Effects of shell disease syndrome on the haemocytes and humoral defences of the edible crab, *Cancer pagurus*

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Abstract

Crustaceans affected by shell disease syndrome display characteristic black-spot lesions in regions of exoskeletal degradation. Vogan et al. [Dis. Aquat. Org. (2001)] have shown that the severity of shell disease in the edible crab, *Cancer pagurus*, correlates with an increase in haemocoelic bacterial infections and may therefore serve as an external marker for the internal health of the animal. Therefore, this present study examined if the same crabs with shell disease lesions and varying degrees of bacterial septicaemia also displayed differences in the haemogramme, haemolymph phenoloxidase activity, total protein, copper and urea, as well as haemolymph-derived antibacterial activity, compared to uninfected individuals. A strong correlation was found between the severity of shell disease and a reduction in serum protein, which was further indicative of haemocoelic infection. Levels of copper, urea, phenoloxidase, and antibacterial activity in the haemolymph showed no correlations with the proportion of exoskeletal lesion cover. However, the degree of melanisation of haemolymph samples taken from shell-diseased individuals was less than that seen in disease-free crabs. Total haemocyte counts were unaffected by the disease, although some minor changes were found in the differential counts. Overall, despite having intrahaemocoelic infections, shell disease-affected individuals displayed few changes in the cellular or humoral defence parameters examined. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Shell disease; *Cancer pagurus*; Immune system; Systemic bacterial infections

1. Introduction

Shell disease syndrome is characterised by the appearance of melanised lesions on the exoskeletons of marine crustaceans (for review, see Stewart, 1993). The disease may be...
divided into two stages, each with potentially different causative agents. Firstly, lesion initiation, which involves removal of the externally situated (non-chitinous) epicuticle, may be the result of abrasive damage (Vogan et al., 1999), fighting injuries (Dyrynda, 1998), chemical attack (Schlotfeldt, 1972), or bacterial degradation (Cipriani et al., 1980). Secondly, lesion development and dissolution of the underlying procuticle is thought to proceed largely via the liberation of extracellular chitinases from epibiotic microorganisms (Stewart, 1993). In severe cases of the disease, death of the animal may result either through lesion-site adhesion of successive cuticles leading to the inability to withdraw from the exuviate at moult (Fisher et al., 1978) or through secondary internal infections (Baross et al., 1978).

The pathogenicity of microbial invaders in the crustacean haemocoel ultimately lies in the ability of the organism to evade or circumvent the host defence mechanisms. In crustaceans, as in other invertebrates, these largely consist of innate (non-specific) responses involving an interplay between humoral and cellular responses (Ratcliffe et al., 1985). Briefly, detection of microorganisms within the haemocoel triggers the granule-containing haemocytes to lyse. They release a variety of proteins, which, by a number of different pathways, ultimately lead to melanin formation, stimulation of phagocytosis, nodule formation/encapsulation, and clotting (Söderhäll and Cerenius, 1998). Microbial killing itself, in crustaceans, has been demonstrated to result from action of antimicrobial peptides (Chisholm and Smith, 1992; Roch, 1999) and reactive oxygen species (Bell and Smith, 1993; Song and Hsieh, 1994), but may also involve the generation of reactive nitrogen intermediates (Holmblad and Söderhäll, 1999; Roch 1999).

Alterations in immune reactivity within invertebrates have been shown in response to various external and artificial stimuli, including changes in temperature and salinity, pollutants (Le Moullac and Haffner, 2000), and natural or artificially induced infections (e.g. Stewart and Zwicker, 1972; Ford et al., 1993). For example, Adams (1991) and Sung et al. (1996) demonstrated that crustaceans exhibit immune enhancement in response to the injection of bacteria or microbial products. Noga et al. (1994) found an impaired antibacterial activity in the serum of Callinectes sapidus, which coincided with an increased prevalence of shell disease syndrome. Vogan et al. (2001) have recently demonstrated that severely shell-diseased edible crabs, Cancer pagurus, display systemic haemocoelic bacterial infections in combination with damage to the gills and hepatopancreas. This part of the study reports on the degree to which such infections led to enhancement or suppression in the internal defence reactions of the same specimens of C. pagurus (Vogan et al., 2001), including changes in the haemogramme, serum proteins and immune parameters such as prophenoloxidase and antibacterial activity.

