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Hypercapnic hypoxia compromises bactericidal activity of fish anterior kidney cells against opportunistic environmental pathogens

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Acute hypoxia can cause massive fish and shellfish mortality. Less clear is the role that chronic sublethal hypoxia might play in aquatic animal health. This study tested whether production of reactive oxygen species (ROS) and bactericidal activity of fish phagocytic cells are suppressed under the conditions of decreased oxygen and pH and increased carbon dioxide which occur in the blood and tissue of animals exposed to sublethal hypoxia. Anterior head kidney (AHK) cells of the mummichog, *Fundulus heteroclitus*, were exposed in parallel to normoxic (pO_2=45 torr, pCO_2=3·8 torr, pH=7·6) or hypoxic (pO_2=15 torr, pCO_2=8·0 torr, pH=7·0) conditions and stimulated with a yeast cell wall extract, zymosan, or live *Vibrio parahaemolyticus*. Hypercapnic hypoxia suppressed zymosan-stimulated ROS production by 76·0% as measured in the chemiluminescence assay and by 58·5% in the nitroblue tetrazolium (NBT) assay. The low O_2, high CO_2 and low pH conditions also suppressed superoxide production by 75·0 and 47·3% as measured by the NBT assay at two different challenge ratios of cells:bacteria (1:1 and 1:10, respectively). In addition to its effects on ROS production, hypercapnic hypoxia also reduced bactericidal activity by 23·6 and 72·5% at the 1:1 and 1:10 challenge ratios, respectively. Low oxygen levels alone (pO_2=15 torr, pCO_2=0·76 torr, pH=7·6) did not significantly compromise the killing activity of cells challenged with equal numbers of *V. parahaemolyticus*. At the higher 1:10 AHK:bacteria challenge ratio, low oxygen caused a small (26·3%) but significant suppression of bactericidal activity as compared to aerial conditions (pO_2=155 torr, pCO_2=0·76 torr, pH=7·6). This study demonstrates that while hypoxia alone has detrimental effects on immune function, suppression of phagocytic cell activity is compounded by naturally occurring conditions of hypercapnia and low pH, creating conditions that might be exploited by opportunistic pathogens. These results indicate that the adverse health effects of chronic hypercapnic hypoxia might greatly exceed the effects of low oxygen alone.

Key words: hypercapnic hypoxia, bactericidal activity, ROS production, *Vibrio parahaemolyticus*, *Fundulus heteroclitus*, anterior kidney cells.

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I. Introduction

Worldwide, the extent of hypoxic waters is increasing, and the coastal distribution of many anoxic zones suggest that estuarine ecosystems may be at particular risk from the adverse effects of low dissolved oxygen levels (Diaz & Rosenburg, 1995). In the southeast United States and the Gulf of Mexico shallow coastal regions often experience dissolved oxygen concentrations of less than 3·0 mg l^{-1} (62 torr or 40% air saturation at 25° C) (Breitburg, 1990; Rabalais *et al*., 1994; Burnett, 1997; Summers *et al*., 1997). Oxygen pressures in tidal creeks of South Carolina can fluctuate between 9 and 170 torr (6 and 110% air saturation) over a 24 h period (Cochran & Burnett, 1996), and may decline to as low as 2 torr (1·2% air saturation) in the nearby Savannah River estuary (Winn & Knott, 1992). Hypoxia occurs naturally as a result of aquatic respiration in excess of photosynthesis, and oxygen can fluctuate both seasonally and diurnally (Burnett, 1997). In addition, summer hypoxic events are often exacerbated by an increase in precipitation, which leads to a decrease in salinity in the surface layer of the water column. The resultant vertical stratification minimises oxygen transfer from the surface to the bottom (Taft *et al*., 1980; Reynaud, 1986; Turner *et al*., 1987). Although hypoxia occurs naturally, its magnitude and duration may increase due to eutrophication (Diaz & Rosenberg, 1995; Nixon, 1995). When excess nutrients enter the water, they support the production of large algal blooms and the decomposition of organic matter in the sediments. Both of these events severely deplete oxygen levels in the water column (Taft *et al*., 1980; Justic *et al*., 1993).

Hypoxia is frequently named as the primary cause of mass mortalities of marine life such as recent fish kills in coastal North Carolina (Paerl *et al*., 1999), the Savannah River estuary (Winn & Knott, 1992) and the Gulf of Mexico (Rabalais *et al*., 1994). Where it is not considered a primary cause of mortality, hypoxia may contribute to outbreaks of disease, such as mycobacteriosis in fish (Vogelbein *et al*., 1999) and *Perkinsus* infections in oysters (Anderson *et al*., 1998). Laboratory studies support the notion that organisms living in hypoxic environments may be at increased risk from opportunistic pathogens. Hypoxia increased the susceptibility of tilapia hybrids, *Oreochromis niloticus*, and carp, *Cyprinus carpio*, to infection with *Streptococcus* spp. (Bunch & Bejerano, 1997). Low dissolved oxygen levels also increased mortality in yellowtail fish, *Seriola quinqueradiata*, challenged with *Enterococcus seriolicida* (Fukuda *et al*., 1997) and in blue shrimp *Litopenaeus stylirostris* exposed to *Vibrio alginolyticus* (Le Moullac *et al*., 1999).

Most field studies and laboratory simulations of hypoxia also fail to consider that low dissolved oxygen is almost always accompanied by an increase in carbon dioxide pressure (pCO_2), or hypercapnia (Burnett, 1997). These elevated levels of CO_2 derived from respiration drive a decrease in pH, or acidosis, in hypoxic waters. For example, Cochran & Burnett (1996) reported that pCO_2 varied from 0·3 to 12 torr in South Carolina tidal marshes. Over the same 24 h period, pH ranged from 6·5 to 7·6. Recently, Mikulski *et al.* (2000) demonstrated that hypercapnic hypoxia (low O_2, high CO_2 and low pH) increased mortality of the shrimp, *Litopenaeus vannamei*, after *Vibrio parahaemolyticus* infection, while low dissolved O_2 alone did not significantly alter

mortality. With the exception of this one study, the impact of hypercapnic hypoxia on susceptibility to infectious diseases remains relatively unstudied.

