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Experimental infection of Pacific white shrimp *Litopenaeus vannamei* with Necrotizing Hepatopancreatitis (NHP) bacterium by per os exposure

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ABSTRACT: Necrotizing Hepatopancreatitis Bacterium (NHPB), which causes Necrotizing Hepatopancreatitis, was successfully transmitted in individually isolated Kona stock *Litopenaeus vannamei* through per os exposure. Animals (140) were individually exposed orally to a 0.05 g piece of an NHPB-infected hepatopancreas and 120 control animals were each exposed to a 0.05 g piece of NHPB-negative hepatopancreas. Shrimp were maintained in Sterilite® containers with approximately 4 l of artificial seawater at 30‰ salinity and 30°C for 60 d. Mortality of infected shrimp was observed from Day 16 to Day 51 post-exposure. Infected animals sustained reduced feeding activity and displayed empty guts. Some infected animals developed a pale hepatopancreas noticeable through the carapace. Survival probabilities fit a Weibull distribution and parametric survival analysis revealed lowered survival due to NHPB infection. Median survival time of NHPB-infected animals was 34.5 d. After correcting for background daily mortality in the controls, mean acute daily mortality of NHPB was estimated at 0.09, a value much lower than that estimated for other diseases in Kona stock *L. vannamei* such as White Spot Syndrome Virus (0.40) and Taura Syndrome Virus (0.30). A chronic, or carrier, state was not demonstrated in NHPB epizootics because all NHPB-positive animals experienced mortality and no animals surviving to 60 d post-exposure were diagnosed NHPB-positive through PCR or histology.

KEY WORDS: Necrotizing Hepatopancreatitis · NHP · Shrimp disease · Shrimp aquaculture

INTRODUCTION

First identified in Texas in 1985, Necrotizing Hepatopancreatitis (NHP) is a severe bacterial disease of cultured penaeid shrimp that produces significant pathology and up to 95% mortality in affected ponds (Johnson 1990). Since 1985, NHP has been observed in Peru, Ecuador, Venezuela, Brazil, Panama and Costa Rica. NHP is also known as Granulomatous Hepatopancreatitis, Texas Necrotizing Hepatopancreatitis, Texas Pond Mortality Syndrome and Peru Necrotizing epatopancreatitis. Species affected by NHP are *Litopenaeus vannamei*, *L. setiferus*, *L. stylirostris*, *Farfantepenaeus aztecus* and *F. californiensis* (Lightner 1996).

The causative agent of NHP is the Gram-negative, pleomorphic, obligate intracellular pathogen NHP Bacterium (NHPB), a member of the α-Proteobacteria initially described as 3 microorganisms: a pleomorphic rod-shaped rickettsia-like bacterium, a helical form of a mollicute-like bacterium and a filamentous mollicute bacterium (Krol et al. 1991). Further morphological analysis produced evidence for a single bacterium present in 2 distinct forms (Frelier et al. 1992, Lightner et al. 1992). The predominant form is rod-shaped, rickettsia-like, lacks flagella and occasionally exhibits a transverse constricted zone indicative of replication by binary fission (Frelier et al. 1992). The helical form possesses 8 flagella on the basal apex and 1 or possibly 2 flagella on the helix crest. Both variants of NHPB are

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small; the rod-shaped form is 0.25 by 0.9 µm and the helical form is 0.25 by 2 to 3.5 µm. An indistinct, intermediate form that exhibits morphology of both variants is observed occasionally in infected hepatopancreas epithelial cells, which suggests development from the replicative rod-shaped form into the helical form (Lightner et al. 1992).

Gross signs of NHPB infection in penaeid shrimp include reduced feed intake, empty guts, soft shells, flaccid bodies, heavy surface fouling by epicommensal organisms, black or darkened gills, chromatophore expansion giving the appearance of darkened edges on pleopods, lethargy and marked atrophy of the hepatopancreas. The shrimp hepatopancreas is the only site of infection for NHPB. In shrimp infected by NHPB, the hepatopancreas may appear pale with a whitish center rather than the normal tan to orange color. In some severe NHPB infections, the hepatopancreas may be pale with black streaks, indicating melanization of hepatopancreatic tubules, and the hepatopancreas may appear soft and watery with a fluid-filled center (Lightner 1996).

