INTRACELLULAR OSMOREGULATION IN THE ESTUARINE MOLLUSC *VILLORITA CYPRINOIDES* VAR. COCHINENSIS (Mollusca: Bivalvia) Hanley

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CERTIFICATE

This is to certify that the thesis entitled ***INTRACELLULAR OSMOREGULATION IN THE ESTUARINE MOLLUSC VILLORITA CYPRINOIDES VAR. COCHINENSIS (Mollusca: Bivalvia) Hanley*** *is an authentic record of the research work carried out by Mr. Vinu Chandran R., under my supervision and guidance in the Department of Marine Biology, Microbiology and Biochemistry, Cochin University of Science and Technology, in partial fulfillment of the requirements for the degree of* ***Doctor of Philosophy*** *in Marine Biology of Cochin University of Science and Technology, and no part thereof has been presented for the award of any other degree, diploma or associateship in any university.*

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Abbreviations used

%	:	Percentage
µg	:	Microgram
µL	:	Microlitre
µM	:	Micromoles
ADP	:	Adenosine Diphosphate
Ala	:	Alanine
ANOVA	:	Analysis of Variance
Arg	:	Arginine
Asp	:	Aspartic acid
ATP	:	Adenosine Triphosphate
cm	:	Centimeter
d	:	Day
Dia.	:	Diameter
DNPH	:	Dinitrophenyl hydrazine
Eg.	:	Example
Fig.	:	Figure
FRP	:	Fibre reinforced plastic
g	:	Grams
Glu	:	Glutamic acid
Gly	:	Glycine
GPT	:	Glutamate Pyruvate Transaminase
h	:	Height
His	:	Histidine
hr	:	Hour
i. e.	:	That is
LAP	:	Leucine Amino Peptidase
kg	:	Kilogram
M	:	Molar
min	:	Minutes
Ml	:	Milli Litre
N	:	Normal
nm	:	Nano metre
NPS	:	Ninhydrin positive substance
Pi	:	Inorganic Phosphate
ppt	:	Parts per thousand
RVD	:	Regulatory Volume Decrease
RVI	:	Regulatory Volume Increase
sp.	:	Species
TCA	:	Tricarboxylic Acid
Temp.	:	Temperature
TonE	:	Tonicity-responsive Element
UV	:	Ultraviolet
Var.	:	Variety
wt.	:	Weight
x g	:	No. of times gravity

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Preface

Life is a unique phenomenon on the planet Earth and is extremely complex. A suite of metabolic processes makes life possible. Diverse types of ecosystems exist in the biosphere. Even though the environmental conditions vary from ecosystem to ecosystem, organisms have specialized to thrive successfully in them. Each type of organism is gifted with myriad adaptations to survive in its habitat, and is able to withstand changes in the environmental conditions. Capability of adaptation to the environment is one of the most remarkable gifts of nature. Being extremely intricate and uncertain, it is quite difficult to unravel life processes and phenomenon of adaptation is also no exception.

The present piece of work strives to investigate the mechanisms of adaptation of a bivalve that is exposed to the most dynamic and unstable environments – the estuary. Even though this work could answer many of the questions related to intracellular osmoregulation of a tropical estuarine bivalve, it raises more questions that are to be addressed by science.

Chapter 1

INTRODUCTION

1.1 General Introduction

Biosphere renders two major habitats for supporting life, namely, the aquatic and the terrestrial. Among these, the former predominates in area and volume and the available evidence indicates that life originated in this habitat. Like other environments, the successful colonization of aquatic environment demands environmental tolerance. Study of the physiological compulsions for combating environmental oscillations are considered together as a subdivision of comparative physiology, the state-of-the-art environmental or ecological physiology, which addresses nature's enigmatic phenomenon of adaptation.

Aquatic environment can be broadly divided into freshwater and marine environments. Typical freshwater and oceanic environments are comparatively stable over vast areas, which allow biota to enjoy wider distributions, whereas the neritic and estuarine environments are highly dynamic in nature. In addition to nutritional uncertainties, inhabitants of coastal marine and estuarine realm face considerable diurnal and seasonal variations in salinity, which alter their rates of metabolism and activity. In the estuarine realm, where the saltwater and freshwater meet, changes in salinity are more pronounced. Major problems that an animal has to face in this realm are osmotic and ionic changes in the medium. Osmotic content of the cells of a large number of marine euryhaline

invertebrates tends, in general, to be close to that of ambient salinity, and tend also to reflect the environmental variation. But an unlimited variation in the ambient medium can be deleterious. Therefore under an osmotic challenge, some osmoregulatory mechanism is initiated to maintain the optimum concentration. In 1902, Rudolf Hober coined the term *osmoregulation*. Capability of ionic and osmotic regulation is a characteristic feature of all aquatic animals and is essentially vital for estuarine animals. Osmotic and ionic regulation is a result of the active regulation of ions and water in the body either by active uptake from the medium or by removal from the body (Cameron, 1978).

Variations in the salinity of seawater may affect the aquatic organisms through specific gravity control and variations in osmotic pressure. The specific gravity of most soft tissues is close to that of normal seawater. Many bottom-living forms, both attached and motile, have very high specific gravities (eg. *Villorita cyprinoides*). But osmolarity change is the major challenge for animals living in ambient salinity variations. Among the benthic forms, molluscs are one of the successful groups and they occupy all types of aquatic biotopes. Many estuarine species of molluscs are able to withstand fluctuations in salinity of their natural environment. Capability of ionic and osmotic regulation at least in the cellular level is essential to successfully invade aqueous environments of differing chemical milieu. Primitive animals as well as those towards the lower part of the evolutionary tree (*eg.* molluscs) lack the sophisticated mechanism of extracellular ionic regulation. In the course of evolution these organisms have developed and refined an excretory system for combating osmotic problems.

Aquatic organisms are hypoosmotic, hyperosmotic, or hypo-hyperosmotic regulators (Kinne, 1971). Regulation takes place at extracellular level in some

organisms and at intracellular level in others. Capability of osmotic regulation in extracellular fluid differs among animal groups. Very poor regulators have osmotic pressure of their extracellular fluid almost same as that of the external medium (poikilosmotic). According to Florkin (1962), osmotic regulation is of two types, extracellular-fluid anisosmotic regulation, which is the regulation of the blood osmotic pressure and intracellular isosmotic regulation, which is the regulation of the osmotic pressure of the cell. These two are the major physiological means of adaptation to fluctuating salinity (Matsushima, *et al.*, 1987). Krogh (1939) found out that in most estuarine animals, an initial lowering of the salinity is usually accompanied by a drop in the cell content salinity, and the osmoregulation does not come into play until a certain definite drop in the external salinity has taken place. For a particular species, the ability to withstand altered salinity may be determined by inherent genetic ability and other environmental physico-chemical factors.

Integuments of animals are not good barriers to salts or water, at the most, they can be only nearly impermeable to them. As a matter of fact, bivalves cannot completely isolate their soft body tissues during an adverse salinity change. Extracellular regulation in bivalve molluscs relies on both valve closure and ionic regulation. Valve closure is a behavioural tactic and helps the animal in creating a microenvironment within its shells. Some molluscs are capable of regulating their blood ionic contents to a limited extent (Deaton, *et al.*, 1989, Salomao & Lunetta, 1989). Extracellular anisosmotic regulation assists the organism in maintaining an osmotic gradient between its blood and the environmental medium. A drastic change cannot be tackled immediately by intracellular adjustments, even though long term adjustments are made at cellular or body fluid level.

Osmoregulation and osmotic adaptations in molluscs have been subjected to study by many authors (Henry, *et al.,* 1980; Bishop, *et al.*, 1981; Otto & Pierce, 1981; Somero & Bowlus, 1983; Zatta and Cervellin, 1987; Matsushima, *et al.*, 1987, 1989; Deaton, 1987, 1990, 1992; Deaton, *et al.*, 1984, 1989; Gardner & Kathiravetpillai, 1997; Michinina & Rebordinos, 1997; Nirchio & Perez, 1997; George and Damodaran, 1999).

Intracellular regulation constitutes the changes of the osmotic pressure in cellular level to coincide with the ambient medium for volume regulation. The major osmolytes involved and the mechanisms of intracellular osmoregulation in many marine species like *Crassostrea* spp. (Pierce, *et al.*, 1992), *Rangea cuneata* (Henry, *et al.*, 1980, Otto and Pierce, 1981), *Sunetta scripta, Perna viridis,* (George, 1993), etc. have been studied. Data pertaining to the regulatory processes from tropical estuarine animals are deficient.

Adaptation which involves major or minor adjustments to displace or expand tolerance ranges may be physiological or behavioural. However, adaptation to abiotic environmental conditions is not usually restricted to a single mechanism. More commonly, a whole range of interactive adaptations are involved. Many environmental variables are interrelated, even synergistic. Thus adaptations to a particular set of environmental conditions too must necessarily show interrelationships amongst themselves. Such sets of co-adaptive characters have been termed 'adaptive suites' (Bartholomew, 1982) and are perhaps best examined amongst organisms adapted to the greatest extremes of environment. Whatever their mechanisms be for adjusting, expanding or gross displacement of their tolerance ranges, animals and plants can never totally escape from the problem. Tolerance limits may be altered - but never removed; whatever

adjustments may be made, organisms are still restricted in performance and distribution by their tolerance to abiotic conditions. Such restriction may determine limits to distribution: will dictate, if not where an organism does or does not occur, at least where it *cannot* occur. (Putman & Wratten, 1984).

Most of the bivalves are sedentary or are with limited mobility. Any transient, frequent or everlasting salinity change cannot be overcome by the animal by emigration. Like all other organisms, bivalves also regulate their osmotic content by the extracellular anisosmotic regulation and the intracellular isosmotic regulation. Bivalves are considered to be poikilosmotic, having very limited power of extracellular regulation; hence intracellular regulation is the ultimate choice of the animal for long term adaptations. Extracellular regulation comprises mainly of the regulation of ionic composition of the blood or hemolymph, the regulations are made mainly for the inorganic ions.

1.2 Review of Literature

The process of cell osmotic pressure adaptation has been termed by Florkin (1962) as isosmotic intracellular regulation. In 1904, Kelly showed the existence of taurine and glycine in *Pecten* muscle. Deaton and Greenberg (1991) have studied the physiological aspects of adaptation of molluscs to oligohaline waters. Gilles (1975) also have contributed to the understanding of mechanisms of ionic and osmotic regulation. Changes in free amino acid concentrations in tissues of a fresh water pulmonate *Helisoma* was studied by Matsushima *et al.* (1989). Changes in volume regulation of muscle fibres of shore crab *Hemigrapsus* was studied by Bedford (1971). Different salinity tolerance mechanisms in Atlantic and Chesapeake Bay conspecific oysters were studied by Pierce *et al.* (1992).

He discovered that the lesser ability of the Bay oysters to tolerate extremes of salinity than Atlantic oysters is due to their lesser capability to alter the intracellular osmolyte pool. Restoration of cell volume in many euryhaline molluscs is reported by Pierce, *et al.* (1992) and Gainey, (1994). It is observed that the cell volume is not regulated in all situations of a change in salinity. Cell volume is regulated only if the cost of metabolic impairment due to volume change surpasses the cost for volume regulation. As a result, under minor salinity changes, the cell adopts a strategy of tolerance. So it can be found that the volume regulation occurs to avoid volume-related impairment of cellular function (Strange, *et al.*, 1996).

A regular fluctuation of salinity found in estuarine environments has an important physiological implication for osmoconforming animals such as bivalves. Neufield and Wright (1998), opined that the responses of cells to a salinity challenge is the result of two strategies (1) full or partial restoration of cells to their original volume, achieved by adjustments in the intracellular contents of osmotically active solutes or (2) osmotic fluctuations in cell volume. To conduct normal cell functions, any way, the cell cannot allow for large volume changes.

George (1993) studied the intracellular osmoregulation in two marine species, *Sunetta scripta* and *Perna viridis*. Pierce *et al.* (1997) studied the variations in intracellular choline levels and glycine betaine synthesis between Atlantic and Chesapeake Bay conspecific oysters. He concluded that glycine betaine synthesis in Bay oysters is reduced by the availability of a precursor, choline. Unlike oysters (*Crassostrea virginica*) from the Bay, oysters from the Atlantic coast use large intracellular concentration of glycine betaine as an osmolyte to respond to hyperosmotic stress. Gardner & Kathiravetpillai, (1997) have studied

the variation of an enzyme leucine aminopeptidase (LAP) during osmoregulation in blue mussel *Mytilus galloprovincialis* and green mussel *Perna canaliculus*. They substantiated with evidences that LAP variation would be of selective importance in marine bivalves which inhabit environments having salinity fluctuations. Changes in amino acid content in the midgut glands of freshwater bivalve *Lamellidens corrianus* during salinity adaptation has been studied by Jdhay & Lomte, (1990). George & Damodaran (1999) studied the intracellular osmolytes responsible for osmoregulation in the yellow clam *Sunetta scripta*. The work reports that taurine is the most abundant and varied osmolyte. Significant qualitative difference was noticed between tissues, but the variation was quantitative among size groups.

1.3 Experimental animal

Villorita spp. occurs abundantly in the upper reaches of the estuary and backwaters of Kerala. The name *Villorita* was introduced for the first time by Griffith and Pidgeon in 1833. *Villorita cyprinoides* var. cochinensis was named by Hanley in 1866. This clam has a moderately large, thick, ovately triangular, inflated oblique shell, swollen in the umbonal region and in the middle region. The umbons are eroded usually in larger specimens. Shell is sculptured with concentric ridges. The hind area bears three posterior oblique cardinal teeth, of which, the anterior in the right valve and the posterior in the left valve are obsolete.

The shell of *Villorita cyprinoides* variety cochinensis is thick, equivalve, inequilateral and ovately triangular. The posterior end is more elongated and oblique. Lunule is heart shaped, broad and slightly convex in the central part. Ligament is external, thick, rounded, brown in colour and extend to 1/3rd of the posterior margin. The animal is commonly seen in the freshwater end of the estuarine waters. Since

these animals inhabit unstable saline environments, they are capable of tolerating wide salinity changes. The tolerance limit varies among various size groups. The smaller size groups have wider tolerance range than larger size groups. Nair and Shynamma (1975) reported the salinity tolerance range of larger animals to be 4.73×10^{-3}-27.11×10^{-3}. They reported that the smaller size groups can tolerate a salinity range of 0.87×10^{-3}-29.85×10^{-3}. Natural populations according to them have got higher salinity tolerance than those reared in the laboratory. They attributed the higher salinity tolerance to acclimatization.

Several works have been conducted in *Villorita* sp., which is an edible form. Nair and Shynamma (1975) have studied the salinity tolerance of *Villorita cyprinoides* var. cochinensis. Pierce and Greenberg (1972) have studied the initiation and control of free amino acid regulation of cell volume in salinity stressed bivalves. The effect of salinity on toxicity of quinalphos on this animal was studied by Jacob and Menon (1986). They found out that the effective time to kill 50% of the animals (ET_{50}) with pesticides decreased with an increase in salinity. Effect of temperature and salinity on the growth and feeding of *Villorita cyprinoides* was documented by Pannikar and Nair (1993). Reghunathan and Nair (1993) suggested the suitability of *Villorita cyprinoides* in prawn diet. Biochemical composition of *Villorita cyprinoides* var. cochinensis was found out by Lakshmanan and Nambisam (1980).

Villorita is used for human consumption apart from being used as an ingredient of prawn feed. In traditional culture systems, raw meat of *Villorita* is used to feed prawns. Culture of this organism for human consumption as a protein source is now under experiment. The shells of this animal is used widely as a raw material for industries like cement and lime. Knowledge of osmoregulation can contribute

to the ecophysiological adaptations of the organism. The isolation and identification of amino acids from the species can contribute to the dietary requirements of the animal during growth in captivity at different salinities.

1.4 Habitat

V. cyprinoides var. cochinensis commonly known as 'black clam' is found in the west coast estuarine backwaters. They are found to form vast beds near the farthest ends of backwaters. This species has got a peak spawning season in the monsoon i.e.; from late May to August-September when salinity and temperature are considerably low. This is followed by intense gametogenic activity till a secondary spawning occurs from December to January (Reethamma, 1991).

1.5 Scope of the Study

The present investigation is dedicated to understanding the various mechanisms of salinity tolerance in the estuarine clam *V. cyprinoides* var. cochinensis. Eventhough *V. cyprinoides* var. cochinensis and *V. cyprinoides* are found to coexist in the same area, *V. cyprinoides* is reported to tolerate higher salinities than variety cochinensis (Reethamma, 1991). It is worth investigating the aspects of osmoregulation of this animal since it inhabits the freshwater ends of estuaries and has a capability of tolerating wide range of salinity.

The experiments are designed to understand the behavioural, physiological and biochemical aspects of osmoregulation in the estuarine clam *V. cyprinoides* var. cochinensis. Attempt was also made to compare and contrast the observations with that of marine bivalves, which have already been subjected to such studies.

The first aspect focused on was the behavioural response and ionic regulation,

because these are the initial responses of an animal to a salinity change. Kinne, (1971) has pointed out that the capability of various aids for osmoregulation varies with age group. So, two size groups were subjected to experiments. From the data available on marine forms, it is seen that smaller size group have a higher capacity of tolerance to salinity variations (Nair and Shynamma,1975; Thampuran, *et al.*, 1982). Behavioural response to adverse salinity conditions includes valve closure and burrowing into the sediment in benthic bivalves. Duration of valve closure gives a picture of how long the animal will take to tune up its physiological machinery to the altered environment. Once the animal has adapted, it starts normal respiration and activities. Thus behavioural responses are initial and temporary.

Many of the molluscan species studied till date are capable of regulating their blood inorganic-ion concentration to some degree. This contributes to the capability of extracellular-fluid anisosmotic regulation, and in most species the inorganic ion concentration of the blood alone is regulated to adjust the osmotic pressure.

Even though the bivalves are capable of regulating their blood ion concentration to a limited extent, regulation often cannot be achieved to the required level. Hence the ultimate choice of these animals is intracellular isosmotic regulation. Even though mechanisms of intracellular osmoregulation are well documented in marine molluscs, such studies are scarce in the case of tropical estuarine forms. Since estuarine animals are facing daily changes in osmotic concentrations of the medium, their strategy of defense will be different. In marine forms the major ninhydrin positive substance (NPS) regulated is taurine (Pierce, *et al.*, 1992, George and Damodaran, 1999). Being a small organic solute, it can be

accumulated to high intracellular concentrations (Welborn and Manahan, 1995) without perturbing macromolecules (Yancey, *et al.*, 1982). This amino acid is reported to be absent in freshwater and terrestrial counterparts (Awapara, 1962). Since taurine is not a component of proteins, neither it could be synthesized nor degraded. Hence its loss during hyposaline regulation will not result in much energy drain in marine organisms. Earlier authors claim that this is the reason why taurine is preferred (George, 1993).

When exposed to salinity variations in the surrounding water, marine molluscs modify the concentration of intracellular small organic molecules to maintain the cellular volume between appropriate physiological limits (Pierce, 1982, Jdhay and Lomte, 1990). Smaller molecules employed for osmoregulation are mainly free amino acids and in lesser quantity ammonia, methyl amines, trimethyl amine oxides, betaines and sarcosine (Ivanovici, *et al.*, 1981; Pierce, 1982). The mechanism reported for the regulation include (1) Release of free amino acids via protein degradation (2) Transamination reactions (3) *de novo* synthesis and (4) Uptake of dissolved amino acids from the environment (Somero and Bowlus, 1983). It has been demonstrated that lysosomal and proteinase inhibitors prevent the accumulation of free amino acids in the gills of the estuarine mussel *Geukensia demissa* – this suggests a proteolytic pathway for volume regulation in intracellular osmoregulation (Deaton, 1987).

Amino acids are selected carefully for osmoregulation so that the internal physico-chemical processes are not impaired by the change in their concentration. It is already known that taurine is a major component of the NPS pool of marine organisms. But the reasons for the limited role of this amino acid in freshwater and terrestrial forms are obscure. Study on the qualitative and quantitative

composition of NPS in various tissues is done in this estuarine bivalve to find out differences with that of the marine forms and to find out the possible substitutes for taurine. Results obtained can help to partly fill the lacunae in information about the strategies of intracellular osmoregulation of an estuarine form.

Chapter 2

ROLE OF VALVE CLOSURE AND EXTRACELLULAR FLUID ANISOSMOTIC REGULATION

2.1 Role of valve closure

By behavioural mechanism of valve closure, bivalves tend to isolate their body parts from sudden changes in the environment. It is observed that isotonicity between the perivisceral fluid and the medium is not reached immediately after an environmental salinity change. Perivisceral fluid in the euryhaline species offers an ambient environment distinctly different from the immediate environment for the soft parts of the body in changed salinity. Valve closure is one of the mechanisms which they adopt, to maintain the disequilibria with the external environment. Shell closing mechanism may help a given individual to withstand, at least temporarily, a dramatic change in concentration of the external medium. Apart from valve closing, some bottom dwelling forms burrow deep into the sediment during adverse conditions. Actively swimming forms tend to move out of the area. In the case of sedentary bivalves, isolated micro environment generated within the shells give ample opportunity for the physiological machinery to adapt to the adverse condition.

Shell closing mechanism along with extracellular anisosmotic regulation works to maintain a hyperosmotic state in some estuarine bivalves like *Scrobicularia*

plana (Shoffeniels and Gilles, 1971). Valve closure in altered salinities was subjected to study by many workers like Milne (1940), Widdows (1985), Genovea, et al. (1988), Berger (1989), Supriya (1992) and George (1993).

True euryhalinity can only exist if the animal possesses various mechanisms which enables it to cope with the osmotic stress. Euryhalinity in molluscs is mainly attributed to the ability of the animals to regulate their cellular osmotic pressure with respect to changes in blood osmolarity. Organic substances together with sodium and chloride ions play an important part in the intracellular isosmotic regulation process, which enables euryhaline molluscs to cope with the osmotic stress. Any investigation on the osmotic adaptations of bivalves must consider the behavioral response too (Burton, 1983; Akberali and Trueman, 1985). Akberali (1978) observed that *Scorbicularia* closed their valves when transferred from 100% seawater to water of lower salinity. He also observed that the tendency to remain isolated inside the shell was greater when the salinity drop was higher. Not only during salinity changes, but in all types of adverse environmental changes also, animals isolate their soft body parts from the external medium by closing their valves. Davenport (1979b) reported that the isolation of body parts of *Mytilus edulis* has three sequential events. The first event is the closure of exhalent siphon, which ceases the irrigation of mantle cavity. Next is the closure of inhalent siphon, triggered by further decline in salinity of the medium. This is followed by the closure of valves. The closure of the valves renders the animal to sequester its soft body parts from the unfavourable environment. By closing the valves, animal creates a microenvironment within its shells. Milne (1940) have described that the salinity within the mantle cavity of *Mytilus edulis* can be as high as 24×10^{-3} even when the salinity of the external medium is only 0.7×10^{-3}. The effect of valve closure on osmotic process of the internal fluids of

molluscs is well demonstrated by experiments carried out on bivalves (Gilles, 1972). In the salinity tolerance experiments, it is often found that the bivalves regulate the ion concentration to a small extent at salinities closer to optimum. The term anisosmotic extracellular regulation was introduced by Florkin in 1962, and the process involves the regulation of blood osmotic pressure. It is noticed that the blood of marine invertebrates is normally isosmotic with the external medium even in euryhaline species, which can tolerate a wide range of external salinities. If this variation is to be reflected in the cellular level, it will upset the maintenance of homeostasis.

In general, in marine forms, gills and/or kidneys take care of the regulation of inorganic ions in the blood. The regulation of inorganic ions are done extracellularly in these forms. This regulation can be seen to a certain extent in invertebrates also. An increase in the principal inorganic osmolytes in the blood has been reported for hyperosmoregulatory crustacea (Gilles and Pequeux, 1983, Cedomil Lucu and Massimo Devescovi, 1999). The increase in blood osmolarity could be fully attributed to the increase in Na^+ and Cl^- in this case. Instances of osmotic regulation by regulation of inorganic ions have been documented by Deaton, (1981) and Deaton, *et. al.* (1989). But in many cases, the concentration of ions in the blood is maintained at a different level than that in the seawater. In these organisms, there is evidence for regulation of specific ions taking place, where the blood remains isosmotic even in 50% seawater. When the salinity falls further, the osmoticity of blood is maintained at a level approximately equal to the value it had in 50% seawater (Schoffeniels & Gilles, 1971).

Inorganic ions at optimal concentrations are vital for the smooth performance of cellular functions. They serve as cofactors for enzymes and influence the

permeability of biological membranes to other solutes (Burton, 1983; Prusch, 1983). The ability of molluscs to regulate inorganic ions varies with species. Extent of regulation is said to be high in freshwater species and low in marine species (Burton, 1983). In dilute media, blood will be in hyperosmotic state due to the regulation of sodium, potassium and chloride ions. Shumway (1977) found no evidence of ionic regulation for sodium, calcium or magnesium in *Mytilus edulis* and *Crassostrea gigas*. Concentration of sodium in the hemolymph and seawater were almost equal while calcium and magnesium were slightly more concentrated in the hemolymph than in the ambient medium under a hypo osmotic stress. Gilles (1972) reported that *Mytilus edulis* and *Glycymeris glycymeris* can regulate potassium ions in diluted medium so that the concentration is maintained at the same level as in the hemolymph of individuals in normal seawater. In freshwater molluscs, sodium and chloride are generally the most abundant ions, while the concentrations of potassium and magnesium are very low.

Mechanisms responsible for the extracellular anisosmotic regulation include Donnan equilibrium, valve closing, permeability of the cell membranes to salt and water and active uptake of ions. Robertson (1953) pointed out the existence of a passive Gibbs-Donnan equilibrium in some molluscs. By virtue of this, the extracellular fluid is kept slightly hyperosmotic to the ambient medium.

Freshwater molluscs produce urine which is hypotonic to the blood (Florkin, *et al.*, 1972). Despite this, there is a net loss of salts from the body. Active reabsorption of salts from urine and the absorption from food balance the loss.

Blood osmolarity reflects osmotic concentration of the body fluid. A difference in osmolarity of the ambient medium and the body fluid implies an active regulation. Davenport (1979b), investigated the changes in hemolymph osmolarity of *Mytilus*

edulis in diluted seawater. In his experiments, the shells of experimental animals were "propped open" so that they could not close them. Results indicate that even when the shells were kept open, animals could close the exhalent siphon and regulate blood osmolarity to a certain extent. Estuarine crustaceans also regulate their blood ionic concentration (Bindu & Diwan, (1997)). There is little information on the blood osmotic concentration of molluscs, which can tolerate low salinities. Moreover, the few species used in experiments have been exposed to media of salinities down to only 50% that of seawater and not lower than that. In these experiments, blood always remained isosmotic with the surrounding medium. There is evidence that at lower salinities a regulatory process takes place in some species (George, 1993) . In order to study the changes in blood ionic concentration during adaptation of euryhaline bivalves to concentrated media, it is necessary to consider the changes occurring in the mantle cavity fluid (perivisceral fluid). This fluid acts as a balancing system between the blood and the environmental medium. *Villorita cyprinoides* var. cochinensis is an inhabitant of estuaries where recurrent salinity changes are observed. Study on ionic composition of mantle cavity fluid and blood of this animal is imperative to derive a clear picture of its osmoregulatory mechanisms.

For the present study, the experiments were designed both to understand the extent of valve closure and the degree of extracellular anisosmotic regulation. Osmolarity of the mantle cavity fluid and hemolymph was estimated to delineate the variation in osmotic concentration between the two at different salinities. Water content of the three tissues (Adductor muscle, Foot and Mantle) were estimated to verify the dilution of the cellular fluid.

2.2 Materials and Methods

Experimental animals (*Villorita cyprinoides* var. cochinensis) were collected from the Cochin estuary (Poochakkal), near Cherthala, where range of salinity fluctuation during the year is considerable. Salinity variation in this area ranges from 0 x 10^{-3} during late monsoon to around 25 x 10^{-3} during pre-monsoon in a year. Animals collected were transported to the laboratory in plastic bags. In the laboratory, the clams were washed thoroughly to remove adhering mud and algae. Their shell size (maximum length) was measured using vernier calipers and they were sorted into two size groups, 2.5 ± 0.5 cm (small) and 4.0 ± 0.5 cm (large). These animals were then introduced into large FRP tanks, with freshwater for acclimation (Plate 1). Washed sand was provided as the substratum. All acclimation tanks were provided with biological filters and aerators. Half of the freshwater in the tanks was renewed once in two days. Dead animals were identified and removed immediately. The animals were fed with Cyanophycean alga *Synechocystis salina* Wislouch (*ad libitum*) during the acclimation period. Animals were acclimatized in the lab for one month before being used for the experiments.

For the experiments, FRP tanks with well-aerated seawater at different levels of salinity were used. All parameters like dissolved oxygen saturation, temperature, pH and quantity and type of feed were kept constant. Dissolved oxygen content was 6-7 ml/l, temperature, 28°C ± 1 and pH 7.0. The only variable parameter between the tanks was salinity. Seawater of desired salinity was made by dilution of 100% seawater with deionized water. Laboratory acclimatized clams were transferred to different levels of salinity, viz. 5, 10, 15 and 20 x 10^{-3}. Samples of mantle cavity fluid and hemolymph of 5 animals were pooled each time. Initial

sample was taken before transferring the animals to the experimental tanks. Subsequent sampling was done at time intervals 1, 2, 3, 4, 5, 6, 12, 24, 48, 72 and 96 hrs. The sampling was stopped when a prolonged steady state was observed in the ionic content of hemolymph and mantle cavity fluid.

For studying the contribution of extracellular isosmotic regulation, animals exposed to definite salinity for 2 weeks were employed. Experimental salinities ranged from 0 - 20 x 10^{-3} at 5 x 10^{-3} intervals. The two size groups were introduced gradually to experimental salinities viz. 0, 5, 10, 15 and 20 x 10^{-3} with a gradual increase of 5 x 10^{-3} every three days. After the medium attained desired salinity, animals were maintained at that salinity for two weeks. During the experimental period, the salinity of individual tanks were maintained at the desired level ± 0.5 x 10^{-3}. All conditions in all the experimental tanks were the same except salinity.

Clean and dry sterile hypodermic plastic syringes were used to collect mantle cavity fluid and hemolymph samples. Mantle cavity fluid samples were collected first, and were transferred to clean injection bottles. Hemolymph samples were also collected from the same animals, after draining out the mantle cavity fluid and removing the water adhering to the tissues using absorbent paper (George, 1993). Hemolymph was collected from the dorsal part of the animal, adjacent to the adductor muscle.

Pooled samples were centrifuged at 4000 x g for 30 min in a refrigerated centrifuge, to remove suspended particles and blood cells. The supernatant was transferred to a clean test tube and was diluted with distilled water. Sodium and Potassium ion concentration in the samples were estimated by the flame photometric method (Robinson & Ovenston, 1951) using a Flame Photometer

Plate 1

Animals in the acclimation/
experimental tank

Actively filtering animals inside the tank

(Elico, type 22). Chloride content was determined with the help of a Chloride meter (Elico Model EE 34). Osmolarity of the mantle cavity fluid and hemolymph was read in an osmometer (Gonotek osmometer (Osmomat 030)).

Water content of the tissues (adductor muscle, mantle and foot) of *Villorita cyprinoides* var. cochinensis acclimatized to various salinities were estimated from the difference in dry and wet weights of the tissues. Tissues of 3-4 animals were excised, blotted and weighed in an electronic balance. Tissues were then left in an oven at 60°C to obtain constant weight. Dry weight was estimated after the tissues attained a constant weight. A high precision electronic balance was used for weighing the tissues. Results are expressed as percentage of weight of the wet tissue.

2.3 Extracellular anisosmotic regulation

2.3.1 Results

Small sized individuals of *Villorita cyprinoides* var. cochinensis exposed to 5 x 10^{-3} salinity maintained the mantle cavity fluid hypo-ionic to the environment for a period of 6-12 hours (Table 2.1, Fig. 2.1). Large size groups attained homogeneity of the mantle cavity fluid within 4-5 hours of exposure (Table 2.1. Fig. 2.2). In both the size groups, hemolymph concentration of sodium was maintained at a lower level than that of the mantle cavity fluid.

Table 2.1. **Concentration of Na^+ in the mantle-cavity fluid and hemolymph of *V. cyprinoides* var. cochinensis exposed to 5 x 10^{-3} salinity (Expressed as ppm).**

Time of Accl. (hr)	Small Size Group				Large Size Group				Medium
	Mantle Cavity fluid		Hemolymph		Mantle Cavity fluid		Hemolymph		
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
0	21.8 ±	3.1	26.6 ±	9.0	25.9 ±	0.9	27.4 ±	0.4	71
1	44.2 ±	3.2	25.4 ±	2.0	49.8 ±	1.8	33.1 ±	0.5	71
2	50.6 ±	8.3	27.0 ±	1.3	62.4 ±	6.1	34.2 ±	3.0	71
3	56.3 ±	8.9	32.2 ±	8.0	65.0 ±	4.9	36.0 ±	4.0	71
4	62.7 ±	4.7	40.1 ±	2.0	67.9 ±	10.6	40.8 ±	7.0	71
5	65.1 ±	3.1	44.1 ±	7.0	68.8 ±	2.0	42.8 ±	2.0	71
6	64.4 ±	9.0	44.1 ±	1.0	69.4 ±	2.0	44.4 ±	1.2	71
12	66.9 ±	8.8	47.6 ±	1.0	70.8 ±	5.2	49.0 ±	5.2	71
24	70.6 ±	1.2	51.6 ±	8.0	69.4 ±	1.1	53.4 ±	3.5	71
48	70.7 ±	3.1	50.7 ±	6.1	69.9 ±	3.7	52.7 ±	4.1	71

Values are the mean of six observations ± SD.
Each observation is made from a sample of 8-12 animals pooled together.