2. Materials and methods

2.1. Animals

C. pagurus used in experiments were obtained from pots anchored between Oxwich Bay and Pwlldu Head, Gower, UK. After capture, animals were maintained in aerated tanks in a circulating seawater aquarium (15 °C and 35 ‰ S) for ca. 24 h prior to their
use in and during the experimental period. All crabs used in these experiments were intermoult males between 100 and 150 mm carapace width. For each crab, the total percentage cover with black-spot lesions of the visible portion of the exoskeleton was calculated as described in Vogan et al. (1999). Ventral surface percentage cover (i.e. all ventral exoskeletal surfaces with the exception of those on the dactylus, propodus and carpus of each pereiopod) was also calculated, as these regions are most frequently in contact with sediments which contain high numbers of chitinolytic microorganisms, and is therefore a reliable guide to the severity of the disease (Vogan et al., 1999, 2001). These crabs were also used to determine if a correlation exists between the degree of lesion severity and the number of haemocoelic bacteria as reported in Vogan et al. (2001).

2.2. Bleeding regime

A bleeding regime was followed where groups of crabs were bled at the time of low tide during daylight hours for 3 consecutive days. This minimised any possible tidal and diurnal changes in the haemogramme (Truscott and White, 1990) and permitted a wide range of experiments to be performed on the same individuals. Haemolymph was withdrawn using a 19-gauge needle from the unsclerotized membrane between the carpus and the propodus of a cheliped or, if absent, a walking limb (pereiopods 2–5).

2.2.1. Day 1: serum sample preparation

A haemolymph sample of 1 ml was removed into an empty syringe and 200 μl was immediately removed for haemolymph bacterial load determination, as described and reported elsewhere (Vogan et al., 2001). The remaining 800 μl of haemolymph was allowed to clot for 4 h at RT. The clot was then gently disrupted and centrifuged to remove clot debris. The supernatant (serum) was stored at −20 °C for later protein, urea-N and copper content determination.

2.2.2. Day 2: antibacterial activity of haemocyte lysate supernatant

From each crab, 0.5 ml of whole haemolymph was removed into an excess volume of marine anticoagulant (0.45 M sodium chloride, 0.1 M glucose, 30 mM trisodium citrate, 26 mM citric acid, 10 mM EDTA, pH 4.6; Söderhäll and Smith, 1983). As crustacean haemocytes have been shown to contain potent antimicrobial activity (Chisholm and Smith, 1995), a haemocyte lysate supernatant (HLS) was prepared and an antibacterial assay performed using Psychrobacter immobilis (NCIMB 308) as a test organism, as described by Chisholm and Smith (1992). Briefly, P. immobilis cultures that had been grown overnight at 25 °C with shaking in Difco Marine Broth 2216 (Becton Dickinson, Oxford, UK) were washed twice in sterile marine saline (0.58 M sodium chloride, 20 mM calcium chloride, 12 mM potassium chloride, 0.56 mM disodium phosphate, 0.05 M tris (hydroxymethyl) methylamine, pH 7.4) and adjusted to give a stock solution containing $4 \times 10^6$ cells ml$^{-1}$. Antibacterial activity of individual crabs was tested by incubating 900 μl HLS and 100 μl bacterial stock solution (control solutions substituted HLS with 450 μl portions of sterile marine saline and marine broth 2216). At six time intervals (0–4 h), 100 μl aliquots were removed, diluted 10-fold in sterile marine saline and 100 μl
was spread-plated in triplicate onto Difco Marine Agar 2216 (Becton Dickinson). After ca. 48 h incubation at 25 °C, all colonies were counted and antibacterial activity for each time period was calculated as a percentage survival of *P. immobilis* compared to the time zero counts.

