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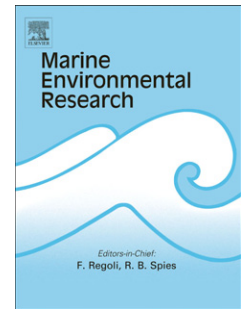
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Effects of temperature on cellular and biochemical parameters in the crab

Carcinus aestuarii (Crustacea, Decapoda)

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Abstract

The effects of temperature on cellular and biochemical parameters of the crab *Carcinus aestuarii* were evaluated. Crabs were kept for 7 days at 4, 17 (reference value) and 30 °C (salinity of 35 psu), and total haemocyte count (THC), haemocyte volume, haemocyte proliferation, phenoloxidase (PO) activity in both haemocyte lysate (HL) and cell-free haemolymph (CFH), CFH total protein and glucose levels, superoxide dismutase (SOD) and catalase (CAT) activities in both gills and digestive gland were evaluated. The lowest and the highest temperature significantly decreased THC, whereas haemocyte volume and haemolymph glucose concentration did not differ significantly among experimental conditions. Haemolymph protein concentration significantly reduced in crabs maintained at 30 °C, when compared with that of animals kept at 4 and 17 °C. Haemocyte proliferation increased significantly in crabs kept at 4 and 30°C, when compared with that of crabs held at 17°C. Likewise, a significantly higher PO activity was recorded in CFH from crabs kept at 4 and 30 °C, than in control crabs. Conversely, PO activity did not vary significantly in HL. With regard to antioxidant enzyme activities, a significant decrease in CAT activity was observed in gills from crabs kept at 4 °C, when compared to that of crabs kept at 17°C and 30 °C. Results obtained demonstrated that the highest and lowest temperature tested influenced crab biological responses, and indicated that *C. aestuarii* modulated its cellular and biochemical parameters (mainly haemocyte proliferation, CFH protein concentrations and CFH PO activity) in order to cope with temperature.

Keywords: crabs; immune parameters; temperature; haemocytes; glucose; antioxidant enzymes

1. Introduction

Crustacea have an open vascular system in which numerous haemocytes freely circulate in haemolymph. Circulating haemocytes are involved in wound repair and defence mechanisms against parasites, viruses and bacteria (Bauchau, 1981). As there is evidence that stress conditions (e.g., variations in temperature and salinity, and exposure to ammonia, sulphide and nitrite) can cause immunomodulation in Crustacea (Le Moullac and Haffner, 2000; Hong et al., 2007; Hsu and Chen, 2007; Romano and Zeng, 2009), increasing interest has recently been addressed to the evaluation of stress effects on immune functions in these invertebrates. Among environmental factors capable of influencing haemocyte-dependent defence mechanisms in Crustacea, temperature is regarded as one of the most important (Le Moullac and Haffner, 2000). For example, Truscott and White (1990) demonstrated that a gradual increase in temperature (from 10 to 20 °C) over a 12 h period significantly increased haemocyte number in *C. maenas* after 5 days, whereas a sudden temperature change did not cause significant alterations. In the same crab species, Chisholm and Smith (1992) observed that seasonal temperature changes affected haemocyte antibacterial activity, with the lowest activity recorded in February and August when water temperatures reached the lowest and highest value, respectively. In the brown shrimp *Penaeus californiensis*, exposure to increased temperature (from 18 °C to 32 °C) affected haemolymph parameters: a decrease in haemocyte pro-phenoloxidase (proPO) system was observed at 32 °C, whereas an increase in haemolymph protein content was recorded at both 28 °C and 32 °C (Vargas-Albores *et al.*, 1998). Temperature has also been shown to negatively affect the haemocyte phagocytic activity of lobsters (Paterson and Stewart, 1973; Steenbergen *et al.*, 1978). In the freshwater prawn *Macrobrachium rosenbergii*, it was observed that total haemocyte count (THC) decreased significantly in prawns kept at 33–34 °C, when compared with THC of animals held at 27–28 °C and 30–31 °C (Cheng and Chen, 2000). In that study, PO activity showed significant differences at all temperatures tested, reaching the highest value at 30–31 °C.

Among decapod species, *Carcinus aestuarii* is particularly abundant in the Mediterranean area, and differs from the Atlantic species *Carcinus maenas* in morphological traits, such as the shape of the copulatory appendages (pleopods) in males, the shape of the frontal area between eyes, and the carapace width to length ratio (Yamada and Hauck, 2001). Surprisingly, *Carcinus* has colonised several regions outside its native ranges, including Australia, Tasmania, South Africa, Japan and both coasts of North America, probably due to their high tolerance to air exposure, starvation, and variations in temperature and salinity (Yamada and Hauck, 2001; Roman and Palumbi, 2004). Along the Italian coasts, *C. aestuarii* has also colonised estuarine areas, such as the Lagoon of Venice and the Lagoon of Orbetello. In the lagoon of Venice, fishing and farming of *C. aestuarii* are important economical activities: fishermen select crabs during moulting, when they lose the carapace and become soft. Local fishermen name this crab moulting stage “moleche” or “moeche”, that are conferred on the market. This is a seasonal activity (mainly in spring and autumn) that follows growth rates of crabs.

