The Impacts of Hypoxia and Hypercapnia on Disease Resistance in Crustaceans

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Abstract. - Many aquatic organisms must adapt to daily or seasonal variations in dissolved oxygen levels. Low environmental oxygen (hypoxia) frequently co-occurs with elevated carbon dioxide (hypercapnia) that is produced by respiration and drives a decrease in water pH. Among its many impacts on animal physiology and behavior, sublethal hypoxia increases the susceptibility of aquatic organisms to infection with pathogens, an effect which is exacerbated by high carbon dioxide and low pH. This brief review presents some of the existing evidence that sublethal hypoxia can directly suppress mechanisms of immune defense in aquatic organisms, with an emphasis on crustacean species. Low oxygen and low pH conditions reduce the rate at which crustaceans clear injected pathogens from their hemolymph and slow the rate at which fish and oyster phagocytes produce reactive oxygen species that are critical to immune defense. The activity of phenoloxidase, an enzyme involved in encapsulation and melanization of pathogens in crustaceans, is also suppressed at the reduced oxygen and pH levels that occur in the tissues of crustaceans held in hypercapnic hypoxia. The rate at which hemocytes engulf pathogens by phagocytosis and the tissues to which hemocyte and pathogens localize may also be impacted in crustaceans held in low oxygen conditions. Progress is being made toward understanding the cellular and biochemical basis for these impacts of sublethal hypoxia on immune defense. This new information promises to enhance our understanding of the integration between the immune system and other physiological systems that are vital to the health of aquatic organisms.

Key Words: hypoxia, hypercapnia, disease resistance, teleost fish, crustaceans, bivalves, disease challenge models, immunity, respiratory burst, bacterial clearance, prophenoloxidase

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Introduction:

Crustaceans, as well as other aquatic organisms, often encounter levels of dissolved oxygen, carbon dioxide and pH that vary widely on a diurnal and seasonal basis. While it is generally recognized that very low levels of dissolved oxygen cause mortalities, the effects of sublethal levels of low oxygen are less well understood. Furthermore, both laboratory and field studies seldom take into consideration the increase in carbon dioxide pressure (Pco_2), or hypercapnia, produced by respiration that often accompanies low oxygen or hypoxia (Burnett 1997). Elevated levels of water CO_2 then drive a decrease in water pH (Cochran and Burnett 1996). This brief review describes our current understanding of the effects of sublethal hypoxia alone and in combination with hypercapnia on the ability of crustaceans to protect themselves against infection. Supportive information gleaned from studies with other aquatic organisms, such as oysters and fish, is also provided where it serves to support or extend the data that are available from work with crustacean species.

Levels of dissolved oxygen in water have been reported in the literature in units of concentration (mg/mL) and pressure (torr or kilopascals). Where possible, data from literature reports have been converted to terms of "% air saturation." As a point of reference, the pressure of oxygen in fully (100%) air-saturated water is 155 torr or 20 – 20.4 kPa, while that of carbon dioxide is 0.23 torr or 0.03 kPa. The concentration of oxygen in 100% air-saturated water varies as

a function of temperature and salinity and is 7.3 mg/mL at 25°C in 30 ppt seawater. For most aquatic organisms, levels of dissolved oxygen from 20 to 50% of air-saturation are considered sub-lethal hypoxia, and levels below 20% are lethal hypoxia. In this review partial pressures of oxygen (Po_2) and carbon dioxide (Pco_2) in tissues or blood are given as kilopascals (kPa), the SI unit for pressure.

