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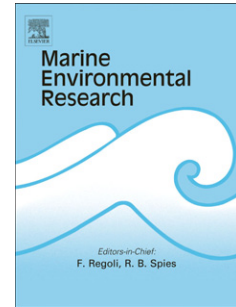
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Effects of temperature on cellular and biochemical parameters in the crab***Carcinus aestuarii* (Crustacea, Decapoda)**

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27 **Abstract**

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

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47

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

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53 1. Introduction

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

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

91 Like other Crustacea (Bauchau, 1981), three main haemocyte types have recently been identified
92 in *C. aestuarii*: granulocytes, with evident cytoplasmic granules, semigranulocytes, with less
93 granules than granulocytes, and hyalinocytes, without evident granules (Matozzo and Marin,
94 2010a). Hyalinocytes are the main phagocytic cells, whereas granulocytes show high phenoloxidase
95 (PO) activity (Matozzo and Marin, 2010b).

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

104 considered as useful indicators of immunesurveillance status in Crustacea (Le Moullac and Haffner,
105 2000), and taking into account that a reduced immunesurveillance can strongly enhance the
106 susceptibility of animals to infections/diseases. In addition, superoxide dismutase (SOD) and
107 catalase (CAT) activities were measured in both gills and digestive gland of crabs maintained at the
108 differing temperatures. SOD and CAT are antioxidant enzymes that play an important role in
109 reducing damage to cells caused by reactive oxygen species (ROS). The aim of the present study
110 was to evaluate whether temperature can affect both immune parameters and antioxidant enzyme
111 activities in an ecologically and economically important crab species.

112

113 **2. Materials and methods**

114 *2.1. Crabs*

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

120

121 *2.2. Exposure to differing temperatures*

122 Prior to experimental temperature exposure, crabs were acclimatised to experimental conditions
123 by progressively increasing or decreasing seawater temperature (2-2.5 °C per day) in the aquaria
124 (50 L) to 4 and 30 °C. Crabs maintained at 17 °C served as controls. Crabs (10 per experimental
125 condition) were kept for 7 days at the three temperatures in three different aquaria provided with a
126 sandy bottom and well-aerated seawater (salinity of 35 ± 1 psu, pH 8.1), and fed with mussels.
127 Seawater temperature, salinity and pH were checked daily, whereas seawater was renewed (50%)
128 every 48 h.

129

130 *2.3. Haemolymph and tissue collection*

131 At the end of temperature exposure, crabs were anaesthetised on ice for 5 min, and the
132 haemolymph (at least 500 μ L per crab) was collected from the unsclerotised membrane of the
133 walking legs using a 1-mL plastic syringe, placed in microcentrifuge tubes on ice and diluted
134 (except for glucose assay) 1:2 in an anticoagulant solution of citrate buffer/EDTA (NaCl 0.45 M,
135 glucose 0.1 M, sodium citrate 30 mM, citric acid 26 mM, EDTA 10 mM, pH 4.6, stored at 4 °C)
136 (Söderhäll and Smith, 1983). For each experimental temperature, 5 pools of haemolymph from 2
137 crabs each were prepared. Pooling was necessary because individuals did not provide enough
138 haemolymph for analyses. Pooled haemolymph were then divided in four aliquots: two aliquots
139 were immediately used to measure THC and haemocyte proliferation, whereas the remaining two
140 aliquots were processed to measure PO activity in both haemocyte lysate (HL) and cell-free
141 haemolymph (CFH), and CFH glucose levels. To obtain CFH, pooled haemolymph was
142 immediately centrifuged at 780 x g for 10 min, and the supernatant (= CFH) was collected. To
143 obtain HL, haemocytes were resuspended in distilled water, sonicated at 0 °C for 1 min, and then
144 centrifuged at 780 x g for 30 min. Both CFH and HL were frozen in liquid nitrogen and stored at –
145 80 °C until analyses.