Two lines of evidence suggest that hypercapnic hypoxia might suppress mechanisms of immune defence that are responsible for killing and clearing infectious pathogens. First, aquatic organisms have only a limited ability to regulate internal levels of dissolved gases and pH by behavioural and physiological mechanisms (Burnett, 1997; Burnett & Stickle, 2001), therefore blood levels of pO_2 and pCO_2 will reflect changes in water pO_2. In rainbow trout, *Oncorhynchus mykiss*, maintained in well-aerated water with pO_2 of 140–155 torr, Nikinma & Solvio (1982) measured blood pO_2 in the dorsal aorta at 90–100 torr. When water oxygen levels decreased to 35–40% air saturation (44–50 torr), the pO_2 in the dorsal aorta declined over 3 h to approximately 20–50 torr. These observations indicate that cells of the immune system are exposed to an internal milieu that reflects the external environment. Second, low dissolved oxygen and low pH suppress the activity of at least one specific biochemical pathway that is involved in defence against infection, the respiratory burst of phagocytic cells. In phagocytic cells, binding a foreign target through an appropriate membrane receptor activates NADPH oxidase and triggers a cascade of biochemical reactions termed the respiratory burst. The respiratory burst generates a complex array of reactive oxygen species (ROS), including superoxide, hydrogen peroxide, hydroxyl radical, perchlorate and, in the presence of nitric oxide, peroxynitrite (Chung & Secombes, 1988; Sharp & Secombes, 1993). In vertebrate phagocytes, ROS provide vital defences against infection (Gabig & Babior, 1981; Chung & Secombes, 1988). 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. In a recent study, Boyd & Burnett (1999) demonstrated that low dissolved oxygen and low pH independently and additively suppressed ROS production by oyster haemocytes. Their data showed clearly that the respiratory burst was sensitive to co-ordinate changes in dissolved gases and acidosis within the levels that occur in tissues of animals exposed to hypercapnic hypoxia. Comparable studies to evaluate the effects of co-ordinate changes in O_2, CO_2 and pH on ROS production and their impact on bactericidal activity have yet to be reported for vertebrate phagocytic cells.

The present study tested the impact of physiologically relevant changes in dissolved gases and pH on the production of ROS and bactericidal activity of phagocytes from a small teleost fish, *Fundulus heteroclitus*. *F. heteroclitus* is extremely abundant in tidal creeks of the eastern United States even during summer months when dissolved oxygen and pH are low and dissolved CO_2 increases. Phagocytic cells from the anterior head kidney (AHK) of *F. heteroclitus* were held under blood gas and pH conditions approximating the tissues of teleost fish living in air-saturated (normoxic) or moderately hypoxic waters. Respiratory burst and bactericidal activities were stimulated with *V. parahaemolyticus*, a well-known opportunistic pathogen of fish, shellfish and humans (Bullock, 1987; Rand & Wiles, 1988; Depaolo *et al*., 1990). This experimental design provided a model in which to assess cellular defence against live pathogen challenge under conditions simulating realistic exposures to hypercapnic hypoxia.

II. Materials and Methods

ISOLATION OF ADHERENT CELLS FROM THE ANTERIOR HEAD-KIDNEY AND PREPARATION OF BACTERIAL CHALLENGE CULTURES

Unless otherwise indicated, all media and media supplements were obtained from Life Technologies (Rockville, MD, U.S.A.) and chemical reagents were purchased from the Sigma Chemical Company (St Louis, MO, U.S.A.).

F. heteroclitus were collected from local tidal creeks on James Island, South Carolina. Fish were held in laboratory aquaria at the Medical University of South Carolina animal holding facility at Fort Johnson, SC, for no less than 14 days prior to use in experiments. Aquaria were equipped with recirculating filtration systems maintained at 21–24° C, pH of 8·0–8·2 and 25–30% salinity with biweekly monitoring of ammonia/nitrite/nitrate levels. Fish were fed a standard diet of Marine Tetra Flakes twice daily.

To isolate adherent cells from AHK of *F. heteroclitus*, animals were anaesthetised with 0·15 g l^{-1} of 3-amino benzoic acid ethyl ester (MS-222) and decapitated. After removal, the AHK was washed for 30 min in L-15 supplemented with 0·21% NaCl, 60 U ml^{-1} penicillin/streptomycin, 100 μg ml^{-1} gentamycin and 2·5 μg ml^{-1} fungizone. AHK tissue was then gently dissociated in Fundulus Media (FM) (L-15, 0·21% NaCl, 5 mM HEPES) with a Teflon pestle and centrifuged for 10 min at 258 × *g*. The pellet of cells was resuspended to the desired density in FM and total cell numbers were determined by haemocytometer count. Differential cell counts were performed with Diff-Quik differential stain (Life Technologies). Viability of phagocytic cells after isolation was measured by exclusion of trypan blue.

ROS and bactericidal assays employed a strain of *Vibrio* sp. (90-69B3) isolated from diseased shrimp in aquaculture (D. Lightner and L. Mohney, University of Arizona). The strain was identified as an atypical *V. parahaemolyticus* using API-2ONE bacterial identification strips (bioMerieux, St Louis, MO, U.S.A.) cultured at 25° C, but does not carry the species-specific genes for thermostable or thermolabile haemolysins. Prior to each assay, bacteria were thawed from stock vials and grown on Tryptic Soy Agar (TSA) + 2·5% NaCl plates for 24 h. Bacterial densities were quantified by optical density ($O.D._{540\ nm}$) in Tryptic Soy Broth (TSB) + 2·5% NaCl and confirmed by enumerating colony forming units on double layer plates consisting of TCBS and Marine Agar.

