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# ASPECTS OF VOLUME REGULATION IN TWO ESTUARINE INVERTEBRATES: *GLYCERA DIBRANCHIATA* (ANNELIDA) AND *MODIOLUS DEMISSUS* (MOLLUSCA)

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A Thesis

Presented to

The Faculty of the Department of Biology

The College of William and Mary in Virginia

In Partial Fulfillment

Of the Requirements for the Degree of

Master of Arts

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by

Ana M. Beardsley

1989

APPROVAL SHEET

This thesis is submitted in partial fulfillment of

the requirements for the degree of

Master of Arts

Ana M. Beardsley

Approved September, 1989

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## ABSTRACT

The bloodworm Glycera dibranchiata is an imperfect volume regulator when exposed to a low salinity stress. During volume readjustment it undergoes two distinct phases: 1) swelling and 2) plateau. There is a very small decrease in volume between the two phases. Also during this period, it has an elevated ammonia exeretion rate whose pattern follows that of the volume readjustment. There are no detectable free amino acids (FAA) excreted in this interval. FAA do pass across the body wall in isolated preparations. The presence of closed nephridia are thought to play a role in this.

In the course of volume readjustment the osmolality of the coelomic fluid drops very rapidly. G. dibranchiata becomes isosmotic at approximately 12 hours after exposure to $18^{o}$/oo. During this time there is little change in the concentration of FAA in the coelomic fluid despite an obvious increase in the volume of the coelomic fluid. These FAA are presumabley degraded and the ammonia is excreted.

The ribbed mussel, Modiolus demissus, also do not excrete intact FAA when exposed to a low salinity stress. The osmolality of the pericardial fluid decreases rapidly to become isosmotic at approximately 12 hours after exposure. During this time there is an increase in concentration of FAA in the pericardal fluid.

Isolated preparations of gill exhibit an increase in the rate of uptake of $\alpha$-amino isobutyric acid (AIB) when subjected to a low

salinity stress. Isolated nephridia exhibit no change in uptake rates. This is presumed to play a role in absence of FAA excretion in M. demissus.

# ASPECTS OF VOLUME REGULATION IN TWO ESTUARINE INVERTEBRATES: *GLYCERA DIBRANCHIATA* (ANNELIDA) AND *MODIOLUS DEMISSUS* (MOLLUSCA)

# INTRODUCTION

Many marine invertebrates tolerate a wide range of salinities. They employ a large pool of intracellular free amino acids (FAA) to help combat salinity stresses (Clark, 1968; Pierce and Greenberg, 1972; Freel et al., 1973; Bartberger and Pierce, 1976; Costa et al., 1980; Henry and Mangum, 1980a; Koenig et al., 1981; Dykens and Mangum, 1984). Cell volume is regulated by altering the size of this pool, increasing as the salinity increases and decreasing as salinity drops. When exposed to a lowered salinity, the permeability of the cell membrane alters. Small osmolytes such as $K^+$ and FAA pass out of the cell. The passage of these substances drags osmotically obligated water out of the cell, reducing the swelling.

Potassium chloride (KCl) is not used as a labile osmolyte exclusively by euryhaline organisms because it is made up of inorganic ions. An increase or decrease in the $[K^+]$ and $[Cl^-]$ in the cell would interfere with the electrochemical gradient across the cell membrane, thereby interfering with the normal functioning of the cell. If employed exclusively the amount of KCl needed by the cell for volume readjustment would be very large. The cost of replacing the KCl would be very great once osmotic steady state has been established. Moreover, many enzymes are sensitive to $Cl^-$ and thus their activities might be altered.

In euryhaline animals FAA are employed in volume readjustment because many lack a net charge, therefore do not interfere with the polarity of the cell. They appear to be easily replaced, either by de novo synthesis or protein catabolism. Finally, FAA are not known to cause large allosteric effects on enzymes and thus they do not greatly influence cell metabolism.

There are five current theories of the fate of these FAA when a euryhaline animal experiences a hypoosmotic shock. They are: 1) FAA are excreted intact into the media, 2) FAA are deaminated in situ to conserve the carbon skeleton and ammonia is excreted, 3) FAA are excreted from the cell, transported to a peripheral site and then metabolized to the carbon skeleton and ammonia, 4) FAA are incorporated into protein, either intra- or extracellular, and 5) FAA are released into the bloodstream and excreted intact.

Volume readjustment has been studied in only a handful of organisms, and fewer still in great detail. Two of the most extensively studied are various species of nereid polychaetes (Oglesby, 1965a, b; Freel et al., 1973; Haberfield et al., 1975; Dykens, 1979; Koenig et al., 1981; Dykens and Mangum, 1984) and the bivalve Modiolus demissus (Pierce 1971a, b; Pierce and Greenberg, 1972; 1973; Bartberger and Pierce, 1976; Shumway et al., 1977; Shumway and Youngson, 1979). Even so, their mechanisms of altering the size of the FAA pool are not fully understood.

Nereis succinea is a euryhaline polychaete known to have a large range of salinity tolerance. It excretes FAA and ammonia in apparently equimolar ratios during its period of volume readjustment following a decrease in salinity. If the two are summed the total More than

accounts for the amount of FAA lost by the animals during this acclimation (Dykens, 1979; Dykens and Mangum, 1984).

Modiolus demissus is a euryhaline bivalve also known to tolerate a wide range of salinities. When subjected to a low salinity stress the FAA pool decreases, both in whole animals (Bartberger and Pierce, 1976; Shumway et al., 1977; Shumway and Youngson, 1979) and isolated ventricles (Pierce and Greenberg, 1972). In this case few FAA are excreted intact into the media, and most are released from the cells into the hemolymph and degraded at an unknown site (Bartberger and Pierce, 1976). This is followed by an increased rate of ammonia excretion, which only lasts through the period of readjustment and then returns to normal in this species, an osmoconformer. The osmoregulating bivalve, Rangia cuneata, maintains an elevated level of ammonia excretion for the duration of the low salinity stress (Henry and Mangum, 1980b). N. succinea, also a regulator of body fluid osmolality, continues to excrete ammonia at an elevated rate after acclimation is apparently complete (Dykens, 1979; Dykens and Mangum, 1984). The excretion of ammonia is believed to play a role in net sodium ($Na^+$) uptake at the gill (Mangum et al., 1978).

The subjects of the present study include the common bloodworm, Glycera dibranchiata, a moderately euryhaline polychaete that has been demonstrated to tolerate salinities ranging from $12.4^{o}/oo$ to $46.5^{o}/oo$ (Machin, 1975). There have been preliminary studies of its volume regulatory abilities, but this has not been fully explored. Machin (1975) established the salinity tolerance and characterized the response to various salinities. When exposed to a low salinity stress, G. dibranchiata undergoes a period of swelling. The weight then

plateaus for a period and then decreases slowly. However, the worms never return to their original weight. The amount of swelling and subsequent decrease in weight appears to be dependent on the magnitude of the salinity stress. The lower the salinity, the greater the degree of swelling and lesser the extent of recovery. G. dibranchiata returns to a state 25 % greater than its original volume, whereas N.succinea returns to a state 10 % greater. Machin (1975) concluded that the animal is an osmoconformer over the salinity range studied.

More extensive work has been done on isolated red coelomocytes. These cells contain hemoglobin and serve to transport and store oxygen (Hoffmann and Mangum, 1970). Their volume regulating capabilities have been characterized (Machin and O'Donnell, 1977; Costa et al., 1980; Costa and Pierce, 1982). They have a very limited ability to regulate volume when exposed to a low salinity. There is a very large increase in volume during the first two minutes of exposure followed by a small shrinking phase, lasting 15 - 120 minutes, depending on the magnitude of the salinity change (Machin and O'Donnell, 1977). The coelomocytes do not regain their original volume at any salinity; in 50 % sea water the coelomocytes return to within 40 % of their original volume. Costa and Pierce (1982) reported that the cells extrude $K^+$ and FAA upon exposure to a hypoosmotic shock. The efflux of $K^+$ is rapid and thought to prevent cell lysis during the first few minutes of hypoosmotic stress (Costa and Pierce, 1982). FAA are used in long term volume readjustment. $Ca^{+2}$ and $Mg^{+2}$ also affect the ability to regulate volume by influencing the permeability of the cell membrane to $K^+$ and FAA. Cells exposed to a low salinity stress in the absence of divalent cations swell less and lose more $K^+$ and FAA than cells exposed to low

salinity in the presence of either or both of these ions (Costa and Pierce, 1982).

G. dibranchiata is a primitively constructed annelid, lacking a closed circulatory system. The red coelomocytes are free in the coelom and are circulated by means of ciliary action and muscle contractions. The animal also possesses closed protonephridia, meaning that there is no direct opening from the coelom to the external environment. This arrangement differs from that of N. succinea, which has metameric, open metanephridia. In this species the FAA are believed to be released into the coelomic fluid and perhaps also directly through the outer cuticle into the bathing medium. Thus it would seem difficult to prevent loss to the external media. Dykens (1979) concluded that there may be increase in ciliary activity of the nephridia during hypoosmotic stress, which has been confirmed by microscopic examination (C.P. Mangum, unpublished observations). This increase in activity would enhance the passage of intact FAA to the external medium.

The second subject of the present study, the ribbed mussel, M. demissus, also possesses nephridia, but intact FAA were not detected in the external medium during low salinity acclimation (Bartberger and Pierce, 1976). The nephridia drain the pericardium, the fluid-filled sac that surrounds the heart. FAA pass into the bathing medium from an isolated ventricle, but do not pass out of the intact animal. Perhaps the nephridia are able to reabsorb FAA and either degrade them or pass them on to a site where this can occur. The giant nephridria of the polychaete Sabella pavonia accumulate and transport large amounts of organic molecules, such as sugars and FAA (Koechlin, 1979;

1980; 1981; 1982; 1984). This process is also $Na^+$ dependent (Koechlin, 1986).

This study was undertaken to further characterize volume regulation in G. dibranchiata and to determine whether the presence of closed nephridia is correlated with the fate of FAA during low salinity stress. M. demissus was also examined to determine the role of the nephridia during hypoosmotic stress.

## MATERIALS AND METHODS

1. Care of Animals

Individuals of Glycera dibranchiata were obtained from local bait stores during fishing season or shipped live from the Maine Bait Co. during off season. The animals were kept in aerated aquaria with sand. Natural sea water was used as a base medium at all times. Appropriate concentrations were made by mixing natural sea water with commercial sea salt or by diluting it with pond water. Natural sea water was collected from VIMS, Gloucester Point, Va. ($18^{o}$/oo) and Wachapreague, Va. ($30.5^{o}$/oo). Worms were acclimated to high salinity ($30^{o}$/oo - $38^{o}$/oo) for 4-7 days prior to experimentation. No worms were kept longer than three weeks.

Modiolus demissus was collected from Indian Field Creek, a subestuary of the York River, Va. Specimens were scrubbed with a wire brush to remove mud and other debris from the valves. They were then placed in aerated aquaria containing natural sea water of high salinity ($28^{o}$/oo - $33^{o}$/oo ). Animals were acclimated to laboratory conditions for at least one week prior to experimentation. No animals were kept longer than 6 weeks. Animals were fed every other day with a commercially prepared food designed for filter feeding invertebrates.

