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REPORT

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

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Abstract Anthropogenic climate change is warming sea water 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 yrs, where intense warming has been identified as one of the main stressors in coastal regions. To determine the influence of warmer conditions on the immunity of an endemic Mediterranean coral species, different immune activity parameters were compared in response to elevated temperature (~ 28 °C) and the presence of a pathogen-associated molecular pattern—*Escherichia coli* lipopolysaccharide (LPS)—as an elicitor of the innate immune response of *Astroides calycularis*. Immune parameters, which included phenoloxidase-like, glutathione peroxidase, lysozyme-like, alkaline phosphatase, and esterase enzyme activity, were measured over time after LPS balneation (0-, 12-, 48-, and 120 h 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 (0 h-time point), 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 sea water, 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 the world over, 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 the effects of warming marine environments (Harvell et al. 2002, 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 yrs, with sea water 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.

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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, b; 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, 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, b; Palmer and Traylor-Knowles 2018). Despite several recent studies focused on elucidating coral immune responses to pathogens, the direct effects of warmer sea water 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, 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, 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 sea water) (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, b) 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, b). 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, b). 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 sea water 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* were located on the upper littoral zone (~ 4–5 m depth) in Capo Zafferano Bay (38° 11′ 11″ N; 13° 53′ 82″ 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 sea water tank to the laboratory.

Experimental design

Four large aquaria inside a temperature-controlled room were supplied with continuous flow-filtered sea water (1 µm) 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 15:9 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^{-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 sea water 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 (sea water 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 sea water temperatures for the two environmental large aquaria ranged from 22.90 ± 0.17 to 23.54 ± 0.01 °C, while for the higher temperature large aquaria the daily averages ranged from 28.00 ± 0.17 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 sea water, equipped with individual air-stones to aerate and mix the sea water, 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 sea water temperature (~ 23 °C) and control (no-LPS); (2) environmental sea water 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 sea water; (3) elevated sea water temperature (~ 28 °C) and control (no-LPS); and (4) elevated sea water temperature with 5 µg ml^{-1} LPS. The colonies were exposed to a constant flow (from the larger aquaria) of 20 µm-filtered sea water, 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 sea water bath using ~ 5 cm blocks. The sea water in each small tank was carefully exchanged with relative-temperature 20 µm sterile filtered sea water, and 5 µg ml^{-1} LPS dissolved in sterile filtered sea water was added to those designated for LPS treatment. The small tanks were kept slightly raised in the sea water baths

throughout the LPS or control treatments (no LPS) so that the sea water within each small tank remained isolated while sea water 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 h, 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 sea water in each small tank was carefully exchanged with relative-temperature 20 µm fresh filtered sea water, 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 h time point) and 12, 48, and 120 h (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.

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 × 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^{-1} 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^{-1}; 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 $U = 0.001\ \Delta A540\ min^{-1}\ mg^{-1}$ protein.

