DM750, Wetzlar, Germany), and images were obtained using an ORMA-Eurotek MDH5 scientific camera (Milan, Italy).

Immunohistochemistry

For the immunohistochemistry assays, dewaxed sections were incubated in a blocking buffer (PBS containing 5% BSA and 1% Tween-20) for 2 h at room temperature, and then with the same primary antibodies indicated above diluted in blocking solution (PBS containing 1% BSA and 1% Tween-20; dilutions recommended by the manufacturer: anti-TLR4 1:200; anti-NF-kB 1:200; anti-HSP70 1:200) overnight at 4°C. All slides were washed before incubation with the secondary antibodies for 90 min at room temperature. The secondary antibodies (the same indicated above) were diluted 1:50 in PBS containing 1% BSA and 1% Tween-20 and incubated for 90 min. The sections were rinsed with the washing buffer (PBS containing 1% Tween-20) and stained with the BCIP/NBT chromogen substrate (Sigma-Aldrich). In all control experiments, primary antibodies were omitted, and sections were incubated only with the secondary antibodies. Slides were analyzed by a light microscope (Leica DM750), and images were obtained using an ORMA-Eurotek MDH5 scientific camera (Milan, Italy).

Statistical analyses

The protein marker expression results from the Western blotting were analyzed through densitometric analysis, using the open-source software Image J (Schneider et al., 2012). Furthermore, the quantification of the immune-positive stained areas (percentage of stained cells) on 6 randomly-chosen fields (45000 μm^2) for each slide, considering all tissue districts of corals (i.e., from oral to aboral ends), were also carried out using Image J software (Schneider et al., 2012). One-way ANOVAs were conducted in order to test differences among experimental groups. When significant differences were found, multiple comparisons using the Tukey post-hoc test were done to highlight differences between treatments. Statistical analyses were carried out using the GraphPad software (Prism 8.0, San Diego CA,

USA). All experiments were performed in triplicate, and the values used were the mean ± standard deviation (SD) resulting from three independent experiments. Differences were considered significant for $p < 0.05$.

Results

Western blot analysis and cross reactivity validation

Western blot assays were performed on protein extracts of *A. calycularis* confirming the presence in sampled tissues of TLR4, NF-kB, and HSP70-immunoreactive bands. The analysis carried out with the anti-TLR4, anti-NF-kB, and anti-HSP70 antibodies revealed bands of approximatively 75, 100, and 70 kDa, respectively, corresponding to the expected molecular weights of these proteins (Fig. S1, S2, S3). The expression of TLR4, NF-kB, and HSP70 was investigated among the different experimental treatments (Fig. 2). In detail, the activity of TLR4 differed significantly among treatments (Fig. 2A; Table 1). At environmental seawater temperature, higher densitometry evaluation of the bands by integrated density values (IDV) were found for the LPS-challenge group of corals compared to unchallenged specimens. However, *A. calycularis* demonstrated a significant change in expression driven by warmer temperature alone, with an increase in IDV comparable to the LPS-challenge group at environmental temperature, while, a significant inhibition was recorded for animals under LPS-challenge and elevated temperature. A similar trend was shown for the NF-kB activity, with significantly higher IDV values for colonies which were LPS-challenged at environmental temperature and LPS-unchallenged at warmer seawater conditions, compared to the other treatments (Fig. 2B; Table 1). Significant differences were found for HSP70 activity (Fig. 2C; Table 1). Under controlled seawater temperature (23°C), a significant up-regulation was recorded for LPS-challenged specimens compared to control colonies. For the elevated temperature condition, a significant and positive modulation in LPS-unchallenged samples was found compared to environmental controls, though it was lower than the specimens exposed to LPS; effectively, the LPS-challenged corals showed significantly higher IDV values compared to controls (both under environmental and elevated temperature conditions), as well as to the LPS-challenged specimens under environmental conditions.

Table 1 *Summary of the one-way ANOVAs carried out among experimental treatments on integrated density values of the investigated markers (TLR4, NF-kB, and HSP70) in the orange coral* A. calycularis.

One-way ANOVA	F	P value	R square
TLR4	66.71	< 0.0001	0.92
NF-kB	57.73	< 0.0001	0.91
HSP70	73.07	< 0.0001	0.93

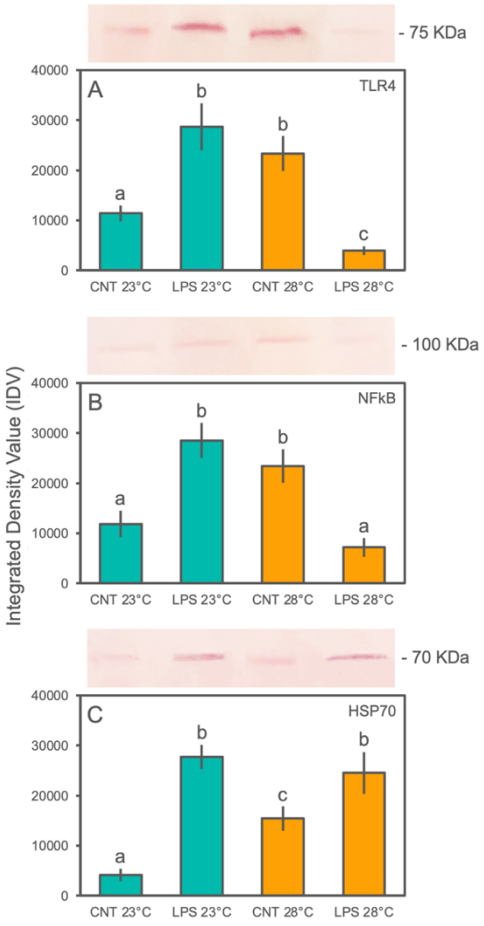

Figure 2 *Western blot representative assay carried out on* A. calycularis *tissue extracts from the experimental treatments. Each nitrocellulose sheet was treated with anti-TLR4, anti-NF-kB, and anti-HSP70, the anti-rabbit IgG-alkaline phosphatase or anti-mouse IgG-alkaline phosphatase secondary antibody, and developed with the BCIP/NBT liquid substrate system (see Supplementary material for full-length blotting images). Plot indicates the relative Integrated*

Density Value (IDV; mean values ± SD) calculated on bands of the Western blot assays. The letters indicate statistically significant differences ($p < 0.05$) between experimental groups.

Histological analysis and immunohistochemistry

Generally, tissues of *A. calycularis* were characterized by an epithelium formed by a large cellular monolayer arranged obliquely with the basal part lying on the mesoglea (Fig. 3A, B), populated by nematocysts even in the innermost portion at the oral end (Fig. 3E, F). No nematocysts were present in the calicoblastic epithelium toward the aboral end. The mesoglea was clearly visible between the epithelium and the gastroderm, consisting of an amorphous fibrous matrix (Fig. 3A, B). Instead, the underlying gastrodermal area of the orange coral was made up of a single, thinner layer, but characterized by smaller cells densely packed together (Fig. 3C, D).

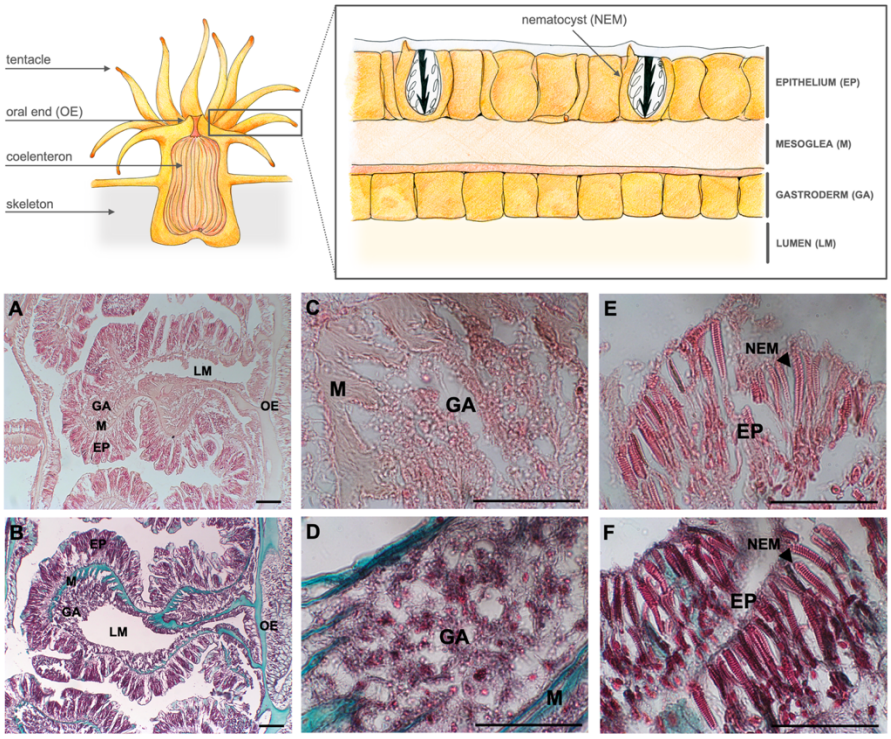

Figure 3 *Schematic anatomical representation and histological sections of adult* A. calycularis *corals showing the different tissues of the gastroderm (GA), mesoglea (M), epithelia (EP) and the specific structures of nematocysts (NEM) at the oral end. Coral sections are shown after staining with: (A - C - E) hematoxylin/eosin and (B - D - F) Gomori trichrome, to act as a reference for structural detail. Scale bar 50 µm*

The immunohistochemistry assays performed on sections of LPS-challenged corals showed a strong signal for TLR4, NF-kB, and HSP70 at environmental temperature, compared to control slides, in both gastrodermal cells as well as in the nematocysts of the epithelium at the oral end (Fig. 4, 5, 6A - F). Immunostaining was also observed in some compartments of the gastrodermal lining of the gut and skeleton (photos not shown). No staining was observed in pre-immune serum controls for either of the primary antibodies or when the primary antibody was omitted. The quantification of the percentage of the immune-positive areas indicated significant differences in the immunostaining for the three markers considered, with higher values for the LPS-challenge treatment compared to control (Fig. 7; Table 2). By contrast, under warmer seawater conditions, the amount of TLR4 and NF-kB immunostaining signals of antibody recognition unveiled that they were constitutively over-expressed in LPS-unchallenged coral tissues (Fig. 4, 5G - L). Staining patterns were similar to the slides of samples exposed to LPS at 23°C, with an extended signal associated with nematocysts in the coral epithelium and gastrodermal cells (Fig. 4, 5G - I). Low immune-positivity was found in tissues of LPS-challenged polyps for both markers (TLR4 and NF-kB) (Fig. 5, 5J - L). Also in this case, the analysis of the percentage of immune-positive areas showed statistically significant differences between experimental treatments (Fig. 7A, B; Table 2).

Table 2 *Summary of the one-way ANOVAs carried out among experimental treatments on immune-positive stained areas (percentage values) of the investigated markers (TLR4, NF-kB, and HSP70) in the orange coral* A. calycularis.

One-way ANOVA	F	P value	R square
TLR4	86.15	< 0.0001	0.94
NF-kB	51.05	< 0.0001	0.90
HSP70	36.82	< 0.0001	0.87

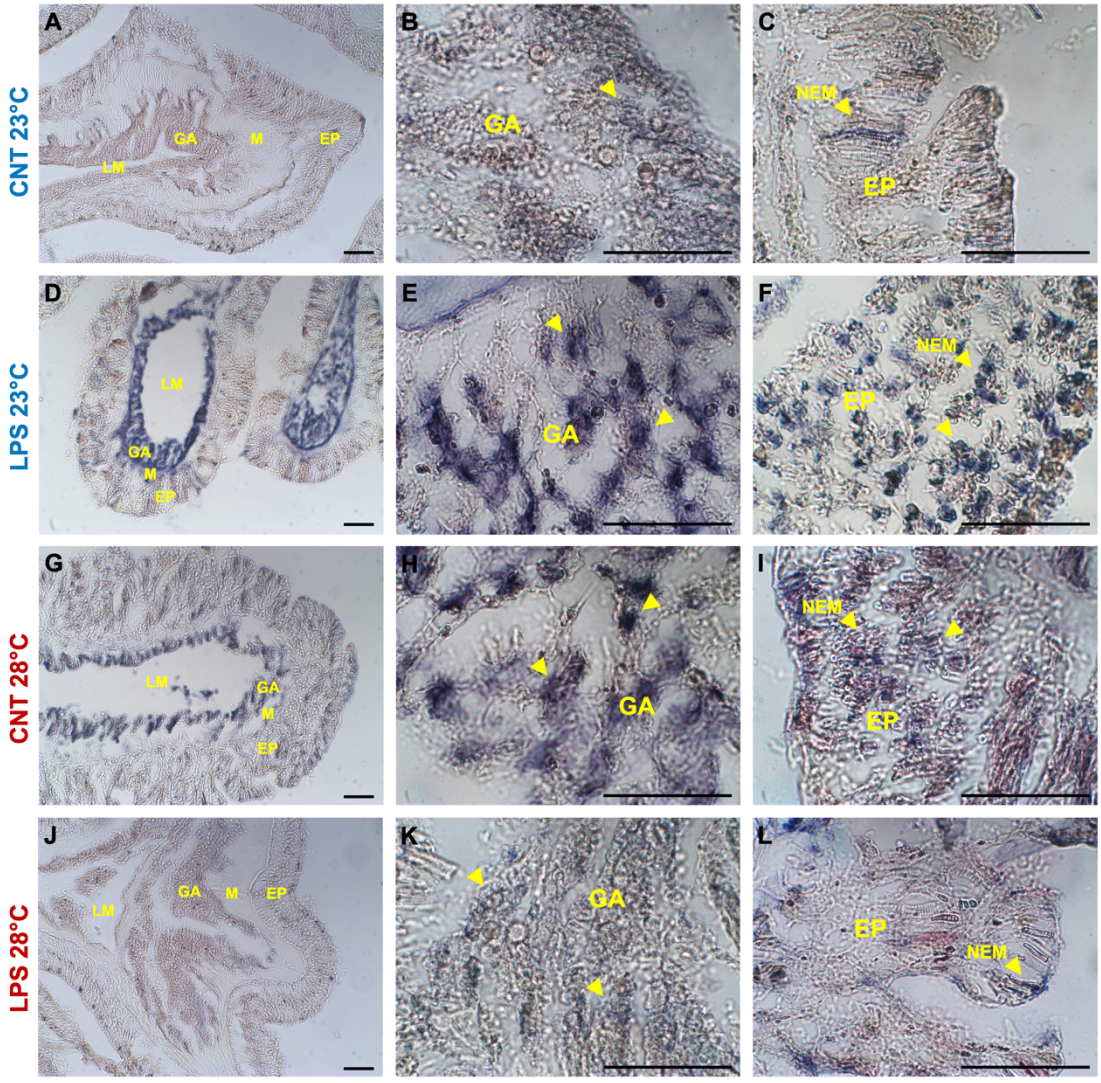

Figure 4 *Immunohistochemical detection of TLR4 in oral end tissues of adult* A. calycularis *corals for the experimental treatments, with the primary antisera anti-TLR4. Yellow arrows indicate immunostaining in tissues or specific structures. Gastroderm (GA); mesoglea (M), epithelia (EP); nematocysts (NEM). Scale bar 50 µm*

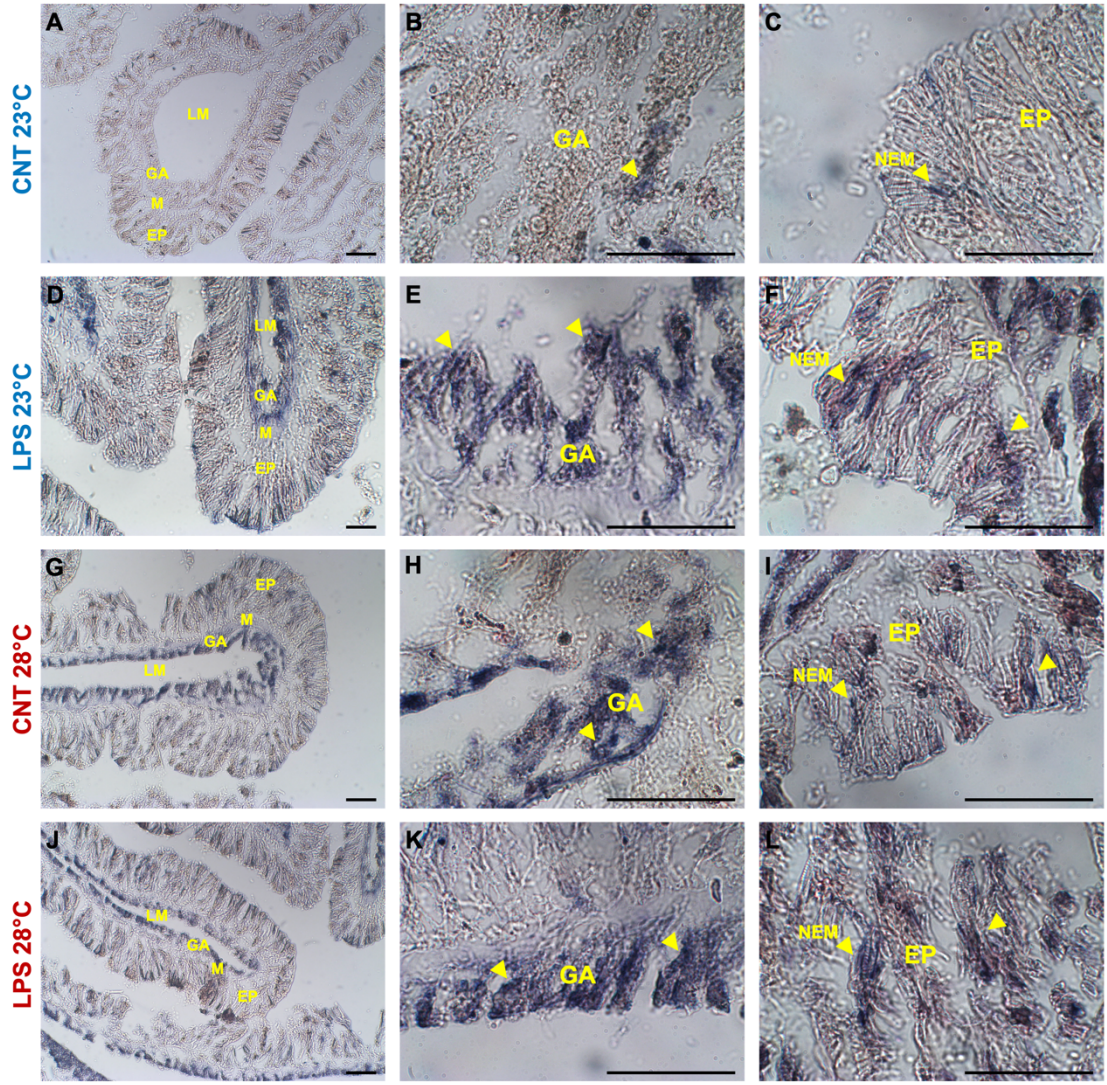

Figure 5 *Immunohistochemical detection of NF-kB in oral end tissues of adult* A. calycularis *corals for the experimental treatments, with the primary antisera anti-NF-kB. Yellow arrows indicate immunostaining in tissues or specific structures. Gastroderm (GA); mesoglea (M), epithelia (EP); nematocysts (NEM). Scale bar 50 µm*

On the other hand, at elevated temperature, the HSP70 marker showed a different staining pattern in the orange coral tissues. A clear immuno-positive signal for both treatments (control and LPS-challenged) was detected (Fig. 6G - L), although the analysis of the percentage areas of stain-cells showed significantly higher values for the samples exposed to LPS (Fig. 7C; Table 2).

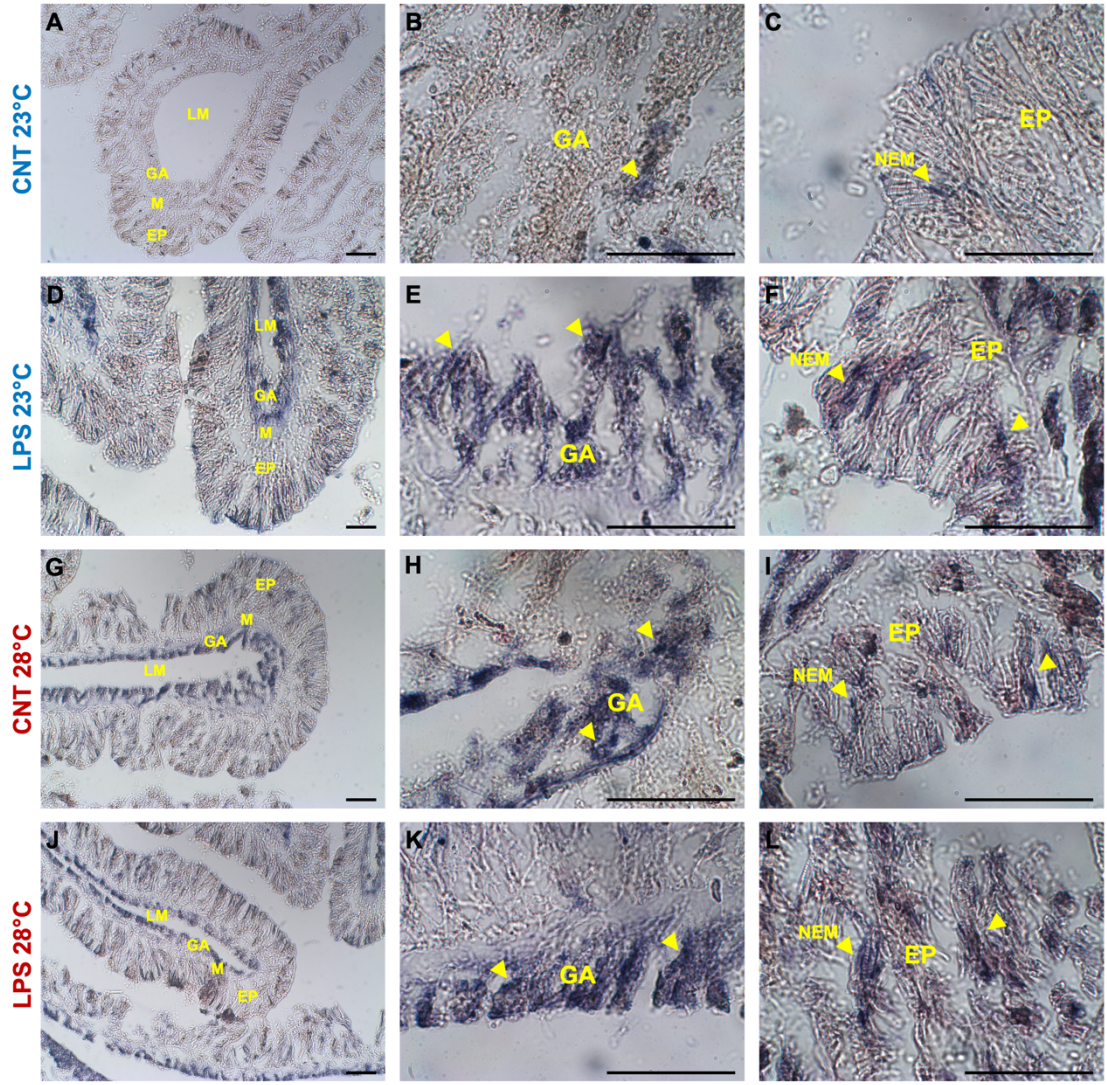

Figure 6 *Immunohistochemical detection of HSP70 in oral end tissues of adult* A. calycularis *corals for the experimental treatments, with the primary antisera anti-HSP70. Yellow arrows indicate immunostaining in tissues or specific structures. Gastroderm (GA); mesoglea (M), epithelia (EP); nematocysts (NEM). Scale bar 50 µm*

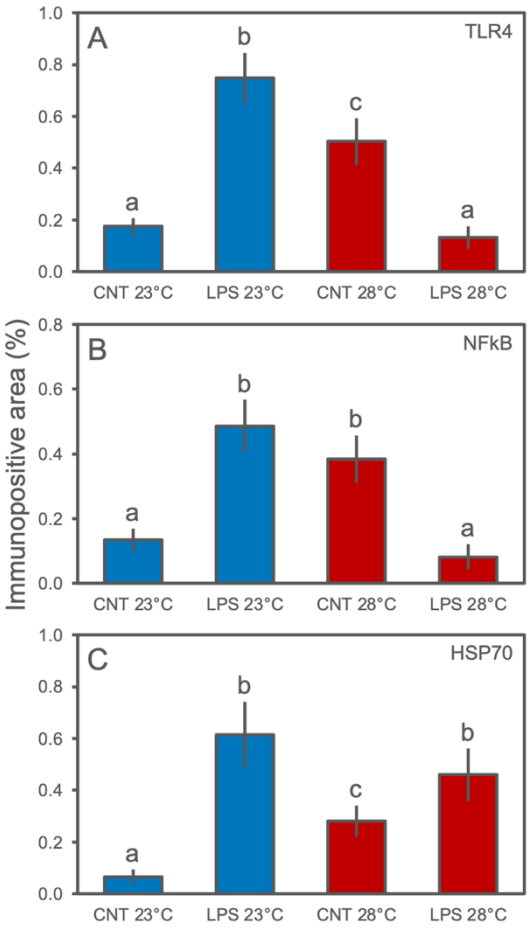

Figure 7 *Quantification of the immune-positive stained areas (percentage of stained cells; mean values ± SD) in* A. calycularis *tissues from slides of the experimental treatments. The analysis was carried out considering all tissue districts of the orange corals (i.e., from oral to aboral ends). The letters indicate statistically significant differences ($p < 0.05$) between experimental groups.*

Discussion

The present study investigated the effects of warmer seawater conditions and LPS-challenge on the expression of selected immunological (TLR4 and NF-kB) and stress (HSP70) markers in the Mediterranean scleractinian species *A. calycularis*. The immune activities of the orange coral 6 hours post LPS-challenge were significantly affected by elevated temperatures. Both these types of abiotic and biotic factors are recognized as being among the most important contributors to the worldwide decline of coral habitats and mass mortality events (Harvell et al., 2002; Gardner et al., 2003; Hughes et al., 2003; Wilkinson, 2004; Garrabou et al., 2019; Garrabou et al., 2022). Indeed, in recent decades, efforts to understand the cellular processes involved in the immunological and physiological responses to environmental stresses that cause mortality have increased. This is significant, especially considering that these animals cannot move to new optimal environmental conditions and that the earliest steps of the organism's response occur at the cellular level (Kültz, 2005; Kenkel et al., 2014).

