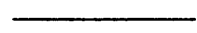


Library

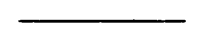
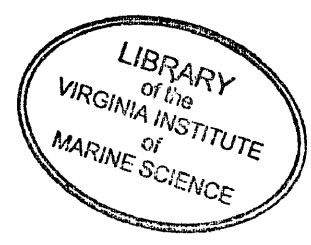
Archives  
VIMS  
Thesis  
O'Farrell  
c.2

OSMOTIC TOLERANCE AND VOLUME REGULATION IN CULTURED CELLS  
OF THE OYSTER PATHOGEN *PERKINSUS MARINUS*



A Thesis  
Presented to  
The Faculty of the School of Marine Science  
The College of William and Mary in Virginia

In Partial Fulfillment  
Of the Requirements for the Degree of  
Master of Arts



by  
Caroline L. O'Farrell  
1995

APPROVAL SHEET

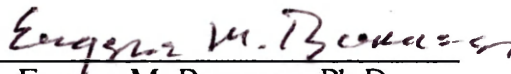
This thesis is submitted in partial fulfillment of  
the requirements for the degree of

Master of Arts

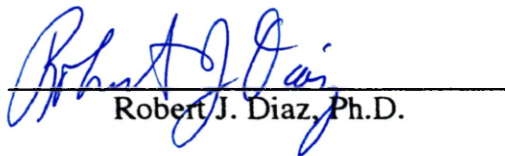


Caroline L. O'Farrell

Approved, August 1995



Eugene M. Burreson, Ph.D.  
Committee Chairman/ Advisor



Robert J. Diaz, Ph.D.



Mohamed Faisal, Ph.D., D.V.M.



Roger L. Mann, Ph.D.



Kennedy T. Paynter, Ph.D.

## TABLE OF CONTENTS

	Page
ACKNOWLEDGEMENTS.....	iv
LIST OF TABLES.....	v
LIST OF FIGURES.....	vi
ABSTRACT.....	vii
INTRODUCTION.....	2
METHODS.....	14
RESULTS.....	31
DISCUSSION.....	48
LITERATURE CITED.....	61
VITA.....	66

## ACKNOWLEDGEMENTS

I am grateful to my major professor, Dr. Gene Burreson, for his support and guidance and for introducing me to a career in fish and shellfish disease research. I also thank my committee members Dr. Bob Diaz, Dr. Mohamed Faisal, Dr. Roger Mann, and Dr. Ken Paynter for their assistance in experimental design, statistical considerations, and critical review of the thesis. I appreciate the many other individuals who assisted my research: Jerome La Peyre for providing daily consultation in cell culture and *P. marinus* expertise; Lisa Ragone Calvo for sharing extensive advice and insight; and Mark Siddall, Nancy Stokes, and Nita Walker for their help. Finally, I would like to thank my family and friends for their continual support and encouragement.

## LIST OF TABLES

Table	Page
1. Media constituents for <i>P. marinus</i> cultures acclimated to 168, 256, 341, 433, and 737 mOsm.....	16
2. Buffered artificial seawater constituents representing treatment osmolalities in osmotic tolerance experiment .....	18
3. Osmolality measurements (mOsm) and salinity equivalents (ppt) of treatment artificial seawater, acclimation media, and isotonic artificial seawater solutions.	19
4. Isotonic artificial seawater constituents for solutions isoosmotic to 168, 341, and 737 mOsm culture media and used in cell size and amino acid experiments .....	23
5. Predicted percent mortalities for osmotic tolerance experiment.....	39

## LIST OF FIGURES

Figure	Page
1. Variations in internal osmotic concentration in response to variations in external osmotic concentration of the environment.....	3
2. Microscopic images of <i>P. marinus</i> cells before and after NIH Image Analysis manipulations. ....	21
3. Mortality of <i>P. marinus</i> cells assessed with neutral red stain .....	25
4. Growth curve of <i>P. marinus</i> cultures acclimated to 168, 256, 341, 433, and 737 mOsm.....	32
5. Mean cell diameter ( $\mu\text{m}$ ) of cultured <i>P. marinus</i> cells acclimated to 168, 341, and 737 mOsm in log phase growth .....	33
6. Mean cell diameter ( $\mu\text{m}$ ) of cultured <i>P. marinus</i> cells acclimated to 168, 341, and 737 mOsm in stationary phase growth.....	35
7. <i>Perkinsus marinus</i> cells acclimated to 168, 341, and 737 mOsm culture media 7 days post-subculture.....	36
8. Percent mortality of cultured <i>P. marinus</i> cells acclimated to 168, 256, 341, 433, and 737 mOsm and placed in treatment osmolalities of 56, 135, 222, 305, 386, and 672 mOsm.....	37
9. Percent mortality of cultured <i>P. marinus</i> cells acclimated to 737 mOsm JL-ODRP-1 media with BSA and without BSA and placed in treatment osmolalities of 56, 135, 222, 395, 386, and 672 mOsm.....	40
10. Mean cell diameter ( $\mu\text{m}$ ) of <i>P. marinus</i> cells acclimated to 737 mOsm and placed in 50% hypoosmotic shock .....	41
11. Standardized mean cell diameter ( $\mu\text{m}$ ) of <i>P. marinus</i> cells acclimated to 737 mOsm and placed in 50% hypoosmotic shock.....	43
12. Standardized mean cell diameter ( $\mu\text{m}$ ) of <i>P. marinus</i> cells acclimated to 737 mOsm and placed in 50% hypoosmotic shock with experiments 1 through 5 pooled and the control experiment.....	44
13. Mean free amino acid totals (nmol) per cell in cultured <i>P. marinus</i> cells acclimated to 168, 341, and 737 mOsm .....	45
14. Mean free amino acid totals (nmol) per unit volume in cultured <i>P. marinus</i> cells acclimated to 168, 341, and 737 mOsm .....	47

## ABSTRACT

Salinity is an important environmental factor which regulates the prevalence and intensity of the oyster pathogen *Perkinsus marinus*. The effect of osmolality changes on *P. marinus* cultured cells was investigated because changes in salinity generate changes in osmolality.

Cells acclimated to the low osmolalities 168 and 256 mOsm (6.5 and 9.7 ppt) began log phase growth 4 days post-subculture while cells acclimated to the higher osmolalities 341, 433, and 737 mOsm (12.7, 16.0, and 27.0 ppt) began log phase growth 2 days post-subculture. During log phase growth, cells acclimated to the higher osmolalities 341, 433, and 737 mOsm had shorter doubling times than cells acclimated to the low osmolalities 168 and 256 mOsm. Osmotic tolerance was assessed by placing cells acclimated to 168, 256, 341, 433, and 737 mOsm into artificial seawater treatments of 56, 135, 222, 305, 386, and 672 mOsm (2.5, 5.3, 8.5, 11.4, 14.4, and 24.7 ppt) for 24 hours. During hypoosmotic shock, percent mortality was lower in groups acclimated to low osmolalities than in groups acclimated to higher osmolalities. In the hypoosmotic treatment of 56 mOsm, cells acclimated to the low osmolality of 168 mOsm had 41% mortality which differed significantly ( $p < 0.001$ ) from the 100% mortality observed for the 737 mOsm acclimated group. The groups acclimated to 341 and 433 mOsm also had 100% mortality in the 56 mOsm treatment. During the hyperosmotic shock of 672 mOsm, all of the acclimated groups had mortalities less than 10%.

The mean diameters of log phase cells acclimated to 168, 341, and 737 mOsm were 11.7, 9.7, and 8.9  $\mu\text{m}$ , respectively. The mean diameters of stationary phase cells acclimated to 168, 341, and 737 mOsm were 8.4, 4.7, and 5.1  $\mu\text{m}$ , respectively. During both log and stationary phase growth, the mean cell diameters of cells acclimated to the low osmolality 168 mOsm were significantly ( $p < 0.05$ ) larger than cells acclimated to the higher osmolalities 341 and 737 mOsm. During both log and stationary phase growth, the mean diameters of cells acclimated to 341 and 737 mOsm did not differ significantly from each other ( $p > 0.05$ ).

Cells acclimated to 737 mOsm and placed in a 50% dilution swelled and returned to baseline size (size before osmotic shock) in 5 minutes with a 13% diameter increase and a 44.5% volume increase. For cells acclimated to 168, 341, and 737 mOsm, the mean total free amino acid (FAA) content (nmol) per cell was  $2.385 \times 10^{-5}$ ,  $4.286 \times 10^{-6}$ , and  $4.957 \times 10^{-6}$ , respectively. The FAA content per unit volume for cells acclimated to 168, 341, and 737 mOsm was  $2.963 \times 10^{-8}$ ,  $9.260 \times 10^{-9}$ , and  $1.202 \times 10^{-8}$ , respectively. For cells acclimated to 341 and 737 mOsm, total FAA content per cell and per unit volume differed significantly from each other ( $p > 0.05$ ), but the 168 mOsm cells had significantly higher levels of FAA than the 341 and 737 mOsm cells ( $p < 0.05$ ). This research determined that *P. marinus* cells gradually acclimated to low osmolalities can withstand both hypo- and hyperosmotic stress. The analyses also indicated that *P. marinus* cells use volume regulatory mechanisms during changes in external osmolalities. These results help explain the fact that low salinities in the Chesapeake Bay tributaries have not eradicated the pathogen from these areas.

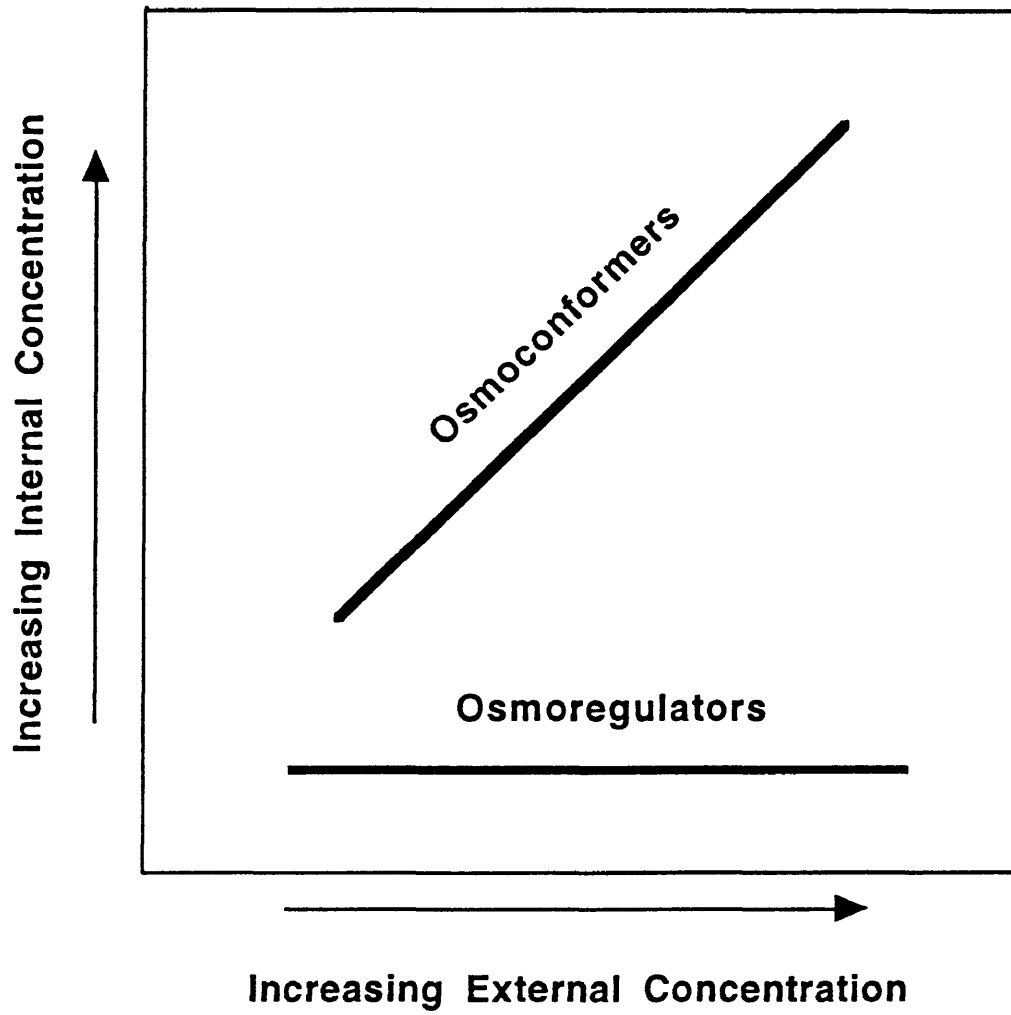
OSMOTIC TOLERANCE AND VOLUME REGULATION IN CULTURED CELLS  
OF THE OYSTER PATHOGEN *PERKINSUS MARINUS*

## INTRODUCTION

Many organisms encounter changes in their environment which cause them to employ biochemical adjustment mechanisms in order to survive. One type of variable environment is the estuarine system which is characterized by a salinity gradient as fresh, riverine input mixes with seawater. In this environment, organisms must adapt to a variety of continuously changing conditions, and to some, changes in salinity are the most important. These changes result in an external environment of variable osmotic concentration which may affect the internal osmotic concentration within an organism. When faced with this situation, an organism must either regulate its internal osmotic concentration or conform to the external environment in order to survive (Fig. 1). Osmoregulation is the process of maintaining a constant internal osmotic pressure despite changes in the external osmotic environment. Osmoconformation occurs when the osmotic pressure inside an organism varies to correspond with the changing external osmotic concentration. Most euryhaline invertebrates are osmoconformers (Pierce 1971a).

Little is known about osmoregulatory capabilities of protozoan parasites living within euryhaline invertebrates, but previous studies with both free living and parasitic protozoa have shown that protozoa have the ability to adjust their cell volumes when faced with external osmotic changes (Kaneshiro et al. 1969, Da Silva and Roitman 1982, Geoffrion and Larochelle 1984, Ahmad and Hellebust 1986, Andre et al. 1988, Cronkite and Pierce 1989, Hellebust et al. 1989, Darling and Blum 1990, and Darling et al. 1990). The eastern oyster, *Crassostrea virginica*, is an osmoconformer, but the osmotic capabilities of the parasites *Haplosporidium nelsoni* (MSX) and *Perkinsus marinus* (Dermo) living within the oyster are unknown (Ford and Haskin 1988). These two

*Figure 1.* Variations in internal osmotic concentration of organisms in response to variations in external osmotic concentration of the environment. Osmoconformers alter their internal concentration to match the environment while osmoregulators maintain a constant internal concentration despite external changes.



common oyster parasites appear to have differing tolerances to hypoosmotic conditions. Ford (1985) reported a reduced prevalence in *H. nelsoni*-infected oysters in salinities lower than 15 ppt. Ford and Haskin (1988) showed destruction of *H. nelsoni* began at 15 ppt with maximum elimination at 9 ppt suggesting that the pathogen is physiologically unable to tolerate low salinities. *Perkinsus marinus* seems to tolerate salinities lower than 12 ppt, but the mechanisms that allow *P. marinus* to tolerate low salinity environments are unknown (Ragone and Bureson 1993). The aim of this study was to examine the osmotic tolerance of *P. marinus* and to examine the process of cell volume regulation in *P. marinus in vitro*. The following literature review describes the cell volume mechanisms used by other protists since some of these strategies may also be utilized by *P. marinus*.

### **Volume Regulation**

When faced with changing salinities, organisms must maintain their ion (i.e. Na<sup>+</sup> and K<sup>+</sup>) gradient as well as their cell volume. Therefore, they must either regulate their internal osmotic concentration or conform to the osmotic concentration of the environment. External osmotic changes may cause a gain or loss of water and thus change the cell volume initially. If solute transfer then occurs, the cell volume is regulated by internal mechanisms to maintain the optimum cell volume. For example, in the horseshoe crab, cells placed in a hypoosmotic shock swelled within the first 2 hours then returned towards baseline size (size before osmotic shock) after 24 hours (Warren and Pierce 1982).

All organisms must continuously maintain their solute concentration gradients (i.e. K<sup>+</sup> and Na<sup>+</sup>) between their internal and external environments, but organisms do not accumulate solutes in direct proportion to the availability of these solutes in the external environment (Hochachka and Somero 1984). They usually keep a higher ratio of K<sup>+</sup> and Na<sup>+</sup> in their intracellular fluids, and thus the body fluids of marine osmoconformers are slightly hyperosmotic to the environment (Gilles 1979, Pierce 1982). Gradients of solutes, water, and volume constancy are maintained by the Donnan equilibrium condition which

reflects a dynamic state. In this condition, a concentration gradient may oppose an electrical gradient. The Donnan condition describes a steady state as one in which the cations inside a cell equal the diffusible plus nondiffusible anions inside and in which the cations outside the cell equal the diffusible anions outside (Prosser 1986). This principle basically explains that the inside of a cell is negative charged compared to the outside. An osmotic gradient exists in that there is a higher osmotic concentration inside the cell than outside, and there is an asymmetrical distribution of diffusible ions. This concentration gradient indicates that diffusible cations like  $\text{Na}^+$  tend to diffuse inward, and because of the osmotic gradient, water tends to diffuse inward (Prosser 1986). An energy-requiring mechanism is needed to maintain an osmotic equilibrium because of the Donnan equilibrium condition (Prosser 1973). This may be a  $\text{Na}^+/\text{K}^+$  pump and/or changes in intracellular free amino acid concentration.

Cell volume regulation occurs in response to the influx or efflux of water in hypo- and hyperosmotic conditions, respectively. In hypo- and hyperosmotic external conditions, animal cells that have flexible plasma membranes usually undergo passive swelling and even lysis, or shrinkage, respectively, unless active mechanisms are utilized (Gilles 1979). Chamberlain and Strange (1989) found that many groups of organisms have been implemented as model systems to answer specific questions about the use of osmotic mechanisms. For instance, bacteria were the focus of studies on the genetic mechanisms of volume regulation. Also, organic osmolyte analyses were performed with some invertebrates, protists, and bacteria. As a result, it has been difficult to compare and contrast volume and osmotic regulatory processes in different organisms, but much of what has been learned is applicable from one cell type to another. One direct intracellular mechanism is the prevention of osmotic flow of water by hydrostatic pressure. For example, bacteria and plant cells have a rigid cell wall that can withstand small osmotic changes by preventing water flow. Another form of a rigid cell wall is shown by the

euryhaline, marine flagellate *Chlamydomonas pulsatilla* which forms resting cysts to avoid cell volume changes (Hellebust et al. 1989).

It has been suggested that some animal cells develop a strong internal hydrostatic pressure in response to external osmotic changes which may cause contractile elements of the plasma membrane or organelles such as contractile vacuoles to assist in maintaining cell volume (Gilles 1979). The use of contractile vacuoles seems to have evolved to protect cells from experiencing lysis in hypoosmotic conditions. In low salinities, *C. pulsatilla* was seen to volume regulate and control its internal osmotic pressure by the use of contractile vacuoles, and the metabolic cost to operate such an organelle was found to be low—only 10% of the energy needed for cell growth (Hellebust et al. 1989). The utilization of contractile vacuoles is usually associated with fresh water protozoa because of the extreme hypoosmotic conditions; however, other studies have shown that the marine ciliate *Miamiensis avidus* also uses a contractile vacuole system to regulate cell volume (Kaneshiro et al. 1969).

In response to cell volume changes, cells have been observed to change in physical appearance. *Leishmania major* promastigotes, which are unicellular parasites living in the gut of the sand fly, change their shape in response to changes in the external osmotic concentration. When the osmolality of the medium was reduced by 50%, cells became shorter and more round as the cell volume increased 1.4 times in just one minute (Darling and Blum 1990, Darling et al. 1990). After several minutes, cell volume and shape returned to normal which suggested an implementation of a volume regulatory mechanism, although none was identified in these studies.

