J Chem Ecol (2007) 33:643–654
DOI 10.1007/s10886-007-9251-z

# The Role of Vanadium in the Chemical Defense of the Solitary Tunicate, *Phallusia nigra*

**Shobu Odate · Joseph R. Pawlik**

Received: 14 August 2006 / Revised: 26 December 2006 / Accepted: 29 December 2006 /
Published online: 31 January 2007


**Abstract** Ascidians (sea squirts) may defend themselves from predators, biofouling competitors, and bacterial infection by producing secondary metabolites or sequestering acid, but many species also accumulate heavy metals, most notably vanadium. The defensive functions of heavy metals in ascidians remain unclear, and to this end, the solitary Caribbean tunicate, *Phallusia nigra*, was studied to localize vanadium in its tissues and to assess the defensive properties of vanadium-containing compounds. As determined by flame atomic absorption spectroscopy, the internal tissues and blood contained the highest vanadium concentrations (mean values of 2,280 and 1,886 ppm dry mass, respectively), followed by the tunic surface (871 ppm dry mass). Results of laboratory feeding assays with the bluehead wrasse, *Thalassoma bifasciatum*, confirmed outcomes of past studies that demonstrated that vanadyl sulfate ($VOSO_4 \cdot 6H_2O$) and sodium vanadate ($Na_3VO_4$) were unpalatable to fish, although these salts do not accurately reflect the chelation environment or oxidation state of vanadium in living tunicates. Fresh preparations of whole tunic, internal tissues, and blood were unpalatable to fish, but freezing and thawing of internal tissues and blood rendered them palatable. Crude organic extracts of whole tunic and internal tissues contained vanadium metabolites (225 and 750 ppm dry mass, respectively) and were palatable to *T. bifasciatum*; crude extracts also exhibited no antimicrobial effects against a panel of four marine bacteria known to be pathogens of marine invertebrates (*Vibrio parahaemolyticus*, *Vibrio harveyi*, *Leucothrix mucor*, and *Deleya marina*). Nonacidic vanadium (+3) complexes neither deterred predation nor inhibited microbial growth, whereas acidic aqua vanadium (+3 and +4) complexes were unpalatable to *T. bifasciatum* and exhibited antimicrobial activity. Difficulties in decoupling low pH from oxidation state and chelation environment of vanadium prevent definitive conclusions about the importance of some vanadium metabolites, but low pH appears to be the principal agent of chemical defense for *P. nigra*.

S. Odate · J. R. Pawlik (✉)
Department of Biology and Marine Biology Center for Marine Science, University of North Carolina Wilmington, Wilmington, NC 28409, USA
e-mail: PawlikJ@UNCW.edu

**Keywords** Tunicate · Ascidian · Predation · Chemical defense · Heavy metals · pH · Vanadium · Acid

## Introduction

Ascidians are soft bodied, sessile fouling invertebrates that are common in benthic marine environments and are widely distributed from the tropics (Hernández-Zanuy and Carballo, 2001) to the poles (Sahade et al., 1998). They secrete a body covering, the tunic, which consists predominantly of polysaccharides and proteins. Calcareous spicules are also found in the tunic of some species (e.g., Lambert and Lambert, 1987). Spicules were once thought to provide a physical defense against predation; however, fish-feeding assays performed with calcareous spicules of tunicates (Lindquist et al., 1992; López-Legentil et al., 2006) have demonstrated that mineralized inclusions have no deterrent effect. Tissue toughness of the tunic and an overall low nutritional value of ascidians have also been thought to deter predation. Tarjuelo et al. (2002) found a positive correlation between palatability and energy content in six ascidian species, but other studies have failed to support this hypothesis (Lindquist et al., 1992).

Putative chemical defenses of tunicates include secondary metabolites and inorganic acids (Pawlik, 1993; Pisut and Pawlik, 2002; Tarjuelo et al., 2002; López-Legentil et al., 2006), but may also include heavy metals, most notably vanadium (Webb, 1939; Ciereszko et al., 1963; Swinehart et al., 1974; Stoecker, 1978, 1980b). Some marine organisms use low pH as a form of chemical defense, ascidians among them (Thompson, 1960). Some ascidians accumulate sulfuric acid (pH≤2.0) in bladder cells located within the tunic (Webb, 1939; Swinehart et al., 1974). Pisut and Pawlik (2002) demonstrated that food pellets with pH≤3.0 were deterrent in feeding assays with the bluehead wrasse, *Thalassoma bifasciatum*.

It has long been recognized that ascidians actively accumulate heavy metals, including manganese, magnesium, iron, molybdenum, niobium, tantalum, chromium, and titanium, but most commonly vanadium (Webb, 1939; Ciereszko et al., 1963). 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 (Rehder, 1999). Vanadium has been identified in trace amounts in 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 $NH_4^+$.

