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THE EFFECTS OF HYPERCAPNIC HYPOXIA ON THE SURVIVAL OF SHRIMP CHALLENGED WITH *VIBRIO PARAHAEMOLYTICUS*

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ABSTRACT Estuarine organisms routinely encounter fluctuations in dissolved oxygen, carbon dioxide, and pH, which can vary both seasonally and diurnally. Such environmental stresses as hypoxia can affect the immune response of invertebrates and vertebrates and have been linked to increased disease incidence. This research investigated the effects of hypoxia, hypercapnia, and low pH on disease susceptibility in both penaeid and palaemonid shrimp. Juvenile penaeid shrimp *Litopenaeus vannamei* and adult grass shrimp *Palaemonetes pugio* were challenged by intramuscular injection with a previously determined LD_{50} dose of a known pathogenic strain of *Vibrio parahaemolyticus*. Mortalities were monitored for shrimp held under normoxia (Po_2 = 150–155 torr, Pco_2 = 0.23 torr, pH = 7.6–8.0 for *L. vannamei*, Po_2 = 150–155 torr, Pco_2 = 0.23 torr, pH = 8.0–8.2 for *P. pugio*) and two levels of hypoxia. The penaeid shrimp were challenged under normocapnic hypoxia (Po_2 = 45 torr, Pco_2 = 0.23 torr, pH = 7.8–8.1) and hypercapnic hypoxia (Po_2 = 30 torr, Pco_2 = 15.2 torr, pH = 6.8–7.0). Grass shrimp were challenged under two levels of hypercapnic hypoxia (Po_2 = 45 torr and 30 torr, Pco_2 = 15.2 torr, pH = 6.7–7.0). Both the juvenile *L. vannamei* and the adult *P. pugio* held under hypercapnic hypoxia at 30 torr oxygen displayed significantly lower 48-hour survival (15.7 and 3.1%, respectively) than animals held in normoxic water (28.7 and 29.4%, respectively). There was no significant decrease in survival in *L. vannamei* under normocapnic hypoxia at 45 torr oxygen or in *P. pugio* under hypercapnic hypoxia at 45 torr oxygen. Total hemocyte count (THC/mL) significantly decreased in adult *L. vannamei* held under hypercapnic hypoxia when compared to normoxic controls. Oxygen level had a significant effect on total hemocyte density; whereas, time and the interaction of time and oxygen did not. The reduction in THC/mL may contribute to an increased rate of mortality in shrimp held under hypoxic conditions and challenged with *V. parahaemolyticus*. These results show that hypercapnic hypoxia decreases survival following bacterial challenge in both *L. vannamei* and *P. pugio* and decreases total hemocyte count in *L. vannamei*. These data provide direct evidence that naturally occurring variations in oxygen, CO_2 and pH can place estuarine organisms at increased risk from opportunistic pathogens

KEY WORDS: LD_{50}, hypercapnia, hypoxia, palaemonid, penaeid, shrimp, *Vibro*

INTRODUCTION

Penaeid and Palaemonid shrimp in estuarine waters frequently encounter levels of dissolved oxygen, carbon dioxide, and pH that vary dramatically on a diurnal and seasonal basis. Shallow coastal regions in the southeast and in the Gulf of Mexico often experience dissolved oxygen concentrations less than 3.0 mg/L (Breitburg 1990, Rabalais et al. 1994, Burnett 1997, Summers et al. 1997). In South Carolina tidal marshes, tidal creek oxygen pressures can fluctuate between 9 and 170 torr (6% and 110% air saturation) within a 24-hour period (Cochran and Burnett 1996). Oxygen levels as low as 1.2% air saturation (approximately 2 torr) have been measured in the nearby Savannah River estuary (Winn and Knott 1992). Moreover, hypoxia is almost always accompanied by an increase in carbon dioxide pressure (Pco_2), or hypercapnia, produced by respiration. Elevated levels of water CO_2 then drive a decrease in water pH. Cochran and Burnett (1996) reported that Pco_2 varies from 0.3 to 12 torr, and pH ranges from 6.5 to 7.6 in South Carolina tidal marshes. Thus, hypoxia and low pH often co-occur in the natural environment (Burnett 1997).

Shrimp raised in aquaculture ponds also experience severe changes in O_2, CO_2, and pH because of high density and nutrient input from feed (Browdy et al. in press, Madenjian 1990). Dissolved oxygen levels are routinely measured in well-managed farm ponds, with the general understanding that low O_2 levels may be lethal to shrimp. Supplemental aeration is used to reduce fluctuations in dissolved oxygen; however, periods of hypoxia and hypercapnia still occur in routine management (Chang and Ouyang 1988, Garcia and Brune 1991).

Although extreme hypoxia or anoxia can cause mass mortalities in estuarine organisms (Garlo et al. 1979, Winn and Knott 1992, Diaz and Rosenberg 1995, Lenihan and Peterson 1998), sublethal hypoxia may have an adverse impact on normal physiological functions in shrimp, such as osmoregulatory capacity (Charmantier et al. 1994) and molting (Clark 1986). Hypoxia also can suppress immune function in shrimp. Direkbusarakom and Danayadol (1998) found that hypoxia (1.8–2 ppm) decreased phagocytosis and bacterial clearance efficiency in the black tiger shrimp, *P. monodon*. In addition, Le Moullac et al. (1999) reported a decrease in hemocyte numbers and respiratory burst activity of *Litopenaeus stylirostris* exposed to severe hypoxia (1 mg O_2/L). These observations suggest that chronic sublethal hypoxia might suppress the ability of shrimp to resist infections with opportunis-

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tic pathogens such as environmental bacteria, viruses, and fungi. Indeed, Le Moullac (1999) demonstrated that the levels of hypoxia that decreased hemocyte numbers and suppressed respiratory burst activity in *L. stylirostris* also increased pathogenicity of *Vibrio alginolyticus* in that shrimp species.

Unfortunately, most studies of hypoxia ignore changes in CO_2 and pH associated with hypercapnic hypoxia (Burnett 1997). Where the effects have been assessed, low pH and high CO_2 enhanced mortality rates of extreme hypoxia (Martinez et al. 1998) and altered metabolic activity (McCulloch 1990). Low pH independently and additively with hypoxia suppressed the respiratory burst of oyster hemocytes (Boyd and Burnett 1999). The latter study strongly suggested that hypercapnic hypoxia suppresses the resistance of wild and aquacultured estuarine organisms against such naturally occurring opportunistic pathogens as bacteria, viruses, and fungi.

In the last 10 years, several highly lethal bacterial pathogens have had a serious impact on both wild and aquacultured populations of penaeid shrimp (Karunasagar et al. 1994, Mohney et al. 1994, Hiney 1995, Liu et al. 1996, Alapide-Tendencia and Dureza 1997, Lavilla-Pitogo et al. 1998). The most frequently reported bacterial infection in penaeid shrimp is vibriosis, caused by bacteria from the family Vibrionaceae (Adams 1991, Sahul Hameed 1995). Bacteria in the family Vibrionaceae comprise 10–50% of the marine heterotrophic bacteria found in coastal waters (Thune et al. 1993). Among several *Vibrio* species associated with this disease, *Vibrio parahaemolyticus* is frequently associated with disease outbreaks in aquaculture (Mohney et al. 1994, Sahul Hameed 1995) and is sometimes found at high densities in coastal waters (Buck 1990, DePaola et al. 1990).

