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Morphology and Haemolymph Composition Changes in Red Sternum Mud Crab (*Scylla serrata*)

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ABSTRACT

Characteristic differences between the abnormal, red sternum mud crab compared to those of normal one were clearly seen. A red sternum mud crab had soft carapace, red chelae and joint, pale hepatopancreas and gill, loose muscle, while its haemolymph contained milky unclotted substance. SDS-PAGE analysis of protein in haemolymph showed an intense band of oxyhemocyanin (~75 kDa) in normal crab, but none in red sternum crab as also confirmed by the absence of 340 nm absorbance of oxyhemocyanin. As for trace elements, the content of copper and zinc in haemolymph was found to be three and four times higher than those in normal crab but calcium, magnesium and iron were higher in red sternum crab. These results indicated that red sternum mud crab had lost oxyhemocyanin and some important elements in haemolymph which were necessary to maintain the normal morphological features of this mud crab.

Key words: mud crab, red sternum, oxyhemocyanin, haemolymph, trace elements, morphology

INTRODUCTION

Mud crab (*Scylla serrata*), an economic aquatic animal, has been well-accepted as high quality food both locally and internationally because of their tasty meat and nutritious value. Hence, the demand for this aquatic animal increases accordingly, especially a soft shell crab which has higher price than the regular one and more subsequent establishments of several soft-shell crab farms. Changes of enzyme activities, epidermal components and trace elements during molting stages of mud crabs were investigated to understand their molting behavior (Salaenoi, 2004; Salaenoi *et al*., 2004). Collecting a large number of mud crab from soft-shell crab farms, we have up to 10% of abnormal crabs with red sternum among the normal population have been found. The high number of these red sternum crab results in the low productivity of mud crab, while the cause of it is still unknown. There are several investigations on the disease of other crustacea having the outer appearances similar to the red sternum crab. They indicated the possible cause of the disease which might may be a parasitic dinoflagellate, *Hematodinium* sp. (Stentiford *et al*., 2002; Pestal *et al*., 2003). These symptoms were collectively called "pink crab disease" (PCD) since the haemolymph and muscle of these affected crabs assumed pink coloration, while the

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meat was irregular in texture. The similar features have been ascribed to *Hematodinium* infections in tanner crabs (*Chionoecetes bairdi* and *C. opilio*), where the term "bitter crab disease" (BCD) was called due to its bitter taste when cooked (Meyers *et al*., 1987). Aside from the outer appearance of these symptoms, there is no report on the changes in composition of trace elements in these sick animals. Some trace metals, i.e., copper, zinc, manganese, magnesium and iron are essential elements that play various physiological roles. The lack or excess of these elements in animals cause a stress that could be manifested in sublethal responses or in causing death (Soegianto *et al*., 1999). Hemocyanin was reported to be a major protein in the haemolymph of normal crab (Terwilliger *et al*., 1999). The change of blood color and characteristic as seen in red sternum crab is possible due to the hemocyanin composition. However, there is no evidence to conclude that red sternum syndrome seen in mud crab is similar in all aspects to bitter crab disease or to pink crab disease. Hence, the study on changes in biochemical components of haemolymph of these red sternum crabs which contributed to the symptoms could tell more about their conditions and how to prevent the disease occurring in crab farming.

MATERIALS AND METHODS

Animal preparation

Red sternum mud crabs, *Scylla serrata* and the normal ones were collected from a soft-shell crab farm in Klung District, Chanthaburi Province. The animals were transferred to the laboratory and kept in an individual aquarium containing 24 ppt salinity seawater. Observations on their morphology were recorded. Prior to experimentation, the animals were anaesthetized in cold water at 4 °C for 1 min. Haemolymph samples were withdrawn from the sinus at the base of the pereiopods and 10% tri-sodium citrate was used as anti-coagulant at the ratio of 5:1. All tissue samples were kept in ice. Haemolymph samples were kept at -20 ^{o}C for further analysis.

