

## RESPONSE OF CELL VOLUME IN *MYTILUS* GILL TO ACUTE SALINITY CHANGE

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### Summary

The response of gill cell volume in *Mytilus californianus* and *Mytilus trossolus* (= *edulis*) to acute changes in salinity was assessed using three independent indicators: optical measurement of lateral cell height, measurement of intracellular water content using radiolabeled tracers and measurement of the contents of the major osmolytes of the gills. Optical measurements indicated significant variation in the response of individual lateral cells of *M. californianus* to acute low-salinity shock. Lateral cell height increased by approximately 20 % shortly after abrupt exposure to 60 % artificial sea water (ASW). Following this initial swelling, we estimate that a substantial regulatory volume decrease (RVD) was present in 25 % of the trials. More commonly, however, an RVD was either absent or minimal: cell height remained elevated for at least 1 h, then returned to the control height when gills were re-exposed to 100 % ASW. Changes in the combined water space of all cells in the gill, measured as the difference between total water space and extracellular space ( $[^{14}\text{C}]$ polyethylene glycol space), indicated that cell volume regulation in the gill as an organ was also absent or minimal. Cell water space was  $2.16 \text{ ml g}^{-1}$  dry mass in isolated gills of *M. californianus* acclimated to 100 % sea water in the laboratory and increased to  $2.83 \text{ ml g}^{-1}$  dry mass after a 6 min exposure to 60 % ASW. Cell water space was still  $2.81 \text{ ml g}^{-1}$  dry mass after 1 h in 60 % ASW and returned to  $2.06 \text{ ml g}^{-1}$  dry mass upon re-exposure to 100 % ASW. Consistent with these

observations, the gill contents of the principal cytoplasmic osmolytes (taurine, betaine and  $\text{K}^+$ ) were unchanged (approximately 450, 250 and  $230 \mu\text{mol g}^{-1}$  dry mass, respectively) following exposure of gills from 100 % ASW-acclimated mussels to 60 % ASW. A decrease in cell water space to  $2.66 \text{ ml g}^{-1}$  dry mass after 4 weeks of acclimation to 60 % ASW corresponded with a 37 % decrease in betaine content; taurine and  $\text{K}^+$  contents were unchanged. The changes in water space and solute content of gills from freshly collected *M. californianus* and *M. trossolus* were also consistent with the absence of volume regulation; cell water space remained elevated for at least 1 h after low-salinity exposure, and solute contents were unchanged after this period. We calculated the potential energetic cost of cell volume regulation for mussels exposed to 12 h of sinusoidal fluctuations between 100 % and 50 % sea water; solute uptake for full volume regulation in all tissues would cost a minimum of approximately 30 % of the standard metabolic rate during the period of salinity increase. The routine absence of substantial cell volume regulation in *Mytilus* gill may reflect the potentially high energetic cost of volume regulation in the face of the large and frequent salinity fluctuations that are regularly encountered by estuarine bivalves.

Key words: *Mytilus californianus*, *Mytilus trossolus*, cell volume regulation, taurine, betaine, mussel, salinity.

### Introduction

Animals in estuarine environments exist under the stress of frequent and large variations in ambient salinity. For osmoconforming animals such as euryhaline mussels, a change in salinity includes a change in the osmotic gradient between the intra- and extracellular media, causing a net flux of water and, consequently, a shrinkage or swelling of cells. Cells from the majority of animal tissues studied counteract such volume changes in a conserved manner by the accumulation or release of selected solutes (Chamberlin and Strange, 1989). The active regulation of cell volume *via* the solute-driven movement of water out of or into the cell is termed regulatory volume decrease (RVD) or regulatory volume increase (RVI),

respectively. Many studies of estuarine bivalves have shown that there is both an immediate increase in the efflux of amino acids (e.g. Pierce and Amende, 1981; Deaton, 1990) and a longer-term decrease in the content of amino acids (e.g. Livingstone *et al.* 1979; Amende and Pierce, 1980) when intact animals or isolated tissues are exposed to a decrease in ambient salinity. These observations are consistent with the overall paradigm of an RVD in bivalves: the swelling of cells is followed by osmolyte release, causing an osmotically obliged efflux of water and the consequential return of cell volume towards normal.

Although these studies indicate that some degree of volume

regulation is present in tissues of at least some bivalves, the response of cell volume in bivalves to salinity changes is not yet fully characterized. Whereas increases in the efflux of amino acids (and other solutes) occurring immediately after exposure to hypo-osmotic media are suggestive of acute volume regulation, the actual cell size is rarely measured and it is not clear that the amount of solute lost is large enough to effect a rapid decrease in cell volume (Wright *et al.* 1987; Wright and Neufeld, 1995). In addition, reports of a substantial loss of solute from bivalve tissues acclimated over a period of days to dilute sea water involve regulatory processes with a time course that is longer than that expected to be involved in acute cell volume regulation (e.g. Baginski and Pierce, 1977; Livingstone *et al.* 1979; Amende and Pierce, 1980). Finally, the repeated loss and gain of solutes with tidal fluctuations in salinity has the potential to incur a large metabolic cost to the animal (Hawkins and Hilbish, 1992), the impact of which has not been examined. Indeed, a recent report indicates that the existence of volume regulation in hepatocytes of a euryhaline elasmobranch is dependent on their energetic status (Jackson *et al.* 1995). Consequently, for bivalves exposed to frequent, large salinity fluctuations, neither the extent nor the physiological impact of short-term cell volume regulation is clear.

In a previous study, we used an optical technique to demonstrate directly that lateral cells from the gill of the marine mussel *Mytilus californianus* are capable of an RVD when exposed to an abrupt decrease in ambient salinity (Silva and Wright, 1994). In the present study, we used the optical technique to confirm that individual lateral cells can exhibit a vigorous RVD, but we also found that an RVD is more frequently absent or minimal in response to an abrupt salinity decrease. Although the optical technique indicated the response of a single cell type to low salinity, we also measured cell water space and osmolyte content in the total gill and observed that a significant RVD was typically absent in the gill as an organ following acute exposure to low salinity. The parallel use of these three independent techniques as indicators of changes in cell volume allowed us to make the most comprehensive assessment to date of the overall response of cell volume in bivalve gills to a salinity change.

## Materials and methods

### Animals

Specimens of *Mytilus californianus* Conrad (7–12 cm in length) for laboratory acclimation at the University of Arizona, USA, were collected by either Bodega Bay Marine Laboratory, Bodega Bay, CA, USA, or Oregon State University, Corvallis, OR, USA, and shipped overnight by air to Tucson, AZ. Mussels were maintained unfed in aerated, recirculating aquaria containing artificial sea water (Instant Ocean) at either 33‰ (940 mosmol l<sup>-1</sup>; 100% artificial sea water) or 20‰ (60% artificial sea water), and mussels were used within 2 months of collection. All studies were performed in artificial sea water (ASW) made from the individual salts (in mmol l<sup>-1</sup>): NaCl, 423; MgCl<sub>2</sub>, 23; MgSO<sub>4</sub>, 26; CaCl<sub>2</sub>, 9; KCl, 9; NaHCO<sub>3</sub>,

2 (Cavanaugh, 1956). We adjusted the pH of ASW made from the individual salts to between 7.6 and 7.8 by the addition of 1 mol l<sup>-1</sup> NaOH or HCl. For the optical studies, mussels were held in tanks maintained at 13 °C, and experiments were performed at room temperature (approximately 23 °C). For the measurements of cell water and solute contents, the temperature at which mussels were maintained and at which experiments were performed was 20 °C. Prior to experiments, we dissected gills from mussels and allowed them to equilibrate for at least 30 min in ASW of the salinity to which the mussels were acclimated. All experiments involving water or solute contents were performed on perfused gill tissue (the procedure for which is described below); optical measurements were performed on non-perfused gill tissue.