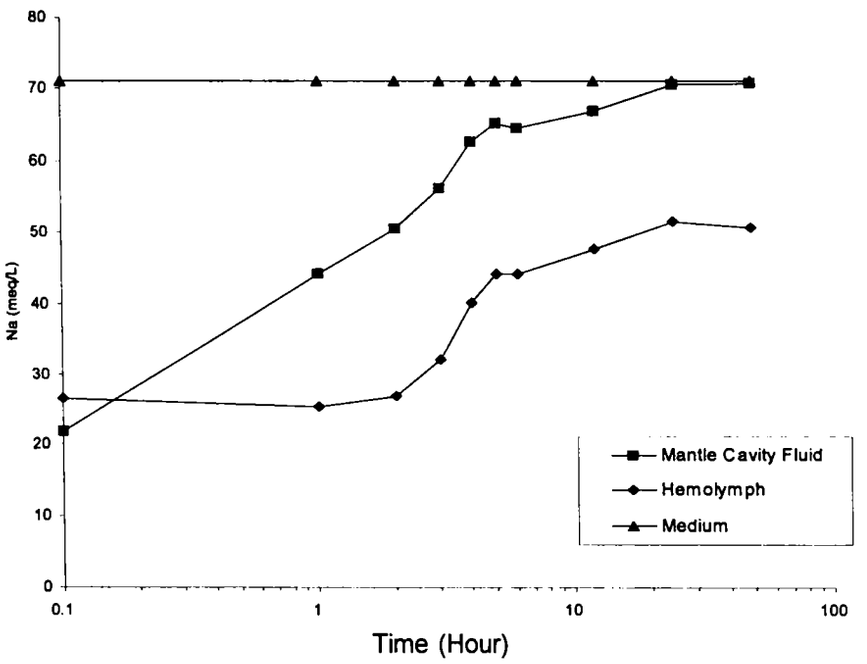


Fig 2.1: Concentration of Sodium in *V. cyprinoides* var. cochinensis (small) exposed to 5 x 10^{-3} ppt salinity

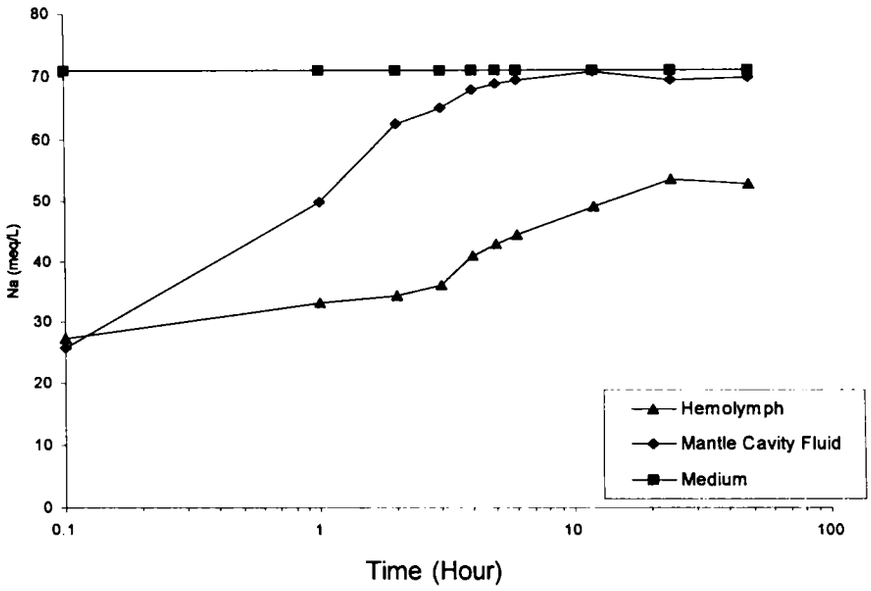


Fig 2.2: Concentration of Sodium in *V. cyprinoides* var. cochinensis (large) exposed to 5 x 10^{-3} ppt salinity

At 10 x 10^{-3} salinity, the hypo-osmoticity of the mantle cavity fluid of small animals was retained for 24-48 hours (Table 2.2, Fig. 2.3). Large animals maintained hypo-osmoticity of mantle cavity fluid for 48 hours. Hypo-osmoticity of hemolymph could be observed throughout the exposure period in both the size groups (Table 2.2. Fig. 2.3 & 2.4).

Table 2.2. Concentration of Na^+ in the mantle-cavity fluid and hemolymph of *V. cyprinoides* var. cochinensis exposed to 10 x 10^{-3} salinity (Expressed as ppm).

Time of Accl. (hr)	Small Size Group				Large Size Group				Medium
	Mantle Cavity fluid		Hemolymph		Mantle Cavity fluid		Hemolymph		
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
0	12.2	± 0.2	23.4	± 2.3	13.8	± 0.5	27.1	± 1.2	141
1	13.5	± 7.4	22.0	± 1.0	42.2	± 12.5	36.9	± 8.5	141
2	18.1	± 19.9	22.0	± 10.8	55.1	± 11.5	43.1	± 6.3	141
3	34.0	± 9.2	28.1	± 9.2	58.9	± 1.9	50.1	± 0.9	141
4	46.1	± 17.7	38.4	± 13.0	65.6	± 1.0	52.0	± 2.3	141
5	65.8	± 10.4	40.9	± 4.0	73.1	± 0.8	60.5	± 3.0	141
6	103.0	± 15.9	83.1	± 0.0	126.6	± 2.0	91.1	± 0.8	141
12	143.0	± 0.0	110.3	± 0.1	144.8	± 0.1	126.6	± 0.2	141
24	138.1	± 0.2	132.0	± 0.4	142.4	± 1.0	135.0	± 0.1	141
48	141.0	± 0.4	133.4	± 0.7	144.0	± 4.0	135.7	± 0.8	141

Values are the mean of six observations ± SD.
Each observation is made from a sample of 8-12 animals pooled together.

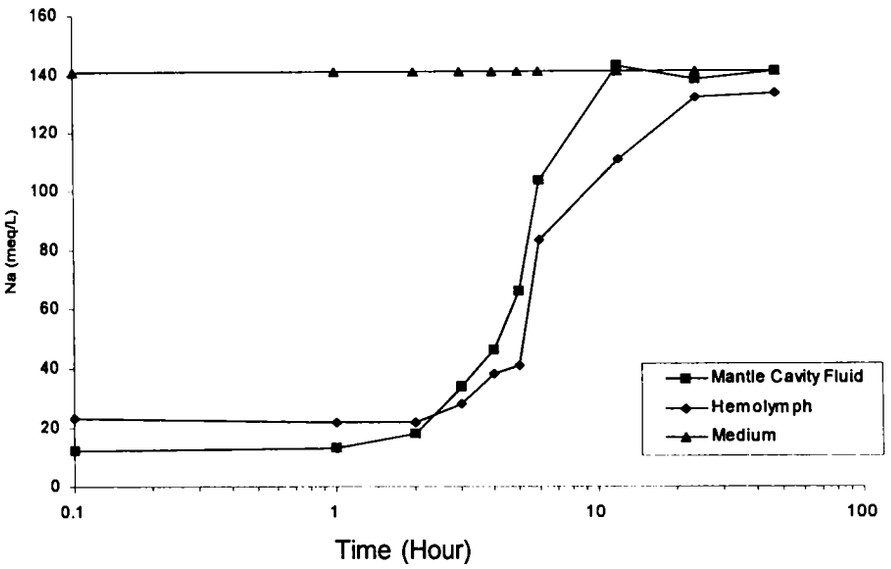


Fig 2.3: Concentration of Sodium in *V. cyprinoides* var. cochinensis (small) exposed to 10 x 10^{-3} salinity

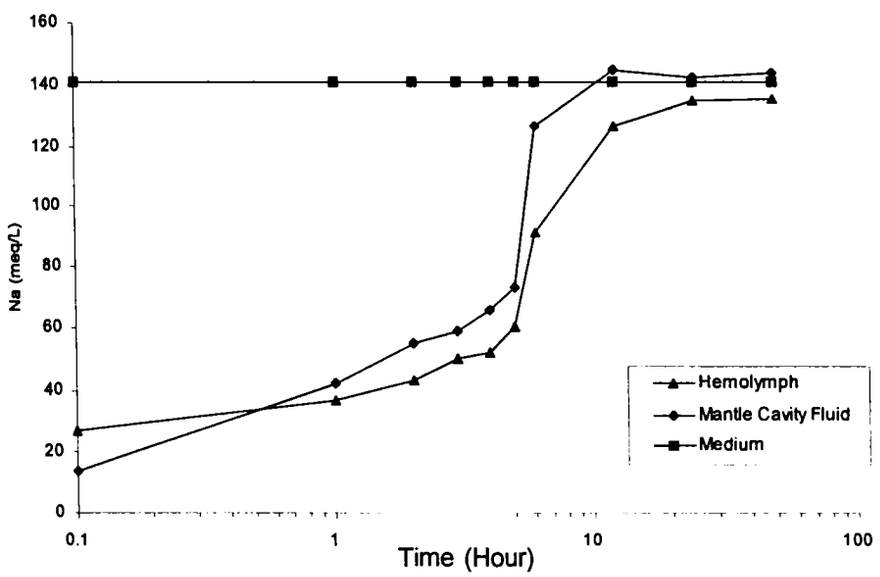


Fig 2.4: Concentration of Sodium in *V. cyprinoides* var. cocninensis (large) exposed to 10 x 10^{-3} salinity

When exposed to 15 x 10^{-3} salinity, small animals retained hypo-osmoticity of the mantle cavity fluid for 48 hours. (Table 2.3. Fig. 2.5). Large animals also attained ionic equilibrium with the mantle cavity fluid in 48 hour. Slight hypo-osmoticityof

hemolymph was observed throughout the exposure period in both the size groups (Table 2.3, Fig. 2.5 & 2.6).

Table 2.3. Concentration of Na^+ in the mantle-cavity fluid and hemolymph of *V. cyprinoides* var. cochinensis exposed to 15 x 10^{-3} salinity (Expressed as ppm).

Time of Accl. (hr)	Small Size Group				Large Size Group				Medium
	Mantle Cavity fluid		Hemolymph		Mantle Cavity fluid		Hemolymph		
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
0	19.3	± 2.5	25.3	± 6.3	19.3	± 3.0	27.1	± 1.1	211
1	34.1	± 6.3	48.8	± 2.5	39.0	± 10.1	34.1	± 2.1	211
2	53.8	± 4.1	53.8	± 4.0	34.1	± 2.8	34.1	± 4.5	211
3	46.4	± 2.1	61.1	± 2.1	48.8	± 9.4	51.3	± 7.1	211
4	58.7	± 1.6	63.6	± 5.4	39.0	± 5.6	41.5	± 5.2	211
5	65.8	± 2.0	64.8	± 1.3	46.4	± 8.5	50.8	± 6.9	211
6	93.6	± 9.7	88.5	± 3.6	58.7	± 2.1	75.4	± 3.8	211
12	149.2	± 5.2	154.0	± 5.0	125.1	± 2.1	165.5	± 3.5	211
24	204.0	± 2.0	194.0	± 9.0	184.2	± 6.8	191.6	± 6.0	211
48	214.3	± 3.0	218.3	± 2.0	213.7	± 5.2	221.1	± 3.0	211
72	211.1	± 4.2	218.3	± 4.3	213.0	± 8.5	224.0	± 8.5	211

Values are the mean of six observations ± SD.
Each observation is made from a sample of 8-12 animals pooled together.

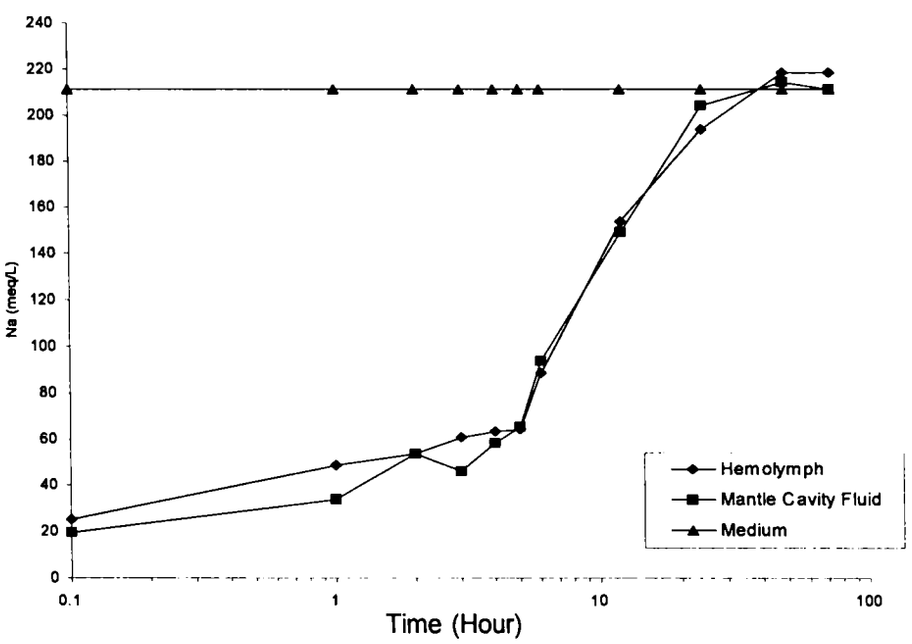


Fig 5: Concentration of Sodium in *V. cyprinoides* var. cochinensis(small) exposed to 15 x 10^{-3} salinity

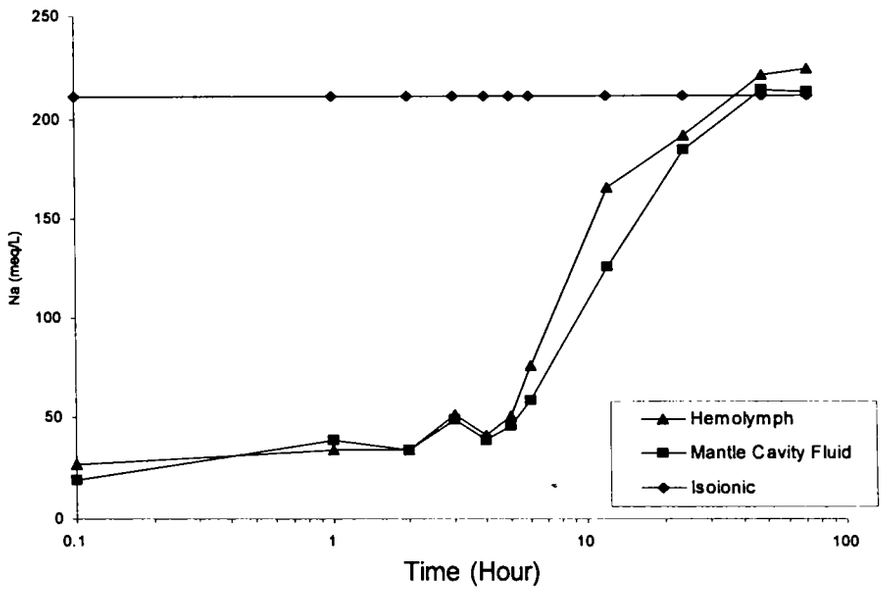


Fig 6: Concentration of Sodium in *V. cyprinoides* (large) exposed to 15 x 10^{-3} salinity

At 20 x 10^{-3} salinity, which is the upper extreme experimented, ionic equilibrium of the mantle cavity fluid with the ambient medium was attained in 48-72 hours in small size group. Isosmoticity of the hemolymph with the mantle cavity fluid was attained between 72 and 96 hours. Hemolymph was hypo-osmotic to the ambient medium till 96 hours (Table 2.4, Fig. 2.7). Large animals also maintained hypo-osmoticity of the mantle cavity fluid for 72 hours. In this case, hemolymph was hypo-osmotic throughout the experiment (Table 2.4, Fig. 2.8).

Table 2.4. Concentration of Na^+ in the mantle-cavity fluid and hemolymph of *V. cyprinoides* var. cochinensis at 20 x 10^{-3} salinity (Expressed as ppm).

Time of Accl. (hr)	Small Size Group				Large Size Group				Medium
	Mantle Cavity fluid		Hemolymph		Mantle Cavity fluid		Hemolymph		
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
0	28.5	± 6.3	31.3	± 5.5	31.3	± 0.2	28.5	± 2.0	331
1	36.9	± 2.3	31.3	± 3.8	31.3	± 6.5	22.9	± 5.2	331
2	59.3	± 2.5	42.5	± 4.4	36.9	± 6.8	36.9	± 1.6	331
3	48.1	± 4.8	39.7	± 0.5	28.5	± 4.2	22.9	± 1.7	331
4	59.3	± 5.5	53.7	± 5.8	64.9	± 3.5	30.5	± 2.9	331
5	73.4	± 0.0	54.9	± 6.9	71.0	± 3.5	45.3	± 3.1	331
6	98.6	± 2.4	62.1	± 8.4	104.2	± 11.1	59.3	± 3.6	331
12	143.5	± 6.0	84.6	± 7.7	241.6	± 9.6	109.8	± 5.2	331
24	238.8	± 9.8	118.0	± 2.8	262.0	± 8.3	236.0	± 6.1	331
48	261.0	± 2.7	241.0	± 8.7	295.0	± 8.7	257.0	± 8.2	331
72	331.3	± 6.9	306.0	± 8.4	340.0	± 8.9	310.5	± 5.7	331
96	331.5	± 8.2	333.6	± 2.5	343.0	± 7.5	310.3	± 2.4	331

Values are the mean of six observations ± SD.
Each observation is made from a sample of 8-12 animals pooled together.

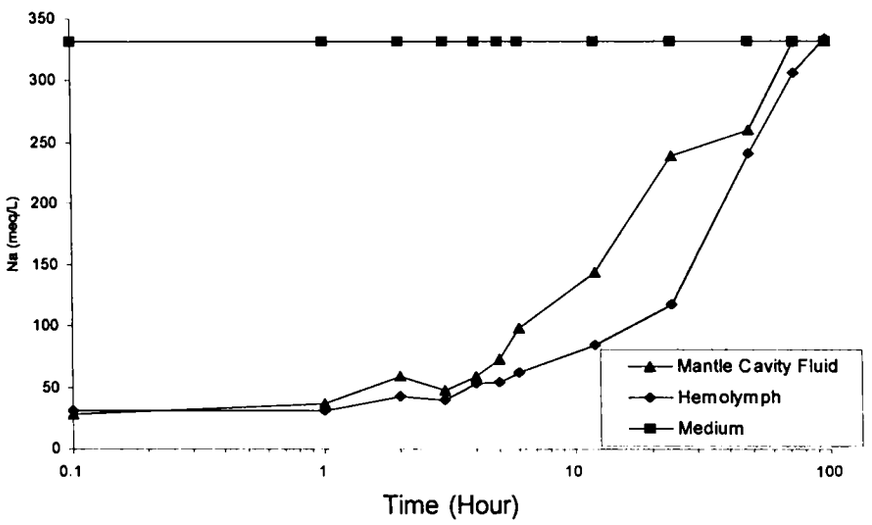


Fig 2.7: Concentration of Sodium in *V. cyprinoides* var. cochinensis (small) exposed to 20 x 10^{-3} salinity

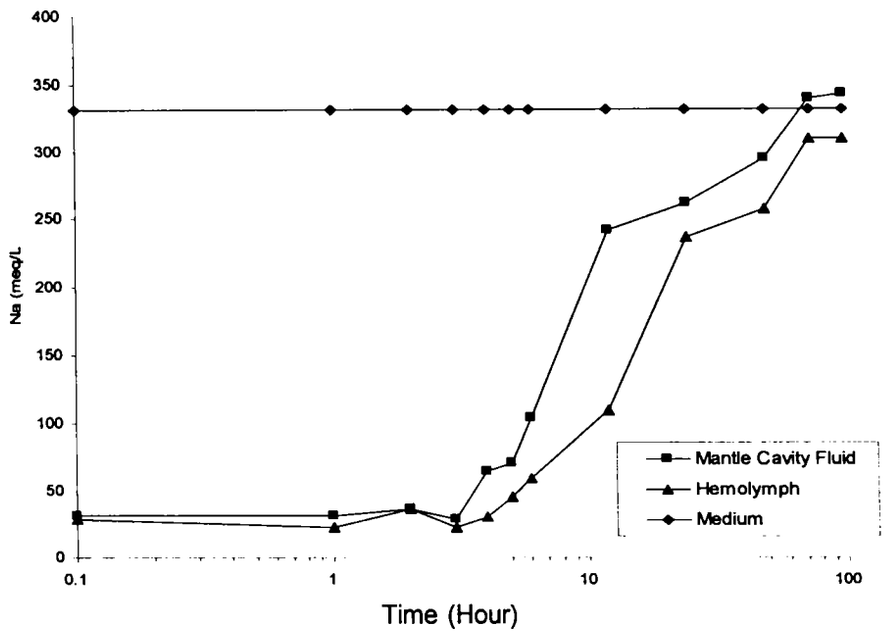


Fig 2.8: Concentration of Sodium in *V. cyprinoides* var. cochinensis (large) exposed to 20 x 10^{-3} salinity

Concentration of chloride in the mantle fluid of experimental animals exposed to salinity 5 x 10^{-3} remained low up to 12-24 hours in both the size groups (Table 2.5, Fig. 2.9 & 2.10). In salinity 10 x 10^{-3}, isosmoticity of mantle cavity fluid with the ambient medium was attained in 24 hours in small size group and between 24 and 48 hours in large group (Table 2.6, Fig. 2.11 & 2.12). When exposed to salinity 15 x 10^{-3}, equilibrium in concentration of chloride with the ambient medium was attained between 24 and 48 hours in both the size groups (Table 2.7, Fig. 2.13 & 2.14). In salinity 20 x 10^{-3}, small size group retained hyposmoticity of mantle cavity fluid up to 72 hours and large size group up to 96 hours (Table 2.8, Fig. 2.15 & 2.16). In all the observations, hemolymph of both the size groups remained slightly hyperosmotic or isosmotic with the mantle cavity fluid.

Table 2.5. Concentration of Cl^- in the mantle-cavity fluid and hemolymph of *V. cyprinoides* var. cochinensis exposed to 5 x 10^{-3} salinity (Expressed as ppm).

Time of Accl. (hr)	Small Size Group				Large Size Group				Medium
	Mantle Cavity fluid		Hemolymph		Mantle Cavity fluid		Hemolymph		
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
0	49.5 ± 3.5		56.0 ± 4.5		50.2 ± 6.3		57.5 ± 6.2		79
1	62.3 ± 0.1		64.0 ± 3.0		62.9 ± 5.8		67.5 ± 1.5		79
2	65.2 ± 3.5		68.3 ± 5.1		67.3 ± 2.1		70.0 ± 5.0		79
3	73.5 ± 2.4		71.3 ± 2.3		70.2 ± 5.2		72.6 ± 3.1		79
4	75.3 ± 6.9		77.0 ± 2.1		75.3 ± 6.7		76.2 ± 2.0		79
5	76.7 ± 2.2		79.7 ± 5.1		76.0 ± 8.1		76.8 ± 8.5		79
6	75.1 ± 4.2		80.0 ± 4.0		76.5 ± 0.6		78.8 ± 6.8		79
12	78.0 ± 4.2		80.3 ± 0.3		78.6 ± 3.6		84.0 ± 3.1		79
24	79.3 ± 8.2		82.3 ± 5.1		80.1 ± 9.0		83.6 ± 3.0		79
48	79.1 ± 2.5		81.3 ± 6.2		79.1 ± 4.3		84.0 ± 1.9		79

Values are the mean of six observations ± SD.
Each observation is made from a sample of 8-12 animals pooled together.

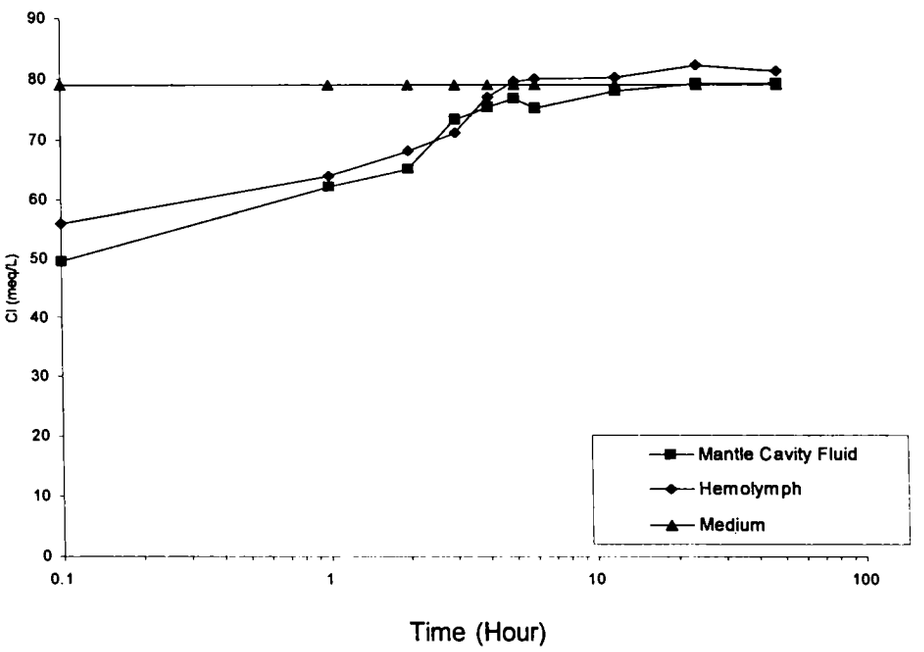


Fig 2.9: Concentration of Chloride in *V. cyprinoides* var. cochinensis (small) exposed to 5 x 10^{-3} salinity

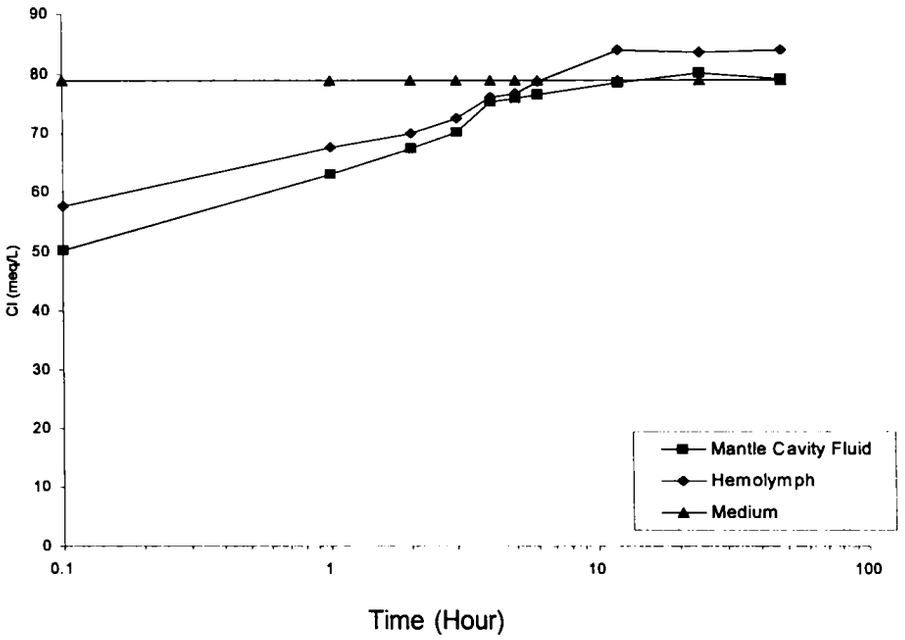


Fig 2.10: Concentration of Chloride in *V. cyprinoides* var. cochinensis (large) exposed to 5 x 10^{-3} salinity

Table 2.6. Concentration of Cl^- in the mantle-cavity fluid and hemolymph of *V. cyprinoides* var. cochinensis exposed to 10 x 10^{-3} salinity (Expressed as ppm).

Time of Accl. (hr)	Small Size Group				Large Size Group				Medium
	Mantle Cavity fluid		Hemolymph		Mantle Cavity fluid		Hemolymph		
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
0.1	48.5 ±	4.3	56.5 ±	9.1	49.4 ±	4.3	57.5 ±	4.9	163
1	63.1 ±	4.1	73.7 ±	8.5	59.6 ±	2.1	71.0 ±	6.8	163
2	73.7 ±	6.8	85.7 ±	6.7	65.3 ±	5.6	85.6 ±	6.1	163
3	87.0 ±	0.8	91.7 ±	3.2	75.4 ±	6.7	90.0 ±	8.1	163
4	94.3 ±	5.3	107.6 ±	4.7	93.7 ±	1.2	98.9 ±	0.9	163
5	95.6 ±	5.6	112.0 ±	6.8	97.2 ±	1.5	105.8 ±	5.6	163
6	120.0 ±	9.8	126.2 ±	11.0	108.5 ±	3.2	121.1 ±	9.1	163
12	156.8 ±	2.7	168.7 ±	2.7	150.7 ±	5.1	166.2 ±	8.9	163
24	164.0 ±	8.2	169.4 ±	5.6	161.3 ±	4.8	173.9 ±	4.1	163
48	163.5 ±	1.7	170.7 ±	9.0	162.7 ±	2.3	175.4 ±	5.1	163

Values are the mean of six observations ± SD.
Each observation is made from a sample of 8-12 animals pooled together.

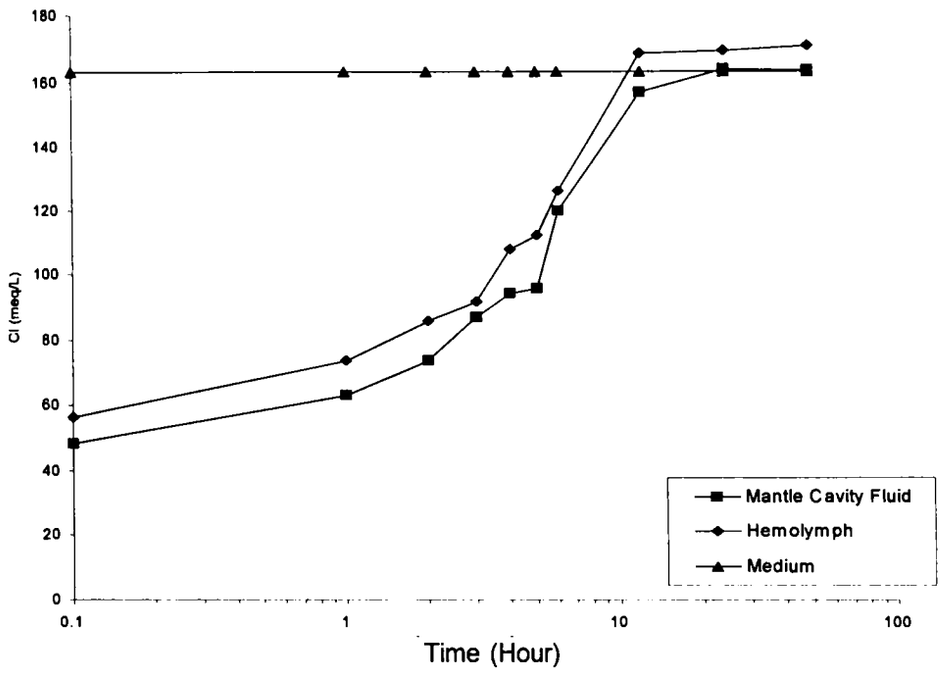


Fig 2.11: Concentration of Chloride in *V. cyprinoides* var. cochinensis (small) exposed to 10 x 10^{-3} salinity

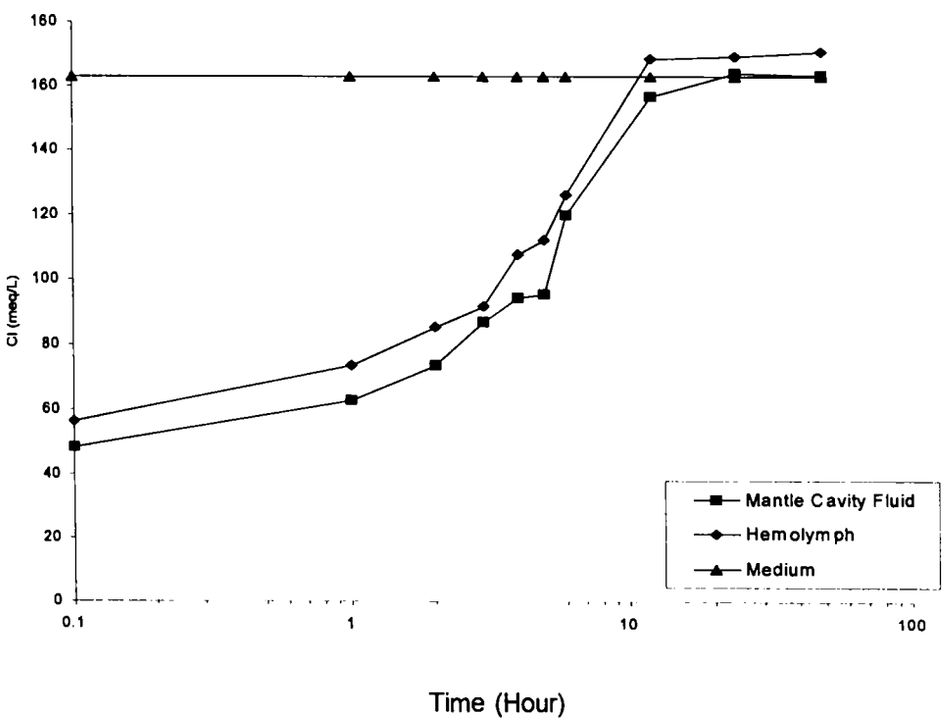


Fig 2.12: Concentration of Chloride in *V. cyprinoides* var. cochinensis (small) exposed to 10 x 10^{-3} salinity

Table 2.7. Concentration of Cl^- in the mantle-cavity fluid and hemolymph of *V. cyprinoides* var. cochinensis exposed to 15 x 10^{-3} salinity (Expressed as ppm).

Time of Accl. (hr)	Small Size Group				Large Size Group				Medium
	Mantle Cavity fluid		Hemolymph		Mantle Cavity fluid		Hemolymph		
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
0	49.1	± 1.5	57.3	± 1.5	48.2	± 1.5	56.1	± 0.0	224
1	48.0	± 0.0	57.6	± 4.5	47.3	± 1.5	55.6	± 4.5	224
2	55.5	± 4.5	55.5	± 1.5	48.0	± 0.0	53.2	± 0.0	224
3	51.0	± 0.0	56.5	± 3.0	54.0	± 0.0	56.4	± 1.5	224
4	61.5	± 4.5	60.0	± 3.0	56.6	± 0.0	56.5	± 1.5	224
5	66.0	± 3.0	83.6	± 0.0	59.4	± 4.5	60.1	± 0.0	224
6	87.0	± 0.0	87.0	± 0.0	82.5	± 1.5	67.5	± 1.5	224
12	210.0	± 3.0	208.5	± 1.5	150.0	± 3.0	126.2	± 1.5	224
24	213.0	± 0.0	222.0	± 0.0	215.0	± 0.0	236.0	± 0.0	224
48	225.0	± 0.0	220.0	± 0.0	225.0	± 4.5	234.2	± 15.0	224
72	225.2	± 0.0	221.0	± 3.2	224.6	± 5.1	230.1	± 5.2	224

Values are the mean of six observations ± SD.
Each observation is made from a sample of 8-12 animals pooled together.