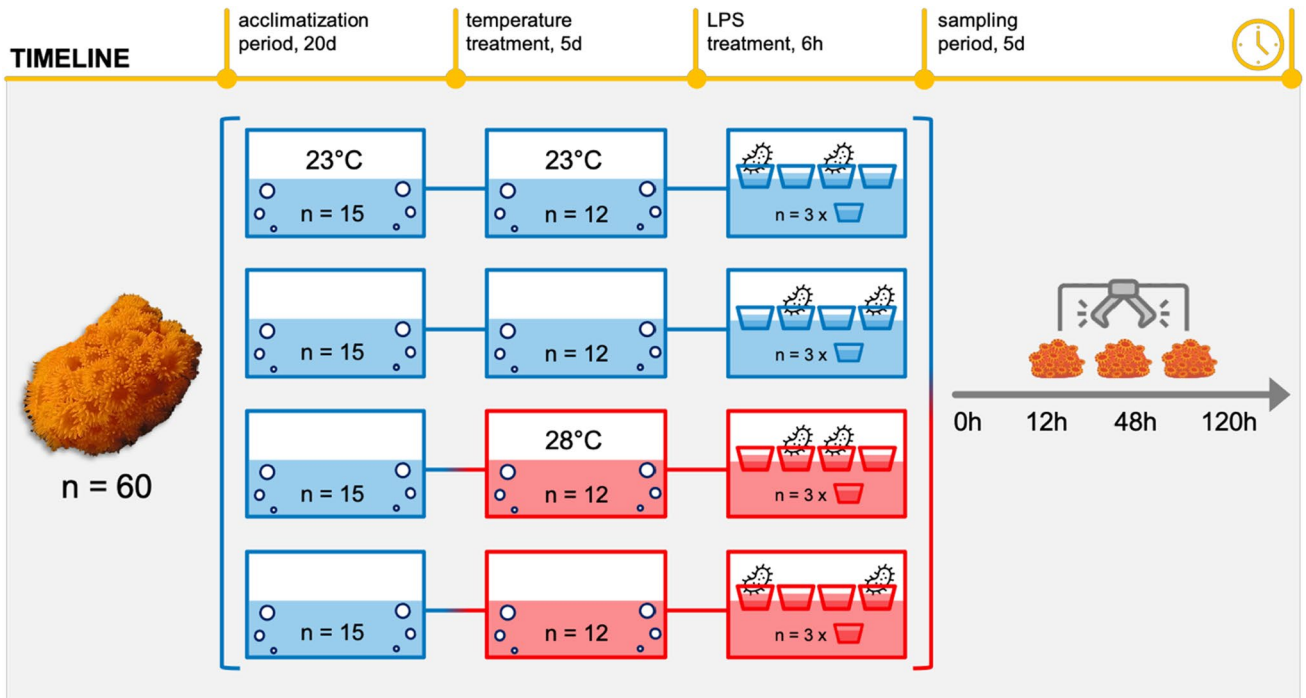

Fig. 1 Schematic drawing summarizing the experimental timeline

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: $U\ mg^{-1} = Abs * V_f / CP$ (V_f, 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 was 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 was 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^{-1} ($U\ min^{-1}$), and U/ml was calculated in accordance with the formula: $U\ ml^{-1} = (\Delta\ abs/min^{-1} * dilution\ factor * 1000) / enzyme\ volume\ buffer$.

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 nonparametric 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 sea water temperature (23 °C) (Table 1; Fig. 2, left). Specifically, differences were found for the 0- and 12 h 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 h 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 sea water temperature (Table S3). The enzyme activity values in LPS-exposed colonies did not vary significantly between sea water 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 sea water temperature (Table 1; Fig. 2A). The increase in PO-like activity was particularly evident immediately at the 0 h time point, when it was approximately threefold greater than LPS-unexposed colonies (pairwise analysis, $p > 0.05$;

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

Immune 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.

PO phenoloxidase, *GPx* glutathione peroxidase, *LYS* lysozyme, *ALP* alkaline phosphatase, *EST* esterase, *CRTL* control, *LPS* lipopolysaccharide, *ENV* environmental temperature, *ELV* elevated temperature

P (MC) = probability level; n.s. = not significant; ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$

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 twofold over time in the elevated temperature treatment, which was absent in the environmental temperature treatment (Fig. 3A). At the 120 h time point, PO-like values were significantly threefold 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 h time point at elevated sea water temperature, which was similar in magnitude to the peak of activity at the 0 h 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 sea water temperature, mean GPx activity was significantly higher in the LPS-treatment compared to the control treatment (Table 1; Fig. 2C). There was a fourfold increase in the activity of this enzyme with LPS at the 0 h time point at environmental temperature, which remained upregulated, compared to controls, for up to 48 h. At elevated sea water 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 h 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-h 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 sea water temperature differed significantly between LPS and control treatments (Table 1; Fig. 2E). In detail, at both the 0- and 12 h time points, LYS-like activity was significantly more than twofold higher in the LPS treatment than in the control treatment (pairwise analysis, $p > 0.01$; Table S3). At the elevated sea water 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 sea water temperature, LYS-like values in LPS-exposed specimens at the 12 h time point were similar to those of the 0 h 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 fourfold over time at the elevated temperature (i.e., 120 h 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 sea water 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 sea water temperature, ALP and EST activity were significantly 2.5- and 3.5-fold higher, respectively, in the LPS-exposed colony at the 0 h time point than in the control treatment (pairwise analysis, $p < 0.05$; Table S1; Fig. 2G, I). At elevated sea water 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 (Fig. 3G, I) and 1.5-fold higher with respect to LPS-elevated

