Chapter 17
Bacteria and Fungi Identified on Horseshoe Crabs, *Tachypleus gigas* and *Carcinoscorpius rotundicauda* in the Laboratory

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Abstract In Malaysia, reduction of horseshoe crab sightings in their natural habitat has prompted studies on the ecology of this invertebrate and potential threats to its survival. In this study, eggs and newly hatched *Tachypleus gigas*, collected from a natural spawning ground in Banting, Selangor (Malaysia), were conditioned and cultured in the laboratory to test the potential of fungal or bacterial infections to occur and impair development and growth. During the culture period, evidence of bacterial and fungal infestation was observed. Identified species included bacteria (*Shewanella putrefaciens*, *Bacillus cereus*, *Corynebacterium* sp. and *Enterococcus faecalis*) and fungi (*Aspergillus* sp., *Aspergillus niger*, *Peniclillium* sp., *Gliocladium* sp.). Eggs infected with bacteria or fungi usually turned reddish, grey or black and ceased to develop. Larvae became passive and coated with black spore and in some cases died soon after molting into the larval stage. The species with greatest potential to affect the growth and survival of horseshoe crab eggs and larvae sampled in this study were *S. putrefaciens* (a Gram-negative bacterium) and *A. niger* (a fungi). The suite of species identified during this study were different from those previously identified on horsehose crabs in culture, suggesting possible species, location, and/or condition-specific variation in communities of infectious microbes brought in from the natural environment and propagated in culture. Infected horseshoe crabs, therefore, may require different management or treatment strategies and additional study.

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17.1 Introduction

Laboratory cultured horseshoe crabs may have less exposure to microbes than horseshoe crabs in the natural environment, nevertheless they are still susceptible to accidentally introduced pathogens (Smith and Berkson 2005; Faizul et al. 2012). Disease agents such as fungi, viruses, bacteria and parasites can affect the development and growth of eggs, larvae and juvenile horseshoe crabs and survival of adults (Nolan and Smith 2009; Smith et al. 2011; Faizul et al. 2012). These disease agents are commonly found in natural environments such as water, soil, animals and plants. Some of these biological agents are not detectable by naked eye and have the ability to reproduce rapidly. It was the aim of this study to identify the microbes found on *Tachypleus gigas* and *Carcinoscorpius rotundicauda*, particularly those suspected in causing negative effects on the development, growth, and survival of horseshoe crabs reared under culture conditions.

17.2 Materials and Methods

Two hundred eggs and larvae of *Tachypleus gigas* and 20 adults of *Carcinoscorpius rotundicauda* were collected from a natural spawning ground at Banting, Selangor, Malaysia and conditioned in 25 ppt of seawater at ambient temperature (26–29 °C) in the laboratory. Experimental studies were carried out at the Marine Laboratory at MTDC (Malaysian Technology Development Corporation), Putra Science Park, Universiti Putra Malaysia, Serdang, Selangor, Malaysia. These eggs and larvae were then held at 30 ppt in rearing trays with daily water changes. Adult *C. rotundicauda* were maintained in a circular fiberglass tank (500 L capacity). The larvae of *T. gigas* were fed *Artemia* nauplii (*ad libitum*) and adult *C. rotundicauda* with shrimp (once a day). Water parameters (temperature, dissolved oxygen (DO), pH, and ammonia) were measured using a YSI DO meter, pH meter, and ammonia API test kit, respectively, once a week. Samplings of 30 eggs and larvae and microscopic observation (under dissecting and compound microscope at 40, 100, 200 and 400× magnification) were carried out weekly. Eggs were incubated until 80 % of the eggs from each tray hatched. *Tachypleus gigas* larvae were observed for a period of 3 months.

To collect microbial contaminants, fungal infected eggs and larvae were swabbed with cotton on the infected areas and plated for culture on triptic soy agar (TSA) + 1.5 % salt media. Resulting bacterial and fungal growth on the agar plate was photographed. Subsequently, these cultures were sent for identification at the Bacteriology Laboratory, Faculty of Veterinary, Universiti Putra Malaysia, Serdang, Selangor.

17.3 Results and Discussion

17.3.1 Bacterial Infection on Tachypleus gigas *Eggs and Larvae*

Species of bacteria found on the eggs and larvae of *T. gigas* were identified as *Shewanella putrefaciens*, *Enteroccus faecalis*, *Bacillus cereus* and *Corynebacterium* sp. The most commonly found species was *Shewanella putrefaciens*, a Gram-negative rod shaped bacteria. Infected eggs usually turned light pink or red (Fig. 17.1). Some of these infected eggs (Fig. 17.2a) continued to develop; however, larvae (Fig. 17.2b) died immediately after hatching.