Like other Crustacea (Bauchau, 1981), three main haemocyte types have recently been identified in *C. aestuarii*: granulocytes, with evident cytoplasmic granules, semigranulocytes, with less granules than granulocytes, and hyalinocytes, without evident granules (Matozzo and Marin, 2010a). Hyalinocytes are the main phagocytic cells, whereas granulocytes show high phenoloxidase (PO) activity (Matozzo and Marin, 2010b).

In the present study, the effects of varying temperatures (4, 17 and 30 °C) on both cellular and biochemical parameters were evaluated for the first time in *C. aestuarii*. The experimental temperatures were chosen taking into account that temperature values close to those tested in this study can be recorded in the Lagoon of Venice during wintertime and summertime, respectively. In particular, the extreme temperatures tested (4 and 30 °C) can be reached in shallow waters (< 1 m) of the lagoon, where crabs are also present. THC, haemocyte volume, haemocyte proliferation, phenoloxidase (PO) activity in both haemocyte lysate (HL) and cell-free haemolymph (CFH), and CFH total protein and glucose levels were measured. These biomarkers were chosen being

considered as useful indicators of immunesurveillance status in Crustacea (Le Moullac and Haffner, 2000), and taking into account that a reduced immunesurveillance can strongly enhance the susceptibility of animals to infections/diseases. In addition, superoxide dismutase (SOD) and catalase (CAT) activities were measured in both gills and digestive gland of crabs maintained at the differing temperatures. SOD and CAT are antioxidant enzymes that play an important role in reducing damage to cells caused by reactive oxygen species (ROS). The aim of the present study was to evaluate whether temperature can affect both immune parameters and antioxidant enzyme activities in an ecologically and economically important crab species.

2. Materials and methods

2.1. Crabs

Intermoult adult male crabs (4 cm mean carapace length) were collected by handmade traps in the Lagoon of Venice. Crabs (50 at least) were then kept in the laboratory in large aquaria (50 L) containing seawater (salinity of 35 ± 1 psu and temperature of 17 ± 0.5 °C) and a sandy bottom. The crabs were fed with mussels (*Mytilus galloprovincialis*) and acclimatised in the laboratory for 5 days before exposure to differing temperatures.

2.2. Exposure to differing temperatures

Prior to experimental temperature exposure, crabs were acclimatised to experimental conditions by progressively increasing or decreasing seawater temperature (2-2.5 °C per day) in the aquaria (50 L) to 4 and 30 °C. Crabs maintained at 17 °C served as controls. Crabs (10 per experimental condition) were kept for 7 days at the three temperatures in three different aquaria provided with a sandy bottom and well-aerated seawater (salinity of 35 ± 1 psu, pH 8.1), and fed with mussels. Seawater temperature, salinity and pH were checked daily, whereas seawater was renewed (50%) every 48 h.

2.3. Haemolymph and tissue collection

At the end of temperature exposure, crabs were anaesthetised on ice for 5 min, and the haemolymph (at least 500 μ L per crab) was collected from the unsclerotised membrane of the walking legs using a 1-mL plastic syringe, placed in microcentrifuge tubes on ice and diluted (except for glucose assay) 1:2 in an anticoagulant solution of citrate buffer/EDTA (NaCl 0.45 M, glucose 0.1 M, sodium citrate 30 mM, citric acid 26 mM, EDTA 10 mM, pH 4.6, stored at 4 °C) (Söderhäll and Smith, 1983). For each experimental temperature, 5 pools of haemolymph from 2 crabs each were prepared. Pooling was necessary because individuals did not provide enough haemolymph for analyses. Pooled haemolymph were then divided in four aliquots: two aliquots were immediately used to measure THC and haemocyte proliferation, whereas the remaining two aliquots were processed to measure PO activity in both haemocyte lysate (HL) and cell-free haemolymph (CFH), and CFH glucose levels. To obtain CFH, pooled haemolymph was immediately centrifuged at 780 x g for 10 min, and the supernatant (= CFH) was collected. To obtain HL, haemocytes were resuspended in distilled water, sonicated at 0 °C for 1 min, and then centrifuged at 780 x g for 30 min. Both CFH and HL were frozen in liquid nitrogen and stored at –80 °C until analyses.

After haemolymph sampling, the carapace was opened, gills and digestive gland were excised, placed in 2 mL Eppendorf tubes, frozen and stored at –80 °C until processing. Gills and digestive gland were thawed on ice and homogenised in four volumes of 0.1 M Tris-HCl buffer, pH 7.5, containing 0.15 M KCl, 0.5 M sucrose, 1 mM EDTA, 1 mM Dithiothreitol (DTT, Sigma) and 40 μ g mL⁻¹ Aprotinin (Sigma), sonicated for 1 min at 0 °C with a Braun Labsonic U sonifier at 50% duty cycles, and centrifuged at 10,000 g for 30 min at 4 °C. Supernatant was collected for enzyme assays.

2.4. *THC and haemocyte volume determination*

THC was determined by a Model Z2 Coulter Counter electronic particle counter/size analyser (Coulter Corporation, FL, USA). Immediately after sampling, 100 μL of pooled haemolymph were added to 19.9 mL of 0.45 μm -filtered seawater. THC values were expressed as the number of haemocytes ($\times 10^6$) mL haemolymph⁻¹, whereas volume of haemocytes were expressed in femtolitres (fL).