SDS-PAGE

The diluted blood samples of red sternum and normal mud crab were mixed with loading buffer (200 mM Tris-HCl pH 6.8, 4% (w/v) SDS, 25% glycerol, 0.04 %(w/v) bromphenol blue, 12% b-mercaptoethanol) at the ratio of 1:2, then boiled for 5 min and subjected to 12.5% SDS-PAGE. The electrophoresis was performed at constant voltage of 100V at room temperature. The protein bands were stained with Coomassie Brilliant Blue R-250.

Oxyhemocyanin absorbance

The diluted blood samples of both red sternum and normal mud crab were measured for their absorbances at the wavelength range of 310-700 nm using UV-visible spectrophotometer (Jasco V550) and subtracted with baseline spectrum. The scanning rate was 100 nm/min, having bandwidth of 1 nm.

Determination of trace elements

Trace elements (Mg, Fe, Cl, Cu, Mn and Zn) were determined using the method of AOAC (1980). The analysis was done by wet ashing, acid hydrolysis under vacuum condition. Approximately 0.2 g of tissue sample was dissolved in 10 ml of acid mixture (conc. HNO_3, conc. H_2SO_4 and conc. $HClO_4$ at the ratio of 5:1:2) in a 75 ml test tube. The sample tube was set in a digesting apparatus under a fume hood and heated at 180-200 °C until the clear solution appeared. After cooling, the solution was diluted with deionized distilled water to make a total volume of 50 ml. It was thoroughly mixed and left for precipitation to occur. The supernatant was collected and kept in a 100 ml polyethylene bottle with a tight cover. Calcium content was determined by using atomic absorption spectrophotometer

(AA-680 ShiMADZU, Atomic Absorption/Flame Emission Spectrophotometer, flame : AIR/C_2H_2).

RESULTS AND DISCUSSION

Morphological observation

Red sternum and normal mud crabs collected from soft shell crab farm were observed (Figure 1). Their characteristics are shown and described in Table 1 and Figure 1(A-H).

The observed characters of red sternum and normal mud crabs were clearly different. Sternum, chelae and joints of red sternum crab were red in color while those of the normal ones were white to pale yellow (Figure 1 B-D). On the contrary, hepatopancreas and gills of the abnormal crabs were pale, soft and unshaped but those of the normal crabs were rigid (Figure 1 E-F). The haemolymph of red sternum crabs was not clotted but formed a milky-like substance, while that of the normal crabs was colorless or pale blue (Figure 1 H). The shells of red sternum crabs were softer than those of normal crabs, while the muscle was loose and had slow motility. They preferred to remain on land and hard surface and their eating ability was also reduced. Diseased crabs displayed signs of acute morbidity, such as drooping limbs and mouthparts. When the red sternum crabs became severely deteriorated, their legs and chelipeds were paralyzed. Molting could not be proceeded as usual and they finally died.

Not only the taste of red sternum mud crabs was found to be bitter than the normal ones but the symptoms found in mud crab (*Scylla serrata*) were also similar to those of infected crab of PCD and BCD. Although *Hematodinium* sp. was found to be the cause of several diseased crabs, i.e., the edible crab (*Cancer pagurus*) (Stentiford *et al*., 2002), tanner crab (*Chionoecetes bairdi*) (Meyers *et al*., 1987), snow crabs (*Chionoecetes opilio*) (Pestal *et al*., 2003), the Norway lobster (*Nephrops norvegicus*) (Field and Appleton, 1995), the velvet swimming crab (*Necora puber*) (Wilhelm and Mialhe, 1996) and blue crab (*Callinectes sapidus*) (Messick, 1994), but other parasites, ciliates, barnacles, virus and yeast were also reported to be the potential pathogens of crabs as well (Stentiford *et al*., 2003).

Similar to both PCD and BCD, red sternum mud crabs were found more often in

Table 1 Characteristics and behaviors of red sternum and normal mud crab.