Isolated *Shewanella putrefaciens* grew within 8 h after inoculation onto the agar media. During egg incubation, *S. putrefaciens* was detected as a visible white film layer covering the water and eggs, with a distinct odor. *S. putrefaciens* is a saprophytic bacterium and commonly found in all types of water samples such as fresh-

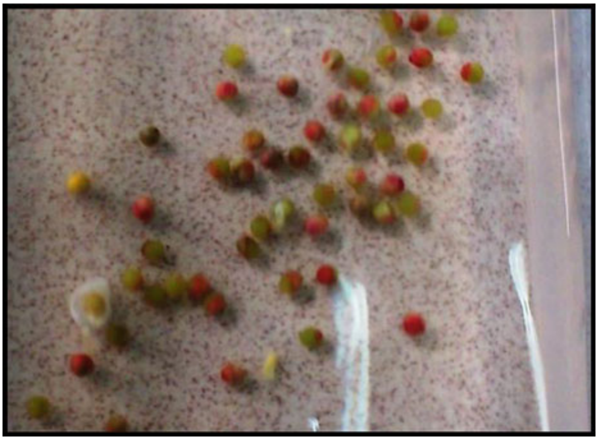

Fig. 17.1 Bacterial infected horseshoe crab (*T. gigas*) eggs that have turned *pink* or *red*

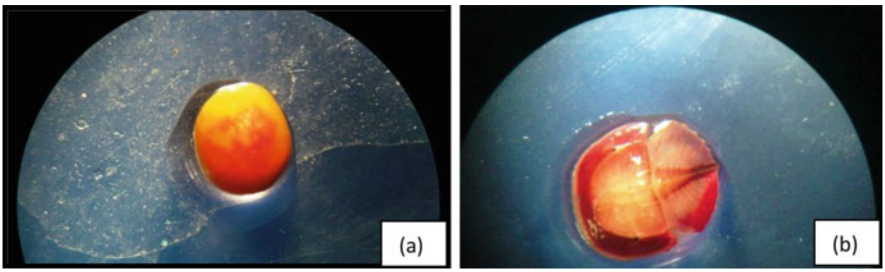

Fig. 17.2 (**a**) Eggs and (**b**) larvae of *T. gigas* infected with *Shewanella putrefaciens*

water, brackish water, marine water, mud water, stagnant water, lake water, river water and sewage water (Chen et al. 1997; Wang et al. 2004). It also is found on frozen poultry carcasses and in petroleum brines, cream, oil emulsions, ground beef, natural gas, milk (Von Graevenitz 1985) and spoiled seafood (The Environment Agency 2012). Clinical signs of skin lesions and septicemia have been found in the proximal intestine of infected perch (Goldschmidt-Clermont et al. 2008). In pufferfish (*Lagocephalus lunaris*), infection has been found in the internal organs and on the skin (Auawithoothij and Noomhorm 2012). European seabass usually displayed characteristics of lethargy, loss of appetite, pale gills, exopthalmus, ulcers over the dorsal part body, haemorrhages (on fins, body, operculum, liver), splenic enlargement, liver and kidney pale and gastro-intestinal tract (Korun et al. 2009). Without water change and proper care, therefore, horseshoe crab eggs may become infected and have potential to contaminate other eggs in the same or nearby incubation trays.

Gram-positive bacteria (Silva et al. 2000), *Enteroccus faecalis*, *Corynebacterium* sp., and *Bacillus cereus* were found on *T. gigas* eggs. These species are different from those previously reported on the American horseshoe crab, *Limulus polyphemus*, including the genera *Vibrio*, *Oscillatoria*, *Pseudomonas*, *Flavobacterium*, *Leucothrix* and *Pasteurella* (Smith et al. 2011). In this study, *E. faecalis* was found on the eggs and larvae of *T. gigas*. Four species from this genus, *E. casseliflavus*, *E. faecium*, *E. mundtii* and *E. raffinosus* have been found in the intestine of farmed fish in Thailand (Petersen and Dalsgaard 2003), occurring in the kidney, liver, spleen and heart of rainbow trout and turbot (Carson et al. 1993; Toranzo et al. 1995). *Bacillus cereus* has been identified as an agent responsible for two types of foodborne diseases (Kenneth 2008). The resulting illnesses are characterized by nausea, vomiting and abdominal cramps or diarrhea with incubation periods from 1 to 16 h, depending on type (Kenneth 2008). *Corynebacterium* sp., a coryneform bacteria (Hindmarch et al. 1990), has been documented to cause exophthalmia or bulging eye in striped bass and salmon (Austin and Austin 1999). *Bacillus cereus* var. *toyoi*, however, is considered a beneficial bacterium and used as a probiotic product for animals and humans (Sanders 2003).