In experiments involving freshly collected *M. californianus* or *M. trossulus* Gould (formerly *M. edulis* L.), specimens were held in aquaria with running sea water (33‰, 10 °C) at Bodega Bay Marine Laboratory. Mussels which we acclimated to 60‰ sea water were held in 201 buckets at 10 °C in which the water [made from natural sea water (SW) and distilled water] was aerated and changed twice daily. One group was fed a daily ration (approximately 3% of body mass) from cultures of the alga *Isochrysis galbana* Parke, while another group was maintained unfed. All other experimental procedures were identical to those for laboratory-acclimated mussels, except that solutions were made from natural SW instead of ASW; experiments were performed at room temperature (approximately 23 °C).

### Optical measurements

For all optical measurements of gill lateral cells, we used an Olympus IMT-2 inverted microscope fitted with an ultra-long distance condenser and either an Olympus 40× objective (numerical aperture 0.55) or a Zeiss 63× objective (numerical aperture 1.25). Small pieces of gill (approximately 3 mm × 5 mm) were mounted in a flow-through chamber (0.15 ml chamber volume) that allowed superfusion with seawater solutions at a rate of 1 ml min<sup>-1</sup>. Since optical resolution of the apical membrane was better when lateral cilia were quiescent, 5-hydroxytryptamine (5-HT) was not included in the solutions. A Lucite ring provided pressure to the ends of the filaments in order to keep the filaments close to the microscope slide and, therefore, within the working distance of the objective. Pressure on the filaments also kept the gill piece from moving during the experiment, allowing us to track the same area on a filament over the course of the entire experiment. We used differential interference contrast (DIC) microscopy in order to obtain an optical section of the tissue at the level of the lateral cells. In each animal test, cell height was followed for a single cell over the course of a trial. We saved and analyzed images using Image-1 computer software (Universal Imaging Corp.).

### Calculation of water spaces

We measured the intracellular water space of the gill as the difference between total and extracellular water spaces.

Extracellular water space was measured using [ $^{14}\text{C}$ ]polyethylene glycol ([ $^{14}\text{C}$ ]PEG; relative molecular mass, 4000). In order to include both the external and vascular spaces in estimations of extracellular water space, we used the gill perfusion technique described by Silva and Wright (1994). Prior to an experiment, we catheterized gill pieces (approximately 75 mg wet mass) *via* the efferent vessel and flushed the vasculature with ASW. Gill pieces were incubated for 5 min in ASW containing  $10\ \mu\text{mol l}^{-1}$  5-HT, thereby activating lateral cilia and facilitating adequate mixing of the bathing medium that is immediately adjacent to the filaments. The gill piece was then placed in 20 ml of ASW containing  $10\ \mu\text{mol l}^{-1}$  5-HT and 19 kBq ( $12.5\ \mu\text{mol l}^{-1}$ ) of [ $^{14}\text{C}$ ]PEG. The gill piece was immediately perfused with 0.75 ml of the ASW solution bathing the external surface; media bathing the external and vascular spaces therefore had identical specific activities for [ $^{14}\text{C}$ ]PEG. The catheter was pulled from the vessel and the gill piece was allowed to incubate for 6 min in the ASW solution unless otherwise indicated. Gill pieces (approximately 20 mg wet mass) were cut from the area bounded by the suture ties, carefully blotted on filter paper, and weighed to the nearest 0.1 mg. Tissue was extracted for at least 1 h in 1 ml of 80 % ethanol before liquid scintillation counting.

Two different methods were used to assess total water space. For some of the measurements of solute contents in the gills of laboratory-acclimated *M. californianus*, total water space was estimated by using  $^3\text{H}_2\text{O}$ .  $^3\text{H}_2\text{O}$  (57 kBq) was included in the 20 ml of ASW bathing the gill (also containing [ $^{14}\text{C}$ ]PEG). Samples were then counted for dual label by correcting for quench and for 'spillover' of counts between the  $^{14}\text{C}$  and  $^3\text{H}$  counting windows. In all other cases, total water space was estimated by comparing the wet mass with the dry mass of tissues. Tissues were dried for at least 2 h at  $60^\circ\text{C}$ ; there was no measurable decrease in tissue mass when tissues were dried for longer. For all experiments on freshly collected mussels at Bodega Bay, we calculated total water space on gill pieces adjacent to those for which extracellular space was calculated. In the comparisons of wet *versus* dry mass in laboratory-acclimated mussels, we calculated total and extracellular water spaces from the same pieces; after incubation in the ASW containing [ $^{14}\text{C}$ ]PEG, the gill pieces were dried for 2 h, weighed and then extracted overnight in  $0.1\ \text{mol l}^{-1}$   $\text{HNO}_3$  for liquid scintillation counting. [ $^{14}\text{C}$ ]PEG space in dried gill pieces ( $0.396 \pm 0.011\ \text{ml g}^{-1}$  wet mass;  $N=3$ ) was identical to [ $^{14}\text{C}$ ]PEG space in gill pieces that had not been dried before extraction ( $0.379 \pm 0.018\ \text{ml g}^{-1}$  wet mass;  $N=3$ ), indicating that there was no loss of [ $^{14}\text{C}$ ]PEG during the drying process and that the extraction of [ $^{14}\text{C}$ ]PEG from dried pieces was complete.

#### Solute contents of the gills

We measured taurine, betaine and  $\text{K}^+$  contents from gill tissue in which the vasculature was perfused with ASW, thereby providing for a known concentration of solutes within the vasculature and a more accurate correction for the amount of solute in the extracellular space (see *Data treatment* below).

Tissue contents of taurine and betaine were estimated using high-performance liquid chromatography (HPLC) (Wolff *et al.* 1989) with a Waters Sugar Pak I column. Taurine content measured using this HPLC technique is equivalent to that measured with ion exchange chromatography (Silva and Wright, 1994). After measuring wet tissue mass, tissue pieces were homogenized in distilled water and allowed to extract overnight at  $4^\circ\text{C}$  in 6 % perchloric acid. Samples were then centrifuged at  $50\,000\text{g}$  for 20 min. The supernatant was titrated to pH 7 with KOH, refrigerated for 2 h, and then centrifuged again at  $50\,000\text{g}$  for 20 min. The resulting supernatant was passed through a Sep-Pak  $\text{C}_{18}$  cartridge and frozen at  $-70^\circ\text{C}$  until used for analysis. As verification of adequate recovery, taurine and betaine contents of several gill pieces were compared with contents of adjacent gill pieces that had had a known amount of taurine and betaine added to the distilled water in which the pieces were homogenized. HPLC analysis for taurine and betaine allowed us to resolve a difference of approximately  $5\ \mu\text{mol g}^{-1}$  dry mass in the taurine and betaine contents of tissue. The resulting recovery was approximately 80 % of the expected amount for both compounds. Since hemolymph concentrations of organic substances are only about  $1\ \text{mmol l}^{-1}$  (Zurburg and DeZwaan, 1981) and most of the hemolymph would be flushed out during perfusion, the calculation of intracellular contents was made on the assumption that all of the measured taurine and betaine was in the intracellular pool.

$\text{K}^+$  content was measured with a flame photometer (Instrumentation Laboratory) from gill pieces extracted in  $0.1\ \text{mol l}^{-1}$   $\text{HNO}_3$  and subjected to three freeze-thaw cycles.  $\text{K}^+$  concentration was also measured in ASW in order to correct for the quantity of  $\text{K}^+$  present in the extracellular space. The use of flame photometry for the measurement of  $\text{K}^+$  allowed us to resolve a difference of approximately  $2\ \mu\text{mol g}^{-1}$  dry mass in the  $\text{K}^+$  content of tissue. For solute measurement in tissues collected at Bodega Bay, samples were shipped on dry ice to Tucson, AZ, USA, and stored at  $-70^\circ\text{C}$  until prepared for analysis.

