A novel bacterial disease of the European shore crab, *Carcinus maenas* – molecular pathology and epidemiology

Fiona Eddy,¹ Adam Powell,¹ Simon Gregory,¹ Linda M. Nunan,² Donald V. Lightner,² Paul J. Dyson,³ Andrew F. Rowley¹ and Robin J. Shields¹

Correspondence

Andrew F. Rowley
a.f.rowley@swansea.ac.uk

¹Centre for Sustainable Aquaculture Research, Department of Biological Sciences, Swansea University, Singleton Park, Swansea SA2 8PP, UK

²Department of Veterinary Sciences and Microbiology, University of Arizona, Tucson, AZ 85712, USA

³School of Medicine, Swansea University, Singleton Park, Swansea SA2 8PP, UK

Several rickettsia-like diseases have been reported in arthropods (insects and crustaceans), some of which result in significant losses of economically important species such as shrimp and crabs. This study reports on the molecular pathology of a recently emerged disease of the European shore crab, *Carcinus maenas*, termed milky disease – named as a result of the unusual milky appearance of the haemolymph (blood). This disease was more prevalent (>26 %) during summer months when the water temperature in a pilot crab farm was approximately 19 °C. The putative causative agent of the disease was a Gram-negative bacterium that could not be cultured on a range of agar-based growth media. Diseased crabs showed significant reductions in free blood cell numbers and total serum protein. Such animals also displayed raised levels of glucose and ammonium in blood. Ultrastructural and *in situ* hybridization studies revealed that the causative agent associated with milky disease multiplied in the fixed phagocytes of the hepatopancreas (digestive gland), ultimately to be released into the haemolymph, where the circulating blood cells showed little response to the presence of these agents. Attempts to induce the infection by short-term temperature stress failed, as did transmission experiments where healthy crabs were fed infected tissues from milky disease affected individuals. Sequence analysis of the 16S rRNA gene from the milky disease bacteria indicated that they are a previously undescribed species of α -proteobacteria with little phylogenetic similarity to members of the order Rickettsiales.

Received 26 March 2007

Revised 18 May 2007

Accepted 28 May 2007

INTRODUCTION

There is increasing interest in the likely effect of global climate change on diseases in both wild and cultured organisms (Harvell *et al.*, 1999, 2002; Lafferty *et al.*, 2004). In particular, aquatic animals are highly sensitive to temperature change and it is widely accepted that elevated temperatures can lead to reduced oxygen tension in the water, higher microbial growth and immunosuppression, resulting in higher prevalence of disease (Le Moullac & Haffner, 2000). For example, brown trout in alpine rivers and streams have been found to show increasing prevalence of proliferative kidney disease caused by the myxozoan parasite *Tetracapsuloides bryosalmonae* over the last 20 years (Hari *et al.*, 2006). These increased temperatures drive the proliferation of *T. bryosalmonae* in its intermediate

bryozoan host (Tops *et al.*, 2006). Equally at very low water temperatures, some aquatic animals may become immunocompromised as a result of a failure of the immune system to mount an effective immune response to bacterial challenge (Chisholm & Smith, 1994).

One potentially important result of climate change will be alterations in the effectiveness of aquaculture as a method of supplementing fish and shellfish for human and animal consumption taken from the wild (Alborali, 2006). Currently, it is thought that at least 15 % of fish and shellfish under aquaculture conditions are lost as a result of infectious and non-infectious diseases. In terms of shrimp production alone, ~US\$3000 million was reported to be lost in one year on a global basis as a result of disease outbreaks (Subasinghe, 1997). Because most aquaculture practices are dependent on the ambient environmental water temperature, long-term alterations in climate are likely to impinge on these activities, with accompanying changes in disease profiles.

Abbreviations: TEM, transmission electron microscopy; THC, total haemocyte count.

Crustaceans, such as shrimps, lobsters and crabs, provide high-quality protein, and marine species also contain omega-3 fatty acids that afford potential health benefits. Therefore, some of these animals have been increasingly subject to farming to supplement those caught by fishing (Wickins & Lee, 2002). One recent candidate for aquaculture development has been the European shore crab, *Carcinus maenas*, which is valued as bait for sport angling and eaten in continental Europe. In addition, these animals have been highlighted for their use as an important indicator species to monitor the health of marine ecosystems (e.g. Hebel *et al.*, 1997). Initial semi-commercial attempts to culture shore crabs have revealed a number of problems, including the appearance of a novel disease state that we have christened 'milky disease' because of the unusual milky nature of the blood. This disease appears to be more prevalent in summer months when water temperatures are higher. The current study investigates the nature of the causative agent of this novel disease, the seasonal effects on disease prevalence, and the interaction between the causative agent and host cells and tissues.

METHODS

Animals. Adult shore crabs (*Carcinus maenas*) affected by milky disease were collected from the Queen's Dock, Swansea, or from the pilot crab farm of J. W. Aquaculture (Research) Ltd based in Swansea, Wales, UK. Some milky disease affected animals were also surveyed from additional, independent experiments designed to determine the dietary needs of shore crabs (Powell & Rowley, 2007) carried out under aquarium conditions. Stock (apparently milky disease-free) collected from the Queen's Dock, were returned to a recirculating sea water aquarium in the university, where they were routinely fed with raw white fish supplemented with mussels or cockles.

Heath status assays. Blood (haemolymph) was withdrawn from crabs by puncturing the unsclerotized membrane with a 21 gauge needle and syringe. For total haemocyte (blood cell) counts (THC), the blood was mixed with an equal volume of ice-cold fixative (5% formaldehyde in 3% sodium chloride solution) and cells counted with a Neubauer haemocytometer. For blood chemistry (total protein, ammonium and glucose) blood was allowed to clot for ~60 min on ice, centrifuged (5000 g; 10 min) and the resulting serum stored at -80 °C. Total protein was determined using a bicinchromic acid assay kit (Pierce & Warriner) using BSA as a standard, while glucose and ammonium concentrations were determined using modifications of the methods of Bergmeyer (1984) and Bolz & Howel (1978) respectively. Glycogen levels in the hepatopancreas (digestive gland) were determined using the anthrone method (Van Handel, 1965) using type II glycogen (Sigma-Aldrich) as the standard. All assays were performed in 96-well microtitre plates and absorbances read on a Anthos II plate reader.

