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Can starvation influence cellular and biochemical parameters in the crab

***Carcinus aestuarii*?**

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Abstract

Crustacea experience periods of starvation during moulting or when limited food availability occurs. The effects of starvation on Crustacea physiological responses have been demonstrated, whereas the effects of starvation on Crustacea immune parameters remain to be more fully studied. In the present study the effects of starvation on immune parameters and antioxidant enzyme activities of the crab *Carcinus aestuarii* were evaluated for the first time. Treated crabs were starved for 7 days, whereas control crabs were fed daily with mussels. Total haemocyte count (THC), haemocyte diameter and volume, haemocyte proliferation, cell-free haemolymph (CFH) glucose and total protein levels, and phenoloxidase (PO) activity in both haemocyte lysate (HL) and CFH were measured in crabs. In addition, superoxide dismutase (SOD) and catalase (CAT) activities were evaluated in both gills and digestive gland from crabs, in order to evaluate whether starvation induced oxidative stress in *C. aestuarii*. THC increased significantly in starved crabs, with respect to controls, whereas no significant variations were observed in haemocyte diameter, volume and proliferation. In CFH of starved animals glucose concentration significantly increased, whereas total protein concentration significantly reduced. A significantly higher PO activity was recorded in HL from starved crabs, than in control crabs. Conversely, PO activity did not vary significantly in CFH. Starvation did not cause significant alterations in antioxidant enzyme activities in both gills and digestive gland. Results obtained demonstrated that starvation influenced crab immune parameters, but did not induce oxidative stress. Results also indicated that *C. aestuarii* can modulate its cellular and biochemical parameters in order to cope with starvation.

Keywords: crabs; *Carcinus aestuarii*; starvation; immune parameters; haemocytes; glucose; antioxidant enzymes

1. Introduction

The green crab *Carcinus aestuarii* (Crustacea, Decapoda) is native of the Mediterranean Sea, and it is very similar to the Atlantic species *Carcinus maenas*. The two species are mainly distinguishable by 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). Owing to natural dispersal, maritime commerce and ballast transport, *Carcinus* species have colonised several regions outside their native areas, showing high tolerance to air exposure, starvation, and variations in temperature and salinity (Yamada and Hauck, 2001). In particular, *C. aestuarii* has also colonised estuarine areas along the Italian coasts, such as the Lagoon of Venice.

Crustacea have an open vascular system in which numerous haemocytes circulate in haemolymph. It has been demonstrated that crustacean haemocytes are involved in important functions, such as wound repair and defence mechanisms against parasites, viruses and bacteria (Bauchau, 1981). Haemocyte-mediated immune defence includes phagocytosis, encapsulation, nodule formation, clotting, agglutination, melanisation and microbicidal activity (Bauchau, 1981; Smith and Söderhäll, 1986; Söderhäll and Cerenius, 1992). Three types of circulating haemocytes are generally recognised in Crustacea: hyalinocytes, the smallest cells without evident granules; semigranulocytes, which contain small granules, and granulocytes, with abundant cytoplasmic granules (Bauchau, 1981). The three haemocyte types were also identified in *C. aestuarii* (Matozzo et al., 2010a). Only hyalinocytes were able to phagocytose yeast cells or Zymosan. All haemocyte types produced superoxide anion, whereas only granulocytes were positive to some hydrolytic and oxidative enzyme activities (Matozzo et al., 2010b).