Hypoxia occurs naturally in shallow coastal regions around the world (Rabalais and Turner 2001). Estuaries and tidal creeks of the southern U.S. and the Gulf of Mexico often experience dissolved oxygen concentrations less than 3.0 mg/L (40% air saturation at 25°C) (Breitburg 1990; Rabalais et al. 1994; Burnett 1997; Summers et al. 1997). Along the South Carolina coast, tidal creek oxygen pressures can fluctuate between 1.2 and 23.0 kPa (6% and 110% air saturation) within a 24-hour period (Cochran and Burnett 1996) and may decline to as low as 0.3 kPa (1.2 % air saturation) in the nearby Savannah River estuary (Winn and Knott 1992). Field monitoring studies have also documented the common occurrence of hypercapnia in coastal areas. For example, Cochran and Burnett (1996) reported that Pco_2 varies from 0.04 to 1.6 kPa and pH ranges from 6.5 to 7.6 on a daily basis in South Carolina tidal marshes.

Aquatic organisms raised in high-intensity pond culture often experience severe changes in O_2, CO_2, and pH due to high animal density and nutrient input from feed (Madenjian 1990; Browdy et al. 2001). 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 low pH still occur in routine management (Chang and Ouyang 1988; Garcia and Brune 1991).

Hypoxia is frequently named as the primary cause of mass mortalities of marine life (Winn and Knott 1992; Rabalais et al. 1994; Lenihan and Peterson 1998; Paerl et al. 1999). Marine organisms may attempt to avoid or mitigate the effects of hypoxia by modifying behavioral or physiological processes such as ventilation and circulation, or altering the expression or oxygen affinity of respiratory pigments when pigments are present. Under conditions of sublethal hypoxia a switch to anaerobic metabolism often occurs (Burnett 1997; Burnett and Stickle 2001), as well as alterations in osmoregulatory capacity (Charmantier et al. 1994) and molting (Clark 1986). Sublethal hypoxia also can increase the incidence of infectious diseases in aquatic organisms, suggesting that low oxygen levels suppress the ability of the host organism to defend itself against infection (see *"Effects of oxygen and carbon dioxide on resistance to infection"* below).

The possibility that increased environmental CO_2 and reduced pH might contribute to the lethal and sublethal effects of hypoxia is seldom considered. Where the effects have been assessed in laboratory experiments, low pH and high CO_2 enhanced mortality rates of extreme hypoxia (Martinez et al. 1998). In addition, McCulloch (1990) found that low pH raised the critical oxygen concentration in the grass shrimp, which is the oxygen concentration below which they are unable to maintain aerobic metabolism independent of ambient oxygen. The critical oxygen concentration in the grass shrimp (*Palaemonetes kadiakensis*) rose from 1.54 mg O_2/L at pH 9.0 to 2.08 mg O_2/L at pH 6.5. Cruz-Neto and Steffensen (1997) reported that hypercapnia increased the

critical oxygen concentration from 3.4 to 5.4 – 6.1 kPa in the European eel, *Anguilla anguilla*. Cochran and Burnett (1996) demonstrated that oxygen uptake was significantly higher at high CO_2 than at low CO_2 in the fish, *Leiostomus xanthurus*. These studies show that hypercapnia can adversely affect mechanisms associated with responses to hypoxia.

Effects of oxygen and carbon dioxide on resistance to infection. Several field studies have 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 the blue crab, *Callinectes sapidus*, collected in areas of the estuary where hypoxia is common, had reduced serum bacteriostatic activity compared to animals that had not been routinely exposed to low oxygen. 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. Hypoxia also may have contributed to reported outbreaks of mycobacteriosis in fish (Vogelbein et al. 1999) and *Perkinsus* infections in oysters (Anderson et al. 1998).

