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Bacterial Septicemia in Juvenile Tiger Shrimp, *Penaeus monodon*, Cultured in Malaysian Brackishwater Ponds

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Abstract

Penaeid bacterial septicemia in juvenile market-size tiger shrimp, *Penaeus monodon*, on three brackishwater pond farms in Johore, Malaysia, is described. Histologically, tissue changes typical of experimental Gram-negative bacterial infections in marine crustaceans were observed in affected shrimp. *Vibrio alginolyticus, V. parahaemolyticus, Pseudomonas* sp., and other Gram-negative bacteria were isolated in low numbers from the hemolymph. The diagnosis and control of penaeid bacterial septicemia are discussed.

Introduction

Culture of marine shrimps in brackishwater ponds is an increasingly important segment of the Malaysian aquaculture industry. It is estimated that 114,000 ha are available for further brackishwater pond development (Abdul-Kadir 1986). With a large market in Japan and USA for frozen shrimp, it is expected that the 1987 Malaysian production of 640.34 tonnes of *Penaeus monodon*

(Anon. 1988), will continue to expand through growth in pond aquaculture. Malaysian shrimp culture systems are extensive or semi-intensive. The development of and transmission of infectious disease are encouraged in intensive culture systems (Lightner 1983, 1985). In the more extensive culture conditions, as found in Malaysia, there is lower disease prevalence. These systems allow shrimp access to natural foods to balance otherwise imperfect compounded diets and reduce individual shrimp interactions minimizing culture stress. Still, disease has been recorded in Malaysian shrimp farms (Anderson et al. 1987; Nash et al. 1988) and can lead to significant economic losses. Viruses, fungi, rickettsia, bacteria and protozoan parasites are all known to cause disease in marine shrimps (Lightner 1983, 1985; Lightner et al. 1985).

In Southeast Asia bacterial infections of shrimp cuticle (shell disease) and generalized septicemias have been observed in the Philippines, Indonesia and Thailand (Primavera and Apud 1977; Nurdjana et al. 1978; Ruangpan 1978). These bacterial conditions are considered to have a worldwide distribution (Johnson 1978; Lightner 1983, 1985). *Vibrio alginolyticus, V. parahaemolyticus, Vibrio* sp., *Pseudomonas* sp., *Aeromonas* sp. and *Flavobacterium* sp. are most commonly isolated from diseased shrimp (Lightner and Lewis 1975; Lightner 1983). This study reports a condition that involved mortalities in pond cultured tiger shrimp, *P. monodon*. These mortalities appeared to be associated with bacterial infections and significantly reduced production in shrimp farms in the State of Johore, Malaysia.

Materials and Methods

Material for this study was obtained during diagnostic visits to three shrimp farms in the State of Johore, Malaysia, during 1986 and 1987. Case histories were obtained by interviewing managers and pond workers. Affected and normal specimens were examined grossly, then dissected and fixed in Davidson's solution. Fixed tissue was stored in 50% ethanol, processed routinely for histology to 5 μm, sections were stained with hematoxylin and eosin, and examined microscopically. Selected sections were stained with Brown and Brenn's modification of Gram's stain (Luna 1968) and the periodic acid-Schiff's reaction. Anatomical and histological terminology used here was based on Young (1959) and Johnson (1980). Bacteriological

examination was done on hemolymph withdrawn from the cardiac hemocoele using a sterile needle and syringe, following surface disinfection with 95% ethanol. Primary isolation was attempted on marine agar or brackishwater (13 ppt) trypticase soy agar (BTSA). Subsequent identification of isolates followed the tables of Cowan (1974) and also utilized the API 20E microtest system.

