THE ECOPHYSIOLOGY OF IRON AND VANADIUM ACCUMULATION BY NORTH ATLANTIC ASCIDIANS

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The ecophysiology of iron and vanadium accumulation

by North Atlantic ascidians

By
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Abstract

Some ascidians (Tunicata, Ascidiacea) accumulate high levels of iron and/or vanadium in their tissues, the latter being very unusual among animals. Previous work has investigated aspects of the vanadium accumulation by Phlebobranch ascidians, including the identification of the "vanadocytes," the oxidation state of the vanadium and the pH in the vacuole in which it is held, and a proposed reduction mechanism for vanadium. The iron dynamics of these animals have received comparatively little attention. There is also a lack of data on the ecophysiological aspects of ascidian metal accumulation. This thesis examines the seasonal variation in vanadium and iron concentrations of two Phlebobranch ascidians, *Ciona intestinalis* and *Ascidia callosa*, which accumulate vanadium and iron, and a Stolidobranch ascidian, *Halocynthia pyriformis*, which accumulates iron only. Experiments examined whether these metal concentrations are responsive to increased food availability and dissolved vanadium concentration.

An HPLC method was developed to simultaneously measure vanadium and iron in large numbers of biological samples. Using this method, a study of the metal concentrations of some tissues of *C. intestinalis* from Woods Hole, USA, found that the vanadium and iron concentrations in the hemocytes and other tissues was higher in March than in the fall or summer sampling periods, corresponding with high activity of a key enzyme of a proposed reductive pathway (G6PDH). A feeding experiment investigated whether food availability/particle concentration affects the vanadium and iron concentrations of *C. intestinalis*. While there were few differences in metal concentrations among high and low food groups, vanadium levels were maintained and iron levels rose compared to animals sampled fresh from Woods Hole. The former may be indicative of low vanadium loss rates and the latter of the importance of dietary sources of iron.

This pattern was confirmed by a year long study of the vanadium and iron concentrations of tissues of two ascidians from Newfoundland, Canada. *A. callosa,* a high level vanadium accumulator, exhibited a peak in the vanadium and iron concentrations of the hemocytes and other tissues in spring prior to the spring plankton bloom. This corresponded with an increase in both the total number of circulating hemocytes and the proportion of cells containing reduced vanadium. *H. pyriformis*, a non vanadium accumulator, exhibited a peak in the iron concentration of some tissues at the spring bloom. A vanadium enrichment experiment indicated that *A. callosa* can respond to increased environmental vanadium concentration. Vanadium concentration of the hemocytes and other tissues initially increased, followed by a marked decline, although remaining elevated above that of control animals. The vanadium exposed animals also displayed an increase in the total number of circulating hemocytes and the number of vanadocytes in all sampling periods. The vanadium levels used in this experiment did not cause an increase in the vanadium concentration of *H. pyriformis*.

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James Watson advises scientists to "avoid boring people." My friends and lab mates in the Driedzic lab are the polar opposite of boring. Their friendship and intellectual engagement made graduate studies a far more enriching experience, inside the lab and out, and we will be friends for life.

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Abbreviations

ASW	artificial sea water
BP	2'2, -bypyridine
DOPA	3, 4-dihydroxyphenylalanine
FSW	filtered sea water
G6PDH	glucose-6-phosphate dehydrogenase
GLM	general linear model
ICPMS	inductively coupled plasma mass spectrometry
MCs	morula cells
NAD	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide reduced form
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	nicotinamide adenine dinucleotide phosphate reduced form
PAR	4-(2-pyridylazo)-resorcinol
RP-HPLC	reverse phase high pressure liquid chromatography
sd	standard deviation
SRCs	signet ring cells
TC	tunichrome
TOPA	3,4,5-trihydroxyphenalanine

Co-authorship statement

With little modification, with the exception of section 1.8, Chapter 1 was published as a book chapter with Joy Stacey as the sole author:

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All other chapters will be submitted for publication with W.R. Driedzic as co-author. Supplement 2 was additionally coauthored with Lindan Tao, Memorial University CREAIT.

Chapter 1: Ecophysiological perspectives on the chemistry and biochemistry of vanadium and iron accumulation by ascidians

1.1 Introduction

The accumulation of vanadium by some ascidian species has attracted the attention of chemists and biochemists for over a century, perhaps owing to the rarity of the phenomenon in the animal kingdom. Some of these benthic marine invertebrates accumulate vanadium at levels up to 350 mM in circulating hemocytes (Michibata *et al.,* 1986; Trivedi *et al.,* 2003). Recent research on the biochemical, physiological, and toxicological effects of vanadium makes the natural accumulation of this metal all the more intriguing (Cam *et al.,* 2000; Chasteen 1999; Crans *et al.,* 2004; Mukherjee *et al.,* 2004; Slebodnick *et al.,* 1997). Ascidians also accumulate high amounts of iron in their tissues but this phenomenon has received relatively little attention. There is much about the accumulation of these metals by ascidians that remains unknown. Neither the phenomenon of vanadium accumulation nor the differences among the major groups have received widespread attention among invertebrate physiologists in recent decades.

1.1.1 Vanadium and iron in the biosphere and biological systems

Vanadium (V) and iron (Fe) are transition metals, in groups 5 and 8 of the periodic table, respectively. Vanadium enters the environment from natural weathering of rock or through pollution from oil processing and combustion. In seawater vanadium is typically found in concentrations of 20 to 35 nM (Butler, 1998b) but can reach locally higher concentrations in polluted areas or after oil spills (Baars, 2002).

Vanadium has a complex oxidation and reduction chemistry. Vanadium can exist in the V(II), V(III), V(IV) and V(V) oxidation states depending upon pH as illustrated in Figure 1.1. The V(III), V(IV) and V(V) oxidation states of vanadium form stable complexes with a variety of ligands (Kustin *et al*, 1983). Under most physiological conditions (pH 6-8), when concentrations are lower than 20 mM, vanadium exists as a mixture of the V(V) (vanadate) species $H_2VO_4^-$, HVO_4^{2-} , $HV_2O_7^{3-}$ and $V_3O_9^{3-}$ (Figure 1.2), often referred to as metavanadate (Chasteen, 1983; Redher, 1991). At higher vanadate concentrations, the $H_2VO_4^-$, HVO_4^2 species predominate (Chasteen, 1983). In seawater, over a pH range of 6-8, vanadate exists as monovanadate, $H_2VO_4^-$ (Wilsky 1990, Rehder, 1991). Vanadate tends to complex with glycols and catechols (Chasteen, 1983). In aqueous solutions, vanadium (IV), vanadyl, exists primarily as VO^{2+} and binds strongly to proteins. Vanadyl is oxidized by air to form vanadate at pH 2-3 and above. In acid, the predominant vanadium species are V^{3+}, VO^{2+}, and *cis*-VO^2 (Figure 1.1). Vanadium(II) is considered to be too reducing to be stable in a biological system (Chasteen, 1983.) Vanadium (III) is not common among biological systems but is the predominant oxidation state in most ascidians that accumulate vanadium (Kustin *et al*, 1983).

Although its exact role is unknown, vanadium is considered to be an essential trace for mammals and birds. Vanadium is present in the active site of enzymes such as the bromoperoxidases and chloroperoxidases of marine algae and fungi and in the amavadine in *Amanita* spp. mushrooms, where it may be involved in electron transfer (Soedjak and Butler, 1990). Vanadate is a structural analog of phosphates and thus inhibits phosphatases, phosphorylases and ATPases, resulting in toxicity in large

doses. However, it also behaves as an insulin mimic and in small doses has been considered as a treatment for diabetes (Cam *et al.*, 2000; Li *et al.*, 1996). For comprehensive reviews of these phenomena, see Crans *et al.* (2004), Mukherjee *et al.* (2004) and references therein.

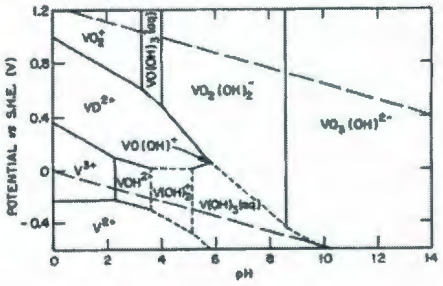

Figure 1.1: The speciation of vanadium as a function of pH. Not all known species are represented on the diagram. (reproduced from Chasteen, 1983).

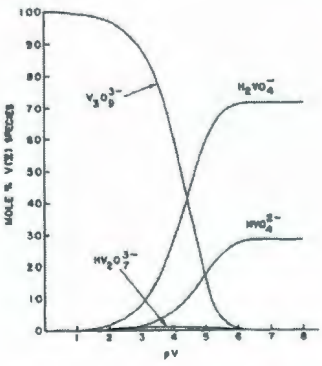

Figure 1.2: Distribution of vanadate species as a function of total vanadium concentration. $pV = \log[V]_{total}$ where $[V]_{total} = 3[V_3O_9^{3-}] + 2[HVO_4^-] + [HVO_4^{2-}]$ (reproduced from Chasteen, 1983).

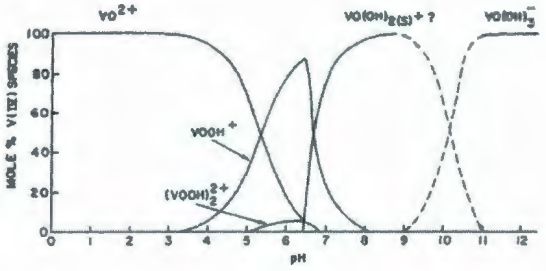

Figure 1.3: Distribution of vanadyl species as a function of pH where the total concentration of vanadium is 10 μM. (reproduced from Chasteen (1983)

Iron's roles in biological systems are well known and for most organisms it is an essential element. In association with proteins, iron is utilized as an oxygen trapper in heme proteins and in enzymes such as the cytochromes (Kendrick *et al.*, 1992). Iron is stored in some organisms as ferritin or bacterioferritin. The concentration of iron in seawater is 0.02 to 1 nM (Butler, 1998a and references therein). Iron is present in the Fe (II) and Fe(III) oxidation states in seawater, with Fe^{2+}, and $FeCO_3$ accounting for 75% and 23% of the total iron content respectively (Milero, Ao, and Aicher, 1995).

1.1.2 Ascidian phylogeny and basic biology

Ascidians are the sessile benthic class Ascidacea of the subphylum Tunicata, Phylum Chordata. While the relationships among the Tunicata and other chordates are undergoing revision (Berrill, 1930; Dehal *et al.*, 2002; Delsuc *et al.*, 2006; Stach and Turbeville, 2002; Turon and Lopez-Legentil, 2004; Zeng and Swalla, 2005), most

authors agree that there are three main groups (suborders) of ascidians: Stolidobranchia, Phlebobranchia and Aplousobranchia. This is based on morphological characteristics such as the complexity of the branchial basket, with aplousobranch ascidians having the simplest with only transverse vessels between rows of stigmata, phlebobranchs having papillae projecting from the transverse vessels and connections to longitudinal vessels, and stolidobranchs having longitudinal vessels and internal longitudinal folds.

The position of the gonads has prompted placing of the stolidobranchs into a separate order, the Order Pleurogona, apart from the phlebobranchs and aplousobranchs which together form the Enterogona. The gonads are positioned on the body wall of the former and associated with the digestive system in the latter (Van Name, 1945). Figure 1.4 is a recent phylogeny constructed using 18S RNA (Zeng and Swalla, 2005). The families Corellidae, Cionidae, Ascidiidae are in the Phlebobranchia whereas the families Pyuridae, Styelidae and Molgulidae are in the Stolidobranchia. There is no consensus on the placement of the families Cionidae or Diazonidae, with some authorities placing these families in the Aplousobranchia (Turon and Lopez-Legentil, 2004). Although detectable in the Stolidobranchia, vanadium has been found in high concentrations only in the aplousobranchs and phlebobranchs, with species of the phlebobranch family Ascidiidae containing the highest amounts (Table 1.1). Members of all groups accumulate high amounts of iron in their tissues.

Phlebobranch and stolidobranch body forms include colonial and solitary (stalked and unstalked) morphs. Representative ascidian morphology is shown in Figure 1.5. The

general physiology of ascidians was reviewed by Goodbody (1974). Ascidians pump water into the body cavity through an incurrent siphon via a ciliated branchial basket or sac. They pump large volumes of water through their branchial cavities, and are capable of very high pumping rates, reaching rates 5 to 10 times higher than those of marine mussels per volume of oxygen consumed (Petersen, 2007). *Ciona* sp. can pump water at a rate of 11 L $h^{-1}g^{-1}$ dry mass (Petersen and Riisgard, 1992). Particles between 0.5 and 100 µm diameter are retained with high efficiency and are trapped in the mucus net, secreted by the endostyle (Fiala-Medioni, 1978a). The mucus and particles trapped within are transported to the esophagus and ingested. Water and feces are squirted out of the body periodically through the exhalant siphon. IN the absence of phytoplankton blooms, inorganic particles can sometimes be the dominant particles consumed.

The circulatory system of ascidians consists of an open ended contractile tubular "heart" which is noted for its periodic reversal of direction of flow. The main nitrogenous waste of protein metabolism of ascidians is ammonia, which they excrete in the exhalant water (Goodbody, 1957, 1965) Members of several families of ascidians store uric acid from purine metabolism in concretions, in large sacs, or in the case of the Ascidiidae, in numerous small vesicles (sometimes referred to as renal vesicles) close to the digestive tract (Goodbody 1965; Lambert *et al.,* 1998; Lambert and Sanamyan, 2001).

Table 1.1: Vanadium and iron concentrations of ascidian tissues from selected representative species from Michibata *et al.* (1986)

	Vanadium concentration ($ng.mg^{-1}$ dry weight)	Iron concentration ($ng.mg^{-1}$ dry weight)
Phlebobranchia		
Family Cionidae *Ciona intestinalis*		
Tunic	1.7 ± 0.4	370.0 ± 39.4
Branchial basket	337.5 ± 47.8	251.4 ± 27.6
Gonad	100.9 ± 7.5	65.8 ± 8.9
Hemocytes	330.7 ± 14.1	424.4 ± 49.4
Plasma	0.4 ± 0.2	89.5 ± 4.8
Family Ascidiidae *Ascidia sydneiensis samea*		
Tunic	30.6 ± 2.6	1432.6 ± 291.1
Branchial basket	730.4 ± 95.2	299.0 ± 46.4
Gonad	339.9 ± 22.1	310.5 ± 56.4
Hemocytes	4675.9 ± 353.5	1702.1 ± 222.2
Plasma	27.6 ± 4.5	114.3 ± 10.8
Stolidobranchia		
Family Styleidae *Styela plicata*		
Tunic		
Branchial basket	2.6 ± 0.5	289.4 ± 20.3
Gonad	0.5 ± 0.1	139.1 ± 23.2
Hemocytes	1.3 ± 0.3	67.4 ± 6.3
Plasma	3.5 ± 0.5	1095.0 ± 117.6
	1.3 ± 0.4	176.3 ±10.2
Family Pyuridae *Halocynthia aurantium*		
Tunic		
Branchial basket	1.3 ± 0.2	83.1 ± 8.1
Gonad	0.8 ± 0.1	155.2 ± 18.4
Hemocytes	0.3 ± 0.04	76.3 ± 16.2
Plasma	2.2 ± 0.2	245.3 ± 18.9
	0.9 ± 0.2	54.0 ± 3.1

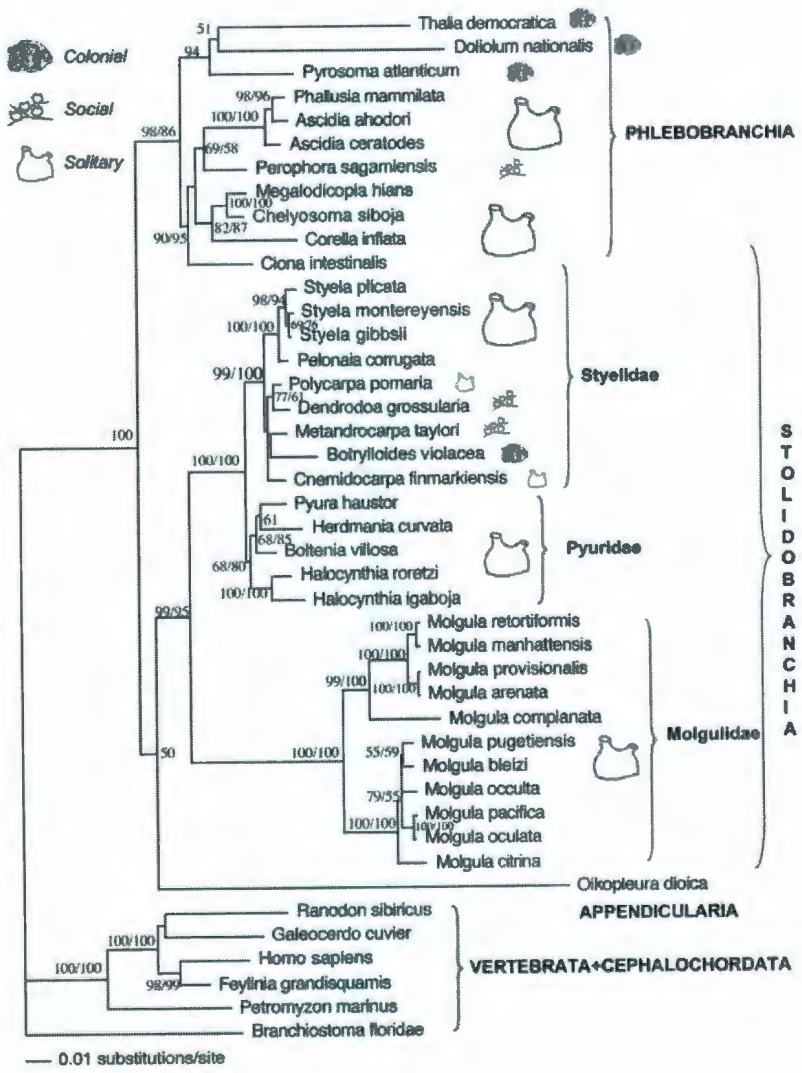

Figure 1.4: Phylogenetic relationships within the Tunicata from analysis of 18S RNA sequences from Zeng and Swalla (2005)

Solitary and colonial ascidians reproduce sexually but sometimes undergo asexual reproduction by budding. Ascidians are hermaphroditic but self-fertilization is unusual, prevented by a variety of physiological and molecular mechanisms (Bates, 2005). Although some species brood their larvae (vivipary), in general ascidians shed their eggs and sperm into the seawater where fertilization takes place. In most parts of the world, spawning is seasonal, usually timed at some interval before the major plankton blooms (Bates, 2005; Goodbody and Fisher, 1974; Howes *et al.,* 2007). After some time, the free-swimming planktonic larvae settle on an appropriate substrate and metamorphose into the sessile adult form, developing the tunic for which the Tunicata are named. Vanadium is present in the eggs (Michibata *et al.*, 1992). Settled larvae begin to accumulate vanadium very early in development (Botte *et al.,* 1979; Nette *et al.*, 1998).

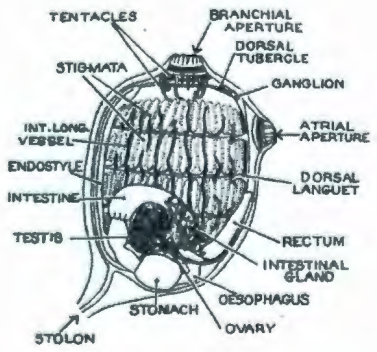

Figure 1.5: General ascidian anatomy. Water is drawn into the branchial cavity through the branchial aperture (inhalant siphon), passes over the branchial basket and is expelled through the atrial aperture (exhalent siphon). Reproduced from Van Name (1945).

1.2 History of the study of vanadium accumulation by ascidians: controversies and consensus

With the development of an array of methods for the quantification of chemical constituents of animal tissues early in the 20th century (Blair, 1908; Finn, 1906; Koch 1910; Perez-Bustamante de Monasterio, 1990) chemists such as Henze began surveys of the levels of vanadium and other metal in the animals. Henze (1911) found that phlebobranch ascidians collected from the Bay of Naples contained large concentrations of vanadium, iron, and sulfur in the coelomic fluid. During the century that followed, research on these animals focused on aspects such as the phylogenetic differences within the Ascidiacea, the oxidation state of vanadium, the identity of the true "vanadocytes", the pH of these vanadocytes, the identity of the reducing agent/pathway and identification of vanadium binding proteins.

Henze's (1911) work revealed that the coelomic fluid of the phlebobranch, but not stolidobranch, ascidians contains large amounts of vanadium which was highest in the members of the family Ascidiidae. Numerous authors have confirmed this finding (Carlisle, 1968; Hawkins *et al.,* 1983; Michibata *et al.*, 1986). Attention then turned to the identity of those cells in the phlebobranch coelomic fluid that contain the vanadium and the nature of the ligation environment in which vanadium is held. Ascidian coelomic fluid contains many cell types responsible for metabolite storage, phagocytosis, immune response, etc. (Endean, 1960). The morula cell, a multi-vacuolated cell resembling a mulberry, was misidentified as the cell type containing the most vanadium as a result of the misinterpretation of the meaning of osmium

tetroxide staining (Webb, 1939; Endean, 1960). The word "vanadocyte" in early literature should be treated with caution as it is often used to refer to the morula cell. The morula cells contain the chromogen tunichrome, a DOPA containing pigment, which gives the coelomic fluid its characteristic yellow to pale orange color. Some *in vitro* experiments with tunichrome suggested that it might be capable of reducing vanadate and since it was thought that vanadium was also present in these cells, tunichrome was suggested as a possible reductant (Bruening *et al.*, 1985; Oltz *et al.*, 1988; Ryan *et al.* 1996).

The use of density gradient centrifugation led to the discovery that the signet ring cell (SRC), so named because a single large vacuole displaces the nucleus to the periphery, contains the most vanadium of the cells in the coelomic fluid (Michibata *et al.,* 1991a). There was some debate regarding the acidity of these cells and whether tunichrome, being absent from them, could still be important in the reduction pathway (Bayer *et al.*, 1992; Bruening, *et al.,* 1985; Lee *et al.,* 1990; Macara *et al.,* 1979a; Ryan, Grant, and Nakanishi, 1996). Experimental evidence employing more sophisticated techniques led to the consensus that the vacuoles of SRCs are acidic and that most of the total coelomic cell vanadium is present there and in the V (III) form (Brand *et al.,* 1987; Dingley *et al.*, 1982; Taylor *et al.*, 1994.) Much of the discussions surrounding tunichrome as the vanadium reducing agent centered on the presence of both in the same cell. The hypothesis of tunichrome as a vanadium reductant now been largely discarded with the identity of the SRCs as the main vanadocytes, but it is possible that vanadium and tunichrome interact in some other way.

After the identity of the SRC as the vanadocyte, attention focused on it for the study of the process of vanadium reduction. Recently, several vanadium binding proteins, referred to as vanabins, have been located and characterized in the plasma and hemocytes (Hamada *et al.,* 2005; Kanda *et al.,* 1997; Ueki *et al.*, 2003a; Yoshihara *et al.*, 2005). Enzymes of the pentose phosphate pathway have been localized within the SRCs (Uyama *et al.,* 1998b; Ueki *et al.*, 2000). In *vitro* evidence suggests that NADPH produced by this pathway may be capable of reducing vanadium (Kanamori *et al.*, 1999). The current hypothesis of vanadium uptake and reduction, articulated by Michibata and colleagues (2003) is that vanadate is taken from up from water through the branchial basket, bound, and partially reduced by plasma vanabins, and then taken into the SRCs where it is reduced by NADPH produced by the pentose phosphate pathway (Figure 1.6). A vacuolar-ATPase generates the proton gradient that allows the vanadium to be held in the V(III) oxidation state (Ueki *et al.,*1998; 2001; 2003c).

Some of the processes involved in the extraction of vanadium from seawater and concentration into SRCs are relatively well studied. However, where and how vanadium exerts its biological action is unknown. Advances in this area are hindered by a lack of thorough knowledge of the interactions between, relationships among, and fate of, the cells in which the vanadium is contained. Many of the ideas regarding the function of vanadium in ascidians have arisen from the observations of the presence of vanadium and hemocytes (or the remnants thereof) in certain areas of the body and vanadium's unpalatability or toxicity.

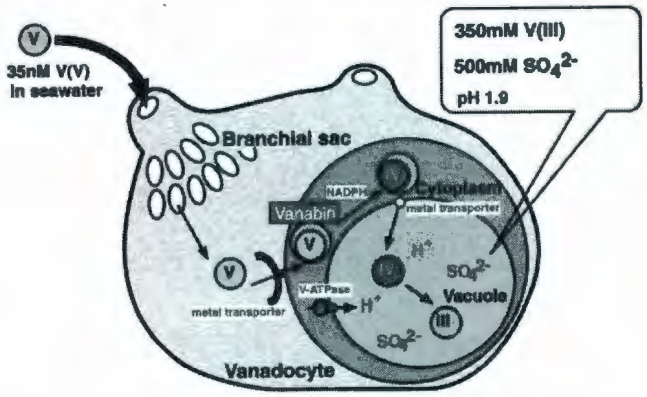

Figure 1.6 (above): Pathway of vanadium accumulation and reduction by ascidians proposed by Michibata and colleagues (2003). Vanadate dissolved in seawater is taken up at the branchial basket, reduced to vanadium (IV) by vanabins and held in a single acidic vacuole as V(III) in the signet ring cells, the low pH of which is maintained by a vacuolar ATPase.

1.3 Proposed roles of vanadium in ascidians

This thesis is not directly focused on the role of vanadium or iron in ascidians. However, no discussion of the phenomenon is complete without mention of some of the proposed functions. Some of the proposed roles of vanadium or iron accumulation might be illuminated by studies of seasonality.

1.3.1 Anti-predatory/antifouling

The use of vanadium as a defense against fouling organisms or predators has been hypothesized by several authors. The rationale has usually been that given the toxicity of vanadate, the presence of vanadium in the tunic of ascidians may be an antifouling/antipredatory defense (Stoecker, 1978). Free vanadate can have toxic effects, being able to perturb even ascidian enzyme systems, but *in vivo* there is little vanadium in the V (V) oxidation state (Guerrieri *et al.,* 1999; Kono *et al.,* 1987; Michibata *et al.,* 1989). Many studies of the palatability and defense aspects of vanadium in phlebobranchs or of other compounds in other ascidians have made use of feeding tests to demonstrate the unpalatability of ascidians. These feeding tests did not adequately mimic field conditions, however, giving predators food pellets treated with ascidian extracts, or the appropriate concentration of vanadium, for example (Tarjuelo *et al.*, 2002; Teo and Ryland, 1994; Stoecker, 1980). However, as shown in Table 1.1, the tunics of most phlebobranchs do not have a high concentration of vanadium in comparison to other tissues. Furthermore, some organisms such as sea urchins do consume the vanadium rich soft body parts of these ascidians, leaving the tunic (Simoncini and Miller, 2007).