Pathogen challenge studies conducted under controlled laboratory conditions provide substantial support for the link between hypoxia and infectious disease. Hypoxia increased the susceptibility of Tilapia hybrids *Oreochromis niloticus* and carp *Cyprinus carpio* to infection with *Streptococcus* spp. (Bunch and Bejerano 1997) and increased mortalities in yellowtail fish *Seriola quinqueradiata* challenged with *Enterococcus seriolicida* (Fukuda et al. 1997). Mortalities were significantly increased in blue shrimp *Litopenaeus stylirostris* exposed at 15% air saturation to *Vibrio alginolyticus* (Le Moullac et al. 1998) as well as in freshwater prawn *Macrobrachium rosenbergii* injected with *Enterococcus* and held at 61% or lower levels of air-saturation (Cheng et al. 2002). In none of these studies were CO_2 and pH considered as possible factors in altering mortality rates. Our laboratory (Mikulski et al. 2000) tested the effect of hypercapnic hypoxia (Po_2 = 4.1 kPa or 20% air saturation, Pco_2 = 2.1 kPa, pH = 6.7-7.0) on the susceptibility of the grass shrimp, *Palaeomonetes pugio*, and the Pacific white shrimp, *Litopenaeus vannamei*, to intramuscular challenge with *V. campbellii* (later reclassified as *Vibrio campbellii* by 16S rRNA sequencing, see Holman et al. 2004) as compared to controls held in air-saturated or normoxic (Po_2 = 20 kPa, Pco_2 = 2.1 kPa, pH = 7.6) water (Fig. 1). After injection with live bacteria, both the juvenile *L. vannamei* and the adult *P. pugio* held under hypercapnic hypoxia displayed significantly lower 48 hour survival (15.7% and 3.1%, respectively) than animals injected with the same dose of V. campbellii and held in normoxic water (28.7% and 29.4%, respectively).

Hypoxia may alter the virulence of a pathogen or suppress the resistance of a host against infection. The question of pathogen virulence under hypoxic conditions was not directly addressed for the *Streptococcus* spp, *Enterococcus seriolicida* or V. *alginolyticus* employed in the laboratory challenge studies cited above. Our report (Mikulski et al. 2000) showed that the *in vitro* doubling time of the *V. campbellii* isolate used in challenge studies was suppressed under the same conditions of hypoxia and hypercapnia that

increased pathogenicity to the shrimp host.

