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Respiratory responses of the salt marsh animals, *Fundulus heteroclitus*, *Leiostomus xanthurus*, and *Palaemonetes pugio* to environmental hypoxia and hypercapnia and to the organophosphate pesticide, azinphosmethyl[1]

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Abstract

In tidal saltmarshes in South Carolina hypoxic (low O_2) and hypercapnic (high CO_2) conditions occur frequently. In the summer, water Po_2 was measured in the upper marshes and over a 24-h period ranged from 9 to 170 torr and Pco_2 ranged from 0.3 to 12 torr. These conditions depend on the stage of the tide and the time of day. The respiratory responses to different levels of Po_2 and Pco_2 of the grass shrimp, *Palaemonetes pugio*, the spot fish, *Leiostomus xanthurus*, and the killifish or mummichog, *Fundulus heteroclitus* living and feeding in the saltmarsh were investigated. Mean oxygen uptake in *P. pugio*, *L. xanthurus*, and *F. heteroclitus* at normoxic Po_2 (130–150 torr) and low Pco_2 (<0.6 torr) was 17.5, 17.1, and 9.5 $\mu mol \cdot g^{-1} \cdot h^{-1}$ and 16.3, 24.5, and 10.5 $\mu mol \cdot g^{-1} \cdot h^{-1}$ at high Pco_2 (=7 torr), respectively. The critical Po_2 for all species was between 30 and 35 torr. Mean whole body lactate concentrations in *P. pugio*, *L. xanthurus*, and *F. heteroclitus* at Pco_2 <0.6 torr are 3.5, 2.4, and 2.3 $\mu mol \cdot g^{-1}$, respectively, in normoxia and 12.3, 4.5, and 11.0 $\mu mol \cdot g^{-1}$ ($p < 0.05$; Dunn's pairwise test) in hypoxia and Pco_2 <0.6 torr. In these saltmarsh animals there appears in general to be no specific effects on oxygen uptake of environmental fluctuations in CO_2 over a wide range of Po_2. The organophosphate pesticide, azinphosmethyl, appears to have no effect on the oxygen uptake of these three species at concentrations of 10 $\mu g \cdot l^{-1}$ for fish and 2 $\mu g \cdot l^{-1}$ for shrimp.

Keywords: Fish; Hypoxia; Lactate; Oxygen; Pesticide; Shrimp

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1. Introduction

Estuaries are the zones of transition between freshwater and marine environments (Ketchum, 1983) that are highly dynamic and variable. Physicochemical factors such as salinity, temperature, oxygen and carbon dioxide tensions, and acidity may change dramatically (Jensen et al., 1993). Estuaries in temperate regions along the east coast of the United States and the Gulf of Mexico contain salt marshes (Knox, 1986). Salt marshes develop in sheltered areas with high sedimentation and are typically dominated by various species of grasses such as *Spartina* spp. and *Juncus* spp. (Knox, 1986). They are important habitats for many animals and serve as nursery areas for many larval and juvenile fishes.

Although salt marsh systems are very productive and serve as an important habitat for the early life stages of fishes, they are challenging environments for the organisms living there. In temperate regions during summer months when temperatures and salinities are highest, the salt marsh will show diel variations in dissolved oxygen (D.O.) often becoming hypoxic at night. In the Chesapeake Bay, D.O. minimas of less than 2 $mg \cdot l^{-1}$ were found to occur significantly more often during the dark hours of warmer months than during the rest of the day (Breitburg, 1990). On days following the most severe declines in oxygen, D.O. concentrations always recovered to at least 3.4 $mg \cdot l^{-1}$ at some point during the day (Breitburg, 1990). Similar but less dramatic fluctuations occurred in the Ala Wai Canal in Oahu, Hawaii where oxygen reached supersaturated values during the day and fell below saturation at night (Laws et al., 1993). These diel fluctuations are believed to be due to photosynthesis carried out during the day followed by respiration in the absence of photosynthesis at night. Thus, during the summer, oxygen concentrations that are already lower due to increased temperature, can decrease to stressful levels during night hours due to normal diel fluctuations.

Carbon dioxide and pH are two environmental parameters often overlooked in studies of hypoxia and respiration. Many factors can affect the pH of the water including humic and tannic acids in rain runoff. Hypercapnia (elevated CO_2 pressure) typically accompanies hypoxia because the processes responsible for oxygen depletion often produce molecular carbon dioxide. Little information is available on the physiological effects of naturally occurring fluctuations in carbon dioxide and pH on animals in combinations with low oxygen. The release of carbon dioxide through community respiration may cause stress to aquatic animals by acidifying the water.

Since CO_2 is one of the main determinants of acid-base status and since CO_2 is highly permeable across body surfaces, elevated water CO_2 will result in acidification of body fluids and tissues. In hypoxic-hypercapnic environments, hyperventilation by an organism will compensate little for a blood acidosis resulting from hypercapnia. The elevated Pco_2 in the water will tend to induce a blood acidosis regardless of hyperventilation. If hypercapnia persists, an organism may compensate for this blood acidosis through the uptake of bicarbonate from the water in exchange for Cl^- (Jensen et al., 1993).

If hypoxia becomes severe enough, organisms may resort to anaerobic mechanisms to sustain metabolism. A common result in many animals of a lack of adequate oxygen is the production of lactic acid. Once oxygen levels return to normal, an increased oxygen uptake repays the oxygen debt and lactic acid is converted back into pyruvate, where it is metabolized aerobically.

South Carolina has a great deal of fertile land near water that is subject to agricultural development. Consequently, there are large discharges of nutrients and pesticides from these fields into the aquatic environment. Several studies have demonstrated the effects of various insecticides on marine organisms (Eisler, 1968; Coppage & Matthews, 1974). Often, concentrations high enough to control target insects are also high enough to produce effects on non-target species. Recently, there has been a trend toward using organophosphate (OP) pesticides on crops. The OP pesticides appear to degrade more rapidly in the environment than other previously used classes of chemicals (e.g., chlorinated hydrocarbons). Aquatic organisms show a wide range of responses to OP compounds depending on the compound, length of exposure, water conditions, and species (Coppage & Matthews, 1974). As a class, OP compounds inhibit esterase activity and act as nerve toxins by blocking transmissions at cholinergic synapses. Death in vertebrate organisms may be caused by blocking neurotransmission in the respiratory organs (Coppage & Matthews, 1974).