### 2.2.3. Day 3: phenoloxidase activity

The phenoloxidase activity of the haemocytes of *C. pagurus* was determined using a method adapted from Smith and Söderhäll (1991) for 96-well microtitre plates. HLS samples were prepared from 0.5 ml of haemolymph as described by Smith and Söderhäll (1991). In the phenoloxidase assay, 100 μl HLS was incubated with 100 μl of 0.1% trypsin (Sigma, Poole, UK; from bovine pancreas EC 3.4.21.4, 11,600 units/mg protein) in mg-CAC buffer (0.1 M sodium cacodylate; 5 mM calcium chloride; 50 mM magnesium chloride; pH 7.0) for 1 h at 20 °C. A further 1 ml of mg-CAC buffer was added and aliquots of 150 μl were transferred in quadruplicate into a 96-well plate. To each well, 25 μl of 0.02 M 1-dopa was added and the phenoloxidase reaction (change in \( \Delta \) OD) was measured at 490 nm on a microplate-reader at time zero and after 20 min. Control wells in which trypsin was replaced with mg-CAC buffer and blank wells containing mg-CAC buffer in place of HLS were run in parallel. Results were calculated as both a \( \Delta \text{OD} \) 490 nm/min/ml haemolymph and a \( \Delta \text{OD} \) 490 nm/min/mg protein.

### 2.3. Haemocyte counts

On each day of bleeding (days 1–3), a separate 0.5-ml sample of haemolymph was taken into an equal volume of marine saline containing 8% formalin (v/v). Total haemocyte counts were established using an improved Neubauer haemocytometer. Differential counts were conducted on slides prepared by placing 100 μl of diluted cell suspension (ca. 3 × 10⁶ cells) into a Shandon cytocentrifuge (170 × g; 5 min). The haemocyte preparations were post-fixed in methanol and stained using May and Grunwald (1:5 dilution of Gurr stain for 1 min) followed by Giemsa (1:25 dilution of Gurr stain for 5 min) (BDH; Merck, Lutterworth, UK). The numbers and relative proportions of haemocyte types were calculated using a minimum number of 250 cells/slide.

### 2.4. Protein determination

Serum and HLS protein concentrations were determined using a bicinchoninic acid assay kit (Pierce and Warriner, Chester, UK) in accordance with the manufacturers instructions for use in a microtitre plate. All samples were replicated in triplicate and calibrated against a bovine serum albumin (BSA) standard curve (100–1000 μg ml⁻¹) run on the same plate.

### 2.5. Urea-nitrogen and copper levels in serum

Urea levels in the serum of crabs were measured using a Urea-N assay kit (Sigma Procedure No. 535). Samples (200 μl) were plated in triplicate onto a 96-well plate and read immediately at 550 nm on a microplate-reader.
Copper content of the serum of both healthy and diseased crabs was measured using dithio-oxamide (rubeanic acid) in a method adapted from Bentley and Hurd (1995). A 200 μl serum sample was diluted five-fold in distilled water. Test samples and sample blanks of 160 μl were both plated in triplicate onto a 96-well microtitre plate. To the test samples, 40 μl of saturated dithio-oxamide in 5% ethanol was added. Sample blanks contained an equivalent volume of 5% ethanol. The microtitre plate was shaken for 30 s and the optical densities determined at 405 nm on a microplate-reader. Copper content of the serum samples were calibrated against a standard curve of cupric sulphate (0–0.1 μmol ml$^{-1}$).

2.6. Data analyses

For statistical analyses, data sets where $n > 10$ were checked for normality using the Kolmogorov–Smirnov test. Non-Gaussian populations and small data sets ($n < 10$) were analysed using non-parametric tests, otherwise, the parametric equivalent was applied.

Correlation calculations were displayed on a continuous scale of lesion severity, whereas bar charts represented discrete groupings (0%, > 0–1%, > 1–5% and > 5%) of black-spot cover. All values are expressed as arithmetic means ± 1 standard error (S.E.) unless otherwise stated. All $n$ values represent the number of individuals tested unless otherwise stated.