In this study, we evaluated the impact of hypoxia and hypercapnic hypoxia on resistance to the opportunistic bacterial pathogen *V. parahaemolyticus* in two commercially and recreationally important species of shrimp. The Pacific white shrimp, *Litopenaeus vannamei,* is the species of choice for penaeid shrimp aquaculture in South Carolina. *L. vannamei* occurs naturally from the Gulf of California to northern Peru (Perez Farfante and Kensley 1997), but is imported for use in aquaculture because of its faster growth over native species (Sandifer et al. 1993). The grass shrimp, *Palaemonetes pugio,* serves an important role in the estuary as a detritivore by consuming and breaking down *Spartina* and aiding in trophic level energy transfer (Welsh 1975). They also serve as prey for many important commercial and recreational fishes and crustaceans, which use the marsh as nursery grounds (Welsh 1975). In the first phase of these experiments, an intramuscular bacterial challenge model with survival endpoint was developed and applied in both shrimp species to determine LD_{50} values for *V. parahaemolyticus.* Then, to evaluate the contribution of hypoxia and hypercapnic hypoxia to disease resistance, survival was monitored in shrimp challenged with LD_{50} doses of *V. parahaemolyticus* and exposed to varying levels of water O_2, CO_2, and pH. Finally, to determine whether hypercapnic hypoxia might alter cellular components of the shrimp immune system over the time course of these bacterial challenges, total hemocyte densities of the hemolymph (THC/mL) were compared in animals exposed to normoxia and hypercapnic hypoxia.

MATERIALS AND METHODS

Experimental Animals

Litopenaeus vannamei (Boone) from specific pathogen-free stocks were provided by the Waddell Mariculture Center in Bluffton, South Carolina, by Island Fresh Seafood in Yonges Island, South Carolina, and by Dixieland Maricultural Farms in Hollywood, South Carolina. Shrimp were maintained in well-aerated recirculating seawater at 28–32 ppt salinity, 23–25 °C, and pH 8.0–8.2. Water quality variables (pH, salinity, and temperature) were measured every other day. Ammonia was monitored twice a month and remained lower than 0.25 mg/L. Animals were fed once daily with shrimp feed (Zeigler Brothers, Inc). All necessary precautions were followed for possessing a nonindigenous shrimp species as outlined in the nonindigenous shrimp possession permit #NI98-0565 granted by the South Carolina Department of Natural Resources.

Grass shrimp *Palaemonetes pugio* (Holthuis) were collected with a dip net in a nearby tidal creek. These shrimp were held in a 50-gallon aquarium at 25–27 ppt salinity and 23–25 °C for at least 2 days before use in an experiment. Animals were fed Marine Tetra Flakes daily.

Bacteria

A known pathogenic strain of *Vibrio parahaemolyticus* (90-69B3) was streaked on a Tryptic Soy Agar (TSA) plate with 2.5% NaCl added and allowed to grow overnight at room temperature. Aliquots (0.5 mL) of the bacteria were stored in freezing media (Tryptic Soy Broth (TSB) + 2.5% NaCl and 20% glycerol) at −70 °C. These aliquots were used as the working stock.

For each assay, *V. parahaemolyticus* was streaked onto TSA + 2.5% NaCl plates from the frozen aliquots and allowed to grow at room temperature for 24 hours before use. A different aliquot was used for each assay to avoid excessive passages of the bacteria on plates. Bacteria were transferred from the plates to 2.5% NaCl buffered with 20 mmol/L HEPES using wooden applicator sticks. Bacterial densities were quantified by optical density (OD) at 540 nm and then serially diluted in the saline to obtain the test dosages. OD values were confirmed by counting colony-forming units on double layer plates (10 mL of marine agar containing the bacterial dilution overlaid onto 10 mL of TCBS agar). OD values of 0.1 and 1.0 were determined to be equal to 1.0×10^8 colony-forming units per mL (CFU/mL) and 1.0×10^9 CFU/mL, respectively. Koch's postulates were satisfied to confirm the pathogenicity and relationship between *V. parahaemolyticus* and vibriosis (Prescott et al. 1996).

The identity of the bacteria used in challenge tests and after each isolation of Koch's postulates was confirmed using Gram strains, motility tests, characterization of growth on TCBS plates, cytochrome oxidase tests, and API-20NE test strips for Gram-negative, nonfermentative bacteria (API resultant bacteria #7276644). Aseptic techniques were used when working with the bacteria. Waste material was either autoclaved or disinfected with 1% chlorine bleach.

LD_{50} Tests for Litopenaeus vannamei

Vibrio parahaemolyticus was streaked onto TSA + 2.5% NaCl plates from the frozen aliquots as described above. Juvenile animals (5.8 to 8.9 cm and weighing from 1.0 to 4.2 g) were injected intramuscularly near the fourth ventral abdominal segment using a Hamilton syringe with 50 μL of bacterial suspension (ranging from 5×10^3 to 5×10^7 CFU/shrimp) or with 2.5% NaCl buffered with 20 mmol/L HEPES without bacteria as a control. Animals were then placed in 3.5 L, wide-mouth, screw-lid, glass jars with 700 mL of filtered (0.45 μm) artificial seawater (ASW) adjusted to 30 ppt. Lids of the test containers were fitted with tubes for in-

coming air and an air release tube (61 cm) with two cotton plugs to contain *Vibrio* aerosol. Seven animals were placed in each jar with three replicates for each dose. LD_{50} tests were performed under normoxic conditions (155 torr oxygen), with low CO_2 (less than 1 torr) and high pH (pH 7.7–7.9), and mortality was recorded at 2, 4, 8, 12, 24, and 48 hours after injection of *Vibrio*. Water was changed in all jars at 12 and 24 hours after feeding and then when necessary in individual jars (i.e., when the water became cloudy because of shrimp mortality). Animals were fed commercial shrimp food (as above) every 12 hours. The *L. vannamei* LD_{50} test was repeated three times. LD_{50} and confidence intervals for both species were calculated using the EPA Trimmed Spearmann–Karber program (Hamilton et al. 1977).

LD_{50} Tests for Palaemonetes pugio

The methods for the *P. pugio* LD_{50} tests were similar to those mentioned above for *L. vannamei,* with a few exceptions. Because *P. pugio* were smaller (2.1 to 3.4 cm and weighing from 0.2 to 0.4 g), only 5 μL of a saline containing bacteria was injected, and the shrimp were held in smaller test chambers with 400 mL of ASW. Water in all experimental jars was changed once every 24 hours. Animals were fed Marine Tetra Flakes (as above) every 12 hours. The *P. pugio* LD_{50} test was repeated two times with different bacterial concentrations for each (ranging from 2.25×10^1 to 2.25×10^5 CFU/shrimp for test 1, and ranging from 5×10^1 to 5×10^5 CFU/shrimp for test 2).

