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How increasing temperature affects the innate immune system of sea urchin *Paracentrotus lividus* (Lamarck, 1816) reared in a RAS system

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ABSTRACT

The purple sea urchin, *Paracentrotus lividus*, is the most exploited and economically important in Southern Europe due to the high value of its gonads. Temperature generally affects several physiological functions of marine invertebrates and the ocean warming has been linked to increasing frequency and severity of disease outbreaks in several echinoderms. Sea urchins have an innate immune system consisting of coelomocytes, the cellular components responsible for the immune response, supported by proteases and lysozymes. The present study aimed to investigate the effect of increasing seawater temperature on the immunological response of *P. lividus*. In this experiment, the animals were exposed to an increase in temperature up to 24 °C for 36 days, after which cellular and humoral immunity parameters were measured. The number of coelomocytes in the animals increased with the temperature rise, mainly the phagocytes and the colourless granulocytes. In the humoral response of the animals, only the concentration of lysozyme responded to the increase in temperature.

Introduction

Global warming poses the greatest threat to marine life, as average seawater temperatures are rising rapidly, reaching new records [1], and are predicted to continue rising [2]. Echinoderms are ectothermic and water temperature is a critical environmental factor that impacts their fitness [2,3], particularly their physiology [4], growth [5], behavior [6] and immune response [2,7]. Echinoderms can tolerate dial and seasonal variations within species' thermal range [8]. However, temperature increases above this threshold may be fatal [3,9-13]. The effects of ocean warming and associated marine heat waves are already evident in echinoderm populations, with disease-related die-offs in numerous species linked to elevated temperatures [3]. For example, *Pisaster ochraceus* has recently experienced significant population declines due to sea star wasting disease as consequence of water temperature increase [3,8]. Another echinoderm, the sea cucumber *Apostichopus japonicus* suffers detrimental effects of high temperatures in the digestive function, immunity and antioxidant defense that result in massive mortality of farmed animals [14,15]. In the case of sea urchins, some disease outbreaks and subsequent high mortalities events were influenced by heightened temperatures [3,16-17]. For instance, in China, the longline cultivation of small *Strongylocentrotus intermedius* has experienced a significant decline in production efficiency due to mass mortality during recent summers [17-19]. Despite these increasing risks, echinoderms can persist in warm seas by shifting their range or by acclimating and adapting [8].

Sea urchins play an essential role in the community structure of many benthic coastal habitats. Through their grazing activity, these animals regulate the abundance and distribution of algae [20]. Like other echinoderm, sea urchins possess an innate immune system that responds rapidly and effectively against a diverse array of pathogens serving as a natural defense mechanism [21-23]. This system operates via both cellular and humoral factors [22]. Coelomocytes are a heterogeneous population of freely moving immune cells found in the coelomic fluid (CF) of sea urchins [22]. They represent the key effectors of the innate immune system of invertebrates. There are four types of coelomocytes: colorless spherule cells, red spherule cells, vibratile cells and phagocytes [2,23,24]. The coelomocytes can act against the entry of invasive microorganisms, inflammation and body injury, by phagocyting, clothing and releasing cytotoxic substances to the CF. The humoral

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component of sea urchin immunity is mediated by bacteriolytic and cytotoxic substances found in the CF [25,26]. Proteases and lysozyme play a crucial role in the defense mechanisms of invertebrates, acting as a barrier to the proliferation of bacteria due to their peptidase activity. Proteases are responsible by the hydrolysis of protein into peptides and amino acids [26]. Lysozymes are ubiquitous components of the animal innate immune system with antibacterial properties, killing bacteria by hydrolysis of their main cell wall polymer, peptidoglycan [27]. Nitric oxide (NO) is produced by the neural cells of the central and peripheric systems and is associated with the immunoreactive activity of epithelial cells and stomach muscles activity [28]. This reactive response molecule has also been proposed as a mediator in response to toxic exogenous stimuli, regarded as a cellular sign of environmental stress in sea urchins [29-31].

The purple sea urchin *Paracentrotus lividus* (Lamarck, 1816) is distributed throughout the Northeastern Atlantic and Mediterranean Sea, from Scotland and Ireland to southern Morocco and the Macaronesia eco-region. This species is the most extensively exploited and economically significant sea urchin in Southern Europe [32]. Their gonads are highly valued and represent a gourmet delicacy in the seafood industry, making them one of the world's most expensive seafood products [32-34]. *Paracentrotus lividus* is considered a sentinel species, recognized as a bioindicator for several reasons: its ecological relevance, benthic and relatively sedimentary lifestyle, rapid response and high sensitivity to several contaminants [35]. This species is abundant in areas with temperatures ranging from 4 to 30 °C, with seasonal fluctuations of up to 15 °C [32] within this range. The thermal requirements for the growth, activity, and gonadal development of *P. lividus* are differentiated and linked to seasonal variations, which significantly impact its reproduction and fitness [2,36]. Previous studies demonstrated that heightened temperatures contribute to increased mortality rates and decreased somatic and gonadal performance in *P. lividus*, particularly in areas near the warmer limits of its distribution [16, 36-38].

