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Effects of Hypercapnic Hypoxia on the Clearance of *Vibrio campbellii* in the Atlantic Blue Crab, *Callinectes sapidus* Rathbun

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Abstract. *Callinectes sapidus,* the Atlantic blue crab, encounters hypoxia, hypercapnia (elevated CO_2), and bacterial pathogens in its natural environment. We tested the hypothesis that acute exposure to hypercapnic hypoxia (HH) alters the crab's ability to clear a pathogenic bacterium, *Vibrio campbellii* 90–69B3, from the hemolymph. Adult male crabs were held in normoxia (well-aerated seawater) or HH (seawater with P_{O_2} = 4 kPa; P_{CO_2} = 1.8 kPa; and pH = 6.7–7.1) and were injected with 2.5×10^4 *Vibrio* g^{-1} body weight. The animals were held in normoxia or in HH for 45, 75, or 210–240 min before being injected with *Vibrio*, and were maintained in their respective treatment conditions for the 120-min duration of the experiment. *Vibrio* colony-forming units (CFU) ml^{-1} hemolymph were quantified before injection, and at 10, 20, and 40 min afterward. Total hemocytes (THC) ml^{-1} of hemolymph were counted 24 h before (−24 h), and at 10 and 120 min after injection. Sham injections of saline produced no change in the bacterial or hemocyte counts in any treatment group. Among the groups that received bacterial injections, *Vibrio* was almost completely cleared within 1 h, but at 10-min postinjection, *Vibrio* CFU ml^{-1} hemolymph was significantly higher in animals held in HH for 75 and 210–240 min than in those held in normoxia. Within 10 min after crabs were injected with bacteria, THC ml^{-1} significantly decreased in control and HH45 treatments, but not in the HH75 and HH210–240 treatments. By 120 min after injection of bacteria, hemocyte counts decreased in all but the HH45 group. These data demonstrate that HH significantly impairs the ability of blue crabs to clear *Vibrio* from the hemolymph. These results also suggest that HH alters the normal role of circulating hemocytes in the removal of an invading pathogen.

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Abbreviations: CFU, colony-forming unit; HH, hypercapnic hypoxia; PPO, prophenoloxidase; THC, total hemocyte count.

Introduction

Where they occur naturally in coastal waters, hypoxia (low oxygen) and hypercapnia (high carbon dioxide) are believed to contribute to outbreaks of infectious disease, such as mycobacteriosis in fish (Rhodes *et al.*, 2001) and infections with a protozoan parasite in oysters (Anderson *et al.*, 1998). The effects of hypoxia and hypercapnic hypoxia (HH) on disease susceptibility are likely to be multiple and complex, changing not only respiration and circulation, but also immune defense (Burnett, 1997). In laboratory-based studies, we and others have shown that hypoxia and HH can increase the rate of mortality in penaeid or palaemonid shrimp injected with live bacterial pathogens (Le Moullac *et al.*, 1998; Mikulski *et al.*, 2000). Here we asked whether levels of dissolved O_2 and CO_2 that increase mortality rates in shrimp also reduce the rate at which live bacteria are removed from the hemolymph of another crustacean species, *Callinectes sapidus* Rathbun, 1896, the Atlantic blue crab.

Crustaceans employ a broad spectrum of soluble (humoral) factors in immune defense, including non-self recognition proteins, immediate defense molecules such as clotting proteins and prophenoloxidase, and antimicrobial peptides (reviewed by Bachère 1998, 2000). Many of these humoral factors are produced, stored, and released from hemocytes—the major cellular components of the crustacean immune system. In addition, hemocytes can adhere to a pathogen, triggering phagocytosis and production of highly toxic reactive oxygen species (Song and Hsieh, 1994; Gargioni and Barracco, 1998; Muñoz *et al.*, 2000).

In well-aerated normoxic water, crustaceans rapidly remove bacteria or other large particles from the hemolymph, with a coordinate drop in the total hemocyte count per milliliter of hemolymph (THC ml^{-1}) (White and Ratcliffe, 1982; Martin *et al.*, 1993). Smith and Ratcliffe (1980) and Martin *et al.* (1993, 1998) have suggested that hemocytes aggregate with injected particles to form nodules that become trapped in small capillary beds of well-vascularized tissues such as the gill and the hepatopancreas. This aggregation is believed to involve non-self recognition proteins, prophenoloxidase (PPO), and antimicrobial peptides. In contrast, van de Braak *et al.* (2002) presented evidence that hemocytes become fixed in peripheral organs before taking up bacteria by phagocytosis.

