1. Introduction

Ascidians are soft bodied, sessile invertebrates that are common in benthic marine environments (Renouf, 1937; Vasseur, 1977; Kuehne, 1997) and are widely distributed from the tropics (Hernandez-Zanuy and Carballo, 2001) to the poles (Goodbody, 1993; Sahade *et al.*, 1998). Chemical defenses have been strongly correlated with a lack of mobility and have arisen to take the place of "fight or flight" responses of more mobile organisms (Bakus *et al.*, 1986 and references cited therein; Dyrynda, 1986; Pawlik, 1993).

Ascidians secrete a body covering, a tunic, which consists predominantly of polysaccharides and proteins. A variety of calcareous spicules have also been found in the tunic of some species (Lowenstam and Abbot, 1975; Lambert and Lambert, 1987; Aizenberg *et al*., 2000). Spicules were once thought to contribute as a physical defense against predation; however, fish feeding assays performed with calcareous spicules of tunicates (Lindquist *et al.,* 1992), siliceous spicules of sponges (Chanas and Pawlik, 1995, 1996), and calcareous sclerites of gorgonians (O'Neal and Pawlik, 2003) have revealed that small, mineralized inclusions do not deter feeding. Tissue toughness of the tunic and an overall low nutritional value of ascidians have also been thought to deter predation on ascidians, but studies have failed to support this hypothesis (as discussed in Lindquist *et al.*, 1992).

Putative tunicate chemical defenses include secondary metabolites and inorganic acids, but may also include heavy metals, most notably vanadium (Henze, 1911; Webb, 1939, 1956; Bertrand, 1950; Goldberg *et al.*, 1951; Ciereszko *et al.*, 1963; Carlisle, 1968; Swinehart *et al.*, 1974). Many marine organisms use low pH as a form of chemical defense (Thompson, 1988). The accumulation or production of sulfuric acid ($pH \leq 2.0$) in bladder cells located within the tunic has been demonstrated in some ascidians (Webb, 1939; Carlisle, 1968; Swinehart *et al.*,

1974). Acids may damage tissue integrity and are, therefore, repellent to many marine organisms (Thompson, 1988). Contact occurs at the point of injury or attack when bladder cells within the damaged tunic of the ascidian are ruptured (Thompson, 1960). Pisut and Pawlik (2002) demonstrated that food pellets with pH $\leq$ 3.0 were deterrent in feeding assays with the bluehead wrasse, *Thalassoma bifasciatum*.

It has long been recognized that ascidians actively accumulate heavy metals (Henze, 1911) including manganese, magnesium, iron, molybdenum, niobium, tantalum, chromium, and titanium, but most notably vanadium (Webb, 1939; Carlisle, 1958, 1968; Levine, 1961; Ciereszko *et al.*, 1963; Kokubu and Hidaka, 1965; Goodbody, 1974; Cheney *et al.*, 1997). Vanadium (V) was first discovered in 1813 by the mineralogist del Rio. Vanadium was initially designated panchromium because it changes color during the transition through various oxidation states. Del Rio withdrew his discovery, believing he had merely found a new "form" of chromium, but the element was subsequently "rediscovered" in 1831 by the chemist Sefström who purified vanadium in the oxide form. Elemental vanadium does not exist in nature, but rather in anionic and cationic form with oxidized states ranging from -1 to +5. The most common oxidation states of vanadium under physiological conditions occur as +3, +4, and +5 (see references in Rehder, 1992, 1999; Barceloux, 1999).

Vanadium has been isolated in trace amounts from bacteria, plants, and animals where it acts to facilitate oxidative and reductive pathways. The vanadium center is necessary for nitrogen-fixing bacteria as part of the vanadium-nitrogenase enzyme complex involved in the reduction of gaseous N_2 to the ammonium ion, NH_4^+. The fly-agaric toadstool accumulates vanadium as a vanadium (+4) complex, amavadine, but the ecological function of amavadine is

unknown. Some seaweed species have vanadium complexes that remove halogens from organic substrates via haloperoxidase enzymes (see references in Rehder, 1992, 1999).

As a necessary component of regulatory enzyme complexes, vanadium has been viewed as an essential trace metal for all living organisms (Mertz, 1981; Rehder, 1992, 1999; Barceloux, 1999). Most trace elements have a range of safe levels; however, harmful effects may occur outside of this range. In mammalian systems, the function of vanadium is not known, but deficiency experiments in animals have revealed reductions of growth, changes in lipid production, and fluctuating levels of reproductive output (Mertz, 1981; Domingo, 1996; Barceloux, 1999). The toxicity of vanadium depends on the mode of entry into the body with ingestion being less toxic than intraperitoneal injection and inhalation, which produce immediate effects (Opresko, 1991). The potency of vanadium also varies with changes in oxidation state, vanadium (+3) is least toxic and vanadium (+5) is the most toxic (Barceloux, 1999).