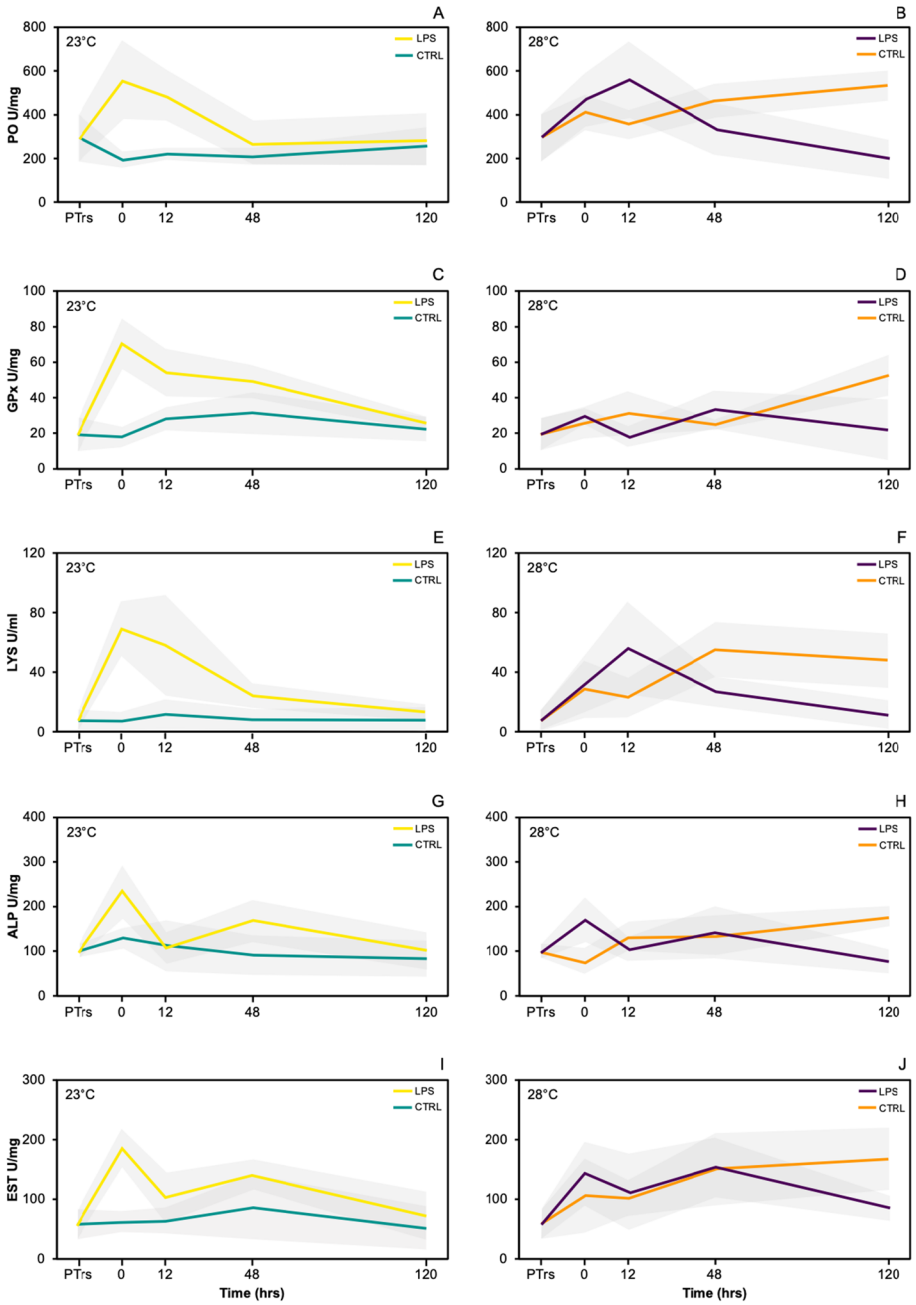

Fig. 2 Mean values (colored lines) of immune enzyme activity ± 95% confidence intervals (gray areas) for control (no LPS) vs LPS-exposed colonies of *A. calycularis* under environmental (23 °C) and elevated (28 °C) sea-water temperatures over time. *PO* Phenoloxidase, *GPx* Glutathione Peroxidase, *LYS*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

values (Fig. 2H, J), at the 120 h time point for both enzymes (Table S1, S2).

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, 2018). An immune response occurred within the 12 h time point in the LPS-exposed sample at environmental sea-water 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 sea water 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.

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

Immune 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	***

PO phenoloxidase, *GPx* glutathione peroxidase, *LYS* lysozyme, *ALP* alkaline phosphatase, *EST* esterase, *CRTL* control, *LPS* lipopolysaccharide, *ENV* environmental temperature, *ELV* elevated temperature

P (MC) = probability level; n.s. = not significant; ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$

Immune activity at environmental sea water temperature

Under controlled environmental conditions, the immune-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, b). 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 h 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 monophenol 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 fourfold higher at 0 h 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 h 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, c, 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