After shrimp received an injection of pathogenic bacteria, those held in hypoxia (Le Moullac *et al.*, 1998) or HH (Mikulski *et al.*, 2000) had higher rates of mortality than animals held under normoxic conditions. Several mechanisms underlie the effects of dissolved gasses on the susceptibility of organisms to a pathogen. Hypoxia and hypercapnia can suppress several key components of the invertebrate immune system that are responsible for killing and clearing bacterial pathogens from tissues. In penaeid shrimp, both hypoxia (Le Moullac *et al.*, 1998) and HH (Mikulski *et al.*, 2000) induced significant decreases in THC ml^{-1} hemolymph while also decreasing resistance to bacterial pathogens. Low O_2 and low pH (induced by high CO_2) independently and additively suppressed *in vitro* production of reactive oxygen species by oyster hemocytes (Boyd and Burnett, 1999), and hypoxia suppressed phagocytosis in the blue shrimp *Litopenaeus stylirostris* (Le Moullac *et al.*, 1998). However, the complexity of the immune responses in the whole organism makes it difficult to attribute changes in disease outcome to any specific defense mechanism.

As an alternative approach to understanding the effects of dissolved O_2 and CO_2 on susceptibility to microbial pathogens in crustaceans, we tested whether hypoxia and hypercapnia can suppress the ability of the blue crab to eliminate live bacteria from its hemolymph. We chose blue crabs because they are hearty organisms that are abundant in estuaries where levels of oxygen and carbon dioxide fluctuate, and they can easily tolerate experimental manipulations such as a bacterial injection and multiple samplings of hemolymph. Animals with prior exposure to HH for 0, 45, 75, or 210–240 min were injected with live *Vibrio campbellii* or saline and were maintained in HH. For control groups, crabs were held in normoxia before and after injection with the same dose of live bacteria or saline. One day before injection (−24 h) and at 10, 20, and 40 min after injection, we monitored the number of colony-forming units (CFU) of bacteria ml^{-1} hemolymph. We also monitored THC ml^{-1} of all experimental animals at −24 h, 10 min, and 120 min postinjection and compared the responses of their circulating hemocytes to bacterial challenge.

Materials and Methods

Male blue crabs were trapped in the creeks of Charleston Harbor, Charleston, South Carolina and transported to the Grice Marine Laboratory where they were held in recirculating seawater at 25 ppt salinity and 24–26 °C. The crabs weighed between 92 and 236 g and were held for a minimum of 3 days prior to experimentation, but no longer than 10 days. The animals were fed frozen fish or shrimp each day, but food was withheld for at least 24 h before the experiments began.

Preparation of the crabs for treatment

A 1-mm hole was drilled in the carapace directly over the heart, creating a port through which saline alone, or saline containing bacteria, was injected directly into the ventricle. The bacteria injected into the heart would then be rapidly distributed throughout the circulatory system. Two similar holes were drilled over the pericardium adjacent to the heart through which hemolymph was withdrawn from the pericardium. Two holes were drilled in case we could not easily withdraw hemolymph from one hole. A thin layer of latex rubber was glued over each hole with cyanoacrylate glue. A needle could be inserted into each hole through the rubber diaphragm and withdrawn easily without causing bleeding. These procedures were performed 2 days prior to experimentation.

Intracardiac (postbranchial) injection of bacteria

The postbranchial point of injection used in the present study is distinct from that used by others who injected pathogens into muscle tissues (Alday-Sanz *et al.*, 2002) or sinuses downstream from the heart (Smith and Ratcliffe, 1980; Martin *et al.*, 1993, van de Braak *et al.*, 2002). Injecting bacteria directly into the single-chambered heart ensures that the bacteria will be rapidly and evenly distributed throughout the circulatory system. The heart of the blue crab distributes hemolymph through seven major arteries (McGaw and Reiber, 2002), and the high cardiac output typical of crabs (McMahon and Burnett, 1990) ensures a uniform distribution throughout the crab's circulatory system, as shown by studies using thermal dilution techniques (Burnett *et al.*, 1981).

The effects of acute exposure to HH on the cardiac output of *C. sapidus* are unknown, but acute hypoxia causes a reduction in heart rate of about 25% (deFur and Mangum, 1979). Cardiac output in crustaceans is often strongly influenced by changes in cardiac stroke volume rather than heart rate, but even with a 25% reduction in cardiac output, mixing and circulation of bacteria injected into the ventricle

would still be rapid. Injection of bacteria into the heart avoids the localization of pathogens that can occur for reasons not specifically associated with normal routes of clearance by the whole organism. For example, injecting pathogens into the infrabranchial sinus, which supplies hemolymph to one or more gills, may bias the observed role of the gill in pathogen clearance. In the present study, sampling hemolymph from the pericardial sinus, which is immediately downstream from the gill, ensured that the bacteria sampled had made a complete circuit through the circulatory system.

Preparation of the pathogen

The bacterial pathogen used in these studies was *Vibrio campbellii* 90–69B3, which was originally isolated from diseased shrimp by D. Lightner and L. Mahone (University of Arizona). The 16S rRNA sequence of this strain places it in the *V. parahaemolyticus/V. harveyi* family with 99% identity to *V. campbellii* (unpubl. data, Eric Stabb, University of Georgia). For each assay, *V. campbellii* 90–69B3 (hereafter referred to as *V. campbellii*) was thawed from frozen aliquots, streaked onto tryptic soy agar (TSA) + 2.5% NaCl, and incubated overnight at 25 °C. A separate aliquot was used for each assay. A wooden applicator stick was used to transfer the bacteria from the culture plate to sterile 2.5% NaCl buffered with 10 mmol 1^{-1} HEPES adjusted to pH 7.6 (HEPES saline). The concentration of *V. campbellii* was adjusted to an optical density of 0.1 at 540 nm (OD_{540nm}). This OD_{540nm} had previously been determined to equal 1.0×10^8 CFU ml^{-1} (Mikulski *et al.*, 2000). The bacterial suspension was then diluted with HEPES saline to obtain the desired dose for injection.