#### Data treatment

Cell water space and solute contents are expressed per gram dry mass. Since both the  $^3\text{H}_2\text{O}$  water technique and the oven-drying technique provide equivalent estimates of the total water space (see Results), we use the term 'dry mass' in the expression of solute contents to include measurements using both techniques. Measurements of solute content and percentage dry mass taken from separate gill pieces were combined to calculate solute content on a dry mass basis. Dry mass was assumed to remain constant during the course of experimental manipulations; changes in water or solute content should therefore reflect accumulation or release of water or solute.  $\text{K}^+$  contents are corrected for the quantity of solute in the extracellular space:

$$\{\text{K}^+\}_i = \{\text{K}^+\}_t - (\text{Ext} \times [\text{K}^+]_e), \quad (1)$$

where  $\{\text{K}^+\}_i$  and  $\{\text{K}^+\}_t$  are the intracellular and total  $\text{K}^+$

contents, Ext is the extracellular water space, and  $[K^+]_e$  is the extracellular  $K^+$  concentration. No correction was necessary for taurine or betaine since these would be flushed from the vasculature during perfusion with ASW.

The statistical procedures used to analyze the data are from Sokal and Rohlf (1981). Treatment effects were tested for statistical significance using analysis of variance (ANOVA). If ANOVA indicated that significant treatment effects existed, pairwise comparisons among means were carried out using the Student–Newman–Keuls procedure. Statistical significance was accepted at  $P < 0.05$ . All data are reported as means  $\pm$  S.E.M.;  $N$  represents the number of mussels tested.

## Results

### Lateral cell height

The apical and basal membrane boundaries of lateral cells are easily tracked *via* DIC microscopy through the changes in salinity by maintaining the focus level on the lateral cilia, which arise only from the lateral cells (Fig. 1). The distance between the apical and basal membranes (i.e. the cell height) of lateral cells in 100 % ASW was  $21.0 \pm 0.6 \mu\text{m}$  ( $N=13$ ) and increased to  $25.2 \pm 0.5 \mu\text{m}$  ( $N=13$ ) 15 min after switching to 60 % ASW. During the subsequent 1 h exposure to 60 % ASW, it was evident that an RVD similar to that observed in a previous study was present in some trials (Fig. 2A; Silva and Wright, 1994). More commonly, however, an RVD appeared to be minimal or absent; cell height did not show a substantial return towards control values (Fig. 2B). As a rough estimate

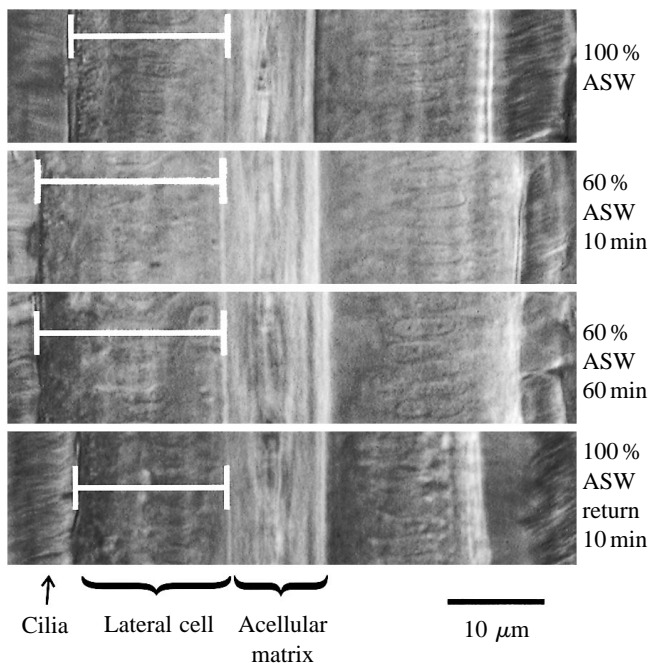


Fig. 1. Images taken using differential interference contrast microscopy showing a typical response of lateral cell height in a gill during a 1 h exposure to 60 % ASW, followed by a return to 100 % ASW.

of the presence of an RVD, we performed a linear regression of cell height on time for each trial from the point of maximal swelling to the maximum time in 60 % ASW (usually 1 h after transfer to low salinity). Of 20 trials (one trial from each animal tested), the calculated regression slope during this period was significantly different from zero ( $P < 0.05$ ,  $t$ -test) and negative in five trials, indicating that an RVD could be resolved in approximately one-quarter of the trials using the optical technique. There was no statistically significant decline in lateral cell height over time in the remaining 15 trials. After 1 h of exposure to 60 % ASW, the decrease in cell height from the initial swelling was  $54.4 \pm 7.0\%$  of the maximum value in the five trials that showed a statistically significant decrease in cell height over time. In the remaining 15 trials, the change in cell height after 1 h in 60 % ASW was  $-1.0 \pm 4.0\%$  of the initial swelling. Each trial represents the response of cell height in a single cell of a mussel; we did not determine whether variability exists between lateral cells within the gill from a single mussel.

When observations obtained from tissues of 20 mussels are pooled (Fig. 3), the cell height after 1 h in 60 % ASW was not

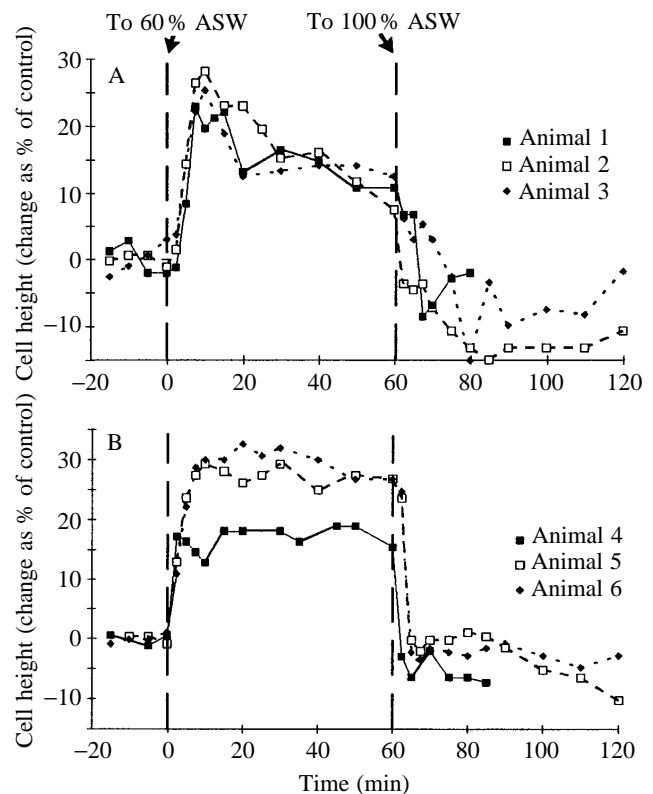


Fig. 2. Individual traces of changes in the height of lateral cells from *Mytilus californianus* acclimated to 100 % ASW, exposed for 1 h to 60 % ASW and subsequently returned to 100 % ASW. (A) Three representative traces from the 25 % of trials in which cell height after the initial swelling decreased significantly with time (see text). (B) Three representative traces from the 75 % of trials in which there was no significant decrease in cell height after the initial swelling. Dates of trials: animal 1, April 19; animal 2, April 27; animal 3, May 6; animal 4, April 21; animal 5, May 12; animal 6, May 12.

