

NOTE

Rickettsia-like organism associated with tremor disease and mortality of the Chinese mitten crab *Eriocheir sinensis*

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ABSTRACT: We report the discovery of a rickettsia-like organism (RLO) in cultured freshwater Chinese mitten crab *Eriocheir sinensis*. The RLO caused tremor disease and was apparently responsible for a mass mortality (30 to 90%) in 2 provinces in southeast China. Moribund crabs were investigated from different districts during outbreaks in 1999 and 2000. With electron microscopy, 3 different pathogens were detected in moribund crabs: a rickettsia-like organism (RLO), virus-like particles (VLP) and a microsporidian-like protozoan (MLP). Based on the high prevalence, infection intensity and cytopathological signs, the RLO was considered to be the probable cause of the high mortality. Both VLP and MLP occurred at low prevalences and were considered secondary infections. The RLO was 0.22 to 0.35 μm in diameter, granular or clavate, bounded by a cell wall and membrane, and possessed no nucleus but a nucleoid was present. When dividing, RLOs occurred in irregular shapes, such as dumbbells, awls, and crescents. The RLOs exhibited a predilection for muscle and connective tissues and were probably transported to various tissues and organs by hemocytes.

KEY WORDS: *Eriocheir sinensis* · Rickettsia-like organism · RLO · Ultrastructure · Pathology · Tremor disease · Epizootic

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Pathogenic diseases caused by rickettsia-like organisms (RLOs) are rare in marine crabs, and have only been reported from *Carcinus mediterraneus* (Bonami & Pappalardo 1980), *Paralithodes platypus* (Johnson 1984) and *lithodes aequispina* (Meyers et al. 1990). Although observed in freshwater amphipoda (Frederici et al. 1974) and redclaw freshwater crayfish (Owen et al. 1992), none have been reported from freshwater crabs. We report pathogenic infections of RLOs in the freshwater Chinese mitten crab *Eriocheir sinensis* causing mortality (about 30 to 90%) and heavy losses of cultured crabs in recent years in southeast China. The disease first appeared in 1994 and spread quickly

in Jiangsu, Anhui, and Zhejiang provinces in southeast China. By 1998, the disease had spread to most aquaculture facilities of mitten crabs in China. Random samplings in Anhui Province in 1998 revealed that prevalence was 34.3% (Wei 1999). Seasonal occurrence of disease was correlated with high water temperatures (19 to 28°C) between July and August. Infected crabs were typically lethargic with loss of appetite and paroxysmal tremors of the pereopod, hence the term 'tremor disease' given by the local fishermen. Infected crabs exhibited signs of weakness, anorexia, paroxysmal intense tremors and death in succession. During epizootics, the disease spread quickly, was difficult to control, and caused heavy losses to the industry. The cytopathology, ultrastructure and basic morphology of this RLO are described in this article.

Materials and methods. From May to September 1999, diseased crabs, weighing 30 to 90 g, with typical 'tremor disease' signs were taken from 6 affected ponds ($\text{O}_2 = 5$ to 7% and 19 to 28°C) in the Sihong and Hongze districts, northern Jiangsu Province, China. Healthy crabs were taken from 2 unaffected ponds ($\text{O}_2 = 7$ to 8% and 20 to 28°C) in Suzhou, southeastern Jiangsu Province. During the same period in 2000, samples were again taken from the same 8 ponds (water temperature and dissolved oxygen were roughly the same as in 1999). At the same time, diseased crabs were sampled from other districts: Luhe, Yixin and Gehu in Jiangsu Province, and Tianchang in Anhui Province. Crabs with signs of weakness or obvious tremor were dissected and sampled.

Blood smears of every crab were collected for Giemsa staining before dissecting. Impression smears of hearts, gills, hepatopancreas, pereopod muscles, thoracic ganglion and gonads from diseased and healthy crabs were also stained with Giemsa. The smears were fixed on film with 10% methanol, air-dried then, placed directly in a glass Coplin jar con-