2.5. *Haemocyte proliferation*

Haemocyte proliferation was evaluated by a colorimetric method using a commercial kit (Cell proliferation Kit II, Roche). The assay is based on the cleavage of the yellow tetrazolium salt XTT to form an orange formazan dye by metabolic active (viable) cells. Briefly, XTT labelling reagent and electron-coupling reagent were thawed at 37 °C and mixed immediately before use to obtain the XTT labelling mixture. Two hundred μL of the mixture were added to 400 μL of haemolymph and incubated for 4 and 6 hrs in a dark humidified chamber at room temperature. Absorbance at 450 nm was then recorded on a Beckman 730 spectrophotometer. Results were expressed as optical density per mL of haemolymph (OD_{450} mL haemolymph⁻¹).

2.6. *Haemolymph glucose and total protein concentrations*

Haemolymph glucose levels were measured using a commercial kit (Quantichrom Glucose Assay Kit, BioAssay System). Briefly, 12 μL of CFH were transferred in centrifuge tubes containing 1200 μL of the reagent provided with the kit. Tubes were heated in a boiling water bath for 8 min, and then cooled in cold water bath for 4 min. Absorbance at 630 nm was then recorded on a Beckman 730 spectrophotometer. Results were expressed as mg glucose mL haemolymph⁻¹.

CFH protein concentrations were quantified according to the Biuret method (Sigma). Results were expressed as mg protein mL haemolymph⁻¹.

2.7. Phenoloxidase activity assay

PO activity was measured in both HL and CFH using L-DOPA (3,4-dihydroxy-L-phenylalanine, Sigma) as substrate in a colorimetric assay. One hundred μL of HL and CFH were added to 900 μL of 1 mg L-DOPA/mL of phosphate buffered saline, pH 7.2, (PBS: 1.37 M NaCl, 0.03 M KCl, 0.015 M KH_2PO_4 , 0.065 M Na_2HPO_4), and incubated for 30 min at 37 °C. Absorbance at 490 nm was then recorded on a Beckman 730 spectrophotometer, and results were expressed as U mg proteins^{-1} . Protein concentrations in both HL and CFH were quantified according to the Biuret method (Sigma). The specific activity of PO in *C. aestuarii* has previously been assayed using N-phenylthiourea (10 mM, Fluka) as a inhibitor (Matozzo and Marin, 2010b).

2.8. SOD activity assay

Total SOD activity was measured in both gills and digestive gland (see 2.3. section for details regarding supernatant obtainment) in triplicate with the xanthine oxidase/cytochrome *c* method according to Crapo et al. (1978). The cytochrome *c* reduction by superoxide anion generated by xanthine oxidase/hypoxanthine reaction was detected at 550 nm at room temperature. Enzyme activity was expressed as U $\text{mg of proteins}^{-1}$, one unit of SOD being defined as the amount of sample producing 50% inhibition in the assay conditions. The reaction mixture contained 46.5 μM $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ (pH 8.6), 0.1 mM EDTA, 195 μM hypoxanthine, 16 μM cytochrome *c*, and 2.5 μU xanthine oxidase. Protein concentrations in homogenised tissues were quantified according to the Biuret method (Sigma).

2.9. CAT activity assay

Gill and digestive gland CAT activity was measured in triplicate following the method of Aebi (1984). Decreases in absorbance of a 50-mM H_2O_2 solution ($\epsilon = -0.0436 \text{ mM}^{-1} \text{ cm}^{-1}$) in 50 mM phosphate buffer (pH 7.8) and 10 μL of tissue supernatant were continuously recorded at 240 nm at 10 sec intervals for 1 min. Results were expressed in U $\text{mg of proteins}^{-1}$, one unit of CAT being

defined as the amount of enzyme that catalysed the dismutation of 1 μmol of H_2O_2 min^{-1} . Protein concentrations were quantified according to the Biuret method (Sigma).

2.10. Statistical analysis

Data were checked for normal distribution (Shapiro-Wilk's test) and homogeneity of variances (Bartlett's test). Results were compared using a one-way ANOVA, followed by a *post hoc* test (Duncan's test). Values were expressed as means \pm standard error. The STATISTICA 9.0 (StatSoft, Tulsa, OK, USA) software package was used for statistical analyses.

3. Results

THC values significantly decreased in crabs kept at 4 °C ($p < 0.05$) and 30 °C ($p < 0.01$), when compared with those of control crabs (17 °C) (Fig. 1). No differences were found between crabs kept at 4 °C and those held at 30 °C. Haemocyte volume did not differ significantly among experimental conditions (293.5 ± 89.5 fL at 4 °C, 278.5 ± 101.4 fL at 17 °C, 291.8 ± 94.5 fL at 30 °C). After 4 and 6 hours of haemocyte incubation in XTT labelling mixture, haemocyte proliferation increased significantly ($p < 0.05$) in crabs kept at 4 and 30 °C, when compared with that of crabs held at 17 °C (Fig. 2).

Although a slight increase in CFH glucose levels was observed in crabs kept at 30 °C, no significant differences were recorded among experimental conditions (Fig. 3). Conversely, total protein concentration significantly ($p < 0.05$) reduced in CFH of crabs maintained at 30 °C (88 ± 11.04 mg mL haemolymph⁻¹), with respect to that of crabs kept at 4 °C (104.26 ± 8.72 mg mL haemolymph⁻¹) and 17 °C (107.36 ± 11.04 mg mL haemolymph⁻¹) (Fig. 4).