To date, the regulation of the TLR-NF-kB pathway performed in corals under bacterial challenge has confirmed that these organisms possess temporally dynamic and responsive cellular machinery to counteract stresses (Fuess et al., 2016; Fuess et al., 2017; Williams et al., 2018; Connelly et al., 2020). Consistently with this, the results of our experiment showed significant activity modulation in corals at environmental temperature, for both TLR4 and NF-kB markers. Immunostaining using specific antibodies on sections of coral colonies exposed to LPS resulted in strong staining of nematocysts in the epithelia compared to control. Dense cellular aggregates lining the gastroderm directly adjacent to the lining of the gut in both oral (facing the mouth and external environment) and aboral (lining the skeleton) tissue were also stained. This wide activation is coherent with the expression levels in LPS-challenged samples detected by the Western blot analysis in the present study. Localization of the TLRs to both the nematocysts and the gastroderm would ensure that pathogens are exposed to receptor proteins regardless of how they enter the corals, thus activating the organism's immune response. Given the binding capabilities of bacterial cell walls by TLRs, this exposure could guarantee an effective means to inhibit further tissue colonization. The activation times observed in this study (i.e., after 6 hours of LPS exposure) are also consistent

with previous observations, which showed up-regulated levels of mRNA encoding several TLR-NF-kB pathway components approximately 4 hours after treatment of a tropical coral species with LPS (Williams et al., 2018). Under healthy control conditions, *A. calycularis* therefore appears to possess the resources necessary to activate the TLR-NF-kB pathway after 6 hours of LPS exposure. These immune pathways are essential in coral response to disease, and generally their tight regulation is a balanced consequence of signaling, organismal conditions, and overall immune strategy (Lazzaro and Rolff, 2011).

Under elevated temperature conditions, a significant up-regulation in TLR4 and NF-kB markers of LPS-unchallenged specimens was shown. Increased expression of several components involved in the TLR-NF-kB pathway has also been described in several anthozoans following heat stress treatment (DeSalvo et al., 2010; Souter et al. al., 2011; van de Water et al., 2015; Mansfield et al., 2017). This provides further evidence that the innate immune system of corals is sensitive to environmental changes (Mydlarz et al., 2008; Palmer et al., 2011; Pinzón et al., 2015). Conversely, when exposed to LPS challenge and warmer seawater conditions simultaneously, a significant suppression in both immune markers' activities was observed, with a lack of extensive immunostaining in both the epithelial and gastrodermal tissues of the corals. Since the TLR-NF-kB signaling pathway also regulates AMP expression, the orange coral may reduce the production of these key molecules and, therefore, be unable to respond effectively to disease under thermal stress. Although no AMPs have yet been identified in *A. calycularis*, genes encoding AMPs have been characterized for several scleractinian corals and in other cnidarians (e.g., Fraune and Bosch, 2007; Fraune et al., 2011; Vidal-Dupiol et al., 2011; Burge et al., 2013; Vidal-Dupiol et al., 2014). Indeed, AMPs have been shown to be crucial in actively regulating and maintaining the health of the tissue-associated bacterial community in several species of anthozoans (Fraune and Bosch, 2007; Franzenburg et al., 2012; Franzenburg et al., 2013). The suppression of these immuno-dynamics could have serious implications for this Mediterranean species during the summer months, when seawater temperatures are higher and the risk of disease outbreaks increases in the basin (Bally and Garrabou, 2007; Vezzulli et al., 2010; Rubio-Portillo et al., 2016; Garrabou et al., 2019). Despite these considerations, a limitation of this work is that it only considers one-time post-exposure to LPS, providing partial information about the organism's responses. Understanding and/or identifying the

relevant timing of immune activities will be crucial for avoiding underestimates of the response capability to stress, and therefore the survival, of these ecologically relevant organisms.

With regard to HSP70, a significant up-regulation was detected in LPS-challenged corals at environmental temperatures. Compared with the control slides, the immunohistochemical analysis showed an effective, wide activation in all tissue districts of the organisms. These findings suggest that changes in HSP70 occur in corals in response to microbial aggressions, also in the absence of thermal stress, further supporting their role in the coral immune response (Brown et al., 2013; Louis et al., 2017). Indeed, several studies conducted on invertebrates (including corals) have already shown that modulations of HSP70 production appear to protect the organism from pathogenic infection (Sung et al., 2008; Baruah et al., 2011; Brown et al., 2013; Louis et al., 2017). For example, evidence that HSP70 enhances resistance to pathogens by priming and enhancing the expression of the pro-phenoloxidase system has already been presented (Baruah et al., 2011). Extracellular HSPs could also represent the ancestral danger signal of cell death or lysis-activating innate immunity (Prohaszka and Fust, 2004). In the present study, an increase in HSP70 protein production following LPS recognition could be an attempt to protect the organism, possibly by activating other constitutive components of the coral effector immune systems (e.g., via activation of the pro-phenoloxidase cascade).

Another novel result from this study is a significant modulation of HSP70 activity at elevated seawater temperature, for both control and LPS-challenged treatments. The unchallenged corals showed significantly higher values than the controls at environmental temperature, though not reaching those of LPS-challenged colonies (both at environmental and elevated temperature). Indeed, under thermal stress, the key role of cytoplasmic HSPs (including HSP70) in several cellular processes has already been widely demonstrated; these include the folding of newly synthesized and misfolded proteins, the stabilization of the cytoskeleton, and protein transfer to other cellular compartments (Mayer, 2013; Balchin et al., 2016). When LPS-challenged, *A. calycularis* showed a significantly up-modulation than control corals, also occurred in terms of increased areal activation of immunostaining tissues. The emerging dynamics suggest that, under warmer seawater conditions, the organism is

able to activate a moderate response to thermal stress alone, probably in order to maintain homeostasis; instead, corals stimulated by LPS are able to further implement their response through the sensitive regulation of HSP70 production similar to that at environmental temperature. However, to confirm such immune dynamics under warmer conditions and pathogen eliciting in corals, studies over longer time intervals and with higher sampling resolution would be required.

In conclusion, these results demonstrate that the immune response of *A. calycularis* exhibited 6 hours post LPS-challenge is significantly affected by warmer seawater conditions. While thermal stress alone stimulated the activation of the coral TLR-NF-kB pathway, responses to LPS under elevated temperature were almost completely suppressed. HSP70 activity was up-modulated for both treatments under thermal stress, with the response of LPS-challenge colonies significantly stronger compared to control corals. These non-linear, temperature-induced responses of the examined markers could be the result of energetic trade-offs between maintaining homeostasis and the costs incurred to implement an effective immune response by the organism, occurring within the predetermined constraints of evolution (Sheldon and Verhulst, 1996; Sadd and Schmid-Hempel, 2009; Lazzaro and Rolff, 2011; Palmer, 2018). Such an immuno-ecological approach represents a challenging path for evaluating immunocompetence and understanding natural patterns of disease among coral species across habitats. This study provides new biological information on an endemic Mediterranean species that can be used to better understand ecological patterns and, therefore, increase the accuracy of predicted responses to future climate-related events.

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Supplementary materials

Table S1 *References and accession number of the sequences used for the sequence alignment of species phylogenetically close to the orange coral* A. calycularis *with the antigenic epitopes of the antibodies employed in the immunoblotting assay.*

Species	Protein	Accession number
Actinia tenebrosa	Toll-like Receptor (TLR)	GenBank ALG40986.1
Homo sapiens	Toll-like Receptor 4 (TLR4)	UniProtKB O00206
Nematostella vectensis	Nuclear Factor kappa B (NF-kB)	GenBank ADQ57372.1
Acropora cervicornis	Nuclear Factor kappa B (NF-kB) ISO1	GenBank UCR60993.1
Acropora cervicornis	Nuclear Factor kappa B (NF-kB) ISO2	GenBank UCR60992.1
Homo sapiens	Nuclear Factor kappa B (NF-kB)	UniProtKB P19838
Actinia tenebrosa	Heat Shock Protein 70 (HSP70)	GenBank XP_031562268.1
Aiptasia diaphana	Heat Shock Protein 70 (HSP70)	GenBank XP_028514728.1
Stylophora pistillata	Heat Shock Protein 70 (HSP70)	GenBank AKC91104.1
Pocillopora verrucosa	Heat Shock Protein 70 (HSP70)	GenBank QDO73499.1
Pocillopora damicornis	Heat Shock Protein 70 (HSP70)	GenBank BAD89541.1
Homo sapiens	Heat Shock Protein 70 (HSP70)	UniProtKB P0DMV8

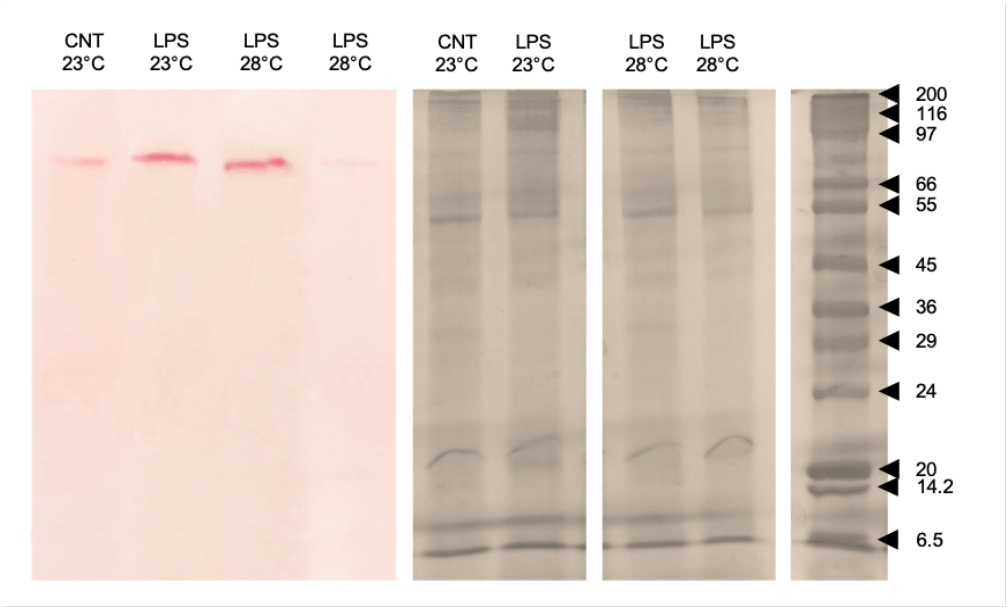

Figure S1 *Representative Western blot assay (left) and SDS-PAGE under non-reducing conditions (right) carried out on A. calycularis tissue extracts from the experimental treatments. Each nitrocellulose sheet was treated with anti-TLR4, the anti-rabbit IgG-alkaline phosphatase secondary antibody, and was developed with the BCIP/NBT liquid substrate system.*

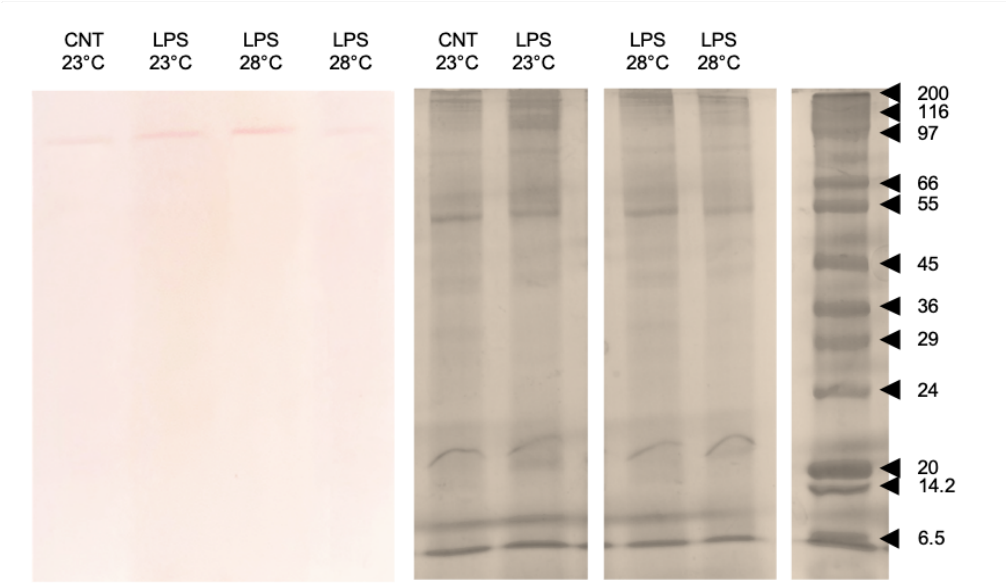

Figure S2 *Representative Western blot assay (left) and SDS-PAGE under non-reducing conditions (right) carried out on A. calycularis tissue extracts from the experimental treatments. Each nitrocellulose sheet was treated with anti-NF-kB, the anti-rabbit IgG-alkaline phosphatase secondary antibody, and was developed with the BCIP/NBT liquid substrate system.*

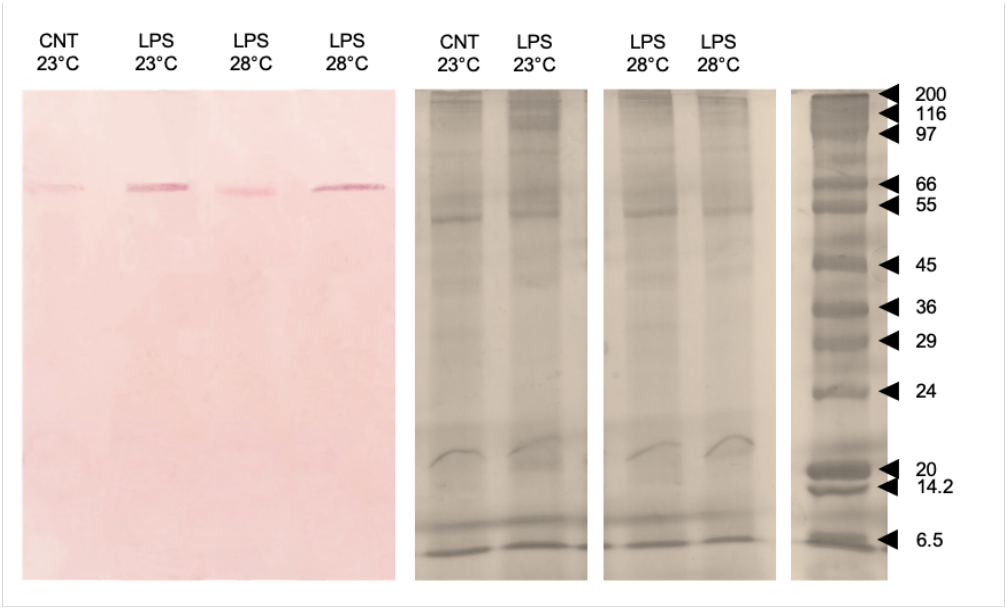

Figure S3 *Representative Western blot assay (left) and SDS-PAGE under non-reducing conditions (right) carried out on A. calycularis tissue extracts from the experimental treatments. Each nitrocellulose sheet was treated with anti-HSP70, the anti-mouse IgG-alkaline phosphatase secondary antibody, and was developed with the BCIP/NBT liquid substrate system.*

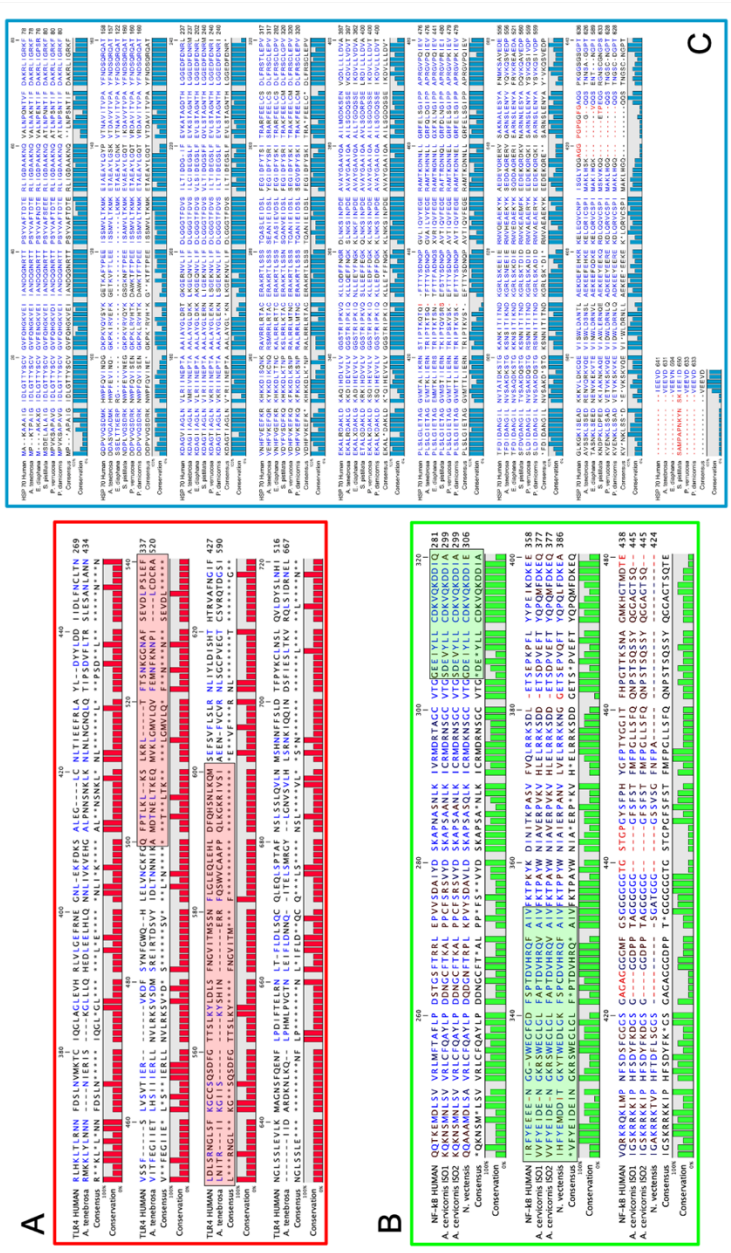

Figure S4 *The sequences of (A) TLR4, (B) NF-kB, and (C) HSP70 used for alignment with species phylogenetically close to A. calycularis, allowing for systematic and comprehensive identification of antigen binding sites. The consensus contained in the colored box (only for TLR4 and NF-kB; for HSP70 the entire protein is considered) improves the understanding of antigenic interactions that determine cross-reaction in the immunoblotting assays performed using* A. calycularis *tissue extracts. The references and the accession number of the sequences used in the alignment are reported in supplementary Table S1.*

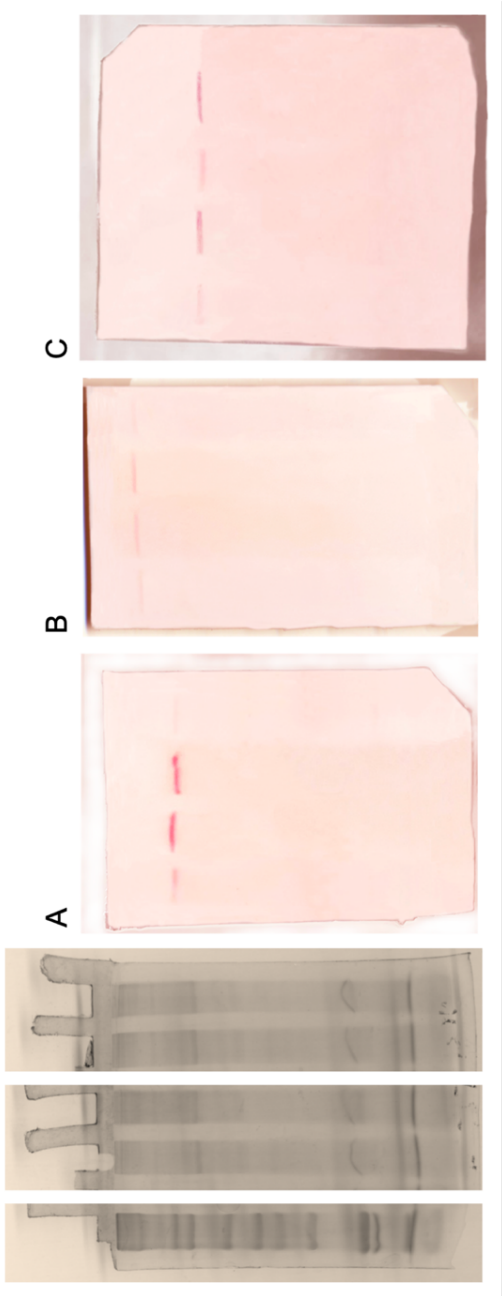

Figure S5 *Full-length representative pictures of Western blot assays and SDS-PAGE under non-reducing conditions carried out on A. calycularis tissue extracts from the experimental treatments. Each nitrocellulose sheet was treated with anti-TLR4 (A), -NFkB (B) and -HSP70 (C), the anti-mouse IgG-alkaline phosphatase secondary antibody, and was developed with the BCIP/NBT liquid substrate system.*

Chapter 2

How does warmer seawater change the sensitivity of a Mediterranean thermophilic coral after immune-stimulation?

Abstract

Anthropogenic climate change is warming seawater worldwide, pushing the limits of tolerance for marine organisms and driving a decline in biodiversity. The risk of thermal anomalies has increased particularly in the Mediterranean region over the last 30 years, where intense warming has been identified as one of the main stressors in coastal regions. To determine the influence of warmer conditions over time in the coral *Astroides calycularis*, different immune activity parameters were compared in response to elevated temperature (~ 28°C) and the presence of a pathogen-associated molecular pattern (PAMP) - *Escherichia coli* lipopolysaccharide (LPS) - as an elicitor of the innate immune response. Immune parameters, which included phenoloxidase-like, glutathione peroxidase, lysozyme-like, alkaline phosphatase, and esterase enzyme activity, were measured after LPS balneation (0-, 12-, 48-, and 120-hour time point). All five enzymes demonstrated constant values under environmental conditions (~ 23°C), indicating a constituent activity. LPS at environmental temperature induced significant upregulation immediately after exposure (time zero), demonstrating an immune response to the pathogen elicitor. Under warmer conditions (~ 28°C), constituent values increased over time, indicating a shift in the immune strategy to maintain homeostasis. However, warmer seawater, within the summer range experienced by this coral species, impaired the immune response to LPS, delaying it over time. These changes in immune strategy indicate that temperature affects coral immunity and, in thermophilic *A. calycularis*, results in an energy trade-off that could maintain its health-state through suboptimal conditions during multiple perturbations, such as summertime diseases.

Keywords: *Astroides calycularis*, LPS, thermal stress, immune related enzymes activities, coral immunity

Introduction

The acceleration of anthropogenic climate change due to global carbon emissions is challenging the survival of marine organisms over the world, as well as the persistence of functional marine ecosystems more generally (Hughes et al., 2018). Rising global sea temperatures, with a concomitant increase in disease outbreaks, have spurred intense study of warming effects on marine environment (Harvell et al., 2002; Harvell et al., 2009; Somero, 2010; Thurber et al., 2020; Burke et al., 2023). In the Mediterranean region, the risk of climate anomalies has increased sharply over the last 30 years, with seawater warming to temperatures beyond the range of normal fluctuations historically experienced by the organisms (Lejeusne et al., 2010; Darmaraki et al., 2019; Garrabou et al., 2022). This shift has induced well-documented disease outbreaks and, consequently, mortality events across varying geographic extents and numbers of affected species (Bally and Garrabou, 2007; Vezzulli et al., 2010; Rubio-Portillo et al., 2016; Garrabou et al., 2019).

The sensitivity of different taxa to disease dynamics can depend on the type of ecosystem considered (Thurber et al., 2020; Burke et al., 2023), and among these, corals are particularly sensitive to changes in sea temperature, as shown by their susceptibility to mortality (e.g., Glynn and D'Croz, 1990; Garrabou et al., 2022) and temperature-driven disease incidence (e.g., Bruno et al., 2007; Walton et al., 2018; Tracy et al., 2019; Howells et al., 2020; Randazzo-Eisemann et al., 2022). Since under warmer stressful conditions resource allocation to defense is diminished (Moret and Schmid-Hempel, 2000; Palmer et al., 2011a; Palmer, 2018a; Palmer, 2018b; Palmer and Traylor-Knowles, 2018), the immune functions (responsible for preventing infection and maintaining the organism's integrity) are increasingly being pushed beyond their physiological limits. Levels of immunity are directly related to the susceptibility of corals to both necrosis and disease (e.g., Palmer et al., 2010; Palmer et al., 2011a), which highlights the ecological relevance of coral immune dynamics during these phenomena. Thus, understanding the complex interplay between warmer conditions, pathogen occurrence, and coral immunity becomes essential (Harvell et al., 2007; Traylor-Knowles and Connelly, 2017; Palmer et al., 2011a; Palmer, 2018a; Palmer, 2018b; Palmer and Traylor-Knowles, 2018). Despite several recent studies focused on elucidating coral immune responses to pathogens, the direct effects of warmer seawater conditions on

specific immune pathways, as well as the timing of activation following pathogen exposure, remain key knowledge-points.

Corals possess many innate immune mechanisms similar to those of other invertebrates (Traylor-Knowles and Connelly, 2017; Palmer and Traylor-Knowles, 2018; Parisi et al., 2020). The presence of phenoloxidase (PO)-like activity has been demonstrated within reef-building corals, gorgonians, and tropical soft corals (Mydlarz et al., 2008; Palmer et al., 2010; Mydlarz and Palmer, 2011). The melanin synthesis pathway is a key component of immunity, responsible for cytotoxic defense (Nappi and Ottaviani, 2000) as well as the formation of an impermeable melanin barrier between healthy host tissue and an invading organism (Nappi, 1973). In particular, this was found to be upregulated in tissues naturally infected with parasites (Palmer et al., 2009) and fungal pathogens (Mydlarz et al., 2008), as well as in visibly impaired tissues (Mydlarz et al., 2009; Palmer et al., 2008; Palmer et al., 2011a). Invertebrate immune responses also produce cytotoxic radicals, such as reactive oxygen species (ROS), which can lead to oxidative stress and cause tissue damage (Traylor-Knowles and Connelly, 2017; Palmer and Traylor-Knowles, 2018). Antioxidants, which readily scavenge oxygen radicals, are enzymes critical to preventing self-damage (Cerenius et al., 2010) and are often abundant during a pathogen infection in corals (Halliwell and Gutteridge, 1999; Lesser, 2006; Palmer et al., 2011a; Palmer et al., 2018a). These include peroxidases, catalases, superoxide dismutase, and fluorescent protein (Halliwell and Gutteridge, 1999; Traylor-Knowles and Connelly, 2017; Palmer and Traylor-Knowles, 2018).

Lysozyme activity is a phylogenetically conserved humoral response in many invertebrate species. It corresponds to the primary and rapid defense of organisms against attacks by pathogens and is a bactericidal hydrolytic enzyme which hydrolyzes the β-1,4 glycosidic bonds of the bacterial cell-wall, destabilizing the membrane (Li et al., 2008). In Anthozoa, lysozyme-like activity has been detected that fulfills the same function (Stabili et al., 2015). In addition to its antibacterial activity, lysozyme has also recently been shown to inactivate some viruses and exert anti-inflammatory action (Leśnierowski and Yang, 2021). The activity and kinetic characteristics of some metabolic regulatory enzymes, closely related to immunity by promoting the organism's homeostasis, are also linked to the adaptive

potential of anthozoans to stress conditions (e.g., warmer seawater) (Parisi et al., 2017). In particular, alkaline phosphatase and esterase are an example of enzymes involved in a wide range of processes involving synthesis and hydrolysis reactions, as well as in various catabolic pathways (Stamatis et al., 1998; Copeland, 2000; Lopes et al., 2011).