Results

History and Clinical Examination

Low level, continuous mortalities of juvenile tiger shrimp were observed in three brackishwater shrimp farms in Johore, Malaysia. The farms all consisted of 0.4 to 1-ha earthen ponds constructed in mangrove areas and supplied by water pumped from surrounding mangrove rivers. Two farms stocked postlarvae (PL) 15 to 25 directly into the growout pond at rates of 25 to 35 $PL \cdot m^{-2}$. The other used a two-stage rearing method where on-farm nursed PL90 were stocked into growout ponds at rates of 15 to 35 $PL \cdot m^{-2}$. The farms would exchange 5 to 10% of pond water daily, with 20 to 50% replacement done when possible if the water condition was poor. Dissolved oxygen concentrations were maintained at 5 $mg \cdot l^{-1}$ or above by paddlewheel aerators. While one farm used by-catch fish occasionally, the shrimp were all fed commercial shrimp pellets at recommended levels (4-12% total pond biomass). Magnesium lime had been used in pond preparation to control hydrogen ion concentrations. On two farms malachite green or teaseed cake had been applied to affected ponds with no effect on mortality rates. The shrimp mortalities first occurred in the fourth production cycle on two farms and in the second in the other farm.

The pattern of mortalities and clinical signs was similar on all three farms. Mortalities would begin towards the end of the growout period as the shrimp approached market size (25-33 g), that is, two to three months after stocking as PL15 or 1.5-2 months after stocking as PL90. At the time of investigation, all ponds containing shrimp of this age were affected. In those affected ponds 0.5 to 7 kg of dead shrimp could be collected from the pond sides each morning. At harvest only 5 to 30% of the expected production was harvested and many shrimp were small and stunted. If the ponds were not harvested, the surviving shrimp would remain undersized and failed to reach

market size after five months of culture. At harvest, an abnormally thick accumulation of fine black detritus was present on the pond bottom, as were dead and putrefied shrimp.

Specimens caught by cast nets from affected ponds exhibited early signs of body reddening, extended branchiostegites (gill covers) and mild erosions of the uropods, pleopods and pereiopods. Often the erosions had a dark brown border. Shrimp were observed swimming lethargically near the water surface. Others were lying at the pond edge, not moving and had lost all escape reflexes. These severely affected specimens had a darkened cuticular color and a fuzzy coating over the carapace and abdomen. Less severely affected ones were pale and had lost their normal shiny translucence to become whitish-opaque in body color. Distinct black or dark brown areas were present in the cuticle of the carapace, abdomen, pleopods, pereiopods, uropods, telson, rostrum and antennae. Frequently this was associated with a marked erosion of antennae, pereiopods and uropods. Occasionally the black cuticular lesions on the carapace and abdomen were pitted in the middle. Gills were often brown in color. There was an absence of midgut contents or the midgut lumen was filled with a watery white liquid. On gross examination of internal organs at post mortem the foregut was empty and, in a few specimens, multiple small black spots were apparent throughout the Y-organ.

Histopathology

Histologically, all specimens examined had a mild to moderate fouling of exoskeletal surfaces with *Epistylis*-like peritrichous ciliates (Fig. 1), unicellular and filamentous algae, and pale brown granular debris. This surface fouling was more severe over gill filaments and the ventrum.

The anorexic state of the shrimp, indicated grossly by empty digestive tracts, was reflected in the hepatopancreas. Hepatopancreatocytes were poorly vacuolated, indicating low lipid and glycogen reserves. Cuticular and epidermal changes were present in all sections examined, though there was a wide variation in the severity and nature of these lesions from specimen to specimen.

Small foci of epidermal necrosis appeared to be the initial change; more usually the necrosis involved a larger area of epidermis. Here the occasional necrotic cell was observed in underlying

connective tissues and hemocytes had begun infiltrating into the affected epidermis. Varying degrees of melanization developed where infiltrated hemocytes had accumulated to the stage where distinct aggregations formed (Fig. 2). Small Gram-negative rod-shaped bacteria could be seen at the edge of these necrotic areas. Occasionally single small granulomas had developed in the subepidermal connective tissue. Melanized epidermal lesions were extensive in some specimens, with a tendency to be distributed in the ventral cuticle, pereiopods, mandibular pulp and scaphognathites (gill beaters). Often cuticular changes were associated with epidermal lesions. Irregular cuticular formation was a general change, but other lesions were restricted to the cuticle immediately above affected epidermis. The cuticle could also be thinner. Usually a migration of hemocytes into the endocuticle had occurred. Invariably these hemocytes were melanized (Fig. 3). Occasionally the epicuticle and exocuticle had ruptured, exposing melanized endocuticle. These most severe cuticular lesions usually showed slight bacterial surface fouling. Present in all shrimp were small bilateral melanized plaques on the inner surface of branchiostegites, which seemed to correspond to the area that was in contact with the scaphognathites.