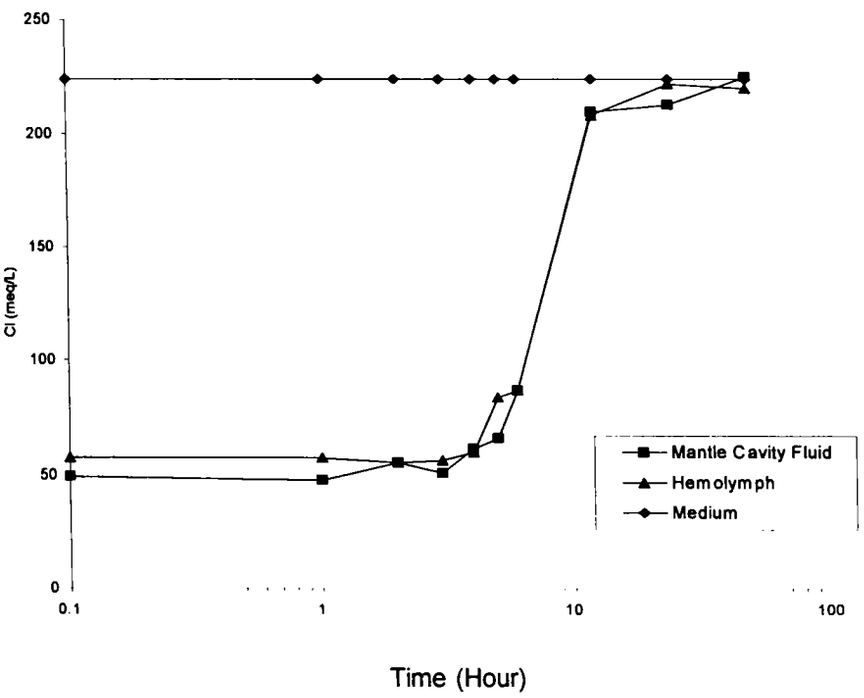


Fig 2.13: Concentration of chloride in *V. cyprinoides* var. cochineisis (Small) exposed to 15 x 10^{-3} salinity

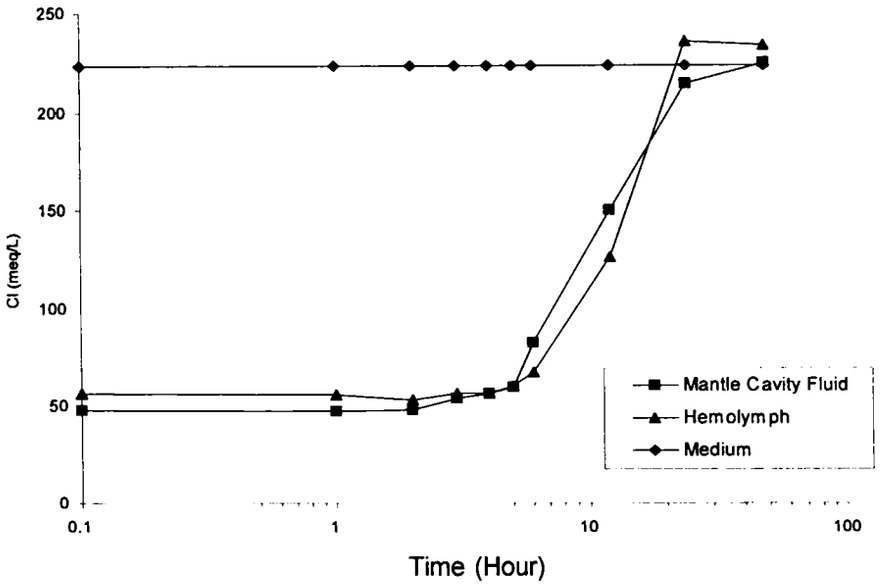


Fig 2.14: Concentration of chloride in *V. cyprinoides* var. cochinensis (Large) exposed to 15 x 10^{-3}salinity

Table 2.8. Concentration of Cl^- in the mantle-cavity fluid and hemolymph of *V. cyprinoides* var. cochinensis at 20 x 10^{-3} salinity (Expressed as ppm).

Time of Accl. (hr)	Small Size Group				Large Size Group				Medium
	Mantle Cavity fluid		Hemolymph		Mantle Cavity fluid		Hemolymph		
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
0	47.7 ±	5.1	57.0 ±	1.6	46.0 ±	6.1	50.2 ±	6.7	348
1	47.2 ±	6.3	63.2 ±	7.8	47.4 ±	11.7	49.3 ±	2.8	348
2	50.7 ±	11.9	70.5 ±	4.6	51.2 ±	8.5	51.0 ±	4.8	348
3	57.4 ±	7.8	56.9 ±	4.8	57.9 ±	6.2	51.1 ±	1.4	348
4	64.2 ±	6.3	70.5 ±	5.4	51.2 ±	1.9	50.2 ±	1.5	348
5	71.9 ±	10.1	70.5 ±	5.4	51.2 ±	8.8	60.1 ±	2.6	348
6	77.7 ±	12.1	75.6 ±	3.7	68.5 ±	9.2	63.5 ±	1.4	348
12	91.2 ±	5.6	138.0 ±	3.9	117.7 ±	4.3	110.1 ±	9.1	348
24	104.7 ±	9.7	185.3 ±	10.0	317.3 ±	4.8	262.0 ±	9.3	348
48	280.3 ±	8.6	310.5 ±	1.3	345.7 ±	6.4	322.9 ±	2.1	348
72	358.3 ±	9.7	350.1 ±	2.7	330.6 ±	2.3	329.5 ±	7.2	348
96	361.3 ±	8.1	356.3 ±	4.9	357.2 ±	2.1	356.1 ±	9.5	348

Values are the mean of six observations ± SD.

Each observation is made from a sample of 8-12 animals pooled together.

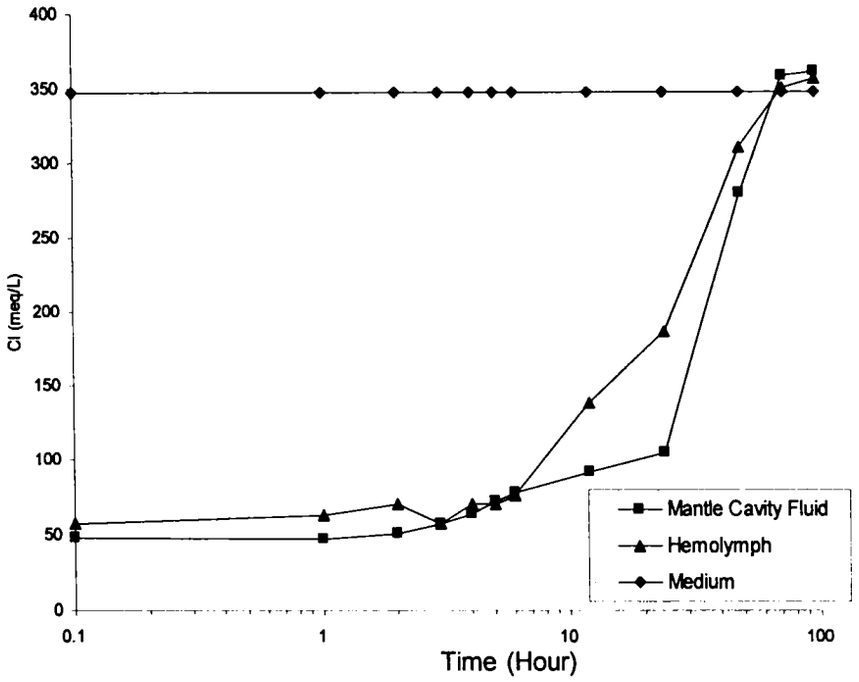


Fig 2.15: Concentration of chloride in *V. cyprinoides* var. cochinensis (Small) exposed to 20 x 10^{-3} salinity

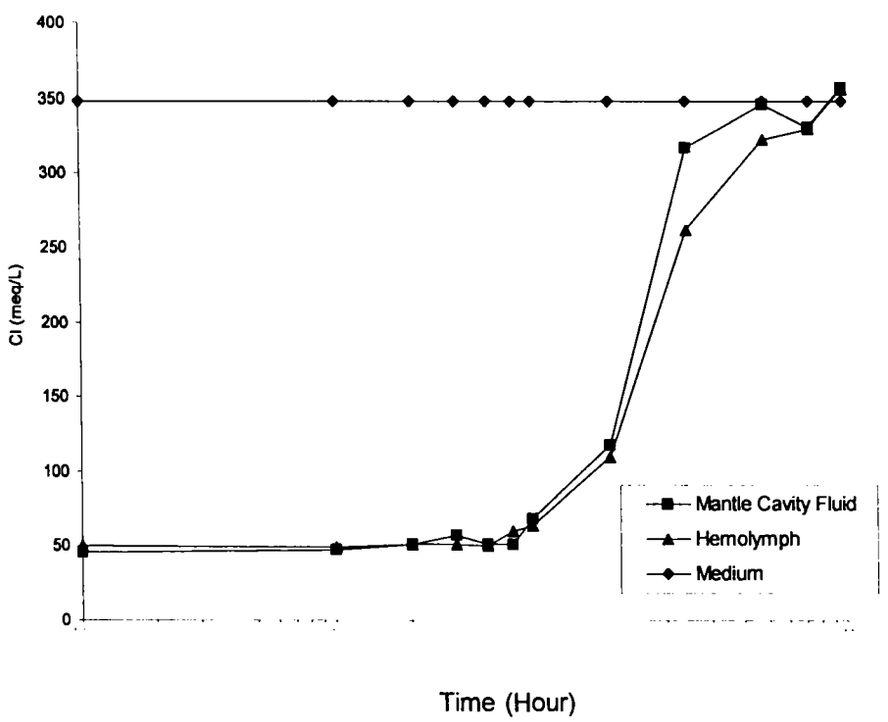


Fig 2.16: Concentration of chloride in *V. cyprinoides* var. cochinensis(Large) exposed to 20 x 10^{-3} salinity

An increase in ionic concentration of Na^+ and Cl^- in the mantle cavity fluid was followed by a corresponding increase in the concentration of these ions in the hemolymph. This was found to be true for both the size groups (Fig. 2.1-2.16). At lower salinities (5×10^{-3} and 10×10^{-3}) concentration of Na^+ in the hemolymph was kept very much lower than that of the ambient medium. But at higher salinities (15×10^{-3} and 20×10^{-3}) the concentration in the hemolymph was slightly lower than that of the ambient medium.

2.3.2 *Discussion*

Bivalves, gifted with osmoconformity, are capable of escaping transient changes in salinity by behavioural responses. Major behavioural means by which bivalves segregate their soft body parts from external medium are by valve closure and burrowing. Valve closure is the immediate response and this helps the animal to streamline the physiological machinery to the altered environment. Therefore studies on the effect of changes in salinity on physiological mechanisms of bivalves should be coupled with studies on behavioral responses (Akberali and Trueman, 1985).

Prolonged valve closure can result in a shoot up of tissue osmolarity, anoxia and accumulation of waste products inside. Hence valve closure is not a permanent solution for escaping from salinity changes. Experiments on ionic concentrations of mantle cavity fluid and hemolymph reveal that animals return to an altered equilibrium after a period of latency. The latent period helps them to make cellular adjustments. It can be postulated that valve closure and activity cessation in altered concentrations represent a resistance mechanism of the organisms to evoke physiological machinery to attain a new steady state.

Villorita cyprinoides var. cochinensis exposed to elevated salinities showed a time lag in attaining ionic equilibrium of mantle cavity fluid with the ambient medium. The time taken to attain homogeneity was more when the salinity was far from the acclimation salinity.

It is quite natural that as the salinity increases, the time taken to adjust also increases. Several authors are of the opinion that the period of valve closure increases with extremes of salinity (Akberali, 1978; Leader, *et. al.*, 1986; George, 1993). But prolonged valve closure can impose a conflict between anoxia and blood osmolarity (Clark & Finley, 1974; de Mahieu, *et. al.*, 1981). In the case of *Villorita cyprinoides*, maximum time taken to attain homogeneity with ambient environment was noticed at salinity 20 x 10^{-3}, which was the upper extreme of salinity, experimented with.

Behavioural response varies with size groups. From the observations made, it can be noticed that towards higher salinities, smaller size group attained osmotic equilibrium with the ambient medium slightly faster than larger animals. Eventhough many workers have reported differences in the period of valve closure between size groups (Kinne, 1971; Skinner & Peretz, 1989), in *Villorita*, this is not prominent as in the case of typical marine forms. Since this animal is an estuarine form and the animal experiences daily fluctuations in salinity, both the size groups may be adapted for faster adjustments. There is a faster metabolic turn over for smaller size group, as evident from oxygen consumption (Chapter 2), small animals have the advantage of faster internal adjustments of osmolarity with the ambient medium. This could be the suitable justification for the faster acclimation of small size group when compared to the large ones.

From the experiments, hypo-osmoticity of the mantle cavity fluid could be observed

for a definite interval of time, in animals transferred to higher salinities. With the progress of time, there is a gradual increase in osmotic concentration in the mantle cavity fluid. Similar to the observations made by George (1993) in the case of *Sunetta scripta* and *Perna viridis*, which are purely marine forms, in the present study also there was a lag in attaining homogeneity of the mantle cavity fluid with the ambient medium. But even in the extreme salinity, i.e. 20 x 10^{-3}, the time taken to attain homogeneity was only 72-96 hours. This is lesser than that observed in the case of purely marine bivalve *Sunetta scripta* studied by George (1993). The probable reason may be that, *Villorita* is exposed to diurnal fluctuations in salinity, unlike marine forms. So their physiological machinery may be adapted for quick adjustments with salinity changes. All euryhaline species of bivalves retain an ambient environment in the mantle cavity for some time when the salinity fluctuation in the environment is greater than the tolerance limit. When the animal is forced to live in the resistance zone, the speed with which it returns to the new equilibrium after exposure is indicative of the perfection of adaptation. As stated earlier, valve closure is not a permanent solution to escape from the alterations in the environment. Moreover it imparts a conflict between blood osmolarity and anoxia. In *Villorita*, hemolymph ionic concentration also increases in accordance with the increase in ionic content of mantle cavity fluid. Hemolymph is hypo ionic to mantle cavity fluid at lower salinity. At higher salinities hemolymph is more or less isoionic to the mantle cavity fluid. This reflects the lack of anisosmotic extracellular regulation at higher salinities. It can be deduced that valve closing mechanism is of help to the animal under transient salinity changes. At the same time a decreased lag in time for attaining homogeneity help this estuarine form to thrive better in an environment where it experiences diurnal changes in salinity.

2.4 Blood osmolarity and ionoregulation

2.4.1 Results

Concentration of all the ions of the hemolymph studied showed an increasing trend with increase in salinity. Values of ion concentrations are given in Table 2.9

Table 2.9. Concentration of Na^+ in the mantle-cavity fluid and hemolymph of *V. cyprinoides* var. cochinensis at various salinities 0 x 10^{-3} to 20 x 10^{-3} (Expressed as ppm).

Salinity	Small Size Group				Large Size Group				Medium
	Mantle Cavity fluid		Hemolymph		Mantle Cavity fluid		Hemolymph		
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
0	21.8	± 3.1	25.3	± 3.2	20.5	± 1.6	23.5	± 5.7	10.0
5	44.1	± 5.3	33.4	± 6.1	45.9	± 5.6	36.8	± 4.2	71.0
10	135.0	± 9.7	42.5	± 8.5	51.0	± 2.7	49.8	± 3.6	141.0
15	211.1	± 4.6	218.3	± 6.7	213.0	± 7.1	223.8	± 8.7	211.3
20	331.5	± 10.1	333.0	± 9.1	343.0	± 5.9	310.0	± 11.3	302.0

Values are the mean of six observations ± SD.
Each observation is made from a sample of 8-12 animals pooled together.

Table 2.9a. Result of Analysis of Variance for concentration of sodium in the hemolymph

Source	Sum of Squares	Degrees of Freedom	Mean of Squares	F-value	Significance
Size groups	1018.92	2	509.46	0.62	NS
Concentration of sodium	190739.80	4	47684.95	57.74	P<0.001
Error	6606.76	8	825.85		
Total	198365.5	14			

NS – Not significant.

- 2.11. Concentration of sodium in the hemolymph of both the size groups at 0 x 10^{-3} was higher than that in the medium. But the hemolymph became hypoionic to the medium at salinity 5 x 10^{-3} and 10 x 10^{-3}. Blood became isoionic at

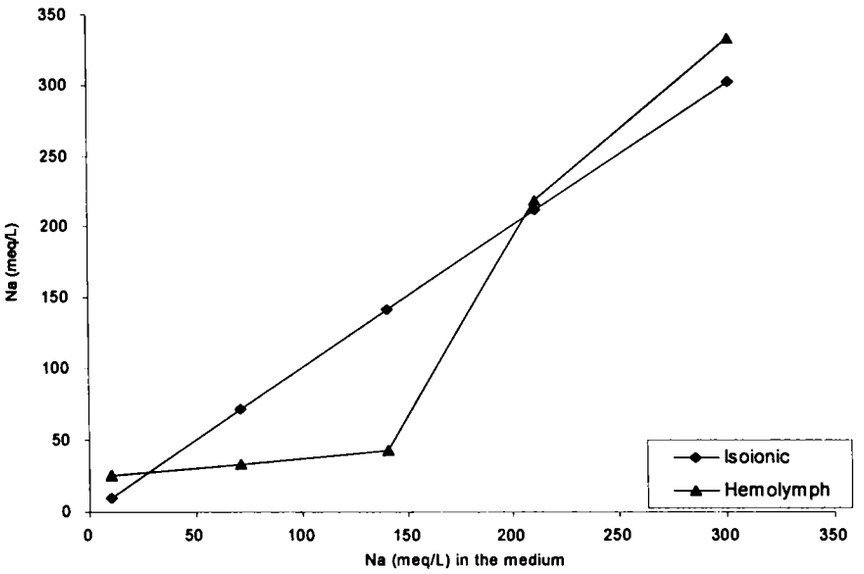


Fig. 2.17 Concentration of Sodium in the hemolymph (Small size group)

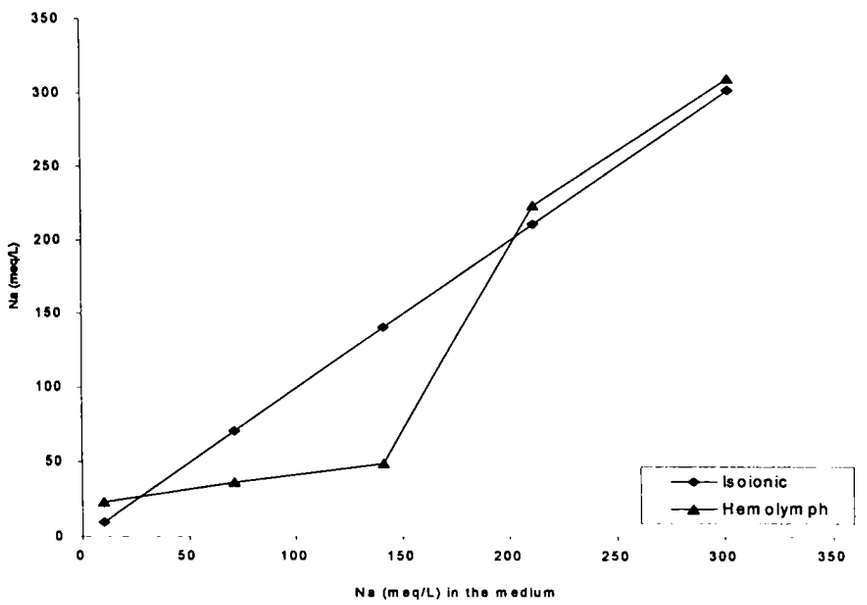


Fig. 2.18 Concentration of Sodium in the hemolymph (Large size group)

15 x 10^{-3} and at 20 x 10^{-3} it was slightly hyperionic to the medium. Variations between large and small size group were not pronounced (Fig. 2.17 & 2.18).

A hyper ionic regulation of potassium was noticed at 0 x 10^{-3} and 5 x 10^{-3} salinity in both the size groups. Variation in concentration from the ambient medium was less in the case of large animals. Results are shown in Table 2.10 and Fig. 2.19 & 2.20. Above 5 x 10^{-3} salinity, in small animals, concentration was almost equal to that of the ambient medium whereas in large size group, a slight hypoionic condition was noticed in hemolymph at salinity 10 x 10^{-3} and 15 x 10^{-3}.

Table 2.10. Concentration of K^+ in the mantle-cavity fluid and hemolymph of *V. cyprinoides* var. cochinensis at various salinities 0 x 10^{-3} to 20 x 10^{-3} (Expressed as ppm).

Salinity	Small Size Group				Large Size Group				Medium
	Mantle Cavity fluid		Hemolymph		Mantle Cavity fluid		Hemolymph		
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
0	1.1	± 0.3	1.7	± 0.2	1.2	± 0.1	1.2	± 0.5	1.0
5	1.7	± 0.4	2.1	± 0.5	1.3	± 0.3	1.7	± 0.2	1.5
10	2.9	± 0.5	2.5	± 0.4	1.6	± 0.3	1.4	± 0.2	2.9
15	3.9	± 0.5	5.1	± 0.1	3.5	± 0.5	3.5	± 0.6	4.4
20	6.5	± 0.2	5.6	± 0.1	5.9	± 0.5	7.8	± 0.7	5.9

Values are the mean of six observations ± SD.
Each observation is made from a sample of 8-12 animals pooled together.

Table 2.10a. Results of Analysis of Variance for concentration of potassium in the hemolymph

Source	Sum of Squares	Degrees of Freedom	Mean of Squares	F-value	Significance
Size groups	0.244	2	0.122	0.18	NS
Concentration of potassium	54.82	4	13.70	19.78	P<0.001
Error	5.54	8	0.69		
Total	60.60	14			

NS – Not significant.

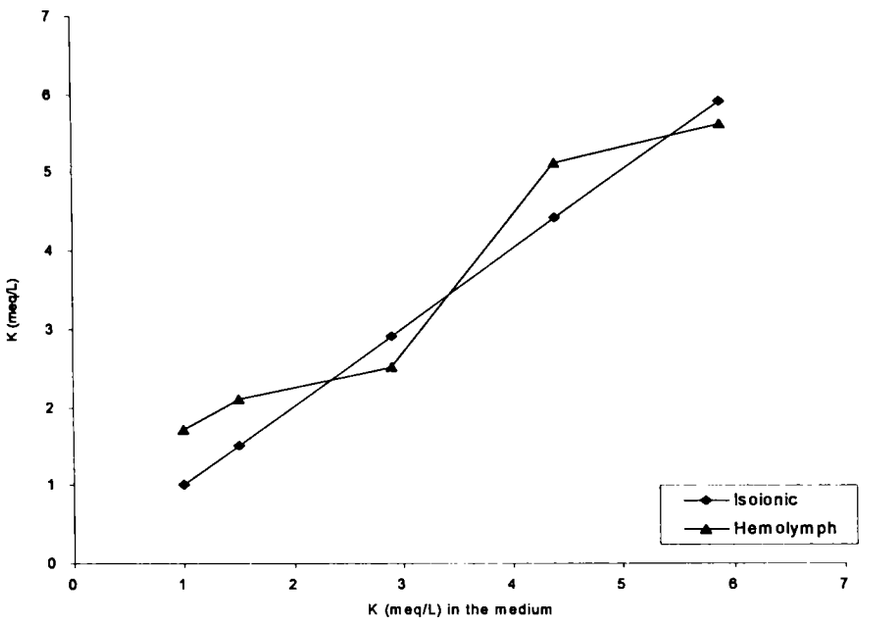


Fig. 2.19 Concentration of Potassium in the hemolymph (Small size group)

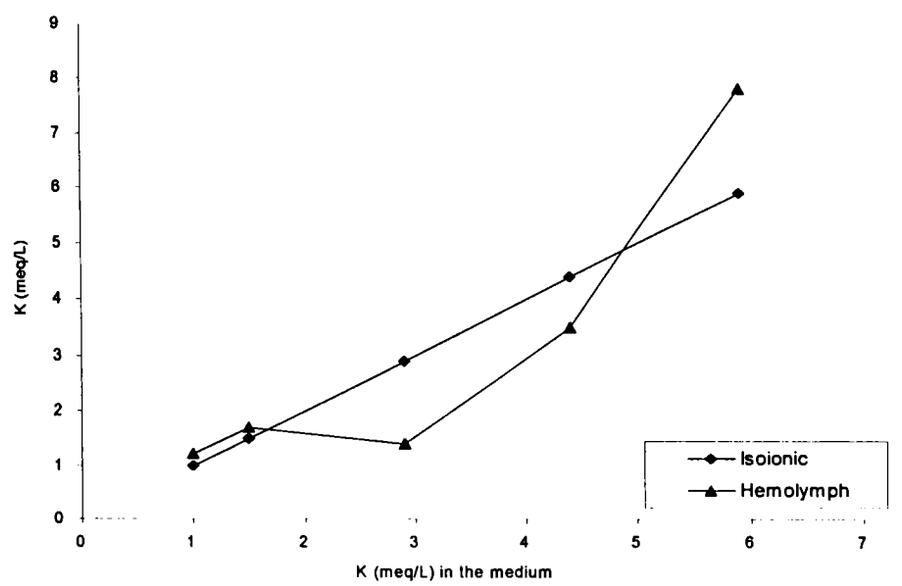


Fig. 2.20 Concentration of Potassium in the hemolymph (Large size group)

Chloride concentration in the extracellular fluid followed a pattern similar to that of potassium in both the size groups. A slight hyper ionicity was noticed in both the size groups in lower salinities (Table 2.11, Fig. 2.21 & 2.22). Increase in ion concentration in the mantle cavity was followed by an increase in the hemolymph also in both the size groups. Hyperionic regulation of chloride unlike sodium and potassium was found in all salinities.

Table 2.11. Concentration of Cl^- in the mantle-cavity fluid and hemolymph of *V. cyprinoides* var. cochinensis at various salinities 0 x 10^{-3} to 20 x 10^{-3} (Expressed as ppm).

Salinity	Small Size Group				Large Size Group				Medium
	Mantle Cavity fluid		Hemolymph		Mantle Cavity fluid		Hemolymph		
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
0	50.2 ±	5.0	88.0 ±	8.0	53.6 ±	6.0	94.0 ±	2.0	50.5
5	79.1 ±	9.0	100.0 ±	2.0	79.1 ±	5.0	84.0 ±	8.0	82.0
10	163.5 ±	7.0	170.7 ±	5.0	162.7 ±	10.0	175.4 ±	10.0	163.0
15	225.0 ±	10.0	256.0 ±	5.0	225.0 ±	9.0	262.0 ±	4.0	241.0
20	341.0 ±	5.0	370.0 ±	7.0	356.1 ±	4.0	357.2 ±	11.0	320.0

Values are the mean of six observations ± SD.
Each observation is made from a sample of 8-12 animals pooled together.

Table 2.11a. Results of Analysis of Variance for concentration of chloride in the hemolymph

Source	Sum of Squares	Degrees of Freedom	Mean of Squares	F-value	Significance
Size groups	2004.06	2	1002.03	8.32	P<0.01
Concentration of chloride	157736.60	4	39434.15	327.35	P<0.001
Error	963.72	8	120.47		
Total	160704.40	14			

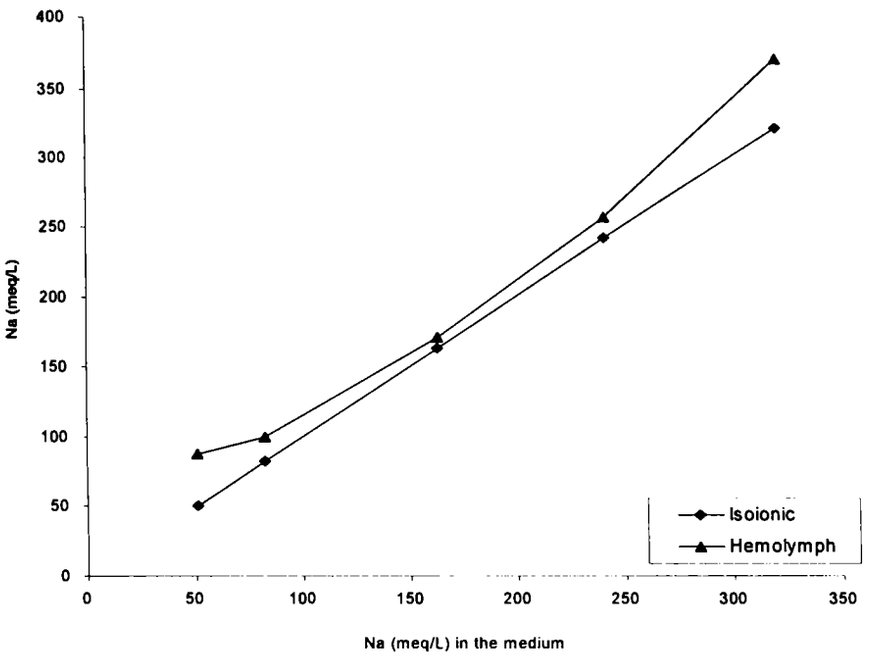


Fig. 2.21 Concentration of Chloride in the hemolymph of *V. cyprinoides* var. cochinensis (Small size group)

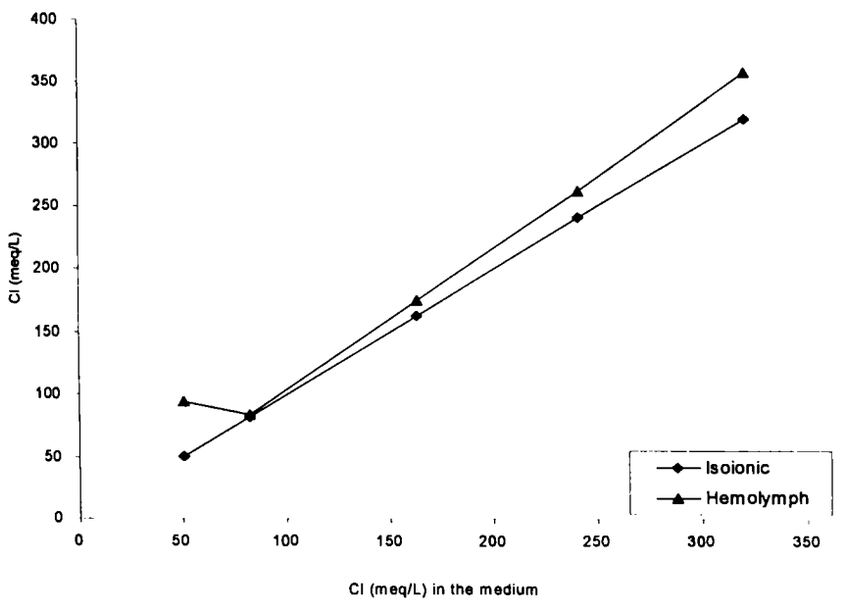


Fig. 2.22 Concentration of Chloride in the hemolymph of *V. cyprinoides* var. cochinensis (Large size group)

Osmolarity of mantle cavity fluid, hemolymph and medium are shown in Table 2.12 and Fig. 2.23 & 2.24. Hemolymph osmolarity of small size group is considerably low at higher salinities. Mantle cavity fluid osmolarity was almost same as that of the ambient medium in both the size groups. Osmolarity of hemolymph followed almost the same pattern as that of the mantle cavity fluid in the case of large size group.

Table 2.12. Osmotic pressure of mantle cavity fluid and hemolymph of *V. cyprinoides* var. cochinensis acclimated to various salinities (Expressed as Milli Osmols/kg Water).

Salinity	Small Size Group				Large Size Group				Medium
	Mantle Cavity fluid		Hesmolymph		Mantle Cavity fluid		Hemolymph		
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
0	20	± 1	87	± 6	30	± 8	63	± 8	0
5	190	± 9	200	± 5	190	± 5	200	± 7	190
10	320	± 3	350	± 8	330	± 7	340	± 7	330
15	430	± 8	380	± 5	450	± 12	450	± 5	450
20	480	± 2	350	± 5	520	± 5	520	± 9	530

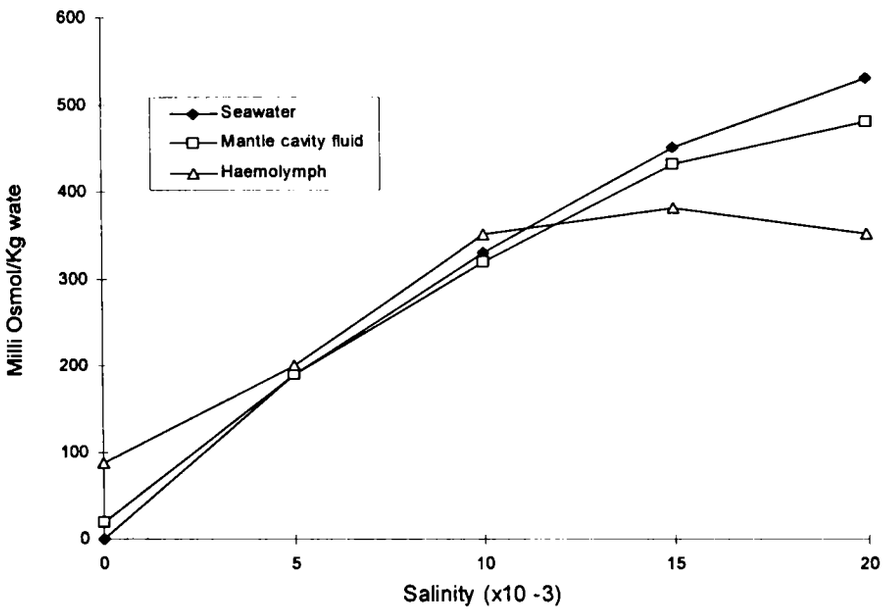


Fig. 2.23 Osmolarity of hemolymph and mantle cavity fluid of small animals

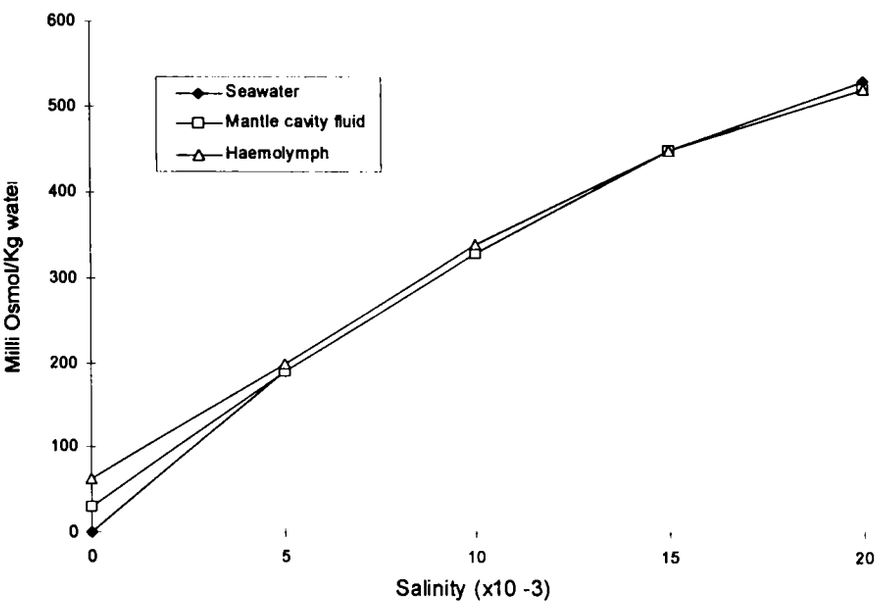


Fig. 2.24 Osmolarity of hemolymph and mantle cavity fluid of large animals

Water content of tissues varied from 70 – 85%. Statistical analysis by student's *t* test showed that there is no significant difference in the water content between salinities except on foot of small size group at salinity 10 x 10^{-3}. Mantle tissue contained more amount of water when compared to adductor and foot in both small and large size groups (Table 2.13 & 2.14, Fig. 2.25 & 2.26).

