Interspecific Comparisons of Capacity for Regulation of Intracellular pH in Molluscan Muscle

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Abstract

The major defenses against intracellular acidosis during anoxia include intracellular buffering and ion exchange of acid-base equivalents between intra- and extracellular compartments. It is unclear whether more anoxia-tolerant species have a higher capacity for such regulatory processes. Using ^{31}*P-NMR methods, we investigated regulation of intracellular pH* (pH_i) *in cardiac muscle of four species of closely related marine gastropod molluscs*—Melongena corona *(high intertidal),* Busycon contrarium *(low intertidal),* Busycon spiratum *(low intertidal) and* Fasciolaria tulipa *(subtidal). When extracellular pH* (pH_e) *was systematically altered, there were minimal changes in* pH_i *in preparations from all four species, indicating similar capacities for defending* pH_i *against alterations in* pH_e. *Using DMO pulses, intrinsic buffering capacity* (β_{int}) *and recovery rate from acid loading were determined, and the rate of ion exchange* ($\mathrm{d}H^+$ *[or* OH^-*]*$/\mathrm{dt}$) *was calculated from these values. Cardiac muscle of one of the most anoxia-tolerant species,* M. corona, *had the highest* β_{int} *and* $\mathrm{d}H^+/\mathrm{dt}$ *values, consistent with its behavior, microhabitat, and anoxia tolerance. Only small differences in these parameters were observed among the other three species. Although our results suggest some adaptive differences among species in capacity for regulation of* pH_i, *it is likely that other facets of the suite of adaptive responses to anoxia are the major determinants of anoxia tolerance.*

Introduction

Intertidal marine invertebrates are frequently exposed to environmental anoxia or hypoxia during air exposure at low tide. During anoxic conditions energy is derived from anaerobic pathways, which leads to an uncoupling of proton-consuming and -producing processes, typically resulting in net proton production. Two general strategies that decrease the rate at which

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protons accumulate during anaerobiosis have evolved in marine invertebrates. A phylogenetically widespread adaptation to anoxia is a depression of metabolic rate, and in marine molluscs the anoxic rate may be <10% of that under aerobic conditions (Shick, De Zwaan, and De Bont 1983; Stickle et al. 1989). In addition, anoxia-tolerant marine molluscs and some marine worm groups utilize metabolic pathways with a lower H^+/ATP ratio (Gnaiger 1980).

Although rates of proton production may be decreased under conditions of reduced oxygen availability, anoxia-tolerant organisms must still compensate for the perturbations to acid-base status that usually accompany prolonged anoxia. Cells regulate intracellular pH (pH_i) by three mechanisms—(1) physicochemical buffering, (2) ion exchange of acid-base equivalents across the plasma membrane, and (3) metabolic production and consumption of acids and bases (Walsh and Milligan 1989). Intracellular buffering serves as the initial defense against challenges to pH_i (Roos and Boron 1981). Previous comparative studies of intrinsic (nonbicarbonate) buffering capacity (β_{int}) have focused on distantly related species or different tissues from the same species. Intrinsic buffering capacity was found to be positively correlated with anaerobic burst activity in skeletal muscle of teleost fishes and marine mammals (Castellini and Somero 1981) and in various tissues of molluscs and crustaceans (Morris and Baldwin 1984). Eberlee and Storey (1984) found the same positive correlation of β_{int} with burst glycolytic activity for a variety of tissues in three species of marine molluscs, but they also concluded that increased buffering capacity was not an important adaptation for anoxia tolerance. Regulation of pH_i by ion exchange mechanisms has been demonstrated for several marine mollusc groups, including gastropods (Wiseman and Ellington 1989), bivalves (Zange, Grieshaber, and Jans 1990; Ellington 1993), and cephalopods (Hemming et al. 1990), but no interspecific comparisons have yet been made of rates of exchange. Metabolic regulation of pH_i has not been demonstrated in marine invertebrates.

Among the physiological adaptations to anoxia available to marine invertebrates, the relative functional value of increased capacity for regulating pH_i is not known. In the present study, we compared buffering capacities and rates of ion exchange among muscle preparations of four species of closely related marine gastropods. The four species of gastropods reside at different positions in the intertidal zone and have different tolerances to anoxic conditions. Our results indicated that one species with a high degree of anoxia tolerance has a greater ability to regulate pH_i. The differences in capacity for regulating pH_i were modest among the other species, however, and it is likely that other factors contribute more significantly to anoxia tolerance in this group.