Tissue changes, previously described as associated with bacterial infections, were observed in the heart, gills, hepatopancreas, antennal gland and connective tissues. Hypertrophied fixed hemocytes with single intracytoplasmic vacuoles containing fine, pale granular material were very prominent on myocardial fibers (Fig. 4a). Hemocyte nests and, less commonly, aggregations were present in the heart and peripheral interstitial tissues of the hepatopancreas (Fig. 4b). At times, hemocytes in nests in these organs contained intracytoplasmic Gram-negative rod-shaped bacteria. At lower intensities, hemocyte nests and aggregatives were also found in gill filament and branch hemal channels, antennal glands and connective tissue surrounding the foregut, heart and below the epidermis. More rarely, gill filaments had a distinct hemocyte infiltration that involved necrosis of filament epithelium and melanization (Fig. 5).

Cell cord degeneration and necrosis were present in the majority of Y-organs. These changes appeared initially as cloudy swelling or hyaline degeneration of cell cord epithelial cells, followed by a mild to marked hemocyte infiltration of cord and interstitial tissue. Advanced lesions with caseation of individual cell cords, showed cord shrinkage, an increased basophilia and scattered karyorrhectic nuclear debris. In some specimens, necrotic cell cords had become compact melanized

granulomas (Fig. 6). These granulomas occasionally contained Gram-negative bacteria and were usually associated with a fibrosis of surrounding interstitial tissue. One specimen had a marked diffuse hemocyte infiltration, edema and fibrocyte proliferation in interstitial tissue associated with a generalized spread of Gram-negative bacteria throughout the Y-organ. From 10 to 60% of cell cords in affected Y-organs would exhibit the above pathological changes.

A number of specimens had more marked hepatopancreatic changes. Here, particularly adjacent to the midgut, interstitial tissues were edematous and had an obvious hemocyte infiltration, as well as an increased number of hemocyte nests (Fig. 7a). Frequently scattered necrotic hemocytes were present in these areas. At times, in these hepatopancreases there was a large number of sloughed hepatopancreatocytes in large tubule, duct and midgut lumens. Rarely, there was a proliferation of Gram-negative bacteria in the lumen of peripheral hepatopancreatic tubules, with epithelial degeneration or coagulative necrosis of the entire tubule.

Other tissue changes were noted much less frequently. Three specimens from two farms had a generalized cloudy swelling degeneration of antennal glad labyrinth epithelium. In affected labyrinths a fine eosinophilic precipitate was present in the organs' lumen (Fig. 7b). Moderately large foci of striated muscle necrosis were infrequently apparent in abdominal muscles. Seventeen per cent (4/23) of shrimp examined from one farm had low numbers of oval eosinophilic intranuclear occlusions in hepatopancreatocytes, indicating a mild *P. monodon* baculovirus (MBV) infection.

Bacteriology

In all affected specimens sampled on the three farms a low or moderate growth of bacteria was obtained from the hemolymph. Based on the morphological and biochemical characteristics the bacteria were considered to resemble most closely: in farm 1, *Vibrio alginolyticus* and *Pseudomonas* sp.; in farm 2, *Vibrio* sp. (most probably *V. alginolyticus*); in farm 3 diseased shrimp, *V. alginolyticus, V. parahaemolyticus, Pseudomonas* sp., *Alcaligenes* sp., and *Moraxella* sp.; and in farm 3 healthy shrimp, *Alcaligenes* sp., *Moraxella* sp. and *Flavobacterium* sp.