The thickness and toughness of the ascidian tunic varies greatly, within both the Stolidobranchia and Phlebobranchia. For example, the tunic of *Ciona intestinalis* is relatively thin compared to that *Ascidia callosa* while that of *Halocynthia pyriformis* is very tough (Figure 1.7). These animals often exist within multispecies communities of invertebrates, the members of which exhibit varying levels of susceptibility to

predation. Predation risk will be influenced by foraging strategies and activities of predators in the context of these complex communities which may include prey other than ascidians, and ascidians with various characteristics including metal concentration and tunic thickness. There are no investigations of predator choice when presented with ascidians of various metal levels and tunic thicknesses and strength and color. Given that predators attack ascidians through their tunics and that hemocytes migrate to the site of an injury, it is possible that the vanadate is antimicrobial.

Figure 1.7 a) *Ciona intestinalis* (Phleobranchia) and
b) *Halcynthia pyriformis* (Stolidobranchia)

1.3.2 Tunic formation and tunichrome

The presence of hemocytes and their remnants in the tunic and their migration to sites of injury have also led to the hypothesis that the metals may be involved in tunic

synthesis (Anderson, 1971; Di Bella *et al.,* 1998; DeLeo *et al.,* 1997; Endean, 1953; Smith, 1970a). There is little discussion about the phylogenetic differences in metal accumulation when this possibility is considered. A secondary hypothesis might be that if a transition metal is required for tunic synthesis, iron might fulfill the same purpose in stolidobranchs.

The earliest protochordate from the Cambrian period had a tunic (Shu *et al.,* 2001). Tunicin is a polymer of sugars and is similar to bacterial cellulose (Krishnan, 1975). It has been hypothesized that tunicin synthesis was a result of lateral transfer of a cellulose synthase gene from bacteria or plankton >500 million years ago (Nakashima *et al.,* 2004; Sasakura *et al.*, 2005). Enzymes of the pentose phosphate pathway have been implicated in the lignification process of plants (Pryke and Rees, 1976). The SRCs of ascidian coelomic fluid contain enzymes of the pentose phosphate pathway (Uyama, *et al.*, 1998b) and phosphoglucomutase and phosphohexose isomerase were found in whole body of *Molgula* sp. (Sable and Calkins, 1954). Since the SRCs migrate to the tunic and expel their contents, it is possible that these enzymes may be involved in tunicin synthesis.

The identity of the "true" vanadocyte influenced the debates over the possible interactions between tunichrome and vanadium. The presence of vanadium in some morula cells and compartment cells, and the presence of the remnants of morula cells in the tunic, allow for the possibility that vanadium and tunichrome interact in the tunic (Nette *et al.,* 1999). Tunichrome is present in very early life stages and is likely essential in early tunic development (Robinson *et al.,* 1986).

A chemical parallel exists between the tunichromes in the tunic of ascidians and similar proteins in other benthic invertebrates. The tunichromes and ferreascidans in ascidians contain DOPA (3, 4-dihydroxyphenylalanine) functional groups which are also important in the proteins forming the byssus in blue mussels and adhesive proteins of barnacles (Taylor *et al.,* 1996; Wiegemann, 2005). Transition metals, especially iron, are involved in cross linking of proteins of the byssus and cement which allow mussels and barnacles to attach to their substrate (Sever *et al.*, 2004). In addition, variation exists among mussels in the metal employed in the cross linking. Some mussels such as *Septifer viragatus* employ manganese while others such as *Mytilus edulis* use iron (Tateda and Koyanagi, 1986). Taylor and colleagues (1994; 1997) pointed to the possibility of a similar function of the iron and vanadium accumulated by ascidians. It is possible that stolidobranch and phlebobranch ascidians use iron and vanadium, respectively, for crossing linking their tunicin, tunichrome or ferreascidans just as different mussel species use different metals in the byssus proteins.

The possible role of vanadium or iron in tunic synthesis is one that might be investigated, to some degree, by a study of the seasonal distribution of these metals in the tissues. Though not definitive, the hypothesis would be supported by an increase in these metals in the tunic during seasons of most active growth, compared to seasons of relatively slow growth.

1.3.3 Anaerobic metabolism and oxygen transport

The parallels between the multiple oxidation states of vanadium and iron and iron's role in oxygen carrying pigments such as hemoglobin, made oxygen transport an attractive hypothesis for the function of vanadium in ascidians. Carlisle (1968) suggested that vanadium, as well as the niobium and manganese found in some ascidians, are responsible for oxygen binding. Invertebrate biology texts still present this hypothesis (e.g. Brusca and Brusca, 2003). However, it is evident that there is no reversible oxygen binding in ascidian blood (Fisher, 1976; Macara *et al.*, 1979b). This negates an oxygen carrying function for either of the metals.

Smith (1989), noting the ability of ascidians to survive hypoxia, suggested anaerobic adaptation as a possible role for vanadium accumulation. A point of clarification needs to be made with respect to terminology from a physiological point of view. Physiologists use "anaerobic" to refer to specific metabolic pathways producing alternative end products of glycolysis such as lactate, or in the case of some invertebrates, the opines (Gade and Grieshaber, 1986; Isani *et al.* 1995). Smith (1989) argues that vanadium, owing to its various oxidation states, might be able to provide "alternative electron and proton sink(s), the final result being to generate a resilient tunic from polymerized tunichrome" and that the decrease in the availability of metals during the time in which ascidians were evolving led to the selection for high affinity metal ion transport systems (Smith, 1989). With regard to electron and proton sinks, it is has been demonstrated that ascidians posses lactate dehydrogenase and produce

lactate during periods of emersion or environmental hypoxia (Brennan *et al.*, 1995). The experimental work has not been completed to test Smith's hypothesis.

1.4 Iron accumulation by ascidians

Perhaps because iron is a common metal in biological systems, the iron accumulation by stolidobrancha and phlebobranchs has received little attention. Some authors have measured iron levels (Table 1.2), but there have been few directed studies of the iron dynamics in ascidians. The purpose of the very high iron concentrations of these animals is also unknown. Oxygen transport is an unlikely role as previously stated. There is a tacit assumption in some of the literature that the iron in stolidobranchs carries out the function fulfilled by vanadium in phlebobranchs. This is a plausible hypothesis if vanadium is involved in some fundamental shared biochemical activity. However, the lack of comparative physiological/metabolic data makes this hypothesis difficult to test.

The iron concentration of ascidian hemocytes and branchial basket is high, and as high as the vanadium concentration in some tissues in phlebobranch ascidians (Table 1.1.). While vanadium concentration is low in the plasma of all ascidians, iron is present in relatively high concentrations (Michibata, 1986). Work by Agudelo *et al.* (1983) with whole blood indicated that the iron is in the Fe(III) oxidation state in stolidobranch ascidian cells, and suggested that most of the iron is associated with the membranes. Experiments with *Pyura stolonifera* and *Styela clava* indicated that iron is primarily obtained from dietary rather than dissolved sources (Hawkins *et al.,* 1980;

Curtin *et al.*1985). This seems plausible considering the relatively high iron content of phytoplankton and the number of cells consumed by ascidians.

Several authors have examined cellular aspects of iron accumulation by stolidobranchs. SRCs have been noted in the stolidobranchs *Boltenia ovifera* and *Halocynthia aurantium* (Agudelo *et al.*, 1983; Smith, 1970b) but they do not contain vanadium and their role in metal accumulation is unknown. Stolidobranch ascidians have morula cells but there is some disagreement over whether they contain iron. While Endean (1953) found iron in the morula cells of *Pyura stolonifera*, Scippa and colleagues (1993) did not find it in the MCs of *Halocynthia papillosa.*

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Phylogenetic relationships may yield clues as to the evolutionary history of vanadium accumulation. To my knowledge, there is no consensus as to the vanadium status of the most recent common ancestor of the ascidian groups. An examination of the relationship of genes for metal binding proteins in stolidobranchs may be informative. Workers have found several iron binding plasma proteins of MW 17000, 31000, 26000, 41000 and 61000 in stolidobranchs (Agudelo *et al.*, Wang, 1983; Martin *et al.*, 1984) but these were identified before the proteomics era and their sequences are not available for comparison with those of the vanabins.

As early as Webb (1939) authors have noted the phylogenetic differences and proposed using vanadium accumulation as a defining characteristic of the Phlebobranchia, and iron-only accumulation as a defining trait of the Stolidobranchia. Hawkins *et al.* (1983) proposed that the predominance of vanadium (IV) be used as a

defining trait of aplousobranch ascidians. This conclusion was based in part, on work with *C. intestinalis*, and its placement, at that time, in the Class Aplousobranchia. As discussed, however, taxonomists have placed this species in the Phlebobranchia. By comparison there has been very little study of the vanadium and iron physiology of aplousobranch ascidians.

1.5 Ecophysiology of ascidians: possible effects on metal dynamics

As sessile ectotherms, adult ascidians cannot move out of unfavorable environmental conditions, and as such, many aspects of their physiology are influenced by physical aspects of their environment (Figure 1.8). Some of these relationships have been well studied because they are important in understanding the impact and role of ascidians in coastal marine ecosystems (Riisgard *et al.*, 1996; 1998). The pumping of water by ascidians supplies a medium for many important physiological functions. The circulation of water provides food, oxygen, iron and vanadium, and removes nitrogenous and digestive wastes (Goodbody, 1974). Because so many physical factors such as water temperature, oxygen concentration, metal concentration, particle type and concentration, and water currents affect pumping rate, and thus the amount of water processed by the animals in any given time period, they directly or indirectly affect the vanadium and iron dynamics. Because these physical factors change over time and space, vanadium and iron uptake, loss and concentration of ascidians are likely to vary on the same scales. Trace metal concentrations can fluctuate in near-

shore environments which are exposed to sporadic high inputs of elements through run off and upwelling events (Lares *et al.*, 2002).

The general effects of size, mass and temperature on metabolic processes in animals are well established (Gillooly *et al.*, 2001). Generally, the overall metabolic rate and the rate of most physiological processes increase with increasing mass and temperature in a non-linear relationship. This is true of the water pumping rate of ascidians (Fiala-Medioni 1978a; 1978b). Metabolic rate, measured by oxygen consumption, and pumping rates generally increase with mass or size with a scaling exponent of 0.7 to 0.8. Mass specific filtration rates decrease with increasing food concentrations while ingestion rates increase (Sigsgaard *et al.*, 2003). This can result in seasonal differences in feeding rates. The feeding rates of *Halocynthia papillosa*, are 3-4 times higher in July than in the winter (Ribes *et al*, 1998). As occurs in other ectothermic invertebrates, ascidian metabolic rates increase during feeding (Thompson and Bayne, 1974; Widdows, 1973). The magnitude of this increase can depend upon the dominant food source which changes with several factors, including the presence of plankton bloom.

Organismal factors can also interact with physical factors to influence vanadium physiology. For example, the timing of growth and reproduction varies spatially. In some areas, such as the North Atlantic, spawning occurs when particle concentrations and temperatures are low, while in the Mediterranean, temperature is still relatively high when spawning occurs (Lambert 2005). The cold water ascidians may experience a lowering of metabolic rate and pumping rate due to low temperatures

prior to spawning, which might influence the vanadium levels in their body and the amount available to put into the eggs compared to Mediterranean ascidians. Branchial basket complexity of ascidians, and the orientation of unstalked ascidians with respect to the water current also affects pumping rate (Bone *et al.*, 2003; Knott *et al.*, 2004), perhaps contributing to variation among ascidian taxa.

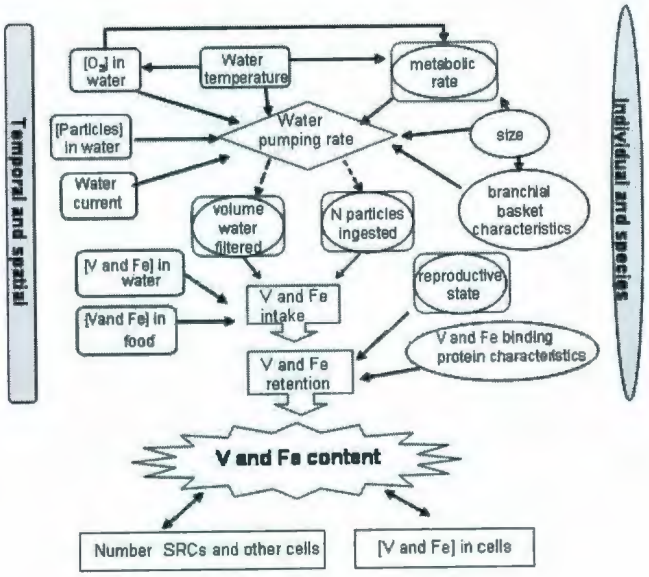

Figure 1.8: Ecophysiological relationships in ascidian biology that may influence vanadium and iron accumulation, and contribute to variation observed at the individual, species and temporal-spatial levels. Factors that affect water pumping rates will influence dissolved exposure to vanadium and iron. Factors that vary on temporal and spatial scales are indicated by a rectangle. Factors that vary on individual and species scale are indicated by round shapes.

1.6. Ecophysiological questions arising from past research

1.6.1 Variability in vanadium and iron concentration

Physiologists are keenly aware of the variation in biological traits, and use the responses of traits to changes in the internal and external environment to reveal information about the causes and mechanisms at work. There is a paucity of knowledge with respect to ecophysiological variation in the vanadium and iron concentrations, uptake and utilization by ascidians at the individual, population or species level.

Given the relationships between physiological and ecological variables highlighted in Figure 1.8, we might expect spatial-temporal and species/individual level variation in the vanadium and iron uptake and concentrations of ascidians. However, many previous studies have not taken these factors into account when reporting metal concentrations or characteristics and there are relatively few studies that focus on both metals. Table 1.2 presents a representative sample of various types of studies of metal accumulation by ascidians. In most cases, data such as the time of year, or the temperature at which animals were sampled or held is not included. This is understandable given that this was not the subject of interest to these researchers. However, if one wants to compare the same species from different parts of the world, for example, it may be important to know at what time of year the samples were taken. One of the most comprehensive surveys was Michibata's (1986) study

comparing stolidobranchs and phlebobranchs from Italy and Japan which did not indicate the time of year that samples were collected.

Several studies have focused on the variation in vanadium accumulation characteristics among ascidians. Frank and colleagues (2003) observed differences in speciation of vanadium between and among species, and among populations, located in two different bays in California. In the 1970s, Swinehart *et al.* (1974) observed inter-annual variation in the vanadium concentration of *Perphora* sp. Recently, Frank and colleagues (2006) detected very little to no vanadium in this species. Goldberg *et al.* (1951) noted differences in the vanadium concentration of *Ciona intestinalis* in April compared to July in California. The chromatographs in Taylor and colleagues (1995) suggest the possibility of seasonal differences in vanadium concentration along with that of the DOPA and TOPA proteins in blood extracts of *Ascidia ceratodes.* Michibata *et al.* (1984) examined variation in vanadium cointent of two species in the genus *Ciona.* There was a higher concentration of vanadium in the blood and branchial basket of *C. intestinalis* than in *C. robusta* (although the latter were collected from different areas of Japan) (Michibata, 1984).

Given that there are inter-annual and seasonal changes in water temperature and particle concentrations, and thus food supply, and that these directly affect pumping rate, there is good reason to suspect that there might be variation in iron and vanadium uptake, loss and concentrations at these scales. If one region experiences a spring plankton bloom at an earlier date than another, for instance, then even members of the

same species from those locations may exhibit some differences in their metal concentrations if they are collected at the same time.

1.6.2 Vanadium uptake and reduction

Ueki *et al.* (2001, 2003 a, b) have characterized vanadium binding proteins and the interaction of these proteins with vanadium. A yet unresolved question is to the relative importance of the contributions of ingested particles and dissolved vanadium to the total vanadium uptake. Phytoplankton and the inorganic particles consumed by ascidians contain some vanadium (Jackson and Morgan, 1978). Toxicological studies have shown that bio-kinetic tracer experiments are most useful in the delineation of the importance of dissolved and dietary pathways of metal uptake and loss. Although appropriate radiotracers of vanadium exist, their availability can be limited in some locations. During the time period of this thesis, for example, radiolabelled vanadium was not available through North American suppliers. This may account for the paucity of these types of studies. There have been no labeled food experiments to determine what proportion of the body load of vanadium is food derived. Recently, it has been found that glutathione-S-transferase from the ascidian gut binds vanadium and copper suggesting that dietary uptake is possible (Yoshinaga *et al.*, 2006).

If the branchial basket is the main site of vanadium uptake there must be membrane transport proteins that allow the vanadium to pass into the lumen, where they bind to the vanabins. Early whole body uptake experiments with *Ciona intestinalis* found

high vanadium uptake rates over the first 2-3 days followed by a slower uptake rate in the following 2-3 days (Goldberg, 1951). This biphasic uptake curve is consistent with this two step process. A study comparing vanadium and iron uptake in *Ascidia ceratodes*, *Ciona intestinalis* and S*tyela montereyensis* showed minimal difference in the uptake of vanadium by *S. montereyensis* and *C. intestinalis* (Cheney *et al.,* 1997). However, no attempt was made to perfuse the tissue of the blood cells. This is a confounding factor as the branchial basket is composed of a series of vessels containing hemocytes which stain positively for vanadium (personal observation). A study of the uptake of branchial baskets with these blood cells is therefore a study of two processes: uptake through the membrane of the branchial basket and accumulation and reduction processes in vanadocytes.

Within the populations of isolated blood cells, Michibata and colleagues (1991) found that compartment cells took up more vanadium (V) and (IV) than the morula or signet ring cells, and that within the whole cell population, the accumulation was biphasic (Michibata *et al.,* 1991b). It is unknown whether the composition of the cell population changed during the course of the uptake experiments but this knowledge might help to resolve the question of the ontogeny of the signet ring cell. Work by Nette *et al.* (2004) suggests that compartment cell vacuoles fuse to form the SRCs .

Table 1.2: Characteristics of representative studies of metal accumulation by ascidians

Species studied	Metals	Study type	Location	Season	T (°C)	Ref
Phlebobranchs						
Ascidia ahodori	Fe, V, Mn	C, O, CT, P	Japan	n.a.	n.a.	91
A. ceratodes	Fe, Mn, V, Cr	UB,O,P	CA, USA	n.a.	15-19	60
	Fe V	C, CT, O, P	CA, USA	sp and sum	n.a.	131
	V	OS, CT	CA, USA	n.a.	10-13	47
	V	CT, OS	CA USA	"all seasons"	n.a.	23
	V	CT, H	CA, USA	Jan-June	n.a.	9
A. dispar	Fe, V, Ti	C, O, CT, P WB	Chile	n.a.	n.a.	113
A. malaca	Fe, V. Mn	C, O, CT, P	Italy	n.a.	n.a.	90
A. nigra	V	C, CT, OS	Bermuda	n.a.	24.5	75
	V	C	Bermuda	Summer	n.a.	130
A. s. samea	Fe, V, Mn	C, O, CT, P	Japan	n.a.	n.a.	90
Perphora annectens	Fe, V	C, OS, UB	CA, USA	n.a	n.a	60
	Fe, V	C, CT, P	CA, USA	sp and sum.	n.a.	131
Ciona intestinalis	Fe, V	C,CT, P	CA, USA	sp and sum	n.a.	131
	Fe, V, Mn	C, O, CT, P	Italy	n.a.	n.a.	90
Ciona savignyi	Fe, V, Mn	C, O, CT, P	Japan	n.a.	n.a.	90
Phallusia mammillata	Fe, V, Mn	C, O, CT, P	Italy	n.a.	n.a.	90
Phallusia julinea	V	OS, CT	Australia	n.a.	10-13	47
Phallusia fumugata	V	CT, C	France(?)	n.a.	n.a.	23
Rhopalaea abdominalis	Fe, V	C, WA	CA, USA	Sp and sum	n.a.	131
Stolidobranchs						
Halocynthia roetzi	Fe, V, Mn	C, O, CT, P	Japan	n.a.	n.a.	90
H. papillosa	Fe, V, Mn	C, O, CT, P	Italy	n.a.	n.a.	90
Herdmania momus	Fe	U, H,	Australia	n.a.	16-18	158
Pyura chilensis	Fe, V, Ti	C, O, CT, P WB	Chile	n.a.	n.a.	113
Pyura stolonifera	Fe, Mn, V, Cr	U,O,P	Australia	n.a.	15-19	60
Pyura sacciformis	Fe, V, Mn	C, O, CT, P	Japan	n.a.	n.a.	90
Polycarpa cryptocarpa	Fe, V, Mn	C, O, CT, P	Japan	n.a.	n.a.	90
Boltenia ovifera	Fe	OS, CT,UB	Mass., USA	n.a.	5-10	1
Styela clava	Fe	OS,CT, UB	Mass., USA	n.a.	5-10	1
Styela plicata	Fe, V, Mn	C, O, CT, P	Japan	n.a.	n.a.	90
Microcosmus sulcatus	Fe, V, Mn	C, O, CT, P	Italy	n.a.	n.a.	90
Molgula manhattensis	Fe	OS, CT, UB	Mass., USA	n.a.	5-10	1
	Fe, V	C, WA, O, Ct	CA, USA	sp and sum	n.a.	131
	Fe V, Mn	C, O, CT, P	Japan	n.a.	n.a.	90

Study type: C- concentration ; UB- uptake/binding; H-hematocrit; CT- coelomic fluid whole; CT-coelomic cell types; O- Organ; P-plasma; OS- oxidation state; WA- whole animal; n.a. not available; sp-spring; sum-summer

Although there is little information on metal toxicology, several papers suggest that the ascidians do not toxicologically take up vanadium. DeCarlat and colleagues (2002) compared metal concentrations of the colonial ascidian *Clavelina ledpaditformis* in polluted and unpolluted Mediterranean harbors and found that there was no difference in vanadium concentration between individuals at the two sites. However, there was significantly more lead and copper in the ascidians at the polluted sites (de Caralt *et al.*, 2002). Few uptake studies have quantified the effects of dissolved vanadium concentration on the uptake rate.

1.6.3 Ontogeny of hemocytes

The hematopoietic tissues of the ascidians are the nodules around the transverse bars of the branchial basket (Ermak, 1976; Wright, 1981). The hemocytes provide many of the same functions as vertebrate blood cells such as immune/inflammatory response (Anderson, 1971; Endean, 1955, 1960; Hecht, 1918; George, 1936; Rowley, 1982, Smith, 1970a, 1970b; Webb, 1939; Wright, 1981). Although density gradient centrifugation has established that the majority of vanadium is found in the SRCs (Michibata *et al.*, 1987; Michibata, Iwata, and Hirata, 1991a), there is evidence that other cell types, such as morula cells and bi-vacuolated cells, contain vanadium (Carlson, 1975; Nette, Scippa, and de Vincentiis, 1998; Nette *et al.*, 1999). As some have proposed, the SRC may be the terminal cell in the lineage (Nette *et al.*, 1999). Other researchers have suggested that MCs are the "excretory" cells for vanadium (Majorta *et al.*, 1994).

In any case, if MCs and SRCs are related cell forms, there is potential to manipulate variables experimentally, or examine temporal or spatial variation, in their relative number and relate the number of cells to the tissue distributions of vanadium. In light of the possibility of variability in vanadium concentration and uptake, we might expect to see more of the presumably "intermediate" forms when vanadium uptake rate is at its highest.

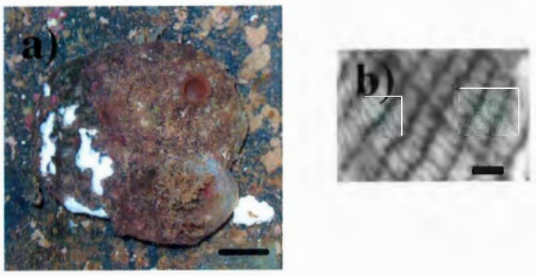

Figure 1.9: a) Living *Ascidia callosa*; an unstalked phlebobranch ascidian (two individuals). The scale bar is 2 cm. and b) the structure of its branchial basket. The scale bar is 2 mm.

There is some evidence for inter- and intra-specific variation in the relative number of SRCs and MCs among vanadium accumulating ascidians (Table 1.3). However there has been no systematic investigation of whether the relative or absolute numbers of these cells correlates with the level of vanadium found in individuals of a species or

among species, or if there is seasonal variation. Is higher blood vanadium associated with a higher hematocrit or is there a higher concentration of vanadium in each cell?

Several researchers have attempted to show the inducibility of SRC production. The total number of hemocytes and the total number of SRCs increased upon treatment of the whole animal with NH_4Cl and several ionophores (Hayashi *et al.*, 1996; Nose *et al.*, 1997). However the concentration of vanadium in the fluid did not increase in the former study. It is unknown whether the average size of the SRCs changed appreciably. Hayashi *et al.* (1996) suggested that the new SRCs arose from the tissue surrounding the gut and might be immature, i.e. that this tissue is hematopoietic. The second possibility is that this tissue is an SRC or vanadium storage tissue. In any case, the exciting implication of these studies is the inducibility of SRC production or utilization.

Table 1.3: Hematocrit and percentage of signet ring cells (SRCs) and morula cells (MCs) in several Phlebobranch ascidians

Species	Hematocrit (cells.mm^{-3})	%SRCs	% MCs	Reference and location
Phallusia mammillata	68000	5	43	(37)England
Ascidia ceratodes	77300	~25	53	(9)California
	na	43-45	57	(98)Florida
	na	68	32	(78)California

1.7 Insights from other invertebrates

There has been relatively little study of the uptake and sequestration of essential metals by other marine invertebrates at the cellular level, with the exception of the crustacean hepatopancreas (Aslamkhan and Ahearn, 2003). However, the search for appropriate bio-indicators of pollution has resulted in much work centered on the toxicological uptake and loss of metals at the organ or whole body level and the physical and physiological factors contributing to the large variability (Wang and Fisher, 1999). Invertebrate metal toxicologists have sometimes studied dynamics of essential elements and have generated data that may be relevant to the current discussions of vanadium accumulation by ascidians. The search for biochemical indicators of metal stress from pollution has resulted in studies of metal-proteins interactions in marine invertebrates.

1.7.1 Metal protein interactions

The accumulation of metals against an apparently steep gradient with environmental concentration is not unusual among marine invertebrates. Some oysters accumulate especially high levels of copper and zinc (400 mM and 1.2 M respectively) under non-polluted conditions, rivaling the concentrations of vanadium in the hemocytes of some ascidians (Pirie *et al.,* 1984). Rapid binding to intracellular ligands helps to decrease the free metal concentration gradient with seawater such that there is less need for active transport at the gill membranes. The high affinity of the sulphydryl (-

SH) groups of proteins and ligands such as reduced glutathione results in a very small free metal pool in most organisms, even at high environmental exposures (Pompella *et al.,* 2003). Complexation with proteins such as metallothioneins or molecules rich in sulphydryl groups is a common trace metal handling strategy amongst marine invertebrates (Roesijadi and Robinson, 1994; Viarengo *et al.,* 1999). Metals can sometimes act as molecular mimics of one another and thus non-essential and toxic metals may cross epithelia via a transport protein normally associated with an essential metal (Bridges and Zalups, 2005). Work on vanadium binding proteins has indicated that they also have a high affinity for copper, but in general ascidians do have large amounts of copper in their bodies (Ueki *et al.,* 2003b). One species did accumulate copper in an enriched environment compared to a pristine environment while vanadium concentration was unaffected (de Caralt *et al,* 2002).