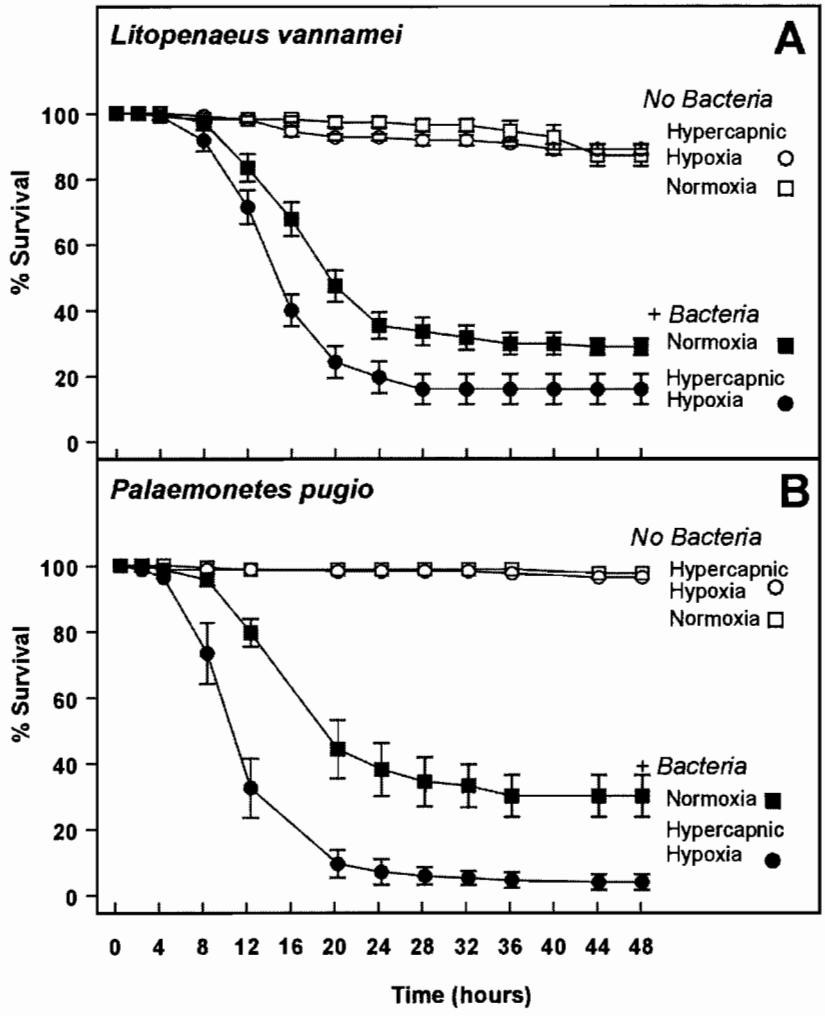

Figure 1.A. *Litopenaeus vannamei* survival following bacterial challenge under well-aerated (Po_2 = 20.0 – 20.4 kPa, Pco_2 = 2.1 kPa, pH = 7.6 – 8.0) and hypercapnic hypoxia (Po_2 = 4.1 kPa or 20% air saturation, Pco_2 = 2.1 kPa, pH = 6.8-7.0) conditions. Shrimp were injected intramuscularly with 50 µL of *Vibrio campbellii* 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 data point. 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 in well-aerated water. **1.B.** *Palaemonetes pugio* survival following bacterial challenge in well-aerated water (Po_2 = 20.0 – 20.4 kPa, Pco_2 = 2.1 kPa, pH = 8.0 – 8.2) and hypercapnic hypoxia (Po_2 = 4.1 kPa or 20% air saturation, Pco_2 = 2.1 kPa, pH = 6.9-7.0) water. Shrimp were injected intramuscularly with 5 µL of *Vibrio campbellii* 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 data point. 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 *V. campbellii* challenge than those held well-aerated water. (From Mikulski et al. 2000).

This aspect of the host-pathogen relationship clearly deserves additional study, however, the limited data that are available suggest that one or more mechanisms of host resistance are more likely to be the target(s) of low oxygen effects.

There are only a few reports comparing the ability of marine organisms to clear a pathogen from their tissues when held under well-aerated and hypoxic conditions. Where it has been measured, the clearance of a pathogen from hemolymph was slowed under hypoxic conditions, an observation that points toward changes in mechanisms that mediate the resistance and, in particular, the immune functions of the host. *Penaeus monodon* exposed to hypoxia (1.8 – 2.0 mg/L) cleared live *Vibrio harveyi* more slowly from the hemolymph than did control animals (6 mg/L) as measured at 30 min after injecting bacteria into the sixth abdominal segment (Direkbusarakom and Danayadol 1998). However, results from the treatment groups were highly variable and the authors questioned the health of some of the animals used in their study. Exposure to 33% air-saturated water for 12-h significantly decreased the ability of the shrimp *M. rosenbergii* to clear live *Enterococcus* injected in the second abdominal segment (Cheng et al. 2002). More recently, our laboratory has reported that exposure of blue crabs to hypercapnic hypoxia at 20% air saturation and 2% CO_2 for 75-min or 210 to 240-min reduced the rate at which the animals cleared the bacterial pathogen *Vibrio* spp. 90-69B3 injected into the pericardium as compared with controls held in 100% air-saturated water (Holman et al. 2004).n the two former studies (Direkbusarakom and Danayadol 1998; Cheng et al. 2002) the absolute numbers of bacteria in the hemolymph were not reported, nor were dissolved CO_2 or pH considered as possible factors in the effects of hypoxia on clearance rates, therefore, the results of these two studies are difficult to compare with the results of Holman et al. (2004). Nonetheless, there is substantial data indicating that the increase in susceptibility of crustaceans to bacterial pathogens under sublethal hypoxia may be correlated with a reduced ability of the host to clear the pathogen from its tissues.