Here, it is hypothesized that the sea urchin immune system could be negatively affected by a short-term increase of environmental temperature of at least 6 °C (in line to would be expected in a summer heat wave). If temperature is constant within the thermal range of the species, it would be expected to observe a stemming adaptation to such conditions. Under this hypothesis, the present study aimed to follow the effect of increasing seawater temperature on the immunological response of *P. lividus* in aquaculture. This was done by quantifying cellular immunity parameters (differential counts of coelomocytes) and humoral immunity (quantification of lysozyme, protease, and NO) in the coelomic fluid of sea urchins subject to different thermal conditions (18 °C and 24 °C).

Materials and methods

Animal collection and maintenance

Sixty-five adult *P. lividus* with a test diameter (TD) of approximately 37.6 ± 0.30 mm (excluding spines) were collected in March 2023 from the intertidal zone of Praia do Abalo (39°22′N; 9°23′W, Peniche, Portugal). Additionally, coelomic fluid from wild *P. lividus* was collected at the same location for comparison with laboratory data. The sea urchins were transported to the Aquaculture Laboratory of MARE - Marine and Environmental Sciences Centre (Polytechnic of Leiria) in isothermal boxes covered with damp cloths, which were kept moist with the local seawater to minimize stress during transport. Upon arrival at the laboratory, the sea urchins were acclimatized and fasted for 11 days at ambient temperature (18.28 ± 0.33 °C).

Temperature selection and trial setup

In the Portuguese west coast, *P. lividus* sea urchins are subject to a seasonal variation of seawater temperature between 14 °C and 18 °C [39,40] with daily variations which upper limit can increase above 24 °C in shallow waters during summer heatwaves. Additionally, in a previous study conducted with sea urchins of the same population, it was observed that when exposed to 24 °C for long periods, these show a reduction in growth rates and gonad development [41]. Considering this, the summer temperature, 18 °C was defined as ambient temperature, and the 24 °C was selected as challenge temperature, because while still within the thermal range of local *P. lividus* population, it results in a reduction physiologic condition.

Before the trial, five individuals were randomly selected to collect the CF and access the initial immune status (T0). The remaining sea urchins ($N = 60$) were randomly distributed across two recirculating aquaculture systems (RAS), each equipped with three 40 L tanks and a 250 L sump ($N = 10$ *per* tank; density = 0.25 ind/L^{-1}; biomass = 5/77 g. L^{-1}). The sea urchins were exposed to two temperature conditions: elevated temperature (system A: 24.33 ± 1.44 °C) and ambient temperature (system B: 18.15 ± 0.57 °C). In system A, the temperature was increased at a rate of 1°C per day until reaching 24°C, which was achieved after 18 days. This elevated temperature was then maintained for the remainder of the trial. The trial lasted for 36 days, during which the sea urchins were fed three times a week with a jellified diet (Table 1) in an amount equivalent to 5 % of the total biomass of each tank.

Coelomic fluid sampling and coelomocytes counts

The collection of CF and immune cell counts was conducted immediately before starting the trial (T0), during the trial when system A attained 24°C (T1, 18 days after the beginning of the trial), and at the end of the trial (T2, 36 days after the beginning of the trial). For the T0 sampling, five sea urchins were used. For the T1 and T2 samplings, thirty individuals were used ($N = 5$ *per* tank).

Coelomic fluid (2 mL) was collected through the peristomial membrane of each sea urchin, using a sterile hypodermic needle (HENKE SASS WOLF 26G- 0.45 × 25 mm) attached to a 1 mL syringe containing 0.1 mL anticoagulant solution (2 mM EGTA, 40 mM HEPES, 1 M NaCl and 1 mM MgCl2). The CF was stored in microtubes containing an anticoagulant solution and kept on ice. Coelomocytes were counted in a 10 μL CF sample under a binocular microscope (Zeiss, Germany) at 400 x magnification, using a Neubauer counting chamber (Marienfeld,

Table 1
Nutritional composition (g per 100 g of dry matter, DM) and formulation of the jellified diet offered to the sea urchins during the increasing temperature trial.

Diet Nutritional Composition	Content (g per 100 g DM)
Crude Protein (CP)	41.27 ± 0.50
Crude Fat (CF)	6.98 ± 0.27
Total Carbohydrates (CH)[1]	50.63
Ash	1.12 ± 0.02
Total Carotenoids (mg/100 g)	28.89 ± 0.60
Ingredient formulation	**Content (g per 100 g DM)**
Pea protein[a]	18.00
Pumpkin[b]	40.00
Nannochropsis sp. powder[c]	22.00
Saccorhiza polyschides powder[d]	8.50
Fish oil[e]	5.00
Agar[f]	6.00
Antioxidant[g]	0.50

[1] Total carbohydrates (CH) were determined by difference: CH =100 – (CP+CF+Ash);
[a] Pea protein 80 g/100 g CP, 9.5 g/100 g CF, 0.9 g/100 g CH Alma & Valor, Lda;
[b] Dried Pumpkin 9 g/100 g CP, 6 g/100 g CF, 50 g/100 g CH;
[c] Nannochloropsis sp. powder 45–55 g/100 g, 15–20 g/100 g CF, 15–20 g/100 g CH Allmicroalgae;
[d] *S. polyschides* powder 14 g/100 g CP, 2 g/100 g CF, 44 g/100 g CH [50];
[e] Fish oil 100 g/100 g CF Lucílio Branco, Lda;
[g] Antioxidant (Vitamin E) Nekton Produkte.