Azinphosmethyl, also known as guthion, gusathion, or carfene, is an organophosphate pesticide used on vegetables, cotton, tobacco, and many other crops. It inhibits acetylcholinesterase activity in marine organisms (Fulton, 1989). By binding to the active site of the acetylcholinesterase molecule, azinphosmethyl inhibits the enzyme from breaking down acetylcholine. Little is known about the presence of azinphosmethyl in nature, but the presence of OP compounds in the environment may be greater than previously believed. Since they degrade more rapidly, it may be necessary to apply them often and in larger quantities (Coppage & Matthews, 1974). Due to increased use of OP compounds, the effects of azinphosmethyl on non-target marine organisms needs to be understood.

Three different types of salt marsh species were used in this study to investigate the respiratory responses to hypoxia and hypercapnia applied simultaneously. Two residents of the marsh are the killifish or mummichog, *Fundulus heteroclitus* (Linnaeus) and the grass shrimp, *P. pugio* (Holthuis), and an estuarine transient living in the marsh during its larval and juvenile stages is the spot fish, *Leiostomus xanthurus* (Lacépède).

There were several objectives in this study. First, the oxygen uptake of these three salt marsh species was investigated in response to combinations of hypoxia and hypercapnia. Oxygen uptake was used to assess the aerobic responses of the organisms. Secondly, whole body lactate concentrations were measured at several combinations of oxygen and carbon dioxide pressures to assess the anaerobic components of metabolism that may be involved in the response. Furthermore, after determining the normal aerobic responses of the three species, the effects of the organophosphate pesticide, azinphosmethyl, on their oxygen uptake were explored.

2. Materials and methods

2.1. Field measurements

In an effort to determine the extremes of environmental variables in the salt marsh, temperature, salinity, oxygen, carbon dioxide, and pH were measured in James Island Creek on Charleston Harbor, South Carolina and two of its tributaries during the summers of 1993 and 1994 (Table 1). Water was sampled in mid-August during consecutive high tides, once in the late evening and again in the early morning. Samples were taken from both the creek channel and among the flooded marsh grasses. Carbon dioxide was determined indirectly from a water sample after measuring the titratable alkalinity in the laboratory (Strickland & Parsons, 1972). Measurements of temperature, salinity, oxygen, and pH were made in the field. Temperature and salinity were measured using a Yellow Springs Instrument (YSI) model 33 S-C-T meter. Dissolved oxygen was measured with a YSI model 58 meter, and the pH was measured with a Jenco model 6009 portable meter. Carbon dioxide pressures in the field were calculated from measurements of temperature, salinity, pH, and total alkalinity using procedures described in Strickland and Parsons (1972).

2.2. Collection and holding of animals

Fundulus heteroclitus (mean wet weight = 1.75 g; weight ranged from 0.61 to 3.69 g) were collected from two sites using minnow traps. Most animals came from Cherry Point on Wadmalaw Island, South Carolina, with the remainder collected from an impoundment located behind the University of Charleston's Grice Marine Biological Laboratory. *Palaemonetes pugio* (mean wet weight = 0.32 g; weight ranged from 0.12 to 1.06 g) were also collected from the Grice Marine Biological Laboratory impoundment as well as from Wilapena Creek on Charles-

Table 1
Field measurements in tributaries of James Island Creek on Charleston Harbor, South Carolina. Water was sampled near high tide in the small channels and in the salt marsh grass

Location		Depth (cm)	Po_2 (torr)	pH	Salinity (‰)	Temp. (°C)	Pco_2 (torr)	Total Alkalinity (meq·l^{-1})
August 3, 1994 (time = 1800–1900)								
Channel	Surface	2	142	7.64	5	32	2.7	2.67
	Bottom	107	78	7.48	13.8	30.5	2.2	1.86
Grass	Surface	2	133	7.11	5	31.5	10.8	3.06
	Bottom	39	53	7.28	11	30.5	5.3	2.68
August 4, 1994 (time = 0630–0730)								
Channel	Surface	2	51	7.08	2	25	10.7	2.58
	Bottom	88	31	6.48	12	28	35.6	3.07
Grass	Surface	2	54	7.11	1.5	25	11.6	2.84
	Bottom	19	20	7.06	3	25	12.7	3.10

ton Harbor. *Leiostomus xanthurus* (mean wet weight = 1.55 g; weight ranged from 0.22 to 3.69 g) were collected from Long Creek on Wadmalaw Island, South Carolina at low tide using a 3 m seine and were transported immediately to the Grice Marine Biological Laboratory and placed in aquaria.

Animals were held in well aerated sea water at 30°C and 25‰ salinity. Ambient Charleston Harbor water was diluted with deionized water as needed to attain the test salinity of 25‰. The pH of the tank water was monitored and maintained above 8.0.

2.3. Respirometry

The oxygen uptake was determined by measuring the depletion of oxygen in one of two closed respirometers with volumes of ≈150 ml and 215 ml. Each respirometer contained ports for oxygen and pH electrodes.

Dissolved oxygen was measured with a Yellow Springs Instrument (YSI) model 58 meter with a YSI model 5730 probe with self stirring sensor tip. The pH of water within the respirometer was measured with a combination pH electrode to assess the carbon dioxide pressures. The relationship between water pH and Pco_2 was determined independently. The outputs from these meters were monitored and recorded using a Sable System data acquisition system.

Animals were placed in the respiration chamber after it was filled with well oxygenated (Po_2 = 200 torr) water at a pH of 8.0–8.2. This water was filtered with a 0.45 μm membrane filter immediately prior to use to remove microbial organisms. For *F. heteroclitus* and *L. xanthurus*, only one animal at a time was used. Because of its smaller size, three to four *P. pugio* shared the respiration chamber. There was no visible difference between the behavior or the activity levels of one grass shrimp in the respirometer compared with four. The measuring chamber was covered with black plastic to reduce any stimulation of the animals from movement in the room. Before measuring oxygen uptake, animals were held in the respirometer for 3 to 4 h. This period was found necessary to allow the animals to become quiescent and for oxygen uptake rates to stabilize. High oxygen (150 to 200 torr) and low carbon dioxide (<1 torr) conditions were maintained by periodically flushing the respirometer with filtered water adjusted to the appropriate gas pressures. In low CO_2 (Pco_2 <1 torr) experiments, the pH was not allowed to fall below 7.9 in the respirometer. In high CO_2 experiments, after the 3 to 4-h period of initial incubation, the chamber was flushed (for ≈15 min) with water that was previously gassed with CO_2 to drop the pH from the low Pco_2 (<1 torr) pH of 8.0–8.2 down to a pH < 7.0 (Pco_2 = 6–8 torr). During the course of oxygen uptake measurements, CO_2 would accumulate in the respirometer lowering the pH. When this occurred in experiments where Pco_2 was held low (<1 torr), the respirometer was flushed with filtered sea water of low Pco_2 and a Po_2 approximately equal to that in the respirometer. When the pH in the respirometer returned to a value of ≈8.2 (very low Pco_2) flushing was ended and measurement of oxygen uptake was resumed. This flushing operation lasted ≈10 min. All experiments were done at 30°C ± 0.1.