Organ, tissue, and observed behaviors	Normal mud crab	Red sternum mud crab
Carapace	Hard	Soft
Chelae and joint	White	Red
Sternum	Hard, white to pale yellow	Soft, red
Haemolymph	Clotted after leaving at room temp. for 1 min, no color to pale blue	
	Unclotted, form a milky-like substance	
Hepatopancreas	Fresh and rigid	Pale and soft
Gill	Fresh, rigid	Pale, soft
Muscle	Rigid	Loose
Strength	Strong, aggressive	Calm, gentle
Locomotive activity	Highly active	Limited movement
Eating behavior	Having good appetite	Lose appetite
Preferred habitat	Mostly stay in water, only sometimes on land	Only stay on land and hard surface

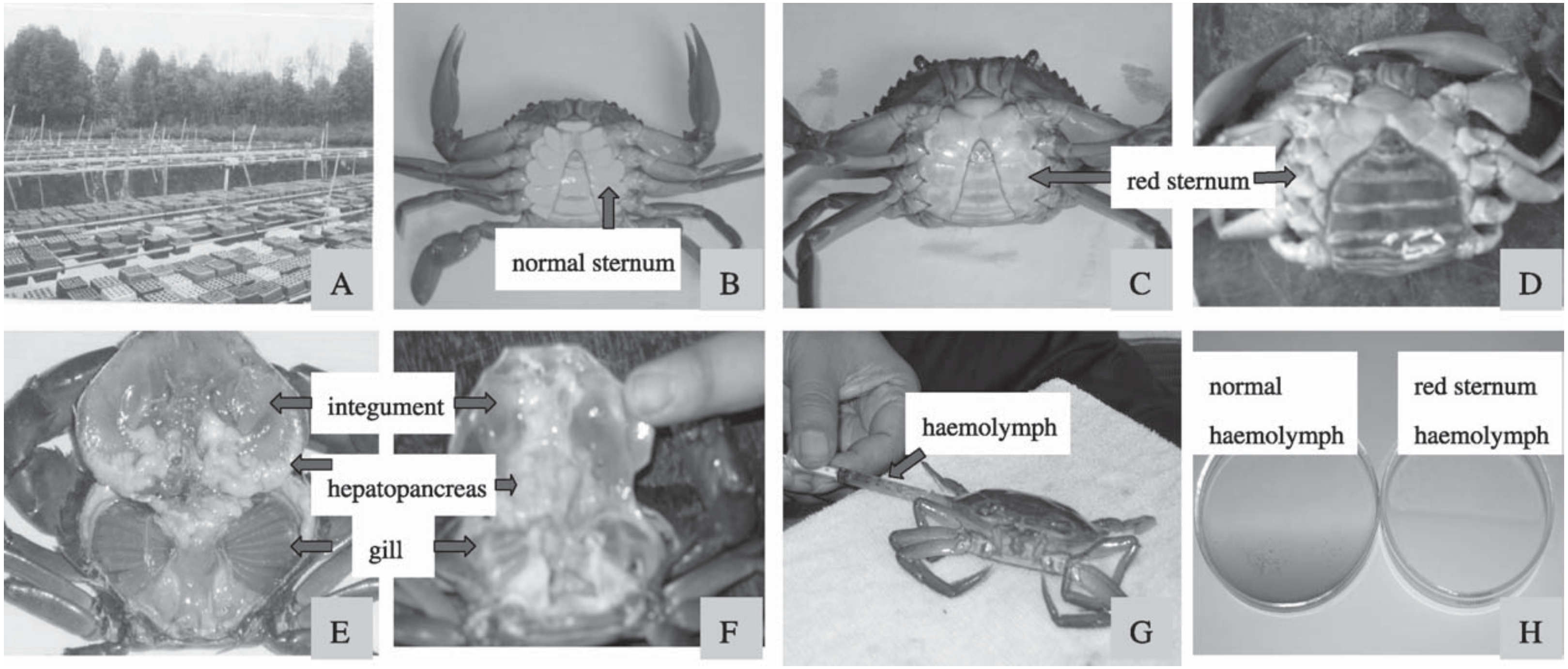

Figure 1 A) A soft-shell crab farm in Klung District, Chanthaburi Province B) A normal mud crab (*Scylla serrata*) C) A red sternum mud crab showing the first sign of red sternum symptom with pink sternum D) The late phase of red sternum showing dark red sternum, chelae, and joint E) and F) Comparing the inner organs of normal (E) and red sternum mud crab (F) Showing deterioration of the integument, gills, and hepatopancreas G) Collecting haemolymph from the base of pereiopods H) Normal transparent haemolymph and milky unclotted haemolymph of a red sternum mud crab

warm, relatively shallow and high salinity water (Burnett, 1992). The ventral sides of their limbs were opaque, bright white color, rather than the normal translucent whitish-gray color. Dorsally, the carapace was slightly pinkish as opposed to the normal orange-tan color. In the case of PCD and BCD, *Hematodinium* consumes oxygen from the crab's blood and tissues which cause it to become weak and lethargic (Meyers *et al*., 1996). The haemolymph of PCD and BCD infected crab is milky white in color rather than the normal translucent light gray. When cooked, the crabmeat had a chalky texture and a bitter aspirin-like flavor (Meyer *et al*., 1987). Stentiford *et al*. (2001) reported on the composition