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Infection with *Hematodinium* sp. in mud crabs *Scylla serrata* cultured in low salinity water in southern China

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ABSTRACT: Dinoflagellates in the genus *Hematodinium* are important parasites of wild marine crustaceans, but are rarely reported in waters with salinities <11 or from cultured crustaceans. Since 2005, the mud crab *Scylla serrata*, which is cultured along the coast of southeastern China, has suffered from an acute epizootic locally known as 'milky disease'. The disease mainly occurrs from September to November. The clinical signs are largely similar to those of crabs suffering from bitter crab disease (BCD) or pink crab disease (PCD), which are caused by parasites of the genus *Hematodinium*. To determine whether *Hematodinium* sp. is a pathogen of milky disease, histopathological examinations of mud crab haemolymph, hepatopancreas, heart and gill were conducted. In addition, previously reported *Hematodinium* molecular probes were applied to infected material. The results indicate that *Hematodinium* sp. is at least one of the main pathogens of milky disease. The salinity in *S. serrata* culture ponds was <9. To our knowledge, this is the first report showing the *Hematodinium* infection in a cultured crustacean in low salinity water.

KEY WORDS: *Scylla serrata* · Milky disease · *Hematodinium* sp. · Histopathology · Polymerase chain reaction · PCR

INTRODUCTION

Among dinoflagellates of the genus *Hematodinium* are important parasites of wild marine crustaceans (Stentiford & Shields 2005). However, due to the lack of distinctive characteristics and the poorly understood life history of the species in this genus, only 2 have been morphologically described. One is *H. perezi*, which was first described from the portunid crabs *Liocarcinus depurator* and *Carcinus maenas* along the French coast (Chatton & Poisson, 1931). A second species, *H. australis*, was described from the sand crab *Portunus pelagicus* in Australia (Hudson & Shields 1994).

Hematodinium is systemically distributed in infected crustaceans. Infection by the disease produces a series of observable negative effects, such as an opaquely discolored carapace, unpalatable flavor, change of meat appearance, and high mortality. These observations strongly suggest a large economic loss to the commercial fishery (Meyers et al. 1987, Taylor & Khan 1995). The nature and extent of *Hematodinium* epizootics can be affected by host size and sex, seasonality, and environmental factors such as ambient salinity (Stentiford & Shields 2005). Available data indicate that *Hematodinium* infections rarely occur below a salinity of 11 (Newman & Johnson 1975, Messick & Sinderman 1992, Messick & Shields 2000) or in cultured crabs.

The mud crab *Scylla serrata* is one of the most valuable shellfish species and the largest crab fishery in China. It is widely cultured in brackish and seawater ponds along the coast of southeastern China. In 2004, the total culture area was about 35 000 ha, and the yield was around 10 000 t. The city of Shantou in Guangdong province, southern China, is one of the main mud crab culture areas with about 7000 ha. Since 2005, cultured mud crabs in this area have suffered from an epizootic locally known as 'milky disease'. The salinity in the mud crab culture ponds is <9 throughout

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the year. The disease breaks out mainly in the fall, from September to November, when the crab is near maturity, and the high mortality (usually >60%) has resulted in large economic losses. Clinical signs of the disease are largely similar to those in *Chinoecetes bairdi*, *Cancer pagurus* and *C. opilio* suffering from a disease named bitter crab disease (BCD) or pink crab disease (PCD), which is caused by parasitic dinoflagellates of the genus *Hematodinium* (Meyers et al. 1987, Taylor & Khan 1995, Stentiford et al. 2002). Despite the low salinity in the culture ponds, *Hematodinium* has been the suspected pathological agent based on the presence of milky crab hemolymph. As a result, histopathological examinations of crab haemolymph, hepatopancreas, heart and gill were conducted. In addition, *Hematodinium*-specific gene primers were applied. Diagnosis and potential treatment of the disease is critical for disease control, which will enhance development of the *Scylla serrata* fishery.

MATERIALS AND METHODS

Sampling and histopathological observation. From September to November 2006 and 2007, a total of 12 batches including 48 healthy and 121 diseased *Scylla serrata* individuals were collected from the 2000 ha Niutianyang culture area in Shantou, Guangdong province, southern China. Upon their arrival at the laboratory, size, weight, sex and any clinical signs were recorded.

After the crabs were anaesthetised by chilling on ice, haemolymph was collected at the axillae of the 5th swimmer leg with a 1.0 ml syringe, and 2 kinds of smears were prepared for each crab. One was immediately examined under a light microscope. The other was stained with hematoxylin and eosin (H & E) after fixation with Bouin's solution, or without fixation.