Challenge Test Design

It was not possible to maintain appropriate levels of oxygen in the jars used for the *L. vannamei* and *P. pugio* LD_{50} tests by directly using Wösthoff gas mixing pumps and individually aerating the jars. This was because of the low output of the pump, the variability in aeration to each jar, and the high oxygen demand of the shrimp. Therefore, a new experimental design was employed for the hypoxic challenge tests for both species. Ten-gallon aquaria were divided into four chambers of equal size to hold the shrimp and one smaller chamber to hold a circulating pump (see below) using Plexiglas drilled with holes to allow water to flow freely among the chambers. Nine L of 30 ppt filtered (0.45 μm) artificial seawater (Crystal Sea marine mix) was added to each tank. A small, submersible pump (Penguin 550) in each experimental tank circulated water among the compartments. Normoxia was maintained by vigorous aeration. Hypoxia was maintained by controlling aeration. The consumption of oxygen by the shrimp lowered the oxygen pressure in the water. Oxygen pressure in the water was monitored using an oxygen electrode and meter (YSI Model 58). Output from the oxygen meter was monitored by a Sable System data acquisition system, which was used to control tank aeration by an air stone at a user-defined setpoint. To control water CO_2 pressures, a Wösthoff gas mixing pump delivered mixtures of CO_2 and nitrogen continuously. The CO_2 and N_2 gas mixture also served to lower the oxygen pressure. At steady state, this system maintained constant oxygen and CO_2 pressures (Fig. 1). Oxygen levels in the chambers remained within 1 torr of the set value.

L. vannamei *Challenge Tests at 45 torr O_2–Normocapnic Hypoxia*

L. vannamei challenge tests were performed under two levels of hypoxia mimicking two different environmental conditions (see Table 1). The first set of tests compared disease susceptibility between animals held under normoxia and animals held under normocapnic (i.e., very low CO_2 pressure) hypoxia with no added CO_2 (treatment 1). This test evaluated the effect of low oxygen only. For these tests, ASW was made hypoxic by bubbling pure N_2 into the first chamber. Gassing the water with N_2 drove off excess CO_2, keeping pH and CO_2 at normoxic levels, with pH 7.8–8.1 and 0.03% CO_2. Normoxia was maintained by bubbling ambient air into the tanks through three air stones.

Using the method described above, juvenile shrimp ranging from 5.7 cm to 9.0 cm (1.0 g to 4.5 g) were injected intramuscularly with 50 μL of bacterial suspension or with 2.5% NaCl buffered with 20 mmol/L HEPES. The bacterial numbers for these tests ranged from 1.8×10^6 CFU/shrimp to 2.25×10^6 CFU/shrimp, which were greater than the previously determined average LD_{50} but were within the 95% confidence interval (Table 2). Nine shrimp with or without injected bacteria were placed in each of the four chambers of the appropriate tanks for a total of 36 animals per tank. Animals were placed randomly in either the hypoxic or normoxic tanks. All four treatments (one per tank) were conducted simultaneously and were counted as one replicate. Mortality was recorded, and dead or moribund animals were removed at 2, 4, 8, 12, 16, 20, 24, 28, 32, 36, 40, 44, and 48 hours after injection challenge. Water was changed in all tanks at 12, 24, and 36 hours, and then whenever necessary in individual tanks. The replacement water for the normocapnic hypoxia treatment was gassed ahead of time to appropriate treatment pressures to avoid a change in oxygen pressure. Animals were fed commercial shrimp food every 12 hours. This challenge test was repeated three times.

L. vannamei *Challenge Tests at 30 torr O_2 + 15.2 torr CO_2–Hypercapnic Hypoxia*

The second set of tests (treatment 2) compared disease susceptibility between animals held under normoxia and animals held under hypercapnic hypoxia at 30 torr oxygen and 15.2 torr CO_2 (4 and 2%, respectively). These tests were conducted as described above (treatment 1); however, the CO_2 and pH were adjusted to mimic hypoxic levels (Table 1). The resulting pH of the hypoxic water was 6.8–7.0. The bacterial concentration used in these tests was 1.125×10^6, which was within the 95% confidence interval previously determined in the LD_{50} tests. This challenge test was repeated three times.

P. pugio *Challenge Tests*

Both of the grass shrimp challenge tests compared disease susceptibility between animals held under normoxia and animals held under hypercapnic hypoxia. These challenge tests were performed under two levels of hypercapnic hypoxia: 45 torr O_2 + 15.2 torr (2%) CO_2 and 30 torr O_2 + 15.2 torr (2%) CO_2. Hypercapnic hypoxia was achieved as described above for both treatments with only the set point for the data acquisition system differing between the two levels of hypoxia (Table 1).

The methods for the *P. pugio* challenge treatments were similar to those mentioned above for the *L. vannamei* treatments, with a few exceptions. For the grass shrimp tests, only 5 μL of a saline containing bacteria was injected, and 10 animals were placed in a chamber for a total of 40 animals per tank. The bacterial concentrations for these tests ranged from 9.10×10^4 to 1.25×10^5 CFU/shrimp. These values were outside of the confidence interval previously determined in the LD_{50} tests because of an experimental error in the original calculation of the confidence intervals. However, because the same bacterial concentration was used for both treatments in a test, the results were unaffected. These challenge tests were each repeated four times.