Effects of oxygen and carbon dioxide on specific mechanisms of immune response. The effects of hypoxia on disease susceptibility and pathogen clearance suggest that one or more mechanisms of host immune defense is/are sensitive to changes in dissolved gasses and pH in the internal tissues of an animal subjected to hypoxic stress. Aquatic organisms have only a limited ability to regulate internal levels of dissolved gasses and pH by behavioral and physiological mechanisms (Burnett 1997; Burnett and Stickle 2001), therefore, blood P_{O_2} and P_{CO_2} will reflect changes in water P_{O_2}. In rainbow trout, *Oncorhynchus mykiss* (formerly *Salmo gairdneri*), maintained in well-aerated water Nikinmaa and Solvio (1982) measured blood P_{O2} in the dorsal aorta at 12.2 – 13.5 kPa. When water oxygen levels decreased to 35-40% air saturation, the P_{O_2} in the dorsal aorta declined over three hours to approximately 2.7 – 6.8 kPa. These observations indicate that the internal milieu of the fish changes in a pattern that reflects the external environment. Similarly, direct measurement of hemolymph P_{O2} in the Eastern oyster, *Crassostrea virginica*, showed that reducing the P_{O2} of the ambient water from 100 to 22% air saturation was associated with a decrease in hemolymph P_{O2} from 4.9 to 1.6 kPa (Boyd and Burnett 1999). The work of deFur et al. (1990) reported that quiescent blue crabs held in well-aerated water had high postbranchial oxygen pressures (P_{O_2} = 13 kPa), which fell

to 2.4 kPa when the ambient Po_2 was reduced to 33% air saturation.

Increasing the pressure of carbon dioxide in the water will result in an increase in Pco_2 within an organism, since carbon dioxide fluxes passively into the water down a pressure gradient. A resulting elevation in circulating pressures of CO_2 and a resulting acidosis have been well-documented in a variety of aquatic organisms (e.g., Cameron 1978; Gaillard and Malan 1983; Lindinger et al. 1984; Pörtner et al. 1998; Perry et al. 1999). The acidosis, termed a respiratory acidosis, can be compensated (i.e., returned to the original pH), but compensation is rarely complete and can take many hours.

It seems reasonable to hypothesize that the dramatic changes in the internal milieu associated with the stress of hypoxia and hypercapnia may have direct impacts on specific oxygen and pH-dependent mechanisms of the immune system. Both cellular and soluble factors play a role in the immune defense of aquatic as well as terrestrial animals. In most, if not all, multicellular organisms there are phagocytic cells, such as hemocytes, coelomocytes, neutrophils or macrophages, which engulf and destroy particulate material or invading microorganisms. A prime target for the immunosuppressive effects of hypoxia is the respiratory burst of phagocytic cells. The respiratory burst is an enzyme cascade that consumes oxygen and produces a complex array of reactive oxygen species (ROS), including superoxide, hydrogen peroxide, hydroxyl radical, perchlorate and, in the presence of nitric oxide, peroxynitrite (Chung and Secombes 1988; Sharp and Secombes 1993). Binding of a phagocyte to its foreign target activates NADPH oxidase, the first member of the respiratory burst. In vertebrate phagocytes, ROS produced by this respiratory burst provide vital defenses against infection (Gabig and Babior 1979; Chung and Secombes 1988).