Tissues of hepatopancreas, heart and gill were fixed in Bouin's solution (4°C) for 12 to 24 h, dehydrated in a graded ethanol series and embedded in paraffin. Sections (4 to 6 µm thick) were excised and stained with H&E, examined under a light microscope (Zeiss Axioplan 2), and representative digital images were captured.

Salinities in the crab culture area were monitored at 6 locations from April to December 2007 with a DREL 2800 (Hach) water quality analysis system. Salinity ranged from 4.41 ± 0.17 (mean ± SE) to 8.24 ± 0.28 (n = 6) throughout the year (Fig. 1).

Molecular identification of *Hematodinium*. Genomic DNA was extracted from haemolymph, hepatopancreas and chela muscles, and purified with the Protocol-BS473 Classic Genomic DNA Isolation Kit (Bio Basic) as described by Stentiford et al. (2002). DNA concentrations were measured with a UV–2501 PC spectrophotometer (Shimadzu) and the values were 0.11 to 0.13 µg µl^{-1}.

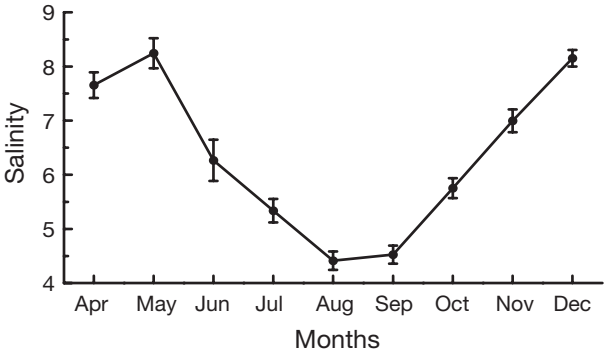

Fig. 1. *Scylla serrata* infected with *Hematodinium*-like parasites. Monthly salinity changes in culture ponds. Means ± SE (n = 6)

Using the isolated DNA as template, a polymerase chain reaction (PCR) was conducted with reported *Hematodinium* primers (Hemat-F-1487 and Hemat-R-1654) as described by Gruebl et al. (2002). Primer concentration was 10 µM. PCR reactions were started with an initial denaturation at 94°C for 5 min, followed by 40 cycles at 94°C for 30 s, 56°C for 30 s and 72°C for 45 s, and with a final extension step at 72°C for 10 min in a total volume of 25 µl containing 1 µl of each primer, 2 µl of template (0.22 to 0.26 µg) and 12.5 µl PCR Master-Mix (TianGen). The PCR products were separated by electrophoresis on a 1.5% ethidium bromide-stained agarose gel and viewed under a UV light source. The expected length of the PCR product was 187 bp (Gruebl et al., 2002). Crabs (n = 5 to 8) were examined for each tissue.

RESULTS

Clinical signs

Scylla serrata suffering from severe milky disease exhibited some gross symptoms such as moribund behavior, death shortly after capture or during transit, a cooked appearance and milky body fluid. The haemolymph lacked clotting ability and chela muscle became liquified. These clinical signs were largely similar to those of crabs suffering from BCD or PCD caused by *Hematodinium* infection (Meyers et al. 1987, Taylor & Khan 1995, Stentiford et al. 2002).

Histopathological changes

In heavily infected individuals, the hemocyte count in haemolymph decreased sharply. Under light micro-

scopy, parasites of various shapes or putative developmental stages were observed in fresh hemolymph smears. Parasites were observed as single round cells, sporonts, dinospores or round trophonts with many refractile granules (Fig. 2a–d), as observed in *Callinectes sapidus* (Shields & Squyars 2000, Stentiford & Shields 2005). In addition, we found uninucleate (Fig. 2e) and multinucleate or dividing parasites (Fig. 2f) as observed in *Hematodinium*-infected *C. sapidus* (Messick 1994).

The hepatopancreas of healthy crabs possessed robust hepatopancreatic tubules as demonstrated by the presence of active R- and B-cells (Johnson 1980) and spongy connective tissues between tubules (Fig. 3a). In advanced milky disease crabs, spongy connective tissues were greatly diminished and became obscured by the proliferating parasite. The epithelium of hepatopancreatic tubules became atrophied, revealing a dilated lumen (Fig. 3b). In contrast to the hearts of healthy crabs (Fig. 3c), numerous parasites were observed in those of heavily infected individuals. The myocardium of heavily infected crabs underwent coagulative necrosis (Fig. 3d). The gill structure of healthy crabs appeared as described by Johnson (1980) (Fig. 3e). In heavily diseased crabs, the respiratory epithelia were greatly diminished as the lamellae became engorged by the proliferating parasite (Fig. 3f).