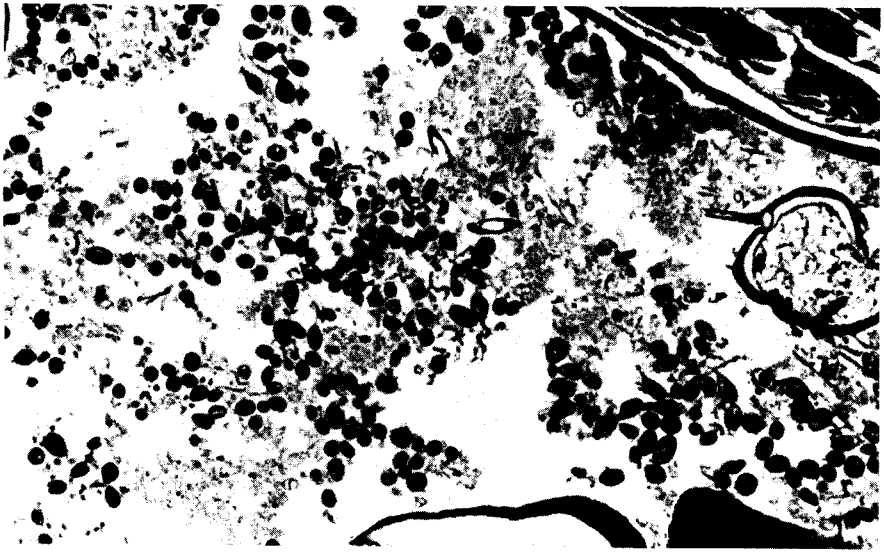

Fig. 1. Epibiont fouling of ventral surface of shrimp with symptoms of bacterial septicemia. H&E, 75 x.

Fig. 2. Epidermal inflammation with hemocytic infiltration and melanization. Note intact cuticle. H&E, 375 x.

Fig. 3. Thinning and infiltration of melanized hemocytes in endocuticle. An increased number of hemocytes are present in sub-epidermal connective tissue. H&E, 188 x.

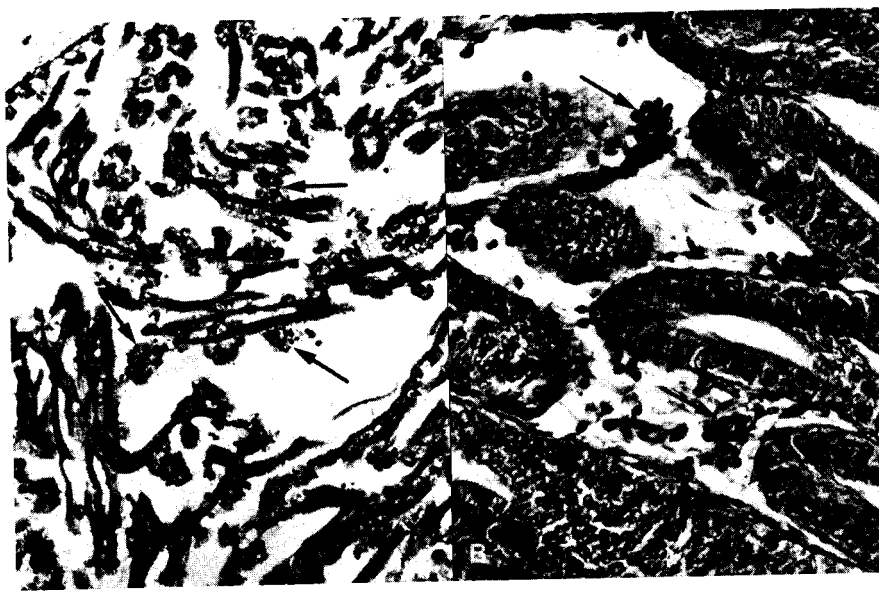

Fig. 4a. Groups of hypertrophied hemocytes on myocardial fibers (arrowed). H&E, 125 x.
Fig. 4b. Hemocyte nests (arrowed) in interstitial tissues of the hepatopancreas. H&E, 250 x.