Germany). Subsequently, the CF samples were centrifuged at 3000 rpm for 10 min (Eppendorf Centrifuge 5810 R, Hamburg, Germany) at 4 °C, the supernatant was collected and frozen at −80 °C for subsequent evaluation of humoral parameters.

Humoral parameters

The concentration of lysozyme in the CF was quantified for each sea urchin using a turbidimetric assay as described by Fernández-Boo et al. [29] with slight modifications. Two solutions were prepared, a 0.05 M sodium hydrogen phosphate (Na_2HPO_4) buffer at pH 6.2, and a bacterial solution (*Micrococcus lysodeikticus* 0.5 mg/L). After preparing these solutions, 50μL of CF was placed in a 96-well microplate (Greiner 96 flat bottom). A total of 300μL of the bacterial suspension was used as a positive control and 300μL of buffer solution served as a negative control. A standard curve was generated by serially diluting 13 solutions of decreasing concentrations of HEWL lysozyme (Hen egg white lysozyme) with a sodium phosphate buffer (0.05 M, pH = 6.2): 20.0 μg/mL; 10 μg/mL; 5 μg/mL; 2.5 μg/mL; 1.125 μg/mL; 0.625 μg/mL; 0.3125 μg/mL; 0.1563 μg/mL; 0.078 μg/mL; 0.0391 μg/mL; 0.0195 μg/mL; 0.0098 μg/mL; 0.0049 μg/mL. The absorbance of the samples was measured at 19 °C and a wavelength of 450 nm, at 30 s and 9.30 min, using an EPOCH 2 microplate spectrophotometer (BioTek Instruments, Inc., United States of America). The amount of lysozyme in each sample was calculated by applying the calibration curve equation, and the results were expressed in μg/mL.

The protease activity in the CF was determined using the azocasein hydrolysis assay, as described by Fernández-Boo et al. [29] with small modifications. Coelomic fluid samples of 50 μL were added to a microtube containing 60 μL of phosphate-buffered saline (PBS) (NaH_2PO_4) at a concentration of 13.9 mg/mL, pH 7 and 125 μL of azocasein ($NaHCO_3$). The samples were then incubated for 24 h at room temperature with continuous stirring. After 24 h, the reaction was stopped by adding 250 μL of 100 mg/mL trichloroacetic acid (TCA) and incubating for 30 min at room temperature. The samples were then centrifuged at 10,000 × *g* for 10 min. Subsequently, 100 μL of each sample was transferred to 96-well microplates (Greiner 96 flat bottom) in duplicate and 100 μL of 40 mg/mL NaOH was added. The absorbance was read at a wavelength of 450 nm using a microplate spectrophotometer. The positive control (60 μL of PBS + 125 μL of azocasein) represents 100 % of the protease activity and the blank control (60 μL of PBS + 100 μL of NaOH) represents 0 % of the protease activity. The percentage of uninhibited trypsin was calculated by the following equation:

$$\%\ non\ inhibited\ trypsin\ = \frac{(sample\ Abs.\ \times\ 100)}{(Abs.of\ the\ reference\ sample)}$$

The Griesse reaction was used to quantify the NO content in CF [42]. First, three solutions were prepared: 2.5 % phosphoric acid (H_3PO_4) solution, 1 % sulfanilamide solution in H_3PO_4, and 0.1 % N-naphthylethylenediamine solution in H_3PO_4. Then, 25 μL of supernatant and 100 μL of 1 % sulfanilamide in 2.5 % phosphoric acid were placed in duplicate microplates (Greiner 96 flat bottom), followed by 100 μL of 0.1 % of N-naphthyl-ethylenediamine in 2.5 % phosphoric acid. A standard curve was prepared by making several dilutions of sodium nitrite ($NaNO_2$) stock solution (69 g/mol), with decreasing concentrations (0.1 M; 0.01 M; 1 mM; 0.5 mM; 0.05 mM; 0.025 mM; 0.0025 mM; 1.25 mM; 0.125 mM). The microplates were then incubated for 10 min at room temperature, and covered with foil. After incubation, the absorbance was read at a wavelength of 540 nm on a microplate spectrophotometer. The NO concentration in the samples was determined by applying the calibration curve equation and the calculated absorbance values. These results were presented in micromolar (μM).