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

153

154 *2.4. THC and haemocyte volume determination*

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

160

161 *2.5. Haemocyte proliferation*

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

170

171 *2.6. Haemolymph glucose and total protein concentrations*

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

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

179

180 2.7. Phenoloxidase activity assay

181 PO activity was measured in both HL and CFH using L-DOPA (3,4-dihydroxy-L-phenyl-
182 alanine, Sigma) as substrate in a colorimetric assay. One hundred μL of HL and CFH were added to
183 900 μL of 1 mg L-DOPA/mL of phosphate buffered saline, pH 7.2, (PBS: 1.37 M NaCl, 0.03 M
184 KCl, 0.015 M KH_2PO_4 , 0.065 M Na_2HPO_4), and incubated for 30 min at 37 °C. Absorbance at
185 490 nm was then recorded on a Beckman 730 spectrophotometer, and results were expressed as U
186 mg proteins^{-1} . Protein concentrations in both HL and CFH were quantified according to the Biuret
187 method (Sigma). The specific activity of PO in *C. aestuarii* has previously been assayed using N-
188 phenylthiourea (10 mM, Fluka) as a inhibitor (Matozzo and Marin, 2010b).

189

190 2.8. SOD activity assay

191 Total SOD activity was measured in both gills and digestive gland (see 2.3. section for details
192 regarding supernatant obtainment) in triplicate with the xanthine oxidase/cytochrome *c* method
193 according to Crapo et al. (1978). The cytochrome *c* reduction by superoxide anion generated by
194 xanthine oxidase/hypoxanthine reaction was detected at 550 nm at room temperature. Enzyme
195 activity was expressed as U $\text{mg of proteins}^{-1}$, one unit of SOD being defined as the amount of
196 sample producing 50% inhibition in the assay conditions. The reaction mixture contained 46.5 μM
197 $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ (pH 8.6), 0.1 mM EDTA, 195 μM hypoxanthine, 16 μM cytochrome *c*, and 2.5
198 μU xanthine oxidase. Protein concentrations in homogenised tissues were quantified according to
199 the Biuret method (Sigma).

200

201 2.9. CAT activity assay

202 Gill and digestive gland CAT activity was measured in triplicate following the method of Aebi
203 (1984). Decreases in absorbance of a 50-mM H_2O_2 solution ($\epsilon = -0.0436 \text{ mM}^{-1} \text{ cm}^{-1}$) in 50 mM
204 phosphate buffer (pH 7.8) and 10 μL of tissue supernatant were continuously recorded at 240 nm at
205 10 sec intervals for 1 min. Results were expressed in U $\text{mg of proteins}^{-1}$, one unit of CAT being

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

208

209 2.10. Statistical analysis

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

214

215 3. Results

216 THC values significantly decreased in crabs kept at 4 °C ($p < 0.05$) and 30°C ($p < 0.01$), when
217 compared with those of control crabs (17 °C) (Fig. 1). No differences were found between crabs
218 kept at 4 °C and those held at 30 °C. Haemocyte volume did not differ significantly among
219 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
220 °C). After 4 and 6 hours of haemocyte incubation in XTT labelling mixture, haemocyte
221 proliferation increased significantly ($p < 0.05$) in crabs kept at 4 and 30 °C, when compared with
222 that of crabs held at 17 °C (Fig. 2).

223 Although a slight increase in CFH glucose levels was observed in crabs kept at 30 °C, no
224 significant differences were recorded among experimental conditions (Fig. 3). Conversely, total
225 protein concentration significantly ($p < 0.05$) reduced in CFH of crabs maintained at 30 °C ($88 \pm$
226 11.04 mg mL haemolymph⁻¹), with respect to that of crabs kept at 4 °C (104.26 ± 8.72 mg mL
227 haemolymph⁻¹) and 17 °C (107.36 ± 11.04 mg mL haemolymph⁻¹) (Fig. 4).

228 A significantly higher PO activity was recorded in CFH from crabs kept at 4 °C ($p < 0.05$) and
229 30 °C ($p < 0.01$), when compared to the control group of crabs maintained at 17 °C (Fig. 5).
230 Conversely, PO activity did not differ significantly in HL from crabs kept at the three experimental
231 temperatures (Fig. 5).