Table 2.13. Water content of tissues of *V. cyprinoides* var. cochinensis (Small) acclimated to various salinities (Expressed as percentage of wet weight).

Salinity	Adductor			Foot			Mantle		
	Mean		SD	Mean		SD	Mean		SD
0	79.9	±	2.1	79.5	±	4.1	84.4	±	5.6
10	85.0	±	7.4	69.7	±	2.9	83.0	±	2.4
20	77.5	±	5.5	76.2	±	5.7	81.1	±	2.4

Table 2.14. Water content of tissues of *V. cyprinoides* var. cochinensis (Large) acclimated to various salinities (Expressed as percentage of wet weight).

Salinity	Adductor			Foot			Mantle		
	Mean		SD	Mean		SD	Mean		SD
0	80.0	±	4.3	79.6	±	3.9	84.5	±	3.4
10	79.7	±	2.4	78.8	±	5.1	83.6	±	5.1
20	74.2	±	3.3	75.6	±	2.9	80.2	±	2.6

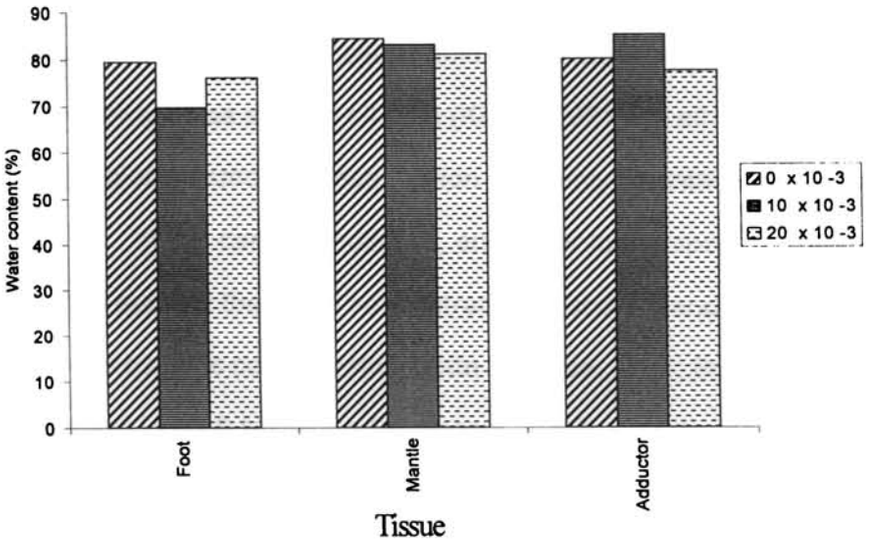


Fig. 2.25 Water content in various tissues of *V. cyprinoides* var. cochinensis (Small) acclimatized to various salinities

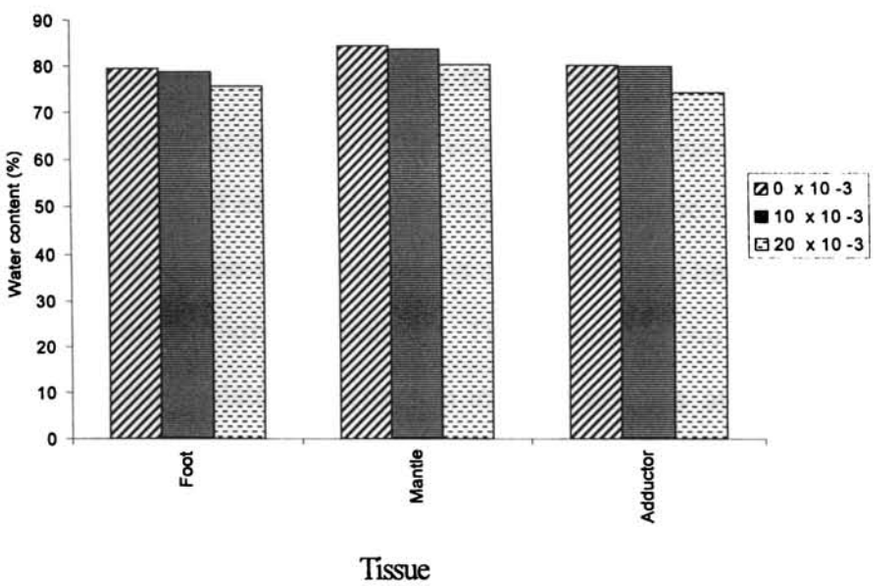


Fig. 2.26 Water content in various tissues of *V. cyprinoides* var. cochinensis (Large) acclimatized to various salinities

2.4.2 *Discussion*

Molluscs are generally poor iono and osmoregulators. In general, they prefer osmoconformity. Many authors have reported limited osmoregulation in molluscs

including bivalves under slight change of salinity from optimum (Freeman & Ringler, 1957; Shumway, 1977). Extracellular fluid anisosmotic regulation observed in all the freshwater species studied till date (Kinne, 1971) In all the species studied, most of the blood osmotic concentration is mainly maintained by sodium and chloride ions. In the case of molluscs, and other lower species, many authors have pointed out that the blood concentration of sodium and chloride follows the trend of blood osmotic concentration (Otto & Pierce, 1981; Burton, 1983; Deaton, 1992; Cedomil Lucu & Massimo Devescovi; 1999). There are discrepancies in the data of blood ionic composition of molluscs.

Concentration of sodium, potassium and chloride ions in the blood of both the size groups of *Villorita cyprinoides* var. cochinensis followed the same pattern of variation of these ions in the ambient medium. No active regulation of the major ions in the hemolymph could be observed. George (1993) had similar observation in the case of *Sunetta scripta* and *Perna viridis*. Since *V. cyprinoides* has only a very limited capability of extracellular ion regulation, it can be considered an iono conformer. A slight hypo-ionic condition is observed in the hemolymph of small animals exposed to higher salinities. This indicates the existence of an extracellular osmoregulatory mechanism in small animals at higher salinities. Concentration of sodium, potassium and chloride was higher than that of the medium in lower salinities (0×10^{-3} and 5×10^{-3}) in both of the size groups. This is in agreement with the data for marine molluscs *S. scripta* and *P. viridis* (George, 1993).

The pattern of regulation of sodium ions in *V. cyprinoides* var. cochinensis is more similar to *S. scripta* than *P. viridis*. *S. scripta* can tolerate salinities as low as 5×10^{-3} (Thampuran, *et. al.*, 1982) whereas *P. viridis* cannot. In *S. scripta*, at

salinities 0 x 10^{-3}, 5 x 10^{-3} and 10 x 10^{-3}, a pronounced hyperionic regulatory mechanism is in operation which maintains the concentration of sodium in the extracellular fluid above that of the ambient medium. Freeman and Ringler (1957) found that in the estuarine clam *Scorbicularia plana,* the hemolymph remains significantly hyperosmotic at 30 x 10^{-3} salinity. In the case of *V. cyprinoides*, pronounced hyper ionic regulation of sodium is seen only at salinity 0 x 10^{-3}. The capability of *V. cyprinoides* to invade freshwater and *S. scripta* to tolerate very low salinity may be due to the hyper ionic regulation of sodium, potassium and chloride ions in the hemolymph. Similar observations have been made in euryhaline molluscs by Schoffeniels and Gilles (1971), Gainey and Greenberg (1977), Deaton (1992) and Roger and Thomas (1997). When osmolarity of the medium increases, it results in an increase in the intracellular osmolarity since the ability to actively regulate ions extracellularly is limited.

Corbiculids invaded freshwater in the relatively recent geological past (Triassic) (Keen & Casey, 1969). Although freshwater bivalves are a highly diverse group, they display striking convergence in some of their physiological adaptation to dilute medium. Osmolarity of body fluid of freshwater bivalves is remarkably low ranging from 35-65 mOsm/Kg (Roger &Thomas, 1997).

Blood osmolarity of *V. cyprinoides* var. cochinensis follows the pattern of osmolarity of the external medium. Small size group has a higher capability of regulating ionic concentration extracellularly compared to large. This is reflected in the osmolarity profile of blood in higher salinities of small size group. This regulation, eventhough limited, can help the animal in reducing the amount of intracellular osmolytes that are to be mobilized for intracellular regulation. Study by George and Damodaran (1999) on *S. scripta* also corroborates this view.

Further, studies on the scope for growth of *S. scripta* has shown that small size group has a wider salinity spectrum for scope for growth compared to larger size groups (Supriya, 1992). Experiments conducted by Neufield and Wright (1998), on the estuarine mussel *Geukensia demissa* also yielded similar results. Blood and mantle fluid osmolarities of *G. demissa* was almost the same at different salinities.

Animals, which are not capable of regulating the water content under a hypo osmotic stress imbibe water and swell. This dilutes the body fluids. In earlier days, it was believed that cells were hyper osmotic to the environment. The basis was the observation that they imbibe water when they are incubated, after arresting metabolism, in an isotonic medium. But studies show that metabolic inhibitors abolish the active transport of cations (Tosteson & Hofman, 1960). A normally metabolizing cell effectively controls water content. Eventhough the cellular water content is regulated, in some species, the cells are tolerant to dilution to a certain extent. Neufield and Wright (1998) reported that the gill tissue of *G. demissa* exposed to cyclical changes in salinity showed no volume regulation, the volume of the tissue changed with a change in salinity. In the present study, it was found that there was no significant change in water content between salinities in various tissues suggesting the efficiency of the animal in water regulation. This shows that the animal is well adapted to survive in an environment where frequent changes in salinity occurs.

It can be concluded that the metabolic and physiological machinery of *V. cyprinoides* is more tuned for a life in estuarine conditions compared to marine forms. The increased capability to adapt to varied salinities is supportive of this view. Generally, in bivalves, Na^+ is the predominant cation, HCO_3^- and Cl^- are the major anions. In order to maintain sufficient levels of these ions in the body fluid, bivalves living in diluted media must actively transport the major ions from the dilute medium. At the same time, it should avoid flooding of ions at higher salinities.

The mechanisms of ion transport open another avenue for detailed study.

Under transient salinity changes the problem is solved by behavioural mechanisms and under prolonged salinity changes ions are regulated by excretion and the problem of water loss is mitigated by intracellular osmoregulation. In the following chapters, the experiments on strategies of intracellular osmoregulation are discussed.

Chapter 3

TOTAL NINHYDRIN POSITIVE SUBSTANCE CONTENT, OXYGEN CONSUMPTION AND AMMONIA EXCRETION DURING INTRACELLULAR OSMOREGULATION

3.1 Introduction

Maintenance of homeostasis requires regulation of osmotic pressure in the body. In euryhaline animals it is a complex process involving a suite of physiological and behavioural responses to oscillating conditions with differing osmoregulatory requirements. Even though the mechanisms of osmoregulation are well understood in many bivalves, very little information is available on the related energetics and physiological processes. Since marine and brackish water bivalves are osmoconformers, each cell must cope with the osmotic stress imposed by salinity change (Pierce, 1971). Estuarine animals have remarkable capacity of cell volume regulation. Voluminous data is available, demonstrating the regulatory volume decrease or regulatory volume increase of tissues (Chamberlin and Strange, 1989) to mitigate change in cell volume due to altered osmolarity of the medium. Tissues of estuarine bivalves do suffer from drastic transient changes in salinity of their body fluids. Since *V. cyprinoides* var. cochinensis was found to be an osmoconformer, it is presumed that it should regulate intracellular level of osmolytes to maintain osmotic balance. Intracellular osmolytes (predominantly amino acids and their derivatives) are derived mainly from metabolic pathways by transamination/amination and protein degradation

(Nirchio and Perez, 1997). Metabolic pathways are manipulated so that the animal can generate more Ninhydrin Positive Substances (NPS) in hypersaline conditions whereas a degradation pathway and passive diffusion of NPS out of the cell is promoted during hyposaline conditions. In many bivalves, apart from the synthesis, active uptake of NPS from the medium have also been pointed out (Somero & Bowlus, 1983). Whether such an absorption of NPS from the ambient medium is taking place in *V. cyprinoides* var. cochinensis is not known. If the animal is relying on synthesis of NPS from metabolic pathways, one might expect a corresponding increase in cellular respiration. This should increase the oxygen demand of the animal. The synthesis of amino acids demands more input of amino nitrogen also resulting in its decreased excretion. Therefore, such increased oxygen demand accompanied by decreased ammonia excretion during the period of acclimation is to be expected since major cellular adjustments are made during this period.

Catabolism of amino acids in a hypo-osmotic environment is documented by Bishop *et al.*, (1983) and Hayashi (1987). Catabolism of NPS yields nitrogenous wastes whereas their production demands nitrogen. Elevation in ammonia excretion along with a reduction in NPS pool is reported by many workers in bivalves during acclimation to lowered salinity. Emerson (1969) reported that a reduction in salinity caused increased excretion of ammonia nitrogen (NH_3-N in) *Macoma inconspicua*. This view was supported by Allen and Garrett (1971) in *Mya areneria* and Bayne (1975) in *Mytilus edulis*. Allen and Garrett (1971) recorded an increase in NH_3-N excretion from 3.22 mg NH_3-N /d at 34 x 10^{-3} salinity to 64.4 mg NH_3-N /d at 17 x 10^{-3} salinity in *Mya areneria*. There are discrepancies in the data regarding the rate of excretion of NH_3-N in animals. Studies conducted so far reveal that the rate of NH_3-N excretion and oxygen consumption do not always show a definite trend.

Oxygen consumption of an animal is influenced by a number of intrinsic and extrinsic factors like size, food, environmental conditions, physiological state of the animal, salinity, other environmental stress, etc. Estimation of the rate of oxygen consumption is a useful tool to assess energy expenditure for adaptation to environmental alterations (Thompson and Bayne, 1972). Swanson (1998) described a higher rate of oxygen consumption at 35 x 10^{-3} salinity than at 15 x 10^{-3} salinity by euryhaline milkfish *Chanos chanos*. Most researchers agree that salinities differing from the internal osmotic concentration involve costs for active regulation. But there is no unity among researchers concerning the magnitude of costs (Morgan and Iwama, 1991, Nordlie, et al., 1991) and very little information is available on the consequences of increased expenditure on life in different salinities. Metabolic regulation of the intracellular organic osmolyte pool involves alterations in the rate of metabolic pathways. Active processes consume energy and a change in metabolic pace will be reflected in the net oxygen demand of the animal. Study on the pattern of ammonia excretion during salinity acclimation can give an account of the pattern of nitrogen metabolism. Coupled with the data on oxygen consumption, this is expected to give a vivid picture of whether an active or passive regulation is taking place. In the present study, excretion of NH_3-N and consumption of dissolved oxygen were estimated in the two size groups of *V. cyprinoides* var. cochinensis during acclimation to different salinities. Total NPS content of the pooled adductor, foot and mantle tissues from acclimated animals were also estimated.

3.2 Rationale

Experiments were designed to study the rate of excretion of ammonia nitrogen of both size groups of *V. cyprinoides* var. cochinensis during acclimation to different

salinities. Rate of consumption of dissolved oxygen was measured simultaneously. Total NPS content of the tissues of acclimated animals was also measured to observe and compare the pattern of NPS accumulation and nitrogen metabolism under salinity stress.

3.3 Materials and Methods

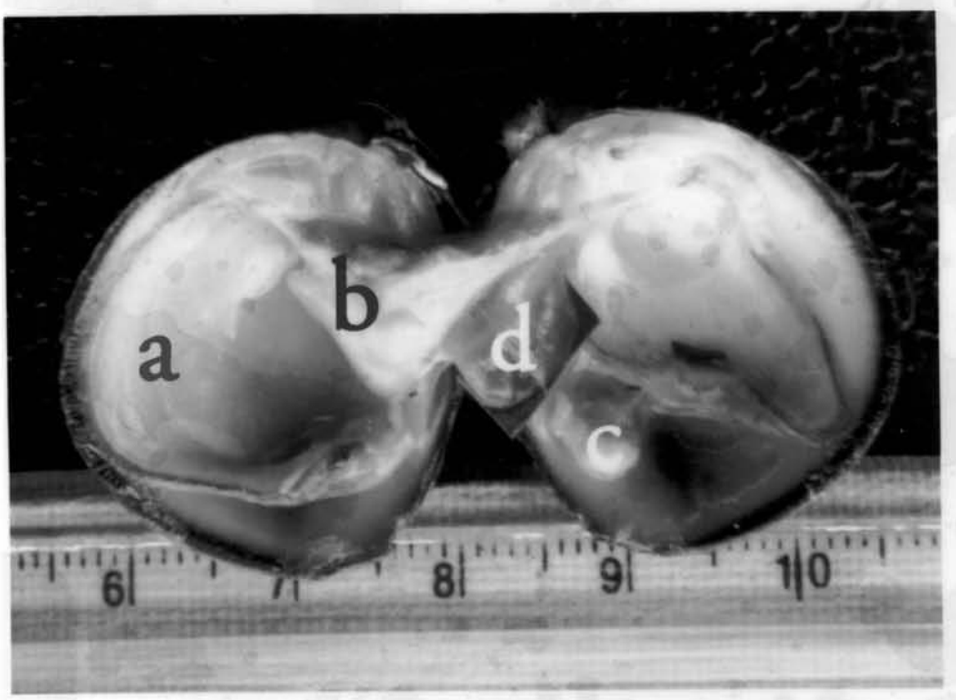
Collection and acclimatization of animals in the laboratory were done as described in section 2.2.

Total amount of Ninhydrin Positive Substances (NPS) in the tissues were estimated from the pooled mantle, adductor muscle and foot tissues excised from the animals (Plate 2) acclimatized to desired salinities for one month. The tissues were excised, blotted and weighed. They were subsequently homogenised in a mortar for seven minutes after adding a definite volume of cold 0.6M perchloric acid. The homogenate was centrifuged twice at 24 hr intervals in a high speed refrigerated centrifuge at 20,000 x g for twenty minutes to remove proteins. Samples were then neutralized. The amount of NPS in the extract was estimated by method of Yemm and Cocking (1995) with glycine as standard.

Rate of oxygen consumption and ammonia excretion were estimated for animals that were exposed to predefined salinities. To give a salinity stress, animals were transferred to water of desired salinity. Sea water of desired salinity was made by dilution of filtered sea water with deionized water. Animals were then left undisturbed for a few hours. After observing that the animals perform normal filtration, estimations for oxygen and ammonia were made for four hours at one hour intervals.

Plate 2

Shell of *V. cyprinoides* var. cochinensis



Shells opened to display the tissues

a. Mantle
b. Foot
c. Adductor
d. Gill

For determining the oxygen consumption, a respirometer devised by Mohan and Cherian (1980) was used. Oxygen was estimated by Winkler's method (Welsh and Smith, 1953). Total amount of ammonia nitrogen in the medium was estimated by phenol hypochlorite method (Solorzano, 1969). After the experiments, the soft body parts of animals were weighed in a high precision electronic balance (wet weight). The results from six separate experiments were statistically analyzed.

3.4 Results

3.4.1 *Total NPS content* :

There was an increase in total NPS content of the tissues with increase in salinity (Table 3.1, Fig. 3.1). At 0 x 10^{-3} and 5 x 10^{-3} salinity, large animals maintained a higher concentration of NPS than the small size group. At salinity 10 x 10^{-3}, tissues of small animals contained more NPS than the large size group. Even at 15 x 10^{-3} the trend was maintained, but the magnitude decreased. The difference was statistically significant only at 0 x 10^{-3} and 10 x 10^{-3} salinity. A reduction in the concentration of NPS was noticed in both the size groups when exposed to 20 x 10^{-3} salinity.

Table 3.1. Total ninhydrin positive substance content of *V. cyprinoides* var. cochinensis acclimated to various salinities.
Values expressed as µM glycine equivalents/g body wet weight of pooled mantle, foot and adductor tissues.

Salinity	Small size group			Large size group		
	Mean		SD	Mean		SD
0	0.69	±	0.05	1.41	±	0.62
5	1.38	±	0.34	2.29	±	0.98
10	4.17	±	0.01	2.23	±	0.05
15	8.32	±	0.50	7.66	±	0.33
20	6.59	±	1.32	6.84	±	0.56

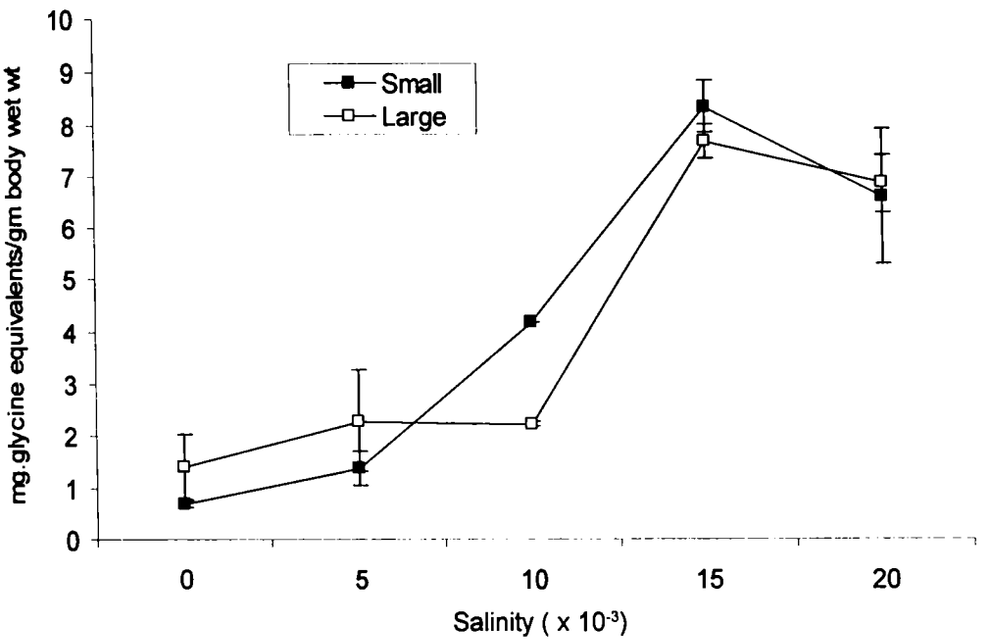


Fig 3.1: Total NPS content of pooled tissues of *V. cyprinoides* var. cochinensis exposed to various salinities

Table 3.2. Correlation matrix - between salinity, ammonia excretion, oxygen consumption and total NPS of Large size group

	salinity	ammonia	oxygen	NPS
salinity	1			
ammonia	-0.9916	1		
oxygen	0.7180	-0.7594	1	
NPS	0.9042	-0.9106	0.4491	1

Table 3.3. Correlation matrix - between salinity, ammonia excretion, oxygen consumption and total NPS of Small size group

	salinity	ammonia	oxygen	NPS
salinity	1			
ammonia	-0.9215	1		
oxygen	0.9654	-0.8750	1	
NPS	0.8777	-0.8143	0.7707	1

3.4.2 *Oxygen consumption*

The rate of oxygen consumption of the two size groups at different salinities are shown in Table 3.4, Fig. 3.2. There is a good correlation between the rate of oxygen consumption and salinity (at 90% confidence level in large size group and 99% in small size group) (Table 3.2 & 3.3). Large and small size groups showed significant difference in oxygen consumption. Large animals consumed more oxygen at 5 x 10^{-3}, 10 x 10^{-3}, 15 x 10^{-3}, and 20 x 10^{-3} where as at 0 x 10^{-3}, smaller animals consumed more oxygen than the larger ones. The rate of oxygen consumption increased with increase in salinity in both the size groups. In small animals, a sharp increase could be observed only at higher salinities, *viz.* 15 and 20 x 10^{-3} (438.88 mL and 909.88 mL O_2/g body dry wt./hr respectively).

Table 3.4. Rate of oxygen consumption of *V. cyprinoides* var. cochinensis during acclimation to various salinities (Rate is expressed as µl/g body dry weight/ hr).

Salinity	Small size group		Large size group	
	Mean	SD	Mean	SD
0	424.00	± 57.22	136.22	± 41.35
5	373.68	± 16.25	536.82	± 39.20
10	383.00	± 35.94	649.00	± 33.33
15	435.88	± 65.05	735.54	± 55.37
20	909.98	± 31.10	1027.86	± 50.12

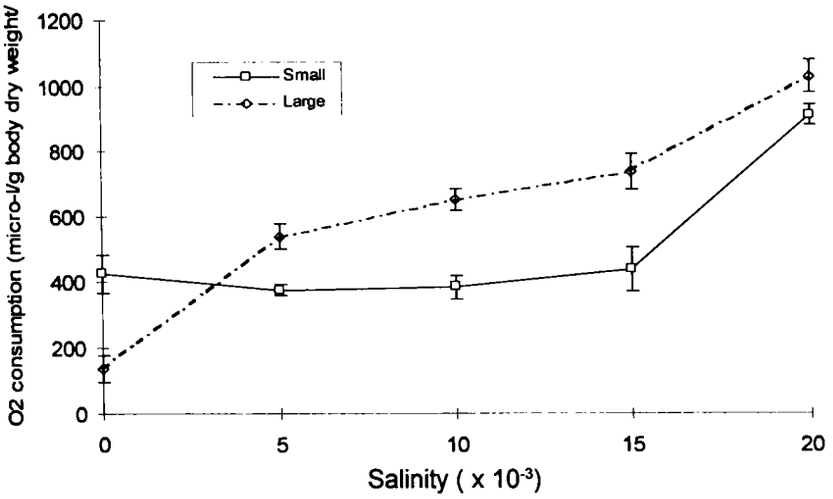


Fig 3.2. Oxygen consumption of *V. cyprinoides* var. cochinensis exposed to various salinities

3.4.3 *Ammonia excretion*

Graphical representation of the data is shown in Fig. 3.3. Rate of excretion of ammonia nitrogen decreased with increase in salinity in both the size groups (Table 3.5). The excretion rate can also be inversely correlated with the increase in total NPS in the tissues (99.9% in both the size groups) (Table 3.2 & 3.3). Small animals excreted more amount of ammonia-nitrogen at salinity 0 x 10^{-3} though no appreciable difference exist between the rate of excretion between small and large size group. Significant difference in the excretion rate between size groups was noted only at salinity 15 and 20 x 10^{-3}. At 10 x 10^{-3} salinity, a reversal in the trend could be observed, i.e. large animals excreted more NH_3-N than the small ones. Small size group showed maximum reduction in ammonia excretion as the salinity increased, from 25.05 mg NH_3-N/g body dry weight/ hr. in 0 x 10^{-3} salinity to 4.76 mg NH_3-N/g body dry weight/hr.in 20 x 10^{-3} salinity. Large size group could not adjust the system to the level as small animals had done.

Table 3.5. **Rate of excretion of NH_3-N of *V. cyprinoides* var. cochinensis during acclimation to various salinities (Rate is expressed as μg NH_3-N/g body dry weight/ hr).**

Salinity	Small size group	Large size group
	Mean SD	Mean SD
0	25.05 ± 6.10	21.66 ± 3.12
5	21.79 ± 3.56	21.05 ± 3.01
10	17.20 ± 2.13	19.90 ± 2.64
15	10.00 ± 2.00	17.25 ± 2.60
20	4.76 ± 0.27	11.61 ± 1.75

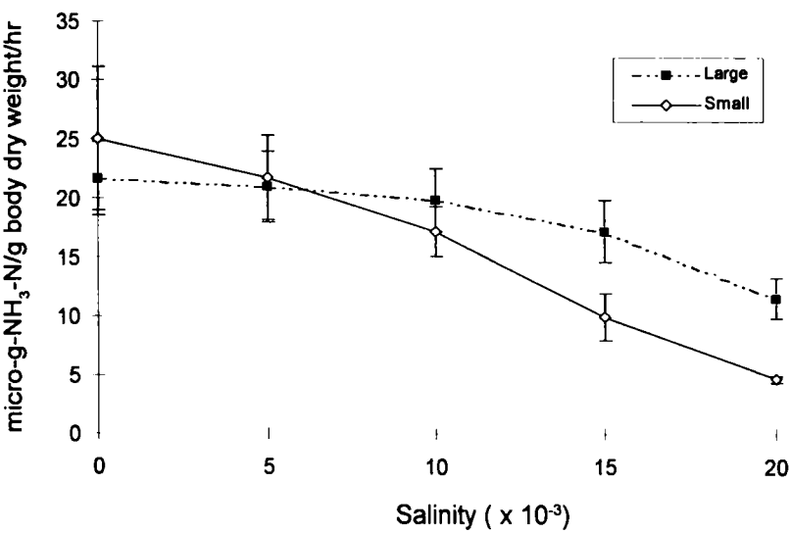


Fig 3.3 **NH_3-N excretion of *V. cyprinoides* var. cochinensis exposed to various salinities**

3.5 Discussion

Present studies revealed that the concentration of total NPS in the tissues of *V. cyprinoides* var. cochinensis increased with an increase in ambient salinity irrespective of the age group. From the results, it is evident that the nitrogenous compounds (especially the NPS) play an important role in maintaining the cell

osmolarity of *V. cyprinoides* var. cochinensis. Further experimentation is necessary to reveal the role of individual components and also the pattern of accumulation in different tissues. Previous studies have already revealed the importance of nitrogenous solutes in the intracellular osmoregulation of marine bivalves (Pierce, et al., 1992; Lange, 1963; Matsushima, et al., 1987, George and Damodaran, 1999). Tissue levels of amino acids and their derivatives vary in response to salinity in bivalves; at the same time, its blood concentration is kept low as discussed in chapter 4. Only in insects, amino acids play a role in maintaining blood osmolarity (Sutcliffe, 1963).

Experiments on the rate of oxygen consumption and ammonia excretion have indicated that the regulation of NPS pool is active during a hypo osmotic stress. A reduction in the excretion of NH_3-N suggests that the components of metabolic NH_3-N pool is recycled for the synthesis of NPS. This view is supported by Karam, et al., (1987). Whether the amino acids are synthesized in a particular tissue and mobilized or whether it is done in the respective tissues is a matter that needs further study. A decrease in total NPS at the highest salinity (*viz.* 20×10^{-3}) observed may be due to a change in choice of osmolyte. Some authors have pointed out that the animal may be switching from free amino acids to some other compounds as intracellular osmolytes for osmoregulation (Schoffeniels & Gilles, 1971). More information on cellular osmolarity is needed to make a conclusion.

Very few records are available on the metabolism and NPS content of osmoregulating estuarine animals. Physiological mechanism of active ion osmolyte regulation involves synchronized and complementary functions of a number of membrane associated ion pumps and other transport related enzymes. Initial step in ion regulation is suggested to be independent of Na^+/H^+ and

Cl^-/HCO_3^- exchange across the apical membranes of the gill (Pequeux and Gilles, 1988). This requires energy in the form of ATP, which is produced by oxidative phosphorylation from ADP, in the mitochondria. Increased oxygen consumption of gills during hyposalinity acclimation is pointed out by Engel and Eggert (1974), Mantel and Farmer (1983), Pequeux and Gilles (1988). Increased oxygen consumption and decreased rate of excretion of ammonia observed in this animal is proof of the active regulatory process. This observation is substantiated by the presence of high concentration of NPS in higher salinities. Moreover, in small animals, the rate of excretion was much lesser than that of the large size group, in small size group, there is a higher concentration of NPS at higher salinities.

From the experiments conducted so far on osmoregulation, it could be observed that there is no similar pattern in oxygen consumption during salinity adaptation among animals. The mollusc *Hydrella* sp. shows a progressive decrease in oxygen consumption when adapted to hyperosmotic media (Hiscock, 1953). Oxygen consumption of *Hydrobia ulvae* was higher at 10% seawater and that of *Mytilus edulis* decreased in both diluted and concentrated media (Bouxin, 1931). Increase in the rate of oxygen consumption by marine animals adapted to low salinity is reported by Pequeux and Gilles (1988). This is attributed to the active transport of ions by gills. Allen (1961) reported a decrease in glycogen content in *Rangea cuneata* exposed to a concentrated media indicating the expenditure of energy to ensure osmoregulation. An increase in oxygen consumption rate can be expected when metabolic rate increases. Allen (1961) reported that when *Rangea cuneata* is placed in concentrated media, there occurs a decrease in muscle glycogen content indicative of the higher expense of energy for metabolism. In *V. cyprinoides* var. cochinensis, the increase in oxygen consumption noticed can be due to increased metabolic rate.

Simultaneously conducted estimations of oxygen consumption, ammonia excretion and total NPS content of tissues of *Villorita cyprinoides* var. cochinensis shows the inter-relationship between these three. The animal inhabits the fresh water ends of estuaries. Estuarine species have broader physiological tolerance than the marine or fresh water species (Pierce, *et. al.*, 1992). Previous reports indicate that the animal is capable of tolerating wide ranges of salinity under laboratory conditions (0 x 10^{-3}-27 x 10^{-3}) (Nair and Shynamma, 1975). Bivalves are not known to regulate the blood osmotic pressure substantially when exposed to osmotic stress. Since they are incapable of extracellular regulation, they make intracellular osmotic adjustments to tide over transient and constant salinity changes. More than 50% of the intracellular osmolytes are composed of amino acids and other NPS (Bricteux, *et. al.*, 1964, Schoffeniels & Gilles, 1971). The NPS content of the tissues of *Villorita* increased with increase in salinity. Previous studies have revealed the importance of nitrogenous solutes in the intracellular osmoregulation of marine bivalves (Lange, 1963, Matsushima, *et. al.*, 1987, Pierce, *et. al.*, 1992, George, 1993). It is evident from the present results that the nitrogenous compounds (especially the NPS) play an important role in maintaining the cell osmolarity of this freshwater-end loving animal also. A decrease in the total NPS content is noticed in salinities higher than 15 x 10^{3}. Eventhough Nair and Shynamma (1975) reported that the animal can tolerate up to 27 x 10^{-3} salinity under experimental conditions and in natural habitats, these animals seldom form and maintain a population beyond 15 x 10^{-3}. The decrease in total NPS content after 15 x 10^{-3} indicate the possibility of the cellular machinery switching over to some other osmolytes other than NPS. This aspect require further probing.

