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EFFECT OF CYCLICAL SALINITY CHANGES ON CELL VOLUME AND FUNCTION IN *GEUKENSIA DEMISSA* GILLS

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Summary

We acclimated the estuarine mussel *Geukensia demissa* to a regime of sinusoidal salinity cycling (12 h cycle between 100 % and 60 % seawater) and correlated changes in the volume of gill cells with changes in several indicators of the functional status of gill cells (rate of O_2 consumption, ATP content and amino acid transport). There was no indication of short-term volume regulation in the gill cells of mussels acclimated to salinity cycling. When exposed to cycling salinity, cell water space consistently increased to approximately 3 ml g^{-1} dry mass during the cycle troughs (60 % seawater) and returned to approximately 2 ml g^{-1} dry mass at the cycle peaks (100 % seawater). In mussels acclimated for 2 weeks to cycling salinity, the gill contents of betaine, taurine and K^+ were unchanged (approximately 240, 230 and 160 μmol g^{-1} dry mass, respectively) between the 60 % and 100 % seawater portions of the salinity cycle. The changes in cell volume did not appear to be associated with large perturbations in the functional status of cells. The rate of O_2 consumption was approximately 100 μl O_2 g^{-1} dry mass min^{-1}, and ATP content was approximately 30 μmol g^{-1} protein, in all salinities to which mussels were exposed. Rates of uptake of taurine, leucine and phenylalanine decreased by approximately 50 % during the first sinusoidal decrease to 60 % seawater, but recovered following re-exposure to 100 % seawater. Uptake rates of all three amino acids were unaffected by any subsequent salinity cycles. These results suggest (1) that the regulation of gill cell volume is normally absent from mussels exposed to repeated, gradual salinity changes, and (2) that any effects of changes in cell volume are not severe enough to justify the energetic expenditure that would be associated with repeated regulation of cell volume. Unlike the response of gill cells to cycling salinity, there was a decrease in the solute contents of ventricles during the salinity troughs compared with the salinity peaks, suggesting that the presence of short-term volume regulation may be more critical in the ventricle.

Key words: *Geukensia demissa*, mussel, salinity, cell volume regulation, gill, amino acid transport.

Introduction

The regular fluctuations of salinity found in estuarine environments have an important physiological implication for osmoconforming animals such as mussels: alterations of cell volume will result from water fluxes that are coupled to changes in external osmolality. The responses of cells to such a challenge can be categorized into two basic strategies: (1) full or partial restoration to the original cell volume, achieved by adjustments in the intracellular contents of osmotically active solutes, or (2) osmometric fluctuation in cell volume. The first strategy, the active regulation of a relatively constant cell volume, is widely observed in animal tissues (Chamberlin and Strange, 1989) and is commonly assumed to be a necessary adaptation for the survival of euryhaline mussels in the estuarine environment (e.g. Pierce *et al.* 1992; Gainey, 1994).

Contrasting with this viewpoint, however, are our recent observations that gill cells from several species of euryhaline mussels typically do not regulate their volume during acute exposure to hypotonic stress (Neufeld and Wright, 1996*a*,*b*). These observations raise two sets of questions. First, it is important to ask to what extent the results of experiments that employ an ‘abrupt change’ protocol are applicable to the response of cells to more gradual or repeated changes in ambient osmolality, i.e. the types of changes to which estuarine animals are more typically exposed. For example, renal proximal cells of rabbits display a very different response to hypotonic stress when the osmotic shift is gradual rather than acute (Lohr and Grantham, 1986). More immediately relevant is Shumway’s (1977) study of intact *Mytilus edulis* in which she noted that the changes in tissue hydration that occur during exposure to a cyclic, gradual change in external osmolality differ markedly depending on whether the animals are acclimated for several weeks to repeated changes in external salinity (of the type associated with tidal changes). Therefore, in the present study, we sought to determine whether cell

volume regulation was elicited by gradual and repeated changes in ambient osmolality.

The second issue arises as a response to our suggestion (Neufeld and Wright, 1996*a*,*b*) that the absence of a volume regulatory response in gill cells during short-term hypotonic stress may be adaptive because it spares the cells the energetic cost necessarily associated with the recovery of solutes lost during a regulatory volume decrease (RVD). That suggestion assumes that the 'benefit' of such energy savings is not outweighed by the 'cost' (in terms of compromised cell function) associated with a change in cell volume. The function of at least some cell types (ventricle and neuron) in mussels is compromised by changes in external osmolality (Pierce and Greenberg, 1972; Willmer, 1978*b*), but the impact of osmotic changes on gill cell function (as measured by ciliary action and O_2 consumption) is often quite small (Van Winkle, 1972; Shumway and Youngson, 1979). It is generally assumed that volume regulation occurs so that cells may avoid volume-related loss of cell function, as noted by Strange *et al.* (1996), but there are in fact comparatively few data on the extent to which changes in cell volume influence cell function in any animal. Therefore, in the present study, we sought to correlate several general parameters of gill cell function with the volume changes that occur during exposure to cyclic osmolality changes.

We used gill and ventricle tissues from the estuarine mussel *Geukensia demissa* to examine the above issues. Our results confirmed that gill cells from *G. demissa* do not regulate either volume or solute content during gradual, transient salinity changes similar to those encountered in the natural environment. Despite the substantial changes in cell volume that occurred during the changes in ambient salinity, we found no systematic effect of cycling salinity on any of the parameters of gill cell function we examined. In contrast, the ventricle, a tissue known to have a profound functional response to hypotonic stress (Pierce and Greenberg, 1972), regulated intracellular solute content in a way consistent with the routine regulation of cell volume. Thus, the strategy to regulate or not to regulate cell volume appears to be tissue-specific, correlating with the magnitude of the effect of cell volume on organ function.

Materials and methods

Animals and tissue

Ribbed mussels (*Geukensia demissa*) Dillwyn were collected at the Whitney Laboratory, St Augustine, FL, USA, and shipped overnight by air on ice to Tucson, AZ, USA. Before starting experiments, mussels were maintained for 2 weeks in an aerated, recirculating aquarium containing 100 % artificial seawater (940 mosmol l^{-1}; Coralife Marine Products) and held at room temperature (approximately 25 °C). For some trials, mussels were held at a constant salinity over the course of the experiments, either in the aquarium with 100 % artificial seawater described above or in a similar aquarium containing 60 % artificial sea water (550 mosmol l^{-1}). For trials testing the effects of cycling salinity, we used a flow-through tank system (40 l). Addition of water from one of two water reservoirs (one containing deionized water, the other containing 200 % artificial seawater) controlled the direction of the salinity change. We used a residential lighting control system (X-10 Home Automation System; X-10, Inc.) to switch low-flow peristaltic pumps (Cole-Parmer) on and off at defined times. The apparatus allowed us to add variable amounts of water every 15 min, thereby creating a reproducible salinity cycle with a sinusoidal pattern (Fig. 1). All mussels were fed a nominal maintenance ration of approximately 1.5 % of body mass per day (Winter, 1978) of a commercial algae paste (Algal Preserve Diet 'C', Coast Seafoods Company, Bellevue, Washington, USA). All experiments were completed within 2 months of collection.