The glutathione metabolism of several marine invertebrates has been studied in the context of toxicological exposure to heavy metals. In some cases glutathione is involved in the transfer of essential metals such as copper to the proteins in which they are active (Brouwer and Brouwer-Hoexum, 1992; Canesi *et al.,* 1999). It does not seem unusual, therefore, that glutathione transferase in the gut of ascidians would bind to vanadium and to copper, suggesting that caution is required when ascribing special vanadium binding function to this enzyme. An interesting related issue is how the presence of vanadium in blood cells and tissues affects the glutathione and metallothionein metabolism of ascidians. An examination of biochemical pathways in other marine invertebrates reveals that the pentose phosphate pathway may also be a source of NADPH (nicotinamide adenine dinucleotide phosphate reduced form) for

the reduction of oxidized glutathione (Rodriguez-Segade *et al.,* 1978). The glutathione biochemistry of ascidians has yet to be fully explored.

The discovery of vanadium accumulation in the branchial crown of the fan worm *Pseudopotamilla occelata* was surprising (Ishii *et al.,* 1994). Perhaps more surprising was the reactivity of antibodies raised to 12.5 and 15 kDa antigens to the vanadium associated proteins of the SRCs of *Ascidia sydneiensis samea* (Uyama *et al.*, 1997; 1998a). There is little known about which amino acids comprise the epitope of the vanadium associated proteins, and the amino acid sequence of the antigen proteins in the polychaete is unknown. Until the sequence is known we cannot be sure that it is, in fact, the same protein. Given the nature of the amino acid residues that are likely to bind to metals, however, the sequence of the polychaete proteins may reveal a great deal about the vanadium associated proteins of ascidians and how they may have been selected for. The branchial crown has a very thin epithelium and is known to be the site of uptake of dissolved metals (Bocchetti *et al.,* 2004; Fattorini and Regoli, 2004). It is possible that these organisms, taken from a habitat that they share with vanadium accumulating ascidians, may accumulate vanadium because it is in locally high concentrations as ascidians die and decompose releasing the contents of their body into the surrounding water. We need to know if the water around aggregations of ascidians can experience transient high levels of dissolved vanadium (especially during low flow situations).

1.7.2 Metal uptake/transport at the whole animal level

Whole body bio-kinetic models have been useful in highlighting the large degree of inter- and intra- species variability in trace metal uptake and the trophic, physiological and physical factors that influence it. These models quantify uptake and loss rates for each exposure and loss pathway, taking feeding and water pumping rates, concentration of metals in different exposure mediums, and other factors into account (Wang *et al.*, 1996).

Perhaps the most relevant and best studied invertebrates relevant to these discussions are the mytillid mussels. The animals are also suspension feeders, with many of the same eco-physiological constraints on feeding and water pumping as ascidians (Baines *et al.*, 2005; Fisher *et al.*, 1996). Whole animal bio-kinetic models have shown that for mussels, the dissolved phase is the most important mode of uptake for most metals (Wang and Fisher, 1999). The models have also elucidated the relationship between size, pumping rate and metal uptake rate. For example, the allometric coefficient for mass specific pumping rate ($2.43 \cdot M^{-0.318}$) is similar to the coefficient for selenium and chromium uptake from the dissolved phase (-0.34 and -0.32) in the mussel *Septifer viragatus* (Wang and Dei, 1999). These models have been used to show how factors such as temperature affect pumping rate and gut passage time, resulting in significant geographic and temporal differences in the trace metal concentrations of *Mytilus edulis* (Cossa *et al.*, 1980). The study of vanadium and iron accumulation by ascidians would benefit from such an approach.

1.8 Objectives of this thesis

This thesis examines the ecophysiology of vanadium and iron accumulation by three North Atlantic ascidians. The ultimate question of the function of vanadium and iron accumulation by ascidians cannot be directly addressed by this type of study. However, a clearer understanding of variation in tissue concentrations vanadium and iron can be exploited in the future to direct experiments that aim at delineating function.

The first task was to develop a method to measure these metals that would be sensitive but economically feasible for a large number of biological samples. Chapter 2 presents the development and validation of an HPLC (high pressure liquid chromatography) technique to measure vanadium and iron in ascidian tissues and an application of that technique to the first study of seasonal variation in these metals in the tissues of *Ciona intestinalis* and the response of the metal levels to changes in feeding rate. I hypothesized that vanadium and iron levels and the activity of G6PDH, an enzyme of the proposed reductive pathway for vanadium, would peak around the time of the spring bloom, given the ecophysiological relationships discussed previously in this chapter. This study found seasonal variation in iron and vanadium concentration, and in the activity of G6PDH, in this low level vanadium accumulator at Woods Hole, Mass, USA.

This finding required confirmation in another phlebobranch species in another geographic location in the North Atlantic, and comparison with the seasonal metal

concentrations of a stolidobranch. Chapter 3 is a study of the seasonal variation in the vanadium and iron concentrations of the tissues of phlebobranch and stolidobranch ascidians (*Ascidia callosa* and *Halocynthia pyriformis* respectively) from Newfoundland and Labrador, Canada. I hypothesized that vanadium and iron levels would peak in both these species at the time of the spring bloom and that in *A. callosa*, this would co-incide with a peak in the number of vanadocytes. This hypothesis was supported.

The observation of a seasonal change in vanadium and iron concentrations of *A. callosa* led to the hypothesis that vanadium accumulation and the proliferation of vanadocytes might be inducible in that species. Chapter 4 investigates the response of the vanadium and iron concentrations of *A. callosa* to increased dissolved exposure. *H. pyriformis* was subjected to the same conditions as an indicator of non-specific uptake. Vanadium levels increased upon dissolved exposure to vanadium for *A. callosa* but not *H. pyriformis*. Chapter 5 presents a summary of the research and possible future lines of research arising from the thesis. Two short supplementary chapters present preliminary work for mass spectrometry sequencing of ascidian vanadium binding proteins and the problems of the formation of the Henze precipitate in hemocyte prepartions of *A. callosa* .

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Chapter 2: Seasonal variation in, and impact of food availability on, vanadium and iron concentrations of *Ciona intestinalis* tissues (Tunicata, Ascidiacea)

2.1 Introduction

Ascidians (Tunicata, Ascidiacea) are unusual among animals in that that some species (members of the suborders Phlebobranchia and Aplousobranchia) non-toxicologically accumulate high levels of vanadium in their tissues (Henze, 1911). The highest concentration of vanadium is found in a reduced form, V(III), in the vacuoles of hemocytes (coelomic cells) called signet ring cells (SRCs), so named because a single acidic vacuole displaces the nucleus to the periphery of the cell. The concentration of vanadium is as high as 350 mM in the SRC vacuoles of some species (Michibata, 1986). Vanadium has also been localized in other hemocytes, and in lower concentrations in other tissues such as the branchial basket and tunic, probably reflecting infiltration of hemocytes into these tissues (Michibata *et al.*, 1986). Ascidians of the sub-order Stolidobranchia contain measurable levels of vanadium, but at several orders of magnitude lower than those found in phlebobranchs (Michibata *et al.*, 1986). Members of both sub-orders also accumulate iron in their tissues. The relationship, if any, between iron and vanadium, within and among these groups, has received little study. The functions ascribed to vanadium include anti-fouling/anti-predator defense (Odate and Pawlik, 2007;Stoecker, 1980), anaerobic adaptation (Smith, 1989) and tunichrome/tunic formation (Taylor *et al.*, 1997), but none have been supported with strong experimental evidence.

There has been very little study of the ecophysiology of vanadium accumulation by ascidians. It is unknown at present whether vanadium is maintained at a constant level

in various tissues over a variety of physiological conditions. There is little known about variation in vanadium concentration, uptake and loss rates over different seasons, the life span of an individual, and with changes in temperature, food supply and so on. Such variation might be exploited when planning experiments that are aimed at addressing function. For example, it would be useful to know if and when vanadium uptake or concentration is highest if one wanted to study the movement of SRCs into certain tissues.

Seasonal variation is a logical starting point for studies of the vanadium dynamics of ascidians. Since ascidians are ectothermic suspension feeders, in most ecosystems, their food supply and environmental temperature are seasonally variable. Accordingly, like many other benthic invertebrates, physiological parameters such as pumping and metabolic rates fluctuate seasonally, which may in turn contribute to seasonal differences in metal physiology and concentration (Coma *et al.*, 2002; Petersen *et al.*, 1995, 1999; Riisgard et *al.*, 1998; Ribes *et al,* 1998). Within a certain range, ascidian water pumping rates increase as concentrations of phytoplankton and other particles increase which may result in increased exposure to dissolved vanadium and iron (Armsworthy *et al.*, 2001; Fiala-Medioni, 1978; Petersen and Riisgard, 1992; Ribes, Coma and Gili, 1998). Although the importance of the contribution of food derived vanadium is unknown, vanadium and iron are present in phytoplankton and ascidians are likely exposed to increased dietary metals during a spring bloom (Unsal, 1982).

Given these factors, I hypothesized that vanadium and iron uptake by ascidians varies temporally, with higher uptake, resulting in an increased concentration of the two metals, during or following the spring bloom than in the winter or fall. I also hypothesized that if vanadium and iron uptake and retention are governed by similar factors, they will show a similar seasonal pattern. I developed an RP-HPLC technique using pre-column chelation with 4-(2-pyridylazo)-resorcinol (PAR) for the determination of vanadium and iron concentrations of several tissues of the ascidian *Ciona intestinalis*. This is a cosmopolitan phlebobranch ascidian that accumulates vanadium to concentrations of 600 μM in its hemocytes, and for which putative vanadium binding proteins have been reported by Michibata and colleagues (Michibata *et al.*, 1986; Trivedi *et al.*, 2003). The animals were collected on six occasions from summer to spring from Woods Hole, Massachusetts (USA).

Although the exact mechanisms of vanadium reduction and compartmentalization in the SRC vacuole are unclear, Michibata and colleagues (Michibata *et al.*, 2003; Yoshihara *et al.*, 2005; Yoshinaga *et al.*, 2006; Ueki *et al.*, 2007) hypothesize that that vanadate, V (V), from seawater is taken up by the branchial basket, and reduced and transported into the SRCs by vanadium binding proteins or vanabins. Given the presence of several enzymes of the pentose phosphate pathway in the SRCs (glucose-6-phosphate dehydrogenase, transkelotase, 6-phosphogluconate dehydrogenase), it was hypothesized that the NADPH produced by these enzymes might participate in the reduction of vanadium (Kanamori *et al.*, 1999; Uyama *et al.*, 1998). Thus, I measured the activity of glucose-6-phosphate dehydrogenase (EC 1.1.1.49) to examine whether the activity of this enzyme co-varies with vanadium concentration.

Finally, in a short feeding experiment, I examined whether vanadium and iron levels vary in response to particle concentration/food borne metals alone.

2.2 Materials and Methods

2.2.1 Chemicals and containers

All chemicals used in the determination of metal concentration were HPLC or MS grade. All containers used in the sampling, digestion and PAR complexation were soaked in 6 M HCl for 24 to 48 hours, rinsed in Milli-Q water (X3) and air dried before use.

2.2.2. Standards

Standards with 100 µM vanadium and 100 µM iron were prepared from 20 mM stock solutions of ammonium vanadate (NH_4VO_3) and iron chloride ($FeCl_3$), respectively, dissolved in 1.6 M HCl. Stock solutions were made monthly and stored at 5 °C. Standard curves did not change over the course of the study.

2.2.3 HPLC method

The HPLC procedure used was adapted from several published methods including those of Tadayon and Massoumi (1999), Tsai and Hsu (1994), and Ming *et al.* (1992).

PAR forms complexes with many metals, including iron and vanadium. These metal-PAR complexes absorb light in the 400-600nm range, and the peak absorption is metal complex specific. These complexes can be separated by HPLC and detected with spectrometry.

The instrument used was a Waters® HPLC equipped with a dual wavelength absorbance detector and a temperature controlled auto-sampler. The column was a 25 mm long Symmetry C_{18} column (5 μm) equipped with a guard column. The mobile phase was 65 mM ammonium phosphate with 35 % MeOH (v/v), and the flow rate was 0.8 ml min^{-1}.

Aliquots of biological samples and standards (typically 200 μl) were brought to ~6.5 pH with ammonium hydroxide. Some biological samples were further diluted to ensure that there was an adequate ratio of PAR: metal for chelation. To this was added 500 μl, 100mM ammonium phosphate (pH 6.5), 500 μl 6mg per 100 ml PAR in 50% MeOH. After 15 minutes, to allow the color to develop, samples and standards were filtered (0.45 μm) into HPLC vials. Injections were monitored at 540 nm for vanadium and 510 nm for iron and other metals. Samples and standards from a sample run were held at 10 °C in the auto-sampler. Four to six standards were injected at the beginning of each run with additional blanks every 6-10 injections and an additional blank and standard injection at the end of each sample run.

To test for interference from other metals that may be present in ascidian tissues, 50 μM vanadium and iron standards were separately, and together, spiked with 50 μM

copper, zinc, manganese, and combinations thereof, and treated according to the HPLC procedure. These were treated as unknowns and the concentrations of iron and vanadium calculated according to the standard curve generated by single element standards.

2.2.4 Digestion of biological samples for metal analysis

Biological samples were glass homogenized in 1.6 M HCl. The homogenates were placed on ice and the protein allowed to precipitate. The samples were centrifuged at 15600 g and the supernatant used for metal analysis. Further acid treatments were applied to the pellet and found not to contain detectable vanadium or iron beyond blank values. The protein pellets dissolved in 300 mM NaOH, brought to pH 6.5 and chelated with PAR in the manner above, and showed negligible absorbance at 510 or 540 nm in a spectrophotometer, indicating negligible remaining iron or vanadium respectively. Samples of each tissue were ground in 1.6 M HCl in 50 μM vanadium, iron, and a mixture of vanadium and iron to examine any matrix effects. Tunic and hemocytes were chosen for the interference study as they represent the expected low and high vanadium levels. A certified reference material, TORT-2 (lobster hepatopancreas, National Research Council of Canada), was glass homogenized (99X dry mass) and reacted as above. While detectable, the vanadium content of TORT-2 (1.64 mg V kg-1) is too low to be reliably extracted by the homogenization methods and volumes employed.

2.2.5 Biological sample collection

Living *C. intestinalis* were collected and shipped from Woods Hole, Massachusetts (USA), to the Ocean Sciences Centre, Logy Bay, Newfoundland and Labrador (Canada), from June 2004 to March 2005. These were shipped in seawater (kept cool with ice packs) around the 15th of each month sampled. Transport required 2 days. Upon arrival, animals were transferred to ambient seawater for observation. Animals that were actively pumping (had open siphons), responded to touch, and were producing fecal pellets were considered to be in good condition. Five or six animals in good condition were transferred to 1 μm filtered seawater for 48 hours to ensure that the branchial basket was devoid of plankton. As the tunic of *C. intestinalis* is transparent, the digestive tract can be visually inspected for the presence of fecal pellets. After 48 hours I observed a consistent lack of fecal pellets visible in the gut of this species. Animals chosen for analysis were 5- 10 cm in total length above the substrate (undisturbed) such that the mean length (~8 cm) did not differ amongst sampling periods ($F_{5,32}$ = 2.83, p=0.048). It was noted that the animals collected in March 2005 were gravid. Animals sampled immediately after arrival and not used in the experiment described in section 2.2.7 were designated as "wild."

Aliquots of coelomic fluid were transferred into pre-weighed vials following cardiac puncture with a ceramic blade. These aliquots were centrifuged (15600 g) for 10 minutes at 5°C and the cell pellets frozen at -20 °C for metal analysis or with liquid nitrogen (and stored at -80 °C) for enzyme analysis (from August 2004 onward).

Other tissues were dissected with a ceramic blade and frozen in the same manner. The tunic sample was taken from the middle region of the long axis of the tunic under the inhalant siphon but not including it. The body wall sample was excised from this region and included a section of musculature and branchial basket.

2.2.6 G6PDH activity measurement

Frozen cell pellets were homogenized in 9, 14 or 19 volumes 100 mM triethanolamine with 2 mM dithiothretol (pH 7.5) on ice followed by centrifugation at 15600 g for 5 minutes to remove cellular debris. The supernatant was used in the measurement of enzyme activity. Glucose-6-phosphate dehydrogenase activity was measured as the production of NADPH at 340 nm under the following conditions: 75 mM Tris pH 7.5, 5 mM G6P, 0.5 mM $NADP^+$. The instrument employed was a Beckman DU880 multiplate reader, situated in a temperature controlled room at 15 °C. The sample compartment was heated to a constant 20 °C.

2.2.7 Feeding experiment

The September sampling point is considered as the time zero for the feeding experiment. Additional animals gathered at this time were immediately reserved for a two month feeding experiment. Twelve animals (mean length ~8.4 ± 0.5 cm; and 8.2 ± 0.7 cm height above substrate) were added to seawater tanks (32 l) with aerated 1μm filtered seawater which were aerated static and held at a constant at 10 °C.

Animals were placed in plastic supports so that they remained upright. There was one tank per experimental group. A 50% water change and fecal pellet removal was completed every 48 hours. Ammonia concentration was monitored over the course of the experiment (with the indophenol blue method) to ensure that water quality was maintained and was found to be satisfactory (<50 μM). There were two treatment groups: high food (500 000 cells l^{-1} $animal^{-1}$) and a low food (250 000 cells l^{-1} $animal^{-1}$) of Shellfish Diet 1800® (Reed Mariculture) every 24 hours. This is a commercial preparation consisting of the marine microalgae *Isochrysis* sp. (25%), *Pavlova* sp. (20%), *Tetraselmis* sp. (20%), *Thalassiosira weisflogii* (30%) and *Nannochloropsis* sp. (5%). The feeding levels were selected to ensure a high filtration rate. They were based on work by Petersen *et al.* (1995). In a feeding study with *Ciona intestinalis* these authors (Petersen *et al.*, 1995) found that filtration rates decreased with increasing food particle concentration from 500-1000 cells ml^{-1} (500000 – 1000000 cells l^{-1}) to 4000-8000 cells ml^{-1} . At one and two months, animals were sampled in the same manner as in the seasonal study. Animals were fed from a single batch (bottle) of Shellfish Diet 1800® throughout the experiment and the algae were mixed before being taken for feeding.

2.2.8 Statistical analysis

Statistical analyses were completed with Systat v. 9 ®. Seasonal tissue vanadium and iron concentrations and G6PDH activity were analyzed by a general linear model (GLM) with the following model:

Tissue concentration or G6PDH acivity = $\beta_0 + \beta_{Time}$ Time + ε

with α =0.05 and "Time" as a categorical variable.

Experimental tissue vanadium and iron concentrations were subjected to a GLM with the following model:

Tissue concentration = $\beta_0 + \beta_{Time}$Time + $\beta_{Feeding\ level}$Feeding level + $\beta_{Feedinglevel*Time}$Feeding level*Time +$\varepsilon$

with α =0.05 and "Time" and "Feeding level" as categorical variables.

Pairwise comparison probabilities among all sampling points for the seasonal data, and among all sampling points and experiment groups for the feeding experiment were generated with the Bonferroni correction and $p<0.05$ was taken as significant. For both GLMs, residuals were visually examined for normality, homogeneity and independence and were found to be satisfactory.

2.3 Results

2.3.1 HPLC characteristics and validation

Figure 2.1 presents typical chromatographs of standard injections of a 1.6M HCl blank, standards and tissues. At pH 6.5, as other authors have found (Ming, Wu, Schwedt, 1992; Tsai and Hsu, 1994), two PAR-metal complexes formed for both vanadium and iron. The retention times under the conditions of this procedure were 4.7 and 8.1 minutes for vanadium [V(PAR) and V(PARH), respectively] and 27 and 32 minutes for iron [Fe(PARH) and $Fe(PARH)_2$ respectively]. The sums of the two peaks for each metal were used for all measurements. The calibration curves for vanadium and iron respectively were:

Area = 17298 + 1016397 * nmol V (linear regression R^2 =99.9%; $p<0.0001$)

Area= 70334 + 919606 * nmol Fe (linear regression R^2=99.9%; $p<0.0001$)

Detection limits were 0.03 nmol for vanadium and 0.05 nmol for iron. Figure 2.1 d shows a sample chromatograph for hemocytes. There was no vanadium detectable in the sample blanks, but there was consistent iron contamination at a level of 0.006 nmol μl^{-1} injected. Homogenization blanks were run at the beginning, middle and end of each sample run, and mean iron blanks were subtracted from the samples. As shown in Table 2.1, there was excellent recovery of vanadium and iron from spiked *C. intestinalis* samples. There was 95% recovery of iron in TORT-2. While vanadium was detectable it could not be reliably quantified using the homogenization methods

employed. There was no interference in vanadium determination from the complex matrix when TORT-2 was homogenized in vanadium standard. The recovery of vanadium and iron was not affected by the presence of zinc, manganese or copper at the concentration of 50 μM of each.

2.3.2 Seasonal iron and vanadium concentrations in C. intestinalis tissues

Figure 2.2 presents the vanadium and iron concentrations of the body wall, tunic and hemocyte pellet over the study period. Hemocyte ($F_{5, 32} = 3.20$ $p=0.027$) and tunic ($F_{5, 32} = 2.97$, $p=0.039$) vanadium levels varied seasonally while body wall ($F_{5, 32} = 1.73$, $p=0.11$) vanadium levels did not. Hemocyte ($F_{5, 32} = 3.27$, $p=0.022$), tunic ($F_{5, 32} = 3.09$, $p=0.032$) and body wall ($F_{5, 32} = 2.81$, $p=0.048$) iron levels varied seasonally.

While the vanadium concentration of the hemocyte pellet was approximately three fold higher than that in the tunic (qualitative comparison), the pattern of seasonal changes was similar for both tissues. There were peaks in vanadium concentration in August and in the March, with the March hemocyte samples exhibiting the highest vanadium concentration (Figure 2.2).

Changes in iron concentration in the tunic and body wall showed a slightly different pattern. The iron concentration in the body wall was lower in November than in the other sampling periods but there was no spike in March (Figure 2.2). The iron concentration of the tunic followed a similar pattern as the vanadium concentration in

that tissue, reaching reached its high levels in August and September and March. The hemocyte iron concentration also peaked in March.

At most sampling points, the vanadium and iron concentration of the hemocytes is higher than that of the other tissues (qualitative comparison). During the July sampling period, the body wall iron concentration, and in August, the tunic iron concentration is higher than that of the hemocytes.

Hemocyte ($F_{3, 21} = 4.26$, $p=0.023$) and body wall ($F_{3, 21} = 4.40$, $p=0.018$) G6PDH activity varied seasonally. The G6PDH activity of the hemocyte pellet showed highest activity in the March (Figure 2.3). The activity was higher in the body wall sample than in the hemocyte pellet, and was higher in that tissue in September and March than the other sampling periods.

2.3.3 Feeding experiment

The mean size (height above substrate) of animals did not differ among experimental groups ($F_{5, 29}=0.39$, $p=0.18$; Table 2.2). Body wall ($F_{5, 29} = 3.25$, $p=0.027$) and tunic ($F_{5, 29} = 2.98$, $p=0.041$) vanadium levels varied with treatment and over time (with significant treatment*time interaction terms in the GLMs) while hemocyte ($F_{5, 29} = 1.028$, $p=0.15$) vanadium levels did not (Figure 2.4). Hemocyte ($F_{5, 29} = 2.92$, $p=0.045$), body wall ($F_{5, 29} = 3.05$, $p=0.038$) and tunic ($F_{5, 29} = 3.14$, $p=0.033$) iron levels varied with treatment and over time ((\with significant treatment*time interaction terms in the GLMs, Figure 2.4).