Experiments were ended when animals could no longer maintain position in the respirometer in response to hypoxia. The volume of the respirometer was determined and the animals weighed. The weight and volume were used to calculate the rate of oxygen uptake. The responses of five or six animals to hypoxia were measured for each treatment of Pco_2.

2.4. Lactate measurements

Whole body lactate concentrations were measured to assess the anaerobic component of these animals' metabolisms. Animals were held overnight in well-aerated experimental aquaria to ensure resting conditions. *Fundulus heteroclitus* and *L. xanthurus* were held in chambers made from 3″ PVC pipe. *Palaemonetes pugio* were held in similar chambers made from 2″ PVC pipe. Six to eight chambers holding one animal each were placed on a screen rack placed into a 10-gallon aquarium filled with water of 25‰ salinity and 30°C. The aquarium was covered in black plastic to prevent outside stimuli from exciting the animals. After sitting overnight, the different experimental aquaria were then gassed with mixtures of nitrogen, oxygen, and carbon dioxide delivered by Wösthoff gas mixing pumps for 4 h. Six different treatments of oxygen and carbon dioxide pressures were used to test the responses of animal tissues to the accumulation of lactate. The oxygen pressures used were ≈150 torr (normoxia), 35 torr (hypoxia, near critical Po_2), and 10 torr (severe hypoxia). These oxygen pressures were used in combination with either low Pco_2 (<1 torr; normocapnia) or high Pco_2 (5–6 torr; hypercapnia). At the end of 4-h, animals were plunged rapidly into liquid nitrogen to freeze the tissues. Animals were then weighed and held in liquid nitrogen until the tissues were assayed for lactate. The responses of 12 to 18 animals were measured for each treatment.

Frozen animals were homogenized in an appropriate volume of 12% $HClO_4$ using a Vertishear Tempest mechanical homogenizer. Homogenates were held on ice. Lactate concentrations were then measured using a standard enzymatic assay modified from Sigma Technical Bulletin No. 826-UV.

2.5. Azinphosmethyl

Animals were dosed with azinphosmethyl for 24 h in 1 l of well aerated 25‰ salinity seawater at 30°C. For fish only one animal at a time was dosed while 3 to 4 shrimp were dosed together. The concentrations of azinphosmethyl used in this study for both shrimp and fish were determined independently. *Palaemonetes pugio* were dosed at 2 $\mu g \cdot l^{-1}$. This figure was based on the calculated 96 hr LC_{50} of 1.64 $\mu g \cdot l^{-1}$ (Key et al., 1994) and a 24 h survival rate of 100% (Peter Key, pers. comm.). Crustaceans are typically more sensitive to organophosphate compounds than marine fishes by several orders of magnitude (Eisler, 1970). *Fundulus heteroclitus* and *L. xanthurus* were dosed at 10 $\mu g \cdot l^{-1}$. This concentration falls within the range of values seen in tidal creeks after fish kills (M.H.

Fulton, pers. comm.), but is still low enough for fish to survive at least 24 h. Since acetone was used as the carrier for azinphosmethyl, all animals were dosed with added acetone to achieve a concentration of 1 $ml \cdot l^{-1}$ acetone in the dosing chamber. As a result, control experiments using 1 $ml \cdot l^{-1}$ of acetone were conducted. Oxygen uptake was measured on dosed animals using water free of azinphosmethyl immediately after the 24-h period. At the end of oxygen uptake experiments, brains were removed from fish and frozen for later measurement of acetylcholinesterase activity. For grass shrimp, the whole animal was frozen and the cephalothorax was assayed for acetylcholinesterase activity. The inhibition of acetylcholinesterase activity was assayed as a measure of the toxicity of azinphosmethyl to the dosed organisms.

Acetylcholinesterase activity was determined using a procedure modified from Ellman et al. (1961) and described by Fulton (1989). In this procedure, acetylcholinesterase activity is determined indirectly by measuring the rate of thiocholine production as the substrate acetylthiocholine is hydrolyzed by acetylcholinesterase. Thiocholine reacts with DTNB (5: 5-dithiobis-2-nitrobenzoate) to produce the yellow anion of 5 thio-2-nitro-benzoic acid (Ellman et al., 1961). This rate of color change is measured at 412 nm using a spectrophotometer. The activity is expressed as nmol product (5 thio-2-nitro benzoic acid) mg $protein^{-1} \cdot min^{-1}$. In order to express this on a protein basis, the total protein content of the homogenate used in the assay was determined. This was done using a modification of the Sigma Technical Bulletin protein assay kit No. P5656.

2.6. Data analysis

For each animal, oxygen uptake as a function of Po_2 was averaged over each decade of Po_2 change (e.g. 150–159 torr, 140–149 torr, etc.). The oxygen uptake values at each decade were then averaged for all animals of a species at each treatment (low Pco_2, high Pco_2, acetone control at low Pco_2, azinphosmethyl treated at low Pco_2, and azinphosmethyl treated at high Pco_2) (Figs. 1–3). Mean oxygen uptake ±SEM in well oxygenated conditions (Po_2 = 130–150 torr) for each species was compared between treatments using a one-way analysis of variance (ANOVA) test for *F. heteroclitus* and *L. xanthurus* and a Kruskal-Wallis test for *P. pugio*. A Kruskal-Wallis test was used on *P. pugio* because these data failed a Kolmogorov-Smirnov test for normality. If differences were found, pairwise multiple comparisons between treatments were made using a Student-Newman-Keuls test. Similar comparisons were made between species at each treatment.