Environmental factors such as temperature and salinity are thought to greatly influence the occurrence of NHPB disease in penaeid shrimp aquaculture. Outbreaks of NHPB in Texas followed temperatures greater than 29 to 31°C in salinities of 20 to 40‰ (Frelier et al. 1992). In 1993, an epizootic of NHPB in Peru followed temperatures greater than 29 to 35°C in salinities of 20 to 38‰ (Lightner & Redman 1994). In addition, conditions similar to those encountered in Texas and Peru were associated with outbreaks of NHPB from 1993 to 1995 in Venezuela, Brazil, Ecuador, Costa Rica and Panama (Lightner 1996).

Experimental transmission of NHPB has been achieved through injection of an isolate of the rod form of the bacterium (Frelier et al. 1993). Attempts to culture NHPB through traditional bacteriological methods have been unsuccessful (Lightner 1996, Braasch et al. 1999); thus, experimental exposure systems have relied upon maintenance of NHPB in live shrimp with injection for transmission. NHPB is not likely to be transmitted in nature by injection; therefore, transmission through the ingestion of infectious material, i.e. cannibalism of dead or dying infected shrimp, may be a more natural route.

The purpose of our study was to develop a more natural exposure protocol by attempting per os transmission of NHPB to *Litopenaeus vannamei*. We describe the per os exposure system, document successful oral exposure and characterize the infection course in laboratory oral exposure. In addition, we test the null hypothesis assumed in epidemic models that the mortality rate is constant for infected animals over time.

MATERIALS AND METHODS

Long-term maintenance of NHPB to *Litopenaeus vannamei*. Since June 2001, NHPB has been maintained long-term in aerated cylindrical tanks that are 183 cm in diameter and 76 cm in height containing 25 cm depth of artificial seawater at 30 ± 2‰ salinity and 30 ± 2°C. The cylindrical tanks are located in a greenhouse and temperature is monitored weekly and salinity biweekly. NHPB was obtained from a shrimp farm in south Texas, USA, in June 2001. Initially, tanks were stocked with approximately 50 susceptible certified Specific Pathogen Free (SPF) *L. vannamei* juveniles ranging in size from 3 to 15 g obtained from the Oceanic Institute in Hawaii and fed commercial pellets daily. This stock, which originated as post-larvae from wild spawns in Sinaloa, Mexico, in 1989, is part of the United States Marine Shrimp Farming Program (USMSFP) and is referred to as USMSFP Kona stock (Pruder et al. 1995, Lotz et al. 2003).

Cephalothoraxes of shrimp infected with NHPB were removed by a transverse cut at the abdomen/cephalothorax junction to insure access to the hepatopancreas (site of NHPB infection) and were placed in the tank with susceptible animals. Susceptible shrimp starved for 3 d were added to the tank approximately every 21 to 28 d. Susceptible shrimp feed upon moribund and dead infected shrimp, continuing the cycle of exposure and infection.

Challenge model of *Litopenaeus vannamei* to NHPB. SPF Kona stock *L. vannamei* juveniles of average size 5.62 g (range 3.00 to 9.27 g) were individually placed in aerated Sterilite® containers (35 cm length by 22 cm width by 12 cm height) and maintained at a depth of 9 cm of 30 ± 1‰ artificial Crystal Sea® Marinemix seawater prepared from reverse osmosis filtered water for a total volume of approximately 4 l. Hepatopancreas excised from NHPB-infected shrimp from the long-term maintenance system were weighed to the nearest 0.01 g and divided into approximately 0.05 g sections, which were introduced into the Sterilite® containers. Susceptible shrimp were starved for 3 d before per os exposure to NHPB.

In Challenge I, 20 shrimp were each exposed orally to a 0.05 g piece from an NHPB-infected hepatopancreas. Twenty control shrimp were exposed similarly to hepatopancreas from NHPB-negative tissue. In Challenge II, 120 shrimp were each exposed orally to 0.05 g of NHPB-infected hepatopancreas and 100 control shrimp were similarly exposed to non-infectious material. In both challenges, shrimp ingested the 0.05 g pieces of hepatopancreas within 1 h. Shrimp were maintained in a waterbath at 30 ± 1°C for 60 d and fed commercial pellets at approximately 5% body weight every other day. Temperature was monitored daily and

salinity weekly. Freshwater was added only to replace water lost by evaporation.