232 With regard to antioxidant enzyme activities, a significant ($p < 0.05$) decrease in CAT activity
233 was observed in gills from crabs maintained at 4 °C, when compared to that of crabs kept at 17 and
234 30 °C (Table 1). No other significant variations in either CAT and SOD activities were recorded in
235 the gill and digestive gland tissues of crabs held at the three experimental temperatures.

236

237 4. Discussion

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

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

264 It has been demonstrated that crustacean haemocytes can divide in haemolymph, and that
265 proliferation can increase significantly after mitogenic or infectious stimulation (Sequeira et al.,
266 1996). Interestingly, the present study demonstrated that extreme temperatures caused a significant
267 increase in haemocyte proliferation, suggesting that the proliferative rate of circulating haemocytes
268 increased in temperature-stressed crabs in order to compensate partially for the reduction in the
269 number of circulating haemocytes. Similar results were recorded in Crustacea subject to different
270 stress conditions. For example, Sequeira et al. (1996) reported a significant increase in the
271 proliferation of circulating haemocytes in *Penaeus japonicus* following injection with
272 lipopolysaccharide, and a six-fold increase in the percentage of proliferating haemocytes was also
273 observed in animals infected with *Fusarium* spp. Likewise, bacteria injection caused a reduction in
274 THC values and a significant increase in haemocyte proliferation in *C. aestuarii* (Matozzo et al.,
275 2011).

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

284 (1998) demonstrated that emersion was a potent stimulator of haemolymph CHH in *Homarus*
285 *americanus*, whereas temperature and salinity variations were less effective. Conversely, increases
286 in water temperature resulted in increased blood CHH in *Cancer pagurus* (Wilcockson et al., 2002).
287 In *Uca minax*, a temperature value of 10 °C caused a significant decrease in haemolymph glucose
288 levels (Dean and Vernberg 1965), whereas in *P. interruptus*, increases in temperature from 20 °C to
289 27 °C induced an increase in haemolymph glucose levels (Ocampo et al., 2003). Powell and
290 Rowley (2008) suggested that increases in haemolymph glucose levels may be due to the
291 mobilisation of glucose from haepatopancreas and/or muscles in stressed crabs. Results of the
292 present study demonstrated that the extreme temperatures tested did not elicit significant variations
293 in haemolymph glucose concentrations. As a consequence, a temperature-induced mobilisation of
294 glucose from haepatopancreas and/or muscles can be excluded in *C. aestuarii*, on the basis of these
295 preliminary results at least.

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

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

310 increase of PO activity in crab CFH, whereas no significant variations in PO activity were recorded
311 in HL. Lobsters (*P. interruptus*) reared at 4 °C had significantly lower PO activity as compared to
312 the lobsters reared in 14 and 19 °C (Gomez-Jimenez et al., 2000). In a recent study, shrimps
313 (*Litopenaeus vannamei*) transferred to 32 °C showed significant decreases in PO activity after 24-
314 96 h, when compared to that of control animals (28 °C) (Cheng et al., 2005). In the same study,
315 shrimps transferred to 20 °C showed a PO activity decreased significantly by 12% and 14% after 24
316 and 48 h, with respect to control shrimps. It is well known that crustacean haemocytes, mainly
317 granulocytes, contain high levels of PO, which can be secreted by haemocytes into the haemolymph
318 where it is involved in melanin deposition around the damaged tissues (Cerenius and Soderhall,
319 2004). We have recently demonstrated that haemocytes from *C. aestuarii* are also involved in PO
320 production (Matozzo and Marin, 2010b). Although a positive correlation between THC and PO
321 activity is generally reported in the literature (see Cheng et al., 2005 and cited references), the
322 present study clearly demonstrated that PO activity increased when THC decreased. We can
323 hypothesise that increased PO activity in haemolymph from temperature-stressed crabs was a
324 physiological response of animals to compensate for lower THC, in order to increase
325 immunosurveillance in both haemolymph and peripheral tissues, corroborating findings by Hauton
326 et al. (1997). They suggested that fluctuations in PO activity are not attributable to changes in
327 haemocyte number. Indeed, *P. stylirostris* increased its PO activity but decreased its THC following
328 hypoxia stress (Le Moullac et al., 1998). A negative correlation between PO activity and THC was
329 also observed in *C. maenas* (Hauton et al., 1995) and in the common shrimp *Crangon crangon*
330 (Smith and Johnston, 1992). Nevertheless, the increase in haemolymph PO activity observed in the
331 present study could be caused by a change in haemocyte sub-populations, as a marked PO activity
332 was found in granulocytes from *C. aestuarii*, but not in hyalinocytes (Matozzo and Marin, 2010b).
333 Further studies are needed to support this hypothesis.

