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A histological study of shell disease syndrome in the edible crab *Cancer pagurus*

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ABSTRACT: Shell disease syndrome is characterised by the external manifestation of black spot lesions in the exoskeletons of crustaceans. In the present study, gills, hepatopancreas and hearts from healthy (<0.05% black spot coverage) and diseased (5 to 15% coverage) edible crabs, *Cancer pagurus*, were examined histologically to determine whether this disease can cause internal damage to such crabs. There was clear evidence of cuticular damage in the gills of diseased crabs leading to the formation of haemocyte plugs termed nodules. Nephrocytes found within the branchial septa of the gills showed an increase in the accumulation of dark material in their vacuoles in response to disease. In the hepatopancreas, various stages of tubular degradation were apparent that correlated with the severity of external disease. Similarly, there was a positive correlation between the number of viable bacteria in the haemolymph and the degree of shell disease severity. Approximately 21% of the haemolymph-isolated bacteria displayed chitinolytic activity. Overall, these findings suggest that shell disease syndrome should not be considered as a disease of the cuticle alone. Furthermore, it shows that in wild populations of crabs shell perforations may lead to limited septicaemia potentially resulting in damage of internal tissues. Whether such natural infections lead to significant fatalities in crabs is still uncertain.

KEY WORDS: Shell disease · Chitinolytic bacteria · Nephrocytes · Hepatopancreas · Haemocytes · Nodules · Edible crab · *Cancer pagurus*

INTRODUCTION

Shell disease syndrome can be described as a progressive degradation of the crustacean cuticle and is characterised externally by the appearance of black spot lesions in the exoskeletal surface. The black colouration of the lesions is a result of melanisation, a defence response triggered by cuticular damage (e.g., Nyhlén & Unestam 1980). Exoskeletal erosion is largely attributed to the chitinolytic activities of microorganisms (for reviews see Getchell 1989, Stewart 1993). However, lesion initiation requires removal of the outermost, non-chitin-containing layer of the cuticle, the epicuticle, and may occur by lipolytic microbial activities (Cipriani et al. 1980), predatory or cannibalistic attacks (Dyrynda 1998), chemical attack (Schlotfeldt 1972) or the abrasive action of sediment or articulated body parts (Young 1991). Although the disease is not believed to be fatal in its initial stages, death is known to result from adhesion of successive moult shells at lesion sites leading to incomplete withdrawal from the exuviate at moult (e.g., Smolowitz et al. 1992). Baross et al. (1978) suggested that death may also result from haemocoelic infections by pathogenic bacteria originating by entry through the lesion site.

Despite numerous reports on crustacean mortality in response to haemocoelic invasions by bacteria (e.g., vibriosis, gaffkaemia) (see review by Stewart 1993), studies that show systemic bacterial infections in combination with shell disease are lacking. Therefore, the current study aimed to investigate whether there are internal changes resulting from haemocoelic septicaemia associated with natural outbreaks of shell disease in the edible crab *Cancer pagurus*.

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MATERIALS AND METHODS

Animals. *Cancer pagurus* (n = 23) used for histological examination and detection of haemolymph bacterial contamination were obtained from pots anchored between Oxwich Bay and Pwlldu Head, Gower, UK. After capture, animals were maintained in aerated tanks in a circulating sea-water aquarium (15°C and 35‰ salinity) for ca 24 h before their use. 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 by Vogan et al. (1999). As the ventral surfaces of crabs are most frequently in contact with sediments that contain high numbers of chitinolytic microorganisms (Vogan et al. 1999), the 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.

Haemolymph sterility. Before bleeding, crabs were surface sterilised at the appropriate site by swabbing with 70% ethanol. Haemolymph (1 ml) was withdrawn from the unsclerotised membrane between the carpus and the propodus of a cheliped or, if absent, a walking limb (pereiopods 2 to 5), using a 19 gauge needle. Of this volume, 200 µl was immediately transferred into an equal volume of sterile marine saline (0.5 M NaCl, 12 mM $CaCl_2 \cdot 6H_2O$, 11 mM KCl, 26 mM $MgCl_2 \cdot 6H_2O$, pH 7.4) and aliquots of 100 µl were spread in triplicate onto Difco Marine Agar 2216 (Becton Dickinson, Oxford, UK). All plates were then incubated inverted at 25°C for up to 10 d and any resultant colonies were counted. Correlation coefficients between haemolymph bacterial number and the percentage lesion cover were assessed using the Pearson's correlation. All variables were initially checked for normality using the Kolmogorov-Smirnov test.

Colonies were randomly selected from the haemolymph spread-plates, re-streaked 3 times on Difco Marine Agar 2216 to ensure strain purity and placed on slopes that were stored at 4°C. All bacterial isolates were streaked onto chitin agar plates (each comprising an underlay containing 50% [v/v] artificial sea-water, 45% [v/v] H_2O, 5% [v/v] 1 M Tris-HCl [pH 7.5] and 90 µM $FeSO_4$; 0.33 mM K_2HPO_4, 1.4 mM NH_4Cl, 2% [w/v] Difco agar and an overlay containing 50% [v/v] colloidal chitin in 45% [v/v] H_2O, 5% [v/v] 1 M Tris-HCl [pH 7.5] and 90 µM $FeSO_4$; and 0.33 mM K_2HPO_4, 1% [v/v] Difco Noble agar), which were incubated at 25°C for up to 14 d. Chitinase activity was detected by the appearance of transparent zones of chitin clearance surrounding areas of bacterial growth. Bacterial smears derived from young cultures (<24 h growth on Difco Marine Agar 2216) were used for Gram stain determinations.