Statistical analyses

The temperatures trial results were presented in the format of mean ± standard deviation (SD), whenever appropriate. Before formal analysis, data collected during the increasing temperature trial were tested for normality and homogeneity of variances using, respectively, the Shapiro-Wilk and Levene tests. Then, to evaluate the effect of temperature (two levels: ambient, 24 °C) and exposure time (three levels: T0, T1, T2) on cellular (cellular density and proportions) and humoral parameters (lysozyme, protease and nitric oxide), it was conducted a fixed factors two-way analysis of variance (ANOVA) and results were presented as F, degrees of freedom (df) and p. In the case of non-compliance with the homogeneity of variances between samples, the Mann-Whitney or the Kruskal-Wallis non-parametric tests were performed (U/H; df; *p*). When statistical differences were found, the Tukey multiple comparison post-hoc test (HSD) was performed. For all cases, significant differences were considered when $p < 0.05$. Statistical analysis was conducted using the IBM SPSS Statistical 28 program.

Results

Density and proportion of coelomocyte cells

In general, the sea urchins subjected to higher temperatures exhibited a higher density of coelomocytes throughout the temperature trial (Fig. 1A) due to the increase of the densities of phagocytes and colourless granulocytes (Table 2). At the beginning of the trial (T0, indicated by the dark grey line in the barplot in Fig. 1A), the mean abundance of coelomocytes was 3.39 ± 1.35 × 10^7 cells/mL. A significant effect of temperature increase was observed (Table 2), with the sea urchins exposed to 24 °C showing higher cell densities at both T1 (46.2 ± 30.5 × 10^6 cells/mL) and T2 (34.9 ± 23.8 × 10^7 cells/mL) compared to those maintained at 18 °C (T1: 16.9 ± 15.6 × 10^6 cells/mL and T2: 14.1 ± 13.3 × 10^6 cells/mL).

Regarding the types of coelomocytes present in the CF, phagocytes were the most abundant cells, followed by spherule colorless cells, with vibratile cells being the least abundant (Table 2). Despite the increase in density, the percentage of phagocytes decreased over the trial period. Starting with 49 %. The sea urchins maintained at 18 °C throughout the trial had the lowest percentage of phagocytes (31 %), while the sea urchins maintained at 24 °C showed a higher percentage of these cells (43 %), although these differences were not statistically significant. The densities of red granulocytes increased significantly with increasing temperature (Table 2) attaining a maximum density of 5.23 ± 4.89 cell/mL at T1 in the sea urchins maintained at 24 °C. Despite that, the proportion of these cells remained relatively stable in both temperatures' conditions. Initially, it was 7 % at T0, increased at T1 (11 % for urchins maintained at 24°C and 12 % at 18°C), and slightly decreased again at T2 in both treatments (approximately 9 %). The density of colorless granulocytes was affected by temperature and by the interaction between temperature and sampling point, but their proportion in total coelomocytes was not affected by these factors (Table 2). At the start of the trial, the density of colorless granulocytes was 10.5 ± 2.60 cell/mL representing 27 % of total coelomocytes at T0. Throughout the experiment, their density increased in both temperature groups, peaking at T1 in the 24 °C group with 16.4 ± 9.74 cell/mL. While the vibratile cells density was not significantly affected by temperature neither time, their proportion were significantly influenced by time elapsed (Table 2). However, their proportion in total coelomocytes decreased with time. Initially representing 12 % of the immune cells, their percentage decreased at T1 in both temperature groups (5 % at 24°C and 3 % at 18°C) and slightly increased by the end of the temperature trial in sea urchins exposed to 18°C (8 %).

Table 2 also shows the percentage of the different types of coelomocytes collected from *P. lividus* sea urchins in the wild. The percentage of phagocytes was on average 25 %. Red and colorless granulocytes had a percentage of 5 % and 20 %, respectively. As for the vibratile cells, the percentage in the coelomic fluid on average was 50 %.