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

336 lipids, DNA and other key molecules (Halliwell and Gutteridge, 1999). Organisms have developed
337 antioxidant systems to protect their biological structures from ROS-mediated damage (Halliwell
338 and Gutteridge, 1999). In antioxidant systems, SOD is the first and most important defence line, as
339 it catalyses the dismutation of the superoxide anion into molecular oxygen and hydrogen peroxide.
340 CAT, with glutathione peroxidase (GPx), is the most important scavenger of H₂O₂ in cells. In our
341 study, the lowest temperature caused a significant reduction of CAT activity in gills. Results
342 obtained in *C. aestuarii* are difficult to explain, mainly considering that information concerning
343 temperature effects on crustacean antioxidant enzyme activities are scarce. In the shrimp *L.*
344 *vannamei* transferred to 32 °C, haemocyte SOD activity decreased significantly after 24 h, when
345 compared to control animal (28 °C); for shrimps transferred to 20 °C, SOD activity decreased
346 significantly by 20% both after 24 and 48 h (Cheng et al., 2005). In the mud crab *Scylla serrata*, the
347 activities of both SOD and CAT in gills were higher in summer and lower in winter (Kong et al.,
348 2008). On the basis of the results obtained, it can be hypothesised that *C. aestuarii* is more
349 susceptible to oxidative stress (with regard to CAT activity at least) when lower seawater
350 temperatures are reached.

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

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363 **Figure legends**

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366 **Fig. 1.** Effects of temperature on THC, expressed as number of haemocytes ($\times 10^6$) mL

367 haemolymph⁻¹, in *C. aestuarii*. Values are means \pm s.e.; n=5, * $p < 0.05$, ** $p < 0.01$.

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369

370 **Fig. 2.** Effects of temperature on haemocyte proliferation, expressed as OD₄₅₀ mL haemolymph⁻¹, in

371 *C. aestuarii*. Values are means \pm s.e.; n=5, * $p < 0.05$.

372

373 **Fig. 3.** Effects of temperature on haemolymph glucose levels, expressed as mg glucose mL

374 haemolymph⁻¹, in *C. aestuarii*. Values are means \pm s.e.; n=5.

375

376 **Fig. 4.** Effects of temperature on haemolymph total protein concentrations, expressed as mg ml

377 haemolymph⁻¹, in *C. aestuarii*. Values are means \pm s.e.; n=5, * $p < 0.05$.

378

379 **Fig. 5.** Effects of temperature on PO activity, expressed as U mg proteins⁻¹, in haemocyte lysate

380 (HL) and cell-free haemolymph (CFH) of *C. aestuarii*. Values are means \pm s.e.; n=5, * $p < 0.05$, ** p

381 < 0.01 .