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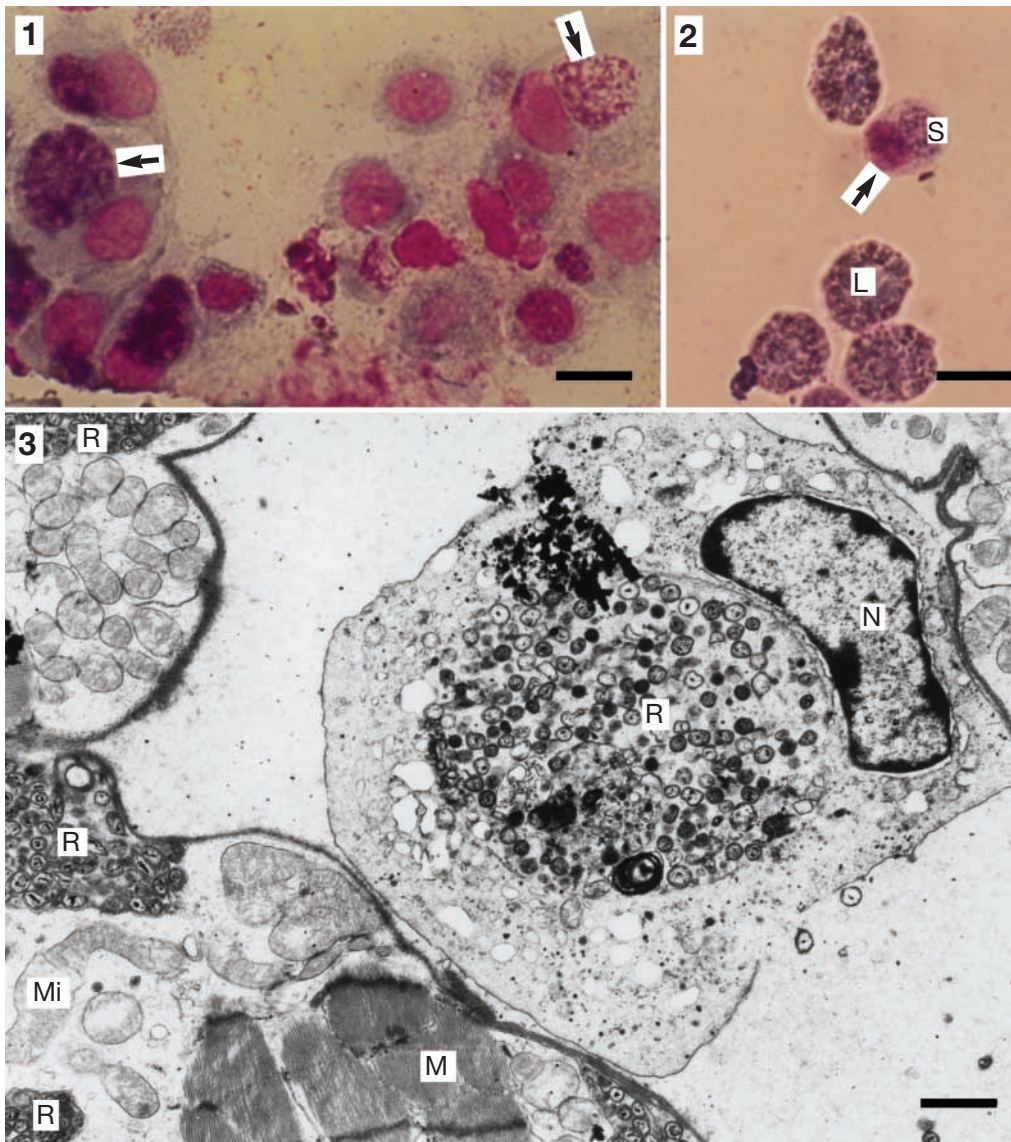
taining Giemsa stain diluted 1:10 with pH 6.8 phosphate buffer for 45 min. The Giemsa stain was prepared using 5 ml of Giemsa stock solution (Giemsa powder 0.7 g, glycerol 50 ml and methanol 50 ml) mixed with 100 ml phosphate buffer pH 6.8 and 5 ml methanol (Du 1998). This method was also used for semi-thin section staining but modified by elongating staining time to 1.5 h and increasing the temperature to 60°C. Inclusion bodies were identified in the smears of diseased crabs using an Olympus BH-2 microscope.