In addition to the direct cell volume regulatory abilities like contractile vacuoles, other mechanisms exist so that cells can conform to external osmotic changes. These indirect mechanisms include the regulation of intracellular osmotic effectors called osmolytes which can be inorganic ions or organic molecules. Adaptation to changes in the osmotic environment relies on the mechanism of volume control by the utilization of

different inorganic ions and organic molecules to act as compensatory osmolytes (Gilles 1988). The general classes of organic osmolytes are amino acids, polyols (glycerol), sugars, methylamines, and urea. Inorganic ions that act as osmolytes include  $\text{Na}^+$ ,  $\text{K}^+$ , and  $\text{Cl}^-$ . The osmolyte used for volume regulation may depend on its involvement in metabolic processes. Osmolytes are utilized if they do not affect the rate of protein turnover, the rate of enzyme reactions, or other important metabolic activities. Often the ease of availability of different osmolytes determine their concentration. The main criterion in utilizing osmolytes is to maintain the standard of solute compatibility; this occurs when the solute regulatory systems work to establish a compatible solute microenvironment for protein structure and function (Hochachka 1983a).

The osmolality of body fluids in animals mostly consists of inorganic ions. In the hemolymph and tissues of marine invertebrates, salts are the predominant constituent (Gilles 1979). Euryhaline invertebrates use inorganic ions and free amino acids as osmolytes, but the contribution of each depends on the species (Pierce 1982). Inorganic ion concentrations may vary with changing external osmotic conditions indicating they may play an important role in cell volume regulatory mechanisms. Studies done with the marine microalga *Brachiomonas submarina* demonstrated that with increasing salinities, the intracellular  $\text{Na}^+$  and  $\text{Cl}^-$  ion concentrations increased, and with these increases in external salinities, the internal osmolality matched the external (Ahmad and Hellebust 1986). A study with the marine ciliate *Miamiensis avidus* reported that the intracellular  $\text{Na}^+$  and  $\text{Cl}^-$  concentrations were maintained at lower concentrations than the external medium over varying salinities, but the intracellular concentrations changed nearly proportionally with the varying external osmolality (Kaneshiro et al. 1969).

Most studies of marine invertebrates have been concerned with the role of free amino acids in intracellular fluid isosmotic regulation, since the concentration of free amino acids in the tissues of marine invertebrates are higher than in fresh water invertebrates. Euryhaline molluscs are osmoconformers and when placed in diluted seawater, transient

cell swelling occurs with subsequent recovery towards the original volume (Pierce and Amende 1981). During the recovery time, there is a controlled efflux of salt and free amino acids from cells and an increase in the catabolism of the intracellular amino acids with the cells then returning to near-normal or baseline size (Hochachka 1983b). Consequently, as salinity decreases, the free amino acid concentrations in muscle also decrease (Gilles 1979). This free amino acid phenomenon has been seen in all of the invertebrate phyla, and in fish, reptiles, and several protozoans. In one study focusing on volume regulation in the axons of Chinese crabs and in the hemocytes of fish, cell volume regulation after swelling occurred in hypoosmotic conditions, but volume control was not seen in hyperosmotic conditions (Gilles 1979). Water flowed outward from the cells, tissue shrank, and the total concentration of free amino acids increased in the crab. Thus, the changes of the free amino acid content were caused by some active intracellular mechanism. Amino acids can be controlled by a change in steady state between the synthesis and degradation of these compounds, a change in amino acid and protein equilibrium, or a change in the rate of transport of amino acids through the cell membrane. During hypoosmotic shock in crustaceans, amino acids were reported to leak from the cells, and the processes of deamination and oxidation of amino acids also occurred (Gilles and Pequeux 1981).

Intracellular amino acid concentrations are controlled by two mechanisms: control of transport in and out of cells and the regulation of amino acid metabolism. In hypoosmotic stress, the turgor pressure within the cell increases, the plasma membrane's permeability increases, and there is an increase in the efflux of amino acids from the cells. In hyperosmotic conditions, there is an increase in intracellular free amino acids caused by variations in catabolic activity which can be affected by changes in intracellular content of inorganic ions. These variations in intracellular catabolic activity consequently increase the amount of free amino acids into the metabolic pool (Gilles 1979).

Studies have been performed using the marine ciliate *Paramecium calkinsi* as a model system to show the use of free amino acids as compensatory osmolytes in hypo- and

hyperosmotic conditions (Cronkite and Pierce 1989). Proline and alanine were the primary free amino acids utilized by the ciliate. Intracellular alanine increased with increasing salinities, and proline and alanine were lost from the cells to the medium with decreasing salinities. In the amoeba *Acanthamoeba castellanii* found in topsoil, an increase in the medium osmolality caused an increase in the cellular free amino acid content, especially proline. A decrease in the medium osmolality stimulated a reduction in the cellular free amino acid concentration as alanine and proline decreased (Geoffrion and Larochelle 1984). In one last example, the study by Kaneshiro et al. (1969) of the marine ciliate *Miamiensis avidus* showed that a decrease in external salinity resulted in a decrease in intracellular free amino acid levels while an increase in external salinity caused an increase in the cellular free amino acid pool. This decrease and increase in the cellular free amino acid pool was not a result of uptake, but of the mobilization of amino acids from proteins or other bound sites within the cell (Kaneshiro et al. 1969).

The widespread use of certain free amino acids as osmolytes does not necessarily indicate that these are the only compatible solutes (Hochachka and Somero 1984). Another important organic osmolyte used when organisms face osmotic changes in the environment is glycerol (Ahmad and Hellebust 1986, Andre et al. 1988). For example, it has been shown that a direct relationship between cellular glycerol and salinity may exist; as salinity increases, glycerol increases and vice versa. In the marine microalga *Brachiomonas submarina*, glycerol was determined to be the major osmoregulatory organic solute as the glycerol content increased almost in a linear fashion with the external salt concentration (Ahmad and Hellebust 1985). The alga *B. submarina* has the ability to accumulate organic solutes like polyhydric alcohol glycerol in response to increasing salinities. When faced with a changing external environment, organisms may not simply employ just one mechanism or one osmolyte to volume regulate. Pierce (1982) performed a comparative study and indicated that volume and osmotic regulatory mechanisms used by cells may be more similar than thought in that they differ in the contribution of the two solute types

(inorganic ions and organic molecules) instead of in the type of solute used. The study with the ciliate *M. avidus* showed an organism can utilize a contractile vacuole, inorganic ions, and organic molecules like free amino acids to volume regulate (Kaneshiro et al. 1969). Thus, a combination of the biochemical adaptation mechanisms previously described may be utilized when an organism encounters external changing osmolalities.

In parasitic protozoans, the ability to osmoconform may be a decisive factor for success in utilizing euryhaline invertebrates as hosts. A parasite may require a certain external osmolality in order to complete the necessary transformations between life stages or for the simple transfer to the next host species (Da Silva and Roitman 1982). For instance, an experiment with the protozoan *Cryptocaryon irritans* found in sea bream examined different salinity treatments in order to control the parasite without killing the host (Colorni 1985). It was shown that tomons (life stage following reproduction of trophonts which become encysted in substrate) could not develop to the tomite life stage (infective period after cyst ruptures) until a salinity range of 25 to 50 ppt was constantly maintained, and all tomons degenerated in salinities of 0 to 10 ppt. Therefore, although the osmoregulatory abilities and osmotic tolerances of protozoan parasites are important in themselves, this information can also be applied in the exploration of host- parasite relationships.

### ***Perkinsus marinus***

*Perkinsus marinus*, a parasite of the eastern oyster, *Crassostrea virginica*, was first discovered in the Gulf of Mexico, and at that time, it was referred to as *Dermocystidium marinum* (Mackin et al. 1950). The parasite has since been reclassified as *P. marinus* in the class Perkinsea in the phylum Apicomplexa by Levine (1978). It was placed in this class because the flagellated zoospores possess an apical complex. The class Perkinsea includes a single genus, *Perkinsus*, and four species: *P. marinus*, *P. olseni*, *P. atlanticus*, and

*P. karlssoni*; these species parasitize marine and estuarine molluscs and often cause high mortalities (Azevedo et al. 1990, McGladdery et al. 1991). *Perkinsus marinus* has been reported in *C. virginica* off the Atlantic west coast from Connecticut to Florida and along the coast of the Gulf of Mexico from Florida to Mexico (Andrews and Hewatt 1957, Mackin 1962, Burreson et al. 1994a). Since the 1950s and especially since 1986, *P. marinus* has been the major cause of death in the eastern oyster in the Chesapeake Bay (Andrews 1988).

*Perkinsus marinus* has a thick cell wall that is present in all stages within oysters. Cell wall formation seems to result partly from material in vesicles from the endoplasmic reticulum as vesicles appear to fuse with the plasmalemma and secrete material into the wall (Perkins 1969). The cell wall is fibrogranular, and vacuoplasts exist that are formed by the synthesis of dense material through the vacuole membrane. Meronts have an eccentric nucleus and large vacuole with a vacuoplast, and the plasmalemma is in close contact with the wall's internal boundary. The life cycle consists of unicellular, uninucleate, coccoid merozoites that divide by progressive cleavage and then spread throughout the oyster tissue (Perkins 1987).

Salinity is believed to be a primary environmental factor which regulates the prevalence and intensity of *P. marinus*. A study by Ray (1954) showed low salinity had a retarding effect on *P. marinus* development when oysters were artificially infected. Mackin (1956) stated that the correlation between high salinity and high prevalence of *P. marinus* resulted because fresh water inflow into estuaries diluted water borne infective elements. Consequently, Mackin (1962) indicated salinity to be an environmental factor that may delay the development of disease and suppress mortality. Likewise, Soniat (1985) suggested that inflow of fresh water may decrease the intensity and prevalence of *P. marinus* infections. In addition, studies reported that infection intensity had a positive correlation with temperature and salinity (Soniat 1985, Soniat and Gauthier 1989, and Crosby and Roberts 1990). In studies performed in the Chesapeake Bay area, salinities

below 6 ppt prevented sporulation, which is the release of free swimming, biflagellated zoospores (Chu and Greene 1989). *Perkinsus marinus* is infective in salinities greater than 15 ppt in the summer and early fall and then remains dormant during the winter. Salinities of 1 to 14 ppt did not kill the pathogen within the oyster, and development of infections did occur at 12 to 15 ppt (Andrews and Hewatt 1957). High prevalences occurred in the 1986 and 1987 drought years as high salinities and temperatures were prolonged (Andrews 1988). Ragone and Burreson (1993) determined that in *C. virginica* the critical range for pathogenicity for *P. marinus* appeared to be between 9 and 12 ppt and that *P. marinus* seemed less virulent below 9 ppt. Also, the study reported that lower salinities (6 and 9 ppt) delayed disease development while infections at higher salinities (12 and 20 ppt) increased in intensity and caused a higher level of oyster mortality.

Little is known about the osmotic tolerance capabilities of *P. marinus* when faced with hypo- and hyperosmotic stress. Few studies have been conducted on the osmotic tolerance of *P. marinus* in the absence of host influences. One experiment was performed on the osmotic capabilities of cultured *P. marinus* cells in which acclimated meronts from 22 ppt were placed in different hypoosmotic conditions; nearly all the cells in 0 and 3 ppt died while less than half died at 9 and 12 ppt (Burreson et al. 1994b). Once again the critical point was shown to be between 6 and 9 ppt. In the Australian *Perkinsus* spp. from infected blood cockles and greenlip abalone, Goggin et al. (1990) found that immersing free prezoosporangia in fresh water killed most within minutes of exposure. But cells surrounded by host tissue survived the immersion in distilled water for 2 hours (Goggin et al. 1990). Thus, host tissue seems to delay the osmotic shock to which the parasite will eventually be exposed.

Little is known about the volume regulatory mechanisms used by *P. marinus*; one possible compensatory osmolyte may be the free amino acid concentration. The free amino acid contribution of *P. marinus* to the free amino acid pool in infected oyster tissue has not been determined, but in *C. virginica*, taurine and aspartic acid concentrations increased with

an increased intensity of parasitism while the total free amino acid concentration decreased with increased parasitic intensity (Soniati and Koenig 1982). These changes in free amino acid concentration can be explained by a study (Paynter et al. 1995) in which uninfected oysters transferred from low salinities to areas of high salinities with high *P. marinus* pressure experienced large increases in glycine, taurine, and total free amino acids until the oysters acquired infections. Once infected, the levels of taurine and total free amino acids decreased in the oysters, which implied that *P. marinus* may have adversely affected the oysters' osmoconformation abilities in hyperosmotic conditions.

The primary objective of this thesis research was to determine the effect of hypo- and hyperosmotic stress on cultured *P. marinus* cells that have been acclimated to different osmolalities ranging from 168 to 737 mOsm (6.5 to 27.0 ppt). This study examined the osmotic tolerances and volume regulatory abilities by placing acclimated *P. marinus* cells in various treatment osmolalities, quantifying mortality, and measuring changes in cell size. Results should help explain the annual variability in distribution and abundance of *P. marinus* and increase the ability to predict these parameters as environmental conditions fluctuate in the future. Based on a review of the literature on cell volume regulation in protozoa, the following hypotheses were developed for *P. marinus*. First, *P. marinus* cells acclimated to low osmolalities have lower mortalities in hypoosmotic stress and higher mortalities in hyperosmotic stress than cells acclimated to high osmolalities. In general, hyperosmotic shock causes lower mortality than hypoosmotic shock. The baseline size (size before osmotic shock) of cells acclimated to low osmolalities is larger than cells acclimated to high osmolalities. Cells swell in hypoosmotic shock and then return to baseline size using volume regulatory mechanisms. Lastly, cells acclimated to high osmolalities have higher levels of free amino acids than cells acclimated to low osmolalities.

## METHODS

### Cultures

*Perkinsus marinus* cultures were initiated following methods described by La Peyre et al. (1993), and *Perkinsus-1* cultures were used in all of the following experiments. Cultures were maintained in a humid atmosphere at 28°C in a 5.0% CO<sub>2</sub> incubator. The original cultures were isolated in February 1992, maintained in JL-ODRP-1 media (La Peyre et al. 1993) (approximately 737 mOsm or 27.0 ppt), and subcultured every 2 to 4 weeks. Cells were transferred to media without bovine serum albumin (BSA) in the summer of 1993 which initiated BSA-free cultures. In January 1994, cells from these BSA-free acclimated cultures were then transferred from 737 mOsm (27.0 ppt) culture media into 168, 256, 341, and 433 mOsm (approximately 6.5, 9.7, 12.7, and 16.0 ppt) media in a gradual step-wise procedure in which cells from 737 were placed into 433, 433 into 341, etc. with the step-wise transfer occurring every 3 days. The final acclimated cultures were subcultured approximately every two to four weeks. Since the BSA-free cultures were acclimated to media of different osmolalities, they are referred to as the "acclimated cultures" or "acclimated groups." The initiation and acclimation of the cultures used in the following experiments were performed by Dr. Jerome La Peyre (Virginia Institute of Marine Science, Gloucester Point, Virginia). The growth curves for the acclimated groups were established with cells approximately 20 generations (1 year) descended from the acclimated cultures. A generation is equivalent to one subculture. Osmotic tolerance experiments used acclimated cultures which were 7-10 generations descended from the initial groups acclimated in January 1994. The cell size experiments

used cells which were approximately 25-30 generations (1 1/2 years) descended from the acclimated cultures.

## **Media**

The culture media were prepared following methods outlined by La Peyre et al. (1993) and included the same constituents as the described JL-ODRP-1 media with the exception that BSA was only added to the media in the culturing of the cells used in the BSA effect experiment. This experiment tested for the effect of BSA media (JL-ODRP-1) on osmotic tolerance following hyper- and hypoosmotic shock. Both cultures were descended from the original *Perkinsus-1* cultures isolated in February 1992, but the BSA-free cells were acclimated to BSA-free media for 2 years while the cells with BSA were acclimated to media with BSA for 3 1/2 years. The osmotic tolerance experiment, the cell size experiments, and the amino acid experiment all used the JL-ODRP-1 media without BSA (BSA-free) to maintain the cultures. Media (100 mL) was prepared prior to each subculture for the different acclimation groups equivalent to 168, 256, 341, 433, and 737 mOsm (6.5, 9.7, 12.7, 16.0, and 27.0 ppt) . In addition to the constituents described by La Peyre et al. (1993), the acclimation media also included basal synthetic sea salts, NaHCO<sub>3</sub>, and KCl (Table 1). In preparing the media, 91.5 mL (BSA-free) or 90.76 mL (BSA) of tissue culture grade (TCG) water was added first for each acclimation group, then NaHCO<sub>3</sub>, KCl, and sea salts. Then, the constituents listed by La Peyre et al. (1993) were added and the final solution filter sterilized. Media was prepared in a sterile biological hood prior to subculturing.

## **Culture Maintenance**

Subculturing occurred every two weeks in a sterile biological hood. For each acclimated group,  $5 \times 10^6$  cells were collected from culture flasks, rinsed twice in culture

Table 1. Media constituents for *P. marinus* cultures acclimated to 168, 256, 341, 433, and 737 mOsm.

media mOsm (ppt)	168 (6.5)	256 (9.7)	341(12.7)	433(16.0)	737(27.0)
TCG water (BSA) (mL)	90.76	90.76	90.76	90.76	90.76
TCG water (BSA-free) (mL)	91.5	91.5	91.5	91.5	91.5
Sea salts (g)	0.3	0.6	0.9	1.2	2.2
NaHCO <sub>3</sub> (g)	0.2	0.2	0.2	0.2	0.2
KCl (g)	0.0061	0.0079	0.0097	0.0115	0.0177

media, and transferred into new flasks with 5 mL culture media following the subculture method described by La Peyre et al. (1993). Cultures in log phase of growth were used in the osmotic tolerance experiment, the media effect experiment, the log phase cell size experiment, and the amino acid experiment. The stationary phase cell size experiment and the hypoosmotic shock cell size experiment used cells approximately 2 weeks post-subculture.

### **Growth Curves**

Growth curves were determined by taking cell counts every day for 12 days starting the day after subculture to determine the time period of log phase growth for the acclimated cultures. For each acclimated group, the amount of time required for 1 doubling was determined from the daily cell counts.

### **Treatment Seawater Solutions**

Buffered artificial seawater (ASW) solutions of 56, 135, 222, 305, 386, and 672 mOsm (approximately 2.5, 5.3, 8.5, 11.4, 14.4, and 24.7 ppt) were prepared by dissolving synthetic basal salts (Sigma Chemical Co.),  $\text{NaHCO}_3$ , and KCl in 97.5 mL of culture grade water (Table 2). After adding these constituents, the mixtures were stirred and the pH adjusted to 7.5 using 1.0 N NaOH or HCl. These solutions were then filter sterilized into 250 mL bottles. All of the treatment solutions, the BSA-free media for the acclimated cultures, and the isotonic seawater solutions (used for cell size and amino acid experiments described later) were analyzed on a vapor pressure osmometer (Wescor) to determine osmolalities (Table 3). To find equivalent ppt measurements for media and treatment seawater, seawater solutions were made with dilutions of a solution containing 5 mg artificial sea salts dissolved in 500 mL distilled water, measured with the osmometer, and a correlation of ppt vs. mOsm determined with Statview 4.01 which also provided a mOsm to ppt conversion formula of the plotted line ( $\text{ppt} = 0.461 + (0.036 * \text{mOsm})$ ). The

Table 2. Buffered artificial seawater constituents representing treatment osmolalities in osmotic tolerance experiment.