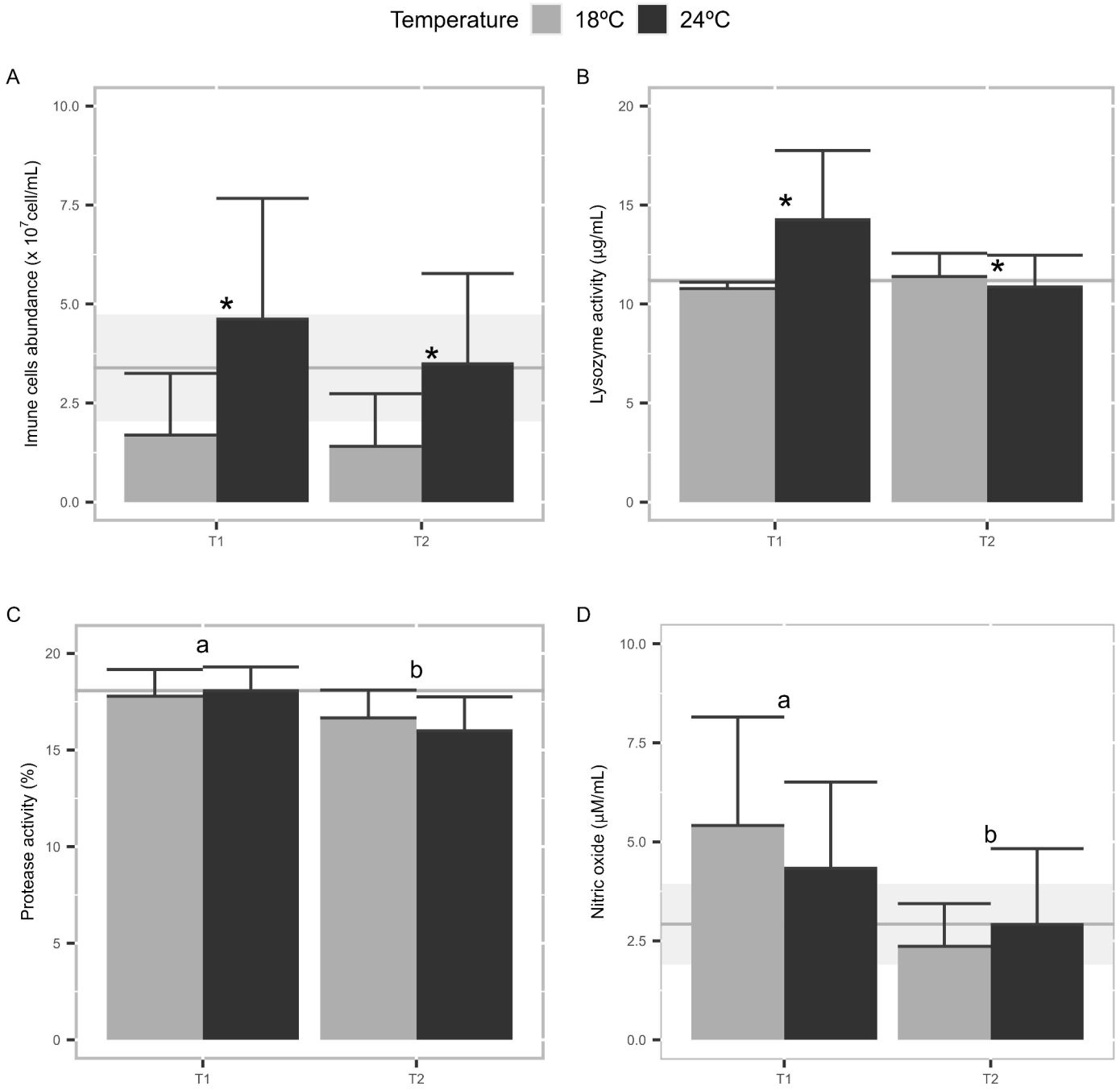

Fig. 1. Mean abundance of coelomocytes (A), Lysozyme activity (B), Protease activity (C) and Nitric oxide (D) concentration in the coelomic fluid of sea urchins *Paracentrotus lividus* evaluated at the start (dashed line) mid (T1) and at the end (T2) of the increasing temperature trial. The bars represent the mean value, and the error bars represent the standard deviation at each sampling point. The symbol * represents statistically significant differences between the 18ºC and 24ºC, and the letters *a* and *b* represent statistically significant differences between sampling points.

Humoral parameters activity

The concentration of lysozyme in the coelomic fluid of sea urchins was significantly influenced by temperature (Table 3). At the beginning of the temperature challenge (T0), the enzyme concentration was 11.19 ± 0.13 µg/ml. By T1, the group of sea urchins exposed to 24 °C, reached the highest concentration, 14.26 ± 3.49 µg/mL, which was greater than the concentration observed in the 18ºC group, 10.78 ± 0.32 µg/mL. At T2, the concentration in the 24 °C group decreased to 10.86 ± 1.61 µg/mL (Fig. 1B).

The protease activity was significantly influenced by the time elapsed from the start of the trial (Table 3). Initially (T0), the enzyme activity was 18.08 ± 0.11 %. This activity remained unchanged at T1, with the group exposed to 24 °C showing activity of 18.07 ± 1.23 %, and 17.79 ± 1.38 %. By T2, there was a slight but significant decrease ($p < 0.001$) in enzyme activity in both treatments, with the 24 °C group decreasing to 16 ± 1.76 %, and the 18ºC group decreasing to 16.67 ± 1.44 % (Fig. 1C).