Prior to the isolation of gill tissue, samples of the mantle cavity fluid were collected from mussels by inserting a needle between the valve halves. Mussels were then opened by cutting the posterior and anterior adductor muscles and gently pulling the valves open until a needle could be inserted into the inter-

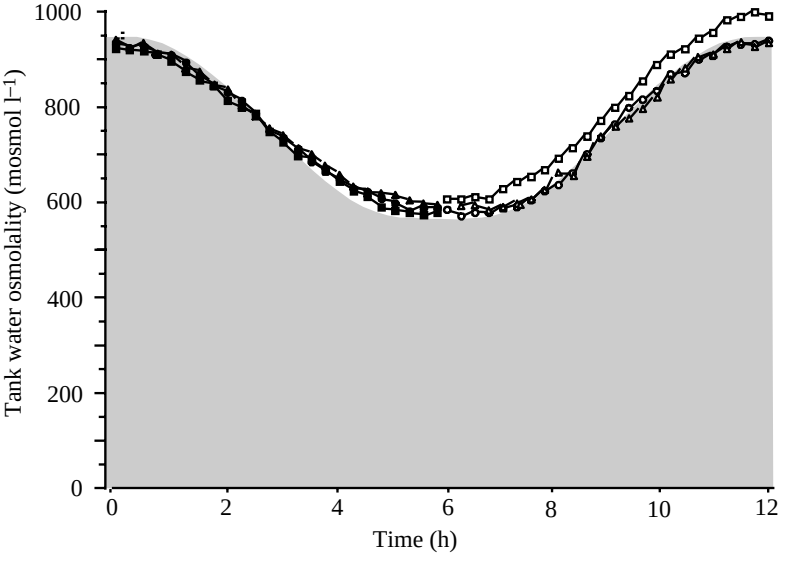

Fig. 1. Profile of sinusoidal salinity change in the water in the mussel holding tank during three periods of salinity decrease and three separate periods of salinity increase. An ideal sinusoidal cycle between 940 mosmol l^{-1} and 550 mosmol l^{-1} is shown by the shaded area.

muscular hemolymph sinus (White, 1937) for collection of a hemolymph sample. We could collect at least 100 µl of hemolymph from approximately two-thirds of the mussels using this method. The osmolality of hemolymph, mantle cavity fluid and artificial seawater (ASW) was measured using a vapor pressure osmometer (Wescor 5500). Gills were then dissected and held in ambient seawater solution until experimentation. All studies on isolated gill tissue were performed in ASW made from the individual salts (in mmol l^{-1}): NaCl, 423; $MgCl_2$, 23; $MgSO_4$, 26; $CaCl_2$, 9; KCl, 9; $NaHCO_3$, 2 (Cavanaugh, 1956). We adjusted the pH of ASW made from the individual salts to between 7.6 and 7.8 using 1 mol l^{-1} NaOH or HCl. All experiments were performed at room temperature (25 °C).

Cell water space

The water space in gill cells was calculated as the difference between the total water content and the extracellular water content (described in more detail by Neufeld and Wright, 1996*a*). Access to the vascular space was provided by the perfusion technique described previously (Silva and Wright, 1994). Briefly, a catheter made from polyethylene tubing pulled to a fine point (approximately 200 µm diameter) was inserted into the efferent branchial vessel and secured with suture thread. By suturing the efferent branchial vessel at a point distal from the catheter, perfusate introduced *via* the catheter was forced through the vascular space of the filaments. Catheterized gill pieces were first perfused with approximately 0.5 ml of ASW of the same salinity as the tank from which mussels had been removed (either 100 % or 60 % seawater; hereafter referred to as the 'acclimation ASW'). After an incubation period of approximately 20 min in acclimation ASW, 5-hydroxytryptamine (5-HT) was added to the bath (to a final concentration of 10 µmol l^{-1}) in order to stimulate the lateral cilia and thereby provide adequate mixing of fluid on the gill surface (Wright, 1979). Following a 5 min exposure to 5-HT, gill sections were transferred to either 100 % or 60 % ASW containing 10 µmol l^{-1} 5-HT and 19 kBq (12.5 µmol l^{-1}) of [^{14}C]polyethylene glycol ([^{14}C]PEG, mean molecular mass 4000). [^{14}C]PEG is not taken up by gill cells and is an effective marker for extracellular space (Neufeld and Wright, 1996*a*). Approximately 0.5 ml of the bathing solution was perfused through the filaments during the first minute, after which the catheter was pulled from the vessel and the gill tissue was allowed to incubate for an additional 5 min. Gill tissue was then gently blotted on filter paper and weighed on tared pieces of aluminum foil. Tissue on the foil was dried for several hours at 35 °C, reweighed to obtain the dry mass, and then extracted overnight in 1 ml of 0.1 mol l^{-1} HNO_3 before scintillation counting.

Cell solute contents

Gill and ventricle tissues for amino acid or betaine analysis were frozen by immersion in liquid N_2 and stored in liquid N_2 until preparation for analysis by perchloric acid (PCA) extraction. The thawed tissue was homogenized in distilled water, extracted overnight at 4 °C in 6 % PCA, and samples were centrifuged at 50 000 ***g*** for 20 min. The supernatant was titrated to pH 7 with KOH and K_2CO_3, refrigerated for 2 h, and then centrifuged again at 48 400 ***g*** for 20 min. The resulting supernatant was passed through a Sep-Pak C_{18} cartridge (Millipore Corp.) and a 0.2 µm filter (Acrodisc, Gelman Sciences).

Free amino acids in the gill and ventricle extracts were measured using ion-exchange chromatography on a Beckman 7300 amino acid analyzer, with lithium citrate buffer and ninhydrin detection. Quantification of betaine was performed using electrospray ionization (ESI) mass spectrometry. The instrumentation used was a TSQ7000 tandem mass spectrometer (Finnigan Instruments, San Jose, CA, USA), equipped with an ESI source coupled to a 1050 HPLC (Hewlett Packard, San Jose, CA, USA). The effluent from the variable-wavelength detector of the HPLC was coupled directly to the ESI source so that both ultraviolet and mass spectrometry data could be obtained from a single flow injection. The ESI source was operated in positive ion mode with a spray voltage of 4.5 kV and a heated capillary temperature of 225 °C. The mass spectrometer was scanned from 50 to 1500 atomic mass units s^{-1} for conventional mass spectra. The flow solvent (in which samples were dissolved) was 0.1 % trifluoroacetic acid in water at a flow rate of 0.3 ml min^{-1}. The ultraviolet wavelength was 215 nm. It should be noted that, prior to freezing, the small quantity of organic substances normally present in the hemolymph (free amino acids, approximately 2–10 mmol l^{-1}; Strange and Crowe, 1979) was flushed from gill sections by perfusing them with acclimation ASW. The measurements of free amino acids and betaine in gill sections therefore reflect concentrations of those organic solutes present in the intracellular pool.