Material and Methods

Specimens and Material

Specimens were obtained from three locations in Florida, in proximity to the Florida State University Marine Laboratory, Turkey Point. *Melongena corona* were collected from a salt marsh near the mouth of the St. Marks River, *Busycon contrarium* and *Busycon spiratum* from an intertidal shoal near Alligator Point, and *Fasciolaria tulipa* from sea grass beds in St. Joseph Bay (table 1). Specimens were maintained in the recirculating seawater system at the Florida State University Marine Laboratory at Turkey Point and, for short periods immediately prior to experiments, in aquaria at the main campus in Tallahassee. Biochemicals were obtained from Sigma Chemical (St. Louis), and all other chemicals were of reagent-grade quality.

TABLE 1

Species of gastropod molluscs (whelks) used in study of pH_i regulation

Species	Activity Levels[a]	Collection Habitat
Melongena corona (crown conch)	Inactive	High intertidal mudflats/ salt marshes
Busycon contrarium (lightning whelk) . . .	Inactive	Low intertidal sand flats
Busycon spiratum (pear whelk)	Active	Low intertidal sand flats
Fasciolaria tulipa (tulip shell)	Highly active	Subtidal sea grass beds

Note. All species are in the superfamily Buccinacea. *Fasciolaria tulipa* is in the family Fasciolariidae, and all other species are in the family Melongenidae. Differences among species in activity level and position of occurrence in the intertidal zone were presumed to reflect differences in tolerance to environmental anoxia.

[a] Criteria are totally subjective and are based on field and laboratory observations of locomotory behavior and habitat. *Melongena corona* and *B. contrarium* appear to feed primarily on sessile bivalve molluscs. In contrast, *F. tulipa* is an active predator that feeds on many highly motile prey items, including bay scallops. *Busycon spiratum* feeds on both sessile and motile prey.

Anoxia Tolerance

To augment anecdotal information from field observations concerning differences in anoxia tolerance among species, we performed a series of experiments to qualitatively assess the relative capacity of each species to tolerate anoxic conditions. Gastropods were individually sealed in 1-quart glass jars filled with anoxic seawater. Prior to each experiment, each specimen's shell was scrubbed to remove any adherent algae. To achieve anoxia, nitrogen gas was bubbled into a large bucket containing seawater, and animals were sealed in jars while fully immersed in the degassed medium. Gastropods were maintained at 22°C in a constant-temperature chamber. At 1-d intervals, a random subset of animals was removed from jars and placed in an aerated aquarium. Those individuals that were alive 24 h after removal from jars were counted as survivors.

Nuclear Magnetic Resonance Procedures

Hearts were dissected from gastropods and immersed in an artifical seawater (ASW) solution of 415 mM NaCl, 10 mM KCl, 9.3 mM $CaCl_2$, 23 mM $MgCl_2$, and 25.5 mM $MgSO_4$ buffered at pH 7.80 with 30 mM HEPES. Two parallel cuts were made across the ventricle of each heart to create a ventricular ring preparation (typically 14–16 mg wet wt). The muscle preparation was suspended with 6-0 surgical silk in a sample chamber of 1.9 mm inside diameter (ID) housed in a homebuilt NMR probe. Muscle preparations were superfused with aerated ASW at a rate of 2–3 mL/min. Artificial seawater was thermostated at 20°C by passing the superfusion tubing through a heat exchanger mounted in the probe head and connected to a Brinkman model RM6 recirculating water bath. The radio frequency coil was a five-turn solenoid of silver-coated copper wire that was wrapped around the sample chamber.

We obtained ^{31}P-NMR spectra at 109.34 MHz on a Bruker WP-270 spectrometer modified with a Tecmag/Libra pulse programmer and acquisition system. Routine spectra were acquired every 4 min by averaging 220 scans collected with a 2.6-μs pulse width (45° tip angle), 0.9-s recycle time, 2,048 data points, 5,000-Hz sweep width, and a 0.205-s acquisition time. Data were processed with two zero fills and an exponential multiplication corresponding to 10 Hz line broadening. To obtain pH_i measurements, the muscle preparations were first loaded with 5–10 mM 2-deoxyglucose (2DG) until a well-resolved 2-deoxyglucose-6-phosphate (2DG6P) peak was observed. The difference in chemical shift between the 2DG6P peak and the arginine phosphate peak was compared to an empirically derived calibration curve to determine pH_i (Wiseman and Ellington 1989).

Energetic Stability of Muscle Preparations

To prove that the muscle preparations were energetically stable in the sample chamber during superfusion and 2DG loading, we monitored the ATP and arginine phosphate pools and pH_i of muscle preparations over a time course comparable to that of our experiments. Procedures for NMR were the same as above except that spectra were acquired every 45 min by averaging 180 scans with a 5.2-μs pulse width (90° tip angle) and a 15-s recycle time (to ensure nuclei were fully relaxed). The first spectrum of each experiment was collected while the muscle preparation was being superfused with ASW only (no 2DG), and the β-ATP and arginine phosphate peaks were directly integrated by MacNMR software. The integrated value of the β-ATP peak from this first spectrum was then used as a reference to determine the relative changes in subsequent β-ATP and arginine phosphate peaks during loading with 2DG. As soon as the 2DG6P peak appeared, pH_i stability was also monitored over the remaining time course.