Diagnostic testing. Moribund shrimp were processed for molecular and histological examination of NHPB infection. A small piece of the hepatopancreas to be used for PCR was excised from the cephalothorax of each moribund shrimp and stored in a 1.5 ml Eppendorf tube at –20°C. Davidson's AFA (alcohol-formalin-acetic acid) fixative was injected into the cephalothorax to ensure thorough fixation of the remaining hepatopancreas for histological analyses and then immersed in preservative for 48 to 72 h. Afterwards, the cephalothorax tissues were cut along the sagittal plane, placed in Tissue-Tek® uni-cassettes and stored in 70% ethanol prior to paraffin embedding, sectioning and staining with Hematoxylin and Eosin (Lightner 1996). Tissues from the animals that were alive at 60 d were preserved in the manner described above. PCR analysis of hepatopancreas followed the protocol of Loy et al. (1996). After DNA extraction and purification, samples were diluted 1:100 and analyzed by a PCR reaction specific for the NHPB (Loy et al. 1996, DiagXotics® 2002).

Statistical analysis. Survival analysis and curve fitting were performed with Systat 10.0 and SPSS 11.5 (SPSS 2002). Survival time is broadly defined as the time to the occurrence of a given event (Lee & Wang 2003), in this case death due to NHPB infection. Given that survival times are subject to random variations, the resulting distribution can be described by a survivorship probability function. Censored data occur when some of the subjects in a study have not experienced the event of interest by the end of the study time or by removal early from the study. In Challenges I and II, Type I censoring occurred when animals were observed for a fixed period of time (60 d), after which the surviving animals were killed. In Type I censoring, the survival times of those animals that survived the experiment are not known exactly but are recorded as at least the length of the study period. Challenge II also included Type I censored observations removed from the study at regular intervals for time-course analysis of NHPB infection. The survival time of these animals is known only to the day of removal from the experiment.

A Weibull distribution was applied to survival and age-specific mortality probabilities. The survival (Eq. 1) and hazard or age-specific mortality function (Eq. 2) of a Weibull distribution include the parameters β (scale), η (shape) and γ (location) at given t (time). Acute state mortality in infected shrimp was corrected for any mortality in control animals (Eq. 3).

$$s(t) = e^{-\left(\frac{t+\gamma}{\eta}\right)^{\beta}} \quad (1)$$

$$h(t) = \frac{\beta}{\eta^{\beta}} \cdot (t+\gamma)^{\beta-1} \quad (2)$$

$$\mathrm{NHPB}_{\alpha} = \frac{h(t)_{\mathrm{Control}} - h(t)_{\mathrm{NHPB}}}{h(t)_{\mathrm{Control}} - 1} \quad (3)$$

Soto & Lotz (2001) suggested that the pathogen-induced mortality rate (α) and the recovery rate (ρ) could be estimated from the following 2 equations describing the dynamics of infected and recovered animals:

$$I_{t+1} = I_t - I_t(\alpha + \rho - \alpha \cdot \rho) \quad (4)$$

$$R_{t+1} = R_t + I_t(\rho - \alpha \cdot \rho) \quad (5)$$

where I is the number of infected animals, R is the number of animals that recover, t is time, α is the rate of mortality and ρ is the rate of recovery of an infected animal. Mathematical models assume α and ρ are constant throughout an epidemic. This equation system allows for estimation of α and ρ by iterating the parameters until the resultant survivorship curve best fits the observed data on infected animals. If there is no recovery of infected animals, then ρ = 0 and Eq. (4) can be estimated by:

$$I_{t+1} = I - I(\alpha) \quad (6)$$

RESULTS

Transmission

A combination of 2 challenges using identical methods for oral exposure of *Litopenaeus vannamei* resulted in successful transmission of NHPB. Challenge I consisted of 20 shrimp, while Challenge II included a larger sample of 120 shrimp (Table 1). Challenge I had a much higher percentage of exposed shrimp that tested NHPB-positive through PCR (60%) than Challenge II (20%) (χ^2 = 14.359, $p < 0.001$, Table 1).