3. Results

3.1. Haemocyte classification

In *C. pagurus*, four different haemocyte types were recognised based on their nuclear morphology, refractile nature of granules and staining characteristics with May-Grunwald/Giemsa stain (Fig. 1A–C). Hyaline cells (H) were ovoid, agranular cells with a large round distinct nucleus that was surrounded by a band of homogeneously pale-staining cytoplasm. Eosinophilic granular cells (EG) were tightly packed with weakly staining eosinophilic granules, which were highly refractile (Fig. 1). Owing to their dense granular packing, these cells tended to be less elongate and had a rounder appearance than the other haemocyte types. The nucleus, when evident, was dense and had an irregular or crenated margin. Basophilic granular cells (BG) were ovoid to spindle-shaped, their cytoplasm contained variable numbers of non-refractile blue-stained granules. The nuclear margin was smooth and the chromatin was less densely packed than the other granular haemocytes. The fourth class contained haemocytes with a mixture of basophilic and eosinophilic granules (BEG), the relative proportions of each being highly variable (Fig. 1). The cell shape varied from ovoid to spindle and the nuclear material was densely packed within a crenated margin.

3.2. Haemocyte counts

At the time equivalent to that of low tide, the average number of circulating haemocytes of all animals tested ($n = 19$) on all 3 days, was found to be $2.55 \pm 0.14 \times 10^7$ cells ml$^{-1}$.
Fig. 1. (A–C) Haemocytes of *C. pagurus* following fixation and staining with May-Grunwald/Giemsa. The four cell types, namely hyaline cells (H), basophilic granular cells (BG), basophilic/eosinophilic cells (BEG), and eosinophilic granular cells (EG) are shown. Note the variable degrees of granular content within the BG and BEG cell types. Scale bar = 10 μm.
(range = $0.87 - 5.31 \times 10^7$ cells ml$^{-1}$). Using the May–Grunwald/Giemsa staining method, the haemogramme was observed to consist of $43.3 \pm 1.4\%$ BG cells, $42.0 \pm 1.3\%$ BEG cells, $7.9 \pm 0.3\%$ EG cells and $6.8 \pm 0.3\%$ H cells (mean values ± 1 S.E., $n=19$). Repeated bleeding over the 3-day period had no significant effect on the total and differential cell counts (data not shown).

No significant correlations were observed between the total numbers of circulating haemocytes and the severity of shell disease on the total (Pearson correlation $P=0.2293$, $r^2=0.0838$, $n=19$) or ventral (Pearson correlation $P=0.0552$, $r^2=0.1996$, $n=19$) surfaces. When the total haemocyte population was divided into the actual numbers of each cell type, significant non-linear correlations in the number of circulating BEG and EG cells (but not H and BG cells) were found when shell disease was expressed as a proportion of the ventral surfaces covered with lesions (Fig. 2). The non-linearity was

![](image1)

Fig. 2. Variation in the differential cell counts with the severity of shell disease cover of the ventral surfaces. Hyaline cells, Pearson correlation $P=0.4065$, $r^2=0.0409$; basophilic granular cells, Pearson correlation $P=0.5958$, $r^2=0.0169$; baso/eosinophilic granular cells, Pearson correlation $P=0.0083$, $r^2=0.3444$; eosinophilic granular cells, Pearson correlation $P=0.0383$, $r^2=0.2412$. 
further reflected as a significant increase in BEG and EG cell populations from >1–5% to 5% cover of the ventral surfaces (Fig. 3). No significant correlations were found between the individual cell types and shell disease over the whole body surface (Pearson correlation $P > 0.05$). However, a significant increase in the BEG cells was seen between the moderately ( > 1–5%) and severely ( > 5%) affected animals ($0.67 \pm 0.11 \times 10^7$ and $1.66 \pm 0.25 \times 10^7$ cells ml$^{-1}$, respectively; Kruskal–Wallis $P=0.0087$, Dunn’s multiple comparison $P<0.05$). Finally, no obvious morphological changes in the haemocytes were observed between control and diseased animals.