Culture of the putative causative agent of milky disease. Attempts to isolate and culture the causative agent of milky disease were performed using various dilutions (0–10 000 fold) of infected haemolymph spread on a variety of solid culture media. These included tryptic soy agar, Marine Agar 2216, Mueller–Hinton agar, brain heart infusion agar, blood agar (5% sheep's blood), fetal calf serum (5% final concentration) supplemented tryptic soy agar and tryptic soy agar supplemented with filter-sterilized (0.22 µm) fresh crab blood (5% final concentration). Where appropriate, 2% sodium

chloride (final concentration) was added to adjust the tonicity of the media. Plates were incubated at temperatures ranging from 15 to 25 °C for >25 days.

Histopathology. Both milky disease-free and affected crabs were killed by the injection of ~5 ml Bouin's sea-water fixative, 5% formaldehyde in sea water or Davidson's sea-water fixative. Tissues (gills, muscle, gonad, hepatopancreas) were removed and left for 24 h in these fixatives, dehydrated and embedded in paraffin wax. Sections (~8 µm thick) were stained with Cole's haematoxylin and eosin, Giemsa, or Pappenheim's stain. Photomicrographs were taken on an Olympus BX50 microscope equipped with a digital camera.

Infectivity trials. Two groups (10 per group) of crabs showing no apparent symptoms of milky disease were fed diets consisting of either whitefish/gelatin homogenate with haemolymph from milky disease affected animals or whitefish/gelatin homogenate with hepatopancreas from milky disease affected individuals. Control animals were fed whitefish and gelatin homogenate only. Animals were monitored over 2 months for signs of disease weekly by examination of blood for THC and turbidity. In addition to these experiments, crabs (~10 per group) were injected with either milky diseased or 'healthy' whole haemolymph (300 µl) and resulting mortality monitored over 48 h. Finally, crabs (10 per group) were held at 25, 15 and 5 °C for 48 h and returned to 'normal' aquarium temperature (15 °C) for >8 weeks. Subsequently, they were fed on a standard whitefish diet and monitored weekly for milky disease symptoms (THC and blood examination for bacteria).

Identification of milky disease bacterium. Haemolymph (~300 µl) from ~10 milky disease affected crabs was diluted in cold 3% NaCl solution, centrifuged (4000 g; 5 min, room temperature) and washed repeatedly in cold sterile 3% NaCl to remove soluble blood products. Bacterial DNA was extracted using a GenElute bacterial genomic DNA kit (Sigma-Aldrich) using the protocol for Gram-negative bacteria. The 16S rRNA gene sequence of the bacterium associated with milky disease was amplified by PCR using the universal primers previously reported (Lane, 1991; Marchesi *et al.*, 1998): 27f (5'-AGAGTTTGATCMTGGCTCAG-3'), 1387r (5'-GGGCGGWTGTA-CAAGGC-3'). The amplification profile consisted of 95 °C for 5 min, 30 cycles of 95 °C 30 s, 60 °C 45 s, 72 °C 90 s and a final extension of 72 °C for 5 min. Ligation and transformations (*Escherichia coli* JM109) for cloning were carried out using the PCR fragment and plasmid vector pGEM-T Easy, following the manufacturer's protocol (Promega). The plasmid was then purified using a Wizard *Plus* SV Minipreps DNA purification system (Promega).

Plasmid digest was carried out using the plasmid DNA and *Eco*RI enzyme (New England Biolabs), incubated at 37 °C for 120 min. A 0.8% agarose gel containing ethidium bromide (0.5 µg ml⁻¹) was run (100 mV, 30 min) to confirm the presence of ~1300 bp product. Plasmid sequencing was performed using M13 universal primers. Sequence reactions were analysed using a Beckman Coulter CEQ capillary sequencer. The sequenced 16S rRNA gene was compared with all sequence data in the GenBank database using the BLAST algorithm to ensure that the origin of the milky disease bacterium sequence was from the 16S rRNA gene and that the sequence was unique. A phylogenetic tree was constructed using sequences of bacterial strains as employed by Loy *et al.* (1996) and Friedman *et al.* (2000). The dendrogram was constructed using MEGA v3.1 (Kumar *et al.*, 2004). The neighbour-joining method of Saitou & Nei (1987) was used with the Kimura two-parameter substitution model (Kimura, 1980), pairwise elimination of incomplete data and bootstrapping of 1000 resamplings.

Cloning of a milky disease bacterium-specific PCR fragment and *in situ* hybridization. *In situ* hybridization was used to show the

