Vol. 75: 265–268, 2007 | DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org | Published May 9

NOTE

Effect of salinity on transmission of necrotizing hepatopancreatitis bacterium (NHPB) to Kona stock *Litopenaeus vannamei*

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ABSTRACT: Elevated salinity and temperature have been observed prior to devastating necrotizing hepatopancreatitis (NHP) outbreaks in several geographically isolated shrimp ponds. These observations have led to the hypothesis that the NHP-bacterium (NHPB) is hindered by reduced salinity, even though the mechanism is not understood. The objective of this research was to examine the effect of salinity on transmission of NHPB. The transmission rate of NHPB was estimated through laboratory experiments whereby individuals of Kona stock *Litopenaeus vannamei* were orally exposed to a dead NHPB-infected shrimp. For each replicate, 12 susceptible shrimp were placed with a dead NHPB-infected shrimp in a 1 m^2 bottom area cylindrical tank maintained at 30°C for a period of 24 h. Four salinities of 10, 20, 30, and 40‰ were replicated 2 times in 2 trials, giving a total of 192 shrimp exposed per os to infective material. In each trial, a negative control group was included at each salinity, giving a total of 96 shrimp exposed orally to uninfected material. After the 24 h exposure period, susceptible shrimp were individually isolated at the same physical conditions for up to 60 d to determine NHPB transmission. The NHPB was transmissible regardless of salinity: nearly a quarter of susceptible shrimp exposed to NHPB at the lowest (10‰) and highest (40‰) salinity examined acquired NHPB. Transmission rates were highest at the intermediate salinities of 20 and 30‰, suggesting that those salinities are optimal for NHPB transmission. The observed association between high salinity and NHP outbreak in a shrimp pond is not explained by these results because reduced transmission occurred at very low and very high salinities.

KEY WORDS: NHP-bacterium · Environmental stress · Shrimp aquaculture · Management

INTRODUCTION

Necrotizing hepatopancreatitis bacterium (NHPB) is a gram-negative *Alphaproteobacterium* that can cause up to 95% mortality in affected shrimp aquaculture ponds (Johnson 1990, Lightner 1996). The NHPB is pleomorphic, present as a smaller rod and a larger flagellated helical form (Krol et al. 1991, Frelier et al. 1992, Lightner et al. 1992). This bacterium is obligately intracellular, residing in the cytoplasm of hepatopancreas epithelial cells. Gross signs of early NHPB infection include reduced feed intake, lethargy, and an empty gastro-intestinal tract. Severe late-stage infections exhibit atrophy and discoloration of the hepatopancreas, and characteristic granulomatous-like lesions of the hepatopancreas tissue (Frelier et al. 1992, Lightner 1996). NHP disease has primarily affected cultured penaeids in the Americas, and reported host species include *Litopenaeus vannamei*, *L. setiferus*, *L. stylirostris*, *Farfantepenaeus aztecus* and *F. californiensis* (Lightner 1996).

Salinity and temperature are environmental factors thought to greatly influence the occurrence of NHP outbreaks in penaeid shrimp aquaculture. Initial NHP

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outbreaks in Texas, USA, were associated with temperatures from 29 to 31°C in salinities of 20 to 40‰ (Frelier et al. 1992). An epidemic of NHPB in Peru was linked to high temperatures of 29 to 35°C, and elevated salinities of greater than 30 to 38‰ preceded the outbreaks for periods of several weeks (Lightner & Redman 1994). In addition, NHPB outbreaks in Venezuela, Brazil, Ecuador, Costa Rica, and Panama from 1993 to 1995 followed physical conditions similar to those encountered in Texas, USA, and Peru (Lightner 1996).

Because epidemics of NHPB in shrimp ponds have been observed following periods of elevated salinity and temperature, a strategy for managing NHP disease has been to reduce salinity in aquaculture ponds (Frelier et al. 1994, 1995, Jory 1997). However, we cannot say for certain that salinity is the determining factor that produces conditions favorable for NHP outbreaks. Thus, a better understanding of the relationship between salinity and NHP disease is greatly needed. In this study, we examine the effect of salinity on transmission of NHPB in experimentally infected Kona stock *Litopenaeus vannamei* in an effort to elucidate the role of salinity in NHP outbreaks.