Histological examination of crab tissue. To determine whether shell disease results in structural changes in internal tissues such as the gills, hepatopancreas and heart, both control (non-diseased) and infected (diseased) crabs were examined histologically (see Table 1).

Selected crabs were killed by injection of ca 50 ml of Bouin's sea-water fixative through the unsclerotised membranes at the base of the fifth pereiopod. Crabs were rapidly dissected and gill, heart and hepatopancreas were placed in an excess volume (ca 25 ml to <1 g tissue) of fixative for storage in the dark at room temperature until processing. Picric acid was removed from the fixed tissue samples by several washes in 70% propan-2-ol that had been previously saturated with lithium carbonate. Tissues were then dehydrated through a graded series of alcohols (70, 90 and 100% propan-2-ol, each for 30 min) and cleared in Histoclear (National Diagnostics, Hull, UK) for 3 h. The specimens were then placed into a 50:50 mixture of Histoclear/

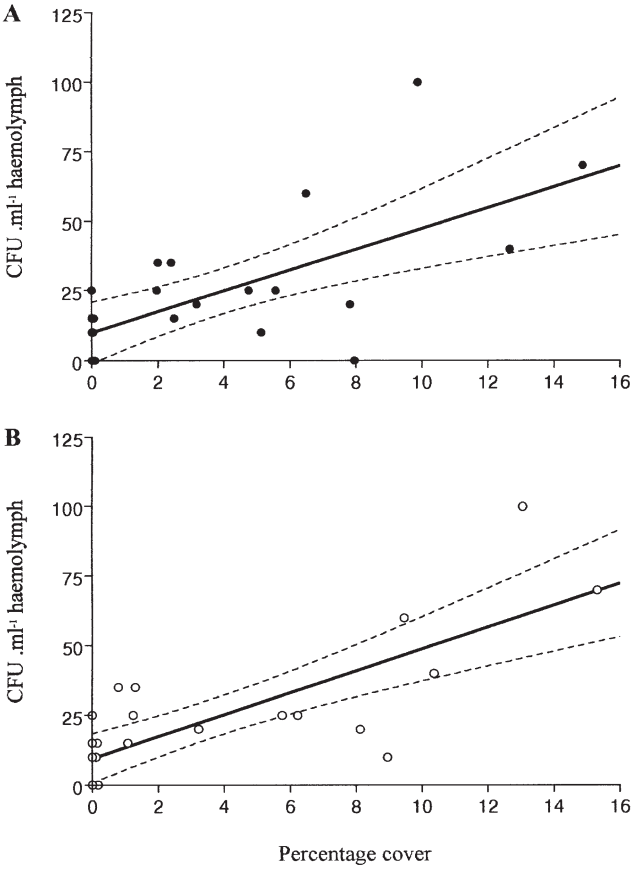

Fig. 1. Correlation of bacteraemia and severity of black spot lesion cover, expressed as (A) a percentage of the total exoskeletal cover (Pearson correlation, $p = 0.0005$, $r^2 = 0.4426$) and (B) ventral surface cover (Pearson correlation, $p < 0.0001$, $r^2 = 0.5973$). Linear regression shown ±95% CI (dashed line)

Table 1. Percentage cover of *Cancer pagurus* cuticle with exoskeletal lesions. D: Diseased animal; ND: Non-diseased animal

Crab #	Total surface lesion cover (%)	Ventral surface lesion cover (%)
D1	7.9	0.2
D2	6.5	9.5
D3	9.9	13.1
D4	4.7	3.4
D5	14.9	15.3
ND1–5	<0.05	<0.05

molten paraffin wax and maintained without vacuum at 60°C for 1 h, after which they were transferred into 3 changes of 100% molten paraffin wax at 2 h intervals at 60°C under vacuum before finally embedding in paraffin wax. Sections (ca 10 µm thick) were cut and stained with Cole's haematoxylin and eosin. In all cases, at least 2 blocks of each tissue (heart, gill or hepatopancreas) were sectioned in 2 to 3 different regions per block. Photographs were taken either with an Olympus digital camera on an Olympus BX50 binocular microscope (Olympus Optical, London, UK) or, for higher power examination, with a Zeiss Photomicroscope II (Carl Zeiss Ltd., Welwyn Garden City, UK).