presence of milky disease causative agent in crab tissues. Briefly, two novel primers were selected to amplify an internal region of the 16S rRNA gene specific to the milky disease bacterium: forward primer MDF (5'-TATTCGGAGGAACACCAGAG-3') and reverse primer MDR (5'-AAGAGCCATGAGGACTTGAC-3'). The PCR amplification profile consisted of 1 cycle at 95 °C for 2 min, 29 cycles of 45 °C, 72 °C and 95 °C for 30 s respectively and 1 cycle at 1 and 2 min at 45 and 72 °C respectively, resulting in a 506 bp product. This product was purified using a QIAquick PCR purification kit (Qiagen) and cloned into pGEM-T Easy as previously described. The cloned fragment was purified and labelled with digoxigenin-dUTP (Roche Diagnostics) and then precipitated overnight at -70 °C, air-dried and rehydrated in distilled water ready for use in the assay. The DIG-labelled probe was tested by *in situ* hybridization following the protocol of Poulos *et al.* (1994) as modified by Nunan *et al.* (2003). Slides of Davidson's fixed hepatopancreas, gonad or alimentary canal were deparaffinized and sections covered with freshly prepared lysozyme (100 µg ml⁻¹) in buffer (0.5 mM Tris/HCl, 0.1 mM NaCl, 0.01 mM EDTA, pH 7.4) and incubated at room temperature for 15 min. Pre-hybridization and overnight hybridization steps were carried out at 37 °C in a humid chamber. After hybridization, slides were washed first in saline sodium citrate solution (0.3 M sodium citrate, 3 M NaCl, pH 7.0), followed by 0.5 × saline sodium citrate, and incubated with 1 µl alkaline phosphatase-labelled anti-DIG antibody (Roche Diagnostics) in 1 ml 0.1 M Tris/HCl, 0.15 M NaCl with 0.5 g blocking reagent (Roche Diagnostics) and incubated at 37 °C for 30 min. Final development of the reaction was carried out at room temperature for 1–3 h with nitroblue tetrazolium and bromochloroindolyl phosphate in pH 9.0 buffer. Sections were briefly (~5 min) counterstained with 5% Bismarck brown, dehydrated, cleared and mounted in DPX.

Transmission electron microscopy (TEM). Milky disease affected and apparently non-diseased crabs were initially bled into an equal volume of ice-cold 2.5 or 5% glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.4 containing sucrose to adjust the osmolarity to 1000 mosmol kg⁻¹. Subsequently, these crabs were sacrificed by the injection of ~5 ml of this fixative and rapidly dissected to remove the gills, hepatopancreas, alimentary canal, gonad and muscle. Small pieces of these tissues and blood cell pellets were fixed for ~90 min in ice-cold 2.5 or 5% glutaraldehyde in 0.1 M cacodylate buffer, washed in ice-cold 0.1 M cacodylate buffer containing sucrose (final osmolarity 1000 mosmol kg⁻¹) and post-fixed for 60 min in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer. Following dehydration in a graded series of ice-cold methanols, tissues were placed in propylene oxide and infiltrated overnight with Araldite resin (Taab). Thick plastic sections (~1 µm) were stained with 1% methylene blue/1% toluidine blue, while thin sections were stained with 10% methanolic uranyl acetate and aqueous lead citrate, and examined using a JEOL transmission electron microscope operated at 80 kV.

RESULTS

Gross pathology and disease prevalence

Crabs affected by milky disease showed no external or behavioural changes that could be readily employed as a method of identifying affected individuals. Instead, during routine sampling, blood was found to exhibit the characteristic milky appearance for this disease (Fig. 1A, B). Examination of blood by phase-contrast microscopy showed the existence of large numbers of bacteria-like particles ~1 µm long together with reduced numbers of

Fig. 1. (A) Shore crab infected with milky disease. Note the characteristic milky appearance of the blood (unlabelled arrow) produced as a result of bleeding from the unsclerotized area at the rear of the crab. (B) Appearance of haemolymph from milky disease affected crabs (1, 2) and non-diseased animals (3, 4). (C) Phase-contrast micrograph of blood from a milky disease affected shore crab. Note the large number of bacteria around the haemocyte. Scale bar, 5 µm.

circulating blood cells (haemocytes) (Fig. 1C). The bacteria in the blood were all Gram-negative. Infected crabs ultimately died, usually within 7 days of initial positive sampling, and although not quantified, such individuals showed increased bleeding times following puncture of the cuticle.

Crabs collected from the wild at all times of the year showed a very low (<1%) prevalence of disease. A long-term survey of crabs from the pilot crab farm [J. W. Aquaculture (Research) Ltd] showed a high prevalence of milky disease in crabs during late summer when water temperatures were high (Fig. 2). There was no significant difference in the prevalence of milky disease between male and female crabs, and there was no difference in the prevalence of disease between crabs of different ages (data not shown). Diseased crabs had significantly higher levels of ammonium in the blood than non-diseased individuals sampled at the same times (18.3 ± 1.8 µg ml⁻¹ blood in diseased crabs compared with 3.4 ± 0.4 µg ml⁻¹ blood in non-diseased animals; mean values $\pm$ SEM, $n=20-81$, $P<0.001$, Student's *t*-test). Crabs maintained under aquarium conditions were also found to exhibit higher

Fig. 2. Effect of season and water temperature on the prevalence of milky disease in adult shore crabs in a pilot crab farm. Each month, 50 crabs (25 male and 25 female) collected at random were surveyed for the presence of 'milky' haemolymph by intrahaemocoelic bleeding and macroscopic examination of haemolymph turbidity.

prevalences of milky disease during the summer months when the water temperature varied between 17 and 21 °C. Salinity and levels of nitrogenous waste were also regularly checked in the aquarium. These were not significantly affected by the elevated temperatures.

Effects of disease on blood cells, serum protein, glucose and ammonium, and hepatopancreatic glycogen

As some of the crabs that were part of an independent dietary trial carried out under aquarium conditions became affected by milky disease, it was possible to follow changes in a variety of blood parameters before and at the first initial diagnosis. The THC of apparently healthy crabs decreased significantly upon development of milky disease (Fig. 3A). Similarly, the amount of total protein in serum also significantly declined before the initial diagnosis of disease (Fig. 3A), while in the case of glucose and ammonium (Fig. 3B), a significant increase in these factors was found at the time of diagnosis. Levels of glycogen in the hepatopancreas (digestive gland) of affected and non-affected crabs were not found to differ significantly (data not shown).