Table 1. Two per os exposure challenges of Kona stock *Litopenaeus vannamei* to NHPB (% NHPB-positive after 60 d, χ^2 = 14.359, $p < 0.001$)

Challenge	# exposed to NHPB	% NHPB-positive after 60 d	Median survival time (d) over 60 d	Days of observed mortality
I	20	60	38.5	18 to 46
II	120	20	34.7	16 to 51
I and II	140	26	34.5	16 to 51

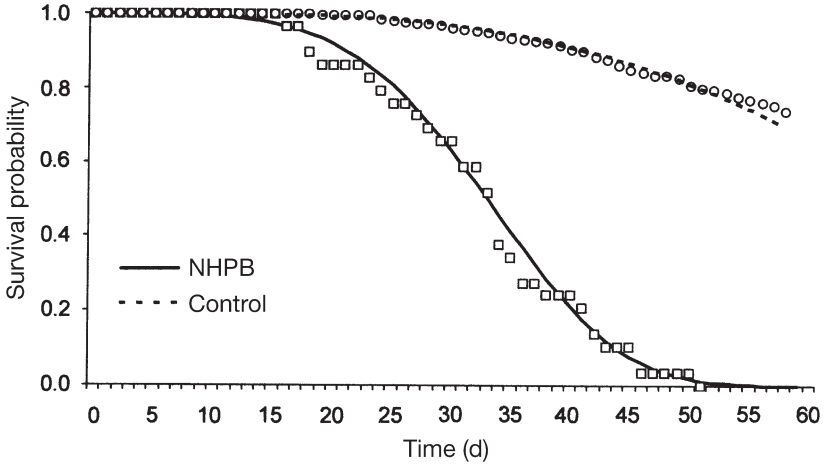

Fig. 1. Survival curve of Kona stock *Litopenaeus vannamei* exposed per os to Necrotizing Hepatopancreatitis Bacterium (NHPB) and controls ($p < 0.001$). □: NHPB; ○: control survival; solid line: Weibull survival function best fit to NHPB survival; dashed line: function fit to control survival. There is a significant difference between the 2 survival curves

Gross signs of NHP infection

The first clinical sign of NHPB infection was the cessation of feeding, which occurred from 9 to 26 d post-exposure. Empty guts observed from 15 to 40 d post-exposure were followed by mortality of NHPB-infected shrimp 1 to 7 d later. Other signs of infection included a grossly pale hepatopancreas that was visible through the carapace several days before mortality.

Survival curves

The survival probabilities of NHPB-infected shrimp and control shrimp were fitted to a Weibull distribution that allows for a more powerful parametric analysis than the nonparametric Kaplan-Meier analysis (Lee & Wang 2003). Weibull parameters estimated for NHPB-infected shrimp are $\beta = 4.33$, $\eta = 37.02$ and $\gamma = 1$. Estimated Weibull parameters for control shrimp are $\beta = 3.44$, $\eta = 74.14$ and $\gamma = -2$.

Survival analysis comparing the survival curves for only the NHPB-positive shrimp in Challenges I and II revealed no difference; therefore, data from both challenges were pooled (Table 1). Mortality due to NHPB began 16 d post-exposure and ended 51 d post-exposure (Fig. 1). Control shrimp experienced a background mortality beginning at Day 19. The median survival time of NHPB-positive shrimp (i.e. time to death of 50%) was 34.5 d, while the median survival time of control shrimp was greater than 60 d. The survival curves generated for NHPB and control animals were significantly different, with lower survival observed in NHPB-positive ($p < 0.001$, Fig. 1).

Survival curve and course of infection

Fig. 2 shows a hypothetical survivorship curve with 3 distinct phases that correspond to prepatent, acute and chronic states of infection. The transition from one state to the next is marked by a change in mortality rate. The prepatent state or incubation period is characterized by a period of asymptomatic disease and a daily survival rate similar to uninfected hosts. During the prepatent state, the pathogen is multiplying. The prepatent infection state begins immediately following exposure to the pathogen and continues until an increase in daily mortality is observed. This point marks the beginning of the acute state. Overt clinical signs of disease and mortality characterize the acute state. In the hypothetical infection, the acute state lasts until a decrease in mortality rate occurs. Chronic state infection occurs when an infected animal does not die from the infection but remains a carrier of the disease. Not all infectious agents have a chronic state.