2. Volume Regulation, Total Ninhydrin Positive Substances and Ammonia Excretion

Twelve worms were selected, carefully blotted dry and weighed on a balance to the nearest 0.01 gm. Six were placed in individual finger bowls containing 300 ml 18°/oo sea water. The other six were placed in bowls containing 300 ml 33°/oo sea water; these were used as the controls. At times ranging from 0 to 120 hours the worms were removed from the water, carefully blotted dry and weighed. Also at these times samples of the water were taken. One tenth ml was assayed for total ninhydrin positive substances (TNPS) as described by Dykens (1979). A 5 ml sample was assayed for ammonia by the phenolhypochlorite method (Solorzano, 1969). The water in the bowls was replaced at each interval with fresh experimental media.

3. Tissue Hydration

Six worms acclimated to 33°/oo sea water were selected and a portion of their body was removed and blotted dry. This was then weighed on a torsion balance and dried at 60°C for 24 hours. Samples were again weighed and tissue hydration calculated by the following formula:

$$\frac{\text{wet weight - dry weight}}{\text{wet weight}} \times 100$$

Twelve animals were placed in an aerated aquarium of 18°/oo sea water and another twelve placed in one containing 33°/oo sea water as controls. Six animals were removed from each aquarium at 1 and 3 hours and tissue hydration determined as above.

4. Red Coelomocyte Volume Regulation (in Whole Animals)

Twelve worms adapted to 38°/oo sea water were selected. Six were placed in individual finger bowls containing 300 ml of 18°/oo sea water; the six controls were placed in bowls containing 38°/oo sea water. At times ranging from 0 to 120 minutes blood samples were taken from each worm using a syringe; the same animals were used throughout the experiment. The samples were then placed into hematocrit tubes and centrifuged at low speed for 1 minute. Tubes exhibiting cell lysis were discarded. Cell volume was calculated by measuring the % of cells to coelomic fluid with vernier calipers.

5. Red Coelomocyte Volume Regulation (Isolated Cells)

Two to three worms acclimated to 30°/oo sea water were sacrificed and the blood was collected in a beaker. Approximately 2 ml of blood was placed into each of two centrifuge tubes and centrifuged at low speed for one minute. The coelomic fluid was drained off the coelomocyte pellet with a pasteur pipette. The pellet in one tube was resuspended with 0.5 ml of 18°/oo sea water and mixed thoroughly; the other tube was resuspended with 0.5 ml 33°/oo sea water as a control. Cell suspensions were kept mixed and aerated by bubbling with a pasteur pipette at frequent intervals. At times ranging from 0 to 60 minutes samples were placed into hematocrit tubes and centrifuged at low speed for 1 minute. Tubes with evident cell lysis were discarded. Cell volume was determined as above. This procedure was repeated several times in an attempt to reduce experimental error.

6. Changes Internal Osmolality and TNPS

Worms adapted to 33°/oo sea water were selected; one half were placed in 18°/oo sea water; the other half were placed in 33°/oo sea water as controls. At times ranging from 0 to 11 hours 9 animals were sacrificed and the blood was collected. The samples were placed in a microcentrifuge and centrifuged for 1 minute. The coelomic fluid was drawn off with a pipette; samples with evident cell lysis were discarded. These samples were assayed for TNPS as described previously and osmolality determined with a Wescor 5500 Vapor Pressure Osmometer.

7. Ammonia and TNPS Excretion by Isolated Tissue

Portions of the body wall from 4 worms acclimated to 33°/oo sea water were excised and placed into a two compartment diffusion chamber. The external side of the body wall faced the upper chamber. In the experiment, both compartments contained 18°/oo sea water; in the control both contained 33°/oo sea water. Samples were taken from the upper compartment at times ranging from 0 to 180 minutes and assayed for ammonia as described previously. The water in the upper compartment replaced at each interval.

In another experiment pieces of tissue were once again placed in diffusion chambers. However, this time the bottom compartment was filled with a 0.9 mM proline solution; proline is one of the major FAA used by the coelomocytes during volume readjustment (Costa et al, 1980). The proline was dissolved in sea water of the appropriate salinity: 18°/oo for the experimental and 33°/oo for the control. At the times stated above samples were taken from the upper chamber and

assayed for TNPS and ammonia by the method previously described. This experiment was repeated with the tissue polarity reversed; the internal face of the tissue faced the upper compartment.

8. Ventilation in Mussels with Propped Open Valves

Six mussels were selected and were propped open by the insertion of a piece of glass tubing between the valves. These animals were then placed in individual bowls containing 1 liter of 33°/oo sea water. Carmine particles were placed in the water so as to make currents more visible. Animals were observed for 1 hour.

9. TNPS Excretion by Propped Open Animals

Twelve mussels were propped open as described above and their mantle cavity drained of excess water. Six were then placed in bowls containing 1 liter of 18°/oo sea water; the other six were placed in bowls containing 1 liter of 37°/oo sea water as controls. At times ranging from 0 to 48 hours water samples were taken and assayed for TNPS as previously. After each sample was taken the water was replaced.

10. TNPS Excretion by the Heart (in vitro)

Mussels acclimated to high salinity were opened and one valve gently removed so as not to damage the heart. The pericardium was removed and a fine piece of surgical silk was tied around each auricle. The heart was then removed from the animal and placed in a shallow dish of 37°/oo sea water until normal beat was resumed. After a spontaneous beat was established the heart was placed in a modified heart chamber,

similar to that used by Welsh and Taub (1948). The chamber was initially filled with 5 ml 37°/oo sea water. The heart was incubated in this medium for 1 hour after the normal beat had resumed and then the water was assayed for TNPS. The water was carefully drained off and replaced with 18°/oo sea water. At times 0.5 - 2 hours after the beat had resumed the water was assayed for TNPS and replaced with fresh experimental media.

11. TNPS Excretion by the Heart (in vivo) and Changes in Internal Osmolality

Mussels acclimated to 28°/oo sea water were selected and their valves propped open as previously described. One half was placed in an aerated aquarium of 14°/oo sea water; the other half was placed in an aerated aquarium of 28°/oo sea water as controls. At times ranging from 0 to 12 hours six animals were removed from each group and sacrificed. A small hole was drilled in the shell in the approximate area of the heart. A sample of the pericardial fluid was carefully taken with a microsyringe. The fluid was then centrifuged for 5 minutes in a microfuge. A portion of the sample was diluted to 1:39 with sea water and read in a Beckman DK-2A Spectrophotometer at 280 nm. Samples exhibiting any absorbence were discarded due to contamination with blood. Clean samples were assayed for TNPS and osmolality determined as previously described.

12. Amino Acid Uptake Rates of Isolated Tissues

Glycera and Modiolus acclimated to 33°/oo sea water were sacrificed and selected tissues removed. From Modiolus, the gill and

nephridia were taken; body wall (free of gills, nephridia and gut), and nephridia were taken from Glycera. Tissues were kept in small petri dishes containing 33°/oo artificial sea water (ASW) until needed. This solution was considered 100 % ASW; the 50 % ASW was made by mixing equal parts of 100% ASW with distilled water.

The incubation solutions were made with 100 % ASW and α-amino isobutyric acid (AIB), a nonmetabolizable amino acid analog, ranging in concentration from 10 mM to 200 mM. Each of the incubation solutions also contained [$^{14}$C] AIB as an indicator of uptake rates. The tissues were incubated as follows: a) 0.5 μCi/ml for Glycera body wall and Modiolus gill, b) 0.75 μCi/0.2 ml for Modiolus nephridia, and c) 0.75 μCi/0.1 ml for Glycera nephridia. The specific activities ranged from 5.5 DPM/nM to 825 DPM/nM.

Pieces of tissue were gently blotted and weighed to the nearest 0.01 mg. They were then placed in 1 ml of the incubation media and set on a shaker to prevent the formation of gradients. Modiolus nephridia were incubated in 200 μl and Glycera nephridia in 100 μl due to the small size of the tissue. Tissues from each of three animals were used for each concentration and incubated for intervals of 15 - 60 minutes. The tissue was then run through a series of three quick ASW washes of 10 seconds each to remove any residual radioactivity. The tissue was placed in a scintillation vial containing 500 μl of a tissue solublizer (Protosol by NEN) and allowed to sit overnight. This was then diluted with 15 ml of scintillation cocktail (Aquasol). The samples were counted in a Beckman Scintillation Counter.

The procedure was repeated for all of the tissues, except the Glycera nephridia, with the substitution of 50 % ASW in the incubation media as a low salinity stress.

13. Statistical Analysis of Data

All data were analyzed using t tests except where noted otherwise in the text.

## RESULTS

### A. GLYCERA DIBRANCHIATA

#### 1. Volume Regulation, TNPS and Ammonia Excretion

Worms transferred from 33°/oo to 18°/oo sea water show a very rapid increase in volume (figure 1). This increase peaks at hr 6. There is a recovery in volume of approximately 10 % between the hours of 6 and 48, with no further net decrease.

The control worms exhibited only slight fluctuations in volume. Over the period of the experiment there were no measurable amounts of TNPS excreted by the worms at either salinity (results not shown). A small amount of color change in the assay was due to the ammonia being excreted. The assay employed is also sensitive to ammonia, which was monitored simultaneously and subtracted from the TNPS values.

Worms exposed to 18°/oo have elevated levels of ammonia excretion over the experimental period when compared to the controls (figure 2). The animals started out with a very high excretion rate at 0.5 hr that dropped drastically at 1 hr. The rate then increased to peak at 3 hours. After this time the rate leveled out for the remainder of the experiment, but remained elevated above that of the controls when the grand means of the ammonia excretion rates from hrs 6 through 120 are compared.

In ammonia excretion the control animals exhibited the same trends as the experimental animals, but they did not excrete the large amounts in the first 18 hours (figure 2). For the remainder of the experiment the rate was fairly constant.

The error bars in figure 2 are quite large due to the variability of the individual animals. When the rates are plotted for each individual, the worms can be seen to follow the same trends in excretion rates while the actual amount of ammonia being excreted differs (figure 3).

2. Mortality due to Size

When exposed to a low salinity stress, worms weighing more than six grams had a higher mortality rate than those weighing less than six grams (table 1).

3. Tissue Hydration

As seen in table 2 the experimental animals experienced an increase in the hydration of the body wall (~7%). The control animals experienced a similar decrease.

4. Changes in Internal Osmolality and TNPS

Figure 4 shows a clear drop in the osmolality of the coelomic fluid of the experimental animals. The greatest decrease occurred within the first 30 minutes of exposure to low salinity. The osmolality continued to drop until hr 6. After this time it leveled off and remained slightly, but not significantly, higher than that of the $18^{o}/oo$ sea water for the remainder of the experiment ($p > 0.05$).

The control animals did not behave as expected. The coelomic fluid initially appeared to be hypoosmotic to the medium. The osmolality increased until hr 12 when it became isosmotic to the media. It remained isosmotic for the remainder of the experiment.