ASW mOm (ppt)	56 (2.5)	135 (5.3)	222 (8.5)	305 (11.4)	386 (14.4)	672 (24.7)
TCG water (mL)	97.5	97.5	97.5	97.5	97.5	97.5
Sea salts (g)	0.0	0.3	0.6	0.9	1.2	2.2
NaHCO <sub>3</sub> (g)	0.1176	0.1176	0.1176	0.1176	0.1176	0.1176
KCl (g)	0.0014	0.0044	0.0061	0.0078	0.0097	0.0156
HEPES (mL)	2.5	2.5	2.5	2.5	2.5	2.5

Table 3. Osmolality measurements (mOsm) and salinity equivalents (ppt) of treatment artificial seawater, acclimation media, and isotonic artificial seawater solutions.

ASW		Media		Isotonic ASW	
mOsm	ppt	mOsm	ppt	mOsm	ppt
56	2.5				
135	5.3	168	6.5	173	6.7
222	8.5	256	9.7		
305	11.4	341	12.7	365	13.6
386	14.4	433	16.0		
672	24.7	737	27.0	740	27.1

Note: -Seawater osmolality was plotted on a regression plot of ppt vs. mOsm from measured values to obtain a formula for calculating approximate ppt from measured mOsm. Equation is  $\text{ppt} = 0.461 + (0.036 * \text{mOsm})$ .

-ASW solutions were prepared buffered artificial seawater solutions used in osmotic tolerance and BSA effect experiments.

-Isotonic ASW solutions were prepared buffered artificial seawater solutions isoosmotic to culture media and used in cell size and amino acid experiments.

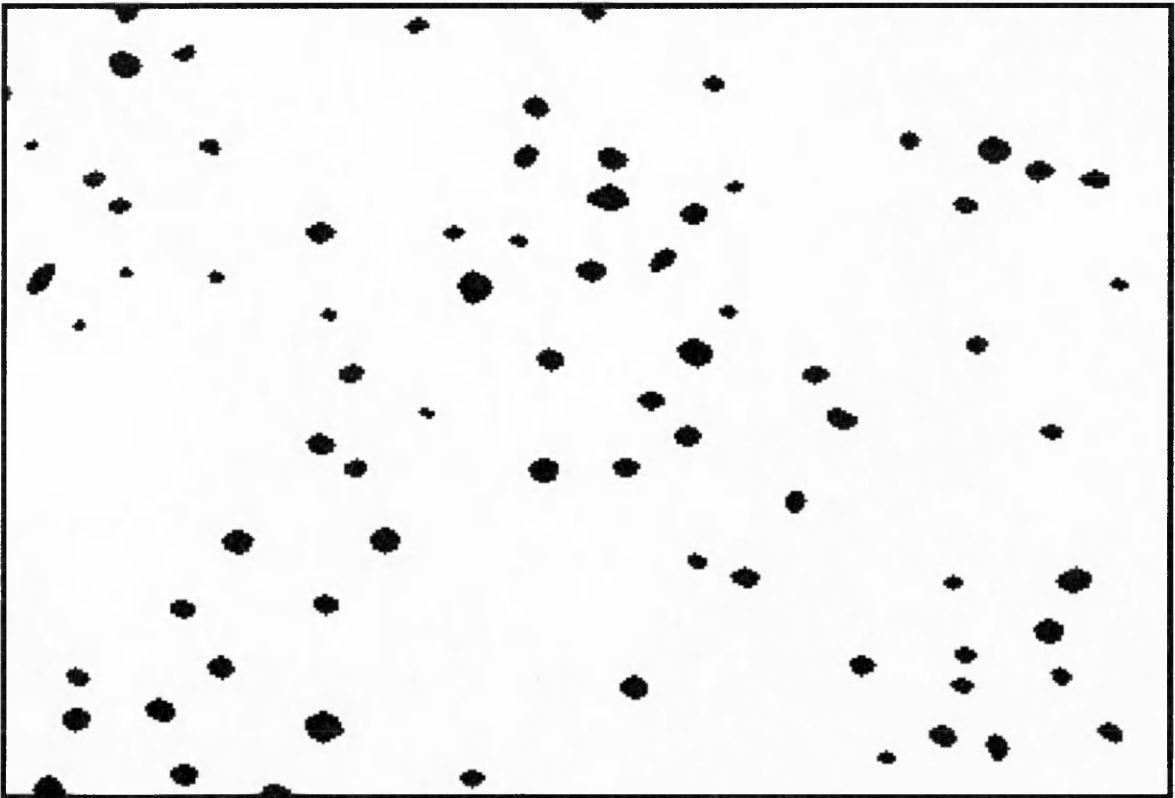
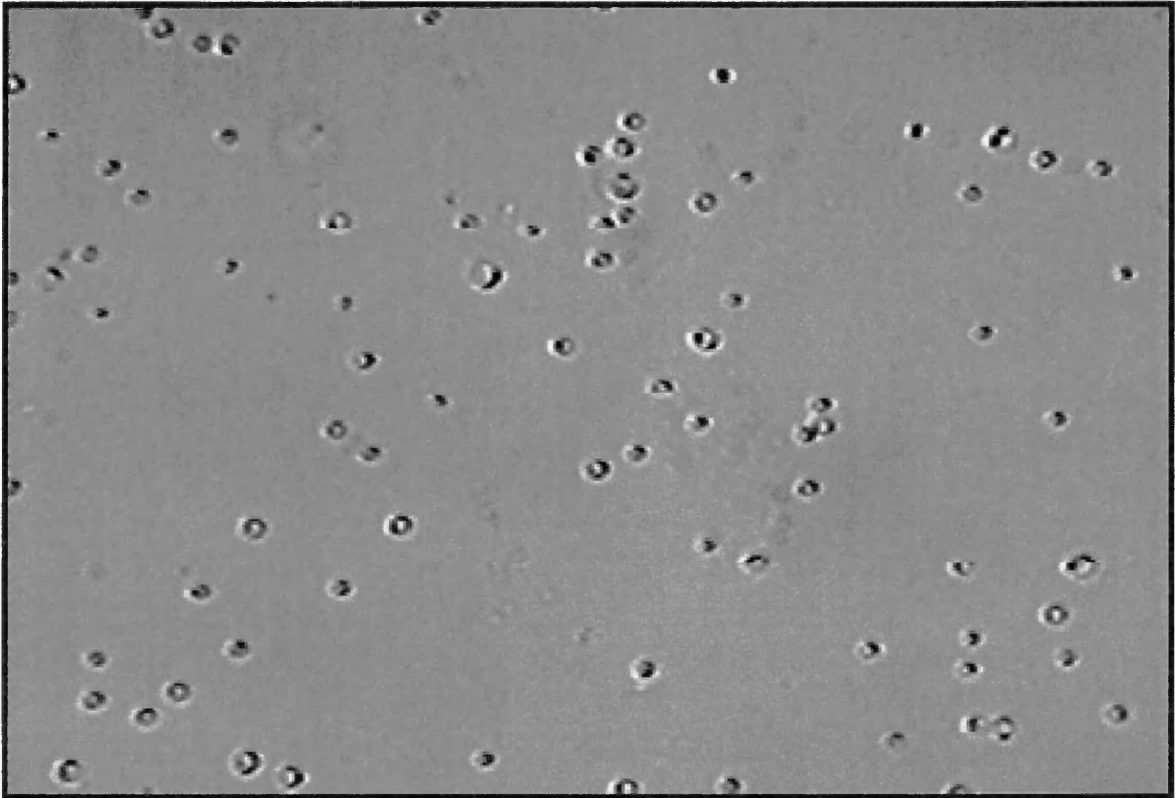
-Media was BSA-free. BSA (JL-ODRP-1) and BSA-free media had same osmolality.

plot did not go through the origin since distilled water did not measure 0.0 mOsm on the osmometer used, so the response of the instrument was then calculated into the formula. A significant correlation existed between salinity and osmolality with  $p < 0.0001$  and  $r^2 = 0.999$ .

### **Cell Size Protocol**

Cell diameters of the acclimated *P. marinus* groups in both log and stationary growth phase as well as size changes following a hypoosmotic shock were measured by using the NIH Image Analysis (Version 1.56) Macintosh computer program for particle size analysis and the MediaGrabber Macintosh program with RasterOps video digitizer board to capture live microscopic images from an inverted Zeiss light microscope (40x objective used in all of the cell size experiments). Generalized cell sizing techniques with image analysis were based on methods described by Weeks and Richards (1993). Images were grabbed as 24 bit color images with MediaGrabber; the microscope had a color video camera (Hitachi) connected to the Macintosh computer to capture live images. The images were saved as 24 bit color images in MediaGrabber until further analysis with NIH Image. To analyze the image, the 24 bit color image was converted to 8 bit gray in the image format option and saved on the desktop where it could be opened from the NIH Image program. Then, it was printed so that comparisons could be made between the manipulated computer image and the saved original image (Fig. 2). The manipulations (menu-command) were as follows: process- equalize, process- find edges, options- density slice, options- threshold, process- binary- make binary, process- binary- set count to 4, process- binary- set iterations to 5, and process- binary- close. To calculate the pixel to micrometer ratio, a stage micrometer was placed on the microscope so that an image of the micrometer with increments was grabbed. The image was measured with NIH Image with the line draw command to measure the length of an increment in pixels. Thus, 140.03 pixels were set to equal 100  $\mu\text{m}$  on the set scale command. Extraneous material in the image that did

*Figure 2.* Microscopic images of *P. marinus* cells acclimated to 737 mOsm. Top image shows before NIH Image manipulations were performed, and bottom image represents the same image after manipulations and just prior to size analysis.



not appear to be cells when compared with the original printed image was erased (eraser in toolbox). Cells that were clumped were separated by cutting and pasting; the cut command used the ellipse highlighter (toolbox). After erasing and separating, the diameters were measured by selecting the analyze particles command with the particles being labeled, outlined, ignored if touching edge, and the counter reset. Under the analyze- options commands, the type of measurement chosen was ellipse major axis to represent diameter since the cells appeared circular. Under the analyze command, show results then displayed the measurements which were copied into the Statview 4.01 program for statistical analyses.

### **Cell Sizes of Acclimated Cultures**

Baseline measurements were initially conducted to determine if the acclimated groups from 168, 341, and 737 mOsm (6.5, 12.7, and 27.0 ppt) varied in cell size. For log growth phase size distributions, cells acclimated to 168, 341, and 737 mOsm media were harvested six days after subculture and transferred to 15 mL microcentrifuge tubes. Cells were declumped by using a 3 mL syringe (25G 7/8 hypodermic needle) and repeatedly withdrawn and passed through the syringe. Cells were centrifuged at 800 x *g* for 15 minutes, the media decanted, and cells resuspended in 10 mL of isotonic seawater consisting of tissue culture water, basal synthetic sea salts, NaHCO<sub>3</sub>, KCl, and HEPES buffer (Table 4). After adding the 173, 365, and 740 mOsm isotonic artificial seawater solutions (Table 3), the cell solutions were stirred with a vortex mixer (Fisher), and a 10 μL sample withdrawn from each group for cell counts using a hemacytometer. A volume containing  $1 \times 10^5$  cells for each acclimated group was calculated, and these volumes were added from each acclimated group (168, 341, and 737 mOsm) to 3 different cell wells in a cell well plate. From each well of each of the three groups, 3 to 4 images were grabbed so that 10 images total were collected from each of the three acclimated groups. Each of these images was analyzed on NIH Image for cell diameter

Table 4. Isotonic artificial seawater constituents for solutions isoosmotic to the 168, 341, and 737 mOsm culture media and used in the cell size and amino acid experiments.

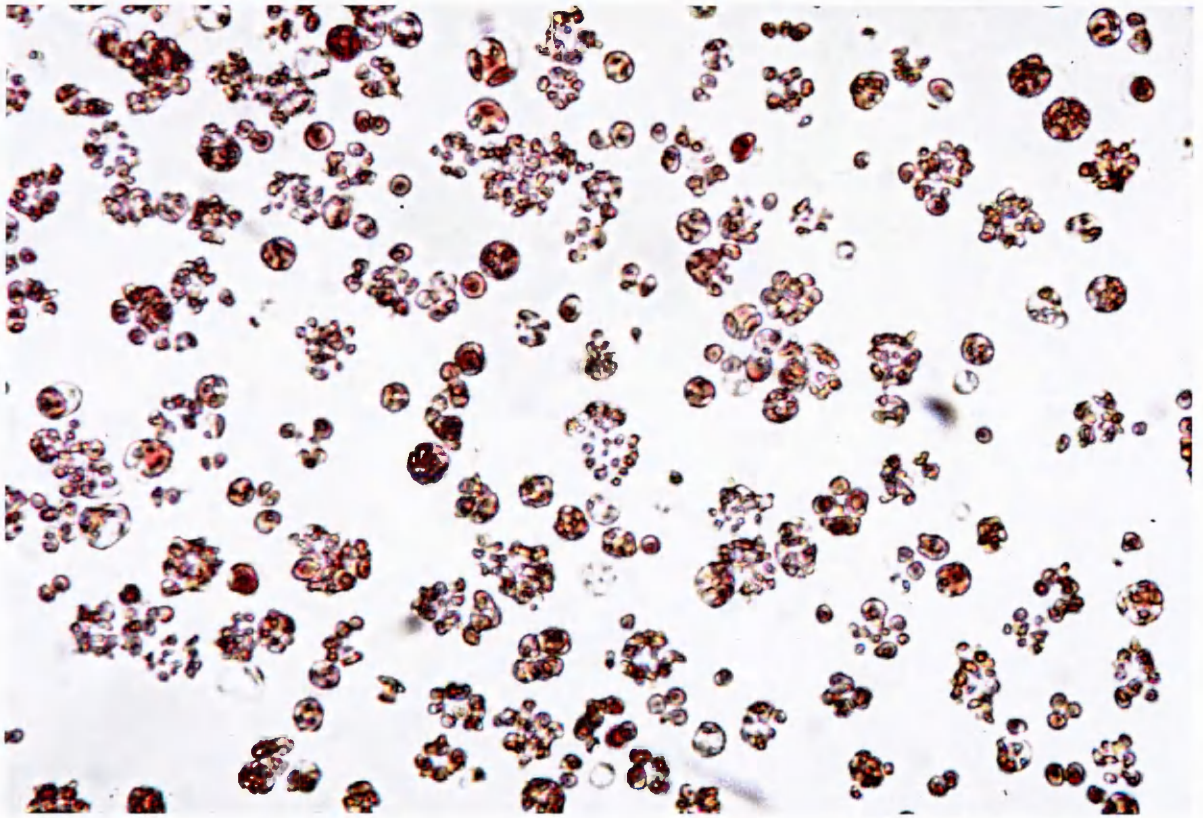
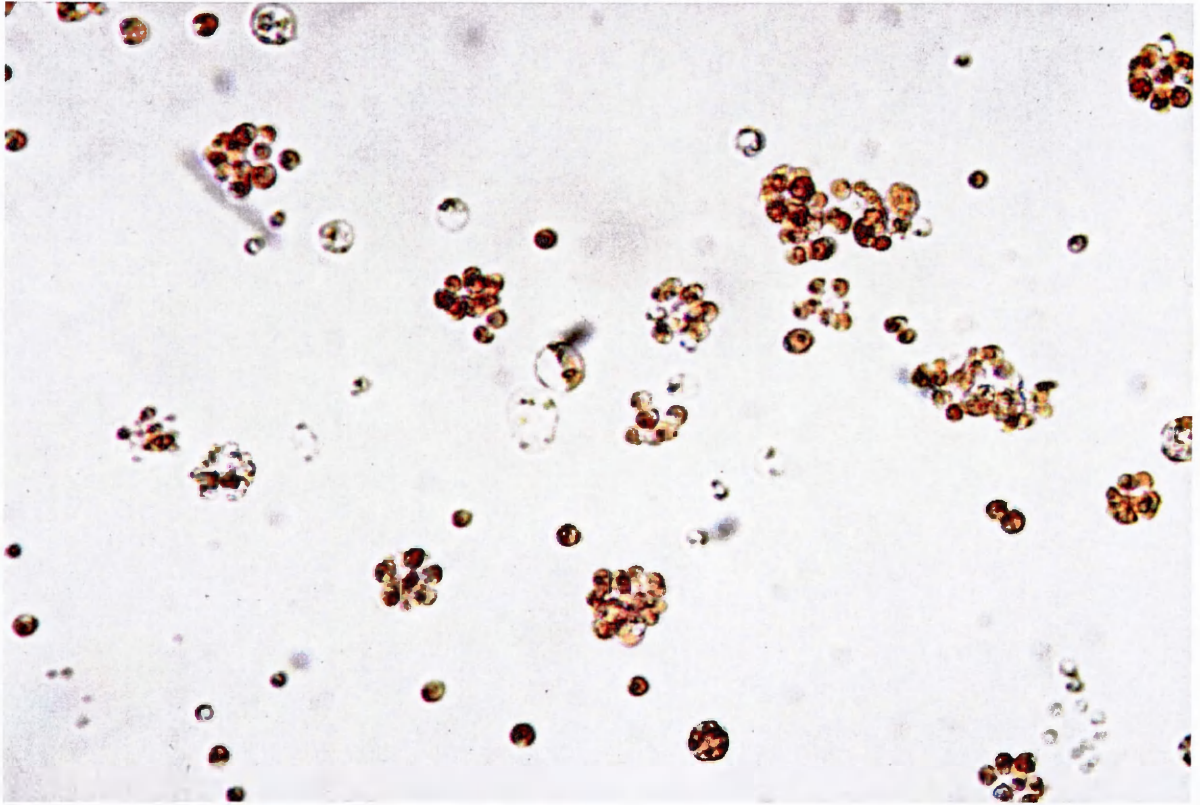
ASW mOsm (ppt)	173 (6.7)	365 (13.6)	740 (27.1)
TCG water (mL)	97.5	97.5	97.5
Sea salts (g)	0.45	1.05	2.35
NaHCO <sub>3</sub> (g)	0.2	0.2	0.2
KCl (g)	0.0061	0.0097	0.0777
HEPES (mL)	2.5	2.5	2.5

measurements, and clumped cells that had not been separated were excluded. This same protocol was followed to measure cells acclimated to 168, 341, and 737 mOsm in stationary phase growth (2 weeks after subculture). A volume of  $1 \times 10^5$  cells was added to 5 different wells from each acclimation osmolality, and a separate image was grabbed from each cell well. The cell diameters were measured with the NIH Image program.

### **Osmotic Tolerance**

Cell density and viability of the *P. marinus* cultures were assessed in each acclimation group (168, 256, 341, 433, and 737 mOsm). Cell density was determined by removing two 10  $\mu$ L aliquots and counting with a hemacytometer (Fisher) and a light microscope (Zeiss). To determine cell viability, a 100  $\mu$ L subsample was placed in a microcentrifuge tube and 10  $\mu$ L of 0.05% neutral red stain added. After 10 minutes, two 10  $\mu$ L aliquots were placed on the hemacytometer. Both live (stained) and dead (unstained) cells were counted for at least 200 cells. After the cell density was determined, the number of cells per mL and the volumes containing  $2.0 \times 10^6$  cells were calculated for each acclimation group. These volumes were added to sterile 15 mL centrifuge tubes for each acclimation group and the volumes raised to 7 mL with the treatment ASW at the osmolality equivalent to the media osmolality. Then, 1 mL of each of these cell suspensions was centrifuged at  $470 \times g$  for 5 minutes. The supernatant was decanted and the pellet resuspended in 1 mL of each of the treatment osmolalities (ASW) in a 24-well tissue culture plate. Thus, acclimated cells from 168, 256, 341, 433, and 737 mOsm (6.5, 9.7, 12.7, 16.0, and 27.0 ppt) media were placed in ASW treatment osmolalities of 56, 135, 222, 305, 386, and 672 mOsm (2.5, 5.3, 8.5, 11.4, 14.4, and 24.7 ppt) for 24 hours in 24-well microtiter plates at 28°C in a non-CO<sub>2</sub> incubator. After the 24 hour incubation, 100  $\mu$ L of neutral red was added to 2 wells at a time and gently mixed with a clean pipette tip. Live cells absorbed the stain while dead cells were clear (Fig. 3). Mortality was assessed by counting live and dead cells in 2-3 random grid fields with an inverted light microscope

*Figure 3.* Mortality of *P. marinus* cells assessed with neutral red stain; stained cells were live and unstained cells were dead. These cells were acclimated to 168 mOsm (6.5 ppt) and placed in 56 mOsm (2.5 ppt) (top) and 672 mOsm (24.7 ppt) (bottom) for 24 hours in the osmotic tolerance experiment.