Since immune constituent levels (continuous baseline immune activity in the absence of an acute perturbation) (Tauber, 2015; Palmer, 2018a; Palmer, 2018b) and the induction/maintenance of an immune response are energetically costly processes (Sheldon and Verhulst, 1996; Armitage et al., 2003; Palmer, 2018b), they may be traded-off against other important life-history traits, such as growth and reproduction (Sadd and Schmid-Hempel, 2009; van der Most et al., 2011). A coral that invests primarily in immunity may be slow growing or have reduced fertility, but it will likely demonstrate higher levels of constituent immunity and/or induce a greater immune response than a species that invests primarily in other traits (Schmid-Hempel, 2003; Schmid-Hempel and Ebert, 2003; Palmer, 2018a; Palmer, 2018b). The constituent immunity and/or immune responses of different coral species (i.e., their immune strategies) can vary depending upon their different life-history characteristics (Palmer et al. 2011a; Palmer, 2018a; Palmer, 2018b). Therefore, although all healthy corals are likely to be immunocompetent (i.e., able to induce a proficient immune response), relative immunocompetence (i.e., the magnitude of a response) (Adamo, 2004; Mydlarz et al., 2016; Palmer and Traylor-Knowles, 2018; Palmer, 2018a) among different corals is likely to vary. Establishing relative immunocompetence among diverse coral species (especially broadening to non-tropical organisms) will provide insights into ecological patterns, such as disease susceptibility, and better establish the ability to cope under adverse environmental conditions.

The aim of this work is to better clarify how warmer seawater affects coral immunity during pathogen eliciting, broadening our understanding of the sensitivity of organisms living in non-tropical habitats; specifically, the constituent immunity and immune responses of a Mediterranean coral species, *Astroides calycularis* (Pallas, 1766) were investigated. *A. calycularis* is commonly found in the central-southern part of the basin and covers relatively large surfaces of vertical rocky reefs, overhangs, and caves below the intertidal fringe (Ingrosso et al., 2018). This azooxanthellate coral occupies both well-lit and dark habitats

and is considered a thermophilic species, thriving at relatively high temperatures (Ingrosso et al., 2018). Previous studies have shown that adult colonies are tolerant of ocean warming and acidification (Movilla et al., 2016; Carbonne et al., 2021). In contrast, natural populations of this orange coral have recently suffered widespread mortality during the summer period, when temperatures reach and exceed 28°C (Gambi et al., 2018; Bisanti et al., 2022). In detail, the PO-like, glutathione peroxidase, lysozyme-like, alkaline phosphatase, and esterase enzymes were studied following exposure to pathogen-associated molecular patterns (PAMPs) at both environmental and elevated temperatures, considered here as immune-activity markers. PAMPs are recognized by pattern recognition receptors, such as lipopolysaccharide (LPS)-binding proteins and peptidoglycan recognition protein, and stimulate invertebrate immune responses (Ratcliffe et al., 1991; Wittwer et al., 1997; Palmer et al., 2011a). In corals, the use of LPS allows us to avoid the well-documented difficulties of infecting coral with a live bacteria strain (Lesser et al., 2007) by testing the induction or suppression of immune pathways by PAMP receptors (Xian et al., 2009; Liu et al., 2011; Palmer et al., 2011a).

Materials and methods

Coral collection

Sixty comparably sized (3.5 ± 1.0 cm colony diameter; 32.2 ± 8.3 polyps per colony) and visibly healthy adult colonies of the orange coral *A. calycularis* from the upper littoral zone (~ 4-5 m depth) in Capo Zafferano Bay (38° 06′ 25″ N; 13° 32′ 10″ E) on the NW coast of Sicily (NW Mediterranean Sea, Italy), and sampled in October 2022. Each colony was carefully removed with the aid of a hammer and chisel and transported in a 1 µm filtered seawater tank to the laboratory.

Experimental design

Four large aquaria inside a temperature-controlled room were supplied with continuous flow-filtered seawater (1µm) at environmental temperature during the sampling periods (23°C), and the 60 coral colonies were equally and randomly assigned among them. Animals were maintained in a 15:9h photoperiod of daily light/dark cycles to match the natural photoperiod at the collection sites (metal halide lights, with levels maintained at 150-250 µmol m-2s-1) and in dimmed light conditions to mimic their natural sciaphilous environment (the tanks were under a canopy of 70% light-reducing shade-cloth). During the 20-day acclimatization period, the corals were fed twice a week with a commercial preparation of plankton (Elos Coral Foods SvC) prior to the experiment (Franzellitti et al. 2018). On day 21, three randomly selected colonies in each large aquarium were snap frozen and stored at -30°C as pre-treatments controls (TPrs). Two aquaria were then randomly selected for the elevated temperature treatment in which the seawater temperature was increased by 1.0 to 1.5°C per day for 3 days and stabilized at 28°C, within the summer range experienced by this coral species (seawater temperature analysis obtained from the Copernicus Marine Environment Monitoring Service, Fig. S1; Buongiorno Nardelli et al. 2013; Bisanti et al. 2022). To regulate the temperature, a submersible heater was placed in each aquarium. The mean (± s. d.) daily seawater temperatures for the two environmental large aquaria ranged from 22.90 ± 0.17°C to 23.54 ± 0.01°C, while for the higher temperature large aquaria the daily averages ranged from 28.00 ± 0.17°C to 28.41 ± 0.19°C during the whole experiment. In the two elevated temperature large aquaria, the daily averages were significantly higher,

by approximately 5°C, than those of the two-environmental temperature large aquaria (ANOVA, MS = 987.3, F = 17275, $p < 0.001$).

Four smaller plastic tanks holding 4.5 - 5 l of seawater, equipped with individual air-stones to aerate and mix the seawater, were submerged within each of the four larger aquaria, for a total of 16 small tanks. Three colonies were placed ~ 5 - 10 cm away from each other in the smaller tanks, and thus did not come into contact with each other. The smaller tanks were thus randomly designated for one of four treatments: (1) environmental seawater temperature (~ 23°C) and control (no-LPS); (2) environmental seawater temperature with 5 µg ml-1 LPS (Palmer et al. 2011a), lyophilized from *Escherichia coli* (ATCC 25922 strain; Chrisope Technologies, Louisiana, USA) and dissolved in sterile filtered seawater; (3) elevated seawater temperature (~ 28°C) and control (no-LPS); and (4) elevated seawater temperature with 5 µg ml^{-1} LPS. The colonies were exposed to a constant flow (from the larger aquaria) of 20 µm-filtered seawater, at either environmental or elevated temperature, for 2 days prior to LPS treatment. Over the LPS exposure, the system was closed, stopping the water circulation in the small plastic tanks by raising each tank's rim slightly above the waterline of the larger seawater bath using ~ 5 cm blocks. The seawater in each small tank was carefully exchanged with relative-temperature 20 µm sterile filtered seawater, and 5 µg ml^{-1} LPS dissolved in sterile filtered seawater was added to those designated for LPS treatment. The small tanks were kept slightly raised in the seawater baths throughout the LPS or control treatments (no LPS) so that the seawater within each small tank remained isolated while seawater temperatures (i.e., ~ 23°C or ~ 28°C) continued to be maintained at the same values as in the large aquaria. The LPS treatment occurred over a period of 6 hours, and the water flow was turned off during incubation, keeping the water moving via air stones in each small tank which flowed vigorously during the entire exposure period.

Once the period of LPS exposure was over, the seawater in each small tank was carefully exchanged with relative-temperature 20 µm fresh filtered seawater, and the system was reopened to allow the natural clearance dynamics of the corals and prevent further immune stimulation. Three colonies were sampled for all treatments immediately upon termination of LPS exposure (0-hour time point) and 12, 48, and 120 hours (5 days) later (Fig. 1). All samples were immediately frozen and stored at -30°C. For the whole duration of the

experiment, no mortality occurred, and all coral branches appeared healthy and without any visible lesions.

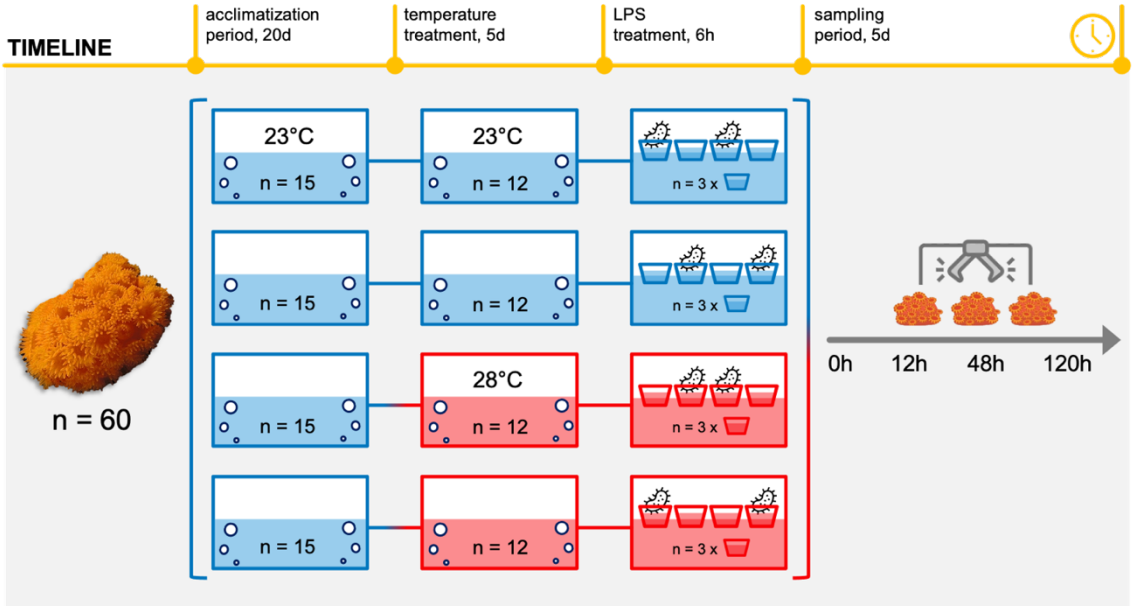

Figure 1 *Schematic drawing summarizing the experimental timeline. Number of biological replicates for each experimental timepoint, n*

Extract preparation and protein concentration

For all orange coral colonies, tissue samples were mechanically removed from frozen specimens and subsequently transferred into polycarbonate tubes with 500 µl TBS-buffer (NaCl 150 mM, Tris-HCl 10 mM, pH 7.4) containing a complete EDTA-free cocktail of protease inhibitors (Sigma-Aldrich) on ice; the resultant coral tissue slurry was then centrifuged (36,200 x g for 20 min at 4°C). The supernatant was collected and protein concentration was measured according to the method found in Bradford (1976). The sample absorbance was read at 595 nm (RAYTO RT-2100C) with TBS as blank, and a calibration curve defined through bovine serum albumin was used to obtain the protein concentration, expressed in mg/ml. Extracts were adjusted to 0.5 mg/ml before performing enzymatic assays.

Phenoloxidase (PO)-like assay

PO-like activity was measured spectrophotometrically according to Winder and Harris (1991), by using L-Dopa (3,4 dihydroxy-L-phenylalanine; Sigma-Aldrich, USA) as a

substrate and 6 mM MBTH (3-methyl-2 benzothiazolinone hydrazone hydrochloride; Sigma-Aldrich, USA) as a specific reagent. 50 µl of coral sample with 50 µl of trypsin from bovine pancreas (1 mg/ml; Sigma-Aldrich, USA) or 50 µl of distilled water, as control, were incubated for 20 min at 20°C in 50 µl reaction mixture (20 mM L-DOPA and MBTH in distilled water). The absorbance was read within 60 min at 5 min intervals by spectrophotometry at 505 nm (microplate reader, RAYTO RT-2100C). PO-like activity was expressed as units (U) per min, where 1 U = 0.001 ΔA540 min^{-1} mg^{-1} protein.

Glutathione peroxidase (GPx) assay

Enzymatic activity was measured according to Ross et al. (2000). In 96-well flat-bottomed plates, 50 µl of sample at standard concentration (0.5 mg/ml) were incubated with 100 µl TMB (3,3′ 5,5′-tetramethylbenzidine; Sigma-Aldrich, USA). The reaction was stopped after 30 min of dark incubation with sulfuric acid (H_2SO_4 2 M). The absorbance was read spectrophotometrically at 450 nm in a microplate reader (RAYTO RT-2100C), and the GPx produced was expressed in U/mg of protein according to the equation: U/mg = Abs * Vf / CP (Vf, final volume of the well; CP, protein concentration of the sample).

Lysozyme (LYS)-like assay

To evaluate lysozyme-like (LYS) activity following Parry et al. (1965), 30 µl of each sample were placed in a 96-well flat-bottomed plate and incubated with 270 µl of bacterial suspension (*Micrococcus lysodeikticus* ATCC 4698, Sigma-Aldrich, USA) in triplicate. 30 µl TBS buffer were replaced in the control sample. The reaction was carried out at 25 °C, and absorbance (450 nm; microplate reader, RAYTO RT-2100C) was measured every 30 s for 10 min. A unit of LYS was defined as the amount of sample causing a decrease in absorbance of 0.001/min (U min^{-1}), and U/ml was calculated in accordance with the formula: U/ml = (Δ abs/min^{-1} * dilution factor * 1000) / enzyme volume buffer.

Alkaline phosphatase (ALP) and esterase (EST) assays

Coral samples were incubated in a 96-well flat-bottomed plate with an equal volume of 4 mM p-nitrophenyl phosphate substrate (Sigma-Aldrich, USA) liquid in 100 mM ammonium bicarbonate containing 1 mM $MgCl_2$ (pH 7.8), for alkaline phosphatase (ALP); esterase (EST) activity was evaluated by incubating the same volume of coral sample with 0.4 mM

p-nitrophenyl myristate substrate (Sigma-Aldrich, USA) in 100 mM ammonium bicarbonate containing 0.5% of Triton X-100 (pH 7.8, 30°C; Sigma-Aldrich, USA). Enzymatic kinetics were evaluated according to Ross et al. (2000) at regular intervals of 5 min to 1 h at 405 nm with a microplate reader (RAYTO RT-2100C). One unit (U) of activity was defined as the amount of enzyme required to release 1 µmol of p-nitrophenol produced in 1 min.

Statistical analyses

To test differences in protein activity between LPS treatments (control and LPS) and temperature treatments (environmental and elevated), univariate and multivariate distance-based permutational non-parametric analysis of variance (PERMANOVAs; Anderson, 2001; McArdle and Anderson, 2001) were performed; the analyses considered: temperature (2 levels), LPS (2 levels), and time (4 levels) as fixed factors. PERMANOVAs were based on Euclidean distance matrix after square root transformation of the data using 9999 permutations under unrestricted permutations of the raw data (univariate tests), or under a reduced model (multivariate test) with a Type III (partial) sum of squares (Anderson, 2001). Due to the restricted number of unique permutations in the pairwise tests, the p-values were obtained from the Monte Carlo samplings. When significant differences were found, a pairwise comparison was done to explore differences among all pairs of the factors. Statistical analyses were carried out using the PRIMER v7+ software (Plymouth Marine Laboratory; Clarke, 1993; Clarke and Warwick, 1994). The measured are expressed as the means ± 95% confidence intervals, resulting from three independent experiments with three specimens in each treatment group.

Results

Overall, the previously described coral immune markers showed significant variation in LPS-exposed enzymatic activity with respect to control specimens at environmental seawater temperature (23°C) (Table 1; Fig. 2, left). Specifically, differences were found for the 0- and 12-hour time points after LPS exposure (pairwise analysis, $p > 0.01$; Table S1). At elevated temperatures (28°C), PERMANOVA showed no significant differences between -LPS and no-LPS colonies (Table 1; Fig. 2, right), although pairwise comparisons revealed significance for the 120-hour time point between LPS-treatments (pairwise analysis, $p > 0.01$; Table S1). The mean activity of the examined enzymes in LPS-exposed specimens varied significantly over time in both temperature treatments, whereas in control colonies, differences were significant only at elevated seawater temperature (Table S3). The enzyme activity values in LPS-exposed colonies did not vary significantly between seawater temperature treatments (Table 2; Fig. 3, left); however, the differences in control colony values were significant (Table 2; Fig. 3, right).

Phenoloxidase (PO)-like activity

The *A. calycularis* specimens demonstrated significantly higher PO-like activity in LPS-exposed colonies than unexposed colonies at the environmental seawater temperature (Table 1; Fig. 2A). The increase in PO-like activity was particularly evident immediately at the 0-hour time point, when it was approximately 3-fold greater than LPS-unexposed colonies (pairwise analysis, $p > 0.05$; Table S1). At elevated temperatures, there was no general difference in PO-like activity between LPS-exposed and control colonies (Table 1; Fig. 2B). However, the mean PO-like values of control corals increased approximately 2-fold over time in the elevated temperature treatment, which was absent in the environmental temperature treatment (Fig. 3A). At the 120-hour time point, PO-like values were significantly 3-fold higher between control and LPS treatments (pairwise analysis, $p < 0.05$; Table S3; Fig. 3A). Compared with control values at the environmental temperature, pairwise PERMANOVA showed significant differences for all experimental time points (Table 2, S2). The LPS-exposed colonies demonstrated peak activity at the 12-hour time point at elevated seawater temperature, which was similar in magnitude to the peak of

activity at the 0-hour time point in LPS treatment at environmental temperature (Fig. 3B). Consistently, PO-like activity across temperature treatments was time-dependent (Table 2).

Glutathione peroxidase (GPx) activity

At environmental seawater temperature, mean GPx activity was significantly higher in the LPS-treatment compared to the control treatment (Table 1; Fig. 2C). There was a 4-fold increase in the activity of this enzyme with LPS at the 0-hour time point at environmental temperature, which remained upregulated, compared to controls, for up to 48 hours. At elevated seawater temperature (Fig. 2D), there were significant differences in GPx activity between controls and LPS-exposed animals (Table 1), although pairwise analysis revealed significant differences only for the 120-hour time point (Table S1). Overall, GPx-control activity levels were significantly higher at the warmer temperature than the environmental temperature (Table 2; Fig. 3C), likely driven by the 48-hour time point, whereas the reverse was true for LPS-exposed colonies (Table 2; Fig. 3D).

Lysozyme (LYS)-like activity

Mean LYS-like activity at environmental seawater temperature differed significantly between LPS and control treatments (Table 1; Fig. 2E). In detail, at both the 0- and 12-hour time points, LYS-like activity was significantly more than 2-fold higher in the LPS treatment than in the control treatment (pairwise analysis, $p > 0.01$; Table S3). At the elevated seawater temperature, there was no difference in LYS-like activity between LPS-exposed and unexposed corals (Table 1; Fig. 2F), indicating no immediate response to LPS. However, at the elevated seawater temperature, LYS-like values in LPS-exposed specimens at the 12-hour time point were similar to those of the 0-hour time point of the LPS-environmental colony (Fig. 3F), suggesting a delayed activity response at warmer temperatures. Also, and similar to PO-like activity, control levels of LYS-like enzymes increased approximately 4-fold over time at the elevated temperature (i.e., 120-hour time point; pairwise analysis, $p < 0.001$; Table S2), and overall were significantly higher than control levels at the environmental temperature (Table 2; Fig. 3E).

Alkaline phosphatase (ALP) and esterase (EST) activity

Mean ALP activity varied significantly over time between both seawater temperatures and LPS treatments (Table 1, 2), whereas the mean EST activity of coral specimens did not show significant differences for either combination of factors (Table 1, 2). At environmental seawater temperature, ALP and EST activity were significantly 2.5- and 3.5-fold higher, respectively, in the LPS-exposed colony at the 0-hour time point than in the control treatment (pairwise analysis, $p < 0.05$; Table S1; Fig. 2G, I). At elevated seawater temperature, there was a significant and increasing trend of activity over time in the control treatment (Table 2); in particular, pairwise analysis revealed significant differences, approximately 2.5-fold higher with respect to control-environmental values (Figure 3G, I) and 1.5-fold higher with respect to LPS-elevated values (Figure 2H, J), at the 120-hour time point for both enzymes (Table S1, S2).

Table 1 *Output of PERMANOVA tests examining the effects of LPS treatments (control vs. LPS) and interaction with sampling time on immune enzyme activity of* A. calycularis *for both environmental and elevated temperatures. Phenoloxidase, PO; Glutathione Peroxidase, GPx; Lysozyme, LYS; Alkaline Phosphatase, ALP; Esterase, EST; Control, CRTL; Lipopolysaccharide, LPS; Environmental temperature, ENV; Elevated temperature, ELV. P (MC) = probability level; n.s. = not significant; ***p < 0.001; **p < 0.01; *p < 0.05.*

Enzyme	**Temperature**				**Temperature x Time**	
	ENV (CRTL vs. LPS)		ELV (CRTL vs. LPS)		CRTL vs. LPS	
	t	**P (MC)**	***t***	**P (MC)**	**Pseudo-F**	**P (MC)**
PO	5.03	**	1.34	n.s.	1.12	n.s.
GPx	7.22	***	2.21	*	5.48	***
LYS	5.88	***	1.07	n.s.	2.35	n.s.
ALP	2.96	**	0.54	n.s.	2.53	*
EST	4.57	***	0.44	n.s.	0.99	n.s.
Total enzymes	4.69	***	1.30	n.s.	1.62	n.s.

Table 2 *Output of PERMANOVA tests examining the effects of temperature treatments (environmental vs. elevated) and interaction with sampling time on immune enzyme activity of* A. calycularis *for both control (no-LPS) and LPS samples. Phenoloxidase, PO; Glutathione Peroxidase, GPx; Lysozyme, LYS; Alkaline Phosphatase, ALP; Esterase, EST; Control, CRTL; Lipopolysaccharide, LPS; Environmental temperature, ENV; Elevated temperature, ELV. P (MC) = probability level; n.s. = not significant; ***p < 0.001; **p < 0.01; *p < 0.05.*

Enzyme	**LPS**				**LPS x Time**	
	Control (ENV vs. ELV)		LPS (ENV vs. ELV)		ENV vs. ELV	
	t	**P (MC)**	***t***	**P (MC)**	**Pseudo-F**	**P (MC)**
PO	9.61	***	0.29	n.s.	7.32	***
GPx	2.68	*	6.31	***	7.28	***
LYS	6.12	***	1.34	n.s.	5.70	***
ALP	2.09	n.s.	1.63	n.s.	5.26	***
EST	4.01	**	0.12	n.s.	2.36	n.s.
Total enzymes	5.69	***	1.42	n.s.	5.20	***

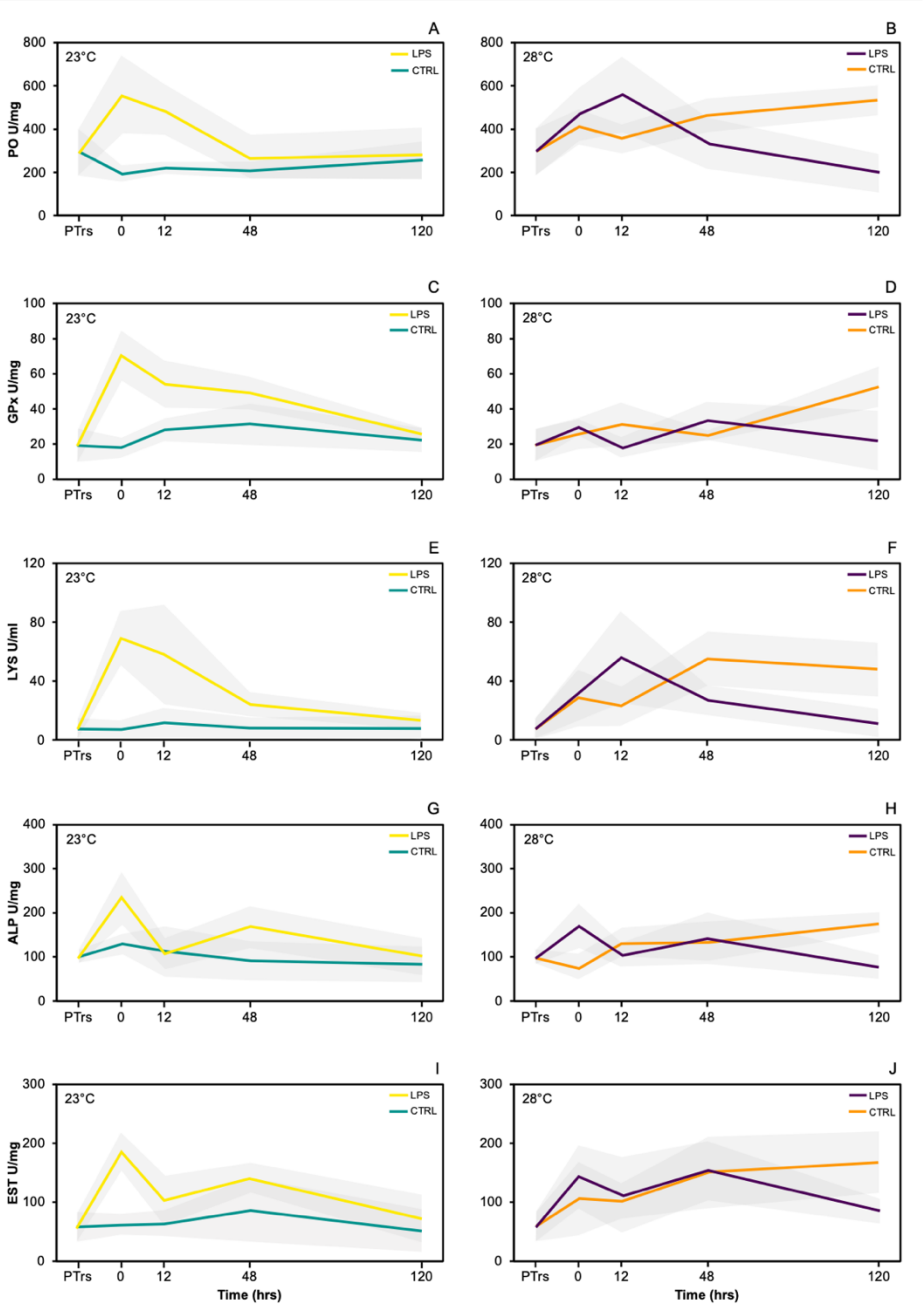

Figure 2 *Mean values (colored lines) of immune enzyme activity ± 95% confidence intervals (grey areas) for control (no LPS) vs LPS-exposed colonies of* A. calycularis *under environmental (23°C) and elevated (28°C) seawater temperatures over time. Phenoloxidase, PO; Glutathione Peroxidase, GPx; Lysozyme, LYS; Alkaline Phosphatase, ALP; Esterase, EST; Control, CRTL; Lipopolysaccharide, LPS; Pre-treatments values, PTrs.*