A significantly higher PO activity was recorded in CFH from crabs kept at 4 °C ($p < 0.05$) and 30 °C ($p < 0.01$), when compared to the control group of crabs maintained at 17 °C (Fig. 5). Conversely, PO activity did not differ significantly in HL from crabs kept at the three experimental temperatures (Fig. 5).

With regard to antioxidant enzyme activities, a significant ($p < 0.05$) decrease in CAT activity was observed in gills from crabs maintained at 4 °C, when compared to that of crabs kept at 17 and 30 °C (Table 1). No other significant variations in either CAT and SOD activities were recorded in the gill and digestive gland tissues of crabs held at the three experimental temperatures.

4. Discussion

It has previously been reported that variations in environmental parameters (e.g., temperature, salinity, oxygen) can influence immune parameters in Crustacea, causing a reduction in immunocompetence (Le Moullac and Haffner, 2000). In the present study, a suite of biomarkers, both cellular and biochemical, were used to evaluate effects of differing temperatures on the crab *C. aestuarii*. Among the available immunomarkers, THC is considered to be a good indicator of stress in Crustacea (Le Moullac and Haffner, 2000). This study clearly demonstrated that temperatures at both the lower and upper extreme for the Lagoon of Venice significantly reduced THC in *C. aestuarii*. A decrease in the number of circulating haemocytes in Crustacea is generally considered a consequence of haemocyte immobilization in gills (or other tissues), as demonstrated in mercury-exposed prawns (Victor et al., 1990) and in crabs after bacterial infection (Martin et al., 2000; Burnett et al., 2006). A similar consequence can be hypothesised for temperature-stressed crabs in the present study. However, further studies -mainly histological - are necessary to support this hypothesis. In *C. maenas*, animals maintained at 5 °C (a temperature close to the extreme values in winter) and 20 °C (a temperature close to the extreme values in summer) showed THC values significantly lower than those of animals kept at 10°C (Brockton and Smith, 2008). Increases in water temperature were shown to rise markedly THC in *C. maenas*, probably owing to the release of haemocytes from hematopoietic organs (Truscott and White, 1990). In the spiny lobster, *Panulirus interruptus*, both cooling rates (3 °C/h and 1.5°C/h) applied to lower water temperature from 19 to 4 °C caused a significant decrease in THC (Gomez-Jimenez et al., 2000). Prawns (*M. rosenbergii*) reared at 27-28 °C and 30-31 °C showed higher THC values with respect to those of

animals reared at 20-21 °C and 33-34 °C (Cheng and Chen, 2000). Low temperature (18 °C) caused a significant drop in THC in *Penaeus stylirostris* compared to prawns kept at 27 °C (Le Moullac and Haffner, 2000). In shrimps (*Litopenaeus vannamei*) transferred to 32 °C, THC values decreased significantly by 40% after 24 h when compared to those of animals kept at 28 °C (control), whereas THC of shrimps transferred to 20 °C decreased significantly by 12% and 16% after 24 and 48 h, respectively (Cheng et al., 2005).

It has been demonstrated that crustacean haemocytes can divide in haemolymph, and that proliferation can increase significantly after mitogenic or infectious stimulation (Sequeira et al., 1996). Interestingly, the present study demonstrated that extreme temperatures caused a significant increase in haemocyte proliferation, suggesting that the proliferative rate of circulating haemocytes increased in temperature-stressed crabs in order to compensate partially for the reduction in the number of circulating haemocytes. Similar results were recorded in Crustacea subject to different stress conditions. For example, Sequeira et al. (1996) reported a significant increase in the proliferation of circulating haemocytes in *Penaeus japonicus* following injection with lipopolysaccharide, and a six-fold increase in the percentage of proliferating haemocytes was also observed in animals infected with *Fusarium* spp. Likewise, bacteria injection caused a reduction in THC values and a significant increase in haemocyte proliferation in *C. aestuarii* (Matozzo et al., 2011).

In Crustacea, an increase in haemolymph glucose levels is considered a response to stress (Hall and van Ham, 1998; Lorenzon, 2005). The release of glucose into the haemolymph is mediated by the crustacean hyperglycaemic hormone (CHH) through the mobilisation of intracellular glycogen stores (Stentiford et al., 2001). Hyperglycaemia has been documented in Crustacea following exposure to a variety of stressors, such as emersion (Durand et al., 2000), cold shock (Kuo and Yang, 1999), anoxia and carbon dioxide (Hall and van Ham, 1998), nitrite (Yildiz and Benli, 2004), and pollutants (Lorenzon et al., 2000). In the present study, extreme temperature did not cause significant variations in glucose levels in haemolymph of *C. aestuarii*. Likewise, Chang et al.