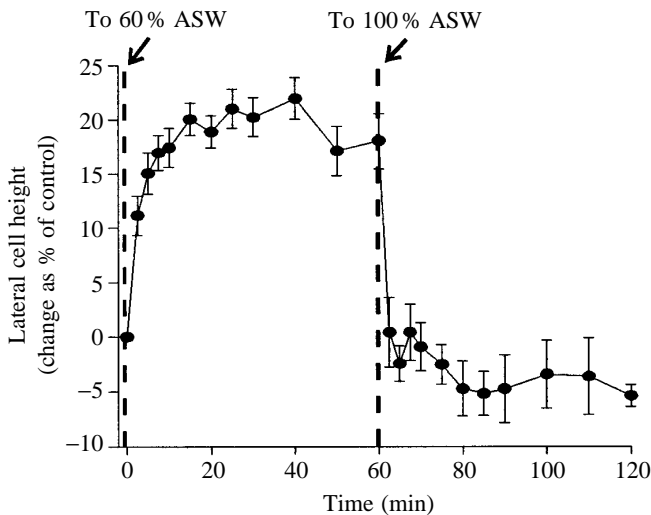


Fig. 3. Pooled data from all optical measurements on *M. californianus* showing lateral cell height after exposure to 60 % ASW, followed by a return to 100 % ASW. Sample sizes range from 10 to 20 for the 60 min exposure to 60 % ASW. Sample sizes range from 4 to 12 for the remaining 60 min period of re-exposure to 100 % ASW. Values are means  $\pm$  1 S.E.M.

significantly different from the maximum cell height achieved following the shift to 60 % ASW ( $P>0.05$ ). Following a return to 100 % ASW after 1 h in 60 % ASW, the cells returned rapidly to the pre-60 % ASW exposure height, followed by an additional slow decrease in cell height. A statistical comparison of the pooled data was carried out between values at five time points: 100 % ASW control, 60 % ASW after 15 min and 1 h of exposure, and 15 min and 1 h after the return to 100 % ASW. Cell heights after 15 min and 1 h in 60 % ASW were not significantly different from each other ( $P>0.05$ ) but were different from cell heights in the 100 % ASW control or return ( $P<0.05$ ). Cell heights after returning to 100 % ASW for either 15 min or 1 h were significantly smaller than the control height ( $P<0.05$ ), although the magnitude of this undershoot was small (approximately 5 %). There was no observed correlation between the degree of an RVD and either the season or site of collection, or the length of time that mussels were acclimated to laboratory conditions.

#### Water spaces

##### Techniques

Since the optical technique measured the volume response of only a single type of cell in the gill, we developed a radiotracer technique to measure the cumulative response of cell water space in the gill as an organ. The fraction of total tissue water that was within cells (i.e. the cell water space) was determined by subtracting the extracellular water space from the measured total water space. The  $^3\text{H}_2\text{O}$  space in non-perfused gill tissue from *M. californianus* rapidly reached equilibrium during incubation in ASW.  $^3\text{H}_2\text{O}$  space was equivalent ( $P>0.05$ , paired  $t$ -test) in 100 % ASW-acclimated mussels after 1 and 10 min

( $0.801\pm0.012$  and  $0.807\pm0.003$  ml g $^{-1}$  wet mass, respectively;  $N=3$  each) of incubation, as was the  $^3\text{H}_2\text{O}$  space in 60 % ASW-acclimated mussels after 1 and 10 min ( $0.808\pm0.027$  and  $0.819\pm0.013$  ml g $^{-1}$  wet mass, respectively;  $P>0.05$ , paired  $t$ -test;  $N=3$  each). In gills taken from mussels acclimated to 100 % ASW,  $^3\text{H}_2\text{O}$  space ( $0.800\pm0.023$  ml g $^{-1}$  wet mass,  $N=3$ ) was identical ( $P>0.05$ , paired  $t$ -test) to water space calculated by comparing the wet mass with the dry mass ( $0.787\pm0.005$  ml g $^{-1}$  wet mass,  $N=3$ ), indicating that the two techniques provided equivalent estimations of total water space in the tissue.

The extracellular water space consisted of two components: the external (sea water) component and the intravascular (hemolymph) component, both of which were determined using the cell-impermeable solute [ $^{14}\text{C}$ ]PEG. In one set of experiments, the external component was measured by incubating non-perfused gill tissue in ASW containing [ $^{14}\text{C}$ ]PEG. The [ $^{14}\text{C}$ ]PEG space increased within 2 min to  $0.150\pm0.015$  ml g $^{-1}$  wet mass, then continued increasing slowly for at least 1 h (Fig. 4). After exposure to ASW containing [ $^{14}\text{C}$ ]PEG, some gill pieces were rinsed for 5 min in ASW lacking [ $^{14}\text{C}$ ]PEG. The [ $^{14}\text{C}$ ]PEG space calculated from rinsed pieces gradually increased over the 1 h period at a rate equivalent to that of the [ $^{14}\text{C}$ ]PEG space in unrinsed pieces ( $P>0.05$ , analysis of covariance; combined slope= $0.126$  ml g $^{-1}$  wet mass h $^{-1}$ ); the difference between the two spaces was constant ( $0.15$  ml g $^{-1}$  wet mass) for the entire 1 h period (Fig. 4). Several tests were performed to investigate whether the gradual accumulation represented an entry of [ $^{14}\text{C}$ ]PEG into the intracellular space of gills (e.g. *via* endocytotic processes) that would cause an overestimation of the extracellular space. [ $^{14}\text{C}$ ]PEG space was compared between perfused gill pieces incubated for short (3 min) or long (1 h) periods in the test solution. [ $^{14}\text{C}$ ]PEG space in perfused pieces incubated for 3 min ( $0.367\pm0.033$  ml g $^{-1}$  wet mass) was not significantly different

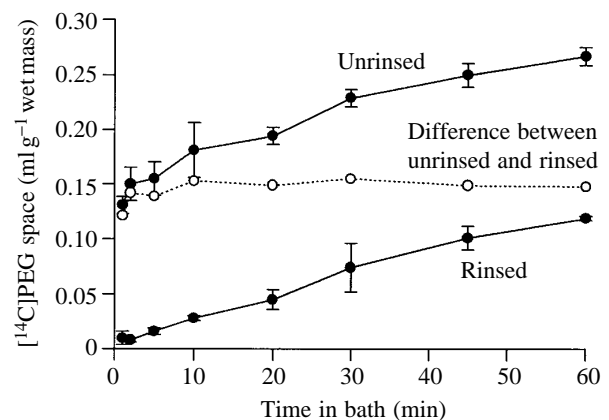


Fig. 4. Time course of measured [ $^{14}\text{C}$ ]PEG space in gill pieces from *M. californianus* acclimated to 100 % ASW. [ $^{14}\text{C}$ ]PEG space was measured in gill pieces that were either rinsed or unrinsed following incubation in ASW containing [ $^{14}\text{C}$ ]PEG (solid lines;  $N=3$  for each condition). The calculated difference between rinsed and unrinsed pieces is also shown (broken line). Values are means  $\pm$  1 S.E.M.

from [ $^{14}\text{C}$ ]PEG space in perfused pieces incubated for 1 h ( $0.401 \pm 0.032 \text{ ml g}^{-1}$  wet mass;  $N=3$ ). The difference in means ( $0.034 \text{ ml g}^{-1}$  wet mass) was less than that expected on the basis of the slope of [ $^{14}\text{C}$ ]PEG accumulation in non-perfused tissue ( $0.122 \text{ ml g}^{-1}$  wet mass), suggesting that there was no continuous accumulation as seen in non-perfused tissue. [ $^{14}\text{C}$ ]PEG therefore probably moves intercellularly between the external and vascular spaces, but is sufficiently restricted to the extracellular space during the 6 min exposure used in the experiments to serve as a measure of extracellular volume. Correction of total tissue water for the volume in extracellular compartments indicated that gill cell water content was  $2.159 \pm 0.070 \text{ ml g}^{-1}$  dry mass in animals acclimated to 100 % ASW.

