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Impact of Exposure to Bacteria on Metabolism in the Penaeid Shrimp *Litopenaeus vannamei*

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Abstract. We hypothesized that aggregation of bacteria and hemocytes at the gill, which occurs as part of the shrimp's antibacterial immune defenses, would impair normal respiratory function and thereby disrupt aerobic metabolism. Changes in oxygen uptake and lactate accumulation were determined in *Litopenaeus vannamei,* the Pacific white shrimp, following injection with either saline (control) or a strain of the gram-negative bacterium *Vibrio campbellii* that is pathogenic in crustaceans. The rate of oxygen uptake was determined during the first 4 h after injection and after 24 h. Injection of bacteria decreased oxygen uptake by 27% (from 11.0 to 8.2 μmol g^{-1} h^{-1}) after 4 h, while saline-injected shrimp showed no change. Decreased oxygen uptake persisted 24 h after *Vibrio* injection. In well-aerated water, resting whole-animal lactic acid levels increased in shrimp injected with bacteria (mean = 2.59 μmol lactate g^{-1} $\pm$0.39 SEM, $n = 8$) compared to saline-injected control shrimp, but this difference did not persist at 24 h. Exposure to hypercapnic hypoxia (Pco_2 = 1.8 kPa, Po_2 = 6.7 kPa) also resulted in significant whole-body lactic acid differences (mean = 3.99 and 1.8 μmol g^{-1} tissue in *Vibrio* and saline-injected shrimp, respectively). Our results support the hypothesis that the crustacean immune response against invading bacteria impairs normal metabolic function, resulting in depression of oxygen uptake and slightly increased anaerobic metabolism.

Introduction

Crustacean gills make an important contribution to immune defense (Martin *et al.,* 1993, 2000; Burgents *et al.,* 2005a, b) and have well-established functions in gas exchange and ion regulation. Foreign particles and bacteria are rapidly removed from the hemolymph, followed by their accumulation and eventual encapsulation at the gills (Martin *et al.,* 1998, 2000). The presence in the gill of nodules composed of hemocytes aggregated with bacteria has been associated with disease in crustaceans for a number of years (Cornick and Stewart, 1968; Fontaine and Lightner, 1974; Smith and Ratcliffe, 1980; White and Ratcliffe, 1982). In recent studies of shrimp given intramuscular doses of bacteria bearing genetically marked plasmids, the gill retained a higher proportion of culturable bacteria than hepatopancreas, heart, or lymphoid organ by 240 min post-injection (Burgents *et al.,* 2005a, b). Although it remains unclear whether the gills are the main site of bacterial elimination in crustaceans (van de Braak *et al.,* 2002), this organ appears to play an important role in trapping hemocytes and foreign particles.

There may be considerable respiratory consequences associated with localization of bacteria and hemocytes at the respiratory surface. Burnett *et al.* (2006) recently reported that injection of bacteria impaired respiratory function in *Callinectes sapidus,* the Atlantic blue crab. The decline in respiratory performance was manifested in decreased oxygen uptake, reduced post-branchial partial pressures of oxygen, and increased hydrostatic pressures across the gills (Burnett *et al.,* 2006). Martin *et al.* (2000) suggested that large numbers of pathogens aggregate with hemocytes in the hemolymph, resulting in vessel occlusions and decreased hemolymph flow, especially flow to or within the gill. Although materials sequestered at the gill appear to be eliminated during ecdysis (White and Ratcliffe, 1982; Martin *et al.,* 1998, 2000), bacterial infection prior to molting may limit normal hemolymph flow across the gills and impair respiratory function. Decreased hemolymph flow to

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the gill and the resulting internal hypoxia (Burnett *et al.,* 2006) may be particularly noteworthy when considering recent findings that oxygen-dependent mechanisms are important in immune response in crustaceans (Mikulski *et al.,* 2000; Burgents *et al.,* 2005a; Tanner *et al.,* 2006) and that low levels of environmental oxygen can impair the rate at which bacteria are cleared from the hemolymph (Holman *et al.,* 2004).