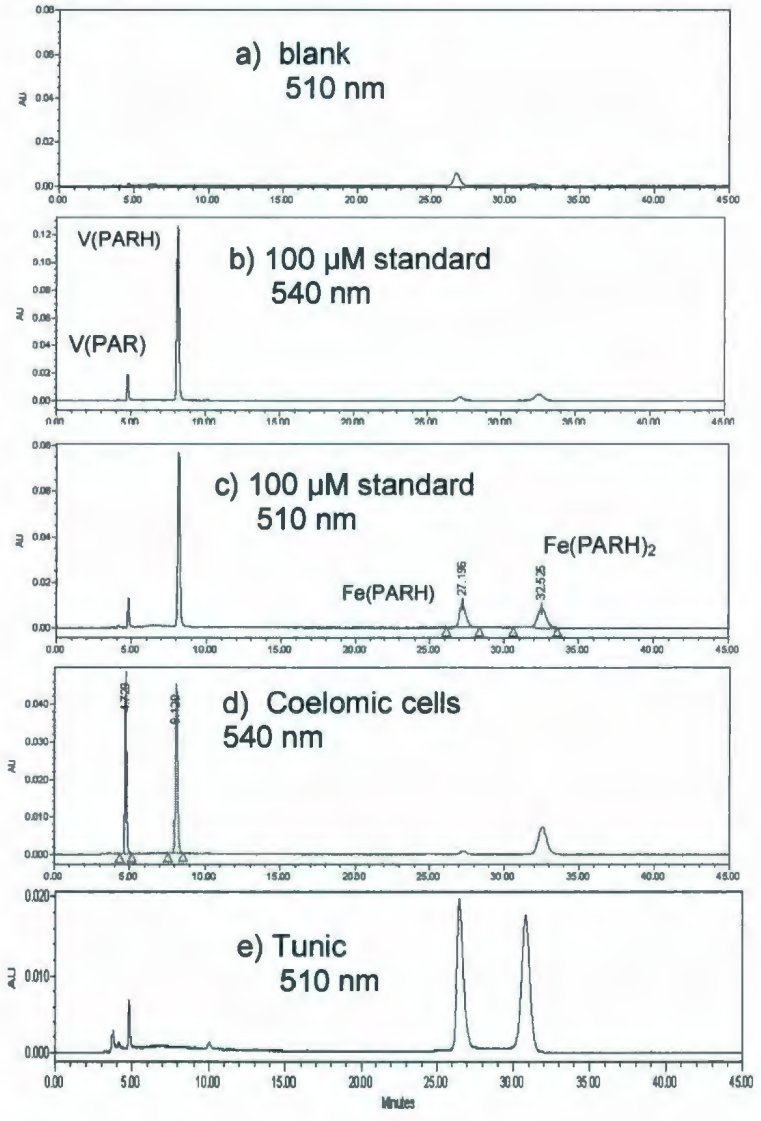


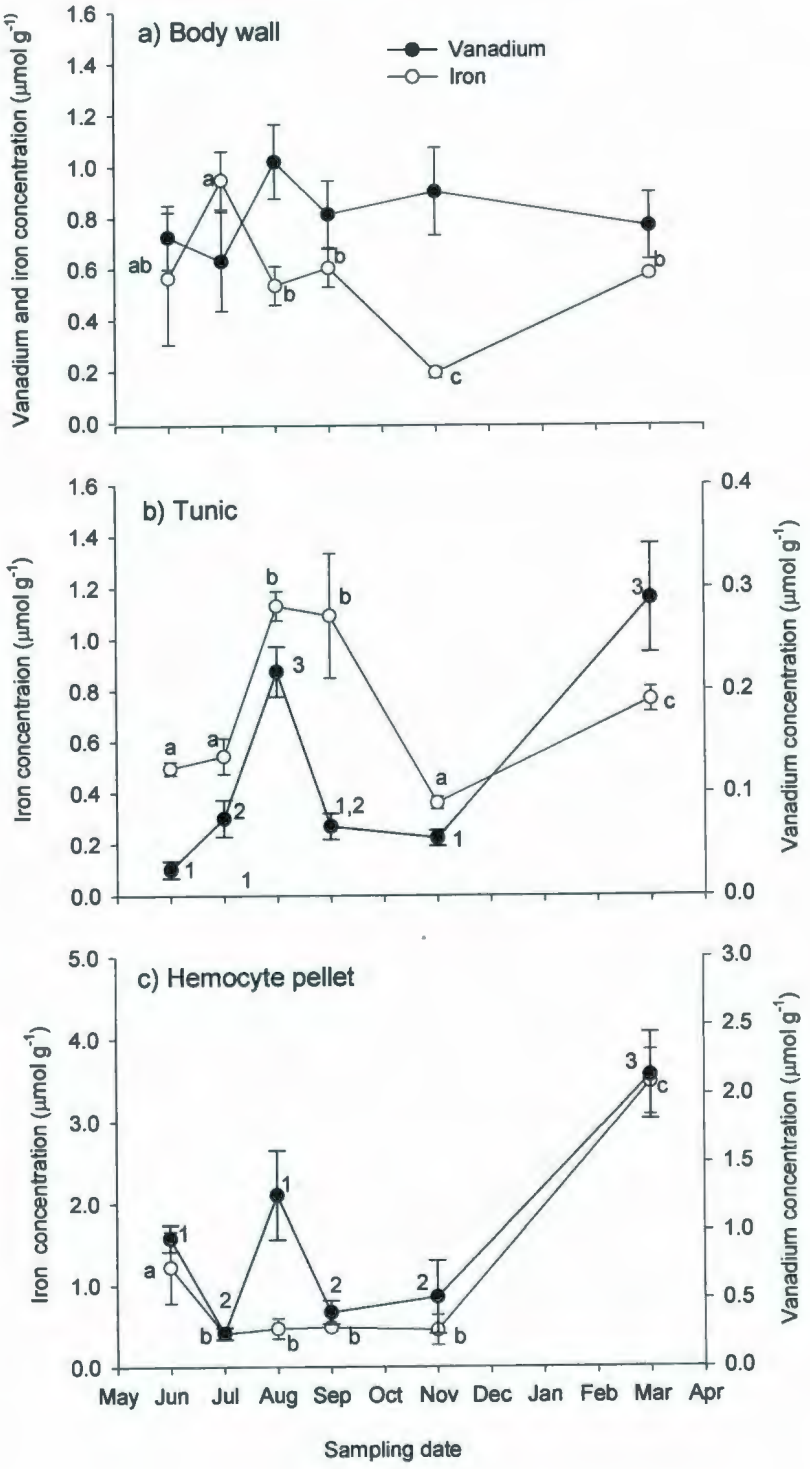
Figure 2.1: Typical RP-HPLC chromatographs for standard-PAR , and tissue sample-PAR chelates monitored at 540nm and/or 510nm

Table 2.1: Recovery of vanadium and iron in reference materials, biological samples homogenized in standards, and standard mixtures (mean ± standard deviation, n=5) with RP- HPLC of PAR chelates.

Recovery of metals (%) from mixtures of reference materials and standards

Sample	Vanadium	Iron
TORT-2 (105 mg kg^{-1} iron and 1.64 mg kg^{-1} vanadium as homogenized)	Below limits of quantification	95.2 ± 3.7
TORT-2 in 50 μM V and 50 μM Fe	99.2 ± 2.1	97.8 ± 3.2
Hemocytes in 50 μM V and 50 μM Fe	101 ± 2.8	98.3 ± 2.5
Body wall in 50 μM V and 50 μM Fe	98.8 ± 2.3	99.2 ± 3.8
Tunic in 50 μM V and 50 μM Fe	102.6 ± 3.1	98.4 ± 3.4
50 V μM and 50 μM Fe *with* 50 μM Mn, 50 μM Cu, 50 μM Zn	99.4 ± 1.5	98.6 ± 2.8

Figure 2.2 (following page) : Seasonal iron and vanadium concentrations (μmol g^{-1} wet mass of the body wall, tunic, and hemocyte pellet of *Ciona intestinalis*, collected from Woods Hole, Mass. (June 2004 – March 2005). The bars indicate the standard deviation from the mean. Data were analyzed by GLM (Tissue concentration = β_0 + β_{Time} Time + ε with α =0.05 and "Time" as a categorical variable. Pairwise comparison probabilities among all sampling points for each metal were generated with the Bonferroni correction and $p<0.05$ was taken as significant. Different numbers refer to significant differences in the vanadium concentration and different letters refer to significant differences in the iron concentrations. Lack of letters or numbers indicates no significant differences


a) Body wall
Vanadium
Iron
Vanadium and iron concentration (µmol g-1)
b) Tunic
Iron concentraion (µmol g-1)
Vanadium concentration (µmol g-1)
c) Hemocyte pellet
Iron concentration (µmol g-1)
Vanadium concentration (µmol g-1)
May Jun Jul Aug Sep Oct Nov Dec Jan Feb Mar Apr
Sampling date

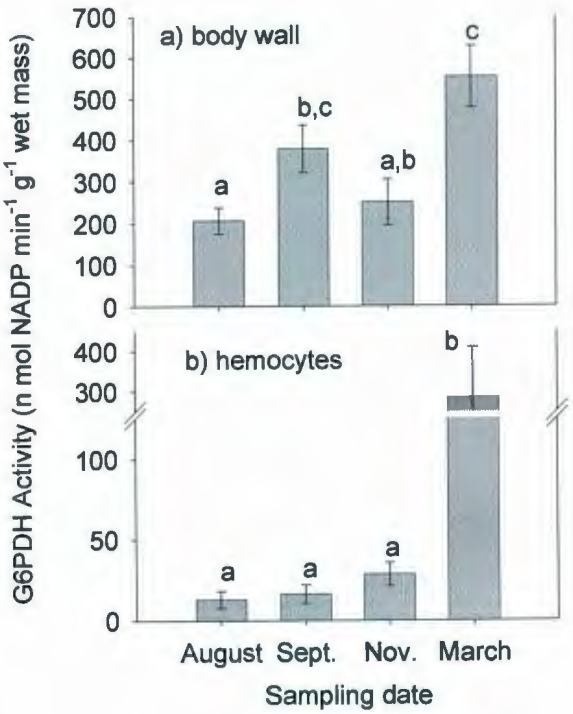


Figure 2.3 (above): Mean (±sd) seasonal G6PDH activity (nmol NADPH min^{-1} g^{-1} wet mass) in *C. intestinalis* body wall and hemocyte preparations at 20 ° C. Data were analyzed by GLM (G6PDH activity = $\beta_0 + \beta_{Time}$ Time + ε) with α =0.05 and "Time" as a categorical variable. Pairwise comparison probabilities among all sampling points were generated with the Bonferroni correction and $p<0.05$ was taken as significant. Different letters indicate significant differences between sampling points.

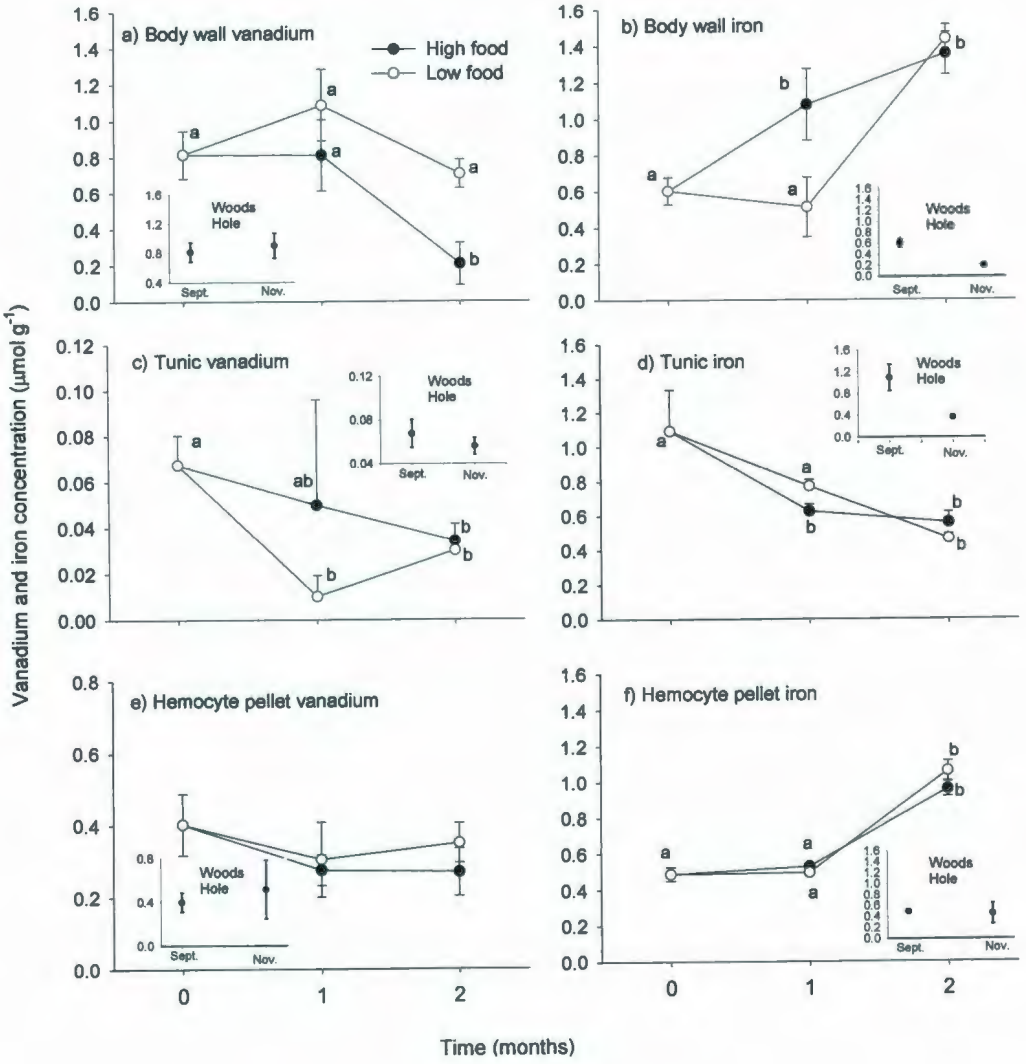
Iron concentrations of the body wall and hemocytes increased about two fold over the course of the feeding experiment in both experimental groups (Figure 2.4). After one month, the iron concentration of the body wall was higher in the high food group than in the low food group, but the groups did not differ at 2 months. During the experiment, iron concentrations in the body wall and hemocytes rose to higher levels than those sampled within days after collection from Woods Hole (designated as "wild") at the same time (November) in the seasonal study. The tunic iron levels declined in both the experimental and wild animals over the course of the feeding experiment and were higher in the low food group at one months but not two months.

Body wall vanadium levels remained constant over the course of the experiment in the low food groups as they did in Woods Hole at that time. In contrast, the high food group showed a significant decrease in body wall vanadium concentration at two months. The low food treatment group had four-fold higher vanadium concentration at the end of the experiment than did the high food treatment. Tunic vanadium declined in the low food groups from the beginning compared to one and two months. Hemocyte vanadium concentration did not change over the course of the experiment and did not differ between treatment groups.

Table 2.2: Mean height (± sd) above substrate (cm) of animals over time during the feeding experiment. Mean height did not vary with time or treatment (Height = $\beta_0 + \beta_{Time}$Time + $\beta_{Feeding\ level}$Feeding level + $\beta_{Feedinglevel*Time}$Feeding level*Time +ε ; $F_{5, 29}$=0.39, p=0.18)

Time	Height above substrate (cm)	
	High food	Low food
Time 0 (animals sampled in Sept.)	8.3 ± 0.9	
Experimental start (n=12)	8.4 ± 0.5	8.2 ± 0.7
One month (n=12)	8.8 ± 0.4	8.7 ± 0.3
Two months (end =6)	9.5 ± 0.6	8.9 ± 0.3
Final % increase with respect to initial height	12%	8%

Figure 2.4 (a-f; following page): Metal concentrations of *C. intestinalis* tissues (mean ± sd), exposed to high and low food conditions, after 1 and 2 months Experimental tissue vanadium and iron concentrations were analyzed GLM (Tissue concentration = $\beta_0 + \beta_{Time}Time + \beta_{Feeding\ level}Feeding\ level + \beta_{Feedinglevel*Time}Feeding\ level*Time + \varepsilon$) with $\alpha = 0.05$ and "Time" and Feeding level" as categorical variables. Pairwise comparison probabilities among all sampling points and experiment groups were generated with the Bonferroni correction and $p<0.05$ was taken as significant. Different letters indicate significant differences between sampling points and groups. Lack of numbers or letters indicates no significant differences. Inserts show the metal concentrations of animals collected directly from Woods Hole at time zero (September) and two months later (November), n=6 at each sampling point .


a) Body wall vanadium
High food
Low food
b) Body wall iron
c) Tunic vanadium
d) Tunic iron
e) Hemocyte pellet vanadium
f) Hemocyte pellet iron
Woods Hole
Sept.
Nov.
Vanadium and iron concentration (µmol g-1)
Time (months)

2.4 Discussion

2.4.1 HPLC quantification of metals in ascidian tissues

The HPLC method applied in this work proved to be especially useful for routine quantification of iron and vanadium in large numbers of biological samples, especially in the ascidian tissues which are relatively rich in these metals. Many of the techniques commonly used today for the quantification of metals such as inductively coupled plasma mass spectrometry (ICPMS) use equipment that is relatively expensive and not available in the average laboratory, making long term studies logistically and economically impractical. An additional advantage of the present method over ICPMS is that it is not affected by the relatively complex sample composition. The limits of detection of the available spectrophotometric methods for the determination of these metals can be inadequate for biological samples when the amount of biological material is small and when pooling of individual samples is undesirable. In the present study, I wished to obtain estimates of individual variability where the harvestable mass of hemocytes from a single ascidian was usually ~50 mg and was required to be used for both metal and enzyme analysis.

Amongst ascidians that accumulate vanadium, *C. intestinalis* is considered to be a low level accumulator (Michibata *et al.*, 1986). As such, it was an ideal subject for this study as detection limits suitable for work with this species will be adequate for other ascidians which accumulate vanadium at higher concentrations. Samples which are lower in vanadium and iron may still be quantified in this manner, with the addition of

a sample concentration method prior to HPLC as has been applied to other methodologies.

2.4.2 Vanadium and iron concentrations in C. intestinalis

The hemocyte vanadium concentration found in *C intestinalis* in this study is comparable to the vanadium concentration found by Michibata *et al.* (1984; 1986) using neutron activation analysis (Table 2.3). These authors assumed 90% water content for tunic and hemocytes in some of their own calculations and this conversion was therefore used to calculate the wet mass concentration for the purpose of comparison. There was an approximate five fold difference in hemocyte vanadium and iron concentrations between *C intestinalis* from Japan and Italy found by Michibata *et al.* (1984, 1986), compared to the eight-fold differences observed during different time periods in this study. The hemocyte iron and tunic vanadium and iron concentrations during the peak periods in the present study are significantly higher than values recorded in either of these studies by Michibata *et al.* (1984, 1986). Instead, the highest levels of vanadium and iron in the tunic in this study are closer to levels in some of the *Ascidia* spp. (Table 2.3). Unfortunately, as has been common in the study of vanadium in ascidians, the season and temperature were not noted so a season to season comparison is not possible.

Table 2.3: Mean (± sd) vanadium and iron concentrations (μmol g^{-1} wet mass, assuming 90% water content) found in the hemocytes and tunic *Ciona intestinalis* and *Ascidia* sp. and the range of mean values of the present study (as μmol g^{-1} wet mass)

Reference	Species/ Location	Hemocyte vanadium (μmol g^{-1})	Hemocyte iron (μmol g^{-1})	Tunic vanadium (μmol g^{-1})	Tunic Iron (μmol g^{-1})
Michibata *et al.*, 1986	*C. intestinalis* Italy	0.650 ± 0.027	0.759 ± 0.088	0.003 ± 0.0008	0.662 ± 0.070
Michibata *et al.*, 1984	*C. intestinalis* Japan	3.096 ± 0.664	0.140 ± 0.009	0.005± 0.0010	0.157± 0.012
Michibata *et al.*, 1986	*Ascidia malaca,* Italy	8.730 ± 1.002	1.326 ± 0.172	0.168± 0.0075	3.015 ± 0.347
Michibata *et al.*, 1986	*A. ahodori,* Japan	41.462 ± 3.903	3.047 ± 0.397	2.415± 0.0275	0.639 ± 0.025
Present study	*C. intestinalis*				
minimum	Woods Hole,	**0.247**	**0.408**	**0.026**	**0.360**
maximum	USA	**2.131**	**3.473**	**0.290**	**1.130**

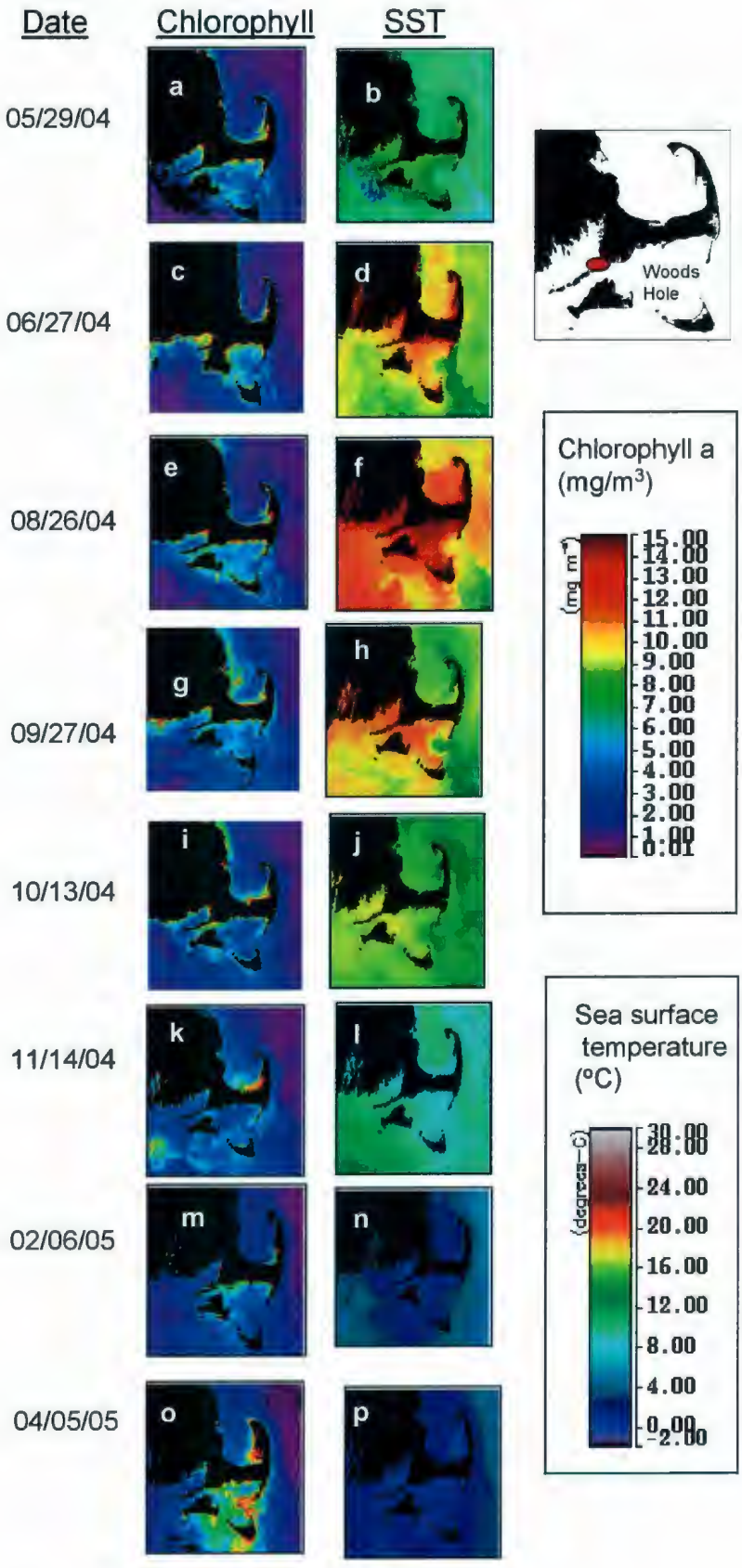
2.4.3 Seasonal changes in metal concentrations

Vanadium and iron concentrations of the hemocytes and tunic showed significant variation among the sampling periods, with notable peaks in the spring. Vanadium concentration of the hemocytes and tunic, and iron concentration in the tunic were also high in the summer. Ecophysiological factors such as water pumping, feeding, and growth rates are implicated here but the responsible factor(s) cannot be separated. This is because temperature and particle concentrations are both different at this time of year: spring blooms occur in the sampling area when sea surface temperatures are low, typically 0 to 5 °C (Figure 2.5). In the summer, surface temperatures are high (typically ~10-15 °C) but particle concentrations are relatively low. On an acute time scale, at low algal concentrations, water pumping rates increase positively with temperature in *C. intestinalis* and other ascidians (Petersen, Mayer, and Knudsen, 1999; Petersen and Riisgard, 1992). At a constant temperature, increases in particle concentrations above a minimum threshold result in adjustment of pumping rate to maintain constant ingestion rates. The seasonally-acclimated pumping rate is likely the net balance of the effects of temperature and particle concentration.

Thus, both low temperature and very high particle concentrations may result in a decrease in water pumping rates. However, bio-kinetic models of metal uptake by other planktivores suggest that, while a lower water pumping rate results in a slower flow (and thus volume) of water through the animal, it might also lead to an increase in the residence time of water in the branchial cavity and thus increase the efficiency of uptake for a given volume of water filtered (Wang and Fisher, 1999).

Carver *et al.* (2003) have found high growth rates in the summer period compared to late fall and winter in *C. intestinalis* in Nova Scotia, Canada. The peak in tunic vanadium and iron concentration in the summer in the present study may be associated with active tunic growth. This is consistent with the suggested link between hemocyte vanadium and iron and tunic or tunichrome formation (Taylor and Molinski, 1991; Taylor, Kammerer, and Bayer, 1997).

Figure 2.5 (a-f, following page): Monthly average chlorophyll *a* (Chl *a*) and sea surface temperatures (SST) during the seasonal study at Woods Hole, with several additional periods. The land mass is represented by black. The data include days for which satellite data were available and un-obscured by cloud cover. Data were provided by NASA, Goddard Space Centre. Images were generated from level 2 data processed with the SeaDAS program.


Date
Chlorophyll
SST
05/29/04
06/27/04
08/26/04
09/27/04
10/13/04
11/14/04
02/06/05
04/05/05
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
p
Woods Hole
Chlorophyll a (mg/m3)
(mg m-3)
15.00
14.00
13.00
12.00
11.00
10.00
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.01
Sea surface temperature (°C)
(degrees C)
30.00
28.00
24.00
20.00
16.00
12.00
8.00
4.00
0.00
-2.00

The peak in hemocyte and body wall G6PDH activities coincide with the peak in hemocyte vanadium concentration. Since G6PDH is an important enzyme in the pentose phosphate pathway, these results are consistent with the hypothesis that NADPH produced via the pentose phosphate pathway provides reducing equivalents for vanadium reduction (Uyama *et al.*, 1998). The higher activity in the body wall may indicate a higher rate of vanadium reduction in the cells in the branchial basket (which forms part of the section) than the cells in the coelomic space during this period as the transverse bars of the branchial basket are hematopoietic (Ermak, 1976; Wright, 1981). An alternative hypothesis is that the pentose phosphate shunt may produce NADPH for the reduction of glutathione, given that reduced glutathione has been shown to be important in metal homeostasis in other species (Ataullakhanov *et al.*, 1981; Brouwer and Brouwer-Hoexum, 1992; Dickinson and Forman, 2002; Rodriguez-Segade *et al.*, 1978)

The iron in ascidian tissues has received little attention, perhaps owing to the familiarity of its known general physiological roles in non-vanadium accumulating organisms compared to the novelty of non-toxicological vanadium accumulation. Well established roles for iron include its participation in the electron transport chain and its role as an oxygen carrier in hemoglobins. Iron does not function as an oxygen carrier in ascidian coelomic fluid as it exhibits no reversible oxygen binding (Macara *et al.*, 1979), and no role has been ascribed to the high concentrations of iron observed. The presence of such high concentrations of iron in both major groups of ascidians illustrates the importance of examining the ecophysiology of iron

accumulation as well as vanadium accumulation in order to determine if both metals fulfill the same physiological function.

In the present study iron and vanadium followed a very similar seasonal pattern in the tunic and hemocytes. This may point to parallel uptake and retention dynamics, or similar or complimentary functions. Both metals may be involved in the tunic growth process, for example. Iron is found in complexes with 3,4-dihydroxyphenylalanine containing peptides in "ferreascidans", but the species distribution and biochemistry of these compounds have not been thoroughly investigated (Taylor *et al.*, 1995). The ferreascidans parallel the byssus forming proteins in marine mussels which are cross-linked with different metals, iron or manganese, depending on the species (Taylor *et al.*, 1994; Tateda and Koyanagi, 1986). Comparisons of iron and vanadium in ecophysiological comparative studies may reveal clues about vanadium's function.

2.4.4 Feeding experiment

While there is some evidence that vanadium may bind to glutathione transferase and be taken up in the gut, (Yoshinaga *et al.*, 2006), the feeding experiment suggests that food is unlikely a major source of vanadium. The differences in food availability were not consistently associated with different vanadium levels, with the exception of the body wall at one time point. However, it is possible that the feeding levels were not sufficiently different to produce a difference in metal concentrations. Future

experiments should involve feeding levels that differ more than those used in this study.

Despite limitations which prevent drawing mechanistic conclusions, a comparison of the feeding experiment data with the seasonal data is informative. Body wall vanadium concentration decreased in the experiment compared to animals sampled directly from Woods Hole, but the hemocyte vanadium levels remained constant, and were not significantly different than those sampled directly from Woods Hole. Exposure to dissolved vanadium was limited for experimental animals due to the water renewal regime i.e. the water renewal rate of the experiment was much lower than the pumping capacity of the ascidians. The maintenance of hemocyte vanadium levels by experimental animals may have resulted from higher residence time of water in the branchial cavity (which may have compensated for the smaller volume of water exposure), renewal from a storage tissue, and/or low loss rates.