Intracellular K^+ content was measured using flame photometry (Instrumentation Laboratory). Extraction of K^+ from gill pieces for analysis was performed by subjecting the gill pieces to three freeze–thaw cycles in 0.1 mmol l^{-1} HNO_3. The amount of K^+ in the extracellular space (calculated from the known extracellular space and the K^+ concentration in ASW) was subtracted from the total K^+ to give the intracellular K^+ content.

Lateral cell height

We used the optical system described previously (Silva and Wright, 1994; Neufeld and Wright, 1996*a*) to measure the height of lateral cells from the gill epithelium during a 1 h acute exposure to 60 % ASW. Briefly, differential interference contrast (DIC) microscopy was employed to take optical sections of gill cells, using an Olympus IMT-2 inverted microscope equipped with an ultra-long-distance condenser and an Olympus 40× objective (numerical aperture 0.55). Tissue was held in a flow-through chamber (0.15 ml chamber volume) that allowed superfusion with seawater solutions at a rate of 1 ml min^{-1}.

Rate of oxygen consumption

The rate of O_2 consumption of isolated gill tissue was measured using a Clark-type O_2 electrode (YSI). Suture thread was secured around the efferent branchial vessel of a gill piece (approximately 10 mg). The thread was then clamped between

the chamber wall and stopper, allowing the tissue to remain suspended in the chamber (volume approximately 1.5 ml) away from the stir bar and electrode surface. After calibration of the electrode system and equilibration for approximately 10 min in acclimation ASW, 5-HT was added (to reactivate lateral cilia) to a final concentration of 10 μmol l^{-1}, and oxygen consumption was monitored for 10 min. Preliminary tests indicated that the rate of O_2 consumption by gill tissue is linear at O_2 levels above 50 % saturation (data not shown), and at no time did the O_2 content of the experimental solutions drop below this value. The tissue was then weighed, dried at 35 °C for several hours, and then reweighed to obtain the dry mass.

The rate of oxygen consumption was calculated as: $\dot{V}_{O_2}$ = (rate of change in percentage O_2 × oxygen content)/dry mass. Oxygen content was taken as 4.83 ml O_2 l^{-1} for 100 % ASW and 5.17 ml O_2 l^{-1} for 60 % ASW (Cameron, 1986).

ATP content

Given the labile nature of the ATP pool in cells, we opted to standardize ATP to protein content rather than risk altering the ATP content by blotting and weighing tissues. Protein content was identical (P>0.05; N=8 each) in mussels acclimated to 100 % (0.50±0.02 g protein g^{-1} dry mass) and 60 % seawater (0.49±0.02 g protein g^{-1} dry mass) (means ± S.E.M.), indicating that standardization to protein content provided an accurate reflection of any changes in ATP content due to salinity changes. Gill tissue was homogenized in 5 % trichloroacetic acid (TCA), extracted for 20 min on ice, then frozen in liquid nitrogen until further analysis. Wijsman (1976) found that ATP extracted from mussel tissues using TCA is stable under these conditions. For analysis, thawed samples were centrifuged for 4 min at 16 000 ***g***. The precipitate was then resuspended with 1.5 mol l^{-1} NaOH for later analysis for protein content using the Bio-Rad protein assay (Bio-Rad Laboratories). For ATP analysis, the supernatant was diluted 200-fold in 25 mmol l^{-1} Hepes buffer (pH 7.75), giving a final ATP concentration of 50–100 ng ml^{-1}. ATP was quantified with a luminometer (Bio-Orbit, model 1251) using the luciferin–luciferase reaction; measurements of ATP standards indicated that this assay was linear between 0.1 and 200 ng ml^{-1}.

Amino acid uptake

Measurement of amino acid accumulation used the general procedure described previously (Neufeld and Wright, 1995). Gill tissue was first preincubated in ASW containing 10 μmol l^{-1} 5-HT to activate the lateral cilia. For the measurement of taurine uptake, two gill pieces were then transferred for 2 min to 20 ml of acclimation ASW containing 10 μmol l^{-1} 5-HT, 19 kBq of [^{3}H]taurine and enough unlabeled taurine to give a final concentration of 0.5 μmol l^{-1}. Following exposure to the labeled substrate, the tissue was rinsed for 5 min in ice-cold acclimation ASW to wash any taurine from the external surface. The tissue was blotted on filter paper, weighed and then extracted overnight in 80 % ethanol before scintillation counting.

For the measurement of uptakes of phenylalanine and leucine, two gill pieces were incubated for 1 min in 20 ml of acclimation ASW containing 50 nmol l^{-1} (19 kBq) [^{14}C]phenylalanine and 0.2 nmol l^{-1} (19 kBq) [^{3}H]leucine in combination. Tissues were rinsed for 5 min in ice-cold acclimation ASW, blotted on filter paper, weighed and then extracted for 1 h in 80 % ethanol. Tissue was moved to another vial for a second extraction with ethanol, after which more than 99 % of the ethanol-soluble radioactivity had been removed from the tissue (data not shown). The ethanol-insoluble fraction was determined by extracting the remaining tissue piece overnight in 1 ml of Solvable tissue solubilizer (Packard Instruments), which was neutralized with 100 μl of glacial acetic acid before scintillation counting. Total uptake of phenylalanine or leucine was taken as the sum of labeled amino acids in the ethanol-insoluble and ethanol-soluble fractions.

Statistics

All gill data were standardized to dry mass, except for ATP contents which were standardized to protein content. For calculation of solute contents and amino acid incorporation and uptake, dry mass was calculated on the basis of the percentage total water in separate tissue pieces. Because of the small amount of tissue available for analysis, we did not determine percentage hydration of ventricles in the present study and expressed ventricle solute concentrations on the basis of wet mass. Osmolalities of tank water, mantle cavity water and hemolymph were compared at each time-point by analysis of variance (ANOVA) followed by pairwise comparisons using the Student–Newman–Keuls test (Zar, 1984). For the other variables measured, an ANOVA was initially performed. Independent F-tests (Sokal and Rohlf, 1981) were then used to make the following *post-hoc* planned comparisons. (1) A single comparison was made between the control group (acclimation to constant 100 % seawater) and the 'cycling salinity group' (consisting of all the groups measured during cycling salinity). This tested for an overall effect of cycling salinity on the measured parameter. (2) Individual comparisons were made between the 60 % and 100 % seawater exposure for each salinity cycle or acclimation condition as a test of whether the measured parameter was dependent on salinity at that particular time point.

Values are presented as means ± S.E.M.