The black sea squirt *Phallusia nigra* (= *Ascidia nigra*) is a large solitary phlebobranch ascidian that ranges from Florida to Texas and throughout the West Indies on pilings and sea walls. In the field, *P. nigra* does not show signs of predation, and the tunic is free of fouling organisms (Goodbody, 1962; Hirose et al., 2001). 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*, vanadium is stored primarily as vanadium (+3)(~70%), but vanadium (+4) is also present (~30%; Webb, 1939; Swinehart et al., 1974; Kustin et al., 1976; Frank et al., 2003). The coordination chemistry of the metal has yet to be completely clarified (Dingley et al., 1982; Bruening et al., 1985). X-ray absorption spectroscopic (XAS) investigations of the blood cells of this tunicate suggest that vanadium resides in more than one ligand environment and includes both V (+3) and V (+4) complexed with water molecules and V (+3) chelated to

proteins containing catechol and pyrogallol moieties. These ligand environments include low molecular weight proteins derived from the amino acids 3,4-dihydroxyphenylalanine (DOPA) or 3,4,5-trihydroxyphenylalanine (TOPA) (Bruening et al., 1985; Frank et al., 2003).

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 mass, but some ascidians have been shown to accumulate vanadium to 3,000 ppm (Ciereszko et al., 1963; Swinehart et al., 1974). 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) (Dingley et al., 1982; Brand et al., 1989; Michibata et al., 2003). Vanadium is sequestered primarily in blood cells called vanadocytes (Kustin et al., 1976; Brand et al., 1989; Hirose, 1999; Hirose et al., 2001; Frank et al., 2003), but can also be found in high concentrations in a thin layer located near the exterior tunic surface (Stoecker, 1978). The oxidation state of vanadium is influenced by both the pH of the localized environment and the chelating ligands (Rehder, 1999). The mechanism of vanadium uptake, reduction, transfer, and accumulation has yet to be clarified (Michibata et al., 2003).

Stoecker (1978, 1980a,b) proposed that heavy metals, particularly vanadium, provide ascidians with antipredatory and antifouling defenses. The importance of vanadium as an antipredatory chemical defense was examined by 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 1,250–3,333 ppm dry mass (~100 ppm wet tissue mass). However, vanadium in ascidian tissues occurs in biochemical complexes that differ from those found in salts used for feeding assays (e.g., Bruening et al., 1985; Frank et al., 2003).

Low pH is coupled with heavy metal accumulation and storage in adult ascidians. In an attempt to decouple the relationship between inorganic acids and vanadium, we compared two nonacidic vanadium (+3) complexes to two acidic aqua vanadium (+3 and +4) complexes in laboratory assays by testing for antipredatory and antimicrobial activity. For comparison, components of the vanadium complexes and vanadium salts were tested for antipredatory and antimicrobial activity. In addition, *P. nigra* tissues and blood samples, both fresh and previously frozen, and crude organic extracts of *P. nigra* whole tunic and internal tissues were also assayed. In this study, we addressed the following questions: (1) Does vanadium act as a chemical defense of *P. nigra*? (2) Are the deterrent properties of vanadium affected by complexation environment and associated pH?

## Methods and Materials

Whole individuals of *P. nigra* were collected with substrate intact along sea walls and pilings in the Port Largo Canal, Key Largo, FL, USA, in October 2002 and January 2003. Individuals were collected by self-contained underwater breathing apparatus (SCUBA) at depths between 3 and 7 m. Substrate attached to the basal portion of tunicates was carefully removed and each individual was immediately weighed and a volume determined by submerging the animal in a graduated cylinder and recording the amount of seawater displaced.

Blood of *P. nigra* was isolated as described in Brand et al. (1989). In brief, blood was extracted by heart puncture with a sterile 3-ml syringe and an 18.5-gauge wide bore hypodermic needle. The blood collected from each individual was placed in a sterile 2-ml cryogenic vial. Extracted blood was flash frozen in an ethanol and dry ice slurry (~−70°C) to limit oxidative effects. The tunic of each individual was then dissected from the internal

tissues, and the volume and mass of each were determined. After dissection, both the tunic and internal tissues were rinsed with distilled water and frozen in separate containers. Specimens of *P. nigra* were collected for the purpose of obtaining samples of the tunic bladder cell layer. This layer was separated from the rest of the tunic by using a sterile scalpel (Stoecker, 1978; Hirose, 1999; Hirose et al., 2001). The volume and mass of the tunic bladder cell layer were recorded. After dissection, the bladder cells were flash frozen and stored as previously stated.