#### Response to salinity changes

Cell water space in gills of *M. californianus* acclimated in the laboratory to 100 % ASW was  $2.833 \pm 0.070 \text{ ml g}^{-1}$  dry mass after isolated gills were exposed for 6 min to low salinity, representing an increase of 31 % compared with the cell water space in 100 % ASW (Fig. 5). Where it occurs, the RVD in lateral cells reaches a steady state within 1 h (see Fig. 2 in Silva and Wright, 1994). However, cell water space was still  $2.807 \pm 0.070 \text{ ml g}^{-1}$  dry mass after 1 h in 60 % ASW, implying that the regulation of gill cell volume was minimal over this period. When isolated gills were returned for 6 min to 100 % ASW after a 1 h exposure to 60 % ASW, cell water space returned to  $2.058 \pm 0.089 \text{ ml g}^{-1}$  dry mass (Fig. 5), as expected in the absence of volume regulation. Short-term changes in cell water

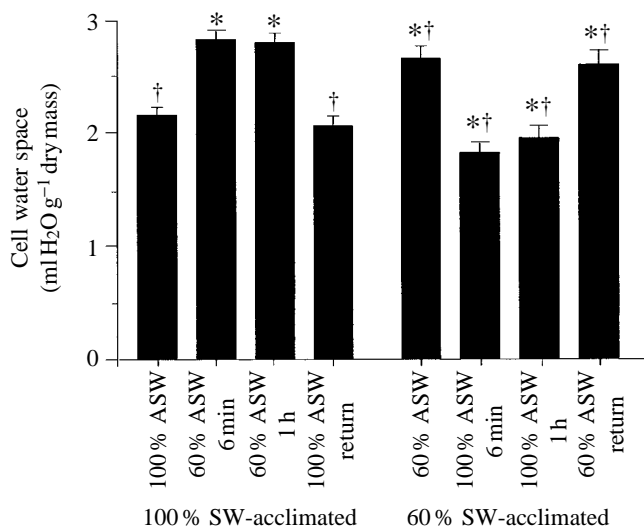


Fig. 5. Cell water space in laboratory-acclimated *M. californianus* gills during acute exposure to ASW either hypo- or hyperosmotic to the acclimation salinity ( $N=11-12$  for mussels acclimated to 100 % ASW;  $N=6$  for mussels acclimated for 4 weeks to 60 % ASW). \*Cell water space was significantly different ( $P<0.05$ ) from cell water space in gills from mussels acclimated to 100 % ASW. †Cell water space was significantly different ( $P<0.05$ ) from cell water space in 100 % ASW-acclimated mussels after 6 min in 60 % ASW. Values are means  $\pm 1$  S.E.M.

space of freshly collected *M. californianus* and *M. trossolus* gills paralleled the responses seen in laboratory-acclimated *M. californianus*. In both *M. californianus* and *M. trossolus* exposed to 60 % natural SW, the cell swelling after 3 min (30 % and 39 %, respectively) was not significantly different from the cell swelling after 1 h (23 % and 55 %, respectively) (Fig. 6).

The cell water space in *M. californianus* acclimated in the laboratory for 4 weeks to 60 % sea water was significantly

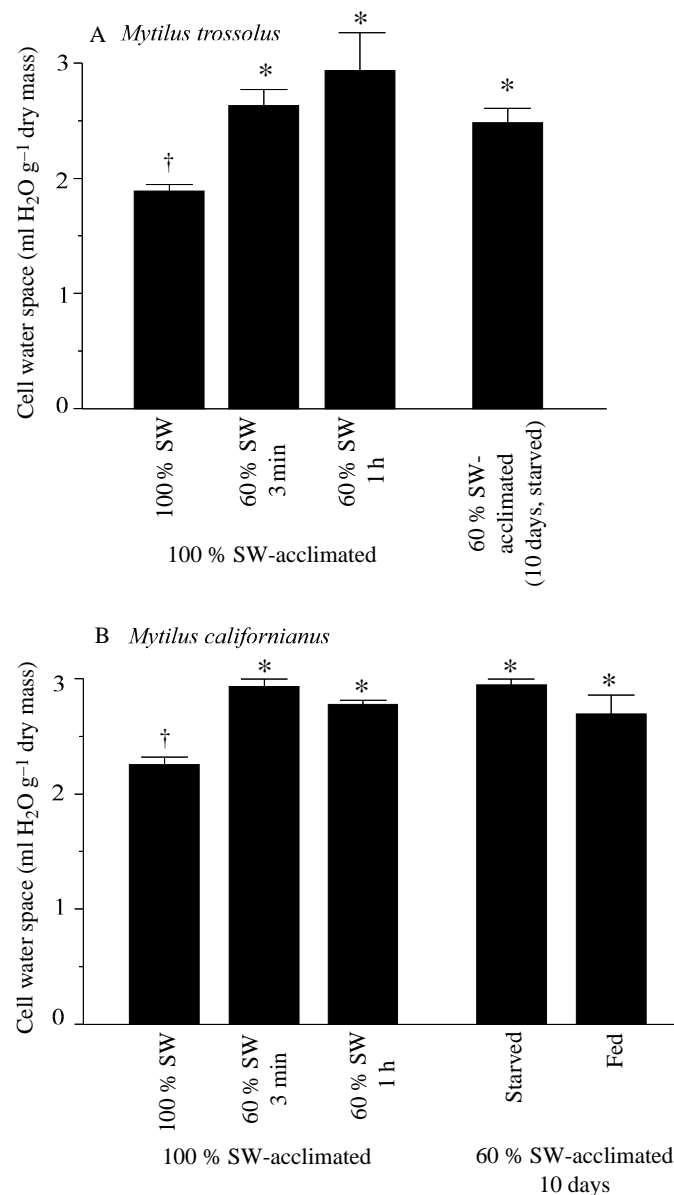


Fig. 6. Cell water space in freshly collected *M. trossolus* (A) and *M. californianus* (B) gills during acute and chronic exposure to hypo-osmotic sea water ( $N=3-6$ ). 'Starved' mussels were maintained without feeding during the acclimation period. 'Fed' mussels were fed a daily ration of algae during the acclimation period. \*Cell water space was significantly different ( $P<0.05$ ) from cell water space in gills from mussels acclimated to 100 % natural SW. †Cell water space was significantly different ( $P<0.05$ ) from cell water space from 100 % natural SW-acclimated mussels after 3 min in 60 % natural SW. Values are means  $\pm 1$  S.E.M.

smaller than the cell water space in 100 % ASW-acclimated mussels exposed for 6 min to low salinity (Fig. 5); this long-term RVD represented a return of 26 % from the maximal swelling. Therefore, unlike the response following an acute exposure to dilute sea water, an RVD was observed over a period of 4 weeks. After 60 % ASW-acclimated mussels had been acutely exposed to 100 % ASW, cell water space decreased by 32 %, to a value significantly smaller than that of the mussels acclimated to 100 % ASW, and remained at this level for at least 1 h (Fig. 5). Cell water space was still elevated for both *M. californianus* and *M. trossolus* after 10 days in 60 % natural SW (Fig. 6). There was no clear long-term effect of feeding status on cell volume: cell water space from 60 %-acclimated *M. californianus* that were fed an algal diet was not significantly different from cell water space in unfed 60 %-acclimated *M. californianus* (Fig. 6B).

#### Solute contents

In gills from *M. californianus* acclimated to 100 % ASW in the laboratory, the solute contents of taurine, betaine and  $K^+$  were  $396 \pm 36$ ,  $226 \pm 24$  and  $229 \pm 21 \mu\text{mol g}^{-1}$  dry mass, corresponding to intracellular concentrations (calculated from measurements of cell water space) of 183, 105 and  $106 \text{ mmol l}^{-1}$ , respectively. Taken together, these three solutes account for 42 % of the osmotically active solutes expected in the cytoplasm of bivalves acclimated to  $940 \text{ mosmol l}^{-1}$  sea water; the remainder of the osmotic pressure presumably consists of inorganic ions and minor amino acids (Silva and Wright, 1994). The gill cell contents of taurine, betaine and  $K^+$

were not significantly different from those of laboratory-acclimated (100 % ASW) *M. californianus* after acute exposure to 60 % ASW (Fig. 7), indicating that there was no significant loss of the primary osmolytes. When *M. californianus* were laboratory-acclimated for 4 weeks to 60 % ASW, the betaine content of the gills decreased significantly by 37 %, while taurine and  $K^+$  contents were unchanged. Taurine, betaine and  $K^+$  contents subsequently remained unchanged for at least 1 h after gills from 60 % ASW-acclimated *M. californianus* were exposed to 100 % ASW (Fig. 7).