of *Hematodinium*-infected in the Norway lobster (*Nephrops norvegicus*) tissues and suggested that disruptions in the normal carbohydrate and amino acid profiles of these tissues might cause the bitter taste of the meat. However, PCD and BCD had no impact on people who ate infected crab, but the parasites could be detected under a microscope (Meyers *et al*., 1996). Once crab got infected, *Hematodinium* grew rapidly inside the crab (up to 10^6 parasites/ml of blood) over the course of 3 to 6 weeks, the crab's blood changed to a milky-white color and lost its clotting ability (Stentiford *et al*., 2002). Although the outer appearances of red sternum, PCD and BCD were quite similar, it could not be conclude that the causes of these symptoms would be the same. Further investigation to clarify its cause needs to be done.

SDS-PAGE

The blood protein of red sternum mud crab was compared to that of normal mud crab using SDS-PAGE analysis. There were several intense protein bands found in the ranges of 66-97 kDa in the normal crab haemolymph, while the haemolymph of red sternum mud crab had no band at all in this range (Figure 2). Since the molecular weight of oxyhemocyanin is 75 kDa and this substance was the main component of blood protein found in white shrimp (*Penaeus vannamei*) (Figueroa-Soto and Barca, 1997), the distinct band of 66-97 kDa suggested the existence of oxyhemocyanin in normal mud crab haemolymph and the gradual disappearance of this band (Figure 2) could be the clear marker of changing in blood component of this symptom as shown by the unclotted and milky-like substances.

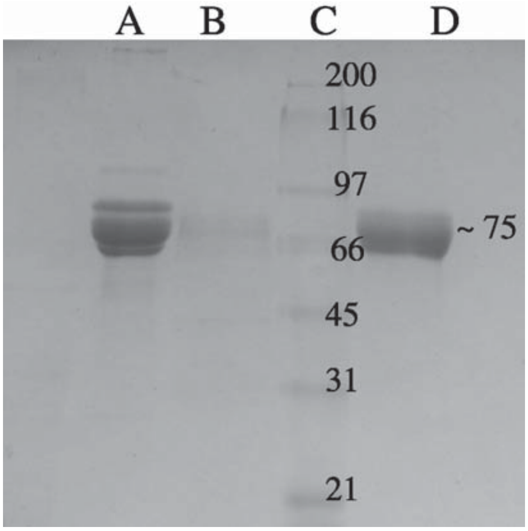

Figure 2 Lane A) intense protein band of 75 kDa in normal haemolymph Lane B) disappearance of 75 kDa band in haemolymph of late phase red sternum symptom Lane C) molecular markers Lane D) less intense band of 75 kDa in haemolymph of mud crab having first sign of red sternum symptom.