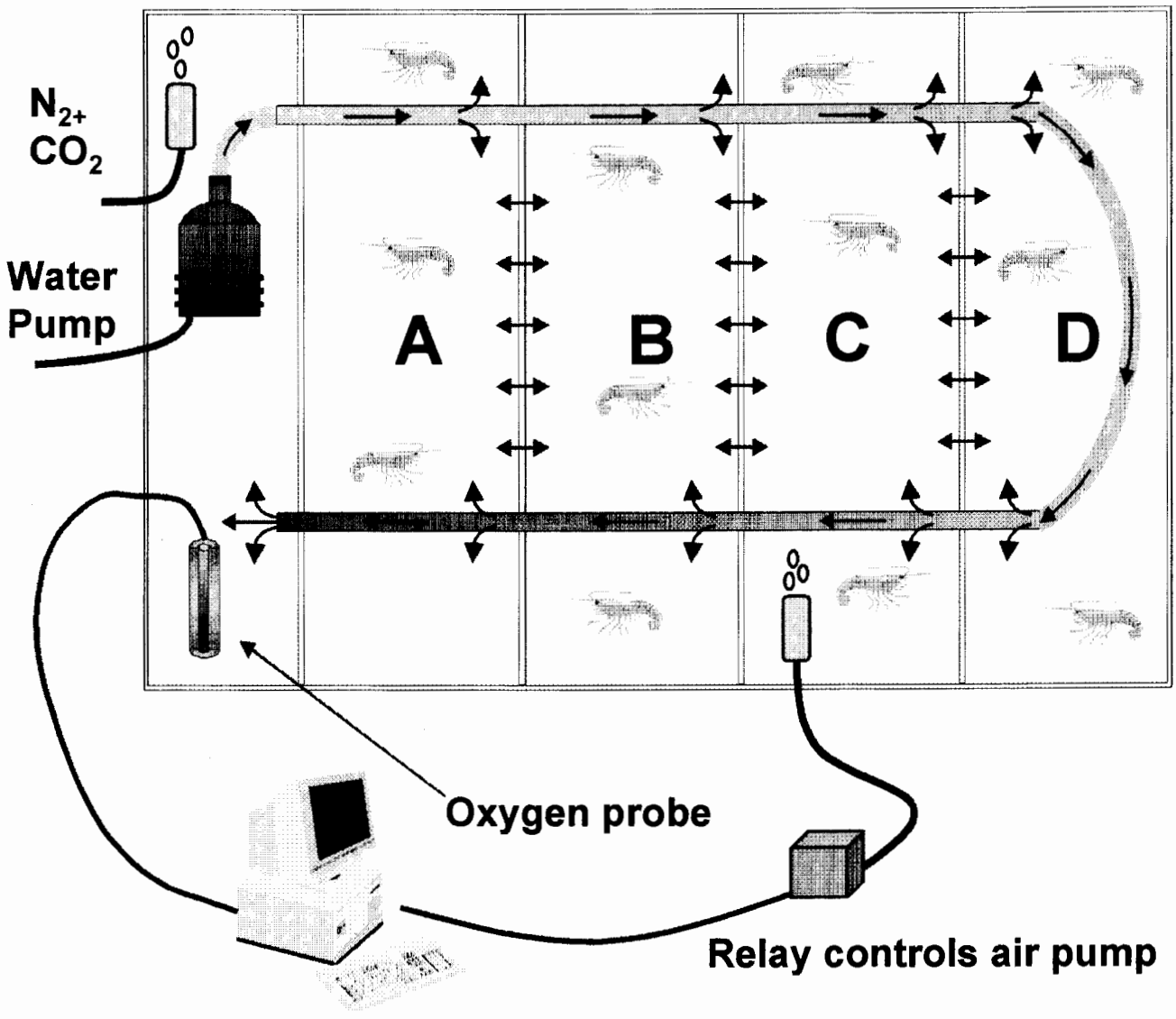

Figure 1. Schematic drawing of the tanks used to hold animals under different gas pressures. Oxygen pressure was measured with an oxygen electrode and adjusted using an air pump controlled by a computer. The infusion of air was opposed by gassing with mixtures of nitrogen and carbon dioxide (depending on the experiment). The tank was divided into compartments, and the water was circulated using a submersible pump.

Statistical Analysis for Challenge Tests

Challenge tests were performed using a full factorial design with bacteria and oxygen as the effect variables. Each challenge test produced four survival curves: normoxia without bacteria, normocapnic hypoxia (hypoxia with very low CO_2) or hypercapnic hypoxia (hypoxia with elevated CO_2) without bacteria (depending on the treatment in question), normoxia plus bacteria and normocapnic hypoxia or hypercapnic hypoxia plus bacteria. Using the statistical program JMP IN (SAS Institute, Inc.), a quadratic polynomial was fitted to each curve to obtain an intercept, response coefficient, and response coefficient^2 for each line. The combined coefficient and coefficient^2 parameters were then analyzed as the response variables in a multivariate analysis of variance (MANOVA) with bacteria, oxygen, and the interaction of bacteria and oxygen (bacteria*oxygen) as the x values. Differences in the coefficient and coefficient^2 revealed differences in the survival rate of shrimp among treatments. The intercepts were not analyzed, because differences in the intercept were artifacts of fitting a quadratic polynomial to a survival curve and did not reveal information about the rate of survival. Four MANOVA tests were run, one for each suite of tests: *L. vannamei* at 45 torr oxygen and <1 torr CO_2, *L. vannamei* at 30 torr oxygen with 15.2 torr CO_2, *P. pugio* at 45 torr oxygen with 15.2 torr CO_2, and *P. pugio* at 30 torr oxygen with 15.2 torr CO_2. Results of the MANOVA tests revealed if oxygen level/CO_2 treatment (normoxia vs. hypoxia), bacteria (absence vs. presence) and/or the interaction of the two (bacteria*oxygen) had a significant effect on ($P < 0.05$) shrimp survival following bacterial challenge. Univariate analysis of variance tests (ANOVAs) were then run to see if the significance found in the MANOVA was attributable to coefficient, coefficient^2, or both.

Total Hemocyte Count

The impact of hypercapnic hypoxia at 30 torr oxygen, 15.2 torr CO_2 and pH 6.9–7.1 on total hemocyte count/mL hemolymph in

TABLE 1.

Water quality variables used in the challenge tests and total hemocyte count (THC) assay.

Vibrio Challenge Tests	O_2			CO_2		
	torr	% air sat.	mg/L	torr	%	pH
Litopenaeus vannamei						
Normoxia (control)	150–155	21	7.29	0.23	0.03	7.6–8.0
Normocapnic hypoxia treatment 1	45	6	2.12	0.23	0.03	7.8–8.1
Hypercapnic hypoxia treatment 2	30	4	1.41	15.2	2	6.8–7.0
Palaemonetes pugio						
Normoxia (control)	155	21	7.29	0.23	0.03	8.0–8.2
Hypercapnic hypoxia treatment 1	45	6	2.12	15.2	2	6.9–7.0
Hypercapnic hypoxia treatment 2	30	4	1.41	15.2	2	6.9–7.0
Total hemocyte count						
Normoxia (control)	150–155	21	7.29	0.23	0.03	8.0–8.2
Hypercapnic hypoxia	30	4	1.41	15.2	2	6.9–7.1
Ranges of variables observed in nature	0–285	0–38.6	0–14	0.23–35.6	0.03–4.7	6.5–8.3

O_2 and CO_2 are presented several ways for comparison with water quality data in the literature. The following references were used to report the environmental ranges listed for O_2, CO_2, and pH: Breitburg, 1990, Winn and Knott, 1992; Rabalais et al., 1994; Cochran and Burnett, 1996.

Litopenaeus vannamei was measured over the 48 hours to replicate the time period of the challenge tests. At time zero, shrimp were placed randomly in normoxic or hypercapnic hypoxia tanks. Hemolymph from individual adult *L. vannamei* was withdrawn from the ventral sinus at the base of the fourth or fifth walking leg at a specified time point (4, 8, 16, 24, or 48 hours) into a 1.0 mL syringe with a 26-gauge needle. Hemolymph was diluted with an anticoagulant solution (AS) described by Lee et al. (1995): 20% filtered seawater, 30 mmol/L trisodium citrate, 0.1 mmol/L glucose, 26 mmol/L citric acid, 10 mmol/L EDTA at pH 4.6. Total hemocyte counts were performed using a hemocytometer, taking into account the dilution of the hemolymph with AS during bleeding. Twenty shrimp were bled at 4, 16, 24, and 48 h (10 each from normoxia and hypercapnic hypoxia); 22 shrimp were bled at 8 h (11 each from normoxia and hypercapnic hypoxia). Individual shrimp were used only once. Mortality was monitored throughout the experiment.

TABLE 2.

48-Hour LD_{50} values for *Litopenaeus vannamei* and *Palaemonetes pugio* for *Vibrio parahaemolyticus*.