Tunic and internal tissues were chopped into small pieces and exhaustively extracted in a mixture of 1:1 dichloromethane is to methanol (DCM:MeOH) followed by 100% MeOH using previously described methods (Pawlik et al., 1995). Frozen samples of tunic, tunic bladder cells, internal tissues, and blood were freeze dried until a constant mass was attained. Subsamples of whole tunic, internal tissues, tunic bladder cell layer, blood samples, and crude extracts of tunic and internal tissues were digested in hot concentrated 2:1 nitric acid is to sulfuric acid ($HNO_3$:$H_2SO_4$) (Sigma-Aldrich, trace metal grade) in a 25-ml Teflon digestion vessel. The concentration of vanadium from each sample was measured with flame AAS by using the standard curve method with a Perkin Elmer 3110 AAS and nitrous oxide ($N_2O_2$) (Kustin et al., 1976; Stoecker, 1978).

A solution of vanadium chloride ($VCl_3$, Fisher Scientific) in 1 M HCl was used to simulate the hexaaqua V (+3) component of *P. nigra* blood cell chemistry. The V (+3) fraction of the blood chelated by DOPA or TOPA-like ligand was simulated by V (acetylacetonate)$_3$ (Strem Chemicals Inc.) and $K_3$[V(catecholate)$_3$]·1.5 $H_20$ (Cooper et al., 1982), which was prepared as needed. The V (+4) fraction of the blood cells was reproduced as $VOSO_4$·6 $H_2O$ in 1 M HCl (Frank et al., 2003). The vanadium (+3) tris (catecholate) complex, $K_3$[V(catecholate)$_3$] (molecular weight=519.55), was synthesized by using the methods described by Cooper et al. (1982). All operations were carried out with standard Schlenk techniques under nitrogen ($N_2$) atmosphere. Vanadium (+4) catecholate and vanadium (+3) catecholate complexes can be distinguished from one another by the presence of a V=O stretch at 977 $cm^{-1}$ within the infrared (IR) spectra of V (+4) tris(catecholate) and the absence of this band in corresponding V (+3) tris(catecholate) complexes (Selbin, 1966). The techniques of infrared spectroscopy with a potassium bromide pellet (KBr) and ultraviolet (UV) spectroscopy were used to confirm the presence and coordination of vanadium in all synthesized vanadium compounds. Reaction products were purified by recrystallization.

Stock solutions of each vanadium-containing compound were prepared on the basis of the molecular weight of the vanadium fraction in proportion to the molecular weight of the entire vanadium complex. Three stock solutions of each vanadium complex with vanadium concentrations of 1,000, 2,000, and 4,000 μg/ml were prepared relative to known vanadium concentrations in tissues of *P. nigra*. The pH of each stock solution, and of ascidian blood, was determined by pipetting 30 μl of solution onto analytical pH strips (EM-reagents 9580, 9581, and 9590). The pH of fresh and frozen tissues was also determined by using analytical pH strips by pressing the test strip to the surface of the tissue with light pressure. Each stock solution and tissue type was measured three times to determine a mean pH value; the pH values were consistent and did not vary with each reading.

Fish feeding assays were performed with the bluehead wrasse, *T. bifasciatum*, a generalist Caribbean reef predator, as described in Pawlik et al. (1995). Vanadium complexes, reaction precursors, tissue and blood extracts were incorporated into a food matrix composed of freeze-dried squid mantle (5 g), alginic acid (Sigma-Aldrich, Na-salt form) (3 g), and distilled water (100 ml) (see Pisut and Pawlik, 2002 for discussion of food value of ascidian tissues). The mixture was stirred until homogenous and then packed into a

3.0-ml syringe and extruded into a 0.25-M calcium chloride ($CaCl_2$) solution, which caused the squid matrix to harden. After a few minutes, the spaghetti-like strand was removed from the $CaCl_2$ solution and rinsed with deionized water. The resulting string was cut with a razor blade into 4.0-mm long pellets. Control pellets were made in the same manner with the addition of the same solvent as used to dissolve the metabolite or extract for the corresponding treatment. Some highly basic compounds reacted with the squid and alginic acid matrix and caused it to harden before being extruded into the $CaCl_2$ solution. These compounds were instead incorporated into a food matrix composed of freeze-dried squid mantle (5 g), carageenan (3 g) (Sigma-Aldrich, type 1 commercial grade), and distilled water (100 ml). The mixture was stirred until homogenous and heated in a microwave until boiling. The mixture was then quickly remixed and poured onto a clean bench top and allowed to harden. Once the carageenan mixture had cooled, the resulting gel was cut with a razor blade into 4 mm long pellets. Control pellets were made in the same manner with the addition of the same solvent as used to dissolve the metabolite or extract for the corresponding treatment.

Five 30 gallon aquaria were divided into 24 cells, and groups of two to four yellow-phase *T. bifasciatum* were placed in each cell. Groups of fish were randomly chosen during feeding assays and offered a control food pellet, followed by a treatment pellet. If the fish rejected the treatment pellet, another control pellet was offered to determine whether the fish had stopped feeding; groups of fish that would not eat control pellets were not used in assays. A pellet was considered rejected if not eaten after a minimum of three attempts by one or more fish to take it into their mouth cavity or if the pellet was approached and ignored after one such attempt. Fish were fed 10–30 min before assays to limit effects due to extreme hunger. Uneaten pellets were removed from tanks after each assay. By using the Fisher exact test, a significant difference in the number of treated and control pellets eaten was determined for an individual 10-replicate assay if 4 or more pellets were rejected ($P \leq 0.043$, one-tailed test). Therefore, treatments were considered deterrent if the mean number of pellets eaten was $\leq 6$ (Pawlik et al., 1995).