Relationship between Extracellular pH (pH_e) and pH_i

We examined the ability of cardiac muscle of each species to regulate pH_i over a range of pH_e's. The pH of the superfusate was systematically altered, and the pH_i of the muscle preparation was allowed to equilibrate with each new pH_e for 20–25 min. The pH_i calculated from the final spectrum at each pH_e was considered to be the equilibrium value. Extracellular pH values used were 8.0, 7.6, 7.2, 6.8, 6.4, and 6.0.

Acid-Loading Experiments

To examine the intrinsic buffering capacity and the rate of exchange of acid-base equivalents ($dH^+[\text{or } OH^-]/dt$) during regulation of pH_i, we performed acid-loading experiments using the weak acid 5,5-dimethyloxazolidine-2,4-dione (DMO; Keifer 1981). Intracellular pH was monitored while loads of 30 or 60 mM DMO were being imposed on muscle preparations. The DMO-ASW solution was the same as normal ASW except that DMO was substituted for an equimolar amount of NaCl. Intrinsic buffering capacity, expressed as $\mu mol\ H^+ \cdot pH^{-1} \cdot mL$ intracellular $water^{-1}$, was calculated as

$$\beta_{int} = [DMO^-]_{in}/\Delta pH,$$

where $[DMO^-]_{in}$ is the concentration of protons loaded into the cells and was calculated from the Henderson-Hasselbalch relation assuming a pKa for DMO of 6.16, and ΔpH is the decrease in pH$_i$ on acid loading. To avoid overestimating β_{int} because of recovery occurring during the acidification process, the ΔpH value was calculated back to time zero of acid loading by multiplying the recovery rate (dpH$/dt$) by the number of minutes to maximal acidification and adding that pH$_i$ change to the ΔpH value. Since ASW was air equilibrated, [HCO_3^-] was <1 mM, so the contribution of this species to total buffering capacity is negligible. Since dissociated DMO would have contributed to buffering capacity in the cells by the time recovery measurements were made, the rate of acid-base exchange was calculated as

$$dH^+/dt = d\mathrm{pH}/dt(\beta_{int} + \beta_{DMO}),$$

where β_{DMO}, calculated as 2.3[DMO$^-$]$_{in}$, is the buffering contribution of DMO$^-$ in the cells (Keifer and Roos 1981). Recovery rate was determined from the slope of the line generated from a least-squares linear regression of the first four data points following acidification, beginning at the point of minimum pH$_i$.

Statistical Analyses

ANOVA was used to compare the β_{int} and dH^+/dt values among species at 30 and 60 mM DMO. The Wilkes-Shapiro test was used to test for normality, and Bartlett's test was used to examine homogeneity of variances. Fisher's protected least significant difference test was used to examine differences among species pairs for variables that were significantly different in the ANOVA.

Results

Anoxia Tolerance

Melongena corona and *Busycon contrarium* displayed no mortality during or after immersion in anoxic seawater, even after 4 d (fig. 1). *Fasciolaria tulipa* was the least tolerant of anoxia and began to show mortality by day 2 and showed 100% by day 4, while *Busycon spiratum* was intermediate in anoxia tolerance (fig. 1). The results agree with field observations in that the species that occur highest in the intertidal zone and have the lowest activity levels tend to have the greatest tolerance to anoxic conditions. All of the surviving specimens demonstrated normal behavior in aquaria within 24 h of removal from jars.

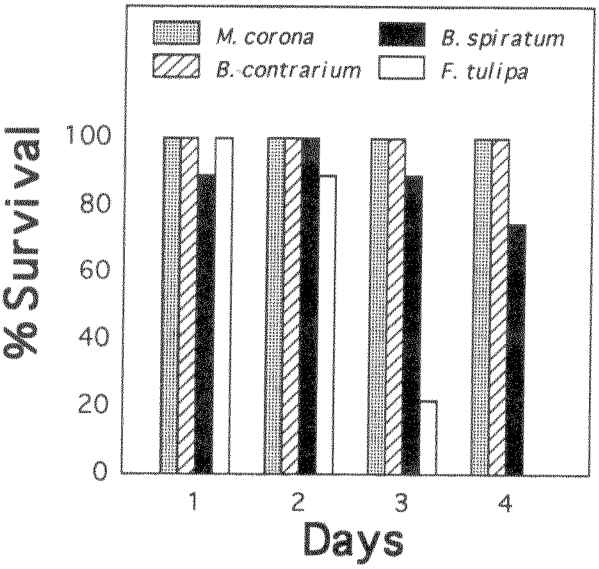

*Fig. 1. Survival of four species of marine gastropods during immersion in anoxic seawater. Data are expressed as percentage of survival for the group of animals removed (*n *= 9 for each day sampled for each species).*