In this study, *E. faecalis* and *B. cereus* were found on the dorsal and ventral part of the prosoma of *T. gigas* larvae. The infection by these bacteria may cause stress on horseshoe crabs by impairing movement of the animal and slowing the blood clotting reaction, and in some cases infection may lead to mortality (Bang 1956). Pathogenic microbes spread in many ways to attack the host and simultaneously avoid its immune defense (Jiang et al. 2007). Bacterial infections in horseshoe crabs can colonize and penetrate the carapace, eyes and gill surfaces, affecting an entire system, involving the deeper tissues of the organs, gills and circulatory sinuses and resulting in extensive tissue necrosis that can cause mortality (Smith et al. 2011).

Hemolymph factors are responsible for the natural innate immune system in horseshoe crabs and are divided into two types: hemocyte and hemoplasma (Kouno et al. 2008). The hemocytes of horseshoe crabs contain various cysteine-rich cationic peptides (polyphemusin, tachyplesin, tachystatin, tachycitin and big defensin), which have inhibitory effects on the growth of bacteria (Gram-positive and Gram-negative bacteria) and fungi (Silva et al. 2000). Blood cells from *T. tridentatus* contain tachycitin (antibacterial substance) to react against both Gram-positive and Gram-negative bacteria. Fresh hemolymph of *T. gigas* exhibited antibacterial activity against *Escherichia coli*, *B. cereus*, and *Vibrio parahaemolyticus*, but demonstrated only partial antibacterial activity against *B. subtillis* and *Sterptococcus aureus* (Shakiba et al. 2011). Hence, horseshoe crabs may become infected despite their innate immune defenses, particularly under stressed conditions or if the immune system is otherwise compromised.

17.3.2 Fungal Infection on Tachypleus gigas *Eggs and Larvae*

Fungus was found to affect the development of *T. gigas* eggs and larvae. Fungus attached on larvae when the spore separated in the water (Fig. 17.3). Fungal infection found on the eggs and larvae of *T. gigas* were *Aspergillus* sp., *Aspergillus niger*, *Penicillium* sp. and *Gliocladium* sp. The fungus *Gliocladium sp*. was found only on eggs of *C. rotundicauda*. Eggs infected by *A. niger* will turn to black, and the eggs cease to develop (Fig. 17.4).

Aspergillus niger is not only found on eggs of *T. gigas*, but also commonly found in the environment where crabs live. On natural spawning grounds, *T. gigas* eggs can be found in highly organic sands at the mangrove areas where *A. niger* is in

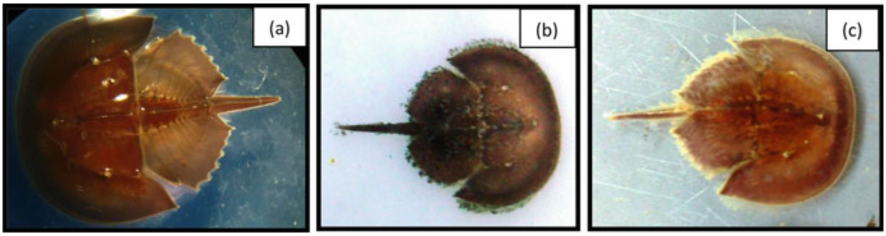

Fig. 17.3 Comparison between horseshoe crabs *T. gigas* that are not infected (**a**) and those that are infected (**b**) and (**c**) by *Aspergilus niger* in culture

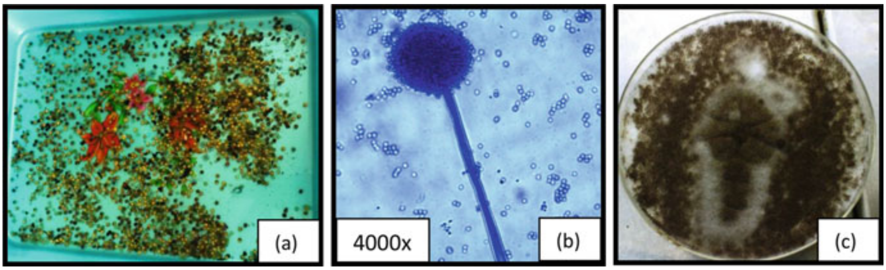

Fig. 17.4 (**a**) *Tachypleus gigas* eggs infected with *Aspergillus niger* during incubation, (**b**) morphology of *A. niger*, (**c**) *A. niger* on agar plate

abundance. *Tachypleus gigas* eggs can tolerate temperatures (28–31.9 °C; Christianus and Saad 2010) in the range suitable for *Aspergillus niger* (6–47 °C). *Aspergillus niger* also is capable of growing at a very wide pH range, from 1.4 to 9.8 (Gautam et al. 2011). The growth ability in various temperature and pH ranges as well as the abundant amount of conidiospores allow the continuous spread of this fungus in horseshoe crab habitats.