Histopathology

Of the tissues examined, the hepatopancreas (digestive gland) of milky disease affected crabs showed the most prominent changes in structure following infection. The hepatopancreas of crabs consists of tubules containing a variety of epithelial cells together with extra-tubular haemal spaces containing blood vessels, fixed phagocytes and freely circulating haemocytes (Fig. 4A). In the majority of milky disease affected individuals these tubules showed no obvious morphological changes, while large numbers of swollen cells were found in the haemal space containing

Fig. 3. (A) Changes in total haemocyte counts and total serum protein, and (B) serum glucose and serum ammonium levels prior to the initial identification of milky disease in crabs. Values shown are means $\pm$ SD, $n=12-17$. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ compared with time 0.

fibrous-like material (Fig. 4B). Only in a small number of cases was any tubule damage found and here this was accompanied by haemocyte encapsulation of the damaged tubules (Fig. 4C). Occasionally, haemocyte accumulations, termed nodules, were found in the haemal space; some of these were seen to contain debris and bacteria-like particles (Fig. 4D).

The only other tissue to show any changes in histology following infection was the gills, where fixed cells termed nephrocytes became more vacuolar and filled with debris (not shown). All other tissues examined (gonad, alimentary canal and muscle) were structurally normal (not shown).

Culture of the putative milky disease agent

Attempts to isolate and grow the potential causative agent on a wide range of agar-based culture media failed despite the use of long incubation periods and a range of dilutions of the infected haemolymph. These procedures were attempted using haemolymph from several different milky disease affected crabs. In all cases, the plates were devoid of colonies apart from the occasional contaminant ($<0.001\%$ of the total number of bacteria seen in blood by direct

Fig. 4. Histopathological changes in crabs affected by milky disease. (A) Histological section of the hepatopancreas of an apparently healthy crab showing the epithelial cells (EP) of the tubule and underlying haemal space containing fixed phagocytes (FP), blood vessels (BV) and free haemocytes (H). Lumen of tubule (L). Scale bar, 20 μ m, haematoxylin/eosin stained. (B) Hepatopancreas from a crab with milky disease showing the densely stained bodies in the haemal space (*). Scale bar, 20 μ m, haematoxylin/eosin stained. Inset shows the fibrous appearance (*) of the material in these dense bodies. Thick plastic section, methylene blue/toluidine blue stained. Scale bar, 5 μ m. (C) Adjacent hepatopancreatic tubules from a milky disease affected crab showing the normal tubular structure on the left and necrotic structure on the right hand side. Note the sheath of haemocytes (unlabelled arrow) around the necrotic tubule. Haematoxylin/eosin stained. Scale bar, 50 μ m. (D) Nodule found in the haemal space of an infected crab. The central necrotic core (C) is surrounded by a sheath of flattened haemocytes (SH). Thick plastic section stained with methylene blue/toluidine blue. Scale bar, 10 μ m.

microscopy). Such colonies were not found on a routine basis from milky disease affected crabs.

Transmission of disease and temperature stress experiments

Apparently disease-free crabs fed hepatopancreas from milky disease affected crabs failed to show any changes in mortality or haemolymph coloration indicative of milky disease for up to 8 weeks post-challenge. Such crabs were also free from bacteria in the haemolymph as observed in milky disease affected crabs. Furthermore, during this time, the THC of these individuals did not differ from that of crabs fed similar amounts of hepatopancreas from non-milky disease affected animals (data not shown). Intrahaemocoelic injection of apparently healthy crabs with blood (100–300 μ l containing $\sim 10^8$ bacteria) from milky disease affected individuals resulted in large mortalities (100 %) within 24 h of injection. Similarly, injection of crabs with the same volume of cell-free haemolymph (i.e. with neither haemocytes nor bacteria) from milky disease affected crabs resulted in 100 % mortality at 24 h post-challenge. In the case of crabs injected with the same volume of whole blood from uninfected crabs, no mortalities were observed 24–48 h post-challenge. Histopathological analysis of crabs injected with whole haemolymph from milky disease affected crabs failed to reveal the characteristic swollen cells in the hepatopancreas as seen in milky disease affected crabs collected from the wild. The explanation for the death of such crabs was unclear and the pathology unlike that of 'normal' infections.

Laboratory trials with various temperature stress parameters failed to induce the appearance of milky disease in such crabs in comparison with those crabs held under 'standard' aquarium conditions.

Identification of the causative agent of milky disease

Using the full-length and the *in situ* hybridization probes, the causative agent of milky disease was found to be a member of the α -proteobacteria with limited similarity to some members of the *Rhodobacteriales*. BLAST searches on either the full-length or the *in situ* hybridization probes showed 88 % similarity with several uncultured marine bacteria (DQ009292, Brown *et al.*, 2005; DQ071145, Lau & Armhurst, 2006). Phylogenetic analysis showed that the causative agent of milky disease is unlike all other rickettsia-like diseases of crustaceans reported to date and distinct from other members of the order *Rickettsiales* (Fig. 5).

In situ hybridization

The DNA probe showed a strong positive hybridization signal in the swollen cells surrounding the tubules of the

Fig. 5. Dendrogram showing the evolutionary relationship between the milky disease bacterium sequence (MD) with various α - and γ -proteobacteria including the causative agents of a range of arthropod rickettsial diseases including necrotizing hepatopancreatitis of shrimp (NHP bacterium, U65509) and *Coxiella cheraxi*, a causative agent of rickettsiosis in the redclaw (*Cherax quadricarinatus*) (Tan, 1998; Tan & Owens, 2000). The numbers at the branch points are the bootstrap values. Based on Loy *et al.* (1996) and Friedman *et al.* (2000).

hepatopancreas from all of the milky disease affected animals tested (Fig. 6A–C). The epithelial cells in the tubules and free haemocytes in the haemal space, however, were free from signal (Fig. 6A, C). Similarly, the epithelial cells in the alimentary canal and the gonadal cells were also lacking signal (Fig. 6D). In these latter tissues, the only reaction product was found in the connective tissue and the blood vessels (Fig. 6D). The probe failed to hybridize with other known vibrio and rickettsia-like infected tissues of other crustaceans, or tissue from apparently non-infected crabs (not shown).