The production of NO was significantly influenced by the time elapsed between sampling time-points (Table 3). Initially, at the start of the trial (T0), the NO concentration was 2.92 ± 1.02 µM/mL. By T1, there was an increase in NO production in both groups. Specifically, in the 18 °C group, sea urchins produced an average of 5.41 ± 2.74 µM/mL, while in the 24ºC group they produced 4.33 ± 2.18 µM/mL. At T2,

Table 2
Density and proportion of total and families of coelomocytes in the coelomic fluid of *Paracentrotus lividus* sea urchins from wild population and subject to the increasing temperature trial. Table includes statistical output on the ANOVA analysis on the effect of temperature in the immune response indicators.

	wild	18°C			24 °C		ANOVA			
		T0	T1	T2	T1	T2	Factors	Statistic test	df	*p*
	Cell density (10^6 cells/mL)									
Total Coelomocytes	37.5 ± 80.9	33.9 ± 13.5	16.9 ± 15.6	14.1 ± 13.3	46.2 ± 30.5*	34.9 ± 22.8*	°C	F=31.286	1	<0.001
							T	H=2.425	2	0.298
							°C x T	F=0.214	1	0.645
Phagocytes	9.30 ± 1.29	17.3 ± 6.62	7.97 ± 8.46	4.77 ± 4.69	22.3 ± 13.6	16.1 ± 10.9	°C	F=18.315	1	<0.001
							T	F=1.519	2	0.227
							°C x T	F=7.109	4	<0.001
Red Granulocytes	1.80 ± 1.08	2.50 ± 1.38^{ab}	2.17 ± 1.98^{ab}	1.43 ± 1.45^{b}	5.23 ± 4.89^{a}*	3.00 ± 2.50^{b}*	°C	U=726.5	2	0.008
							T	F=1.644	2	0.202
							°C x T	H=10.381	1	0.034
Colorless granulocytes	7.30 ± 7.48	10.5 ± 2.35^{ab}	5.33 ± 3.37^{b}	6.70 ± 5.62^{b}	16.4 ± 9.74^{a}	14.0 ± 7.51^{a}	°C	U=857.0	2	< 0.001
							T	F=0.03	2	0.969
							°C x T	H=23.283	4	< 0.001
Vibratile cells	19.1 ± 4.97	3.60 ± 1.77	1.43 ± 1.81	1.23 ± 1.54	2.30 ± 2.29	1.83 ± 1.89	°C	F=0.667	1	0.417
							T	F=2.366	2	0.102
							°C x T	F=1.725	4	0.156
	Cell Proportion (% of Total Coelomocytes)									
Phagocytes	25.20 ± 0.04	49.37 ± 0.07	41.50 ± 0.23	31.49 ± 0.21	44.68 ± 0.18	43.38 ± 0.17	°C	F=3.321	1	0.073
							T	F=2.008	2	0.143
							°C x T	F=0.303	4	0.584
Red Granulocytes	4.68 ± 0.02	6.90 ± 0.04	12.19 ± 0.07	8.80 ± 0.07	11.41 ± 0.06	9.01 ± 0.06	°C	F=0.024	1	0.876
							T	F=1.824	2	0.170
							°C x T	F=0.074	4	0.787
Colorless granulocytes	19.84 ± 0.03	27.39 ± 0.04	36.72 ± 0.18	40.81 ± 0.15	38.55 ± 0.15	42.29 ± 0.14	°C	F=0.255	1	0.616
							T	F=2.653	2	0.079
							°C x T	F=0.010	4	0.921
Vibratile cells	50.28 ± 0.06	11.89 ± 0.02^{a}	9.59 ± 0.12^{a}	7.91 ± 0.08^{b}	5.36 ± 0.05^{a}	5.32 ± 0.04^{b}	°C	F=0.218	1	0.642
							T	H=8.386	2	0.015
							°C x T	F=9.161	4	0.057

Data is presented as mean value ± SD, * indicate differences between temperatures (°C) superscript a and b indicate significant statistical differences ($p < 0.05$) between sampling points (T).

Table 3
Lysozyme, protease and nitric oxide concentration in the coelomic fluid of *Paracentrotus lividus* sea urchins from wild population and subject to the increasing temperature trial. Table includes statistical output on the ANOVA analysis on the effect of temperature in the immune response indicators.

	18°C			24 °C		ANOVA			
	T0	T1	T2	T1	T2	Factors	Statistic test	df	*p*
Lysozyme (μg/mL)	11.19 ± 0.13	10.78 ± 0.32	11.39 ± 1.18	14.26 ± 3.49*	10.86 ± 1.61*	°C	F=5.605	1	0.022
						T	H=2.124	2	0.346
						°C x T	H=6.950	4	0.139
Protease (%)	18.08 ± 0.11	17.79 ± 1.38^{a}	16.67 ± 1.44^{b}	18.07 ± 1.23^{a}	16.00 ± 1.76^{b}	°C	F=2.480	1	0.621
						T	H=8.697	2	<0.001
						°C x T	F=1.448	1	0.234
Nitric Oxide (μM/mL)	2.92 ± 1.02	5.41 ± 2.74^{a}	2.36 ± 1.08^{b}	4.33 ± 2.18^{a}	2.91 ± 1.92^{b}	°C	F=1.273	1	0.435
						T	H=7.989	2	0.018
						°C x T	H=8.602	4	0.072

Data is presented as mean value ± SD, * indicate differences between temperatures (°C) superscript a and b indicate significant statistical differences ($p < 0.05$) between sampling points (T).

there was a significant reduction in NO production ($p = 0.001$) in both the 18 °C and 24 °C treatments. The average NO concentration was 2.36 ± 1.08 μM/mL in the 18 °C group and 2.91 ± 1.92 μM/mL in the 24 °C group (Fig. 1D).