In the current investigation, we examined metabolic responses of *Litopenaeus vannamei* (Boone), the Pacific white shrimp, following exposure to the bacterium *Vibrio campbellii.* We hypothesized that immune defense mechanisms alter normal respiratory function, disrupting aerobic metabolism. We compared the changes in oxygen consumption in shrimp that had been injected with sublethal doses of this crustacean pathogen to those in shrimp injected with saline. The impact of bacterial injection on whole-body lactate levels of shrimp resting in normoxic and hypoxic waters was also measured.

Materials and Methods

Pacific white shrimp, *Litopenaeus vannamei* (weighing about 3–5 g) were purchased from Waddell Mariculture Center, Bluffton, South Carolina, and maintained at the Grice Marine Laboratory, Charleston, South Carolina, in recirculating seawater at 30 ppt salinity and 23–25 °C for a minimum of 2 weeks. Shrimp were fed daily with commercial shrimp pellets (Rangen Inc., Buhl, ID). A photoperiod of 12 h light to 12 h dark was maintained in all housing facilities. Animals were not fed for 24 h before and during all experiments.

Injection of bacteria

About 18 h prior to injection, *Vibrio campbellii* (90-69B3, originally isolated by D. Lightner and L. Mahone, University of Arizona) was streaked onto a Tryptic soy agar plate supplemented with 2.5% NaCl and kept overnight at 25 °C. Immediately prior to injection, bacteria were suspended in a 2.5% NaCl, 10 mmol l^{-1} HEPES buffer (pH = 7.5) diluted to an optical density at 540 nm equivalent to 1×10^8 colony forming units per milliliter (Mikulski *et al.,* 2000). The bacterial suspension (3.3 μl g^{-1} body weight) was injected into the third abdominal segment of each animal for a final injection dose of approximately 1×10^5 CFU g^{-1} animal. The bacterial dose was about one-half of the LD_{50} dose for this species (Mikulski *et al.,* 2000) and comparable on a per-weight basis to that used in previous studies examining the respiratory effects of bacterial exposure in *Callinectes sapidus,* the blue crab (Burnett *et al.,* 2006). Injections typically took less than 30 s using sterile technique and a microliter syringe (Hamilton). Injections using identical volumes of sterile buffered saline (2.5% NaCl, 10 mmol l^{-1} HEPES, pH = 7.5) were used as controls against injections of bacteria.

Metabolic measurements

Oxygen uptake was measured using flow-through respirometry at 25 °C. Fasted shrimp were placed inside of a 260-ml plastic chamber and the Po_2 of filtered seawater (45-μm nylon filter, 30 ppt, pH = 7.8) entering and leaving the chamber was measured every 5 s using a Sable System (Las Vegas, NV) data acquisition system and micro-oxygen electrode (Yellow Springs Instruments, Yellow Springs, OH). The design of the animal chamber and the oxygen sampling system conformed to the principles of a single-chamber system as defined in Frappell *et al.* (1989). The time constant for the washout of oxygen in the animal and electrode chambers was 46.5 min ($\tau_1 + \tau_2$), whereas the time constant for the electrode circuit alone was less than 1 min (τ_2). Because τ_2 was small with respect to $\tau_1 + \tau_2$, the system could be considered a single chamber in which $\tau_2 = 0$. Flow rate typically ranged between 18 and 20 ml min^{-1} in order to maintain Po_2 differences of about 2.3 to 4.8 kPa between water entering and leaving the chamber. We accounted for the washout characteristics of the respirometry system by applying the Z transformation (Bartholomew *et al.,* 1981).

Each shrimp was placed in a respirometer and left undisturbed for 2 h. Oxygen uptake was then measured for 30 min prior to the injection of either *Vibrio* or a saline solution. Shrimp were then removed from the chamber, injected with a saline (control) or a saline containing bacteria (according to the protocol described above), and returned to the respiratory chamber. This process took less than 3 min. After injection, oxygen uptake was measured continuously for the first 4 h. After 4 h, shrimp were removed from the respiratory chamber and held overnight in well-aerated aquaria at 25 °C and 25 ppt salinity. Approximately 22 h after injection, shrimp were returned to the respiratory chamber, left undisturbed for 2 h while the chamber was flushed continuously, and oxygen uptake was measured for the final 30 min.