In the last few years, efforts have been addressed to the evaluation of stress effects in Crustacea, considering that many species are commercially-important and disease outbreaks may cause a decline in both natural and farmed populations. In this context, it has been

demonstrated that environmental factors can cause immunomodulation in Crustacea (LeMoullac and Haffner, 2000). Among stressors, starvation is worthy of consideration. Crabs experience periods of starvation during moulting (Lipcius and Herrnkind, 1982; Sanchez-Paz et al., 2006) or when limited food availability occurs (Crothers, 1967). Feeding generally resumes postmoult, when the exoskeleton hardens (Sanchez-Paz et al., 2006). Although it has previously been demonstrated that starvation can cause several physiological, metabolic and behavioural changes in Crustacea (Sanchez-Paz et al., 2007), little attention has been addressed to the evaluation of the effects of starvation on immune responses of Crustacea. To fill this gap, in the present study the effects of 7 days' starvation on immune parameters and antioxidant enzyme activities in gills and digestive gland from the crab *C. aestuarii* were evaluated for the first time. Considering that a good nutritional status is generally associated with the maintenance of an efficient immune system in animals (Biao et al., 2008), we posed two questions:

- i. can starvation influence immune parameters and antioxidant status in *C. aestuarii*?
- ii. can *C. aestuarii* modulate its immune parameters to cope with starvation?

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 and kept in the laboratory in large aquaria containing seawater (salinity of 35 ± 1 psu and temperature of 17 ± 0.5 °C) and a sandy bottom. The crabs were fed on alternate days with mussels (*Mytilus galloprovincialis*) and acclimatised in the laboratory for 5 days before the experiments. Only crabs without obvious injuries or infection were used for experiments.

2.2. *Experimental setup*

Treated and control crabs (12 starved and 12 fed crabs, respectively) were kept for 7 days in two distinct aquaria at the same experimental conditions described above. Every 24 h, both treated and control crabs were transferred to two distinct aquaria fitted with partition boards forming 12 individual boxes each (Fig. 1A). Half a mussel (4 cm mean shell length, 1.5 g mean fresh weight) was supplied daily to each control crab, whereas treated crabs did not receive food (Fig. 1B, C). The maintenance of each control crab in separate boxes allowed animals to eat quietly, avoiding assault by the other crabs during feeding. After one hour, both fed and starved crabs were moved again to the two unpartitioned aquaria.

2.3. *Haemolymph and tissue collection*

Crabs were anaesthetised on ice for 10 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 Eppendorf tubes on ice and diluted (except for glucose determination) 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). Six pools of haemolymph from two crabs each were prepared. Pooling was necessary because individuals did not provide enough haemolymph for analyses. Pooled haemolymph was then divided in four aliquots: two aliquots were immediately used to measure THC, haemocyte diameter and volume, and haemocyte proliferation; 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 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 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. In due time, gills and digestive gland were individually 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 $40\text{ }\mu\text{g mL}^{-1}$ Aprotinin (Sigma), sonicated for 1 min at 0°C with a Braun Labsonic U sonifier at 50% duty cycles, and centrifuged at $10,000\text{ g}$ for 30 min at 4°C . Supernatants were collected for enzyme assays.

2.4. THC and haemocyte diameter and volume determination

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

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 $400\text{ }\mu\text{L}$ of haemolymph and incubated for 4 and 6 hrs in a dark humidified chamber. Absorbance at 450 nm was then recorded on a Beckman 730 spectrophotometer. Results were expressed as optical density per ml of haemolymph ($\text{OD mL haemolymph}^{-1}$). Protein concentration was quantified according to the Biuret method (Sigma) using bovine serum albumin (BSA) as standard.

2.6. *Haemolymph glucose and total protein concentrations*

CFH 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 $\text{mL haemolymph}^{-1}$.

CFH protein concentrations were quantified according to the Biuret method (Sigma). Results were expressed as mg protein $\text{mL haemolymph}^{-1}$.

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. One hundred μL of HL and CFH were added to 900 μL of L-DOPA (1 mg mL^{-1} in phosphate buffer, PBS) 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).

2.8. *SOD activity assay*

Total SOD activity was measured in both gill and digestive gland homogenised (see 2.3. section) 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 μ U 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 μ L of tissue supernatant were continuously recorded at 240 nm at 10 sec intervals for 1 min. Results were expressed in U mg proteins⁻¹, one unit of CAT being defined as the amount of enzyme that catalysed the dismutation of 1 μ mol of H_2O_2 min⁻¹. Protein concentrations were quantified according to the Biuret method (Sigma).