To confirm this result, spectroscopic analysis was used to reveal the different spectra of haemolymph of red sternum and normal mud crab. In Figure 3, the spectrum of normal crab haemolymph showed the maximum absorbance at 340 nm representing the absorbance of oxyhemocyanin (Terwilliger *et al*., 1999) but it was absent in the red sternum crab haemolymph.

Haemocyanin (Hc) is a copper-

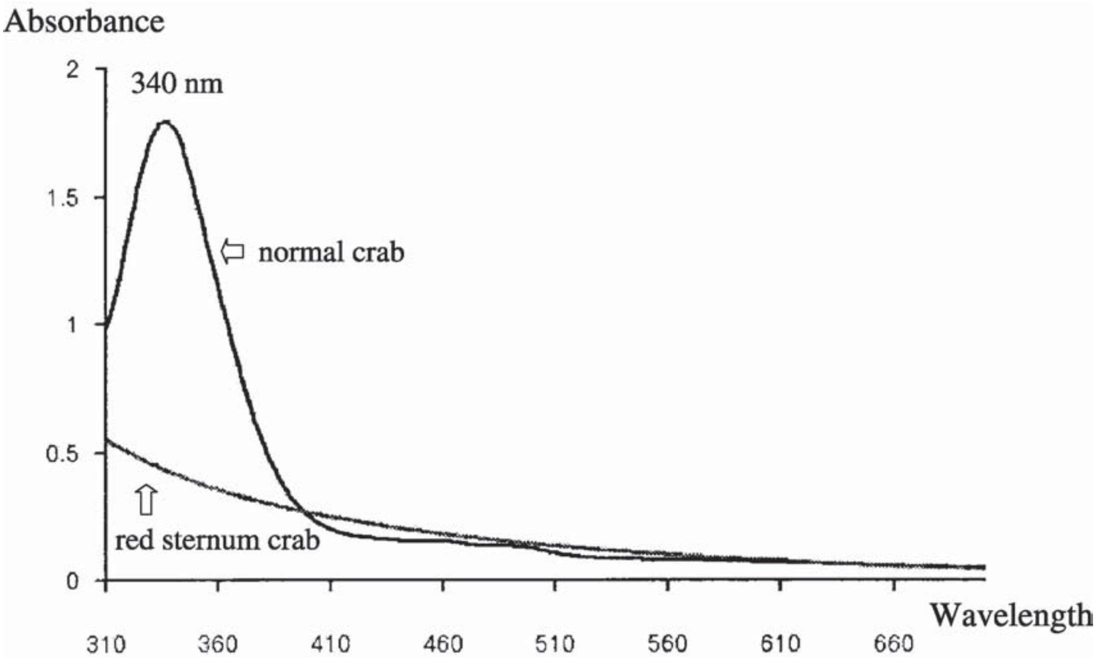

Figure 3 Spectrum profile of protein absorbance at 310 nm - 660 nm. The maximum absorbance at 340 nm of normal hacmolymph indicated the existence of oxyhymocyanin compared to the absence of this compound in red sternum crab.

containing protein, capable of transporting more than 80% of the O_2 delivered to the tissue (Ainslie, 1980). It presents at high concentrations in crustaceans, comprising 90-95% of the total plasma protein in rockpool prawn (*Palaemon elegans*) and white shrimp (*Penaeus vannamei*) (Cariolou and Flytzanis, 1993). Besides transporting oxygen, hemocyanin also involves in the transport of metals and amino acids (Weeks and Rainbow, 1992). Therefore, the lack of hemocyanin in the haemolymph of red sternum mud crab affects the function of oxygen binding and the activities of metals. That is why red sternum mud crab prefers to stay on land for acquiring more oxygen. Taylor and Spicer (1987) investigated that aquatic animals experienced hypoxic conditions more often than air breathers and would adapt themselves by moving away from that environment or raise posture with the anterior part of the body above the water surface.