To assess the effect of basic (high) pH on palatability, food pellets were made as before with carageenan and the addition of 1 N sodium hydroxide (NaOH) that provided the desired pH, as indicated by pressing pellets against analytical test strips. Control pellets were made in the same manner, but did not include the addition of base. The mixture was stirred and microwaved for 30 sec. The heated mixture was poured onto a clean bench top and allowed to harden. Food pellets were cut from the hardened gel. Feeding assays were performed as before; however, the pH of food pellets was determined with analytical test strips both before assay and after pellets were rejected to assure consistency in pellet pH values. The pH of successive assays was increased within a range starting at pH 7.0 to 14.0. Control pellets had a pH of 7.0.

Antimicrobial activity was assessed for vanadium-containing compounds and reaction precursors and crude organic extracts of *P. nigra* tunic and internal tissues. Each compound and tissue extract was tested against an assembly of four marine bacteria that included: *Vibrio harveyi* (Microbial Identification, Inc., MIDI, Newark, DE, USA), *Deleya marina* (American Type Culture Collection (ATCC) 35142), *Vibrio parahaemolyticus* (ATCC 27969), and *Leucothrix mucor* (ATCC 25906). The bacterium *V. harveyi* was isolated and identified as part of a previous study by Kelly et al. (2003) and has been recognized as an opportunistic pathogen of certain fish and marine invertebrates. The marine microbe *D. marina* is a common surface fouling bacterium, and both *V. parahaemolyticus* and *L. mucor* are known marine pathogens that can cause disease in marine invertebrates (Kelly et al., 2003). Standard disc-diffusion antimicrobial assays were performed by inoculating each

bacterial test strain onto the surface of a YP agar medium plate: peptone (1.0 g $l^{-1}$) and yeast extract (1.0 g $l^{-1}$) in filtered seawater (32–36‰) with the addition of agar (15 g $l^{-1}$) (Newbold et al., 1999). Culture concentrations were measured before each antimicrobial bioassay by using a Milton Roy Spectronic 20 D at 500 nm. Only cultures that had an absorbance between 0.2 and 0.5 were used to inoculate bioassay media plates. A 100-µl aliquot of inoculated broth was spread with a sterile glass rod over the entire agar surface to form an evenly distributed film. Compounds were dissolved in solvent and tested by pipetting 30 µl onto a sterile circular paper disc (Becton Dickinson, 6×0.9 mm, disc volume=30 µl) and allowing the solvent to evaporate. Crude organic extracts were dissolved in methanol before transfer to paper disc. All solvents used to dissolve air-sensitive materials were degassed of oxygen before use, and all stock solutions were stored under inert atmosphere with purified nitrogen. After pipetting the stock solution onto a paper disc, the solvent was evaporated under nitrogen. Stock solutions were monitored for color change that would indicate oxidation events. If oxidation occurred, the solutions were remade before use in the assay. The discs were placed on the surface of the inoculated agar (up to nine per plate). By pressing lightly on the disc with an analytical pH strip, the pH of each disc was checked once the discs had rehydrated after being placed on the agar plate. All strains were incubated at room temperature (22°C), and a confluent film developed over the entire agar surface after 24 hr. Clear halos (zones) surrounding the discs denoted areas of inhibited microbial growth. The computer-imaging program Image J (National Institutes of Health, USA, http://rsb.info.nih.gov/ij/) was used to calculate area of inhibition zones by subtracting the area of the disc (28.27 $mm^2$) from the total area of clear zone. Compounds that exhibited inhibition zones (total area of clear zone minus the area of the disc) of greater than or equal to 50 $mm^2$ were considered to possess acute antimicrobial activity. Statistical analyses were not used to assign significance of differences between treatments because the disc diffusion assay is used as a qualitative measure of antibacterial activity (Newbold et al., 1999).