2.10. Statistical analysis

Results were compared by the Student's *t* test. Values are expressed as means $\pm$ standard error. The STATISTICA 5.5 (StatSoft, Tulsa, OK, USA) software package was used for statistical analyses.

3. Results

THC values significantly ($p < 0.05$) increased in starved crabs, when compared with those of control crabs (Fig. 2). Conversely, no significant differences were found in haemocyte diameter between starved and fed crabs ($8.04 \pm 1.4 \mu\text{m}$ and $8.41 \pm 1.6 \mu\text{m}$, respectively; $n=6$), as well as in haemocyte volume ($287.5 \pm 102.4 \text{ fL}$ and $280.2 \pm 127.5 \text{ fL}$, respectively; $n=6$).

After 7 days' starvation, haemocyte proliferation did not differ significantly between starved and control crabs, both at 4 and 6 hours of haemocyte incubation in XTT labelling mixture (Fig. 3).

An opposite pattern of variation was observed between glucose and total protein levels in CFH from fed and starved crabs (Fig. 4). A significant increase ($p < 0.05$) in CFH glucose

levels was observed in starved crabs (0.018 ± 0.0005 mg mL haemolymph⁻¹; n=6), with respect to controls (0.012 ± 0.0003 mg mL haemolymph⁻¹; n=6). Conversely, total protein concentration significantly ($p<0.05$) reduced in CFH of starved crabs (56.11 ± 11.63 mg mL haemolymph⁻¹; n=6), with respect to that of fed crabs (81.74 ± 8.16 mg · mL haemolymph⁻¹; n=6),

HL PO activity was significantly higher in starved crabs than in controls (Fig. 5). Conversely, PO activity did not differ significantly in CFH from starved and control crabs (Fig. 5).

With regard to the antioxidant enzyme activity, no significant variations of SOD and CAT activities were observed in both gills and digestive gland of starved and control crabs (Table1).

4. Discussion

Crustacea are generally characterised by a constant feeding activity. However, they can alternate periods of feeding and fasting during development, which occurs through moulting (ecdysis) and results in growth by sequential steps (Sanchez-Paz et al., 2006). Differing feeding behaviour can be recognised during crustacean moulting: during intermoult, they feed actively; prior to moulting, feeding declines and stops completely during moulting; feeding begins again in postmoult when the exoskeleton hardens (Phlippen et al., 2000). Animals experiencing such cycles of food intake may have a peculiar adaptation capability for use and storage of energy sources.

In the present study, starvation influenced immune parameters in crabs. In particular, THC values significantly increased in starved crabs. To this regard, results from the literature are controversial. For example, in *Homarus americanus*, prolonged fast caused a reduction of about 40% in THC values (Stewart et al., 1967), whereas in *L. vannamei* THC did not differ in starved and fed animals after 7 days' treatment, but it reduced in starved shrimps after 14

days (Pascual et al., 2006). The total number of circulating haemocytes was unaffected by the addition of chitin to the diet (a fish-based diet supplemented with 0, 5 or 10% chitin) in *C. maenas* (Powell and Rowley, 2007). Sequeira et al. (1996) suggested that increases in THC in Crustacea may be due either to more active mobilisation of haemocytes from tissues to haemolymph or to a faster division of circulating haemocytes. Conversely, the decrease in the number of circulating haemocytes 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). However, a reduction in cell proliferation rate can be also hypothesised. In the present study, a faster division of circulating haemocytes can be excluded, as haemocyte proliferation - as well as cell size - did not differ significantly between starved and control crabs. As a consequence, it is highly probable that the increased number of circulating haemocytes in *C. aestuarii* was due to an active mobilisation of haemocytes from tissues to haemolymph. A more active mobilisation of haemocytes from tissues to haemolymph probably allowed starved crabs to have mature cells in circulation at low energy costs, without waiting for maturation of new haemocytes originating from the division of existing haemocytes. Indeed, haemocyte proliferation requires energy expenditure, not convenient during starvation. In addition, we have recently demonstrated that carbohydrates are stored in large quantities in all haemocyte types of *C. aestuarii* (Matozzo et al., 2010a). Therefore, it can be hypothesised that the number of circulating haemocytes increased in starved crabs to allow them to have an important energy reserve to be mobilised during starvation.