(1998) demonstrated that emersion was a potent stimulator of haemolymph CHH in *Homarus americanus*, whereas temperature and salinity variations were less effective. Conversely, increases in water temperature resulted in increased blood CHH in *Cancer pagurus* (Wilcockson et al., 2002). In *Uca minax*, a temperature value of 10 °C caused a significant decrease in haemolymph glucose levels (Dean and Vernberg 1965), whereas in *P. interruptus*, increases in temperature from 20 °C to 27 °C induced an increase in haemolymph glucose levels (Ocampo et al., 2003). Powell and Rowley (2008) suggested that increases in haemolymph glucose levels may be due to the mobilisation of glucose from haepatopancreas and/or muscles in stressed crabs. Results of the present study demonstrated that the extreme temperatures tested did not elicit significant variations in haemolymph glucose concentrations. As a consequence, a temperature-induced mobilisation of glucose from haepatopancreas and/or muscles can be excluded in *C. aestuarii*, on the basis of these preliminary results at least.

In the present study, a significant decrease in haemolymph total protein levels was recorded in *C. aestuarii* maintained at 30 °C. In *H. americanus*, significant differences in total protein concentrations were observed between animals kept at 6 °C and those maintained at 15 °C (Lorenzon et al., 2007). In the present study, it can be hypothesised that crabs maintained at 30 °C used haemolymph proteins to cope with high temperature, considering that proteins are the primary source of energy in Crustacea (Helland et al., 2003; Sanchez-Paz et al., 2007). The results obtained suggested that *C. aestuarii* exploited proteins as an immediate energy source during high temperature stress, while it accumulated glucose in haemolymph to be used after.

The role of PO in immune reactions of Crustacea has extensively been reviewed by Söderhäll and Cerenius (1998). The zymogen proPO occurs within the granulocytes of decapods; upon activation by limited proteolysis, the active form of the enzyme - which is responsible for melanin deposition - can be released into the haemolymph (Cerenius and Söderhäll, 2004). We chose to measure PO activity in both CFH and HL of *C. aestuarii* to follow enzyme variation pattern in both media. In the present study, both the lowest and highest temperature tested caused a significant

increase of PO activity in crab CFH, whereas no significant variations in PO activity were recorded in HL. Lobsters (*P. interruptus*) reared at 4 °C had significantly lower PO activity as compared to the lobsters reared in 14 and 19 °C (Gomez-Jimenez et al., 2000). In a recent study, shrimps (*Litopenaeus vannamei*) transferred to 32 °C showed significant decreases in PO activity after 24-96 h, when compared to that of control animals (28 °C) (Cheng et al., 2005). In the same study, shrimps transferred to 20 °C showed a PO activity decreased significantly by 12% and 14% after 24 and 48 h, with respect to control shrimps. It is well known that crustacean haemocytes, mainly granulocytes, contain high levels of PO, which can be secreted by haemocytes into the haemolymph where it is involved in melanin deposition around the damaged tissues (Cerenius and Soderhall, 2004). We have recently demonstrated that haemocytes from *C. aestuarii* are also involved in PO production (Matozzo and Marin, 2010b). Although a positive correlation between THC and PO activity is generally reported in the literature (see Cheng et al., 2005 and cited references), the present study clearly demonstrated that PO activity increased when THC decreased. We can hypothesise that increased PO activity in haemolymph from temperature-stressed crabs was a physiological response of animals to compensate for lower THC, in order to increase immunosurveillance in both haemolymph and peripheral tissues, corroborating findings by Hauton et al. (1997). They suggested that fluctuations in PO activity are not attributable to changes in haemocyte number. Indeed, *P. stylirostris* increased its PO activity but decreased its THC following hypoxia stress (Le Moullac et al., 1998). A negative correlation between PO activity and THC was also observed in *C. maenas* (Hauton et al., 1995) and in the common shrimp *Crangon crangon* (Smith and Johnston, 1992). Nevertheless, the increase in haemolymph PO activity observed in the present study could be caused by a change in haemocyte sub-populations, as a marked PO activity was found in granulocytes from *C. aestuarii*, but not in hyalinocytes (Matozzo and Marin, 2010b). Further studies are needed to support this hypothesis.

Reactive oxygen species (ROS), such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^\cdot), can be dangerous for organisms, being able to induce oxidative damage to

lipids, DNA and other key molecules (Halliwell and Gutteridge, 1999). Organisms have developed antioxidant systems to protect their biological structures from ROS-mediated damage (Halliwell and Gutteridge, 1999). In antioxidant systems, SOD is the first and most important defence line, as it catalyses the dismutation of the superoxide anion into molecular oxygen and hydrogen peroxide. CAT, with glutathione peroxidase (GPx), is the most important scavenger of H_2O_2 in cells. In our study, the lowest temperature caused a significant reduction of CAT activity in gills. Results obtained in *C. aestuarii* are difficult to explain, mainly considering that information concerning temperature effects on crustacean antioxidant enzyme activities are scarce. In the shrimp *L. vannamei* transferred to 32 °C, haemocyte SOD activity decreased significantly after 24 h, when compared to control animal (28 °C); for shrimps transferred to 20 °C, SOD activity decreased significantly by 20% both after 24 and 48 h (Cheng et al., 2005). In the mud crab *Scylla serrata*, the activities of both SOD and CAT in gills were higher in summer and lower in winter (Kong et al., 2008). On the basis of the results obtained, it can be hypothesised that *C. aestuarii* is more susceptible to oxidative stress (with regard to CAT activity at least) when lower seawater temperatures are reached.