While iron levels in the body wall and hemocytes did not differ between treatment groups, both experimental groups exhibited a rise in iron concentration during the experiment compared to the animals harvested from Woods Hole. The latter did not experience high phytoplankton levels while this experiment was being carried out. Given the high concentrations of iron in phytoplankton cells, this suggests that diet may be a more important source of iron than it is for vanadium. Iron levels are in the millimolar range in some marine phytoplankton, being especially high in *Thalassiosira* sp. (Ho *et al.,* 2003) which made up 30% of the phytoplankton fed to the animals in the experiment.

2.4.5 Conclusions and future directions

This work is the first to study seasonal variation in vanadium and iron levels in ascidian tissues. I find, for the first time, evidence of significant increases in metal concentration around the time of the spring plankton bloom. This study was not able to identify the reason for this increase. Now that a period of higher vanadium concentration has been identified, investigations can be undertaken to determine whether this is a result of changes in factors such as pumping rate, loss rates or changes in vanadium utilization. It will be important to determine whether the patterns observed occur among other phlebobranch ascidians in the North Atlantic, especially those which accumulate higher levels of vanadium, such as those of the genus *Ascidia*. It is also important to know whether iron in stolidobranch tissues varies in the same manner. If iron is fulfilling a similar function, or if iron uptake is affected by similar ecophysiological factors, one might expect a similar seasonal pattern in concentration.

C. intestinalis is an attractive study animal as it is very widely geographically distributed (Dybern, 1965). Comparisons of the seasonal vanadium dynamics in areas such as the northwestern Atlantic and Scandinavia to areas where the environmental fluctuations in temperature and food supply are much different, such as the Mediterranean, would be very fruitful in separating temperature and food supply effects. Laboratory experiments will be able to determine the relationships between physiological parameters and vanadium uptake and concentration.

2.5 References

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Chapter 3: Seasonal changes in the vanadium and iron concentrations of a phlebobranch (*Ascidia callosa*) and stolidobranch (*Halocynthia pyriformis*) ascidian in the Northwest Atlantic

3.1 Introduction

The accumulation of vanadium in the absence of environmental contamination from pollution is very rare in the animal kingdom. In specialized cells of the coelomic fluid of some ascidians, however, vanadium is present at concentrations up to 350 mM, and is found in lesser, but high, concentrations in other tissues (Henze, 1911; Michibata *et al.*, 1986; Turon and Lopez-Legentil, 2004). Members of the suborders Phlebobranchia and Aplousobranchia exhibit this trait, while members of the Solidobranchia do not (Carlisle, 1968; Hawkins *et al.*, 1983; Michibata *et al.*, 1986). Iron is found in high concentrations in members of all suborders (Agudelo *et al.*, 1983; Endean, 1953; Michibata *et al.*, 1986). The vanadium dynamics of the phlebobranch ascidians have received the most attention, especially in the last decade (see Michibata *et al.*, 2002; 2003).

Vanadium is present in very low, but detectable, amounts in stolidobranch ascidians, in the highest amounts and predominantly in the V(III) oxidation state in phlebobranchs, and in high amounts and predominantly in the V(IV) oxidation state in aplousobranchs. Together with morphological and molecular characteristics, (Stach and Turbeville, 2002; Turon and Lopez-Legentil, 2004; Zeng and Swalla, 2005), the vanadium concentration and predominant oxidation state of vanadium have been suggested as defining characteristics of the three groups (Hawkins *et al.*, 1983). It is unknown whether vanadium provides a novel function in the groups that accumulate it, whether iron performs a similar function as vanadium, or whether vanadium

accumulation is an ancestral or derived trait. While some aspects of the uptake and reduction of vanadium have been characterized, including the identification of vanadium binding proteins or vanabins (Michibata and Uyama, 1990; Michibata et *al.*, 1998; Ueki et al., 2003; Uyama et al., 1998; Yoshihara et al., 2005), the iron physiology of no ascidian group has received a great deal of attention (Agudelo *et al.*, 1983; Endean, 1953; Curtin et *al.*, 1985). One parallel with vanadium is that iron is found in the reduced, Fe (II) state (Agudelo et *al.*, 1983). A comparative study of the iron accumulation by ascidians, and the similarities and differences with that of vanadium may therefore be informative with respect to both metals.

There is little known about the variation in vanadium and iron dynamics under different physical and physiological parameters. Studies of the ecophysiology of vanadium or iron accumulation by ascidians may provide information regarding sources of variability that might, in the future, be exploited to investigate function. As ascidians are ectothermic suspension feeders relying on a seasonal phytoplankton food supply, seasonal variation is an obvious starting point. Iron and vanadium are present in both the seawater and the food supply of ascidians. Vanadate dissolved in seawater is considered the primary source of vanadium for ascidians and is present at a higher standing concentration than iron (Butler, 1998). While vanadium is also present in some phytoplankton, the iron concentration of phytoplankton is higher, suggesting that diet may be a significant source of iron (Ho *et al.*, 2003). In the past, few authors have directly investigated seasonality in ascidian metal concentrations, and few have noted the season in which samples were gathered.

Pumping and circulation of water by ascidians fulfills multiple physiological functions and leads to both exposure to dissolved and dietary iron and vanadium. The effects of temperature and particle concentrations on pumping rate indicate that there might be seasonal variation in metal concentration. A preliminary study of vanadium and iron concentration of the low level vanadium accumulator *Ciona intestinalis* (Chapter 2 of this thesis) showed that the concentration of both metals peaks in the spring period while the water is still cold. Accordingly, this chapter examines the seasonal variation of the vanadium and iron concentrations of *Ascidia callosa*, a phlebobranch ascidian, accumulating large amounts of vanadium and iron, and *Halocynthia pyriformis*, a stolidobranch ascidian, accumulating only iron, from the Northwest Atlantic. I hypothesized that similar seasonal patterns of vanadium and/or iron concentration will occur in these species with peaks before the spring bloom. I examine the vanadium and iron concentration of the branchial basket, tunic, muscle, and renal vesicles (*A. callosa* only) along with the hemocytes, to determine whether the relative tissue distributions changes seasonally.

Vanadium is found primarily in the signet ring cells in phlebobranch ascidians, in the reduced V(III) state. It is also found in other cell types, some of which are considered intermediate or precursor cell types, such as compartment cells or morula cells (Nette *et al.*, 1999). Vanadium(III) can be stained with 2,2' bypyridine in living hemocytes of phlebobranchs (Nette *et al.*, 1999). In this study this procedure is used to determine whether 1) the frequency and proportion of these cells varies seasonally and coincides with variation in vanadium concentration of *A. callosa* and 2) increases in vanadium

concentration are associated with changes in the number of signet ring cells or with changes in the vanadium concentration of the cells.

3.2 Materials and Methods

3.2.1 Animal collection

Ascidia callosa and *Halocynthia pyriformis* were collected from the Admiral's Cove, Avalon Peninsula of Newfoundland (November 2005-November 2006). Animals were gently removed from substrate by divers and transported in coolers with seawater to the Ocean Sciences Centre, Logy Bay, Newfoundland on the same day. They were transferred to ambient flowing seawater. Sampling was generally conducted monthly. In the March-April period tissues were sampled at two week intervals. Six animals of each species were placed in FSW (filtered seawater, 1 μm) for 48 hours before use to ensure that the gut and branchial basket were empty. A similar size and mass range was chosen for sampling in each sampling period (with no statistical difference among sampling periods) with the mean mass (± sd) and length of 55 ±15 g ($F_{9, 58}$= 2.33, p=0.039), 50 ± 12 mm ($F_{9, 58}$= 2.61, p=0.012), for *A. callosa* and 33 ±10 g ($F_{9, 58}$= 2.51, p=0.021), 45± 9 mm ($F_{9, 58}$= 2.57, p=0.017) for *H. pyriformis.*

3.2.2 Tissue Sampling

Coelomic fluid was obtained via cardiac puncture. It was drawn into a syringe containing 40 mM cysteine in ASW seawater (ASW- 500 mM NaCl, 8.0 mM KCl, 29 mM Na_2SO_4, 75 mM Tris, pH 7.8) in an approximately ~1:1 ratio to prevent clotting (total volume 500-2000 µl). Several aliquots of this diluted coelomic fluid were immediately used for image analysis of the cells. The remainder was divided into aliquots and transferred into pre weighed, acid washed vials for metal analysis. Other tissues were dissected for metal analysis including the siphon retractor muscle, renal vesicles (*A. callosa* only), tunic (1 mm thick strips from the proximal edge of *A. callosa* and a section of the entire tunic for *H. pyriformis*), and branchial basket (50% of the entire branchial basket). These were frozen at -20 °C until further analysis.

3.2.3 Image analysis

Aliquots of coelomic fluid of *A .callosa* (typically 10 µl) were mixed 1:1 with a ASW solution saturated with 2,2' by-pyridine on a gridded microscope slide. This compound forms a purple complex with reduced vanadium (Nette *et al.*, 1998, 1999). Images (1260x960 pixels) covering one or two 1 mm^2 squares were captured at with a Pixera Viewfinder camera, at a total magnification of 0.16 µm $pixel^{-1}$. Usually 750 to 1000 cells greater than 2 µm were counted. Cells stained with 2,2,' bypyridine were sized. The counted cells were categorized as follows: stained SRCs, stained multi-vacuolated cells, unstained morula cells, and "other" types (including pigment cells,

compartment cells, gametocytes and others.). Images were analyzed with the program UTHSCSA ImageTool v.3 ® (University of Texas Health Sciences Centre).

3.2.4 HPLC analysis of vanadium and iron

This was carried out as in Chapter 2. After approximately 2/3 of sampling was completed, sample analysis was started and continued with samples chosen randomly.

3.2.5 Vanadium and iron concentration of Ficoll separated signet ring cells of A. callosa

At the mid-March sampling, hemocytes were harvested from 4 individuals in the manner described above. The cells were centrifuged at 800 g for 10 minutes and re-suspended in ASW with 20 mM cysteine. A discontinuous gradient of Ficoll 400 (12%, 18%, 29%, and 36%) in ASW was prepared and layered in a plastic tube. The cell suspensions were layered on top and allowed to separate by gravity at 5^{o} C for two hours. Layers were carefully pipetted from the top. Small samples of each layer were examined for cell composition. The third layer, the SRC layer (29% Ficoll) was centrifuged at 15600 g for 10 minutes and the resultant pellet was homogenized in acid and the vanadium and iron concentration of determined by the PAR- HPLC method.

3.2.6 Statistical analysis

Statistical analyses were completed with Systat v. 9 4 ®. Seasonal tissue vanadium and iron concentrations were analyzed by GLM with the following model:

$$\text{Tissue concentration} = \beta_0 + \beta_{\text{Time}} \text{Time} + \varepsilon$$

with $\alpha = 0.05$ and "Time" as a categorical variable.

Pairwise comparison probabilities among all sampling points for the seasonal data, were generated with the Bonferroni correction and $p<0.05$ was taken as significant. Residuals were visually examined for normality, homogeneity and independence and were found to be satisfactory.

3.3 Results

3.3.1 Vanadium and iron concentration of A. callosa tissues

A. callosa hemocyte ($F_{9, 58} = 2.44$, $p=0.028$), branchial basket ($F_{9, 58} = 2.32$, $p=0.039$), muscle($F_{9, 58} = 2.42$, $p=0.029$) and tunic ($F_{9, 58} = 2.38$, $p=0.033$) vanadium levels varied seasonally while renal vesicle ($F_{9, 58} = 1.12$, $p=0.15$) vanadium levels did not.

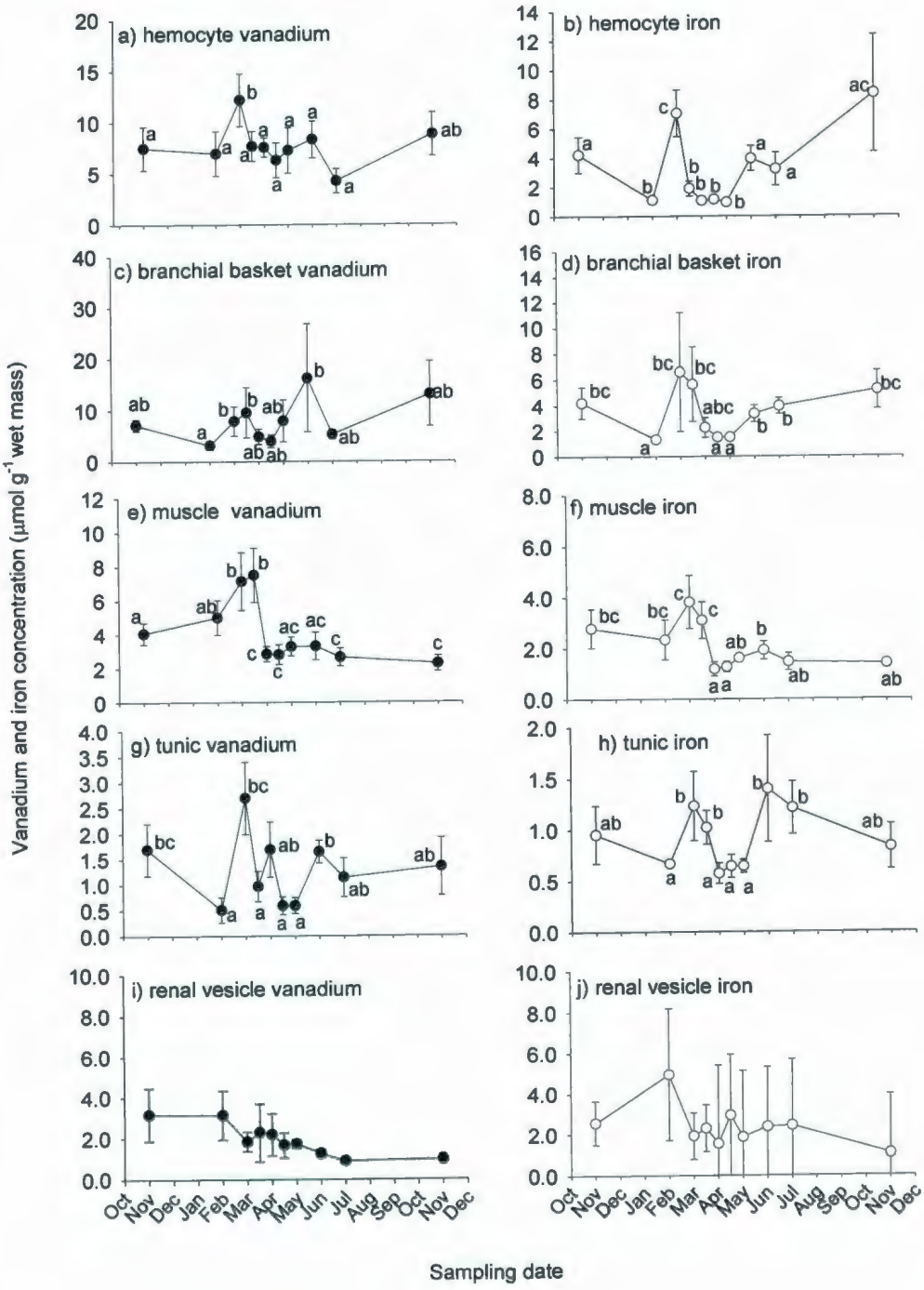
Vanadium concentration of the *A. callosa* hemocytes was higher in the peaked in March (~12 μmol g^{-1}) compared to other times of year. The average over all other

sample periods was 7.0 μmol g^{-1} (Figure 3.1 a); this and comparisons like it involving global means are qualitative, not statistical. Branchial basket vanadium concentration exhibited a series of small seasonal increases in February to March, June and November (Figure 3.1 c). Muscle vanadium concentration reached 5.0 μmol g^{-1} in the March sampling period compared to an average of 3.3 μmol g^{-1} over all other sampling periods (Figure 3.1 e). Tunic vanadium levels also exhibited a series of small peaks in March and June (Figure 3.1 g). The vanadium concentration of the renal vesicles was 1.9 μmol g^{-1} over the sampling year.

A. callosa hemocyte ($F_{9, 58} = 2.32$, p=0.038), branchial basket ($F_{9, 58} = 2.21$, p=0.049), muscle ($F_{9, 58} = 2.25$, p=0.045) and tunic ($F_{9, 58} = 2.40$, p=0.031) iron levels varied seasonally while renal vesicle ($F_{9, 58} = 0.58$, p=0.20) iron levels did not.

The iron concentration of the hemocytes peaked in March and November, reaching 7.5 and 8.4 μmol g^{-1}respectively compared to an average of 2.1 μmol g^{-1} over all other sampling periods (Figure 3.1 b). The iron concentration of the branchial basket was 3.6 μmol g^{-1} averaged among all sample periods, reaching a high of 6.1 μmol g^{-1} over the March sampling periods. The iron concentration of muscle averaged 3.5 μmol g^{-1} in the two March sampling periods, compared to 1.7 μmol g^{-1} in the other sampling periods (Figure 3.1 f). Tunic iron reached 1.1 μmol g^{-1} in March and 1.3μmol g^{-1} June/July (Figure 3.1 h). The iron concentration of the renal vesicles was 2.4 μmol g^{-1} averaged over all the sampling points.

Figure 3.1 (following page): Seasonal changes in iron and vanadium concentration in tissues of *Ascidia callosa* in Newfoundland, determined by RP-HPLC. The data presented are the mean ± sd of n=6 for each sampling period. Data were analyzed by GLM (Tissue concentration = $\beta_0 + \beta_{Time}$ Time + ε with α =0.05 and "Time" as a categorical variable. Pairwise comparison probabilities among all sampling points were generated with the Bonferroni correction and $p<0.05$ was taken as significant. Different letters refer to significant differences in the vanadium or iron concentration. Lack of letters indicates lack of significance in the overall model.


a) hemocyte vanadium
b) hemocyte iron
c) branchial basket vanadium
d) branchial basket iron
e) muscle vanadium
f) muscle iron
g) tunic vanadium
h) tunic iron
i) renal vesicle vanadium
j) renal vesicle iron
Vanadium and iron concentration (µmol g-1 wet mass)
Oct Nov Dec Jan Feb Mar Apr May Jun Jul Aug Sep Oct Nov Dec
Sampling date

3.2.2 Cell types in A. callosa

Figure 3.2 illustrates some of the cell types observed in *A. callosa*. Unstained signet ring cells were so rare that they could not be reliably quantified, and were probably an artifact of the staining procedure. Figure 3.3 shows the seasonal changes in the hemocyte population in *A. callosa*. The number of stained signet ring cells ($F_{9, 58}$ = 2.61, p=0.012), unstained multivacuolated cells (($F_{9, 58}$ = 2.57, p=0.015), other cells ($F_{9, 58}$ = 2.32, p=0.039) and total number of cells ($F_{9, 58}$ = 2.46, p=0.026) varied seasonally while the number of stained multivacuolated cells ($F_{9, 58}$ = 0.472, p=0.21) did not vary seasonally.

The number of vanadium-containing cells, as evidenced by the number of stained signet ring cells showed a sharp increase, tripling, during March and decreasing to pre-peak levels by early April (Figure 3.3a). The number of non-vanadium containing cells (both multivacuolated cells and "other" cells) and the total number of cells followed a similar pattern (Figure 3.3 b, d, e).

Examination of the percentage of each cell type in reference to the total number of cells reveals a similar seasonal pattern (Figure 3.4). The proportion of stained signet ring cells ($F_{9, 58}$ = 2.34, p=0.037) stained multivacuolated cells ($F_{9, 58}$ = 2.22, p=0.048), unstained multivacuolated cells ($F_{9, 58}$ = 2.26, p=0.044) and other cells ($F_{9, 58}$ = 2.33, p=0.038) varied seasonally.

The percentage of stained signet ring cells reached 22 % in early March, while the percentage of "other cells" decreased to ~40% in March from 70% in November. The percentage of unstained multi-vacuolated cells decreased to 12% in the early April period from 40% in early March. This cell type as a proportion of the total cell population peaked in March (40%) and May (37%).

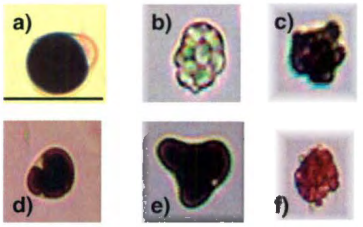
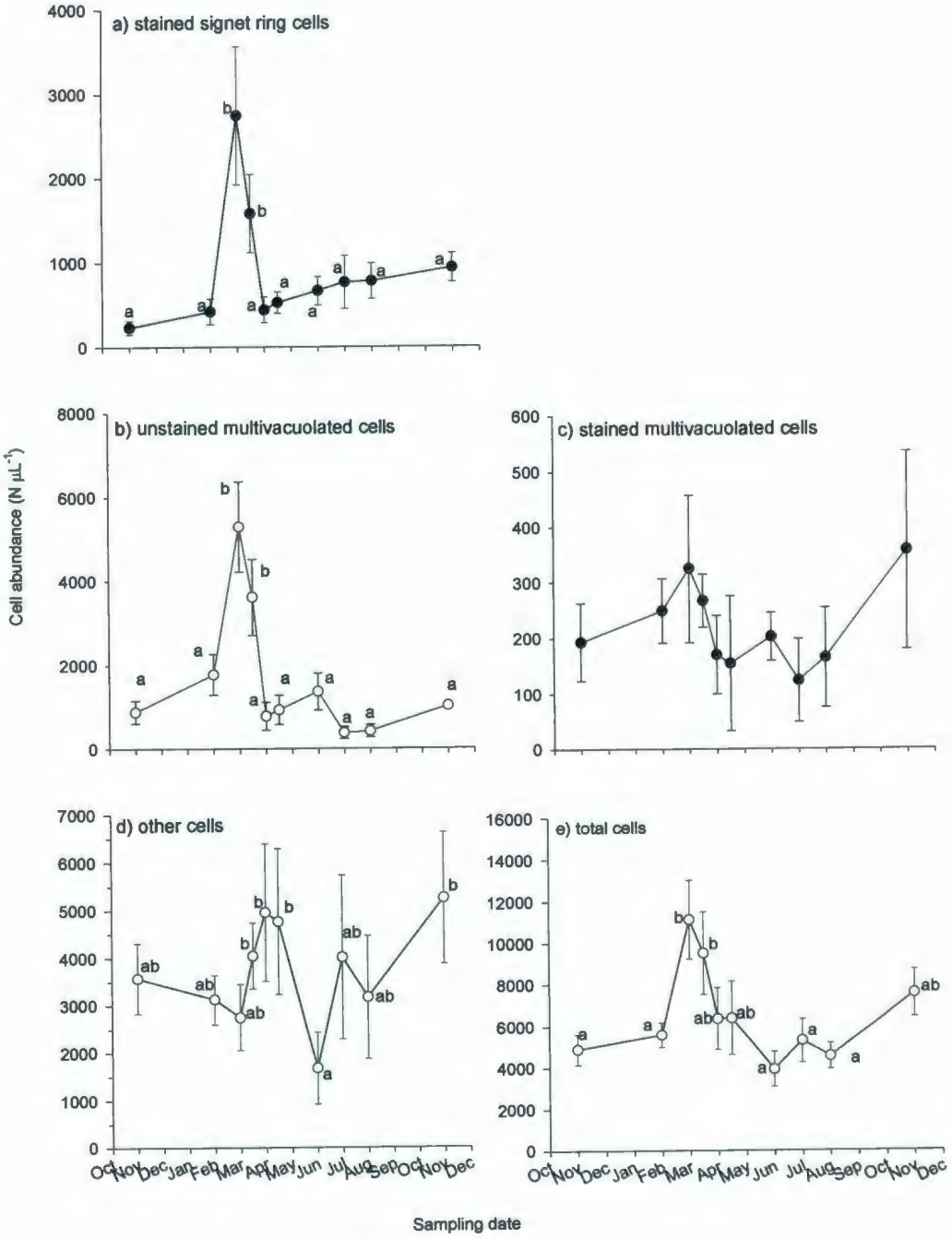


Figure 3.2 (above): Some of the cell types observed in *A. callosa*. a) Stained signet ring cell, b) unstained morula cells, c) stained morula cell, d) stained bi-vacuolated cell, e) stained tri-vacuolated cell, and f) pigment cell. The scale bar in a) represents 10 μm and applies to all panels.

Figure 3.3 (following page): Seasonal changes in the number of circulating hemocytes in the coelomic fluid of *Ascidia callosa* in Newfoundland, determined by image analysis of living cells. Stained refers to cells that stained purple with 2,2'by-pyridine. The data presented are the mean ± sd of n=6 for each sampling period. Data were analyzed by GLM (Response= $\beta_0 + \beta_{Time}$ Time + ε) with α =0.05 and "Time" as a categorical variable. Pairwise comparison probabilities among all sampling points were generated with the Bonferroni correction and $p<0.05$ was taken as significant. Different letters refer to significant differences in the various responses among sampling points. A lack of letters indicates no significance in the overall model.


a) stained signet ring cells
b) unstained multivacuolated cells
c) stained multivacuolated cells
d) other cells
e) total cells
Cell abundance (N µL-1)
Sampling date
Oct Nov Dec Jan Feb Mar Apr May Jun Jul Aug Sep Oct Nov Dec

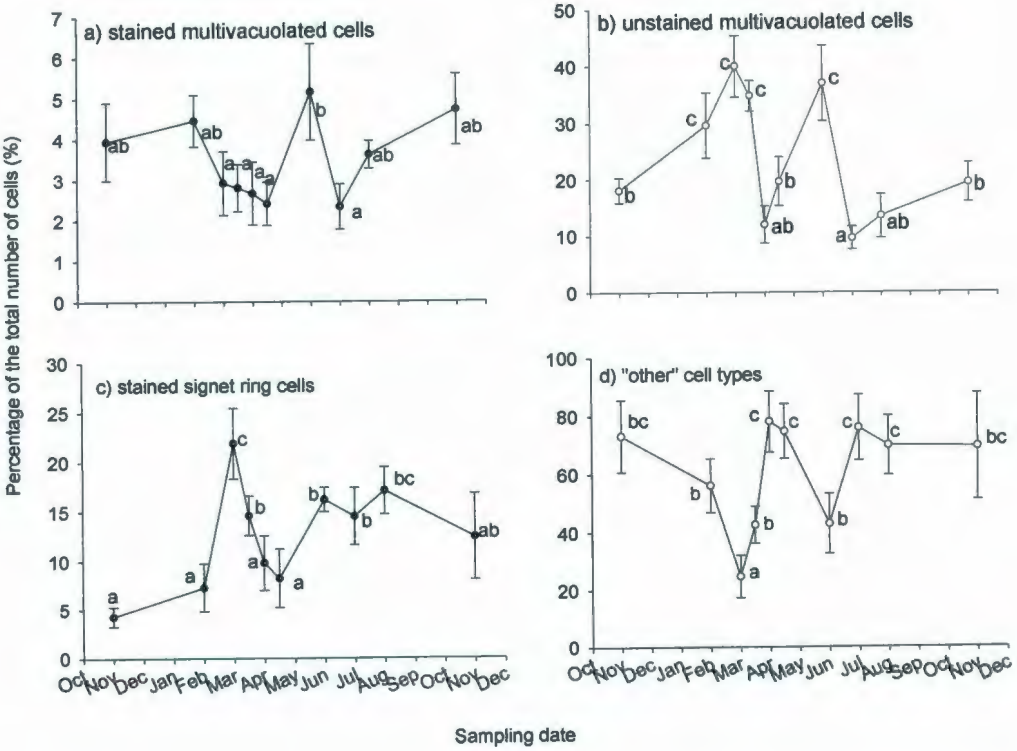


Figure 3.4 (above): Seasonal changes in the relative proportion of circulating hemocytes types in the coelomic fluid of *Ascidia callosa* in Newfoundland, determined by image analysis of living cells. "Stained" refers to cells that stained purple with 2,2'bypyridine. The data presented are the mean ± sd of n=6 for each sampling period. Data were analyzed by GLM (Response= $\beta_0 + \beta_{Time}$ Time + ε)with α =0.05 and "Time" as a categorical variable. Pairwise comparison probabilities among all sampling points were generated with the Bonferroni correction and $p<0.05$ was taken as significant. Different letters refer to significant differences in the various responses among sampling points.