MATERIALS AND METHODS

Experimental design. ***Trial 1:*** Four salinity treatments of 10, 20, 30, and 40 ± 2‰ were each replicated 3 times, giving a total of 12 treatments. The 3 replicates at each salinity consisted of 2 replicates in which susceptible shrimp were orally exposed to a dead NHPB-positive shrimp and 1 replicate in which susceptible shrimp were exposed to an uninfected dead shrimp. A total of 12 cylindrical tanks of 1000 l volume and 1 m^2 bottom surface area were filled to approximately 10 cm with artificial Crystal Sea® Marinemix seawater prepared from reverse osmosis filtered water. The assignment of salinity and exposure of NHPB-positive or -negative control was randomized for each replicate. The exposure protocol followed Soto et al. (2001). A total of 144 susceptible juveniles of Kona stock *Litopenaeus vannamei* (Pruder et al. 1995, Lotz et al. 2003) (mean weight 12.1 ± 0.2 g, range 6.8 to 18.3 g) were divided into 12 cylindrical tanks, giving a total of 12 shrimp in each tank. The density of 12 shrimp m^{-2} is comparable to mean densities of *L. setiferus* in wild populations (Zimmerman & Minello 1984, Soto & Lotz 2001). In order to reduce possible variation, all susceptible shrimp in this experiment were male. Shrimp were gradually acclimated over 1 wk from 22‰ salinity to appropriate salinities of 10, 20, 30, or 40‰. Cylindrical tanks were aerated and maintained at 30 ± 2°C. Susceptible shrimp were fasted 3 d prior to exposure. Susceptible shrimp were exposed orally (an effective mode of NHPB exposure) to NHPB-infected shrimp (Vincent et al. 2004). Eight shrimp were obtained from a long-term exposure system of NHPB to individuals of Kona stock *L. vannamei* (Vincent et al. 2004), killed, weighed to nearest 0.01 g, and a small piece of hepatopancreas was excised from the cephalothorax and stored in a 1.5 ml microcentrifuge tube at –20°C for PCR analysis. The weight of the hepatopancreas sample was recorded prior to molecular analysis for detection and quantification of NHPB. On Day 0, each of the 8 exposure shrimp were randomly placed into 1 of the 8 cylindrical tanks corresponding to 2 replicates at each salinity. The remaining 4 cylindrical tanks were a negative control for each of the 4 salinities. Four shrimp obtained from a non-NHPB infected shrimp stock were killed, weighed to the nearest 0.01 g, and placed into the appropriate negative control tank for each salinity on Day 0.

During the 24 h exposure period for each replicate, susceptible shrimp generally consumed the entire cephalothorax and in most cases the entire carcass. After the 24 h exposure period, individual shrimp were randomly placed into aerated Sterilite® containers (35 cm length × 22 cm width × 12 cm height) in a total volume of approximately 4 l of artificial seawater maintained at the same salinity as the exposure phase. Shrimp were maintained in a waterbath at 30 ± 2°C for 60 d, and freshwater was added to the individual containers to replace evaporation. Temperature was monitored daily and salinity weekly. Individual shrimp were fed Rangen 45/10 (Buhl) commercial pellets at approximately 5% body weight every 2 d.

The day of post-exposure mortality was recorded for shrimp that died over the 60 d period. Moribund and dead shrimp were weighed to the nearest 0.01 g and cut transversely at the junction of the cephalothorax and abdomen, and a small piece of the hepatopancreas of approximately 25 mg was excised from the cephalothorax and stored in a 1.5 ml microcentrifuge tube at –20°C for molecular detection of NHPB. Shrimp surviving to 60 d were killed and processed in like manner.

Trial 2: The procedure described in Trial 1 was repeated in Trial 2. Mean weight of the 144 susceptible males of Kona stock *Litopenaeus vannamei* was 12.8 ± 0.2 g (range 7.5 to 18.2 g).