One shrimp injected with *V. campbellii* died during the study. Metabolic data for this shrimp are not reported. There were no other incidental shrimp mortalities.

Total body lactate levels were determined to assess the impact of bacteria and the subsequent immune response on anaerobic metabolism. Animals injected with *V. campbellii* or sterile saline (according to the injection protocol described above) were placed in plastic mesh cages ($14 \times 9 \times 6$ cm, mesh size 0.7×0.7 cm) with clear acrylic plastic floors. Cages were designed to ensure that each animal could be rapidly frozen in liquid nitrogen with little or no change in their activity, thereby minimizing lactate accumulation due to capture. Cages were fitted with a wooden

dowel handle so that the entire cage could be quickly removed from the aquarium and placed in liquid nitrogen. Once shrimp had been injected and placed in individual cages, they were immersed in 17-1 glass aquaria containing well-aerated seawater (20.7 kPa Po_2 and <0.06 kPa Pco_2) at 25 °C. In one set of experiments, shrimp remained in well-aerated seawater for either 4 or 24 h post-injection before being flash-frozen as described above. In a second set of experiments, shrimp were placed in individual cages in the aquaria, as described above, and exposed to hypercapnic hypoxia for 4 h. These shrimp were initially placed in an aquarium with well-aerated water, and nitrogen was mixed with carbon dioxide (Cameron gas mixing pump GF-3) and bubbled into the aquarium to achieve a Po_2 of 6.7 kPa and Pco_2 of 1.8 kPa. It took about 40 min to achieve the final gas pressures of oxygen and carbon dioxide, and shrimp were held in these conditions for an additional 3.3 h. Frozen shrimp were weighed and homogenized in 100 ml of cold 12% $HClO_4$, using a tissue homogenizer. Homogenates were centrifuged and lactate concentrations measured colorimetrically at 340 nm (Sigma Technical Bulletin No 862-UV). Total body lactate concentrations were calculated as μmol lactate per gram wet weight.

Hemocyanin concentrations were determined on hemolymph drawn from the base of the periopods and diluted 101-fold with 10 mmol l^{-1} EDTA at pH = 10. Absorbance was measured at 335 nm, using an extinction coefficient of 26.9 absorbance units mg^{-1} ml^{-1} (Nickerson and Van Holde, 1971). Hemocyanin concentrations were determined on shrimp that had been injected with saline or *Vibrio* as described above, and the animals were left undisturbed in well-aerated water for 4 h (n = 18 for each group).

Statistical analysis

A repeated-measures ANOVA was used to compare oxygen uptake as a function of time (pre-injection and through 24 h) and treatment (saline-injected *versus Vibrio*-injected groups). A significant difference revealed by the repeated measures ANOVA resulted in a comparison of pre-injection oxygen uptake and oxygen uptake after injection within a treatment group and a separate comparison between the treatment groups at different times after injection.

To test for differences in whole-body lactate concentrations resulting from injection and handling, we used an ANOVA to compare shrimp 4 and 24 h after injection with shrimp that were not injected. We also used an ANOVA to test for differences between saline-injected controls and *Vibrio*-injected shrimp held in well-aerated (normoxic) water at 4 and 24 h. Finally, we used an ANOVA to test for differences between shrimp held in hypercapnic hypoxia during the 4-h post-injection period and those held in well-aerated water for 4 h. If experimental groups differed significantly, pairwise comparisons were performed using the Holm-Sidak method.