Discussion

Recent attention has been given to the implications of climate change on aquaculture due to the significant contribution of this sector to global food security, and nutrition [43]. Key aspects of aquaculture such as growth, feed utilization, product quality, welfare, disease treatment and mortality are affected by climate changes [2,44].

In sea urchins, coelomocytes play crucial roles in various cellular responses such as phagocytosis, encapsulation, cytotoxicity, opsonization, and production of antimicrobial agents in response to immunological challenges [25]. These activities are performed by different cell types within the coelomocyte population [22]. In this study, the increase of water temperature to 24 °C between T0 and T1 resulted in a significant increase in the density of coelomocytes in the CF, mainly the phagocytes and colorless granulocytes. However, maintaining the high temperature subsequently led to a decrease in coelomocyte densities in the exposed group. These findings align with previous research by Murano et al. [45], where thermal stress initially increased coelomocyte density in *P. lividus* followed by a decline over time. Similar results were also obtained for the sea cucumber *Parastichopus regalis* exposed to 23 °C for two weeks, suggesting that different echinoderms have identical immune response when subject to heat stress [46].

Phagocytes are identified as the predominant immune cell type in sea urchin CF [22]. The density of these cells increased with increasing

temperature followed by a non-significant density reduction indicating an activation of immune response followed by an adaptation within species' thermal range. Interestingly, their percentages were not significantly affected by the elevated water temperature in line with the results obtained by Branco et al. [47] for *Echinometra lucunter*, an intertidal sea urchin from tropical regions. Phagocytes functions include detecting, trapping, and eliminating foreign materials and they are activated in response to the presence foreign particles. Since during the trial were not introduce foreign particles into the body of the sea urchin, we are not able to conclude if increasing temperature effectively trigger significant phagocytic activity. In the study conducted on *P. regalis* [46], it was observed that, despite the increase in the coelomocyte's density, the phagocytic activity decreased with increasing temperature.

Red and colorless granulocytes in sea urchins are associated with inhibiting bacterial growth and exhibiting cytotoxicity [48,49]. Both families of coelomocytes showed the same pattern of increasing densities with increasing temperature followed by reduction with time course, more pronounced in the red granulocytes. As for phagocytes, increasing temperature favors the production of these families of immune cells followed by an adaptation to thermal stress. However, the percentages of these immune cells were not significantly altered by the increasing water temperature. These cells are often observed near damaged spines, encapsulated bacteria and infested epidermal tissue of sea urchins [50], not present in these experimental conditions. Therefore, the thermal stress alone was not sufficient to induce significant changes in the percentages of these immune cell populations.

There is no experimental evidence for the function of vibratile cells. However, some *in vitro* observations suggest that these cells may play a role in defense mechanisms by degranulating during coagulation events [51]. Other studies indicate that vibratile cells could be involved in clot formation with their granules aiding the circulation of CF through the body cavity [52,53]. In the present study, the density of vibratile cells was not influenced by temperature, while its percentage in the CF of sea urchins decreased over time and was lower in animals exposed to 24 °C compared to the control group. Similarly, in a study conducted by Queiroz et al. [51], sea urchins *Arbacia lixula* exposed to *Escherichia coli*, showed a sharp decrease in vibratile cells within the first 24 h, after which the cell count remained stable. These authors noted that during the inflammatory response, vibratile cells and red granulocytes change shape by releasing their contents. A similar process may have occurred in the present study, where the vibratile cells degranulated in response to the thermal stress (temperature), releasing their content and leading to a decrease in their density in the CF of sea urchins exposed to 24 °C.

Among all the immune cells, vibratile cells showed the most significant differences between wild and captive environments. In the wild, *P. lividus* have a higher percentage of vibratile cells in their CF than in captivity. This can be attributed to the clotting function of the vibratile cells. When sea urchins are caught in the wild, they can lose their tube feet, causing damage to their bodies. In such injuries, vibratile cells form a clot over the wound, thereby increasing their density in response to the damage.

Lysozyme plays an important role in the modulation of the immune system and in anti-bacterial activities [54]. In the present study, lysozyme activity was significantly increased in sea urchins exposed to 24 °C compared to those at the control temperature as a consequence of the significant increase in the density of colourless granulocytes responsible by the production of this cytotoxic molecule. These results are consistent with findings obtained in another group of echinoderms such as sea cucumbers [15,55], who observed that lysozyme activity in the coelomic fluid of *A. japonicus* was significantly affected by increasing temperature.