(Zeiss) with a 10x10 mm ocular micrometer grid. Percent mortality was determined by dividing the number of dead cells by the total number of cells counted. This experiment was repeated three times.

### **BSA Effect**

Due to the fact that the results obtained for the osmotic tolerance experiment differed from the previous *in vitro* osmotic tolerance study (Burreson et al. 1994b), the osmotic tolerance experiment was repeated with cells from media with and without BSA. Thus, the same protocol as the osmotic tolerance experiment was followed using cells acclimated to 737 mOsm BSA media (referred to as JL-ODRP-1 media) and cells acclimated to 737 mOsm BSA-free media. These cells were placed in the same osmolality treatments as described above for 24 hours, and mortality was determined with neutral red to assess whether the BSA constituent affected osmotic tolerance. Three groups of BSA and three groups of BSA-free cells were used (each group from a different flask for both media types).

### **Cell Size After Hypoosmotic Shock**

Cell diameter changes following a hypoosmotic shock were measured with MediaGrabber and NIH Image Analysis systems. Cells acclimated to 737 mOsm (27.0 ppt) were harvested 2-3 weeks after subculture (stationary growth phase), declumped with a 3 mL syringe (25G 7/8 hypodermic needle), and centrifuged at  $800 \times g$  for 15 minutes. The media was decanted, and isotonic seawater was added to obtain a volume of 10 mL. After using a hemacytometer for a cell count, a volume containing  $1 \times 10^5$  cells was added to a cell well. A volume of 173 mOsm artificial seawater was added to the well to result in a 50% dilution of the original seawater solution. Before adding this calculated volume of hypoosmotic shock solution, an image was grabbed to represent time 0 or before the shock. Ten to twenty seconds after adding the shock, a second image was grabbed as

time 1. Images were then grabbed at 1, 3, 5, 7, 10, 12, 15, 20, 30, 45, and 60 minutes after the shock for the first replicate. The microscope was left untouched throughout the experiment so that the same cells from the same plane were captured as images and thus the same population experiencing the shock would be represented. These images were analyzed with NIH Image to determine cell diameters at each time interval. This experiment was repeated five times. The first replicate used cells 19 days post-subculture. The second replicate used cells from a different culture 18 days post-subculture and included time points of 0, 10-20 seconds, 1, 3, 5, 10, 15, 20, and 30 minutes. The third, fourth, and fifth replicates used cells 20 days post-subculture and were performed consecutively on the same day, with cells from the same flask, and the time zero was recorded only at the start of replicate five. Replicates three, four, and five included images grabbed at 10 to 20 seconds, 1, 3, 5, 10, 15, 20, and 30 minutes. The control experiment used cells 21 days post-subculture and followed the protocol described above without adding the shock solution. Seawater was not added to the cells in the well, and images were grabbed at 0, 1, 3, 5, 10, 15, 20, and 30 minutes. In a separate experiment, cell viability was assessed with the vital stain neutral red before the shock and 30 minutes after adding the shock solution.

### **Amino Acid Content**

Acclimated cells from 168, 341, and 737 mOsm (6.5, 12.7, and 27.0 ppt) were analyzed for intracellular free amino acid content on an automated amino acid analyzer (Beckman, System Gold) using methods described by Paynter et al. (1995). The cells used for these analyses were seeded  $5 \times 10^7$  per 50 mL of media when subcultured. Two samples of cells acclimated to 168 mOsm containing  $1.77 \times 10^8$  and  $1.23 \times 10^8$  cells, respectively, were collected and frozen in liquid nitrogen 4 days after subculture. A sample acclimated to 341 mOsm of  $2.2 \times 10^9$  cells was harvested and frozen 7 days after subculture, and another sample from 341 mOsm of  $1.04 \times 10^9$  cells was harvested and

frozen 4 days after subculture. A sample from 737 mOsm media of  $1.27 \times 10^9$  was harvested and frozen 7 days post-subculture, and another sample from 737 mOsm of  $1.23 \times 10^9$  cells was harvested and collected 4 days post-subculture. These volumes of cells were analyzed as separate samples on the amino acid analyzer.

First, the cells were harvested from their culture flasks, placed in 50 mL centrifuge tubes, and centrifuged at  $800 \times g$  for 20 minutes. The media was decanted and the cell pellets transferred to 15 mL centrifuge tubes. Then, the pellet solutions were raised to 10 mL with isotonic artificial seawater and centrifuged at  $800 \times g$  for 20 minutes. The seawater was decanted, 10 mL of isotonic seawater added, and the solution centrifuged again at  $800 \times g$  for 20 minutes. The seawater was decanted and a cell count determined with a hemacytometer (Fisher). Then, the cells were centrifuged once again at  $800 \times g$  for 20 minutes, the seawater decanted, and the pellets transferred to microcentrifuge tubes after adding 500  $\mu$ L of isotonic seawater. The microcentrifuge tubes were centrifuged at  $800 \times g$  for 20 minutes and the seawater decanted so that only the cell pellets remained. These tubes were placed in liquid nitrogen to freeze and then were stored in a  $-90^\circ\text{C}$  freezer.

After 6 freeze-thaw cycles which included transferring the samples back and forth between  $-20^\circ\text{C}$  freezer to  $4^\circ\text{C}$  refrigerator, 500  $\mu$ L of distilled  $\text{H}_2\text{O}$  were added to each of the 6 samples (2 each from 168, 341, and 737 mOsm acclimated cultures) which were then sonicated for 5 seconds a total of three times. The samples were placed in the  $-20^\circ\text{C}$  freezer, removed after frozen, allowed to thaw, and again sonicated for 5 seconds each of 3 times. The samples were then centrifuged for 15 minutes at  $16000 \times g$ . To each microcentrifuge tube, 500  $\mu$ L of 95% EtOH was added and the tubes shaken to mix. With the caps open and a marble placed on top of each tube, the samples were placed in a  $100^\circ\text{C}$  water bath for 15 minutes. The samples were then centrifuged again at  $16000 \times g$  for 15 minutes. The supernatant was decanted into Erlenmeyer flasks and placed in a deep freezer ( $-80^\circ\text{C}$ ) for 30 minutes. Then, the flasks were placed in a lyophilizer overnight. The next day lithium buffer was added to dissolve the residue. The samples were then

placed in the automated amino acid analyzer and 20  $\mu\text{L}$  subsamples analyzed from each of the 6 samples.

### Statistical Analyses

In the osmotic tolerance experiment, logistic regression analyses with SAS proc catmod and logistic procedures were utilized to examine the response of the population (acclimated group) to the treatment osmolality and to calculate predicted mortalities with 95% confidence intervals for each of the acclimated groups at each treatment osmolality. A logistic regression model was chosen to represent the binary response of mortality (live versus dead). The actual live and dead cell counts were used to calculate percent mortalities and in a comparison of proportions from independent samples test described by Fleiss (1981). This comparison test was used to determine if the 168 mOsm population experienced a significantly different mortality response in the 56 mOsm treatment solution than the 737 mOsm acclimated group. In the cell size of acclimated cultures study, mean cell diameters were calculated, and the effect of media or acclimation osmolality on cell diameter was shown by a one-way analysis of variance. Significant differences between populations were determined by using the Tukey-Kramer *post hoc* multiple comparison test. The results from the cell sizes after hypoosmotic shock study were analyzed with the nonparametric Kruskal-Wallis test to first examine the effect of the grouping variable experiment. Then, to separate out the effect of experiment, a mean center standardization was used by subtracting from each data point the mean cell diameter (from each experiment). A second Kruskal-Wallis test was run on the standardized data to examine if experiment had a significant effect on cell diameter. Then, the experiments were pooled and a third Kruskal-Wallis used to determine if time had a significant effect on cell diameter. Lastly, the Tukey-Kramer multiple comparison *post hoc* analysis with a  $p < 0.05$  significance level was implemented to determine at which time points the mean cell diameters were significantly different from each other. In the amino acid content study,

mean total free amino acid content was calculated on both a per cell and a per unit volume basis. To test for a significant effect of the media or acclimation osmolality on total free amino acid content, a one-way analysis of variance was utilized. Significant differences in total free amino acid content between populations were determined with the Tukey-Kramer *post hoc* multiple comparison test.

## RESULTS

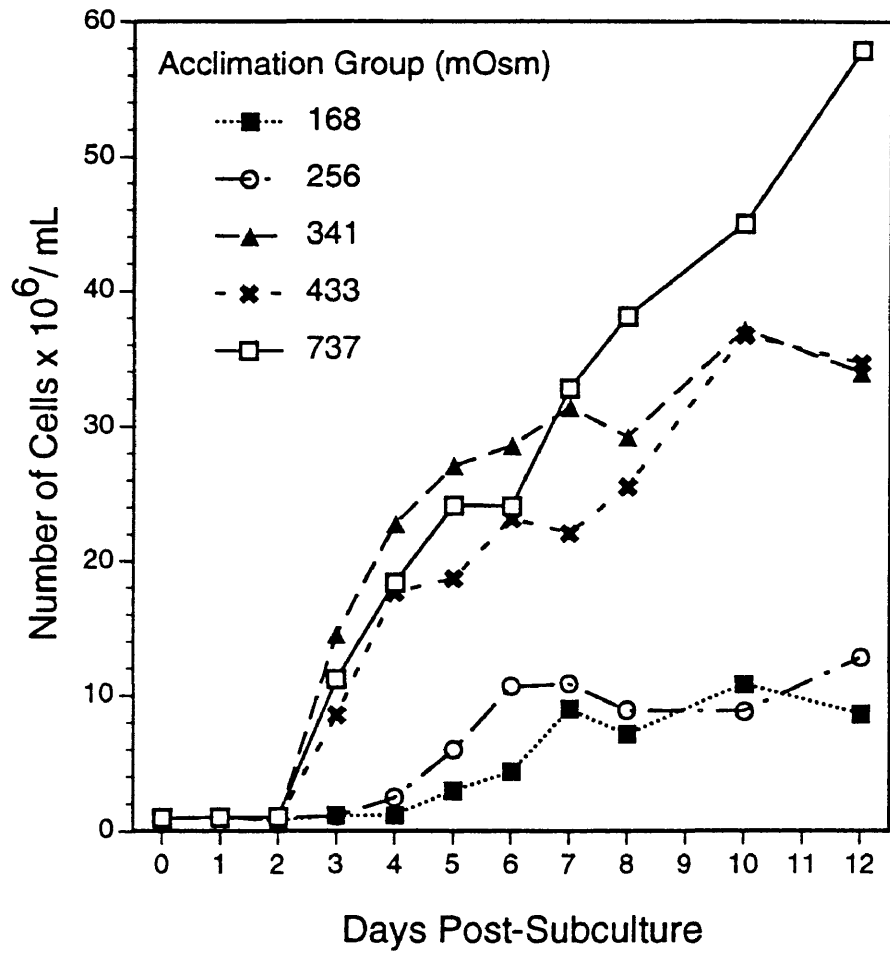
### Growth Rate

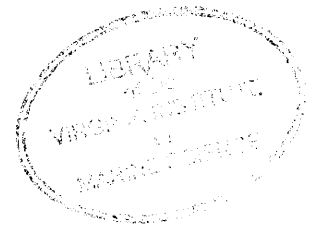
The results of the growth rate study indicated that the time of log phase growth began approximately 2 days post-subculture for *P. marinus* cultures acclimated to 341, 433, and 737 mOsm (12.7, 16.0, and 27 ppt) culture media; in 168 and 256 mOsm (2.5 and 5.3 ppt) media, cultures began log phase growth approximately 4 days post-subculture (Fig. 4). The groups acclimated to the higher osmolalities of 341, 433, and 737 mOsm had shorter doubling times compared to the groups acclimated to the low osmolalities of 170 and 256 mOsm. For the 168 mOsm cells, 35.2 hours were required for 1 doubling and 35.7 hours for the 256 mOsm cells. For the higher osmolality cells from 341, 433, and 737 mOsm, 1 doubling required 22.8, 25.9, and 24.4 hours, respectively. During log phase, cells acclimated to 168 and 256 mOsm appeared to divide by slowly growing as a large parent cell with numerous smaller cells inside that were then released. Cells acclimated to the higher osmolalities 341, 433, and 737 mOsm appeared to follow more of a binary fission type of multiplication with single cells dividing at a fast rate.

### Cell Sizes of Acclimated Cultures

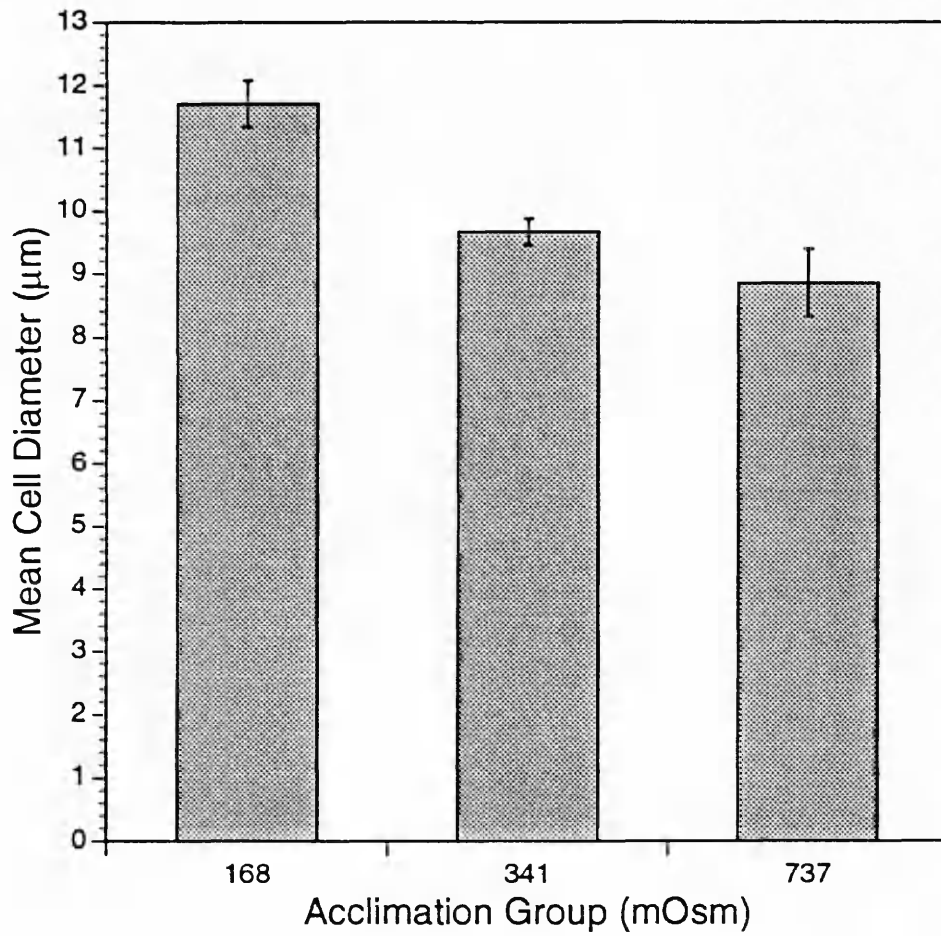
The mean diameters of log phase *P. marinus* cells acclimated to 168, 341, and 737 mOsm (6.5, 12.7, and 27.0 ppt) were 11.7, 9.7, and 8.9  $\mu\text{m}$ , respectively (Fig. 5). A one-way ANOVA showed that acclimation osmolality had a significant effect on diameter ( $p=0.0001$ ). A *post hoc* analysis demonstrated that at the  $p<0.05$  level of significance, the

*Figure 4.* Growth curve of *P. marinus* cultures acclimated to 168, 256, 341, 433, and 737 mOsm (6.5, 9.7, 12.7, 16.0, and 27.0 ppt ) and seeded  $5 \times 10^6$  cells/ mL.





*Figure 5.* Mean cell diameter ( $\mu\text{m}$ ) of cultured *P. marinus* cells acclimated to 168, 341, and 737 mOsm (6.5, 12.7, and 27.0 ppt) in log phase growth. Error bars = standard error.



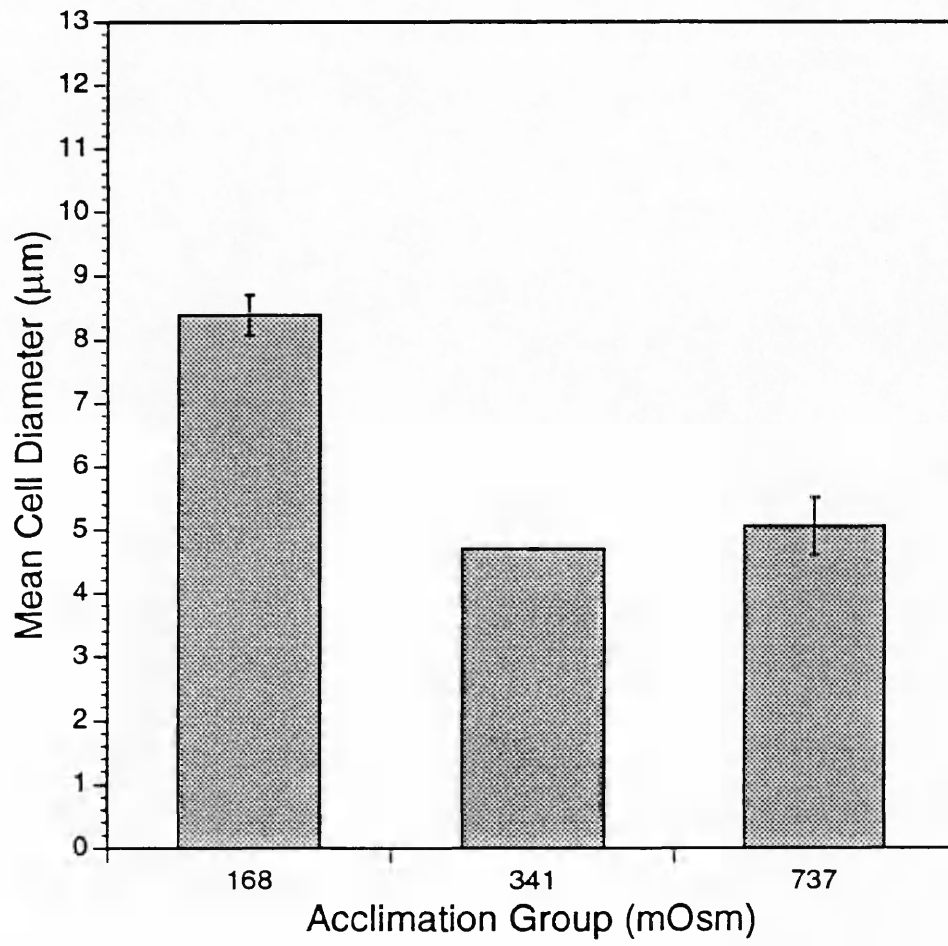
168 and 341 mOsm as well as the 168 and 737 mOsm acclimation groups were significantly different from each other in diameter (168 mOsm were significantly larger), but the 341 and 737 mOsm groups did not differ significantly in diameter. The mean diameters of stationary phase *P. marinus* cells acclimated to 168, 341, and 737 mOsm were 8.4, 4.7, and 5.1  $\mu\text{m}$ , respectively (Fig. 6). A one-way ANOVA indicated that the acclimation osmolality had a significant effect on diameter ( $p=0.0003$ ). A *post hoc* analysis at a  $p<0.05$  significance level showed that the 168 and 341 mOsm as well as the 168 and 737 mOsm groups were significantly different from each other in diameter, but the 341 and 737 mOsm groups did not differ significantly in diameter (Fig. 7). Cells acclimated to 168 mOsm appeared to have a thicker cell wall and a larger vacuole than cells acclimated to higher osmolalities.