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507

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

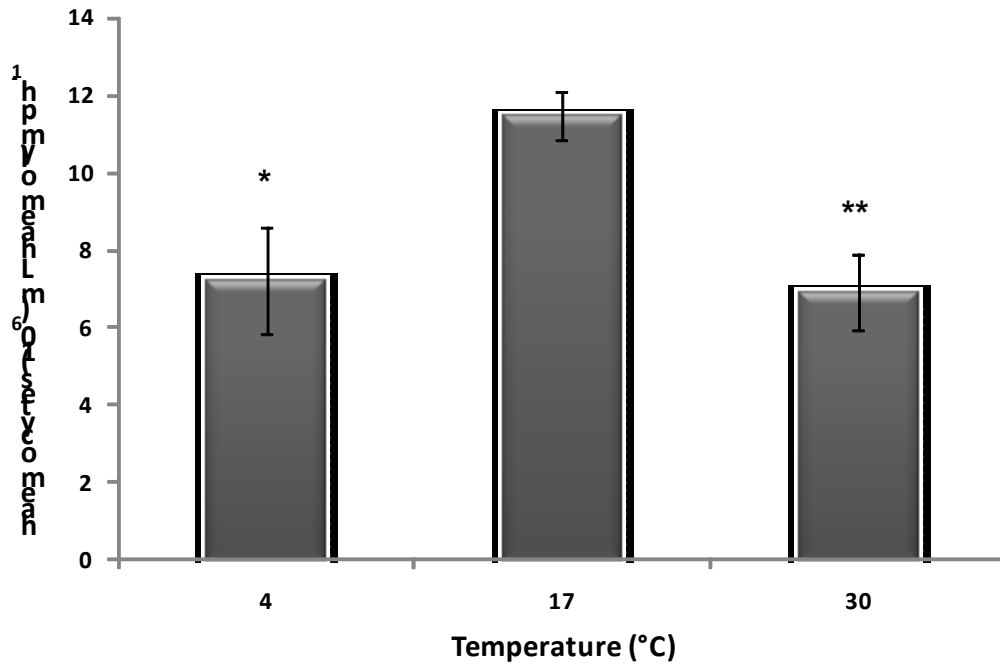


Fig. 1

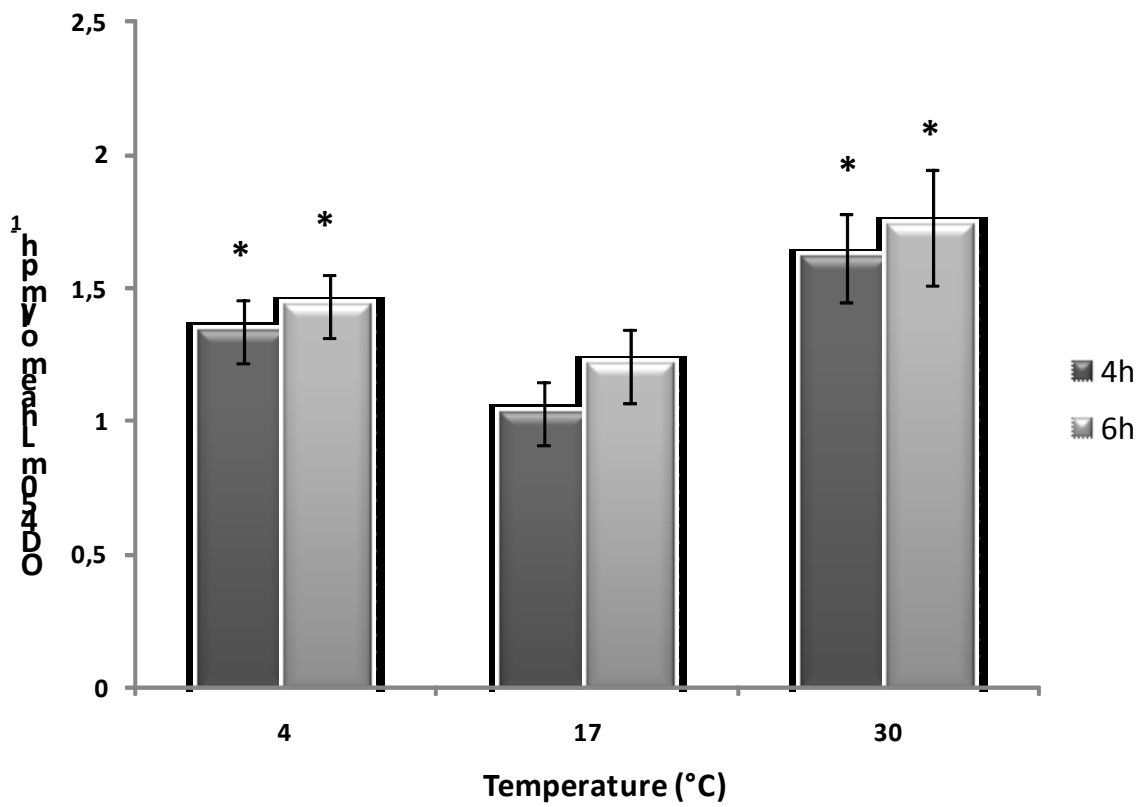


Fig. 2

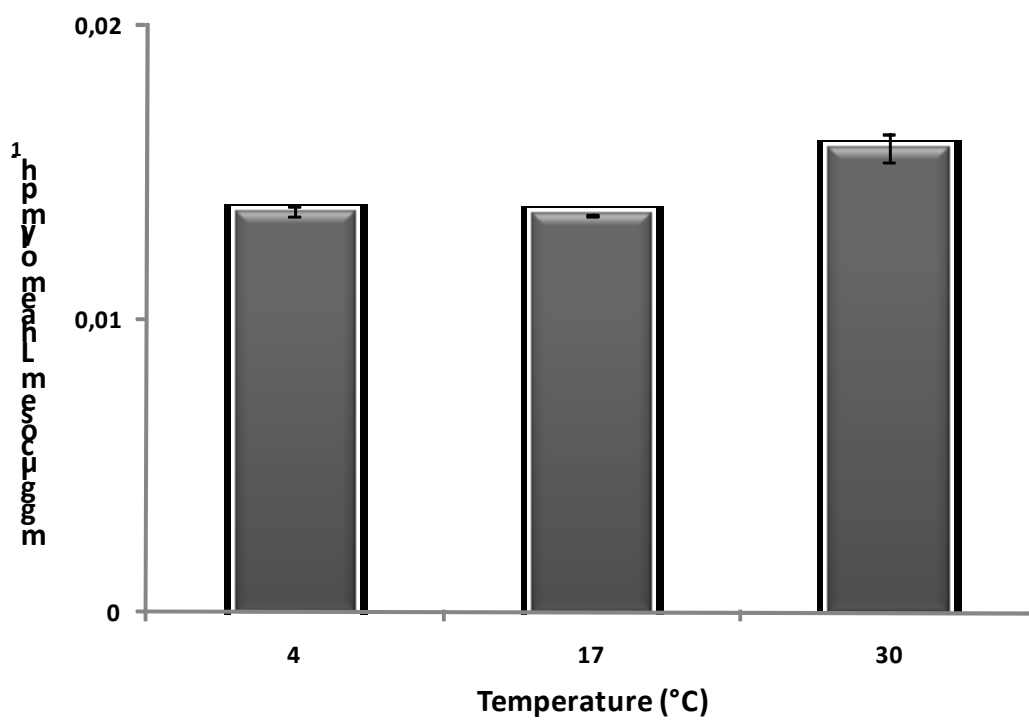