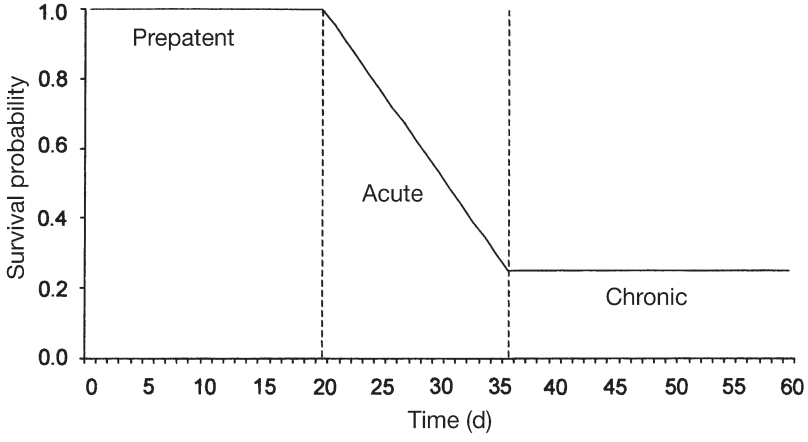

Fig. 2. Course of infection including prepatent, acute and chronic disease states in a hypothetical survival curve. Vertical dashed lines mark points where mortality changes, signifying transition from one infection state to the next on the survival curve. Prepatent and chronic states are characterized by low mortality, whereas the acute state is characterized by high mortality

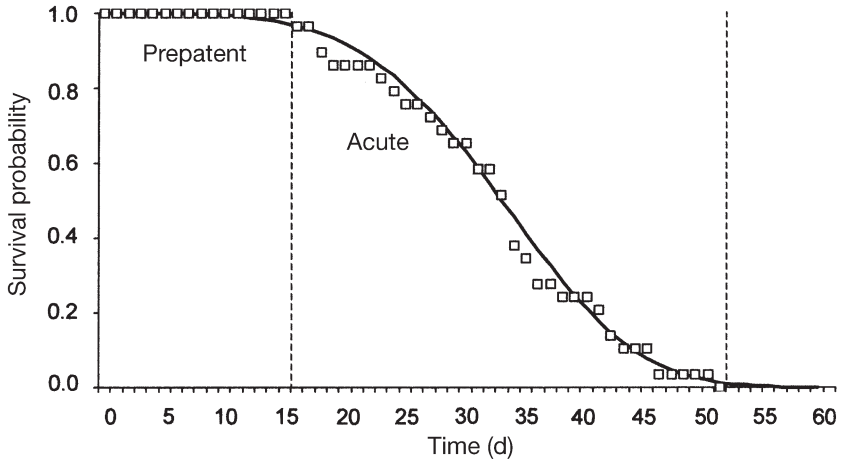

Fig. 3. Course of infection with prepatent and acute disease states in relation to survival curve of Kona stock *Litopenaeus vannamei* exposed per os to NHPB. □: NHPB survival; solid line: Weibull survival function best fit to NHPB survival. Disease states prepatent and acute are separated by a vertical dashed line

We observed only prepatent and acute states for the course of infection in *Litopenaeus vannamei* exposed orally to NHPB (Fig. 3). The prepatent state or incubation period of NHPB began after exposure and lasted to 15 d post-exposure. Mortality, and thus the acute state, began at 16 d post-exposure and continued to 51 d post-exposure. During the acute state, shrimp experienced an elevated daily probability of mortality. However, no subsequent decrease in mortality was noted. Of the exposed shrimp alive at the end of the 60 d period, none were diagnosed as NHPB-positive through PCR. It is possible that some shrimp alive at 60 d post-exposure that tested negative by PCR included those that once carried an NHPB infection but had since lost the infection. However, histological analysis in those shrimp alive at 60 d post-exposure did not reveal any clinical sign of past infection. Within our limits of detection, no NHPB-infected shrimp were considered to have either lost infection or remained a carrier of NHPB infection. Thus, a chronic state was not detected and the recovery rate (ρ) is considered to be 0.

Mortality rate in acute infection state

The daily mortality rate during the acute state of NHPB infection was not constant but increased over time. The probability of death at the beginning of the acute state was lower than that near the end. The age-specific mortality probabilities are shown in Fig. 4. Although epidemic models assume a constant rate of acute mortality, the observed rates were not constant over time. However, for modeling purposes, the mean of the acute state daily mortality can be used as an estimate of NHPB acute state daily mortality. After correcting for background mortality, the adjusted mean acute state daily mortality from NHPB was 0.09.