### Osmotic Tolerance

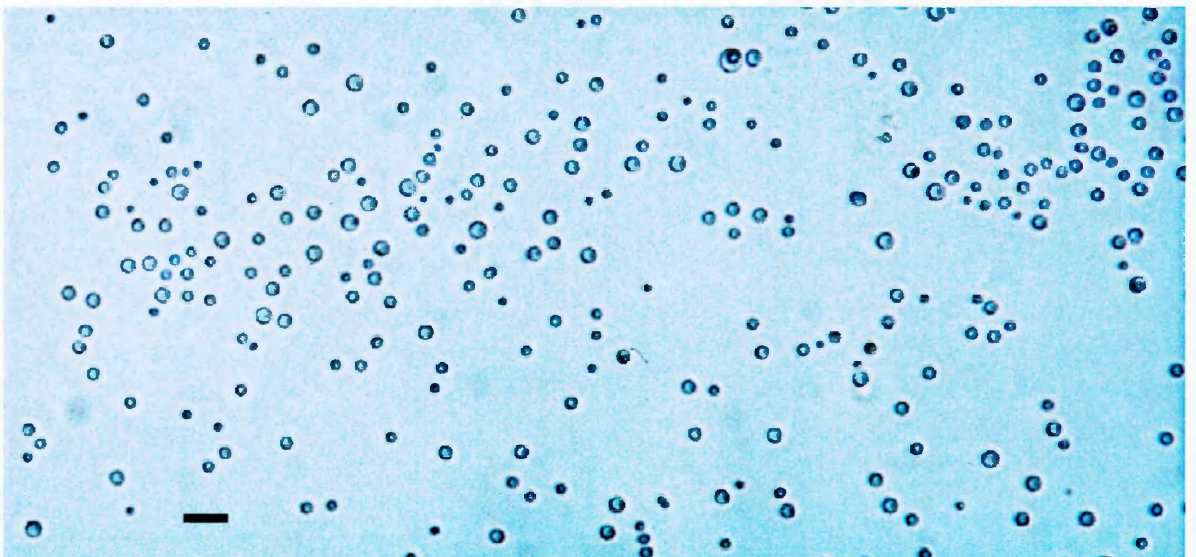
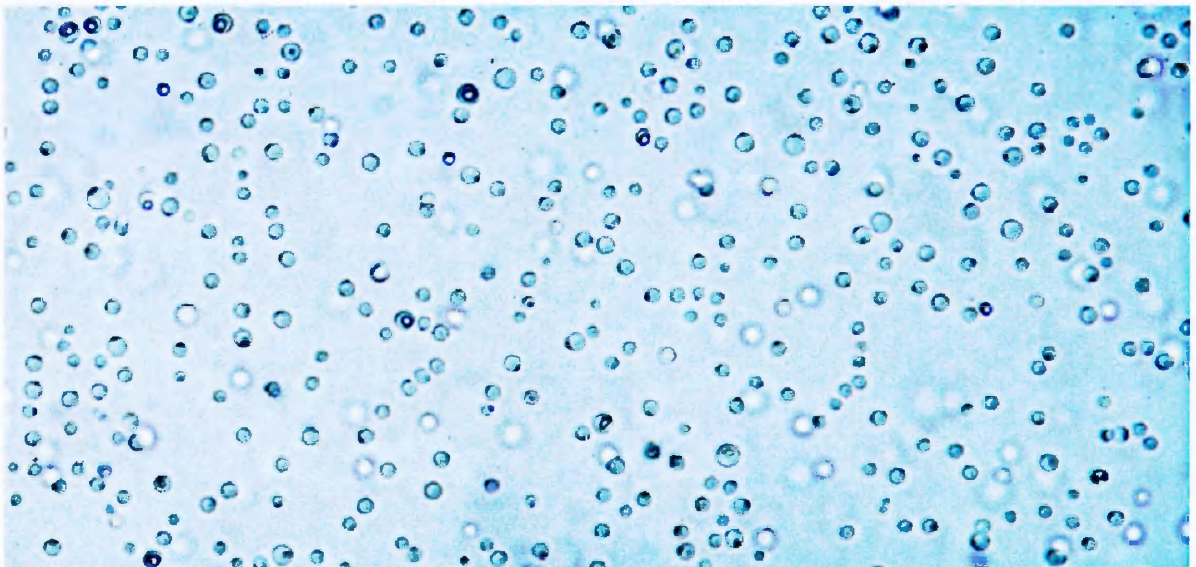
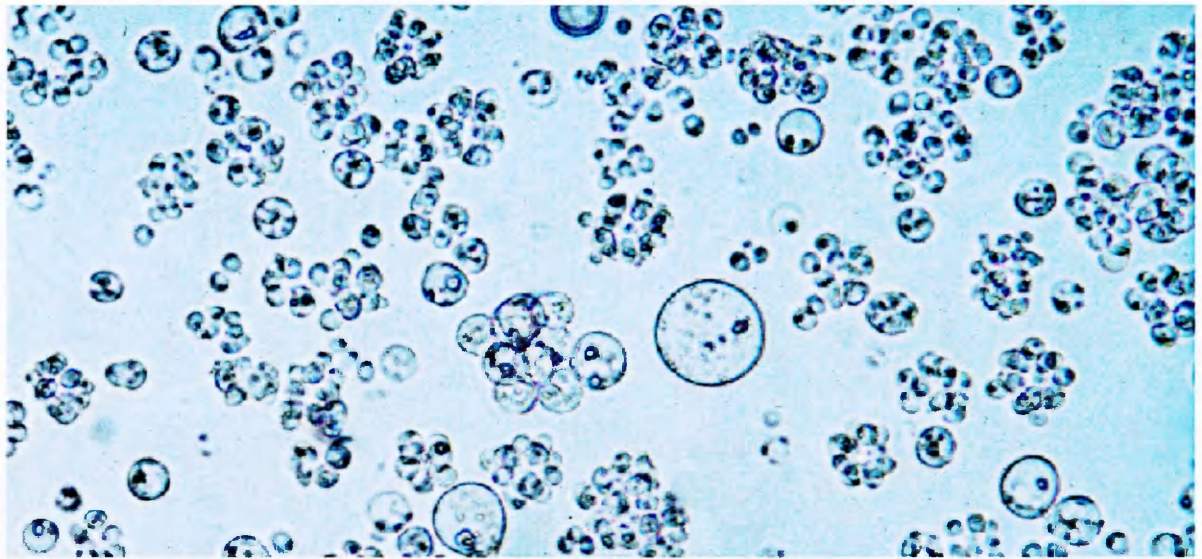
The mean viabilities of the cultures acclimated to 168, 256, 341, 433, and 737 mOsm (6.5, 9.7, 12.7, 16.0, and 27.0 ppt) prior to osmotic shock were 88.2%, 96.2%, 99.1%, 99.3%, and 98.8%, respectively. The results of the osmotic shock experiment showed that during hypoosmotic shock, the percent mortality was lower in groups acclimated to low osmolalities than in groups acclimated to higher osmolalities (Fig. 8). For example, in the extreme hypoosmotic shock of 56 mOsm (2.5 ppt), cells acclimated to an osmolality of 168 mOsm had 41% mortality; this mortality was significantly different ( $p<0.001$ ) from the 100% mortality observed for the 737 mOsm group at the 56 mOsm treatment osmolality. The groups acclimated to 341 and 433 mOsm also had 100% mortality in the 56 mOsm treatment. Conversely, during the hyperosmotic shock of 672 mOsm (24.7 ppt), groups acclimated to low osmolalities as well as high osmolalities all experienced mortalities of less than 10% (Fig. 8).

Predicted mortalities from logistic regressions indicated that in low osmolality

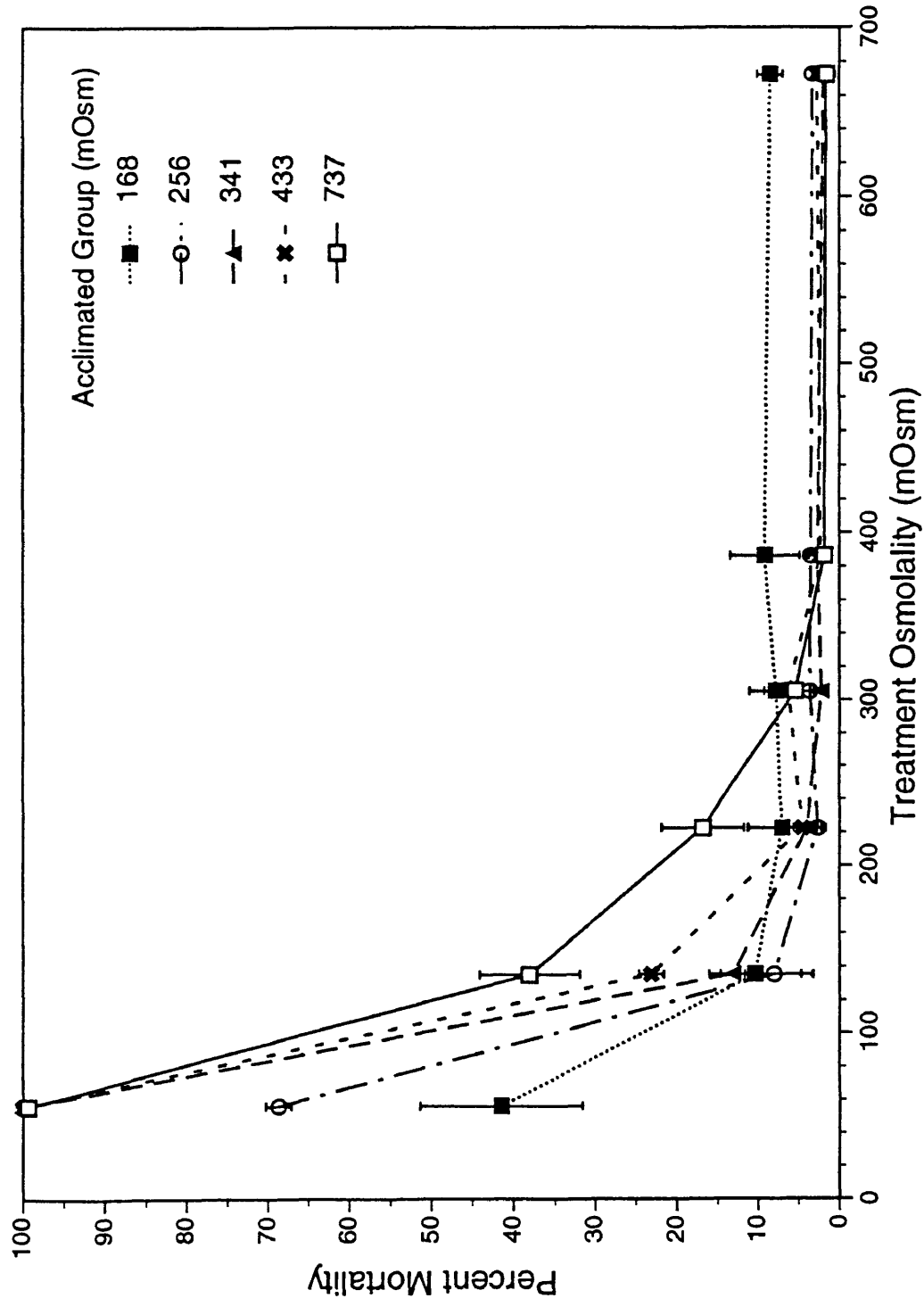
*Figure 6.* Mean cell diameter ( $\mu\text{m}$ ) of cultured *P. marinus* cells acclimated to 168, 341, and 737 mOsm (6.5, 12.7, and 27 ppt) in stationary phase growth. Error bars = standard error.



*Figure 7. Perkinsus marinus* cells acclimated to 168 mOsm (top), 341 mOsm (middle), and 737 mOsm (bottom) culture media 7 days post-subculture. Bar = 10  $\mu$ m and refers to all cells.



*Figure 8.* Percent mortality of cultured *P. marinus* cells acclimated to 168, 256, 341, 433, and 737 mOsm (6.5, 9.7, 12.7, 16.0, and 27.0 ppt) and placed in treatment osmolalities of 56, 135, 222, 305, 386, and 672 mOsm (2.5, 5.3, 8.5, 11.4, 14.4, and 24.7 ppt) for 24 hours. Error bars = standard error.



treatments the groups acclimated to 168 and 256 mOsm had lower mortality than the groups acclimated to 341, 433, and 737 mOsm (Table 5). For example, in the treatment osmolality of 56 mOsm, the 168 mOsm population predicted mortality was 30.6%, and for the 737 mOsm population, it was 87%, an increase of almost 3 times. In the 672 mOsm treatment, the predicted mortalities for all of the acclimated groups was less than 5%.

### **BSA Effect**

Both the groups acclimated to media with BSA (JL-ODRP-1) and media without BSA (BSA-free) showed similar percent mortalities during osmotic shock (Fig. 9). For example, in the 56 mOsm (2.5 ppt) treatment, percent mortality for both the BSA-free and JL-ODRP-1 groups was 100%, and in the 672 mOsm (24.7 ppt) treatment, the BSA-free had 2.2% mortality and the JL-ODRP-1 had 1.5% mortality. Both of the cultures had the same significant relationship ( $p < 0.0001$ ) of mortality as a function of osmolality as determined by logistic regression. At the 95% confidence level, overlapping confidence intervals for both the slope and intercept values indicated that the media types were not significantly different.

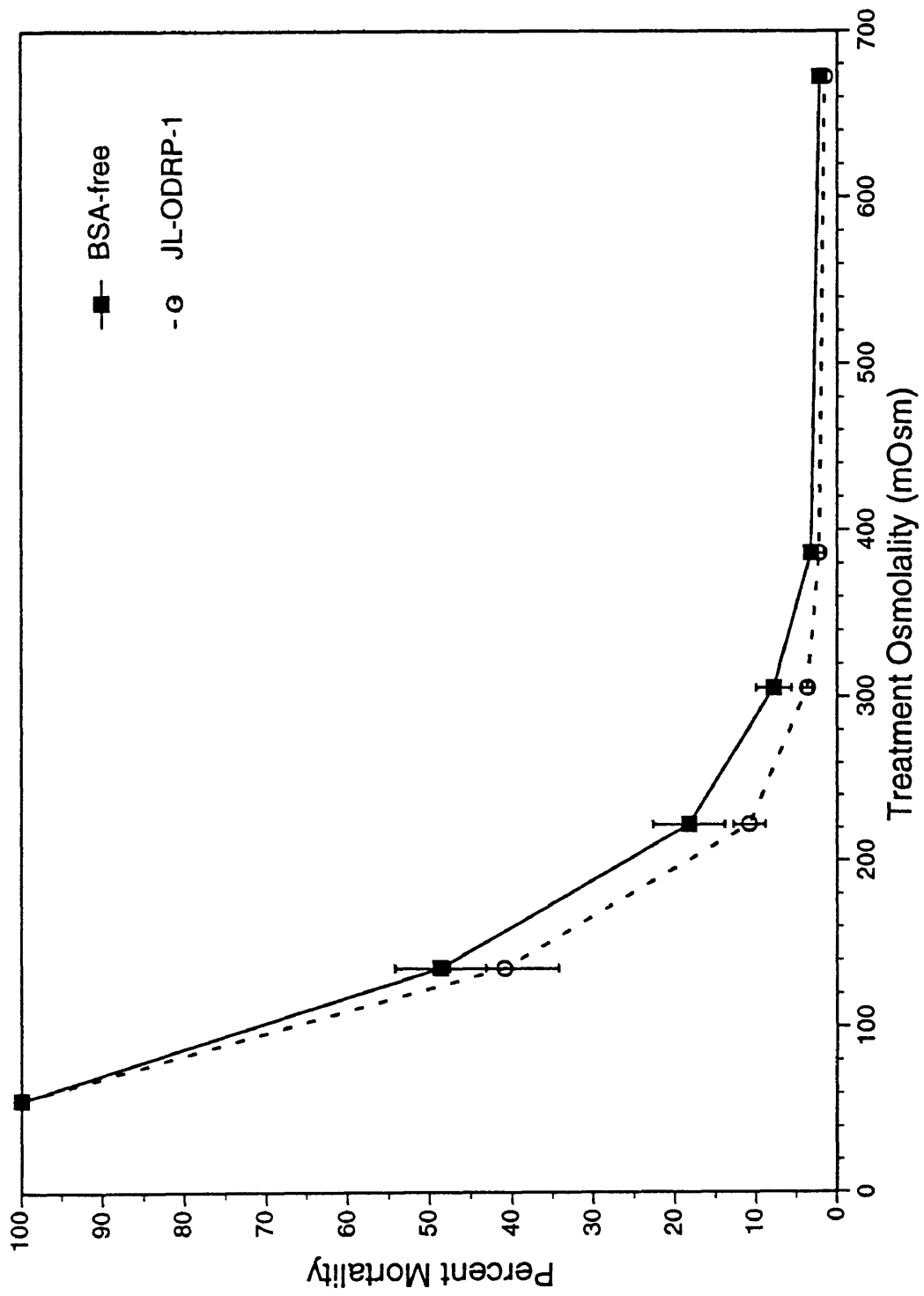
### **Cell Size After Hypoosmotic Shock**

The results of the viability test indicated a 99% viability at time 0 before the shock and 97% viability 30 minutes after the shock. The initial results of experiments 1 through 5 showed variable results with no significant trend; missing values and outliers were included in the initial analysis as equal data points (Fig. 10). For example, values at time 0 were not obtained for experiments 3, 4, and 5. The data point at 5 minutes for experiment 4 was 4.2  $\mu\text{m}$  and appeared to be an outlier. A definite size change was difficult to establish with each experiment considered separately. From the first nonparametric analysis, it was difficult to examine the effect of osmolality on cell size because of the variability between experiments; experiment had a significant effect on cell diameter ( $p < 0.0001$ ). After

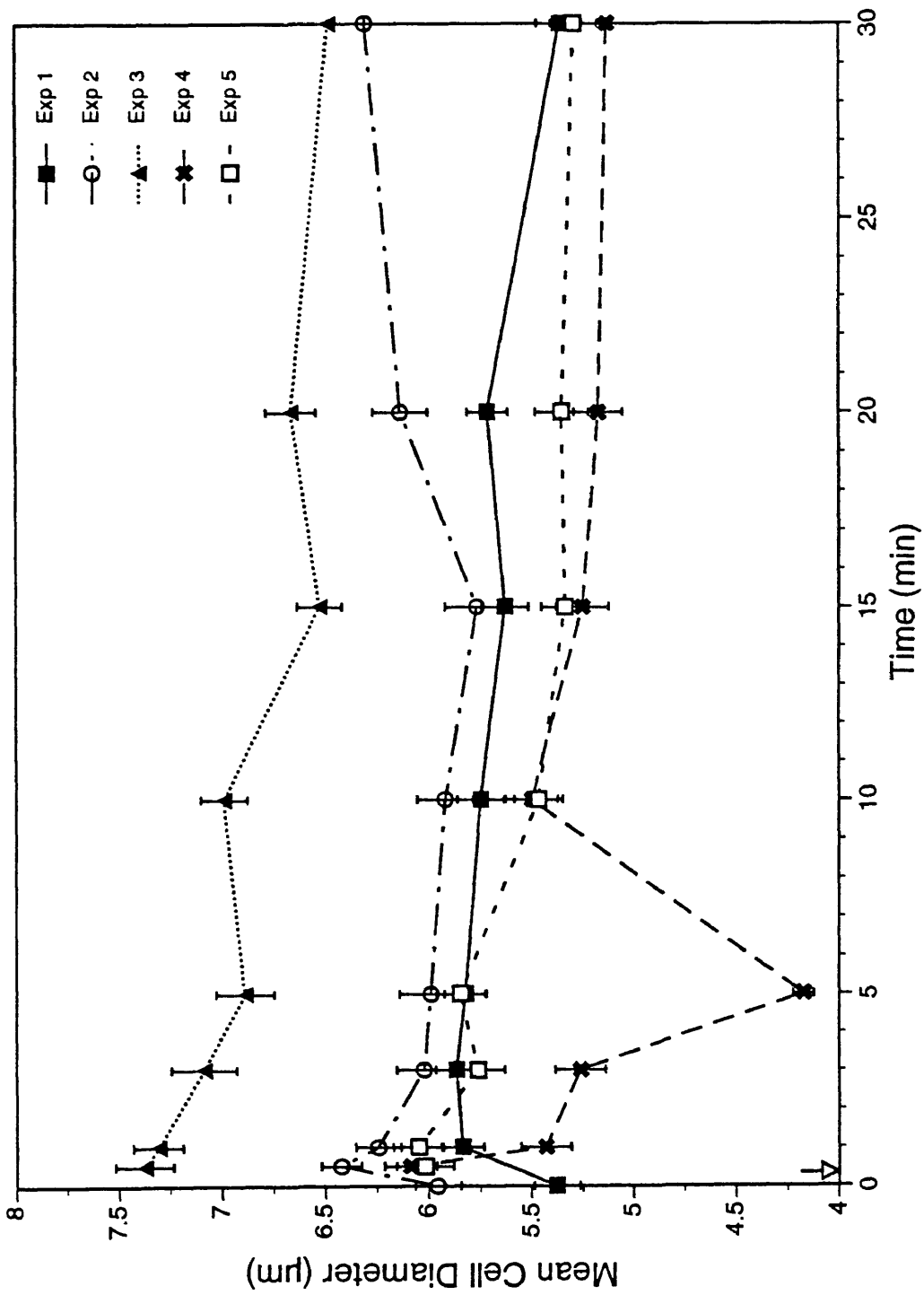
Table 5. Predicted percent mortalities of *P. marinus* with 95% confidence intervals denoted by upper (UL) and lower (LL) limits calculated for the osmotic tolerance experiment.