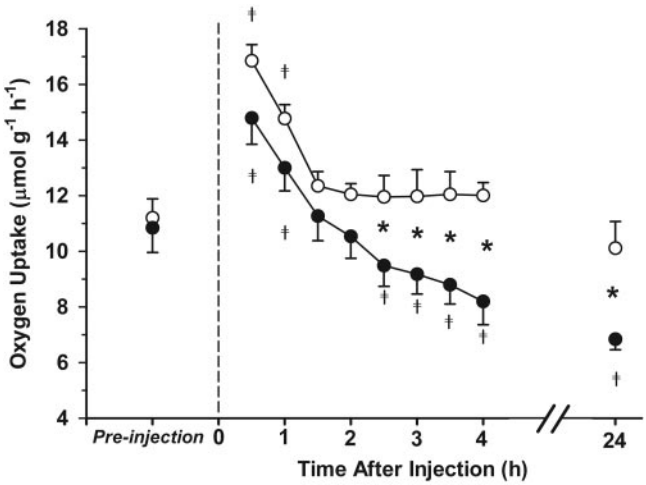

Figure 1. The rate of oxygen uptake (μmol g^{-1} h^{-1}) of *Litopenaeus vannamei* following injection with *Vibrio campbellii* (closed circles) compared to saline injection (open circles). Oxygen uptake was measured in a flow-through respirometry chamber thermostated to 25 °C. Pre-injection values represent average oxygen uptake for 30 min prior to injection. The dashed line represents the injection time. Values post-injection represent averages over 30-min intervals for 6 to 9 shrimp ± standard error. Significant differences between saline (control) and *Vibrio* injection occurred after 2.5 h (*; $P < 0.05$ repeated-measures ANOVA). Differences in oxygen uptake from pre-injection values at different times are indicated by crosses.

All data are reported as mean ± standard error unless noted otherwise. Statistical analyses were done using SigmaStat 3.0.

Results

Injection of *Vibrio campbellii* into shrimp abdominal muscle significantly reduced the rate of oxygen uptake in whole animals (Fig. 1). There was a 27% decrease in oxygen uptake after 4 h and almost a 40% decrease in oxygen uptake after 24 h in *Vibrio*-injected animals when compared to pre-injection oxygen uptake rates (Fig. 1). Oxygen uptake for *Vibrio*-injected animals was significantly lower than both pre-injected and saline-injected values after 2.5 h and remained lower for 24 h post-injection ($P < 0.05$; Holm-Sidak). Although removal from the respiratory chamber and injection itself caused an initial increase in oxygen uptake for about 1 h, there was no significant difference between oxygen uptake in *Vibrio*- and saline-injected animals during this initial elevation.

Lactate levels in whole animals injected with saline were not significantly different after 4 h (mean = 1.35 μmol lactate g^{-1} ±0.18, n = 6) or 24 h (mean = 1.87 μmol lactate g^{-1} ± 0.13, n = 8) from shrimp receiving no injection at all (mean = 1.32 μmol lactate g^{-1} ± 0.18,

$n = 6$) (ANOVA, $P = 0.052$), indicating that there was no effect of injection and handling on lactate levels. We analyzed the whole-body lactate data in shrimp held in normoxia, assessing the effects of treatment (saline- *vs. Vibrio*-injection) and time (4 h *vs.* 24 h; Fig. 2). There was a significant difference ($P = 0.001$) between control (saline-injected) and *Vibrio*-injected (mean = 2.59 μmol lactate $g^{-1} \pm 0.39$, $n = 8$) shrimp at 4 h, but this difference disappeared ($P = 0.08$) at 24 h (two-way ANOVA using the Holm-Sidak method). There was a significant difference ($P = 0.041$) between control (mean = 1.83 μmol lactate $g^{-1} \pm 0.44$, $n = 8$) and *Vibrio*-injected (mean = 3.99 μmol lactate $g^{-1} \pm 1.30$, $n = 8$) shrimp held in hypercapnic hypoxia for 4 h, but whole-body lactate levels in *Vibrio*-injected shrimp held in normoxia were not different ($P = 0.177$) from those of *Vibrio*-injected shrimp in hypercapnic hypoxia (two-way ANOVA using the Holm-Sidak method).

To examine the possibility that decreased oxygen uptake in *Vibrio*-injected shrimp might be due to loss of functional hemocyanin, hemolymph hemocyanin concentrations were measured in shrimp injected with saline or *Vibrio.* After 4 h, mean hemocyanin concentration in saline-injected shrimp was 60.3 mg ml^{-1} (± 3.7, $n = 18$), and this was not significantly different (Student's t test) from the mean hemocyanin concentration in *Vibrio*-injected shrimp (58.1 $\pm$ 3.9 mg ml^{-1}, $n = 18$).

