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Comparative LC-MS based non-targeted metabolite profiling of the Chinese mitten crab *Eriocheir sinensis* suffering from hepatopancreatic necrosis disease (HPND)

Tianheng Gao[b,d,1], Yu Xu[a,1], Kai Wang[a], Yanfei Deng[a], Yuanjie Yang[a], Quanping Lu[a], Jianlin Pan[a,c,d], Zhiqiang Xu[a,c,d,*]

[a] Freshwater Fisheries Research Institute of Jiangsu Province, Nanjing 210017, China
[b] Institute of Marine Biology, College of Oceanography, Hohai University, Nanjing 210098, China
[c] The Jiangsu Provincial Platform for Conservation and Utilization of Agricultural Germplasm, Nanjing 210014, China
[d] Jiangsu Key laboratory for Conservation and Utilization of freshwater Fisheries Germplasm Resources, Nanjing 210017, China

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ABSTRACT

Hepatopancreatic necrosis disease (HPND) is a newly emerged disease in the Chinese mitten crab, and has caused significant economic damage to crab farmers. However, the exact pathogenesis of crab HPND has not been elucidated. To understand the metabolic shifts in the pathogenesis of the disease, we used LC-MS for metabolite profiling of the hepatopancreas of crabs with HPND. Diseased (DC) and healthy crabs (HC) from the ponds with disease occurrence, and healthy crabs (HHC) from adjacent ponds free from HPND, were analyzed. Histopathologic characteristics and potential pathogens in the hepatopancreas of the healthy and diseased crabs were preliminary investigated. Cellular damage or necrosis, including cell swelling, rupturing, vacuole formation and nuclear fragmentation were observed in diseased crabs. Pathogen screening revealed that, *Hepatospora eriocheir*, a parasite which was ever deemed to be the agent of the disease before, was unlikely the cause of HPND. Partial least squares discriminant analysis (PLS-DA) score plots revealed a significant metabolic difference between DC and HC in positive ionization modes (R^2 = 0.96807, Q^2 = 0.74558), while HC and HHC were grouped closely together. Fourty-five differential metabolites were identified and used for further functional pathway analyses. Two potential pathogenic factors, including fatty acid metabolic abnormalities and high concentrations of propamocarb (a widely used pesticide in vegetables), were found to be likely associated with HPND in the Chinese mitten crab. The identified metabolites and regulation pathways, and screening of potential pathogens in diseased crabs, should provide useful information for the prevention of the disease in the future.

1. Introduction

Chinese mitten crab, *Eriocheir sinensis* is an important aquaculture species in China and has caused considerable ecological damage as a recent invader in North America and Europe. Chinese mitten crab is considered the most nutritious and delicious crustacean by Chinese consumers, and thus has high economic value in China. Chinese mitten crab farming accounts for almost two thirds of global crab production, and is a leading component of the freshwater aquaculture industry in China (Paterson, 2009). In 2015, the annual production of Chinese mitten crab reached 770,000 tons, which was worth approximately 6.6 billion dollars (Fishery Bureau of Ministry of Agriculture PRC, 2016). In the 1990s, due to high stocking density and poor management, frequent outbreaks of various diseases caused significant economic losses in the crab aquaculture industry (Wang and Gu, 2002). During the past 20 years, crab farmers have realized the importance of ecological regulation in crab aquaculture. By adopting the concepts of ecological aquaculture with lower stocking density and widely cultivated aquatic plants, the incidence of crab diseases has long been controlled and maintained at a relatively low level. Recently, a kind of serious disease caused hepatopancreatic necrosis disease (HPND) has emerged in the main crab production regions (mainly in Xinghua, Jiangsu Province) of China since 2015 (Ding et al., 2016; Pan et al., 2017; Shen et al., 2017). This disease mostly occurs from the third molting to the harvest during crab farming, and most of the diseased crabs are males (Chen et al., 2017; Shen et al., 2017). The incidence rate in aquaculture ponds with

* Corresponding author at: Freshwater Fisheries Research Institute of Jiangsu Province, 79 Chating East Street, Nanjing 210017, China.
E-mail address: zhiqiangx@163.com (Z. Xu).
[1] These authors contributed equally to the study.

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HPND varies from 20% to 30%. The hepatopancreas of diseased crabs appears white, atrophied and necrotic, and the muscle appears to have atrophy and edema. Although most of the crabs with HPND will not die until the harvest, there is no market value for diseased crabs (Pan et al., 2017; Shen et al., 2017). In 2016, the disease spread rapidly throughout crab aquaculture regions, and the disease-affected regions comprised approximately 30% of the total crab aquaculture area. HPND has become the principal bottleneck for the development of crab aquaculture in China.

Many researchers have focused on revealing the risk factors for crab HPND, but pathogenesis is still not understood. Ding et al. (2016) claimed that *Hepatospora eriocheir*, a kind of microsporidian first described by Wang and Chen (2007) in the crabs with tremor disease, was detected in the hepatopancreas of diseased crabs, and claimed that this microsporidian caused HPND in the Chinese mitten crab. However, *H. eriocheir* has also been detected in natural populations of Chinese mitten crab. Although *H. eriocheir* was detected in up to 70% of the Thames population, crabs infected with a large number of this parasite did not appear to display external symptoms of disease (Stentiford et al., 2011). More recently, Shen et al. (2017) used a meta-transcriptomic approach to compare the microbiota of Chinese mitten crab suffering from HPND, and has not found any differences in microsporidial communities in the hepatopancreas between diseased and healthy crabs. Using electron microscopy observations, Pan et al. (2017) also did not detect microsporidians in crabs suffering from HPND. Hepatopancreas is a key organ in nutritional metabolism, energy storage, and other life activities in crustaceans (Huang et al., 2015). Therefore, many hepatopancreas disease-related factors, which include aquaculture environments, nutrition feeding or genetic degeneration, are speculated to be associated with HPND. Some drug residues, such as pyrethroid pesticides, which are usually used as crab pond clearing drugs, have also been suspected to be associated with HPND (Pan et al., 2017; Yang et al., 2016). However, all these speculations have yet to be accurately further confirmed.