In conclusion, the present study demonstrated that both the lowest and the highest temperature tested influenced immune parameters in crabs. Conversely, responses provided by antioxidant enzymes were not indicative of particular differences due to variation in temperature values. In any case, the present study also indicated that *C. aestuarii* modulated its cellular and biochemical parameters in order to cope with temperature (see for example haemocyte proliferation and CFH PO activity). This suggested that when crabs suffer temperature stress in the environment, they can respond promptly by modulating immune parameters. However, both results of the present study and those from the literature suggest differing immunomodulation patterns in crustacean species depending on their thermal optimum.

Figure legends

Fig. 1. Effects of temperature on THC, expressed as number of haemocytes ($\times 10^6$) mL haemolymph⁻¹, in *C. aestuarii*. Values are means \pm s.e.; n=5, * $p < 0.05$, ** $p < 0.01$.

Fig. 2. Effects of temperature on haemocyte proliferation, expressed as OD₄₅₀ mL haemolymph⁻¹, in *C. aestuarii*. Values are means \pm s.e.; n=5, * $p < 0.05$.

Fig. 3. Effects of temperature on haemolymph glucose levels, expressed as mg glucose mL haemolymph⁻¹, in *C. aestuarii*. Values are means \pm s.e.; n=5.

Fig. 4. Effects of temperature on haemolymph total protein concentrations, expressed as mg mL haemolymph⁻¹, in *C. aestuarii*. Values are means \pm s.e.; n=5, * $p < 0.05$.

Fig. 5. Effects of temperature on PO activity, expressed as U mg proteins⁻¹, in haemocyte lysate (HL) and cell-free haemolymph (CFH) of *C. aestuarii*. Values are means \pm s.e.; n=5, * $p < 0.05$, ** $p < 0.01$.

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ACCEPTED MANUSCRIPT

HIGHLIGHTS

- Effects of temperature to the crab *Carcinus aestuarii* were evaluated.
- Various cellular and biochemical biomarkers were measured.
- Both high and low temperature influenced crab biological responses.

Tab. 1. SOD and CAT activities, expressed as U mg proteins⁻¹, in gills and digestive gland from crabs (*C. aestuarii*) kept at different temperatures. Values are means \pm s.e.; n = 10. Asterisk denotes significant differences with respect to both control (17 °C) and 30 °C-exposed crabs; * $p < 0.05$.

Experimental temperatures	Gills		Digestive gland	
	SOD activity	CAT activity	SOD activity	CAT activity
4 °C	2.22 \pm 0.24	4.77 \pm 1.59*	0.81 \pm 0.18	1.97 \pm 0.49
17 °C	1.91 \pm 0.27	8.05 \pm 2.12	0.63 \pm 0.19	2.64 \pm 1.37
30 °C	2.10 \pm 0.27	10.68 \pm 1.86	0.97 \pm 0.39	2.22 \pm 1.07