Results

Mantle cavity and hemolymph osmolality

Because the mussels could isolate themselves physically from the salinity changes by valve closure, we monitored mantle cavity and hemolymph osmolalities to determine the degree to which tissue was exposed to the imposed salinity regimen. In all cases, hemolymph osmolality was not significantly different from mantle cavity osmolality (P>0.05; Fig. 2). At the peak salinity (100 % seawater) during cycling, the osmolalities of the mantle cavity water and hemolymph were identical (P>0.05) to ambient salinity. At the cycle trough (60 % seawater), although the osmolalities of mantle cavity water and hemolymph were consistently higher (P<0.05) than the ambient salinity, at an average of approximately 650 mosmol l^{-1}, they were

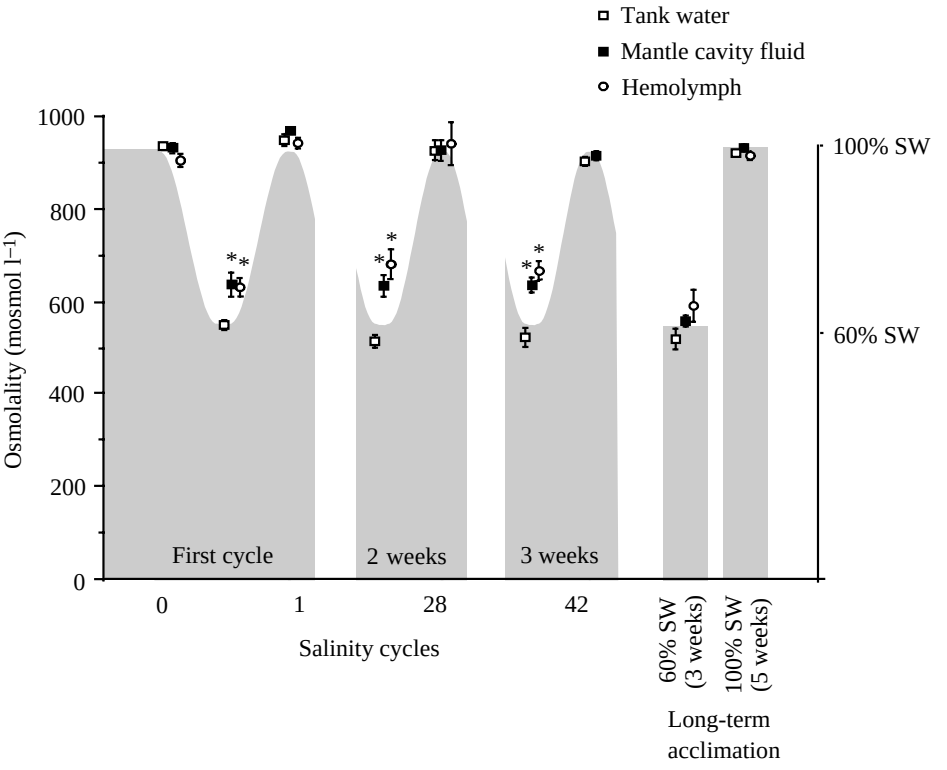

Fig. 2. Osmolality of tank water, mantle cavity fluid and gill hemolymph from mussels acclimated for various times to either constant or cycling salinity. *Osmolality of mantle cavity fluid or hemolymph was significantly (P<0.05) different from tank water osmolality at that time-point. Data are expressed as means ± 1 S.E.M., N=3–6. SW, seawater. The shaded area shows the salinity profile of the tank water.

significantly lower than at peak salinity (Fig. 2). These results confirm that mussels were osmoconformers and that tissues were exposed to the cyclical changes in osmolality. In mussels acclimated long-term to either 100 % or 60 % seawater, the mantle cavity osmolality was not significantly different (P>0.05) from the acclimation salinity (Fig. 2).

Cell water space of gills

The cell water space of gills in mussels acclimated to 100 % seawater was 1.99±0.06 ml g^{-1} dry mass, a value similar to that measured in our previous studies of mussel gills (Neufeld and Wright, 1996*a*,*b*). In all mussels exposed to cycling salinity, cell water space behaved in a manner consistent with a lack of cell volume regulation: cell space increased to approximately 3 ml g^{-1} dry mass during each low-salinity exposure and returned to approximately 2 ml g^{-1} dry mass with each re-exposure to 100 % seawater (Fig. 3). In addition, when gills were abruptly exposed to the ‘opposite’ salinity for 6 min, cell

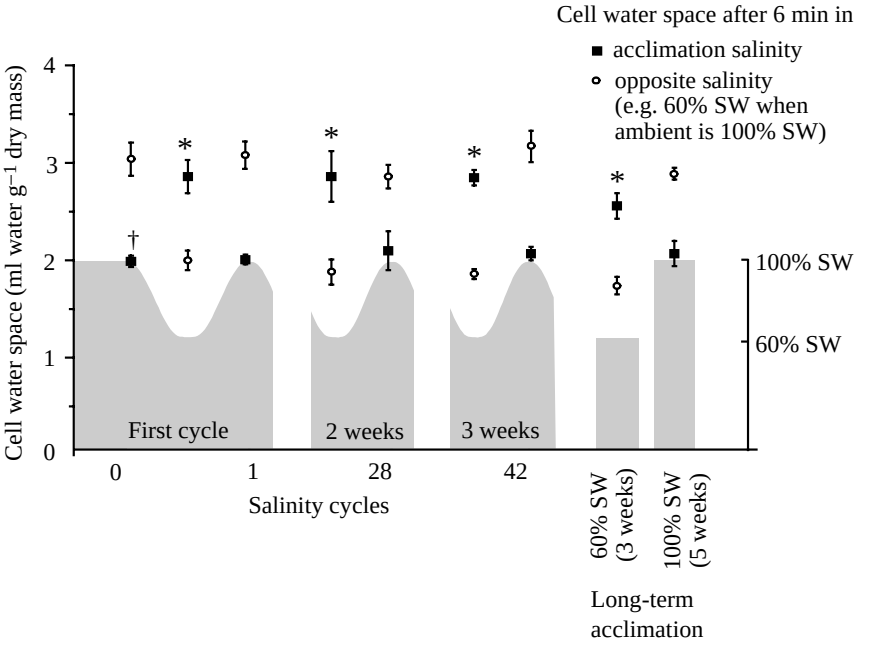

Fig. 3. Cell water space in isolated gills from mussels acclimated for various times to either constant or cycling salinity. Cell water was measured in gill pieces either in the tank salinity from which mussels were taken (‘acclimation salinity’; filled symbols) or after abrupt exposure to the ‘opposite salinity’ (open symbols) (e.g. 60 % seawater if mussels were taken during the 100 % seawater period of the cycle). †F-test indicates cycling salinity had a significant (P<0.05) effect on cell water space compared with the control. *F-test indicates that the cell water space was significantly different (P<0.05) between 60 % and 100 % seawater for that salinity cycle or acclimation condition. Data are expressed as means ± 1 S.E.M., N=5 or 6 in all conditions, except for long-term acclimation to 100 % seawater (SW), where N=3. The shaded area shows the salinity profile of the tank water.