Amino acids and other derivatives for osmoconformation are generated in the cytoplasm either by amination of ketoacids or from other metabolic sources, and

this require manipulations in the metabolic pathways. There exists a degradation-synthesis balance between organic compounds in the regulation of osmotic effector pool (Karam, *et. al.*, 1987). Metabolic regulation of NPS pool in response to salinity variations of external medium involves changes in glycolysis, TCA cycle reactions, transaminases and metabolite shuttling between cellular compartments (Emerson, 1969). Higher rate of oxygen consumption is observed during adaptation to higher salinity. The metabolic machinery of the cellular system is accelerated when a stress is imparted, and this accounts for the higher rate of oxygen consumption. According to categories of metabolic responses referred to by Kinne (1971) for marine individuals, it can be seen that many organisms have a metabolic rate which varies directly with changing salinities. The decreased excretion rate of ammonia may be due to the decreased catabolism of amino compounds or enhanced removal of NH_4^+ from the blood for the synthesis of amino acids and their derivatives for isosmotic regulation as suggested by Karam *et. al.* (1987). A reduction in NH_4^+ excretion with an increase in salinity is reported by Lemos *et. al.* (2001) in the crustacean *Farfantepenaeus paulensis*. The isosmotic intracellular regulation of many euryhaline molluscs is paralleled by the modification in ammonia excretion. Emerson (1969) observed a significant increase in the excretion of NH_3-N in *Macoma inconspicua* adapted to 50% sea water. Conversely, *Villorita,* when adapted to higher salinity showed a decrease in NH_3-N excretion. This observation fortifies the hypothesis of the existence of a degradation-synthesis balance for intracellular osmoregulation in this animal. The lesser rate of excretion of NH_3-N is well correlated with the increased NPS in the tissues. However, the cellular regulation mechanisms of the osmolytes in

euryhaline species is not simple and is yet to be clarified (Somero and Bowlus, 1983). The qualitative composition of the NPS pool of different tissues is to be focused on to gain more information on the regulatory mechanisms.

Chapter 4

INTRACELLULAR-FLUID ISOSMOTIC REGULATION

4.1 Introduction

Capability of bivalves to regulate ionic concentration of extracellular fluid is limited, and is confined to extremes of variations in salinities. They tend to be osmoconformers than regulators in most of the cases. In the absence of a physiological mechanism of regulation, it is necessary for the organism to develop some alternate method to survive in the estuarine environment. The animal overcomes abrupt salinity changes by behavioural mechanisms. Such mechanisms include shell closure, secretion of mucus, burrowing and withdrawal of soft body parts (Kinne, 1971). This can help the animal only for a short period and if the altered salinity condition persists for a prolonged period, the animal has to switch to some regulatory mechanisms, or succumb to the changes that it cannot tolerate. Behavioural mechanisms are often used as an interim measure for gradual internal adjustments. Some species, in addition to their behavioural responses, are found to regulate their blood inorganic ions (Davenport, 1979b, Deaton, 1981, Burton, 1983, Deaton *et al.*, 1989, Salomao and Lunetta, 1989). But this capability is very limited and is not relied upon by many of the bivalves. Intracellular regulation of osmolytes is found to be the major mechanism which helps the bivalves to maintain homeostasis. Even though the idea of intracellular

osmoregulation was not accepted by earlier physiologists, recent studies have established beyond doubt the capability of maintaining isosmoticity with ambient medium in many animals. Osmotic equilibrium between cell and extracellular fluid minimizes the osmotic transfer of water across the cell membrane. Thus the cell volume is regulated. Regulation of intracellular osmolytes is the underlying mechanism of salinity tolerance of euryhaline bivalve molluscs (Lange, 1963, Schoffeniels and Gilles, 1972, Deaton and Greenberg, 1991). The body fluids and tissues of most marine bivalve molluscs are isosmotic with the surrounding seawater, and the concentration of mineral salts of the extracellular fluid is normally close to that of the environment (Prosser, 1973). But, the amino acids/derivatives are recruited to the internal organic osmolyte repository, for cell volume and osmotic regulation. Concentration of the intracellular inorganic osmolytes like Na^+, K^+ and Cl^- and also the extracellular osmolytes like urea (Elasmobranchs) cannot be altered in the cells because they disrupt the ionic equilibrium and compounds like urea can interfere with normal protein function. Amino acids are the commonly found intracellular osmolyte since they are not found to disrupt the metabolic functions of the cell.

4.2 Amino acid composition

Osmoconformers, especially bivalves rely on intracellular level adjustments for maintaining osmotic equilibrium. The role of both organic and inorganic ions in intracellular osmoregulation (Allen, 1961, Schoffeniels and Gilles, 1972) have been studied extensively for years. Now it is established that all types of cells use both inorganic and organic osmolytes to some degree to regulate cell water in the face of external osmotic variation (Pierce & Politis, 1990). Molluscs usually modify the concentration of intracellular small organic molecules to maintain the

cellular volume within physiological limits (Pierce, 1982; Jdhay & Lomte, 1990). Molecules that are most modified are the ninhydrin positive substances (NPS), which are composed mainly of free amino acids. Minor components of the NPS pool include methyl amines, trimethylamine oxide, betaines, sarcosine and to a lesser extent ammonia (Ivanovici, *et al.,* 1981; Pierce, *et al.*, 1992). Among molluscs, the major intracellular osmolytes reported are free amino acids (George & Damodaran, 1999). Depending upon the variations in external salinity, there is a concordant change in the concentration of the intracellular osmolytes also. Amino acids for adaptation to hyper osmotic medium are derived from various metabolic processes (chapter 2). Cells of some extremely salt tolerant species often use another type of low molecular weight organic osmolytes like glycine betaine. During adaptation to a lower salinity, the intracellular osmolytes are removed from the intracellular milieu by passive diffusion out of the cell, synthesis of large molecular weight compounds, catabolism and oxidation. Adjustments in the intracellular concentration of amino acids may occur either by passive osmosis or by intervention of an active phenomenon which regulates the amount of the intracellular osmotic effectors (Somero & Bowlus, 1983). Then the changes in cellular volume due to water exchange would be avoided to a certain degree. The speed of acclimation to hyperosmotic and hypo-osmotic environment varies among animals (Pierce, 1971). This indicates the presence of different mechanisms in hyperosmotic and hypo-osmotic regulatory processes. It is reported that only euryhaline species have the capability of volume regulation and stenohaline forms lack this ability.

Cell-volume regulation appears to play an important part in the ecology of euryhaline species. Since the cells are observed to be in osmotic equilibrium with the surrounding medium, it must be assumed that in these species the

intracellular water content is controlled by a mechanism which regulates the intracellular osmolyte concentration. There is a large body of literature demonstrating that cells of most tissues studied to date can invoke a regulatory volume decrease (RVD) or regulatory volume increase (RVI) in response to osmotic stress (Chamberlin and Strange, 1989). By the regulatory volume change, the functional consequences of a change in cell volume due to osmolarity change are mitigated to a certain degree. Unlike mammalian tissues that are bathed in body fluid of more or less constant osmolarity, cells of estuarine bivalves are exposed to large and frequent fluctuations in osmolarity of hemolymph resulting from salinity change. Under this situation, as the body fluid fluctuates in osmolarity, it is essential for the animal to prevent this from occurring in the cellular level. This is achieved through intracellular regulation. It is observed that the amino acid content is higher in the muscle of seawater species than in freshwater species. Betaine and taurine, though found in large amounts in seawater species are not found in freshwater forms (Simpson, *et. al.,* 1959). Studies conducted by Pierce, *et al.* (1992) on Atlantic and Bay conspecific oysters (*Crassostrea virginica*) have shown that conspecific Bay oysters which are stenohaline have a lower survival potential under varied osmolarities. This is attributed to the absence of a major intracellular osmolyte, glycine betaine in these animals. The fundamental enzyme systems are also different in the two species. Choline, a precursor of glycine betaine is reported to be the limiting factor in Bay oysters (Pierce, *et al.,* 1997). Conspecific Bay oysters lacked the capability to synthesise/ regulate glycine betaine. From this observation it can be attributed that even among single species, there exists habitat-specific variation in the capability of osmoregulation.

Considerable work has been done in intracellular osmoregulation of marine molluscs. Most of the studies dealing with the isosmotic intracellular regulation have been performed by submitting the animals to a hypoosmotic stress. Very few experiments have been performed on the adaptation of euryhaline mollusc to a hyper osmotic medium. A freshwater-end loving mollusc, in nature experiences increase in salinity of the ambient medium. There are few experiments designed to address this. Since it is established that the mechanism varies from province to province, animal to animal, and even in the same animal from tissue to tissue, it is worth experimenting with the intracellular osmoregulatory tactics of an estuarine mollusc. Present experiments were designed to understand the strategies of intracellular osmoregulation, particularly the variation of intracellular osmolytes under osmotic stress in *V. cyprinoides*. Qualitative and quantitative variations of amino acids during a hyperosmotic stress is studied in the adductor, foot and mantle tissues of the two size groups.

4.3 Materials and Methods

Experimental animals were obtained from Poochakal, near Cherthala during the month of April. Acclimation in the laboratory was done as discussed in section 2.2. The animals were fed with blue-green algae, *Synechocystis salina* Wislouch (*ad libitum*) during the acclimation period.

Experimental tanks were slightly smaller FRP tanks with clean sand and seawater of desired salinity. Seawater of desired salinity was made by diluting 100% seawater with deionized water. Initially, animals in the acclimation tanks were transferred into experimental tanks with seawater of salinity 3×10^{-3}. A rise in salinity of 5×10^{-3} was given in every three days till the desired salinity is reached. This was to avoid a sudden osmotic shock. All parameters like dissolved oxygen,

temperature, pH and rate of feeding were same in all the tanks and the only variable parameter was salinity. The animals were exposed to salinities 5×10^{-3}, 10×10^{-3}, 15×10^{-3} and 20×10^{-3} for one month before sacrificing them for experimental purpose.

After giving hyper osmotic stress, acclimated animals (both small and large size groups) were taken out of water. Foot, mantle and adductor muscle tissues were then dissected out. Dissected tissues from 10-15 animals were blotted and weighed (wet weight) and pooled for homogenization. Tissues were then homogenized in 0.6 M perchloric acid, for 7 min. The homogenate was centrifuged in a refrigerated centrifuge (temp. 0° C) at 20,000 x g for 30 min. Supernatants were saved and were used for chromatographic separation and quantification of amino acids.

Separation of amino acids was done by ion-exchange chromatography using strong cation exchange resin, Dowex-50 (50 x 8% DVB) 200-400 mesh form by methods of Moore and Stein (1951) and Jayaraman (1981). Commercial resin was suspended in 4 N HCl (12.5 gL^{-1}) for 15 min. The suspension was then filtered through Whatman No. 1 filter paper with distilled water till a neutral pH was attained. Then the resin was suspended in distilled water and was allowed to settle. Lighter particles were decanted. Then the resin was again suspended in 0.2 N citrate buffer (pH 2.0) for equilibration. After filtering off the buffer, a slurry was made and was poured into the column (60 cm h x 0.9 cm dia), which was enclosed in a thermostatic jacket (Plate 3). Care was taken not to trap air bubbles while pouring. Slurry was poured in installments to obtain 20 cm beds each time. Citrate buffer (about 200 ml) was allowed to pass through the freshly set up column. The ion exchange capacity of the column was taken into account

Plate 3: Setup for the semiautomated separation of amino acids

a. Reservoir
b. Jacketed column with Dowex-50 resin
c. Peristaltic pump
d. Fraction collector
e. Thermostatic circulation bath

while loading samples. The pH of the samples was adjusted to 1.0. Definite volumes of samples were introduced into the surface of the column without disturbing the surface, using a pipette. The flow was turned on till the level of the sample reached the surface of the column. At this point the amino acids were bound to the column. About 10 ml of buffer was added to the top of the column and elution was started using buffer of pH 3.1. Initial elution was carried out by citrate buffer, pH 3.1, 0.2 N Na^+ (Moore and Stein, 1951), at temperature 30° C. After allowing about 300 ml of buffer to pass through the column, the temperature was elevated to 50° C. Final elution was done with buffer of pH 5.1. The technique was standardized to get full separation of the targeted amino acids.

Fractions of 4 ml were collected in 20 ml serially numbered test tubes. An automatic fraction collector (LKB 2112 Rediarc) was used for the purpose. Definite volumes of the fractions were analyzed quantitatively for amino acids using modified fluorescence technique with O-phthalaldehyde (Roth, 1971). Secondary amino acids were read after reaction with sodium hypochlorite. For this, definite volumes of eluted fractions were pipetted out into serially numbered test tubes. 1ml of solution A (see below) was added to the tubes. After 3 minutes, 1ml of solution B (O-pthalaldehyde solution) was added and the fluorescence was read in a Hitachi (model F-3010) fluorescence spectrophotometer at excitation wavelength 335nm and emission wavelength 425nm.

Composition of :

Boric-Carbonic acid buffer

1. 0.384 M Sodium carbonate
2. 0.216 M Boric acid
3. 0.108 M Potassium sulphate – total 3 L was prepared, pH≈10.

Solution A:

1. 500ml of Boric-Carbonic acid buffer.
2. 0.2ml of sodium hypochlorite, mixed well and filtered.

Solution B:

1. 400mg *O*-pthalaldehyde.
2. 7ml of Ethanol.
3. 1ml 2-mercaptoethanol, made up to 500ml with boric acid buffer solution.

A solution with known concentration of amino acids was also run. A graph was plotted with fluorescence in the y-axis and tube number in the x-axis. Area of individual peaks was calculated (internal standard technique) to quantify amino acids. Three samples were analyzed qualitatively and quantitatively for amino acid content in an amino acid analyzer (Shimadzu). Results were compared with those obtained by semi-automated separation.

4.4 Results

Variation of total intracellular NPS content in response to changes in salinity was discussed in detail in chapter 3. In this chapter, an attempt has been made to study the qualitative and quantitative variations of NPS during a hyperosmotic stress in the major tissues *viz.* adductor, foot and mantle,. Changes in the amino acid content in different age groups during the osmotic stress are also studied. Qualitative and quantitative details of amino acids separated from various tissues are given in Tables 4.1-4.6, Fig. 4.1-4.6. Concentration of individual amino acids is expressed as μmoles/gm wet tissue weight.

From the data obtained by HPLC analysis, the major amino acids which appear to have the role as intracellular osmolytes are alanine, glycine, histidine, arginine, glutamic acid and aspartic acid in the order of their importance (Table 4.7 & 4.8). Hence a detailed study on the quantitative variation of these amino acids has

been carried out. The presence of taurine in different salinities in the different tissues was also checked, since it was reported to be the major intracellular osmolyte in marine bivalves. Taurine was not found in detectable levels in any of the tissues in any salinities.

Among all the tissues in both the size groups studied, foot tissue of small size group at salinity 15 x 10^{-3} showed the highest content of total amino acids and the minimum was observed in the mantle tissue of small size group at salinity 5 x 10^{-3}. When large size group is considered, the maximum content was observed in the adductor muscle at salinity 15 x 10^{-3} and the minimum in mantle tissue at 5 x 10^{-3}.

Comparison between the different tissues of both the size groups showed that the adductor muscle of large size group had a higher content of amino acids than the adductor muscle of small size group. The foot and mantle of small size group had higher amino acid content when compared to the respective tissues of the large size group.

A preliminary scrutiny of the data showed that alanine and glycine were the dominant and the most variable amino acids compared to the other amino acids separated. Histidine was preferred next to alanine and glycine. Aspartic acid was the least preferred in all the tissues of both the size groups. When the average among different salinities of the two size groups was compared, concentration of aspartic acid was higher in the small size group in all the three tissues.

Taking into account all the salinities experimented, when the adductor muscle of small size group is considered, alanine was the predominant amino acid, followed

by glycine, histidine, glutamic acid, and arginine. Aspartic acid was found only in minimal quantities. In the large size group also, alanine was the predominant amino acid in this tissue followed by glycine, histidine, glutamic acid, arginine and aspartic acid.

The order of preference in the foot tissue of small size group was alanine, glycine, histidine, followed by glutamic acid and arginine in equal amounts. Aspartic acid was found only in minimal quantities. In the large size group, glycine was predominant, followed by alanine, histidine, glutamic acid, arginine and aspartic acid in that order.

Mantle tissue of small size group also showed high concentration of alanine followed by glycine, histidine, glutamic acid, arginine and aspartic acid. In large size group, alanine is replaced by glycine as the predominant osmolyte. Glycine was followed by histidine, alanine, arginine, glutamic acid and aspartic acid in the order of preference.

In general, when the salinity increased from 0×10^{-3} to 10×10^{-3}, there was no appreciable increase in the total quantity of amino acids studied in all the three tissues. An exception to this observation was the adductor muscle of large sized animals where there was a significant increase in amino acid content as the salinity increased from 0×10^{-3} to 5×10^{-3}. From salinitiy 5×10^{-3} to 10×10^{-3} total amino acid concentration increased except in the foot muscle of large size group where a marginal decrease in concentration was observed. When the salinity increased from 10×10^{-3} to 15×10^{-3}, all the three tissues of both the size groups showed an increasing trend in the concentration of free amino acids. This trend was reversed in all the tissues of both the size groups when the salinity increased

from 15 x 10^{-3} to 20 x 10^{-3}.

Quantitative variation of individual amino acids in relation to salinity in the two size groups and the differences between tissues are discussed below, in the order of dominance of individual amino acids in respective tissues.

4.4.1 *Adductor muscle*

Alanine showed an increasing trend from 0 x 10^{-3} to 20 x 10^{-3} in the adductor muscle of small size group. The concentration of this amino acid did not show a decrease at 20 x 10^{-3} in small sized animals whereas in the adductor muscle of large size group, its concentration increased with a change in salinity from 10 x 10^{-3} to 15 x 10^{-3} and the concentration decreased as the salinity increased further from 15 x 10^{-3} to 20 x 10^{-3}. From salinities 0 x 10^{-3} to 10 x 10^{-3}, the variation of alanine content in this tissue of large size group did not follow a steady increasing trend with the increase in salinity. Average concentration of this amino

Table 4.1. Concentration of amino acids in adductor muscle of *V. cyprinoides* var. cochinensis (Small) acclimated to different salinities.
Values expressed as µM per gram wet weight of tissue.

Free Amino Acids	0 x 10^{-3}		5 x 10^{-3}		10 x 10^{-3}		15 x 10^{-3}		20 x 10^{-3}	
	Conc	%	Conc	%	Conc	%	Conc	%	Conc	%
Asp	0.06	0.09	0.19	0.40	0.42	0.66	0.63	0.39	1.93	1.85
Glu	11.83	18.26	0.36	0.73	0.38	0.60	0.56	0.35	6.48	6.22
Ala	11.85	18.29	16.18	33.18	20.51	32.35	63.15	39.42	79.10	75.91
Gly	22.87	35.31	18.77	38.48	35.46	55.94	63.16	39.42	10.04	9.64
His	5.41	8.36	12.94	26.53	6.22	9.81	32.00	19.97	2.62	2.52
Arg	12.75	19.68	0.33	0.68	0.41	0.65	0.71	0.45	4.02	3.86
Total	64.77		48.77		63.39		160.21		104.20	

Values are the mean of three observations
Each observation is made from a sample of 8-12 animals pooled together.

Table 4.2. Concentration of amino acids in adductor muscle of *V. cyprinoides* var. cochinensis (Large) acclimated to different salinities.
Values expressed as μM per gram wet weight of tissue.

Free Amino Acids	0×10^{-3}		5×10^{-3}		10×10^{-3}		15×10^{-3}		20×10^{-3}	
	Conc	%	Conc	%	Conc	%	Conc	%	Conc	%
Asp	0.45	2.02	0.36	0.31	0.32	0.22	0.56	0.35	0.43	0.28
Glu	1.04	4.69	1.08	0.94	0.61	1.53	3.88	0.67	3.01	1.97
Ala	7.73	34.85	55.65	48.61	16.62	65.71	166.60	18.13	63.88	41.82
Gly	0.97	4.37	31.33	27.37	64.98	19.20	48.67	70.88	31.61	20.69
His	11.51	51.87	25.65	22.41	8.82	12.73	32.28	9.62	53.28	34.88
Arg	0.49	2.20	0.40	0.35	0.32	0.60	1.53	0.35	0.54	0.35
Total	22.18		114.47		91.68		253.52		152.76	

Values are the mean of three observations.
Each observation is made from a sample of 8-12 animals pooled together.

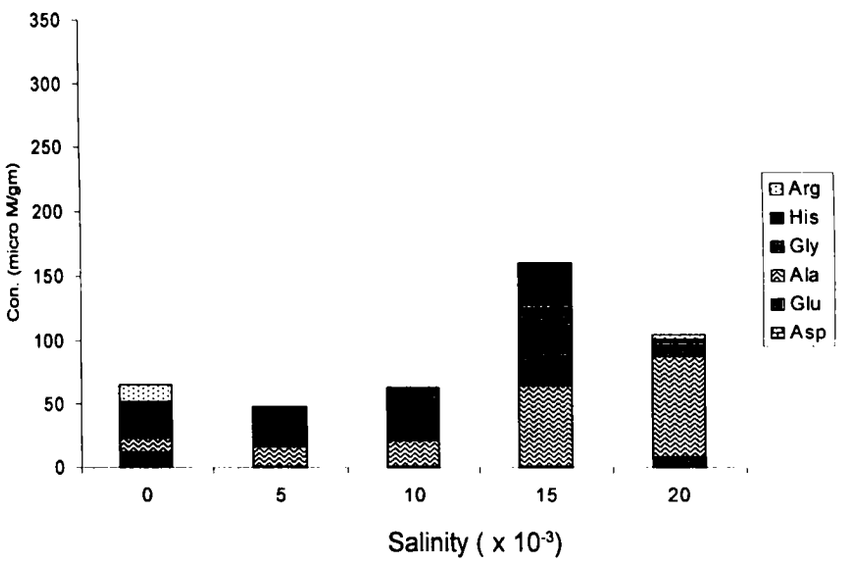


Fig. 4.1 Concentration of amino acids in the adductor muscle of small size group

acid was 38.2mM/g in small size group and 62.1mM/g in large group (Table 4.8).

Glycine content was high in the large size group (35.5mM/g) compared to small (30.1mM/g) in this tissue. In both the size groups, the amino acid increased with an increase in salinity from 0×10^{-3} to 15×10^{-3} salinity and at 20×10^{-3} it showed

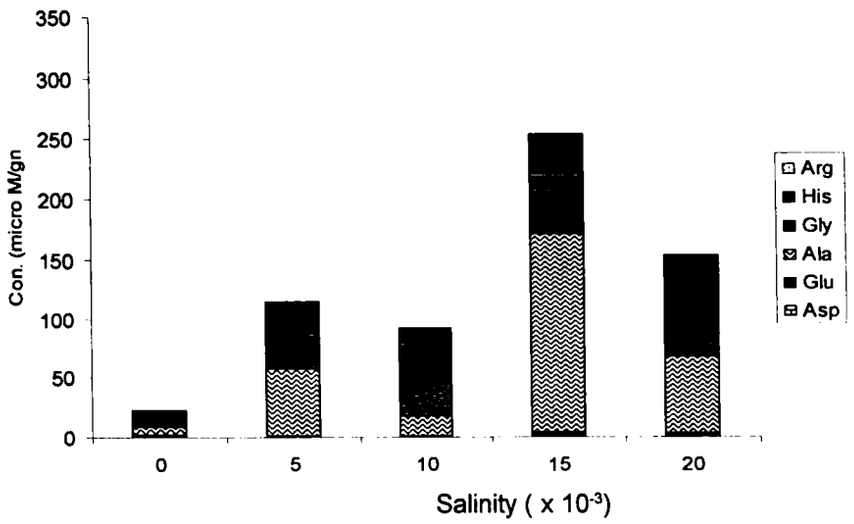


Fig 4.2 Concentration of amino acids in the adductor muscle of large size group

a decline in its concentration.

Histidine was the third predominant amino acid in the NPS pool of this tissue of both the size groups. Concentration of histidine increased from salinity 0 x 10^{-3} to 5 x 10^{-3} and, from 5 x 10^{-3} to 10 x 10^{-3} a decrease was noticed in the small size group, whereas a steady increase in concentration with an increase in salinity from 0 x 10^{-3} to 10 x 10^{-3} and from 15 x 10^{-3} to 20 x 10^{-3} was observed in the large size group. Decrease in concentration was observed as the salinity changed from 10 x 10^{-3} to 15 x 10^{-3} in this group. In the small size group, a marked increase in concentration was observed when the salinity increased from 10 x 10^{-3} to 15 x 10^{-3}. Its concentration decreased as the salinity increased further from 15 x 10^{-3} to 20 x 10^{-3} in small size group.

Glutamic acid ranked fourth in the adductor muscle of small size group, at the same time it was the fifth preferred among the amino acids studied in the same tissue of large size group. Glutamic acid content in the small size group showed a decrease as the salinity increased from 0 x 10^{-3} to 5 x 10^{-3}. When the salinity

increased from 5 x 10^{-3} to 15 x 10^{-3}, the content did not show appreciable variation. This was followed by a further increase in concentration from 15 x 10^{-3} to 20 x 10^{-3}. Unlike the small size group, large, showed a steady increase in concentration of glutamic acid as the salinity increased from 0 x 10^{-3} to 10 x 10^{-3} and from 10 x 10^{-3} to 20 x 10^{-3}, there was a decrease.

Arginine content was low in the adductor tissue of small size group compared to other amino acids except in salinity 0 x 10^{-3}. At 0 x 10^{-3} salinity, the arginine concentration was 12.8 mmoles/gm, and showed a decrease at 5 x 10^{-3}. As the salinity increased from 5 x 10^{-3} to 20 x 10^{-3} a steady increase in arginine concentration was noticed in this size group and this change is more conspicuous when the salinity changed from 15 x 10^{-3} to 20 x 10^{-3} salinity. Concentration was less and the variation with salinity was not prominent in the large size group.

Aspartic acid was of least importance in the adductor tissues of both the size groups. Appreciable quantity of this amino acid could only be found at 20 x 10^{-3} salinity in the small size group. Variation in concentration was marginal as the salinity changed in both the size groups.

4.4.2 *Foot*

It can be seen from the Table 4.8 that alanine is the predominant osmolyte for intracellular adjustments for the foot tissue of small size group (84.0mM/g). If an average is worked out (Table 4.7) for different salinities for this tissue of small size group, it accounts for 54.2% (for large size group the average is 37.5%). Concentration of alanine in the small size group increased with an increase in salinity from 0 x 10^{-3} to 15 x 10^{-3} and beyond 15 x 10^{-3} showed a decrease. The increase which was noticed in the small size group from 0 x 10^{-3} to 5 x 10^{-3} was not reflected in the large size group. In the large size group, from 0 x 10^{-3} to

Table 4.7 **Average percentage composition of amino acids among salinities in the different tissues of the two size groups of *V. cyprinoides* var. cochinensis**

Free Amino Acids	Small size group			Large size group		
	Adductor	Foot	Mantle	Adductor	Foot	Mantle
Asp	0.7	0.9	0.8	0.6	0.9	0.9
Glu	5.2	1.2	1.4	2.0	2.4	3.3
Ala	39.8	54.2	63.4	41.8	37.5	22.7
Gly	35.8	17.8	17.9	28.5	43.8	42.1
His	13.4	24.9	14.9	26.3	12.4	17.8
Arg	5.1	1.0	1.6	0.8	3.0	7.6

Table 4.8 **Average concentration of amino acids among salinities in the different tissues of the two size groups of *V. cyprinoides* var. cochinensis**
Values expressed as μM per gram wet weight of tissue.

Free Amino Acids	Small size group			Large size group		
	Adductor	Foot	Mantle	Adductor	Foot	Mantle
Asp	0.6	0.9	0.4	0.4	0.5	0.3
Glu	3.9	1.2	2.0	1.9	2.4	2.9
Ala	38.2	84.0	86.9	62.1	25.1	6.9
Gly	30.1	41.7	32.5	35.5	45.4	33.5
His	11.8	31.4	17.1	26.3	8.7	7.9
Arg	3.6	1.2	0.5	0.7	1.5	4.4

5 x 10^{-3}, the content showed a decrease. In this size group, the increase was noticed when the salinity increased from 5 x 10^{-3} to 15 x 10^{-3}, and again as the salinity varied from 15 x 10^{-3} to 20 x 10^{-3}, there was a decrease in concentration of this amino acid.

It appears that a variation in preference of amino acid exists in the foot tissue of large size group. Glycine was the major amino acid of choice for large size group. The average contribution of glycine to the total amino acid studied in all the salinities is 43.8%. At the same time, for small size group, the average contribution of this amino acid was 17.8%. The contribution increased from 4% at 0 x 10^{-3} to 63% at 20 x 10^{-3} salinity (Table 4.7) in the large size group.

Table 4.3. Concentration of amino acids in foot tissue of *V. cyprinoides* var. cochinensis (Small) acclimated to different salinities.
Values expressed as μM per gram wet weight of tissue.

Free Amino Acids	0×10^{-3}		5×10^{-3}		10×10^{-3}		15×10^{-3}		20×10^{-3}	
	Conc	%	Conc	%	Conc	%	Conc	%	Conc	%
Asp	0.71	1.19	1.52	2.13	0.53	0.27	1.41	0.47	0.54	0.31
Glu	1.09	1.82	1.90	2.66	1.85	0.94	0.84	0.28	0.46	0.27
Ala	32.07	53.55	42.52	59.58	103.50	52.49	141.27	46.78	100.63	58.47
Gly	1.33	2.22	2.31	3.24	64.53	32.72	122.59	40.60	17.83	10.36
His	23.69	39.56	22.35	31.32	26.15	13.26	34.99	11.59	49.69	28.87
Arg	1.00	1.66	0.76	1.06	0.63	0.32	0.87	0.29	2.96	1.72
Total	59.89		71.37		197.19		301.96		172.12	

Values are the mean of three observations.
Each observation is made from a sample of 8-12 animals pooled together.

Table 4.4. Concentration of amino acids in foot tissue of *V. cyprinoides* var. cochinensis (Large) acclimated to different salinities.
Values expressed as μM per gram wet weight of tissue.

Free Amino Acids	0×10^{-3}		5×10^{-3}		10×10^{-3}		15×10^{-3}		20×10^{-3}	
	Conc	%	Conc	%	Conc	%	Conc	%	Conc	%
Asp	0.63	1.69	0.36	1.38	0.43	0.49	0.60	0.42	0.53	0.42
Glu	0.77	2.05	0.34	1.29	0.99	1.12	1.16	0.83	8.65	6.8
Ala	28.12	75.16	9.59	36.82	26.41	30.01	39.78	28.32	21.47	17.07
Gly	1.50	4.01	8.21	31.50	52.31	59.43	85.43	60.81	79.68	63.36
His	5.32	14.23	5.57	21.39	4.36	4.95	13.07	9.30	15.02	11.95
Arg	1.07	2.87	1.98	7.62	3.52	4.00	0.46	0.32	0.40	0.32
Total	37.42		26.05		88.03		140.49		125.76	

Concentration showed an increasing trend from 0×10^{-3} to 15×10^{-3} and a marginal decrease was observed at 20×10^{-3} in large size group (Table 4.8). In small size group, glycine was of second preference and there was a steady increase in concentration of this amino acid from 0×10^{-3} to 15×10^{-3} and the increase was

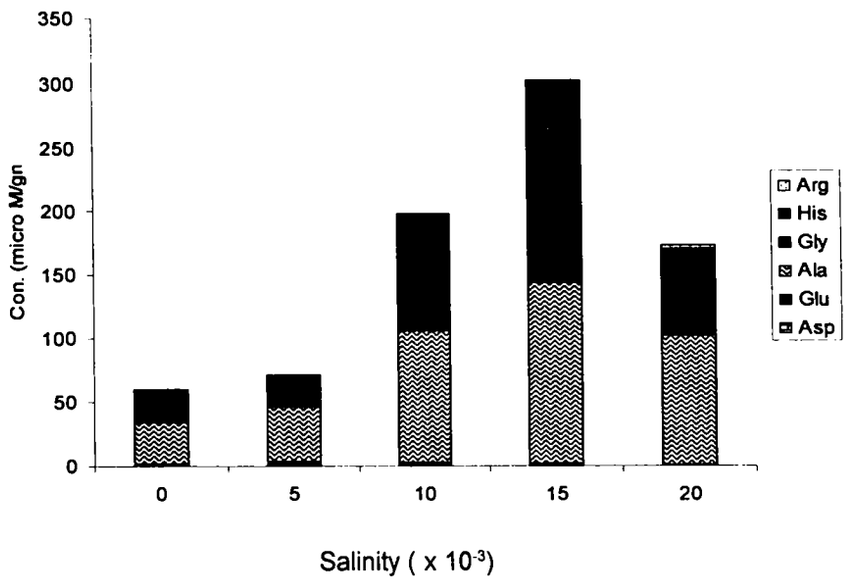


Fig 4.3 Concentration of amino acids in the foot of small size group

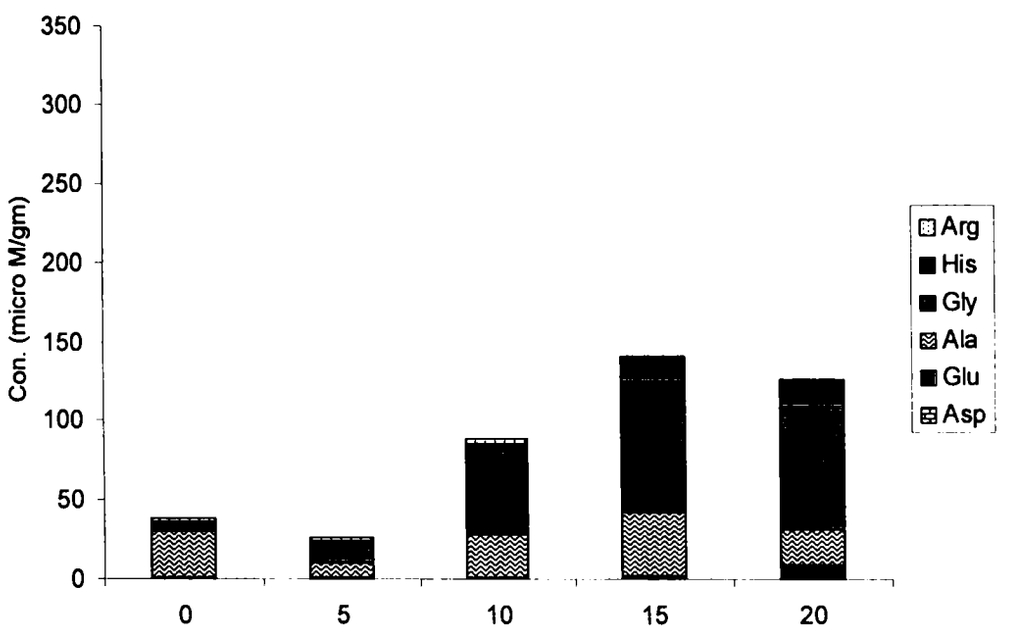


Fig 4.4: Concentration of amino acids in the foot of large size group

prominent from 5 x 10^{-3} to 10 x 10^{-3}. Concentration decreased considerably as the salinity increased further from 15 x 10^{-3} to 20 x 10^{-3} in this group.