Density gradient separation was successful in isolating a population of signet ring cells for determination of vanadium and iron concentrations. The third layer contained ~90% SRCs and the vanadium concentration was found to be comparable to that of *A. sydneiensis samea* (Table 3.1).

3.2.3 Vanadium and iron concentrations of H. pyriformis tissues

Using the present methodology and homogenization volumes, vanadium was detectable but below the limits of quantification in *H. pyriformis* hemocytes. *H. pyriformis* branchial basket ($F_{9, 58} = 2.26$, $p=0.044$) and tunic ($F_{9, 58} = 2.34$, $p=0.037$) vanadium levels varied seasonally while muscle ($F_{9, 58} = 0.688$, $p=0.043$) vanadium levels did not. *H. pyriformis* hemocyte ($F_{9, 58} = 2.22$, $p=0.048$), muscle ($F_{9, 58} = 2.28$, $p=0.043$) and tunic ($F_{9, 58} = 2.20$, $p=0.049$) iron levels varied seasonally while branchial basket ($F_{9, 58} = 0.148$, $p=0.24$) iron levels did not.

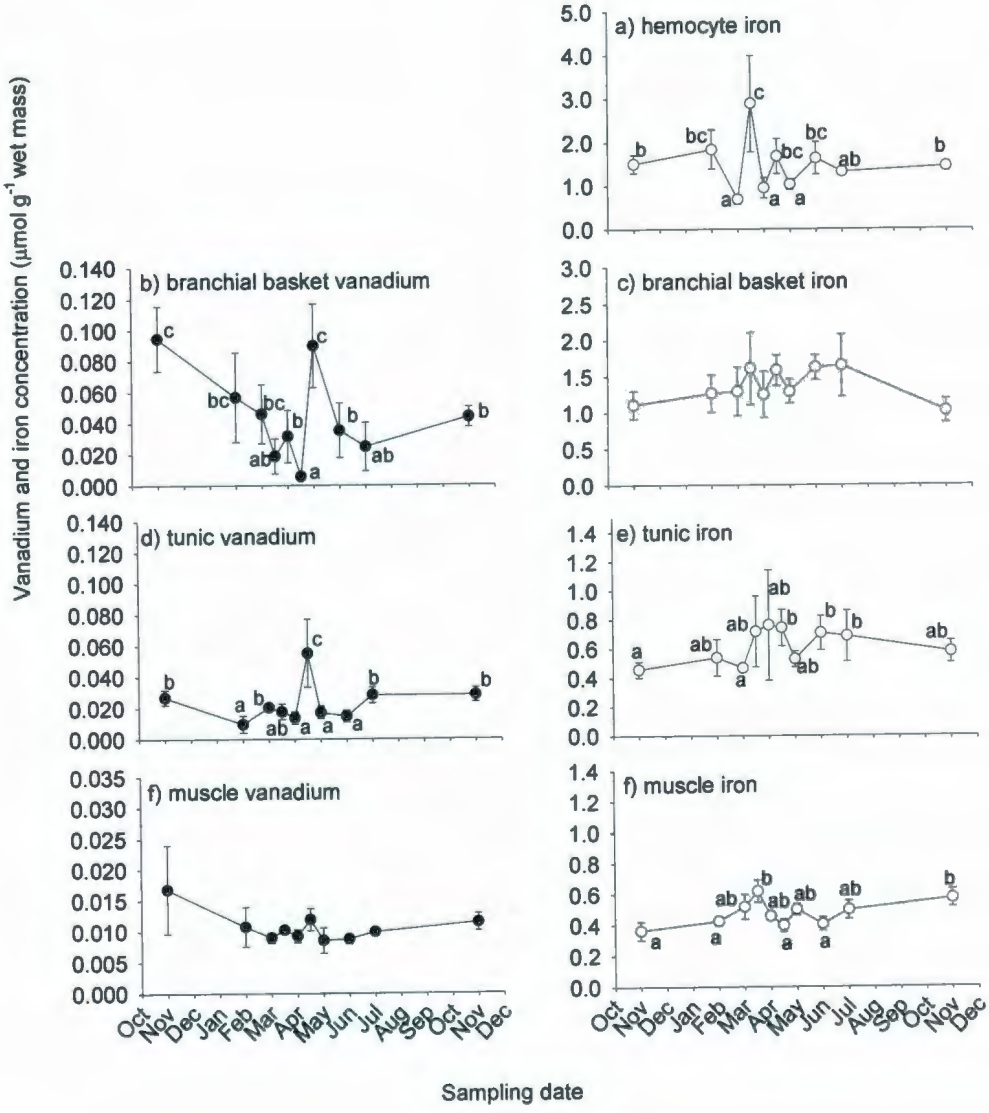
The iron concentration of the hemocytes (Figure 3.5) was 1.5 μmol g^{-1} over the entire year and did not exhibit a pronounced seasonal peak. Vanadium concentration of the branchial basket was quantifiable and peaked in November (0.095 μmol g^{-1}) and in May (0.089 μmol g^{-1}). Branchial basket iron concentration was relatively constant with a mean concentration of 1.4 μmol g^{-1} over the year. Tunic vanadium concentration was higher in late April (0.055 μmol g^{-1}) than at any other time (0.020 μmol g^{-1}). The iron concentration of the tunic did not exhibit a clear peak and was 0.62 μmol g^{-1} over all sampling period. Muscle vanadium showed no seasonal

variation (annual mean of 10.6 μmol g^{-1}). The muscle iron concentration was highest in March but did not exhibit a clear seasonal peak.

Table 3.1: Mean vanadium and iron concentrations of the signet ring cells (n=4) of *Ascidia callosa* in March, separated by discontinuous Ficoll density gradient separation

Signet ring cells in Layer 3 (% of total cells)	92.0 ± 4.0
Iron concentration of layer 3 (μmol/g))	35.0 ± 8.2
Vanadium concentration of layer 3 (μmol/g)	212.1 ± 18

Figure 3.5 (following page): Seasonal changes in iron and vanadium concentrations in tissues of *Halocynthia pyriformis* in Newfoundland, determined by RP-HPLC. The data presented are the mean ± sd of n=6 for each sampling period. The data presented are the mean ± sd of n=6 for each sampling period. Data were analyzed by GLM (Response= $\beta_0 + \beta_{Time}$ Time + ε) with α =0.05 and "Time" as a categorical variable. Pairwise comparison probabilities among all sampling points were generated with the Bonferroni correction and $p<0.05$ was taken as significant. Different letters refer to significant differences in the various responses among sampling. The absence of letters indicates a lack of significance in the overall model.


a) hemocyte iron
b) branchial basket vanadium
c) branchial basket iron
d) tunic vanadium
e) tunic iron
f) muscle vanadium
f) muscle iron
Vanadium and iron concentration (µmol g-1 wet mass)
Sampling date
Oct Nov Dec Jan Feb Mar Apr May Jun Jul Aug Sep Oct Nov Dec

3.4 Discussion

3.4.1 Comparison with other species in the genera

Table 3.2 presents the vanadium and iron concentrations of two Japanese species in the each of the genera of the present study (Michibata *et al.*, 1986). For comparison with the present study, the data were converted from dry mass to wet mass based on 90% water content. Generally, the vanadium and iron concentrations of the North American species were similar to those of the Japanese species. The hemocyte vanadium and iron concentrations of *A. callosa* were similar to those of *A. sydneiensis samea*. The branchial basket vanadium concentrations are more similar to those of *A. ahodori*, while the iron levels in *A. callosa* were much higher than either of these species. The vanadium concentrations of the tunic and muscle were higher in *H. pyriformis* than *H. aurantium* and *H. roetzi*, but were similarly low in comparison to levels found in the phlebobranch ascidians by the methodology. The vanadium concentration of the branchial basket was several orders of magnitude higher in *H. pyriformis* than the species collected in Japan or Italy by (Michibata *et al.* 186).

The level of intraspecific variation in the metal concentrations in this study is data is higher than that of Michibata *et al.* (1986) and other studies that use dry mass as a denominator. Michibata *et al.* (1986) measured metal concentration of freeze dried samples with neutron activation analysis which is a more sensitive technique than the one used in this study. The assumption of 90% water content may not be uniformly appropriate given possible differences in water content among samples. Despite these

limitations, the comparisons reveal largely similar vanadium and iron concentrations of the North Atlantic ascidians with the Japanese congeners. A season to season comparison might be informative but is not possible.

Table 3.2 Vanadium and iron concentrations of *Ascidia* spp. and *Halocynthia* spp. from Michibata *et al* (1986), based on 90% water content.

Species	Vanadium concentration ($\mu mol\ g^{-1}$ wet mass)	Iron concentration ($\mu mol\ g^{-1}$ wet mass)
Ascidia sydneiensis samea		
Coelomic cells	9.179 ± 0.694	1.619 ± 0.332
Branchial basket	1.434 ± 0.187	0.535 ± 0.083
Tunic	0.060 ± 0.005	2.564± 0.521
Ascidia ahodori		
Coelomic cells	41.462 ± 3.903	3.047± 0.397
Branchial basket	12.868 ± 0.650	0.860± 0.0797
Tunic	2.415 ± 0.0275	0.639 ±0.025
Halocynthia aurantium		
Coelomic cells	0.0043± 0.0004	0.4391 ± 0.0338
Branchial basket	0.0016 ± 0.0002	0.2778± 0.0329
Tunic	0.0025± 0.0004	0.1489 ± 0.0145
Halocynthia roetzi		
Coelomic cells	0.0071± 0.0008	0.5728 ± 0.0447
Branchial basket	0.0037 ± 0.001	0.0822 ± 0.0150
Tunic	0.0114 ± 0.0049	0.0906 ± 0.0238

3.4.2 Seasonality in metal concentrations in A callosa and H. pyriformis

There is seasonal variation in the iron and vanadium concentrations in some tissues of *A. callosa* and *H. pyriformis*. As occurred in *C. intestinalis* (Chapter 2 of this thesis), the hemocyte, tunic and muscle vanadium concentrations of *A. callosa* peaked in early spring. Iron concentration of the hemocytes and tunic of this species showed similar seasonal variation. That this occurred in different years and different sampling locations in two species within the North Atlantic suggests that it is general. The data do not suggest a bulk movement of vanadium from one tissue or the hemocytes to the other tissues, as the peaks in vanadium concentration of the hemocyte pellet coincide with, rather than precede the peaks in vanadium concentration of muscle and tunic. Rather the data indicate an increase in the number of hemocytes in these tissues at that time, corresponding with a general increase in the number of hemocytes.

The vanadium concentration of the branchial basket and tunic of *H. pyriformis* also peaked during the spring period. Although the seasonal pattern is not as strong in *H. pyriformis* as in *A. callosa*, the increase in the spring may indicate that similar processes are responsible for this increase in vanadium concentration. In the year covered by this study, the spring bloom occurred in mid April (Figure 3.6), as is generally the case in this area (Pomeroy and Deibel, 1986; Pomeroy *et al.*, 1991; Putland, 2000). Thus the peaks in vanadium and iron concentration occurred when the water was still cold (~1°C), and before the spring phytoplankton bloom.

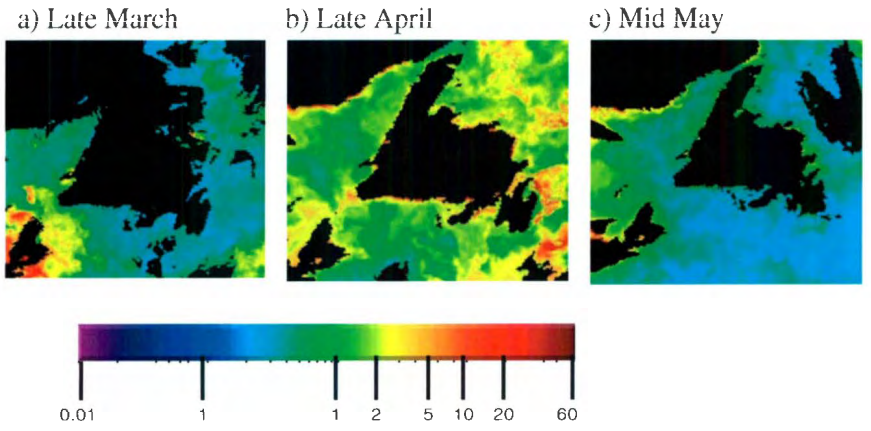


Figure 3.6: Satellite images showing the timing of the spring phytoplankton bloom around the island of Newfoundland. These images are NASA generated weekly composites of days on which cloud cover did not prevent data gathering. The scale below the maps in mg chlorophyll *a* m^{-3}. Data provided by the SeaWiFS Project, NASA/Goddard Space Flight Center and ORBIMAGE.

Given that pumping rates decrease with decreasing temperature and particle concentrations (Fiala -Medioni, 1978; Petersen *et al.*, 1999), the total dissolved vanadium exposure from water passing over the branchial basket was probably lower during March than in months with higher temperature and particle concentrations. Bio-kinetic models employed by toxicologists suggest several possible explanations for the increase in metal concentration in light of these factors (Wang *et al.*, 1996). A release from a storage site that was not sampled, or a decrease in loss rates might result in an increase in the net vanadium and iron retention and thus higher vanadium and iron concentration. In addition, uptake efficiency may be higher compared to

times during which the pumping rate is higher. A higher residence time of water in the branchial basket may result in higher efficacy of uptake from a given volume of water, perhaps offsetting the decrease in total volume of water exposure. Vanadium levels were relatively high in the November as well, as this may result from similar phenomena as there is also a fall phytoplankton bloom in this area of the North Atlantic.

Although there is scant data on the metal loss rates of ascidians, work on other suspension feeders indicates that metal loss rates decrease with decreasing temperature. For example, loss rates of zinc, an essential metal, from the bivalve mollusk *Mytilus edulis* in the Baltic Sea were higher in warm water in summer than in cold water in winter (Dahlgaard, 1986). Such a phenomenon may contribute to the pattern observed in the current study. Once the spring bloom starts, while pumping rates and total metal exposure may increase, loss rates may increase and efficiency of uptake decrease.

These relationships between temperature, pumping rate and particle concentration apply to iron and vanadium, and to both ascidian species. The seasonal patterns were not identical between the two species or the two metals of this study, however. Vanadium concentration peaked only in the branchial basket and tunic of *H. pyriformis* and at a later date than in *A. callosa*. Whereas the iron concentration of *H. pyriformis* tissues exhibited little seasonal variation, there was a clear increase in the iron concentration of the hemocytes tunic and muscle of *A. callosa* in spring.

The existence of a seasonal peak in vanadium concentrations of *H. pyriformis* in some tissues implies that similar utilization, uptake and/or loss processes may be involved, as is the case for iron. However, the lag in the peak in the vanadium concentration may be related to a general physiological difference between the two species such as seasonal pumping rates. One would expect a portion of the iron pool to be relatively constant due to its role in the activity of enzymes and the cytochromes for instance. That the iron concentration of *H. pyriformis* exhibited less seasonal variation than in *A. callosa* suggests that an additional factor may influence the iron concentration of *A. callosa*. The diet of ascidians would likely be higher in iron during the spring and fall phytoplankton blooms, perhaps contributing to the patterns observed in *A. callosa*. As suggested in Chapter 2, an increase in phytoplankton availability may result in an increase in iron concentration. The iron concentration of the tunic of *A. callosa* peaked in the spring and in November, after the fall bloom, while in *H. pyriformis* this occurred only in the spring.

It is possible that a physiological factor may affect the pattern observed in these species, in addition to the discussed environmental influences. Specimens of both species were gravid when the vanadium levels peaked. As a result, a general increase in vanadium concentration and signet ring cells for reproductive processes cannot be ruled out. Botte *et al.* (1979) found that the vanadium concentration in the ovary of *Ciona intestinalis*, *Ascidia malaca*, and *Polycarpa gracilais* (Stolidobranchia) follows the same pattern as the rest of the body in terms of relative vanadium concentration. However, they determined that the vanadium was localized in the inter-ovarian cells, not within the oocytes themselves (Botte *et al.*, 1979). Nette *et al.* also demonstrated

that the first accumulation of vanadium (III) occurs five days after fertilization. (Nette *et al.*, 1998). This observation, together with the observation that the peak in vanadium and iron co-occur with, rather than precede the appearance of gravid individuals, suggests that the spring peaks in vanadium and iron is not necessarily directly linked to input of metals to the oocytes, but co-incident with maturation.

3.4.3 Cell types in A. callosa

The number of circulating hemocytes per volume of coelomic fluid in *A. callosa* was generally lower than that found in studies of other phlebobranchs but the relative proportions of cell types was similar to other phlebobranchs (Table 1.3). The entire hemocyte population of this species tripled in the spring period (Figure 3.2). The vanadium-containing signet ring cells and non-vanadium containing morula cells were largely responsible for the increase. The increase in "other cell types" lagged behind the increase in signet ring cells and morula cells. The 'other cell' types were generally small compartment cells, approximately 2 μm in diameter, as well as a small number of pigment cells. The fate of the vanadium containing and other cells responsible for the peak in vanadium concentration in the spring is unknown. They may have migrated to a tissue or part of a tissue not sampled in this study.

The only other study found by the author on seasonal variation in the hemocytes of vanadium accumulating ascidians was that of *A. ceratodes* in Bodega Bay, California (Biggs and Swinehart, 1979). Biggs and Swinehart (1979) found significant variation

among individuals in the total cell counts of 47 400-144 500 cells mm^{-3}. *A ceratodes* coelomic fluid was composed of approximately 53% morula cells (which they misidentified as vanadocytes). In contrast to this study, there was no significant seasonal variation in any of the cell types from January to June.

The increase in the number of vanadium containing cells in March was greater in magnitude than the change in the vanadium concentration of the hemocyte pellet. This might indicate that there was less metal per individual cell than at other times of year. It is also possible that the changing composition of the cell pellet resulted in a slight change in the water content, a phenomenon not affecting the image analysis of cell types.

3.4.4 Conclusions

This study has shown that there is seasonal variation in vanadium and iron levels in *H. pyriformis* and *A. callosa* and that, in general, the concentrations of both metals peak in the spring before the major phytoplankton bloom. Although this study cannot provide an explanation for this, future study of the vanadium and iron uptake and loss rates during the spring compared to other times of the year may reveal the cause. There was a general proliferation of hemocytes during the spring, and an increase in the number of vanadium containing cells in *A. callosa*. This study points to the need for a detailed study of the fate of the hemocytes after the peak in the spring. If vanadium and/or iron were involved in tunic synthesis to, a histological study of the

tunic and other tissues might be informative. The tunic accounts for about 50% of the total mass in *A. callosa*. The growing edge of the tunic was sampled in this study. It is possible that the hemocytes migrated to the outer edge of the tunic, which was not sampled. *Ascidia callosa* is an ideal animal for such a study of the tunic as the thickness and rigidity of the tunic makes sectioning an easy task.

3.7 References

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Chapter 4: Increased environmental vanadium concentration increases the vanadium concentration of tissues and the number of circulating vanadocytes in *Ascidia callosa* (Ascidiacea, Phlebobranchia)

4.1 Introduction

Ascidians of the sub-order Phlebobranchia non-toxicologically accumulate vanadium in their tissues, especially in hemocytes (coelomic cells) called signet ring cells (SRCs). In these cells, the vanadium reaches concentrations of over 300mM and is held in the reduced state in a single acidic vacuole (pH <2) (Anderson *et al.*, 1991; Henze, 1911; Michibata *et al.*, 1986; 2002). Ascidians of both sub-orders accumulate iron. Ascidians of the sub-order Stolidobranchia do not accumulate vanadium. The function of these metals in ascidians is unknown. While there has been some progress in understanding how vanadium is taken up by the branchial basket, and reduced and concentrated in the hemocytes, very little data exists on the factors that may influence vanadium accumulation by phlebobranch ascidians and how vanadium and iron accumulation are related (Michibata *et al.* 1998; Ueki et *al.* 2003; Yoshihara, et *al.* 2005).

Chapters 2 and 3 of this thesis reported peaks in the vanadium concentration of two species of phlebobranch ascidians from two separate locations in the North Atlantic over the course of 2-4 weeks in the spring (March-April), with a corresponding increase in the number of circulating vanadocytes. Multiple ecophysiological factors are/might be changing at this time of year which may contribute to this phenomenon. These include changing day length, temperature, food availability, and their effects on water pumping rate, reproductive state (animals are typically gravid at the time of the peak in metal concentration), and environmental vanadium concentration. There is

relatively little data on the latter, perhaps due to the methodological difficulties in measuring very low levels of vanadium in seawater (Crompton, 2002).

The experiment described in this chapter examines whether the vanadium concentration of ascidians can respond to elevated environmental vanadium levels on the time scale of the increase observed in the seasonal studies, outside of the spring reproductive period. Little is known about if and how ascidian vanadium concentrations respond to increases in environmental concentrations of vanadium. Few studies of invertebrate metal toxicology include both ascidians and/or vanadium. The studies that exist have found that the iron, copper, and lead concentration of ascidians in polluted waters is higher than in pristine waters while the vanadium concentrations does not differ (de Caralt *et al.*, 2002; Monniot *et al.,* 1994). The experiment of Nose *et al.* (1997) found no increase in the vanadium concentration or number of vanadocytes of *A. sydneiensis samea* upon treatment with 1 mM Na_3VO_4 for 20 hours, but a marked increase upon treatment with ionophores, which may disrupt the transmembrane gradient which is thought to help keep the concentrations of the vanadium containing vacuole acidic. However these experiments were carried out in filtered seawater. As ascidian pumping rates decrease when particle concentrations are extremely low, the flow of the medium through the organism, and thus the total dissolved exposure may have been low (Fiala-Medioni, 1978; Petersen *et al.*, 2002; Randlov et *al.,* 1979).

I subjected *Ascidia callosa* and *Halocynthia pyriformis* collected from Newfoundland Canada, in the non- reproductive season (fall), to a chronic low level of vanadium

enrichment to determine whether vanadium concentration of the hemocytes, branchial basket, renal vesicles, muscle or tunic would rise in response to increased environmental levels. A low level of vanadium enrichment was chosen so as not to invoke a toxicological response so that the responses or lack thereof observed would be more reflective of natural conditions. Hemocyte populations were examined to determine whether this would be accompanied by a rise in the number of vanadocytes. *Halocynthia pyriformis*, a stolidobranch, was subjected to the same experimental conditions because vanadium enrichment in tissues of this species would indicate whether levels were not high enough to cause toxicological uptake. I hypothesized that the vanadium concentration of the hemocytes and other tissues of *Ascidia callosa* would rise over time compared to that of the control, and that the vanadium concentration of *H. pyriformis* tissues would show no such enrichment. Iron and vanadium are both taken up by the branchial basket of ascidians (Cheney *et al.*, 1997). Since little is known about this process in terms of the nature of the membrane transport proteins, for example, I also measure iron concentrations of the tissues of each species. If vanadium competes for a similar transport protein, the iron concentration of the vanadium enriched group might change compared to the control group.

4.2 Materials and Methods

4.2.1 Animals

Halocynthia pyriformis and *Ascidia callosa* were diver collected from the Avalon Peninsula of Newfoundland (Admiral's Cove) Canada in November 2007. They were transported in seawater filled coolers on the same day to the Ocean Sciences Centre, Logy Bay, Newfoundland and maintained in flowing ambient seawater (~10 °C) until the start of the experiment.

4.2.2 Experimental setup

Five animals of each species (representing a similar size range as those subjected to control and vanadium enriched conditions) were removed from ambient seawater, placed in 1 µm filtered sea water (FSW) for 24 hours, and sampled as time 0 in the experiment (Table 4. 1). Four aerated static experimental tanks (19 l) were set up as follows: *H. pyriformis* - vanadium enriched (8 animals), *H. pyriformis- control* (8 animals), *A. callosa-* vanadium enriched (8 animals), and *A, callosa* - control (8 animals). Control and experimental groups included an equal size and mass range of animals. All tanks were subjected to a ½ water change every 48 hours, and maintained at 10°C, under constant low light intensity. Fecal pellets were removed with every water change. The enriched tanks were started with 9.5 µmol vanadium (as ammonium vanadate) in 19 l of FSW (for a final concentration of 500 nM). The control tanks were started with the same concentration of ammonium chloride. At

each 50% water change, 2.375 µmol of ammonium vanadate or ammonium chloride in 9.5 l FSW were added to the experimental and control tank, respectively. Figure 4.1 illustrates the theoretical vanadium concentration and content in the tanks with this regime. The regime was chosen as a compromise between maintaining a similar dose added at each water change subsequent to the first and the vanadium content available to each animal when animals were removed for sampling part way through the experiment. Tanks were all fed the same food ration, 500 000 cells $L^{-1}animal^{-1}$, every 24 hours, of the commercially available Shellfish Diet®.

Four animals of each group were sampled at 14 and 42 days. Animals were placed in FSW for 24 hours before sampling to ensure that the adherent experimental medium was removed from the body surfaces, and that the branchial basket and gut were free from plankton and feces. Animals from each group were depurated in separate tanks. The length (*A. callosa* $F_{9, 58} = 1.44$, p=0.12; *H. pyriformis*: $F_{9, 58} = 2.00$, p=0.069) and mass (*A. callosa* $F_{9, 58} = 1.82$, p=0.085; *H. pyriformis*: $F_{9, 58} = 0.47$, p=0.21) of specimens did not differ statistically among experimental groups or over time in either species in the model.

4.2.3 Tissue sampling

Coelomic fluid was obtained via cardiac puncture. Fluid was drawn into a syringe containing 40 mM cysteine in artificial sea water (ASW: 500 mM NaCl, 8.0 mM KCl, 29 mM Na_2SO_4,75 mM Tris, pH 7.8) to prevent clotting (1:1, total volume 500-2000 µl). Samples were mixed prior to be divided into aliquots. Several aliquots of

this diluted coelomic fluid were immediately used for image analysis of the cells. The remainder was divided into aliquots and placed in pre-weighed, acid washed vials for metal analysis. Other tissues were dissected for metal analysis including siphon retractor muscle, renal vesicles (*A. callosa* only), tunic (1 mm thick strips from the proximal edge of *A. callosa* and a section of the entire tunic for *H. pyriformis*), and branchial basket (50% of the entire branchial basket). These were frozen at -20 °C until analysis.

Table 4.1: Whole animal mass (g) and maximum length (mm) of animals (mean ± sd) used in the experiment.