Trace elements

Trace elements in haemolymph of red sternum mud crab were compared to those of normal mud crab by using atomic absorption spectrophotometer. The differences of major elements in both crabs are shown in Table 2.

Most of the elements measured in both crabs were quite different except chloride. The contents of calcium, magnesium and iron in red sternum crab haemolymph were higher than that in normal crab. In contrast to the content of copper, manganese and zinc in the red sternum crab were lower. Although the increase in calcium content was only 8.99 %, magnesium was found to increase at 58.68 % in the red sternum mud crab. Calcium and magnesium are involved in several physiological processes, i.e., nerve conduction, muscle contraction and blood coagulation as well as activate alkaline phosphatase which is important for calcification and mineralization during molting

Table 2 Trace elements content in haemolymph of normal and red sternum mud crab.

Type of element	Trace elements content in haemolymph (ppm)	
	Normal crab	Red sternum crab
Calcium	342.15	372.90
Magnesium	252.68	400.95
Iron	2.46	10.80
Chloride	715	728
Copper	122.55	37.44
Manganese	6.76	3.44
Zinc	23.76	5.78

cycle in crustacean (Chen *et al*., 2000). Since calcium is an important constituent of skeletons and many other rigid mechanical structures, the nerve of animals received excessive level of calcium will make them move slowly, which explains the limited movement found in red sternum crabs. However, the overall balance of ion concentration in cells is important, the excess of magnesium content and the increase in calcium in red sternum mud crab may affect the function of hemocyanin.

The iron content in red sternum mud crab haemolymph was found to be four times higher than that in normal crab. Iron is a constituent of several intracellular enzyme systems, notably as the cytochromes (Rainbow, 1997). Although the total amount of iron in organism is not really much, the excess iron in haemolymph of red sternum mud crab can possibly hinder the enzyme activities. On the other hand, copper content in red sternum mud crab was three times lower than that in normal crab. It suggested that the decrease of copper led to the reduction of metabolic function of hemocyanin (Mangum, 1992).

Manganese is important for the development of bones. It also functions as the activator of enzyme systems, but the connection with the deficiency symptoms in crustacean is not entirely clear (Rainbow, 1997). Manganese content in red sternum crab was found only half of that in normal crab. This effect might result in the non-rigid carapace of red sternum crab. On the other hand, zinc content was four times lower in red sternum than in normal crab. The decrease of zinc could result in the decrease of enzyme activities because zinc is a constituent of many important enzymes, including carbonic anhydrase and several peptidases which play important roles in CO_2 exchange (Bottcher and Siebers, 1993). The inefficient functions of these enzymes also explained the preferable behavior of red sternum mud crabs to live on land. In addition, one of the most quantitatively important ions in animals is chloride. The results showed that chloride content in red sternum mud crab remained at the same level as that of normal crab indicating its importance in keeping the osmotic balance for the animals.

CONCLUSION

The morphological characteristics of red sternum mud crabs were distinctively seen by the red color of sternum, the milky-like haemolymph and the deteriorations of gill, integument and hepatopancreas as well as the bitter taste of meat and the preferable habitat on land. The disappearance of hemocyanin in red sternum mud crab was illustrated by SDS-PAGE and confirmed by the absence of 340 nm spectrum. Changes of trace elements in the red sternum haemolymph could be divided into 2 groups. The increase in calcium, magnesium and iron and the decrease in copper, manganese and zinc, all of which contributed to the changes in physiological functions and apparent behaviors of mud crab.

This was the first investigation on red sternum mud crab. There was still no conclusive evidence that the causes of symptoms found in mud crab were the same as those of pink crab disease (PCD) or bitter crab disease (BCD). However, the changes in biochemical components of haemolymph and other tissues of red sternum crab could help elucidate these symptoms. Further investigations on lactate, enzymes, pigments, carbohydrate, protein and glycoprotein in red sternum crabs would be of great interest.

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