## Results

Mean vanadium concentrations in tissues, blood samples, and crude organic extracts of *P. nigra* ranged from 2,280 ppm dry mass (63 ppm wet mass) in internal tissues to 225 ppm dry mass (7 ppm wet mass) in crude organic extracts of the ascidian tunic (Table 1). Assay foods prepared from tissue and blood of *P. nigra* were variable in their palatability to the

**Table 1** Flame atomic absorbance spectroscopy results of the vanadium content (ppm ± SD) of freeze dried *P. nigra* tissues, blood samples, and crude organic extracts of whole tunic and internal tissues

| Sample | *N* | Vanadium Content (ppm) | |
|---|---|---|---|
| | | Dry Mass | Wet Mass |
| Whole tunic | 6 | 259±157[a] | 5±3 |
| Tunic bladder cell layer | 7 | 871±390[b] | 18±8 |
| Internal tissues | 5 | 2,280±822[c] | 63±23 |
| Blood | 7 | 1,886±567[c] | 38±12 |
| Crude extract, internal tissues | 4 | 750±289 | 31±12 |
| Crude extract, tunic | 4 | 225±126 | 7±4 |

Wet mass concentrations were converted from dry mass. Significant differences in concentrations for tissues and blood are shown as different superscript letters (one-way ANOVA, $N$=3, $F$=41.02; Ryans–Einot–Gabriel–Welsch Multiple Range Test, homogeneous subsets at $P$=0.05).

generalist consumer fish, *T. bifasciatum*, with fresh material highly deterrent, while frozen material was readily eaten (Fig. 1). Both freshly dissected whole tunic tissue (pH ~ 2.0) and previously frozen tunic (pH=7) were unpalatable to *T. bifasciatum*. Freezing and thawing neutralized low pH of tissues but did not affect the vanadium content. In all cases, fishes

| Treatment | Vanadium Concentration µg/ml | Food Pellet pH |
|---|---|---|
| Whole Tunic (fresh) | 5 ± 3 | 2.0 |
| Whole Tunic (frozen) | 5 ± 3 | 7.0 |
| Soft Body (fresh) | 63 ± 23 | 7.0 |
| Soft Body (frozen) | 63 ± 23 | 7.0 |
| Blood (fresh) | 38 ± 12 | 2.0 |
| Blood (frozen) | 38 ± 12 | 7.0 |
| Crude Extract: Tunic | 7 ± 4 | 5.0 |
| Crude Extract: Soft Body | 31 ± 12 | 5.0 |
| $V(acetylacetonate)_3$ | 1000 | 6.5 |
| $V(acetylacetonate)_3$ | 2000 | 6.5 |
| $V(acetylacetonate)_3$ | 4000 | 6.5 |
| $K_3[V(catecholate)_3]$ | 1000 | 11.0 |
| $K_3[V(catecholate)_3]$ | 2000 | 11.0 |
| $K_3[V(catecholate)_3]$ | 4000 | 11.0 |
| $VCl_3$ in 1M HCl | 1000 | 0.0 |
| $VCl_3$ in 1M HCl | 2000 | 0.0 |
| $VCl_3$ in 1M HCl | 4000 | 0.0 |
| $VOSO_4$ in 1M HCl | 1000 | 0.0 |
| $VOSO_4$ in 1M HCl | 2000 | 0.0 |
| $VOSO_4$ in 1M HCl | 4000 | 0.0 |
| $VOSO_4$ | 1000 | 2.2 |
| $VOSO_4$ | 2000 | 1.9 |
| $VOSO_4$ | 4000 | 1.6 |
| $Na_3VO_4$ | 1000 | 11.0 |
| $Na_3VO_4$ | 2000 | 11.0 |
| $Na_3VO_4$ | 4000 | 11.0 |

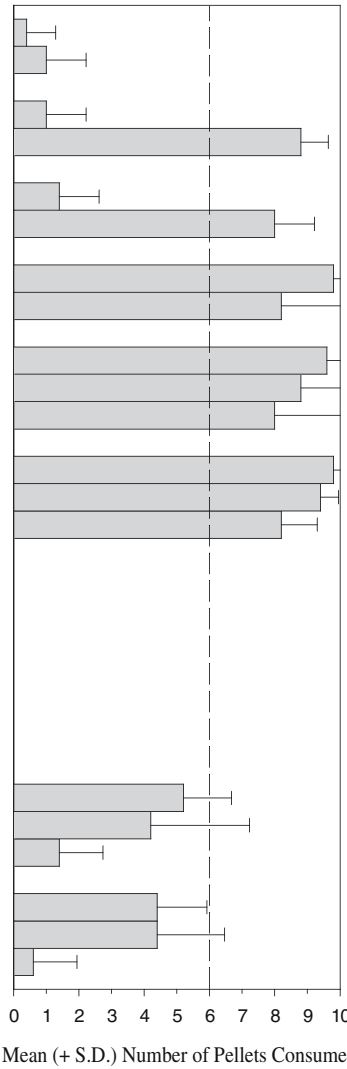


**Fig. 1** Consumption by the bluehead wrasse, *T. bifasciatum*, of food pellets treated with tissue and blood of *P. nigra*, vanadium complexes, and vanadium salts. Compounds were considered deterrent if the mean number of pellets eaten for any individual assay were less than or equal to 6 ($P \leq 0.043$, Fisher exact test, one-tailed), as indicated by the *dotted line* on the graph. Mean + SD shown for five replicate assays of each treatment. Vanadium concentration and pH of food pellet are shown for each treatment

approached and mouthed the pieces of the tunic but did not eat them. Food pellets made with finely chopped tunic tissue, fresh and frozen, were also mouthed and the squid matrix consumed; however, the bulk of the tunic tissue was rejected. Freshly dissected soft body internal tissues (pH=7) were unpalatable to *T. bifasciatum*, whereas frozen and thawed tissues were consumed, but in this case, there was no change in pH after freezing and thawing. Fishes rejected freshly collected *P. nigra* blood (pH ~ 2) incorporated into food pellets, whereas frozen and thawed blood (pH=7) was palatable.