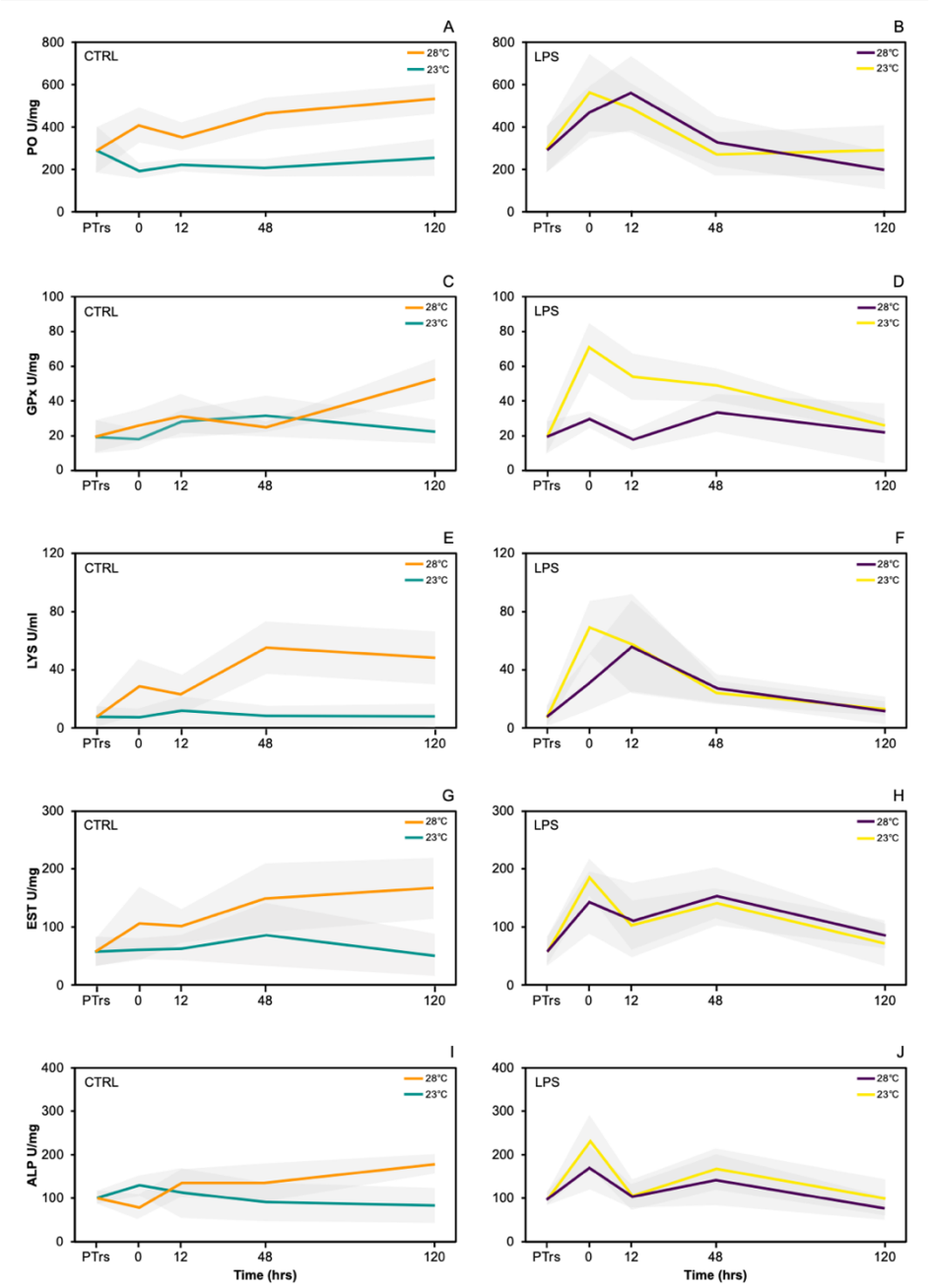

Figure 3 *Mean values (colored lines) of immune enzyme activity ± 95% confidence intervals (grey areas) for environmental (23°C) vs elevated (28°C) temperature-exposed colonies of* A. calycularis *under control (no LPS) and LPS treatments over time. Phenoloxidase, PO; Glutathione Peroxidase, GPx; Lysozyme, LYS; Alkaline Phosphatase, ALP; Esterase, EST; Control, CRTL; Lipopolysaccharide, LPS; Pre-treatments values, PTrs.*

Discussion

The orange coral *A. calycularis* showed consistent activity of all immune-enzymes in the control treatment (no-LPS) at 23°C, demonstrating the presence of a stable constituent immunity under controlled environmental conditions (van de Water et al., 2016; Palmer, 2010; Palmer, 2018b). An immune response occurred within the 12-hour time point in the LPS-exposed sample at environmental seawater temperature, as demonstrated by the upregulation of the investigated enzymes. Under warmer conditions (28°C), control colonies showed a gradual ramping up of constituent values, and the immune response to pathogen elicitation was altered relative to environmental temperatures. The elevated seawater temperature, within the natural summer range experienced by this coral, would appear to have an enhancing effect on the constituent immunity but a suppressive impact on the immune response to LPS exposure.

Immune activity at environmental seawater temperature

Under controlled environmental conditions, the enzymatic values of the *A. calycularis* colonies remained significative and constant throughout the duration of the experiment, representing the constituent immunity responsible for maintaining homeostasis in the absence of an acute perturbation (Palmer, 2018a; Palmer, 2018b). Such an investment could represent an advantageous strategy for organisms living in environments characterized by biotic and abiotic fluctuations and perturbations, such as infralittoral zones, thus favoring tolerance and promoting survival (Palmer and Traylor-Knowles, 2018; Palmer, 2018b). On the other hand, an immune response was observed at the 0-hour time point under LPS treatment, demonstrated by the significantly increased activity of the five enzymes relative to their constituent values. These results confirm the involvement of PO-like and GPx enzymes in the immune response of a non-tropical species (Palmer et al., 2011a; Palmer, 2018a), and are consistent with ALP and EST activity recorded in other cnidarians subjected to bacterial injection (Trapani et al., 2016). Furthermore, an alteration of LYS-like activity following LPS elicitation in corals was demonstrated for the first time.

Upon activation by PAMP detection, the coral PO-like cascade employs several compounds to hydroxylate mono-phenol and diphenol substrates in melanin polymeric deposits that produce highly cytotoxic defenses and create barriers to infection (Traylor-

Knowles and Connelly, 2017; Palmer, 2018a). Consistent with the observed PO-like upregulation, high PO activities have been documented in lesions associated with white syndrome disease in an Indo-Pacific coral species (Palmer et al., 2011b). These enzymes are activated in response to bacterial challenge (Palmer et al., 2010; Palmer and Traylor-Knowles, 2018) and have shown diverse reactions in different tropical species in response to experimentally-exposed pathogen stress, indicating that coral species can modify this system for their immune needs (Palmer et al., 2011a). Concurrent with the increased activity of this cytotoxic immune pathway, there is a corresponding heightened antioxidant activity of GPx, an enzyme that scavenges hydrogen peroxide (Traylor-Knowles and Connelly, 2017; Palmer and Traylor-Knowles, 2018; Palmer, 2018a). GPx activity, which plays a key protective role, was approximately 4-fold higher at 0-hour time point than constituent levels, suggesting a tight regulation due to the potential for cytotoxic self-harm. This is consistent with the induction of oxidative stress conditions during an immune response as a result of the oxidative burst and as a product of the upregulation of PO-like activity (Halliwell and Gutteridge, 1999; Palmer et al., 2011a), which are primary sources of oxidative stress during an invertebrate immune response (Nappi and Ottaviani, 2000; Sadd and Siva-Jothy, 2006; Palmer and Traylor-Knowles, 2018). Likewise, at the 0-hour time point, an increase in LYS-like activity has been demonstrated, which triggers an innate immune response in coral that probably acts both directly by damaging the bacterial cell-walls and through a stimulating effect on phagocytosis (Leclerc, 1996; La Corte et al., 2023). In several marine invertebrates, this non-specific immune molecule plays a key role in the control of humoral bacteriolytic activity (Leclerc, 1996; Dhainaut and Scaps, 2001).

In light of these results, at a healthy environmental temperature, *A. calycularis* seems to possess the resources necessary to mount an immune response and re-establish homeostasis over time. Inducing a rapid and effective immune response to efficiently isolate a pathogenic outbreak is essential to restoring homeostasis and thus promoting survival. The involvement of proteolytic cascades could allow a timely and more rapid immune activation, pending transcriptomic responses (Palmer, 2018a). Indeed, the immediacy of the coral immune response is a hallmark of invertebrate innate immunity (Palmer et al., 2011a; Palmer et al., 2011c; Palmer, 2018b). Understanding and/or identifying the relevant timing of immune activity is fundamental for avoiding underestimates of the response capability to stress, and

therefore the survival, of these ecologically relevant organisms (e.g., climate change-related stressor).

Immune activity under warmer conditions

The elevated seawater temperature, within the natural summer ranges experienced by the species, had an enhancing effect on the constituent immunity of *A. calycularis* while almost suppressing the immune response of colonies exposed to LPS. These results suggest a modulation in immune strategy under warmer temperature relative to environmental seawater conditions. Consistent with previous findings on tropical species (Palmer et al., 2011a; Palmer, 2018a), this provides further corroboration that coral immune systems are responsive to environmental changes (Mydlarz et al., 2008; Pinzón et al., 2015; Palmer 2011c; Palmer, 2018a). Such a shift in immune strategy is probably the result of physiological trade-offs, environmentally dependent (e.g., warmer seawater conditions) and phenotypically plastic, that occur within the limits of maintaining an optimal health-state and balancing the costs of achieving it (Sadd and Schmid-Hempel, 2009; Lazzaro and Rolff, 2011; Mydlarz et al., 2010; Palmer, 2018a; Palmer, 2018b). The gradually boosting of constituent immunity over time at elevated temperatures may represent a viable low-cost strategy to maintain the organism's homeostasis and reduce demand for costly and dangerous immune responses (Lee, 2006; Palmer, 2018a; Palmer, 2018b). Such a constituent immunity, with the environmental cue of warmer seawater in a tolerant coral, seems appropriate given the higher disease risk reported for summer periods (e.g., Cerrano et al., 1999; Vezzulli et al., 2010; Rubio-Portillo et al., 2016). Therefore, this orange coral has the potential to adapt to modulate immunity - i.e., enhance constituent immune levels - in order to increase survival chances under warmer conditions (Mydlarz et al., 2010; Palmer, 2018b).

At the elevated seawater temperature, the immune response to LPS elicitation occurred at the 12-hour time point with both PO- and LYS-like activities. In this temperature-induced delay of the immune response, enhanced activity was present but limited for ALP and EST, and was absent for GPx. The missing immune response at the time zero under warmer conditions could suggest that mounting an immune response is too costly for this coral (Palmer et al., 2011a; Palmer, 2018a). While variable over time, GPx activity did not demonstrate an evident trend to combined treatments of elevated temperature and LPS

exposure. However, the temperature ranges considered in this experiment may not have induced oxidative stress conditions (e.g., Jin et al., 2016), and/or an alternative strategy may have been used to mitigate autoimmune risk (Cerenius et al., 2010; Palmer, 2018a). Two, possibly interacting, hypotheses of how seawater temperature might trigger immune modulation include: (1) during periods of warmer conditions, coral cells release "danger" components, such as nitric oxide and uric acid (Hawkins et al., 2014), which modulate immunity (Gallucci et al., 1999; Palmer, 2018b); (2) the increase in seawater temperature is the cue in itself detected by an anthozoan endocrine-like system (Tarrant, 2015) which signals the immune activation.

After the immune-response peak of LPS-exposed colonies, immune activity levels fell below constituent immunity levels (48-hour time point), indicative of the high energy expenditure of an immune response (Palmer et al., 2011a; Palmer, 2018a; Palmer, 2018b). The immune response, albeit delayed, appears to come at the expense of enhanced constitutive levels and suggests that *A. calycularis* may be more vulnerable to threats after acute immune activity (e.g., caused by pathogen elicitation) under warmer seawater conditions. Such a pattern of immune-dynamics could have implications for this habitat-forming coral during the summer period, when seawater temperatures are higher and the risk of pathogen load and virulence increases (Harvell et al., 1999; Harvell et al., 2002; Mydlarz et al., 2006; Bally and Garrabou, 2007; Vezzulli et al., 2010; Vezzulli et al., 2013).

To date, studies on intra- and interspecific differences in heat-stressed corals have shown highly variable outcomes (e.g., Pinzón et al., 2015; van de Water et al., 2016; Palmer et al., 2010; Palmer et al., 2011a; Palmer, 2018b). As with other invertebrates, the ability of coral to deliver an optimal immune response and maintain healthy constituent immune levels depends on the energy available (Sheridan et al., 2014; Palmer and Traylor-Knowles, 2018). However, given the high energetic cost (as well as the autoimmune risk) of mounting an immune response (Lee, 2006; Palmer, 2018b), the benefits must outweigh the costs incurred by implementing it and/or the risk of not doing so (Lazzaro and Rolff, 2011; Palmer, 2018a Palmer, 2018b). The energy trade-offs are therefore likely to contribute to the variations in immunity observed within coral species (e.g., Palmer, 2010; Palmer et al., 2011a; van der Most, et al. 2011; Wright et al., 2017). However, it is increasingly apparent that the

environmental context (i.e., the Life History of an organism) needs to be considered with measurements of immunity (Mydlarz et al., 2006; van de Water et al., 2015; Wright et al., 2017), given that innate immune responses and responses to environmental factors (generally called "stress responses") are closely intertwined. In this regard, an organism's immune strategies under warmer condition are probably the consequence of energetic compromises acting at different physiological (e.g., different molecular pathways), ecological, and evolutionary scales (Sadd and Schmid-Hempel ,2009; Palmer, 2018b).

This study shows that *A. calycularis*, affected by warmer temperatures, seems to be capable of physiological adjustments in order to promote homeostasis and survival in response to environmental signals. Given the worrying global trend, and particularly in the Mediterranean area (Lejeusne et al., 2010; Darmaraki et al., 2019), a comprehensive approach to coral health from an immunological perspective will provide deeper insight into survival mechanisms under climate change. In research efforts to unravel key components of climate resilience, it becomes imperative to contemplate the entirety of coral diversity across the range of sensibilities in order to effectively conserve, restore, and manage marine habitats.

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Supplementary materials

Table S1 *Output of PERMANOVA pairwise tests examining the effects of LPS treatments (control vs. LPS) at each sampling time on immune enzyme activity of* A. calycularis *for both environmental and elevated temperatures. Phenoloxidase, PO; Glutathione Peroxidase, GPx; Lysozyme, LYS; Alkaline Phosphatase, ALP; Esterase, EST; Control, CRTL; Lipopolysaccharide, LPS; Environmental temperature, ENV; Elevated temperature, ELV; P (MC) = probability level; n.s. = not significant; ***p < 0.001; **p < 0.01; *p < 0.05.*

Immune enzyme		**Temperature**			
		ENV (CRTL vs. LPS)		ELV (CRTL vs. LPS)	
	Sampling time	***t***	**P (MC)**	***t***	**P (MC)**
PO	0	3.87	*	0.75	n.s.
	12	4.44	*	2.16	n.s.
	48	1.16	n.s.	1.81	n.s.
	120	0.44	n.s.	5.90	**
GPx	0	6.73	**	0.60	n.s.
	12	3.48	*	1.91	n.s.
	48	2.34	n.s.	1.50	n.s.
	120	0.84	n.s.	2.93	*
LYS	0	6.34	**	0.20	n.s.
	12	2.41	n.s.	1.89	n.s.
	48	2.89	*	2.67	n.s.
	120	1.03	n.s.	3.50	*
ALP	0	3.47	*	3.15	n.s.
	12	0.13	n.s.	1.28	n.s.
	48	2.34	n.s.	0.21	n.s.
	120	0.62	n..s.	5.80	**
EST	0	6.68	**	0.89	n.s.
	12	1.58	n.s.	0.27	n.s.
	48	1.72	n.s.	0.08	n.s.
	120	0.71	n.s.	2.90	*
Total enzymes	0	5.04	**	1.40	n.s.
	12	2.94	**	1.78	n.s.
	48	1.79	n.s.	1.38	n.s.
	120	0.63	n.s.	4.41	**

Table S2 *Output of PERMANOVA pairwise tests examining the effects of temperature treatments (environmental vs. elevated) at each sampling time on immune enzyme activity of* A. calycularis *for both control (no LPS) and LPS samples. Phenoloxidase, PO; Glutathione Peroxidase, GPx; Lysozyme, LYS; Alkaline Phosphatase, ALP; Esterase, EST; Control, CRTL; Lipopolysaccharide, LPS; Environmental temperature, ENV; Elevated temperature, ELV; P (MC) = probability level; n.s. = not significant; ***p < 0.001; **p < 0.01; *p < 0.05.*

Immune enzyme		**LPS**			
		CRTL (ENV vs. ELV)		LPS (ENV vs. ELV)	
	Sampling time	***t***	**P (MC)**	***t***	**P (MC)**
PO	0	4.76	**	0.84	n.s.
	12	3.62	*	0.66	n.s.
	48	5.85	**	0.77	n.s.
	120	4.97	**	0.30	n.s.
GPx	0	1.38	n.s.	5.66	**
	12	0.43	n.s.	4.91	**
	48	1.05	n.s.	2.19	n.s.
	120	4.48	*	0.43	n.s.
LYS	0	2.09	n.s.	2.80	*
	12	1.00	n.s.	0.07	n.s.
	48	4.74	**	0.42	n.s.
	120	3.97	*	0.25	n.s.
ALP	0	2.67	n.s.	1.40	n.s.
	12	0.66	n.s.	0.01	n.s.
	48	1.43	n.s.	0.73	n.s.
	120	4.13	*	0.90	n.s.
EST	0	1.33	n.s.	1.32	n.s.
	12	1.99	n.s.	0.21	n.s.
	48	1.51	n.s.	0.46	n.s.
	120	3.59	*	0.58	n.s.
Total enzymes	0	2.81	*	1.54	n.s.
	12	1.98	n.s.	0.96	n.s.
	48	3.13	**	0.80	n.s.
	120	4.00	**	1.04	n.s.

Table S3 *Output of PERMANOVA pairwise comparison between sampling times on immune enzyme activity of* A. calycularis *for both control (no LPS) and LPS samples at each temperature treatment (environmental and elevated). Phenoloxidase, PO; Glutathione Peroxidase, GPx; Lysozyme, LYS; Alkaline Phosphatase, ALP; Esterase, EST; Control, CRTL; Lipopolysaccharide, LPS; Environmental temperature, ENV; Elevated temperature, ELV; P (MC) = probability level; n.s. = not significant; ***p < 0.001; **p < 0.01; *p < 0.05.*

Immune enzyme		**Temperature**							
		ENV				**ELV**			
		CRTL		LPS		CRTL		LPS	
	Time	***t***	**P (MC)**	***t***	**P (MC)**	***t***	**P (MC)**	***t***	**P (MC)**
PO	0, 12	1.13	n.s.	0.65	n.s.	1.02	n.s.	0.85	n.s.
	0, 48	0.51	n.s.	2.72	n.s.	0.94	n.s.	1.53	n.s.
	0, 120	1.32	n.s.	2.44	n.s.	2.29	n.s.	3.49	*
	12, 48	0.51	n.s.	2.79	*	2.10	n.s.	2.11	n.s.
	12, 120	0.78	n.s.	2.36	n.s.	3.67	*	3.65	*
	48, 120	1.01	n.s.	0.22	n.s.	1.35	n.s.	1.81	n.s.
GPx	0, 12	2.30	n.s.	1.62	n.s.	0.69	n.s.	3.25	*
	0, 48	1.97	n.s.	2.47	n.s.	0.19	n.s.	0.79	n.s.
	0, 120	0.97	n.s.	5.99	**	3.59	*	0.77	n.s.
	12, 48	0.46	n.s.	0.62	n.s.	0.99	n.s.	2.47	n.s.
	12, 120	1.20	n.s.	4.03	*	2.49	n.s.	0.43	n.s.
	48, 120	1.27	n.s.	4.56	**	4.61	*	1.10	n.s.
LYS	0, 12	2.22	n.s.	0.57	n.s.	0.44	n.s.	1.32	n.s.
	0, 48	0.20	n.s.	4.40	*	1.99	n.s.	0.38	n.s.
	0, 120	0.16	n.s.	5.84	**	1.46	n.s.	1.79	n.s.
	12, 48	1.75	n.s.	1.89	n.s.	2.77	n.s.	1.73	n.s.
	12, 120	1.62	n.s.	2.57	n.s.	2.16	n.s.	2.66	n.s.
	48, 120	0.02	n.s.	2.29	n.s.	0.53	n.s.	2.20	n.s.
ALP	0, 12	0.54	n.s.	3.80	*	2.55	n.s.	2.22	n.s.
	0, 48	1.50	n.s.	1.52	n.s.	2.13	n.s.	0.80	n.s.
	0, 120	1.98	n.s.	3.81	*	5.42	**	3.25	*
	12, 48	0.60	n.s.	2.01	n.s.	0.07	n.s.	1.30	n.s.
	12, 120	0.84	n.s.	0.24	n.s.	2.31	n.s.	1.51	n.s.
	48, 120	0.24	n.s.	2.12	n.s.	1.70	n.s.	2.37	n.s.
EST	0, 12	0.17	n.s.	3.03	*	0.11	n.s.	0.75	n.s.
	0, 48	0.86	n.s.	2.19	n.s.	0.99	n.s.	0.26	n.s.
	0, 120	0.47	n.s.	4.39	*	1.50	n.s.	1.96	n.s.
	12, 48	0.75	n.s.	1.45	n.s.	1.40	n.s.	1.01	n.s.
	12, 120	0.56	n.s.	1.05	n.s.	2.17	n.s.	0.76	n.s.
	48, 120	1.04	n.s.	2.85	*	0.45	n.s.	2.47	n.s.
Total enzymes	0, 12	1.04	n.s.	1.79	n.s.	1.05	n.s.	1.18	n.s.

0, 48	1.04	n.s.	2.67	*	1.40	n.s.	1.10	n.s.
0, 120	1.22	n.s.	3.32	**	2.33	*	2.93	*
12, 48	0.70	n.s.	2.25	*	1.73	n.s.	1.82	n.s.
12, 120	0.86	n.s.	2.10	*	2.70	*	3.05	*
48, 120	0.82	n.s.	1.63	n.s.	1.38	n.s.	2.05	*

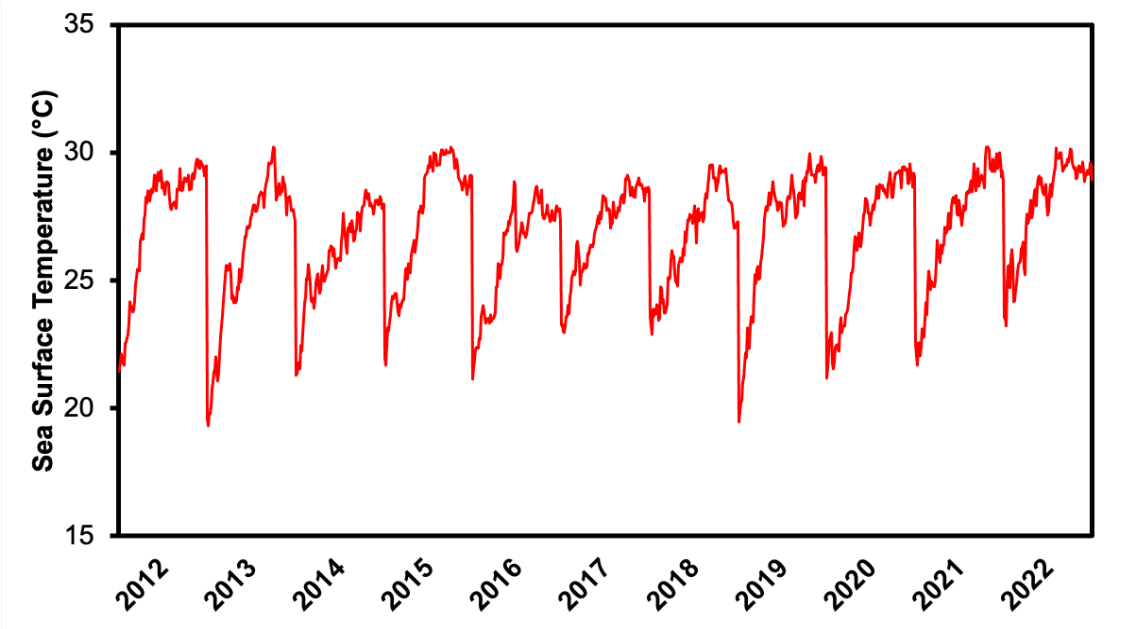

Figure S1 *Sea surface temperature at the sampling site during the summer period (satellite-derived daily average values from 1 June to 31 August) over the last decade (2012-2022), obtained from the Copernicus Marine Environment Monitoring Service (product ID, SST_MED_SST_L4_NRT_ OBSERVATIONS_010_004).*

Chapter 3

Seasonal immune activity and microbiome responses to injury show the detrimental impacts of ocean warming on temperate corals

Abstract

Among the main drivers of critical changes in marine environments is global warming, largely caused by anthropogenic activities. Growing evidence suggests that climate change is rapidly and detrimentally impacting ecosystems worldwide, challenging the survival of marine organisms, including corals. For the first time, we used field-transplant experiments to investigate the seasonal effect of increasing seawater temperatures on the injury responses of several immune-related enzymes (phenoloxidase, glutathione peroxidase and lysozyme) and host-associated bacterial communities in two endemics Mediterranean zooxanthellate coral species: the colonial *Cladocora caespitosa* and the solitary *Balanophyllia europaea*. Our results show that both corals demonstrated strong enzymatic responses in each immune parameter to injury under winter conditions. However, during the summer period, there was an alteration (significant in at least one parameter) of enzymatic activities, outlining a general pattern of enhanced effects of elevated temperatures on constitutive activities but a suppressive impact on injury responses. While winter corals showed relative stable bacterial communities, under summer warming conditions, several taxa of coral-associated microbiota generally associated with coral diseases shifted in abundance and composition. These changes suggest that environmental cues of warming seawater could affect coral health and survival, potentially hindering their ability to recover following physical disturbances under predicted climate change conditions.