Acclim. (mOsm)		Treatment Osmolality (mOsm)					
		56	135	222	305	386	672
	LL	28.6	21.4	15.3	11.9	7.9	1.8
168	% Mort.	30.6	22.6	16.2	12.8	8.9	2.4
	UL	32.6	23.8	17.2	13.8	9.8	3.0
	LL	50.0	22.4	7.2	3.1	0.8	0.008
256	% Mort.	52.5	23.8	8.1	3.7	1.1	0.016
	UL	55.1	25.3	9.1	4.3	1.3	0.024
	LL	84.5	33.3	3.9	0.7	0.055	<0.001
341	% Mort.	86.2	35.5	4.6	1.0	0.085	<0.001
	UL	87.9	37.7	5.3	1.2	0.114	<0.001
	LL	81.9	40.9	9.0	2.5	0.35	<0.001
433	% Mort.	83.7	42.9	9.9	3.0	0.45	<0.001
	UL	85.4	44.9	10.9	3.4	0.55	<0.001
	LL	85.5	51.4	15.1	5.0	0.82	0.002
737	% Mort.	87.0	53.4	16.4	5.7	1.0	0.003
	UL	88.5	55.4	17.7	6.4	1.2	0.004

*Figure 9.* Percent mortality of *P. marinus* cells acclimated to 737 mOsm (27.0 ppt) JL-ODRP-1 media with BSA (JL-ODRP-1) and 737 mOsm (27.0 ppt) JL-ODRP-1 media without BSA (BSA-free) and placed in treatment osmolalities 56, 135, 222, 305, 386, and 672 mOsm (2.5, 5.3, 8.5, 11.4, 14.4, and 24.7 ppt) for 24 hours. Error bars = standard error.



*Figure 10.* Mean cell diameter ( $\mu\text{m}$ ) of *P. marinus* cells acclimated to 737 mOsm (27.0 ppt) and placed in 50% hypoosmotic shock (arrow). Replicate experiments 1 through 5 are shown. Arrow indicates actual time of shock; time represents time following shock. Error bars = standard error.

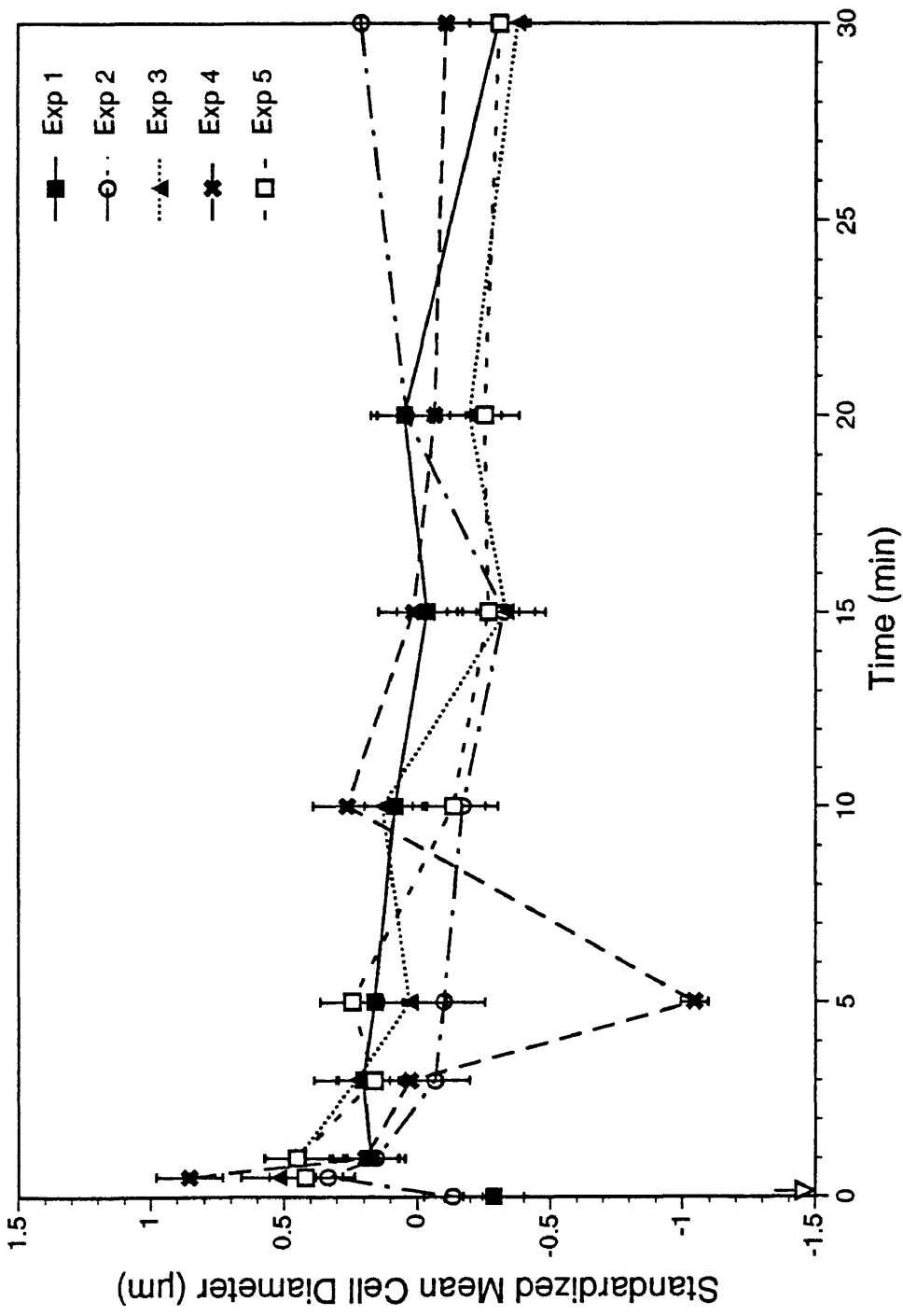


implementing a mean center standardization by subtracting the overall mean diameter of each experiment from each value in that experiment, the responses of each experiment were not as variable (Fig. 11). Nonparametric analysis after standardization indicated that experiment did not have a significant effect on cell diameter ( $p=0.8976$ ). Since the effect of experiment was no longer significant, the experiments were pooled, and the overall response to hypoosmotic shock indicated an initial swelling followed by a return to baseline size (Fig. 12). When placed in the 50% dilution treatment, acclimated *P. marinus* cells from 737 mOsm experienced an initial swelling between time 0 and time 1 which was approximately between 0 and 30 seconds following hypoosmotic shock. Cells swelled and returned to baseline size in about 5 minutes. The mean diameter change during swelling was 0.7  $\mu\text{m}$ . Since the initial mean cell diameter was 5.7  $\mu\text{m}$ , the percent diameter increase during initial swelling was approximately 13% which was a 44.5% change in cell volume. The last nonparametric analysis on the pooled, standardized data showed that time had a significant effect on cell diameter with a tied p-value of  $<0.0001$ . The *post hoc* analysis with a  $p<0.05$  level of significance indicated that significant differences existed between the following time points: 0 and  $<30$  seconds,  $<30$  seconds and 5 minutes,  $<30$  seconds and 15 minutes,  $<30$  seconds and 20 minutes, and  $<30$  seconds and 30 minutes.

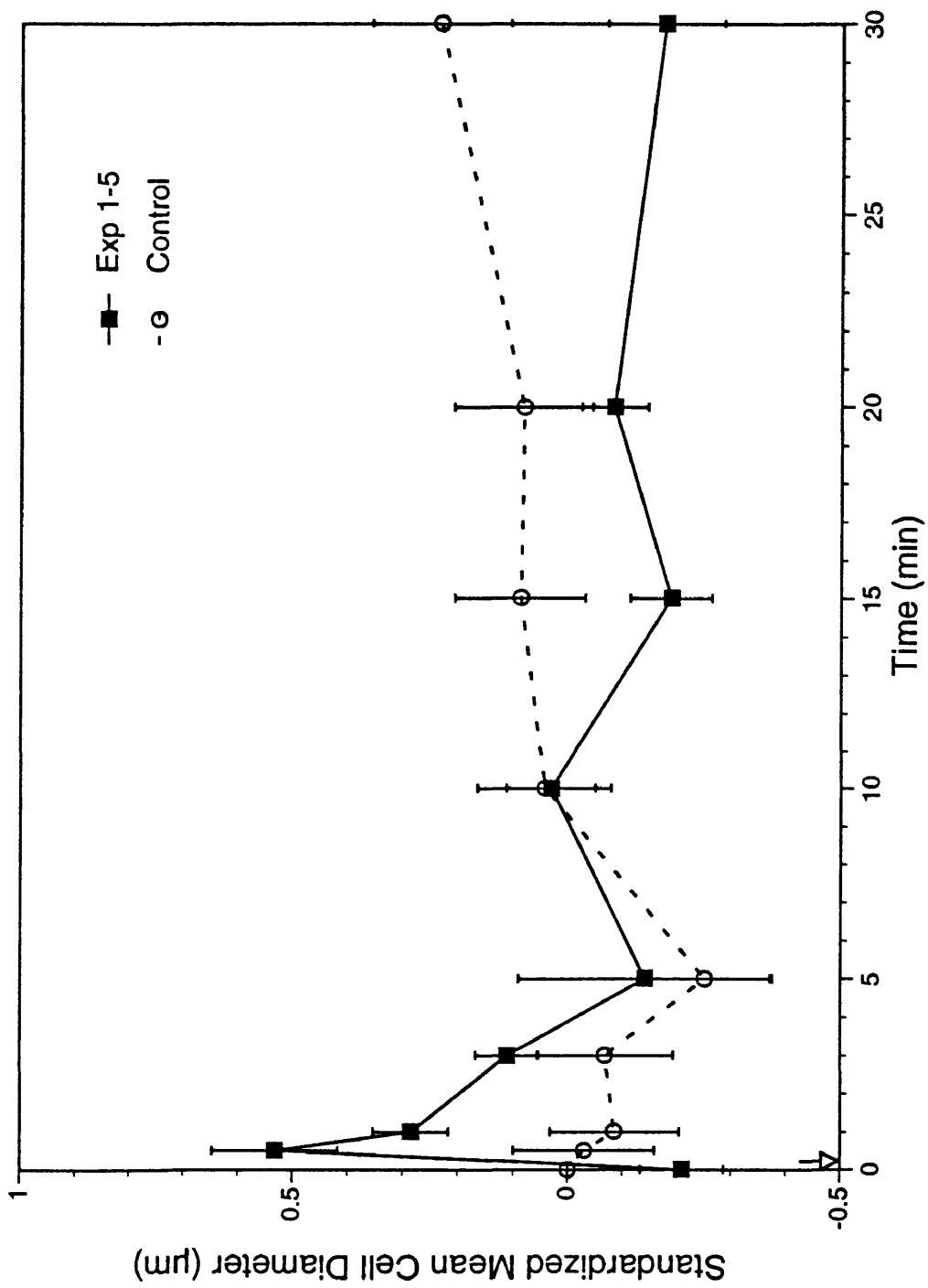
### **Amino Acid Content**

The 168 mOsm (6.5 ppt) acclimation group had a higher total free amino acid content than the 341 and 737 mOsm (12.7 and 27.0 ppt) acclimation groups in total free amino acid content on a per cell basis and on a per unit volume basis. The mean free amino acid totals (nmol) per cell for 168, 341, and 737 mOsm acclimated groups were  $2.385 \times 10^{-5}$ ,  $4.286 \times 10^{-6}$ , and  $4.957 \times 10^{-6}$ , respectively (Fig. 13). The mean free amino acid totals (nmol) per unit volume for 168, 341, and 737 mOsm acclimated groups were

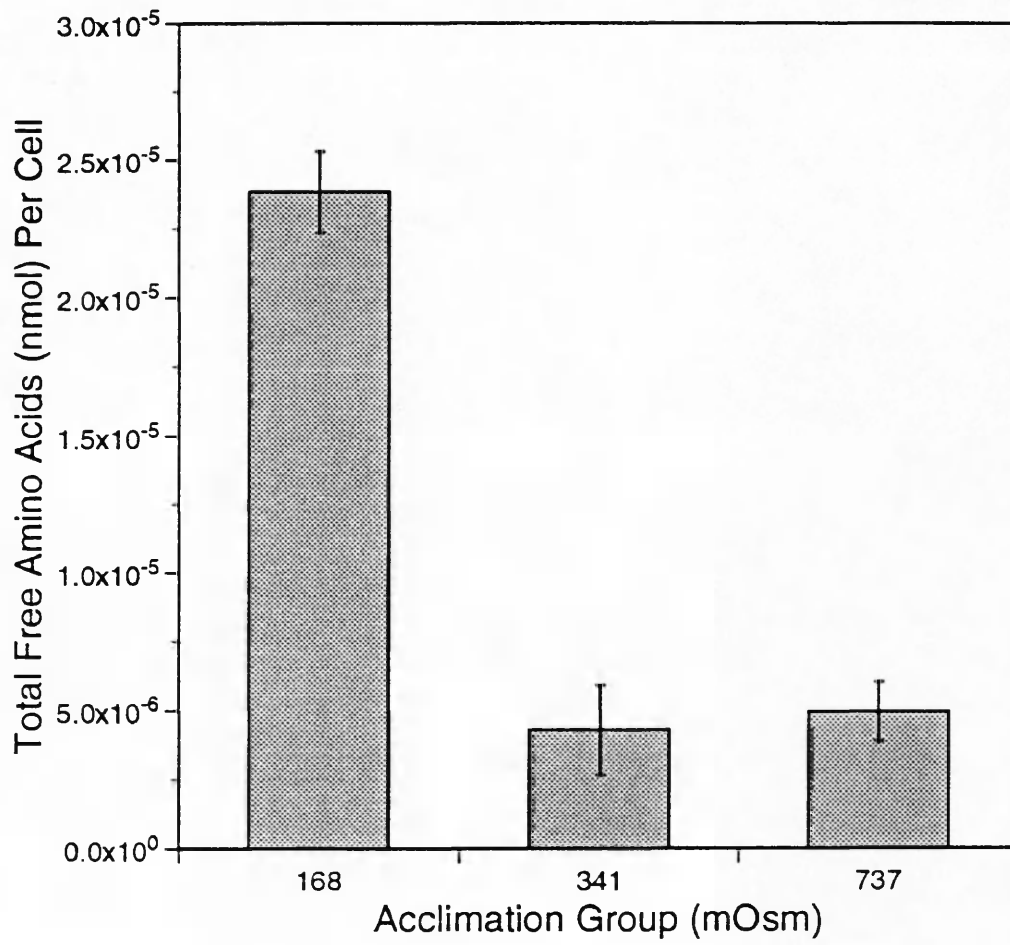
*Figure 11.* Standardized mean cell diameter ( $\mu\text{m}$ ) of *P. marinus* cells acclimated to 737 mOsm (27.0 ppt) and placed in 50% hypoosmotic shock. Replicate experiments 1 through 5 were standardized by subtracting the overall mean diameter of each experiment from each value in that experiment. Arrow indicates actual time of shock; time represents time following shock. Error bars = standard error.



*Figure 12.* Standardized mean cell diameter ( $\mu\text{m}$ ) of *P. marinus* cells acclimated to 737 mOsm (27.0 ppt) and placed in 50% hypoosmotic shock (arrow) with experiments 1 through 5 pooled (Exp 1-5) and the control experiment (control). Arrow indicates actual time of shock; time represents time following shock. Error bars = standard error.