In Crustacea, the increase in haemolymph glucose levels is considered an important response of animals to stress (Jussila et al., 1997; 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 recorded following exposure of Crustacea to differing

stressors, such as emersion (Durand et al., 2000), cold shock (Kuo and Yang, 1999), anoxia (Hall and van Ham, 1998) and pollutants (Lorenzon et al., 2000). In the present study, starvation caused a significant increase in glucose levels in haemolymph of *C. aestuarii*. Conversely, in *C. maenas*, starved animals showed lower blood glucose concentrations than fully-fed crabs (Dissanayake et al., 2009). In the same crab species, Powell and Rowley (2007) observed that the addition of chitin (one of the main complex polysaccharides of the cuticle of arthropods) had no significant effect on the serum concentrations of glucose. In *L. vannamei*, the starved animals showed significantly lower glucose concentration than control shrimps, with a constant reduction in the plasma glucose concentration from the beginning (4h) until the end of the experiment (120h) (Sanchez-Paz et al., 2007). According to Powell and Rowley (2008), the higher concentrations of glucose measured in the present study in haemolymph from starved crabs compared to fed animals may be due to the mobilisation of glucose from haepatopancreas and/or muscles to haemolymph in stressed crabs. Nevertheless, a mobilisation of glucose from haemocytes to the haemolymph cannot be excluded in *C. aestuarii*, considering both the high number of circulating haemocytes measured in starved crabs and the role of *C. aestuarii* haemocytes in carbohydrate transport (Matozzo et al., 2010a).

After 7 days' starvation, a significant decrease in haemolymph total protein levels was recorded in *C. aestuarii* (56.11 mg protein mL haemolymph⁻¹ in starved crabs, and 81.74 mg protein mL haemolymph⁻¹ in fed crabs). In *C. maenas*, the addition of chitin (5 to 10%) to a fish-based diet did not cause significant effects on the serum concentrations of proteins (Powell and Rowley, 2007). In the copepod *Calanus finmarchicus*, the protein content showed a moderate decline during the first 10 days of starvation, suggesting that this species cope with starvation utilizing endogenous reserves different from proteins; however, during the next 21 days, total protein content was drastically reduced (Helland et al., 2003). In *L. vannamei*, food deprivation resulted in a reduction of shrimp plasma protein concentration,

which reached the lowest levels (76.65 mg mL^{-1}) after 48 h starvation (Sanchez-Paz et al., 2007). In the present study, it can be hypothesised that starved crabs used high amounts of haemolymph proteins to cope with starvation, considering that proteins are the primary source of energy in Crustacea (New, 1976; Sanchez-Paz et al., 2007). The results obtained suggested that *C. aestuarii* may have exploited proteins as an immediate energy source during starvation, while it accumulated glucose in haemolymph to be used after, when protein levels reduced.

The role of PO in immune reactions of Crustacea has been reviewed by Söderhäll and Cerenius (1998). 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). In the present study, PO activity significantly increased in HL of starved crabs, whereas no significant variations in PO activity were recorded in CFH. 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 of *C. aestuarii* are also involved in PO production (Matozzo et al., 2010b). A positive correlation between THC and PO activity is reported in the literature (see Cheng et al., 2005, and related references). Likewise, a positive relationship between THC and HL PO activity can be suggested in the present study, both the immune parameters increasing in starved crabs. We also hypothesised that increased PO activity in HL from starved crabs was a physiological response of animals in order to reduce energy expenditure due to release of PO from haemocytes to haemolymph.