Considerable variation of histidine could not be observed as the salinity varied from 0 x 10^{-3} to 10 x 10^{-3} in the foot tissue of both the size groups. But a steady increase in concentration could be observed as the ambient salinity increased from 10 x 10^{-3} to 20 x 10^{-3} in both small and large size groups. Percentage contribution of histidine to the NPS pool of the foot tissue of small size group was higher (average = 24.9%) when compared to that of large size group (average = 12.4%). On an average between salinities, histidine content in this tissue was higher in small size group compared to large.

Arginine content also was higher in the small size group compared to large. Arginine was preferred next to histidine (fourth) in the small size group and next to glutamic acid (fifth) in the large group. Concentration of arginine showed a slight decrease in the foot tissue of small size group from salinity 0 x 10^{-3} to 10 x 10^{-3} and the concentration increased from 15 x 10^{-3} to 20 x 10^{-3}. At 20 x 10^{-3} the concentration was considerably higher compared to that in other salinities (Table 4.3). In large size group, this amino acid was preferred next to glutamic acid. The concentration of arginine increased steadily from 0 x 10^{-3} to 10 x 10^{-3} and from 10 x 10^{-3} to 20 x 10^{-3} the concentration of this amino acid showed a decline.

Glutamic acid and arginine were present in almost equal amounts in the small size group, whereas in the large size group glutamic acid was more abundant. The concentration of glutamic acid in the NPS pool of the foot tissue of small size group increased during a change in salinity from 0 x 10^{-3} to 5 x 10^{-3}, from 5 x 10^{-3} to 10 x 10^{-3}, the concentration remained constant and further increased from salinity 10 x 10^{-3} to 20 x 10^{-3} (Table. 4.3). In the large size group, glutamic acid content showed a decreasing trend from 0 x 10^{-3} to 5 x 10^{-3} salinity and increased with an increase in salinity from 5 x 10^{-3} to 20 x 10^{-3}.

Aspartic acid was the least preferred in this tissue of both size groups and it

showed no definite trend in concentration with an increase in salinity in either of the size groups.

4.4.3 *Mantle*

In the mantle tissue, concentration of alanine was considerably high in small size group (86.9mM/g) compared to that of large (6.9mM/g). In the small size group, there was no notable variation in concentration as the salinity increased from 0 x 10^{-3} to 5 x 10^{-3}, but a steady increase in concentration could be observed from 5 x 10^{-3} to 15 x 10^{-3} salinity. A decrease in concentration was noticed at 20 x 10^{-3}. In large size group, alanine was the third predominant amino acid, first and second being glycine and histidine respectively. A clear trend in variation as in small size group could not be observed in the large size group.

Contribution of glycine to the intracellular osmolyte pool of the mantle tissue of large animals was considerably high (33.5mM/g) whereas in the small size group, it was preferred only next to alanine. Glycine contributed to, on an average of 67.1% to the total NPS pool of the mantle tissue of large size group at 15 x 10^{-3}. During the salinity change from 0 x 10^{-3} to 10 x 10^{-3}, the concentration of glycine was more or less the same and was low compared to other salinities. Concentration showed a sharp increase from 10 x 10^{-3} to 15 x 10^{-3} and decreased beyond this salinity. Glycine contributed comparatively less to the intracellular osmolyte pool of mantle of small size group (average = 17.9%). Concentration in small size group increased with an increase in salinity from 0 x 10^{-3} to 15 x 10^{-3}. Beyond 15 x 10^{-3} a decrease in concentration was noticed. Quantity per unit gram tissue of this amino acid was more in large size group compared to small.

Table 4.5. **Concentration of amino acids in mantle tissue of *Villorita cyprinoides* (Small) acclimated to different salinities.**

Values expressed as μM per gram wet weight of tissue.

Free Amino Acids	0×10^{-3}		5×10^{-3}		10×10^{-3}		15×10^{-3}		20×10^{-3}	
	Conc	%	Conc	%	Conc	%	Conc	%	Conc	%
Asp	0.36	1.50	0.33	2.11	0.34	0.21	0.38	0.13	0.49	0.24
Glu	0.48	2.03	0.20	1.30	0.97	0.60	7.50	2.56	0.64	0.32
Ala	16.11	67.73	10.29	66.24	104.30	64.56	206.72	70.51	97.10	47.92
Gly	0.45	1.88	1.82	11.74	53.41	33.06	66.24	22.59	40.60	20.04
His	5.57	23.40	2.28	14.65	1.96	1.21	11.75	4.01	63.70	31.44
Arg	0.82	3.46	0.61	3.96	0.59	0.36	0.60	0.20	0.09	0.04
Total	23.79		15.53		161.57		293.19		202.62	

Table 4.6. **Concentration of amino acids in mantle tissue of *Villorita cyprinoides* (Large) acclimated to different salinities.**

Values expressed as μM per gram wet weight of tissue.

Free Amino Acids	0×10^{-3}		5×10^{-3}		10×10^{-3}		15×10^{-3}		20×10^{-3}	
	Conc	%	Conc	%	Conc	%	Conc	%	Conc	%
Asp	0.27	1.40	0.35	2.02	0.15	0.73	0.25	0.17	0.35	0.32
Glu	0.14	0.75	0.07	0.40	1.04	5.00	7.85	5.37	5.25	4.75
Ala	7.54	39.31	6.33	36.01	5.41	26.10	7.86	5.37	7.22	6.54
Gly	7.82	40.77	6.78	38.54	3.72	17.95	98.22	67.17	51.00	46.17
His	2.96	15.46	3.12	17.75	6.97	33.62	9.52	6.51	17.15	15.52
Arg	0.44	2.31	0.93	5.29	3.44	16.60	7.52	5.14	9.50	8.60
Total	19.18		17.59		20.73		146.23		110.47	

Values are the mean of three observations.
Each observation is made from a sample of 8-12 animals pooled together.

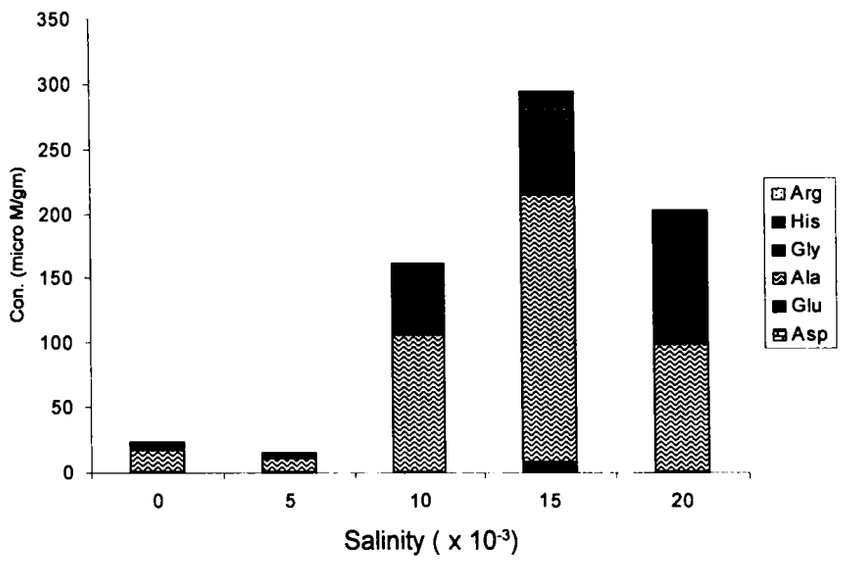


Fig 4.5. Concentration of amino acids in the mantle of small size group

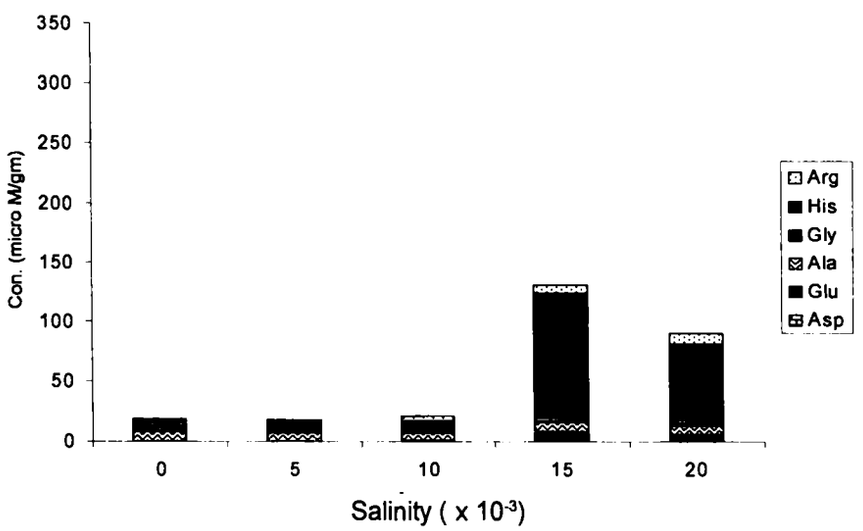


Fig 4.6: Concentration of amino acids in the mantle of large size group

Histidine was the second amino acid of preference for this tissue of the large size group whereas it was third, next to glycine for the small size group. In the large size group, the content of this amino acid increased steadily as the salinity increased from 0 x 10^{-3} to 20 x 10^{-3}. There was no decline in concentration of this amino acid observed in any of the salinities. In the small size group, its concentration decreased slightly as the salinity increased from 0 x 10^{-3} to 10 x 10^{-3}. As the salinity increased from 10 x 10^{-3} to 20 x 10^{-3}, there was a steep increase in concentration. The average content was high in the small size group (17.1 mM/g) compared to large (7.9mM/g).

On an average, glutamic acid content was high in the mantle tissue of large size group compared to small size group. This amino acid was preferred next to histidine in small size group whereas it was the fifth in rank in large size group and stood next to arginine. As the salinity increased from 0 x 10^{-3} to 5 x 10^{-3}, the change in concentration of glutamic acid in both the size groups was only marginal. But there was a sharp increase in concentration from salinity 5 x 10^{-3} to 15 x 10^{-3} in both the size groups. From salinity 15 x 10^{-3} to 20 x 10^{-3}, there was a decrease in concentration and the decrease was more in small compared to that of large.

Concentration of arginine was high in large size group compared to that of small. On an average, the percentage contribution was 7.6 in large and 1.6 in small size groups. In large size group, the concentration showed a steady increase as the salinity went up from 0 x 10^{-3} to 20 x 10^{-3} whereas a decreasing trend in concentration with rise in salinity from 0 x 10^{-3} to 10 x 10^{-3} was observed in the small size group. From 10 x 10^{-3} to 15 x 10^{-3}, the concentration remained almost

the same in small group and a marked decrease in concentration was observed when the salinity increased from 15 x 10^{-3} to 20 x 10^{-3}.

In the mantle tissue, contribution of aspartic acid was almost equal in both the size groups. Concentration of aspartic acid did not vary much in the tissue with a change in salinity.

4.5 Results of Analysis of Variance

Two way ANOVA test was performed to check the statistical significance of the above observations. Results are shown in the ANOVA tables (Tables 4.9 - 4.20). To check the difference between individual observations in a group, Least Significant Difference (LSD) was calculated and compared with the difference in means of the groups.

4.5.1 *Total amino acids*

Results showed that the concentration of amino acids in small size group at higher salinities (10 x 10^{-3}, 15 x 10^{-3} and 20 x 10^{-3}) is significantly different from that at 0 x 10^{-3}. Comparison of the total amino acids studied, between tissues showed that the variation is insignificant (Tables 4.21 & 4.22).

In large size group, ANOVA showed significant difference in NPS content between tissues and between salinities (Table 4.22). Difference in concentration was insignificant between salinities 0 x 10^{-3} to 10 x 10^{-3} and between 15 x 10^{-3} and 20 x 10^{-3}. But the NPS content was significantly high at 15 x 10^{-3} and 20 x 10^{-3} salinity compared to lower salinities. When all the three tissues in all the salinities are considered, it was found that, concentration of total amino acids studied was significantly high in the adductor muscle of large size group (LSD between

Table 4.9 Results of Analysis of Variance for concentration of Aspartic acid in small size group of *V. cyprinoides* var. cochinensis

Source	Sum of Squares	Degrees of Freedom	Mean of Squares	Variance Ratio	Significance
Tissues	0.800	2	0.400	1.32	NS
Salinity of the medium	0.783	4	0.196	0.65	NS
Error	2.424	8	0.303		
Total	4.007	14			

NS – Not significant.

Table 4.10 Results of Analysis of Variance for concentration of Glutamic acid in small size group of *V. cyprinoides* var. cochinensis

Source	Sum of Squares	Degrees of Freedom	Mean of Squares	Variance Ratio	Significance
Tissues	19.390	2	9.695	0.650	NS
Salinity of the medium	26.668	4	6.667	0.447	NS
Error	119.329	8	14.916		
Total	165.387	14			

NS – Not significant.

Table 4.11 Results of Analysis of Variance for concentration of Glycine in small size group of *V. cyprinoides* var. cochinensis

Source	Sum of Squares	Degrees of Freedom	Mean of Squares	Variance Ratio	Significance
Tissues	377.921	2	188.960	0.458	NS
Salinity of the medium	12824.490	4	3206.123	7.763	P<0.01
Error	3303.892	8	412.987		
Total	16506.30	14			

NS – Not significant.

Table 4.12 Results of Analysis of Variance for concentration of Alanine in small size group of *V. cyprinoides* var. cochinensis

Source	Sum of Squares	Degrees of Freedom	Mean of Squares	Variance Ratio	Significance
Tissues	7476.457	2	3738.229	3.487	NS
Salinity of the medium	29209.700	4	7302.425	6.810	P<0.05
Error	8575.312	8	1071.914		
Total	45261.47	14			

NS – Not significant.

Table 4.13 Results of Analysis of Variance for concentration of Histidine in small size group of *V. cyprinoides* var. cochinensis

Source	Sum of Squares	Degrees of Freedom	Mean of Squares	Variance Ratio	Significance
Tissues	1023.473	2	511.737	1.950	NS
Salinity of the medium	1763.819	4	440.955	1.680	NS
Error	2099.518	8	262.440		
Total	4886.811	14			

NS – Not significant.

Table 4.14 Results of Analysis of Variance for concentration of Arginine in small size group of *V. cyprinoides* var. cochinensis

Source	Sum of Squares	Degrees of Freedom	Mean of Squares	Variance Ratio	Significance
Tissues	26.477	2	13.239	1.405	NS
Salinity of the medium	41.714	4	10.428	1.106	NS
Error	75.401	8	9.425		
Total	143.592	14			

NS – Not significant.

Table 4.15 Results of Analysis of Variance for concentration of Aspartic acid in large size group of *V. cyprinoides* var. cochinensis

Source	Sum of Squares	Degrees of Freedom	Mean of Squares	Variance Ratio	Significance
Tissues	0.1366	2	0.0683	7.98	P<0.05
Salinity of the medium	0.0800	4	0.0200	2.34	NS
Error	0.0684	8	0.0086		
Total	0.2851	14			

NS – Not significant.

Table 4.16 Results of Analysis of Variance for concentration of Glutamic acid in large size group of *V. cyprinoides* var. cochinensis

Source	Sum of Squares	Degrees of Freedom	Mean of Squares	Variance Ratio	Significance
Tissues	2.0814	2	1.041	0.2190	NS
Salinity of the medium	68.635	4	17.16	3.6110	NS
Error	38.014	8	4.751		
Total	108.7304	14			

NS – Not significant.

Table 4.17 Results of Analysis of Variance for concentration of Glycine in large size group of *V. cyprinoides* var. cochinensis

Source	Sum of Squares	Degrees of Freedom	Mean of Squares	Variance Ratio	Significance
Tissues	478.35	2	239.175	0.449	NS
Salinity of the medium	11085.84	4	2771.46	5.214	P<0.05
Error	4252.60	8			
Total	15816.79	14			

NS – Not significant.

Table 4.18 Results of Analysis of Variance for concentration of Alanine in large size group of *V. cyprinoides* var. cochinensis

Source	Sum of Squares	Degrees of Freedom	Mean of Squares	Variance Ratio	Significance
Tissues	2452.70	2	1226.35	1.255	NS
Salinity of the medium	19692.35	4	4923.09	5.037	P<0.05
Error	7818.78	8	977.35		
Total	29963.81	14			

NS – Not significant.

Table 4.19 Results of Analysis of Variance for concentration of Histidine in large size group of *V. cyprinoides* var. cochinensis

Source	Sum of Squares	Degrees of Freedom	Mean of Squares	Variance Ratio	Significance
Tissues	698.93	2	349.46	4.77	P<0.05
Salinity of the medium	1210.40	4	302.60	4.13	NS
Error	586.24	8	73.28		
Total	2495.56	14			

NS – Not significant.

Table 4.20 Results of Analysis of Variance for concentration of Arginine in large size group of *V. cyprinoides* var. cochinensis

Source	Sum of Squares	Degrees of Freedom	Mean of Squares	Variance Ratio	Significance
Tissues	37.74	2	18.87	2.81	NS
Salinity of the medium	18.41	4	4.60	0.69	NS
Error	53.74	8	6.71		
Total	109.89	14			

NS – Not significant.

Table 4.21. Results of Analysis of Variance for concentration of sum of NPS in small size group of *V. cyprinoides*

Source	Sum of Squares	Degrees of Freedom	Mean of Squares	Variance Ratio	Significance
Tissues	13791.10	2	6895.54	3.43	NS
Salinity of the medium	88482.60	4	22120.65	11.00	P<0.005
Error	16087.40	8	2010.93		
Total	118361.00	14			

Table 4.22. Results of Analysis of Variance for concentration of sum of NPS in large size group of *V. cyprinoides* var. cochinensis

Source	Sum of Squares	Degrees of Freedom	Mean of Squares	Variance Ratio	Significance
Tissues	8478.47	2	4239.24	5.06	P<0.05
Salinity of the medium	48679.80	4	12169.65	14.54	P<0.001
Error	6698.05	8	837.26		
Total	63856.30	14			

NS – Not significant.

tissues = 42.27), whereas the difference in the contents between foot and mantle was statistically insignificant in this size group. In the small size group, the difference between tissues was insignificant.

4.5.2 *Alanine*

Variation in small size group was insignificant at salinities 0 x 10^{-3} to 10 x 10^{-3}. At higher salinities (15 x 10^{-3} to 20 x 10^{-3}) the concentration was significantly high (LSD = 61.75). The apparent decrease in concentration as the salinity increased from 15 x 10^{-3} to 20 x 10^{-3}, was not statistically significant.

In the large size group, the quantity of alanine was significantly high at salinity 15 x 10^{-3}. Difference in quantity in other salinities is insignificant (LSD = 58.96). Difference between tissues was insignificant in both the size groups.

4.5.3 *Glycine*

In small size group, increase in quantity of glycine in the tissues was significant when the salinity increased from 5 x 10^{-3} to 10 x 10^{-3}. The decrease in concentration when the salinity was raised from 15 x 10^{-3} to 20 x 10^{-3} was also statistically significant (LSD = 38.33).

In large animals, the increase in quantity at 15 x 10^{-3} and 20 x 10^{-3} salinities was significant. A decrease in average concentration from salinity 15 x 10^{-3} to 20 x 10^{-3} was insignificant (LSD = 43.49). In both the size groups, the difference between tissues was not significant.

4.5.4 *Histidine*

In the small size group, differences in content of this amino acid was insignificant between different salinities and between the three tissues studied.

On the other hand significant difference in content between salinities (LSD = 16.15) and between tissues (LSD = 12.51) could be observed in the case of large sized animals. Histidine content was significantly high at higher salinities (15 x 10^{-3} and 20 x 10^{-3}). Between tissues, the higher concentration in the adductor smuscle was significant compared to mantle and foot tissues. Difference between mantle and foot tissues was insignificant.

4.5.5 *Other amino acids*

Variation in concentration of glutamic acid, arginine and aspartic acid was

statistically insignificant between salinity and between tissues in both the size groups, except in large size group, where the higher quantity observed in the foot tissue was significant (LSD = 0.135) compared to the adductor and mantle.

4.6 Discussion

Variation in concentration of the total ninhydrin positive substances in the intracellular fluid in response to a variation in salinity indicates the existence of a mechanism for the regulation of intracellular osmolytes. In the present study, a significant increase in the concentration of total free amino acids was noticed in response to an increase in ambient salinity in both the size groups. This substantiates the role of amino acids and their derivatives as intracellular osmolytes. Estuary is one of the most dynamic types of habitats where variations in salinity are frequent. In order to successfully inhabit this realm, ability to tolerate changes in salinity is a prerequisite. *Villorita cyprinoides* that inhabit the freshwater end of estuaries also is no exception. They can tolerate salinities from 0 - 27 x 10^{-3}. Immediate response to a rapid salinity change is behavioural, predominantly by closing the valves and lasted for only up to 72-96 hrs for this animal (chapter 2). Present observations indicate that extracellular-fluid anisosmotic osmoregulation is operational only at lower salinities in *V. cyprinoides*. This limited capability of regulation is reported by George (1993), in the case of *Sunetta scripta* and *Perna viridis*. At higher salinities, concentration of ions in the hemolymph is not much different from that of the ambient medium. Osmolarity of the hemolymph is not higher than that of the ambient medium. (Figs. 2.23 & 2.24). These confirm that the efficiency of the animal for extracellular-fluid anisosmotic regulation at higher salinities is not effective to maintain the conducive osmolarity of the hemolymph. This also makes it clear that amino acids have no significant role in

maintaining the high osmolarity of hemolymph. Pierce and Greenberg, (1972), Hoyaux, *et al.*, (1976), Livingstone, *et al.*, (1979) and Pierce (1982), have pointed out the role of ninhydrin positive substances in intracellular osmoregulation. It is also reported that not all amino acids function as intracellular osmolytes. There exists a selection of intracellular osmolytes for osmoregulation.

For both the size groups studied, correlation between salinity and total ninhydrin positive substance concentration was positive (Tables 3.2 & 3.3). Variation in concentration between size groups was insignificant. Osmoconformers rely on intracellular compatible osmolytes for permanent adjustments to elevated salinity. Similar regulation of intracellular osmolytes by euryhaline bivalves is reported by Deaton, et. al., (1989) and Deaton & Greenberg, (1991). In marine bivalves, major osmotic constituents of the hemolymph are mainly inorganic ions. Free amino acids and their derivatives form the major osmotic effectors in the cells. This is true in the case of *V. cyprinoides* also. In the marine forms *S. scripta* and *P. viridis* studied by George, (1993), adductor muscle was found to have maximum concentration of ninhydrin positive substances. Foot ranked second and mantle third in almost all salinities tested. In the case of *V. cyprinoides*, foot muscle was found to contain maximum amount of ninhydrin positive substances, mantle stood second and adductor third in small size group. The difference in concentration between foot and mantle tissue was marginal. In the case of large sized animals, adductor muscle contained more ninhydrin positive substances than mantle. Variation between size groups was insignificant whereas in *S. scripta* and *P. viridis*, small animals showed a higher amount of total ninhydrin positive substances. Marine bivalves are not as frequently exposed to salinity variations as *V. cyprinoides*. For an estuarine organism that is exposed to large and frequent swings in salinity, the reduction or elimination of cell volume regulation would

represent a substantial saving of metabolic energy. Active cell volume regulation is not carried out in all types of tissues equally for these reasons. For example, the functional cost of not regulating volume of gills may be modest: because functional impairment of isolated gill cilia of *Geukensia demissa* exposed to 20 x 10^{-3} salinity from 30 x 10^{-3} salinity is only 15% (Van Winkle, 1972). Hence it is advantageous for the animal to regulate the cell volume of tissues those are functionally impaired to a significant extent under a salinity stress. Variation of ninhydrin positive substances between size groups is insignificant. The probable reason is that they experience salinity changes more frequently than marine forms.

A reduction in total ninhydrin positive substance concentration was noticed in all the tissues of both the size groups beyond salinity 15 x 10^{-3}. During the adaptation of *Tegula funebralis* to 160% seawater, there is a reduction in the cellular osmotic pressure imparted by the free amino acids (Florkin, 1972). However the osmolarity of the cell increased at 160% seawater also. This made him conclude that some other organic compounds act in the isosmotic regulation in hyperosmotic media. A clear picture of the mechanism for osmoregulation above 15 x 10^{-3} salinity could be derived only if the details of the tissue osmolarity is known. It is probable that the animal is switching from free amino acids to some other compounds as intracellular osmolytes at this salinity. A decrease in cellular osmotic pressure imparted by free amino acids at higher salinities is also reported by Allen (1961). He observed a change in the intracellular osmolyte during isosmotic regulation in concentrated media in *Rangea cuneata*.

Variations in concentration of amino acids in the cytoplasm can impede the normal cellular function. Increase in total ninhydrin positive substances at 15 x 10^{-3} salinity is around 3 – 12 times the concentration of control (salinity 0 x 10^{-3}). Studies on

the quantitative composition of the amino acid pool have revealed that amino acids are selected in such a way that they do not disrupt the metabolic harmony of the cell.

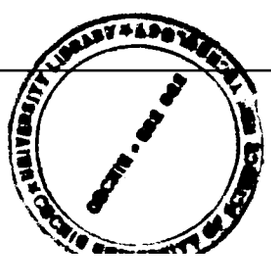
To study the contribution of individual amino acids, qualitative separation and estimation of amino acids were done. Results showed that the major varied osmolytes in the tissues of *V. cyprinoides* were glycine and alanine. Arginine, histidine, glutamic acid and aspartic acid were found to vary, but their contribution was less. As stated earlier, animals are very much selective in the choice of intracellular osmolytes. Glycine and alanine are the commonest amino acids and can be obtained easily. Amino acids of low molecular weight are more soluble and add to the total osmotic pressure of the cell without considerable increase in viscosity of the cellular fluid, when compared to other high molecular weight amino acids. In addition to that, the accumulated osmolytes should not change the pH of the cellular fluid since pH is a crucial factor for the cellular reactions. pKa values of these amino acids are close to neutrality. These may be the reasons for the preference of these amino acids for osmotic adjustments. Ability of the euryhaline bivalves to conform osmotically depends upon the degree of tolerance and manipulation of intracellular osmolyte repository by the intracellular system. Accumulation of intracellular osmolytes is a technique to resist water loss and thus maintaining cell volume. In *Sunetta scripta*, which is a marine bivalve, George and Damodaran (1999) described taurine, glycine alanine, proline aspartate and glutamate as the major components of the NPS pool. Taurine dominated glycine in quantity in the foot and mantle tissues. *V. cyprinoides* employs lesser number of amino acids when compared to the marine forms studied. Moreover this animal has a lesser choice since taurine is not available at its disposal. Glycine seems to be a common organic osmoprotectant in osmoconforming bivalves. Similar

observations were made by Douglas and Stephen, (1996) in the estuarine mussel *Geukensia demissa*. Hoyaux, *et al.* (1976) and George, (1993) have pointed out the importance of glycine as intracellular osmolyte. Hoyaux, *et al.* (1976) reported that the relatively high amount of intracellular glycine may be characteristic of bivalves of littoral area. The present observation and the reports by Douglas and Stephen, (1996) fortifies the importance of glycine as intracellular osmolyte not only in marine bivalves but in estuarine forms also.

Alanine also has equal importance as glycine as osmoprotectant in *V. cyprinoides*. Alanine is found in lesser proportion in the foot and mantle of large animals. It is found in all tissues and the concentration was found to vary in response to salinity. This amino acid has also been reported in many marine animals by authors like Pierce, *et al.*, (1992), George and Damodaran (1999). De Zwaan and Van Marrewijk (1973) suggested the conversion of ammonia into alanine catalyzed by alanine dehydrogenase. In molluscs, during anaerobiosis, pyruvate formed is transaminated to alanine. Succinate is also formed in equimolar concentrations as that of pyruvate. These accounts for the fact that there is an accumulation of both succinate and alanine in the mantle tissue of *Rangea cuneata* incubated anaerobically (Stokes & Awapara, 1968). Thus during the period of valve closure, the animals are benefited in two ways, (1) anaerobic respiration and (2) intracellular amino acid buildup. Large specimens of *V. cyprinoides* seem to prefer glycine over alanine for osmoregulation. This is reflected from the higher concentration of glycine in the mantle and foot tissue of this size group. From a physico-chemical viewpoint, glycine is a smaller molecule when compared to alanine. It is more efficient as an osmolyte since the accumulation of equal number as alanine needs lesser energy for synthesis and also results in lesser buildup in viscosity.

Histidine also seems to have a role as an intracellular osmolyte. The concentration of this amino acid was found to be high at higher salinities irrespective of the tissues. This suggests that the amino acid may be useful in some way or the other at higher salinities. It seems that there may be some functions for these amino acids other than osmoprotection.

Taurine (2-aminoethane sulphonic acid) prevents cell swelling and increases cell viability (Pasantes-Morales, *et al.*, 1984). Recent data suggests an important participation of taurine in the transmission of the osmotic information. Taurine is an amino acid mainly known for its involvement in cell volume regulation, as it is one of the major inorganic osmolytes used by cells to compensate for changes in extracellular osmolarity. In the supraoptic nucleus, taurine is highly concentrated in astrocytes, and released in an osmodependent manner through volume-sensitive anion channels. (Nicolas Hussy, *et al.,* 2000). This amino acid which is not a component of the proteins is a major osmolyte in marine forms but is absent in *V. cyprinoides.* Its role is taken over by other amino acids. Awapara (1962) has reported the absence of taurine in freshwater and terrestrial molluscs whereas in the marine counterparts, a high content of taurine was observed. Marine forms always need a high quantum of amino acids to keep osmolarity high. Taurine which is a non essential amino acid is a good candidate for meeting such high demands. In freshwater forms, such high quantities are not required or the animals may be lacking the metabolic system to procure taurine. Studies carried out on *Mytilus galloprovincialis* and *Perna canaliculus* (Gardner & Kathirvetpillai, 1997), *Mercenaria mercenaria* (Koehn, *et al.*, 1980), *Geukensia demissa* (Garthwaite, 1986, 1989), *Crassostrea virginica* (Buroker, 1983, Rose, 1984), *C. rhizhophorae* (Nirchio, *et al.*, 1991) and *C. angulata* (Michinina & Rebordinos, 1997) provide substantial evidence that there exists variation in



genetic capability for selecting the osmolytes for osmotic regulation in different environments which is important for the survival of the animal. Thus, by genetic selection, nature has carefully perfected the tricks of the trade.

Chapter 5

MECHANISMS OF REGULATION OF INTRACELLULAR OSMOLYTES

5.1 Introduction

Water provides the medium for metabolic reactions inside the cell. Solutes, especially inorganic ions are as equally important as water for an organism. They act as cofactors in many enzymatic reactions, and also influence the permeability of biological membranes to other solutes. Enzymes have a role in maintaining water content of the cell and preventing loss of water from the cell during hyper osmotic stress. Even in osmotic equilibrium, osmotic constituents of the cell and that of the extracellular fluid are different. Osmolytes of the cells that are regulated mostly are free amino acids and their derivatives, even though potassium ions, bicarbonate ions and phosphate compounds are also regulated to a limited extent. Optimum concentration of inorganic and organic constituents of cells is maintained by various mechanisms involving membrane permeability, synthesis or degradation and active transport.

5.2 Mechanisms of regulation of amino acids and derivatives

When exposed to a hypertonic environment, cells throughout the biological spectrum, spanning from bacteria to vertebrates, accumulate certain small organic solutes termed compatible (non-perturbing) osmolytes (Yancey *et al.*, 1982). It is proven beyond doubt that the level of amino acids and similar compounds are regulated in response to osmotic variations in environment.

Intracellular-fluid isosmotic regulation is likely to be achieved in a number of ways. Control of amino acid concentration by regulating the deamination in different tissues and/or in a specific organ following release from other tissues appears to be an important mechanism facilitating cellular osmotic adjustments under conditions of osmotic stress in tissues of euryhaline invertebrates (Kinne, 1971). During hyperosmotic stress, mechanisms involving modification of the oxidation or deamination rate of amino acids are in operation. On hypo osmotic stress the nitrogenous osmolytes are removed from the cell and they are catabolized rapidly. A major constituent which is suggested during loss of ninhydrin positive substance by nitrogen excretion in marine molluscs is taurine. Conversely during hyperosmotic regulation, there is an active uptake, *de novo* synthesis and proteolysis to augment the yield of osmolytes. Mobilization of amino acids across the cell membrane is passive during hypo osmotic adjustment and active during hyper osmotic regulation.

Metabolic regulation of ninhydrin positive substance pool in response to salinity variations involves manipulations in glycolytic pathway, TCA cycle reactions, activity of transaminases and metabolic shuttling between cellular compartments. Mitochondrial transport and oxidation of organic osmolytes may supplement the removal of osmolytes in response to a hypo-osmotic stress.

A marked variation in the activity of enzymes of the concerned metabolic pathways and transport makes the regulation of osmolytes possible. Apart from covalent modification of the enzymes, the induction or repression of the enzymes may also be involved in the regulation (Yamauch *et al.*, 1993, Uchida *et al.*, 1993). Recent research is focused on the molecular mechanisms of osmolyte regulation. It is indicated that the critical event during a hypertonic stress is the stimulation of transcription and that the changes in mRNA abundance and transport activity

are consequences of the stimulation. Cloned gene of 28kb size (BGT1 gene) is reported to be involved in the process of transport of compatible osmolytes and has three promoter sites, each regulated similarly by hypertonicity (Kwon and Handler, 1995). A tonicity responsive element (TonE) is also found in the gene. In contrast to the slow onset of accumulation of organic osmolytes, when shifted to hypertonic medium, in many animals, the loss of osmolytes occurs rapidly when exposed to a medium of lower tonicity. Initially a swelling occurs when transferred to hypo osmotic medium, but in over 10-20 min almost all cells shrink back nearly to their original size. This shrinkage in response to swelling resulting from osmotic shock is termed regulatory volume decrease (RVD). It is as a result of the loss of water secondarily to the loss of cell electrolytes like K^+, Cl^- and organic osmolytes. During RVD, compatible osmolytes exit via a different pathway than that involved in their uptake. A few laboratories (Roy and Malo, 1992, Kirk *et al.*, 1992, Pasantes-Morales *et al.*, 1994) have independently proposed that the efflux of compatible osmolytes occurs via the volume-sensitive chloride channel that is activated during RVD.