The oxygen pressure at which animals could no longer regulate their oxygen uptake, the critical oxygen pressure or P_{crit}, was calculated for each animal of the three species at each treatment condition. The critical oxygen pressure was determined by calculating regression lines for the two distinctly different parts of the relationship between oxygen uptake and Po_2, the horizontal high Po_2 segment and the sharply sloped low Po_2 segment. The critical Po_2 (P_{crit}) was designated as the intersection point of the two lines. An average P_{crit} ± SEM for each species at

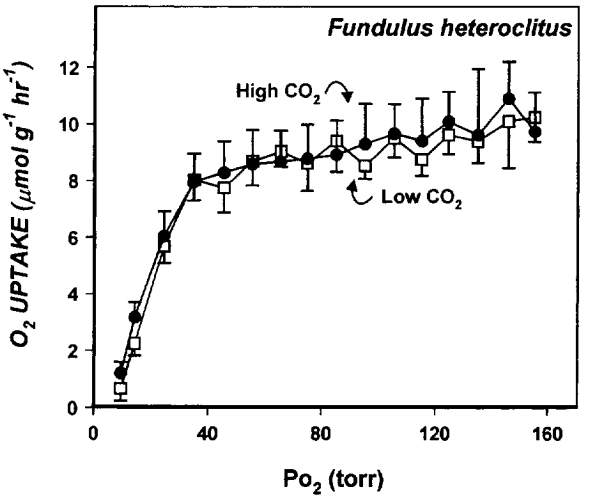

Fig. 1. Mean (±SEM) oxygen uptake $\dot{M}o_2$ (μmol·g^{-1}·h^{-1}) for each decade of Po_2 for *Fundulus heteroclitus* subjected to both low (<1 torr) and high (6–8 torr) CO_2 pressures at 30°C. Error bars show +SEM for high CO_2 and −SEM for low CO_2.

a treatment was calculated and comparisons of these mean values were made using a one-way ANOVA.

Whole body lactate concentration for each species was averaged for each of the six different treatments and compared using a one-way ANOVA. Pairwise

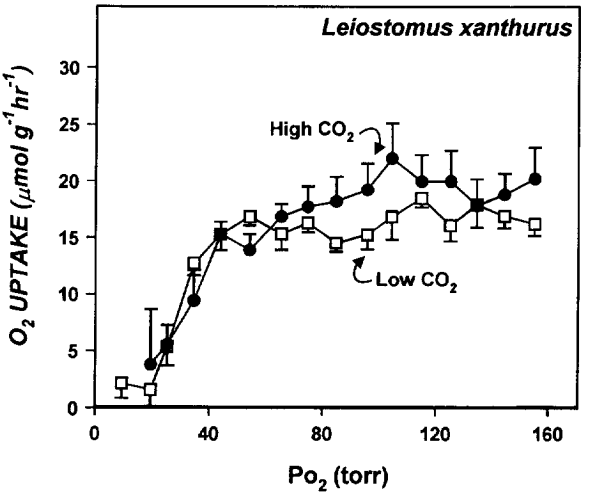

Fig. 2. Mean (±SEM) oxygen uptake $\dot{M}o_2$, (μmol·g^{-1}·h^{-1}) at each decade of Po_2 for *Leiostomus xanthurus* subjected to both low (<1 torr) and high (6–8 torr) CO_2 pressures at 30°C. Error bars show +SEM for high CO_2 and −SEM for low CO_2.

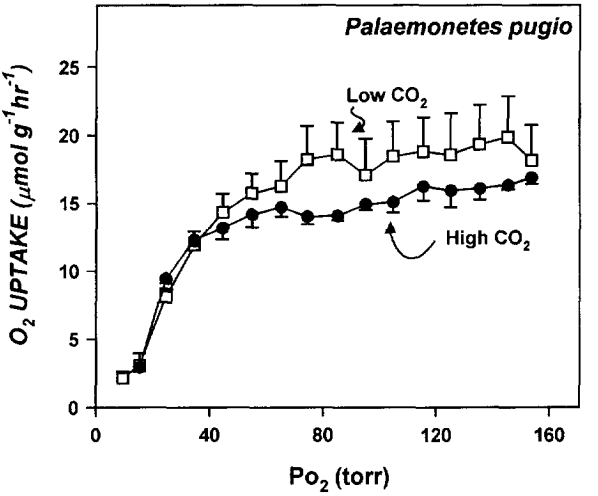

Fig. 3. Mean (±SEM) oxygen uptake $\dot{M}o_2$, (μmol·g^{-1}·h^{-1}) at each decade of Po_2 for *Palaemonetes pugio* subjected to both low (<1 torr) and high (6–8 torr) CO_2 pressures at 30°C. Error bars show +SEM for low CO_2 and −SEM for high CO_2.

multiple comparisons between treatments for each species were made using a Dunn's test.

Acetylcholinesterase activities in the brain tissues of fishes and in the cephalothorax of grass shrimp were compared between control animals and animals treated with azinphosmethyl using a Mann-Whitney Rank Sum test for *F. heteroclitus* and *P. pugio* and a Student's *t*-test for *L. xanthurus*. In addition, the percent of acetylcholinesterase inhibition between control and treated animals was calculated.

3. Results

3.1. Oxygen uptake

No differences were found in the oxygen uptake of *F. heteroclitus* ($p = 0.87$; one-way ANOVA) or *P. pugio* ($p = 0.23$; Kruskal-Wallis) between treatments in well oxygenated conditions ($Po_2 = 130$–150 torr) (Figs. 1, 3 and 4). *Leiostomus xanthurus*, however, showed a difference between treatments ($p = 0.001$; one-way ANOVA) (Fig. 2). Oxygen uptake was significantly higher ($p < 0.05$; Student-Newman-Keuls) in the high CO_2 treatment (pH < 7.0) compared with all other treatments (Fig. 4).