Assessment of baseline conditions

One day (*i.e.*, −24 h) before each bacterial challenge experiment, two 100-μl samples of hemolymph were withdrawn through the pericardial sampling port. One sample was used to determine whether the crab had detectable levels of live, culturable bacteria in the hemolymph, as measured in the CFU assay. The other sample was used to determine the THC ml^{-1} in the hemolymph. To measure CFU ml^{-1}, one part of hemolymph was diluted with 9 parts HEPES saline. A 150-μl sample of this mixture was suspended in marine agar and plated over TSA and TCBS (thiosulfate citrate bile sucrose) agar plates. TSA supports the growth of a wide range of bacteria; TCBS agar is more selective, and supports the growth of a few species of *Escherichia* and *Vibrio*, including *V. campbellii.* Plates were incubated at 25 °C for 24 h, at which time the number of bacterial colonies was counted and recorded. For each hemolymph sample, bacterial colonies on three replicate plates were counted and averaged. CFU ml^{-1} was calculated according to the formula CFU $plate^{-1} \times 10$ dilution factor/0.15 ml, where CFU is the average number of bacterial colonies counted on three replicate TCBS plates for each 0.15-ml hemolymph sample diluted 10-fold in saline prior to plating. Only crabs whose hemolymph had no CFU on TSA or TCBS plates at the −24 h time point were used in these experiments. The frequency with which CFU are detected in the hemolymph of crabs collected from the field varies considerably with the season. During early to midsummer, when these experiments were performed, about 10% to 15% were positive for CFU in the hemolymph at −24 h and were rejected for experimental use. The same assay was used to determine CFU ml^{-1} in hemolymph samples taken from crabs after injection of *V. campbellii* or saline, except that the marine agar containing the diluted hemolymph sample was plated only on TCBS plates. This provided a measure of assurance that the bacterial colonies being counted in the hemolymph arose from the injected bacteria.

The THC ml^{-1} in a hemolymph sample was determined as follows. Hemolymph (100 μl) was drawn into a syringe containing 900 μl of ice-cold 10% neutral buffered formalin (Mix and Sparks, 1980). After mixing, an aliquot of the hemocyte suspension was transferred to a hemocytometer for direct counting. Hemocytes were counted in three separate aliquots of each hemolymph sample and averaged for each crab at each time point in these experiments.

Experimental protocol

In a typical experiment (Fig. 1), a crab was transferred to a 17-l glass aquarium in which oxygen and carbon dioxide levels were regulated (Mikulski *et al.*, 2000). For all experiments, the seawater was at first aerated vigorously; and for treatments in normoxia, crabs were held in this well-aerated water (20.7 kPa P_{O_2} and <0.06 kPa P_{CO_2}) throughout the experiment. At the start of all HH treatments, the oxygen and the carbon dioxide pressures of the water were regulated (Mikulski *et al.*, 2000) to achieve values of 4 kPa P_{O_2} and 1.8 kPa P_{CO_2} within 20 to 30 min. The crabs were held in HH for one of three durations prior to injection of bacteria (45, 75, and between 210 and 240 min), and they remained in these HH conditions throughout the experiment (Fig. 1). The crabs were injected with *V. campbellii* suspended in HEPES saline. The bacterial suspension (between 40 μl and 140 μl, depending on the size of the crab) was injected directly into the ventricle. Control animals from the normoxia treatment were injected with the same dose of bacteria.

Crabs were injected with 2.5×10^4 bacteria g^{-1} body weight, to achieve a circulating dose of 1.0×10^5 *V. campbellii* ml^{-1} of hemolymph, assuming a hemolymph volume of 25 ml 100 g^{-1} body weight (Gleeson and Zubkoff, 1977). The dose is slightly below the LD_{50} for