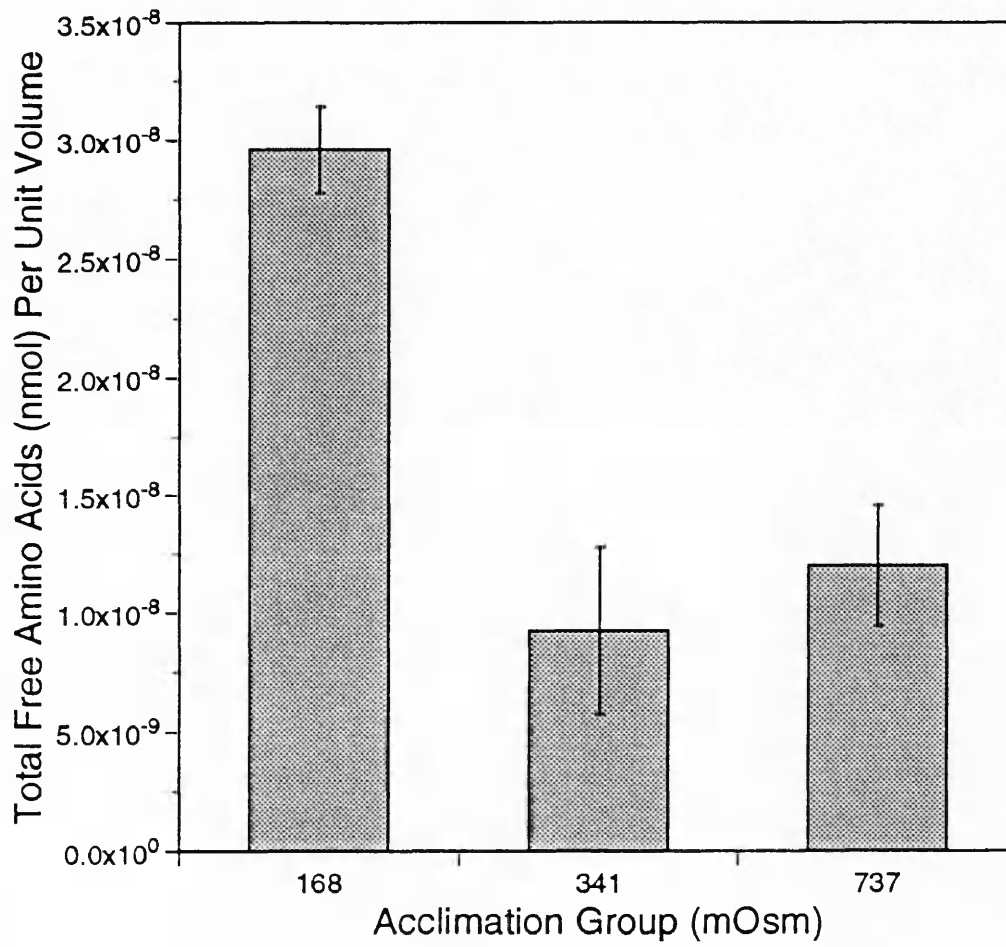


*Figure 13.* Mean free amino acid totals (nmol) per cell in cultured *P. marinus* cells acclimated to 168, 341, and 737 mOsm (6.5, 12.7, and 27.0 ppt). Error bars = standard error.



$2.963 \times 10^{-8}$ ,  $9.260 \times 10^{-9}$ , and  $1.202 \times 10^{-8}$ , respectively (Fig. 14). A one-way analysis of variance showed that acclimation osmolality had a significant effect on total free amino acid content for both the per cell ( $p=0.0036$ ) and the per unit volume ( $p=0.0242$ ) basis. *Post hoc* analyses at a  $p<0.05$  significance level on both the total free amino acid content per cell and per unit volume showed that the 168 mOsm acclimation group had free amino acid levels significantly higher than the 341 and 737 mOsm groups, but the 341 and 737 mOsm groups did not differ significantly from each other.

*Figure 14.* Mean free amino acid totals (nmol) per unit volume in cultured *P. marinus* cells acclimated to 168, 341, and 737 mOsm (6.5, 12.7, and 27.0 ppt). Error bars = standard error.



## DISCUSSION

The results from this research indicate that continuous cultures of *P. marinus* can be maintained in osmolalities as low as 168 mOsm (6.5 ppt). Furthermore, *P. marinus* cells acclimated to low osmolalities of 168 and 256 mOsm are larger and have a slower growth rate than cells acclimated to higher osmolalities (>256 mOsm). When acclimated, these low osmolality cells can withstand even lower osmolalities of 56 mOsm (2.5 ppt) for at least 24 hours. Cells maintained in osmolalities ranging from 168 to 737 mOsm are tolerant of hypo- and hyperosmotic conditions in the treatment range of 56 to 672 mOsm. Thus, this thesis research shows that cultured cells of *P. marinus* can survive both hypo- and hyperosmotic stress. During hypoosmotic stress, cells increased in diameter followed quickly by a return to baseline size (size before osmotic shock) which indicates a volume regulatory response. While the volume regulatory mechanisms implemented by *P. marinus* are currently unknown, the results of the free amino acid content study suggest that free amino acids may not be the primary osmolyte used. As a result of these experiments, it was demonstrated that *P. marinus* can survive changing osmotic conditions and actively use volume regulatory mechanisms to do so. This response may explain why *P. marinus* continues to persist in the low salinity areas of the Chesapeake Bay.

### **Growth Rate**

Cultured *P. marinus* cells acclimated to osmolalities of 341, 433, and 737 mOsm (12.7, 16.0, and 27.0 ppt) media reached log phase growth before cells acclimated to lower osmolalities of 168 and 256 mOsm (6.5 and 9.7 ppt). In addition, cells acclimated to the higher osmolalities had greater rates of multiplication (shorter doubling time) during log

phase than cells acclimated to the low osmolalities. These results correspond to a study with trypanosome cultures, in which high osmolality media was reported to generate greater multiplication rates than low osmolality media (Da Silva and Roitman 1982). Acclimated *P. marinus* cells from 168 and 256 mOsm did not reach log phase growth until 4 to 5 days post-subculture while the cells acclimated to 341, 433, and 737 mOsm began log phase after 2 to 3 days post-subculture. Thus, cells acclimated to the low osmolalities were in stationary phase longer before beginning log phase than the cells acclimated to the higher osmolalities which indicated that growth was reduced at low osmolalities. Continuous cultures which were acclimated to osmolalities as low as 168 mOsm were maintained for over 12 months indicating *P. marinus* can survive and grow in low osmolalities.

*Perkinsus marinus* infections in oysters are present at low salinities but do not proliferate until salinities are greater than 12 ppt (Ragone and Bureson 1993). Thus, even though the cultured cells have been shown to proliferate in low osmolalities, infections may not be intensifying at a rate detectable by routine diagnostic methods. In low salinities, the oysters may also be killing the pathogen at a higher rate than in higher salinities which would decrease the detectable level of infection. This phenomenon may be the result of increased oyster hemocyte activity in low salinities and low temperatures since hemocytes are reported to be the primary defense response in marine bivalves (Fisher 1988). The rate of hemocyte locomotion in eastern oysters has been shown to decrease in high salinities with the highest rate of locomotion occurring in the lowest test salinity of 6 ppt (Fisher and Newell 1986). The low salinities in the environment may be keeping the proliferation rate of *P. marinus* low enough so that the oyster can eliminate the pathogen at a rate equal to the pathogen's proliferation. Furthermore, *P. marinus* infections in oysters can persist in low salinities, even as low as 3 ppt (Chu et al. 1993).

## Cell Sizes of Acclimated Cultures

In addition to differences in growth rates, size differences between the acclimated groups were also observed. Cells acclimated to the low osmolality of 168 mOsm (6.5 ppt) were significantly larger than cells acclimated to the high osmolalities of 341 and 737 mOsm (12.7 and 27.0 ppt) during both log and stationary phase growth. The cells acclimated to the high osmolalities of 341 and 737 mOsm, however, were not significantly different from each other in size. The size distribution supported my hypothesis that cells acclimated to low osmolalities are larger than cells acclimated to high osmolalities. The difference in cell size may be due to an increased water content required to match the low osmolality of the dilute external medium. A study with red coelomocytes of the euryhaline polychaete *Glycera dibranchiata* showed cells acclimated to a lower osmolality had a higher "body-wall-tissue water" content and greater cellular volume than cells acclimated to a higher osmolality (Costa et al. 1980). An experiment with the amoeba *Acanthamoeba castellanii* indicated that the amount of intracellular water increased when cells were placed in a severe hypoosmotic shock (Geoffrion and Laroche 1984). As a result, the gradual acclimation of the *P. marinus* cells from high to low osmolality media may have caused an increase in size as water initially diffused into the cells during hypoosmotic acclimation, and cells may not have been able to return to baseline size during volume regulation. During acclimation to low osmolalities, cells must maintain their optimal ion concentrations for normal metabolic activities, so water is continuously diffusing into the cell while the cell is simultaneously controlling the efflux of both water and osmolytes. Consequently, this continuous influx and efflux during acclimation may have caused the low osmolality acclimated *P. marinus* cells to become larger in size than the higher osmolality acclimated cells.

Cells must maintain certain levels of metabolites in order to survive in the stress of a low osmolality environment. These levels of solute attract water molecules because of simple diffusion, and therefore, an increased intracellular water content results. Studies on

the erythrocytes of the bivalve *Noetia ponderosa* by Amende and Pierce (1980a) and Smith and Pierce (1987), and a report on the euryhaline ciliate *Paramecium calkinsi* by Cronkite and Pierce (1989) indicated that cells may not completely return to baseline (size before osmotic shock) after volume regulation. Size differences observed in this study may also be attributed to the stress of the low osmolality environment causing the cells in that media to develop a thicker cell wall or increase their intracellular metabolites. Cells acclimated to the low osmolality appeared to have a larger vacuole and may have had higher levels of nutrients which were needed to survive the stress of the low osmolality environment. The low osmolality cells did have higher free amino acid levels which represents an increase in the intracellular solute which consequently pulls water into the cell to act as the necessary solvent.

Cells acclimated to 168 and 256 mOsm were characteristically different from cells acclimated to 341, 433, and 737 mOsm in respect to growth, size, and osmotic tolerance. The observations appear to suggest that the cells acclimated to 168 and 256 mOsm behave similarly. Since the cells acclimated to the lower osmolalities had lower rates of multiplication, they most likely shifted their cellular metabolism from cellular activities such as growth to instead support their survival in the low osmolality conditions. The diversion of normal cellular metabolism may account for their larger size as they may not have been able to efflux organic molecules as quickly as cells acclimated to the higher osmolalities. The cells acclimated to higher osmolalities grew at a faster rate, experienced higher mortality in extreme hypoosmotic shock, and were smaller in size. This corresponds with the report that *P. marinus* infections increase at high salinities of 12 and 20 ppt and cause a higher level of oyster mortality than infections at lower salinities (Ragone and Burreson 1993).

## Osmotic Tolerance

The osmotic tolerance study indicated that *P. marinus* cells acclimated to low osmolalities experienced reduced mortality relative to the groups acclimated to higher osmolalities when placed in extreme hypoosmotic conditions. Cells acclimated to the low osmolalities 168 and 256 mOsm (6.5 and 9.7 ppt) experienced lower mortality in low osmolality treatments than cells acclimated to the higher osmolalities 341, 433, and 737 mOsm (12.7, 16.0, and 27.0 ppt). This mortality response supported my hypothesis that cells acclimated to low osmolalities would have lower mortality in hypoosmotic stress than cells acclimated to high osmolalities. Since the cells were already acclimated to the stress of a low osmolality environment, they were able to withstand an extreme low osmolality of 56 mOsm (2.5 ppt) better than cells acclimated to much higher osmolalities. Approximately 60% of the 168 mOsm acclimated cells survived the extreme low osmolality of 56 mOsm for at least 24 hours.

In this osmotic tolerance study, all of the acclimated groups had low mortalities (<10%) after hyperosmotic stress. This response did not confirm my hypothesis that cells acclimated to low osmolalities would have higher mortalities in hyperosmotic stress than cells acclimated to high osmolalities. Consequently, *P. marinus* was more tolerant of hyper- than hypoosmotic shock. Furthermore, the stressor did not seem to be the magnitude of the shock, but instead the type of stress (hypo- or hyper-) and the actual osmolality of the stress. For example, the 737 mOsm cells placed into 222 mOsm treatment (a difference of 515 mOsm) had much higher mortality than cells from 168 mOsm placed into 672 mOsm (a difference of 504 mOsm). Although the magnitude of the stress was about the same, the hypoosmotic rather than the hyperosmotic environment was more stressful. Also, cells acclimated to 737 mOsm and placed into 386 mOsm treatment (a difference of 351 mOsm) had much lower mortality than cells from 433 mOsm placed into 56 mOsm treatment (a difference of 377 mOsm). Although the magnitude of both of the

hypoosmotic shocks was similar, the actual osmolality of the treatment seemed to be the stressor that induced higher mortality.

The results from the osmotic tolerance experiment differ from the study by Burreson et al. (1994b) which reported much higher mortality levels after hypoosmotic shock. For example, the authors reported that cells acclimated to 650 mOsm and placed in 136 mOsm treatment had close to 100% mortality; in this thesis study, cells acclimated to 737 mOsm and placed in 135 mOsm treatment had only 38% mortality. These two studies differed in that the study by Burreson et al. (1994b) used cultures acclimated to media with BSA (JL-ODRP-1) while this study used cells acclimated to BSA-free media, but the media effect experiment of this thesis research showed no significant difference between the two types of media. Other factors may have contributed to the differences in mortality. This study used cells 4 days post-subculture while the previous study used cells 7 days post-subculture; perhaps the cells were healthier and less susceptible to mortality 4 days post-subculture than after 7 days. Furthermore, the cells in the previous study were kept in a non-CO<sub>2</sub> incubator for one week prior to use which may have stressed the cells making them more susceptible to mortality after osmotic shock. The CO<sub>2</sub> component is necessary to maintain a buffered media solution; without the CO<sub>2</sub>, the pH of the media fluctuates. Growth rates are reduced in cultures that have been transferred to a non-CO<sub>2</sub> incubator when compared to cultures maintained in a 5.0% CO<sub>2</sub> incubator (La Peyre, personal communication). Cultures in a 5.0% CO<sub>2</sub> incubator had a doubling time of 22 hours whereas cultures in a non-CO<sub>2</sub> incubator had a doubling time of 48 hours, and cultures did not regain the original high growth rates until the second subculture in the non-CO<sub>2</sub> environment (La Peyre, personal communication). Thus, the non-CO<sub>2</sub> environment may have stressed the cells in the study by Burreson et al. (1994b) which would explain the higher mortalities reported following hypoosmotic shock.

The osmotic tolerance experiment shows that *P. marinus* cells acclimated to low osmolalities can survive low osmolality conditions for at least for 24 hours. This seems to

indicate that merely transplanting infected oysters to low salinity areas will not eradicate the disease. Perhaps by transferring oysters directly from high to extreme low salinities, *P. marinus* cells may be killed as observed during the osmotic tolerance experiment of this thesis research, but it must be taken into account that oysters close their valves tightly during hypoosmotic shock and then gradually osmoconform to the environment which allows *P. marinus* cells to gradually acclimate as well. When oysters were transferred from 20 to 10 ppt, the oysters closed their valves within 5 minutes and did not reopen for at least 20 hours (Hand and Stickle 1977). This response was also demonstrated by marine mussels in which valve closure was cited to be the primary response of volume control during initial osmotic shock and short term salinity changes until the organism must interact with the environment for food and oxygen which causes volume regulatory mechanisms to then be utilized (Pierce 1971a).

In addition to the results of this thesis research, studies have shown that both low salinities in the environment as well as low osmolality treatments in culture are correlated with lowered *P. marinus* infection intensity and progression (Ray 1954, Mackin 1962, Soniat 1985, Soniat and Gauthier 1989, Crosby and Roberts 1990, Paynter and Burreson 1991, and Burreson et al. 1994b). An *in vitro* study by Ford and Haskin (1988) with the oyster parasite *H. nelsoni* showed that it is highly intolerant of low salinities with destruction of parasites occurring at 15 ppt and maximum damage at 9 ppt, and Ford (1985) demonstrated that infections in oysters disappear after a 2 week exposure to 10 ppt seawater. Despite recent reports of decreasing salinities during spring freshets in the Chesapeake Bay, *P. marinus* does not seem to have a strict point of salinity intolerance and continues to persist at high prevalences. The fact that low salinities in Chesapeake Bay tributaries have not eradicated the pathogen from these areas may be explained by the results in this osmotic tolerance study which indicated *P. marinus* can survive extreme low osmotic conditions. Fluctuations in stream flow, which indicate changes in salinity due to the inflow of freshwater, have not greatly affected the abundance of *P. marinus* in the

James River. In the low salinity areas of the James River, *P. marinus* was reported to persist even during months when salinities approached 0 ppt in March and April of 1993. Despite exposure to low osmotic conditions, prevalence the following summer was not affected (Burreson and Ragone Calvo 1994). High stream flows in February and March, 1994 had little effect on abundance of *P. marinus* the following summer and fall (Burreson and Ragone Calvo 1994). In the low salinity area of Deep Water Shoal in the James River, salinities were less than 6 ppt from November to June, 1994, even 0 ppt from February through April, and still the prevalence of *P. marinus* reached 60% in October, 1994 (Ragone Calvo and Burreson 1995). Therefore, these reports indicate that the prevalences and infection intensities of *P. marinus* in low salinity areas are not decreasing substantially, and consequently, the overall abundance of *P. marinus* in the Chesapeake Bay is not abating.

### **Cell Size After Hypoosmotic Shock**

During hypoosmotic stress, *P. marinus* cells swelled within one minute and returned towards baseline size in five minutes. This response supported my hypothesis that cells swell and then return to baseline size following hypoosmotic shock. In this experiment, the cells followed a typical cell volume response by experiencing an initial swelling and then shrinkage back towards baseline. Typical responses were evidenced in the study with coelomocytes of the euryhaline polychaete *Glycera dibranchiata* by Costa et al. (1980) that reported swelling within 2 minutes and recovery beginning in less than 5 minutes following hypoosmotic exposure. Similarly, a study with red blood cells of the clam *Noetia ponderosa* reported an initial swell within 5 minutes and a recovery beginning within 10 minutes after hypoosmotic stress (Smith and Pierce 1987). Moreover, Darling et al. (1990) determined that *Leishmania major* promastigotes swelled with 1 minute and returned towards baseline in 10 minutes, and Cronkite and Pierce (1989) found that the

marine ciliate *Paramecium calkinsi* swelled within 5 minutes and started volume recovery within 10 minutes of hypoosmotic exposure.

Likewise, the results reported here indicate that *P. marinus* cells utilize volume regulatory mechanisms to compensate for osmotic changes in the external medium. These mechanisms enabled cells to survive a 50% dilution of the external medium as cells swelled within 1 minute and returned to baseline in 5 minutes following hypoosmotic shock. Also, the analysis indicated that *P. marinus* cells do not form a cyst or utilize a thick cell wall to resist swelling during sudden external osmolality changes. The size at the maximum swell was significantly different from the initial size and the final baseline size. The mean size change during swelling was 0.7  $\mu\text{m}$  which was approximately a 13% increase in diameter and approximately a 44.5% increase in volume. The erythrocytes of the clam *N. ponderosa* exhibited a similar pattern when cells acclimated to 935 mOsm were placed in a hypoosmotic shock of 560 mOsm; the cells swelled thereby increasing their volume by 50% within 5 minutes followed by a gradual return towards baseline (Smith and Pierce 1987).

When an organism encounters changing external osmolalities, it must maintain its ion gradient in addition to its cellular volume to satisfy the constraints of the Donnan equilibrium condition. During osmotic shock, cells may initially extrude inorganic ions such as  $\text{Na}^+$  to decrease the electrical gradient as well as maintain osmotic equilibrium (Prosser 1986). The use of inorganic ions may adversely affect important enzyme activities, and other more efficient, less metabolically expensive osmolytes are also used in cell volume regulation. An organism evolves its cell volume response so that an optimal solute microenvironment is attained for the cellular macromolecules. In other words, an organism develops its osmolyte system in order to obtain optimal metabolic activity. How an organism evolves its particular volume regulatory response system is not known; instead, research focuses on the trigger of changing the external osmolality and the response of variations in the intracellular levels of organic molecules and inorganic ions.

To examine the volume regulatory responses in *P. marinus*, volume changes following hypoosmotic shock were observed. Since the cells in this study did swell and return to baseline size, the results suggest that *P. marinus* utilizes volume regulatory mechanisms during changing external osmolalities. Energy-requiring mechanisms were employed to maintain osmotic equilibrium due to the constraints of the Donnan equilibrium condition. But to define the specific volume regulatory mechanisms used by *P. marinus*, the levels of inorganic ions and organic molecules before and after osmotic shock must be measured.