RESULTS

Haemolymph sterility

Bacteria were found in the haemolymph of 78% of crabs tested (n = 23). Of the 5 crabs with sterile haemolymph, all but 1 had intact limbs. Of the 18 crabs with bacteria in the haemolymph, 12 had more than 1 limb missing, 1 had the dactylus of a walking limb missing, 2 had chelipeds that were dwarf regenerates and 3 had all limbs intact. As the percentage of the exoskeleton covered with shell disease lesions increased, so too did the number of viable bacteria circulating in the haemolymph (Fig. 1). From the cultures of haemolymph-derived isolates (n = 19) 58% were Gram negative, 42% Gram positive and 21% positive for chitinase activity. All chitinolytic isolates were Gram negative.

Histopathology

Table 1 shows the levels of shell disease among the diseased *Cancer pagurus* used for histopathological examinations. All control (non-diseased) animals had <0.05% of their body surfaces covered with shell disease lesions (i.e., superficial minor infections).

Gills

Cancer pagurus has 9 gills on each side of the branchial chamber. The phyllobranchiate structure consisted of pairs of flattened lamellae branching from the central (branchial) stem, which had the afferent and efferent haemal channels on each end (Figs 2 & 3). Each lamella was surrounded by a thin epithelium,

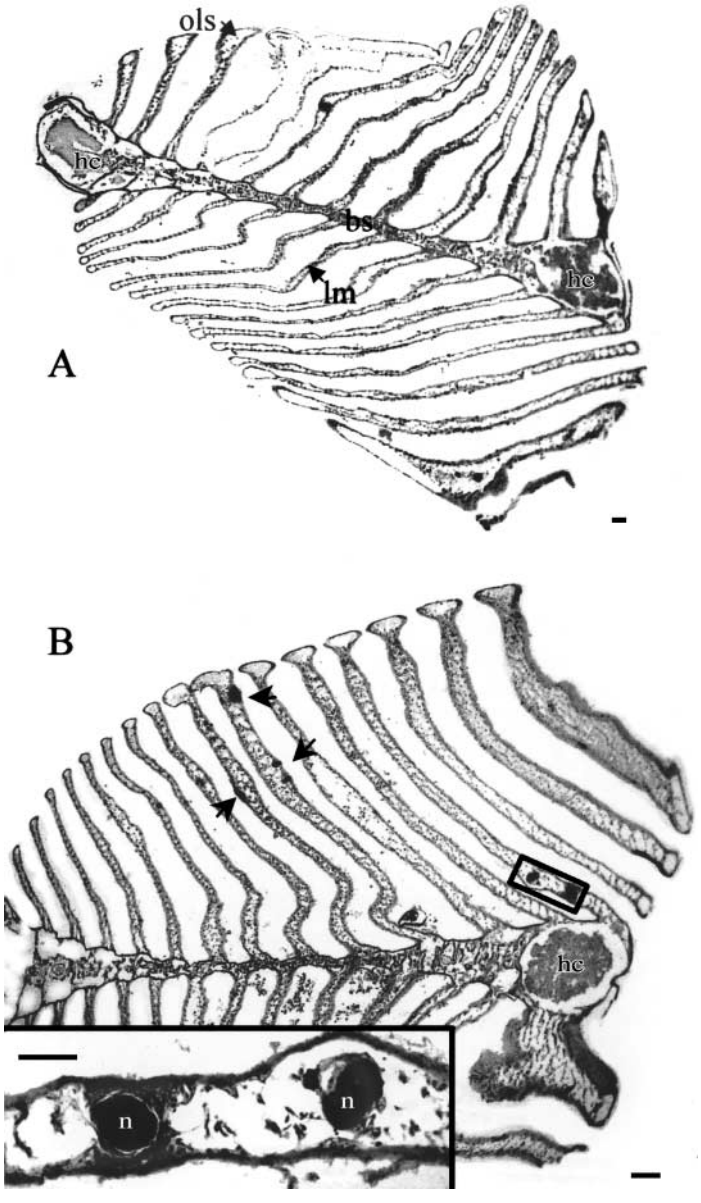

Fig. 2. Low power micrographs of the gills of *Cancer pagurus*. (A) Gill from an uninfected crab showing haemal channels (hc), branchial stem (bs), lamellae (lm) and outer lamellar sinuses (ols). (B) Gill from shell diseased crab (D2) showing haemocytic nodules (unlabelled arrows) in lamellae. Boxed region shows area enlarged in inset. Inset shows 2 haemocytic nodules (n) in the lamellal space. Scale bars = 200 µm (A,B) and 100 µm (inset)