Energetic Stability of Muscle Preparations

Examination of fully relaxed phosphorus spectra revealed that ATP levels were stable and unchanged in muscle preparations from all species while mounted in the NMR probe for >3.5 h (fig. 2). This was also true for pH_i (data not shown). Arginine phosphate levels gradually declined somewhat during 2DG loading, with the decrease in arginine phosphate levels being equivalent to the corresponding increase in 2DG6P. The formation of 2DG6P has a well-established effect of trapping some inorganic phosphate in cells. No inorganic phosphate peak was observed. These results indicate that our superfusion and 2DG-loading procedures had no appreciable adverse effects on the muscle preparations, which were maintained in an energetically favorable condition during our experiments.

Nuclear Magnetic Resonance Experiments

A typical ^{31}P-NMR spectrum of a ventricular ring preparation is shown in figure 3. The arginine phosphate and 2DG6P resonances required for measurement of pH_i are well resolved in 4 min of acquisition.

The four species of gastropods demonstrated similar abilities to regulate pH_i over a range of pH_e's (fig. 4). Intracellular pH was constant at a pH_e of

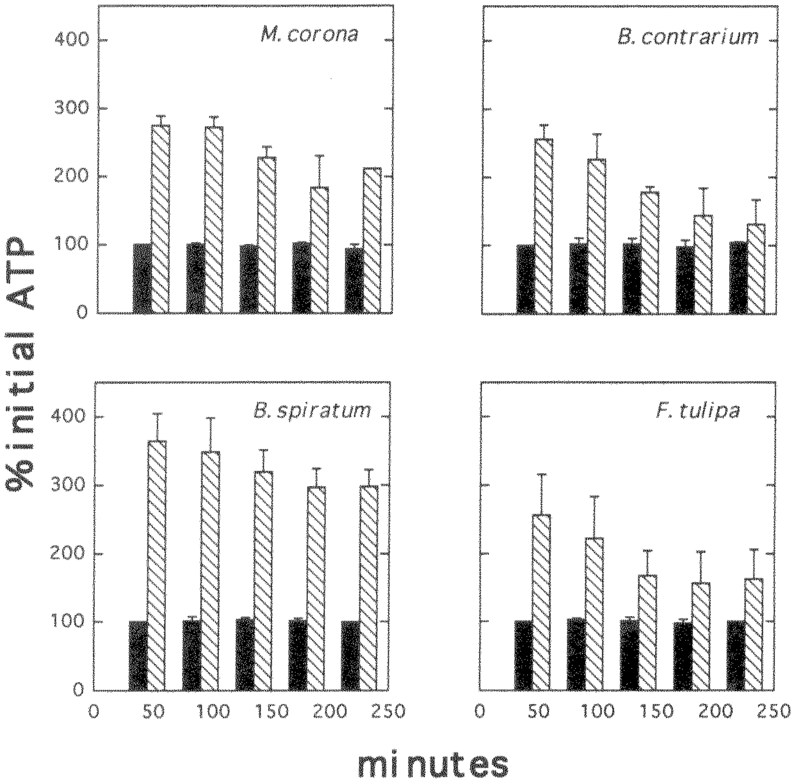

Fig. 2. Stability of high-energy phosphates in ventricular ring preparations from four species of marine gastropods during superfusion and loading with 2DG6P. Solid bars are ATP; hatched bars are arginine phosphate. The initial levels of ATP and arginine phosphate were measured during superfusion with normal ASW. The decline in arginine phosphate levels corresponds to loading with 2DG6P. Data represent means ± 1 SD (n = *4 for each species).*

7.2 or higher for all species but showed a modest decline at pH$_e$'s of 6.8 and lower. Despite this decline, all species maintained pH$_i$ well above the iso-pH line, indicating that pH$_i$ is well regulated even at unusually low pH$_e$'s.