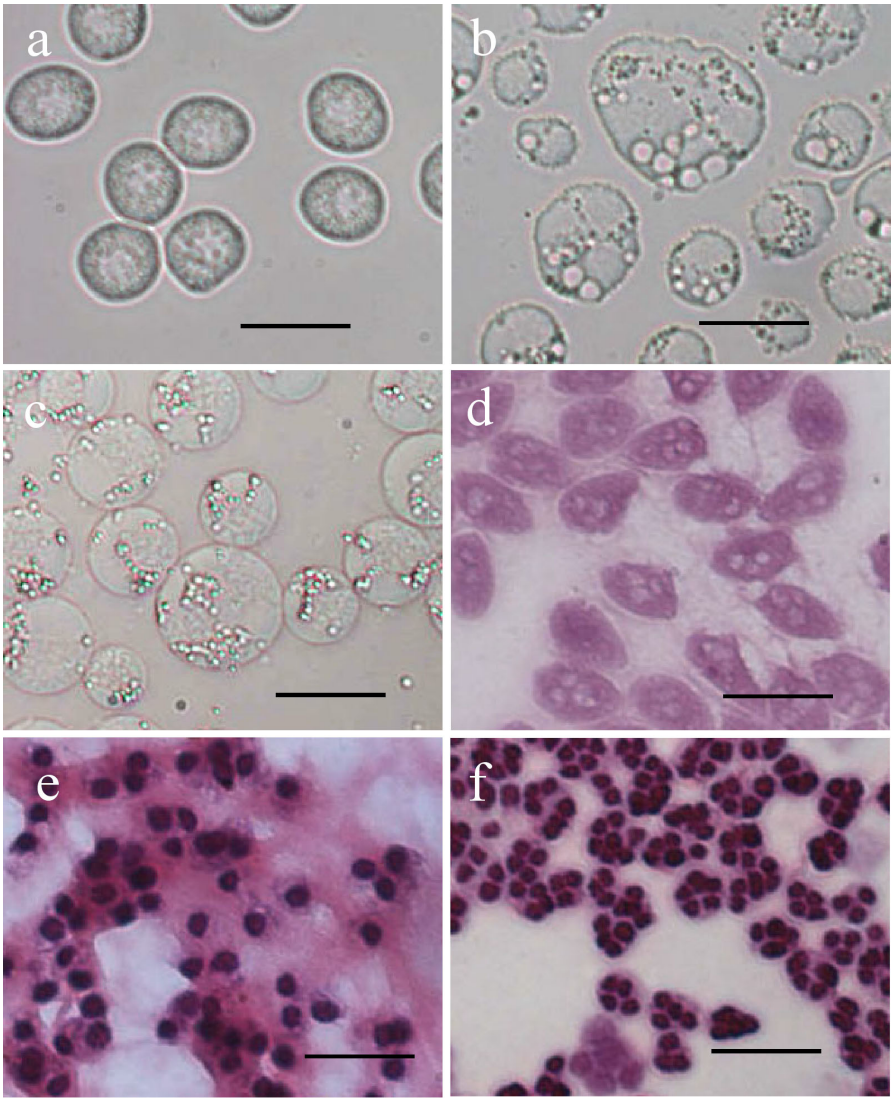

Fig. 2. *Scylla serrata* infected with *Hematodinium*-like parasites. Light micrographs of haemolymph from heavily infected mud crabs showing different shapes or developmental stages of the parasites. (a) Round cells; (b) sporonts; (c) round trophonts with many refractile granules; (d) dinospores; (e) uninucleate parasites; (f) multinucleate or dividing parasites. (a–c) Direct observation of fresh smears; (d) smear stained with hematoxylin and eosin without fixation; (e, f) smears stained with hematoxylin and eosin after fixation with Bouin's solution. Scale bars = 20 µm