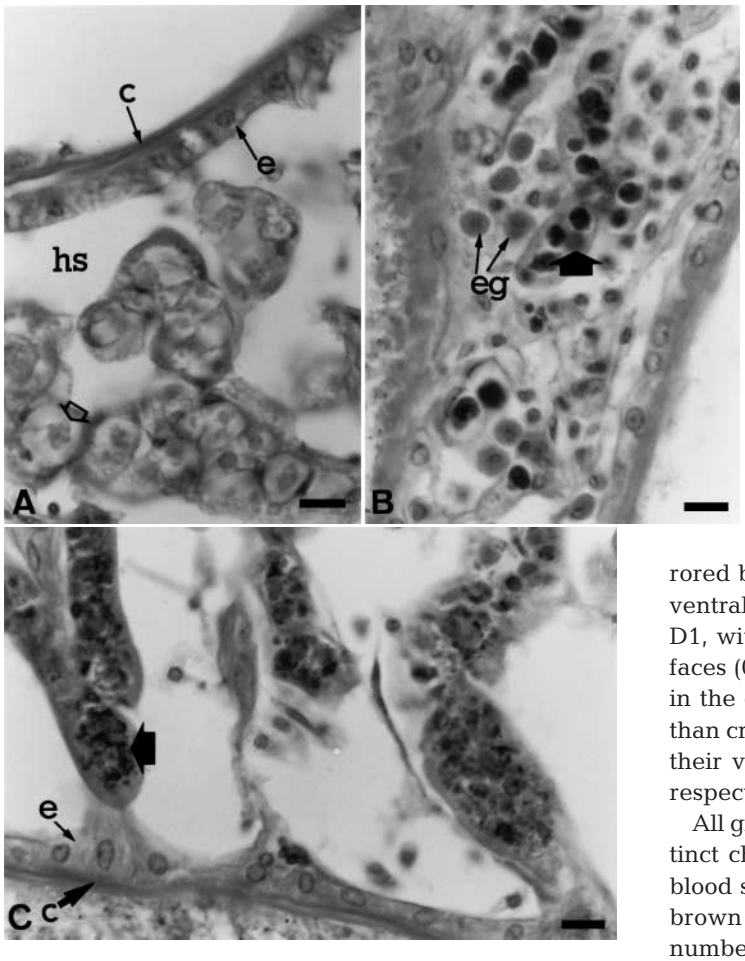

Fig. 3. Appearance of nephrocytes in the gill branchial stem of uninfected and shell-diseased crabs. (A) Nephrocytes in control crab containing a small amount of brown material in the central vacuoles (unlabelled arrow). Note outer cuticle (c) with underlying epithelium (e) and haemal space (hs). (B,C) Nephrocytes from infected crabs (D3 and D4, respectively). Note dense brown deposits inside these cells (unlabelled arrows) and accumulation of haemocytes including eosinophilic granular haemocytes (eg). Outer cuticle (c) and epithelium (e). Scale bars = 10 µm

which enclosed a haemolymph-filled cavity. The tips of the lamellae were slightly splayed to accommodate the outer lamellar sinus, which, unlike other haemal sinuses of the lamellae, contained nephrocytes. The epithelium of the branchial stem was covered by a thin cuticle. The centre of each branchial stem contained haemocytes and fixed nephrocytes (termed podocytes by some authors) (Fig. 3A). In control crabs, the nephrocytes were seen to be highly vacuolate and the material contained within the central vacuole generally appeared pale and translucent (Fig. 3A). However, some nephrocytes had small quantities of a pale brown material within their vacuoles.

Distinct histological changes in the gill were observed in crabs with shell disease lesions on their body surface. In particular, lesions appeared on the outer surfaces of the lamellae, and haemocyte accumulations (nodules) were observed occluding the haemal sinuses at such sites (Figs 2B & 4A,B,C). Associated with these epithelial lesions was an infiltration of haemocytes including many eosinophilic granular cells. On a number of occasions, nodules were found in close association with epithelial haemocytic plugs (Fig. 4A,B,C). Removal of the plug, as an artefact during histological preparation, revealed a complete breach of the epithelium (Fig. 4B). Such nodules showed characteristic necrotic, melanised centres surrounded by concentric layers of flattened haemocytes. It was also noted that the severity of histopathological changes in the gills was mirrored by the degree of exoskeletal lesion cover on the ventral surfaces of *Cancer pagurus*. For example, crab D1, with only minor lesion damage on its ventral surfaces (0.2% lesion cover), although displaying nodules in the gills, had far less extensive damage to the gills than crabs D2, D3 and D4, which had severe lesions on their ventral surfaces (9.5%, 13.1% and 3.4% cover, respectively).

All gills from shell disease-affected crabs showed distinct changes to the nephrocytes found in the central blood sinus. These cells were enlarged and filled with brown pigment (Fig. 3B,C). In some cases greater numbers of haemocytes, particularly eosinophilic granular cells, were found in association with the nephrocytes (Fig. 3B).

Hepatopancreas

Histologically, each tubule of the hepatopancreas was surrounded by haemal spaces that contained haemocytes and apparent blood vessels (Fig. 5A). The hepatopancreatic tubules were enclosed by a basal lamina and contained a central lumen (Fig. 5A,B). Three types of epithelial cells have previously been recognised in these tubules: R-cells, F-cells and B-cells (Ceccaldi 1998) (Fig. 5A,B,C,D). R-cells, the most common cell type observed, were characterised by variable numbers of cytoplasmic vacuoles. The F-cells were distinguished by their deeply staining basophilic cytoplasm. Unlike the other cells of the tubules, mature B-cells bordered the lumen with a vacuolar apical complex and had no apparent connection to the basal lamina (Fig. 5B). Their nuclei and cytoplasm were forced to the basal peripheries of the cell by a large central vacuole that contained variable amounts of brown material.