### **Amino Acid Content**

The free amino acid levels in cells acclimated to the low osmolality of 168 mOsm were significantly higher than in those acclimated to the high osmolalities of 341 and 737 mOsm. As in the cell size relationship, the 341 and 737 mOsm osmolality groups did not differ significantly from each other. These differences in free amino acid levels did not support my hypothesis that cells acclimated to high osmolalities have higher free amino acid levels than cells acclimated to low osmolalities. Several studies have revealed that organisms which employ free amino acids as osmolytes have higher levels in hyperosmotic conditions than in hypoosmotic conditions. If free amino acids are the primary osmolyte used by *P. marinus*, one would expect to observe this same pattern. Hence the atypical high levels of free amino acids in the low osmolality cells observed in this study suggest that free amino acids may not be the primary osmolyte used by *P. marinus*. Other organic molecules like glycerol or inorganic ions like Na<sup>+</sup> or K<sup>+</sup> may be used as the main compensatory osmolyte with free amino acids contributing to a much lesser degree if at all. If other molecules do act as the primary osmolyte, then their contribution to the osmolyte pool would be much greater than the free amino acid levels measured in the acclimated *P. marinus* cultures, and the levels of the primary osmolytes would probably differ by an order of magnitude in concentration.

In organisms that have been shown to utilize free amino acids as important compensatory osmolytes, the levels of free amino acids decreased when the organisms were placed in low salinities (Kaneshiro et al. 1969, Pierce 1971b, Pierce and Greenberg 1972, Pierce and Greenberg 1973, and Costa et al. 1980, and Costa and Pierce 1983). In the marine ciliate *Paramecium calkinsi*, free amino acids are used as osmolytes, and it was reported that cells acclimated to low osmolalities have a free amino acid level an order of magnitude lower level than cells acclimated to higher osmolalities (Cronkite and Pierce 1989). This study also examined the free amino acid levels within the bacteria which the ciliate consumed. Over a wide range of salinities, the free amino acid levels in the bacteria varied little, and all were within the same order of magnitude which led the authors to suggest that free amino acids were not the primary osmolyte used (Cronkite and Pierce 1989). In other organisms, free amino acids have been determined to be the primary osmolytes when during osmotic shock, their intracellular levels change dramatically and their contributions as osmolytes are greater than that of inorganic ions or other organic molecules. For instance, following a hypoosmotic shock, the free amino acid pool in the amoeba *Acanthamoeba castellanii* was observed to decrease six fold which was greater than changes in the inorganic ion levels (Geoffrion and Larochelle 1984). Future studies with *P. marinus* should examine the free amino acid content after hypoosmotic shock, and results may show decreased levels for all acclimated groups despite differences in the levels between the groups before shock. Even if free amino acids do contribute to the compensatory osmolyte pool in *P. marinus*, inorganic ions such as Na<sup>+</sup> and K<sup>+</sup> and other organic molecules such as glycerol may also act as osmolytes since organisms have been reported to use a combination of osmolytes during volume regulation (Kaneshiro et al. 1969, Pierce 1982, Ahmad and Hellebust 1985, Ahmad and Hellebust 1986, and Smith and Pierce 1987).

In addition to intracellular free amino acid levels changing by influx and efflux, the concentrations are also regulated by intracellular amino acid metabolism. Organisms must

maintain a certain threshold limit of amino acids for normal metabolic processes, and the stress of the low osmolality conditions may cause that threshold limit to be higher which may explain why the low osmolality cells had higher free amino acid levels than the higher osmolality cells. The higher levels observed in the low osmolality cells may also be the result of increased protein catabolism or decreased protein synthesis. Either of these two processes may be caused by the stress of the low osmolality environment. Gilles (1979) noted that variations in intracellular catabolic activity increase the amount of free amino acids into the intracellular free amino acid pool. Observing changes in the free amino acid concentrations in *P. marinus* before and after osmotic shock would indicate whether these molecules contribute to the compensatory osmolyte pool during cell volume regulation.

## Conclusions

The results of these experiments help explain why *P. marinus* continues to persist in the upper portions of the Chesapeake Bay tributaries despite periods of low salinities. These studies indicate that *P. marinus* cells gradually acclimated to low osmolalities can withstand extreme low osmotic conditions and use volume regulatory mechanisms to adapt to the changing external osmolality. Since *P. marinus* cells in oysters are usually found within hemocytes, future studies should examine how oyster tissue and hemocytes may delay the osmotic shock to which *P. marinus* cells may be exposed. The actual osmotic shock may be delayed due to the protective layer of host tissue and hemocytes. In addition, measurements of other osmolytes should be obtained in acclimated *P. marinus* cultures before and after osmotic shock to determine their role in volume regulation. By quantifying ion concentrations in cells placed in different osmolalities, it could be determined if internal ion distributions passively follow the levels in the external environment, or if the levels are actively regulated by volume regulatory mechanisms. In conclusion, it has been determined that *P. marinus* can withstand osmotic shock and use volume regulatory

mechanisms to do so. Therefore, transferring oysters to low salinity areas or waiting for prolonged low salinities should not be used as methods to eradicate *P. marinus* infections.

## LITERATURE CITED

- Ahmad, I. and J.A. Hellebust. 1985. Salinity responses of the marine microalga *Brachiomonas submarina* Bohlin. *Exp. Mar. Bio. Ecol.* 88: 45-53.
- Ahmad, I. and J.A. Hellebust. 1986. The role of glycerol and inorganic ions in osmoregulatory responses of the euryhaline flagellate *Chlamydomonas pulsatilla* Wollenweber. *Plant Physiol.* 82: 406-410.
- Amende, L.M. and S.K. Pierce. 1980a. Cellular volume regulation in salinity stressed molluscs. The response of *Noetia ponderosa* (Arcidae) red blood cells to osmotic variation. *J. Comp. Physiol.* 138: 283-289.
- Amende, L.M. and S.K. Pierce. 1980b. Free amino acid mediated volume regulation of isolated *Noetia ponderosa* red blood cells: control by  $Ca^{2+}$  and ATP. *J. Comp. Physiol.* 138: 291-298.
- André, L., Nilsson, A., and L. Adler. 1988. The role of glycerol in osmotolerance of the yeast *Debaryomyces hansenii*. *J. Gen. Microbiol.* 134: 669-677.
- Andrews, J.D. 1988. Epizootiology of the disease caused by the oyster pathogen *Perkinsus marinus* and its effects on the oyster industry. *Amer. Fish. Soc. Spec. Publ.* 18: 47-63.
- Andrews, J.D. and W.G. Hewatt. 1957. Oyster mortality studies in Virginia. II. The fungus disease caused by *Dermocystidium marinum* in oysters of Chesapeake Bay. *Ecol. Monogr.* 27: 1-25.
- Azevedo, C., Corral, L., and R. Cachola. 1990. Fine structure of zoosporulation in *Perkinsus atlanticus* (Apicomplexa: Perkinsia). *Parasitology* 100: 351-358.
- Burreson, E.M. and L. Ragone Calvo. 1994. Status of the major oyster diseases in Virginia- 1993. A summary of the annual monitoring program. *Mar. Res. Rept.* 93-5. Virginia Institute of Marine Science, Gloucester Pt., VA.
- Burreson, E.M., Alvarez, R.S., Martinez, V.V., and L.A. Macedo. 1994a. *Perkinsus marinus* (Apicomplexa) as a potential source of oyster *Crassostrea virginica* mortality in coastal lagoons of Tabasco, Mexico. *Dis. Aquat. Org.* 20: 77-82.
- Burreson, E.M., Ragone Calvo, L.M., La Peyre, J.F., Counts, F., and K.T. Paynter. 1994b. Acute osmotic tolerance of cultured cells of the oyster pathogen *Perkinsus marinus* (Apicomplexa: Perkinsida). *Comp. Biochem. Physiol.* 109A, 3: 575-582.

- Chamberlain, M.E. and K. Strange. 1989. Anisosmotic cell volume regulation: a comparative review. *Amer. J. Physiol.* 257: C159-C173.
- Chu, F.E. and K.H. Greene. 1989. Effect of temperature and salinity on *in vitro* culture of the oyster pathogen, *Perkinsus marinus* (Apicomplexa: Perkinsea). *J. Invert. Pathol.* 53: 260-268.
- Chu, F.E., La Peyre, J.F., and C.S. Burreson. 1993. *Perkinsus marinus* infection and potential defense-related activities in eastern oysters, *Crassostrea virginica*: salinity effects. *J. Invertebr. Pathol.* 62: 226-232.
- Colomi, A. 1985. Aspects of the biology of *Cryptocaryon irritans*, and hyposalinity as a control measure in cultured gilt-head sea bream *Sparus aurata*. *Dis. Aquat. Org.* 1: 19-22.
- Costa, C.J., Pierce, S.K., and M.K. Warren. 1980. The intracellular mechanism of salinity tolerance in polychaetes: volume regulation by isolated *Glycera dibranchiata* red coelomocytes. *Biol. Bull.* 159: 626-655.
- Costa, C.J. Costa and S.K. Pierce. 1983. Volume regulation in the red coelomocytes of *Glycera dibranchiata*: an interaction of amino acid and K<sup>+</sup> effluxes. *J. Comp. Physiol.* 151: 133-144.
- Cronkite, D.L. and S.K. Pierce. 1989. Free amino acids and cell volume regulation in the euryhaline ciliate *Paramecium calkinsi*. *J. Exp. Zool.* 251: 275-284.
- Crosby, M.P. and C.F. Roberts. 1990. Seasonal infection intensity cycle of the parasite *Perkinsus marinus* (and an absence of *Haplosporidium* spp.) in oysters from a South Carolina salt marsh. *Dis. of Aquat. Org.* 9: 149-155.
- Darling, T.N. and J.J. Blum. 1990. Changes in the shape of *Leishmania major* promastigotes in response to hexoses, proline, and hypoosmotic stress. *J. Protozool.* 37(4): 267-272.
- Darling, T.N., Burrows, C.M., and J.J. Blum. 1990. Rapid shape change and release of ninhydrin-positive substances by *Leishmania major* promastigotes in response to hypoosmotic stress. *J. Protozool.* 37(6): 493-499.
- Da Silva, J.B.T. and I. Roitman. 1982. Effect of temperature and osmolarity on growth of *Crithida fasciculata*, *C. hutneri*, *C. luciliae thermophila*, and *Herpetomonas samuelpessoai*. *J. Protozool.* 29(2): 269-272.
- Fisher, W.S. 1988. *Disease Processes in Marine Bivalve Molluscs*. Environmental influence on host response. Environmental influence on bivalve hemocyte function. *Amer. Fish. Soc. special publ.* 18: 225-237.
- Fisher, W.S. and R.I.E. Newell. 1986. Salinity effects on the activity of granular hemocytes of American oysters, *Crassostrea virginica*. *Biol. Bull.* 170: 122-134.
- Fleiss, J.L. 1981. *Statistical Methods for Rates and Proportions*. John Wiley and Sons: New York, pp. 138-141.

- Ford, S.E. 1985. Effects of salinity on survival of the MSX parasite *Haplosporidium nelsoni* (Haskin, Stauber, and Mackin) in oysters. *J. Shellf. Res.* 5(2): 85-90.
- Ford, S.E. and H.H. Haskin. 1988. Comparison of *in vitro* salinity tolerance of the oyster parasite, *Haplosporidium nelsoni* (MSX) and hemocytes from the host, *Crassostrea virginica*. *Comp. Biochem. Physiol.* 90A(1): 183-187.
- Geoffrion, Y. and J. Larochelle. 1984. The free amino acid contribution to osmotic regulation in *Acanthamoeba castellanii*. *Can. J. Zool.* 62: 1954-1959.
- Gilles, R. 1979. Intracellular organic osmotic effectors. In *Mechanisms of Osmoregulation in Animals*. Wiley Press: New York. pp. 111-154.
- Gilles, R. 1988. Comparative aspects of cell osmoregulation and volume control. *Renal Physiol. Biochem.* 3(5): 277-288.
- Gilles, R. and A. Pequeux. 1981. Cell volume regulation in crustaceans: relationship between mechanisms for controlling the osmolality of extracellular and intracellular fluids. *J. Exp. Zool.* 215: 351-362.
- Goggin, C.L., Sewell, K.B., and R.J. Lester. 1990. Tolerances of *Perkinsus* spp. (Protozoa, Apicomplexa) to temperature, chlorine, and salinity. *J. Shellf. Res.* 9(1): 145-148.
- Hand, S.C. and W.B. Stickle. 1977. Effects of tidal fluctuations of salinity on pericardial fluid composition of the American oyster *Crassostrea virginica*. *Mar. Biol.* 42: 259-271.
- Hellebust, J.A., Merida, T., and I. Ahmad. 1989. Operation of contractile vacuoles in the euryhaline flagellate *Chlamydomonas pulsatilla* (Chlorophyceae) as a function of salinity. *Mar. Biol.* 100: 373-379.
- Hochachka, Peter W. 1983a. *The Mollusca: Vol. 2. Environmental Biochemistry and Physiology*. Academic Press, Inc.: New York, pp. 77-95.
- Hochachka, Peter W. 1983b. *The Mollusca: Vol. 1. Metabolic Biochemistry and Molecular Biomechanics*. Academic Press, Inc.: New York, pp. 244, 289-293.
- Hochachka, P.W. and G.N. Somero. 1984. *Biochemical Adaptation*. Princeton University Press: Princeton, pp. 304-310.
- Kaneshiro, E.S., Holz, G.G., Jr., and P.B. Dunham. 1969. Osmoregulation in a marine ciliate, *Miamiensis avidus*; regulation of intracellular free amino acids. *Biol. Bull.* 137: 161-169.
- La Peyre, J. F. personal communication.
- La Peyre, J.F., Faisal, M., and E.M. Burreson. 1993. *In vitro* propagation of the protozoan *Perkinsus marinus*, a pathogen of the Eastern oyster, *Crassostrea virginica*. *J. Euk. Microbiol.* 40(3): 304-310.
- Levine, N.D. 1978. *Perkinsus* gen. n. and other new taxa in the protozoan phylum Apicomplexa. *J. Parasitol.* 64(3): 549.

- Mackin, J.G. 1956. *Dermocystidium marinum* and other microorganisms in Louisiana. Proc. Nat. Shell. Assoc. 46: 116-133.
- Mackin, J.G. 1962. Oyster disease caused by *Dermocystidium marinum* and other microorganisms in Louisiana. Tx. Inst. of Mar. Sci. Publ. 7: 132-229.
- Mackin, J.G., Owen, H.M., and A. Collier. 1950. Preliminary note on the occurrence of a new protistan parasite, *Dermocystidium marinum* n. sp., in *Crassostrea virginica* (Gmelin). Science 111: 328-329.
- McGladdery, S.E., Cawthorn, R.J., and B.C. Bradford. 1991. *Perkinsus karlssoni* n. sp. (Apicomplexa) in bay scallops *Argopecten irradians*. Dis Aquat. Org. 10: 127-137.
- Paynter, K.T. and E.M. Bureson. 1991. Effects of *Perkinsus marinus* infection in the eastern oyster, *Crassostrea virginica*: II. Disease development and impact on growth rate at different salinities. J. of Shellf. Res. 10(2), 425-431.
- Paynter, K.T., Pierce, S.K., and E.M. Bureson. 1995. Levels of intracellular free amino acids used for salinity tolerance by oysters (*Crassostrea virginica*) are altered by protozoan (*Perkinsus marinus*) parasitism. Mar. Biol. 122: 67-72.
- Perkins, F.O. 1969. Ultrastructure of vegetative stages in *Labyrinthomyxa marina* (= *Dermocystidium marinum*), a commercially significant oyster pathogen. J. Invert. Pathol. 13: 199-222.
- Perkins, F.O. 1987. Protistan parasites of commercially significant marine bivalve molluscs- life cycles, ultrastructure, and phylogeny. Aquaculture 67: 240-243.
- Pierce, S.K., Jr. 1971a. Volume regulation and valve movements by marine mussels. Comp. Biochem. Physiol. 39A: 103-117.
- Pierce, S.K., Jr. 1971b. A source of solute for volume regulation in marine mussels. Comp. Biochem. Physiol. 38A: 619-635.
- Pierce, S.K. 1982. Invertebrate cell volume control mechanisms: a coordinated use of intracellular amino acids and inorganic ions as osmotic solutes. Biol. Bull. 163: 405-419.
- Pierce, S.K. and L.M. Amende. 1981. Control mechanisms of amino acid-mediated cell volume regulation in salinity-stressed molluscs. J. Exp. Zool. 215: 247-257.
- Pierce, S.K., Jr. and M.J. Greenberg. 1972. The nature of cellular volume regulation in marine bivalves. J. Exp. Biol. 57: 681-692.
- Pierce, S.K., Jr. and M.J. Greenberg. 1973. The initiation and control of free amino acid regulation of cell volume in salinity-stressed marine bivalves. J. Exp. Biol. 59: 435-440.
- Prosser, C. Ladd. 1973. *Comparative Animal Physiology*. W.B. Saunders Co.: Philadelphia, pp. 4-10, 32-34, 71-73.
- Prosser, C. Ladd. 1986. *Adaptational Biology: Molecules to Organisms*. John Wiley and Sons: New York, pp. 341-389.

- Ragone, L.M. and E.M. Burreson. 1993. Effect of salinity on infection progression and pathogenicity of *Perkinsus marinus* in the eastern oyster, *Crassostrea virginica* (Gmelin). J. Shellf. Res. 12: 1-7.
- Ragone Calvo, L. and E.M. Burreson. 1995. Status of the major oyster diseases in Virginia- 1994. A summary of the annual monitoring program. Mar. Res. Rept. 95-5. Virginia Institute of Marine Science, Gloucester Pt., VA.
- Ray, S.M. 1954. Biological studies of *Dermocystidium marinum*, a fungus parasite of oysters. Rice Inst. Pamphlet, 114 pp. (Monograph in Biology Special Series Issue).
- Smith, L.H., Jr. and S.K. Pierce. 1987. Cell volume regulation by molluscan erythrocytes during hypoosmotic stress:  $\text{Ca}^{2+}$  effects on ionic and organic osmolyte effluxes. Biol. Bull. 173: 407-418.
- Soniat, T.M. 1985. Changes in levels of infection of oysters infected by *Perkinsus marinus*, with special reference to the interaction of temperature and salinity upon parasitism. Nw. Gulf Sci. 7(2): 171-174.
- Soniat, T.M. and J.D. Gauthier. 1989. The prevalence and intensity of *Perkinsus marinus* from the mid northern Gulf of Mexico, with comments on the relationship of the oyster parasite to temperature and salinity. Studies in Zool. and Bot. 27(1): 21-27.
- Soniat, T.M. and M.L. Koenig. 1982. The effects of parasitism by *Perkinsus marinus* on the free amino acid composition of *Crassostrea virginica* mantle tissue. J. Shellf. Res. 2(1): 25-28.
- Warren, M.K. and S.K. Pierce. 1982. Two cell volume regulatory systems in the *Limulus* myocardium: An interaction of ions and quaternary ammonium compounds. Bio. Bull. 163: 504-516.
- Weeks, P.J.D. and L. Richards. 1993. Morphometric analysis of *Chlorella fusca* var. *vacuolata*: a comparison of image analysis and coulter counting. Binary 5: 29-36.

## VITA

Caroline L. O'Farrell

Born in Madison, Wisconsin, 29 May 1970. Graduated from Southfield Christian High School, Southfield, Michigan, in 1988. Earned a B.S. in Biology/ Pre-Medicine from Oral Roberts University, Tulsa, Oklahoma, in 1993. Interned at the Virginia Institute of Marine Science in Gloucester Point, Virginia from June to August 1990, at the University of Texas Medical Branch- Marine Biomedical Institute in Galveston, Texas from June to August 1992, and at the Bermuda Biological Station in Bermuda from January to August 1993. Entered the Masters program of the College of William and Mary, School of Marine Science in 1993. Employed as a graduate research assistant at the College of William and Mary, School of Marine Science from August 1993 to August 1995.