For light and electron microscopies, 1 to 2 mm³ portions of gill, guts, hepatopancreas, cardiac muscle, pereopod muscle, thoracic ganglion and gonad were fixed in 4% glutaraldehyde in phosphate buffer for 4 h at 4°C, washed for 2 h at 4°C in the same buffer, then postfixed in buffered 2% osmium tetroxide for 2 h at 4°C. The tissues were dehydrated through a graded

series of acetone and embedded in Epon 812. Semithin sections for light microscopy (LM) were stained with 1% methylene blue and Giemsa stain respectively. Ultrathin sections were double stained with uranyl acetate and lead citrate, and observed with a Hitachi H-600 transmission electron microscope (TEM).

Results. All of the 46 diseased crabs from affected ponds were infected varyingly by the RLO. One crab was infected by virus-like particles (VLP), and 6 were infected by a microsporidian-like protozoan (MLP). No parasite or disease agent was detected in 23 crabs examined from the 2 unaffected ponds.

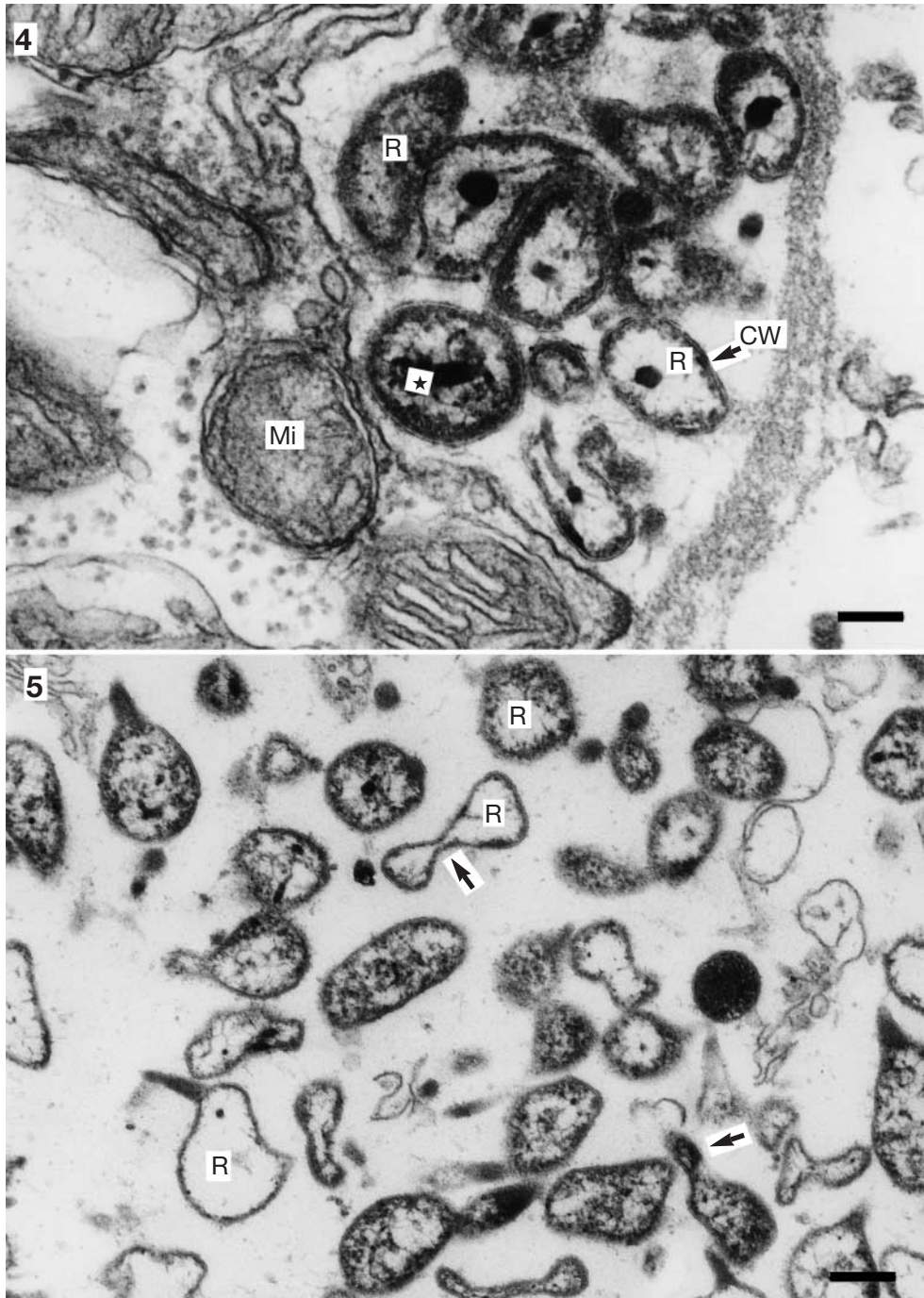
From blood smears and tissue impression smears, inclusions that stained pink to bluish-purple with Giemsa were observed in the cytoplasm of small granular hemocytes (Fig. 2) and connective tissue (Fig. 1) of infected crabs. The percentage of the cell with inclu-