Coelomic fluid TNPS from the same animals exhibited little change under experimental conditions (figure 5); it appeared to be fairly constant for most of the experiment but with a slight increase at 12 hours. Once again the control animals showed unexpected trends. The TNPS concentration dropped below the initial amount and fluctuated significantly below this value for two hours ($p = 0.05$). At hr 3 the concentration rose above the initial and experimental values and remained high. Together with the osmolality results, these data suggest that the control animals were not at osmotic equilibrium with the $33^{o}/oo$ water prior to the experiment. However, the osmolality of both their acclimation medium and that used during the experiment was measured and it did not differ.

There is no significant difference in the values for TNPS between the experimental and control animals ($p = 0.05$).

5. Red Coelomocyte Volume Regulation (in Whole Animals)

Both the experimental and control animals appeared to exhibit a decrease in red cell volume that never quite returned to the original volume (figure 6). The experimentals appear to recover more than the control coelomocytes. The was no significant difference between the experimental and the control coelomocyte responses ($p = 0.05$).

6. Red Coelomocyte Volume Regulation (Isolated Cells)

The data in figure 7 represent individual runs of cells pooled from two animals. They were not averaged together due to the variability of the response. In one run red coelomocytes exposed to sea water appear to show a decrease in size, whether exposed to low or control salinity. This shrinking phase occurred within the first 2 minutes of exposure to the test medium.

In another run the experimental cells swelled slightly in the first 2 minutes, then decreased in size. This shrinking phase lasted for 20 minutes and the cells went below their intial volume. At 40 minutes the cells had swelled to a greater volume than at 2 minutes. The control cells from the same group also swelled (at 10 minutes) and then shrunk to less than the original volume.

The data may reflect an osmotic disequilibrium of the worms as seen previously. This is not certain because the internal osmolality was not measured on the worms used in this part of the experiment.

7. Ammonia and TNPS Excretion by Isolated Tissue

The first part of this experiment was to determine if isolated tissue exhibited the same response as the whole animal in terms of high initial ammonia excretion when exposed to a low salinity. Body wall transferred to $18^{o}/oo$ sea water in the diffusion chamber exhibited a high rate of ammonia excretion for the first 15 minutes (figure 8). This rate then decreased but remained elevated when compared to the excretion rate of tissue from the same animal exposed to $33^{o}/oo$ sea water. The amount of ammonia excreted varied from worm to worm, but appears to follow the same trends.

No measurable amounts of proline were seen to pass across the body wall, regardless of the polarity, in either salinity (figures 9 and 10). There are two peaks in ammonia excretion seen at approximately 15 and 60 minutes. The second peak may be a delayed response to the salinity change, but was not seen in tissue exposed to $18^{o}/oo$ sea water on both sides. This may indicate that the proline from the bottom half of the chamber was deaminated and the ammonia excreted. The control tissue had a very low excretion rate for the duration of the experiment.

8. FAA Uptake by Isolated Tissue

The body wall of G. dibranchiata showed a decrease in the rate of uptake when subjected to a decrease in salinity (table 3). Both the $J_{max}$ (the maximal rate of uptake) and $K_t$ (the concentration at which the rate of uptake is half-maximal) decrease.

The results presented for the nephridia from G. dibranchiata are highly suspect. The data is variable and trends in correlation between treatments and isotope uptake could not be determined. Problems occurred with the experiment using Glycera nephridia, which are very tiny. It took several hours to extract enough nephridia from a worm to conduct an experiment. The state of the tissue after removal was also uncertain, i.e. whether it was intact or had other tissue associated with it. The small size also made transferring the tissue through the necessary solutions difficult and it was often lost. Carryover of the label may be partly responsible for part of this. There might also be a variable extracellular retention due to labeled media trapped in the protonephridial duct.

## B. MODIOLUS DEMISSUS

### 1. Ventilation of Propped Open Animals

Mussels with glass tubing inserted between their valves appear to ventilate. The carmine particles were observed to enter and exit the valves in the area of the siphons.

### 2. TNPS Excretion of Propped Open Animals

No measurable TNPS were detected in the incubating media of either the experimental or control animals.

### 3. TNPS Excretion by Heart (in vitro)

An isolated ventricle had an elevated rate of TNPS excretion when transferred to a lower salinity (table 4). This experiment was successfully performed only once; there was no successful control. Out of 60 animals only one heart continued to beat for the duration of the experiment. The same apparatus was tried on hearts taken from *Mercenaria mercenaria* to determine whether the problem was technical or related to the tissue. All four *M. mercenaria* hearts used beat for several hours in the apparatus, thus showing that the procedure and apparatus were not at fault.

### 4. TNPS Excretion by Heart (in vivo) and Changes in Internal Osmolality

Changes in the osmolality of the pericardial fluid exhibited clear trends. The experimental animals exhibited a drop in internal osmolality over the course of the experiment (figure 11); the greatest

decrease occurred between hours 2 and 3. They become isosmotic with the external media in approximately 12 hours. The control animals stayed slightly hyperosmotic to the external media for the course of the experiment.

Both experimental and control animals exhibited no significant changes ($p > 0.05$) in pericardial TNPS concentration for the first three hours of the experiment (figure 12). At hr 3 the experimental group experienced an sharp increase in TNPS concentration that continued for the duration of the experiment. The control animals also experienced an increase, but this did not occur until hr 6 and it was not as dramatic. The concentration then leveled out for the remainder of the experiment.

The number of animals represented by each point is small and varies. This was not due to a lack of animals; over 200 animals were sacrificed during this part of the experiment. Problems occurred in taking samples from the pericardium; they were often contaminated with blood. These samples gave extremely high TNPS readings and were discarded. The samples represented on the graph are those that I am reasonably sure were not contaminated.

5. FAA Uptake by Isolated Tissues

When isolated gill tissue of M. demissus was exposed to a decrease in salinity (100 % ASW to 50 % ASW) there was an increase in the uptake rate of AIB (table 3). Both $J_{max}$ and $K_t$ changed with the change in salinity, but stayed in the same ratios.

Isolated nephridia tissue from the same organism exhibited a different response to the low salinity stress. There appears to be no

change in the uptake rate of AIB (table 3) under hypoosmotic conditions. Neither $J_{max}$ or $K_t$ are significantly different ($p = 0.05$).

The data presented in Table 3 were analyzed by the use of Lineweaver-Burke plots. All the systems studied exhibited typical Michaelis-Menten kinetics and were saturable.

## DISCUSSION

### 1. GLYCERA DIBRANCHIATA

Glycera dibranchiata shows a very limited capacity to regulate volume after a hypoosmotic shock. Present observations show larger worms have less ability to regulate volume and were more likely to die before completion of the experiment, usually within 60 hours. Machin (1975) reported no difference in the ability to regulate volume due to size; his size range was 2.7 - 6.5 gm initial weight. A large majority of worms in the present investigation were bigger (7 - 10 gm). This indicates that the ability to volume regulate is related to size.

When compared with the even more euryhaline polychaete, N. succinea, the rate of readjustment in G. dibranchiata is much slower; N. succinea takes 12-48 hours to readjust its volume, depending on the magnitude of the salinity change (Dykens, 1979; Dykens and Mangum, 1984). N. succinea also goes through four distinct phases during volume readjustment: 1) swelling, 2) plateau, 3) shrinking, and 4) steady state. G. dibranchiata only exhibits two clear phases: 1) swelling and 2) steady state. The shrinking between the two phases is so small that it is difficult to delineate clearly. N. succinea approaches the original weight far more closely than G. dibranchiata. Both are imperfect volume regulators, but Nereis has a better ability to readjust than does Glycera.

G. dibranchiata volume regulation differs from that of N. succinea in other ways. N. succinea excretes a pulse of FAA into the medium during a low salinity stress (Dykens, 1979; Dykens and Mangum, 1984). At no time were measurable amounts of FAA detected in the media containing Glycera, in spite of a clear increase in the coelomic fluid FAA. The present investigation also shows that FAA do not cross the isolated body wall in either direction. Qafaiti and Stephens (1988) also found no leakage of FAA across the body wall. This would indicate that the FAA do not cross the body wall during hypoosmotic stress and that they have another fate. There is a decrease in size and a change in the composition of the FAA pool of the body wall and coelomocytes with a decrease in salinity (Costa et al., 1980). Chien et al., (1972) reported that both sides of the isolated body wall of G. dibranchiata are capable of taking up FAA from the media; however, the internal surface had a significantly greater uptake rate. Qafaiti and Stephens (1988) also found that the FAA were not translocated across the body wall from the external media.

N. succinea exhibits an ammonia excretion pattern that closely resembles that of its volume readjustment (Dykens, 1979; Dykens and Mangum, 1984). The pattern of ammonia excretion in Glycera also seems to follow that of its volume readjustment. There are two phases: 1) an increase in rate and 2) steady state. In animals transferred to low salinity the rate is elevated above that of the controls. Both the experimental and control animals exhibit a high initial excretion rate followed by a sharp decrease, but this pattern is common in a variety of taxa (Mangum, personal communication).

Upon exposure to dilute media worms visibly swell and cease all unnecessary movement. There also appears to be a decrease in blood cells circulating through the gills. Tissue hydration studies do not show a large increase. Costa et al. (1980) found that tissue hydration increased from 70.6 % to 83.2 % after 2 weeks at 374 mosm/Kg $H_2O$. Tissue hydration in N. succinea increases from 76 % to 85 % when adapted to a low salinity (10 % ASW) (Freel et al., 1973). This small difference, coupled with the sharp decrease in internal osmolality may indicate that the increase in weight is due largely to an increase in the volume of coelomic fluid.

There were few changes in the concentration of TNPS present in the coelomic fluid of the experimental animals. Since it appears that the volume of the coelomic fluid increases this would indicate that the amount of FAA are also increasing. There is no dilution of the TNPS. These FAA are presumably being released from the tissues into the coelomic fluid. A source of these may be from the coelomocytes which are known to release FAA under hypoosmotic conditions (Costa et al., 1980). In the present study, ammonia was not measured because of the small size of the samples. Clark (1968) found that ammonia comprises less than 10 % of the TNPS of the coelomic fluid.

The results of the red coelomocyte volume regulation portion of this study are inconclusive. The method employed showed a decrease in volume for both the control and experimental preparation. Amende and Pierce (1980a) employed the same technique using Noetia ponderosa red blood cells and obtained clear evidence of swelling. With a different method the coelomocytes of G. dibranchiata exposed to isosmotic sea water have been demonstrated to shrink over time (Machin and O'Donnell,

1977; Costa et al., 1980). A Coulter counter was used to monitor changes in cell volume and the same sample was used throughout the entire experiment. My method required a new sample at each reading.

Data for in vivo coelomocyte volume regulation do not show a significant difference between experimental and control animals due to the large error bars. If these are ignored trends are apparent. The initial decrease in the hematocrit for both groups can be attributed to a decrease in the number of coelomocytes present in the coelom. The control group continues to decrease for this same reason. The experimental gro up does show a recovery. This recovery could be due to the swelling of the coelomocytes since it is presumable that the cell number is not increasing.