DISCUSSION

The long-term maintenance and challenge models of NHPB in *Litopenaeus vannamei* through per os exposure were documented. The long-term system provides for a continuous supply of NHPB infections for experiments. The challenge model allows short-term observation of NHPB infection in susceptible animals from a single transmission event under controlled conditions. Although background mortality was observed, we were able to remove background mortality from estimates of NHPB-induced acute state daily mortality.

Due to the lack of *in vitro* culture methods for NHPB, *in vivo* maintenance is necessary. NHPB infection is maintained continuously *in vivo* by adding naïve susceptible individuals to a population of shrimp experiencing NHPB disease. In this long-term maintenance system, the number of NHPB infections decreases in the population either from removal of infected animals

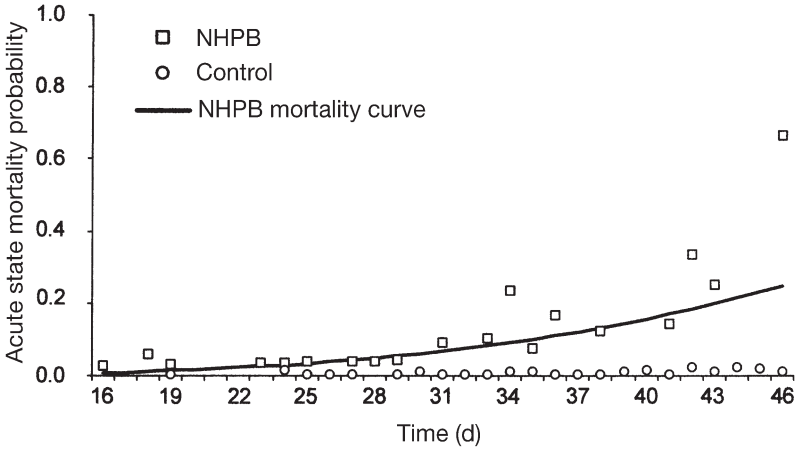

Fig. 4. Probability of daily mortality during acute infection state in Kona stock *Litopenaeus vannamei* exposed per os to NHPB and controls. □: NHPB; ○: control daily mortality; solid line: the NHPB mortality curve generated from best fit of the Weibull hazard function. The estimated average daily mortality from NHPB infection is $\alpha = 0.09$

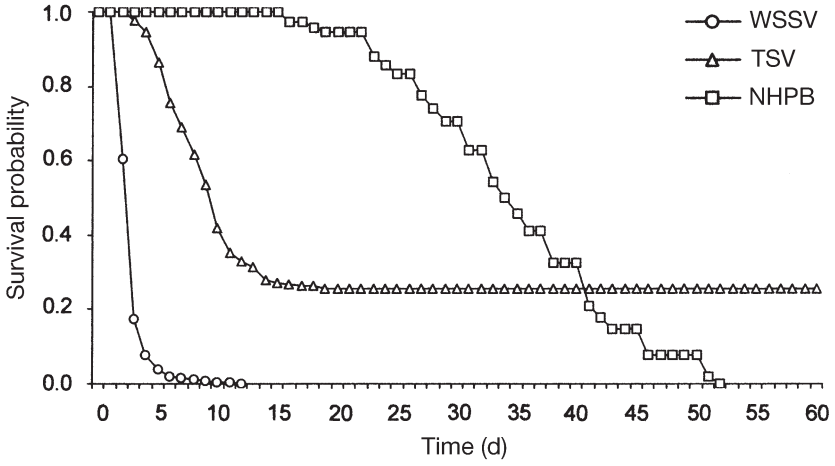

Fig. 5. Comparison of NHPB survival curve to those generated for the viral pathogens WSSV (White Spot Syndrome Virus) and TSV (Taura Syndrome Virus) in Kona stock *Litopenaeus vannamei*. NHPB survival generated in our study is also shown. Data for WSSV and TSV from J. M. Lotz (unpubl.)

through cannibalism or for experimental purposes. This presents difficulty in obtaining a large amount of infectious material. In our 2 challenges, a difference was observed between the percent of exposed shrimp that tested NHPB-positive through PCR, with a higher percent NHPB-positive in Challenge I than in Challenge II. The larger amount of NHPB infectious material needed for Challenge II may have caused heavily infected material to be diluted with lightly infected material in our attempt to expose a larger number of shrimp. Thus, the difference in percent of NHPB-positive shrimp between the 2 challenges may have been related to dose.