Detection of milky disease by PCR

To determine if the probe developed for *in situ* hybridization routinely gave positive results for haemolymph from milky disease affected crabs, extracted DNA from the

haemolymph of 10 diseased crabs and 50 apparently healthy crabs was run on agarose gels. In all cases, no characteristic 506 bp signal was present in the samples from healthy crabs while all diseased crabs gave this positive signal (not shown).

Transmission electron microscopy (TEM)

The TEM studies revealed significant presence of milky disease causative agent in the fixed phagocytes found in the connective tissue around the tubules of the hepatopancreas. In uninfected animals, these cells were found to be attached external to the endothelial cells that line the blood spaces (see Figs 4A and 7A). Groups of fixed phagocytes were in turn surrounded by an unusual perforated basement membrane with pores 80–200 nm in diameter (Fig. 7A inset). Many of the fixed phagocytes contained

Fig. 6. *In situ* hybridization assay results for hepatopancreas (A–C) and gonad (D) of milky disease affected crabs. (A, B) Positive hybridization signal (dense purple-black reaction product) in the dense bodies within the haemal space of an infected crab. Scale bars=100 μ m (A) and 10 μ m (B). (C) Free haemocytes in the haemal space of an infected crab. Note lack of hybridization signal in these cells (unlabelled arrows). Scale bar, 10 μ m. (D) Gonadal tissue showing lack of reaction product in the epithelial cells and immature spermatozoa (S). Positive hybridization signals are shown, however, by the blood vessels (BV) and haemal spaces around the gonadal cells. Scale bar, 200 μ m.

cytoplasmic electron-dense granules and a single vacuole containing homogeneously dense material of unknown origin (Fig. 7A). No bacteria were seen in any of these cells from uninfected animals. In milky disease affected animals, the fixed phagocytes showed evidence of bacterial multiplication directly in the cytoplasm. Such cells typically contained a single vacuole containing many bacteria together with myelin whorls, while in the peripheral cytoplasm bacteria were also found either alone or in groups (Fig. 7B–D). Such bacteria did not appear to be within phagocytic vacuoles. In highly infected fixed phagocytes, bacteria were observed apparently in the process of release into the surrounding area (Fig. 7D). Ultimately, the fixed phagocytes apparently disintegrated and the area bounded by the perforated basement membrane became swollen and replete with bacteria (Fig. 7E). In some cases, bacteria were observed ‘escaping’ through the slit regions of the basement membrane to gain entry to surrounding blood sinusoids and other tissues (Fig. 7F).

Although many free haemocytes (blood cells) were commonly found in the extra-tubular region of the

Fig. 7. TEM of the hepatopancreas from healthy (A) and milky disease affected (B–F) crabs. (A) Fixed phagocyte from an apparently milky disease-free crab showing the characteristic dense granules (G), large vacuole containing electron-dense material (V) and the surrounding basement membrane with slit-like appearance (arrows) as also seen in the higher-power micrograph in the inset. Scale bar, 1 μ m; inset scale bar, 200 nm. (B, C) Fixed phagocyte from milky disease affected crab showing bacteria and associated myelin whorls (M) in the large vacuole, as well as bacteria in the peripheral cytoplasm. Basement membrane (arrow). Scale bars=1 μ m (B) and 200 nm (C). (D) High-power micrograph showing the apparent escape of bacteria from an infected fixed phagocyte (arrows). Scale bar, 200 nm. (E) Low-power micrograph showing area bounded by the basement membrane (unlabelled arrow) enclosing numerous free bacteria and remnants of fixed phagocytes also with bacteria (R). Scale bar, 2 μ m. (F) High-power micrograph showing the apparent transit of a bacterium through the slit-like pores of the basement membrane (BM) surrounding a group of heavily infected, effete, fixed phagocytes. Scale bar, 200 nm.

hepatopancreas (see Fig. 4A) and in contact with the causative agent of the disease, only on rare occasions was

there any interaction between these cells and the surrounding bacteria. TEM examination of pelleted blood cells from the haemolymph of milky disease infected animals revealed that despite the large numbers of bacteria free in the blood, the haemocytes remained normal and all of the main types (hyaline, semi-granulocytes and granulocytes; Bauchau, 1981) were observed (Fig. 8A). In a limited number of cases (<0.01 % of cells examined) bacteria were seen within the cytoplasm of some haemocytes (Fig. 8A inset). In such occurrences, these bacteria were invariably associated with myelin and other debris but remained structurally intact. Finally, despite the majority of the haemocytes displaying normal ultrastructural features, a few blood cells in circulation showed signs of apoptotic-like breakdown (Fig. 8A).

Fig. 8. TEM of haemolymph from milky disease affected crabs. (A) Low-power micrograph showing semi-granular (SG), granular (G) and hyaline (H) haemocytes surrounded by numerous bacteria. Scale bar, 1 µm. The inset shows cytoplasm of a haemocyte containing bacteria and myelin debris (M). Scale bar, 200 nm. (B) Apparently necrotic haemocyte with fragmented nucleus (*) and patchy cytoplasm. Scale bar, 1 µm.