To determine bacterial growth curves under aerial, normoxic and hypoxic conditions, 100 ml glass bottles containing 45 ml TSB + 2·5% NaCl were placed on a shaker and held at a constant temperature of 25° C. For 1 h prior to adding bacteria, the bottles were gassed with 21·0% oxygen and 0·1% CO_2 for aerial conditions, 6% O_2 and 0·5% CO_2 for normoxic conditions and 2·0% O_2 and 1·0% CO_2 for hypoxic conditions. To compare growth curves under normal and acidic conditions, TSB + 2·5% NaCl was brought to a pH of 7·6 or 7·0. Growth tests were initiated by adding 5×10^7 bacteria to each bottle and measuring $O.D._{540\ nm}$ as a function of time.

Table 1. Test conditions used to represent physiologically correct tissue levels of dissolved oxygen and carbon dioxide and pH under aerial, normoxic and hypoxic conditions in teleost fish

Test condition	pO_2	pCO_2	pH
Aerial	155 torr/8·43 mg l^{-1}	0·76 torr	7·6
Normoxic	45 torr/2·45 mg l^{-1}	3·8 torr	7·6
Hypoxic	15 torr/0·82 mg l^{-1}	8·0 torr	7·0
Hypoxic pO_2 only	15 torr/0·82 mg l^{-1}	0·76 torr	7·6

In experiments to test the effects of low oxygen alone, AHK cells were exposed to conditions listed as 'hypoxic pO_2 only'.

OBTAINING PHYSIOLOGICALLY RELEVANT HYPOXIC, HYPERCAPNIC AND ACIDIC CONDITIONS

FM were gassed to appropriate gas pressures and pH using Wösthoff gas mixing pumps as indicated in Table 1. All media were gassed for 3 h the day before running the assay and kept at 2–8° C overnight. For normoxic conditions simulating tissues of animals held in air-saturated water, the medium was gassed with 6% O_2, 0·5% CO_2 and 93·5% N_2. To obtain hypoxic conditions resembling tissues of animals held in hypercapnic hypoxia, the medium was gassed with 2·0% O_2, 1·0% CO_2 and 97·0% N_2. The final pH for the aerial and normoxic conditions was 7·6 while the final pH for the hypoxic condition was 7·0. Prior to performing functional assays, buffer curves were measured in FM with varying amounts of HEPES.

ASSAYS FOR ROS PRODUCTION

Production of ROS by phagocytes stimulated with zymosan was quantified using luminol-enhanced chemiluminescence (CL) as described by Van Dyke *et al.* (1977) and Trush *et al.* (1978). Luminol was prepared according to the method of Scott & Klesius (1981) and stored in 1·0 ml portions at −20° C. One ml of the AHK cell suspension ($5·5 \times 10^5$ cells ml^{-1}) was allowed to adhere to each 10 ml glass scintillation vial at 25° C under aerial conditions for 7 h. Nonadherent cells were removed by replacing the media, while the adherent cells were incubated overnight at 25° C under aerial conditions. Preliminary studies showed that approximately 20% of AHK failed to adhere to glass or plastic surfaces. The adherent AHK population consisted on average of 70% macrophages and 28% granulocytes. Prior to assay, the media was replaced with 675 μl of medium previously gassed at the appropriate combination of O_2, CO_2 and N_2. Heat inactivated serum (200 μl) from *Sciaenops ocellatus*, the red drum, was added to each of the vials to opsonise zymosan. Size limitations of *F. heteroclitus* precluded the use of homologous serum in these assays and preliminary studies indicated that red drum serum provided comparable opsonic activity in this assay, as well as the nitroblue tetrazolium and bactericidal assays. The vials were capped with rubber stoppers and gassed for 3 min under the specified conditions (normoxic, hypoxic). Luminol (25 μl) was added to each vial using a 25 μl Hamilton syringe. CL was quantified using a Wallace 1414 liquid scintillation counter set for single photon monitoring at

10 s intervals. After assuring that background levels of CL had stabilised, 100 μl of the zymosan suspension (Austin & Paynter, 1995) was added to the vials and CL was measured for 100 min. Total CL was calculated as the area under the curve post-zymosan exposure using the final background count as baseline for the curve.

Alternatively, production of intracellular superoxide was measured using the nitroblue tetrazolium (NBT) assay as modified by Secombes (1990) from Pick & Mizel (1981). AHK ($2{\cdot}0 \times 10^5$) in 200 μl FM, 2 mM $CaCl_2$, were placed in individual wells of a 96 well flat-bottom plate (Fisher Scientific, Springfield, IL, U.S.A.), and incubated at 25° C under aerial conditions. After 7 h the medium was replaced with 150 μl of fresh FM, 2 mM $CaCl^2$, removing approximately 50 000 non-adherent cells $well^{-1}$. The cells were then incubated overnight at 25° C under aerial conditions. Prior to running the assay, the medium in each well was replaced with 150 μl FM, 2 mM $CaCl_2$, gassed to normoxic or hypoxic conditions along with 50 μl of heat inactivated red drum serum and 25 μl of NBT from a stock of 3·0 mg ml^{-1} made in phenol red-free L-15. Superoxide production was initiated by adding 25 μl of the stimulant, either zymosan or bacteria, to each well, to a final ratio of 1:100 phagocytes:zymosan, and either 1:1 or 1:10 phagocytes:bacteria. Assay plates were centrifuged for 10 min at 258 × *g*, then placed under the specific gassed environments for 60 min. The plates were centrifuged at 258 × *g* for 3 min and the media replaced with 120 μl 2 M KOH and 140 μl DMSO to dissolve the reduced NBT. Superoxide production was reported as $O.D._{620\ nm}$ minus NBT reduction caused by zymosan or bacteria alone. NBT can also be reduced by unstimulated cells through the action of the diaphorase enzyme. Thus, the amount of reduction that was performed by the superoxide anion was confirmed by adding superoxide dismutase (SOD) to some wells in each plate.