Figs. 1 to 3. *Eriocheir sinensis*. Fig. 1. Light micrograph of impression smears from the thoracic ganglion of an infected crab with a typical tremor. Rickettsia-like organism (RLO) colonies in the cytoplasm of connective cells stained pink to bluish-purple with Giemsa stain (arrows). Scale bar = 10 µm. Fig. 2. Light micrograph of a blood smear from infected crab stained with Giemsa. An RLO colony showing a pink color with Giemsa stain (arrow) can be seen inside a small granular hemocyte (S) from the hemolymph. The cells around it are large granular hemocytes (L). Scale bar = 10 µm. Fig. 3. TEM micrograph of cardiac muscle tissue from an infected crab, showing large numbers of RLOs (R) within vacuoles of hemocytes and in muscle cells (M). The nucleus (N) of the host hemocyte is laterally compressed. (Mi) indicates mitochondria in the muscle cell. Scale bar = 1.5 µm

sions was about 20 to 40% in the smears from the crabs with signs of weakness and 50 to 80% in smears from crabs with signs of obvious tremor. There was a small difference in the color of inclusions in smears of hemocytes and impression smears of tissues. The former were pink while the latter were more bluish-purple.

Observations by TEM revealed that the Giemsa-positive inclusions were microcolonies of a RLO within membrane-bounded vacuoles (Fig. 3). The RLO had a prokaryote character, with no nucleus but contained a nucleoid (Fig. 4). The internal structure had an electron-dense center, with filiform structures around it.



Figs. 4 & 5. *Eriocheir sinensis*. Fig. 4. TEM micrograph of pereopod muscle from an infected crab at high magnification showing the fine structure of RLO (R), the cell wall (CW) and the nuclear area (★). Mi: mitochondria. Scale bar = 170 nm. Fig. 5. TEM micrograph of a hemocyte from an infected crab showing electron-lucent RLOs (R) with irregular shapes, budding and binary fission (arrows). Scale bar = 294 nm

The RLOs varied in size between 0.22 and 0.35 μm in diameter when in rounded form and $0.2 \times 0.9 \mu\text{m}$ when bacilliform. Without exception, the RLOs were located in muscle and connective tissues within the gonad, the pereopods, midgut, hepatopancreas nerves, heart (Fig. 3), and gills of moribund crabs.

Two forms of RLO were often detected in the hemocytes. One was electron-dense and spherical-shaped (Fig. 4); the other was electron-lucent irregular-shaped with budding and binary fission (Fig. 5). Many RLOs were contained in membrane-bound vacuoles in hemocytes (Fig. 3). When dividing, RLOs were irregular in shape, resembling dumbbells, awls, or crescents (Fig. 5). When mature, they were apparently released by the rupture of the host cell.

In all diseased crabs, RLO colonies were observed inside hemocytes (Fig. 2). Giemsa staining showed that the percentage of hemocytes that possessed RLO inclusions increased with the severity of disease. Ultrastructure revealed that RLO were mainly distributed in the connective tissues of heart and guts from crabs with signs of weakness, and could be detected additionally in nerves and muscles from crabs with signs of obvious tremor. RLOs were likely carried to other tissues and organs by infected hemocytes.

In the body muscle tissues from crabs with tremor, the occurrence of RLO was similar to that in cardiac and gut muscle cells. Most RLOs were in the connective tissue capsule, the cytoplasm of pereopod muscle cells, and in connections between nerves and muscle cells (myoneural junctions). In muscle cells, RLOs were dispersed in twos or threes in the sarcoplasmic reticulum. In the digestive system, RLOs were mainly present in the connective tissues underlying the epithelial cells and muscular layer. RLOs were also present in neurogliocytes of the thoracic ganglion.