Table 1. *Contents of amino acids, betaine and K^+ in mussel gills during cycling salinity or acclimation to constant salinity*

	Control	Cycling salinity				Constant salinity	
		First salinity cycle		2 weeks of cycling salinity			
	100 % seawater	60 % seawater	100 % seawater	60 % seawater	100 % seawater	60 % seawater	100 % seawater
Taurine	227.2±18.7	222.0±16.8	235.5±15.9	243.2±13.0	225.8±24.1	178.7±11.3*	240.5±0.6
Glycine	40.3±4.7†	18.5±3.0	18.8±1.8	10.6±1.8	18.3±13.6	5.8±1.4*	31.1±5.2
Alanine	18.7±1.7	12.5±1.2	14.3±3.2	18.5±4.3*	32.7±14.0	6.5±0.7	10.2±1.3
Glutamate	8.9±2.5	9.1±3.7	4.7±2.9	12.6±3.6	5.5±7.8	11.5±1.4	9.1±1.0
Other amino acids‡	13.8±3.6	10.8±3.6	6.1±0.8	32.2±7.8	28.4±6.7	21.4±3.5	8.6±4.7
Betaine	311.6±53.5†	147.4±23.4	110.5±5.9	239.6±26.3	246.6±45.0	162.2±28.2*	644.3±172.4
K^+	132.4±4.5	145.0±6.2	144.2±4.6	155.8±6.9	177.0±20.4	120.4±7.7	128.7±6.4
Total	752.9±59.9	565.3±31.8	544.7±17.0	712.4±36.1	734.2±41.9	506.5±43.8*	1072.5±181.6

Values are $\mu mol\ g^{-1}$ dry mass.

†*F*-test indicates a significant ($P<0.05$) effect of cycling salinity compared with the control; **F*-test indicates that solute contents were significantly different ($P<0.05$) between 60 % and 100 % seawater for that salinity cycle or acclimation condition.

Data are expressed as means ± 1 S.E.M.; sample sizes range from 3 to 5.

‡Includes phosphoserine, phosphoethanolamine, threonine, serine, γ-aminobutyric acid and ornithine.

water space changed to a value similar to that in the mussels gradually taken to that salinity over the course of the cycle (Fig. 3). For example, the cell water space in a gill from a mussel taken from the 60 % seawater portion of a cycle and abruptly exposed to 100 % seawater was similar to the cell water space in gills from mussels taken from the 100 % seawater portion of the cycle.

A prolonged exposure to reduced salinity did, however, result in a regulatory decrease in cell water space. After 3 weeks of acclimation to 60 % seawater, the mean cell water space ($2.56\ ml\ g^{-1}$ dry mass) was midway between the mean cell water spaces of mussels exposed to 100 % and 60 % seawater during salinity cycling (Fig. 3).

Solute content

The major osmolytes present in the gill were taurine, betaine and K^+, with the other amino acids detected (primarily glycine, alanine and glutamate; Table 1) constituting approximately 11 % of the measured osmotically active compounds. Cycling salinity had little effect on the contents of individual solutes. Exposure of mussels to cycling salinity caused an overall decrease ($P<0.05$) in betaine and glycine contents, and alanine content was higher in 100 % seawater than in 60 % seawater after 2 weeks. These minor changes were not, however, reflected by any significant changes in total solute content: there was no overall effect ($P>0.05$) of cycling salinity on total solutes, and neither was there a difference ($P>0.05$) in total solute content between the 60 % and 100 % seawater portions of the salinity cycles. The absence of substantial changes in cell solute contents thus agrees with the observed osmometric behavior of cell water space during salinity cycling, indicating that cell volume regulation was not invoked in parallel with each salinity cycle.

Unlike the short-term decreases in salinity that occur during a cycling regime, long-term acclimation to 60 % seawater caused a significant decrease ($P<0.05$) in the total solute content, relative to long-term acclimation to 100 % seawater (Table 1). The decrease in total solute content was due to individual decreases ($P<0.05$) in the levels of the three major organic osmolytes (taurine, glycine and betaine). The lowered solute content after long-term acclimation to 60 % seawater thus agrees with the long-term regulatory volume decrease suggested by the measurements of cell water space (Fig. 3).

Lateral cell height

As an additional check for the presence of volume regulation in mussels acclimated for 3 weeks to cycling salinity, we used the optical technique employed more extensively in previous studies (Silva and Wright, 1994; Neufeld and Wright, 1996*a*,*b*) to test for the presence of acute volume regulation in individual lateral cells of gills. Gills from mussels acclimated for 3 weeks to cycling salinity were taken at the peak of the salinity cycle and abruptly exposed for 1 h to 60 % ASW. Lateral cell height after 15 min in 60 % ASW was 18.3±1.4 % ($P<0.05$ relative to control; $N=3$) greater than control height (in 100 % ASW). Lateral cell height was still 23.3±3.8 % ($P<0.05$; $N=3$) greater than control height after 1 h in 60 % ASW, and returned to within −0.6±1.7 % of control cell height after a 15 min re-exposure to 100 % ASW. Thus, acute volume regulation was absent from individual lateral gill cells of mussels acclimated to cycling salinity.

Oxygen consumption

We measured the rate of oxygen consumption as a first approximation of the state of cellular energy metabolism in mussel gills. In gills from mussels acclimated to 100 % seawater, the rate of oxygen consumption of gill pieces was $107.2\pm4.4\ \mu l\ O_2\ g^{-1}$ dry mass min^{-1} ($N=6$). Exposure of animals to cycling salinity had no overall effect ($P>0.05$) on the rate of O_2 consumption by gill tissue (Fig. 4). There was, however, a higher ($P<0.05$) rate of O_2 consumption during the 60 % seawater portions of the first salinity cycle and of the

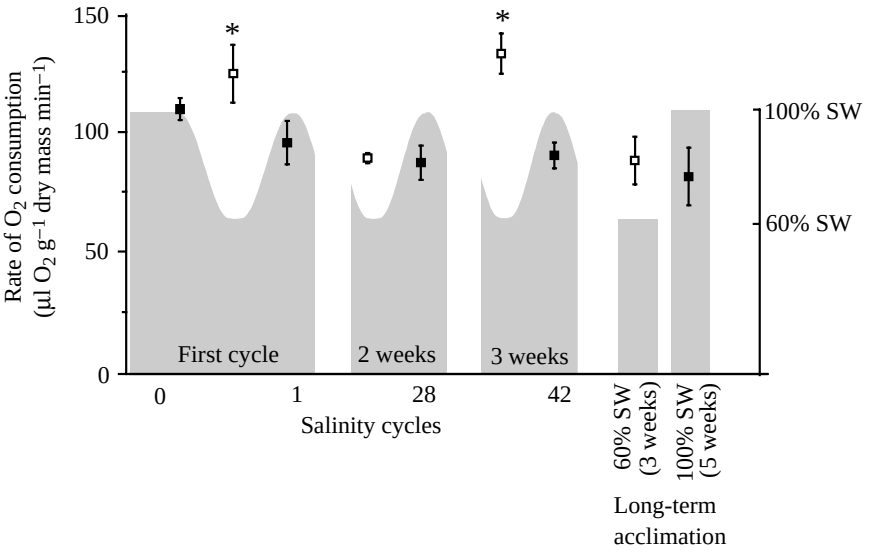

Fig. 4. Rate of O_2 consumption in isolated gills from mussels acclimated for various times to either constant or cycling salinity. Rates of O_2 consumption from mussels in 100 % and 60 % seawater are shown as filled and open symbols, respectively. An *F*-test indicated that cycling salinity had no significant (P>0.05) effect on the rate of O_2 consumption compared with the control. **F*-test indicates that the rate of O_2 consumption was significantly different (P<0.05) between 60 % and 100 % seawater for that salinity cycle or acclimation condition. Data are expressed as means ± 1 S.E.M., N=5 or 6 in all conditions, except for long-term acclimation to 100 % seawater (SW), where N=3. The shaded area shows the salinity profile of the tank water.

salinity cycle at 3 weeks. No significant differences (P>0.05) in rates of O_2 consumption were observed between animals held for 5 weeks in 100 % and for 3 weeks in 60 % ASW (Fig. 4).