Test	48-hour LD_{50}	95% Confidence Interval
Litopenaeus vannamei		
1	6.04×10^5 CFU/shrimp	2.69×10^5–1.36×10^6
2	1.37×10^6 CFU/shrimp	7.09×10^5–2.64×10^6
3	5.89×10^5 CFU/shrimp	2.90×10^5–1.19×10^6
Mean	8.54×10^5 CFU/shrimp (3.06×10^5/g wet weight)	
Palaemonetes pugio		
1	1.46×10^4 CFU/shrimp	6.22×10^3–3.44×10^4
2	2.16×10^4 CFU/shrimp	2.16×10^4–3.88×10^4
Mean	1.81×10^4tCFU/shrimp ($6.08t\times 10^4$/g wet weight)	

The LD_{50} values are presented as colony forming units (CFU) per shrimp and per gram shrimp wet weight.

A two-way ANOVA test was performed using time, oxygen, and the interaction of time times (*) oxygen as the x values and total hemocyte count (THC) as the response (y) value ($\alpha = 0.05$). An *a posteriori* *t*-test was used to compare means at 48 hours.

RESULTS

LD_{50} Tests

Vibrio parahaemolyticus has dose-response pathogenicity to both *Litopenaeus vannamei* and *Palaemonetes pugio*. Dead or moribund shrimp exhibited signs of Vibriosis, including opaqueness of the abdominal muscle, lethargy, expansion of the chromatophores, and abdominal flexure that peaked at the third abdominal segment (Lightner 1988). LD_{50} values for *L. vannamei* ranged from 5.89×10^5 to 1.37×10^6 CFU per shrimp with an average value of 8.54×10^5 CFU/shrimp (Table 2, $n = 3$). The 95% confidence interval ranged from 2.69×10^5 to 2.64×10^6 CFU/shrimp. Concentrations of bacteria used for the challenge tests remained within these confidence intervals.

LD_{50} values for *P. pugio* were 1.46×10^4 CFU/shrimp and 2.16×10^4 CFU/shrimp, with an average value of 1.81×10^4 CFU/shrimp (Table 2, $n = 2$). The 95% confidence interval ranged from 6.22×10^3 to 3.88×10^4 CFU/shrimp.

Challenge Tests

Control survival

L. vannamei control survival was greater than 77.8, 88.9, and 86.1% in the experiments testing normoxia, normocapnic hypoxia at 45 torr oxygen, and hypercapnic hypoxia at 30 torr oxygen, respectively. *P. pugio* control survival was greater than 92.5, 97.5, and 95% in the experiments testing normoxia, hypercapnic hypoxia at 45 torr oxygen, and hypercapnic hypoxia at 30 torr oxygen, respectively (Fig. 2 and 3). These results show that the levels of hypoxia used were not lethal to either organism.

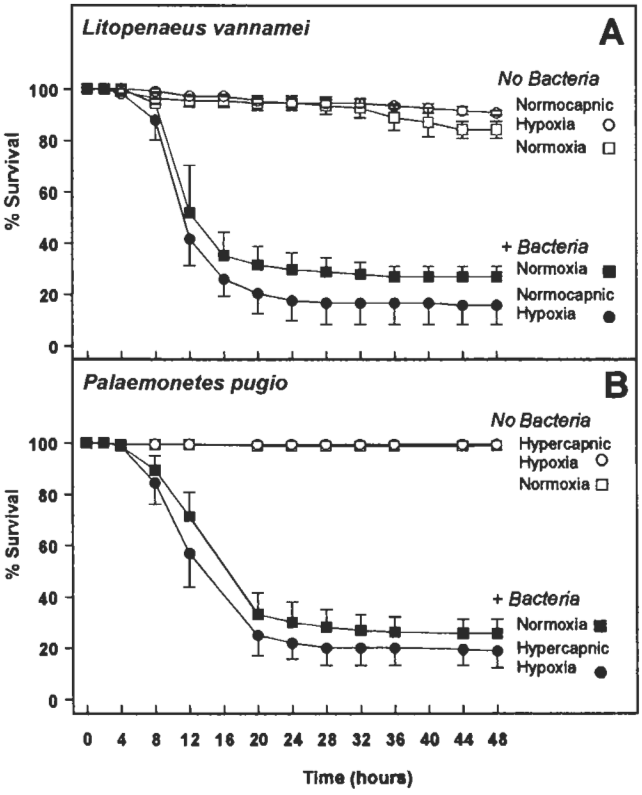

Figure 2. A. *Litopenaeus vannamei* survival following bacterial challenge under normoxia (Po_2 = 150–155 torr, Pco_2 = 0.23 torr, pH 7.6–8.0) and normocapnic hypoxia (Po_2 = 45 torr, Pco_2 = 0.23 torr, pH 7.8–8.1). Shrimp were injected intramuscularly with 50 µL of *Vibrio parahaemolyticus* bacterial suspension (1.8×10^6 CFU/shrimp) or with HEPES buffered 2.5% NaCl for controls. There were 36 shrimp per treatment. Values at each time point are the mean (n = 3 experiments); standard errors are indicated except where the error is small and falls within the width of the datapoint. The effects of oxygen/CO_2 treatment and the interaction of bacteria*oxygen/CO_2 treatment on disease susceptibility were not significant as determined by a MANOVA (P = 0.6478 and P = 0.3594). B. *Palaemonetes pugio* survival following bacterial challenge under normoxia (Po_2 = 150–155 torr, Pco_2 = 0.23 torr, pH 8.0–8.2) and hypercapnic hypoxia (Po_2 = 45 torr, Pco_2 = 15.2 torr, pH 6.9–7.0). Shrimp were injected intramuscularly with 5 µL of *Vibrio parahaemolyticus* bacterial suspension (1.0×10^5 CFU/shrimp) or with HEPES buffered 2.5% NaCl for controls. There were 40 shrimp per treatment. Values at each time point are the mean (n = 4 experiments); standard errors are indicated except where the error is small and falls within the width of the datapoint. The effects of oxygen/CO_2 treatment and the interaction of bacteria*oxygen/CO_2 treatment on disease susceptibility were not significant as determined by a MANOVA (P = 0.7379 and P = 0.7412).