A typical acid-loading experiment is presented in figure 5. The decrease in pH$_i$ following superfusion with DMO-ASW usually occurred within 4 min for all species (mean time to maximum acidification was 5.6 min). Values for β_{int}, dpH/dt, and dH^+/dt are presented in table 2. *Melongena corona* clearly had the highest values for the three measured parameters at both DMO concentrations. Among the other three species, dpH/dt was fairly constant, and although the β_{int} and dH^+/dt values generally fell into the

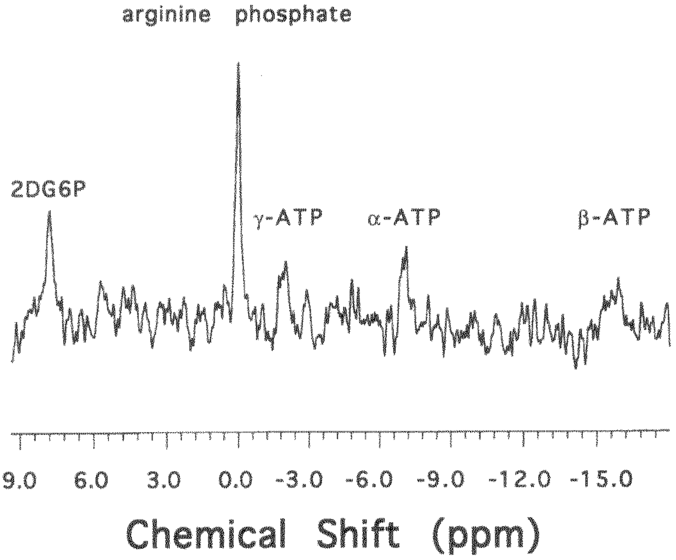

Fig. 3. A typical ^{31}*P-NMR spectrum of a* Busycon spiratum *ventricular ring preparation (16 mg) acquired over a 4-min time course. The arginine phosphate resonance was set to 0 ppm and was used as an internal chemical shift reference. Intracellular pH was calculated from the chemical shift of 2DG6P. The three ATP resonances are also visible.*

expected order based on habitat and anoxia tolerance, the differences among these species were slight. It is expected that all of the measured parameters increase with increased acidification, and this was generally the case. The $d\mathrm{pH}/dt$ values for *M. corona* and *B. contrarium,* however, were lower at 60 mM DMO than at 30 mM. The lack of a positive relationship between extent of acidification and $d\mathrm{pH}/dt$ for these two species was probably because the acidification was fairly mild and the variance associated with the measurements was high. Additional experiments with 90 mM DMO (S. T. Kinsey and W. R. Ellington, unpublished data) resulted in $d\mathrm{pH}/dt$ values for these two species that were higher than those for the 30 and 60 mM DMO experiments, as would be expected.

The data were $\log_{10}$ transformed to meet the assumptions of normality and homogeneity of variances before statistical analysis. ANOVAs showed that β_{int} and $d\mathrm{H}^+/dt$ were significantly different among species in both the 30 and 60 mM DMO experiments (table 3). The paired comparisons tests showed that *M. corona* was significantly different ($P < 0.05$) from all other species in both β_{int} and $d\mathrm{H}^+/dt$ at both DMO concentrations. In the 60 mM DMO experiments, β_{int} values of *B. contrarium* were significantly different at $P < 0.10$ from *B. spiratum* and *F. tulipa.*

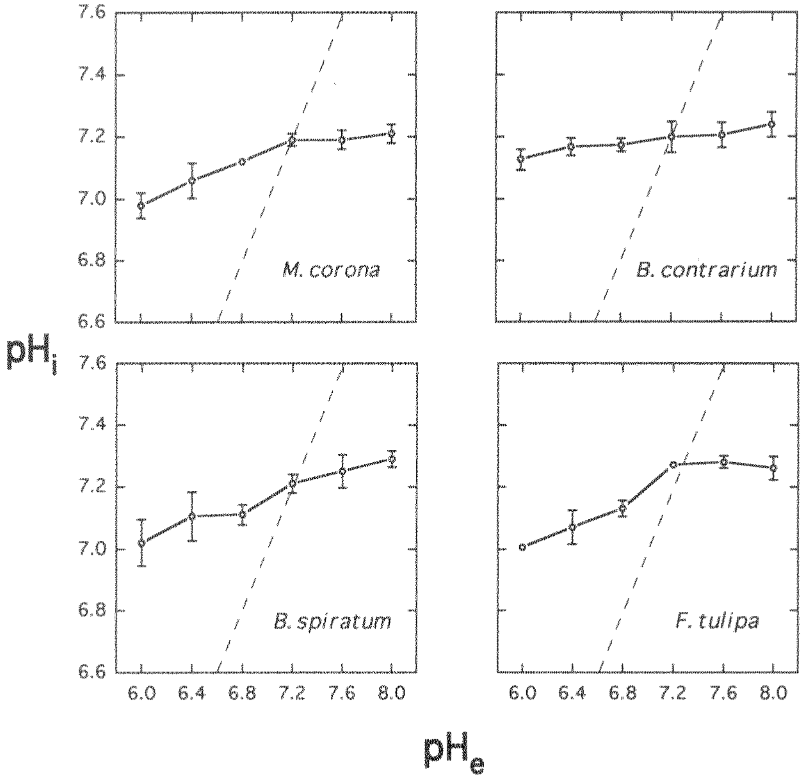

*Fig. 4. Relationship between pH_e and pH_i in four species of marine gastropod ventricular ring preparations. The dashed line is the iso-pH line. Data represent means ± 1 SD (*n = *3 for each species).*