Protease activity plays a crucial role in innate immune mechanisms against pathogens, particularly in invertebrates where these enzymes serve as a key effector of immune response [2] in addition to its digestive function. Interestingly, in the present study, protease activity was not influenced by the temperature, contrasting with the findings reported by Gallo et al. [2], where protease activity increased progressively with higher temperature. In the present study, the sea urchins were fed a jellified protein-rich diet that may have promoted the production and the transfer of proteases from the gastric tube to the circulating CF, masking the effect of increasing temperature in this immune factor.

Nitric oxide serves as a biomarker for reactive nitrogen species (RNSs) and has been implicated in inducing apoptosis and DNA damage in animal cells and tissues under environmental stress [56]. In the study by Gallo et al. [2] with *P. lividus* sea urchins subjected to heat stress, it was found that NO concentration increased steadily with increasing temperature. However, in contrast to protease activity, the production of NO was not significantly affected by heat stress in their study. In the current study, the NO production in sea urchins exposed to increased temperature reduced with the course of time. The levels of NO ranged from 2.36 to 5.41 μM/mL across different conditions, indicating that the temperature increase did not induce a significant increase in NO production.

The cellular and humoral components of echinoderms immune system act in cooperation against external stress, particularly against pathogenic agents and injuries [22]. Our results indicate an activation of immune response of sea urchins with increasing temperature through the production of phagocytes, colorless granulocytes and the consequent production of lysozyme. These results indicate that the immune system of *P. lividus* holds some level of tolerance to increasing temperature near the upper limit of its thermal range. The thermotolerance is, most probably, a result from the adaptation to the intertidal habitat that is environmentally demanding. Nevertheless, the possible activation of immune response can also increase the production of NO and other reactive oxygen intermediates causing oxidative damage [46] if not followed by increasing densities of red granulocytes rich in echinochrome A. The stabilization in the number of red granulocytes and the reduction in the number of vibratile cells may compromising the clothing and anti-oxidant responses in a more demanding environmental. Other studies refers to this unclear effect of environmental stress, like temperature increase or pH reduction [2,57].

Current global changes are altering fishing practices due to habitat and biodiversity losses and affecting aquaculture productivity. For echinoids in particular, ocean warming is a major environmental factor controlling their development, physiology and genetic expression [58]. Excessive warming alters basic physiological processes, influences negatively the reproduction [59], impairs juvenile development and ultimately increases mortality in several populations [38,60]. Increasing temperature may benefits the growth of pathogenic agents while alters the complex functioning of sea urchin immune system. *Paracentrotus lividus* is an intertidal species naturally adapted to short-term changes of environmental conditions. The present study indicates that its immune system is activated and adapts to temperature in the upper level of its thermal range. However, such immune response requires to be challenged in the presence of a pathogen agent to prove its' efficiency. Such results would be key to understand if these animals can adapt to the cumulative effects of increasing water temperatures and high production densities.

Conclusion

Temperature is a critical environmental factor that significantly impacts physiological processes, including the immune defense of sea urchins. In the study involving *P. lividus* exposure to 24 °C, it was observed an increase in total coelomic cells (mainly phagocytes and colorless granulocytes) and lysozyme concentration. These results indicate that the sea urchins' immune defenses reacted promptly to the thermal stress, recognizing it as a potential threat to their health. On the other hand, the non-significant increase in the nitric oxide production without the increase in red granulocytes densities indicates a level of oxidative stress that may have consequent physiologic damage. Moreover, the reduced response of NO and the lack of response of protease

suggests that these parameters might not be sensitive indicators for evaluating *P. lividus* defense against thermal stress in this context. The maintenance of cells proportions across trial indicate that the temperature rise to the upper limit of species thermal level do not deregulate immune response. Only vibratile cells proportion decreased with increasing temperature, indicating that clot function can be compromise in such conditions.

The study highlights how different immune parameters in sea urchins respond differently to environmental stressors like temperature. While some parameters, such as total coelomic cells and lysozyme concentration, reflect an immediate immune response to thermal stress, others, like protease activity may not be as indicative in this regard. Understanding these different responses is crucial for assessing the overall health and resilience of sea urchin populations under changing environmental challenges in aquaculture production.

Several methods have been proposed to minimize the effects of climate change in the context of aquaculture industry. One method can be to increase species resistance to thermal stress through feeding the animals with diets holding anti-oxidant properties. Another method could be select the genetic and phenotypic characteristics of population from intertidal habitats through selective breeding to obtain stocks more resistant to environmental stresses [61,62].

In conclusion, in a future scenario of rising water temperatures in the context of climate change in aquaculture, the immune system of the *P. lividus* species would react punctually to this external threat and eventually adapt to such conditions.

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CRediT authorship contribution statement

Ana Filipa Rodrigues: Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation. **Sílvia Lourenço:** Writing – review & editing, Visualization, Formal analysis, Data curation, Conceptualization. **Ana S. Gomes:** Writing – review & editing, Methodology, Investigation. **Carolina F. Tchobanov:** Writing – review & editing, Methodology, Investigation. **Ana Pombo:** Writing – review & editing. **Teresa Baptista:** Writing – review & editing, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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