Metabolomics, a newly established omics technique that focuses on small molecular metabolites (< 1000 Da), has been used to explore the physiological responses of living organisms to environmental conditions (Tim and Andrea, 2016; Viant, 2007). In general, metabolites are sensitive to environmental changes and can reveal changes on the physiological level (Patti et al., 2012). Metabolomic techniques have been used to resolve issues related to nutrition and diet, disease, and environmental toxicology in many aquaculture species (reviewed by Andrea and Tim, 2016; Ji et al., 2016). For example, the effect of bacterial infection on the metabolomics changes in Atlantic salmon *Salmo salar* has been studied; exposure to *Aeromonas salmonicida* induced a characteristic biochemical response, which can be used to determine the health status of salmon (Solanky et al., 2005). In red abalone *Haliotis rufescens* with withering syndrome, the influence of food availability, temperature, and bacterial infection on metabolic status was measured by metabolomics analysis. The metabolic data correlated well with histological measurements, supporting the metabolomics approach for characterizing pathological events in aquaculture species (Rosenblum et al., 2005). In *Apostichopus japonicas*, divergent metabolic responses were investigated in natural skin ulceration syndrome-diseased (SUS) and *Vibrio splendidus*-challenged samples. The results showed that the metabolic biomarkers induced by *V. splendidus* were not usable for the prediction of SUS disease in practice (Shao et al., 2013).

In this study, we hypothesized that multiple physiological processes of the crabs with HPND are influenced by potential pathogenic factors, such as pathogen infections, environmental toxins or nutrition. Consequently, the metabolome profiles in the hepatopancreas of the diseased crabs should also be affected by these factors. Significant changes in certain metabolic pathways may be responsible for the onset and development of HPND. To address this hypothesis, high throughput LC-MS based metabolomic workflows were used to obtain the global non-targeted metabolome profiles that are altered in the hepatopancreas of crabs with HPND. The identified metabolites and key regulation pathways, and screening potential pathogens in diseased crabs, may help us to identify the exact pathogenic factors of HPND, and provide useful information for the prevention of the disease.

2. Materials and methods

2.1. Sampling and preparation of tissue samples

In August 2016, two types of crab samples, which included 10 healthy crabs (HC; 172.3 ± 4.1 g) and 10 diseased crabs (DC; 167.2 ± 4.2 g) with typical symptom of HPND, were randomly sampled in the ponds with disease occurrence in the Xinghua area (the main HPND endemic area in China). Another 10 healthy crabs roughly of the same size (HHC; 169.3 ± 3.2 g) were also randomly sampled from the adjacent crab ponds free of the disease. All the crabs in the three groups of samples were males. For histopathological examination, the hepatopancreas of each sample was dissected and placed immediately into Davidson's alcohol formalin acetic acid fixative. Fixed samples were processed to wax in a vacuum infiltration processor using standard protocols. Sections were cut to a thickness of 3–5 μm and stained with haematoxylin and eosin (H&E). Stained sections were analyzed by light microscopy (Nikon Eclipse 80i). For potential pathogen screening by PCR amplification, the hepatopancreas from each crab was collected under sterile conditions and preserved in 100% ethanol at −20 °C until DNA extraction. The hepatopancreas of each crab was dissected and immediately frozen in liquid nitrogen for subsequent metabolite extraction and metabolite profiling analysis.

2.2. Preliminary screening for potential pathogens in the crab hepatopancreas

The unstained wet smear of the hepatopancreas from each sample was first microscopically examined for the presence of parasites (Nikon Eclipse 80i). Potential bacterial infections in the hepatopancreas of healthy and diseased crabs were preliminary investigated by streaking on nutrient agar plates and TCBS agar plates. The isolated monoclonal colonies were first classified according to colony color, edge style and shapes, and then cell morphology of the identified bacteria were further examined under microscope using Gram stain method. After further isolation and purification, only representative bacterial strains were selected as candidates for further physiology-biochemistry tests and 16S rRNA analysis (Drancourt et al., 2000).

Two pairs of primers were designed according to previous reports to examine the presence of two other proposed potential pathogens, which included white spot syndrome virus (WSSV) and *H. eriocheir* (Mendoza-Cano and Sánchez-Paz, 2013; Stentiford et al., 2011). Total genomic DNA was extracted from the hepatopancreas of each crab for the molecular detection of potential pathogens using an EasyPure Genomic DNA Kit (Transgen, Beijing, China). PCR reactions were performed for all samples using KOD Dash DNA Polymerase according to the manufacturer's instructions (Toyobo, Japan). The PCR products were purified using an EasyPure PCR Purification Kit (TransGen, Beijing, China), and directly sequenced by using the Sanger method with a 3730xl DNA Analyzer (Thermo Fisher Scientific, USA).

2.3. Metabolite extraction and metabolite profiling analysis

Ten crabs from each kind of samples were chosen as a test set to identify metabolites whose levels were significantly different. Hepatopancreas tissue of each sample was homogenized with mortar and pestle in liquid nitrogen; after which 150 mg homogenized hepatopancreas tissue was precisely weighted and used for subsequent metabolite extraction. Metabolites from each crab were extracted with 1 mL of cold MeOH/H_2O (8,2, *v*/v). The metabolite extract of each