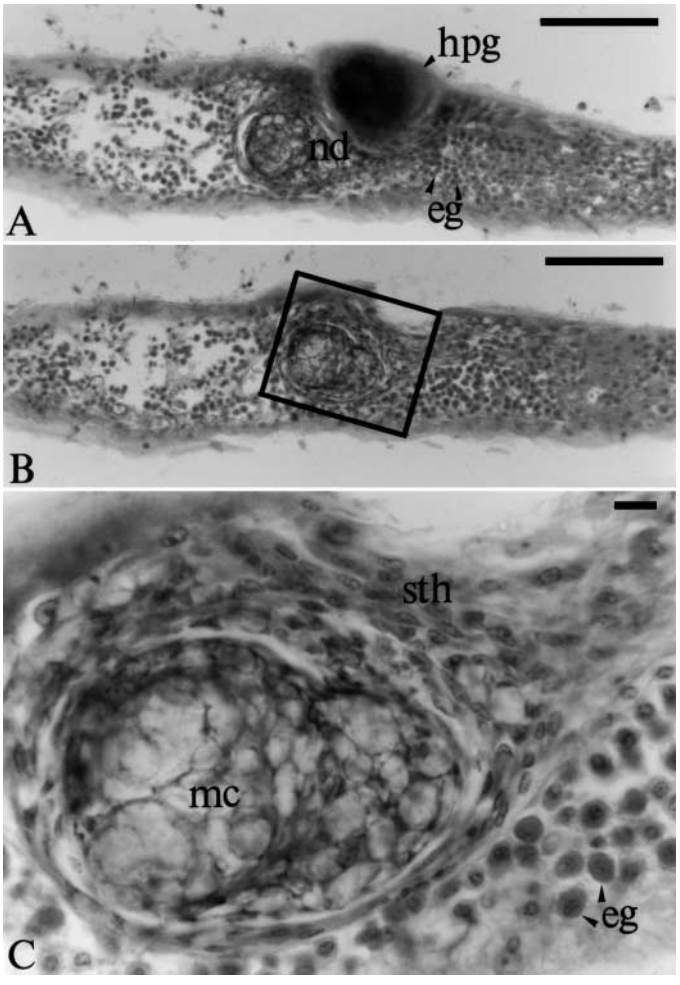

Fig. 4. Section through a nodule formed in response to cuticular damage in the gill of crab D1. (A,B) Adjacent sections through nodule (nd) and associated haemocyte plug (hpg). Note large number of eosinophilic granular haemocytes (eg) in the area of the nodule. Boxed area shows region enlarged in Fig. 4C. (C) High power micrograph showing the structure of the nodule with its melanised core (mc), sheath of flattened haemocytes (sth) and adjacent eosinophilic granular haemo-cytes (eg). Scale bars = 100 µm (A,B) and 10 µm (C)

Shell-diseased animals showed varying degrees of necrosis of their hepatopancreatic tubules (Figs 5C,D & 6A,B,C). Furthermore, it should be noted that the degree of tubular damage was not uniform within the hepatopancreas and sections from individual crabs contained tubules apparently in different stages of epithelial cell breakdown. In general, the hepatopancreas of crabs with a greater degree of external lesions was more damaged than that of less affected crabs. In some diseased animals the number of B-cells markedly increased, as did the quantities of their vacuolar material (Fig. 5C,D). In more severely affected crabs, epithelial cell boundaries were often not as distinct as those observed in control animals and the cytoplasm was more patchy (compare Fig. 5B with Fig. 6A). In places, the basal lamina appeared to have become detached from the epithelial cells and developed a crenate appearance. In the R-cells the nuclei became pyknotic or lacking (Fig. 6A). As the apparent severity of necrosis increased, haemocytic nodules appeared in the haemal spaces (Fig. 6B). In the most severely affected animals, particularly Crab D5, a massive destruction of the epithelial cells was observed and in some areas, virtually all that remained of the original tubule was the basal lamina (Fig. 6C). A distinct lack of free haemocytes was also observed in the intertubular spaces.

Heart

No structural differences were detected in the myocardium or the epicardium between control and shell-diseased crabs although a number of haemocyte nodules were observed in the cardiac lumen of shell-diseased crabs, particularly those displaying marked cuticular damage in the gills (e.g., Crabs D2 & D3).

DISCUSSION

The current study found a positive correlation between shell disease severity and the degree of infection of the haemolymph with culturable bacteria. Although a number of studies report high numbers of bacteria within the body cavities of apparently healthy crustaceans (Colwell et al. 1975, Sizemore et al. 1975, Ueda et al. 1993), this study has shown that the existence of bacteria in the body cavity may have apparent detrimental effects on the animal. Vogan et al. (1999) showed that shell-diseased male *Cancer pagurus* displayed a significantly higher percentage of limb loss than their disease-free counterparts. The present study has shown that haemolymph infection frequently co-occurs with limb loss suggesting a potential route of microbial entry. However, bacteria may also gain entry to the haemocoel through breach of the cuticle either at the gill lamellae (e.g., see Fig. 4) or through other external lesions.

This study found distinct changes from the normal histology of the gill and hepatopancreas in animals displaying the characteristic black spot lesions of shell disease. A grading in histopathological change was observed with the most severe internal damage associated with crabs that showed the highest amount of external damage in regions surrounding the branchial chambers.