3.3. Serum protein, copper and urea levels

Protein levels in the serum decreased with an increase in the severity of shell disease. Highly significant negative correlations were found in association with total body lesion

![Graphs showing changes in differential cell counts within different shell disease severity groups of the ventral surfaces.](image)

Fig. 3. Changes in the differential cell counts within different shell disease severity groups of the ventral surfaces. Hyaline cells, Kruskal–Wallis $P=0.2982$; basophilic granular cells, Kruskal–Wallis $P=0.6243$; baso/eosinophilic granular cells, Kruskal–Wallis $P=0.0104$, Dunn’s multiple comparison post-test $P<0.05$ indicated by letters; eosinophilic granular cells, Kruskal–Wallis $P=0.0123$, Dunn’s multiple comparison post-test $P<0.05$ indicated by letters. Mean values from day 1 bleed ± 1 S.E., $n=3–8$. 


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cover and ventral surface lesion cover (Pearson correlation \( P = 0.0077 \) and \( P = 0.0061 \), respectively). When divided into disease severity groups, significant differences were only observed between the non-diseased (0% lesion cover) and the most severely diseased (> 5% lesion cover) animals when related to shell disease infections of their ventral surfaces (Fig. 4).

The mean value of urea measured in all serum samples was found to be 1.57 ± 0.15 mg dl⁻¹ (mean value ± 1 S.E., \( n = 26 \)). Urea levels showed no relationships with percentage of shell disease cover when expressed as total body cover or ventral surface cover (data not shown; Pearson correlation \( P > 0.05 \); Kruskal–Wallis \( P > 0.05 \), \( n = 26 \)).

The scatter plots for serum copper displayed no significant correlation with shell disease severity (data not shown; Pearson correlation \( P > 0.05 \), \( n = 26 \), for the total and ventral surfaces). However, severity groupings revealed a significant increase in copper

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**Fig. 4.** Changes in serum protein levels within different shell disease severity groups, expressed as a percentage of total exoskeletal cover (A) and ventral surface cover (B). Mean values ± 1 S.E. Significant differences (Kruskal–Wallis \( P < 0.05 \); Dunn’s multiple comparison post-test \( P < 0.05 \)) between groups indicated by letters. Sample sizes are shown in parentheses.
levels between 0% lesion cover and >1–5% lesion cover over the total external exoskeletal surface (Fig. 5A). A different pattern was observed when animals were grouped by the percentage cover of lesions on their ventral surfaces with a significant increase between the 0% and >0–1% classes (Fig. 5B).

3.4. Prophenoloxidase (PPO) and antibacterial activity of HLS

For *C. pagurus*, the average PPO activity in HLS was found to be $2.89 \pm 0.18$ units (where 1 unit represents a change in OD min$^{-1}$mg$^{-1}$ protein; mean value $\pm$ S.E., $n = 19$). The activity showed no significant correlation against the severity of shell disease (data not shown; Pearson correlation $P > 0.05$) and no significant variation among shell
disease severity groups, regardless of whether the results were standardised for protein (data not shown; Kruskal–Wallis \( P > 0.05 \)). It was noted, however, that the degree of melanisation of serum samples taken from grossly infected individuals was less than that observed in control animals. This was not further investigated.

HLS was observed to be an extremely effective agent in preventing the growth of \( P. \) immobilis (Fig. 6A,B). For all shell disease severity groups, the portion of viable bacteria significantly decreased through time (two-way ANOVA \( P < 0.001 \) for the total and ventral surfaces). Percentage survival values of approximately zero (i.e. 100% bacterial stasis) were observed in all animals, regardless of their shell disease status after only 1 h (Fig. 6A,B). There were no significant differences between the severity groups examined (two-way ANOVA \( P > 0.05 \) for the total and ventral surfaces).