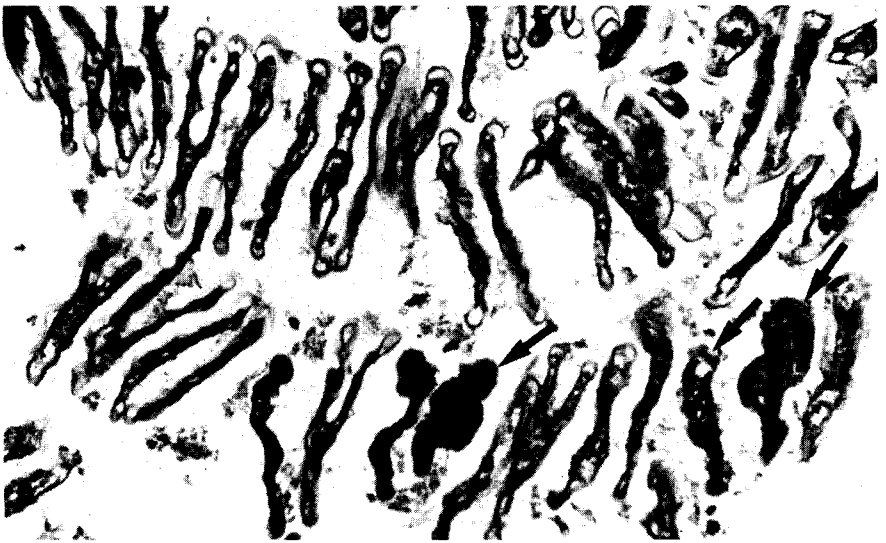

Fig. 5. Hemocyte infiltration, necrosis and melanization in gill filaments (arrowed). H&E, 188 x.

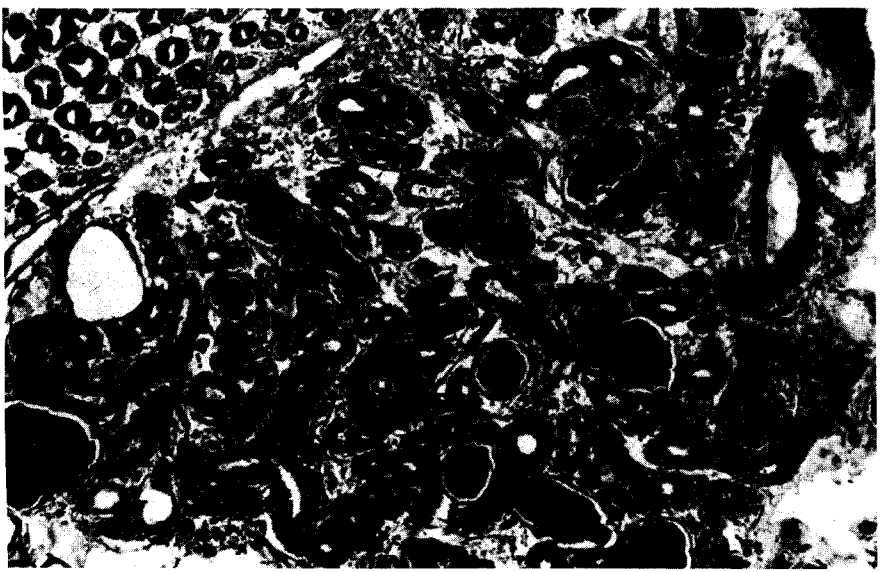

Fig. 6. Sclerotic and compact melanized cell cords in the Y-organ. H&E 75 x.

Fig. 7a. A marked edema and hemocyte infiltration of hepatopancreatic interstitial tissues. H&E, 125 x.

Fig. 7b. Cloudy swelling degeneration of antennal gland labyrinth epithelium. H&E, 125 x.