BACTERICIDAL ACTIVITY OF ADHERENT AHK CELLS

The bacterial killing assay described by Sharp & Secombes (1993) was performed with slight modifications. Adherent AHK cells were prepared as described for the NBT assay in FM without $CaCl_2$. Prior to running the assay, the FM was again replaced with 150 μl FM gassed to pO_2 and pCO_2 as described above, along with 40 μl of heat-inactivated serum. To test the effect of oxygen alone, the levels of CO_2 and pH were maintained at 0·1% and 7·6, respectively, while the oxygen level was decreased from aerial levels (21·0%) to hypoxic levels (2·0%). The assay was initiated by adding 50 μl of *V. parahaemolyticus* suspensions to achieve the indicated final ratios of phagocytes:bacteria. Plates were spun for 10 min at 258 × *g*, then placed under the specific gassed environments for 180 min. At this point, the medium was removed, AHK were lysed with 50 μl 0·1% Tween for 10 min to release any live bacteria and 100 μl TSB 2·5% NaCl was added to each well. Surviving bacteria were allowed to grow out at 23° C for 9 h. Then, 10 μl of a 5 mg ml^{-1} solution of 3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide (MTT) in distilled H_2O was added to each well. After 15 min, 10% SDS in 0·01 N HCl (100 μl) was added to each well to dissolve reduced formazan and the optical density of test wells measured at 560 nm. $O.D._{560\ nm}$ values of surviving bacteria

in test wells were normalised to parallel wells containing 10 μl of 5 mM trifluoroperazine (TFP) to inhibit the respiratory burst. The normalised value, expressed as a percent and subtracted from 100%, provided a killing index (KI) using the following formula:

$$\mathrm{KI} = \left\{1{\cdot}0 - \left(\frac{\mathrm{O.D.}_{P+B} - \mathrm{O.D.}_{P}}{\mathrm{O.D.}_{P+B+TFP} - \mathrm{O.D.}_{P}}\right)\right\} \times 100\%,$$

where $\mathrm{O.D.} = \mathrm{O.D.}_{560\ \mathrm{nm}}$, P=phagocytes and B=bacteria.

STATISTICAL ANALYSIS

For each treatment *n* refers to the number of animals tested. In the NBT and bactericidal assays, AHK cells from individual animals were assayed in triplicate wells under each of the treatment conditions. To measure ROS production by CL, a single assay for each individual fish was performed in parallel under hypoxic and normoxic conditions. To determine if a treatment significantly affected the processes being evaluated, statistical analysis was performed using the program SigmaStat (Jandel Corporation, San Rafael, CA, U.S.A.). Unless otherwise indicated, experimental data met the assumptions of normality so that experimental groups could be compared using a one-way, paired *t*-test. For data that did not meet the assumptions of normality, responses were compared with the nonparametric Wilcoxon signed rank test. For both statistical tests, a value of $P<0{\cdot}05$ was considered significant.

III. Results

EFFECTS OF PHYSIOLOGICAL LEVELS OF HYPERCAPNIC HYPOXIA ON ROS PRODUCTION

To evaluate the impact of hypercapnic hypoxia on respiratory burst activity of adherent AHK in *F. heteroclitus*, *in vitro* ROS production was compared under dissolved gas and pH conditions that might occur in the blood and tissues of animals held in fully aerated water (normoxia) and moderately hypoxic waters (hypercapnic hypoxia) (Nikinmaa & Solvio, 1982). The respiratory burst was stimulated with zymosan, an extract of yeast cell wall. ROS production was measured with the luminol-enhanced CL assay that detects a wide range of oxyradicals. CL activity was reduced by 76·0% under hypoxic conditions ($0{\cdot}37 \pm 0{\cdot}08 \times 10^6$ cpm) as compared to normoxia ($1{\cdot}54 \pm 0{\cdot}42 \times 10^6$ cpm) (Fig. 1a). This significant decrease in ROS production ($P=0{\cdot}002$, $n=10$) confirmed that the strong respiratory burst response elicited by zymosan can be suppressed by changing parameters of dissolved gases and pH within tissue-level extremes that occur in organisms subjected to moderate hypercapnic hypoxia.

Although zymosan rapidly elicits intense ROS production by many types of phagocytic cells, live bacteria induce considerably weaker responses under the same *in vitro* test conditions (Johnston, 1981). Changes in pO_2, pCO_2 and pH within the physiologically relevant range might not suppress the low levels of ROS production stimulated by live bacteria. Furthermore, many live

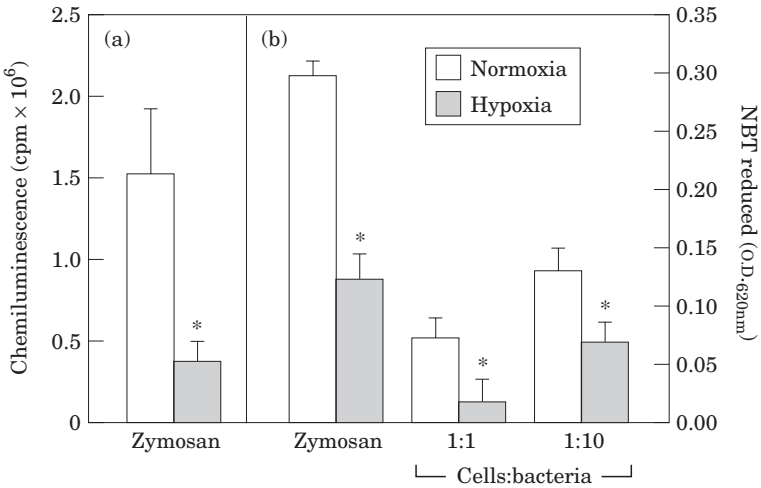

Fig. 1. Production of ROS by mummichog AHK held under conditions simulating tissue levels of O_2, CO_2 and pH in normoxic and hypoxic environments. (a) Total ROS production was measured by a chemiluminescence assay in response to stimulation with zymosan. ROS production was significantly suppressed under hypoxic conditions (P=0·002, n=10). (b) Intracellular superoxide production was measured by NBT assay following stimulation with zymosan or live *V. parahaemolyticus* 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 ± S.E. error.