Fig. 6. The effect of \( C. \) pagurus HLS on the survival of \( P. \) immobilis after 0–2-h incubations (mean values ± 1 S.E.). Crabs are divided into shell disease severity groups based on (A) total and (B) ventral surface cover. After 4 h, the controls had risen to 205% survival and all disease severity groups were approximately zero (data not shown).
4. Discussion

In this study, shell disease had no clear effects on haemocyte counts, PPO activity or antibacterial bacterial activity of the HLS. This is particularly surprising since, in a previous study (Vogan et al., 2001), individuals exhibiting shell disease were shown to have internal infections. There was a linear relationship between the bacterial load of the haemolymph and the severity of shell disease. This study (Vogan et al., 2001) also revealed breakdown of the hepatopancreas and damage to the gills associated with nodule formation (haemocyte clumping).

Our current study divided the haemocyte population of *C. pagurus* into four groups, one of which was agranular (hyaline, H) and the remaining three granular (basophilic granular, BG; basophilic/eosinophilic granular, BEG; eosinophilic granular, EG). Previous investigations into decapod haemocytes have tended to categorise the blood cells into only three types: (i) the agranular hyaline cell, (ii) semigranular or small granular cell and (iii) granular, refractile or large granular cells (Johansson et al., 2000). These divisions are in agreement with the current investigation since other studies define the semi or small granular population to consist of both purely basophilic granular and a mixture of basophilic and eosinophilic granular cell types (Hose et al., 1990).

Smith and Söderhäll (1983), Söderhäll and Smith (1983) and Hose et al. (1990) all suggest an overlapping function between the granule-containing cell types of decapod crustaceans, with the semigranular cells (BG and BEG) mainly involved in phagocytosis, and granular cells (EG) largely responsible for PPO activity. More specifically, *C. pagurus* has been reported to have a PPO activity (expressed as Δabsorbance 490 nm/min) of 0.009 and 0.038 in the semigranular cells and granular cells, respectively (Söderhäll and Smith, 1983). Therefore, it is surprising that in the current study, no significant increases in PPO activity were observed (in HLS derived from the total cell population) despite elevations in the BEG and EG haemocyte populations. Such increases in the numbers of BEG and EG cells do not, however, necessarily imply an increase in PPO activity.

As in the current study with *C. pagurus*, potent neutralising abilities of neat HLS against *P. immobilis* has been reported for *C. maenas* (Chisholm and Smith, 1992), the squat lobster *Galathea strigosa*, the Norway lobster *Nephrops norvegicus* and the common shrimp *Crangon crangon* (Chisholm and Smith, 1995). These, combined with other studies (Noga et al., 1996; Xylander et al., 1997) have shown that the haemolymph-associated antibacterial factors of decapod crustaceans are synthesized and mainly reside in the haemocytes. More specifically, Chisholm and Smith (1992) and Xylander et al. (1997) have demonstrated that this activity resides exclusively in the granule-containing haemocytes of *C. maenas* and *Astacus leptodactylus* (a fresh water crayfish).

Noga et al. (1994) determined that in *Callinectes sapidus*, an increase in the prevalence of shell disease coincided with a decrease in serum antibacterial activity against *Escherichia coli* D31. For these studies, they used crabs from a variety of areas within the Albemarle–Pamlico estuary in North Carolina that differed in a number of environmental and biological factors (salinities, pollution levels, prey abundance and type, turbidity and substratum type). The authors suggested that the difference in immunocompetence between animals from different sites may have been due to any of
these environmental variables, but these ultimately lead to an increase in the prevalence of shell disease. The study also found that in sites that displayed higher prevalences of exoskeletal lesion occurrence, diseased individuals showed lower levels of serum antibacterial activity than their non-diseased counterparts. In the current study, no attempt was made to determine if a correlation exists between serum antibacterial activity and the severity of shell disease in animals from the same sample site because initial experiments showed that the level of such activity was highly variable from one crab to another, irrespective of the disease status of such animals (unpublished observations). Furthermore, as the antibacterial activity of the serum may result from the degranulation or damage of haemocytes, it was deemed more appropriate to use HLS preparations rather than serum.