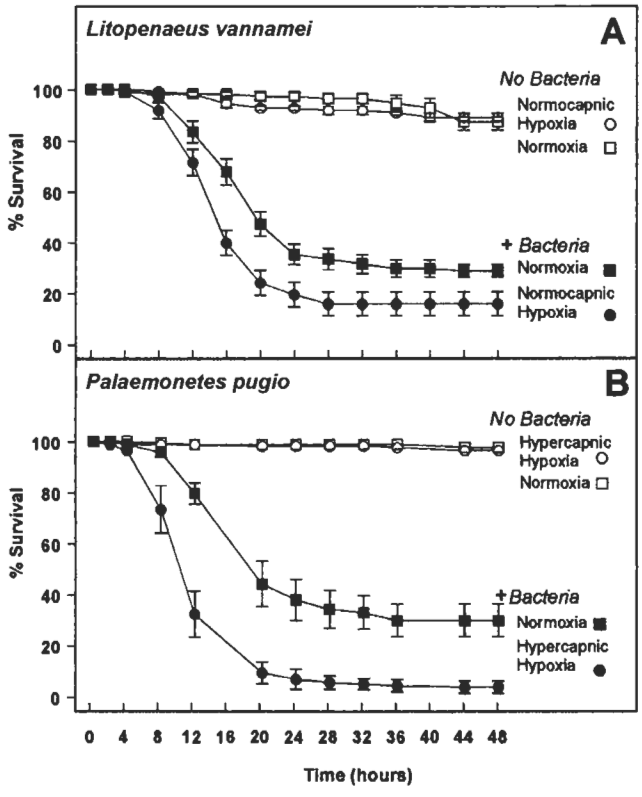

Figure 3. A. *Litopenaeus vannamei* survival following bacterial challenge under normoxia (Po_2 = 150–155 torr, Pco_2 = 0.23 torr, pH 7.6–8.0) and hypercapnic hypoxia (Po_2 = 30 torr, Pco_2 = 15.2 torr, pH 6.8–7.0). Shrimp were injected intramuscularly with 50 µL of *Vibrio parahaemolyticus* bacterial suspension (1.125×10^6 CFU/shrimp) or with HEPES buffered 2.5% NaCl for controls. There were 36 shrimp per treatment. Values at each time point are the mean (n = 3 experiments); standard errors are indicated except where the error is small and falls within the width of the datapoint. The effects of oxygen/CO_2 treatment and the interaction of bacteria*oxygen/CO_2 treatment on survival were significant as determined by a MANOVA (P = 0.0009 and P = 0.0493). Animals held under this level of hypercapnic hypoxia were more susceptible to *Vibrio* challenge than those held under normoxia. B. *Palaemonetes pugio* survival following bacterial challenge under normoxia (Po_2 = 150–155 torr, Pco_2 = 0.23 torr, pH 8.0–8.2) and hypercapnic hypoxia (Po_2 = 30 torr, Pco_2 = 15.2 torr, pH 6.9–7.0). Shrimp were injected intramuscularly with 5 µL of *Vibrio parahaemolyticus* bacterial suspension (9.10×10^4 CFU/shrimp) or with HEPES buffered 2.5% NaCl (controls). There were 40 shrimp per treatment. Values at each time point are the mean (n = 4 experiments); standard errors are indicated except where the error is small and falls within the width of the datapoint. The effects of oxygen/CO_2 treatment and the interaction of bacteria*oxygen/CO_2 treatment on survival were significant as determined by a MANOVA (P = 0.0113 and P = 0.0095). Animals held under this level of hypercapnic hypoxia were more susceptible to *Vibrio* challenge than those held under normoxia.

L. vannamei—normocapnic hypoxia at 45 torr oxygen

These challenge tests examined the effect of moderate hypoxia only (normocapnic hypoxia) without added Co_2 (hypercapnia) on *L. vannamei* survival (Fig. 2A). The effects of oxygen and the interaction of bacteria*oxygen were not significant at Po_2 = 45 torr as determined by a MANOVA (P = 0.6478 and P = 0.3594, respectively). The effect of bacteria alone was significant ($P \leq 0.0001$). Subsequent ANOVA tests revealed that the significance was due to differences in the coefficient and coefficient^2 ($P <$ 0.0001 for both). These results indicated that differences in *L. vannamei* survival were attributable to the injection of bacteria over the injection of saline, and not to differences in oxygen levels of the water.

P. pugio—hypercapnic hypoxia at 45 torr oxygen + 15.2 torr (2%) CO_2

These *Vibrio* challenges tested the effect of hypercapnic hypoxia on *P. pugio* survival at a moderate level of hypoxia (45 torr

or 6% O_2) (Fig. 2B). The effects of oxygen/CO_2 treatment and the interaction of bacteria*oxygen/CO_2 treatment were not significant ($P = 0.7379$ and $P = 0.7412$, respectively). The effect of bacteria was significant ($P < 0.0001$) and was attributable to differences in the coefficient and coefficient^2 ($P < 0.0001$ for both, ANOVA). These results show that there was no additional disease susceptibility in *P. pugio* held under this level of hypercapnic hypoxia than those held in normoxic water.

L. vannamei—hypercapnic hypoxia at 30 torr oxygen + 15.2 torr (2%) CO_2

These challenge tests investigated the effects of hypercapnic hypoxia on *L. vannamei* at a more severe level of hypoxia (30 torr or 4% O_2) (Fig. 3A). The effects of oxygen/CO_2 treatment, bacteria, and the interaction of bacteria*oxygen/CO_2 treatment were significant ($P = 0.0009$, $P < 0.0001$ and $P = 0.0493$, respectively) and were attributable to differences in the coefficient and coefficient^2. These results show that *L. vannamei* held under this level of hypercapnic hypoxia experienced a higher rate of mortality from *Vibrio* challenge than shrimp held under normoxic conditions. Average survival at 48 h for animals in normoxia was 28.7 ± 2.4% standard error (SE) versus 15.7 ± 4.6% SE for those in hypercapnic hypoxia.

P. pugio—hypercapnic hypoxia at 30 torr oxygen + 15.2 torr (2%) CO_2

These *Vibrio* challenges tested the effect of hypercapnic hypoxia on *P. pugio* at a more severe level of hypoxia (30 torr or 4% O_2) (Fig. 3B). The effects of oxygen/CO_2 treatment, bacteria, and the interaction of bacteria*oxygen/CO_2 treatment were significant ($P = 0.0113$, $P < 0.0001$, and $P = 0.0095$, respectively) and were attributable to differences in the coefficient and coefficient^2. These results show that *P. pugio* held under this level of hypercapnic hypoxia experienced higher mortality rates from bacterial challenge than animals held under normoxic conditions. Average survival at 48 h for normoxia was 29.4 ± 6.4% SE versus 3.1 ± 2.4% SE for hypercapnic hypoxia.

Total Hemocyte Count

Total hemocyte count significantly decreased in adult *L. vannamei* held under hypercapnic hypoxia when compared to animals held under normoxia over 48 hours. THC/mL was reduced in hypercapnic hypoxia by 60.7, 34.1, 34.3, 40.4 and 16.7% at 4, 8, 16, 24, and 48 hours, respectively, in relation to the normoxia value at the same time point (Fig. 4). A two-way ANOVA indicated that there was a significant effect of oxygen level/CO_2 treatment ($P < 0.0001$) on THC/mL; however, there was no significant effect of time ($P = 0.2907$) or the interaction between time and oxygen/CO_2 treatment ($P = 0.2276$). An *a posteriori* *t*-test used to compare means between oxygen levels at 48 hours revealed that although oxygen level/CO_2 treatment was significant in the two-way ANOVA, THC/mL was not significantly different between the two treatments at 48 hours ($P = 0.3207$, Fig. 4).