FAA do appear to efflux into the coelomic fluid; however, they are not excreted intact into the media nor do they efflux across the isolated body wall during hypoosmotic stress. It is reasonable to suggest that the presence of closed protonephridia contributes to the FAA impermeability. Costa et al. (1980) reported a decrease in the amount of FAA present in the body wall after a two week exposure to low salinity. The composition of the FAA pool also changes. Measuring the five major constituents of the FAA pool, there is a decrease of 390 mole/gm-dry wt over the two week period (Costa et al., 1980). An increase of 165.4 mole/gm-dry wt of ammonia was lost from the worms during a five day interval. If one assumes that three times this amount would be excreted in 14 days, then this would more than account for the decrease in FAA seen by Costa. This phenomenon is similar to that seen in N. succinea, which excretes an excess of FAA and ammonia.

The trend seen in the influx rates of the body wall of G. dibranchiata agrees with that found in the literature. There is a decrease in the uptake rate with a decrease in salinity. The increase in the $K_t$ value would seem to indicate a decrease in the affinity of the transport enzyme for the amino acid. The transport of alanine by the body wall was reported to occur by two separate pathways, both of which are $Na^+$ dependent (Stevens and Preston, 1980a, b). This dependency could account for the decrease in transport with the decrease in salinity.

2. MODIOLUS DEMISSUS

M. demissus also does not excrete measurable amounts of FAA into the external media upon exposure to a hypoosmotic stress. Mussels do exhibit an increased rate of ammonia excretion in the intervals 12 - 24 hours, 24 - 36 hours and 48 - 60 hours after initial exposure; after this time the rate returns to that of the controls (Bartberger and Pierce, 1976). The levels of FAA in the hemolymph rise within 4 hours and the ammonia levels increase at 24 hours (Bartberger and Pierce, 1976). The present findings show that the concentration of FAA also increases in the pericardial fluid, corresponding to the trend seen by Bartberger and Pierce (1976). The hypothesis that the FAA are not degraded intracellularly, but that they are released intact from the cells is substantiated by the work of Pierce and Greenberg (1972) and the response of isolated ventricles to low salinity.

Davenport (1979) reported that another mechanism in Mytilus edulis may combat low salinity stress. Even though the mussel was visibly gaping and the siphon extended, the excurrent siphon was closed. This

would reduce the exchange rate between the mantle cavity and the external medium, thus reducing the magnitude of the hypoosmotic shock. Davenport criticized the use of propped open valves. The present observations, however, clearly indicate that water was circulating through the mantle cavity of M. demissus. Using freshly collected mussels, Strange and Crowe (1979) found that M. demissus exposed to 60% sea water rapidly opened up and resumed active ventilation. Samples of the mantle cavity fluid and hemolymph showed that the animals had nearly reached osmotic equilibrium with the external medium within three hours. Samples of the pericardial fluid taken from laboratory acclimated animals in the present study also show a rapid decrease in pericardial osmolality, although 12 hours was required to reach steady state with the medium. The animals were also observed to resume ventilation soon after exposure.

The use of laboratory acclimated animals has also been in question. The time that animals are kept before experimentation varies from one week (Shumway, 1977) to at least three weeks (Pierce and Greenberg, 1972). Strange and Crowe (1979) reported a difference in the behavior of laboratory acclimated animals and freshly collected ones; laboratory animals immediately closed their shells when exposed to low salinity and did not show any osmotic changes after six hours. Fresh mussels, however, began active pumping very shortly after exposure to low salinity. They proposed that the difference was related to the nutritional and metabolic state of the animals. The mussels used in this study were fed with a commercially prepared food for filter feeding invertebrates. There were no differences observed in the response of the animals with respect to the period of time they

were held in the lab. Wright and Stephens (1977) also report no difference in the experimental results obtained from fresh Mytilus californianus and those held in the laboratory for eight weeks.

The trend in amino acid uptake rates for the isolated gill tissue from M. demissus differs from that in the literature. Results from whole animal and isolated tissue experiments show a decrease in the rate of amino acid influx as the salinity decreases (Stephens, 1964; Stephens and Virkar, 1966; Anderson and Bedford, 1973; Wright et. al., 1987). Results from this study indicate an increase in the uptake rate. Pa rt of this discrepancy may be due to the use of an amino acid analog. AIB was used in these experiments because it is nonmetabolizable and would not be broken down or incorporated into cellular components. It is also not normally present in the cells thus avoiding the complications of an intracellular pool. There are reports of at least four separate pathways for amino acid uptake in bivalve gill (Stewart, 1978; Wright, 1985; Bishop et. al., 1983). An unreported amino acid transport system may be responsible.

Wright (1979) reported that the cilia on the lateral surface of isolated gills from M. californianus cease to beat upon removal from the animal. This caused an overestimate in the value of $K_t$. However, Crowe et al. (1977) reported that the cilia on the gills isolated from M. demissus appear to continue to beat actively. This was not done under hypoosmotic conditions so there may be a diminished activity of the lateral cilia due to stress. This factor may have caused the observed increase. However, these unstirred boundary effects should have been reduced by the use of the shaker during the incubation period.

In the present experiments the labeling of extracellular space was counteracted by multiple washings of the tissue in unlabeled ASW. Wright and Stephens (1977) reported that influx rates are overestimated due to the labeling of the extracellular space and corrected for it by using $^{14}C$ inulin.

Uptake of ions, amino acids, and other organic compounds has been documented for the nephridia of annelids (Koechlin, 1979; 1981a; 1981b; 1982; 1984; Smith and Ruppert, 1986), and molluscs (Suzuki, 1988). The data from the present study indicate that this is also true of M. demissus. This is substantiated by the presence of a transport system and the absence of detectable amounts of FAA being excreted into the media.

The interesting finding is the lack of change in the influx rate with a drop in salinity. This lack of change and differing values of $J_{max}$ and $K_t$ from that of the gill may indicate a different transport system.

Both G. dibranchiata and M. demissus are able to tolerate a range of salinities and employ a large pool of FAA to combat the stress. These osmolytes are not excreted intact from either animal in detectable amounts, but there is an increase in the rate of ammonia excretion. This suggests that the FAA are deaminated. The nephridia are believed to be responsible for the prevention of loss of the FAA to the external media. In at least M. demissus the nephridia are capable of taking up FAA from solution.

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Figure 1. The time course of volume readjustment by Glycera dibranchiata acclimated to 33°/oo and exposed to 18°/oo. N = 6, mean ± s.e.

■ Experimental, 18°/oo

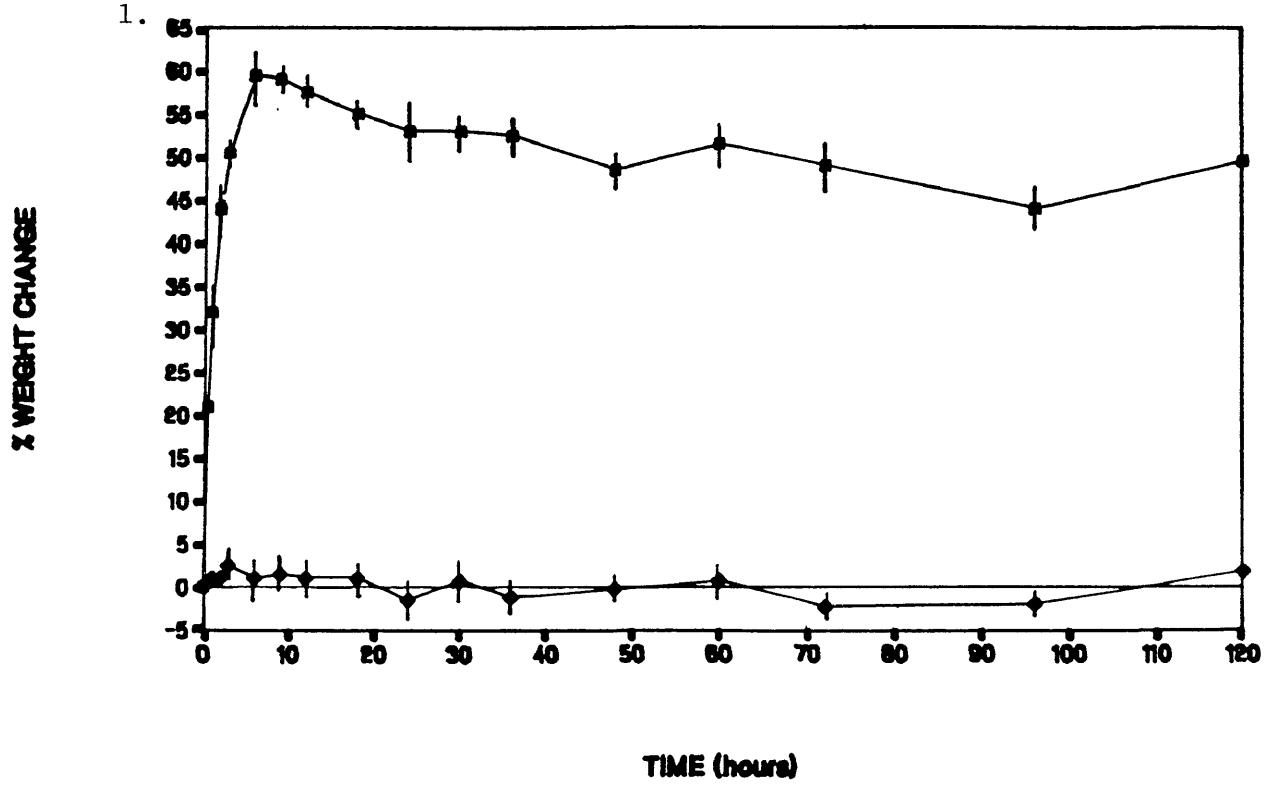
◆ Control, 33°/oo

Figure 2. The pattern of ammonia excretion by Glycera dibranchiata during the course of volume readjustment, 33°/oo to 18°/oo. N = 6, mean ± s.e.

■ Experimental, 18°/oo

◆ Control, 33°/oo

1.