Increased degradation of amino acids by deamination in response to a hypo osmotic stress yields ammonia. This appears to have added advantage in sodium transport. It is reported that active uptake of sodium by gills implicates an exchange with NH_4^+ ions involving some enzyme systems. (Roger & Thomas, 1997).

Increase in amino acid concentration of isolated tissues incubated in hyperosmotic media, which did not initially contain amino acids, favours the idea of an intracellular mechanism of synthesis of osmotic solutes (Bedford, 1971). It seems that a mechanism involving changes in intracellular metabolism works in the control of the concentration of various amino acids. This metabolic mechanism may be involved in the control of the steady state between amino acids and proteins and/

or control of synthesis-degradation rates of amino acids. Siebers *et. al.*, (1992) have suggested a transport of amino acids from hemolymph into the cells during a hyperosmotic stress. Conte *et al.*, (1973) have suggested active uptake of ^{14}C leucine by *Artemia salina* nauplii. But in a medium devoid of or containing very low concentration of amino acids, metabolic synthesis appears to be in operation. Bedford (1971) showed that the increase in amino nitrogen occurring in isolated foot muscle of the mollusc *Melanopsis trifasciata* submitted to hyperosmotic stress, is paralleled by an increase in total nitrogen. This suggests a *de novo* synthesis of amino acids rather than proteolysis.

In vitro experiments conducted by Huggins and Boulton (1971) clearly demonstrated that muscle extracted from a crab acclimated to 40% seawater utilizes glutamic acid faster than the same tissue isolated from a 100% seawater acclimated crab. Changes in the activity of leucine amino peptidase and ninhydrin positive substances in *Crassostrea rhizophorae* on exposure to extremes of salinity were studied by Nirchio *et. al.* (1997). Results suggest that the enzmye leucine amino peptidase plays an important role in the cell volume regulation of the marine bivalves. The study also revealed that the response of the enzyme in the hypo osmotic environment suggests the possibility that it could be associated with other metabolic functions, distinct from cell volume regulation.

Experiments on isolated tissues by Dutchateau and Florkin (1962) have shown that hormonal control is not primarily implicated in the regulation of the cellular osmotic pressure. Manipulation of degradation-synthesis balance and/or intermediary metabolism is found to accomplish the task of intracellular osmotic pressure build up.

Immediate regulation of amino acid pool and consequent change in the metabolism

strongly suggest a change in the protein content, especially of low molecular weight proteins of the tissues. It is worth experimenting with the changes in protein composition of osmotically stressed animals to have an idea about the role of protein metabolism during osmotic regulation.

The accumulation or removal of intracellular osmolytes is coupled with metabolic cycle manipulation. Study of the enzyme involved will be of great importance in understanding the mechanisms that are operational and the pathways that are modified for regulation. Regulatory mechanisms under hypo-osmotic and hyperosmotic stress has been pointed out by (Zurburg and De Zwaan (1981), Bishop *et al.* (1983), Somero and Bowlus (1983), Hayashi (1987) and Nirchio and Perez (1997). Deaton *et al.* (1984) and Deaton (1987) have found out that protein is a source of amino nitrogen during hyper osmotic regulation and that volume regulation will be hampered by lysosomal inhibitors and proteinase inhibitors. Role of amino acids, eventhough less in number, in the intracellular osmoregulation of this animal is proven. The next step is to find out the source of intracellular osmolytes, in this animal.

The study discussed here was designed to verify the role of the enzymes glutamate pyruvate transaminase, cathepsin and acid phosphatase, which are involved in various metabolic pathways in an event of osmotic stress. Protein content of the tissues at various salinities was also studied to substantiate the observations.

Work carried out by George (1993) and the experiments conducted in *Villorita cyprinoides* could vindicate the existence of extracellular anisosmotic regulation in molluscs exposed to slightly different salinity from their optimum. Active transport of ions for ionic regulation is carried out by gills and kidneys. Enzymes like Na^+-K^+ ATPase are reported to vary indicating regulation. Activity of Na^+-K^+

ATPase in the sea bass *Dicentrarchus labrax* which can tolerate salinity ranging from freshwater to 50×10^{-3} - 60×10^{-3} was studied by Jensen, *et. al.*, (1998). An increase in activity of this enzyme was reported during high or low salinity adaptation. The enzyme is primarily involved in the active transport of Na^+ and K^+ through the gill membrane. In the present study, variation of Na^+-K^+ ATPase in the gills of *V. cyprinoides* under different salinities was studied to understand the level of Na^+-K^+ regulation.

5.3 Materials and Methods

Bivalves for the experiments were collected from Poochakal estuary, near Cherthala and were acclimatized as described in section 2.2. Enzyme analysis was done during the acclimation period, 5-6 days after transferring the animals to the desired salinity (ie. 10×10^{-3} or 20×10^{-3}). In the previous experiments conducted, it was found that foot tissue and mantle tissue presented the maximum and minimum amount of ninhydrin positive substance respectively. So these tissues were selected for the study of enzymes. Protein content of dried tissues was determined by Lowry's method (Lowry *et al.*, 1951). Tissues were dried overnight in a hot air oven (60° C) and were powdered and weighed. Definite quantity of powdered tissue was dissolved in 0.1 N NaOH for estimation of protein.

5.3.1 Glutamate Pyruvate Transaminase

Assay of glutamate pyruvate transaminase (GPT) was carried out in phosphate buffer (11.3g of anhydrous Na_2HPO_4 and 2.7g of anhydrous KH_2PO_4 dissolved in 1 litre of distilled water) containing alanine and µ-keto glutarate as substrates. Buffered substrate was prepared as follows.

Dissolved 900mg of alanine and 15mg of µ-keto glutarate in 9ml of water. 1N

NaOH was added to attain complete dissolution. Adjusted the pH to 7.4 and made up to 50ml with phosphate buffer. To 0.5ml of substrate solution incubated for 5 min at 37 °C, 0.1ml of enzyme extract was added. Incubated the mixture for 30min in water bath at 37 °C. Removed from the bath and added 0.5ml of 2,4-dinitrophenyl hydrazine (2,4-DNPH) solution (DNPH solution was prepared by dissolving 19.8mg of 2,4-dinitrophenyl hydrazine in 10ml of hot concentrated HCl, and diluting the solution to 100ml with water). In the control, enzyme extract was added after incubation for 30min. To 0.3ml of standard pyruvate solution (prepared by dissolving 220g of sodium pyruvate in 500ml of buffer) 0.2ml of buffered substrate was added. Blank was 0.5ml of buffered substrate. 0.1ml of water and 0.5ml of DNPH colour reagent was added to blank and standard. After 20 min, added 0.5ml of 0.4N NaOH solution. Absorbance at 510nm was measured after 10 min. The experiment was repeated several times.

5.3.2 Na^+-K^+ ATPase

Animals were acclimatized in 0, 10 and 20 x 10^{-3} salinity for two weeks and were kept unfed for four days before experiments. Gill tissue was dissected out and was cut in to pieces. It was homogenized in 0.25M cold sucrose solution. Cell fractionation of the gill homogenate was done according to the modified method of Davis (1970). The homogenate was centrifuged at 3000 x g for 15 min and the supernatant was centrifuged at 12,000 x g for 30 min and lastly supernatant was centrifuged again at 24,000 x g for 30 min at 4°C in a refrigerated centrifuge. The precipitate after final centrifugation was suspended in 0.25M sucrose and was used as the enzyme solution.

5.3.2.1Assay of enzyme activity

Na^+-K^+ activated, Mg-dependent ATPase (Total ATPase) was determined using

the reaction mixture containing 60mM NaCl, 20mM KCl, 2mM $MgCl_2$, 30mM Tris-HCl (pH-7.5) and 2.5mM ATP in 0.8ml. The Mg^{2+}-ATPase was measured in the presence of 0.25M sucrose in place of NaCl and KCl. The reaction mixture was preincubated for 5min at 37°C before adding 0.2ml of heavy microsomal suspension (10-60mg protein/ml). Enzyme reaction was carried out at 37°C for 15min. The Na^+-K^+ ATPase activity was calculated in terms of the difference of the total ATPase and Mg^{2+}-ATPase values. After incubation, 2ml of 10% trichloroacetic acid was added to the reaction mixture and the supernatant was separated by centrifugation at 13,000 x g for 10 min. The inorganic phosphate (Pi) liberated from ATP was measured by the Fiske-SubbaRow method (1925) using a Hitachi UV-Visible spectrophotometer (model 200-20) at 660nm. The specific activity of Na^+-K^+ ATPase is defined as milli moles of Pi formed per mg of enzyme protein during one hour. The protein content was determined by the method of Lowry *et al.*, (1951) using bovine serum albumin as standard.

5.3.3 Acid Phosphatase

Foot and mantle tissues from large and small size groups were excised during the period of acclimation. Each tissue was homogenized separately in 10% volume of 0.25 M cold sucrose solution in an electric mortar for 7 min. The homogenate was centrifuged at 3,000 rpm at 4° C in a refrigerated centrifuge for 10 min to remove cellular debris. Again the supernatant was centrifuged for 30 min at 13,500 rpm to remove intact lysosomes. Acid phosphatase activity in the soluble fraction (supernatant after removal of lysososmes) was estimated.

5.3.3.1 Assay of enzyme activity:

Acid Phosphatase activity was assayed following the method of Anon (1963). 0.5 ml of substrate (400 mg% p-nitrophenol phosphate in distilled water) and 0.5

ml of 100 mM citrate buffer (pH 4.5) were incubated for 3 min at 37° C. Enzyme sample was added and the reaction mixture was incubated for 30 min at 37° C. Reaction was stopped by the addition of 4 ml of 0.1 N NaOH. The yellow colour formed by the liberation of p-nitrophenol was read at 410 nm. The activity of the enzyme was quantified from the calibration curve prepared for *p*-nitrophenol. The protein content of the enzyme extract was estimated following the method of Lowry *et al.* (1951). Activity of acid phosphatase was calculated as mg *p*-nitrophenol liberated per hour per gram protein.

5.3.4 Lysosomal Lability Index

Foot and mantle tissues from large and small size groups were excised during the period of acclimation as mentioned above. Homogenized in 10 volumes (10ml solution for 1g tissue) of 0.25 M cold sucrose solution in an electric mortar for 7min. The homogenate was centrifuged at 600 x g at 4° C in a refrigerated centrifuge for 15min. The pellet was resuspended in 10 volumes citrate buffer (Nuclear fraction). The supernatant was centrifuged for 30min at 15,000 x g to remove intact lysosomes. The supernatant was saved (soluble fraction). Lysosomal pellets were resuspended in 10 volumes citrate buffer containing 0.1% Brij-35 (Lysosomal fraction). Acid phosphatase activity in the fractions was estimated as discussed above. Protein content of all the fractions were estimated by Lowry's method (Lowry *et al.*, 1951).

5.3.5 Cathepsin-D

Animals were acclimatized in the lab at 0 x 10^{-3} salinity for one month and fed on blue-green alga *Synechocystis salina*. Animals were then given a salinity stress by transferring them to sea water of desired salinitiy made by dilution of 100% sea water with deionized water. Small and large animals were exposed to 10 x

10^{-3} and 20 x 10^{-3} salinities. During the acclimation process (i.e. 5-6 days after transferring the animals to the desired salinity), animals were taken out and tissues were excised. Excised tissues were weighed and homogenized in distilled water for the estimation of cathepsin.

5.3.5.1 Assay of enzyme activity:

Cathepsin-D activity in the tissues was estimated as per the procedure of Anson (1938). Acid denatured hemoglobin was used as substrate. 1.0 ml of enzyme extract was added to the substrate and was incubated at 25° C for 10 min. Reaction was stopped by adding 5% TCA. To the control, enzyme extract was added after the addition of TCA. The solution was centrifuged at 3000 g for 10min to remove precipitated proteins. The catabolic products present in the supernatant were estimated by Lowry's method (Lowry *et. al.*, 1951).

Experiments were repeated several times. Student's *t*-test was employed for comparison of the results obtained. Significant values are marked by asterisks (*) in the tables.

5.4 Results

Activity of acid phosphatase increased with an increase in salinity in the mantle tissue of both the size groups. The mantle at salinity 20 x 10^{-3} showed highest activity of acid phosphatase in the two size groups studied. Foot tissue showed a reduction in enzyme activity when compared to the control (0 x 10^{-3}), in both the size groups. Activity of the enzyme in the foot of large animals at 20 x 10^{-3} salinity was not significantly different from the control.

Increase in activity in the mantle of small size group at salinity 10 x 10^{-3} was not significantly different from that of the control.

Significant difference in activity between size groups was observed in foot at salinities 10 x 10^{-3} and 20 x 10^{-3}. In both the salinities, tissues of large size group showed higher activity when compared to small size group. But the higher activity was statistically significant only in the case of mantle at salinity 20 x 10^{-3}. In this

Table 5.1. Activity of Acid Phosphatase in various tissues of *V. cyprinoides* var. cochinensis (Small) during acclimation to different salinities.

Values expressed as mg p-Nitrophenol formed/mg protein/hr ± SD.

Salinity	Foot			Mantle		
	Activity		% of control	Actiivity		% of control
	Mean	SD		Mean	SD	
0	0.83	± 0.105	100.0	1.06	± 0.33	100.0
10	0.26	± 0.054	31.2*	1.24	± 0.05	116.8
20	0.28	± 0.110	33.6*	1.83	± 0.14	172.8*

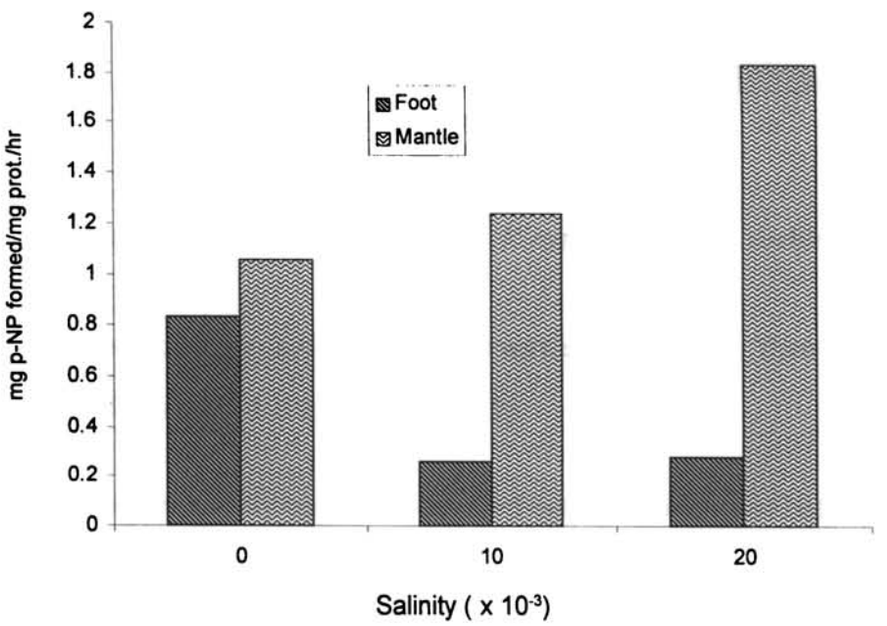


Fig 5.1: Activity of acid phosphatase in *V. cyprinoides* var. cochinensis (Small)

Table 5.2. Activity of Acid Phosphatase in various tissues of *V. cyprinoides* var. cochinensis (Large) during acclimation to different salinities.

Values expressed as mg p-Nitrophenol formed/mg protein/hr ± SD.

Salinity	Foot			Mantle		
	Activity		% of control	Activity		% of control
	Mean	SD		Mean	SD	
0	1.24 ±	0.41	100.0	1.08 ±	0.10	100.0
10	0.39 ±	0.03	31.7*	1.25 ±	0.02	115.3*
20	1.04 ±	0.05	84.1	1.52 ±	0.05	140.6*

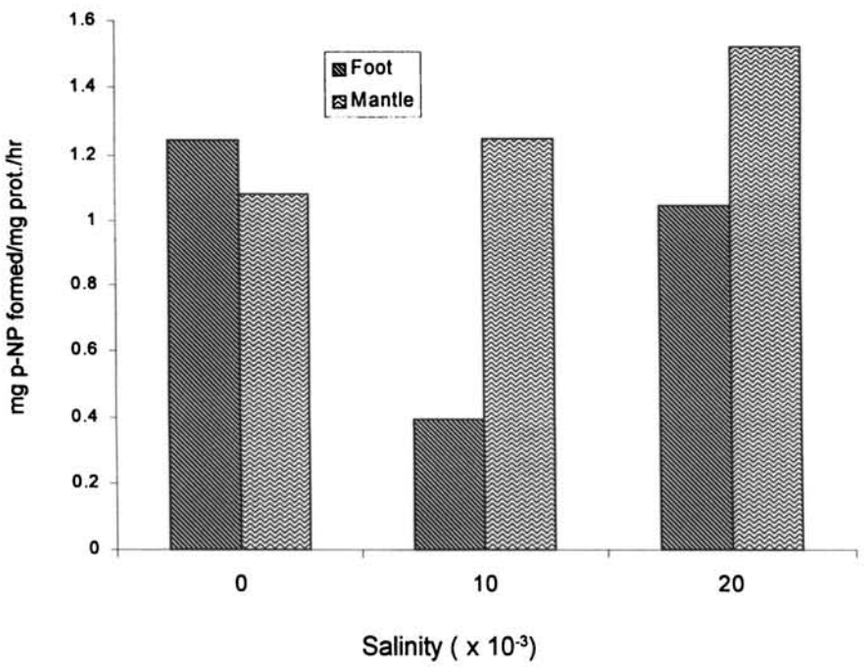


Fig 5.2: Activity of acid phosphatase in *V. cyprinoides* var. cochinensis (Large)

case, the activity in small size group was slightly higher than that in large size group (Tables 5.1 & 5.2, Fig. 5.1 & 5.2).

Difference in activity could be observed between tissues of both the size groups in elevated salinities.

Lysosomal Lability Index (LLI) is a measure of the lysosomal lysis and is obtained

by calculating the ratio of the amount of acid phosphatase present in the soluble fraction to that in the lysosomal fraction in the tissue. In general it is observed that the lysosomal lability increased as the salinity increased to 20 x 10^{-3} in the two tissues of both the size groups, except in the foot of small size group. Foot tissue of small size group showed maximum lability at salinity 10 x 10^{-3} and a slight decrease was observed at salinity 20 x 10^{-3}. Maximum lability was found in

Table 5.3. Lysosomal Lability Index (LLI) of *V. cyprinoides* var. cochinensis (small size group) during acclimation to different salinities.

Salinity	Foot	Mantle
	Mean SD	Mean SD
0	0.27 ± 0.09	0.18 ± 0.07
10	0.48 ± 0.13*	0.05 ± 0.00*
20	0.28 ± 0.06	0.34 ± 0.09*

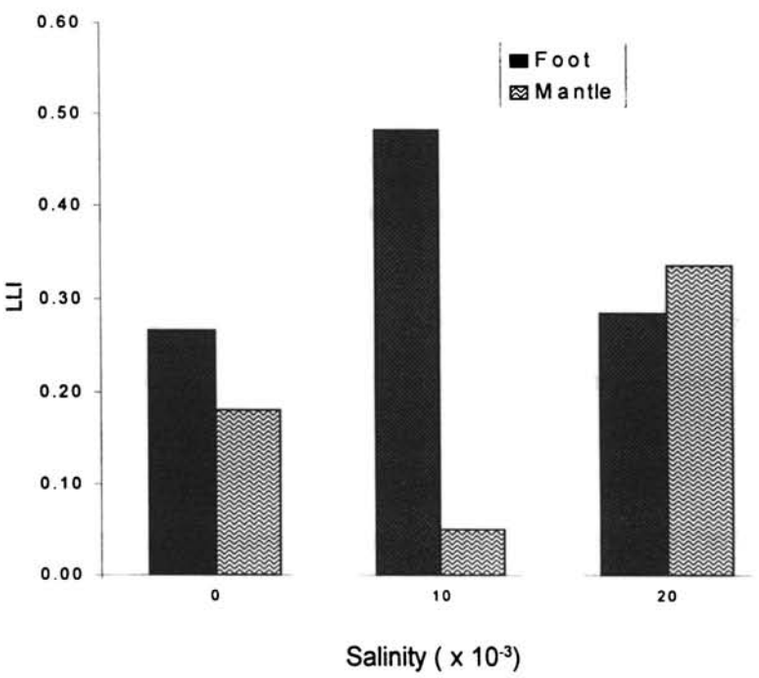


Fig 5.3: Lysosomal Lability Index *V. cyprinoides* var. cochinensis (Small)

Table 5.4. Lysosomal Lability Index (LLI) of *Villorita cyprinoides* (large size group) during acclimation to different salinities.

Salinity	Foot		Mantle	
	Mean	SD	Mean	SD
0	0.26 ±	0.08	0.12 ±	0.04
10	0.05 ±	0.01*	0.08 ±	0.01
20	0.97 ±	0.24*	0.13 ±	0.02*

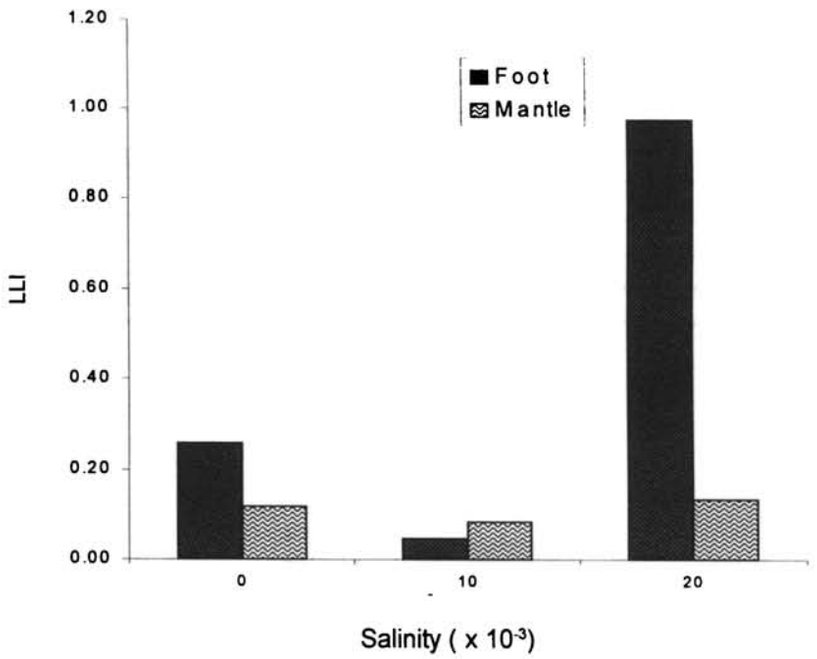


Fig 5.4: Lysosomal Lability Index *V. cyprinoides* var. cochinensis (Large)

foot tissue of large size group at salinity 20 x 10^{-3}. It was observed that the increase in lability is more in the foot tissue in both the size groups (Tables. 5.3 & 5.4, Fig. 5.3 & 5.4).

Glutamate pyruvate transaminase (GPT) activity in both the tissues of small size group increased as the salinity increased to 10 x 10^{-3}. Activity showed a decline as the salinity increased further from 10 x 10^{-3} to 20 x 10^{-3}, but in the case of mantle, it was higher than the control. In large size group, foot showed a slight reduction in activity at 10 x 10^{-3} salinity ($P<0.05$). At 20 x 10^{-3} salinity, significant

difference in activity could be observed only in the mantle of small size groups.

Differences in GPT activity between size groups could be noted in foot at 10 x 10^{-3} and 20 x 10^{-3} salinities and mantle at 0 x 10^{-3} and 10 x 10^{-3} salinities. Foot tissue of small size group showed higher activity, when compared to large size

Table 5.5. Activity of Glutamate Pyruvate Transaminase in various tissues of *V. cyprinoides* var. cochinensis (Small) during acclimation to different salinities.

Values expressed as mg hydrazone formed/mg protein/hr ± S D.

Salinity	Foot		Mantle	
	Activity	% of control	Activity	% of control
	Mean SD		Mean SD	
0	17 ± 2	100.0	2 ± 1	100.0
10	20 ± 1	117.6*	20 ± 3	1000.0*
20	16 ± 2	94.1	11 ± 2	550.0*

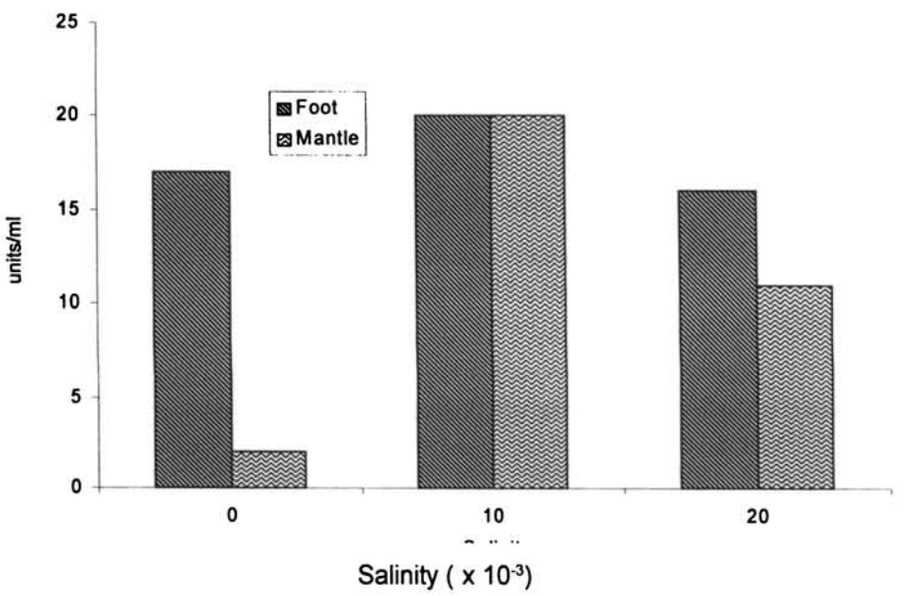


Fig 5.5: Activity of glutamate pyruvate transaminase in *V. cyprinoides* var. cochinensis (Small)

Table 5.6. Activity of Glutamate Pyruvate Transaminase in various tissues of *V. cyprinoides* var. cochinensis (Large) during acclimation to different salinities.

Values expressed as mg hydrazone formed/mg protein/hr ± SD.

Salinity	Foot		Mantle	
	Activity	% of control	Activity	% of control
	Mean SD		Mean SD	
0	16 ± 4	100.0	12 ± 5	100.0
10	10 ± 3	62.5*	11 ± 3	91.7
20	13 ± 1	81.3	11 ± 3	91.7

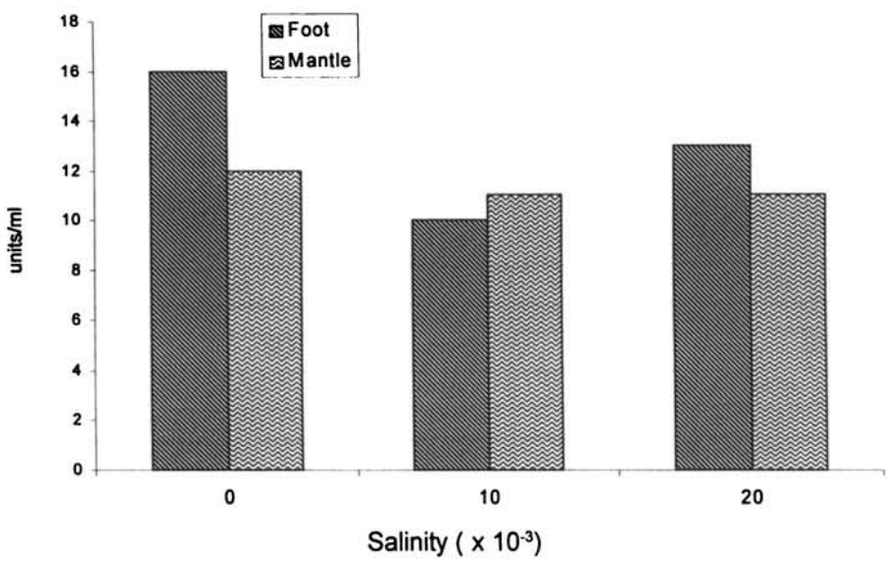


Fig 5.6: Activity of glutamate pyruvate transaminase in *V. cyprinoides* var. cochinensis (Large)

group. There was a difference in activity among the mantle tissue of large and small size groups at control conditions. Mantle of large size groups showed a higher content of GPT (Tables 5.5 & 5.6, Fig. 5.5 & 5.6).

Between tissues in the same size group, significant difference in activity was noticed only in small size groups at 0 x 10^{-3} and 20 x 10^{-3} salinities. Foot showed higher activity than mantle in these salinities ($P<0.01$).

Activity of cathepsin in the foot tissue of small size group showed an increase at 20 x 10^{-3} salinity. A steady increase in activity of this enzyme could be observed in the mantle tissue of the same size group. There was no significant difference in activity at 10 x 10^{-3} when compared to that of control. On the contrary, increase in activity was significantly high in both the tissues of large size group at salinities 10 x 10^{-3} and 20 x 10^{-3} compared to that of control. Mantle tissue of large size group at 20 x 10^{-3} showed a seven fold increase in cathepsin activity (Tables 5.7 & 5.8, Fig. 5.7 & 5.8). Variation in activity between size groups was significant

Table 5.7. Activity of Cathepsin in various tissues of *V. cyprinoides* var. cochinensis (Small) during acclimation to different salinities.
Values expressed as mg tyrosine formed/mg protein/hr ± SD.

Salinity	Foot			Mantle		
	Activity		% of control	Activity		% of control
	Mean	SD		Mean	SD	
0	0.126 ±	0.006	100.0	0.109 ±	0.030	100.0
10	0.108 ±	0.020	85.7	0.126 ±	0.021	116.0
20	0.174 ±	0.011	138.1*	0.198 ±	0.051	182.3*

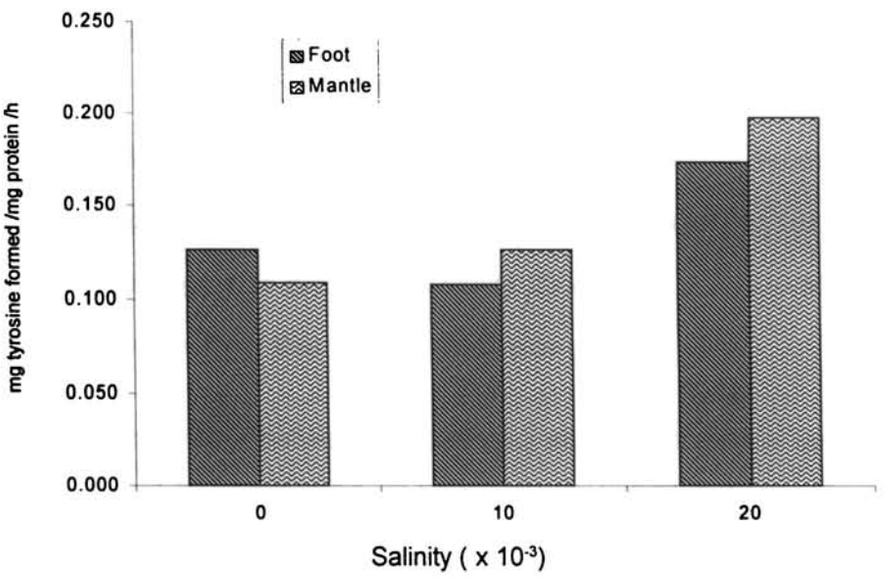


Fig 5.7: Activity of cathepsin in *V. cyprinoides* var. cochinensis (Small)

Table 5.8. Activity of Cathepsin in various tissues of *V. cyprinoides* var. cochinensis (Large) during acclimation to different salinities.

Values expressed as mg tyrosine formed/mg protein/hr ± SD.

Salinity	Foot			Mantle		
	Activity		% of control	Activity		% of control
	Mean	SD		Mean	SD	
0	0.026 ±	0.002	100.0	0.035 ±	0.002	100.0
10	0.058 ±	0.001	225.6*	0.078 ±	0.010	224.1*
20	0.103 ±	0.042	397.7*	0.271 ±	0.006	777.6*

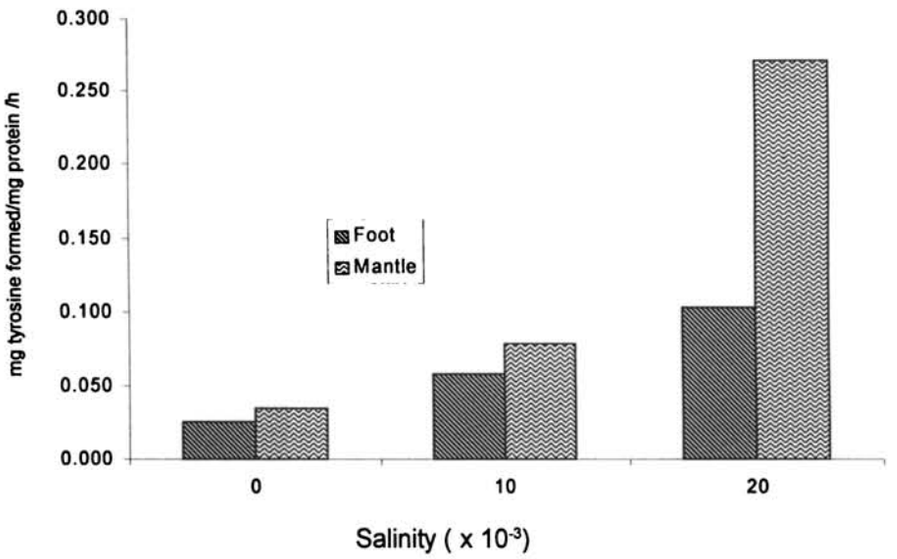


Fig 5.8: Activity of cathepsin in *V. cyprinoides* var. cochinensis (Large)

for both foot and mantle at all salinities experimented. Tissues of small size group except foot tissue at 20 x 10^{-3} salinity showed higher activity when compared to large size group. At salinity 20 x 10^{-3} foot tissue of large animals showed higher activity than that of small size group.