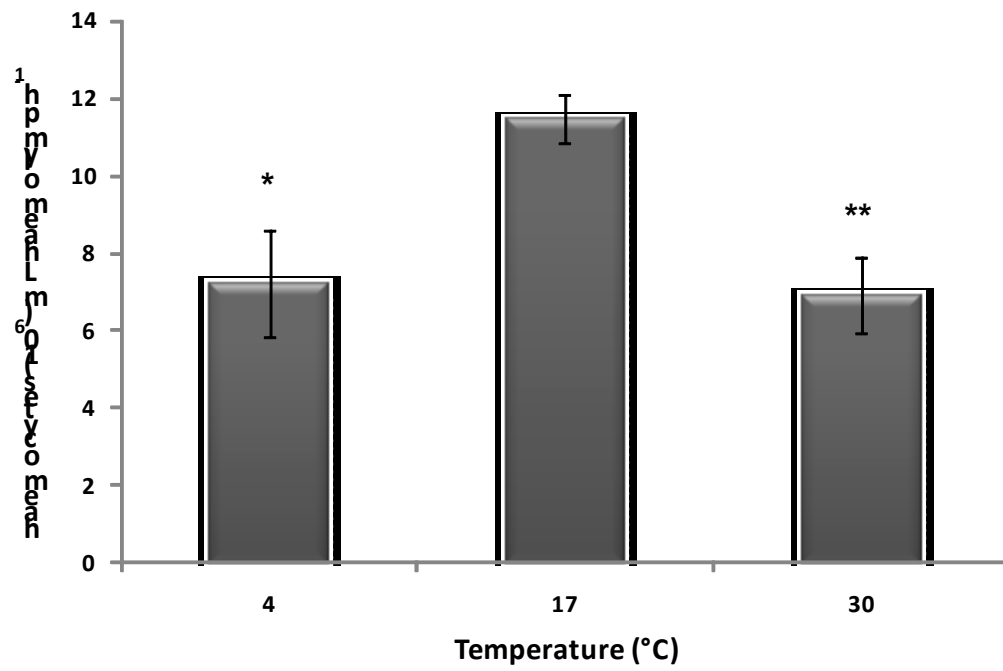


Fig. 1

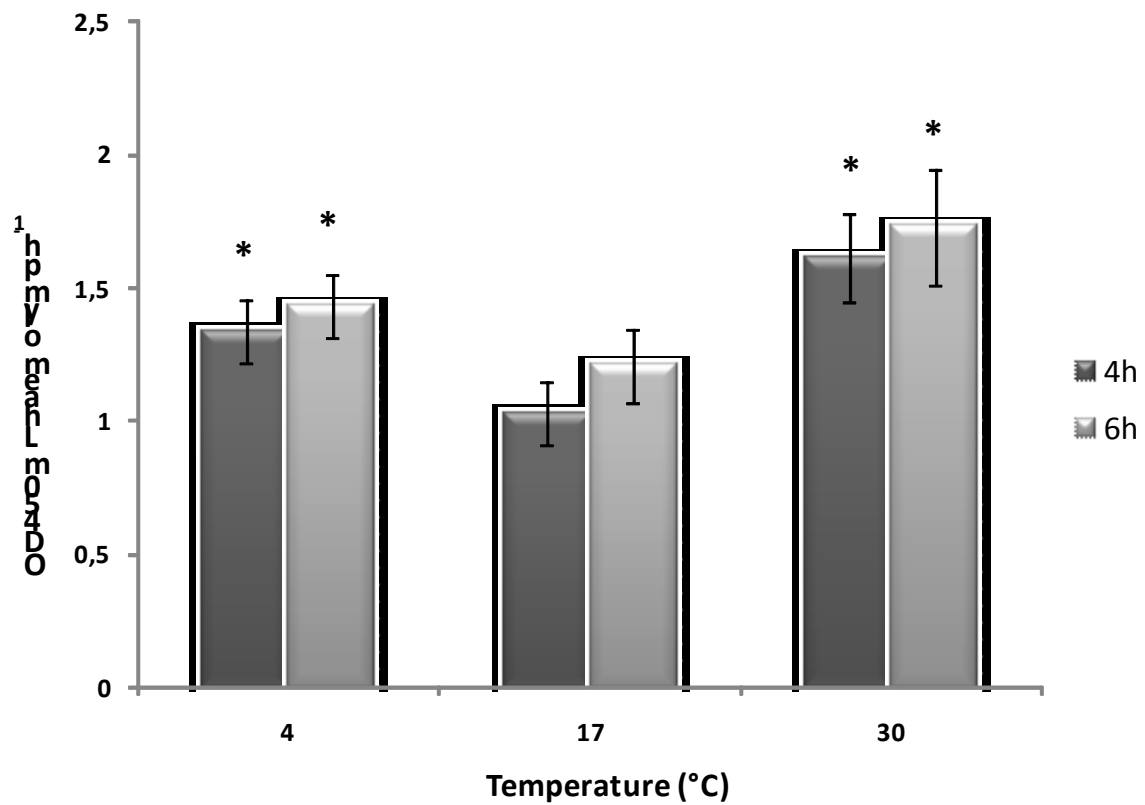


Fig. 2

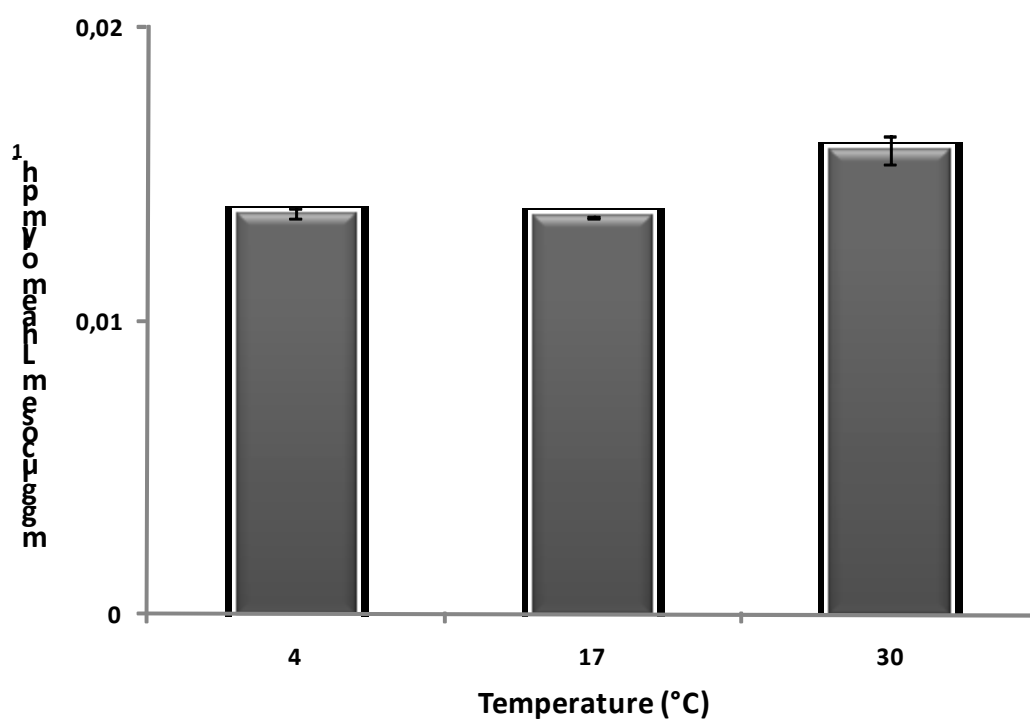