Discussion

Aerobic metabolism in shrimp responds considerably when shrimp are injected with the bacterium *Vibrio campbellii.* Oxygen uptake declines by 27% after 4 h, and this response persists for at least one day (Fig. 1). Only a small fraction of this decrease in metabolism appears to be offset by a switch to anaerobic mechanisms, as judged by the significant but small accumulation of lactate in the tissues of shrimp injected with bacteria. In all of our treatments, shrimp remained quiescent, and so differences in metabolism are apparently not due to changes in behavior. This large and sustained metabolic depression also cannot be attributed to the establishment of a persistent bacterial infection. Shrimp, like other crustaceans, rapidly eliminate sublethal doses of injected bacteria, with less than 0.1% of injected bacteria still detectable by microbial culture techniques from the hepatopancreas, gill, hemolymph, lymphoid organ, and heart by 4 h after an intramuscular exposure (Burgents *et al.*, 2005a).

It is surprising that the injection of bacteria resulted in such a large and sustained metabolic depression (Fig. 1). Burnett *et al.* (2006) report that injection of *Vibrio campbellii* into *Callinectes sapidus* resulted in a 19% decline in post-branchial partial pressure of oxygen, indicating significantly reduced internal oxygen levels associated with injection. Several studies have reported that marine invertebrates are able to reduce overall metabolic rates when faced with hypoxia (for reviews, see Grieshaber *et al.,* 1994; Burnett, 1997; Guppy, 2004). In organisms that undergo severe and extended metabolic depressions by as much as 85% to 100% of basal metabolic rate, oxygen deprivation is a common trigger (Hand and Hardewig, 1996). Further studies are needed to determine if shrimp have alternate routes of anaerobic metabolism or are able to down-regulate overall metabolism when faced with internal hypoxia due to bacterial exposure. However, if shrimp are able to excrete lactate, as previously reported in isopods (de Zwaan and Skjoldal, 1979) and crayfish (Head and Baldwin, 1986), then the large difference in metabolism between the two treatments may disappear. This possibility should be explored.

The large decrease in oxygen uptake and significant increase in lactic acid induced by injection of bacteria in shrimp resting in air-saturated water suggests that bacterial infection may have an even greater effect on metabolism and survival in shrimp living in hypoxic environments. By itself, environmental hypoxia can have a profound impact on the immune response in invertebrates. Both the phagocytic activity of hemocytes (Direkbusarakom and Danayadol, 1998) and the total hemocyte number (Le Moullac *et al.,* 1998) are depressed in crustaceans exposed to hypoxia. Production of reactive oxygen species by isolated oyster hemocytes is depressed when hemocytes are exposed to hypercapnic hypoxia (Boyd and Burnett, 1999), and the activity of phenoloxidase is inhibited at the reduced levels of oxygen that occur within the tissues of *C. sapidus* held in sublethal hypoxia (Tanner *et al.,* 2006). Reduced delivery of oxygen to the tissues of an animal in response to injection of bacteria, as demonstrated in the present study, would compound the impacts of environmental hypoxia or hypercapnic hypoxia on metabolism and survival.

The levels of lactate measured in the whole bodies of shrimp in the present study are similar to those found in *Palaemonetes pugio,* the grass shrimp (Cochran and Burnett, 1996). The small, but significant increase in whole-body lactate concentrations in *Vibrio*-injected shrimp suggests that oxygen delivery cannot meet energy demand. This situation may be similar to oxygen uptake limitations resulting from exercise, where hypoxic tissues in exercising crustaceans are characterized by simultaneous lactate accumulation and removal (Full and Herreid, 1984). Elevated lactate levels in *Vibrio*-injected shrimp exposed to hypercapnic hypoxia (above critical oxygen pressures, Burgents and L. Burnett, unpubl. data; Fig. 2) are consistent with a limitation in oxygen diffusion in a situation that is more challenging for the shrimp.