DISCUSSION

The current study has revealed the nature of a novel disease of a marine decapod crustacean recently found to occur during summer months when high water temperatures prevail. It is well established that crustaceans, like all aquatic animals, are highly sensitive to environmental degradation such as elevated or depressed temperature, reduced oxygen tension and changes in the concentrations of xenobiotics or waste products in the water (see Le Moullac & Haffner, 2000 for a review). Temperature has been found to have both direct and indirect effects on the immune systems of crustaceans resulting in immune suppression and enhanced disease prevalence and severity. In particular, Chisholm & Smith (1994) found that temperature affected the antibacterial activity of the haemocytes of *C. maenas*. They noted that lowest levels of this antibacterial activity coincided with the extremes of environmental temperature in February and August. Similarly, phagocytosis, another important defence mechanism of crustaceans, is temperature dependent. For instance, Wang & Chen (2006) observed that the rate of clearance and phagocytosis of *Photobacterium damsela* by haemocytes of the tiger shrimp, *Penaeus monodon*, was inhibited when these animals were moved from 22 to 34 °C. The respiratory burst and superoxide dismutase activities of the haemocytes were also depressed following this change in water temperature. Changes in temperature will also have a direct effect on the growth and survival of parasites and pathogens, where relatively small changes in environmental temperature in temperate waters, such as those inhabited by shore crabs (*C. maenas*), can cause profound changes in seasonal patterns of disease (Harvell *et al.*, 2002). In the current study, attempts to induce milky disease by temperature elevation with or without challenge with milky disease affected material failed, suggesting that either longer-term exposure is required or that a further seasonal variable (e.g. reduced oxygen tension, high levels of waste products, seasonal hormonal levels) is responsible for the increased prevalence of disease observed in the wild and under aquarium conditions during the summer months.

One of the characteristic changes found in crabs in early stages of milky disease was the rapid decline in the THC. There is no obvious explanation for this reduction, as the blood cells in circulation failed to respond to the presence of the bacteria and the limited damage seen histologically in the hepatopancreas with accompanying haemocyte encapsulation on its own could not account for the reduction. Other possible causes could include elimination by elevated apoptosis or depression of haemopoiesis. As well as the change in THC, there was also significant elevation in the amounts of glucose and ammonia in the blood of milky disease affected crabs. Both environmental stress and infection have been shown to cause a similar elevation in glucose in other crustaceans (e.g. Hall & van Ham, 1998; Yoganandhan *et al.*, 2003). For example, shrimps infected with white spot syndrome virus show

rapid increases in haemolymph glucose and total carbohydrate (Yoganandhan *et al.*, 2003).

There are several reports of 'rickettsia-like' diseases of crustaceans but only in some of these cases has the nature of the causative agent been elucidated. For example, one form of rickettsiosis in the freshwater crayfish *Cherax quadricarinatus* is caused by *Coxiella cheraxi* (Tan & Owens, 2000). Further ultrastructural studies of this disease in *Ch. quadricarinatus* revealed multiplication of the causative agent in the foregut columnar epithelial cells, antennal gland cells, free haemocytes and fixed phagocytes in the hepatopancreas (Romero *et al.*, 2000). In the case of necrotizing hepatopancreatitis in the Pacific white shrimp, *Penaeus vannamei*, originally thought to be a 'rickettsia-like' disease, the causative agent has been shown to be most closely related to *Caedibacter caryophila* and *Holospora obtusa*, both bacterial endosymbionts of protozoans (Loy *et al.*, 1996). This latter disease is characterized by the parasitization of the epithelial cells of the tubules in the hepatopancreas by these bacteria. In the current study, there was no indication of the presence of the milky disease causative agent in these epithelial cells either in thin sections for electron microscopy or in histological sections prepared for *in situ* hybridization. Furthermore, unlike rickettsiosis of *Ch. quadricarinatus*, there was no indication of any infection in the midgut and only very limited interaction with the free haemocytes in circulation. Also, despite the causative agent of milky disease showing some of the key characteristics of rickettsial infections (e.g. intracellular multiplication directly in cells, Gram-negative pleomorphic rod-shape bacterial morphology), it was unlike all of the rickettsial-like diseases identified to date in arthropods (insects and crustaceans) in terms of its identity based on 16S rRNA sequencing (see Fig. 5). The causative agent has limited relatedness to bacteria within the *Rhodobacteriaceae* that include a number of pathogens of marine organisms including *Roseovarius crassostreae*, the probable causative agent of juvenile oyster disease (Boettcher *et al.*, 2005). These forms, however, do not show the characteristic obligative intracellular multiplication and resulting lack of *in vitro* cultivation on agar-based media, as seen in many of the *Rickettsiales* and the presumptive causative agent of milky disease.

The current study has unfortunately been unable to fully demonstrate Koch's postulates in terms of disease transmission. For example, feeding of apparently healthy crabs with hepatopancreas from crabs affected with milky disease failed to induce any of the characteristic symptoms of the disease, such as milky haemolymph with free bacteria and hepatopancreatic parasitization. Intrahaemocoelic injection of healthy crabs with blood from milky disease affected crabs resulted in their rapid (>24 h) mortality but without any of the key symptoms of the disease as seen in natural infections in the wild or under aquarium conditions. The finding that injection of cell-free haemolymph from milky disease affected crabs into normal crabs also caused their rapid death rules out the possibility of a rapid

septicaemia-like infection as the cause of this mortality. More likely, there could be toxic factors, such as bacterial products or waste products from the damaged hepatopancreas, free in the blood that caused such rapid death. Whatever the explanation, the pathology observed following intrahaemocoelic challenge is so radically unlike that seen in natural infections that it is of limited relevance to our understanding of the natural disease. Further long-term experiments involving repeated feeding of material from milky disease affected crabs may be needed to gain a better understanding of how the disease is spread.

The failure to cultivate the bacteria associated with milky disease was also a limiting factor in the elucidation of the nature of the causative agent of the disease. For example, it could be argued that the sequencing of the product obtained after cloning could have resulted from other bacterial contaminants in the haemolymph associated with the disease that were unrelated to the 'true' causative agent. The finding that few other contaminating bacteria could be demonstrated in the haemolymph from milky disease affected crabs probably refutes this as a viable explanation. Furthermore, the bacterial DNA was extracted on several separate occasions from a variety of infected crabs, always with similar results, and all crabs affected by milky disease showed the characteristic 506 bp product on agarose gels following PCR amplification.