Among the three species, *P. pugio* showed the highest mean oxygen uptake for each treatment except in high CO_2 where *L. xanthurus* was the highest ($p < 0.05$; Student-Newman-Keuls) (Fig. 4). Oxygen uptake in *P. pugio* was significantly

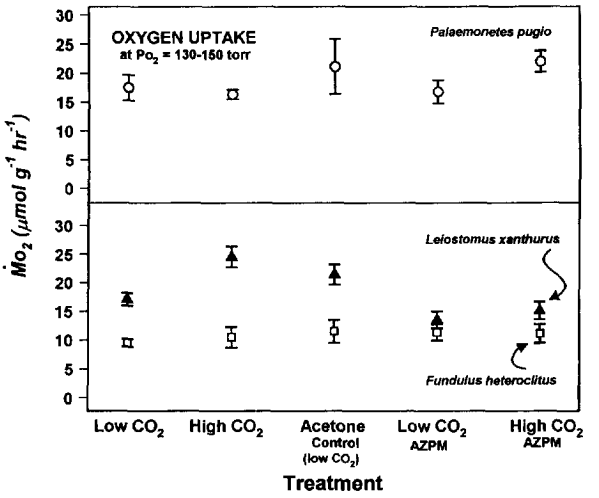

Fig. 4. Mean oxygen uptake (±SEM) in *Fundulus heteroclitus* (□), *Leiostomus xanthurus* (▲), and *Palaemonetes pugio* (○) at Po_2 = 130–150 torr, 25 ppt salinity, 30°C and different treatments.

higher ($p < 0.05$; Student-Newman-Keuls) than *F. heteroclitus* at low CO_2, high CO_2, and azinphosmethyl treated high CO_2. The other treatments showed no significant difference ($p > 0.07$; Student-Newman-Keuls). *Leiostomus xanthurus* had the highest average oxygen uptake of the two fish species and was significantly higher ($p < 0.05$; Student-Newman-Keuls) at the low CO_2 and high CO_2 treatments.

Comparisons of critical Po_2 between treatments (Fig. 5) revealed no significant differences ($p > 0.2$; one-way ANOVA). Similarly, there was no difference between species within a treatment.

3.2. Whole body lactate measurements

Palaemonetes pugio showed significantly higher ($p < 0.05$; Dunn's test) mean lactate concentrations at very low oxygen levels (10 torr) compared to levels at low oxygen and normoxia (Fig. 6). There was no statistical difference between the high or the low CO_2 treatments at each oxygen level.

Fundulus heteroclitus showed significantly higher lactate concentrations (Fig. 6) at low CO_2 and very low O_2 conditions ($p < 0.05$; Dunn's test) than all other treatments except for the high CO_2 and very low O_2 treatment ($p > 0.05$; Dunn's test). The high CO_2 and very low O_2 treatment was only significantly different from the high CO_2 and normoxia treatment ($p < 0.05$; Dunn's test).

Leiostomus xanthurus proved to have the most variable lactate concentrations (Fig. 6), showing significant differences ($p < 0.05$; Dunn's test) between the low CO_2 and very low O_2, low CO_2 and low O_2, and between the high CO_2 and very

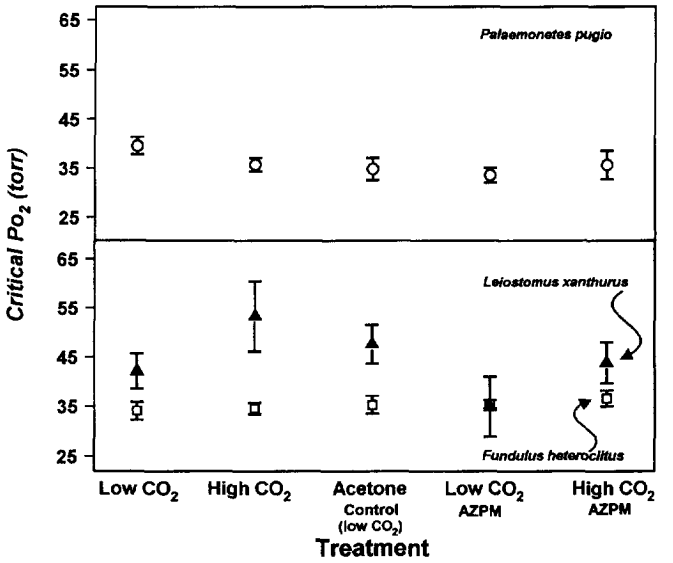

Fig. 5. Mean critical Po_2 (torr) ± SEM for *Fundulus heteroclitus* (□), *Leiostomus xanthurus* (▲), and *Palaemonetes pugio* (○) at 25 ppt salinity, 30°C, and different treatments.

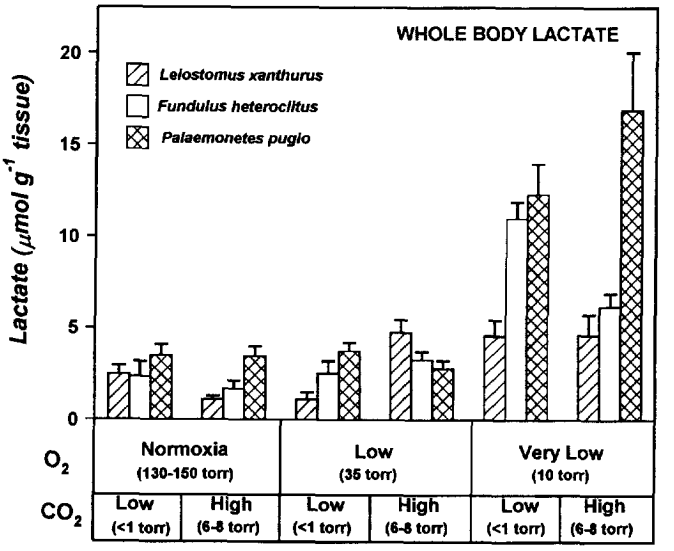

Fig. 6. Mean (+SEM) whole body lactate concentrations (μmol lactate · g^{-1} tissue) for *Fundulus heteroclitus*, *Leiostomus xanthurus*, and *Palaemonetes pugio* at 25 ppt salinity, 30°C and plotted for each condition of oxygen (O_2) and carbon dioxide (CO_2).