% WEIGHT CHANGE
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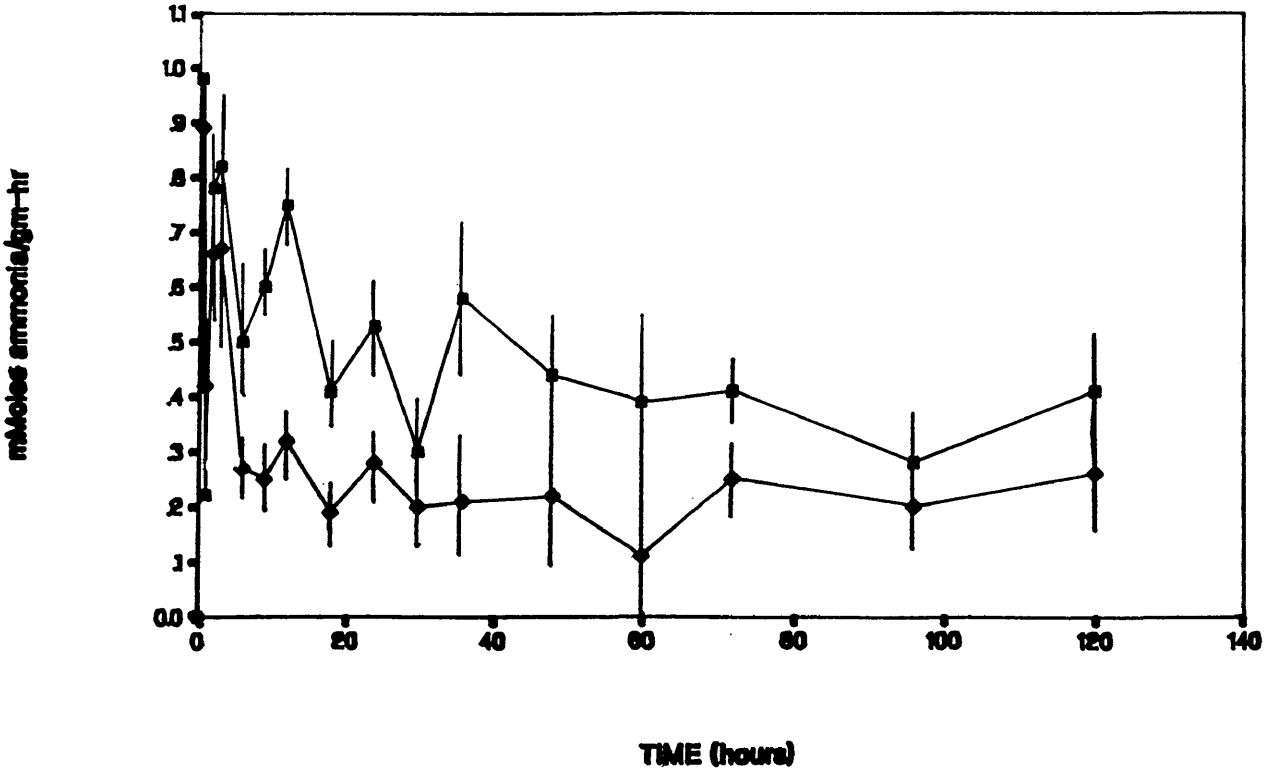
TIME (hours)

2.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TIME (hours)

Figure 3. Ammonia excretion rates of individual Glycera dibranchiata exposed to 18°/oo sea water.

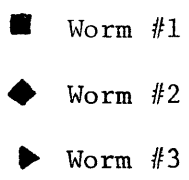
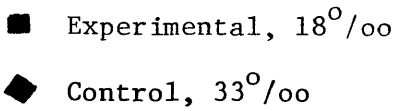



Figure 4. Changes in the coelomic fluid osmolality of Glycera dibranchiata during the course of volume readjustment, 33°/oo to 18°/oo. N = 9, mean ± s.e.



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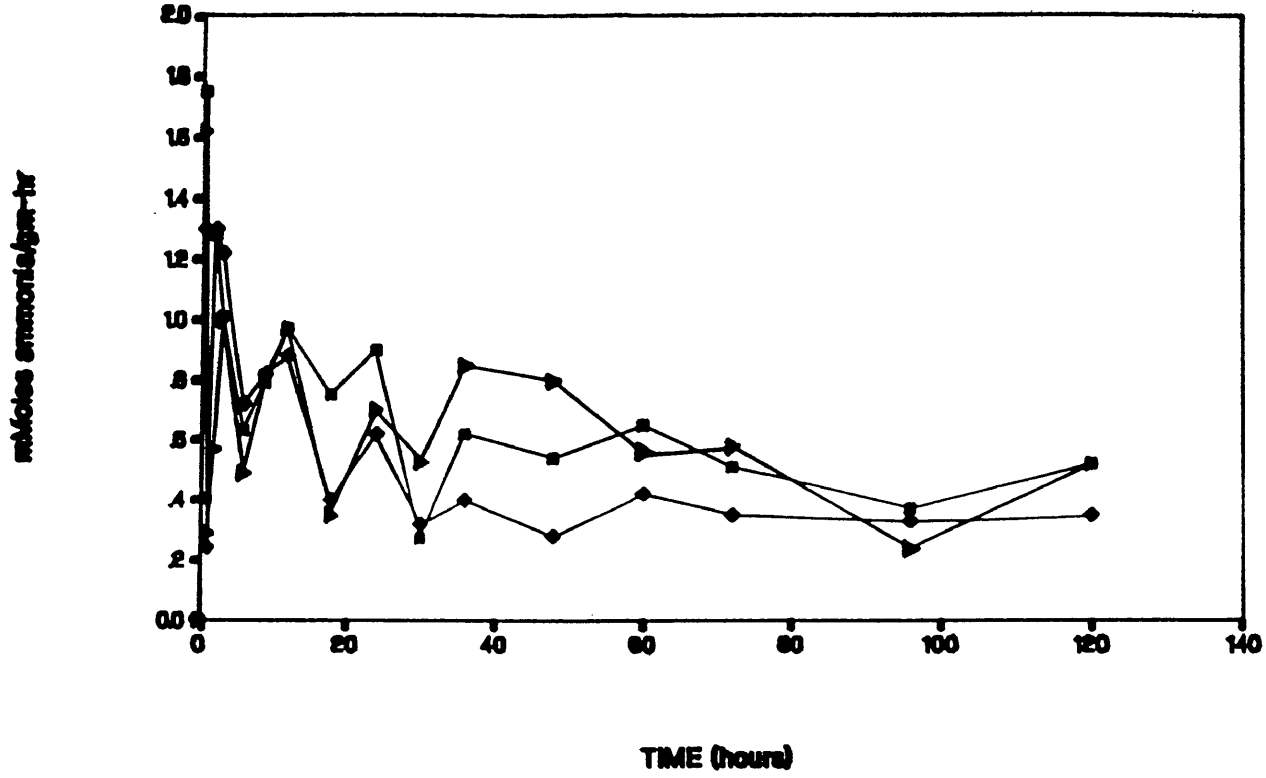
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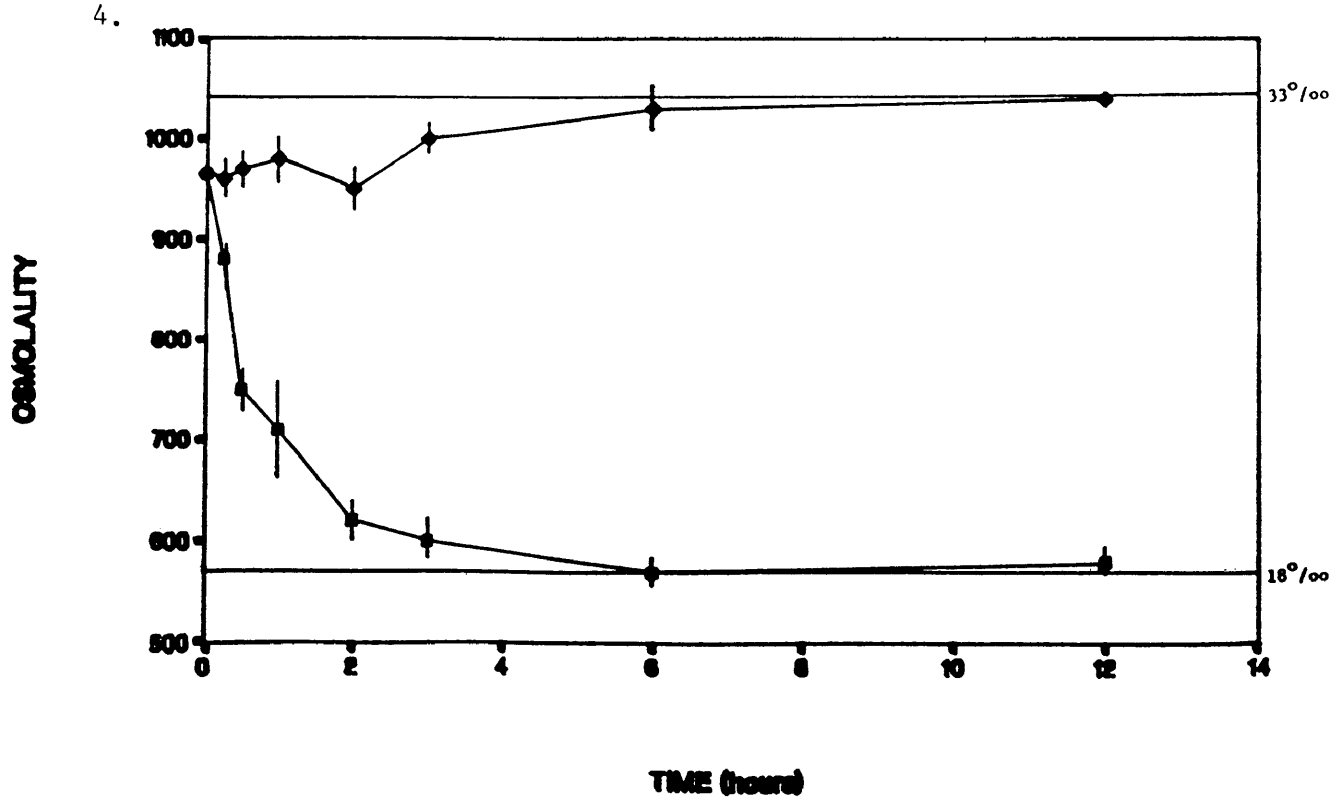
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18°/oo
TIME (hours)

Figure 5. Changes in the coelomic fluid TNPS concentration of Glycera dibranchiata during the course of volume readjustment, $33^{o}/oo$ to $18^{o}/oo$. N = 9, mean $\pm$ s.e.