Fig. 3

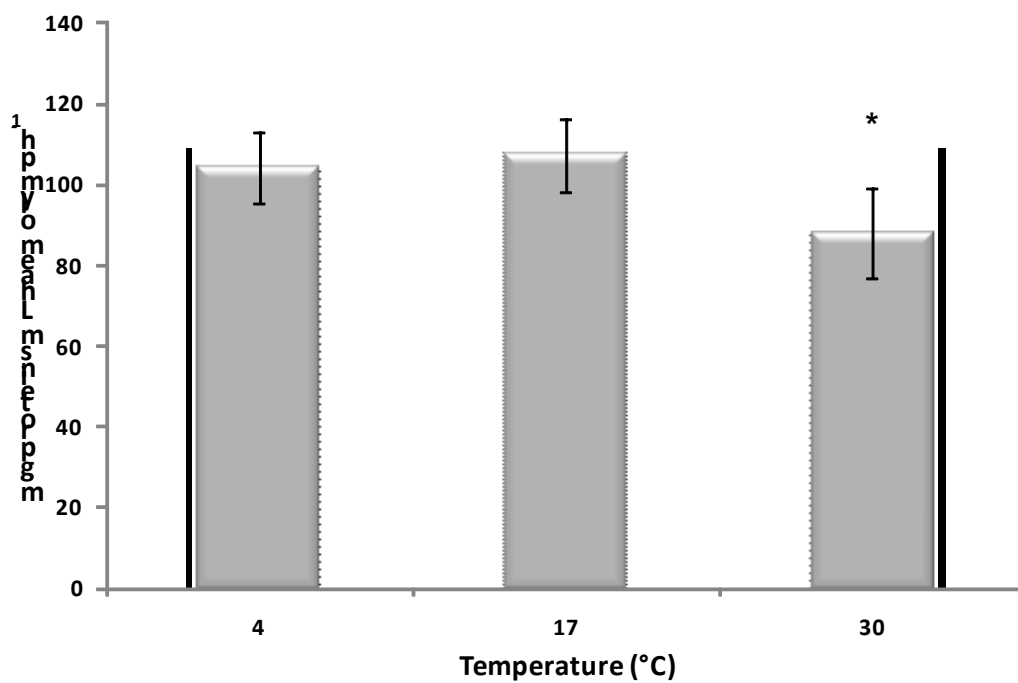


Fig. 4

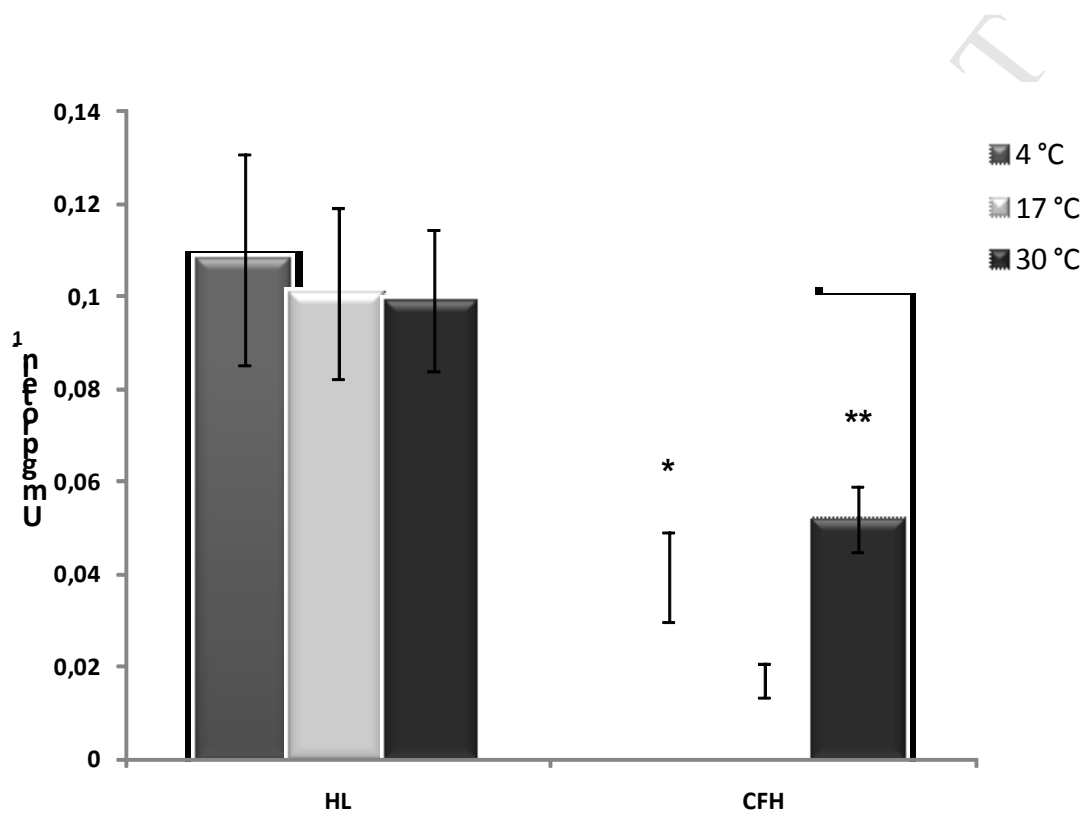


Fig. 5