The concentration of vanadium in seawater is between 0.0003 and 0.003 parts per million (ppm). Most marine organisms contain vanadium concentrations of 1 to 3-ppm dry weight, whereas some ascidians have been shown to accumulate vanadium to 3000-ppm (Ciereszko *et al.*, 1963; Rummel *et al.*, 1966; Swinehart *et al*., 1974). Three suborders of the class Ascidiacea concentrate heavy metals from seawater: Stolidobranchia concentrate heavy metals other than vanadium, such as iron, whereas Aplausobranchia and Phlebobranchia both accumulate vanadium, but reduce the metal to different oxidation states, +4 and +3 respectively. The presence and valence state of vanadium in the body tissues of some ascidians has been used as a taxonomic character (Webb, 1939; Hawkins *et al*., 1983).

The black sea squirt *Phallusia nigra* (= *Ascidia nigra*) (Abbot *et al.*, 1997) is a solitary Phlebobranch ascidian that ranges from Florida to Texas and throughout the West Indies, is

frequently found attached to rocks, pilings and sea walls of marinas in the Florida Keys, and is easily distinguished from other Western Atlantic tunicates by its matte black color. In the field, *P. nigra* does not show signs of predation and the tunic is often clear of fouling organisms (Goodbody, 1962; Hirose *et al.*, 2001; Odate, personal observation).

In comparison to other ascidians, *P.nigra* has some of the highest recorded vanadium concentrations and is one of the best studied with respect to vanadium chemistry. In *P.nigra*, accumulated vanadium has been shown to be stored primarily as vanadium (+3), ~70%, but also vanadium (+4), ~30% (Webb, 1939; Boeri and Erhenberg, 1954; Rezaeva, 1964; Swinehart *et al.*, 1974; Carlson, 1975; Kustin *et al.*, 1976; Frank *et al.*, 1999, 2001, 2003). The coordination chemistry of the metal has yet to be completely clarified (Carlson, 1975; Dingley *et al.,* 1982; Bruening *et al.*, 1985; Frank *et al.*, 1986). X-ray absorption spectroscopic (XAS) investigations of the blood cells of this organism by Frank *et al.* (1998), implied that vanadium resides in more than one ligand environment and included both V (+3) and V (+4) complexed with water molecules as well as V (+3) chelated to proteins containing catechol and pyrogallol moieties. These ligand environments suggests complexation with low molecular weight proteins derived from the amino acids 3, 4-dihydroxyphenylalanine (DOPA) and/or 3, 4, 5-trihydroxyphenylalanine (TOPA) (Bruening *et al.*, 1985; Oltz *et al.*, 1988; Bayer *et al.*, 1992; Frank *et al.*, 2001, 2003). Diverse arrays of DOPA/TOPA-like proteins, such as tunichrome-class molecules, are known to exist within ascidain blood chemistry (Macara *et al.,* 1979; Oltz *et al.*, 1988, 1989; Azumi *et al*., 1990; Taylor and Bayer, 1997; Taylor *et al.*, 1994, 1997). The V (+3) fraction included the acidic aqua vanadium ion, $[V(H_2O)_6]^{+3}$ and two *non*-acidic chelated vanadium species (Frank *et al.*, 2001). Frank *et al.* (2003) revealed the *non*-acidic V (+3) forms

to be V(acetylacetonate)$_3$ and [V(catecholate)$_3$]$^{-3}$. The V (+4) fraction included the acidic oxovanadium ion, [VO(H_2O)$_5$]$^{+}$ (Frank *et al.*, 2003).

In seawater, vanadium exists as vanadate ($H_2VO_4^-$/HVO_4^{-2}), vanadium (+5). In *P. nigra*, vanadium (+5) is accumulated from seawater, reduced to V (+4), and then stored as V (+3) (Carlson, 1975; Dingley *et al.*, 1982; Frank *et al.*, 1986; Brand *et al.*, 1989; Michibata *et al.*, 2003). The oxidation state of vanadium is influenced by both the pH of the localized environment in which the vanadium resides and the chelating ligands (see reviews Rehder, 1995; Meier *et al.*, 1995).). The mechanism of vanadium uptake, reduction, transfer, and accumulation has yet to be fully clarified (Rummel, *et al.*, 1966; Danskin, 1978; Cheney *et al.*, 1997; see reviews Kustin and Robinson, 1995; Michibata *et al.*, 2002, 2003).