ATP content

The ATP content of gill tissue was used as another indicator of the metabolic status of the gill cells. The ATP content of tissue from control mussels acclimated to 100 % seawater was 29.7±4.5 µmol ATP g^{-1} protein. There was no significant effect (P>0.05) of long-term exposure to cycling salinities (Fig. 5), suggesting that repeated exposure to reduced salinity, and the increase in cell volume that accompanied each exposure, had no detrimental effect on the energy status of these cells. Long-term acclimation to 60 % seawater had no significant effect (P>0.05) on ATP content compared with long-term acclimation to 100 % seawater. At a protein content of 0.50±0.02 g protein g^{-1} dry mass (N=8) in 100 % seawater, the ATP content was approximately 15 mmol ATP g^{-1} dry mass (equal to approximately 3 µmol ATP g^{-1} wet mass), similar to that reported for *M. edulis* tissues (Wijsman, 1976). Because cell volume fluctuated between 2 and 3 ml g^{-1} dry mass in 100 % and 60 % seawater, respectively, the ATP concentration in gill cells evidently fluctuated between approximately 5 and 7.5 mmol l^{-1}.

Amino acid uptake

Amino acid uptake in gill cells is the integrated consequence of a suite of cellular parameters, including membrane integrity, membrane potential and the maintenance of an inward Na^+ gradient (Wright and Pajor, 1989). We therefore measured uptake rates of taurine, leucine and phenylalanine as indirect

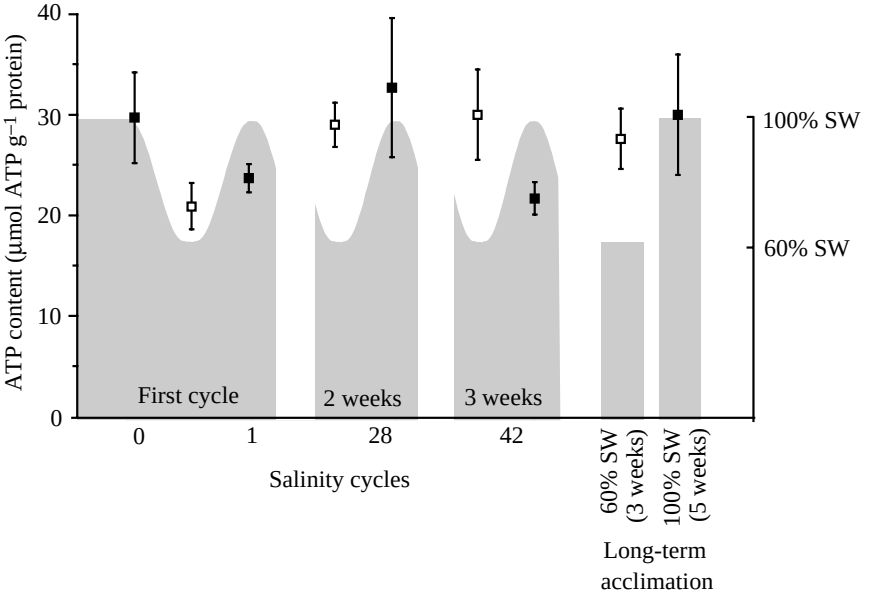

Fig. 5. ATP content in gill pieces from mussels acclimated for various times to either constant or cycling salinity. ATP contents from mussels in 100 % and 60 % seawater are shown as filled and open symbols, respectively. Data are expressed as means ± 1 S.E.M., N=5 or 6 in all conditions, except for long-term acclimation to 100 % seawater (SW), where N=3. *F*-tests indicated that cycling salinity had no significant (P>0.05) effect on ATP content, nor was ATP content significantly different (P>0.05) between 60 % and 100 % seawater for a salinity cycle or acclimation condition. The shaded area shows the salinity profile of the tank water.

indicators of the influence of repeated osmotic stress, and the accompanying unregulated changes in cell volume, on general cell viability. Taurine, one of the primary osmolytes present in gill cells, was taken up at a rate of $95.9\pm10.9\,nmol\,g^{-1}\,dry\,mass\,2\,min^{-1}$ (N=6) (at an ambient concentration of $0.5\,\mu mol\,l^{-1}$) in gills from mussels in 100 % seawater prior to start of the cycling experiments (Fig. 6A). During the first salinity cycle, taurine uptake at 60 % seawater was approximately half (P<0.5) of that observed at 100 % seawater, as expected because of the decrease in extracellular [Na^+] that activates taurine uptake (Silva and Wright, 1992). Unexpectedly, after exposure to cycling salinity for 2 or 3 weeks, there was no difference (P>0.05) in taurine uptake between the 60 % and 100 % seawater portions of the salinity cycle. In addition, taurine uptake in mussels acclimated for 3 weeks to 60 % seawater was not significantly different from that of mussels acclimated for 5 weeks to 100 % seawater (P>0.05).

The effect of acclimation to cycling salinity on leucine and phenylalanine uptake appeared to be similar to that observed for taurine; uptake of both leucine and phenylalanine was depressed (P<0.05) during the trough of the first salinity cycle,