Hypoxic conditions can suppress the production of ROS by phagocytic cells. Low O_2 levels (Edwards et al. 1984; Feldman et al. 1990) and low pH (Hackam et al. 1996; Leblebicioglu et al. 1996) limit ROS production by mammalian macrophages and neutrophils. The respiratory burst of fish phagocytes was suppressed when the cells were held under dissolved gas and pH conditions existing in the tissues of animals in hypercapnic hypoxia (Boleza et al. 2001). Superoxide production stimulated by the yeast cell-wall extract, zymosan, in phagocytic cells of the mummichog, *Fundulus heteroclitus*, was reduced by 76% in hypercapnic hypoxia (Po_2 = 2.0 kPa, Pco_2 = 1.1 kPa, pH = 7.0), as compared to the activity of cells held at dissolved gas and pH conditions (Po_2 = 6.1 kPa, Pco_2 = 0.5, pH = 7.6) typical of animals in air-saturated water (Fig. 2, Boleza et al. 2001). Superoxide production in response to live *V. campbellii* was inhibited by as much as 75% in hypercapnic hypoxia compared to air-saturated conditions. In the same study, hypercapnic hypoxia reduced the bactericidal activity of the *F. heteroclitus* phagocytes against live *V. campbellii* by 72.5% (Boleza et al. 2001). The independent effects of low dissolved oxygen and low pH on zymosan-stimulated ROS production were demonstrated by Boyd and Burnett (1999) in phagocytic cells (hemocytes) of the oyster (*C. virginica*). ROS production was reduced by 46% when hemocytes were held at the low oxygen levels (1.47 kPa) that occur in the hemolymph of oysters exposed to sublethal hypoxia, as compared to hemocytes held in the oxygen pressures experienced by oysters in well-aerated water (Po_2 = 5.2 kPa). Hemolymph pH is also an important factor. At pH 7.1, ROS production was reduced by

52% when compared to hemocytes held at pH 7.6. The effects of low oxygen and low pH were additive, with ROS production of hemocytes suppressed by 67% in conditions simulating hypercapnic hypoxia compared to cells held at oxygen and pH levels reported for oysters in well-aerated water (Boyd and Burnett 1999). Although comparable studies have yet to be reported for a crustacean species, a respiratory burst response can be activated in shrimp hemocytes (Muñoz et al. 2000) and we suggest that this response will also be sensitive to oxygen and pH.

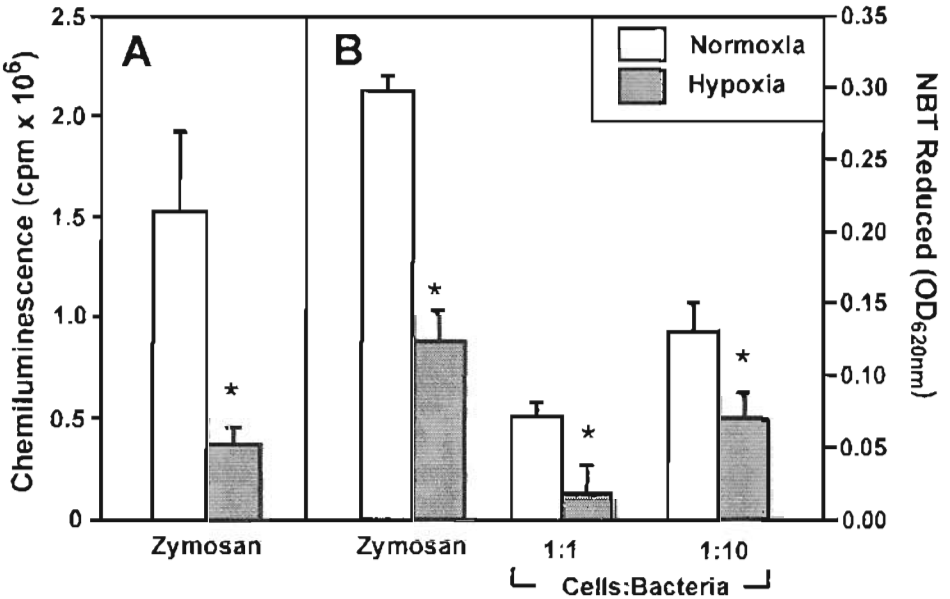

Figure 2. Production of ROS by anterior head kidney macrophages of the fish *Fundulus heteroclitus* held under conditions simulating tissue levels of O_2, CO_2 and pH in well-oxygenated (Po_2 = 6.1 kPa, Pco_2 = 0.5, pH = 7.6) and hypoxic (Po_2 = 2.0 kPa, Pco_2 = 1.1 kPa, pH = 7.0) environments. (A) Total ROS production was measured by a chemiluminescence assay in response to stimulation with zymosan. The ROS production was significantly suppressed under hypoxic conditions ($p = 0.002$, $n = 10$). (B) Intracellular superoxide production was measured by NBT assay following stimulation with zymosan or live *Vibrio campbellii* at phagocyte:bacteria ratios of 1:1 and 1:10. Hypercapnic hypoxia significantly suppressed the response to zymosan ($p = 0.001$, $n = 5$) and to live bacteria ($p = 0.016$, 1 to 1; $p < 0.001$, 1 to 10; $n = 5$). Values for A and B are means ± standard error. (From Boleza et al. 2001).