Diagnostic methods. NHPB in hepatopancreas tissue was extracted and analyzed by PCR (Loy et al. 1996a). Real-time PCR was used to quantify the initial dose of NHPB in hepatopancreas tissue of exposure shrimp, and to quantify lethal load in moribund and dead shrimp (Vincent & Lotz 2005).

Statistical analysis. For each replicate, the transmission rate of NHPB from a dead infected shrimp (β_d) was estimated as the proportion of susceptible shrimp that acquired NHPB after a 24 h exposure to a dead

infected shrimp (Soto et al. 2001). Mean β_d of NHPB at different salinities were compared by ANOVA using Systat version 11.

RESULTS

NHPB dose in exposure shrimp

For each replicate, the dose of NHPB in *Litopenaeus vannamei* exposed to susceptible shrimp was determined through real-time PCR (Table 1). Lethal NHPB loads were identified as being greater than 1×10^5 copies mg^{-1} hepatopancreas tissue. NHPB dose (expressed as copies mg^{-1} of hepatopancreas tissue) ranged 2.2×10^7 to 1.3×10^8 for 14 of the replicates. In Trial 2, the initial dose for 1 of the 40‰ replicate was moderate at 2.3×10^4 copies mg^{-1}, and no NHPB was detected in the tissue sample for a replicate at 20‰.

Transmission rate of NHPB from a dead host (β_d)

β_d occurred at each salinity (Table 2). At the lowest salinity of 10‰, NHPB transmission ranged from 1 to 5 shrimp (of 12 exposed), with a mean β_d of 0.21. At 20‰, 6 to 8 shrimp (of 12) acquired NHPB, with a mean β_d of 0.61. Transmission of NHPB at 30‰ ranged from 7 to 8 shrimp (of 12), with a mean β_d of 0.63. Transmission at 40‰ ranged from 1 to 6 shrimp (of 12) resulting in a mean β_d of 0.27.

Table 1. Dose of necrotizing hepatopancreatitis bacterium (NHPB) (copies mg^{-1} hepatopancreas tissue) exposed per os to susceptible individuals of Kona stock *Litopenaeus vannamei* at different salinities. nd: NHPB not detected in tissue sample

Trial	Replicate	NHPB dose (copies mg^{-1})			
		10‰	20‰	30‰	40‰
1	1	2.6×10^7	3.5×10^7	5.6×10^7	1.0×10^8
	2	2.2×10^7	3.5×10^7	1.3×10^8	9.5×10^7
2	1	1.2×10^8	nd	6.5×10^7	2.3×10^4
	2	3.9×10^7	8.7×10^7	3.3×10^7	7.8×10^7

Table 2. Transmission rates of necrotizing hepatopancreatitis bacterium (NHPB) exposed per os to Kona stock *Litopenaeus vannamei* at different salinities. Results given as no. of shrimp (of 12) that acquired infection after exposure to a single dead NHPB-infected shrimp for 24 h, and the resulting transmission rate (β_d). nd: transmission rate not determined because initial exposure shrimp was not NHPB-positive

Trial	Replicate	Transmission rate from a dead infected shrimp (β_d)			
		10‰	20‰	30‰	40‰
1	1	2/12 = 0.17	8/12 = 0.67	7/12 = 0.58	3/12 = 0.25
	2	1/12 = 0.08	6/12 = 0.50	8/12 = 0.67	3/12 = 0.25
2	1	2/12 = 0.17	nd	7/12 = 0.58	1/12 = 0.08
	2	5/12 = 0.42	8/12 = 0.67	8/12 = 0.67	6/12 = 0.50
Mean β_d (95% CI)		0.21 (0.11–0.35)	0.61 (0.43–0.77)	0.63 (0.47–0.76)	0.27 (0.15–0.42)