In *M. californianus* and *M. trossolus* freshly collected from natural SW at Bodega Bay, the solute content followed a pattern similar to that for laboratory-acclimated mussels; there was no significant change in taurine, betaine or  $K^+$  contents in gill tissue exposed to 60 % SW for 1 h (Fig. 8). Over the long term, there was no significant change in osmolyte content in unfed *M. californianus* after 10 days of acclimation to 60 % natural SW (Fig. 8B); there was a significant decrease in betaine content in fed *M. californianus* acclimated to low salinity. In *M. trossolus*, there was a significant decrease in both taurine and betaine content after 10 days of acclimation to 60 % natural SW (Fig. 8A).

#### Discussion

Mussels from the genus *Mytilus* include species that are exposed to large and frequent salinity changes associated with changing tides or climatic conditions. Although one species that we studied, *M. californianus*, is most commonly found on

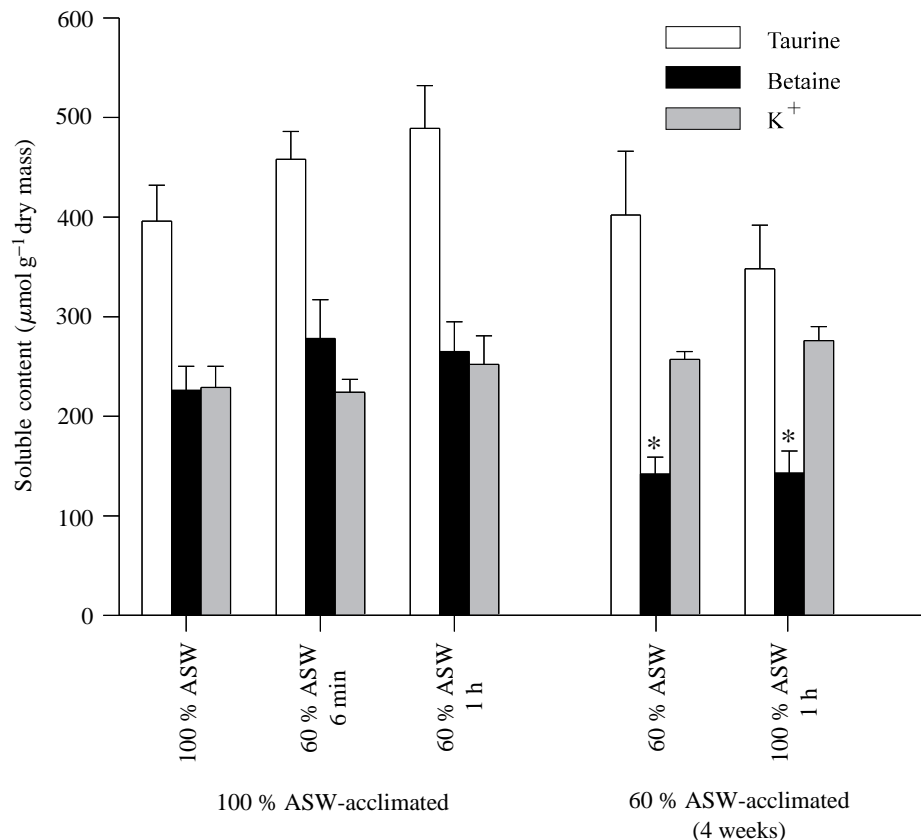


Fig. 7. Solute contents in gills of laboratory-acclimated *M. californianus* during acute exposure to ASW either hypo- or hyperosmotic to the acclimation salinity ( $N=5-7$ ). \*Solute contents were significantly different ( $P<0.05$ ) from solute contents in mussels acclimated to 100 % ASW. Values are means  $\pm 1$  S.E.M.

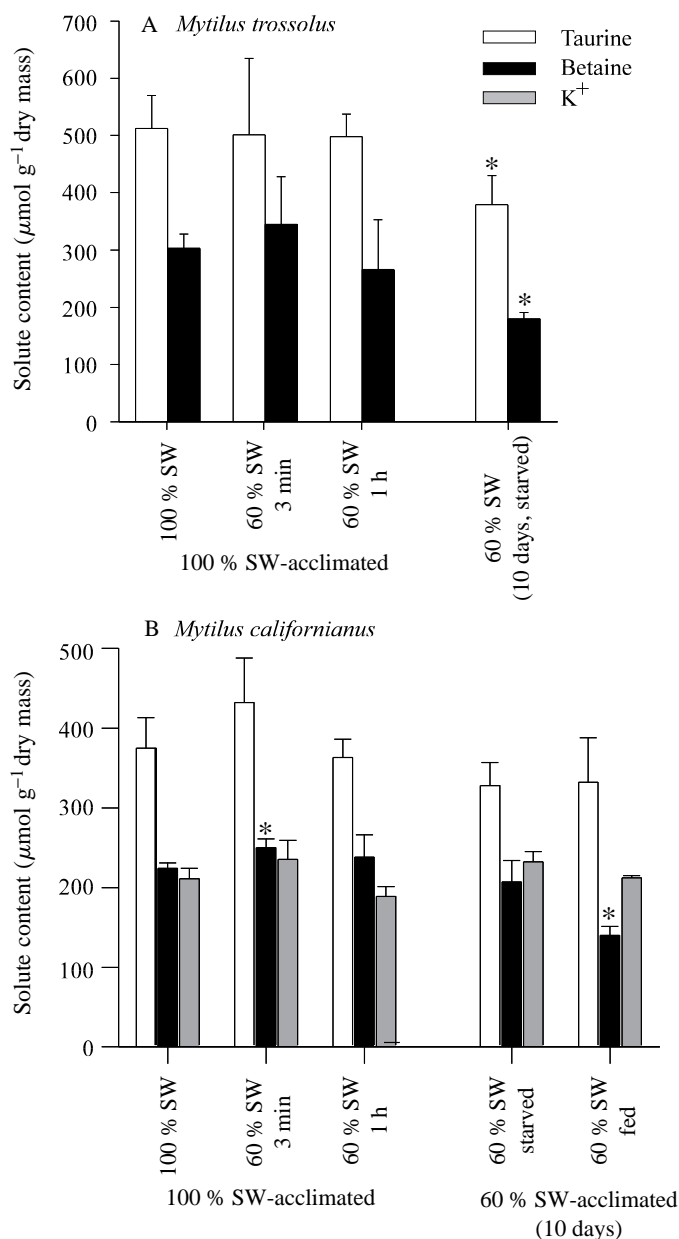


Fig. 8. Solute contents in gills of *M. trossolus* (A) and *M. californianus* (B) collected fresh from 100 % natural SW and exposed for short or long periods to 60 % natural SW ( $N=3-5$ ). 'Starved' mussels were maintained without feeding during the acclimation period. 'Fed' mussels were fed a daily ration of algae during the acclimation period. \*Solute contents were significantly different ( $P<0.05$ ) from solute contents in mussels acclimated to 100 % natural SW. Values are means  $\pm 1$  S.E.M.

the open coast where there is little change in salinity, it survives many months of exposure to low salinity in laboratory conditions (Fox *et al.* 1936; Young, 1941). The other species studied, *M. trossolus*, inhabits estuarine environments where it remains with the shell gaped open during naturally occurring salinity changes, at least to an ambient osmolality equal to 50 % sea water (Stickle and Denoux, 1976; Shumway, 1977).

Hemolymph osmolality changes with external salinity in these osmoconformers (Stickle and Denoux, 1976; Shumway, 1977), so that tissues are not buffered from the changes in ambient salinity as they are in osmoregulating animals. The cells of the gill epithelium are in direct contact with the external medium and are therefore exposed to the immediate effects of changes in ambient salinity, making the gill, in particular, a good tissue for establishing the response of cell volume to a salinity shock in these animals.