It should be noted that Le Moullac et al. (1998) reported a significant reduction in superoxide production by hemocytes from *L. stylirostris* that had been exposed to severe hypoxia (15% air saturated water) for 24 hours. Similarly Cheng et al. (2002) reported an 11% decrease in superoxide production by hemocytes of *M. rosenbergii* exposed for 24-h to 35% air-saturated water. These assays for superoxide production, however, were conducted under aerial conditions (100 air saturation) rather than at tissue-level oxygen conditions, so the results from Le Moullac et al. (1998) and Cheng et al. (2002) are difficult to compare to those of Boyd and Burnett (1999) and Boleza et al. (2001).

The means by which oxygen and pH regulate ROS production have not been clarified. It is likely that pH alters a variety of enzymatic activities associated with the

respiratory burst cascade (Boyd and Burnett 1999). The potential cellular target for the action of low oxygen is less apparent. Many mammalian oxidases, including the cytochrome oxidases and NADPH oxidase, have a particularly high affinity for oxygen (de Groot and Littauer 1989). As a result, significant inhibition of enzyme activity usually requires an extracellular Po_2 of 0.3 kPa or less (de Groot and Littauer 1989). Therefore, it is unlikely that low oxygen is substrate limiting for NADPH oxidase. More intriguing is the possible involvement of a low affinity cell-surface oxygen receptor that coordinates a general cellular response to hypoxia through the hypoxia inducible transcription factor, HIF-1 (Ratcliffe et al. 1998). Evidence for such an oxygen receptor linked to activation of HIF-1 suggests the involvement of a flavoprotein-linked oxidase, similar but not identical to NADPH oxidase (Bunn et al. 1998).

There may well be other cellular immune mechanisms which are sensitive to oxygen stress. Direkbusarakom and Danayadol (1998) found that hypoxia significantly decreased phagocytic uptake by hemocytes of the black tiger shrimp, *P. monodon*. Animals were held at approximately at 25 – 30% air saturation for six hours prior to injection of a yeast (*Saccharomyces cerevisiae*) suspension. Hemocytes sampled 30-min post-injection were examined to determine the percentage of cells that had taken up yeast particles. This value was expressed as phagocytic rate (PR). PR in control shrimp reared under low oxygen conditions was slightly, but significantly reduced compared to that of animals held under air-saturated conditions. The PR was also reduced in *M. rosenbergii* exposed to 23– 61% air-saturated conditions for as long as 120 hours, however, the phagocytic activity was measured under aerial conditions (Cheng et al. 2002).

Le Moullac et al. (1998) documented a significant decline in total hemocyte count (THC)/mL hemolymph in *L. stylirostris* exposed to 15% air-saturated water for 24 hours. Mikulski et al. (2000) also found a significant decrease in THC/mL within 4 hours after exposing *L. vannamei* to 22 - 25% air-saturated water. In comparison, Cheng et al. (2002) saw no decrease in THC/mL of *M. rosenbergii* held for 120 hours over a range of O_2 levels from 61% to 23% air-saturation, nor did Holman et al. (2004) observe significant changes in hemocyte count over 6 h exposure of blue crabs to hypercapnic hypoxia (4 kPa O_2; 20% air saturation), 1.8 kPa CO_2, pH 6.7 – 7.1). Differences in the duration and the severity of hypoxic exposure may explain in part the discrepancies among the results of these four studies. However, changes in THC/mL may well prove important to understanding the effects of hypoxia on the immune response.