■ Experimental, $18^{o}/oo$

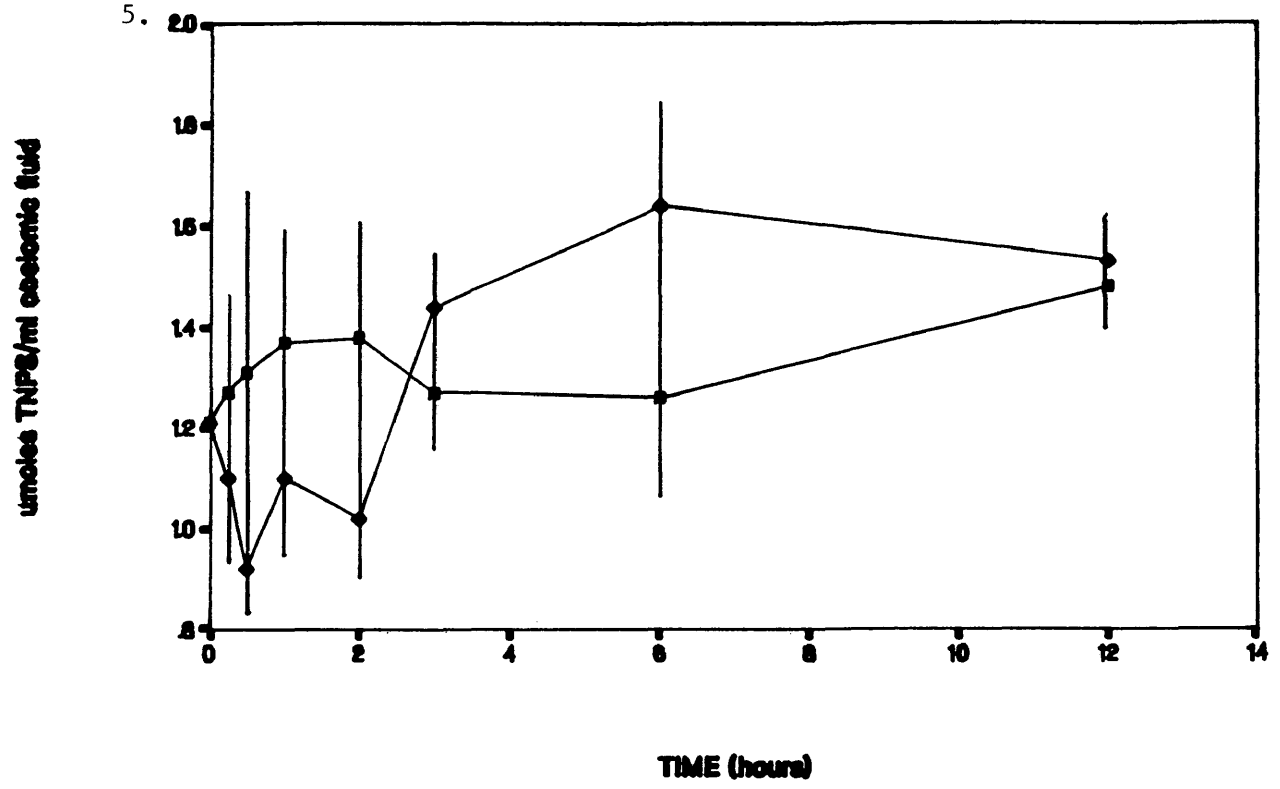
◆ Control, $33^{o}/oo$

Figure 6. Time course of volume readjustment of the red coelomocytes taken from worms exposed to a low salinity stress, $37^{o}/oo$ to $18^{o}/oo$. N = 6, mean $\pm$ s.e.

■ Experimental, $18^{o}/oo$

◆ Control, $37^{o}/oo$

5.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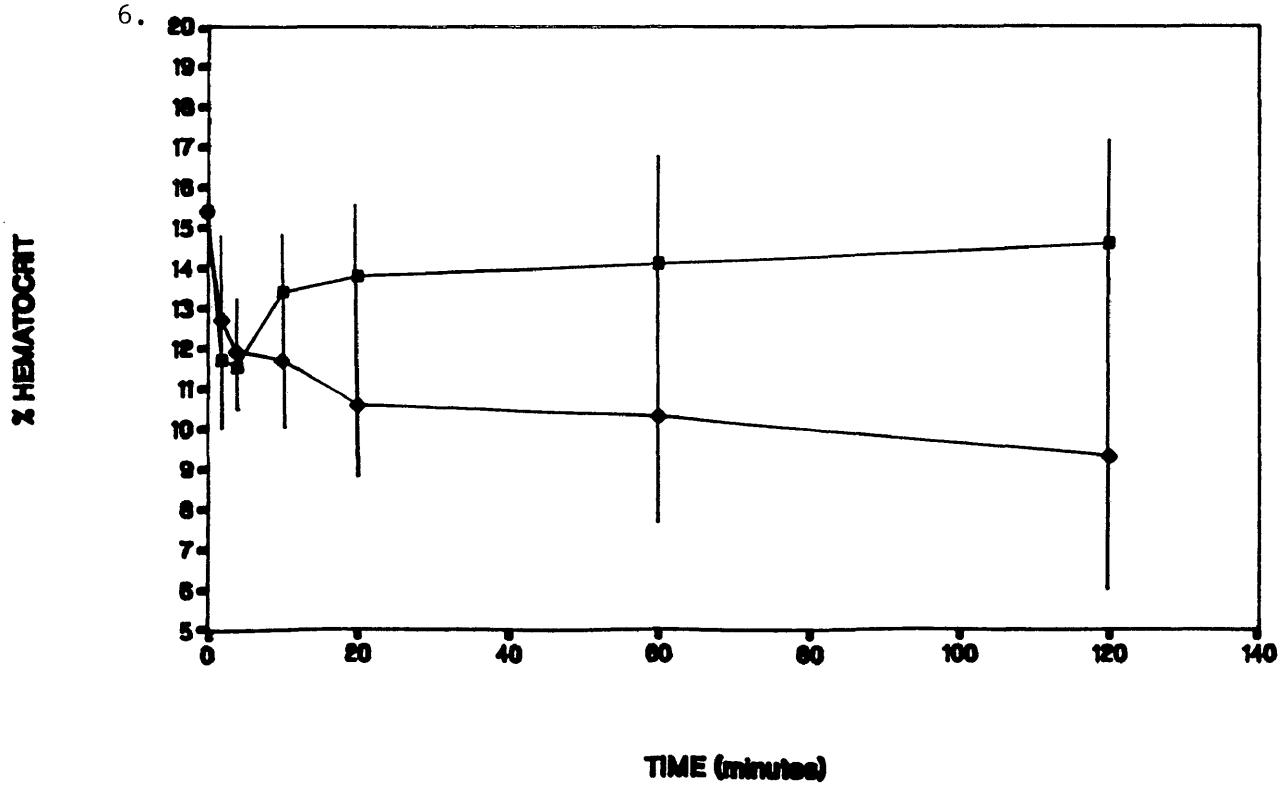
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140
TIME (minutes)

Figure 7. Volume readjustment by red coelomocytes isolated from Glycera dibranchiata, $33^{o}/oo$ to $18^{o}/oo$.

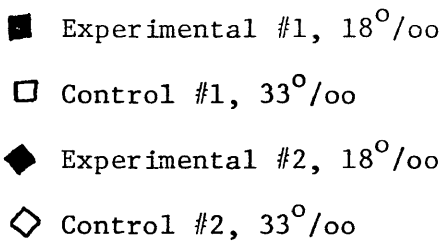
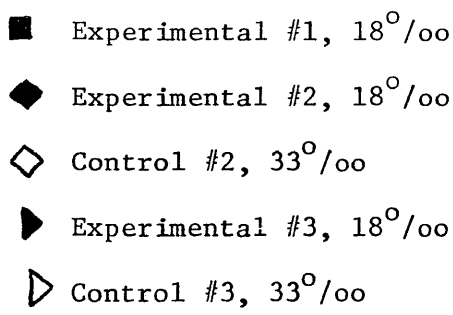
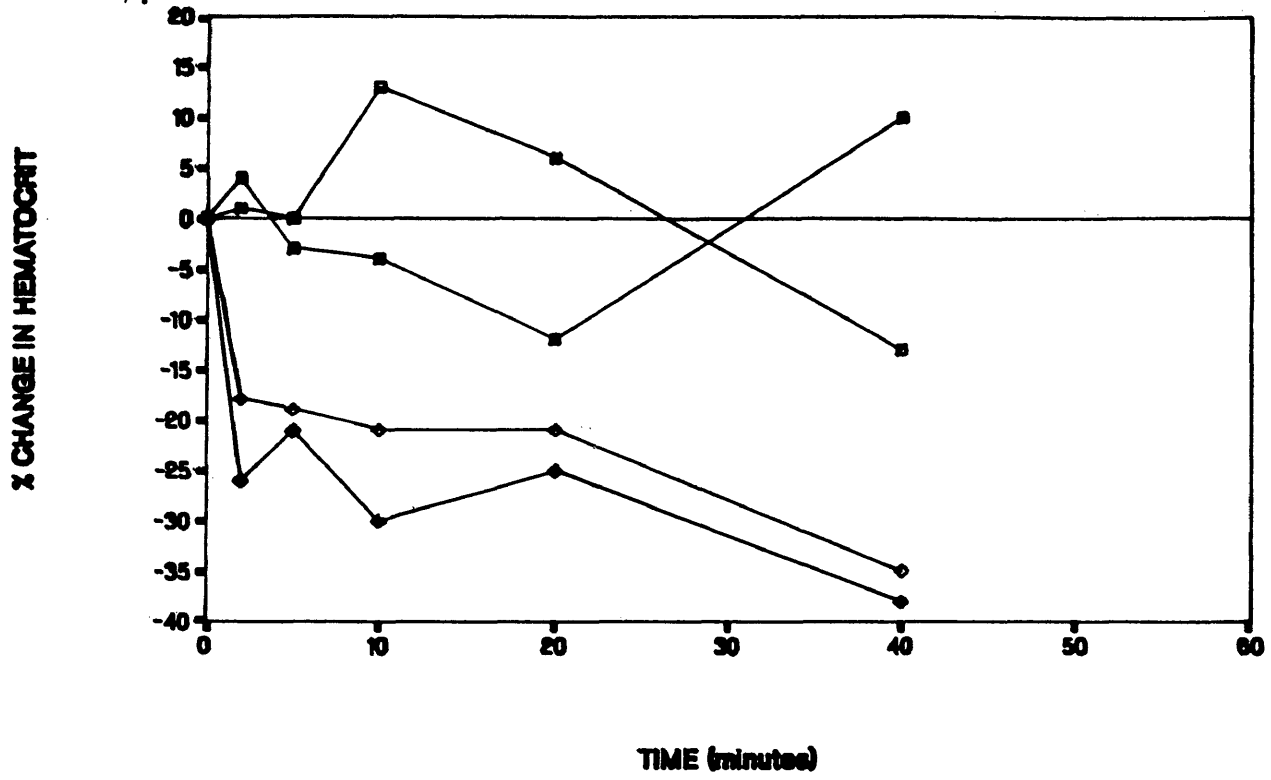



Figure 8. The percent change in ammonia excretion by isolated body wall of Glycera dibranchiata, exposed to a low salinity stress, $33^{o}/oo$ to $18^{o}/oo$.



7.


% CHANGE IN HEMATOCRIT
20
15
10
5
0
-5
-10
-15
-20
-25
-30
-35
-40
0
10
20
30
40
50
60
TIME (minutes)

8.