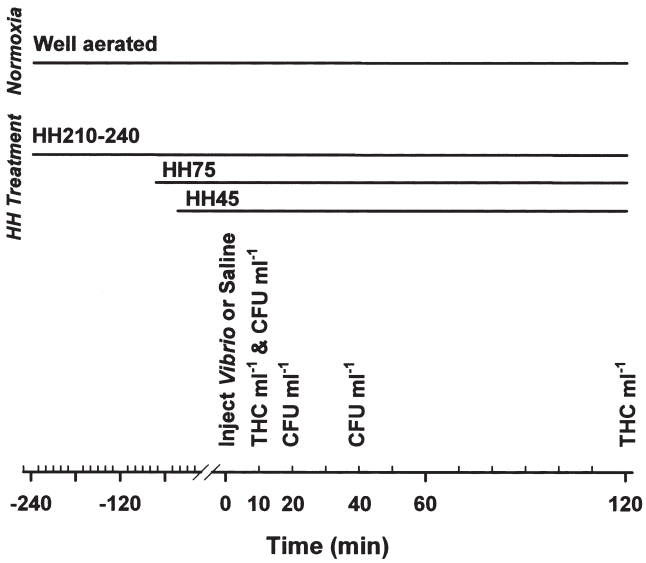

Figure 1. An illustration of the experimental design, indicating the timing of exposure to hypercapnic hypoxia (HH), bacterial injection, and hemolymph withdrawal along with subsequent analyses. Crabs were placed in experimental tanks and HH treatments were initiated at different times prior to the injection of bacteria, which is indicated at time = 0. For example, in the HH210–240 treatment, HH was initiated between 210 and 240 minutes before injection and maintained until 120 min after injection. The normoxia group was held in well-aerated water before and after the injection of bacteria. For the sham injection treatment, crabs were held in one of the HH treatments or in normoxia, then were injected with HEPES-saline at time = 0, and maintained in the same treatment condition for 120 min after injection. Hemolymph samples were taken from animals in all treatment groups at the same time points and for the same assays as illustrated.

juveniles of the penaeid shrimp *Litopenaeus vannamei* (Mikulski *et al.*, 2000).

After the bacteria were injected, animals in HH treatments were maintained in HH and those in normoxic treatments were held in normoxia. Hemolymph was sampled from the pericardium of each crab at 10, 20, 40, and 120 min. Preliminary experiments indicated that these time points would be optimal for discerning the impacts of HH on hemocyte counts and bacterial clearance. At the 10-min time point, two hemolymph samples were withdrawn from the pericardium. One unfixed sample was used to quantify the *Vibrio* CFU ml^{-1} remaining in the hemolymph, as described above. The other sample was fixed in formalin and used to quantify THC ml^{-1} hemolymph, as described above. At the 20-min and 40-min time points, hemolymph was sampled, and the number of CFU ml^{-1} was determined again. Finally, a sample of hemolymph taken at 120 min was fixed in formalin to monitor THC ml^{-1}. The crab was then removed from the aquarium, frozen, and ultimately autoclaved and discarded.

To control for the effects of injection and hemolymph sampling, sham experiments were performed for each treatment (normoxia, HH45, HH75, and HH210–240) by injecting a sterile solution of HEPES saline into the ventricle as described above. Hemolymph was sampled at the time points indicated above (Fig. 1), and *V. campbellii* and hemocytes were quantified. During all injections and samplings, the animals remained submerged in the aquarium, either in normoxic or HH water, with minimal disturbance. The crab was near the top of the 17-l aquarium on a plastic platform, where it was completely immersed and free to move. To inject bacteria or sample hemolymph, the crab remained immersed but was lifted so that the injection or sampling ports on its carapace were raised to the surface of the water.

Aseptic techniques were used when working with the bacteria, and all waste material was autoclaved or disinfected with 2% chlorine bleach. Experimental tanks were rinsed with 2% bleach daily, and the filtration systems were rinsed with fresh water daily.

Data analysis

SigmaStat 3.0 software was used to perform all statistical analyses. To determine whether the amount of bacteria in the hemolymph changed as a function of time after injection, a one-way ANOVA was performed on the *V. campbellii* CFU ml^{-1} hemolymph at 10, 20, and 40 min within each treatment group. All tests for normality (Kolmogorov-Smirnov test) failed; therefore, a Kruskal-Wallis ANOVA on ranks test was used. When differences within a treatment group were detected, the Student-Newman-Keuls method was used for multiple comparisons between individual time points.

To determine whether there were differences at individual times among treatment groups, a one-way ANOVA was performed on CFU ml^{-1} data at 10, 20, and 40 min across all treatment groups (normoxia, HH45, HH75, and HH210–240). As above, all tests for normality (Kolmogorov-Smirnov test) failed, so a Kruskal-Wallis ANOVA on ranks test was used. When the test indicated a significant effect of treatment within a time, a Dunn's test was used, because of unequal sample sizes, to compare HH treatments with the normoxic value.

To determine whether there were differences in THC ml^{-1} at one day prior to the initiation of the experiments, a one-way ANOVA was performed for the crabs across all normoxic and HH treatments. For subsequent analysis, THC ml^{-1} data at 10 and 120 min were normalized to the −24 h counts for an individual crab. A one-way ANOVA was performed on normalized THC ml^{-1} for each normoxic and HH treatment group as a function of time after sham and bacterial injections. All tests for normality (Kolmogorov-Smirnov test) or equal variances failed and, therefore, a Kruskal-Wallis ANOVA on ranks test was used. When a significant effect of time within a treatment group was indicated, a comparison of 10-min and 120-min counts with

−24 h counts was performed using Dunnett's test (equal sample sizes).