Time	*A. callosa*		*H. pyriformis*	
	V enriched	Control	V enriched	Control
Start (n=5)	35 ± 3.2 g 62 ± 4 mm		25 ± 4.9 g 50 ± 2 mm	
14 days (n=4)	37 ± 2.8g 51 ± 5 mm	34 ± 3.2 g, 53 ± 5 mm	19 ± 3.9 g, 37 ± 8mm	20 ± 2.2 g, 36 ± 6mm
42 days (n=4)	29 ± 3.6 g, 47 ± 5 mm	30 ± 4.1 g, 48 ± 6 mm	20 ± 3.1 g, 40 ± 7mm	18 ± 3.2 g, 40 ± 3mm

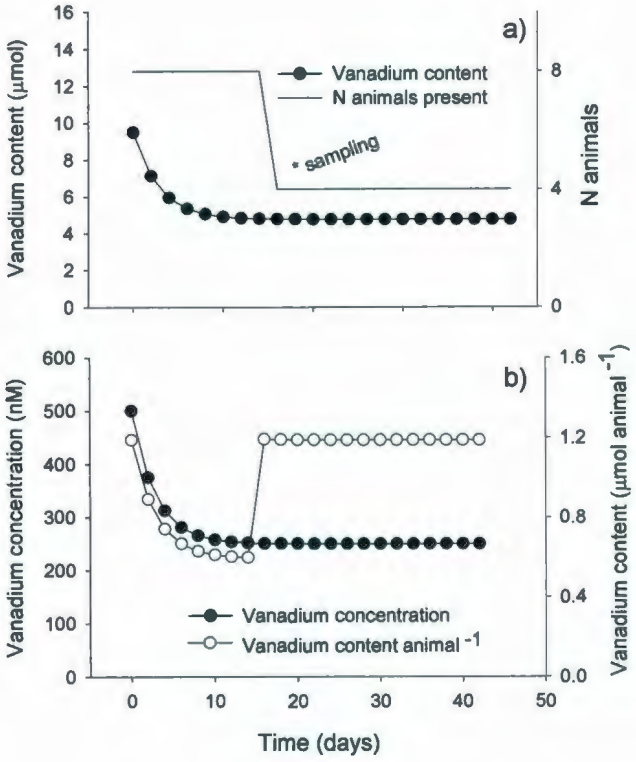


Figure 4.1(above): The theoretical content (a) and concentration (b) of vanadium (V) as ammonium vanadate above control levels, in the vanadium enriched groups, assuming no uptake of vanadium by the animals. The control groups have the same concentration and content of chloride as ammonium chloride.

4.2.4 HPLC determination of vanadium and iron concentration

Vanadium and iron concentration were assayed as in chapter 2 of this thesis. All reagents were HPLC grade or higher, and all containers and tools were acid washed and MQ rinsed before use.

4.2.5 Image analysis of coelomic cells

Aliquots of coelomic cells (10 µl) were mixed in equal parts on a gridded slide with saturated 2,2' bypyridine in ASW, and viewed at 200X. This compound forms a purple complex with reduced vanadium (Nette *et al.,* 1998; 1999). Images (1260x960 pixels) covering one or two 1 mm^2 squares were captured with a Pixera Viewfinder camera, with a total magnification of 0.16 µm $pixel^{-1}$. Typically 750 to 1000 cells greater than 2 µm were counted. Cells stained with 2,2,' bypyridine were sized. The counted cells were categorized as follows: stained SRCs, stained multi-vacuolated cells, unstained morula cells, and "other" types (including pigment cells, compartment cells, amoebocytes and others). Images were analyzed with the program UTHSCSA ImageTool v.3 ®(University of Texas Health Sciences Centre).

4.2.6 Statistical analysis

Statistical analyses were completed with Systat v. 9 4 ®. Tissue vanadium and iron concentrations and cell count data were analyzed by GLM with the following:

Tissue concentration = $\beta_0 + \beta_{Time}$Time + $\beta_{Vanadium\ level}$Vanadium level + $\beta_{Vanadium\ level*Time}$Vanadium level*Time +$\varepsilon$

with α =0.05 and "Time" and :Vanadium level" as categorical variables.

Pairwise comparison probabilities among all sampling points and experiment groups for were generated with the Bonferroni correction and $p<0.05$ was taken as significant. For all GLMs, residuals were visually examined for normality, homogeneity and independence and were found to be satisfactory.

4.3 Results

4.3.1 Vanadium concentration of tissues

A. callosa hemocytes ($F_{5, 51}$ = 3.23, p=0.012), branchial basket ($F_{5, 51}$ = 3.04, p=0.024), tunic ($F_{5, 51}$ = 2.68, p=0.047), muscle ($F_{5, 51}$ = 2.93, p=0.031) and renal vesicle ($F_{5, 51}$ = 3.01, p=0.026) vanadium content varied significantly between treatments and over time (with significant treatment*time interaction terms in these GLMs). *H. pyriformis* branchial basket ($F_{5, 51}$ = 2.98, p=0.028) vanadium varied significantly between treatments and over time (with significant treatment*time interaction terms in these GLMs) but tunic ($F_{5, 51}$ = 0.123, p=0.21) vanadium and muscle ($F_{5, 51}$ = 0.594, p=0.18) vanadium did not.

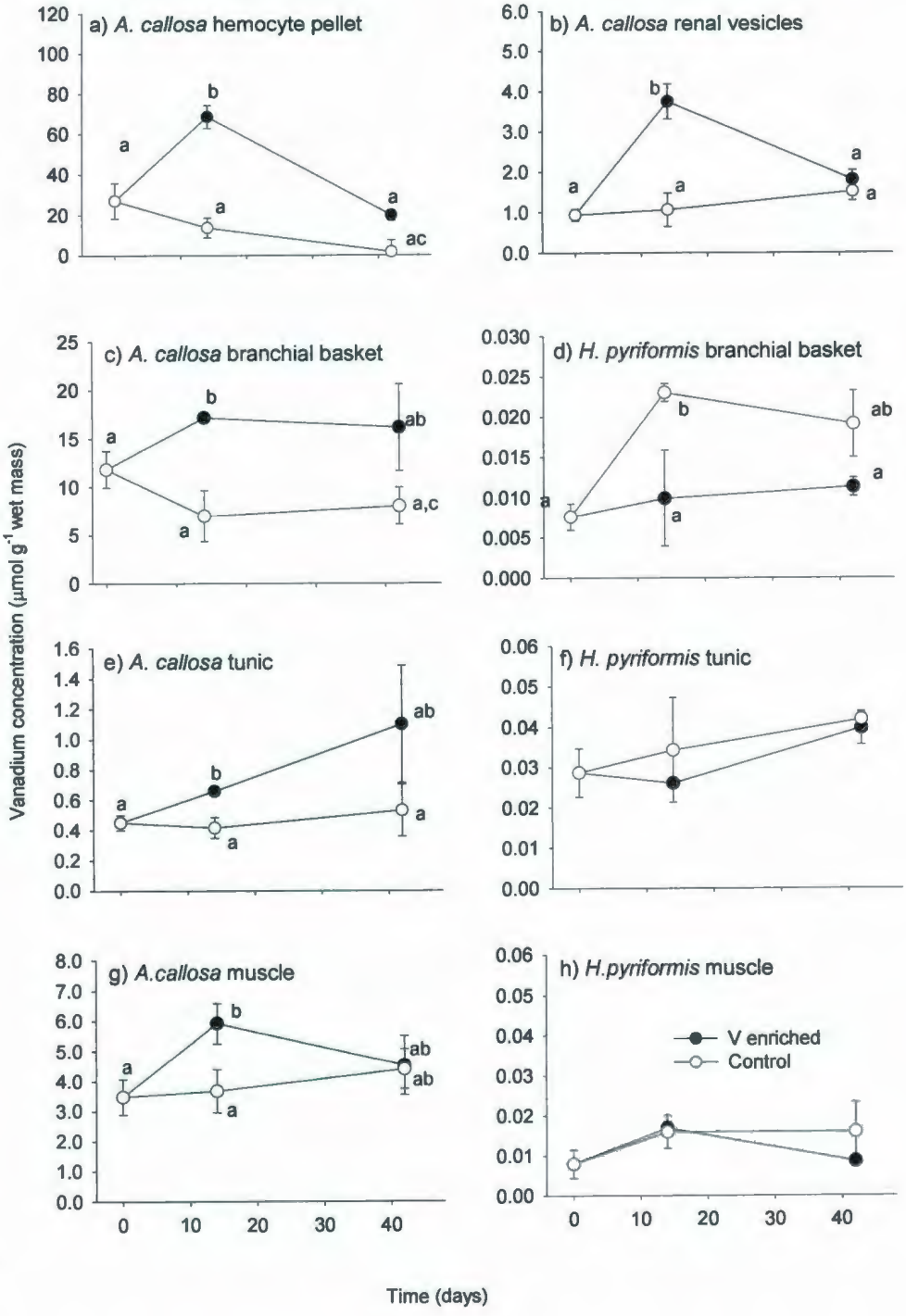
The vanadium concentration of the hemocytes of *A.callosa* subjected to enriched vanadium tripled in the first two weeks of the experiment while that of the control decreased from day 0 to day 42 (Figure 4.2 a). The vanadium concentration of the hemocyte pellet in the enriched group returned to a level similar to the start of the experiment at day 42 but was still higher than that of the control group. Vanadium remained un-detectable in *H. pyriformis* hemocytes throughout the experiment. *A. callosa* renal vesicle vanadium concentration increased by day 14 from the beginning, and was elevated above control. The latter remained constant over the course of the experiment (Figure 4.2 b). The experimental renal vesicle vanadium concentration decreased to control levels by day 42. This tissue is not present in *H. pyriformis.*

The branchial basket vanadium concentration of *A. callosa* increased from day 0 to day 14 but did not change between day 14 and day 42. The vanadium enriched group had a higher concentration than the control at day 14 and 42 (Figure 4.2 c). The branchial basket vanadium in *H. pyriformis* remained relatively constant for the enriched group while in the control it increased, and remained higher than the experimental group until the end of the experiment (Figure 4.2 d).

The *A. callosa* tunic vanadium concentration increased slightly in the vanadium enriched group over the course of the experiment, and was higher than that of the control at day 14. The vanadium concentration of the control group remained constant (Fig 4.2 c). The vanadium concentration of *H. pyriformis* tunic remained constant over time in both treatment and control groups (Figure 4.2 f). *A. callosa* muscle vanadium concentration was higher at day 14 than the start of the experiment and was

higher in the experimental than in the control group (Figure 4.2 g). By the end of the experiment, the muscle vanadium concentration of the enriched group returned to starting levels, and was not different from those of the control group. There was no change over time or between experimental groups in the *H. pyriformis* muscle vanadium concentration at day 14 (Figure 4.2 f).

Figure 4.2 (following page): Vanadium concentration (mean ± sd) of *A. callosa hemocytes*, renal vesicles (branchial basket, tunic and muscle, and *H. pyriformis* branchial basket, tunic and muscle. Experimental animals were subjected to elevated levels of dissolved vanadium as shown in Figure 4.1. Data were analyzed by GLM with the following: Tissue concentration = $\beta_0 + \beta_{\text{Time}}\text{Time} + \beta_{\text{Vanadium level}}\text{Vanadium level} + \beta_{\text{Vanadium level*Time}}\text{Vanadium level*Time} + \varepsilon$ with $\alpha = 0.05$ and "Time" and :Vanadium level" as categorical variables. Pairwise comparison probabilities among all sampling points and experiment groups for were generated with the Bonferroni correction and $p<0.05$ was taken as significant. Different letters indicate significant differences among all sampling points and times. A lack of letters indicates no significant difference in the overall model.


a) A. callosa hemocyte pellet
b) A. callosa renal vesicles
c) A. callosa branchial basket
d) H. pyriformis branchial basket
e) A. callosa tunic
f) H. pyriformis tunic
g) A.callosa muscle
h) H.pyriformis muscle
V enriched
Control
Vanadium concentration (µmol g-1 wet mass)
Time (days)

4.3.2 Iron concentration of tissues

A. callosa hemocytes ($F_{5, 51}$ = 3.22, p=0.013), branchial basket ($F_{5, 51}$ = 2.98, p=0.045), tunic ($F_{5, 51}$ = 2.81, p=0.039), muscle ($F_{5, 51}$ = 2.66, p=0.048) and renal vesicle ($F_{5, 51}$ = 2.87, p=0.035) iron levels varied between treatments and over time (with significant treatment*time interaction terms in these GLMs). *H. pyriformis* hemocytes ($F_{5, 51}$ = 3.42, p=0.046), muscle ($F_{5, 51}$ = 2.70, p=0.044), tunic ($F_{5, 51}$ = 2.73, p=0.038) and branchial basket ($F_{5, 51}$ = 2.82, p=0.036) iron levels varied between treatment and over time (with significant treatment*time interaction terms in these GLMs).

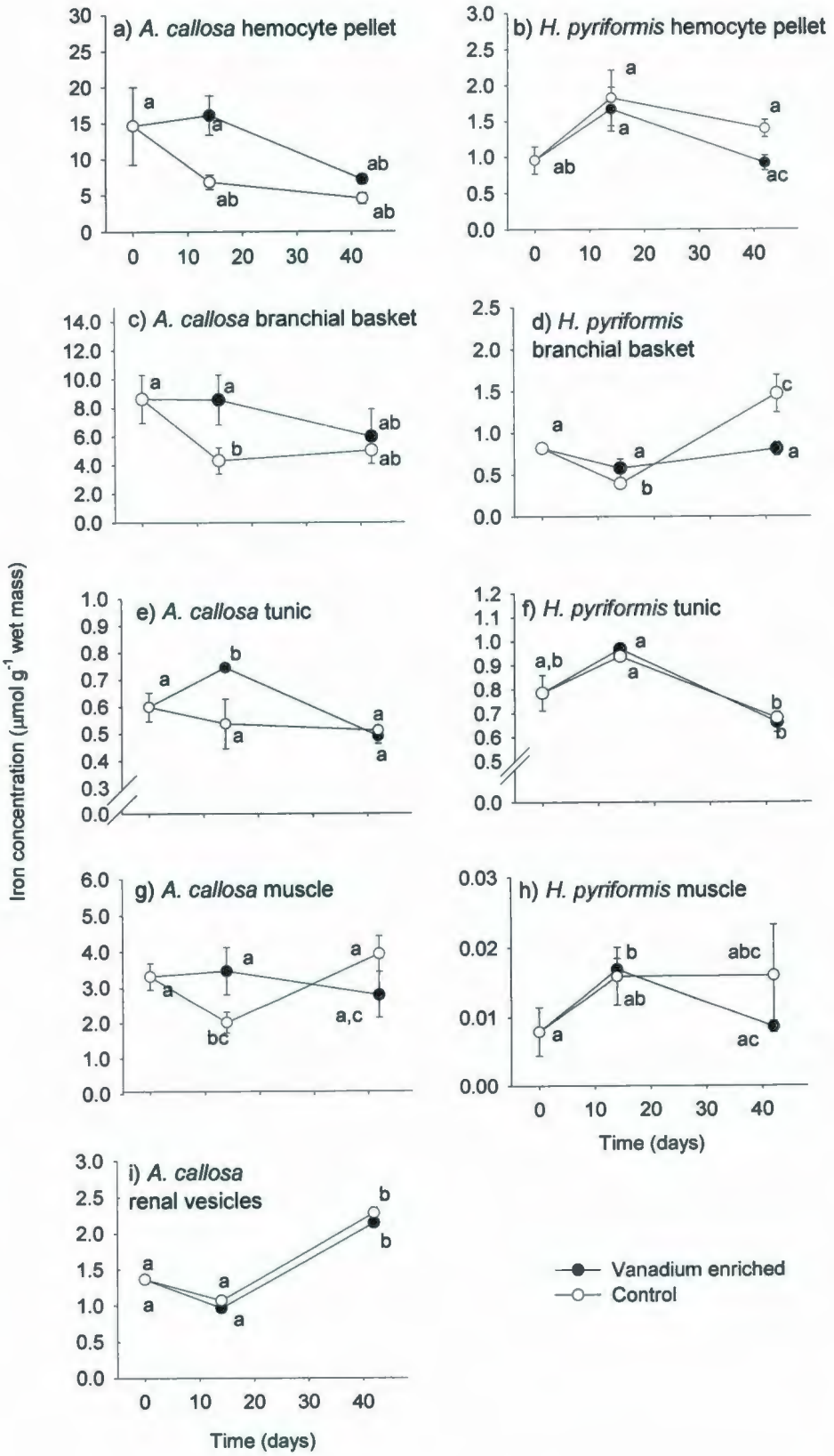
The iron concentration of the hemocytes of the control groups of both species remained relatively constant over the time course of experiment but decreased at day 42 compared to day 0 in the *A. callosa* control group (Figure 4.3 a, b). Hemocyte iron concentration was elevated in the *A. callosa* vanadium enriched group compared to the control, especially at day 14 (Figure 4.3 a).

Iron concentration of the *A. callosa* branchial basket remained constant over the course of the experiment in both groups, and at day 14 was ~2 times higher in the vanadium enriched group than in the control (Figure 4.3 c). The branchial basket iron concentration of *H. pyriformis* remained constant over the experiment in the vanadium enriched group, but showed a marked increase in the control group at the 42 day point compared to the earlier time points (2-3 fold) and was higher (~ 2 fold) than the vanadium enriched group (Figure 4.3 d).

The tunic iron concentration of the *A. callosa* control group remained constant over the course of the experiment (Figure 4.3 e). It was higher in the vanadium enriched group, than at day 0 and compared to the control (~1.5 fold) at day 14. *H. pyriformis* tunic iron levels remained relatively constant over the course of the experiment and did not differ between experimental groups (Figure 4.3 f). *A. callosa* muscle iron concentration was higher in the experimental group compared to the control at day 14 (Figure 4.3g) There was no difference in the iron concentration of *H. pyriformis* muscle between sampling points or experimental groups (Figure 4.3 h)

A. callosa renal vesicle iron did not change from day 0 to day 14 in either group but did increase (~2 fold) in both groups by the end of the experiment (Fig 4.32 i).

Figure 4.3 (following page): Iron concentration (mean ± sd) in hemocytes, renal vesicles, branchial basket, tunic and muscle of *A. callosa and H. pyriformis* . Data were analyzed by GLM with the following: Tissue concentration = $\beta_0 + \beta_{Time}$Time + $\beta_{Vanadium\ level}$Vanadium level + $\beta_{Vanadium\ level*Time}$Vanadium level*Time +$\varepsilon$ with α =0.05 and "Time" and :Vanadium level" as categorical variables. Pairwise comparison probabilities among all sampling points and experiment groups for were generated with the Bonferroni correction and $p<0.05$ was taken as significant. Different letters indicate significant differences among all sampling points and times. A lack of letters indicates no significant difference in the overall model.


a) A. callosa hemocyte pellet
b) H. pyriformis hemocyte pellet
c) A. callosa branchial basket
d) H. pyriformis branchial basket
e) A. callosa tunic
f) H. pyriformis tunic
g) A. callosa muscle
h) H. pyriformis muscle
i) A. callosa renal vesicles
Iron concentration (µmol g-1 wet mass)
Time (days)
Vanadium enriched
Control

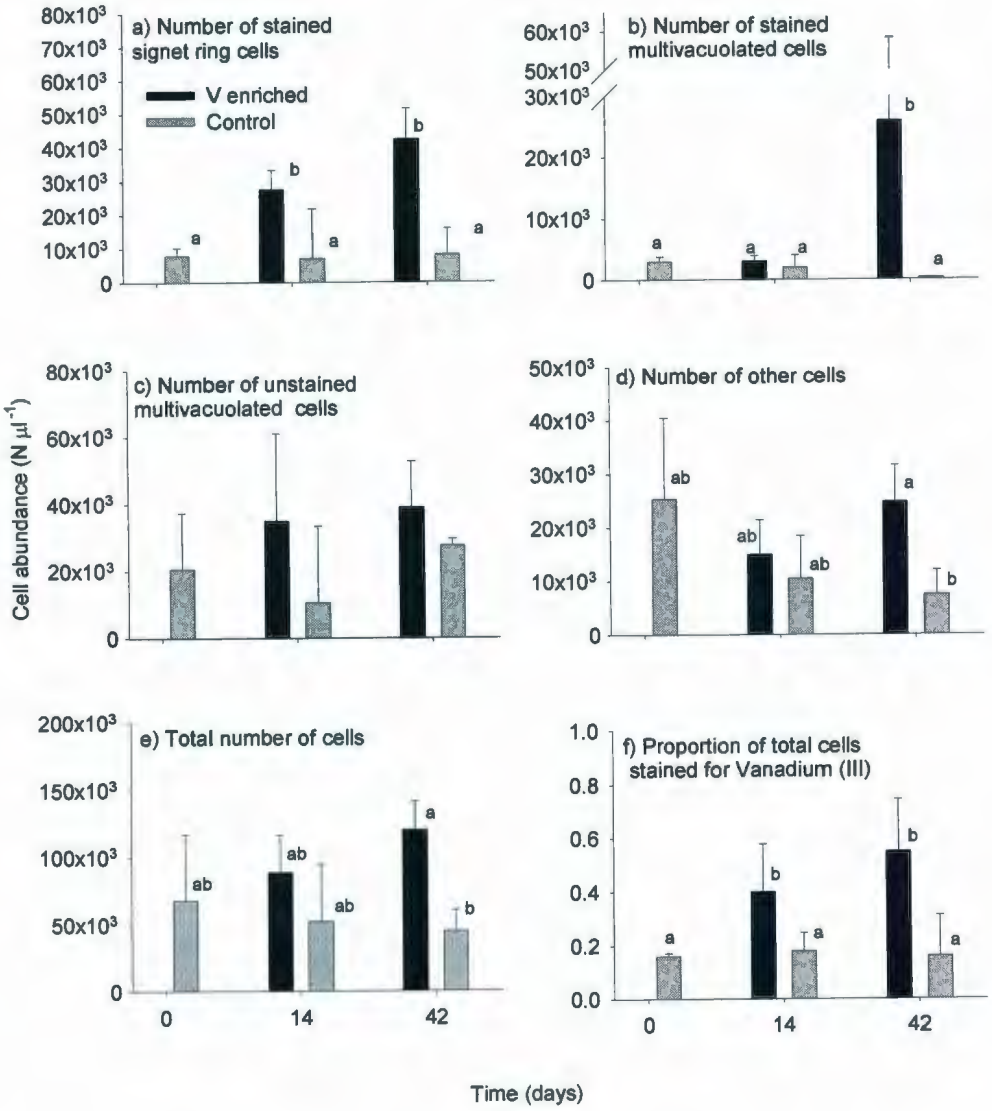
4.3.3 Cell numbers and types in the coelomic fluid of Ascidia callosa

The number of *A. callosa* stained SRCs ($F_{5, 51}$ = 3.07, p=0.022), stained multivacuolated cells ($F_{5, 51}$ = 2.93, p=0.031), other cells ($F_{5, 51}$ = 2.81, p=0.039) and total cells ($F_{5, 51}$ = 2.87, p=0.035) varied between treatments and over time (with significant treatment*time interaction terms in these GLMs) while the number of unstained multivacuolated cells ($F_{5, 51}$ = 1.065, p=0.15) did not vary with treatment or over time. The proportion of stained signet ring cells ($F_{5, 51}$ = 3.07, p=0.022) varied with treatment and over time (with significant treatment*time interaction terms in these GLMs).

Figure 4.4 presents some of the cell types observed in the coelomic fluid of *A. callosa.* There was an approximately four-fold increase in the number of stained signet ring cells in the vanadium enriched group from the start of the experiment, while there was no change in the number of these cells in the control group (Figure 4.4 a). There was also a three fold increase in the number of vanadium containing multi-vacuolated cells by the end of the experiment, which was also higher than control levels (Figure 4.4 b). There were no significant differences between experimental groups or over time in the number of unstained morula cells (Figure 4.4 c) At the end of the experiment the number of "other cells" also was higher in the animals exposed to vanadium enrichment compared to the control (Figure 4.4 d). The proportion of all cells staining for vanadium (III), increased during the experiment and was higher in the experimental compared to the control. The number of unstained morula cells and the

total number of cells were not different over time in either of the groups or between the enriched and control groups (Figure 4.4 f).

Figure 4.4 (following page): Cell composition in *A. callosa* coelomic fluid from animals exposed to ammonium vanadate (V enriched) and ammonium chloride (control) . "Stained" refers to cells stained purple with 2,2' by-pyridine. Living cells were counted using image analysis. (Data are presented as mean ± sd, n=5, 4 and 4 for the 0, 14 and 42 day sampling points respectively). Data were analyzed by GLM with the following: Tissue concentration = $\beta_0 + \beta_{Time}Time + \beta_{Vanadium\ level}Vanadium\ level + \beta_{Vanadium\ level*Time}Vanadium\ level*Time + \varepsilon$ with $\alpha = 0.05$ and "Time" and :Vanadium level" as categorical variables. Pairwise comparison probabilities among all sampling points and experiment groups for were generated with the Bonferroni correction and $p<0.05$ was taken as significant. Different letters indicate significant differences among all sampling points and times. A lack of letters indicates no significant difference in the overall model.


a) Number of stained signet ring cells
V enriched
Control
b) Number of stained multivacuolated cells
c) Number of unstained multivacuolated cells
d) Number of other cells
e) Total number of cells
f) Proportion of total cells stained for Vanadium (III)
Cell abundance (N µl-1)
Time (days)
0
14
42

4.4 Discussion

4.4.1 Vanadium concentration

The lack of enrichment of *H. pyriformis* tissues with vanadium in the vanadium exposed group compared to the control indicates that the concentration of vanadium chosen for this experiment was not high enough to cause toxicological uptake, and indicates that depuration procedures were sufficient to remove adherent vanadium. Vanadium concentrations in this experiment were about 100X that found in natural seawater. However, the normal pumping rates of ascidians at medium particle concentrations in the natural setting are more than 10-100 fold higher than the water turnover rate of 9.5 L every 48 hours in this experiment (Fiala-Medioni, 1978). Given that vanadium exposure is a product of the rate of pumping and the concentration of vanadium in the water (among other factors), the total vanadium exposure was not higher than would be experienced under field conditions.

The vanadium concentration of the hemocytes, renal vesicles, branchial basket and muscle of *A. callosa* tissues were 2 to 4 times higher in the vanadium enriched group than in the control group after 14 days of exposure, but returned to starting levels by day 42. However, the vanadium concentration of the hemocytes and branchial basket remained greater than those of the control group at day 42. These sampling times were chosen to be similar to the time course of the natural increase observed in the spring. These results indicate that the mechanisms of vanadium uptake can respond to increased environmental concentrations in less than 14 days, outside the spring

reproductive period. The experiment was carried out in the early fall, when vanadium concentration of these tissue was previously found to be lower than in spring (Chapter 3 of this thesis). The co-occurrence of the spring peak in vanadium concentration in the seasonal studies with sexual maturation might suggest that the two phenomena may be related and that the increased vanadium concentration is used in the production of eggs. However, the results of this experiment do not support this contention. Animals were not gravid in the fall and did not become gravid during this experiment.