bacteria, including *V. parahaemolyticus*, generate a large signal in the CL assay that obscures ROS production by phagocytes. To assess ROS production in response to challenge with live *V. parahaemolyticus*, another common test for production of oxygen radicals was employed, the NBT assay. This test detects intracellular production of superoxide anion, the first oxyradical generated by the respiratory burst cascade. The NBT assay (Fig. 1b) confirmed that hypercapnic hypoxia depressed zymosan-stimulated superoxide production by 58·5% (P<0·001, n=5). Average $O.D._{620\,nm}$ values declined from 0·299 ± 0·028 under normoxia to 0·124 ± 0·034 under the low oxygen conditions. Furthermore, the NBT assay could be used to assess the impact of hypercapnic hypoxia on superoxide production in response to live *V. parahaemolyticus* (Fig. 1b). When challenged with an equal number of bacteria (1:1), AHK held under hypercapnic hypoxia ($O.D._{620\,nm}$=0·018 ± 0·012) generated 75·0% less superoxide than those held under normoxia ($O.D._{620\,nm}$=0·072 ± 0·012). At a 10-fold excess of bacteria (1:10), phagocytes under low oxygen conditions ($O.D._{620\,nm}$=0·069 ± 0·014) produced 47·3% less superoxide than cells held under normoxia ($O.D._{620\,nm}$=0·131 ± 0·017). Addition of SOD to the test wells in each assay plate indicated that 98% of NBT reduction in normoxic wells and 70% of NBT reduction in hypoxic wells can be attributed to superoxide production, rather than to endogenous diaphorase activity. Thus, although AHK appeared to be poorly activated by the live bacterial challenge, conditions of low pO_2, high pCO_2 and low pH significantly impaired the production of superoxide at two different effector-to-target ratios (1:1, P=0·016, n=5; 1:10, P<0·001, n=5).

EFFECTS OF HYPERCAPNIC HYPOXIA ON BACTERICIDAL ACTIVITY

Having established that hypercapnic hypoxia can suppress ROS production, experiments were intiated to compare the ability of AHK to kill live *V. parahaemolyticus* under normoxic *v.* hypoxic conditions. To measure bactericidal activity, AHK were mixed with live bacteria for 9 h. After this period of bacterial uptake and killing, the kidney cells were lysed and live bacteria released from the cells were cultured and quantified by a colorimetric assay. In this assay format, increased bacterial killing activity correlated with a decrease in optical density of test wells.

Killing activity of adherent AHK against *V. parahaemolyticus* was compared under hypoxic and normoxic conditions at two different effector-to-target ratios. When AHK cells were challenged with an equal number of bacteria (challenge ratio 1:1) or 10-fold excess bacteria (challenge ratio 1:10), AHK preparations from individual animals demonstrated marked differences in their ability to kill *V. parahaemolyticus* (Fig. 2a and b, respectively). To combine data from animals within a treatment group, $O.D._{560\,nm}$ values of surviving bacteria in test wells were normalised to optical densities of parallel wells containing trifluoroperazine to inhibit all bactericidal activity associated with the respiratory burst. The normalised values subtracted from 100% provided a killing index that could be compared among individual animals. At the 1:1 challenge ratio, conditions of low oxygen, high CO_2 and low pH reduced the killing index to $66{\cdot}1 \pm 13{\cdot}2\%$ compared with $86{\cdot}5 \pm 5{\cdot}9\%$ under normoxia. Although small, the suppressive effect of hypercapnia hypoxia (23·6%) was significant as indicated by the Wilcoxon signed rank test ($P=0{\cdot}016$, $n=10$) (Fig. 3). Hypercapnic hypoxia suppressed the killing index of AHK to an even greater extent (72·5%) when the cells were challenged at the higher dose of 1 phagocyte:10 bacteria (Fig. 3). At this high challenge ratio bactericidal activity under normoxic conditions averaged $64{\cdot}1 \pm 10{\cdot}3\%$, which was significantly higher than the activity under low oxygen conditions ($17{\cdot}6 \pm 9{\cdot}8\%$, $P=0{\cdot}002$, $n=10$).

BACTERICIDAL ACTIVITY AGAINST *V. PARAHAEMOLYTICUS* AS A FUNCTION OF OXYGEN ALONE

The respiratory burst is oxygen-dependent, therefore, suppression of oxygen radical production and bactericidal activity observed under conditions of hypercapnic hypoxia might be driven predominantly, or exclusively, by dissolved oxygen. To test this possibility, bactericidal activity was compared under low (15 torr) and high (155 torr) pO_2 (hypoxic and aerial pO_2, respectively) while maintaining constant pCO_2 (0·76 torr) and pH (7·6). As observed in previous tests, killing activity varied dramatically among the AHK preparations from individual animals (Fig. 4a and b). At the 1:1 challenge ratio, the average killing index under low oxygen was $69{\cdot}7 \pm 15{\cdot}1\%$ compared with $90{\cdot}1 \pm 8{\cdot}8\%$ under aerial oxygen, however, the two treatment groups could not be distinguished by statistical testing (Wilcoxon signed rank, $P=0{\cdot}297$, $n=10$). At the 1:10 challenge ratio, the killing index under low oxygen ($60{\cdot}4 \pm 10{\cdot}7\%$) was 26·3% lower than under high oxygen conditions ($81{\cdot}9 \pm 8{\cdot}5\%$), a difference that was statistically significant ($P=0{\cdot}006$, $n=10$) (Fig. 5), but smaller than the