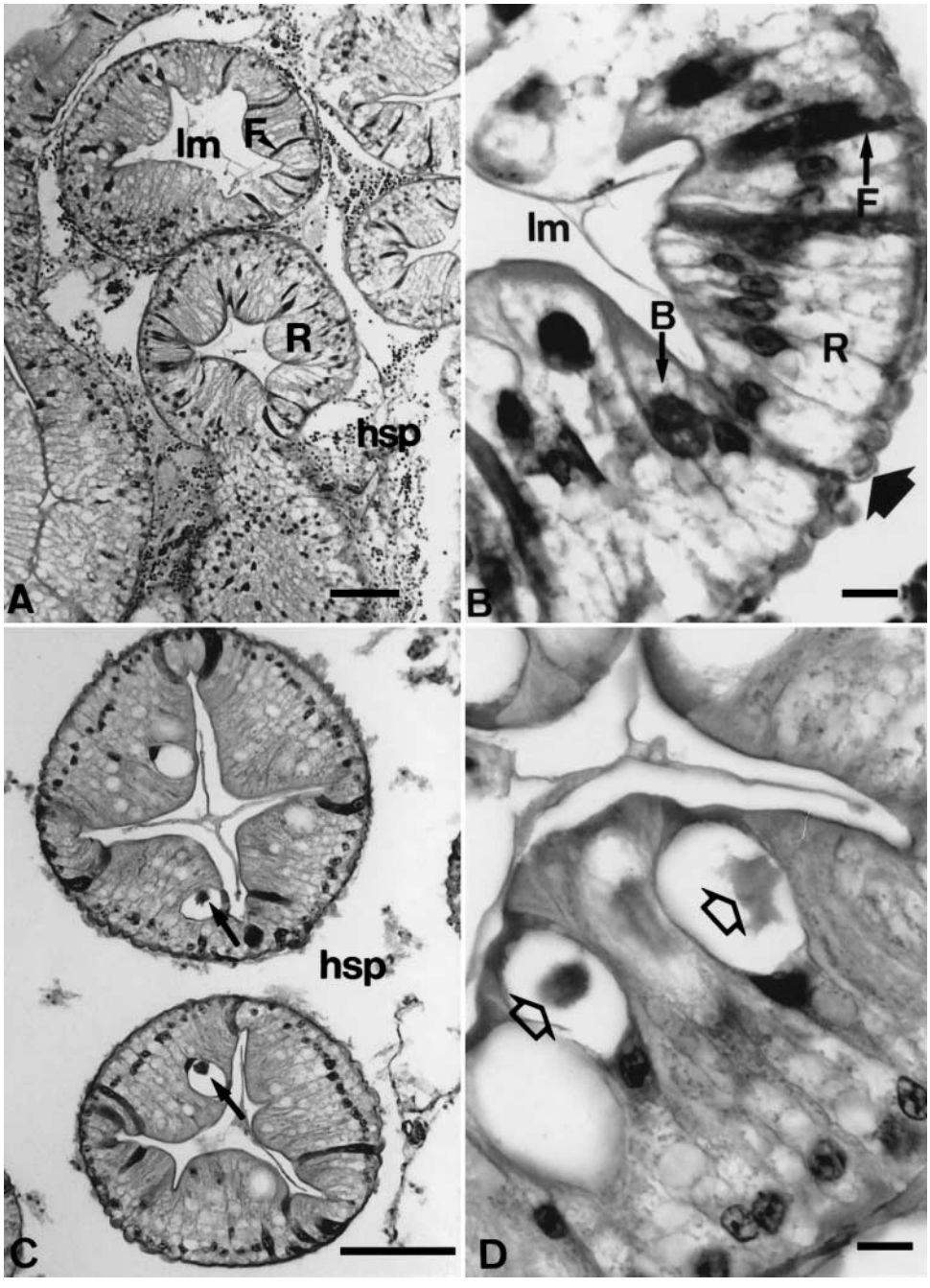

Fig. 5. Sections through the hepatopancreas of uninfected and shell-diseased crabs. (A,B) Low and high power micrographs from uninfected crab showing tubules containing R-cells (R), F-cells (F) and B-cells (B) with underlying basal lamina (unlabelled arrow), lumen of tubules (lm) and inter-tubular (haemal) spaces (hsp). (C,D) Low and high power micrographs of hepatopancreas from infected (D3) crab showing increase in number of B-cells and associated debris (unlabelled arrows) in their vacuoles. Haemal space (hsp). Scale bars = 100 µm (A,C) and 10 µm (B,D)

Comely & Ansell (1989) investigated the prevalence of shell disease and its occurrence in combination with gill damage in 5 species of crabs. *Liocarcinus puber*, *L. corrugatus* and *L. depurator* were all found to have erosion of the dorsal carapace in combination with necrosis of the gills. However, on no occasion was gill damage observed in *Cancer pagurus* or *Carcinus maenas*. The present results show damage to the gills of *C. pagurus* with a correlation between severity of gill damage and percentage cover of exoskeletal lesions on ventral surfaces.