In the same feeding assays with *T. bifasciatum*, crude organic extracts of ascidian tissues, vanadium complexes, and vanadium salts were also variably deterrent, with the greatest level of unpalatability associated with low pH (Fig. 1). Crude organic extracts of internal tissues contained high vanadium concentrations (750 ppm dry mass=31 ppm wet mass, Table 1), but were palatable to *T. bifasciatum* (Fig. 1). The nonacidic vanadium (+3) compounds bound to DOPA/TOPA-like chelation environments were also tested, and V (acetylacetonate)$_3$ and $K_3$[V(catecholate)$_3$] were palatable to *T. bifasciatum* at all of the concentrations assayed. Food pellets with vanadyl sulfate were unpalatable to *T. bifasciatum*, but at the highest pH assayed (pH=2.2), a mean of five pellets were eaten, with decreasing palatability as the vanadium content was increased and the pH decreased. Food pellets made with the vanadium salt sodium vanadate at all concentrations (pH=11) were unpalatable to *T. bifasciatum* (Fig. 1). This effect was not attributable to high pH because food pellets treated with NaOH to generate high pH were only unpalatable to *T. bifasciatum* at pH≥13.0 (Fig. 2). The pH of food pellets treated with NaOH was tested after rejection by assay fishes and had not changed.

By using a standard disc-diffusion assay, crude organic extracts of tunic and internal tissues, vanadium complexes, and vanadium salts were assessed for antimicrobial activity against a panel of four marine bacteria known to infect marine invertebrates. Crude organic extracts of dissected tunic and internal tissues ($N$=3 for each tissue type vs. each bacteria) of four individual tunicates did not exhibit any antimicrobial activity against the panel of marine bacterial lines tested (data not shown). The nonacidic vanadium (+3) complexes, V (acetylacetonate)$_3$, and $K_3$[V(catecholate)$_3$] did not exhibit any antimicrobial activity against the entire panel of marine bacteria; however, the acidic aqua vanadium (+3 and +4) complexes inhibited microbial growth (Table 2). Although assayed at equivalent vanadium concentrations, the two vanadium salts had different effects on the panel of bacteria.

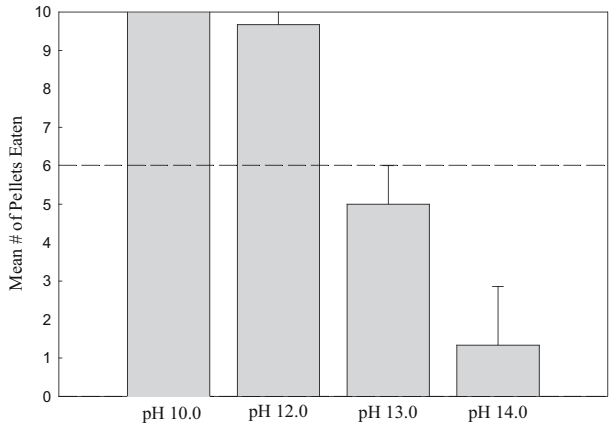


**Fig. 2** Consumption by *Thalassoma bifasciatum* of food pellets treated with sodium hydroxide. Fish consumed ten control pellets in all cases. Treatments were considered deterrent if the number of pellets eaten was less than or equal to 6 ($P$≤0.043, Fisher exact test, one-tailed), as indicated by the dotted line on the graph. Mean + SD shown for three replicate assays of each treatment

Sodium vanadate at all concentrations (pH=11) did not inhibit microbial growth, whereas vanadyl sulfate (pH 1.9 to 3.6) did produce clear zones of inhibition (Table 2)

## Discussion

The presence of low pH and heavy metals in the tissues of ascidians has long been recognized. Stoecker (1978) first hypothesized that inorganic acids and heavy metals, most notably vanadium, provided ascidians with effective antipredatory and antifouling defenses. Parry (1984) disputed Stoecker's results, but arguments by Parry (1984) were correlative and based on the existence of other tunicate species in similar habitats that contained neither vanadium nor acid, yet remained uneaten or fouled. Parry (1984) did not consider the presence of secondary metabolites as defenses that might function in the absence of vanadium and sulfuric acid. Stoecker (1980a) conducted feeding assays with fish and crabs with the salts vanadyl sulfate and sodium vanadate. These salts putatively represented the oxidation state of vanadium when it was released from the ascidian during a predation event. The results of the feeding assays reported herein that used the bluehead wrasse, *T. bifasciatum*,