VLPs were found in the gill epithelium of 1 moribund crab also infected by RLOs. The crab was collected from the Sihong district. VLPs were not detected in other tissues of the crab or in other moribund crabs. The VLP was spherical, 100 nm in diameter, and could form an inclusion body in the cytoplasm. An MLP was found only in the hepatopancreas of 6 moribund crabs that were also infected by RLOs. All the microsporidian-infected crabs came from only 1 pond in Sihong district and they were found in both years of sampling. Certain characteristics, such as the lack of a parasitophorous vacuole, the lack of distinct numbers of spores occurring in spore packets, and the small size (1.0 to 1.2 μm), suggested that the microsporidian might be a member of the genus *Ameson*.

Discussion. The etiological agent of 'tremor disease' in *Eriocheir sinensis* appeared to be a RLO that was always present in diseased crabs. By contrast, VLPs and an MLP were found only occasionally and were

not considered to be involved in the etiology of the disease because of their low prevalences and sites of infection. We consider the VLP and MLP as secondary invaders that infected crabs weakened by infection with RLOs.

RLOs were observed systemically in *Eriocheir sinensis* and the resulting histopathology might explain the gross signs of the disease. RLO infection of the musculature and connective tissue of pereopods, heart and guts could have been the cause of weakness and anorexia. RLOs in the thoracic ganglion (Fig. 1) and myoneural junctions could have affected nerve transmission leading to paroxysmal tremor of the pereopods.

There are few reports of pathogenic RLOs causing serious diseases in crustaceans (Vago et al. 1970, Frederici et al. 1974, Lightner et al. 1985, Brock et al. 1986, Owens et al. 1992), and even fewer have been reported from crabs. Mass mortality of shrimp has been associated with RLOs (Krol et al. 1991, Lightner et al. 1992, Loy & Frelier 1996), and infections in blue king crabs *Paralithodes platypus* (Johnson 1984, Meyers et al. 1990), golden king crabs *Lithodes aequispina* (Meyers et al. 1990), and green shore crabs *Carcinus mediterraneus* (Bonami & Pappalardo 1980) were thought to be fatal. Prevalences from aquaculture systems have rarely been reported. In blue crabs *Callinectes sapidus*, the prevalence of RLO was 2.3% in a Maryland shedding facility, but heavy infections were not fatal (Messick & Kennedy 1990), and were associated with little pathology (Messick 1998). However, the infection in the Chinese mitten crab is significant due to the high prevalence in pond systems and the rapid mortality associated with the disease. These conditions are similar to those reported for epizootics in shrimp aquaculture systems (Frelier et al. 1993).

The morphological descriptions of RLOs in crustaceans are varied. As in tremor disease, they mostly infect connective tissues and hemolymph. Unlike the RLO reported by Johnson (1984), Bower et al. (1996), Messick (1998), Brett & Prior (1999) and Drobne et al. (1999), we did not find RLOs in the epithelium of the hepatopancreas although they were found in the underlying connective tissues. Our cytopathological study revealed that RLOs only infected hemocytes, muscle, and connective tissues but not epithelial cells. Like the RLO in redclaw crayfish (Romero et al. 2000), we also found RLO colonies inside small granular hemocytes of the hemolymph (Fig. 2).

The morphology of RLOs in the mitten crab had typical characteristics of rickettsias. Characteristics such as transverse binary division in cytophagous vacuolus (Fig. 5), formation of inclusion bodies (Figs. 1 to 3) or single appearance in the cytoplasm, and inhibition of phagosome-lysosome fusion, suggested that this RLO may be a member of the genus *Ehrlichia* (Wen 1999).

However, further studies on immunology and molecular biology (Tan & Owens 2000) will be needed to determine its relationship to other RLOs.

Although the RLOs had electron-dense and electron-lucent forms (Fig. 5), they differed from those of Chlamydia-like organisms (CLOs). The condensed forms and elementary bodies of CLOs are usually devoid of organelles except the nuclear area (Sparks et al. 1985), whereas the electron-dense forms of our RLO were ribosome-rich in the periphery of the body. In addition, the reticulate (initial), intermediate and ellipsoidal bodies of CLOs are less densely packed, contain more cytoplasmic organelles and are larger than the elementary bodies (Sparks et al. 1985). By contrast, the electron-lucent forms of our RLOs lacked ribosomes and they were similar in size to the electron-dense forms, not 6 times larger. Another difference between CLOs and RLOs is that CLOs are commonly spherical or oval and never bacilliform as some of the RLOs described here.

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