Reactive oxygen species (ROS), such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($\text{OH}^\cdot$), can be dangerous for organisms, as they can induce oxidative damage to lipids, DNA and other key molecules. Organisms have antioxidant

systems to protect their biological structures from ROS-mediated damage (Halliwell and Gutteridge, 1999). In antioxidant systems, SOD is the first defence line, as it catalyses the dismutation of O_2^- into molecular oxygen and H_2O_2 . CAT, with glutathione peroxidase (GPx), is the most important scavenger of H_2O_2 in cells. In the present study, starvation did not cause significant alterations in enzyme activities in both gills and digestive gland from crabs, suggesting that no oxidative stress occurred in *C. aestuarii*. In a recent study, antioxidant status has been evaluated in *C. maenas* by measuring the combined reducing power of the electron donating antioxidants present in the haemolymph of starved and fed crabs (Dissanayake et al., 2008). Similarly to what observed in the present survey, 7 days' starvation did not induce significant alterations in antioxidant status of *C. maenas*. However, after 14 days, starved crabs had significantly lower antioxidant status compared with diet-restricted and fully fed crabs (Dissanayake et al., 2008). These results (the present and those from the literature) suggested that crabs can suffer reductions in antioxidant status after prolonged starvation only.

In conclusion, the present study demonstrated that starvation influenced immune parameters in crabs, but it did not induce significant alterations in antioxidant enzyme activities. It was also observed that *C. aestuarii* modulated its cellular and biochemical parameters in order to cope with starvation. This may explain - partially at least - the great adaptability of *C. aestuarii* and its widespread out of the native area (Chen et al., 2004). In any case, the long-term effects of starvation on cellular, physiological and biochemical parameters of *C. aestuarii* – and of crabs in general - need to be more fully investigated in further studies.

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Figure legends

Fig. 1. A graphical description of an experimental tank fitted with partition boards forming 12 individual boxes (A). Half a mussel was supplied daily to each control crab (B), whereas treated crabs did not receive food (C).

Fig. 2. THC values, expressed as number of haemocytes ($\times 10^6$) mL haemolymph⁻¹, in fed and starved crabs. Values are means $\pm$ s.e.; n=6, * p <0.05.

Fig. 3. Haemocyte proliferation, expressed as OD mL haemolymph⁻¹, in fed and starved crabs. Haemolymph samples were incubated for 4 and 6 h in XTT labelling mixture. Values are means $\pm$ s.e.; n=6.

Fig. 4. Cell-free haemolymph glucose and total protein concentrations, expressed as mg mL haemolymph⁻¹, in fed and starved crabs. Values are means $\pm$ s.e.; n=6, * p <0.05.

Fig. 5. PO activity, expressed as U mg proteins⁻¹, in haemocyte lysate (HL) and cell-free haemolymph (CFH) from fed and starved crabs. Values are means $\pm$ s.e.; n=6, * p <0.05.

Research Highlights

The effects of starvation on cellular and biochemical parameters of the crab *Carcinus aestuarii* were evaluated for the first time.

Results obtained demonstrated that starvation influenced crab immune parameters, but did not induce oxidative stress.

Results also indicated that *C. aestuarii* can modulate its cellular and biochemical parameters in order to cope with starvation.

Tab. 1. SOD and CAT activities, expressed as U/mg proteins, in gills and digestive gland from fed (control) and starved crabs. Values are means $\pm$ s.e.; n = 12.

	Gills		Digestive gland	
	SOD activity	CAT activity	SOD activity	CAT activity
Fed crabs	3.82 $\pm$ 0.22	2.45 $\pm$ 1.50	0.36 $\pm$ 0.15	3.20 $\pm$ 1.96
Starved crabs	3.25 $\pm$ 0.36	2.98 $\pm$ 1.83	0.62 $\pm$ 0.17	1.78 $\pm$ 0.11

Fig. 1 **Fig. 2**

Fig. 3

Fig. 4

Fig. 5