Keywords: Climate change, injury, field study, coral immunity, Mediterranean Sea

Introduction

Global climate change is the main threat currently facing marine ecosystems as oceans have been progressively warming over the past four decades at unprecedented rates. Compared to 1850 and 1900, global surface temperatures have increased by 0.8°C and 1.3°C in the period 2010-2019, respectively (IPCC, 2021). Therefore, unless drastic reductions in CO_2 and other greenhouse gas emissions occur, sea warming is expected to result in increases of 1.5 - 2°C during the 21st century (IPCC, 2021). In this environmental context, coral health is declining worldwide due to thermal stress impacts (Hoegh-Guldberg et al., 2007), mainly disrupting the symbiotic partnership with dinoflagellate algae of the *Symbiodiniaceae* family (i.e., coral bleaching), and thus compromising coral survival (Glynn et al., 1993; LaJeunesse et al., 2018).

Many biological processes and key life functions, including sexual reproduction, growth, regeneration, and immune functions can be affected by warming seawater (Gagliano et al., 2007; Palmer, 2018; Carbonne et al., 2024; Sani et al., 2024; Bisanti et al., 2024). Among these processes, coral response to physical injury is a critical challenge as it is both an entry point for pathogens that activate the innate immune system and an energy demand for tissue repair. The increasing severity and/or frequency of reports showing loss of tissue integrity (Garrabou et al., 2019), including physical damage following extreme events (e.g., severe storms) and escalating coastal development, as well as more localized impacts (e.g., destructive fishing techniques and ghost nets, anchoring, diving activities, invasive species, and outbreaks of corallivorous species) (Bavestrello et al., 1998; Kružić et al., 2008; Kružić et al., 2013; Teixidò et al., 2013; Kersting et al., 2014; Casado et al., 2015), has already prompted investigations of the ability of the coral immune system to respond to injury (van de Water et al., 2015; Palmer, 2018; La Corte et al., 2023). To restore tissue integrity after injury, corals have a wound healing process similar to that of more complex organisms (Palmer et al., 2011a; La Corte et al., 2023). In this process, the innate immune system aids in wound sealing, microbial regulation, and tissue regeneration (Palmer et al., 2011a; Palmer and Traylor-Knowles, 2012; Parisi et al., 2021). Thus, determining how rising seawater temperatures affect immune functions in response to injury within and among corals (e.g.,

species with different growth forms, colonial versus solitary) is the next critical step in understanding how these organisms will respond under future climate change scenarios.

Corals possess a range of innate immune and stress response mechanisms as defenses against biotic and abiotic disturbances (Palmer and Traylor-Knowles, 2012; Traylor-Knowles and Connelly, 2017; Parisi et al., 2020). The melanin synthesis pathway (i.e., phenoloxidase cascades) is a key component of immunity, responsible for cytotoxic defense (Nappi and Ottaviani, 2000) and the formation of an impermeable melanin barrier between healthy host tissue and an invading organism (Nappi, 1973). Oxidative stress induced by immune functions, as well as that experienced under warmer water and bright light conditions, can be mitigated by increased coral antioxidant activity (Palmer and Traylor-Knowles, 2012; Traylor-Knowles and Connelly, 2017), including peroxidase, catalase, superoxide dismutase and fluorescent proteins (Halliwell and Gutteridge, 1999; Palmer and Traylor-Knowles, 2012; Traylor-Knowles and Connelly, 2017). In addition, lysozyme activity has recently been detected in corals, representing a bactericidal hydrolytic enzyme that hydrolyzes the β-1,4 glycosidic bonds of the bacterial cell-wall, destabilizing the membrane (Li et al., 2008; Stabili et al., 2015; Bisanti et al., 2024). Lysozyme has also been shown to inactivate some viruses and exert anti-inflammatory action (Leśnierowski and Yang, 2021).

Injured tissues generally present different bacterial communities than healthy tissues (Sunagawa et al., 2009; Cárdenas et al., 2012; Vezzulli et al., 2013; Meyer et al., 2014; Roder et al., 2014; Shirur et al., 2016). Under non-stressful conditions, maintaining an optimal array of microbial symbionts (including the endosymbiotic dinoflagellate, *Symbiodinium* spp., and bacteria) are critical to coral health and survival (Bourne et al., 2016). *Symbiodinium* fixes carbon via photosynthesis, providing carbon- and sulfur-based nutrients to coral holobionts (Rohwer et al., 2002); coral-associated bacteria, in addition to nitrogen fixation (Lema et al., 2012) and sulfur cycling (Raina et al., 2009), produce antimicrobial compounds (Nissimov et al., 2009; Shnit-Orland and Kushmaro, 2009; Kvennefors et al., 2012) and exclude pathogen bacteria by occupying available microbial niches (Rohwer et al., 2002). These communities include thousands of bacterial and archaeal phylotypes in species-specific associations over broad geographic and temporal scales, populating the diverse habitats of

coral anatomical compartments (Apprill et al., 2016). Furthermore, microbiomes are generally considered capable of modifying their composition and functionality in response to environmental conditions (van de Water et al., 2018; Biagi et al., 2020; Sani et al., 2024). Therefore, considering the interactions among host immunity, microbial symbionts, and the environment becomes fundamental to understanding the response of corals to physical injury.

Here, for the first time, we studied the effects of *in situ* exposure to different seasonal temperatures (winter versus summer) on physical injury responses of several immune-related enzymes (phenoloxidase (PO), glutathione peroxidase (GPx), and lysozyme (LYS) and host-associated bacterial communities in two endemic Mediterranean zooxanthellate species: the colonial coral *Cladocora caespitosa* (Linnaeus, 1767) and the solitary coral *Balanophyllia europaea* (Risso 1826), transplanted onto artificial platforms at Capo Zafferano Bay (north-western coast of Sicilian, Italy). Compared to laboratory experiments, transplantation techniques can more adequately represent real-environment conditions since organisms are maintained in natural settings under environmental conditions that are difficult and/or impossible to simulate ex situ (e.g., nutrients, currents, irradiance). This work provides crucial new insights into the effects of global seawater warming on the health and survival of corals in the Mediterranean basin.

Materials and methods

Field activities and experiment periods

Twenty similar-sized samples of each coral species, *C. caespitosa* (fragments) and *B. europaea* (polyps), were collected from Capo Zafferano Bay (38° 06′ 25″ N; 13° 32′ 10″ E; NW coast of Sicily, Italy) during February and July 2024 (Fig. 1a). Coral samples were placed in a container with fresh seawater and transported in a temporary boat wet lab. Upon arrival, *C. caespitosa* fragments and *B. europaea* polyps were randomly assigned to each treatment (control and injury) and glued onto ceramic tiles with a non-toxic bicomponent epoxy coral glue (Milliput, Wales, UK). The number of coral polyps/fragments (i.e., replicates) per treatment/season were 5 (n = 40 per species). Coral samples were mounted on platforms at ~ 5 m depth in the sampling site, mimicking their natural orientation, and placed at least ~ 5 cm away from each other, without coming into contact. After 10 days to

allow coral recovery from sampling and acclimatation to transplant conditions, injuries were inflicted on designated samples using bone-cutters to score a ring approximately 2.5 mm deep (at least 1 cm away from all fragment margins) on *C. caespitosa* fragments, or a longitudinal line along the entire oral axis on *B. europaea* polyps. All corals were sampled 6 h after injury, immediately frozen at -80 °C, and returned to the Marine Immunobiology Laboratory of the University of Palermo for analysis. No mortality occurred throughout the whole experiment, and the corals appeared healthy, without visible paleness (loss of zooxanthellae).

In situ temperature data were collected in triplicate during the experiment using HOBOs Pendant temperature loggers (Onset Computer Corporation) in the following experimental periods (average daily-mean temperature ± standard deviation): February 15 - February 25, 2024 (15.22 ± 0.04°C); July 15 - July 25, 2024 (28.51 ± 0.55°C) (Fig. 1b). Sea-surface temperature data series for the period August 2023 - August 2024 at the sampling site were obtained using Copernicus Marine Environment Monitoring Service (CMEMS) products (Buongiorno Nardelli et al., 2013) (Fig. 1b).

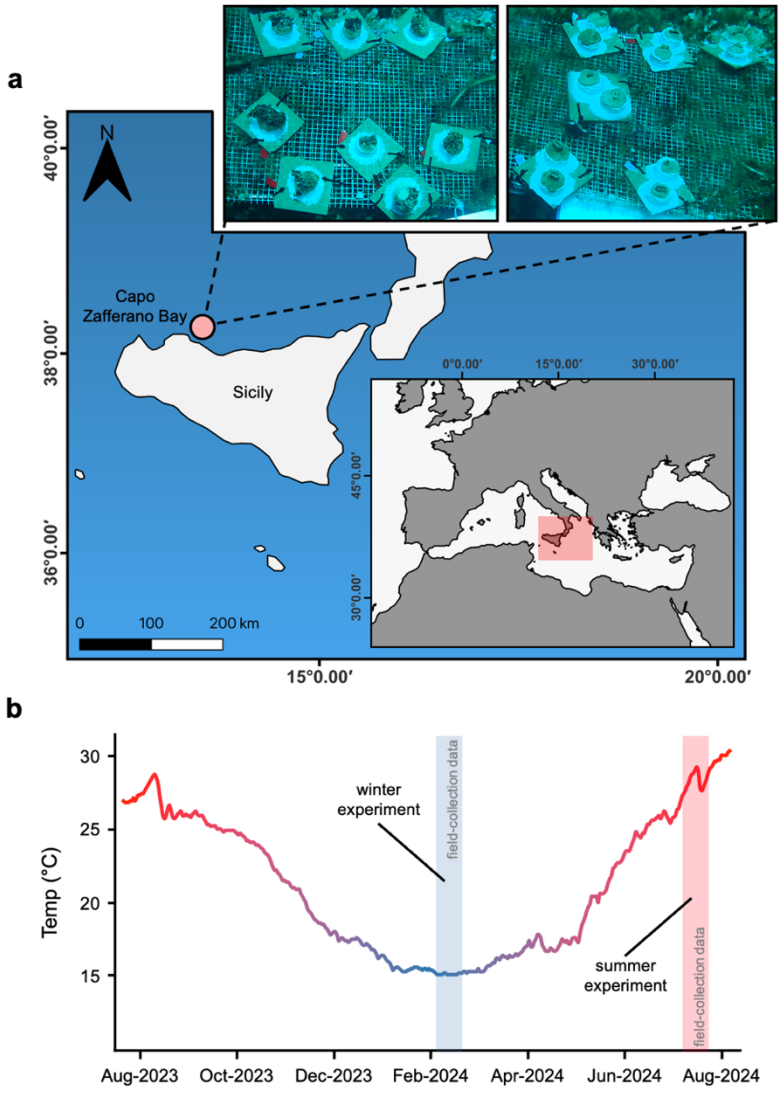

Figure 1 *(**a**) Experimental site located at Capo Zafferano Bay (NW coast of Sicily, Italy). The pictures show representative images of transplanted corals. (**b**) Experimental timeline showing in situ winter and summer experiments, overlaid on a graph of environmental temperature changes.*

Extract preparation and protein concentration

Coral-tissue samples surrounding the lesions were excised under ice using an air-gun in TBS-buffer containing an EDTA-free cocktail of protease inhibitors (Sigma-Aldrich); the resultant coral slurry was then centrifuged (36,200 x g for 20 min at 4°C). The supernatant was collected and protein concentration was spectrophotometrically measured in triplicate (Bradford, 1976). The sample absorbance was read at 595 nm (RAYTO RT-2100C) using TBS as a blank, and a calibration curve defined through bovine serum albumin was used to

obtain the protein concentration (mg/ml). Extracts were adjusted to 0.5 mg/ml before performing enzymatic assays.

Phenoloxidase assay

PO activity was measured spectrophotometrically using L-Dopa (3,4 dihydroxy-L-phenylalanine; Sigma-Aldrich, USA) as a substrate and MBTH (3-methyl-2 benzothiazolinone hydrazone hydrochloride; Sigma-Aldrich, USA) as a specific reagent. 50 µl of coral sample and 50 µl of trypsin from bovine pancreas (1 mg/ml; Sigma-Aldrich, USA) were incubated for 20 min at 20°C in a 50 µl reaction mixture (5 mM L-DOPA and 20.7 mM MBTH in distilled water). Absorbance was read by spectrophotometry for 60 min at 5 min intervals at 505 nm (microplate reader, RAYTO RT-2100C). PO activity was calculated as units (U) per min, where 1 U = 0.001 ΔA540 min^{-1} mg^{-1} protein (Winder and Harris, 1991).

Glutathione peroxidase assay

GPx activity was measured in 96-well flat-bottomed plates, with 50 µl of coral sample incubated with 100 µl TMB (3,3′ 5,5′-tetramethylbenzidine; Sigma-Aldrich, USA). The reaction was stopped after 30 min of incubation in the dark with sulfuric acid (H_2SO_4 2 M). Absorbance was read spectrophotometrically at 450 nm on a microplate reader (RAYTO RT-2100C), and the GPx produced was quantified in U/mg of protein according to the equation: U/mg = Abs * V_f/CP (V_f, final volume of the well; CP, protein concentration of the sample) (Ross et al., 2000).

Lysozyme assay

Coral LYS activity was evaluated following Parry et al. (1965): 30 µl of sample (or TBS buffer for control) were placed in a 96-well flat-bottomed plate and incubated with 270 µl of bacterial suspension (*Micrococcus lysodeikticus* ATCC 4698, Sigma-Aldrich, USA) in triplicate. The reaction was started by incubation at 25°C, and absorbance (450 nm; microplate reader, RAYTO RT-2100C) was measured every 30 s for 10 min. A unit of LYS was defined as the amount of sample causing a decrease in absorbance of 0.001/min (U min^{-1}), and U/ml was calculated according to the formula: U/ml = (Δ abs/min^{-1} * dilution factor * 1000)/enzyme volume buffer.

Microbial DNA extraction and Illumina High-Throughput analysis of the 16S rRNA gene

To extract coral-associated microbial DNA, five samples of both *C. caespitosa* (fragments) and *B. europaea* (polyps) from each experimental treatment were pooled together, and total DNA was extracted using the QIAamp UCP Pathogen Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions, except in the last step, where the elution was repeated twice with 50 µL of DNase- and RNase-free water. The concentration and quality of extracted DNA were checked using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA).

The V3-V4 region of the 16S rRNA gene of bacteria and archaea was sequenced on a Miseq Illumina sequencer with 300 bp paired-end reads. PCR amplification was performed in a 25 µl reaction volume containing 10 ng of DNA, 1 × MightyAmp buffer ver. 2, 0.5 µl of MightyAmp DNA polymerase (TaKaRa Bio, Japan), 0.25 mM of Pro341F/Pro805R primers (Takahashi et al., 2014). PCR consisted of 95°C for 5 min, followed by 36 cycles of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 60 sec for each cycle extension, and 72°C for 10 min for the final extension. Sequences obtained from Illumina sequencing were processed using the QIIME2 software package, version 2023.7.0 (Delhoumi et al., 2020). Operational taxonomic units (OTUs) were clustered with 97% similarity cutoff. Taxonomy was assigned using trained sequences from Silva database, version 138. The 16S rRNA gene sequences were deposited into the NCBI short reads archive (SRA) database under BioProject number PRJNA1168933.

Data analysis

All data analyses were conducted using PRIMER v7+ software (Plymouth Marine Laboratory; Clarke 1993; Clarke and Warwick 1994) and PAST 3.0 software (Microsoft Disk Operating System; Redmond, Washington, U.S.). Normalized data of coral enzymatic activities were analyzed using univariate and multivariate distance-based permutational non-parametric analysis of variance (PERMANOVA; Anderson, 2001; McArdle and Anderson, 2001); the analyses considered: season (2 levels: winter vs. summer), and injury (2 levels: injury vs. control) as fixed factors. PERMANOVA tests were based on Euclidean distance matrix after square root transformation of the data using 9999 permutations under unrestricted permutations of the raw data (univariate tests), or under a reduced model

(multivariate test) with a Type III (partial) sum of squares (Anderson, 2001). Due to the restricted number of unique permutations in the pairwise tests, the p-values were obtained from the Monte Carlo samplings. When significant differences were found, a pairwise comparison was done to explore differences among all pairs of the factors. Diversity indices were also calculated (Shannon, Simpson, Dominance and Evenness indices), based on the sequencing data of coral-associated microbiota.

Results

Immune-enzymatic activities

Overall, immune-related activities of the colonial coral *C. caespitosa* showed non-significant effects for the season and injury factors, but a highly significant effect ($p < 0.001$) for the interaction factor (Tab. 1). Pairwise analysis revealed statistically significant variations in coral responses between seasons ($p < 0.001$), as well as significant differences between injured and control fragments in both winter and summer experiments ($p < 0.001$). There were generally significant effects ($p < 0.001$) for season and factor interaction on enzymatic activities of the solitary coral *B. europaea*, while there was no significant effect for the injury factor (Tab. 1). Highly significant differences emerged for both controls and injured corals in seasonal comparison (PERMANOVA pairwise analysis, $p < 0.001$). Even within the season factor, there were significant differences.

Table 1 *Output of PERMANOVA tests examining the effects of season (winter vs. summer) and injury (injury vs. control), and their interaction with immune-related enzyme activities of both coral species C. caespitosa and B. europaea. Phenoloxidase, PO; glutathione peroxidase, GPx; lysozyme, LYS. P (MC) = probability level; n.s. = not significant; ***p < 0.001; **p < 0.01; *p < 0.05.*

Enzyme	Season				Injury				Season x Injury			
	C. caespitosa		*B. europaea*		*C. caespitosa*		*B. europaea*		*C. caespitosa*		*B. europaea*	
	Ps-F	Ps-F	Ps-F	P(MC)	Ps-F	P(MC)	Ps-F	P(MC)	Ps-F	P(MC)	Ps-F	P(MC)
PO	0.04	n.s.	0.22	n.s.	2.80	n.s.	4.34	n.s.	251.3	***	6.29	n.s.
GPx	0.86	n.s.	42.69	***	2.78	n.s.	0.04	n.s.	72.93	***	46.83	***
LYS	1.33	n.s.	25.33	**	0.00	n.s.	0.19	n.s.	66.48	***	47.82	***
Total	0.92	n.s.	15.15	***	1.59	n.s.	2.42	n.s.	100.5	***	25.01	***

Phenoloxidase activity

There were non-significant overall effects of season and injury factors on PO activity for the colonial coral *C. caespitosa*, but there was a highly significant effect ($p < 0.001$) for the interaction factor (Tab. 1). PERMANOVA pairwise analysis revealed significantly higher PO activity in injured fragments than in controls during the winter period, with differences of about 3-fold greater between the experimental groups (Fig. 2). Instead, under summer

conditions, the PO activity of injured corals decreased significantly, by approximately 3-fold, compared to controls 6 h after cutting, which showed values comparable to those of winter injured corals (PERMANOVA pairwise analysis; Fig. 2).

PO activity in the solitary coral *B. europaea* showed no significant overall effects of season or injury factors, nor for the interaction of factors (Tab. 1). Despite the higher mean normalized PO values of injured polyps compared to controls in the winter experiment, there were no significant differences among experimental groups (PERMANOVA pairwise analysis; Fig. 2). No statistical difference between treatments of summer coral was recorded, although injured polyps had significantly lower mean normalized PO values than those injured in winter (PERMANOVA pairwise analysis; Fig. 2).

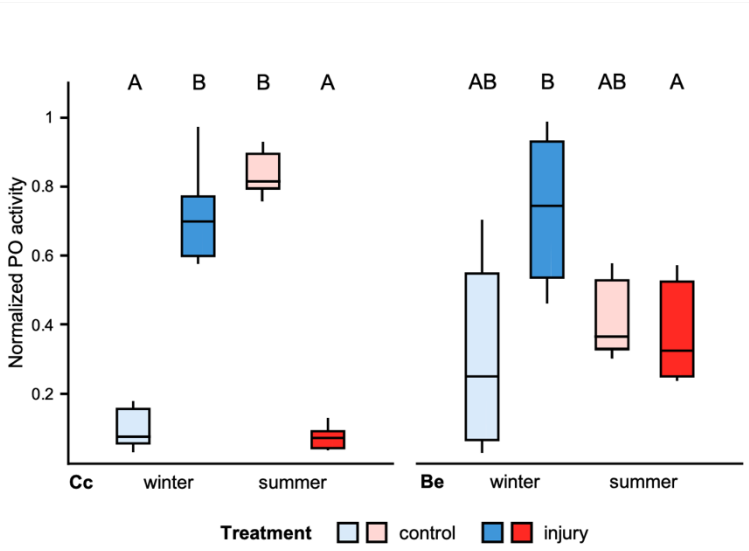

Figure 2 *Boxplots of normalized phenoloxidase (PO) activity (mean ± standard deviation) of both coral species C. caespitosa (Cc) and B. europaea (Be) in response to season (winter vs. summer) and injury (injury vs. control), with the middle horizontal line showing mean normalized values. Letters represent the differences between treatment groups found during the PERMANOVA pairwise analysis ($p < 0.05$).*

Glutathione peroxidase activity

The GPx activity of *C. caespitosa* corals showed no significant overall effects of either season or injury factors, however there were highly significant effects ($p < 0.001$) for their interaction (Tab. 1). Under winter conditions, injured fragments of this colonial species increased significantly, about 2-fold, compared to control group (PERMANOVA pairwise analysis; Fig. 3). Conversely, normalized GPx values of control corals were significantly

higher in the summer experiment, with 4-fold lower activity in injured fragments that was similar to the winter values of controls (PERMANOVA pairwise analysis; Fig. 3).

There were highly significant overall effects ($p < 0.001$) of season and interaction factors on the GPx activity of *B. europaea* corals, but no effect of the injury factor (Tab. 1). Even for this solitary species, PERMANOVA pairwise analysis revealed significant, 2-fold greater, normalized values of injured polyps compared to controls in winter (Fig. 3). During the summer period, there were significantly lower GPx values in injured polyps with a 3-fold higher control activity, but these were comparable to normalized values of winter controls (PERMANOVA pairwise analysis; Fig. 3).

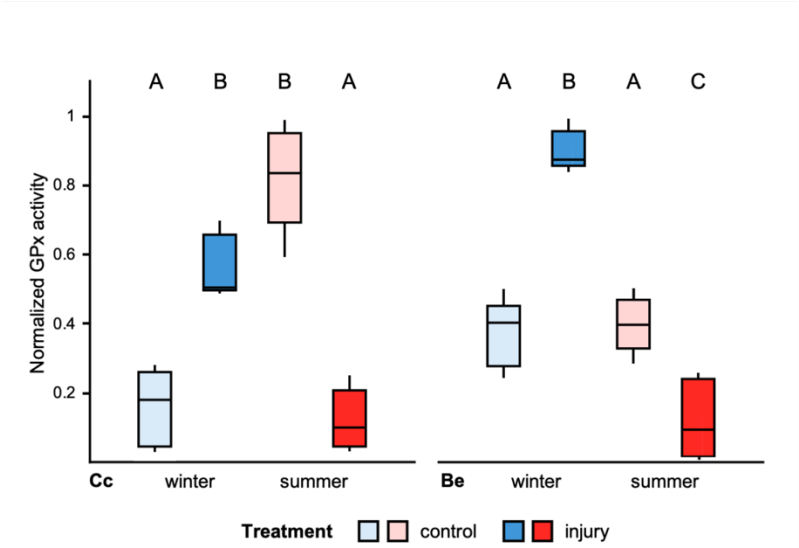

Figure 3 *Boxplots of normalized glutathione peroxidase (GPx) activity (mean ± standard deviation) of both coral species C. caespitosa (Cc) and B. europaea (Be) in response to season (winter vs. summer) and injury (injury vs. control), with the middle horizontal line showing mean normalized values. Letters represent the differences between treatment groups found during the PERMANOVA pairwise analysis ($p < 0.05$).*

Lysozyme activity

No significant overall effects of season and injury factors were found on LYS activity in the *C. caespitosa* species, while there were highly significant effects ($p < 0.001$) for the interaction of factors (Tab. 1). Responses of colonial corals during the winter experiment showed a significantly higher enzymatic activity (approximately 5-fold greater) in injured fragments than controls (PERMANOVA pairwise analysis; Fig. 4). The LYS values of controls increased significantly, by approximately 5-fold, under summer conditions, which

was unmatched in the winter period; in contrast, injured coral activity was significantly lower 6 h after cutting (PERMANOVA pairwise analysis; Fig. 4).

There were significant overall effects of the season ($p < 0.01$) and interaction factors ($p < 0.001$) on the LYS activity of *B. europaea* polyps, but no effect of the injury factor (Tab. 1). In winter, PERMANOVA pairwise analysis revealed significant, 2-fold greater, normalized values of injured polyps compared to controls (Fig. 4). Increased normalized values of LYS occurred in controls, with an approximately 2.5-fold greater activity then injured polyps during the summer period (PERMANOVA pairwise analysis; Fig. 4).

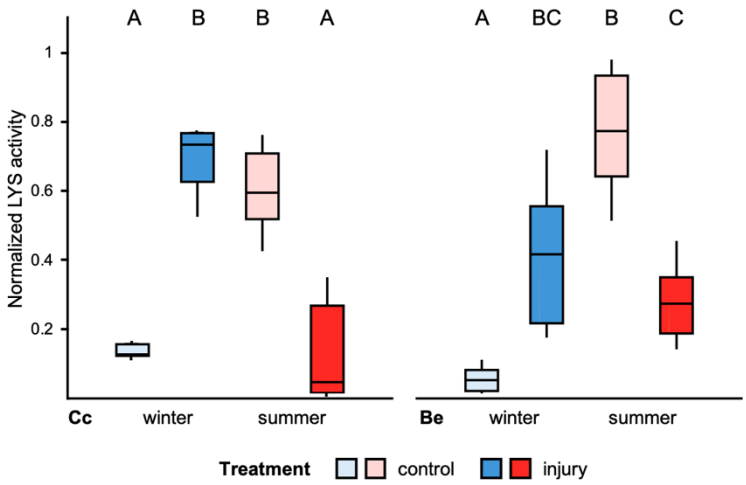

Figure 4 *Boxplots of normalized lysozyme (LYS) activity (mean ± standard deviation) of both coral species C. caespitosa (Cc) and B. europaea (Be) in response to season (winter vs. summer) and injury (injury vs. control), with the middle horizontal line showing mean normalized values. Letters represent the differences between treatment groups found during the PERMANOVA pairwise analysis ($p < 0.05$).*

Coral-associated bacterial communities

Overall, the OTUs associated with *C. caespitosa* ranged from 148 to 317, while from 103 to 228 OTUs were associated with B. europaea (Tab. 2). The Shannon diversity index showed slight variations, with coral-associated microbiota diversity increasing in C. caespitosa, and decreasing in *B. europaea* after injuries in both experimental periods (Tab. 2). Conversely, the dominance index decreased in *C. caespitosa*, and increased in *B. europaea* after injuries during the winter and summer experiments.