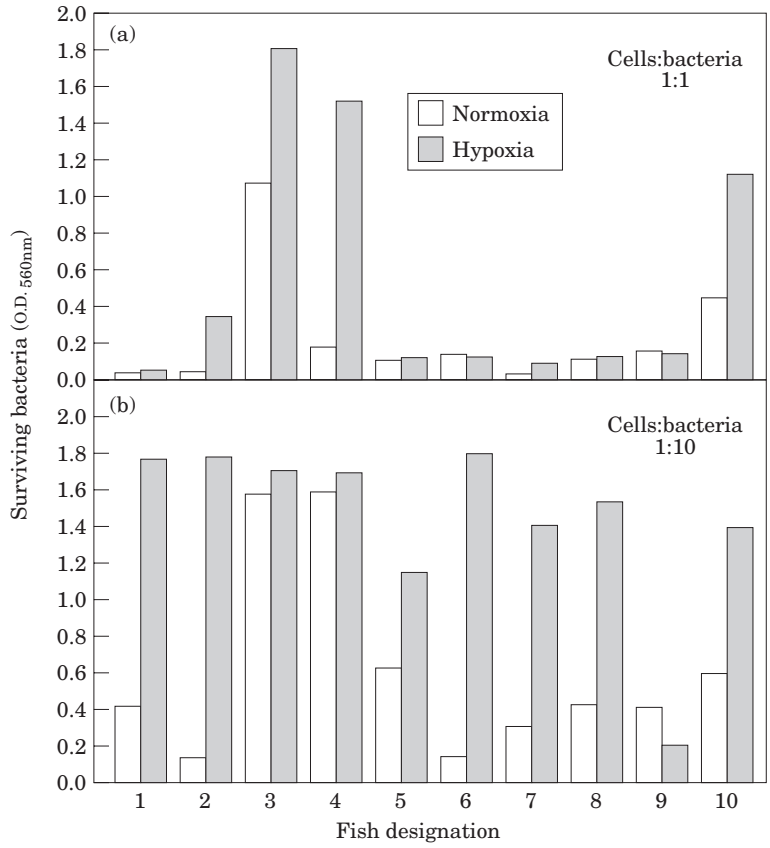

Fig. 2. Bactericidal activity of AHK cells from individual *F. heteroclitus* against live *V. parahaemolyticus* under normoxic and hypoxic conditions, at two phagocyte: bacterium ratios (a) 1:1 and (b) 1:10. Phagocytes were incubated with bacteria for 3 h at which point medium was removed and phagocytes lysed, releasing surviving bacteria. Surviving bacteria were quantified after a 9 h growth period by measuring reduction of MTT by $_{O.D.560\ nm}$. Each data point represents the average of three determinations for AHK preparations from individual fish.

72·5% suppression observed under conditions of hypercapnic hypoxia (Fig. 3). These data suggest that although low oxygen alone can reduce bactericidal activity, physiological levels of high CO_2 and low pH that co-occur with low O_2 lead to a more extensive suppression of bacterial killing activity in adherent AHK cells.

The test conditions of hypercapnic hypoxia and the levels of TFP used in these studies did not reduce viability of adherent AHK cells or enhance growth rates of the challenge bacteria. As measured by trypan blue exclusion, cell viability was greater than 98% after a 3 h incubation in the presence of TFP and greater than 96% after a 3 h exposure to hypercapnic hypoxia (data not shown). The conditions of low dissolved oxygen and high carbon dioxide used in the bactericidal assay slightly suppressed the growth rate of *V. parahaemolyticus*, as might be expected for this facultative aerobe, while decreasing the pH of culture medium from 7·6 to 7·0 had no effect on bacterial

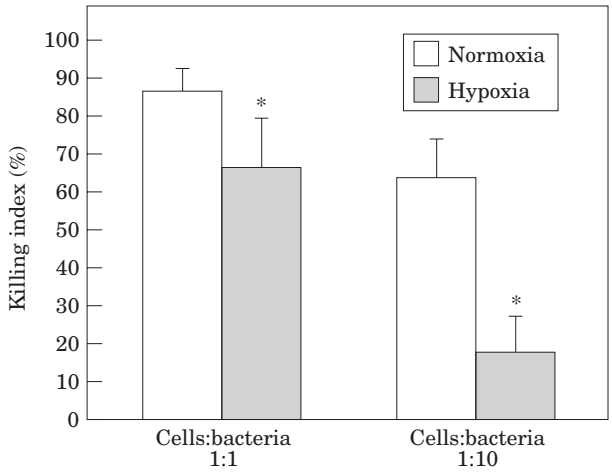

Fig. 3. Mean killing index of *F. heteroclitus* AHK cells under normoxic and hypoxic conditions at two phagocyte:bacteria ratios. Phagocytes were incubated with bacteria for 3 h at which point medium was removed and phagocytes lysed, releasing surviving bacteria. Surviving bacteria were quantified after a 9 h growth period by measuring reduction of MTT at $O.D._{560\ nm}$. To calculate killing index, $O.D._{560\ nm}$ values were normalised to parallel wells containing the inhibitor TFP, expressed as a percent and subtracted from 100%. The indicated values are mean killing index ± S.E. Bactericidal activity of the hypoxic group was significantly lower than the normoxic group at both phagocyte:bacteria ratios tested (Wilcoxon signed rank test, P=0·016 for 1:1 ratio, n=10; P=0·002 for 1:10 ratio, n=10).

growth (Fig. 6a and b). Therefore, the observed suppression of bactericidal activity by hypercapnic hypoxia cannot be explained as a secondary effect of decreased phagocyte viability or increased bacterial growth rate. These data further strengthen the conclusion that the changes in dissolved gases and pH that accompany hypoxia can compromise bactericidal activity of phagocytic cells from a teleost fish.