Discussion

Researchers have successfully employed ^{31}P-NMR spectroscopy as a noninvasive probe of pH_i transients in a number of invertebrate muscle tissues (e.g., Ellington 1983; Hamm and Yue 1987; Zange et al. 1990). Previous studies have also demonstrated that accurate measures of intracellular buffering capacity can be obtained with ^{31}P-NMR (Wiseman and Ellington 1989; Zange et al. 1990). Our use of small-diameter muscle preparations (1.9-mm-ID sample chamber) allowed for rapid diffusion of DMO into cardiac tissues, inducing a rapid acidification during acid-loading experiments (fig. 5). The rapid diffusion of DMO into the ventricle tissue reduced the likelihood that buffering capacity values were overestimated because of recovery occurring simultaneously with acidification (Boron 1977; Keifer 1981). The short time course to acidification also makes extrapolating to time zero of acid loading less likely to be biased by the nonlinear nature of the recovery

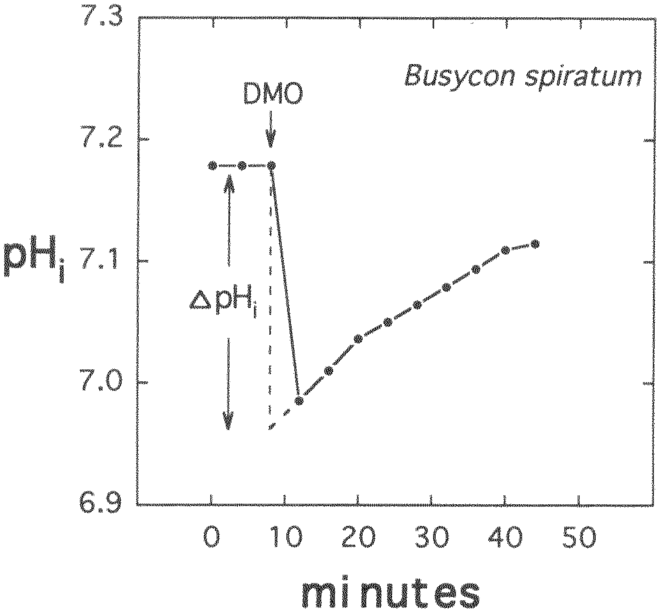

Fig. 5. Typical acid-loading experiment used to calculate β_{int}, d*pH*/dt, and dH^+/dt *for ventricular ring preparations of marine gastropods. The treatment in this experiment was 60 mM DMO. The decrease in* pH_i *with acid loading* (ΔpH) *is used to estimate* β_{int}. *The dashed line is an example of how* ΔpH *was calculated back to time zero of acid loading. The recovery rate* (d*pH*/dt) *is determined from the slope of the line beginning at the minimum* pH_i.

process. The favorable diffusion conditions in the sample chamber and the energetic stability of the gastropod ventricle preparations over the time course of our experiments (fig. 2) make this system well suited for our experimental design.

The relative constancy of pH_i for all species over a pH_e range from 8.0 to 6.0 indicates that protons are not in electrochemical equilibrium across the plasma membrane. These results are similar to those found with fluorescent pH probes in hemocytes of the squid *Sepiateuthis lessoniana* (Hemming et al. 1990) and cardiac myocytes of the bivalve *Mercenaria campechiensis* (Ellington 1993), and with ^{31}P-NMR on the anterior byssus retractor muscle (ABRM) of the mussel *Mytilus edulis* (Zange et al. 1990). The proton disequilibrium found in marine molluscs suggests that there is a continuous export of protons or import of base equivalents across the plasma membrane (Zange et al. 1990), a process that may be partially facilitated by the fact that pH_e is higher than pH_i (Ellington 1993). All four species of gastropods in this study regulated pH_i as the transmembrane proton gradient was decreased and eventually reversed (at pH_e's <7.2), which suggests that ion

TABLE 2

Intrinsic buffering capacity (β_{int}), recovery rate from acid loading (dpH/dt), and rate of transport of acid-base equivalents (dH$^+$/dt) for ventricular ring preparations of marine gastropods