Results

Clearance of bacteria from the hemolymph

The theoretical maximum *Vibrio* CFU ml^{-1} hemolymph after injection is 1×10^5 ml^{-1}. This value is based on the known number of bacteria injected and assumes a homogeneous distribution of bacteria in the hemolymph as well as a hemolymph volume of 25 ml per 100-g crab (Gleeson and Zubkoff, 1977). Patterns of bacterial clearance were similar in all treatment groups: when the crabs were injected with bacteria, *V. campbellii* CFU ml^{-1} hemolymph declined precipitously to very low levels within 10 min after injection and became almost undetectable after 40 min (Table 1, Fig. 2). Even though most of the bacterial clearance occurred before the 10-min measurement, the decrease between the CFU ml^{-1} at 10 min and the value at 40 min was significant in all treatment groups. Comparisons between 10 and 20 min and between 20 and 40 min revealed significant differences in some, but not all, cases (Table 1, Fig. 2).

Comparisons of different treatments at single time points after injection of *V. campbellii* revealed significant differences in CFU ml^{-1} at 10 min between the normoxic treatment and the HH75 treatment and between the normoxic treatment and the HH210–240 treatment (Kruskal-Wallis ANOVA on ranks and Dunn's multiple comparison procedure, $P = 0.002$); but there were no differences between the normoxic and the HH45 treatments (Fig. 2). With treatment as a variable, differences were detected 20-min postinjection (Kruskal-Wallis ANOVA on ranks, $P = 0.027$), but no differences among the individual treatments were found using Dunn's multiple comparison procedure. No differences were detected among treatments at 40-min postinjection when treatment was used as a variable (Kruskal-Wallis ANOVA on ranks, $P = 0.131$). No bacterial colonies were detected at any time in the hemolymph samples from animals that received sham injections of saline (data not shown).

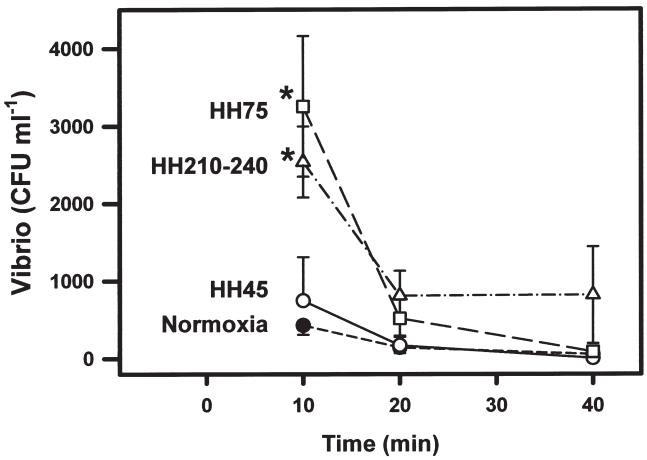

Figure 2. Colony-forming units (CFU) ml^{-1} of *Vibrio campbellii* circulating in the hemolymph at different times after injection plotted as a function of treatment in well-aerated normoxic water or in hypercapnic hypoxia water (PCO_2 = 1.8 kPa, PO_2 = 4 kPa). Crabs were exposed to normoxia or to hypercapnic hypoxia (HH) for different times (given in minutes), then injected with bacteria at time = 0, and for the subsequent duration of the experiments were held in well-aerated (normoxia) or HH water. At time = 0, crabs were injected with 2.5×10^4 *V. campbellii* g^{-1} body weight to achieve a theoretical circulating concentration of 100×10^3 CFU (colony-forming units) ml^{-1} hemolymph. Levels of bacteria in hemolymph (CFU ml^{-1}) are shown at 10, 20, and 40 min after injection. Mean values ± standard error are shown. Significant differences between the normoxic treatment and the HH treatment occurred only at 10 min after injection and are indicated by an asterisk (*).

Table 1

Statistical analysis of the colony-forming units (CFU) ml^{-1} of Vibrio campbellii *in the hemolymph of crabs at different times following injection*

Treatment	*n*	*P* value: Kruskal-Wallis ANOVA	*P* value: Pairwise comparisons using Student-Newman-Keuls method		
			10 and 20 min	10 and 40 min	20 and 40 min
Normoxia	8	0.005	NS	<0.05	NS
HH45	4	0.005	NS	<0.05	<0.05
HH75	7	<0.001	<0.05	<0.05	<0.05
HH210–240	9	0.008	<0.05	<0.05	NS

Tests for normality failed on one-way ANOVA; therefore, data within a treatment group were tested for differences using a Kruskal-Wallis ANOVA on ranks. Values at different time intervals within a treatment were compared pairwise using the Student-Newman-Keuls method. HH, hypercapnic hypoxia; NS, nonsignificant.