IV. Discussion

In the present study, the changes in blood levels of O_2, CO_2 and pH that occur in fish exposed to sublethal hypoxia significantly suppressed the antibacterial activity of AHK. The level of hypercapnic hypoxia that suppressed bactericidal activity also reduced production of superoxide by zymosan-activated AHK cells as measured by the CL and NBT assays (Fig. 1). This observation is consistent with a previous report by Boyd & Burnett (1999) who showed that low pH and low dissolved oxygen independently and additively suppressed ROS production by oyster haemocytes. The respiratory burst of vertebrate phagocytes is also sensitive to acidosis and to low levels of oxygen within the physiological range. For example, exudative rat neutrophils exposed to a pH drop from 7·5 to 6·8 displayed a decrease in internal pH and ROS production (Hackam *et al.*, 1996). Similarly, Feldman *et al.* (1990) found that superoxide production by mouse macrophages at 10% oxygen was less than half of what it was at 15% oxygen. These studies and others

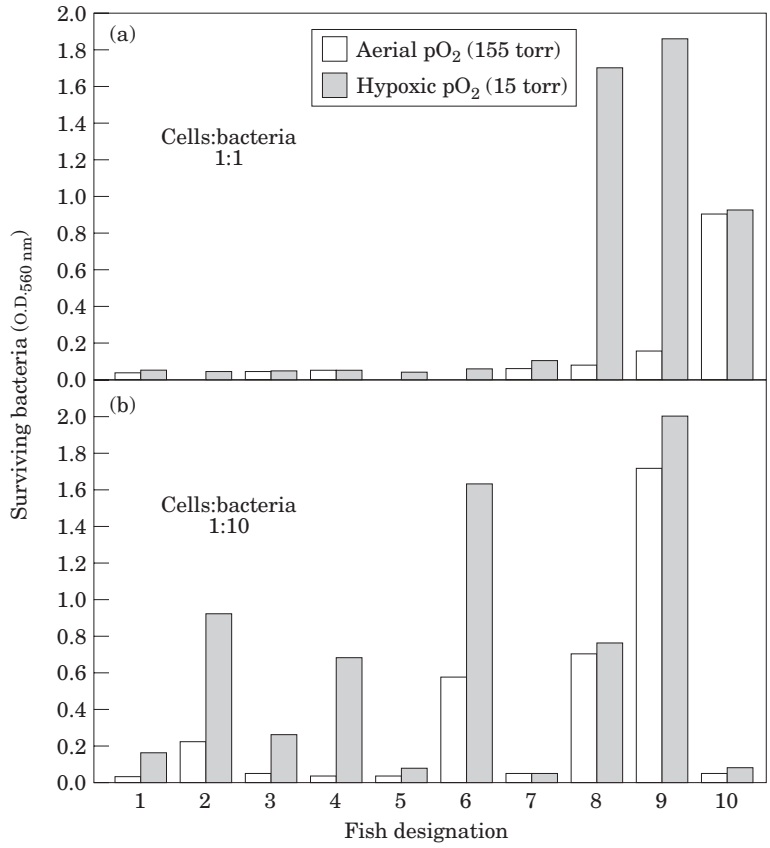

Fig. 4. Bactericidal activity of AHK cells from individual *F. heteroclitus* against live *V. parahaemolyticus*, as a function of oxygen only. Activity was compared under aerial and hypoxic pO_2 conditions at two phagocyte:bacterium ratios (a) 1:1 and (b) 1:10. Aerial values of pO_2 were used to maximise the potential for observing effects of oxygen alone. To simulate hypoxia, oxygen level was decreased to 15 torr, while maintaining pCO_2 at 0·76 torr and pH at 7·6. Phagocytes were incubated with bacteria for 3 h at which point media was removed and phagocytes lysed, releasing surviving bacteria. Survival of bacteria was quantified after a 9 h growth period by measuring reduction of MTT by optical density at $O.D._{560\ nm}$. Each data point represents the average of three determinations for AHK preparations from individual fish.

(reviewed by Boyd and Burnett, 1999) are consistent with, but do not prove the possibility that, the observed decrease in bactericidal activity in the present study might be caused by direct additive impacts of low O_2/high CO_2/low pH on the respiratory burst.

It should be noted that live *V. parahaemolyticus* were less effective than zymosan in activating AHK production of ROS. The poor ability of live bacteria to elicit ROS production compared with the potency of zymosan has been reported by others (e.g. Nagelkerke *et al.*, 1990; Belotsky *et al.*, 1998), and may reflect the pathogen's own ability to evade recognition by phagocytes or inhibit the respiratory burst directly (Ellis, 1999). Nonetheless, in the present

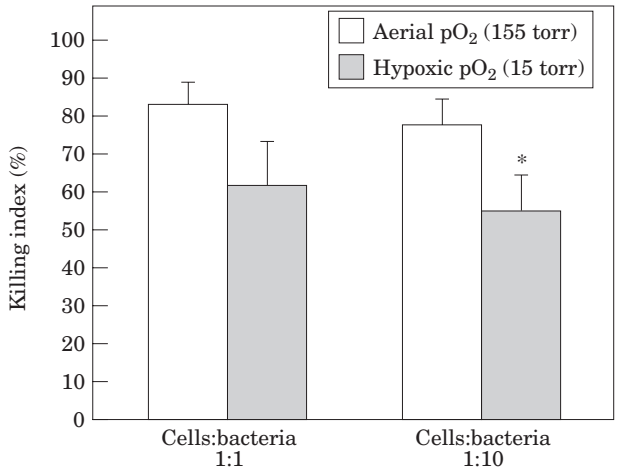

Fig. 5. Mean bactericidal activity of *F. heteroclitus* AHK against live *V. parahaemolyticus*, as a function of oxygen only. Activity was compared under aerial and hypoxic pO_2 conditions at two phagocyte:bacterium ratios, 1:1 and 1:10. Aerial values (15 torr) were tested to maximise the potential for observing effects of oxygen alone. To simulate pO_2 under hypoxia, oxygen level was decreased to 15 torr, while maintaining pCO_2 at 0·76 torr and pH at 7·6. To calculate killing index, $O.D._{560\ nm}$ values were normalised to parallel wells containing the inhibitor TFP, expressed as a percent and subtracted from 100%. The indicated values are mean killing index ± S.E. Although bactericidal activities under aerial and low oxygen conditions at the 1:1 cell:bacterium challenge ratio could not be distinguished by the Wilcoxon signed rank test (P=0·297, n=10), killing activity was significantly reduced under low oxygen at the 1:10 challenge ratio (P=0·006, n=10).

work, AHK cells challenged at 1:1 or 1:10 phagocytes:bacteria and held under hypoxic conditions produced significantly lower levels of superoxide than AHK held under normoxia. This reduction was observed after subtracting baseline superoxide production by unchallenged cells under the two levels of oxygen (see Materials and Methods). Further evidence for the role of superoxide in killing of *V. parahaemolyticus* by AHK comes from the observed increase in bacterial survival in the presence of the NADPH oxidase inhibitor TFP. The optical density of this positive control under hypoxic and normoxic conditions was used to normalise killing activity. For example, in Fig. 3 the average killing index 86·5% means that AHK under normoxia with a functional NADPH oxidase suppressed 86·5% of total bacterial outgrowth that occurred in the presence of TFP, as measured by $O.D._{560\ nm}$. These observations argue in favour of an important role for ROS in bactericidal activity and its modulation by hypercapnic hypoxia.