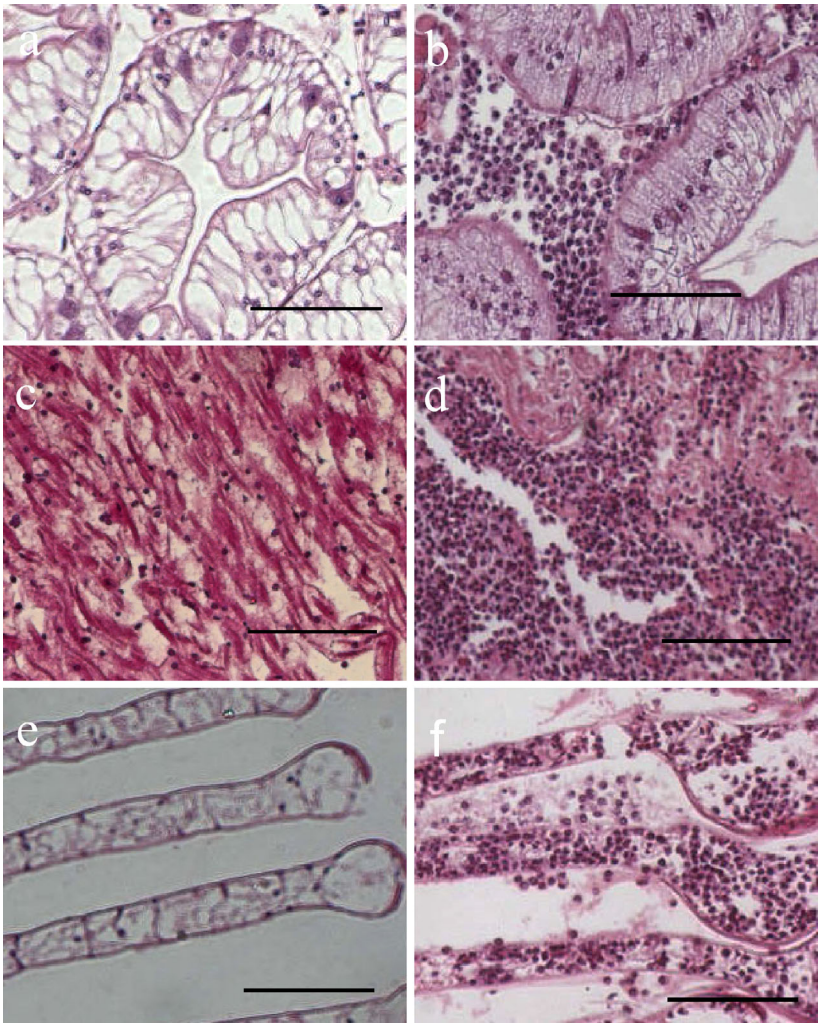

Fig. 3. *Scylla serrata.* Light micrographs of (a,b) hepatopancreas, (c,d) heart, and (e,f) gill. Compared with the tissue structure of healthy crabs (a,c,e), tissue from heavily infected individuals (b,d,f) is severely damaged and filled with *Hematodinium*-like parasites. Scale bars = 100 µm

Molecular evidence of *Hematodinium* in milky diseased crabs

In the PCR amplification with reported *Hematodinium* primers (Gruebl et al. 2002), an expected band of about 187 bp was obtained from all the genomic DNA samples of hepatopancreas, muscle and haemolymph of *Scylla serrata* with milky disease. While diagnostic bands were absent from hepatopancreas of healthy individuals (Fig. 4).

DISCUSSION

Parasitic dinoflagellates in the genus *Hematodinium* have gained attention as pathogens of commercially important crustaceans. Infections have been described from several wild marine crustacean hosts in Europe, USA and Australia (see 'Introduction'), but have seldom been reported in cultured crustaceans. Recently, *Hematodinium* infections have been reported in cul-

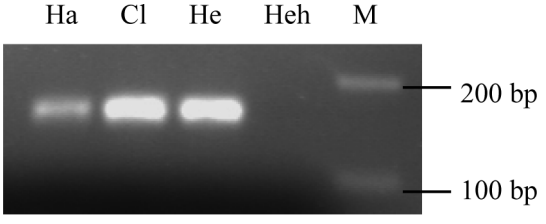

Fig. 4. *Hematodinium*-like diagnostic bands detected by PCR in haemolymph (Ha), claw muscle (Cl) and hepatopancreas (He) from infected mud crab *Scylla serrata*. Diagnostic bands for the parasite are not in hepatopancreas (Heh) from a healthy individual. M: DNA marker