There is a large literature reporting results obtained using a wide variety of animal tissues that consistently display a volume regulatory response shortly after cell swelling or shrinkage caused by an osmotic shock (Chamberlin and Strange, 1989). This ability to regulate cell volume is found not only in animal tissues that normally experience large osmotic changes, such as the mammalian kidney medulla (Morgan, 1977) and tissues of some estuarine invertebrates (Costa *et al.* 1980), but also in tissues that are rarely exposed to aniso-osmotic media, such as proximal kidney tubules (Lohr and Grantham, 1986). Given the salinity fluctuations normally encountered by the gill tissues of estuarine mussels, we expected gill cells to demonstrate a well-developed ability to regulate cell volume. We did, in fact, routinely observe a vigorous RVD in a previous study of the response to acute exposure to dilute sea water of lateral ciliated cells from the gill of *M. californianus* (Silva and Wright, 1994). In extending those observations to include a much larger sample size we have, however, come to the conclusion that the lateral cell RVD occurs in only about 25 % of the trials. The more common response, observed in 75 % of the trials, was the lack of a significant RVD in lateral gill cells. Substantial variation in the size of the response of lateral cells to an osmotic challenge is indicated by both the present study and the previous study (Silva and Wright, 1994). It is worth emphasizing that, in the present, expanded study, we not only examined a larger sample size but also included (1) *M. californianus* collected at several times of the year from two locations (central California and Oregon), and (2) both *M. californianus* and *M. trossolus* immediately after collection from the coast of California.

Since our optical observations indicated the response to low salinity only of lateral cells (a specialized cell representing a small fraction of the entire gill), we used two additional, independent techniques to assess the response of the gill as an organ to a salinity change. The first technique, measurement of cell water space using radiolabeled tracers, indicated a lack of volume regulation in the gill as a whole; the combined water space of gill cells remained swollen or shrunken for 1 h after an acute exposure to low or high salinity, respectively. In addition, if regulation of cell volume had occurred during acute hypo- or hyperosmotic shock, there should have been an undershoot or overshoot, respectively, of cell volume when gills were returned to their control salinities after the 1 h salinity shock. Instead, cell water space returned to its control value when gills were returned to control salinity after a 1 h salinity shock, providing additional evidence that volume regulation was absent or minimal.

Following acute exposure to dilute sea water, the increase in

gill cell water content (Fig. 5) was not as large as that expected if the cells were behaving as 'perfect osmometers' (i.e. a 67% increase in cell water following a shift from 100% to 60% sea water would be predicted); cell volume increased by an average of 31% in laboratory-acclimated *Mytilus californianus*. The increase in lateral cell height (approximately 20%, Fig. 3) was likewise smaller than expected, although this is at least partially because cell height measurements do not take into account changes in the other dimensions of the cell and include both the water space and the space occupied by dry mass (Silva and Wright, 1994). Although the failure to achieve the predicted increase in cell water could be construed as an indirect indication that volume regulation had occurred, we consider this possibility to be unlikely. First, it would necessitate a rate of response that far exceeds the time course of the lateral cell RVD which, when present, has a half-time to a new steady-state cell height of 10–12 min (Silva and Wright, 1994). Second, the absence of an undershoot of cell water space following the return to 100% ASW (Fig. 5) implies that, within 6 min of the return to 100% ASW, cell solute content was equal to that of the control condition. Again, this would necessitate a rate of response, in this case the active accumulation of several hundred milliosmoles of solute per liter of cell water, that would be unprecedented. Instead, we consider it more likely that

failure to achieve an osmometric increase in cell volume is indicative of a non-ideal behavior of the cell water space, an observation that has been reported in many systems (see Garner and Burg, 1994).

An RVD or RVI must involve the loss or accumulation, respectively, of osmotically active solutes. The degree of cell volume regulation in the gill as a whole should, therefore, correlate with the degree of change in the concentrations of major solutes. As expected for a system lacking substantial volume regulation, the contents of the major osmolytes (taurine, betaine and  $K^+$ ) in *M. californianus* and *M. trossulus* gills were unchanged during an acute salinity change. All solutes were measured from catheterized gills, so that any solutes released into the blood were flushed from the vasculature during perfusion and therefore were not reflected in measurements of tissue solute contents. It is worth emphasizing that the absence of a significant solute loss from gill tissue exposed to dilute sea water was also noted in our previous study (Silva and Wright, 1994). Indeed, in acknowledging the inconsistency of this result with the presence of an RVD in lateral cells, we had suggested that the regulatory response may be restricted to only a few cell types, with the remaining cells of the gill exercising a quantitatively more limited response to a change in ambient salinity.

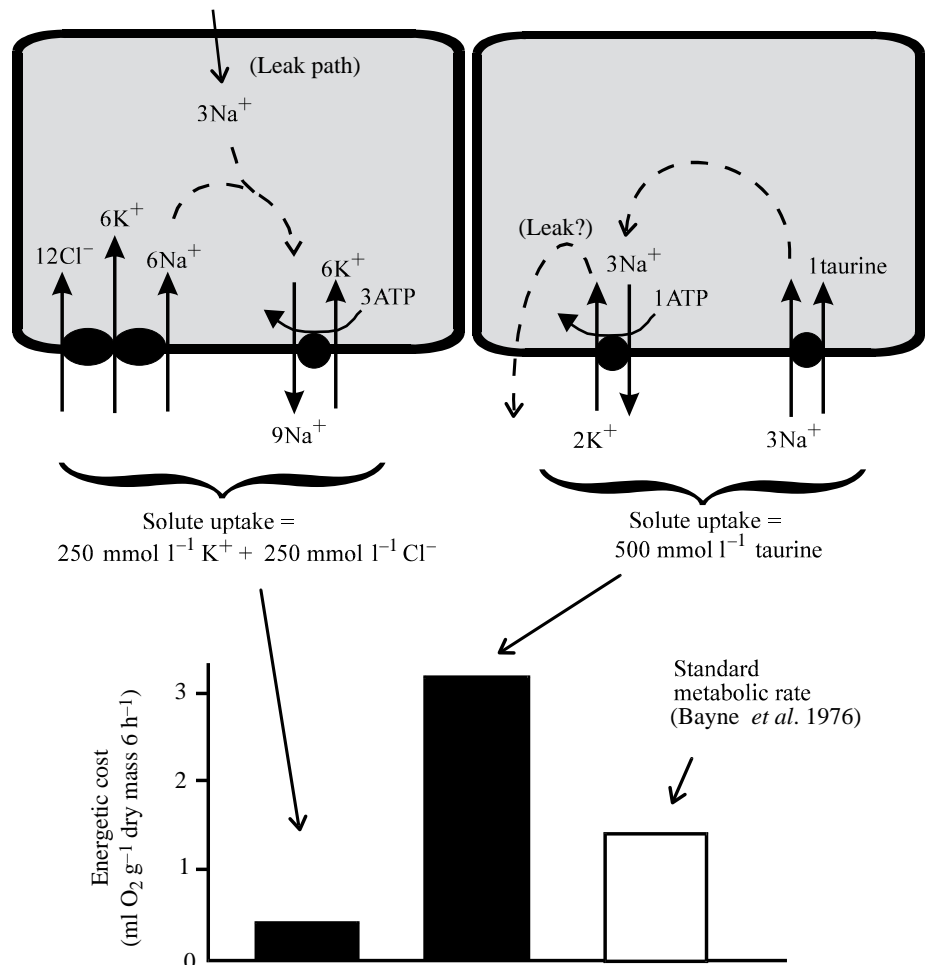


Fig. 9. Calculation of the potential energetic cost in volume-regulating tissue exposed to a 12 h sinusoidal cycle between 100% and 50% SW. The cost of solute uptake during the 6 h period of salinity increase was estimated under two scenarios: one assuming re-accumulation of  $K^+$  and  $Cl^-$  via the 'cheapest' mechanism, the other assuming re-accumulation of taurine. Conversion of solute uptake (in  $mmol\ solute\ l^{-1}\ cell\ water\ 6\ h^{-1}$ ) to energetic cost (in  $ml\ O_2\ g^{-1}\ dry\ mass\ 6\ h^{-1}$ ) was made using the following assumptions: dry mass =  $0.2\ g\ g^{-1}\ wet\ mass$ , cell water =  $0.35\ ml\ g^{-1}\ wet\ mass$ , production of 6 mmol of ATP from 1 mmol of  $O_2$ , and  $1\ mmol\ O_2 = 22.4\ ml\ O_2$ .