Hemocyte counts

The THC ml^{-1} in hemolymph of crabs prior to treatment (−24 h) was the same across all treatment groups (ANOVA, $P = 0.557$; Figs. 3 and 4). No treatment group that received a sham injection ($n = 17$) showed a significant difference in circulating hemocyte counts at any time (Kruskal-Wallis ANOVA on ranks, $P > 0.155$, Fig. 3). THC ml^{-1} of animals held in normoxia (well-aerated conditions) declined significantly 10 and 120 min after injection with *V. campbellii* (Table 2; Fig. 4). Circulating hemocyte counts declined in the HH 45 (hypercapnic hypoxia 45-min) treatment group 10 min after injection of *V. campbellii,* but at 120 min, the count was not detectably different from the baseline count at −24 h (Table 2, Fig. 4). When crabs were held in HH for 75 min and then injected with *V. campbellii*, there was no change in THC ml^{-1} after 10 min, but the hemocyte concentration declined significantly after 120

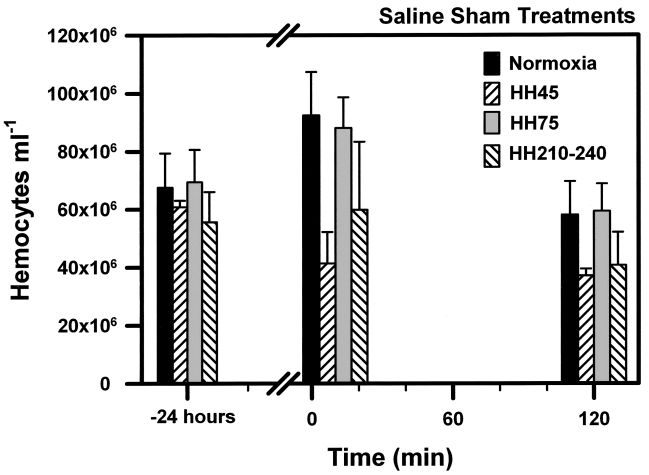

Figure 3. Total hemocyte counts (THC) ml^{-1} of hemolymph in crabs one day prior to treatment (−24 h) and 10 and 120 min after injection of saline in four treatment groups. Normoxia = crabs in well-aerated water, HH treatments = hypercapnic hypoxia (P_{O_2} = 4 kPa, P_{CO_2} = 1.8 kPa) administered for 45, 75, and 210–240 min before the saline injection at time = 0. No significant differences were detected within any treatment between pre-injection (−24 h) THC ml^{-1} and postinjection values at 10 and 120 min. Mean values + standard error are shown.

min. The same pattern of response occurred when crabs were incubated in HH for 210–240 min and then injected with *V. campbellii* (Table 2; Fig. 4).

Discussion

Minutes after being injected with *Vibrio campbellii*, blue crabs rapidly remove the bacteria from their hemolymph.

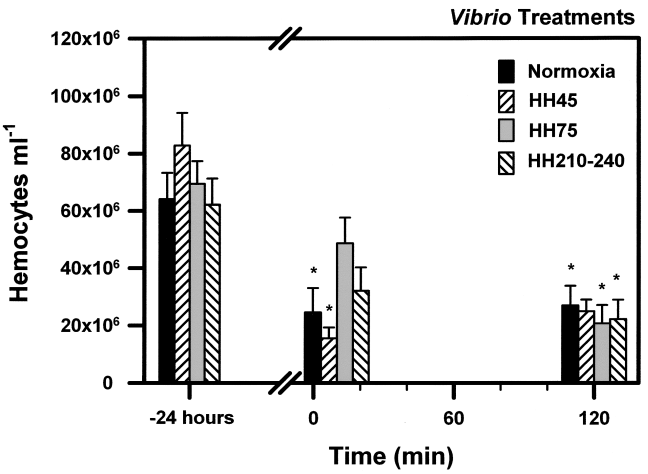

Figure 4. Total hemocyte counts (THC) ml^{-1} of hemolymph in crabs before treatment (−24 h) and 10 and 120 min after injection of *Vibrio campbellii* in four treatment groups. Normoxia = crabs in well-aerated water, HH treatments = hypercapnic hypoxia (P_{O_2} = 4 kPa, P_{CO_2} = 1.8 kPa) administered for 45, 75, and 210–240 min before the injection of bacteria. Significant differences within a treatment between pre-injection (−24 h) THC ml^{-1} and postinjected values at 10 and 120 min are indicated by an asterisk (*). Mean values + standard error are shown.

Table 2

Statistical analysis of total hemocyte counts ml^{-1} (THC ml^{-1}) following injection of Vibrio campbellii

Treatment	*n*	*P* value: Kruskal-Wallis ANOVA	*P* value: Comparison with pretreatment using Dunnett's method	
			10 min	120 min
Normoxia	8	0.002	<0.05	<0.05
HH45	4	<0.001	<0.05	NS
HH75	7	<0.001	NS	<0.05
HH210–240	9	0.044	NS	<0.05

Data were normalized to pretreatment (−24 h) THC ml^{-1} and compared as a function of time. Tests for normality or equal variances failed on one-way ANOVA; therefore, data within a treatment group were tested for differences using a Kruskal-Wallis ANOVA on ranks. THC ml^{-1} at 10 and 120 min after injection were then compared to pretreatment numbers using Dunnett's method. HH, hypercapnic hypoxia; NS, nonsignificant.