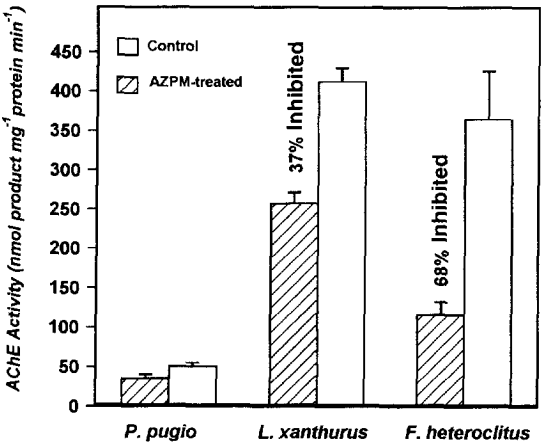

Fig. 7. Mean acetylcholinesterase activity (+SEM) for both control and azinphosmethyl-treated animals held in well-aerated water at 25 ppt salinity and 30°C. *Fundulus heteroclitus* and *Leiostomus xanthurus* were dosed with azinphosmethyl at 10 $\mu g \cdot l^{-1}$. *Palaemonetes pugio* was dosed at 2 $\mu g \cdot l^{-1}$. The % inhibition is shown above dosed values.

low O_2 and the high CO_2 and normoxia treatments. There was also a significant difference between the high and low CO_2 and low O_2 treatments ($p < 0.05$; Dunn's test).

3.3. Acetylcholinesterase inhibition

There was no statistical difference in acetylcholinesterase activity between control and azinphosmethyl-treated animals in *P. pugio* ($p = 0.64$; Mann-Whitney Rank Sum; Fig. 7). However, there was a significant decrease in activity for animals dosed with azinphosmethyl in both *F. heteroclitus* ($p = 0.002$; Mann-Whitney Rank Sum) and *L. xanthurus* ($p = 0.0001$; Student's *t*-test; Fig. 7).

4. Discussion

Fluctuations in oxygen and carbon dioxide pressures in the estuary reflect changes in the overall metabolism of the biological community where increasing oxygen and decreasing carbon dioxide pressures during the daylight are due to net photosynthesis over respiration, and nighttime decreases in oxygen and increases in carbon dioxide are due to net respiration (Kemp & Boynton, 1980; Lingeman & Ruardij, 1981). Nighttime oxygen pressures in waters of the salt marsh can be quite low (Table 1), producing adverse effects on the organisms living there.

However, it is quite rare to see mortality among animals that live in these communities due to low dissolved oxygen (Kramer, 1987). Carbon dioxide pressures measured in the field were high and variable. Our field measurements reported in Table 1 were made ≈3 days after significant rainfall. Interestingly, even in the shallow creeks after 3 days of tidal flushing when the creeks would be nearly dry at low tide, the freshwater appeared to remain layered over the saltier water. This result suggests that the bottom water remains isolated from the fresher surface water in both the creek channel and the salt marsh. The result of the isolation of the bottom water is that it tends to be more hypoxic and more hypercapnic.

Animals have several responses to hypoxic environments, both behavioral and physiological. The most common behavioral response is simply to avoid the hypoxic water. The three species in this study all appear capable of using some type of avoidance (Lewis, 1970; Welsh, 1975; Hales & Van Den Avyles, 1989). Evading hypoxia is not always an option, and organisms must be able to adapt to or to tolerate hypoxic conditions. Animals are typically placed into one of two groups based on their ability to maintain oxygen uptake as a function of environmental Po_2. An organism is considered an oxygen conformer if its oxygen uptake varies directly with the environmental Po_2. An oxygen regulator has an oxygen uptake that is independent of the environmental Po_2 (Herreid, 1980). Animals are, however, neither complete oxygen conformers nor complete oxygen regulators (Mangum & VanWinkle, 1973). There may be a hypoxic Po_2 at which an oxygen regulator becomes sufficiently stressed, becoming an oxygen conformer. This Po_2 is considered the organism's critical oxygen tension or P_{crit} (Herreid, 1980). It was possible to identify critical oxygen tensions for each of the three species in this study (Figs. 1–4). In many studies, researchers have not identified the oxygen regulating ability of animals because adequate time to recover from handling stress has not been allowed before oxygen uptake measurements were made (Welsh, 1975; Subrahmanyam, 1980; Dillon, 1983; Moser & Hettler, 1989). Animals that lack a sufficient recovery period will appear to be oxygen conformers. This was confirmed in our preliminary experiments when there was little recovery time allowed before measuring oxygen uptake.

In nature, the grass shrimp, *P. pugio*, has shown an avoidance response to hypoxia. During periods of low dissolved oxygen, they will become air exposed (Welsh, 1975). *Palaemonetes pugio* showed a similar response in the laboratory when held in very low oxygen (10 torr) conditions. Under these conditions, shrimp would use their tail movements to flip out of the water and adhere to the sides of the holding chamber. This may be an avoidance response to hypoxia that occurs in the field.

At Po_2 values near air saturation (130–150 torr) *P. pugio* was an oxygen regulator. Dillon (1983) found that the oxygen uptake for *P. pugio* was 3.0 to 3.5 μl O_2 mg dry $wt^{-1} \cdot h^{-1}$ (46.5 μmol O_2 g wet $wt^{-1} \cdot h^{-1}$) at 25°C and 17 ‰ salinity, while Welsh (1975) found that the oxygen uptake of *P. pugio* was 2.5 mg O_2 g dry $wt^{-1} \cdot h^{-1}$ (62.5 μmol O_2 g wet $wt^{-1} \cdot h^{-1}$) at 30°C. Both of these values were calculated for the dry weight of shrimp and had to be adjusted to wet weight

for comparison sake. Wet weight values of shrimp were converted to dry weight after taking several shrimp of known wet weight and drying them for 1 h in an oven at 60°C ± 1.0. The estimates of oxygen uptake of Welsh (1975) and Dillon (1983) were much higher than in this study. The differences may be real because little or no recovery time from stress was allowed in their studies. Furthermore, based upon their experimental methods (Welsh, 1975; Dillon, 1983), the animals were probably excited. In both situations, *P. pugio* was found to be an oxygen conformer for all Po_2 values.

There was no difference in oxygen uptake in *P. pugio* in normoxia at high or low Pco_2. The hemocyanin of *P. pugio* has a CO_2 specific effect (Mangum & Burnett, 1986) resulting in an increase in oxygen affinity with increasing CO_2, i.e. CO_2 affects oxygen affinity independently of pH (the Bohr effect). However, this CO_2 effect does not appear to enhance oxygen uptake when there is an excess of available oxygen.