Several *in vitro* and *in vivo* studies in crustaceans 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). Martin et al. (1998) presented evidence that these nodules 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 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). It is interesting that localization of these hemocyte:bacteria aggregates to the gill vasculature would assure that cellular immune defenses in

crustaceans are initiated in the relatively oxygen-enriched environment of the branchial tissues. Movement of hemocytes to the gill might be a useful strategy to optimize immune defenses under hypoxic conditions. However, the trapping of nodules in the gills may interfere with respiratory function and could ultimately have negative effects on immune function and other oxygen-dependent functions.

In addition to cellular effectors, the immune systems of both vertebrates and invertebrates rely on soluble factors, including anti-bacterial peptides, pattern-recognition molecules such as mannan-binding protein, and beta-glucan binding protein, as well as cytolytic cascades to mediate the elimination of pathogens. A well-studied soluble mechanism of immune defense among arthropods is the prophenoloxidase (PPO) cascade. The end product of the PPO cascade is melanin, which barricades wounded tissues, hardens and darkens post-molt carapace, and minimizes bacterial and fungal infections through encapsulation (Söderhäll 1982; Smith and Söderhäll 1991; Söderhäll and Cerenius 1998). The terminal enzyme of the PPO cascade is phenoloxidase (PO). Using oxygen as a proton acceptor, PO converts phenols such as L-3,4-dihydroxyphenylalanine (L-DOPA) to quinones such as dopachrome, which can then be rearranged into the end product melanin (Aspán et al. 1995). The regulation of this PO reaction has been studied primarily through the activation of PPO with detergents (Decker et al. 2001), temperature (Hauton et al. 1997), protease inhibitors (Ashida 1971; Söderhäll 1981), and divalent cations (Sung et al. 1998). Where it has been examined, the optimum pH for PO in crustaceans lies between 8.0 and 9.0 (Gollas-Galván et al. 1999; Cárdenas and Dankert 2000). Cheng et al. (2002) reported a significant decline in PO activity of hemocytes from the shrimp *M. rosenbergii* exposed for 24 hours to 35% air-saturated water. In contrast, Le Moullac et al. (1998) found that PO activity increased in the blue shrimp during hypoxia (15% air saturation for 24 h). An important consideration is that PO activities in the latter two studies (Le Moullac et al. 1998; Cheng et al. 2002) were measured under aerial conditions. Our laboratory is currently investigating the sensitivity of PO from the blue crab within the physiologically relevant ranges of oxygen and carbon dioxide pressures and pH. In these *in vitro* studies, PO was isolated from blue crabs held in air-saturated conditions. This proenzyme was activated and assayed under a broad range of oxygen pressures (Po_2 = 5 – 21% air saturation) and pH (7.0 – 7.8) conditions that occur within the internal milieu of crustaceans exposed to well-aerated water and to hypoxia. Preliminary data strongly suggested that PO activity was significantly suppressed at low O_2. Enzyme activity was also suppressed at low pH conditions that occur in the tissues of normoxic and hypoxic animals (Tanner et al. 2004).

Summary. In this review, we have documented some of the sensitivities of the immune system in crustaceans and other marine organisms to physiologically-relevant changes in oxygen and pH. Both the respiratory burst and the prophenoloxidase cascade are suppressed at dissolved gas and pH conditions that occur in hypoxic animals. The corresponding sensitivities of other mechanisms of immune defense remain to be studied. The biochemical mechanisms that underlie these sensitivities have yet to be clarified and are worthy of further investigation. Information garnered from these experiments promises to enhance our understanding of the integration between the immune system and

other physiological processes that are vital to the health of aquatic organisms.

Acknowledgements

We wish to thank our students, particularly J. Boyd, C. Mikulski, J. Holman, C. Tanner, for their contributions to the work described here. Major support was provided by USDA CREES 99-35204-8555 and NSF IBN-0212921. Contribution #264 from the Grice Marine Laboratory.

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