It is unknown whether the vanadium concentration reached higher levels in the tissues at some point before day 14 of the experiment. Goldberg and colleagues (1951) found a significant uptake of dissolved radiolabelled vanadium (^{48}V) by *Ascidia ceratodes* and *Ciona intestinalis* over several days. These studies are consistent with the present study, although they were completed with non feeding animals, and not directly comparable.

The lack of change in the vanadium concentration of the *A. callosa* control group throughout the experiment is indicative of relatively low vanadium loss rates in this group as has been suggested by other authors (Anderson, 1991; Goldberg, 1951). It is unknown whether the decrease in the vanadium concentration in the experimental group towards the end of the experiment was due to a higher loss rate of vanadium or to a movement of vanadocytes into another tissue. Using "natural concentrations" of vanadium, enriched with ^{48}V, Anderson and colleagues (1991) showed maximum incorporation of the radiolabelled vanadium into hemocytes after approximately 20

days of whole animal exposure with a decrease thereafter but they did not examine other tissues.

Qualitatively, the pattern of change in vanadium concentrations was similar in the hemocytes, the renal vesicles and the muscle, increasing at day 14 and declining by day 42. The tunic vanadium concentration, however, tended to increase toward the end of the experiment. The tunic typically represents greater than 25-50% of the wet mass of whole *A. callosa*, and this may represent a large concentration of vanadium. Further experiments are required to examine the possibility that the tunic was the destination tissue and whether the increase in the whole tunic vanadium can account for the decrease in hemocyte vanadium at day 42. I sampled small pieces of tissue from the proximal edge of the tunic which is presumably the growing edge. A section through the entire tunic could have shown whether the distribution of hemocytes changes throughout the experiment or differed between the experimental groups.

4.4.2 Hemocytes

The initial number of signet ring cells in the coelomic fluid of *A. callosa* in this study was lower than that found in the fluid of control animals in the study of *A. s. samea* by Nose and colleagues (1997). However, the proportions of signet ring cells (SRCS) and morula cells (MCs) are well within the 5-68% SRCs and 24-57% MCs found in the coelomic fluid of species of *Phallusia* and *Ascidia* by other authors (Endean, 1960; Biggs *et al.* 1979; Oltz *et al.*, 1989; Lee *et al.*, 1990; Nette *et al.* 2004).

The increase in both the number of circulating cells containing vanadium and the relative proportion of these cells in the experimental group indicates a proliferation of these cells from hemapoetic tissue or a morphological change in, and uptake of, vanadium by other cells. The total number of cells did increase in the enriched group compared to the control by the end of the experiment. This is in contrast to the lack of increase in the number of vanadocytes in found A. *sydneiensis samea* upon immersion in 1 mM vanadium found in a study by Nose and colleagues (1997). The different time scale (20 hours) and lack of food may account for this difference.

At the end of the present experiment, the number of vanadium containing cells was still high while the vanadium concentration of the whole pellet was low. This suggests lower vanadium content per cell. There was also a very large increase in the number of vanadium containing multi-vacuolated cells at the end of the experiment. Assuming no procedural artifact (photos were captured over approximately the same time period for each animal), this might indicate a proliferation of new vanadocytes. Nette *et al.* (1999) suggested that the SRC is the terminal cell in a lineage from compartment and bivacuolated cells based on observation of coalescence of vacuoles in multi-vacuolated cells. Work with isolated cells of *A. gemmata* supports this as compartment cells take up more vanadium (IV) and vanadium (V) than either signet ring cells or morula cells (Michibata *et al.*, 1991).

4.4.3 Iron concentration of tissues

The higher iron concentration of the hemocytes, branchial basket, tunic and muscle in the *A. callosa* experimental group than in the control at day 14 indicates that the iron uptake and retention mechanisms are not inhibited by increased vanadium levels. The reason that iron concentration would be higher in the presence of higher vanadium levels is unknown. While the dissolved iron exposure was low due to the low water turnover rate, the food supply provided a high source of iron.

By contrast, the iron concentration of the *H. pyriformis* hemocyte pellet and branchial basket was higher in the control group than in the experimental group by the end of the experiment, indicating a possible negative impact of vanadium on iron uptake or a positive impact on iron loss rates. Given that the hemocytes of the *H. pyriformis* vanadium enriched group did not incorporate enough vanadium to be detectable by the methods used in this experiment, and that there was no enrichment of vanadium in other tissues, it can be concluded that vanadium was not incorporated into the tissues of this species. Further knowledge of the uptake of iron and vanadium at the level of the branchial basket is required to understand impacts of vanadium on iron transport in ascidians.

4.4.4 Future directions

This study is the first to show that vanadium accumulation and signet ring cell proliferation or release is inducible by higher environmental vanadium concentrations in phlebobranch ascidians, and that vanadium levels in the circulating fluids return to normal after a period of time. The increased uptake occurs on a time scale of weeks to months or less. The additional vanadium must be transported to a sink tissue or be excreted between two weeks and two months. Experiments that focus on the release and fate of the signet ring cells from the initial active uptake phase and the subsequent decline will reveal much about the uptake dynamics of vanadium and perhaps, function.

4.8 References

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Chapter 5: Summary and conclusions

Henze's pioneering work in 1911 revealed that phlebobranch ascidians contain large amounts of vanadium and iron in their tissues. While vanadium is important in certain enzymes in some fungi and algae, the non toxicological accumulation of vanadium is rare in the Animal Kingdom. Vanadium, able to exist in multiple oxidation states, was first thought to be involved in oxygen transport, in a role analogous to iron in hemoglobin. However, it was shown that there is no reversible oxygen binding in ascidian blood. Other functions such as chemical defense have been proposed, but none have been fully tested.

Since the 1970s, when vanadium was found to inhibit ATPases, the natural accumulation of the element by ascidians has received more attention. After some debate and discussion of the chemical nature of the vanadium in ascidians, it was established that vanadium is maintained in highest concentrations in the hemocytes called signet ring cells, in which the vanadium occurs in a reduced state in a single acidic vacuole. Michibata and colleagues in Japan have completed undertaken the bulk of the work over the last two decades in characterizing vanadium binding proteins or vanabins, and suggesting a possible reduction pathway.

From the point of view of invertebrate ecology, there was little work characterizing sources of variation in vanadium concentration. Researchers have always completed measurements on multiple animals, and measurements were made on genera from different parts of the world, but there has been no focused study of ecophysiological variability. Studies of metal dynamics in other species have been aided by the comparative ease in obtaining suitable radioactive tracers which enabled

measurements of concentration, uptake and loss and the effects of physical and physiological factors. This is not true for ^{48}V or ^{49}V. This may contribute to the lack of knowledge surrounding whether vanadium concentration, uptake, loss rates and partitioning among tissues, change with individual characteristics such as metabolic rate, age, reproductive state, or size, or with environmental traits such as temperature, food availability, vanadium concentration in the environment and oxygen availability, to name a few. These types of data, while not directly addressing function, may lead to the identification of factors that can be manipulated in experiments that address function.

Aside from a few studies, iron accumulation by aplousobranch, stolidobranch and phlebobranch ascidians has received scant attention. Given that there is no reversible oxygen binding in ascidian coelomic fluid, neither vanadium nor iron is used for oxygen transport. A major question regarding whether vanadium accumulation is an ancestral trait has yet to be addressed. It is also unknown whether iron and vanadium fulfill a similar function in t ascidians. Accordingly, it will be important to study the iron dynamics of all groups in order to fully understand the metal physiology of ascidians.

This thesis is a first step in the study of the ecophysiological variation in iron and vanadium concentrations of ascidians. Given the effects of food availability and temperature on physiological processes related to metal uptake in ascidians, seasonal variation was a logical starting point. The first step was the development of an assay for vanadium and iron that would be economically feasible for the number of

biological samples involved. An RP-HPLC method involving pre column chelation with PAR was adapted for use on acid extracts of biological tissues. This method gave vanadium and iron levels that were comparable to those found in ascidians using different methodologies such as neutron activation analysis.

The first species to which this technique was applied was *Ciona intestinalis* from Woods Hole, Mass which exhibited a peak in iron and vanadium concentration around the time of the spring bloom, with a concurrent peak in G6PDH, an enzyme of Michibata's proposed reduction pathway. The spring bloom period is characterized by high particle concentrations and low temperatures. While low temperature tends to decrease pumping rates, high particle counts have the opposite effect, and a combination of these factors may have been responsible for the peak in both metals. It appears that both metals are affected by these factors as both exhibit a similar seasonal pattern.

A feeding study was undertaken to determine whether a difference in food availability associated with the bloom might be responsible for the increase in both metals, whether through the effects of increased particle concentration on pumping rate and thus dissolved exposure, or through direct dietary exposure from phytoplankton ingested. While there were few differences in iron or vanadium concentration between high and low food groups, the iron concentration of some tissues did increase over the course of the experiment to reach values greater than those animals sampled directly from Woods Hole. This is likely reflective of the importance of diet as a source of iron. The vanadium concentration of the coelomic cells did not decrease over the

course of the experiment, despite a limited water renewal regime which restricted exposure to dissolved vanadium. This is indicative of relatively low vanadium loss rates.

A confirmation of the seasonal peak in another phlebobranch species and comparison with a stolidobranch was then necessary. Accordingly, Chapter 3 examined the seasonal variation in *Ascidia callosa* and *Halocynthia pyriformis* from the Avalon Peninsula, Newfoundland. Sampling was conducted more frequently around the spring bloom to get a better understanding of the time course of the hypothesized increase in the spring. A similar pattern was observed in the vanadium and iron concentration of *A. callosa* from Newfoundland as in *C. intestinalis* from Woods Hole. The formation of the Henze precipitate (see supplementary chapter 1) in *A. callosa* hemocyte homogenates prevented G6PDH measurement in this species. There was a slight peak in the iron concentration of the hemocytes and muscle of *H. pyriformis* and in vanadium concentration of the branchial basket and tunic which occurred at a later date than the peak in *A. callosa*. This may indicate that similar dynamics are driving the accumulation of these metals in both species in the spring. The concurrence of this phenomenon with the gravid reproductive state of the animals in the spring raises questions as to whether the two phenomena are linked, that is, that the increases in vanadium levels are destined for the eggs of pre-spawning individuals. However, the results of the enrichment experiment in Chapter 4 indicate that the two are not necessarily linked.

I examined living hemocytes of *A. callosa*, stained with 2,2' bypyridine, to determine whether there was seasonal variation in the number of vanadium (V(III)) containing cells and whether there was an increase in vanadium as a result of an increase in the number of vanadium containing cells. It was evident that there was a general increase in the total number of circulating hemocytes and the number of vanadium containing signet ring cells. Most significant, however, was the increase in the proportion of vanadium containing signet ring cells, which is an indication of either a proliferation of these cells, a release from a storage site, or a metamorphosis from a precursor cell to the signet ring cell.

To determine whether ascidians could respond to increases in vanadium availability in the environment on the same time scale as the period of the peak in vanadium concentration in the spring, and outside the spring reproductive period, a vanadium enrichment experiment was conducted with both *A. callosa* and *H. pyriformis* (Chapter 4). The vanadium concentration was 100X that of seawater, but the total exposure was low as the water renewal regime was much less than the pumping capacity of the animals. Vanadium concentration of the hemocytes of *A. callosa* tripled over the first 14 days but had returned to normal (though still elevated above the control) by day 42. The vanadium concentrations of the branchial basket and tunic (approaching significance) remained elevated at day 42. A remarkable increase in the number and proportion of vanadium containing cells accompanied this increase. The vanadium concentration of the hemocytes decreased to starting levels by day 42. However, the number of these cells remained high, suggesting that they contained less vanadium per cell. The vanadium concentration of tunic suggests that this tissue

might be the repositories of this vanadium. Study of sections of the entire thickness of the tunic and the number of hemocytes in the branchial basket over the course of this type of experiment would be informative here.

The lack of vanadium accumulation by *H. pyriformis* confirms that the levels of vanadium enrichment used in this experiment were not high enough to induce toxicological uptake and that *H. pyriformis* does not have the same uptake processes for vanadium to be able to harness sporadic increases. The increase observed in *H. pyriformis* in the seasonal study must rely on the total volume of water pumped through the body of the animal and not response to a sudden input.

The hemocytes, branchial basket and tunic iron concentrations of *A. callosa* were higher, while the hemocyte and branchial basket iron concentrations of *H. pyriformis* were lower in the vanadium enriched groups compared to those of the control groups. The dietary iron to which control and vanadium enriched groups were exposed through phytoplankton and the theoretical dissolved exposure were the same. If the main source of iron is the diet, this suggests that the iron uptake was enhanced by the presence of vanadium in *A. callosa* but decreased in *H. pyriformis.* This decrease may have been controlled by feeding behavior if the vanadium enriched *A. callosa* individuals increased pumping rates while *H. pyriformis* decreased pumping rates compared to that of the control animals. For this to occur, individuals of both species would have to be able to sense the dissolved vanadium concentration and respond accordingly. To the author's knowledge, there is no work which indicates whether this is possible.

This thesis is the first work to show clear seasonal variation in the vanadium and iron concentrations of phlebobranch and stolidobranch ascidians. The demonstration of seasonal variation and the response of ascidians to short term increases in environmental vanadium have identified two factors which can be harnessed or manipulated to further investigate the dynamics of their metal accumulation. Researchers in the field may focus on the period surrounding the spring bloom as a time of high activity of the vanadium and iron sequestration machineries. Future research should trace the production or release of signet ring cells and the destinations of the cells or their contents. We should investigate the similarities and differences in the responses of stolidobranch and phlebobranch ascidians to vanadium enrichment at the cellular as well as whole animal level. As one of the first works to consider the vanadium and iron accumulation of ascidians from an ecophysiological point of view, hopefully it will stimulate more study into how the phenomenon relates to the basic biology of the animals.

Supplementary 1: Henze precipitate in *A. callosa* hemocyte homogenates

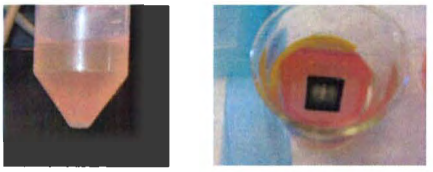
S1.1 Introduction

Since the enzyme G6PDH is on of the enzymes of the proposed reductive pathway, it was desirable to measure its activity in A. *callosa* in the seasonal study (Chapter 3) and in the vanadium enrichment experiment (Chapter 4). However, the formation of the Henze precipitate upon lysis of the hemocytes under the homogenization protocol described in Chapter 3 prevented measurement of this enzyme. Upon homogenization in the buffer a thick bluish green precipitate formed (Figure S1.1). Although there was some evidence of activity, i.e the production of NADPH, observed as a temporary erratic increase in absorption at 340nm, the whole homogenate could not be used for enzyme assays. It absorbed and scattered light such that a linear response was not obtained, and eventually after several minutes precipitated out in the reaction well, upon which all increase in absorption stopped. Upon centrifugation to remove this precipitate, the supernatant was found to contain no activity at all. This chapter reports the steps that were taken to prevent the formation of this precipitate so that the enzyme could be measured.

S1.2 Methods

Coelomic fluid was gathered as described previously. The fluid was centrifuged at various speeds, the plasma pipetted off and the cells then sonicated under the conditions in Table S1.1. When the homogenization conditions did not produce a severe significant greenish blue precipitate, an attempt was made to use the homogenate in a reaction by adding it to a well with the usual reaction mixture (75

mM Tris, pH 7.5, 5mM G6P, o.5 mM $NADP^+$) unfortunately, precipitate formation occurred seconds after addition of homogenate to the wells.



a) b)

Figure S1.1: Normal color of *A. callosa* coelomic fluid, and acidic homogenate (a) and color of homogenate dialyzed against a pH 7.5 buffer showing blue-green precipitate formation (b).

Table S1.1 (following 2 pages) Qualitative assessment of the extent of Henze precipitate formation upon homogenization of *A. callosa* hemocytes with various buffers, pHs and additives. The qualitative scale is from * no precipitate formation to **** severe precipitate formation (dark blue green solids). Each condition was tested with the coelomic cells of two animals.

Buffer	pH	Additional reagents	Severity of precipitate formation
100 mM acetate	3.5	none	*
		2 mM DTT	*
		1M KCl	*
		DTT + 1M KCl	*
		0.5% Tween 20	*
		0.5% Triton X 100	*
		500mM EDTA	*
	4.5	none	**
		2 mM DTT	**
		1M KCl	**
		DTT + 1M KCl	**
		0.5% Tween 20	**
		0.5% Triton X 100	**
		500mM EDTA	**
100mM phosphate	6.0	none	***
		2 mM DTT	***
		1M KCl	***
		DTT + 1M KCl	***
		0.5% Tween 20	***
		0.5% Triton X 100	***
		500mM EDTA	***
	6.5	none	****
		2 mM DTT	****
		1M KCl	****
		DTT + 1M KCl	****
		0.5% Tween 20	****
		0.5% Triton X 100	****
		500mM EDTA	****
	7.0	none	****
		2 mM DTT	****
		1M KCl	****
		DTT + 1M KCl	****
		0.5% Tween 20	****
		0.5% Triton X 100	****
		500mM EDTA	****
100mM Tris	4.5	none	*
		2 mM DTT	*
		1M KCl	*
		DTT + 1M KCl	*
		0.5% Tween 20	*
		0.5% Triton X 100	*
		500mM EDTA	*
	5.0	none	*
		2 mM DTT	*
		1M KCl	*
		DTT + 1M KCl	*
		0.5% Tween 20	*
		0.5% Triton X 100	*
		500mM EDTA	*
	6.0	none	***
		2 mM DTT	***
		1M KCl	***
		DTT + 1M KCl	***
		0.5% Tween 20	***
		0.5% Triton X 100	***
		500mM EDTA	***

Table S1.1 continued

Buffer	pH	Additional reagents	Severity of precipitate formation
100 mMTris	7.0	none	****
		2 mM DTT	****
		1M KCl	****
		DTT + 1M KCl	****
		0.5% Tween 20	****
		0.5% Triton X 100	****
		500mM EDTA	
	7.5	none	****
		2 mM DTT	****
		1M KCl	****
		DTT + 1M KCl	****
		0.5% Tween 20	****
		0.5% Triton X 100	****
		500mM EDTA	
100mM imidazole	6.5	none	***
		2 mM DTT	***
		1M KCl	***
		DTT + 1M KCl	***
		0.5% Tween 20	***
		0.5% Triton X 100	***
		500mM EDTA	
	7.0	none	****
		2 mM DTT	****
		1M KCl	****
		DTT + 1M KCl	****
		0.5% Tween 20	****
		0.5% Triton X 100	****
		500mM EDTA	
	7.5	none	****
		2 mM DTT	****
		1M KCl	****
		DTT + 1M KCl	****
		0.5% Tween 20	****
		0.5% Triton X 100	****
		500mM EDTA	
100mM triethanolamine	7.0	none	****
		2 mM DTT	****
		1M KCl	****
		DTT + 1M KCl	****
		0.5% Tween 20	****
		0.5% Triton X 100	****
		500mM EDTA	
	7.5	none	****
		2 mM DTT	****
		1M KCl	****
		DTT + 1M KCl	****
		0.5% Tween 20	****
		0.5% Triton X 100	****
		500mM EDTA	

S2.2 Results and Discussion

As shown in Table S1.1, no experimental condition with a pH >5 was successful in preventing the interfering precipitate. When non-precipitate forming homogenates were added to a pH 7.5 reaction mixture (75 mM Tris, pH 7.5, 5 mM G6P, 0.5 mM $NADP^+$) , precipitates were almost immediate. Addition of these to a reaction buffer of pH 5.5 (75 mM Tris, pH 5.5, 5 mM G6P, 0.5 mM $NADP^+$) showed no enzyme activity at all. The low pH that prevents the precipitate formation is outside the optimal pH range recorded for this enzyme in most species. This will likely be a problem for most enzyme measurements in the whole hemocyte population this species. If unable to solve this problem, one could isolate populations of signet ring cells in which to measure the G6PDH, as it is the morula cells that are responsible for the precipitate (Frank *et al.*, 1995; Frank and Hodgson, 2000). This may be impractical as it may require the coelomic fluid from several animals to make a single measurement. A second alternative may be to measure the expression of the gene for the enzyme but this is not the same as an in *situ* activity of the protein.

There is a second problem with the measurement of NADH or NADPH based enzymes in this species with which future investigators should be familiar. Incubation of the homogenate at all the pH values tested and all of the buffers tested results in a linear decrease of the absorbance at 340nm. This may be a reduction of the NADPH, which can be significant when activity of the forward reaction of G6PDH is low. These phenomena present interesting challenges to future students of the biochemistry and physiology of these animals.

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Supplementary 2: Preliminary experiments in preparation for sequencing *A. callosa* plasma vanadium binding proteins

S2.1 Introduction

In light of the seasonal and experimentally induced changes in iron and vanadium concentration and the hemocyte population, preliminary experiments aimed at finding the vanadium binding protein (vanabin) sequence of *A. callosa* were undertaken. This sequence could be compared with those of other phlebobranch ascidians. This would enable investigations of seasonal changes in vanabin concentration. In preparation for sequencing of plasma vanabins of *A. callosa* plasma was subjected to immobilized metal affinity chromatography. The resultant proteins were exposed to a range of denaturants and enzymatic digestions to determine the best conditions for mass spectrophotometric analysis of protein sequence.

S2.2 Vanadium affinity chromatography

Coelomic fluid was gathered via cardiac puncture from several very large specimens and spun at 15 600 g for 12 minutes. Approximately 2 ml of plasma was recovered from each animal. Each sample was dialyzed twice against 200 ml of binding buffer (Pierce Slide-a-Lyzer ®dialysis cassette, 3500 Da pore size) for 4 hours, with fresh buffer after 2 hours. After dialysis, the samples were pooled and then subdivided into separate tubes as necessary for affinity chromatography.

Metal affinity media (BioRad Profinity® uncharged immobilized metal affinity media) was prepared according to the manufacturer's instructions. The media was used in a slurry method as in Trivedi et al. (2003). Briefly, approximately 0.75 ml of

media was added to a 3 ml centrifuge tubes and rinsed with 5 volumes of deionized water by gentle agitation on a tilt table, followed by settling and removal of the water with a pipette. In the same way, the media was the equilibrated with 5 volumes of equilibration buffer (50 mM sodium acetate, 0.3 M NaCl, pH 4.0) The column was charged with five volumes of a 300 mM vanadyl sulfate ($VOSO_4$), followed by 5 volumes of equilibration buffer and 10 volumes of deionized water. The column was then washed with 5 volumes of starting buffer (50 mM NaH_2PO_4, 0.3 M NaCl, pH 7.8) followed by 5 volumes of binding buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole). The protein concentration of the eluted sample was approximately ~50 $\mu g\ ml^{-1}$ as determined by the Coomassie Blue method.

Dialyzed plasma samples were added to the media and agitated gently for 4 hours. After this time, the media was allowed to settle for two hours and the unbound sample pipetted off. The column was then washed with 5 volumes of wash buffer. The bound sample in each tube was eluted with 1 ml elution buffer (50 mM NaH_2PO_4, 300 mM NaCl; 500 mM imidazole, pH 3.5).

Samples were reduced by incubation with dithiothretol (DTT, final concentration 50 μM) at 60 C for 1 hour, alkylated with 2-Iodoacetamide (IAA, 250 μM final concentration) in the dark for 1 hour at room temperature, followed by additional DTT to react with excess IAA. Samples were then divided and subjected to one or a combination of denaturants, as described in Tables 1 and 2 below. 'Heat" refers to denaturing by immersion in 95 ^{o}C water for 1 hour. The samples were then dialyzed against 50 mM ammonium bicarbonate (Lys-C (approximately 1:100 w/w enzyme to

substrate) and trypsin (approximately 1:100 w/w)) were applied for 16 or 24 hours at 37 °C with gentle mixing and shielded from light. Lys-C (EC 3.4.21.5) hydrolyses peptide binds on the carboxyl side of the lysine residues. Trypsin (EC 3.4.21.4) hydrolyses the carboxyl side of arginine and lysine. The digestions were stopped by adding 0.1 % trifluoracetic acid (TFA) and freezing the samples. The digests were subjected to nanoESi-MS/MS analysis at Memorial University of Newfoundland's Genomics and Proteomics facility as described below.

S2.3 Protein Identification by nanoESI-MS/MS Analysis, Lidan Tao, Memorial University CREAIT

Peptide separation was conducted using a DIONEX UltiMate3000 Nano LC System (Germering, Germany). 250 fmol of protein digest was loaded onto a C18 precolumn (LC Packing, Sunnyvale, CA) for desalting and concentrating. Peptides were then eluted from the precolumn and separated on a nanoflow analytical C18 column (PepMap 75 μm i. d., LC Packing, Sunnyvale, CA) at 180 nl/min using a gradient. The mobile phases consisted of (A) 0.1%FA/0.01% TFA/2% ACN and (B) 0.08%FA/0.008% TFA/98% ACN. A gradient of 0% B for 10min, 0-60% B in 55 min, 60-90% in 3 min,90% B for 5 min was applied. Including the regeneration step, one run was 106 min. An ABI QSTARXL (Applied Biosystems/MDS Sciex, Foster City, USA) hybrid quadrupole TOF MS/MS system equipped with a nanoelectrospray source (Protana XYZ manipulator) was used for peptide sequence analysis. The nanoelectrospray was generated from a PicoTip needle (10 μm i.d., New Objectives,

Wobum, USA) at 2400 V. The peptide tandem mass spectra were searched against the NCBInr database and a customer-designed database using the MASCOT search engine with a precursor mass tolerance of 0.2 Da and a fragment ion mass tolerance of 0.2 Da. Cysteinecarbamidomethylation was set as a fixed modification, and methionine oxidation was allowed as variable modifications. Peptides were considered identified if the Mascot score was over the 95% confidence limit based on the 'identity' score of each peptide.

S 2.4 Results and Discussion

The results of the digestion experiment are outlined in Tables S3.1 and S3.2 below. Generally digestion with Lys-C produced more peptides in the 700-2000 range than the trypsin digestion. The only peptide identified of all those found was actin. The peptides produced by the digestions were likely those of vanadium binding proteins bound to actin. As a result it is recommended that gel separation of the proteins that are isolated by affinity chromatography is necessary to separate the actin before sequencing.

Table S2.1 The number of unidentified peptides (mass to charge ratio of 600-2000) generated after vanadium affinity chromatography of *Ascidia callosa* plasma after subjection to various denaturing conditions and digestion for 16 or 24 hours with endoproteinase Lys-C

Denaturing conditions	Time with enzyme	Number of peptides in common with at least one other condition with charge to mass ratio		
		600-2000	700-2000	2000-5000
No denaturant	16	37	21	8
No denaturant	24	28	16	4
Heat	16	57	41	8
Heat	24	57	44	9
Acetonitrile	16	44	28	14
Acetonitrile	24	58	43	6
Trifluoroethanol	16	58	40	11
Trifluoroethanol	24	57	41	6
Heat + 10% Acetonitrile	16	52	36	7
Heat + 10% Acetonitrile	24	43	30	12
Heat + trifluoroethanol	16	46	33	4
Heat + trifluoroethanol acid	24	44	38	6