Table 2 *Number of reads, OTU and diversity indices calculated on the 16S rDNA metagenomic sequencing of coral-associated microbial communities (C. caespitosa and B. europaea) in response to season (winter and summer) and injury (injury and control). OTUs and diversity indices were calculated from filtered reads (excluding OTUs with less than 0.05% abundance and OTUs classified as chloroplasts)*

	C. caespitosa				*B. europaea*			
	Winter		**Summer**		**Winter**		**Summer**	
	cnt	inj	cnt	inj	cnt	inj	cnt	inj
OTUs	195	148	306	317	208	228	195	103
Dominance	0.036	0.018	0.032	0.021	0.037	0.067	0.043	0.051
Simpson	0.963	0.981	0.967	0.978	0.962	0.932	0.956	0.948
Shannon	4.227	4.386	4.673	4.892	4.118	3.872	3.975	3.554
Evenness	0.351	0.542	0.349	0.420	0.295	0.210	0.273	0.339

Microbial communities associated with *C. caespitosa* were characterized mainly by Gammaproteobacteria, Alphaproteobacteria, Cyanobacteria, Bacteroidota (this latter phylum was abundant only in the summer period). During the winter experiment, Chloroflexota was the dominant phylum in both control and injured corals (Fig. 5a). The abundance of Proteobacteria did not change after injuries compared to control corals, but an increase in the proteobacterial families Rhodobacteraceae and Nitrincolaceae occurred in summer (Fig. 6a). Vibrionaceae were among the few identified families and increased after injuries compared to controls under elevated temperature conditions. Despite Vibrionaceae being mostly unclassified at the species level, the identified taxa included *Vibrio coralliilyticus* and *Vibrio mediterranei*, with *V. coralliilyticus* being more abundant during the summer experiment in injured corals (control 0.04%; injury 0.17%). The phylum Bacteroidota was mainly made up of Flavobacteriaceae and Saprospiraceae in both control and injured corals, especially in summer (Fig. 5a, 6a). Cyanobacteria, mainly consisting of Coleofasciculaceae and Prochloraceae in both winter and summer periods, decreased after injury (Fig. 5a, 6a). Some other phyla, such as Verrucomicrobiota, Nitrospirota, Firmicutes and Campylobacteriota, showed greater abundance in injured corals compared to controls (Fig. 5a). Arcobacteraceae (Campylobacteriota) were also among the most abundant

identified families characterizing the injured corals under elevated temperature conditions (Fig. 6a).

B. europaea coral-associated microbial communities were dominated by Proteobacteria (Gamma and Alphaproteobacteria) and Bacteroidota (Fig. 5b). Moreover, Cyanobacteria were among the most abundant phyla in winter only (Fig. 5b). While the overall relative abundance of these groups remained almost unaltered after injury, other, less represented, phyla increased (Actinobacteria, Firmicutes, Fusobacteriota, and Desulfobacteriota) or decreased (Spirochaetota) in one or both seasons (Fig. 5b). The most abundant families within Gammaproteobacteria were Vibrionaceae and Alteromonadaceae, while the Rhodobacteraceae family was the most abundant in Alphaproteobacteria (Fig. 6b). The Bacteroidota phylum was mostly made up of Flavobacteriaceae (Fig. 6b). Compared to the controls, injured corals showed a higher abundance of families affiliated with Vibrionaceae (Gammaproteobacteria), Prolixibacteraceae and Marinifilaceae (Bacteroidota), Fusobacteriaceae (Fusobacteriota), Fusibacteriaceae (Firmicutes) (Fig. 6b). A lower abundance of Spirochaetaceae (Spirochaetota) under elevated temperature conditions, and of Alteromonadaceae (Gammaproteobacteria) in both winter and summer experiments, were observed in injured corals (Fig. 6b). Vibrionaceae were mostly composed of unclassified Vibrio spp. Identified species included *Vibrio crassostreae* in winter, and both *V. coralliilyticus* and *V. mediterranei* in summer. Notably, an increase in *V. mediterranei* abundance occurred in summer after injury (from control 0.6% to injury 1.2%).

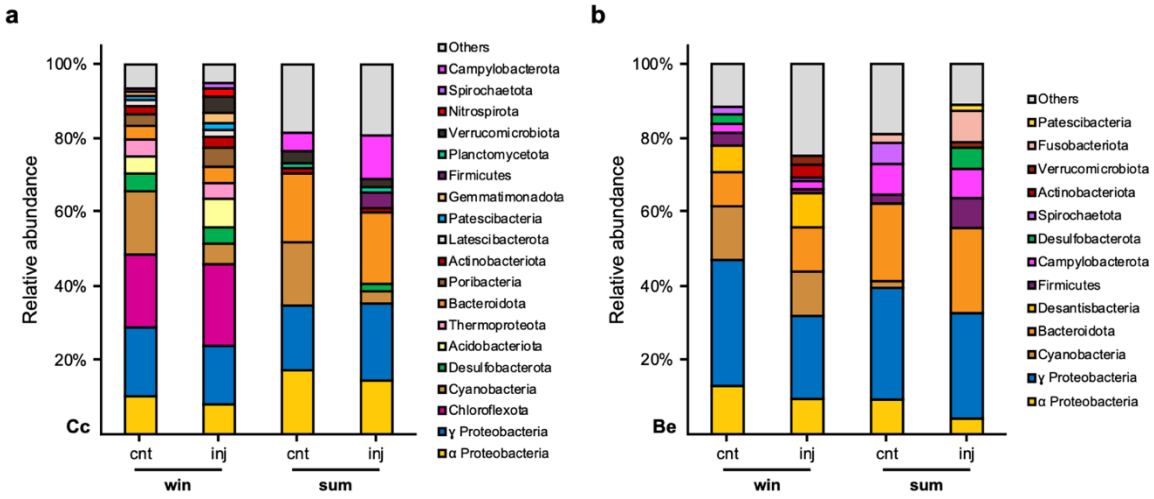

Figure 5 *Composition of (a) C. caespitosa (Cc) and (b) B. europaea (Be) associated microbial communities at phylum level in response to season (winter and summer) and injury (injury and control). Only taxa with relative abundance ≥ 1% are shown. Others includes unclassified taxa and identified taxa < 1%.*

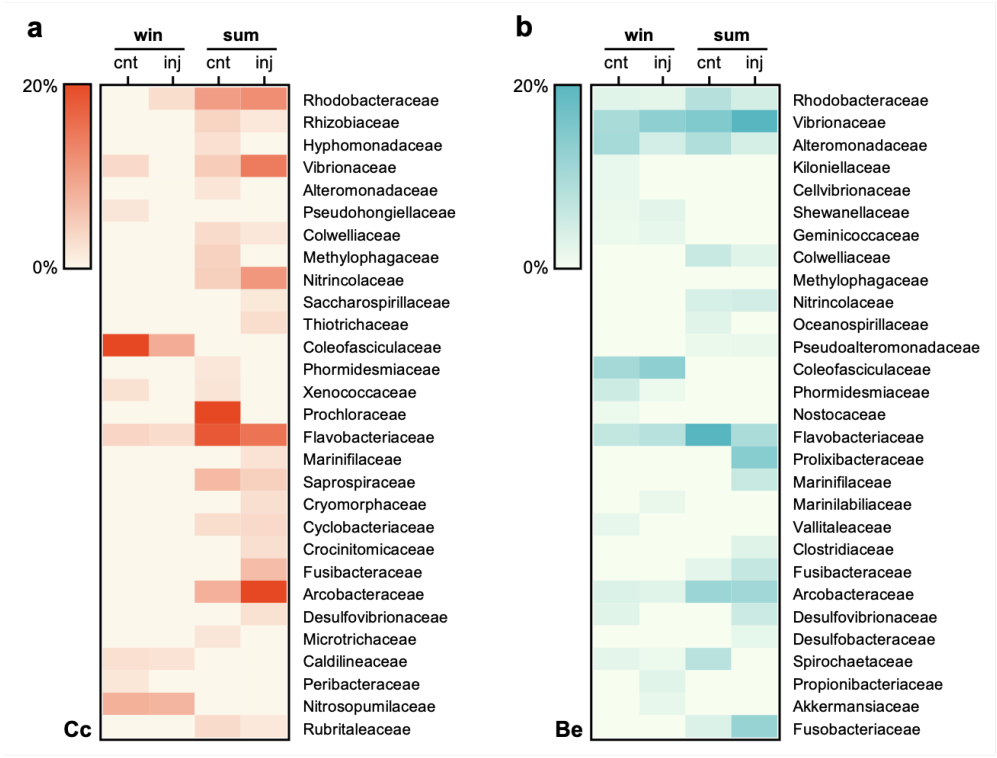

Figure 6 *Heatmaps of (a) C. caespitosa (Cc) and (b) B. europaea (Be) associated microbial communities at family level in response to season (winter and summer) and injury (injury and control). Only taxa with relative abundance ≥ 1% are shown.*

Discussion

Both endemic Mediterranean species considered here, the colonial coral *C. caespitosa* and the solitary coral *B. europaea*, demonstrated an enzymatic response for each immune parameter to injury under winter conditions. Our findings confirm recent studies reporting the presence of immune-related enzyme activity, including PO, antioxidants and LYS within several coral species from both tropical and temperate habitats (Mydlarz et al., 2010; Palmer et al., 2010; Palmer, 2018; Bisanti et al., 2024). Furthermore, both coral species showed a strong alteration (significant in at least one parameter) in enzymatic responses during the summer period, outlining a general pattern of enhanced effects of elevated temperature on constitutive activities, but a suppressive impact on injury responses. The microbiota associated with the two coral species were also different, with notable differences between winter and summer experiments. These results are consistent with variable seasonal patterns in microbiome compositions observed in coral species (Sharp et al., 2017; Haydon et al., 2022). Overall, coral-associated microbial communities shifted in the abundance and composition of several taxa associated with coral disease or identified as opportunistic pathogens, such as Proteobacteria, Bacteroidota, and some other less represented taxa (e.g., Firmicutes, Spirochaetota and Campylobacterota). Nevertheless, changes in community compositions in response to experimental variables (i.e., season and injury) were more evident at lower taxonomy levels. These alterations of enzymatic activity and microbial communities demonstrate that environmental cues of elevated temperature affect coral health, with subsequent critical ecological-implications for the survival of these important marine organisms during the summer periods.

Immune-enzymatic responses

Under winter conditions, PO activity was significantly induced by injuries in the colonial coral *C. caespitosa*, and higher but non-significant enzymatic values were found in the solitary coral *B. europaea*. Our results are consistent with the degranulation timing of melanin-containing cells observed in other tropical species (Palmer et al., 2011a; Palmer, 2018). Some POs (e.g., the tyrosinase-type pathways) are highly cytotoxic, and potentially play a pathogen-killing or sterilization role at the injury site (van de Water et al., 2015). Instead, other less cytotoxic types (e.g., the laccase-type pathway) are likely involved in

structural reinforcement of wounded coral tissue (Palmer et al., 2011a; La Corte et al., 2023). There was also increased and significant activity of the antioxidant GPx (a hydrogen peroxide-scavenging enzyme) for both coral species during the colder period. Serving a protective role, the peroxidase enzymes are important during invertebrate immune responses, which often induce oxidative bursts and can lead to self-harm (Halliwell and Gutteridge, 1999; Nappi and Ottaviani, 2000; Sadd and Siva-Jothy, 2006).

Concurrent with these enzymatic increases, both *C. caespitosa* and *B. europaea* corals showed significantly LYS activation 6h after injuries. This non-specific immune molecule controls humoral bacteriolytic activity in marine invertebrates (Leclerc, 1996; Dhainaut and Scaps, 2001), acting both directly by damaging the invading pathogen cell-walls and as a phagocytosis stimulator (Leclerc, 1996; La Corte et al., 2023). In corals, LYS fulfills the same functions (Stabili et al., 2015), and have already been observed as pathogen eliciting (Bisanti et al., 2024). Although enzymatic activities over time (i.e., homeostasis re-establishment) were not considered in our study, the overall winter pattern suggests that these coral species possess the required resources to mount an effective enzymatic response. Inducing a rapid and effective response to efficiently seal a wound is essential to reducing the likelihood of infection and thus promote coral survival. Indeed, the response immediacy of the coral immune system is a hallmark of invertebrate innate immunity (Palmer et al., 2011b; Palmer, 2018).

Elevated summer temperatures have significantly boosted the constitutive values of all immune-related parameters in *C. caespitosa*, while only the constitutive LYS activity was significantly enhanced in *B. europaea*. Both coral species instead experienced a suppression of enzymatic activity when injured in the summer period compared to winter responses of injured corals. To date, intra- and interspecies studies on heat-stressed corals have shown highly variable outcomes (e.g., Pinzón et al., 2015; van de Water et al., 2016; Palmer et al., 2010; Palmer et al., 2011b; Palmer, 2018). However, the shifted patterns (significant in at least one parameter) observed here corroborate that coral immune activities are generally responsive to environmental changes in an attempt to foster tolerance and promote survival through both biotic and abiotic fluctuations and perturbations (Mydlarz et al., 2008; Pinzón et al., 2015; Palmer, 2018).

Boosted constitutive values of *C. caespitosa* with elevated seawater temperatures may represent a viable low-cost strategy for maintaining homeostasis and reducing demand for costly immune activations (Lee, 2006; Palmer, 2018). Given the higher disease and bleaching risks during the summer months, this coral can potentially modulate its activities, i.e., enhance constitutive levels, in order to increase survival chances under warmer conditions (Mydlarz et al., 2010; Palmer, 2018). For example, inducing PO may be particularly beneficial since melanin is a photoprotective brown to black pigment found within coral cells (Meredith et al., 2006), providing photoprotection for *Symbiodinium* and mitigating coral bleaching (Palmer et al., 2008). The ramping up of constitutive enzymatic activities with the environmental cue of elevated temperatures has already been documented for other tropical and temperate corals (Palmer, 2018; Bisanti et al., 2024). Conversely, the constitutive activity of melanin-synthesis pathways did not increase in *B. europaea*, and there was no enhancement of H_2O_2 scavenging by coral peroxidase. Thus, temperature conditions during the summer experiment may not have been adverse enough to warrant the enzymatic responses of this coral species, as they were not upregulated nor were other immune parameters preferentially used (Jin et al., 2016; Palmer, 2018).

The missing enzymatic increase 6h after injury under summer conditions suggests that mounting an immune-related response would be too costly for both coral species (Palmer et al., 2011b; Palmer, 2018). Injury responses appear to come at the expense of constitutive enzymatic activity and suggest that corals may be more vulnerable to disease and bleaching after physical injury during warmer water conditions. There were similar missing or delayed patterns when *Porites cylindrica* was injured with elevated seawater temperatures in various PO and antioxidant coral activities (Palmer, 2018). But these temperature effects were not universal; for example, basal expression levels of immune genes (involved in the TLR-NFkB pathway and complement system) did not affect injury response after 24h under moderate thermal stress of another tropical species (van de Water et al., 2015). As with other invertebrates, corals' ability to implement responses, maintaining sufficient healthy levels, likely depends on energy compromises acting at different physiological (e.g., different molecular pathways), ecological, and evolutionary scales (Sadd and Schmid-Hempel, 2009; Lazzaro and Rolff, 2011; Mydlarz et al., 2010).

Coral-associated bacterial communities

During the winter experiments, injuries did not cause changes in the overall structure of the microbial communities of *C. caespitosa* and *B. europaea*, which remained relatively stable as observed for other tropical corals (van de Water et al., 2015; Shirur et al., 2016). Instead, summer conditions revealed a different coral-associated microbiota in both species compared to winter corals, and between controls and injured corals. Notably, there were changes in the abundance of taxa generally associated with coral stress and disease or identified as opportunistic pathogens. Elevated seawater temperatures potentially cause shifts in coral bacterial communities towards pathogenic species (Bourne et al., 2008; Littman et al., 2011; Ritchie, 2006), resulting in increased disease prevalence in coral species.

Clear changes in the abundance of Rhodobacteraceae, Vibrionaceae, Alteromonadaceae, and Flavobacteriaceae occurred in injured corals of both species under summer conditions, while few obvious differences were found during the winter experiments. Rhodobacteraceae is a common bacterial family in the marine environment involved in free radical scavenging which reduces the oxidative stress-induced damage to coral symbionts (Lu et al., 2022), such as those produced in warmer seawater. In the summer experiments, both coral species also showed increasing Vibrionaceae occurrence, and in particular of Vibrio spp. including well-known pathogens, especially after injuries. Among them, *V. coralliilyticus* has been already implicated as a causative agent of coral diseases known as white syndromes (Sussman et al., 2008; Ushijima et al., 2014). The virulence of this pathogen is temperature-dependent (Ben-Haim et al., 2003; Kimes et al., 2012), affecting both the coral host and Symbiodinium (Sussman et al., 2009), with critical implications for coral health during the summer months. The Arcobacteraceae, which include sulfide-oxidative species producing both sulfate and filamentous sulfur, and which may help detoxify the surrounding sulfidic microenvironment around corals, were abundant in injured summer corals, and have previously been associated with increases in coral diseases under thermal stress conditions (Wright et al., 2017; Rubio-Portillo et al., 2018; Howard et al., 2023).

The Nitrincolaceae (potential producers of nitrates, nitrous oxide, and dinitrogen) were observed as coral microbiota members under hypoxic stress and poor host health conditions.

They may play a role as opportunistic heterotrophs and increase their abundance in coral-associated microbial communities in response to tissue necrosis, exploiting the subsequent supply of organic matter (Howard et al., 2023). Similarly, other groups, such as Fusobacteriaceae (Fusobacteriota) and Fusibacteraceae (Firmicutes), showing higher abundances in response to injuries, may take advantage of available organic matter. For example, Fusibacteraceae increased in both *C. caespitosa* and *B. europaea* after summer injuries. Members of this family are able to degrade and utilize DNA or molecular subcomponents of DNA as a nutrient/energy source in marine environments (Wasmund et al., 2021). The Prolixibacteraceae, highly abundant only in polyps of injured *B. europaea* during the summer period, were almost entirely composed of the genus *Roseimarinus*. This genus was previously reported as being prevalent in diseased colonies of tropical corals, suggesting a link to the presence of abundant organic matter (Thome et al., 2021; Verhoeven et al., 2018).

Conclusion

This is the first transplant experiment conducted in natural environments aimed at assessing seasonal injury impacts on Mediterranean scleractinian species (the colonial coral *C. caespitosa* and the solitary coral *B. europaea*), in terms of immune-related enzymatic activities and coral-associated bacterial communities. We showed that both coral species seem to implement effective enzymatic responses during the winter experiments. However, warmer summer conditions led to altered immune-related activities (significant in at least one parameter), with increased constitutive enzymatic values but a suppression of injury responses. Microbial communities of both species varied among seasons with different patterns between control and injured corals, especially under summer conditions, while winter corals showed relatively stable bacterial communities. Changes occurred mainly in the abundance of opportunistic-pathogen taxa or bacteria generally associated with coral stress and disease. This emerging seasonal dynamic from our findings, with the impairment of immune-related enzyme activities concurrent with shifting from a healthy microbiome toward a potentially coral pathobiome condition, may have critical implications for Mediterranean temperate corals during the summer months when seawater temperatures are higher and agents triggering physical injuries increase. Our findings contribute to a growing body of evidence showing how rising seawater temperatures may be detrimental to significant components of shallow temperate ecosystems following physical injury, potentially affecting coral health. A thorough understanding of the interactions among hosts, microbial symbionts, and environmental conditions will be the crucial challenge in understanding the drivers of coral survival and resilience in the future.

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Chapter 4

Stress memory regulates immuno-enzymatic enhancement and bleaching resistance via epigenome variation in a Mediterranean hermatypic coral, *Cladocora caespitosa* (Linnaeus, 1767)

Abstract

Stress memory is a key ecological and evolutionary response for sessile organisms under changing environmental conditions, but the ubiquity of this phenomenon among coral species and habitat is unknown. We exposed colonies of the Mediterranean coral *Cladocora caespitosa* to two short-term thermal profiles (constant high and pulse) and quantified the genome-wide DNA methylation level before stress testing 75 days later. Here we demonstrate the durable protective effects of a short-term thermal profile, which significantly improved immune tolerance and bleaching resistance. We found a significative relationship between genomic methylation levels and accumulated thermal stress by corals, preliminary suggesting an epigenetic regulation dynamic of temperate coral tolerance in response to climate change. Our results represent new mechanistic insights into the stress memory of Mediterranean corals, supporting a role for DNA methylation in crucial cryptic complexity of plasticity avenues.

Keywords: Climate change, priming, thermal acclimatation, coral immunity, epigenetics, DNA-methylation

Introduction

Global warming represents a challenge for marine organisms, which can respond to fast environmental changes through non-genetic mechanisms involving changes in phenotype, without changes to the underlying DNA sequences (Hackerott et al., 2021; Drury et al., 2022). Stress memory, a transient "priming" stimulus that leads to a modified response to a subsequent "triggering" stress exposure (also called pre-exposure or preconditioning; Putnam and Gates, 2015; Hawkins and Warner, 2017; NASEM, 2019; Martell, 2023), becomes particularly important in the context of rapid environmental changes because it occurs within the time of a generation (Hilker et al., 2016; Putnam et al., 2017; Hackerott et al., 2021). These short-term responses can bridge the gap between reproductive events, during which genetic adaptation occurs (Munday et al., 2013), potentially allowing organisms to cope with rapid climate change. This phenomenon is well-documented in marine plants (Kishimoto et al., 2019; Nguyen et al., 2020), but has also been observed in planktonic and benthic invertebrates (Klein et al., 2017; Li et al., 2020), and may be particularly important for long-lived, sessile organisms living near their thermal limits such as corals (Coles et al., 1978; Putnam et al., 2017).

Coral health is declining worldwide due to a complex network of local and global environmental drivers (Pandolfi et al., 2003), with the impacts of climate change (e.g., warming seawater and ocean acidification) representing a serious threat to their long-term survival (Hoegh-Guldberg et al., 2007). Thermal stress has been identified as the main factor affecting coral survival (Lough, 2011), as it disrupts the symbiotic partnership with the dinoflagellate algae of the Symbiodiniaceae family (i.e., coral bleaching), thus compromising the coral health-state (Glynn et al., 1993; LaJeunesse et al., 2018). The frequency and severity of coral bleaching events have increased in the last few decades around the world (Hughes et al., 2018; Garrabou et al., 2022) and massive bleaching is expected to occur annually by mid-century, under current emissions scenarios (Van Hooidonk et al., 2016).

Stress memory will play a crucial role as a mechanism by which sessile organisms can respond to rapid climate change (Palumbi et al., 2014; Hackerott et al., 2021). Coral priming to thermal stress can result from changes in the symbiotic algal community (Silverstein et

al., 2015), microbiome composition (Ziegler et al., 2019), and gene expression patterns (Rivera et al., 2021; Drury et al., 2022), allowing for differential responses in growth, morphology, skeletal characteristics and bleaching tolerance (e.g., Todd, 2008; Drury et al., 2017; Drury and Lirman, 2021; Martell, 2023). Interestingly, colonies exposed to environmental stress display a variety of epigenetic marks that regulate gene expression and affect the phenotype (Putnam et al., 2016; Dixon et al., 2018; Eirin-Lopez and Putnam, 2019), and are influenced by the community dynamics of coral symbionts (Hackerott et al., 2021; Rodriguez-Casariego et al., 2022).

Epigenetic mechanisms act as a link between environmental conditions and genetic processes, dynamically altering and in some cases perpetuating gene activation (Cavalli and Heard, 2019; Hackerott et al., 2021). As carriers of environmental information, epigenetic regulation underlies the creation and maintenance of stress memory, within the limits inherent to the organism's genome (Adrian-Kalchhauser et al., 2020). Epigenetic modifications include post-translational modifications of histones (Lämke et al., 2017; Fabrizio et al., 2019), reduced nucleosome occupancy (Brzezinka et al., 2016), DNA methylation (Wibowo et al., 2016), as well as genetic regulation by small RNAs (Stief et al., 2014). In particular, the regulation of DNA methylation appears to be involved in the reduction of spurious transcription in corals (Putnam et al., 2016; Liew et al., 2018; Dixon et al., 2018) and other cnidarians (Li et al., 2018), which could be fundamental in the activation of stress tolerance in primed colonies. Seasonal patterns of DNA methylation have also been reported in *Acropora cervicornis* (Rodríguez-Casariego et al., 2020), suggesting a role for epigenetic modifications in mediating seasonal acclimation in corals (Jurriaans and Hoogenboom, 2020).

Stress memory, priming, or stress-hardening have all been identified as promising research avenues in corals conservation (van Oppen et al., 2015; NASEM, 2019). A key question is how the stress dose (i.e., temporal and magnitude variability) affects coral functional traits, which is complicated given the intricate network of interactions between the host animal, symbionts, and the microbiome. In fact, previous work has documented both negative and positive feedbacks between these components (Kellett et al., 2005; Calosi et al., 2008; Gerken et al., 2015), which reflects the presence of complex “hermetic” barriers

(Putnam et al., 2017) where a small dose of stress may benefit an organism, but higher doses will cause deleterious effects (Calabrese et al., 2007; Mattson, 2008; Carelli and Iavicoli, 2016). Our understanding of priming duration and its ability to support thermal tolerance has been limited by experimental approaches, that so far have assessed only short-term phenotype responses (Bellantuono et al., 2012; Bay and Palumbi, 2015). Nevertheless, some recent experiments have used longer recovery periods (up to 4 months), and found that persistent changes in gene expression accounted for improved physiological performance, mainly in apoptosis and cell-death pathways (Ainsworth et al., 2016; Majerová et al., 2021; Drury et al., 2022). The importance of relative temperature variability is also object of debate, as regimes with greater variations may give corals greater thermal tolerance than constant exposures (Oliver and Palumbi, 2011; DeMerlis et al., 2022), but this effect is not universal (Bay and Palumbi, 2015; Klepac and Barshis, 2020; Drury et al., 2022).

Here, we make use of artificial thermal profiles in a laboratory setting to induce warming tolerance associated with stress memory in the zooxanthellate coral *Cladocora caespitosa* (Linnaeus, 1767). This species is the only endemic coral in the Mediterranean Sea forming localized bioconstructions comparable to tropical reefs, of key relevance for marine conservation. Using this approach, (1) we investigate the effectiveness of two temperature profiles in inducing performance benefits in *C. caespitosa*, assessed by changes in innate immune responses and symbiont photosynthetic efficiency, and (2) we run a genome-wide analysis to investigate the role of DNA methylation as an epigenetic control mechanism underlying stress memory. We considered as proxy of immune response the activity of several immune-related enzymes: the non-specific lysozyme, which act in the primary and rapid defense against pathogens and some viruses, exerting an anti-inflammatory action (Li et al., 2008; Leśnierowski and Yang, 2021; La Corte et al., 2023); the phenoloxidase, responsible for cytotoxic defense and melanin depositions with photoprotective effects on the algal endosymbionts and bleaching mitigation (Nappi and Ottaviani, 2000; Palmer et al., 2010); the glutathione peroxidase, a scavenger antioxidant enzyme that counteracts the effects of reactive oxygen species (ROS) under thermal stress conditions (Lesser, 2006; Hawkins et al., 2014; Parisi et al., 2020). This work provides new insights into the use of artificially induced stress memory to increase thermal tolerance in threatened coral species,

and sets a baseline for relevant priming dose that could induce beneficial responses by organisms.