Crustacean gill nephrocytes are generally believed to be involved in the removal, processing and detoxification of substances from the haemolymph (e.g., Smith & Ratcliffe 1981, Lawson et al. 1994). An increase in the number of nephrocytes in the gill has been observed in response to exposure to heavy metals (Soegianto et al. 1999a,b). Rather than a change in the actual number, the animals in the present study had a massive accumulation of dark brown material in the central vacuoles of the nephrocytes. Similar observations have been reported for *Carcinus maenas* fol-

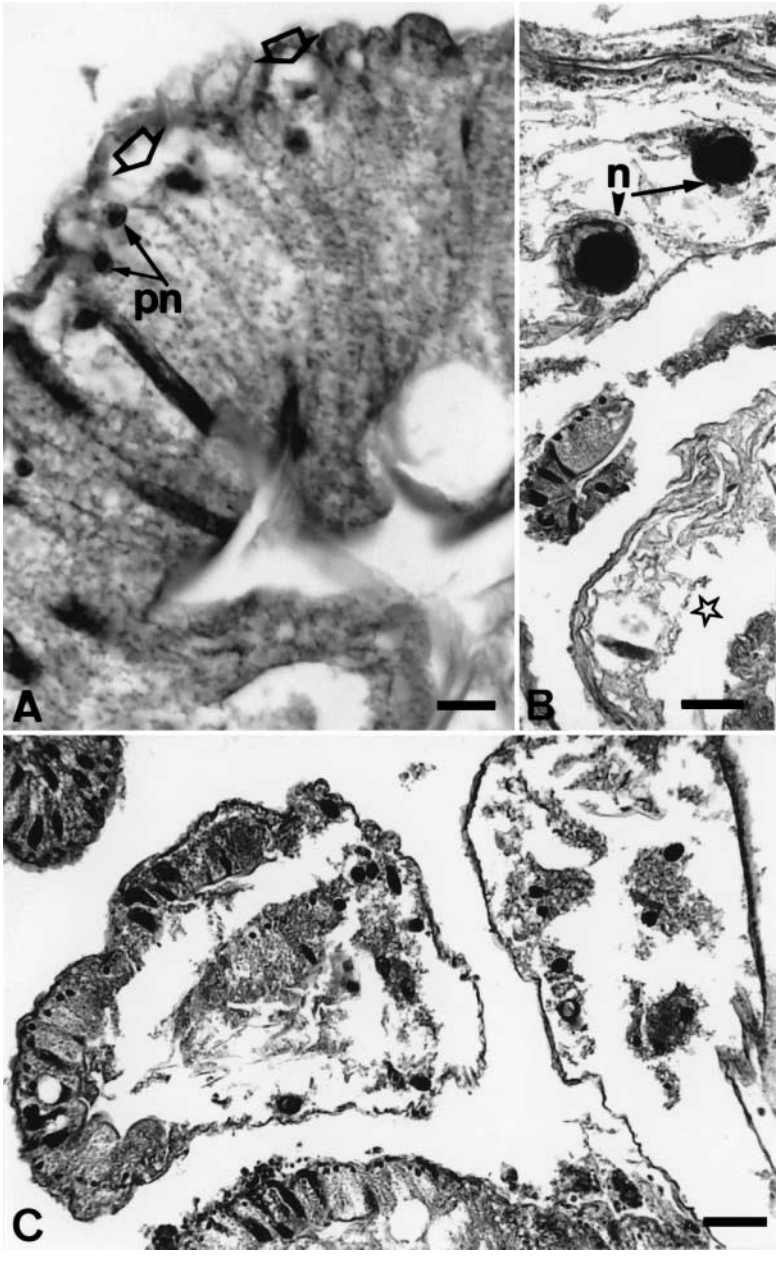

Fig. 6. Micrographs showing degradation of hepatopancreatic tubules in shell-diseased crabs. (A) High power micrograph showing tubule disorganisation in crab D2 consisting of pyknotic nuclei (pn), indistinct cell boundaries and spaces underlying the basal lamina (unlabelled arrows). (B) Nodules (n) in connective tissue region of hepatopancreas from crab D5. Note underlying tubule destruction (☆). (C) Tubule disruption in crab D2. Scale bars = 10 µm (A) and 50 mm (B,C)

lowing intrahaemocoelic injection of bacteria (Smith & Ratcliffe 1981). These authors concluded that the vacuolar material, although not bacterial in nature, was likely to be composed of cellular debris generated during the haemocytic response to the presence of bacteria in the haemocoel. This explanation corresponds with the present observation of lesion formation, haemocytic infiltration and nodule formation in the gill lamellae of *Cancer pagurus*, suggesting that the observed nephrocytic changes in shell-diseased animals might be directly due to systemic bacterial infections.