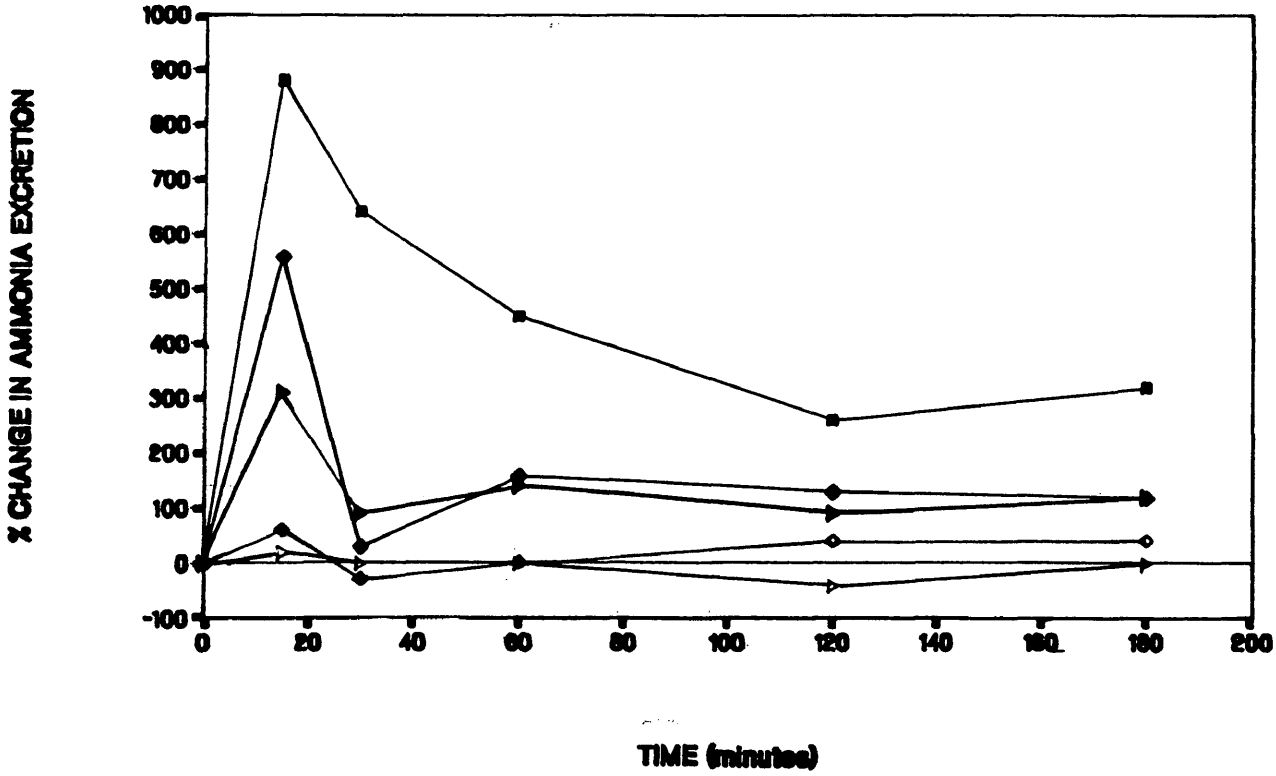

% CHANGE IN AMMONIA EXCRETION
1000
900
800
700
600
500
400
300
200
100
0
-100
0
20
40
60
80
100
120
140
160
180
200
TIME (minutes)

Figure 9. Percent change in ammonia excretion by isolated body wall of Glycera dibranchiata placed in a diffusion chamber with 0.9 mM proline present at the internal face of the tissue.

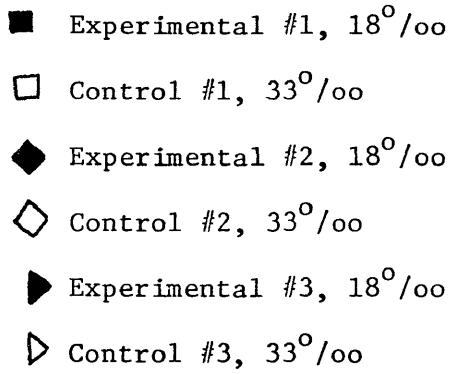
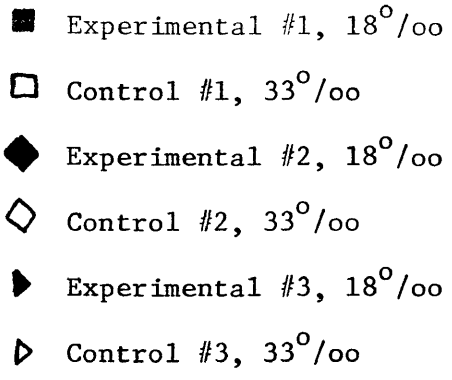
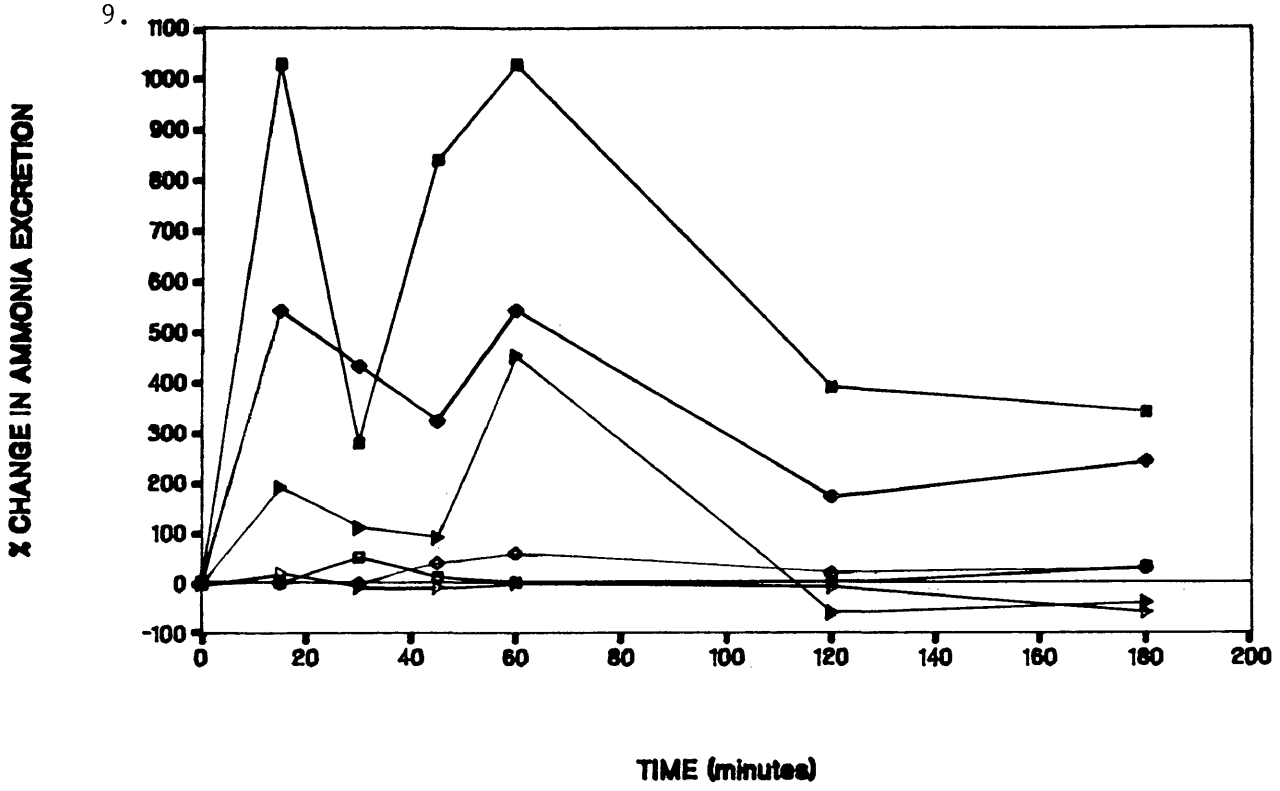



Figure 10. Percent change in ammonia excretion by isolated body wall of Glycera dibranchiata placed in a diffusion chamber with 0.9 mM proline present at the external face of the tissue.



9.


% CHANGE IN AMMONIA EXCRETION
1100
1000
900
800
700
600
500
400
300
200
100
0
-100
0
20
40
60
80
100
120
140
160
180
200
TIME (minutes)

10.

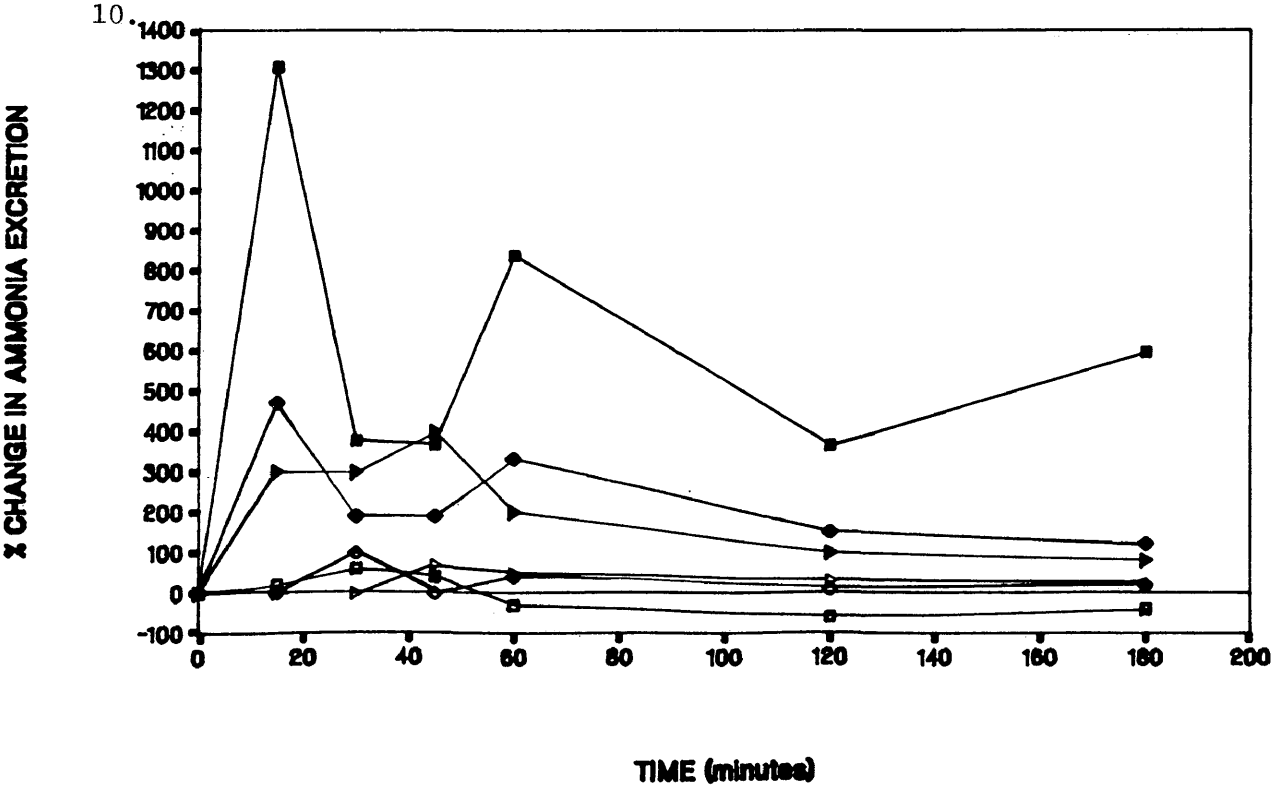

% CHANGE IN AMMONIA EXCRETION
1400
1300
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
-100
0
20
40
60
80
100
120
140
160
180
200
TIME (minutes)

Figure 11. Changes in the osmolality of the pericardial fluid of Modiolus demissus during the course of volume readjustment, $28^{o}/oo$ to $13^{o}/oo$. N = 6, mean $\pm$ s.e.