Difference in activity between tissues was significant only in large size group and in small size group, no significant difference in cathepsin activity between foot and mantle tissues could be observed. In the large size group, mantle tissue showed higher activity compared to foot.

Significant difference in activity of Na^+-K^+ ATPase could be observed between salinities in the gills of both small and large size groups. Activity of the enzyme

Table 5.9. Activity of Na^+-K^+ ATPase in the gills of *V. cyprinoides* var. cochinensis during acclimation to different salinities.

Values expressed as μ moles of Pi formed/mg protein/hr ± SD.

Salinity	Small		Large	
	Activity	% of control	Activity	% of control
	Mean SD		Mean SD	
0	1.056 ± 0.012	100.0	0.874 ± 0.018	100.0
10	0.046 ± 0.008	4.4*	2.963 ± 0.037	339.0*
20	0.224 ± 0.014	21.2*	3.880 ± 0.012	443.9*

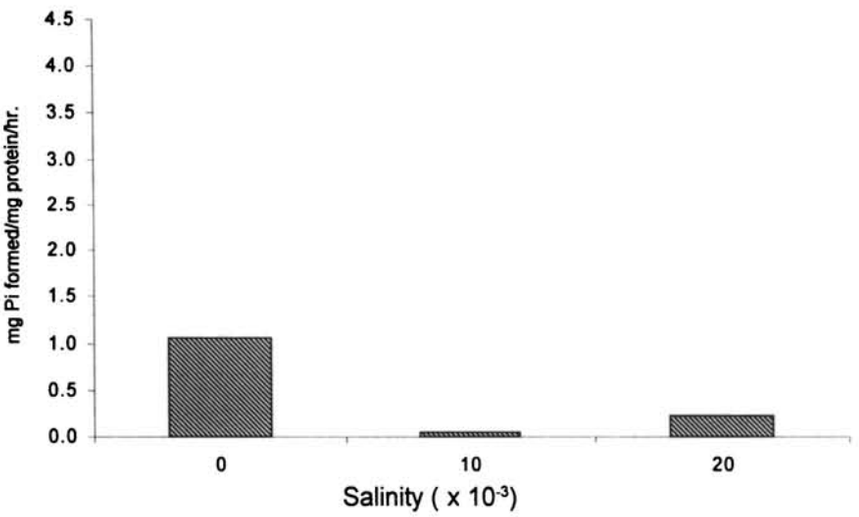


Fig 5.9: Activity of Na+-K+ ATPase in *V. cyprinoides* var. cochinensis (Small)

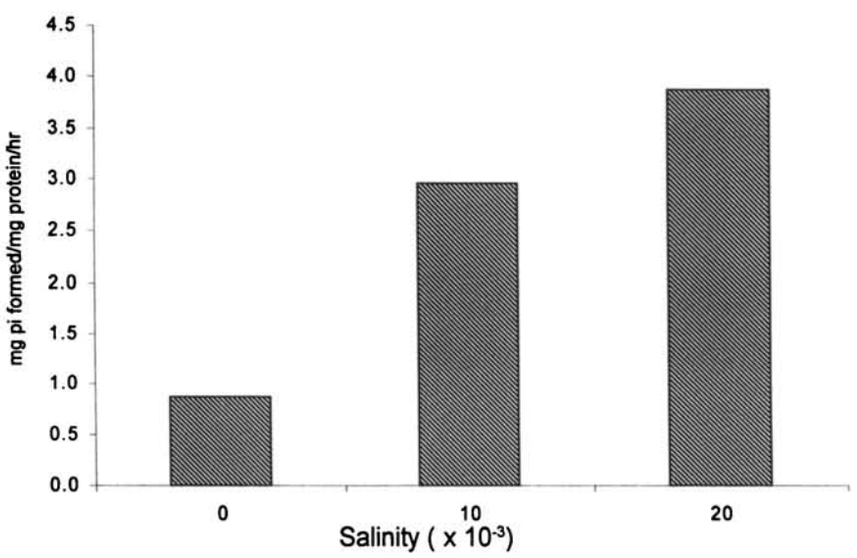


Fig 5.10: Activity of Na+-K+ ATPase in *V. cyprinoides* var. cochinensis (Large)

decreased slightly with an increase in salinity in small size group. The least activity was found at salinity 10 x 10^{-3} in small size group. In the large size group, there was a marked increase in activity of this enzyme with an increase in salinity (Tables 5.9, Fig. 5.9 & 5.10).

From the comparison of activity of Na^+-K^+ ATPase between size groups, it could be observed that the enzyme activity was higher in large size group than in the small size group.

Protein content, in general showed a decrease in the three tissues of both the

Table 5.10. Protein content of tissues of *V. cyprinoides* var. cochinensis (Small size group) acclimated to various salinities. Expressed as percentage of dry weight.

Salinity	Adductor		Foot		Mantle	
	Mean	SD	Mean	SD	Mean	SD
0	92.3	± 5.1	80.0	± 5.1	91.2	± 1.5
10	94.7	± 1.4	77.3	± 1.5	74.0	± 0.9
20	81.3	± 3.6	70.9	± 2.2	70.6	± 3.2

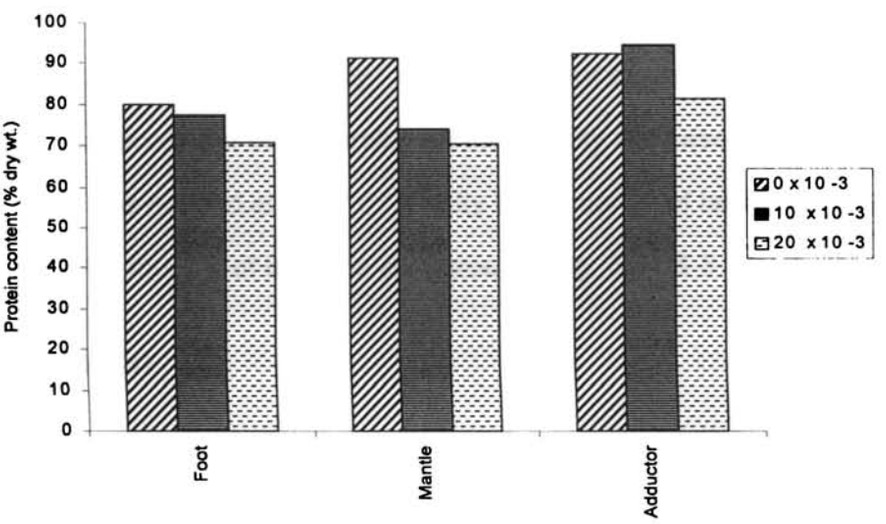


Fig 5.11: Protein content in various tissues of *V. cyprinoides* var. cochinensis (Small) acclimatized to various salinities

Table 5.11. Protein content of tissues of *V. cyprinoides* var. cochinensis (Small size group) acclimated to various salinities.

Expressed as percentage of dry weight.

Salinity	Adductor		Foot		Mantle	
	Mean	SD	Mean	SD	Mean	SD
0	77.0	± 6.5	82.8	± 1.9	85.8	± 1.4
10	94.7	± 1.9	82.8	± 1.1	88.8	± 2.6
20	88.8	± 2.4	79.9	± 3.5	79.9	± 1.4

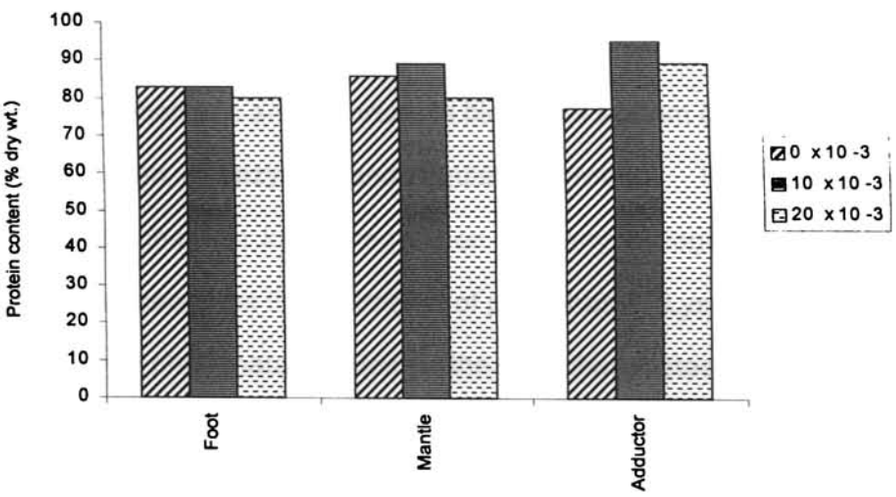


Fig 5.12: Protein content in various tissues of *V. cyprinoides* var. cochinensis (Large) acclimatized to various salinities

size groups at 20 x 10^{-3} salinity. Protein content of mantle tissue of the small size group decreased considerably as the salinity increased. A slight increase in content was noticed at 10 x 10^{-3} salinity in the mantle of large size group and in the adductor muscle of both the size groups (Fig. 5.11 & 5.12).

5.5 Discussion

Regulation of intracellular osmolytes is the tactic for osmoregulation in most of the euryhaline bivalves. Eventhough intracellular osmolyte pool is composed of both organic and inorganic components, the most manipulated are the organic

components. It is known that the quality and quantity of organic osmolyte components inside the cell is different from that of the extracellular fluid. The advantage of regulation of organic nitrogenous osmolytes over regulation of inorganic osmolytes is that they do not interfere with protein structure and function over a wide range of their concentration (Yancey *et al.*, 1982; Somero and Bowlus, 1983). Therefore, they are called compatible or non-perturbing osmolytes (Kwon & Handler, 1995).

Protein phosphatases are important in biological system because they catalyze phosphorylation of enzymes and regulatory proteins. Phosphorylation is an important mechanism in the regulation of many biological processes. Acid phosphatase catalyses the hydrolysis of phosphates. Lysosomes are the major source of acid phosphatase. Most of the acid phosphatases are non-specific in nature. An increase in acid phosphatase activity during hyper osmotic stress indicates a regulatory mechanism involving phosphorylation. In the present study, mantle tissue showed an increase in the activity of acid phosphatase with increase in salinity. Mantle tissue is very thin and has a large surface area in contact with the medium and hence is more vulnerable to osmotic rupture under stress. Eventhough foot muscle contains a higher amount of ninhydrin positive substance, very rapid buildup of NPS is necessary in the case of mantle tissue. This may be achieved by the increased activity of enzymes and also lysosome mediated lysis. Variation in mechanisms of osmoregulation between tissues of the same animal is also pointed out by Kettunen, *et. al.* (2001). Deaton, *et. al.* (1984); Hawkins and Hilbish, (1992); Weber, *et. al.* (1992) have pointed out that protein breakdown generates the ninhydrin positive substance during hyperosmotic volume regulation. Protein concentration of the mantle tissue decreased considerably compared to foot tissue, especially in small animals. This substantiates lysosome

mediated proteolysis in the mantle tissues of small animals during hyperosmotic stress. George (1993) also has reported an increased lysosome activity in *S. scripta* and *P. viridis* acclimatized to higher salinities. Labilization of lysosomes as a high salinity adaptation in marine molluscs, accompanied by an increased production of amino acids from proteins mediated by lysosomal proteases, has been reported by Bayne *et. al.*, (1981) and Stickle *et al.*, (1985). A reduction in activity of acid phosphatase is noted in the foot tissue of small animals. With the present data, it is difficult to explain this observation.

Glutamate pyruvate transaminase catalyses the transamination between glutamate and pyruvate to yield alanine and μ-keto glutaric acid. U.P. Kelavkar and H.S. Chhatpar (2001) have found that the yeast *Aspergillus repens* grown in a medium with sodium chloride exhibited an increased activity of glutamate dehydrogenase (http://www.fgsc.net/fgn39/ kelav.html). In the present study, GPT activity increased to a significant extent in mantle tissue of small animals at 10 x 10^{-3} salinity. Beyond this salinity, a decrease in activity was noticed. As discussed in the previous chapter, alanine is one of the major intracellular osmolytes. A considerable amount of buildup of alanine during hyperosmotic stress was observed in the mantle tissue of small animals. This can be correlated with the increased activity of GPT. GPT helps in the conversion of pyruvate to alanine by transamination. This can help the animal to remove the pyruvate from the metabolic pool and to shift the equilibrium and yield more pyruvate from glycolysis or from other pathways. In the foot tissue of small size group, activity was high at 0 x 10^{-3} and 20 x 10^{-3} salinity, compared to that of mantle in these salinities. This observation indicates a pathway of transamination for generation of ninhydrin positive substances in small animals. Since small animals are in the phase of growth and their metabolic pathways are more dynamic than in large animals, transamination may be the most suitable means of intracellular osmolyte

generation. Detailed investigation is required for deriving a vivid picture in this respect.

In the case of large animals, no correlation of GPT activity with salinity could be observed even though enzyme activity was found in both the tissues.

Cathepsins cleave proteins into smaller fragments which are then hydrolysed to their amino acid units. Cathepsins are usually lysosomal enzymes with a molecular weight of 58,000. It has been already proven that in molluscs, protein breakdown occurs under hyperosmotic stress. This proteolysis is in part mediated by lysosomes. Proteolysis complemented by amino acid synthesis enhances the input to the ninhydrin positive substance pool.

Our studies on cathepsin activity in mantle and foot tissues of *V. cyprinoides* revealed an increase in activity of the enzyme with salinity of acclimation in both small and large size groups. Activity in the tissues studied, of small size group was high in all the salinities. Variation in activity with salinity was not pronounced. It seems that in small size groups, cathepsin mediated proteolysis is of lesser magnitude when compared to that of large size groups. In the case of large animals, a 8-10 fold increase in activity could be observed at 20 x 10^{-3} salinity, whereas in the case of small animals, the increase was only 1.7 fold. This suggests that large animals depend more on a proteolytic pathway for the buildup of intracellular organic osmolyte pool during a hyper osmotic stress.

Studies on protein content of tissues in relation to salinity adaptation support the degradation-synthesis hypothesis of amino acid/derivatives during salinity acclimatization. Mantle tissues of small animals showed maximum reduction in protein content with increase in salinity. In the case of large animals, the difference in protein concentration was inconsistent. In *Mytilus edulis*, Bishop *et al.*, (1981)

showed a 10-15% decrease in the protein concentration of tissues studied during hyperosmotic stress. But the variation between size groups is not known. Nirchio and Perez, (1997) found that the increase in amino acid pool of *Crassostrea rhizophorae* exposed to hyper saline media produced a decrease of about 20% in concentration of protein.

The presence of an ATPase (ATP phosphohydrolase, E.C. 3.6.1.3) was first demonstrated in the crab's myelinated nerves, which is activated by Na^+, Mg^{2+} and K^+ (Skou, 1957). It is well known that freshwater organisms have relatively higher hemolymph osmoconcentration of ions. A decrease in activity of Na^+-K^+ ATPase with an increase in salinity is reported by Jensen, *et. al.* (1998). This reflects the decreased necessity of active intake of ions from the concentrated medium. On the other hand, Jensen *et al.*, (1998) has reported that the activity of Na^+-K^+ ATPase remained constant with an increase in salinity in the European sea bass *Dicentrarchus labrax* (L.). These observations indicate that the activity of the enzyme is modified in accordance with the requirement of the animal. For osmoregulation, active absorption by gill plays an important role as do renal salt reabsorption and water secretion (Mantel & Farmer, 1983, Lucu, 1990). Shiro Horiuchi (1977) reported that in the crab *Cardisoma guanhumi*, the enzyme is involved in an ionic regulatory mechanism. A slight increase in the activity of this enzyme is indicative of the ability of the animal to regulate transport of ions across the gill membrane in response to a change in ionic concentration in the external medium. O. Kinne (1971) opined that Na^+-K^+ ATPase activity is not implicated in the mechanism, which allows euryhaline animals to withstand rapid changes in salinity. They rather may be part of a mechanism, which assists osmoregulation during long-term acclimation. Hence the manipulation in the activity of this enzyme is not of great help for adjusting to short-term salinity changes; rather it can help

the animal during seasonal changes in salinity.

A synoptic overview of the results indicates the importance of enzymes in intracellular osmoregulation. It can be seen that the system in totality is tuned to cope up with the altered salinity. Apart from the regulation of inorganic ions by Na^+-K^+ ATPase activity, intracellular organic osmolyte pool is strengthened by the activity of various enzymes. It is reported that the activity of enzymes are modified by the inorganic ions like Na^+ and K^+. Further investigation is required to unravel the mechanism. Recent studies on osmoregulation are focused on the molecular levels of regulatory aspects. Osmolarity responsive elements have already been isolated. Since salinity acclimation is a sum of behavioural, morphological and biochemical changes, a detailed study of the mechanisms is essential. An understanding of the mechanisms at molecular level can help in rendering a thorough explanation for euryhalinity.

Chapter 6

SUMMARY AND CONCLUSIONS

Among the aquatic biomes, estuary is one of the most dynamic and unstable. Organisms surviving in this environment are specialists in adapting to the ever changing physico-chemical factors of the environment. Osmotic variation is the most challenging for estuarine animals. Osmoticity of the medium varies with tidal cycle, season and region. Strategies of osmotic and ionic regulation will be more efficient in the organisms dwelling in this regime. In the process of adaptation, behavioral, extracellular regulatory and intracellular regulatory mechanisms work together. It is absolutely logical to state that these mechanisms of estuarine animals will be coordinated and streamlined for faster adaptation to the changing ambient environment.

Experimental animals used for the present study, *Villorita cyprinoides* var. cochinensis, are widely distributed in the upper reaches of the Cochin estuary. This clam is used for human consumption and as feed in aquaculture. Shells of the clam are used as raw material for cement and lime industry. In the present project, an attempt was made to study the salinity tolerance mechanisms of the species and to compare and contrast the data obtained with marine forms, which had been subjected to study by other workers.

The first aspect investigated was the behavioral adaptation since this helps the animal in escaping and protecting its soft tissues from drastic and transient changes in salinity. Valve closure helps in streamlining the physiological machinery to the altered environment. As the animals were transferred to higher salinity, they closed their valves and gradually the valves were opened resulting in an increase in ionic content of the mantle cavity fluid. Increase in ionic content was then reflected in the hemolymph. In the present study, it was found that the time taken for acclimation to higher salinities was more in the aged animals and the younger ones could acclimatize faster. In the case of both small and large size group, the period of valve adduction was maximum in salinity 20 x 10^{-3} followed by 15 x 10^{-3}, 10 x 10^{-3} and 5 x 10^{-3} salinities respectively. It is clear that valve closure is adopted by the different size groups for preventing the exposure of soft body parts to change in salinity. Gradual exposure of the soft tissues helps in conditioning the cellular machinery to reach a new equilibrium with the altered environment. Compared to *Sunetta scripta*, a typical marine form studied, it could be observed that the time taken by *V. cyprinoides* to adapt to the extreme salinity is lesser. Thus the period of valve closure is lesser in *V. cyprinoides*. This helps the animal to minimize the difficulties (anoxia and accumulation of metabolic wastes) arising from valve closure. This will be of great help to the animal, since it lives in the estuaries and is exposed to diurnal fluctuations in salinity besides the drastic seasonal variations.

Ionic concentration and osmolarity of the mantle fluid and hemolymph showed an increase with an increase in ambient salinity. It was found that at lower salinities, the animal is capable of extracellular ion regulation irrespective of the age, whereas at higher salinities the capability is limited and is more efficient in the small size group. Acclimation experiments have showed that the total intracellular free NPS

concentration increased with an increase in acclimation salinity in pooled tissue samples analysed of both the age groups. A reduction in the rate of excretion of ammonia-nitrogen and an elevated rate of oxygen consumption were noticed at higher salinities. Ionic content and the osmolarity of the blood suggests that the contribution of amino acids on blood osmolarity is meagre, at the same time, they contribute considerably to the tissue osmolarity. Above 15 x 10^{-3} salinity, a reduction in free NPS content was noticed. This suggests the switch over to an alternate mechanism or different osmolyte for intracellular osmoregulation at higher salinities. Even though the NPS content decreased above 15 x 10^{-3} salinity, oxygen consumption continued to increase and the ammonia excretion continued to decrease. This strongly supports the view that an alternative mechanism is in operation at higher salinities. It could also be inferred that the nitrogenous wastes are recycled rather than expelled at elevated salinities. Some other mechanism is reported to be in operation at elevated salinities which require further studies in this animal.

It was found that there was no significant difference in the concentration of total NPS between size groups. This is because of the fact that these animals are estuarine and are exposed to more frequent and drastic fluctuations in salinity, whereas the marine forms suffer lesser fluctuations. Small and large size groups were equally competent in the capability of osmotic regulation whereas in marine forms, large size groups acclimatized slower than the smaller group to a changed salinity.

Tissue specific differences were observed in both qualitative and quantitative content of amino acids. Foot tissue was found to contain higher concentration of amino acids compared to other tissues followed by mantle and adductor. In the case of marine forms, adductor muscle contained high content of amimo acids followed by foot and

mantle. In both estuarine and marine forms studied it could be observed that mantle employs a lesser quantity of amino acids compared to adductor and foot. It is observed that the regulation of cell volume is not carried out equally in all types of tissues, rather the regulation is prioritized to those tissues which are functionally impaired to a considerable extent. This prioritization saves substantial amount of energy. The difference in the amino acid content of various tissues may be attributed to this.

Qualitative analysis have shown that the amino acids for osmoregulation are selected carefully so that they do not interfere with the normal cellular functions. Simpler and non-essential amino acids with pKa values close to neutrality (alanine and glycine) were found to be abundant. The role of these amino acids as osmoprotectants in marine animals have already been reported. Alanine is formed by utilizing ammonia and the reaction is catalysed by alanine dehydrogenase. Pyruvate also is converted to alanine by transamination or amination reactions. Pyruvate is a product of anaerobic respiration. This has special significance since during the periods of valve closure, pyruvate formed can be converted to alanine which helps in the removal of pyruvate and at the same time yielding an osmolyte. Compared to the marine forms, the number of amino acids selected for osmoregulation was less in *V. cyprinoides*. Taurine which is an abundant osmolyte did not contribute to the osmolyte pool of *V. cyprinoides*.

Studies on the mechanisms of the regulation of intracellular osmolytes revealed that various enzymes related to metabolism contributed to the manipulation of the NPS pool. Acid phosphatase was found to play an important role in the mantle tissue, the tissue that is thin and that needs rapid NPS build-up. Increased activity of acid phosphatase also indicates an increased lysosome lysis. Other lysosomal enzymes, preferably proteases, also may contribute to the build-up of intracellular osmolarity.

Activity of Glutamate Pyruvate Transaminase also increased with an increase in salinity. This can contribute to the increased alanine concentration in the tissues, since GPT converts pyruvate to alanine. Cathepsin, which is a proteolytic enzyme was found to play a role in both the size groups studied. In the large size group, the increase was around ten fold. Proteolytic pathway of NPS generation was further supported by the studies on protein concentration in the tissues. Maximum reduction in protein concentration was found in the mantle tissue, where a rapid buildup in osmolytes is needed. Study also revealed that gill Na^+-K^+ ATPase activity is manipulated according to the requirement of the animal.

It can be concluded that the capability of salinity tolerance is an aggregate of both the capabilities of extracellular anisosmotic and intracellular isosmotic regulations in osmoconforming animals. Both the mechanisms are found to be complementary – at salinities closer to optimum, the former predominates and at more altered salinities, intracellular regulation is at help. Intracellular osmolytes are quantitatively less diverse in *V. cyprinoides* var. cochinensis, when compared to the marine forms. But the time taken for acclimation to a salinity stress is lesser in *Villorita* compared to the marine forms like *Sunetta scripta*. These suggest that the osmoregulatory machinery of estuarine animals is more streamlined for a successful life in the estuarine regime.

6.1 Future Avenues of Research

Since the regulation of osmolarity in the tissues is the basis of water regulation, osmolarity of the tissues should be studied to derive a vivid picture of the mechanisms of regulation. In the present study, it was found that the concentration of total NPS decreased beyond salinity 15 x 10^{-3}. The mechanisms and osmolytes used at higher salinities beyond this salinity is worth experimenting. Gills play an important role in the

regulation of inorganic ions and it is reported to be the main site ATPase mediated ion transport. More investigations are to be carried out in this tissue.

The ultimate aim of water regulation is to regulate the cell volume. It is observed that there occur slight changes in cell volume even in osmoregulators. Ultrastructural studies are to be undertaken for understanding the volume change during osmotic stress in different tissues. These studies can also help in revealing the changes brought about in the cellular organelles like lysosomes, which were found to have a role in the osmoregulatory process.

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Short Communication

Oxygen consumption, ammonia excretion and total ninhydrin positive substances in black clam *Villorita cyprinoides* (Pelecypoda) exposed to various salinities

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Changes in the rate of oxygen consumption, ammonia excretion and the tissue content of total ninhydrin positive substances in the black clam, *Villorita cyprinoides*, were studied during the exposure of the animal to salinities 0, 5, 10, 15 and 20 ppt. The content of total ninhydrin positive substances was found to increase with an increase in ambient salinity, maximum concentration was found at 15 ppt. Rate of oxygen consumption also showed an increase with increase in salinity. Rate of excretion of ammonia nitrogen decreased with an increase in ambient salinity. The role of ninhydrin positive substances in the osmoregulation is depicted and the existence of a degradation-synthesis balance for the regulation of osmotic effector pool is suggested.

Salinity fluctuation is one of the major challenges posed by brackish water organisms. Maintenance of homeostasis requires regulation of osmotic pressure in the body. Since marine and brackish water bivalves are osmoconformers, each cell must cope with the osmotic stress imposed by salinity change[1]. Several works conducted in marine bivalves have shown that nitrogenous osmolytes, especially free amino acids are the major and varied osmolytes during osmotic adaptation. The study is an attempt to understand the pattern of oxygen consumption and ammonia excretion of black clam *Villorita cyprinoides*, during salinity stress which will point to the strategy of intracellular osmoregulation. The animal, commonly known as the black clam, has got economic importance both as food and as raw material for industries like cement.

Specimens of *Villorita cyprinoides* (Gray) (Pelecypoda) were collected from Poochakkal estuary, near Cochin, cleaned and sorted into two size groups based on shell length—small (2.5 ± 0.5 cm) and large (3.5 ± 0.5 cm). Animals were acclimatized in the lab at 0 ppt salinity for one month and were fed on cyanophycean alga *Synechocystis salina*. Total amount of Ninhydrin Positive Substances (NPS) in the tissues were estimated from the pooled mantle, adductor muscle and foot tissues excised from the animals acclimatized for one month to desired salinities, 0, 5, 10, 15 and 20 ppt. Seawater of above salinities were made by dilution of filtered seawater with deionized water. The tissues were excised, blotted, weighed and homogenised in a mortar for 7 minutes after adding a definite volume of 0.6*M* perchloric acid. The homogenate was then centrifuged twice at 24 h intervals in a high speed refrigerated centrifuge at 20,000 g for 20 minutes to separate and remove proteins. The amount of NPS in the extract was estimated by ninhydrin method[2] with glycine as standard.

To give a salinity stress, animals ere transferred to water of desired salinity (0, 5, 10, 15 and 20 ppt) prepared as mentioned above. Animals were then left undisturbed for a few hours. After observing that the animals were performing normal filtration, estimations for oxygen and ammonia were made for 4 h at 1 h intervals. For determining the oxygen consumption, apparatus devised by Mohan & Cherian[3] was used. Oxygen was estimated by Winkler's method[4]. Total amount of ammonia nitrogen in the medium was estimated by phenol hypochlorite method[5]. The results from six separate experiments were statistically analyzed.

There is an increase in total NPS content of the tissues with increase in salinity (Fig. 1, Table 1). The difference in the total NPS content between size groups is insignificant in all salinities except at 10 ppt. At 10 ppt salinity (Table 2), tissues of small animals contained more NPS than the larger ones. Results of the rate of oxygen consumption of the two size groups at different salinities are shown in Fig. 2. The rate of oxygen consumption increased with increase in salinity in both the size groups. Large and

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Table 1—Correlation matrix-between salinity, ammonia excretion, oxygen consumption and total NPS of large size groups and small size groups

Large size groups	*Salinity*	*Ammonia*	*Oxygen*	*NPS*
Salinity	1			
Ammonia	–0.9215	1		
Oxygen	0.9654	–0.8750	1	
NPS	0.8777	-0.8143	0.7707	1
Small size groups				
Salinity	1			
Ammonia	–0.9916	1		
Oxygen	0.7180	–0.7594	1	
NPS	0.9042	–0.9106	0.4491	1

Table 2—Results of *t*-test between size groups

Salinity (ppt)	Oxygen consumption	Ammonia excretion	Total NPS content
0	9.1150 [a]	1.1060	2.6063 [b]
5	8.5966 [a]	0.3573	1.9728
10	12.1347 [a]	1.7787	78.9383 [a]
15	7.8439 [a]	4.9421 [a]	2.4619
20	4.4687 [a]	8.6509 [a]	0.3832

a-highly significant, b – significant at 95% level, no symbol-not significant

small animals showed significant difference in oxygen consumption. Large animals consumed more oxygen at 5, 10, 15, and 20 ppt where as at 0 ppt, small animals consumed more oxygen than the large ones. There is good correlation between the rate of oxygen consumption and salinity (at 99% confidence level in large size group and 90% in small size group) (Table 1). Rate of excretion of ammonia nitrogen decreased with increase in salinity in both the size groups (Fig. 3). This is inversely correlated with the increase in total NPS in the tissues (99.9% in both the size groups) (Table 1). Significant difference in the excretion rate between size groups was noted only at salinity 15 and 20 ppt (Table 2).

Villorita cyprinoides inhabits the fresh water ends of estuaries. Estuarine species have broader physiological tolerance than the marine or fresh water species[6]. Previous report[7] indicates that the animal is capable of tolerating wide ranges of salinity under laboratory conditions (0-27 ppt). Bivalves are not known to regulate blood osmotic pressure substantially when exposed to osmotic stress. Since their capability of extracellular regulation is less, they make intracellular osmotic adjustments to tide over transient and constant salinity changes. More than 50% of the intracellular osmolytes are composed of amino acids and other NPS[8,9]. NPS content of the

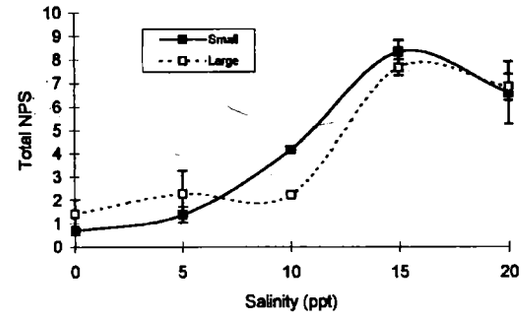

Fig. 1—Variation in total NPS (mg glycine eq/g wet body weight) in the pooled samples of Mantle, Foot and Adductor muscle tissues of *Villorita cyprinoides* acclimatized to different salinities.

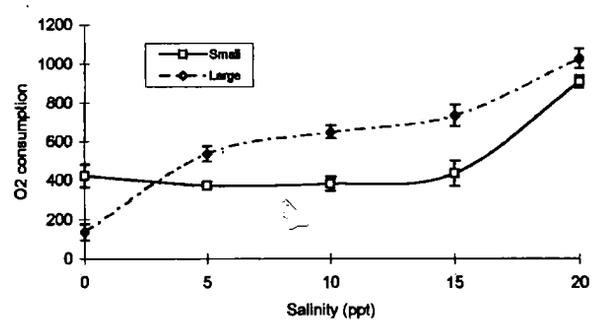

Fig. 2—Rate of oxygen consumption (μL O_2 consumed/g dry body weight/h) of *Villorita cyprinoides* at different salinities during acclimation.

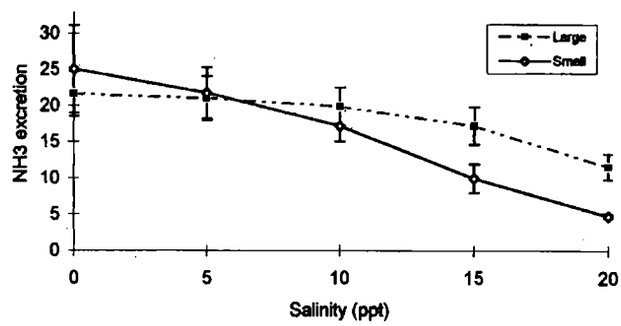

Fig. 3—Rate of excretion of NH_3-N (μg /g dry body weight/h) by *Villorita cyprinoides* at different salinities during acclimation.

tissues of *Villorita* increased with increase in salinity (Fig. 1, Table 1). Previous studies have already revealed the importance of nitrogenous solutes in the intracellular osmoregulation of marine bivalves [6, 10-12]. It is evident from the present results that the nitrogenous compounds (especially the NPS) play an important role in maintaining the cell osmolarity of this freshwater loving animal also. A decrease in the total NPS content is noticed after 15 ppt salinity.

Eventhough Sivankutty & Shynamma[7] reported that the animal can tolerate up to 27 ppt salinity under experimental conditions, in natural habitats, these animals are seldom found beyond 15 ppt. The decrease in total NPS content after 15 ppt indicates the possibility of breakdown of the cellular machinery responsible for maintaining higher levels of intracellular NPS. This aspect requires further probing.

When an osmotic stress is imparted, metabolic machinery is accelerated, and this accounts for the higher rate of oxygen consumption. Decreased excretion rate of ammonia may be due to the decreased catabolism of amino compounds or enhanced removal of NH_4^+ from the blood for the synthesis of amino acids and their derivatives for isosmotic regulation as suggested by Karam *et al*[13]. Isosmotic intracellular regulation of many euryhaline molluscs is paralleled by the modification in ammonia excretion. Emerson[14] observed a significant increase in excretion rate of NH_3-N in *Macoma inconspicua* adapted to 50% seawater. Conversely, *Villorita,* when adapted to higher salinity showed a decrease in NH_3-N excretion. This observation suggests the possibility of existence of a degradation-synthesis balance for intracellular osmolyte regulation in both large and small size groups of this animal. The lesser rate of excretion of NH_3-N is well correlated with the increased NPS in the tissues. Catabolism of amino acids in a hypo osmotic environment is documented by Bishop *et al*,[15] and Hayashi[16]. However, the cellular regulation mechanisms of the osmolytes in euryhaline species is not simple and is yet to be clarified[17]. The qualitative composition of the NPS pool of different tissues is to be focused to know more about the regulatory mechanisms.

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