tured *Portunus trituberculatus* (Xu et al. 2006, 2007b) and *Scylla serrata* (Xu et al. 2007a) in eastern China. The present study showed that the clinical signs and histopathological changes in haemolymph, hepatopancreas, heart and gill in *S. serrata* with milky disease were similar not only to those observed in crustaceans suffering from BCD or PCD, which were infected by *Hematodinium* (Meyers et al. 1987, Taylor & Khan 1995, Stentiford et al. 2002), but also to those in *P. trituberculatus* and *S. serrata* (Xu et al. 2006, 2007a,b). The results suggest that *Hematodinium* sp. is at least one of the main pathogens of milky disease. This was further supported at the molecular level by the expected amplification of the PCR band with the *Hematodinium* primer set (Gruebl et al. 2002) in the tissues of diseased crabs but not in healthy individuals (Fig. 4). Comparing the reports of Xu et al (2006, 2007a,b) with results of the present study, it is apparent that the *Hematodinium* infection synchronously broke out in cultured crabs grown in both eastern and southern China. These are the first reports of a *Hematodinium* infection in crustaceans in China.

Several factors, including host factors (size or maturity status, age, sex, moult condition), seasonality and environmental factors (temperature, salinity), may affect the epidemiology of *Hematodinium* (Stentiford & Shields 2005). What should be noted is that the available data show *Hematodinium* to be most prevalent in crabs from high salinity waters (Messick & Shields 2000, Stentiford & Shields 2005, Xu et al. 2006, 2007a,b); it is rarely reported below 11 salinity (Newman & Johnson 1975, Messick & Sinderman 1992, Messick & Shields 2000). In the present study, however, *Hematodinium*-infection broke out in *Scylla serrata* cultured at salinities below 9. The characteristics of the parasite and the infection mechanism need to be further clarified.

The natural or *in vivo* life cycle of *Hematodinium* parasites is not fully understood. However, the parasite (isolated from *Nephrops norvegicus*) has been grown in culture (Appleton & Vickerman 1998), and partial life cycles of parasitic dinoflagellates from the host species *Chionoecetes bairdi* and *C. sapidus* are known (Meyers et al. 1987, Shields & Squyars 2000). Furthermore, different life cycle stages of *Hematodinium* parasites from *C. sapidus*, *C. opilio* and *N. norvegicus* have been reported (Messick 1994, Taylor & Khan 1995, Appleton & Vickerman 1998, Shields & Squyars 2000, Stentiford & Shields 2005). In this study, different developmental stages of parasites were observed in the haemolymph smears while only one type of parasite was found in histological sections of *Scylla serrata* with milky disease. Similar results have also been reported in other *Hematodinium*-infected crustaceans, which suggests that future studies need to resolve the differences between life history stages of *in vitro* cultures or in haemolymph and those observed in histological sections (Stentiford & Shields 2005).

The present study revealed severe histopathological changes in the hepatopancreas, gills and heart of *Scylla serrata* with milky disease, similar to reports from some other *Hematodinium*-infected crustaceans (Meyers et al. 1987, Field et al. 1992, Hudson & Shields 1994, Messick 1994, Stentiford et al. 2002, Sheppard et al. 2003, Wheeler et al. 2007). In *Hematodinium* infections, parasitic congestion and disruption of the gills, heart and other tissues lead to respiratory dysfunction or decrease of metabolic function and finally to host death. This may account for the high mortality of mud crabs with milky disease.

A range of methods, such as the observation of clinical signs, microscopic examination, indirect immunofluorescent antibody technique (IFAT), PCR- and ELISA-based diagnostic methods have been developed for the detection and assessment of *Hematodinium* infections in crustaceans. An external assessment of the opaquely discolored carapace proved to be a simple and rapid field diagnostic tool for *Hematodinium* infection of heavily infected *Scylla serrata*, but was not useful for detecting low-level infections, as reported for some other crabs (Meyers et al. 1987, Field et al. 1992, Shields & Squyars 2000, Stentiford et al. 2001, 2002, Pestal et al. 2003). Microscopic determination of haemolymph smears is a reliable, cost-effective and permanent method for the diagnosis of *Hematodinium* infection in crabs (Stentiford & Shields, 2005). Similarly, histopathological examination and PCR-based molecular diagnostic techniques are applicable to *Hematodinium* infection in *S. serrata* as in other crabs (Field et al. 1992, 1998, Field & Appleton 1995, Dawe 2002, Gruebl et al. 2002, Stentiford et al. 2002, Pestal et al. 2003, Sheppard et al. 2003, Shields et al. 2005).

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