Epidemic models assume constant mortality over time for each state of infection. However, the probability of mortality associated with the acute state of NHPB was not constant and in fact increased over time. The development of epidemic models incorporating this observed change in mortality rate over time may provide a better predictive model of disease dynamics.

The course of an NHPB infection in Kona stock *Litopenaeus vannamei* exhibited different dynamics than other virulent infectious diseases of shrimp such as White Spot Syndrome Virus (WSSV) and Taura Syndrome Virus (TSV) (Soto & Lotz 2001, Lotz & Soto 2002, Lotz et al. 2003). In comparison with WSSV and TSV, NHPB exhibited a much longer prepatent state that is indicative of slower development and a longer time to reach a lethal infection than either WSSV or TSV (Fig. 5). WSSV (2 to 11 d post-exposure) and TSV (3 to 18 d post-exposure) exhibit relatively short acute states of infection (Soto & Lotz 2001, Lotz & Soto 2002, Lotz et al. 2003) whereas the acute infection in NHPB begins nearly 13 d later and lasts over 20 d longer than the acute state of either WSSV or TSV. In addition, WSSV and TSV produce lethal infections at a faster rate than NHPB.

The TSV survival curve reaches a point where daily survival probabilities of infected shrimp increase, indicating the transition from the acute to the chronic state of infection (Fig. 6). Animals in the chronic state are considered survivors of TSV infection; however, these survivors remain carriers and are sources for infection of susceptible animals. The acute state of WSSV and NHPB remains until all infected animals are dead. Therefore, in our laboratory work, neither WSSV nor NHPB infections contain a significant chronic or carrier state and all infected animals succumb to disease.

The absence of a chronic state may be a characteristic of NHPB infection in *Litopenaeus vannamei*. A chronic state is observed in *L. vannamei* infected with TSV but is rare or absent in WSSV and NHPB infections. The absence of a chronic state in NHPB infection suggests disease dynamics in regard to infection states that are more similar to that of WSSV than TSV.

Cannibalism of dead and dying shrimp infected with NHPB appears to be a natural mode of transmission in

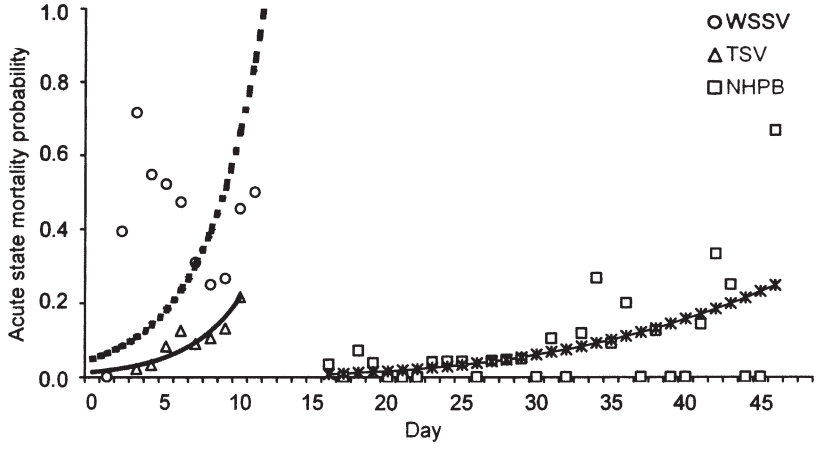

Fig. 6. Comparison of NHPB acute state daily mortality to that of the viral pathogens WSSV and TSV in Kona stock *Litopenaeus vannamei*. □: NHPB daily mortality generated in our study; ✱: NHPB acute state mortality curve. Data for WSSV (○) and TSV (△) from J. M. Lotz (unpubl.). Dashed line: daily mortality curve of acute state for WSSV; solid line: TSV

the shrimp pond environment. Estimates of additional epidemiological parameters of NHPB, such as probability of transmission and the probability of decay of infectious material or how long NHPB remains infectious in a dead or dying shrimp (Soto & Lotz 2001), may allow for the use of a mathematical model for predicting the dynamics of NHPB in an aquaculture pond.

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