■ Experimental, $13^{o}/oo$

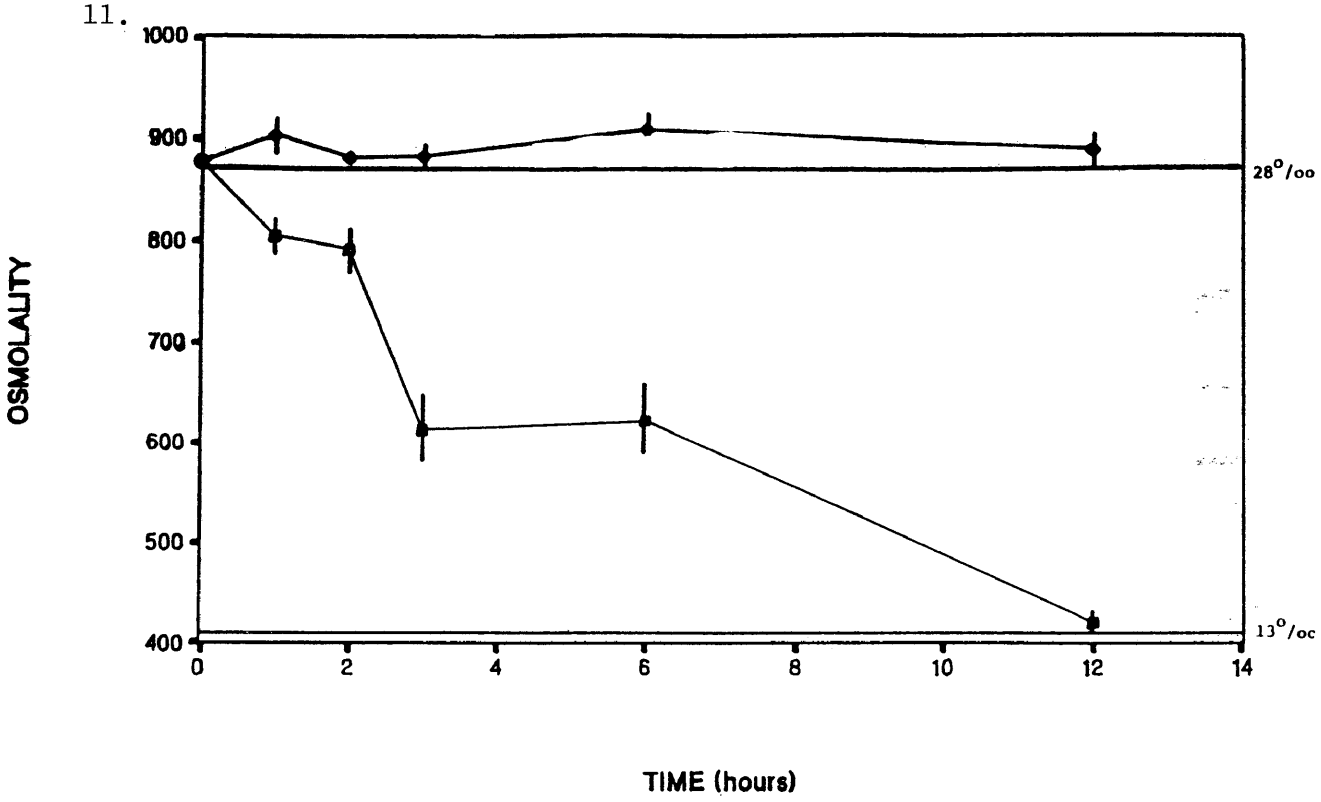
◆ Control, $28^{o}/oo$

Figure 12. Changes in the TNPS concentration of the pericardial fluid of Modiolus demissus during the course of volume readjustment, $28^{o}/oo$ to $13^{o}/oo$.

■ Experimental, $13^{o}/oo$

◆ Control, $28^{o}/oo$

11.


OSMOLALITY
1000
900
800
700
600
500
400
0
2
4
6
8
10
12
14
28°/oo
13°/oc
TIME (hours)

12.

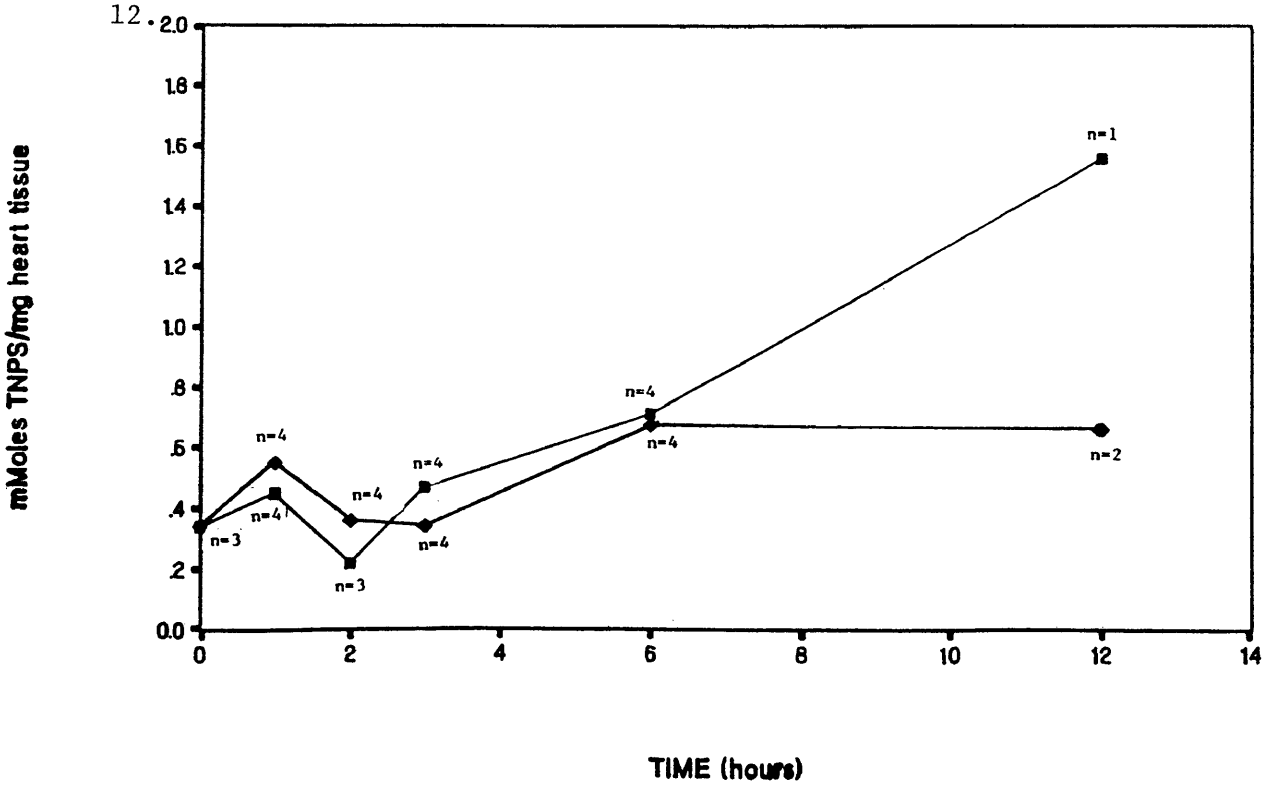

mMoles TNPS/mg heart tissue
2.0
1.8
1.6
1.4
1.2
1.0
.8
.6
.4
.2
0.0
0
2
4
6
8
10
12
14
n=1
n=2
n=4
n=4
n=4
n=4
n=4
n=4
n=3
n=4
n=3
TIME (hours)

**Table 1. The mortality rate of worms with respect to their size.**

| | Number alive at 30 hrs | Number alive at 60 hrs | Number alive at 120 hrs | % Mortality |
|---|---|---|---|---|
| Wet weight > 6 gm; N=7 | 6 | 4 | 3 | 57 % |
| Wet weight < 6 gm; N=16 | 15 | 14 | 12 | 25 % |

Table 2. Changes in percent tissue hydration of G. dibranchiata body wall with a decrease in salinity ($33^{o}$/oo to $18^{o}$/oo). N=6; mean $\pm$ s.e.

| Time | $18^{o}$/oo | $33^{o}$/oo |
|---|---|---|
| initial | 73.9 % $\pm$ 1.8 | 73.9 % $\pm$ 1.8 |
| 1 hour | 76.0 % $\pm$ 2.3 | 68.9 % $\pm$ 4.0 |
| 3 hours | 80.6 % $\pm$ 2.7 | 66.3 % $\pm$ 5.7 |

Table 3. Influx rates of α-amino isobutyric acid into various isolated tissues at different salinities. N = 3; mean ± s.e.

| Tissue | 18°/oo | 33°/oo |
|---|---|---|
| M. demissus | | |
| gill | $J_{max}$ = 7.95 + 4.08 nM/min $mg^{-1}$ | 1.81 + 0.65 nM/min $mg^{-1}$ |
| | $K_t$ = 538.5 + 68.2 µM | 107.3 + 11.3 µM |
| nephridia | $J_{max}$ = 0.75 + 0.32 nM/min $mg^{-1}$ | 1.08 + 0.7 nM/min $mg^{-1}$ |
| | $K_t$ = 152.8 + 13.2 µM | 156.4 + 42.9 µM |
| G. dibranchiata | | |
| body wall | $J_{max}$ = 2.80 + 2.14 nM/min $mg^{-1}$ | 10.16 + 4.06 nM/min $mg^{-1}$ |
| | $K_t$ = 144.7 + 13.1 µM | 789.9 + 29.1 µM |
| nephridia * | | $J_{max}$ = 0.88 + 0.67 nM/min |
| | | $K_t$ = 179.0 + 96.3 µM |

*: N = 2. The nephridia were too small to weigh so the values are not real rates.

Table 4. TNPS excretion of isolated ventricles from M. demissus in response to a decrease in salinity ($28^{o}$/oo to $13^{o}$/oo). N=1.

| | |
|---|---|
| initial rate ($28^{o}$/oo) | 0.00* |
| 1/2 hr at $13^{o}$/oo | 0.04* |
| 1 hr at $13^{o}$/oo | 0.06* |

*: value represents the absorbance of sample after adding the reagents.

# VITA

## ANA MARIE BEARDSLEY

Born in Fayetteville, North Carolina, 15 May 1963. Graduated from Liberty Center High School, Liberty Center, Ohio in 1981. Received an Associate of Science from Richard Bland College, Petersburg, Virginia in 1983. Received a Bachelor of Science degree in Biology with a minor in Mathematics from the College of William and Mary in 1985. Entered Master's program in Biology at the College of William and Mary in January 1986. Completed M.A. degree in September 1989. Employed at the Virginia Institute of Marine Science, the College of William and Mary, as a marine scientist in the Genetics Laboratory.