**Table 2** Antimicrobial activity [mean area of inhibition (standard deviation), $mm^2$] of vanadium compounds and vanadium salts vs. bacterial test strains using standard disc diffusion assay

| Compound | Vanadium Concentration (μg/ml) | Solution pH | Disc pH | *Vibrio parahaemolyticus* Mean Area of Inhibition | *Deleya marina* Mean Area of Inhibition | *Leucothrix mucor* Mean Area of Inhibition | *Vibrio harveyi* Mean Area of Inhibition |
|---|---|---|---|---|---|---|---|
| V (acetylacetonate)$_3$ | 4,000 | 7.0 | 4.0 | 0 | 0 | 0 | 0 |
| V (acetylacetonate)$_3$ | 2,000 | 7.0 | 5.0 | 0 | 0 | 0 | 0 |
| V (acetylacetonate)$_3$ | 1,000 | 7.0 | 6.0 | 0 | 0 | 0 | 0 |
| K3[V(catechol)$_3$] | 4,000 | 11.0 | 10.0 | 0 | 0 | 0 | 0 |
| K$_3$[V(catechol)$_3$] | 2,000 | 11.0 | 10.0 | 0 | 0 | 0 | 0 |
| K$_3$[V(catechol)$_3$] | 1,000 | 11.0 | 9.0 | 0 | 0 | 0 | 0 |
| $VCl_3$ in 1 M HCl | 4,000 | 0.0 | 2.5 | +++ | +++ | +++ | +++ |
| $VCl_3$ in 1 M HCl | 2,000 | 0.0 | 3.3 | + | + | + | + |
| $VCl_3$ in 1 M HCl | 1,000 | 0.0 | 3.6 | + | + | + | + |
| $VOSO_4$ in 1 M HCl | 4,000 | 0.0 | 1.6 | + | +++ | +++ | +++ |
| $VOSO_4$ in 1 M HCl | 2,000 | 0.0 | 1.9 | + | + | + | + |
| $VOSO_4$ in 1 M HCl | 1,000 | 0.0 | 2.2 | + | + | + | + |
| $VOSO_4$ | 4,000 | 1.6 | 1.9 | + | +++ | +++ | +++ |
| $VOSO_4$ | 2,000 | 1.9 | 2.5 | + | + | + | + |
| $VOSO_4$ | 1,000 | 2.2 | 3.6 | + | + | + | + |
| $Na_3VO_4$ | 4,000 | 11.0 | 10.0 | 0 | 0 | 0 | 0 |
| $Na_3VO_4$ | 2,000 | 11.0 | 9.0 | 0 | 0 | 0 | 0 |
| $Na_3VO_4$ | 1,000 | 11.0 | 8.0 | 0 | 0 | 0 | 0 |

0 No activity, +clear zone of inhibition present, +++clear zone of inhibition greater than or equal to 50 $mm^2$

corroborate those of Stoecker (1980a) that used the fishes *Fundulus heteroclitus* (common killifish), *Abudefduf saxatilis* (sergeant major), and *Haemulon sciurus* (blue striped grunt). However, salts of vanadium do not accurately reflect vanadium as it is found in the tissues and blood of *P. nigra*. Furthermore, pH and vanadium accumulation and storage in adult ascidians are coupled, making it difficult to determine the chemical deterrence of the individual components.

This study is the first to attempt to decouple the chemical deterrence of vanadium from low pH in assessing the chemical defense of an ascidian. The presence of vanadium did not always deter predation or inhibit microbial growth (Fig. 1, Table 2), whereas low pH consistently did both. Whole tunic deterred feeding despite having a low concentration of vanadium, regardless of whether it was assayed at pH 2 or 7 (Fig. 1), suggesting a physical defense of the outer body covering. Crude extracts of tunic and internal tissues that contained vanadium but were not acidic neither deterred predation nor inhibited microbial growth. Vanadium species decoupled from acidity by chelation to DOPA/TOPA-type ligands did not deter predation or inhibit microbial growth; however, acidic aqua vanadium (+3 and +4) complexes were deterrent in both assay systems.