In the light of all the evidence accumulated to date for *Mytilus* gill, we draw two conclusions. First, both the present study and the previous study (Silva and Wright, 1994) indicate that lateral cells of the gills of *M. californianus* and *M. trossolus* contain the cellular 'machinery' required to execute a vigorous RVD following acute exposure to dilute sea water. Second, and we think more importantly, the ability to regulate volume is commonly not invoked in individual lateral cells, and any volume regulation by individual cells is typically not reflected by the gill as an organ. When viewed in terms of the entire gill, any volume regulation by individual cells appears to involve an insignificant loss of solute and, consequently, an insignificant alteration of cell volume in the gill as a whole.

There are few other direct measurements of cell size in bivalves with which our optical and radiotracer measurements can be directly compared. The most extensive evidence for RVD is in red blood cells of the clam *Noetia ponderosa*, where cell volume (measured using a Coulter counter) rapidly returns to about 50% of the control volume after an initial hypotonically induced swelling (Amende and Pierce, 1980). In contrast, data presented by Gainey (1994) for intracellular space in the posterior adductor muscle of several bivalve species (by comparing total water with extracellular space) indicate a variable response of cell volume to long-term salinity acclimation. Comparing intracellular water content with the relative osmotic concentration (see Table 3 in Gainey, 1994), cell volume regulation appears to be present in the posterior adductor muscle of *Geukensia demissus*. Cells in the posterior adductor muscle of *Mytilus edulis*, however, behaved as near-osmometers while the change in cell water space in *Modiolus modiolus* upon salinity acclimation was greater than that expected for a perfect osmometer. Comparisons of wet versus dry mass in various bivalves indicate that tissue water space follows a pattern suggestive of volume regulation, increasing after exposure to low salinity then returning towards control levels, and are cited as evidence of volume regulation in isolated tissues (Strange and Crowe, 1979; Deaton, 1990) and whole animals (Pierce, 1971; Gainey, 1978). However, changes in tissue water space may not accurately reflect the response of the intracellular water compartment alone (Gainey, 1994), since the change in total tissue water can also reflect a change in the size of the extracellular water compartment (Livingstone *et al.* 1979).

There is a larger body of literature chronicling short-term changes of solute content in various bivalves (e.g. Amende and Pierce, 1980; Bishop *et al.* 1994), but it is not clear whether the reported changes in solute content are great enough to effect a substantial change in cell volume. For instance, in *Noetia ponderosa* red blood cells, the intracellular amino acid content shows a large percentage decrease (18%) after 2 h in 50% sea water, but the starting concentration of amino acids (60 mmol l<sup>-1</sup>, calculated from the reported cell volume of the *Noetia ponderosa* red blood cells; Amende and Pierce, 1980) is relatively low and so could provide for only a small percentage of the osmotic pressure of a cell. The increases in amino acid efflux that are seen in other bivalve tissues after a

salinity decrease are also smaller than would be expected if substantial volume regulation were present (Wright *et al.* 1987; Wright and Neufeld, 1995).

The longer-term changes in osmolyte levels observed in the present study are consistent with many studies correlating long-term changes in salinity with free amino acid content in numerous bivalves (e.g. Bricteux-Gregoire *et al.* 1964; Pierce and Greenberg, 1972; Hoyaux *et al.* 1976; Livingstone *et al.* 1979; Amende and Pierce, 1980). In the present study, *M. californianus* acclimated in the laboratory to 60% ASW showed a decrease in betaine content of 84  $\mu\text{mol g}^{-1}$  dry mass compared with those acclimated to 100% ASW, which corresponds to a decrease of 0.15 ml cell water g<sup>-1</sup> dry mass (assuming 545  $\mu\text{mol}$  total solute ml<sup>-1</sup> cell water in 60% sea water). Cell water space after 4 weeks in 60% ASW was, in fact, 0.15 ml g<sup>-1</sup> dry mass smaller than the cell water space 1 h after exposure to 60% ASW. Likewise, the combined 256  $\mu\text{mol g}^{-1}$  dry mass decrease in betaine and taurine contents observed in freshly collected *M. trossolus* acclimated for 10 days to 60% natural SW is equivalent to a decrease of 0.47 ml cell water g<sup>-1</sup> dry mass. This corresponds well to the 0.46 ml cell water g<sup>-1</sup> dry mass decrease in cell water space observed over long-term acclimation to this salinity, although the difference in cell water space after 1 h and 10 days in 60% SW was not statistically significant, probably because of variability in the water space measurements. It is notable that the presence and magnitude of the change in free amino acid content during a salinity decrease is dependent on tissue (Baginski and Pierce, 1977), species (e.g. Hoyaux *et al.* 1976), population (Pierce *et al.* 1992), and acclimation to constant or fluctuating salinities (Livingstone *et al.* 1979; Shumway *et al.* 1977). Indeed, in some cases, no long-term change in amino acid content is observed after a change in acclimation salinity (Kluytmans *et al.* 1980; De Vooy, 1991).

Given the widespread occurrence of cell volume regulation in response to osmotic shocks in other tissues, it may seem surprising that the gill in an animal such as *Mytilus*, which is naturally exposed to large and regular changes in salinity, does not show a substantial degree of volume regulation. However, the absence of volume regulation may reflect the energetic cost of the processes typically involved in volume regulation. In fact, it is interesting to speculate that it may be 'too expensive' to regulate cell volume routinely. While release of solutes would occur down electrochemical gradients, solute re-accumulation during the period of salinity increase would be 'uphill' and ultimately require energetic expenditure. We calculated that the energetically cheapest alternative for uptake is by utilizing K<sup>+</sup> and Cl<sup>-</sup> via concerted action of the Na<sup>+</sup>/K<sup>+</sup>/2Cl<sup>-</sup> cotransporter, the Na<sup>+</sup>/K<sup>+</sup> ATPase and a Na<sup>+</sup> leak path (Fig. 9). If all cells in the animal regulated volume during a 12 h sinusoidal shift in salinity of the type encountered under tidal conditions, solute uptake would pose energetic demands corresponding to approximately 30% of the 'standard' metabolic rate (representative of the metabolic rate of mussels in the natural environment; Bayne *et al.* 1976). Losses and re-accumulations of organic osmolytes (i.e. taurine) pose even

greater energetic costs (Fig. 9). The use of organic compounds for short-term regulation is less likely, since large changes in solute content usually occur over a period of days (e.g. Baginski and Pierce, 1977; Livingstone *et al.* 1979; Amende and Pierce, 1980).

We do not suppose that the specific pathways or their location in the polarized epithelium of gill cells outlined in Fig. 9 necessarily represent those used for the solute flux associated with the steady-state maintenance of cell volume during repeated exposure to tidal fluctuations in salinity (although each of the pathways indicated has been observed in one or more molluscan systems). Nevertheless, the metabolic costs of moving solutes can be estimated objectively. It is our intent, therefore, to emphasize that this cost may be sufficiently large to serve as a limiting factor in defining evolutionary strategies of physiological design. This is an issue that has received comparatively little attention (Hawkins and Hilbish, 1992). The present results suggest that the nature of the physiological response to osmotic stress in bivalves should be revisited.

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