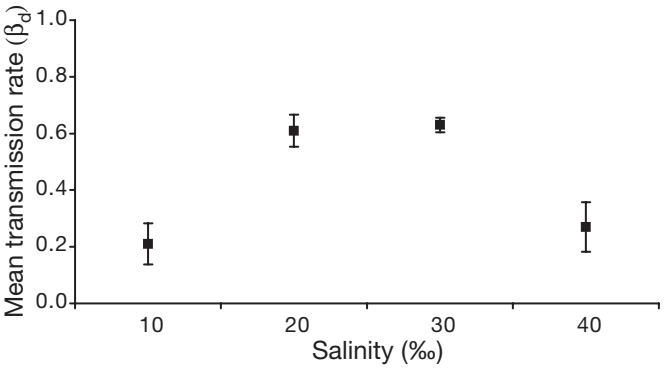

Fig. 1. Mean transmission rate of necrotizing hepatopancreatitis bacterium (NHPB) from a dead infected shrimp (β_d) exposed per os to Kona stock *Litopenaeus vannamei* at salinities of 10, 20, 30 and 40‰ (ANOVA, p = 0.001). Error bars indicate SE; β_d estimated as proportion of susceptible shrimp (of 12) that acquired NHPB infection after exposure to a dead infected shrimp for 24 h

Mean β_d differed significantly among salinities (Fig. 1; ANOVA, p = 0.001). Mean β_d was significantly lower at 10 and 40‰ than at 20 or 30‰, but there was no difference in mean β_d between 10 and 40‰ or between 20 and 30‰. β_d increased from 10‰ to peak at 20 and 30‰ before decreasing at 40‰.

DISCUSSION

Outbreaks of NHP have been linked to periods of elevated salinity and temperature (Frelier et al. 1992, Lightner & Redman 1994, Lightner 1996). The present study examined the effect of salinity on transmission of NHPB under experimental laboratory conditions. Susceptible shrimp were exposed to dead shrimp carrying lethal loads of NHPB that ranged from 10^7 to 10^8 copies mg^{-1} hepatopancreas tissue (Vincent & Lotz 2005). The NHPB was transmissible to juvenile Kona stock *Litopenaeus vannamei* regardless of salinity. Although the mean β_d of NHPB from a dead shrimp was lower at the lowest (10‰) and highest (40‰) salinities, nearly a quarter of susceptible shrimp became infected with NHPB after contact with a dead infected shrimp at

these low and high salinities. Thus, low (or high) salinity does not appear to stop NHPB transmission by cannibalism.

Transmission of NHPB was highest at 20 and 30‰, similar to that previously observed at 30‰ and 30°C (A. Vincent & J. Lotz unpubl. data). Because NHPB transmission peaked at intermediate salinity, this suggests an optimal salinity range for NHPB transmission. Additional studies are warranted to examine NHPB transmission below 10‰ to see if this holds true.

The results do not explain the observed association between high salinity and NHP outbreaks, because reduced transmission occurred at low as well as high salinities. These results suggest that a correlation between salinity and outbreaks is not explained by transmission alone. However, salinity may affect outbreaks in other ways. In addition, factors such as temperature or reservoir hosts may be involved in an NHP outbreak in a shrimp pond. Although no reservoir host has been reported for NHPB, it has been proposed through molecular phylogeny that epicommensal ciliates may be a part of this bacterium's life cycle (Loy et al. 1996b). Another possibility is that an interaction of several physical characteristics of a shrimp pond creates favorable conditions for NHP outbreaks. While interaction between environmental factors is possible, salinity's effect on transmission alone does not appear to be a determining factor in NHP outbreaks. Although NHPB transmission was lower at reduced salinity, susceptible shrimp were still able to acquire NHPB infection after contact (through cannibalism) with a dead infected shrimp. Because of the dynamics of NHPB infection, the introduction of infected shrimp into a susceptible population of shrimp could produce an NHP outbreak (A. Vincent & J. Lotz unpubl. data); however, the character of the outbreak could differ at various salinities. Further studies to investigate the effect of salinity and temperature on survival of NHPB-infected shrimp and to identify potential reservoir hosts for NHPB infection in the shrimp pond environment are warranted.

Acknowledgements. We thank V. Breland and M. Turner for technical assistance and 4 anonymous reviewers for comments regarding the manuscript. This study was partially funded by the United States Department of Agriculture CSREES Grant No. 2002-38808-01381.

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Editorial responsibility: Timothy Flegel, Bangkok, Thailand

Submitted: June 15, 2006; Accepted: February 2, 2007
Proofs received from author(s): March 29, 2007