The presence of large numbers of milky disease bacteria in the blood of crabs resulted in little sign of any haemocytic responses. Crabs, like invertebrates in general, rely heavily on phagocytosis as a mechanism of pathogen recognition and destruction (Ratcliffe *et al.*, 1985). Of the three types of haemocytes reported (hyaline, semi-granular and granular cells; Bauchau, 1981) the hyaline and semi-granular cells are considered to be actively phagocytic (Bauchau, 1981). The ultrastructural studies in the present work revealed very few examples of uptake of the milky disease bacteria despite their close association. Indeed, few of the circulating haemocytes showed any changes in morphology such as increased pinocytotic or pseudopod formation that might be associated with a response to the bacteria. Our understanding of how microbial pathogens of arthropods resist phagocytosis is limited to a few studies mainly with insects (e.g. Au *et al.*, 2004). Despite the lack of haemocytic response to the milky disease bacteria, damage to the hepatopancreatic tubules, potentially as a result of infection, resulted in the walling off of these tissues by the haemocytes, hence showing that the general ability of these cells to recognize damaged or 'foreign' material is unaltered in milky disease affected animals.

The main site for replication of the bacteria associated with milky disease appears to be the fixed phagocytes found in the hepatopancreas. These cells have previously been characterized in a variety of crustaceans and their phagocytic activity identified (Johnson, 1980; Factor & Naar, 1990; Sagrista & Durfort, 1990). The current study apparently showed early stages during which the bacteria

replicated either within a large vacuole or directly in the cytosol of these cells followed by release into the surrounding area. Ultimately, the remnants of the fixed phagocytes became surrounded by large numbers of bacteria held within the extracellular space bounded by the unusual perforated basement membrane. Presumably, the bacteria escape through these perforations to gain entry to the general haemocoel and to give the characteristic milky colour indicative of this disease. Whether the milky disease bacteria are capable of parasitizing other cells within crabs is unclear, but there was no evidence for their presence in the midgut epithelial cells or the epithelial cells of the hepatopancreatic tubules, unlike the situation in other rickettsia-like infections of crustaceans (Loy *et al.*, 1996; Romero *et al.*, 2000).

Overall, the current study has revealed the nature of this novel disease of the European shore crab, *C. maenas*. However, many questions still remain unanswered, including how the disease is normally transferred in the wild and whether other economically important species of crustaceans, such as the edible crab, *Cancer pagurus*, are also susceptible to infection. The fact that the causative agent cannot be maintained in the absence of susceptible cells, coupled with the lack of available crustacean cell lines, will unfortunately hamper further studies. Whether the causative agent of milky disease can replicate in other established cell lines, such as those from insects or even fish, would be worth investigating.

ACKNOWLEDGEMENTS

This work was supported by Natural Environment Research Council CASE awards to F.E. (NER/S/C/2004/12711) and S.G. (NER/S/C/2004/) and a University of Wales Swansea training bursary to A.P. The assistance of Bob Porter, School of Biological Sciences, University of Bristol, with the TEM studies is also acknowledged.

REFERENCES

- Alborali, L. (2006). Climatic variations related to fish diseases and production. *Vet Res Commun* **30** (Suppl. 1), 93–97.
- Au, C., Dean, P., Reynolds, S. E. & French-Constant, R. H. (2004). Effect of the insect pathogenic bacterium *Photobacterium* on insect phagocytes. *Cell Microbiol* **6**, 89–95.
- Bauchau, A. G. (1981). Crustaceans. In *Invertebrate Blood Cells*, vol. 2, pp. 386–420. Edited by N. A. Ratcliffe & A. F. Rowley. London: Academic Press Inc.
- Bergmeyer, H. V. (1984). *Methods of Enzymatic Analysis. Metabolites I: Carbohydrates*, 3rd edn. Weinheim: Verlag Chemie.
- Boettcher, K. J., Geaghan, K. K., Maloy, A. P. & Barber, B. J. (2005). *Roseovarius crassostreae* sp. nov., a member of the *Roseobacter* clade and the apparent cause of juvenile oyster disease (JOD) in cultured Eastern oysters. *Int J Syst Evol Microbiol* **55**, 1531–1537.
- Bolz, D. F. & Howel, J. A. (1978). *Colorimetric Determination of Non-Metals*. London: Wiley.
- Brown, M. V., Schwalbach, M. S., Hewson, I. & Fuhrman, J. A. (2005). Coupling 16S-ITS rDNA clone libraries and automated ribosomal intergenic spacer analysis to show marine microbial diversity: development and application to a time series. *Environ Microbiol* **7**, 1466–1479.
- Chisholm, J. R. S. & Smith, V. J. (1994). Variation in antibacterial activity in the haemocytes of the shore crab, *Carcinus maenas*, with temperature. *J Mar Biol Assoc UK* **74**, 82–979.
- Factor, J. A. & Naar, M. (1990). The digestive system of the lobster, *Homarus americanus*. II. Terminal hepatic arterioles of the digestive gland. *J Morphol* **206**, 283–291.
- Friedman, C. S., Andree, K. B., Beauchamp, K. A., Morre, J. D., Robbins, T. T., Shields, J. D. & Hedrick, R. P. (2000). ‘*Candidatus Xenohalotus californiensis*’, a newly described pathogen of abalone, *Haliotis* spp., along the west coast of North America. *Int J Syst Evol Microbiol* **50**, 847–855.
- Hall, M. R. & van Ham, E. H. (1998). The effects of different types of stress on blood glucose in the giant tiger prawn *Penaeus monodon*. *J World Aquacult Soc* **29**, 290–299.
- Hari, R. E., Livingstone, D. M., Siber, R., Burkhardt-Holm, P. & Güttinger, H. (2006). Consequences of climatic change for water temperature and brown trout populations in alpine rivers and streams. *Global Change Biol* **12**, 10–26.
- Harvell, C. D., Kim, K., Burkholder, J. M., Colwell, R. R., Epstein, P. R., Grimes, D. J., Hofmann, E. E., Lipp, E. K., Osterhaus, A. D. M. E. & other authors (1999). Emerging marine diseases – climate links and anthropogenic factors. *Science* **285**, 1505–1510.
- Harvell, C. D., Mitchell, C. E., Ward, J. R., Altizer, S., Dobson, A. P., Ostfeld, R. S. & Samuel, M. D. (2002). Climate warming and disease risks for terrestrial and marine biota. *Science* **296**, 2158–2162.
- Hebel, D. K., Jones, M. B. & Depledge, M. H. (1997). Responses of crustaceans to contaminant exposure: a holistic approach. *Estuarine Coastal Shelf Sci* **44**, 177–184.
- Johnson, P. T. (1980). *Histology of the Blue Crab, Callinectes sapidus. A Model for the Decapoda*. New York: Praeger Publishers.
- Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* **16**, 111–120.
- Kumar, S., Tamura, K. & Nei, M. (2004). MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform* **5**, 150–163.
- Lafferty, K. D., Porter, J. W. & Ford, S. E. (2004). Are diseases increasing in the ocean? *Annu Rev Ecol Evol Syst* **35**, 31–54.
- Lane, D. J. (1991). 16S/23S rRNA sequencing. In *Nucleic Acid Techniques in Bacterial Systematics*, pp. 115–175. Edited by E. Stackebrandt & M. Goodfellow. Chichester: Wiley.
- Lau, W. W. Y. & Armhurst, E. V. (2006). Detection of glycolate oxidase gene *glcD* diversity among cultured and environmental marine bacteria. *Environ Microbiol* **8**, 1688–1702.
- Le Moullac, G. & Haffner, P. (2000). Environmental factors affecting immune responses in Crustacea. *Aquaculture* **191**, 121–131.
- Loy, J. K., Dewhirst, F. E., Weber, W., Frelie, P. F., Garbar, T. L., Tasca, S. I. & Templeton, J. W. (1996). Molecular phylogeny and in situ detection of the etiologic agent of necrotizing hepatopancreatitis in shrimp. *Appl Environ Microbiol* **62**, 3439–3445.
- Marchesi, J. R., Sato, T., Weightman, J. I., Martin, T. A., Fry, J. C., Hiom, S. J. & Wade, W. G. (1998). Design and evaluation of useful bacterium specific PCR primers that amplify genes coding for bacterial 16S rRNA. *Appl Environ Microbiol* **64**, 795–799.
- Nunan, L. M., Noble, B., Le Groumelle, M. & Lightner, D. V. (2003). Experimental infection of *Penaeus vannamei* by a rickettsia-like bacterium (RLB) originating from *P. monodon*. *Dis Aquat Organ* **54**, 43–48.