For animals held in well-aerated water, this rapid clearance of bacteria is associated with a significant decline in the concentration of circulating hemocytes. The present study demonstrates that bacterial clearance from the hemolymph of blue crabs is reduced when the animals are held in water that is hypercapnic and hypoxic. Moreover, this reduced ability to clear bacteria is associated with a slower decline in circulating hemocytes after injection of bacteria. Taken together, these findings suggest that the low-oxygen and acidic internal milieu of a blue crab exposed to hypercapnic hypoxia may impair the mechanisms responsible for clearing pathogens from its hemolymph.

The rapid removal of bacteria from the hemolymph of the blue crabs in the present study confirms findings in other crustacean species (Merrill *et al.*, 1979; Smith and Ratcliffe, 1980; White and Ratcliffe, 1982; Martin *et al.*, 1993; van de Braak *et al.*, 2002). The efficiency and the rate of clearance vary with the particular pairing of host and bacterial species. For example, Martin *et al.* (1993) found that the gram-positive bacteria *Bacillus cereus*, *B. subtilis*, and *Aerococcus viridans* were cleared to undetectable levels from the hemolymph of the penaeid shrimp *Sycionia ingentis* within 10 min of injection. The gram-negative bacteria *Pseudomonas fluorescens* and *Vibrio alginolyticus* were reduced, but not eliminated: 7.8% and 23%, respectively, of the bacteria were still free in the hemolymph one hour after injection. The different rates at which bacteria are cleared may reflect the binding specificity of hemolymph components such as lectins (Vargas-Albores *et al.*, 1993, 1997), anti-microbial peptides (Schnapp *et al.*, 1996; Destoumieux *et al.*, 1997; Khoo *et al.*, 1999; Bartlett *et al.*, 2002), the prophenoloxidase (PPO) cascade (Aspàn *et al.*, 1995), and hemocytes (Gargioni and Barracco, 1998).

The present study demonstrates that the rate of bacterial

clearance from the hemolymph of blue crabs is reduced when the animals are held in water that is hypercapnic and hypoxic. The systemic respiratory responses of blue crabs to hypoxia are well-documented and are typical of brachyuran crabs (Burnett, 1992, 1997). A quiescent blue crab in well-aerated water has the high postbranchial oxygen pressures (P_{O_2} = 13 kPa) typical of many water-breathers (deFur *et al.*, 1990). Hemolymph passes through the tissues, where oxygen is consumed, and returns to the infrabranchial sinus just before it passes through the gills, where it is oxygenated. Oxygen pressures of prebranchial hemolymph are as low as 1 kPa (Booth *et al.*, 1982). Thus, a hemocyte circulating freely in the hemolymph is exposed to a wide range of oxygen pressures. During hypoxia, oxygen pressures in the hemolymph fall to levels that, depending on the severity of the ambient P_{O_2}, may be very low. deFur *et al.* (1990) reported oxygen pressures of 2.4 kPa in the postbranchial hemolymph when the ambient P_{O_2} of the water was 6.7 kPa. The ambient P_{O_2} used in the present study was lower (4 kPa), forcing the hemolymph oxygen pressures to fall below 2.4 kPa. The main point is that the mechanisms associated with the clearance of bacteria must operate in a very low-oxygen environment within the crab when the animal is exposed to hypoxic water.

In the present study, at 10 min after bacterial injection, crabs in the HH75 and HH210–240 treatments had significantly greater numbers of *V. campbellii* in the hemolymph than the control crabs had (Fig. 2). Thus, the process of bacterial clearance is somehow inhibited by hypercapnic hypoxia. The bacteria are ultimately cleared in all treatments, but it is the rate of clearance that is compromised. These data support the idea that rapid clearance of live bacteria, whether by bactericidal mechanisms in the hemolymph or by physical trapping and removal to peripheral sites, contributes to disease resistance in crustaceans by limiting the spread of free pathogens to other tissues. The effects of hypoxia on bacterial clearance in crustaceans have received limited attention. *Penaeus monodon* exposed to hypoxia (1.8–2.0 mg l^{-1}, P_{O_2} = 5.4–6.4 kPa) cleared live *V. harveyi* more slowly from the hemolymph than did control animals (Direkbusarakom and Danayadol, 1998). However, results from the treatment groups were highly variable, and the authors questioned the health of some of the animals. The ability of the freshwater prawn *Macrobrachium rosenbergii* to clear live *Enterococcus* was significantly decreased by exposure to hypoxia (P_{O_2} = 4.4 and 7.0 kPa) for 12 h (Cheng *et al.*, 2002). Neither study (Direkbusarakom and Danayadol, 1998; Cheng *et al.*, 2002) reported the absolute numbers of bacteria in the hemolymph. Since bacteria are generally cleared from crustacean hemolymph within minutes to hours following injection, the results of these two studies are difficult to compare with the present work.