The antibacterial activity of AHK from individual animals varied dramatically under conditions of normoxia (Figs 2 and 4). This variation is consistent with other reports that bactericidal activity, phagocytosis and ROS production can vary among individual fish. These differences may arise from differences in age, sex, history of pathogen and contaminant exposure and stress levels of these wild, outbred animals (Warinner *et al.*, 1988; Nagelkerke *et al.*, 1990; Secombes, 1990; Roszell & Anderson, 1994, 1997). Furthermore, the

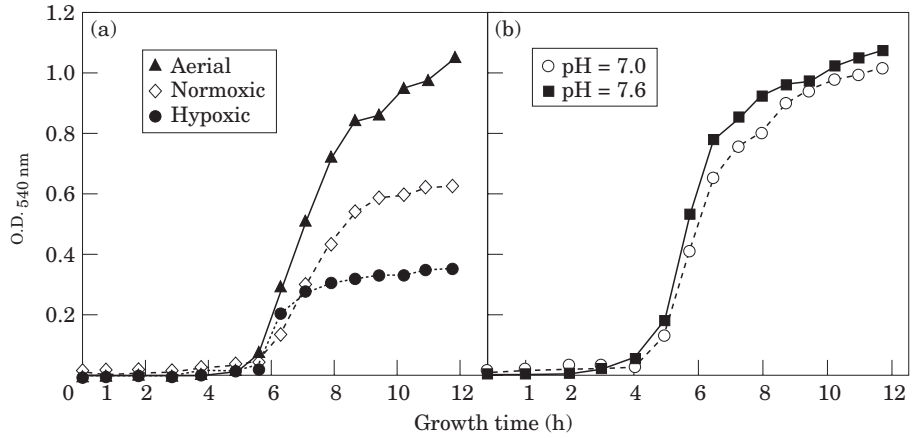

Fig. 6. Growth of *V. parahaemolyticus* under conditions simulating an aerial, normoxic or hypoxic environment with respect to (a) levels of dissolved oxygen and carbon dioxide only, or (b) pH only. Cultures were inoculated into TSB 2·5% NaCl at the indicated conditions and monitored for growth at $O.D._{540\ nm}$ over 12 h. Data points represent the average of two replicates for each growth curve. Hypoxic conditions that suppressed bactericidal activity of AHK cells [(a), pO_2=15 torr, pCO_2=8·0 torr; (b), pH=7·0] did not enhance growth of *V. parahaemolyticus*.

relative involvement of oxygen-dependent and/or oxygen-independent mechanisms in immune defence may have varied among these individuals. In support of the latter explanation, the suppressive effects of hypercapnic hypoxia varied dramatically among individuals (Figs 2 and 4) when reported as $O.D._{560\ nm}$. However, when these values were normalised against the contribution of the respiratory burst component to killing activity, as determined with the TFP positive control, the effects of low oxygen became more consistent among individuals, as reflected in Figs 3 and 5.

The present study also suggested that oxygen-mediated defence capability became more crucial to the AHK cells and the effects of hypercapnic hypoxia became greater at a higher multiplicity of infection. When phagocytes were presented with relatively low numbers of a potential pathogen, e.g. the 1:1 cell:bacterium challenge of the present study illustrated in Fig. 3, hypercapnic hypoxia caused only a small (23·6%), although significant, suppression of bactericidal activity. At the higher 1:10 challenge ratio, hypercapnic hypoxia had a greater effect (72·5%) on the ability of AHK cells to kill *V. parahaemolyticus*.

It is likely that hypercapnic hypoxia impacts more than one enzymatic and transport activity associated with the respiratory burst. For example, in a cell-free system, the enzymatic activity of NADPH oxidase activity is optimal between pH 7·0 and 7·5 (McPhail *et al.*, 1985), with significant reductions in superoxide production at pH<6·8 (Swallow *et al.*, 1993). Boyd & Burnett (1999) suggested that the pH specific reduction in superoxide production may be focused at a point prior to activation of NADPH oxidase. The potential cellular target for the action of low oxygen is less apparent. Among various oxidases, the cytochrome oxidases, including NADPH oxidase, have a

particularly high affinity for O_2 (de Groot & Littauer, 1989). As a result, significant inhibition of enzyme activity usually requires an extracellular pO_2 of 2 torr (de Groot & Littauer, 1989). Therefore, it is unlikely that low oxygen is substrate limiting for NADPH oxidase activation. More intriguing is the possible involvement of a low affinity cell-surface oxygen receptor that co-ordinates a general cellular response to hypoxia through the hypoxia inducible factor (HIF)-1 transcription factor (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).

The results of the current study suggest that periodic or chronic sublethal hypoxia could adversely effect disease resistance in teleost fish. Aquatic habitats impacted by urban development, agricultural run-off and industrial discharge often experience low levels of dissolved oxygen and high pH, in addition to a broad array of anthropogenic compounds. By themselves, many common organic and metal contaminants can suppress bactericidal activity and ROS production in teleost fish (Roszell & Anderson, 1994, 1996; Zelikoff, 1998). In the future, efforts to protect the health of aquatic organisms must consider the critical role of dissolved carbon dioxide and pH, along with dissolved oxygen, in supporting a fully-functional immune defence against infectious pathogens.

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