Anaerobic metabolism in *Vibrio*-injected shrimp supplements aerobic metabolism, but only in a minor way. We estimated the relative energetic contribution of lactate in ATP equivalents (1 mol lactate = 1.5 mol ATP) compared

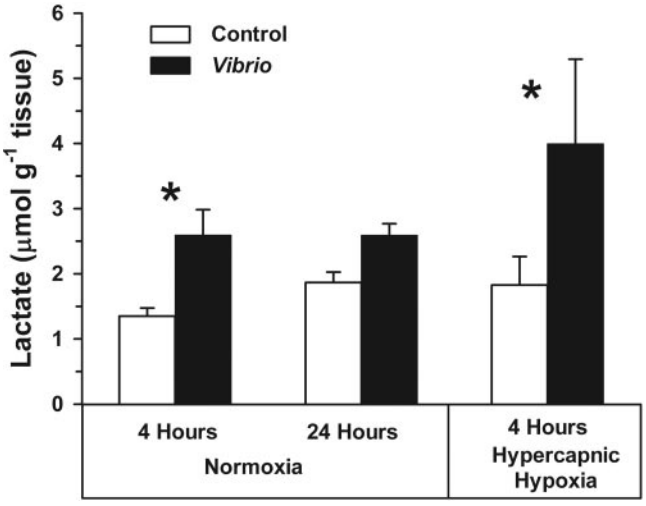

Figure 2. Whole-animal lactate (μmol g^{-1} tissue) in *Litopeneaus vannamei* measured at 4 and 24 h after injection with *Vibrio campbellii* or saline (control). In one set of experiments, shrimp were exposed to normoxia (well-aerated) seawater at 30 ppt and 25 °C. Shrimp injected with *Vibrio* had significantly higher whole-body lactate levels than saline-injected shrimp 4 h (indicated by *; $P < 0.05$ two-way ANOVA using the Holm-Sidak method), but not 24 h following injection. In the second set of experiments, shrimp were exposed to hypercapnic hypoxia (Pco_2 = 1.8 kPa, Po_2 = 6.7) at 25 °C for 4 h following injection. Whole-body lactate was also significantly elevated in *Vibrio*-injected shrimp compared with saline-injected controls (indicated by *; $P = 0.041$ two-way ANOVA using the Holm-Sidak method). Whole-body lactate 4 h after injection was not different between normoxia and hypercapnic hypoxia treatments in *Vibrio*-injected shrimp. Mean values ± standard error are shown for 6 shrimp in the 24-h normoxia treatment and 8 shrimp for all others.

to the energy contribution of aerobic pathways (1 mol oxygen = 6.33 mol ATP; if glycogen is the fuel source, as suggested by Hohnke and Scheer, 1970). Four hours after injection, control shrimp took up 12.0 μmol O_2 g^{-1} h^{-1}, equivalent to 76.0 μmol ATP. *Vibrio*-injected shrimp took up 8.2 μmol O_2 g^{-1} h^{-1}, which is equivalent to 51.9 μmol ATP. If only the difference in whole-body lactate between the two treatments is considered, then 1.24 μmol lactate is produced in *Vibrio*-injected shrimp above that of the controls (2.59–1.35 = 1.24 μmol lactate g^{-1}). If it is assumed that 1.24 μmol lactate is produced in 1 h instead of over 4 h (a more conservative approach), then 1.9 μmol ATP g^{-1} h^{-1} makes up the anaerobic contribution. Clearly, anaerobic metabolism under these conditions contributes little to the overall production of energy. On the basis of these calculations, there appears to be a 29% reduction in overall metabolism as a result of *Vibrio* injection.

There is evidence that the respiratory pigment hemocyanin can have antimicrobial, antifungal, and antiviral activity (Destoumieux-Garzón *et al.,* 2001; Lee *et al.,* 2003; Zhang *et al.,* 2004). We found no evidence for the loss of hemocyanin that might be associated with the formation of an antimicrobial peptide fragment from the intact respiratory pigment, thereby limiting oxygen uptake in *Vibrio*-injected shrimp.

Overall, we found that shrimp experience a large depression in aerobic metabolism and a small but significant increase in resting anaerobic metabolism following exposure to bacteria. Our results provide a plausible explanation for the increased susceptibility of crustaceans to infectious disease in hypoxic environments and lend support for further investigations to determine how reduced ATP production associated with exposure to bacteria may impact overall activity and performance.

Acknowledgments

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