Variations in total haemolymph protein levels in crustaceans have been found in relation to the stages of the moult cycle (e.g. Depledge and Bjerregaard, 1989), males and females, and females with and without egg-masses (Horn and Kerr, 1963). Serum protein levels in *C. maenas* (Uglow, 1969) and *Homarus americanus* (Stewart et al., 1967) have also been shown to decrease in response to starvation. The present study used only intermoult males that were actively feeding and, hence, eliminated the aforementioned reasons for the observed decline in protein levels with the severity of shell disease. In lobsters (*H. americanus*) decreases in both serum glucose and a “fibrinogen-like protein” have also been reported in response to systemic bacterial infections (Stewart et al., 1969; Stewart and Cornick, 1972). The significant decrease in protein concentrations found in the current study following shell disease correlates with the previously reported increase in the number of bacteria isolated from the haemolymph of externally lesioned crabs (Vogan et al., 2001). It is important to note that the same crabs were employed in both serum protein and haemolymph bacterial load estimations. Hence, it is justified to propose such a correlation. It would be of interest to determine if all serum proteins, including the recently discovered clotting proteins (Sritunyalucksana and Söderhäll, 2000), are equally affected in haemocoelic bacterial infections following shell disease.

In aquatic decapods, ammonia, amino acids and urea form the three main end products of nitrogenous metabolism, accounting for 60–90%, <10% and <5%, respectively, of the total nitrogen excreted (Regnault, 1987). An increase in urea excretion has been reported in response to salinity stress in *Scylla serrata* (Chen and Chia, 1996), temperature stress in *Macrobrachium rosenbergii* (Chen and Kou, 1996), ammonia stress in *Penaeus japonicus* (Chen and Chen, 1997) and chemical exposure in *Oziotelphusa senex senex* (Reddy et al., 1994). However, in the present study, no significant increases in the levels of urea were detected in severely shell diseased *C. pagurus* (the stressed state). This is particularly surprising, when combined with the finding of damage to the lamellar epithelium in the gills of shell-diseased crabs (Vogan et al., 2001), since the gill epithelium is the major site for ammonia excretion (Regnault, 1987) and impairment could feasibly result in hyperammonemia, with a possible switch to the ureogenesis (Reddy et al., 1994).

Dithio-oxamide has been classically used to identify haemocyanin, the copper-containing, plasma-residing respiratory pigment of crustaceans on electrophoresis gels (Horn and Kerr, 1969). In the current study, like that of Bentley and Hurd (1993), the method was adapted to quantify total haemolymph copper in the test organism. The
haemolymph of control *C. pagurus* is likely to have two principal sources of copper, haemocyanin and exogenous, or waterborne, copper. In *C. maenas*, copper that has been absorbed across the gill epithelium is believed to bind in a labile manner to haemocyanin itself for transport to and storage in the hepatopancreas (Rtal and Truchot, 1996). Although waterborne copper causes extensive damage to the gill epithelium (e.g. Nonnotte et al., 1993), once in the haemolymph, the protein bound form displays a greatly reduced toxicity (Rtal et al., 1996). Hence, in the present study, the elevated copper levels found in correlation with the early stages of shell disease are unlikely to cause the animal any harm. More importantly, the current investigation revealed no significant increases in serum copper levels in the most severely diseased animals, indicating that free copper does not appear in the haemolymph during the proposed hepatopancreatic breakdown in such individuals (Vogan et al., 2001). Although the hepatopancreas serves as a storage organ for copper in a number of crustaceans (reviewed by Gibson and Barker, 1979), such a function has apparently not been demonstrated for the edible crab, *C. pagurus*. Clearly, further investigations involving the measurement of hepatopancreatic copper, the identification of the sites of haemocyanin synthesis and the status of copper measured (i.e. free vs. bound to plasma proteins) should be carried out before inferences can be made into the links between shell disease and copper levels in *C. pagurus*.

In summary, the severity of shell disease in *C. pagurus* does not appear to cause any dramatic changes to any of the immune parameters tested. This is unexpected in light of an earlier study (Vogan et al., 2001) where the same individuals displayed intrahaemocoelic bacterial infections.

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