The tunic of *P. nigra* is stratified and contains 3 distinct layers (Endean, 1960; De Leo *et al.*, 1981; Stoecker, 1980a; Hirose, 1999; Hirose *et al.*, 2001). The outermost layer of the tunic is made up mostly of cells containing pigment granules. The distinct black coloration of *P. nigra* tunic is due in large part to these deposits (Stoecker, 1980a). Beneath the pigmentary layer is a layer of tightly packed, highly vacuolated bladder cells. Bladder cells contain acidic fluid (pH < 2) (Stoecker, 1978, 1980b; Hirose, 1999; Hirose *et al.*, 2001) and are concentrated more towards the pigmentary layer and decrease in number further from the tunic exterior (De Leo, 1981; Stoecker, 1980a; Hirose, 1999). The innermost layer of the tunic is the ground layer that consists mainly of a gelatinous matrix and is low in cell density (Hirose, 1999).

A network of blood vessels extends throughout the tunic (Hirose, 1999). Based on morphological characteristics between 9 and 11 different cell types have been described in ascidian blood (De Leo *et al.*, 1981; Hirose *et al.*, 1994; Michibata *et al.*, 2002). Vanadium is sequestered primarily in blood cells called vanadocytes (Bielig *et al.*, 1966; Carlson, 1975;

Kustin *et al.*, 1976; Botte *et al*., 1979; De Leo *et al.*, 1981; Brand *et al.*, 1989; Hirose, 1999; Hirose *et al.*, 2001; Frank *et al.*, 2001, 2003), but can also be found in high concentrations in a thin layer located near the exterior tunic surface (Stoecker, 1978). In vanadocytes, vanadium is stored in the +3 oxidation state in association with low pH (Michibata and Uyama, 1990; Michibata *et al.*, 1987, 1990, 1991, 2002, 2003).

Vanadocytes can migrate from blood vessels, move through the ground layer, and have been shown to accumulate at the pigmentary layer (Anderson, 1971). Vanadocytes accumulate at the sites of tunic injury and have been hypothesized to function in the regeneration and synthesis of tunic tissue (Endean, 1955c, 1960; Smith and Dehnel, 1970; Wardrup, 1970; Anderson, 1971; Goodbody, 1974; Hirose *et al*., 1997; Taylor *et al.*, 1997, and references cited therein). Hecht (1918) showed that blood coagulation in ascidians is triggered by exposure to seawater. Endean (1961) observed vanadocytes releasing contents in the tunic matrix producing fiber-like proteins that span areas devoid of tunic substance. Stoecker (1978) proposed that the vanadium-rich deposit present at the tunic surface of *P. nigra* is formed by the degeneration of vanadocytes. Vanadocytes turn blue-black during the process of degeneration (Hecht, 1918; Goodbody, 1962; Smith and Dehnel, 1970). The migration and degeneration of vanadocytes at the tunic exterior explains the high concentration of vanadium found at the tunic surface (Stoecker, 1978

Stoecker (1978, 1980a,b) proposed that heavy metals, in particular vanadium, provided ascidians with anti-predatory and anti-fouling defenses. The significance of vanadium in anti-predatory chemical defense of ascidians was examined using feeding assays with fish and crabs incorporating the vanadium salts, vanadyl sulfate ($VOSO_4 \cdot 6H_2O$) and sodium vanadate ($NaVO_3$). These compounds had a minimum deterrent concentration of *ca.*100-µg/g dry mass (1250-3333-

μg/g wet tissue mass). However, vanadium in ascidian tissues occurs in biochemical complexes that differ from those found in salts used for feeding assays. The relationship between secondary metabolites, low pH, and vanadium complexes in ascidians remains unclear.

Low pH and heavy metal accumulation and storage in adult ascidians are coupled properties. In an attempt to decouple the relationship between inorganic acids and vanadium we compared two *non*-acidic vanadium (+3) complexes to two acidic aqua vanadium (+3 and +4) complexes in laboratory assays testing anti-predatory and anti-microbial activity. For comparison, components of the vanadium complexes as well as vanadium salts and crude organic extracts of *P. nigra* tissues were also tested for anti-predatory and anti-microbial activity. In addition, *P. nigra* tissues and blood samples, both fresh and previously frozen, as well as crude organic extracts of *P. nigra* whole tunic and soft body tissues were also assessed for anti-predatory activity. In this study we addressed the following questions: (1) Does vanadium act as a chemical defense of *Phallusia nigra*? (2) Is the deterrence of vanadium affected by complexation environment and associated pH?