The occurrence of melanised nodules in the gills, heart and hepatopancreas suggests systemic bacterial infections in these animals (Johnson 1976, Smith & Ratcliffe 1980). The observed breaches in the lamellar epithelium combined with haemocytic infiltration and nodule formation around these foci identify a potential route of invasion by microorganisms. Exposure to sub-lethal levels of cadmium (Victor 1993, Soegianto et al. 1999a), lead (Victor 1994) and copper (Soegianto et al. 1999b) were shown to induce necrotic lamellar lesion formation and haemocytic infiltration but more important, in a few cases, secondary bacterial invasions in decapod gills (Couch 1977, Soegianto et al. 1999a). Smith & Ratcliffe (1980) remarked that the crustacean gills are the most permeable part of its integument, hence vulnerable to injury and microbial invasion. In decapods, the epidermal cells of the gill lamellae, unlike the rest of the integument, possess only an ultra-thin covering of chitinous cuticle (Figs 2 & 3). Thus, although this thin covering is more vulnerable than the rest of the integument, its breach would still be have to be through active chemical attack (Victor 1993, 1994, Soegianto et al. 1999a,b), abrasive injuries or the extracellular enzymatic activities of microorganisms (Lightner & Fontaine 1975). Morado et al. (1988) showed erosion of the arthrodial membrane between the propodus and pereiopods of Dungeness crabs *Cancer magister* that coincided with the appearance of nodules in the gills. Although such erosion is not classified as shell disease, the pathology also suggests invasion across the cuticle and resultant septicaemia leading to nodule formation in the gills.

Extensive vacuolation of the hepatopancreas has been reported in response to chemical (Anderson et al. 1997, Bhavan & Geraldine 2000) and bacterial exposure (e.g., Bowser et al. 1981). For example, Anderson et al. (1997) stated that the R-cells were

responsible for the increase in vacuolation in response to chemical exposure. By contrast, Bowser et al. (1981) reported an increase in the vacuolation of the B-cells following intrahaemocoelic injections with *Vibrio* spp. Robertson et al. (1998), who investigated the pathogenicity of *Vibrio harveyi* on *Penaeus vannamei* larvae, showed that necrotic bundles formed in the hepatopancreas of grossly infected animals. Interestingly, although not stated by the authors, the 'necrotic bundles' appear to be located within the B-cells and are very similar in appearance to those observed in the present study.

Another condition found in the haemal spaces between the hepatopancreatic tubules of decapods with bacterial infections is haemocytic infiltration or whirling (Edgerton & Owens 1999, Ruangpan et al. 1999). The results from the current study, although indicative of a limited systemic bacterial infection, do not show a massive infiltration of haemocytes in the hepatopancreas. Despite the lack of haemocytic infiltration, the infection still has its impact on the hepatopancreas with destruction of the tubular structure seen in severely infected crabs.

Lightner (1974) noted the rapid autolytic post-mortem changes of the hepatopancreas in penaeid shrimps that highlight the importance of rapid fixation of this tissue. Therefore, care must be taken in the interpretation of the apparent damage seen in the present study to the hepatopancreas. Poor fixation, however, is not considered to be the sole cause of the damage seen in the current study since intrahaemocoelic injections of large volumes of fixative were used to sacrifice crabs and histologically the hepatopancreas of all control (non-diseased) animals appeared normal. Furthermore, Bouin's sea-water fixative (as used in our study) rapidly penetrates and fixes all tissues. Variation in the quality of histological integrity that could have been argued to be a marker of poor penetration of fixative (e.g., peripheral areas of the block look structurally similar to tissues in the centre of the block) was not observed. Initial experiments using 5% buffered sea-water formalin injected via the same route as used in the present work resulted in poor preservation of the hepatopancreas (Costa-Ramos & Rowley pers. obs.). Importantly, however, the appearance of the hepatopancreas in these formalin-fixed tissues was significantly different from that observed in the present study. Finally, of significance to this discussion is the finding that a correlation exists between the degree of hepatopancreas damage and the level of external shell disease. Taken together these observations suggest that the changes observed are more likely to result from disease than from post mortem or fixation artefacts. However, it cannot be ruled out that at least some of the damage observed is an artefact of histology.

In *Cancer pagurus*, severe necrosis of the hepatopancreas has been found following injection of a *Vibrio* sp. isolate taken from a shell disease lesion (Costa-Ramos, Vogan & Rowley pers. obs.). Similarly, Johnson (1976), Vogt (1997), Edgerton & Owens (1999) and Ruangpan et al. (1999) also showed damage of the hepatopancreas in combination with bacterial infections. Release of an exotoxin from Gram-negative bacteria in the tubules of *Palaemon elegans* was believed to be the cause of destruction of the hepatopancreas (Vogt 1997). Bacterial toxins may cause initial lysis of cells, but once the R-cells have disintegrated, autolysis of the tubules may be exacerbated by the liberation of digestive enzymes.

In summary, shell disease, normally defined as an external infection with limited mortality, has been shown to coincide with low levels of systemic bacterial infections that may lead to damage of internal tissues. Animals with severe levels of the disease are more likely to incur rupture of the cuticle, through exoskeletal lesions of the visible body surface, through the thin cuticle of the gills or across the fracture plane after limb autotomy. Whether these invasions result in increases in mortality of *Cancer pagurus* among natural populations remains to be determined, but will ultimately depend on the virulence of the microbial invaders.

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