[DMO] (mM)	*n*	Initial pH$_i$	ΔpH	β_{int} (μmol · pH^{-1} · mL^{-1})	*d*pH/*dt* (pH units · min^{-1})	*d*H$^+$/*dt* (μmol · mL^{-1} · min^{-1})
Melongena corona:						
30	4	7.22 ± .04	.11 ± .01	43.3 ± 5.7	.0080 ± .0029	.452 ± .150
60	6		.16 ± .02	56.8 ± 12.0	.0063 ± .0029	.485 ± .167
Busycon contrarium:						
30	4	7.20 ± .04	.14 ± .00	30.9 ± 3.5	.0053 ± .0020	.228 ± .096
60	6		.20 ± .02	42.9 ± 6.5	.0042 ± .0009	.267 ± .040
Busycon spiratum:						
30	4	7.22 ± .07	.18 ± .04	29.1 ± 7.0	.0047 ± .0018	.186 ± .049
60	8		.23 ± .03	35.4 ± 6.8	.0049 ± .0016	.269 ± .065
Fasciolaria tulipa:						
30	4	7.25 ± .04	.19 ± .02	27.0 ± 6.4	.0047 ± .0025	.174 ± .071
60	5		.25 ± .03	33.7 ± 6.6	.0048 ± .0023	.249 ± .080

Note. Values were determined from acid-loading experiments using 30 or 60 mM DMO. Initial pH$_i$ values are means of all experiments (30 and 60 mM treatments). Values for ΔpH are the extent of acidification on acid loading and were used to calculate β_{int} (see text). The data are presented as means ± 1 SD.

TABLE 3

ANOVAs of β_{int} and transport of acid-base equivalents (dH^+/dt) by species

Source	df	Sum of Squares	Mean Square	*F* Value	*P* Value
β_{int} by species in 30 mM DMO experiments:					
Species	3	.106	.035	4.332	.0275*
Error	12	.098	.008		
β_{int} by species in 60 mM DMO experiments:					
Species	3	.217	.072	9.723	.0004*
Error	20	.149	.007		
dH^+/dt by species in 30 mM DMO experiments:					
Species	3	.442	.147	4.932	.0186*
Error	12	.359	.030		
dH^+/dt by species in 60 mM DMO experiments:					
Species	3	.197	.066	3.470	.0354*
Error	20	.379	.019		

Note. The data were $\log_{10}$ transformed prior to analysis to meet assumptions of normality and homogeneity of variances.

* Significant difference among species ($P < 0.05$).

exchange mechanisms are operating to regulate pH_i. Two recent studies have shown that a $HCO_3^-:Cl^-$ exchanger is likely responsible for regulation of pH_i in *M. edulis* ABRM (Zange et al. 1990) and *M. campechiensis* cardiac myocytes (Ellington 1993).

The buffering capacities obtained in our study are in agreement with those found for other marine gastropods. Morris and Baldwin (1984) reported β_{int} values for *Nassarius coronatus* and *Strombus gibberulus* of 60 and 56 μmol · pH^{-1} · g wet wt^{-1}, respectively. Eberlee and Storey (1984) obtained a β_{int} value of 34 μmol · pH^{-1} · g wet wt^{-1} for *Busycon canaliculatum* heart, and Graham and Ellington (1985*a*) found β_{int} to be 39 μmol · pH^{-1} · g wet wt^{-1} for *Busycon contrarium* heart. However, all of these β_{int} values are actually much higher than those for the species in the present study (except for *Melongena corona*) when converted to units of μmol · pH^{-1} · mL intracellular $water^{-1}$. Assuming intracellular water is 54.8% of wet weight (Wiseman and Ellington 1989), each of the above β_{int} values would be increased by 82%. The higher buffering capacities can be explained by the fact that all of these studies utilized the homogenate titration method for determining β_{int} (Bate Smith 1938), which has been shown to yield overestimates of buffering capacity (Wiseman and Ellington 1989; Pörtner 1990). Buffering capacity values obtained from isolated tissues of marine molluscs by ^{31}P-NMR are similar to those found in our study. The β_{int} value of the ABRM of *M. edulis* was determined to be 26.5 μmol · pH^{-1} · mL intracellular water^{-1} (Zange et al. 1990), and the radula protractor muscle of *B. canaliculatum,* approximately 30 μmol · pH^{-1} · mL intracellular water^{-1} (Wiseman and Ellington 1989) on the basis of NMR measurements. Our β_{int} values were also similar to that found for cardiac myocytes of *M. campechiensis* (40 μmol · pH^{-1} · mL intracellular water^{-1}), which was obtained by fluorescence imaging of the entrapped pH probe BCECF (Ellington 1993). Our dpH/dt and dH^+/dt values were similar to those reported for *M. edulis* (Zange et al. 1990) and *M. campechiensis* (Ellington 1993).