The genus *Aspergillus* with 260 species has been studied for several centuries (Samson and Varga 2009). *Aspergillus niger* is a filamentous ascomycete having the ability of fast growth, black color, and having broad pH tolerance (Pitt and Hocking 1997; Perrone et al. 2007; Perfect et al. 2009). *Aspergillus* spp. in carp species infect a variety of body parts from the head and eyes to abdomen and fins, including symptoms such as eroded scales, hemorrhaging along the body surface, body distension, and tissue erosion (Iqbal et al. 2012). *Aspergillus niger* may also produce certain mycotoxins having nephrogenic, heptocarciinogenic, and immunological properties (Gautam et al. 2011). Mycotoxins such as ochratoxin are important food-safety agents (Sweeney and Dobson 1998). *Aspergillus niger* is most commonly used by citric acid and pectinase producers and serves as a model fungal fermentation process (Li and King 1963; Rombout and Pilnik 1978; Kerns et al. 1987; Baker 2006). If inhaled in sufficient quantity (Baker 2006; Gautam et al. 2011), *A. niger* also can infect the lungs of humans (aspergillosis), but the U.S. Food and Drug Administration (USFDA) considers the fungus as generally recognized as safe (GRAS) (Gautam et al. 2011).

The fungus *Penicillium sp*. was also found on eggs of *C. rotundicauda*. *Penicillium corylophilum* has been found infecting the kidney and swim bladder of red snapper (*Lutjanus campechanus*) where it can cause the kidney and swim bladder to enlarge (Blaylock et al. 2001). *Penicillium* spp. infection on goldfish head, pelvic and abdomen of goldfish (*Carassius auratus*) can cause eroded scales, hemorrhaging over body surface and moderate body distension (Iqbal et al. 2012). Fungal secondary metabolites (citrinin and patulin) are produced by fungal strains belonging to the genus *Aspergillus* and *Penicillium*. These toxins contaminate cereals and fruits (Bennett and Klich 2003) and also have been detected in monascus (natural dietary supplements to prevent cardiovascular disease in Asia) fermentation products (Wei et al. 2003; Liu et al. 2005). Kidney is one of the target organs of citrinin in animals, including dogs, rats, and rabbits (Kogika et al. 1993; Kumar et al. 2007; Singh et al. 2007).

In general, fungi are organisms depending on other organisms for their supply of nutrients, known as eukaryotic organisms (Ozdal et al. 2012). Fungi play an important role in the recycling of nutrients in the ecosystem, and most fungi are living on dead organic material or known as saprophytes (Eduard 2007). Conidia (asexual spores) are attached to the surface of the host and release extracellular enzymes including lipases, chitinase and proteases that help break the host's chitinous exoskeleton (Ozdal et al. 2012). Mycotoxin (basis of their toxicity) production is dependent on a number of factors such as water humidity, substrate, strain of mold, temperature, the presence of chemical preservatives and microbial interactions (Chagas et al. 2000; Varga et al. 2005). Fungi are known to attack all life stages of fish including eggs, fry, fingerlings, adult and brood stock (Iqbal et al. 2012). Fungal infections can cause low production in fish culture and low productivity of fry (Kwanprasert et al. 2007). Unfortunately, most treatments using antibiotics for bacterial infections are generally ineffective. Accordingly, infected horseshoe crabs become inactive, anorectic and eventually die from the infection.

17.4 Conclusion

During the culture period, evidence of microbial infestation was observed, including bacteria (*Shewanella putrefaciens*, *Bacillus cereus*, *Corynebacterium* sp. and *Enterococcus faecalis*) and fungi (*Aspergillus* sp., *Aspergillus niger*, *Peniclillium* sp., *Gliocladium* sp.). Eggs infected with bacteria or fungi usually turned reddish, grey or black and ceased to develop. Larvae became passive and coated with black spore, and in some cases died soon after molting into the larval stage. The species with greatest potential to affect the growth and survival of horseshoe crab eggs and larvae sampled in this study were *S. putrefaciens* (a Gram-negative bacterium) and *A. niger* (a fungi). The species identified during this study were different from those previously identified on *Limulus polyphemus* in culture, suggesting possible species, location, and/or condition-specific variation in communities of infectious microbes brought in from the natural environment and propagated in culture. Infected horseshoe crabs, therefore, may require microbe-specific management or treatment strategies and additional study to ensure successful rearing in culture.

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