The impacts of hypercapnia alone or in combination with hypoxia on bacterial clearance in crustaceans have been largely neglected. Mikulski *et al.* (2000) reported that hypercapnic hypoxia at 4 kPa O_2, 2 kPa CO_2, and a pH range of 6.8 to 7.0 decreased survival following bacterial challenge in both *Litopeneaus vannamei* and the grass shrimp *Palaemonetes pugio.*

The decline in circulating hemocytes that accompanies the clearance of injected bacteria in crabs from the normoxia treatment is consistent with the role of this cell type in immune defense in crustaceans. This effect mirrors similar declines in the hemocyte counts of crustaceans that have been injected with foreign substances including lipopolysaccharide, β-1,3 glucan (laminaran), or bacteria (Smith and Söderhäll, 1983; Persson *et al.*, 1987; Martin *et al.*, 1993; van de Braak *et al.*, 2002), although this decline is not universally observed (Destoumieux *et al.*, 2000; Cheng and Chen, 2001). Following injection of foreign matter into crustacean tissues, hemocytes move into the injection site to seal the wound and to trap and kill invading bacteria by mechanisms such as melanization (Fontaine and Lightner, 1974; van de Braak *et al.*, 2002). Several studies using *in vitro* and *in vivo* measurements have shown that hemocytes, in the presence of foreign particles, rapidly associate with each other to form aggregates, or nodules (Smith *et al.*, 1984; Martin *et al.*, 1998). Aggregate formation may be mediated by lectins such as LPS-binding protein and β-glucan-binding proteins (Vargas-Albores *et al.*, 1993, 1997), by components of the prophenoloxidase cascade (Aspàn *et al.*, 1995), or by other unidentified receptors on hemocytes. Martin *et al.* (1998) presented evidence that these aggregates grow in size by the adhesion of hemocytes until they become trapped in the narrowest diameter vessels of the body. The extensive capillary network of the gill that supports respiration and osmotic regulation appears to play an important role in the trapping and removal of these nodules (Fontaine and Lightner, 1974; Johnson, 1976; Smith and Ratcliffe, 1980), although this is not observed in all cases (van de Braak *et al.*, 2002). Aggregates of hemocytes with or without associated bacteria have also been reported to accumulate in the heart, the hepatopancreas, and the connective tissues of crabs (Smith and Ratcliffe, 1980) and lobsters (Factor and Beekman, 1990) and in the lymphoid organ of penaeid shrimp (van de Braak *et al.*, 2002).

No significant changes in the concentration of hemocytes occurred in the hemolymph of crabs in the HH75 and HH210–240 treatments at 10 min after injection of bacteria (Fig. 4). It is possible that low pH and low O_2 inhibited aggregate formation by slowing or blocking the interactions of hemocytes with bacteria or with each other. An alternative possibility is that hypercapnic hypoxia may have induced changes in the process by which aggregates were removed from circulation. Several important components of the innate immune system involve oxygen, including respiratory burst reactions that generate toxic reactive oxygen

species and reactive nitrogen species as well as phenoloxidase, the terminal enzyme of the PPO cascade. Inhibition of the production of reactive oxygen species was observed in *Crassostrea virginica*, the Eastern oyster, and was related specifically to low levels of oxygen and pH, which occur naturally in the tissues of oysters exposed to hypercapnic hypoxia (Boyd and Burnett, 1999). Phagocytic activity (Direkbusarakom and Danayadol, 1998) and respiratory burst activity (Le Moullac *et al.*, 1998) were decreased in hemocytes of *Penaeus monodon* exposed to hypoxia (25%–30% and 15% air saturation, respectively) as compared to controls in air-saturated water. The latter study also reported an increase in PPO activity associated with low O_2 conditions. Hemocyte phagocytosis, respiratory burst, and PPO activity were significantly reduced in *Macrobrachium rosenbergii* exposed for 24 h to hypoxia (35% air saturation; Cheng *et al.*, 2002). Note that these tests of immune function in *P. monodon* and *M. rosenbergii* were conducted *in vitro* under air-saturated conditions and, therefore, do not necessarily reflect changes in the *in vivo* activity of the enzymes involved.

In the present study, pre-exposure to hypercapnic hypoxia for 75 or 210–240 min significantly reduced the rate at which blue crabs removed bacteria from their hemolymph. These results support the concept that oxygen-dependent mechanisms play an important role in the physical removal and killing of bacterial pathogens. The accumulation of hemocytes and bacteria in the gill that has been observed by others (Smith *et al.*, 1984; Martin *et al.*, 1998) may be an efficient means to assure optimal P_{O_2} for immune reactions such as the respiratory burst and the PPO cascade. A potential liability of this accumulation in the gill is that aggregates of hemocytes or hemocytes and bacteria might impede hemolymph flow in the microvasculature of the gill lamellae, reducing hemolymph oxygenation and pH. This line of reasoning leads to the suggestion that tissues of the blue crab, and other crustaceans, may become hypoxic as a consequence of mounting an immune defense against a new pathogen or during an active infection. Prior exposure of the animal to hypercapnic hypoxia may reduce the rate of aggregate formation, minimize occlusion of capillaries in the gill, and help to maintain oxygenation of internal tissues, while increasing exposure of internal tissues to bacterial pathogens circulating in the hemolymph. In this study we did not directly test the interplay of respiration, acid-base balance, and immune defense, but these results do provide a framework for testing and understanding how hypercapnic hypoxia may contribute to the outbreaks of infectious disease in coastal waters.

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