- Poulos, B. T., Mari, J., Bonami, J. R., Redman, R. & Lightner, D. V. (1994).** Use of non-radioactively labelled DNA probes for the detection of baculovirus from *Penaeus monodon* by *in situ* hybridisation on fixed tissues. *J Virol Methods* **49**, 187–194.
- Powell, A. & Rowley, A. F. (2007).** The effect of dietary chitin supplementation on the survival and immune reactivity of the shore crab, *Carcinus maenas*. *Comp Biochem Physiol A Mol Integr Physiol* **147**, 122–128.
- Ratcliffe, N. A., Rowley, A. F., Fitzgerald, S. W. & Rhodes, C. P. (1985).** Invertebrate immunity: basic concepts and recent advances. *Int Rev Cytol* **97**, 184–350.
- Romero, X., Turnbull, J. F. & Jiménez, R. (2000).** Ultrastructure and cytopathology of a rickettsia-like organism causing systemic infection in the redclaw crayfish, *Cherax quadricarinatus* (Crustacea: Decapoda), in Ecuador. *J Invertebr Pathol* **76**, 95–104.
- Sagrista, E. & Durfort, M. (1990).** Ultrastructural study of hemocytes and phagocytes associated with hemolymphatic vessels in the hepatopancreas of *Palaemonetes zariquieyi* (Crustacea, Decapoda). *J Morphol* **206**, 173–180.
- Saitou, N. & Nei, M. (1987).** The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Subasinghe, R. (1997).** Fish health and quarantine. In *Review of the State of World Aquaculture 1997* (FAO Fisheries Circular 886). Rome: FAO.
- Tan, C. K. (1998).** *The characterization of a Rickettsiales isolated from Cherax quadiacarinatus*. PhD Thesis, James Cook University, Australia.
- Tan, C. K. & Owens, L. (2000).** Infectivity, transmission and 16S rRNA sequencing of a rickettsia, *Coxiella cheraxi* sp. nov., from freshwater crayfish *Cherax quadricarinatus*. *Dis Aquat Organ* **41**, 115–122.
- Tops, S., Lockwood, W. & Okamura, B. (2006).** Temperature-driven proliferation of *Tetracapsuloides bryosalmonae* in bryozoan hosts portends salmonid declines. *Dis Aquat Organ* **70**, 227–236.
- Van Handel, E. (1965).** Microseparation of glycogen, sugars and lipids. *Anal Biochem* **11**, 266–271.
- Wang, F.-I. & Chen, J.-C. (2006).** The immune response of tiger shrimp *Penaeus monodon* and its susceptibility to *Photobacterium damsela* subsp. *damsela* under temperature stress. *Aquaculture* **258**, 34–41.
- Wickins, J. F. & Lee, D. O'C. (2002).** *Crustacean Farming: Ranching and Culture*, 2nd edn. Oxford, Blackwell Science.
- Yoganandhan, K., Thirupathi, S. & Sahul Hameed, A. S. (2003).** Biochemical, physiological and haematological changes in white spot syndrome virus-infected shrimp, *Penaeus indicus*. *Aquaculture* **221**, 1–11.

Edited by: D. M. Gordon