The differences observed between *M. corona* and the other three species in β_{int} and dH^+/dt are to be expected if the regulation of pH_i is an important adaptation to environmental anoxia. *Melongena corona* are high intertidal, and at the collection site animals appear to remain buried in anoxic sediments for several months during the winter (Bowling 1992). If this logic were applied to the other three species, which also demonstrate differences in habitat (table 1) and anoxia tolerance (fig. 1), it would be expected that differences in capacities for pH_i regulation would be observed among these species as well. But although the β_{int} and dH^+/dt values generally fell into the predicted order for these three species, the differences were not significant. This suggests either that the comparison is weak or that there are other adaptations more important than increased capacity for pH_i regulation that allow some species to exploit more anoxic habitats. We believe the comparison is valid. All species were collected from nearby locations and maintained under identical conditions, so potential complicating factors, such as temperature or seasonal differences among collection locations or differences in physiological condition, were controlled. Specimens of all species were roughly uniform in size, and the characteristics of the ventricle preparations were similar among species. Further strengthening the comparison are the close phylogenetic relationships between the species and the strong evolutionary and functional homology among the ventricles of the four species.

A possible factor that may be a more important adaptive component of anoxia tolerance than increased capacity for pH_i regulation is the metabolic rate of proton production. Considering that activity level appears to be decreased in species higher in the intertidal zone, it is reasonable to assume that activity level may be indicative of the relative dependence on aerobic metabolism and/or the ability to reduce metabolic demand during anoxia. Therefore, differences in the rate of proton production alone may be sufficient to allow for the observed differences in anoxia tolerance among the three species with similar capacities for pH_i regulation. *Melongena corona* appears to be the only species that has further increased its capacity for pH_i regulation despite having a presumed well-developed capacity for metabolic arrest.

To evaluate the adaptive value of having a higher capacity for regulating pH_i, the problem must be viewed in the context of energy production during environmental anoxia. Metabolite data for in vitro preparations of perfused *B. contrarium* ventricles are available (Ellington 1981, 1983; Graham and Ellington 1985*a*, 1985*b*). On the basis of established proton stoichiometries (Pörtner, Heisler, and Grieshaber 1984; Pörtner 1987) and metabolite data for anoxic *B. contrarium* ventricles (Graham and Ellington 1985*a*), we estimate that the amount of proton production during early anoxia (3 h) is 2.6 μmol $H^+ \cdot$ g wet wt^{-1}. This value converts to a rate of 0.014 μmol $H^+ \cdot$ g wet $wt^{-1} \cdot min^{-1}$ or 0.026 μmol $H^+ \cdot$ mL intracellular $water^{-1} \cdot min^{-1}$.

This calculated rate of metabolic proton production for *B. contrarium* is 10 times lower than the corresponding dH^+/dt values (0.228 and 0.267 μmol $H^+ \cdot$ mL intracellular $water^{-1} \cdot min^{-1}$ for 30 and 60 mM DMO, respectively). However, since proton exchange rates are positively correlated with proton load in the cell (Pörtner, Vogeler, and Grieshaber 1986), the imposition of an acute acid load may have elicited maximal exchange of acid-base equivalents across the cell membrane. On DMO loading, we induced a pH_i drop of about 0.2 pH units in 5.6 min, whereas anoxic *B. contrarium* ventricles undergo a drop of this magnitude over a 3–4 h period (Ellington 1983; Graham and Ellington 1985*a*). Clearly, in vivo rates of acid-base exchange would be much lower than those measured in our experiments because of the lower rates of metabolic acid loading during anoxia. To fully address the question of differences in acid-base exchange rate among species, a full accounting of the effect of pH_i on dpH/dt is necessary for each species, but that is beyond the scope of this study. But even if our measured rates of exchange were near maximal, the fact that maximal dH^+/dt is much greater than the rate of proton production during anoxia at least offers the possibility that increased capacity for pH_i regulation in *B. contrarium* (relative to *Busycon spiratum* and *Fasciolaria tulipa*) is not necessary to allow this species to exploit its present habitat. A decreased rate of proton production may be functionally more important from an adaptive point of view than are potential changes in pH_i regulatory elements. This is a particularly appealing adaptive scenario for the *B. contrarium, B. spi-*

ratum, and *F. tulipa* comparison, in which the species show substantial differences in activity and habitat but few or no differences in pH_i regulation. The above conclusion does not diminish the significance of the high β_{int} and dH^+/dt values characteristic of *M. corona.* The extreme physical features of this species' habitat likely result in more frequent and severe bouts of anoxia necessitating additional adaptations involving enhancement of capacity for regulation of pH_i.

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