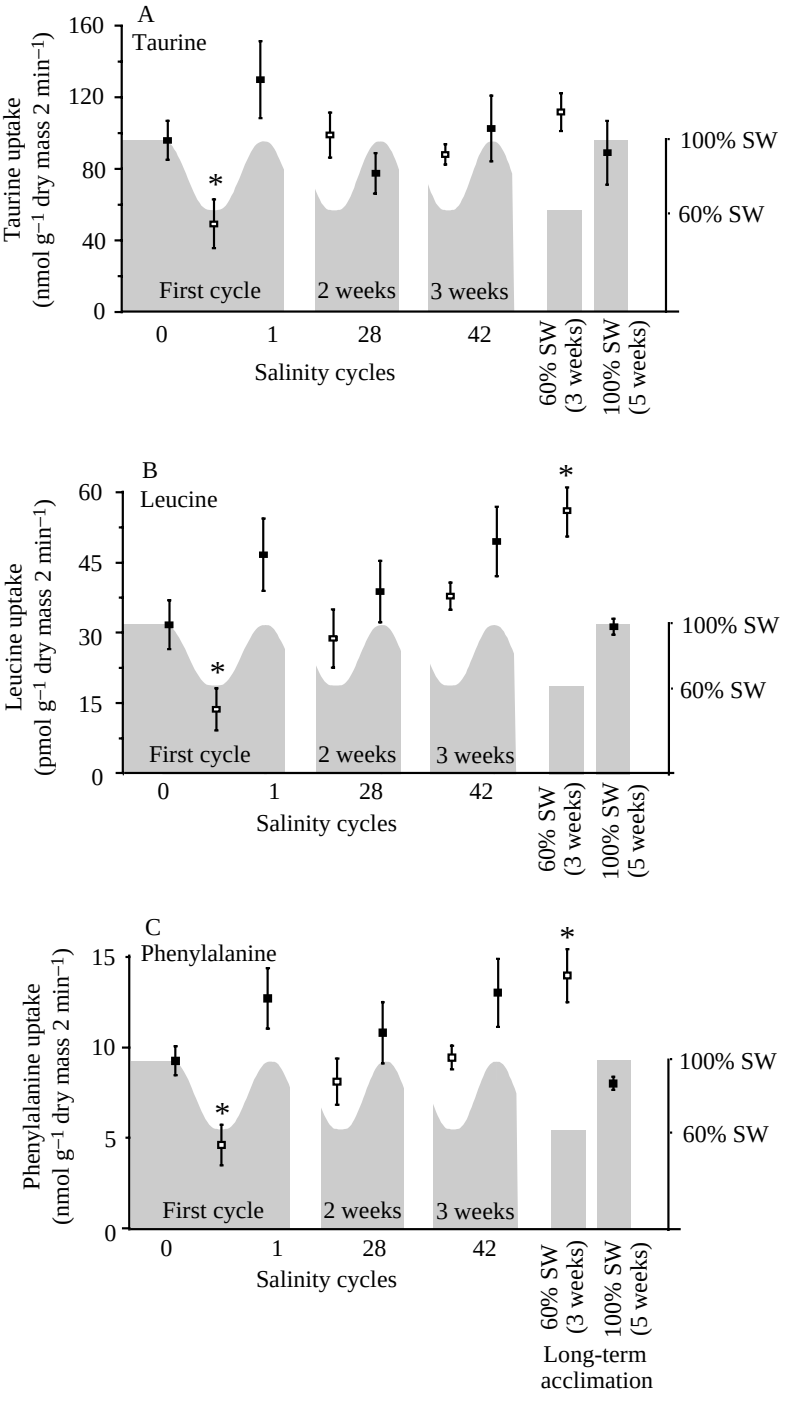

Fig. 6. Uptake rates for (A) taurine, (B) leucine and (C) phenylalanine in gill pieces from mussels acclimated for various times to either constant or cycling salinity. Taurine uptake was from a total external concentration of $0.5\,\mu mol\,l^{-1}$, leucine uptake was from a total external concentration of $0.2\,nmol\,l^{-1}$ and phenylalanine uptake was from a total external concentration of $50\,nmol\,l^{-1}$. Uptake rates from mussels in 100 % and 60 % seawater (SW) are shown as filled and open symbols, respectively. F-tests indicated that cycling salinity had no significant (P>0.05) effect on amino acid transport compared with controls. *F-test indicates that the amino acid transport was significantly different (P<0.05) between 60 % and 100 % seawater for that salinity cycle or acclimation condition. Data are expressed as means ± 1 S.E.M., N=5 or 6 in all conditions, except for long-term acclimation to 100 % seawater, where N=3. The shaded area shows the salinity profile of the tank water.

Table 2. *Contents of amino acids and betaine in the ventricle during cycling salinity or acclimation to constant salinity*

	Control	Cycling salinity			
		First salinity cycle		2 weeks of cycling salinity	
	100 % seawater	60 % seawater	100 % seawater	60 % seawater	100 % seawater
Taurine	41.0±2.5	26.1±2.6*	41.2±4.7	31.9±3.0*	43.3±3.8
Glycine	13.0±1.4†	6.5±1.6	7.1±1.1	6.5±0.6	5.4±0.4
Alanine	10.4±0.9	6.0±1.0	9.3±1.6	12.3±1.1	15.8±2.6
Glutamate	1.1±0.6	0.9±0.6	0.8±0.5	1.7±0.5	2.7±0.6
Other amino acids‡	1.0±0.2	0.7±0.1	1.1±0.3	5.3±2.8	2.7±0.9
Total amino acids	66.4±3.4	40.2±6.5*	59.4±7.1	57.8±4.5	69.9±6.8
Betaine	No data	39.0±5.5	No data	25.2±3.2*	37.0±4.5

Values are $\mu mol\ g^{-1}$ wet mass.

†F-test indicates that cycling salinity had a significant (P<0.05) effect, compared with the control, on the solute content; *F-test indicates that solute contents were significantly different (P<0.05) between 60 % and 100 % seawater for that salinity cycle.

Data are expressed as means ± 1 S.E.M.; sample sizes range from 3 to 6.

‡Includes phosphoserine, phosphethanolamine, aspartate, threonine, serine, β-alanine, γ-aminobutyric acid and ornithine.

but there was no salinity dependence of uptake after 2 or 3 weeks of acclimation to salinity cycling (Fig. 6B,C; P>0.05). There was a higher rate of uptake for both leucine and phenylalanine (P<0.05) after long-term acclimation to 60 % seawater in comparison with long-term acclimation to 100 % seawater.

Ventricle solutes

The percentage hydration of ventricles from mussels acclimated to 100 % seawater does not change significantly (P>0.05) after 4 h of exposure to 60 % seawater (Neufeld and Wright, 1996*b*). Solute contents standardized to wet mass should, therefore, provide a sufficiently accurate indicator of any changes in solute content of the ventricle. As in gill tissue, the bulk of the organic osmolytes present in *G. demissa* ventricles was represented by two compounds, taurine and betaine, with glycine and alanine also present at substantial levels (Table 2). The content of total amino acids in ventricles roughly paralleled the cyclical changes in salinity; the lowest amino acid contents were from mussels during the 60 % seawater portion of the salinity cycles. The change in total amino acid content appeared to be due mainly to fluctuations in the taurine content. As in gill tissue, exposure to cycling salinity produced a sustained decline in glycine content in the ventricle. Two observations suggest that betaine content may also fluctuate with the repeated salinity changes: (1) after 2 weeks of acclimation to cycling salinity, betaine content was significantly higher (P<0.05) at the salinity peak (100 % seawater) than in the salinity trough (60 % seawater), and (2) the betaine content of ventricles from mussels exposed to cycling salinity was much lower than that determined in our previous study for mussels held in constant 100 % seawater ($110\ \mu mol\ g^{-1}$ dry mass; Neufeld and Wright, 1996*b*). The pattern of changes in the contents of major osmolytes therefore suggests that the ventricle responds to frequent salinity changes with a greater degree of volume regulation than that observed in the gills.