Lactate concentrations in *P. pugio* fell within the range of 1–4 $\mu mol \cdot g^{-1}$ as found in other crustaceans (Agnew & Jones, 1986; Fig. 6). Phillips et al. (1977) found a lactate concentration in lobster tail muscle of around 2.7 $\mu mol \cdot g^{-1}$. In the shore crab, *Carcinus maenas*, pooled tissue lactate levels ranged from 4.49 ± 3.28 $\mu mol \cdot g^{-1}$ at normoxia, and up to 20.3 ± 4.5 $\mu mol \cdot g^{-1}$ in anoxia (Hill et al., 1991). Gäde (1984) found a resting tissue lactate level in crayfish of 1.25 $\mu mol \cdot g^{-1}$. After subjecting crayfish to hypoxia, tissue lactate increased to 16 to 19 $\mu mol\ g^{-1}$. This was similar to the tissue lactate levels seen by Albert & Ellington (1985) in the stone crab, *Menippe mercenaria*, after exposure to severe hypoxia (16 to 20 $\mu mol\ g^{-1}$). The lactate concentrations measured in *P. pugio* exposed to extreme hypoxia were similar to other crustacean studies. Whole body lactate concentrations increased from a resting level of 3.5 ± 0.59 $\mu mol \cdot g^{-1}$ to 12.3 ± 1.67 $\mu mol \cdot g^{-1}$ under severe hypoxia (Fig. 6).

Of the three species studied, *F. heteroclitus* was the most tolerant of very low oxygen. Fish of the genus *Fundulus* are often seen living in tidal pools in extremely hypoxic conditions. In the lab, *F. notatus* have been able to survive in anoxic water for 24 h (Lewis, 1970). It appears that mummichogs do not use an avoidance response per se when faced with hypoxia. They are morphologically adapted to utilize a phenomena known as aquatic surface respiration (ASR). A thin layer of water exists at the surface in which diffusion of oxygen from air provides significant oxygen in an otherwise hypoxic water mass (Kramer & Mehegen, 1981). *Fundulus heteroclitus* and other members of the family Cyprinodontidae, as well as some other families, have small mouths dorsally oriented and heads dorsoventrally flattened that allow them to utilize ASR efficiently as they swim at the surface (Lewis, 1970). In the guppy, *Poecilia reticulata*, which is morphologically similar to the mummichog, Kramer & Mehegen (1981) found that at oxygen levels above 50 torr, fish spend little time at the surface but below this oxygen level they spent more time at the surface. At $Po_2 = 4$ torr, fish spent 90% of their time at the surface engaging in ASR. In this study, when *F. heteroclitus* were held in hypoxic conditions for lactate experiments, fish were often seen near the surface probably trying to use ASR. This

behavior was viewed as an avoidance response allowing animals to move to an area of better oxygenated water.

The oxygen uptake rate of *F. heteroclitus* (9.48 ± 0.7 μmol $O_2 \cdot g^{-1} \cdot h^{-1}$) in air saturated water and low Pco_2 was within the range of values seen by others. Respiration rates for *Fundulus* sp. ranged from 0.073 ml $O_2 \cdot g^{-1} \cdot h^{-1}$ (2.8 μmol $O_2 \cdot g^{-1} \cdot h^{-1}$) at 25°C and 19 ‰ salinity in *F. grandis* (Subrahmanyam, 1980) to 0.6 ml $O_2 \cdot g^{-1} \cdot h^{-1}$ (22.6 μmol $O_2 \cdot g^{-1} \cdot h^{-1}$) at 29°C and 30 ‰ salinity in *F. heteroclitus* (Targett, 1978). The range of these values is quite large and is likely because in most of the experiments done by others, the animals were held only minutes in the respirometer before measurements were made. Hoss (1967) calculated an oxygen uptake in a 1 g mummichog from an experimentally derived equation to be 0.237 ml $O_2 \cdot g^{-1} \cdot h^{-1}$ (9.4 μmol $O_2 \cdot g^{-1} \cdot h^{-1}$). This equation was based on experiments where fish were acclimated in the respirometer for 48 h. This calculated value is very similar to the rate we found experimentally. *Fundulus heteroclitus* showed no difference in oxygen uptake in normoxic conditions at low or high CO_2.

Fundulus heteroclitus had relatively low whole body lactate concentrations at both normoxia and low (35 torr) oxygen levels (i.e. near P_{crit}) with an increase in lactate at very low (10 torr) oxygen (Fig. 6). These values fall within those seen in tissues of other fishes. In the mudskipper, *Boleophthalmus boddaerti*, which lives in hypoxic waters most of the time, muscle lactate levels ranged from 1.3 ± 0.2 μmol $\cdot g^{-1}$ in normoxia to 2.0 ± 0.3 μmol g^{-1} in hypoxia (Chew & Ip, 1992). Burton & Heath (1980) found muscle lactate values for trout and bluegill in normoxia to be 1.07 mg $\cdot g^{-1}$ (11.9 μmol g^{-1}) and 0.92 mg $\cdot g^{-1}$ (10.5 μmol g^{-1}), respectively. During hypoxia, lactate increased in both species to 2.42 mg $\cdot g^{-1}$ (26.9 μmol g^{-1}) and 1.75 mg $\cdot g^{-1}$ (19.4 μmol g^{-1}).

For *L. xanthurus*, no laboratory documentation of avoidance exists. However, juvenile spot are usually found in tidal creeks and deeper pools moving out of the marsh areas at low tide (Subrahmanyam, 1980). This distribution pattern may suggest a behavioral avoidance of more hypoxic waters. When collecting *L. xanthurus* from tidal creeks during the warmer summer months, we typically found them further downstream in deeper waters than during sampling in the early spring. Spot are not morphologically adapted to use aquatic surface respiration as are *F. heteroclitus*. They might attempt it under hypoxic conditions, but it would likely be an inefficient mechanism for use over an extended period.