Fig. 3

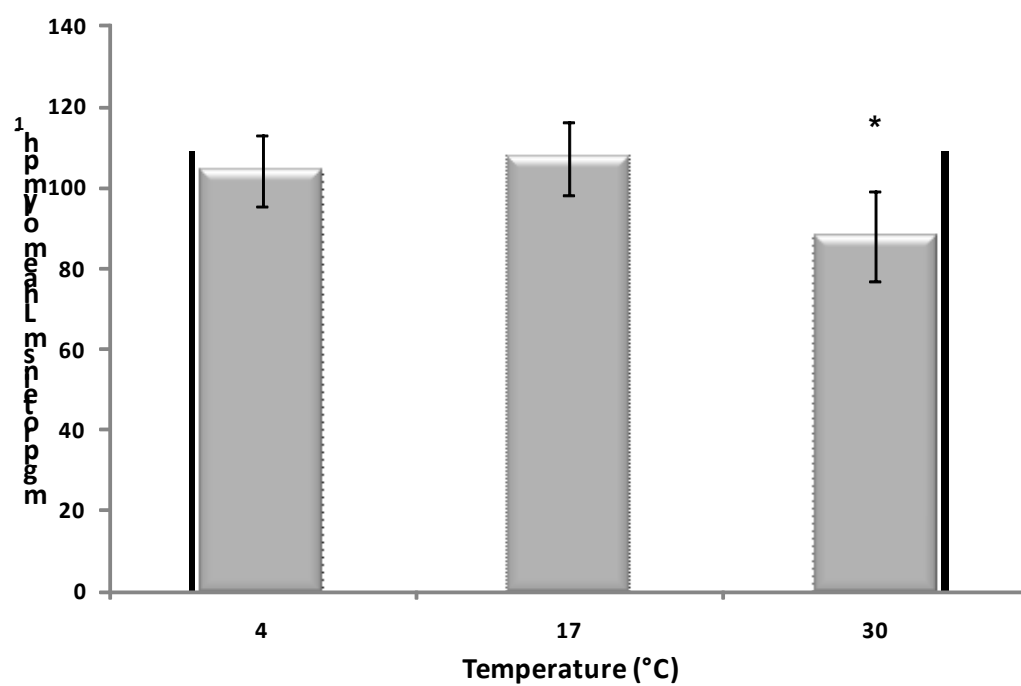


Fig. 4

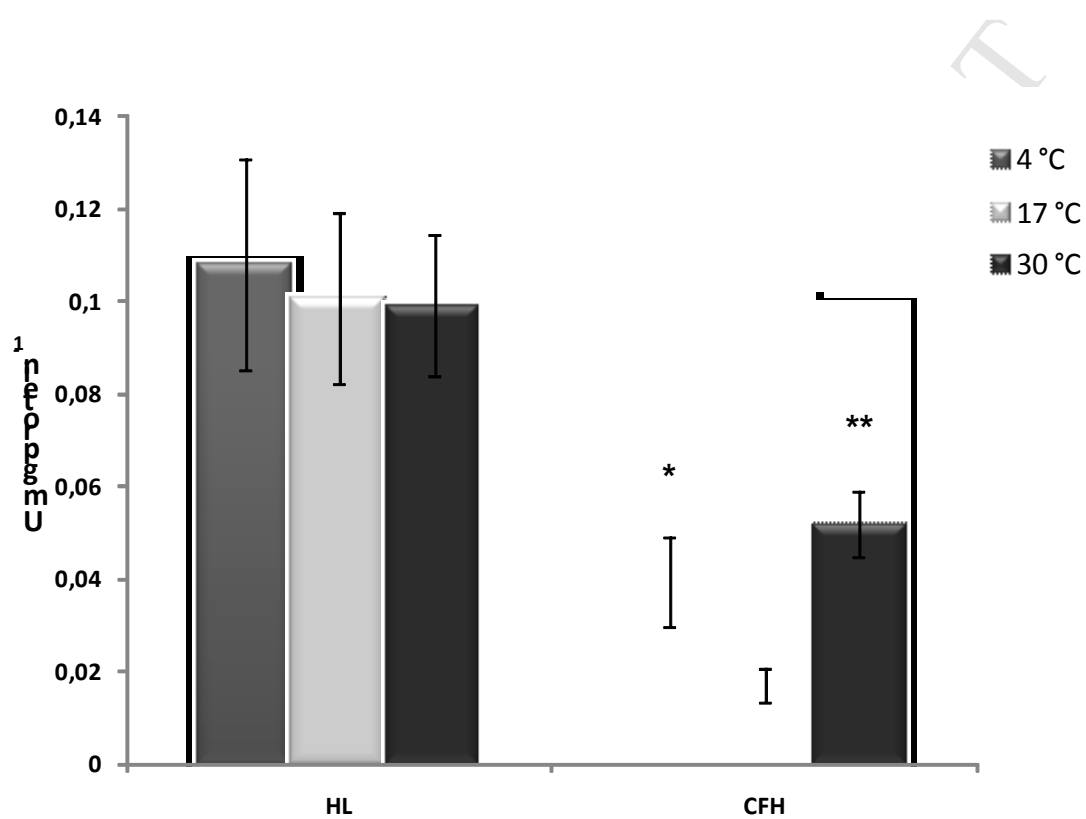


Fig. 5