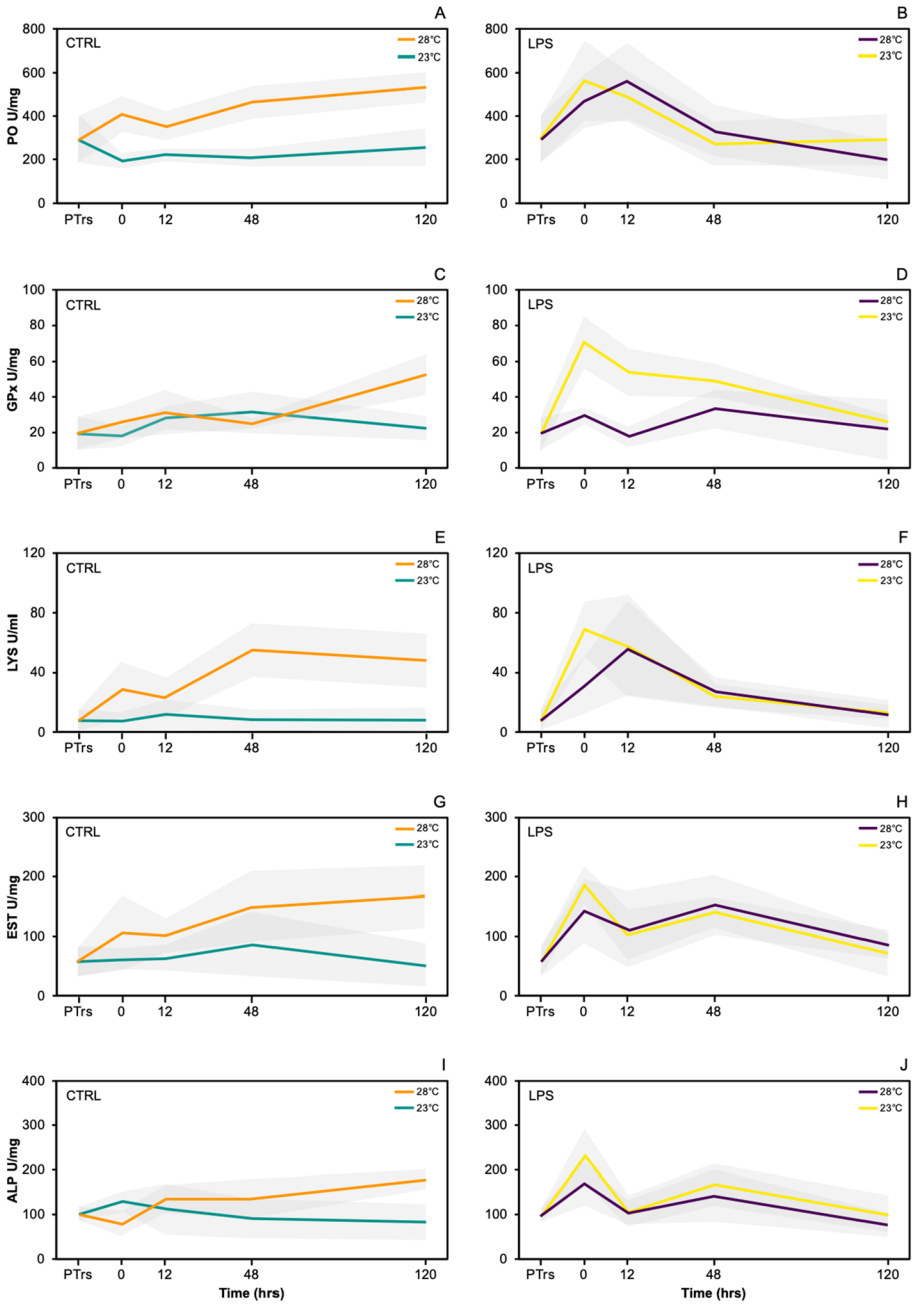

Fig. 3 Mean values (colored lines) of immune enzyme activity ± 95% confidence intervals (gray areas) for environmental (23 °C) vs elevated (28 °C) temperature-exposed colonies of *A. calycularis* under control (no LPS) and LPS treatments over time. *PO* Phenoloxidase, *GPx* Glutathione Peroxidase, *LYS*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

these ecologically relevant organisms (e.g., climate change-related stressor).

Immune activity under warmer conditions

The elevated sea water 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 sea water conditions. Consistent with previous findings on tropical species (Palmer et al. 2011a, 2018a), this provides further corroboration that coral immune systems are responsive to environmental changes (Mydlarz et al. 2008; Pinzón et al. 2015; Palmer 2011c, 2018a). Such a shift in immune strategy is probably the result of physiological trade-offs, environmentally dependent (e.g., warmer sea water 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, b). The gradual 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, b). Such a constituent immunity, with the environmental cue of warmer sea water in a tolerant coral, seems appropriate given the higher disease risk reported for summer periods (e.g., Cerrano et al. 2000; 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 sea water temperature, the immune response to LPS elicitation occurred at the 12 h 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 0 h time point 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 sea water 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 sea water 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 h time point), indicative of the high energy expenditure of an immune response (Palmer et al. 2011a; Palmer 2018a, b). 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 sea water conditions. Such a pattern of immune-dynamics could have implications for this habitat-forming coral during the summer period, when sea water temperatures are higher and the risk of pathogen load and virulence increases (Harvell et al. 1999, 2002; Mydlarz et al. 2006; Bally and Garrabou 2007; Vezzulli et al. 2010, 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, b). 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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Declarations

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