Leiostomus xanthurus also showed the ability to regulate their oxygen uptake at high Po_2 values. Their respiration rate (17.1 ± 2.2 μmol $O_2 \cdot g^{-1} \cdot h^{-1}$) fell within the range of values for other fishes. Spot, however, showed a significantly higher oxygen uptake at high CO_2 than at low CO_2 (Fig. 4). This increased rate might be due to the presence of a Root effect in spot hemoglobin. Bonaventura et al. (1976) found that spot hemoglobin shows an extreme sensitivity to pH and anion concentration. When the pH is decreased from 7 to 6, the hemoglobin will unload 30% of its bound oxygen (Root effect). In a Root effect, the oxygen carrying capacity of the hemoglobin is decreased preventing oxygen saturation in the hemoglobin from occurring. Therefore, a decrease in pH will mean that spot

hemoglobin binds less oxygen, forcing spot to work harder to maintain tissue oxygen requirements. This work could occur at any of the steps involved in transporting oxygen from the environment to the tissues (e.g. ventilating gills or pumping blood). Increased gill ventilation would not likely provide enough enhancement in oxygenation at the gills to be useful. It will not compensate for the decreased carrying capacity of the hemoglobin and will increase the transmission of the environmental hypercapnia to the blood causing a further drop in the blood pH (Heisler, 1993). The fish cannot greatly alter the rates of branchial or tissue diffusion since they are determined by physical conditions beyond control of the animal (e.g. gill surface area, permeability coefficients, etc). Fish may, however, increase their heart rate to circulate more oxygen to the tissues. Because of this increased cardiac work, the animal would increase its oxygen uptake. *Fundulus heteroclitus* possesses a normal Bohr effect but no Root effect has been identified (Greaney & Powers, 1978; Meid & Powers, 1978; Powers et al., 1986). In a normal Bohr effect, the hemoglobin oxygen affinity is decreased but the actual capacity for binding oxygen is unchanged.

Leiostomus xanthurus also showed relatively low whole body lactate concentrations at both normoxia and low (35 torr) oxygen levels (i.e. near P_{crit}) with a slight increase at very low (10 torr) oxygen (Fig. 6). These values fall within values seen in the tissues of other fishes. It is interesting to note that under very low oxygen conditions spot fish still had relatively low lactate concentrations. There was often mortality of spot early in the 4 h of exposure to very low O_2 (10 torr) suggesting that *L. xanthurus* may have difficulty using anaerobic mechanisms. This result may also reflect the behavioral ecology of spot. By remaining in deeper pools and creeks and avoiding shallows or tidal pools, spot avoid severe hypoxia in nature reducing the need for a well adapted anaerobic metabolism component.

The similarity of the P_{crit} values of the three species is probably due to the fact that all three animals are found in the salt marsh. Kalinin et al. (1993) found a P_{crit} of 32 torr for *Hoplias malabaricus*, a freshwater bottom dwelling fish inhabiting lentic environments and experiencing periodic hypoxia. This value is similar to the value found in this study for *F. heteroclitus* (34 torr). The P_{crit} of these three species appears to be unaffected by Pco_2, and the transition from regulator to conformer is likely only in response to declining Po_2. While Pco_2 can increase significantly during hypoxia (Table 1), it appears to have little effect on oxygen uptake. This result is not surprising for *P. pugio*, which has a specific CO_2 effect that increases hemocyanin oxygen affinity (Mangum & Burnett, 1986). While CO_2 specific effects of hemoglobin oxygen affinity (independent of pH) have not been documented for *L. xanthurus* and *F. heteroclitus*, it would not be surprising to find that CO_2 specifically increases oxygen affinity.

The inhibition of acetylcholinesterase activity by azinphosmethyl in *F. heteroclitus* (68%) was lower than that found by Fulton (1989) (80–90%). Differences in the treatment of animals after dosing may have caused this decrease. In our study, oxygen uptake was determined after dosing using water free of azinphos-

methyl and then animals were sacrificed. These experiments could last from 6 to 9 h depending on the size of the animal and respirometer volume. The time the fish spent in the respirometer can be considered an acetylcholinesterase recovery time and could be a sufficient recovery period to reverse the inhibitory effects of azinphosmethyl to only 68%. Weis and Khan (1991) found that some fish species showed a rapid increase in brain acetylcholinesterase activity after dosing during the first 24 h. This increase in acetylcholinesterase activity leveled off to a much slower rate until full activity was reached several days later. This short-term recovery rate appears species specific. The short term recovery rate in *F. heteroclitus* is unknown.

Leiostomus xanthurus also showed a low inhibition of acetylcholinesterase activity (37%). This can again be a function of recovery time as mentioned above, but the low inhibition is likely due to the fact that spot fish, in general, show less sensitivity to azinphosmethyl than does *F. heteroclitus*. The LC_{50} of spot is 50 $\mu g \cdot l^{-1}$ (Mayer, 1987) compared to that of *Fundulus* of 36.9 $\mu g \cdot l^{-1}$ (Fulton & Scott, 1991). Thus, a greater concentration of azinphosmethyl might be needed to see a greater acetylcholinesterase inhibition. *Palaemonetes pugio* showed a 31% inhibition of acetylcholinesterase activity. However, in this and other studies the variability of acetylcholinesterase activity in grass shrimp between animals has been very large and should be viewed with caution. This variation could be due in part to sensitivity differences of differently sized shrimp.

Even though acetylcholinesterase inhibition was detected in this study, it did not affect the P_{crit} or the ability of *F. heteroclitus* or *P. pugio* to regulate oxygen uptake. *Leiostomus xanthurus* did show a decreased oxygen uptake from the control high CO_2 treatments when dosed and subjected to high CO_2. Although acetylcholinesterase activity in tissues was monitored only in animals in well-aerated water, the influence of azinphosmethyl on oxygen uptake in *L. xanthurus* at high CO_2 suggests that CO_2 treatment affects the uptake and/or efficacy of azinphosmethyl. Coppage & Matthews (1974) suggested that organophosphate compounds caused death in higher vertebrates by blocking neurotransmission in the respiratory organs. This may still be the ultimate cause of death. Typically, when concentrations are lethal to organisms, the animals show loss of muscular control with increased twitching and muscular contractions (Meyer, 1965). This might inhibit the animal's ability to ventilate, preventing gas exchange and lead to death.

The three species in this study are well adapted to live in an environment where periodic hypoxia and hypercapnia are normal. They are able to regulate their oxygen uptake over a wide range of oxygen pressures, and at some critical Po_2 they are no longer able to regulate oxygen uptake. Moderately high Pco_2 had different effects on the oxygen uptake of the three species. Uptake was increased in *L. xanthurus*, but had no effect on *P. pugio* or *F. heteroclitus*. These three species rely little on anaerobic metabolism until faced with extreme hypoxia (10 torr). Under extreme hypoxia, all three species showed an increase in lactate concentrations. These increases were significant in *P. pugio* and *F. heteroclitus*. It

also appeared that the organophosphate pesticide, azinphosmethyl, had no effect on the ability of these organisms to regulate oxygen uptake when used in nonlethal doses.

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