Materials and methods

Experimental design and temperature profiles

Ten large colonies of *C. caespitosa* were collected from Capo Zafferano Bay (38° 06′ 25″ N; 13° 32′ 10″ E; NW coast of Sicily, Italy) during June 2023. Fragments (n = 120) were returned to the Marine Immunobiology Laboratory of the University of Palermo, mounted on labeled plugs, and transferred to indoor mesocosms (Fig. 1). Only colonies belonging to the same natural population were used to limit potential genetic background effects. After 2 weeks of indoor acclimation, corals were exposed to either a control or one of two short-term thermal profiles - constant high and pulse - between 28 and 31°C for 5 days (Fig. 1b) in replicate tanks (n = 2). In order to determine the appropriate maximum temperature of thermal profiles, *C. caespitosa* fragments of opportunity were exposed to increasing peak temperatures over the course of 12 hours, then kept at each temperature for six days. Fragments were exposed to maximum temperatures of 30, 30.5, 31, 31.5, and 32°C, and monitored daily to detect bleaching responses. After the six-day trial, corals in the 31.5°C and higher temperature treatments had visibly bleached. Thus, 31°C was chosen as the maximum temperature for the acclimatization profiles in order to ensure coral fragments in all treatments, including constant high temperature conditions, would be exposed to sub-lethal thermal stress. The pulse treatment varied from the lowest temperature point of 28 to 31°C daily. The constant profile remained at 31°C throughout the treatment period, while the control tanks were kept at ~ 28°C, close to the environmental temperature. Temperatures in the tanks were programmed and controlled using an Haquoss thermo-control system (Aquarialand s.a.s) and recorded throughout the experiment using HOBO Pendant temperature loggers (Onset Computer Corporation). Thermal profiles have accumulated 0.03 - 2.14 degree heating weeks (DHWs; Fig. 1c) had a ~ 12-fold range in rate of change (°C/hr; Fig. 1d). After the priming, a subset of corals (n = 35 fragments per treatment, N = 105) were returned to the collection site on July 25th and mounted on a platform at ~ 5m depth for recovery prior to subsequent stress testing 75 days later. In situ temperatures were recorded during the recovery period using 3 HOBO loggers.

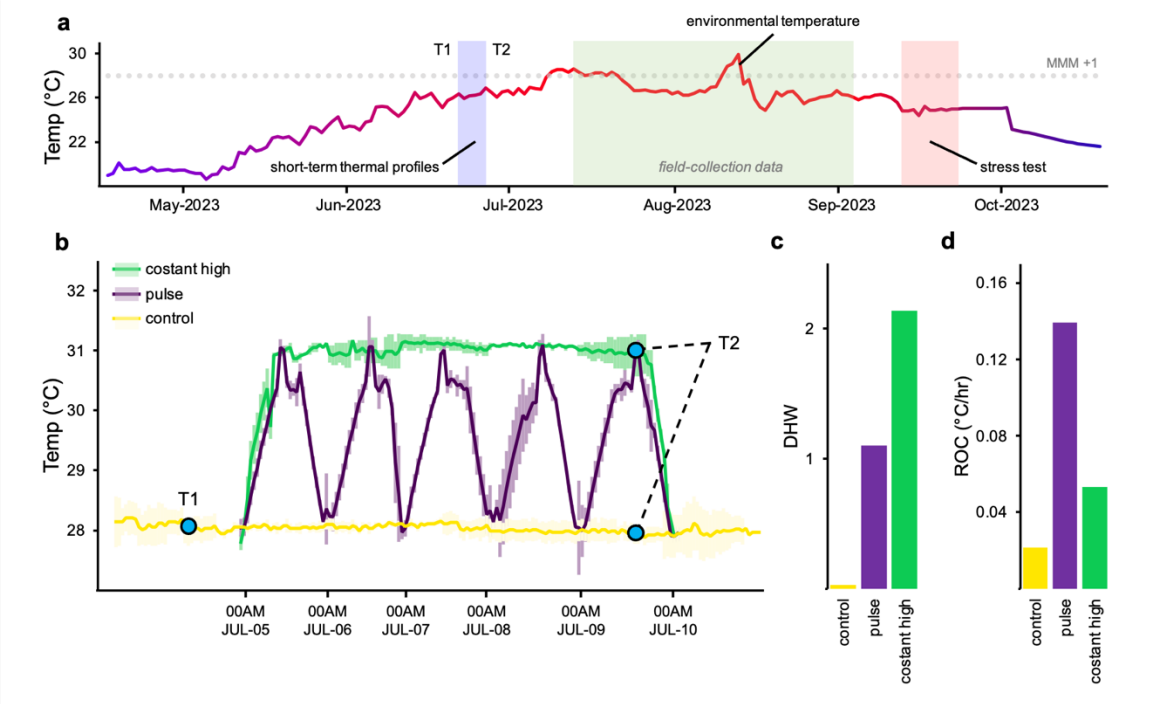

Figure 1 *Experimental Design (**a**) experimental timeline showing short-term thermal profiles, field deployment and stress testing, overlaid on ambient temperatures (**b**) five-day short-term thermal profiles including a control treatment at environmental temperature and two high temperature profiles with different characteristics (**c**) experimental degree heating weeks (DHWs) during short-term thermal profiles (**d**) rate of change (ROC) during short-term thermal profiles, calculated as °C/hr, averaged across replicate tanks. Treatment colors are maintained across figures.*

Epigenomic assessment of DNA methylation

At noon on day 5 of the thermal profiles (Fig. 1b) when all treatments (excluding control) had reached 31°C, we collected coral samples, flash-frozen and stored them at −80 °C until processing. We collected 5 coral samples per treatment (n = 3), for a total of 15 samples. We used the nuclei isolation method to extract DNA, minimizing contamination with symbiont DNA (Voolstra et al., 2017). Cells of *C. caespitosa* were harvested from a fragment of about 5 cm in 50 ml of 0.2 M EDTA solution refrigerated at 4°C using an air-gun. Extracts were passed through a 100-µm and a 40- µm cell strainer (Falcon® Cell Strainers, ThermoFisher) to eliminate as much algal symbionts as possible. Extracts were then centrifuged at 2000g for 10 min at 4°C. The supernatant was discarded, and the resulting pellets were homogenized with a lysis buffer (G2) included in the Qiagen Genomic DNA Isolation Kit (Qiagen). The DNA was extracted following the manufacturer's instructions using

Genomic-tip 100/G (Qiagen) with few changes (Volpes et al., 2023). DNA concentration was determined at the NanoDrop® ND-1000.

Dot Spot Hybridization Analysis was performed to assess genome-wide changes in coral DNA methylation, (Jia et al., 2017; Bellavia et al., 2022). Briefly, 500 µg of genomic DNA were spotted on the Hybond N+ membrane and fixed with U.V. treatment for 2 min. The membranes were soaked in 5% BSA in TBS-T (NaCl 150 mM, Tris-HCl 10 mM, 1% Tween-20, pH 7.4) for 1 h, to block non-specific antibody binding sites. The membranes were rinsed in TBS-T for 5 min three times and incubated with a rabbit anti-5-methylcytosine (5-mC) (InvitrogenTM, MA5-24694; 1:1000 dilution) monoclonal antibody in TBS-T at 4°C overnight. After three more washes, in TBS-T, the membranes were incubated with a secondary anti-rabbit IgG HRP-linked antibody (1:2000, #7074-Cell Signaling) in TBS-T for 1 h at room temperature. The membranes were washed again for 5 min three times in TBS-T and the enzyme substrate was added. The secondary antibody signals were visualized using a chemiluminescence kit (NovexTM ECL Chemiluminescent Substrate Reagent Kit, InvitrogenTM), through a ChemiDoc apparatus (Bio-Rad Laboratories, Hercules, CA, USA), and images were visualised through densitometric analysis using the open-source software Image J (Schneider et al., 2012) as previously reported (Naselli et al., 2024).

Stress testing and coral response variables

Fragments of *C. caespitosa* were collected from the field in September 2023, 75 days after the conclusion of thermal profile exposures, and randomly allocated into two tanks for stress testing. Each tank was heated from 28 to 32°C over 5 days and then maintained at 32°C (Fig. S1). We collected data from randomly chosen coral samples at noon on the day before the temperature ramp (n = 5 for each treatment), on day 1 and every 2 days during stress testing, totaling 5 samples per treatment (n = 3) per timepoint (n = 6), for a grand total of 105 samples. Coral performances were assessed throughout the stress test duration using 4 response variables, of which 3 enzymatic activities related to coral immune activities, namely (1) lysozyme, (2) glutathione peroxidase and (3) phenoloxidase, and (4) the maximum yield of photosystem II (fv/fm) of dark adapted *in hospite* dinoflagellate endosymbionts (Pulse Amplitude-Modulated fluorescence; PAM). Details for each are below.

Extract preparation and protein concentration

Tissue samples of *C. caespitosa* were excised under ice using an air-gun in TBS-buffer containing an EDTA-free cocktail of protease inhibitors (Sigma-Aldrich); the resultant tissue slurry was then centrifuged (36,200 x g for 20 min at 4°C). The supernatant was collected and protein concentration was measured with a Bradford assay (Bradford 1976) as better specified (Schiera et al., 2023). The sample absorbance was read at 595 nm (RAYTO RT-2100C) using TBS as a blank, and a calibration curve defined through bovine serum albumin was used to obtain the protein concentration (mg/ml). Extracts were adjusted to 0.5 mg/ml before performing enzymatic assays.

Lysozyme (LYS) activity

LYS activity was evaluated following Parry et al. (1965). Briefly, 30 µl of each sample were placed in a 96-well flat-bottomed plate and incubated with 270 µl of bacterial suspension (Micrococcus lysodeikticus ATCC 4698, Sigma-Aldrich, USA) in triplicate. 30 µl TBS buffer were instead used in the control sample. The reaction was started by incubation at 25°C, and absorbance (450 nm; microplate reader, RAYTO RT-2100C) was measured every 30 s for 10 min. A unit of LYS was defined as the amount of sample causing a decrease in absorbance of 0.001/min (U min^{-1}), and U/ml was calculated according to the formula: U/ml = (Δ abs/min-1 * dilution factor * 1000)/enzyme volume buffer.

Glutathione peroxidase (GPx) activity

GPx activity was measured according to Ross et al. (2000). In 96-well flat-bottomed plates, 50 µl of sample at standard concentration (0.5 mg/ml) were incubated with 100 µl TMB (3,3′ 5,5′-tetramethylbenzidine; Sigma-Aldrich, USA). The reaction was stopped after 30 min of incubation in the dark with sulfuric acid (H_2SO_4 2 M). The absorbance was read spectrophotometrically at 450 nm in a microplate reader (RAYTO RT-2100C), and the GPx produced was quantified in U/mg of protein according to the equation: U/mg = Abs * V_f / CP (V_f, final volume of the well; CP, protein concentration of the sample).

Phenoloxidase (PO) activity

PO activity was measured spectrophotometrically according to Winder and Harris (1991), by using L-Dopa (3,4 dihydroxy-L-phenylalanine; Sigma-Aldrich, USA) as a substrate and

MBTH (3-methyl-2 benzothiazolinone hydrazone hydrochloride; Sigma-Aldrich, USA) as a specific reagent. 50 µl of coral tissue sample and 50 µl of trypsin from bovine pancreas (1 mg/ml; Sigma-Aldrich, USA) or 50 µl of TBS buffer, as control, were incubated for 20 min at 20°C in a 50 µl reaction mixture (5 mM L-DOPA and 20.7 mM MBTH in distilled water). The absorbance was read for 60 min at 5 min intervals by spectrophotometry at 505 nm (microplate reader, RAYTO RT-2100C). PO activity was calculated as units (U) per min, where 1 U = 0.001 ΔA540 min^{-1} mg^{-1} protein.

Chlorophyll-a fluorescence

A diving-PAM (Walz, Germany) fluorescence was used to measure the maximum quantum yield (fv/fm) of photosystem II, which is commonly used as a measure of damage to the photosynthetic apparatus of the algal symbiont following thermal stress and bleaching (Warner et al., 1999; Jones et al., 2000). Corals were dark-acclimated for 45 min and *fv/fm* measurements were recorded by applying a saturation pulse of actinic light (9000 µmol photon m-2 s-1, 800 ms). The maximum quantum yield was calculated using the formula *fv/fm* = (*fm* - *fo*)/*fm* (Murchie and Lawson, 2013), where *fv* is variable fluorescence, *fm* is the maximum fluorescence and *fo* is the basal fluorescence. Triplicate measurements were recorded for each coral fragment, before the temperature was ramped to assess latent impacts of thermal profiles and confirm that photochemical data had a consistent baseline, in addition to measurements at 6 key time points throughout the experiment. During each PAM measurement session, one area of interest per coral was selected (avoiding fragment edges) and *fo* was kept at values between 200 and 400 prior to applying the saturation pulse. Data were downloaded from the diving-PAM and filtered. *fv/fm* values less than 0.52 were removed from the data set, as lower densities of light-absorbing *Symbiodiniaceae* in thermally stressed coral tissues can cause problems with PAM fluorometry measurements, due to back-scattering of light by the coral skeleton (Wangpraseurt et al., 2019). *fv/fm* values above 0.84 were also removed from the data set, as they are higher than theoretical maximum values of *fv/fm* using PAM fluorometry (Walz, 2020).

Data analysis

All data analyses were conducted in XLSTAT software (Lumivero, 2023.3.0). We compared raw values of response variables before stress testing across thermal profile using

a one-way ANOVA to ensure recovery from thermal profile treatments. Normalized data were analyzed using a linear mixed-effects model with thermal profile and timepoint as fixed effects, and fragment as random intercepts. A post-hoc interaction analysis was used to detect differences between factor levels.

In order to determine which characteristics of temperature profiles may be important for inducing stress memory, we explored the relative importance of total thermal stress accumulated by corals during the temperature profiles and physiological responses to stress testing. We performed polynomial regressions to examine the relationship between the response variables during the stress test and the accumulated DHWs. DHWs were approximated as the time spent above the 28°C (average summer-monthly maximum +1°C at sampling site), a measure of the total accumulation of coral thermal stress above the typical warmest conditions for a location, which is integrative of temperature and duration (Dilworth et al., 2021). We used this value to calculate the experimental DHW (Leggat et al., 2022) for each experimental thermal profile. Seawater temperature data series at the sampling site were obtained from the Copernicus Marine Environment Monitoring Service (CMEMS) products (Buongiorno Nardelli et al., 2013).

Changes in the DNA methylation occurrence was analyzed using a linear mixed-effects model with fixed effects of thermal profile, and random intercepts of fragment. A post-hoc interaction analysis was used to detect differences between factor levels. We also compared coral methylation patterns between treatments using a linear regression to compare levels of DNA methylation with accumulated DHWs during thermal profiles.

Results

Stress testing

We exposed fragments of *C. caespitosa* to two different high-temperature profiles for 5 days before returning them to their sampling site (Fig. 1). We stress tested corals 75 days after the conclusion of thermal profiles, at which point there was no difference in baseline of response variables between treatments (Tab. S1). During the stress test, there was a significant effect only for time point ($p < 0.001$) on LYS activities. Instead, there was a significant effect of thermal treatment ($p < 0.001$, GPx; $p < 0.05$, PO; $p < 0.001$, fv/fm) and time point ($p < 0.001$) on GPx, PO and fv/fm variables, as well as an interactive effect of treatment and time point ($p < 0.05$) on GPx and PO. Mean LYS activities in pulse and constant high corals were also lower than control, but there were no significant differences among treatments (Fig. 2). Corals that were exposed to the pulse and constant high thermal profiles had lower mean GPx and PO activities than those in the control treatments, even though there were significant differences among constant high fragments (Fig. 2). Corals from both constant high and pulse treatments had higher mean fv/fm than the controls, but only the constant high thermal profile significantly differed (Fig. 2).

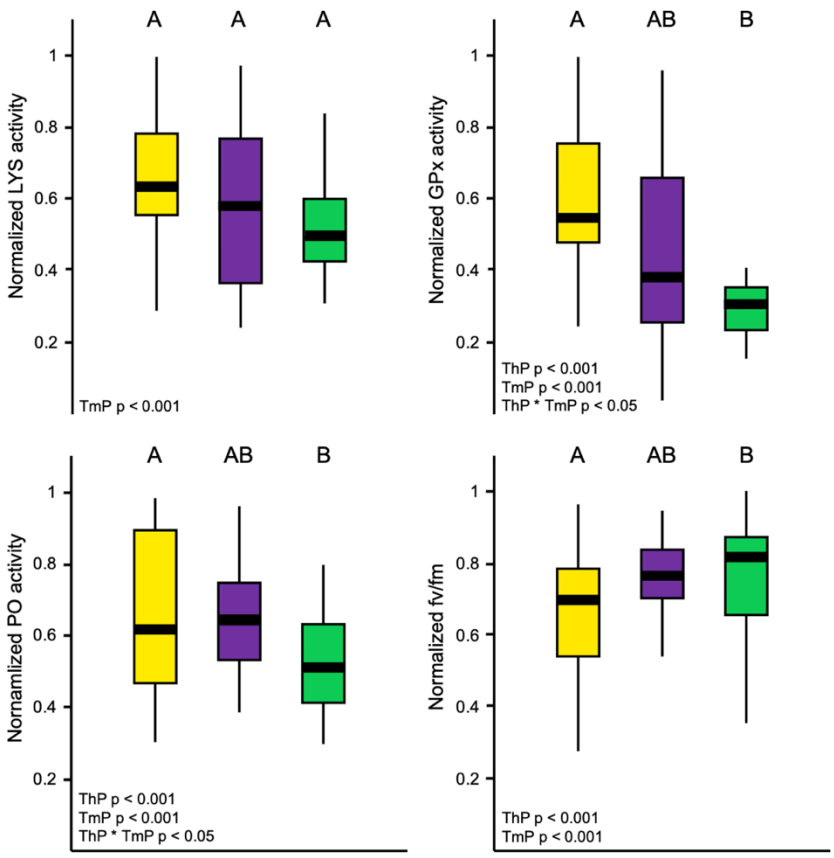

Figure 2 *Boxplots of normalized response variables (lysozyme, LYS; glutathione peroxidase, GPx; Phenoloxidase, PO; maximum quantum yield of photosystem II, fv/fm) by thermal profile treatments averaged over time in fragments of C. caespitosa, with the middle horizontal line showing mean normalized values. Colors indicate thermal treatments: control (yellow), pulse (purple), constant high (green). Letters represent the differences between treatment groups found during post-hoc interaction analysis (p < 0.001). ThP, thermal profiles; TmP, timepoint.*

Corals from the control treatment showed significant relationships in enzymatic variables as accumulated thermal stress increased (LYS: R-square = 0.27, $p < 0.05$; GPx: R-square = 0.64, $p < 0.05$; PO: R-square = 0.37, $p < 0.05$), decreasing steeply after reaching a peak with the highest values found among thermal profiles (Fig. 3). Pulsed corals showed similar significant patterns (LYS: R-square = 0.25, $p < 0.05$; GPx: R-square = 0.50, $p < 0.05$; PO: R-square = 0.36, $p < 0.05$) with an initial rapid increase in enzymatic activity followed by a collapse, with lower peaks compared to control treatment (Fig. 3). Different significant relationships emerged from constant high corals in enzymatic pattern (LYS: R-square = 0.20, $p < 0.05$; GPx: R-square = 0.44, $p < 0.05$; PO: R-square = 0.41, $p < 0.05$), which experienced

lower and almost constant activities as thermal stress increased, compared to other thermal profiles (Fig. 3). There was also a significant relationship between photo-chemical efficiency and accumulated thermal stress by corals (control: R-square = 0.88, $p < 0.05$; pulse: R-square = 0.73, $p < 0.05$; constant high: R-square = 0.59, $p < 0.05$), with less decline in bleaching tendency of corals at constant high compared to control and pulse profiles (Fig. 3).

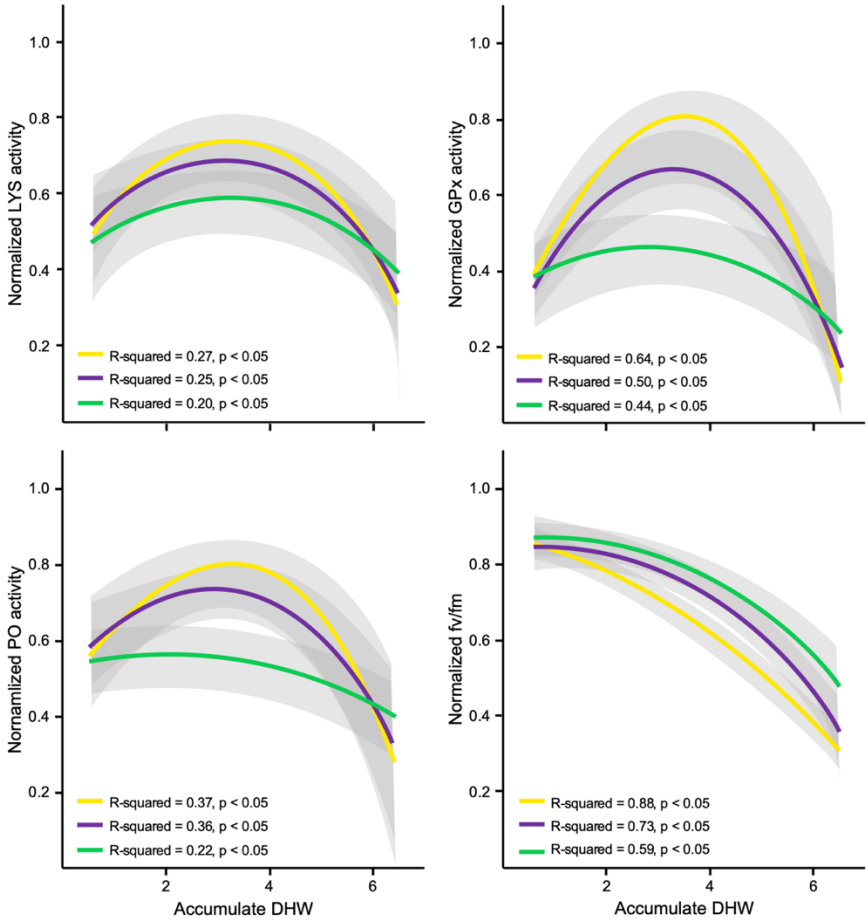

Figure 3 *Polynomial regression plots between normalized response variables (lysozyme, LYS; glutathione peroxidase, GPx; Phenoloxidase, PO; maximum quantum yield of photosystem II, fv/fm) by thermal profiles treatment in fragments of C. caespitosa during the stress test and the accumulated degree heating weeks (DHWs). Colors indicate thermal treatments: control (yellow), pulse (purple), constant high (green). All regression fits include shaded error intervals which represent the 95% CI.*

DNA methylation response to thermal profiles

Starting from the hypothesis that stress memory acts at the level of the regulation of DNA methylation, a spot hybridization of DNA extracted by fragments of *C. caespitosa* was carried

out to assess modifications in the methylation status after the thermal profile treatments, using antibody anti-methyl-cytosine. The pulse and constant high temperature treatments induced a general hyper-methylation of coral genomic DNA, with mean values increasing from 1.6 to 3.1-fold compared to control fragments (Fig. 4a). There was a significant effect of thermal treatments on methylation levels ($p < 0.001$), although a post-hoc interaction analysis revealed significantly higher values only for constant high corals ($p < 0.001$; Fig. 4a). A strong and significant relationship was also found between the accumulated DHWs during thermal treatments and the methylation levels of corals in the corresponding treatments (R-squared = 0.63, $p < 0.001$; Fig. 4b).

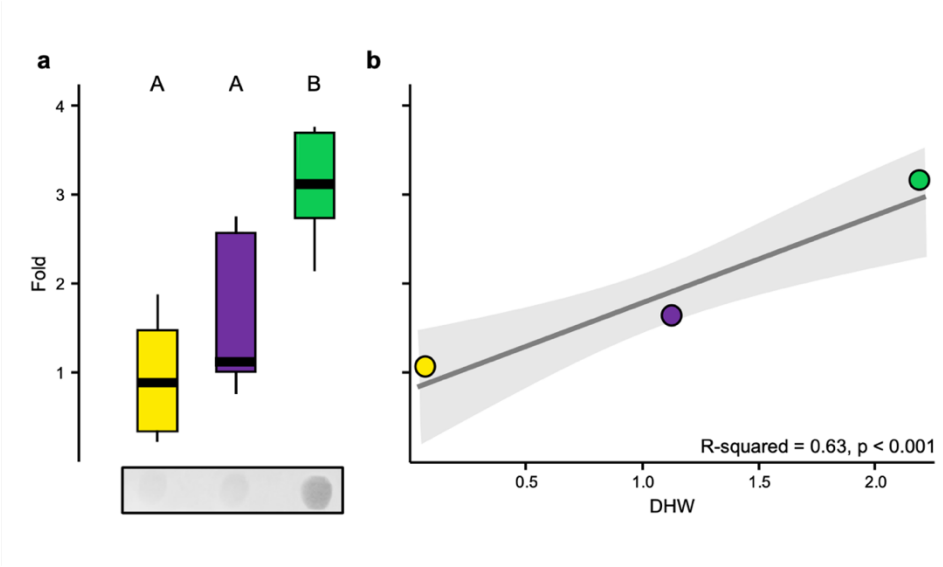

Figure 4 *(a) Representative image and boxplot of densitometric analysis of spot hybridization of genomic DNA from fragments of C. caespitosa by thermal profile treatments using antibody through methyl-cytosine, with the middle line showing the mean values (see Supplementary materials for full-length spot images). Letters represent the differences between treatment groups found during post hoc interaction analysis. (b) Linear regression plot between densitometric data by thermal profile treatments and the accumulated degree heating weeks (DHWs). The gray line represents overall relationship and shaded area is 95% CI of linear regression fit. Colors indicate thermal treatments: control (yellow), pulse (purple), constant high (green).*

Discussion

The resistance of marine ecosystems to ocean warming requires both short- and long-term stress responses. For long-lived sessile organisms that have a limited ability to move towards better environmental conditions, stress memory could play a crucial role, especially in view of the rapid climate changes expected in the near future. Here we show that changes in genome-wide DNA methylation levels during short-term thermal profiles are correlated with a latent, durable boost of thermal tolerance during a subsequent stress test in a reef-forming Mediterranean coral species. This acclimatization phenomenon highlights the plastic potential in temperate coral organisms.

Our study confirms the durability of stress memory-induced tolerance in corals (Middlebrook et al., 2012; Hackerott et al., 2021), by using an acute thermal profile that still elicits ecologically relevant changes after 75 days. We did not re-collect DNA samples after the recovery period, and there is little information available on the persistence of acquired methylation patterns over time; however, corals that undergo environmental activation of DNA methylation have the potential to fix epigenetic marks over time and transfer them to the offspring (Putnam et al., 2016; Liew et al., 2020). The persistence of the response observed here suggests that a moderate thermal stress outside the warmest period of the year could influence subsequent tolerance levels of corals, which has critical ecological implications for the seasonal dynamics on marine ecosystems.