Discussion

Cell volume regulation is observed in a wide variety of cell types (Chamberlin and Strange, 1989) and is frequently assumed to be a physiological basis for euryhalinity (e.g. Pierce *et al.* 1992; Gainey, 1994). In the light of this widely accepted view, our previous observations that cells of bivalve gills do not routinely regulate their volume when exposed to hypotonic stress (Neufeld and Wright, 1996*a*,*b*) was unexpected. It also, however, represented an opportunity to pose some basic questions about the time course of osmotic challenges and about our understanding of the adaptive basis of cell volume regulation in marine bivalves.

The present study examined two issues arising from our earlier observations. First, we tested whether the response of gill cell volume to gradual and repeated changes in ambient osmolality is similar to that occurring when tissues are acutely exposed to osmotic stress. The latter paradigm, though commonly used experimentally (including our previous work), is not representative of the type of exposure to which animals are subjected in estuarine environments (Stickle and Denoux, 1976; Davenport, 1982). This represents a critical issue, since the few studies that have investigated the effect of the time course of an osmotic challenge indicate that, in mammalian cells, the degree of volume regulation decreases as the rate of osmolality change increases (Lohr and Grantham, 1986; Mountian and Van Driessche, 1997). In the present study, however, we found that gill cells responded identically to both acute and gradual exposure to an osmotic challenge (Fig. 3), i.e. cell volume changed in a virtually osmometric manner regardless of the time course of change in external osmolality. Consistent with this conclusion was the observation that there were no systematic changes in cell content of the principal osmotically active solutes (K^+, taurine and betaine) during exposure to cycling salinity (Table 1). Furthermore, acclimation to several weeks of repeated salinity cycling had no effect on the nature of the volume response of gill cells:

they continued to respond osmometrically to changes in ambient salinity (Fig. 3).

The absence of short-term volume regulation in gills needs to be considered in the light of two other observations. First, at least some gill cells (i.e. the ciliated lateral cells) do possess mechanisms to regulate volume rapidly and vigorously in the face of acute hypotonic stress. Activation of the RVD in the lateral cells is observed in approximately 30 % of trials with gills from both *M. edulis* (Neufeld and Wright, 1996*a*) and *G. demissa* (Neufeld and Wright, 1996*b*). The absence of a regulatory response appears not to reflect a lack of capability and thus may reflect an adaptive strategy. That interpretation is supported by Strange *et al.* (1996), who have shown that the selective activation of volume regulation in some cells can be influenced by their metabolic status (e.g. ATP content), a possible reflection of the substantial energetic cost of volume regulation.

The second point that warrants emphasis is that, in contrast to gill cells, the changes in the solute content of the ventricle suggest that ventricular cells did meet the repeated osmotic challenges of salinity cycling with a volume regulatory response (Table 2). This observation is consistent with other studies showing salinity-dependent changes in the ventricular content of organic solutes (Pierce and Greenberg, 1972; Baginski and Pierce, 1977; Neufeld and Wright, 1996*b*). Pierce and Greenberg (1972) showed that *G. demissa* ventricles cease normal contractile activity following acute exposure to dilute seawater and that the recovery of normal activity is associated with the loss of ninhydrin-positive substances, suggesting that the recovery of ventricular function does require the regulation of cell volume. We suggest that, in the case of the ventricle, the benefit to the animal (in continued ventricular function) is worth the metabolic cost arising from repeated regulatory cycles.

To summarize the conclusions associated with the first issue addressed in the present study, the regulation of cell volume is a tissue-specific response to the short-term osmotic challenges associated with gradual, repeated fluctuations in ambient salinity; whereas ventricular cells regulate their volume, gill cells do not.

In the above discussion, we suggested that the adaptive value of a volume regulatory response should be viewed in terms of a balance between the energetic cost of regulation and the functional cost of failing to regulate. As noted previously, most cells examined do routinely regulate their volume, at least to some extent, when faced with a hypotonic challenge (Chamberlin and Strange, 1989). The interpretation of the generality of this cellular response is that that failure to regulate volume must place cells at a disadvantage. The present set of observations requires that this interpretation be re-examined. Gill cells do not regulate their volume during the several hours of exposure to dilute seawater, yet this does not seem substantially to compromise (at least) three parameters of cell function: O_2 consumption, ATP content and amino acid transport (Figs 4–6). The near constancy of these parameters suggests that overall cellular viability is not critically compromised by these profound, but transient, changes in cell volume. We suggest that routine volume regulation in gills to transient osmotic challenges would not be adaptive: the benefit (in terms of protection of cell function) does not warrant the high cost (in terms of energy expended repeatedly to recover solute lost during an RVD). In contrast, ventricular cells do appear to regulate their volume rapidly, thereby maintaining ventricular function and the associated tissue perfusion. Partial volume regulation has also been reported in neurons of several molluscan species (Willmer, 1978*a*; Quinn and Pierce, 1992), serving to mitigate the detrimental effects of large osmotic shocks on neural function (Willmer, 1978*b*). Thus, the presence of volume regulation in specific tissues may correlate with the magnitude of the functional effects of a change in cell volume and the consequential effect on the immediate survival of the organism. A similar principle was suggested for another animal that normally encounters osmotic challenges: wood frogs undergoing regular dehydration selectively maintain a constant hydration only in the internal organs that are presumably more critical to the organism's survival (Churchill and Storey, 1993).

Although the overall health of gill cells was not severely compromised by the volume changes, there is little doubt that specific elements of cell function would be influenced by alterations in the intracellular environment that occur when cell volume increases during hypotonic exposure. For example, the associated dilution of inorganic salt concentration can be expected to influence the activity of some or all enzymes, and many mechanisms associated with the 'iso-osmotic regulation of intracellular fluid' have been described (Gilles and Delpire, 1997). Consequently, it is not surprising that gill cells meet a long-term hypotonic challenge with an RVD effected by a loss of organic osmolytes (taurine and betaine), sparing changes in intracellular K^+ content (Table 1). Previous studies have noted this alteration of gill solute contents in response to long-term changes in osmolality (e.g. Baginski and Pierce, 1977; Livingstone *et al.* 1979). The energetic cost to the animal of this regulation is, however, 'pro-rated' over a long period, thereby ameliorating the ultimate cost of regaining these solutes when the animal is again exposed to full-strength seawater.

In summary, we draw two conclusions about volume regulation in estuarine mussels on the basis of the present study. First, substantial regulation of cell volume is normally absent from *G. demissa* gills during salinity cycling, as it is after a single abrupt salinity change (Neufeld and Wright, 1996*b*). Thus, our results suggest that the active regulation of cell volume in all tissues is not a precondition for the survival of euryhaline mussels in estuarine environments. Second, our results suggest that changes in cell volume do not have substantial effects on the function of all cells. The present study supports our suggestion that volume regulation in estuarine mussels may be restricted to certain tissues for which it is necessary for continued function, thereby reducing for the animal the overall metabolic cost of solute transport associated with volume regulation.

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