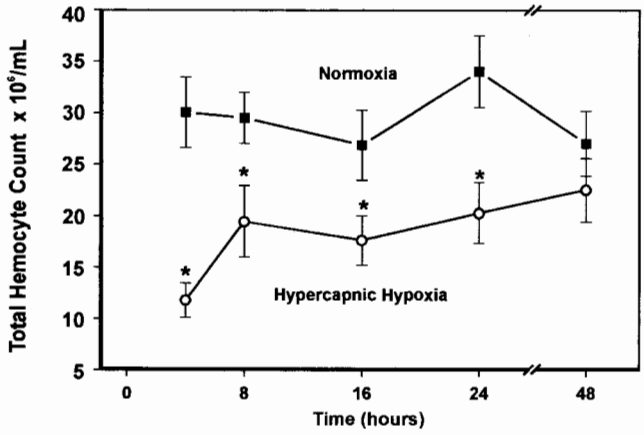

Figure 4. Total hemocyte counts (THC) per mL in *Litopenaeus vannamei* over 48 hours. Shrimp were held under normoxic conditions (closed squares, Po_2 = 150–155 torr, Pco_2 = 0.23 torr, pH 8.0–8.2) or under hypercapnic hypoxic conditions (open circles, Po_2 = 30 torr, Pco_2 = 15.2 torr, pH 6.9–7.1). Individual shrimp were used for each time point and for each test condition (n = 10 shrimp for normoxia and hypercapnic hypoxia at 4, 16, 24, and 48 h, n = 11 shrimp for normoxia and hypercapnic hypoxia at 8 h). A two-way ANOVA indicated that there was a significant effect of oxygen level/CO_2 treatment (P = <0.0001) on THC/mL; however, there was no significant effect of time (P = 0.2907) or the interaction between time and oxygen/CO_2 treatment (P = 0.2276). An *a posteriori* *t*-test revealed that THC/mL was not significantly different between normoxia and hypercapnic hypoxia at 48 hours (P = 0.3207). Values are mean ± standard error (α = 0.05).

DISCUSSION

Estuarine organisms routinely encounter fluctuations in oxygen, carbon dioxide, and pH that may affect their ability to defend against infections. Previous research has linked poor water quality, particularly hypoxia, with increased incidence of infectious disease (Snieszko 1974, Hargis et al. 1989, Landsberg et al. 1998). For example, Landsberg et al. (1998) found that the occurrence of opportunistic protist infections increased in fish subjected to low oxygen conditions. Noga et al. (1994) reported that blue crabs *Callinectes sapidus* collected in areas of the estuary where hypoxia is common, have low serum bacteriostatic activity. This decreased antibacterial activity was correlated with an increase in shell disease. Haley et al. (1967) attributed infections by *Aeromonas liquefaciens* in the threadfin shad *Dorosoma petenense* and the American shad *Alosa sapidissima* to low dissolved oxygen. However, in these field studies, pollutants or other physical factors, such as temperature, may have exacerbated the effects of hypoxia. There have been relatively few studies on the direct effects of hypoxia, hypercapnia, and low pH on disease susceptibility. In the present study, survival following challenge with *Vibrio parahaemolyticus* was depressed in hypercapnic and hypoxic water (Po_2 = 30 torr, Pco_2 = 15.2 torr and pH = 6.8–7.0) in the penaeid shrimp *Litopenaeus vannamei* and in the grass shrimp *Palaemonetes pugio* (Fig. 3A and B). In addition, the THC/mL in *L. vannamei* was reduced under the same conditions (Fig. 4).

The present study used a known pathogenic strain isolated from shrimp with vibriosis. LD_{50} values were reproducible using the same strain and produced consistent mortalities when used in the challenge assays. These are the first reported LD_{50} values for *L. vannamei* and *P. pugio* using *V. parahaemolyticus*.

The 48-h LD_{50} of *V. parahaemolyticus* for *L. vannamei* reported in the present study (Table 2) is similar to the LD_{50} of the same bacterial species for *P. monodon* (3.16×10^5, 95% C.I. 9.60×10^4 to 1.03×10^6 CFU/shrimp). The latter values were calculated from data in Alapide-Tendencia and Dureza (1997) using the trimmed Spearmann–Karber program. Arume (1989) reported LD_{50} values of *Vibrio* isolates to *Litopenaeus stylirostris* ranging from 4.0×10^2 to 3.3×10^4 CFU/g, which is lower than the value

of 3.06×10^5/g wet weight calculated for *L. vannamei* (Table 2). However, the species of *Vibrio* used was not reported. In contrast, *V. parahaemolyticus* had a much higher LD_{50} value for *M. japonicus* juveniles of 4.27×10^7 CFU/shrimp (Vera et al. 1992). This inconsistency in LD_{50} values may be attributable to host specificity and the differences in the size of the animals (Vera et al. 1992, Lee et al. 1996). In addition, virulence of bacteria can vary among strains (Arume 1989, Thune et al. 1993, Wong et al. 1996).

L. vannamei and *P. pugio* were more susceptible to *V. parahaemolyticus* when held under hypercapnic hypoxia at 30 torr oxygen (1.41 mg/L), 15.2 torr (2%) CO_2 and a pH of 6.8 to 7.0 than under normoxia at 150–155 torr oxygen (7.29 mg/L), approximately 0.23 torr CO_2 (0.03%) and a pH of 7.6–8.2 (Figs. 3A and B). This decrease in disease resistance was not attributable to enhanced bacterial growth under these conditions (data not shown). Le Moullac et al. (1999) also found that mortality under hypoxia at 1 mg O_2/L (48%) was significantly greater than control (well-aerated water) mortality (32%) when *L. stylirostris* was challenged with *V. alginolyticus*. However, the levels of CO_2 and the resultant hypoxic pH were not controlled or reported by the investigators. As a result, it is unclear if the animals were subject to hypercapnic hypoxia or to normocapnic hypoxia. In the present study, there was no significant effect on disease susceptibility in *L. vannamei* of normocapnic hypoxia at 45 torr oxygen with less than 1 torr CO_2 or in *P. pugio* under hypercapnic hypoxia at 45 torr oxygen and 15.2 torr CO_2 (Figs. 2A and B).

The level of hypoxia at which disease susceptibility increased (Po_2 = 30 torr) over normoxia in both species may be explained, in part, by the shrimps' critical oxygen tension. The critical oxygen tension for an organism is the oxygen tension below which an organism is unable to maintain its rate of oxygen uptake. Below the critical oxygen tension, organisms may be unable to sustain an internal oxygen level sufficient to defend against infection. Cochran and Burnett (1996) reported a critical Po_2 for *P. pugio* between 30 and 35 torr, which may partly explain differences in susceptibility at 30 torr (1.41 mg O_2/L) and 45 torr oxygen (2.12 mg O_2/L) observed in grass shrimp in this study (Figs. 2B and 3B). On the other hand, Hutcheson et al. (1985) reported a much higher critical Po_2 (approximately 95 torr) for the same species. Nielsen and Hagerman (1998) reported critical Po_2s for *Palaemonetes varians* and *Palaemon adspersus* of 2.4 mg O_2/L (approximately 46 torr) and 2.87 mg O_2/L (approximately 55 torr), respectively, which are both above the highest level of oxygen used in the present experiments. Villarreal et al. (1994) identified a critical Po_2 of 1.3 mg O_2/L (approximately 34 torr) in *L. vannamei*. This value is similar to the value of 1.41 mg O_2/L (30 torr) found to be significant to disease resistance in *L. vannamei* in the present research. In contrast, Rosas et al. (1999) found that juvenile *Litopenaeus setiferus* were oxyregulators down to 4 mg O_2/L (approximately 92 torr), but were oxyconformers between 3 and 2 mg O_2/L (approximately 69 and 46 torr) suggesting that the critical Po_2 lies between those two values. The variability in published critical oxygen pressures may be attributable to many factors including temperature, salinity, activity, molt cycle, size, and experimental technique that can affect the critical Po_2 of a species (Herreid 1980, Dall 1986, Cochran and Burnett 1996).