Significant changes in immune-related patterns after the thermal treatments were found in one of our profiles (constant high temperature) but were latent in the other (pulse temperature). Immune functions sustain organism health and survival, contributing to stress tolerance (Sheldon and Verhulst, 1996; Mydlarz et al., 2006; Palmer, 2018a). However, it has been shown before that temperature can affect coral immune activities and trigger immune-modulations in response to detrimental environmental cues (Lee 2006; Palmer 2018a). Ramping of constitutive enzymatic activities promoting homeostasis under warmer conditions has been documented for both tropical and Mediterranean coral species (Palmer 2018b; Bisanti et al., 2024). Based on this evidence, we have postulated that fragments showing lower immune activation would be less impacted by accumulated thermal stress and, therefore, would show improvement in thermal tolerance (Mydlarz et al., 2010; Palmer

2018a; Yu et al., 2020). Low superoxide dismutase and catalase values in the tissues of primed corals compared to naïve ones have been found to be related with lower ROS damages, promoting higher thermal tolerance in *Acropora pruinosa* (Yu et al., 2020). Here, the lower and constant immune-related patterns of constant high thermal profile indicated a boosted immune-tolerance acquired via stress memory. Activities collapsing beyond the functional thresholds of accumulated thermal stress probably represents the ultimate frontier beyond which corals are no longer able to sustain a response implementation (Palmer 2018a; Palmer 2018b).

Moreover, the bleaching patterns showed little decline in the constant high thermal profile, and a modest benefit in the pulse-treated corals. The ability to improve photo-chemical efficiency has been associated with fine-scale differences in temperature regime, host genotype and relatively stable symbiont community composition (Dilworth et al., 2021). Temperature variability may confer major bleaching tolerance to corals compared to constant exposures (Oliver and Palumbi, 2011; DeMerlis et al., 2022), but these observations are not universal (Bay and Palumbi, 2015; Klepac and Barshis, 2020), and our results suggest that constant high profiles create more beneficial responses. We have not investigated coral genotype and composition of harbored symbiont communities in the natural population of *C. caespitosa* considered in this experiment. However, recent findings suggest that both host genotype and *Symbiodiniaceae* (both tolerant and non-tolerant species) do not intrinsically limit the acquisition of thermal tolerance in *Montipora capitata* colonies (Drury et al., 2022).

Our results support a strong and positive correlation between accumulated total thermal stress and global changes in global DNA methylation. Constant high exposure has elicited a large change in addition of methyl groups (-CH3) to DNA nucleotides, probably related to the lack of cooler and darker night-time conditions, during which corals can respond to DNA alterations (Bertucci et al., 2015). In corals, stress memory activation via DNA methylation may be driven by temperature-related signals, reflective of the surrounding environment, (e.g., Dixon et al., 2018; Dimond and Roberts, 2020; Rodriguez-Casariego et al., 2022). A recent cross-transplantation experiment revealed that colonies showing closer gene methylation-patterns match to local corals reached higher fitness, via balancing between expressions of housekeeping and environmentally responsive genes (Dixon et al., 2018).

Pairing DNA bisulfite sequencing with RNASeq to identify methylation-associated expression patterns may provide further insights into thermal profile features, and allow to identify mechanistic links to coral stress memory.

We documented a 2-fold difference in genomic DNA methylation levels between thermal profile treatments, with an hypermethylation of DNA in corals at constant high profiles. These hypermethylation patterns of genomic DNA are consistent with recent studies showing hypermethylation of genes involved in stress responses of bleaching tolerant corals (Liew et al., 2018; Liew et al., 2020). Increased methylation levels may occur in coral gene-body, intergenic regions, and transposable elements (Rodriguez-Casariego et al., 2022), potentially generating new genetic combinations by selective inhibition of mobile genome elements (Venkataraman et al., 2020; Choi et al., 2020). DNA methylation also underpins changes in symbiont communities, favoring thermal tolerant associations in coral hosts (Rodriguez-Casariego et al., 2022). Thus, DNA hypermethylation in *C. caespitosa* may provide the necessary homeostatic control through changes in transcriptomic responses under dynamic acclimatization mechanisms, such as increased constitutive expression of a variety of genes (Barshis et al., 2013; Putnam et al., 2016).

The results presented here suggest that a long-lasting adaptive effect can be induced by short-term thermal profiles in temperate corals. Thermal tolerance during stress testing resulted in boosted immune tolerance and, consequently, greater coral bleaching resistance. We also document substantial variation in DNA methylation levels in relation to accumulated thermal stress, warranting further investigation on the extent, longevity, and heritability of these epigenetic tags in primed corals. Identification of genomic locations and resulting transcriptional control in a subset of nursery-propagated *C. caespitosa* genotypes is the next step to assess the relevance of epigenetics in survivorship via stress memory. These efforts may prove crucial to averting large-scale losses of corals in the Mediterranean basin, and potentially worldwide, due to future climate change scenarios.

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Supplementary materials

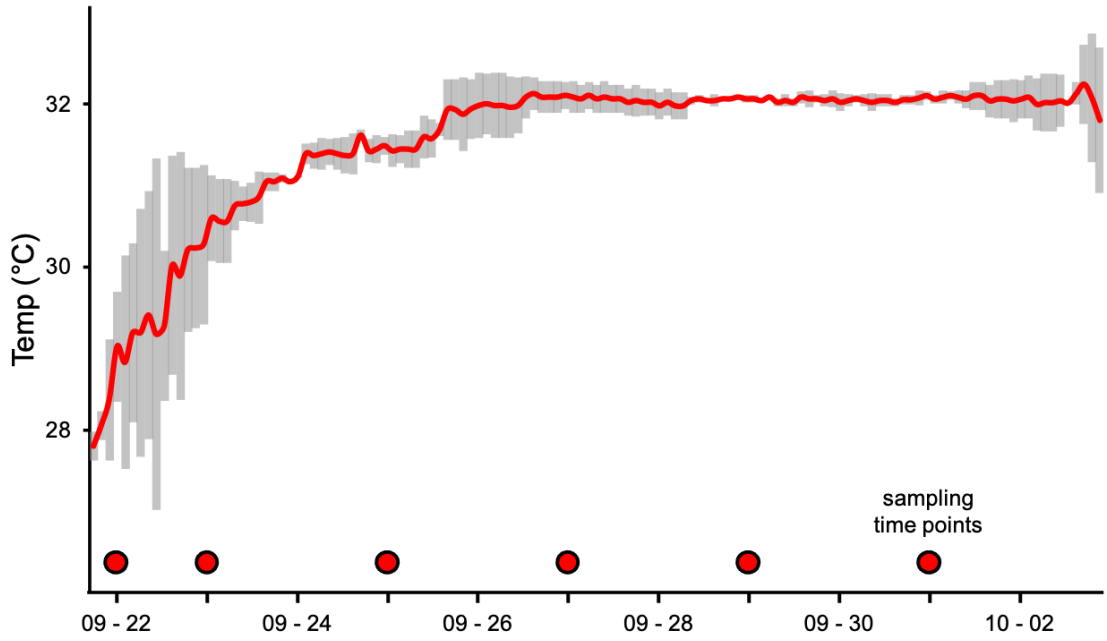


Figure S1 *Timeline of experimental stress-test occurring 75 d after thermal profile treatments. Red line is the average temperature of tanks, and red dots at the bottom represent the sampling time points.*

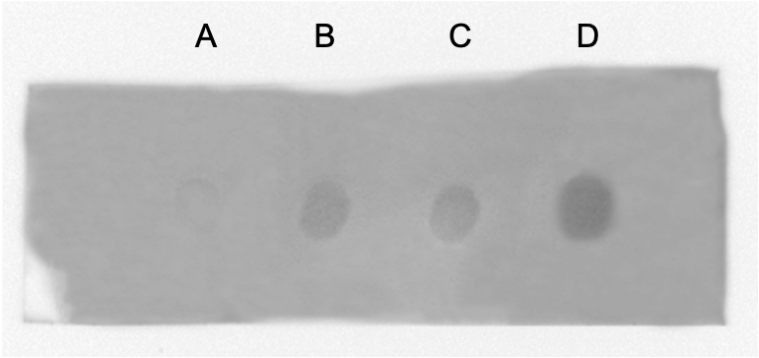
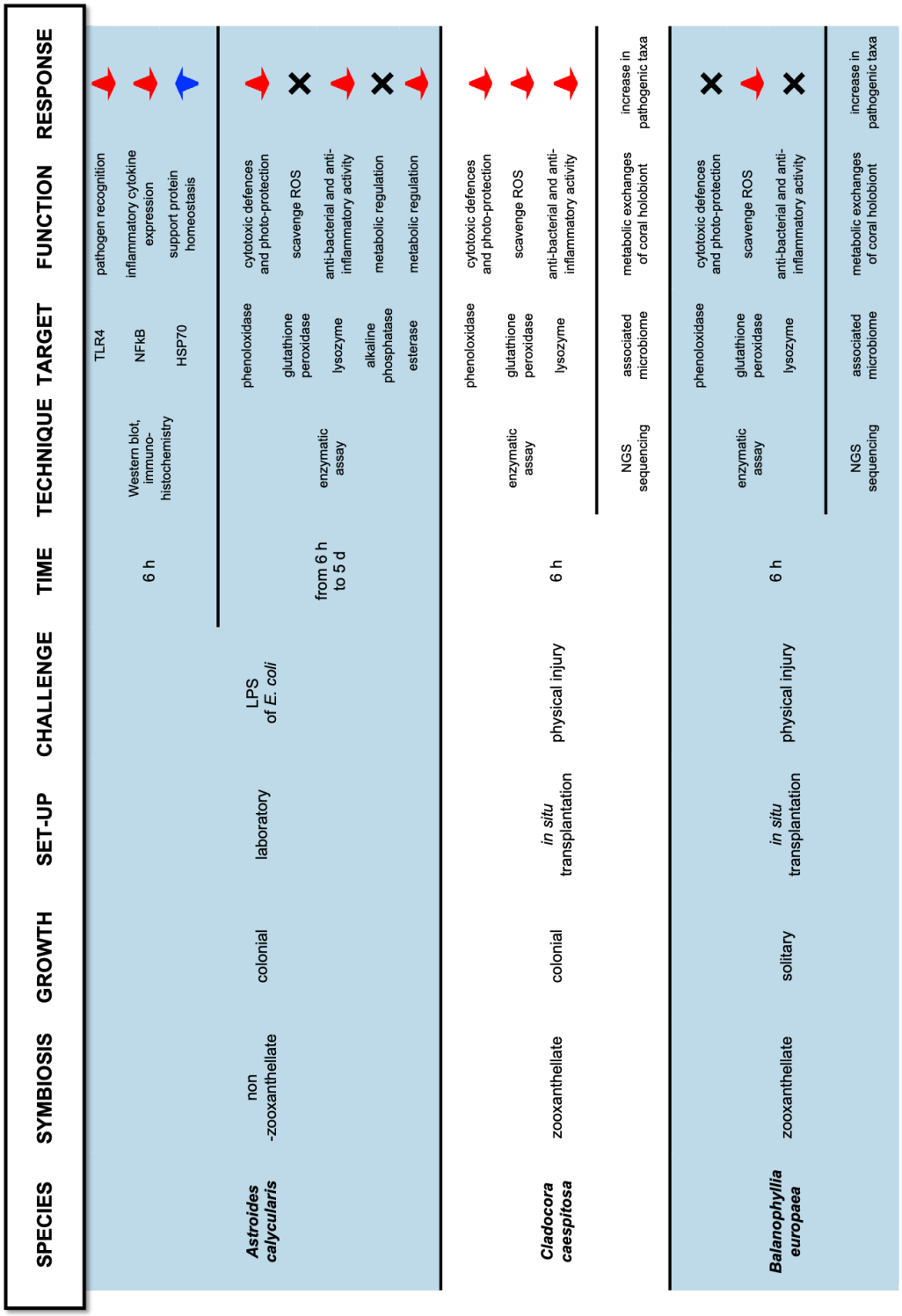


Figure S2 *Representative full-length image of spot hybridization of 5-methyl-cytosine in genomic DNA from fragments of C. caespitosa by thermal profile treatments using antibody through methyl-cytosine (ChemiDoc imaging apparatus). No cross-reactivity was observed in negative controls by the dot blot assay. (A) elution buffer; (B) control; (C) pulse; (D) constant high.*

Final considerations

Addressing the dramatic global loss of coral species is a daunting and bleak task. To date, great efforts have been made to understand how the effects of anthropogenic climate change impact tropical coral reefs worldwide and to unravel the components of climate resilience. Although the Mediterranean region is a climate change hotspot, where the respective impacts of warming are quite pronounced and well-documented (Cramer et al., 2018; Garrabou et al., 2019; Garrabou et al., 2022), much remains to be discovered about the responses of temperate coral species. Here, through a multi-comprehensive approach incorporating elements from fields ranging from biology to ecology, the health of Mediterranean corals under climate change has been considered from an immunological perspective, providing new in-depth insights into impacts and survival mechanisms (the main results are shown in Table 1).

The findings of experiments carried out in the laboratory confirmed the regulation of the TLR-NF-kB pathway, a key component of the invertebrate innate immune system; experiments were also performed on temperate corals under bacterial challenge (Fuess et al., 2016; Fues et al., 2017; Williams et al., 2018; van de Water et al., 2018; Connelly et al., 2020), revealing that they possess a temporally dynamic and responsive cellular machinery for counteracting diseases. Furthermore, the interplay of HSP70 in response to pathogen aggressions further supported its role in immune responses of Mediterranean corals (Brown et al., 2013; Louis et al., 2017). Both laboratory and field experiments revealed that several immune-related enzymes (e.g., phenoloxidase, glutathione peroxidase and lysozyme) are involved in temperate coral responses to pathogen elicitation and physical damage, as had already been observed in tropical species (Mydlarz et al., 2010; Palmer et al., 2010; Palmer, 2018). Overall, temperate corals therefore possess a hierarchically complex, organized set of molecular, cellular and organismal networks involved in immune responses and, under healthy control conditions, appear to be able to activate their correct functioning in response to stressor agents (e.g., pathogen challenges or injuries).



SPECIES	SYMBIOSIS	GROWTH	SET-UP	CHALLENGE	TIME	TECHNIQUE	TARGET	FUNCTION	RESPONSE
Astroides calycularis	non -zooxanthellate	colonial	laboratory	LPS of *E. coli*	6 h	Western blot, immuno-histochemistry	TLR4	pathogen recognition	↓
							NFkB	inflammatory cytokine expression	↓
							HSP70	support protein homeostasis	↑
					from 6 h to 5 d	enzymatic assay	phenoloxidase	cytotoxic defences and photo-protection	↓
							glutathione peroxidase	scavenge ROS	×
							lysozyme	anti-bacterial and anti-inflammatory activity	↓
							alkaline phosphatase	metabolic regulation	×
							esterase	metabolic regulation	↓
Cladocora caespitosa	zooxanthellate	colonial	*in situ* transplantation	physical injury	6 h	enzymatic assay	phenoloxidase	cytotoxic defences and photo-protection	↓
							glutathione peroxidase	scavenge ROS	↓
							lysozyme	anti-bacterial and anti-inflammatory activity	↓
						NGS sequencing	associated microbiome	metabolic exchanges of coral holobiont	increase in pathogenic taxa
Balanophyllia europaea	zooxanthellate	solitary	*in situ* transplantation	physical injury	6 h	enzymatic assay	phenoloxidase	cytotoxic defences and photo-protection	×
							glutathione peroxidase	scavenge ROS	↓
							lysozyme	anti-bacterial and anti-inflammatory activity	×
						NGS sequencing	associated microbiome	metabolic exchanges of coral holobiont	increase in pathogenic taxa

Table 1 *Major results obtained in this scientific research regarding the effects of global sea-warming on temperate corals of the Mediterranean Sea. Red arrows represent downregulation, blue arrows represent upregulation and the crosses represent a neutral effect.*

The implementation of an integrated strategy considering a wide range of functional techniques, histology as well as the use of antibodies towards specific molecular targets has allowed us to show how these coral-response patterns were severely affected by warming conditions. All species considered in this research indicated strong alterations (in almost all immune parameters), outlining a general trend of enhanced effects on constituent immunity but a suppressive impact on injury and/or LPS responses. Comparing the immune activities of the three corals, there were no clear differences in response to elevated temperatures between zooxanthellate and non-zooxanthellate species. However, previous observations on Mediterranean corals have highlighted an increased sensitivity to seawater temperatures of zooxanthellate species, probably due to the inhibition of photosynthesis, with negative effects on growth, regeneration, demography, reproduction and skeletal structural parameters (Goffredo et al., 2008; Goffredo et al., 2009; Caroselli et al., 2011; Fantazzini et al., 2013; Airi et al., 2014; Goffredo et al., 2015; Sani et al., 2024). Indeed, hermatypic corals are highly dependent on their relationship with endosymbiotic dinoflagellates (i.e., *Symbiodiniaceae*), receiving much of their carbon and energy needs from their symbionts (Muscatine et al., 1984; Falkowski et al., 1993; Tremblay et al., 2012). The loss of these endosymbionts, due to detrimental environmental changes, can impact coral energy and carbon balance, leading to death under prolonged and severe stressful conditions (Landsberg et al., 2020; Sikorskaya and Imbs, 2020). But such temperature-related effects are not universal; other comparative experiments demonstrated that warming conditions did not affect net calcification rates in zooxanthellate corals, while showing additive negative impacts on non-zooxanthellate species (Prada et al., 2017).

In laboratory experiments, elevated temperatures plus LPS challenge suppressed the TLR4-NF-kB activation, while HSP70 upregulation appeared in both treatments (LPS-free and LPS-challenge). The activities of several immune-related enzymes (phenoloxidase, glutathione peroxidase, lysozyme, alkaline phosphatase and esterase) were also impaired under warmer conditions and bacterial challenge, with constituent values increasing over time. Likewise, summer field experiments revealed a strong shift in coral enzymatic responses to injury (phenoloxidase, glutathione peroxidase and lysozyme), with an enhancement of constitutive activities but a suppressive impact after physical damages. There were similar missing or delayed immune activities in other tropical corals when

challenged (with both pathogen elicitation and injury) under environmental conditions of elevated seawater temperatures (Mydlarz et al., 2010; Palmer et al., 2010; van de Water et al., 2015; Palmer, 2018). To make matters worse, microbiological analyses from *in situ* experiments revealed a shift in the coral-associated bacterial community during the summer period towards pathobiome conditions. Relevant changes occurred in the abundance of opportunistic pathogenic taxa or bacterial species generally associated with stress and diseases, such as *V. coralliilyticus* and *V. mediterranei* (Sussman et al., 2008; Ushijima et al., 2014; Rubio-Portillo et al., 2020). The immuno-ecological dynamics emerging from these findings, with a general impairment of immune activities concomitant with detrimental microbial shifting in the presence of elevated seawater temperatures, significantly contribute to our understanding of coral health threats and limits and are essential for generating appropriate conservation strategies for the "assisted evolution" of more resilient corals.

Despite the worrying scale of the impacts on the health state of corals shown in this research, these corals also have the ability to acquire and maintain enhanced stress tolerance across transient exposures to a variety of environmental stressors (Pandolfi, 2015; Hilker et al., 2016; Hackerott et al., 2021). Stress memory is a key ecological and evolutionary response for sessile marine organisms under changing environmental conditions, and considering this phenomenon has become critical to better predictions of coral survival chances in future climate change scenarios. Many knowledge gaps still need to be addressed to evaluate the potential for stress-hardening strategies to increase coral resilience, especially given the magnitude of the resources and effort required for their implementation (Hackerott et al., 2021; Martell, 2023).

Given the urgency of characterizing stress memory across coral species and habitats and ascertaining the nature, dynamics and interactions among underlying mechanisms, we used two artificial short-term thermal profiles (constant high and pulse temperature regimes) in a laboratory setting to induce warming tolerance in temperate corals. Furthermore, a genomic analysis was performed to investigate the role of DNA methylation as an epigenetic control mechanism of coral stress memory. The results presented above have indicated a durable protective effect of stress memory in temperate corals, significantly improving immune tolerance and bleaching resistance 75 days after exposure to thermal profiles. Additionally,

a significant relationship was found between genomic methylation levels and accumulated thermal stress in corals, preliminarily suggesting an epigenetic regulation dynamic of coral stress tolerance in response to climate change. This evidence, combined with recent studies revealing that species (including corals) exposed to intense warming events can develop stress memory through epigenetic alterations and subsequent modified gene expression (Maher et al., 2019; Nguyen et al., 2020; Hackerott et al., 2021; Jueterbock et al., 2021; Pazzaglia et al., 2022; Xu et al., 2022), provides a glimmer of hope for the future of corals, potentially enabling them to withstand repeated stressors on both seasonal and annual scales.

Based on the experimental plans and findings presented here regarding the effects of anthropogenic climate change on temperate corals and, consequentially, on their survival mechanisms, future research efforts should be directed toward unravelling the components of stress responses using species across the susceptibility range. Although some dynamics of coral immunity can be conceptualized, such an approach would avoid skewed, misleading and confounding interpretation of results, which likely cannot be accurately generalized to other species or habitats around the world. Additionally, several priorities still need to be addressed before applying coral stress memory techniques to large-scale conservation (see outstanding questions in Figure 1).

Identifying the hormetic dose and temporal regime of primed stimuli will be crucial to informing *ex situ* exposures, as well as to selecting locations with environmental conditions that may confer enhanced stress tolerance. These priming features should be relative to local conditions and/or baseline tolerance thresholds to base their application on temporal, spatial and interspecific differences in coral stress memory. Understanding the persistence of coral stress memory, and therefore the duration of priming enhancement, will also help to maximize the net ecological benefits against the logistical costs of implementing the techniques. The potential trade-offs among functional traits associated with enhanced stress tolerance, such as the inhibition of immune responses, reduction in growth or reproductive output (Jones and Berkelmans, 2010; Jones and Berkelmans, 2011; Palmer, 2018), must also be addressed to evaluate the net physiological benefit to coral performance. Moreover, further description of trans-priming in corals will be essential in order to fully describe the benefits (i.e., increased tolerance toward a specific stressor versus general increased

tolerance of many stressors) and/or potential trade-offs (i.e., increased tolerance toward a specific stressor but reduced tolerance of another) conferred by stress memory. Finally, developing biomarkers of enhanced resilience is essential for informing the marine conservation sector by delineating the mechanistic pathways for establishing and maintaining coral stress memory. While multiple diagnostics and/or response markers can already be used to detect stress in corals (e.g., Louis et al., 2017), biomarkers that predict stress tolerance are lacking. Ideally, stress memory biomarkers should be detectable in primed corals after priming stimuli and be able to predict tolerance during subsequent triggering exposures (Hackerott et al., 2021). Such predictive tools would allow researchers to confirm the success of a priming exposure, isolate primed individuals for targeted propagation, and monitor the persistence of stress memory.

This research has the potential to offer valuable insights for developing conservation strategies in the face of climate change. Protecting corals and Mediterranean biodiversity is the main ecological issue of our time, as it provides a number of contributions to human health, ranging from the more obvious material goods (e.g., food and raw materials) to possibly less evident but still very valuable services (e.g., cultural and recreational services) (Campagne et al., 2015; Paoli et al., 2016; Zuino et al., 2019). Pressing emphasis should be placed on recognizing the connections and scientific evidence that relate nature and biodiversity to human well-being. While this largely hinges on political agendas, scientists can utilize a multidisciplinary framework, combining new immunological information with physiological natural history, to better understand the drivers of endangered coral health and resilience. In doing so, and while pushing for action against global climate change, the ultimate avenue will be to buy enough time to conserve enough coral species to maintain their ecological function.

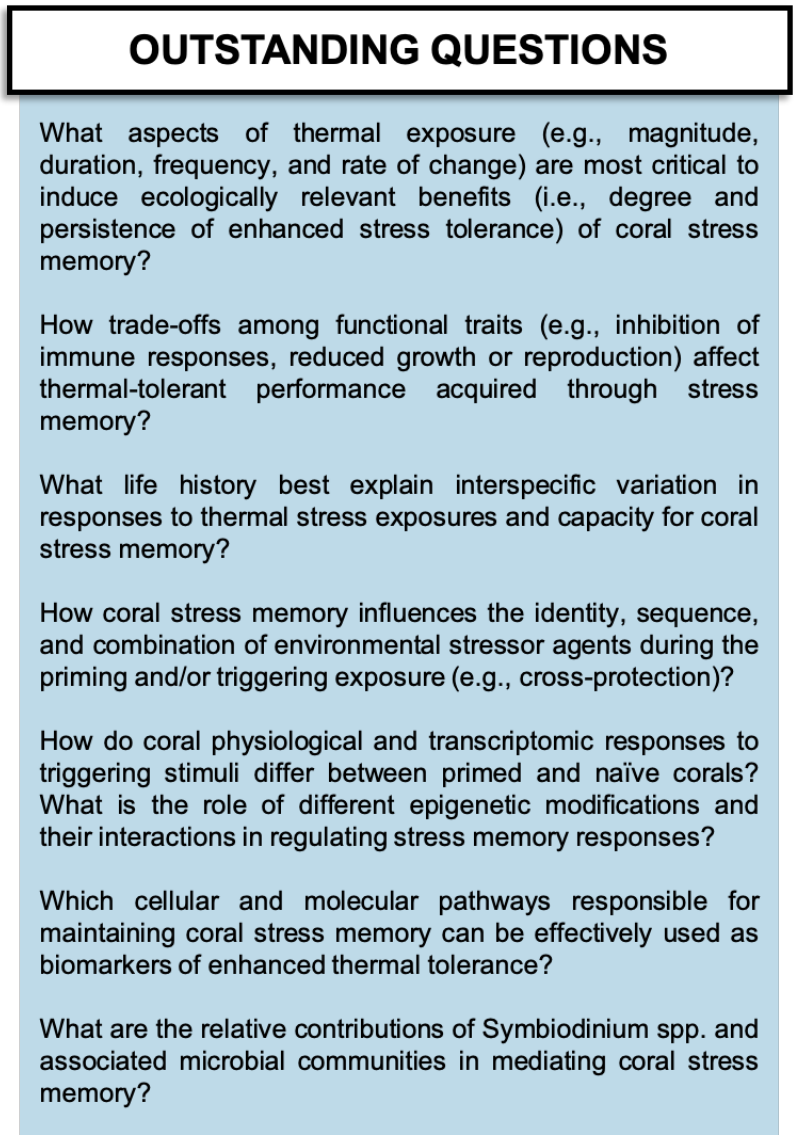


Figure 1 *Future priorities that need to be addressed for the application of coral stress memory to large-scale marine conservation.*

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