Discussion

Initial observations of affected shrimp lead to a tentative diagnosis of shell disease. Shell disease, an infection of the cuticle by chitinoclastic bacteria (Cook and Lofton 1973; Cipriani et al. 1980), is diagnosed on gross signs of brownish eroded areas of cuticle, often beginning as small circular spots (Lightner 1977). Subsequent histopathological examination did not reveal epicuticular erosions characteristic of shell disease in marine crustaceans. Tissue changes previously associated with experimental crustacean bacterial infections, the presence of intracytoplasmic Gram-negative rod-shaped bacteria in hemocytes and to a lesser extent, the isolation of *Vibrio alginolyticus, V. parahaemolyticus* and *Pseudomonas* sp., from hemolymph lead us to diagnose the condition as bacterial septicemia (or vibriosis).

Gross signs exhibited by shrimp examined in this study, including the melanized cuticular lesions, are the same as those described from previous studies on penaeid bacterial septicemias (Lightner and Lewis 1975; Delves-Broughton and Popuard 1976; Lightner 1977). Additionally we observed epibiont fouling of the cuticle (the fuzzy coat over the carapace and abdomen) and gills (brown coloring) in moribund shrimp. The gross signs other workers and ourselves observed are nonspecific, merely indicating a moribund state in diseased shrimp. For example hypoxia (Lightner and Lewis 1975), protozoan epibiont fouling (Lightner 1977), MBV (Lightner et al. 1983) and rickettsial infections (Anderson et al. 1987) can cause similar gross signs as those recorded in bacterial septicemia. Consequently a definitive diagnosis of penaeid bacterial septicemia is considered to depend on the demonstration of numerous Gram-negative motile rod-shaped bacteria in shrimp hemolymph (Lightner 1977, 1983; Sparks 1981). With the exception of *Alcaligenes* sp. and *Moraxella* sp., bacteria isolated from affected shrimp in this study are typical of those isolated from shrimp exhibiting clinical signs of bacterial septicemias (Barkate 1972; Lightner and Lewis 1975; Delves-Broughton and Poupard 1976; Lightner 1983). But our isolations grew in low numbers, similar to levels obtained from healthy shrimp hemolymph. Low numbers of bacteria, including those strains known to cause disease, may be isolated quite normally from apparently healthy shrimp hemolymph and tissues (Vanderzant et al. 1971; Lightner 1977). Thus this light growth of bacteria from hemolymph, and the absence of bacteremias histologically, failed to

fully support a classical diagnosis of bacterial septicemia. Lightner and Lewis (1975) also observed that hemolymph drawn from moribund shrimp with bacterial septicemia had much delayed clotting times and was slightly turbid. This was not present in shrimp examined in this study.

Histologically, adhesion of hemocytes in groups, hemocyte nests and aggregations have been described in experimental Gram-negative bacterial infections in the blue crab, *Callinectes sapidus* (Johnson 1976), shore crabs, *Carcinus maenas* (Smith and Ratcliffe 1980) and lobsters, *Homarus americanus* (Johnson et al. 1981). Hemocyte clumping would be detected in the heart most commonly, followed by the gills, antennal gland, Y-organ and hepatopancreas (Johnson 1976; Johnson et al. 1981). A few bacteria were present at various times after infection in hemocyte nests in the heart and gills, and in hepatopancreas interstitial tissue hemocytes (Johnson 1976). Other tissue changes in the blue crab included necrosis and sloughing of hepatopancreatocytes, extensive focal necrosis of the Y-organ and antennal gland epithelial degeneration (Johnson 1976). Delves-Broughton and Poupard (1976) observed phagocytic activity and bacteria in muscle, gill and hepatopancreas in bacterial septicemia of *P. monodon* in recirculation systems. The histological changes present in naturally infected shrimp in this study follow closely those in experimental infections in other marine crustaceans. Most notable in this study were the prominent hypertrophied hemocytes in hearts and the melanized granulomas in some Y-organs, grossly apparent as small black spots.