It is important to note that the oxygen tensions used in the present study were well above the lethal limits reported for these and similar species. Hopkins et al. (1991) reported an oxygen lethal limit of 1 mg O_2/L (approximately 22 torr) for *L. vannamei*. Allan and Maguire (1991) calculated 98-h and 24-h oxygen LC_{50}s for juvenile *P. monodon* of 0.9 mg O_2/L and 0.6 mg O_2/L (approximately 21 and 14 torr), respectively, demonstrating that the duration of the hypoxia also has an effect. Stickle et al. (1989) showed that *F. aztecus* were much more sensitive to low oxygen than *P. pugio*. The 28-day LC_{50} values were 123 torr (5.94 mg O_2/L) for *F. aztecus* and 46 torr (2.22 mg O_2/L) for *P. pugio*. Differences in disease susceptibility between the two species used in the present study (*L. vannamei* and *P. pugio*) could not be compared statistically because of differences in the size of the shrimp and the bacterial challenge dose; however, they exhibited similar responses to the two levels of oxygen tested (Figs. 2 and 3).

As mentioned previously, studies that investigate the effects of hypoxia on estuarine organisms often do not take into account hypercapnia and the low pH that accompanies it (Hutcheson et al. 1985, Seidman and Lawrence 1985, Allan and Maguire 1991, Charmantier et al. 1994, Direkbusarakom and Danayadol 1998, Nielsen and Hagerman 1998, Le Moullac et al. 1999). Nevertheless, these variables may have contributed, in combination or independently, to the decreased disease resistance observed in the present work. Martinez et al. (1998) reported that the lethal dissolved oxygen concentrations for postlarval and juvenile *L. setiferus* are higher under low pH (pH = 6) than under high pH (pH = 8). In addition, McCulloch (1990) found that low pH raised the critical oxygen concentration from 1.54 mg O_2/L at pH 9.0 to 2.08 mg O_2/L at pH 6.5 for *Palaemonetes kadiakensis*. Cochran and Burnett (1996) demonstrated that oxygen uptake was significantly higher at high CO_2 than at low CO_2 in the spot *Leiostomus xanthurus*. Cruz-Neto and Steffensen (1997) reported that hypercapnia increased the critical oxygen concentration from 25 torr to 40–45 torr in the European eel *Anguilla anguilla*. These studies show that hypercapnia can adversely affect hypoxia tolerance.

Total hemocyte count was significantly reduced in *L. vannamei* held under hypercapnic hypoxia (Po_2 = 30 torr, Pco_2 = 15.2 torr, pH 6.8–7.0) when compared to shrimp held under normoxia (Po_2 = 150–155 torr, Pco_2 = 0.23 torr, pH 7.6–8.0) at 4, 8, 16, and 24 h (Fig. 4). Similarly, Le Moullac et al. (1999) found that THC/mL decreased in *L. stylirostris* exposed to hypoxia at 1 mg O_2/L for 24 hours. Alvarez et al. (1989) also noted a reduction in hemocyte concentration in oysters held under two levels of hypoxia (9 and 80 torr oxygen) for 3 days.

Although not measured in the present study, injection of whole bacteria or isolated cell wall components of bacteria and yeast can trigger a decrease in THC/mL in crustaceans (Hauton et al. 1997, Smith et al. 1983, Lorenzon et al. 1999). Using bacterial lipopolysaccharide to suppress circulating THC, Lorenzon et al. (1999) reported threshold lethal limits for THC of 28.9, 32.9, and 15.3% of the initial circulating cells for *P. elegans, C. crangon,* and *Squilla mantis,* respectively. These observations suggest that *L. vannamei* and *P. pugio* in the present study that were challenged with bacteria while being held under hypercapnic hypoxia may have experienced a greater decrease in THC than the unchallenged adults held under hypercapnic hypoxia alone. The average total hemocyte count in *L. vannamei* in the present study was reduced to 39.3% of the normoxic average 4 hours after placement in hypercapnic hypoxia (Fig. 4). The combined effects of hypercapnic hypoxia and bacterial injection could reduce cell density in shrimp to a level below the minimum necessary for survival. This is a possible explanation for the increase in mortality in shrimp challenged with bacteria and exposed to hypercapnic hypoxia as compared to animals challenged with bacteria and maintained under normoxia in this study. It is important to note, however, that

dead or moribund animals exhibited signs of vibriosis and that low hemocyte number was not the sole cause of mortality. More likely, the low cell numbers in animals held under hypercapnic hypoxia were insufficient to defend against the bacterial challenge.

The decrease in THC/mL observed in this study may contribute to the increase in mortality rate following bacterial challenge under hypercapnic hypoxia, but many other factors also may play a role. Le Moullac et al. (1999) found that respiratory burst activity, as measured by NBT reduction, decreased in *L. stylirostris* subjected to hypoxia (1 mg O_2/L), but that phenoloxidase (PO) activity increased significantly because of a lower amount of inhibitors regulating the prophenoloxidase system. Direkbusarakom and Danayadol (1998) demonstrated that phagocytosis and bacterial clearance efficiency were reduced in *P. monodon* exposed to 1.8–2.0 mg O_2/L. Although Alvarez et al. (1992) reported that phagocytosis by hemocytes of the eastern oyster *C. virginica* was not affected by hypoxia, Boyd and Burnett (1999) demonstrated that reactive oxygen intermediate (ROI) production by hemocytes was significantly depressed under hypoxia in the same species. Boleza (1999) found that ROI production and bactericidal activity of phagocytes in the mummichog *Fundulus heteroclitis* were suppressed under hypercapnic hypoxia. Comparable studies of cellular and acellular bactericidal factors in *P. vannamei* and *P. pugio* under relevant water quality conditions may clarify the defense mechanisms that are sensitive to dissolved gasses and pH.

The results of the present study show that hypercapnic hypoxia at 30 torr O_2, 15.2 torr CO_2 and a pH range of 6.8 to 7.0 decreases survival following bacterial challenge in both *L. vannamei* and *P. pugio* and decreases total hemocyte count in *L. vannamei* (Fig. 3A and B and 4). This has implications regarding the health of these organisms in both the natural environment and in aquaculture. Diaz and Rosenberg (1995) reported that the occurrence of hypoxia in shallow coastal and estuarine systems is increasing worldwide. Dissolved oxygen monitoring by Summers et al. (1997) in the mid-Atlantic and Gulf of Mexico regions suggests that the extent of hypoxia is often substantially underestimated. Thus, conditions that suppress disease resistance may become more prevalent, which could affect the penaeid shrimp fishery and reduce the density of the ecologically important grass shrimp. In addition, a decrease in THC/mL under hypercapnic hypoxia, as observed in this research (Fig. 4), could result in a decrease in immune function and possibly a reduction of the effectiveness of immunostimulants used in aquaculture to prevent outbreaks of disease (Sung et al. 1991, Sung et al. 1996, Itami et al. 1998, Devaraja et al. 1998, Teunissen et al. 1998). Taken together with the well-recognized importance of water oxygen, dissolved CO_2 and concomitant changes in pH that accompany naturally occurring hypoxia should be carefully monitored and regulated to sustain the wild shrimp fishery and optimize farm production.

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