Our failure to obtain heavy bacterial growth from hemolymph, in shrimp with gross signs of a bacterial septicemia and histological lesions characteristic of experimental bacterial infections, suggests that the demonstration of numerous Gram-negative motile rod-shaped bacteria in hemolymph is an insensitive test to confirm a diagnosis of bacterial septicemia in shrimp. A heavy growth of bacteria probably only occurs in shrimp near death when all physiological homeostasis is lost. Similarly, Johnson et al. (1981) found that the limit of detection of bacteria by histological means is between 10^6 and 10^7 bacteria per gram of tissue, much higher numbers than one would expect a virulent strain to be at before their endotoxins are sufficient to cause cell necrosis. Much more sensitive, and the evidence we used to confirm the diagnosis, is the presence of hemocyte nests, and aggregations, particularly in the heart and hepatopancreas, and the infrequent hemocytic intracytoplasmic Gram-negative rods in tissue sections.

Not present in experimental infections, but the most common tissue changes noted in these natural infections were in the epidermis and cuticle. The epidermal and cuticular lesions are unusual and have not been previously described. Grossly the melanized cuticular spots were typical of shell disease. Histologically there was no erosion of outer cuticle; instead the lesion appeared to develop from the epidermal epithelium, then extend to the overlying endocuticle or cause malformation of new cuticle. This is not typical of shell disease histopathology in marine crustaceans (Rosen 1970). The pathogenesis of epidermal necrosis was not clear, though hemocytic infiltration and the occasional bacteria present at the edge of necrotic foci imply that it is of bacterial etiology. In our opinion, bacteria entered via small cracks in the cuticle or via intact exo- and endocuticle following removal of the protective waxy epicuticle. Sparks (1981) also considers small cuticular wounds the likely portal or entry of bacteria in bacterial septicemias. Bacteria then spread from these primary foci to other tissues via circulating hemolymph.

Penaeid bacterial septicemias are secondary infections that follow severe stress, cuticular injury or weakening of shrimp immunity due to other diseases (Lightner 1977, 1983). The increased density of aquatic organisms in aquaculture allows the adaptation and selection of virulent strains of opportunist pathogens already present in the aquatic environment (AQUACOP 1979). It is interesting to note the tendency, in this study, of bacterial septicemia occurring after three production cycles. This would have provided time for virulent strain selection to occur in the pond environments. In these farms, the buildup of pond bottom detritus appeared to be the origin of primary stressors which allowed successful infection by opportunist pathogens. Excessive accumulation of feces and waste food results in deteriorating water quality as a sub-benthic anaerobic layer forms with an associated production of hydrogen sulfide and other toxic substances (Hanson and Goodwin 1977). Another notable aspect of these mortalities was that they began towards the end of growout cycles when pond biomass approaches maximum. At this time shrimp are much more likely to be stressed as oxygen demand is greatest, excretion of nitrogenous metabolites from shrimps is high and significant amounts of organic waste will have accumulated on the pond bottom.

Successful treatment of bacterial septicemia in juvenile shrimp has been reported by addition of antibiotics to the diet (Delves-Broughton 1974; Lightner and Lewis 1975; Lightner 1977; Lightner

et al. 1984). In Malaysia it is not economical to attempt chemotherapy in semi-intensive brackishwater pond culture, particularly in these conditions where a primary husbandry fault existed and would continue to exist after treatment. It was recommended that partial or total harvesting start (to reduce pond biomass) and that daily water exchange be increased once mortalities begin or in the last four weeks of pond culture. Most successful in preventing mortalities in subsequent production cycles were changes in pond preparation. In addition to the usual draining and drying of ponds until the bottom cracked, excessive detritus was physically removed and quicklime (CaO) was applied at the rate of 0.5 $kg \cdot m^{-2}$ of pond. These steps removed any sub-benthic anaerobic layer that would pollute the water and disinfected the pond bottom. Subsequent production from the farms was good and mortalities caused by bacterial septicemias ceased.

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