Localization of chemical defenses to specific regions of an organism is exhibited in terrestrial plants and marine organisms (Zangerl and Rutledge, 1996; Dworjanyn et al., 1999; Van Alstyne et al., 1999) and has also been suggested in tunicates (Pisut and Pawlik, 2002; Tarjuelo et al., 2002). Surface associated defenses may enhance the survival of an organism by providing a mechanism to protect against predation, deter fouling organisms, and control epibiont populations (Wahl et al., 1994). Acidity is important in deterring potential predators and is probably effective against most grazers and browsers (Thompson, 1960; Stoecker, 1980a). Davis and Wright (1989) hypothesized that surface acidity would be neutralized by the buffering capacity of seawater, but Pisut and Pawlik (2002) demonstrated that low pH persists in tissues for some time. Brief localized exposure to low pH may be enough to deter a potential predator (Hirose et al., 2001). Pisut and Pawlik (2002) demonstrated that pieces of the acidic tunic of *Ascidia interrupta* were unpalatable to *T. bifasciatum* in feeding assays, whereas crude organic extracts of the tunic were palatable. Moreover, epibionts are rare on species that have acidic tunics (Stoecker, 1980a).

In the solitary tunicate *P. nigra*, low pH and vanadium are associated with the tunic exterior. Acidic fluid is released from bladder cells localized near the exterior surface when the tunic is bruised or damaged (Hirose et al., 2001; Pisut and Pawlik, 2002). Vanadium is not evenly distributed throughout *P. nigra* tissues (Table 1), corroborating the previous work of Stoecker (1978) and Ciereszko et al. (1963). Vanadium is stored mainly in blood cells called vanadocytes, but is also accumulated at the tunic surface (Kustin et al., 1976; Brand et al., 1989; Hirose, 1999; Hirose et al., 2001; Frank et al., 2003). Interestingly, vanadocytes are able to migrate from blood vessels located in the tunic and accumulate in the pigmented layer located at the tunic surface. However, the coordination environment of vanadium at the tunic surface has yet to be fully clarified.

Acidic tissues are repellant to most fish (Thompson, 1960) and may primarily explain why *P. nigra* is unpalatable to fish predators. Tissues associated with low pH were unpalatable to *T. bifasciatum*; however, these same tissues that were frozen and then thawed exhibited higher pH values and were more palatable, perhaps, because of the buffering capacity of spicules or salts in the tunic. Although this process would not affect the presence of vanadium in these tissues, the oxidation state and ligand coordination associated with vanadium could be affected due to changes in the storage environment and oxidative effects of cell lysis. Non-chelated vanadium (+3) ions are unstable in air or moisture and are rapidly oxidized at pH≥2 (see review in Rehder, 1999). Interestingly, tunic

tissue pieces from both fresh and frozen *P. nigra* were both unpalatable to *T. bifasciatum* despite differences in pH, a result that cannot be readily explained.

In standard disc diffusion assays, crude organic extracts of the tunic and internal tissues of *P. nigra* did not inhibit the growth of any of the bacteria assayed. Nonacidic vanadium (+3) compounds also did not inhibit microbial growth, whereas low pH and acidic aqua vanadium (+3 and +4) complexes did. Although assayed at equivalent vanadium concentrations, the two vanadium salts had different effects on the panel of bacteria. Sodium vanadate (pH=11) did not inhibit microbial growth, whereas vanadyl sulfate (pH 1.9 to 3.6) did produce clear zones of inhibition. Acidic fluids that are released from ruptured bladder cells may be able to kill or inactivate bacteria or other microorganisms that would otherwise gain access to tunic tissues. Difficulties in decoupling low pH from oxidation state and chelation environment of vanadium prevent definitive conclusions about the defensive properties of some vanadium metabolites, but low pH appears to be the principal agent of chemical defense for *P. nigra*.

**Acknowledgments** This research was funded by grants from the NOAA/NURC Program (NOAA-NA96RU-0260), from the National Science Foundation Biological Oceanography Program (OCE-0095724, 0550468), and by a GlaxoWellcome Ocean and Human Health Fellowship. We thank Robert D. Hancock, Richard M. Dillaman, Richard D. Lancaster, Stephen A. Skrabal, and S. Bart Jones for advice and technical support.

## References

BRAND, S. G., HAWKINS, C. J., MARSHALL, A. T., NETTE, G. W., and PARRY, D. L. 1989. Vanadium chemistry of ascidians. *Comp. Biochem. Physiol.* 93B(2):425–436.

BRUENING, R. C., OLTZ, E. M., FURUKAWA, J., and NAKANISHI, K. 1985. Isolation of tunichrome B-1, a reducing blood pigment of the sea squirt, *Ascidia nigra*. *J. Nat. Prod.* 49(2):193–204.

CIERESZKO, L. S., CIERESZKO, E. M., HARRIS, E. R., and LANE, C. A. 1963. Vanadium content of some tunicates. *Comp. Biochem. Physiol.* 8:137–140.

COOPER, S. R., KOH, Y. B., and RAYMOND, K. N. 1982. Synthetic, structural, and physical studies of bis (triethylammonium) tris(catecholato) vanadate(IV), potassium bis(catecholato) oxovanadate(IV), and potassium tris(catecholato) vanadate(III). *J. Am. Chem. Soc.* 104:5092–5102.

DAVIS, A. R. and WRIGHT, A. E. 1989. Interspecific differences in fouling of two congeneric ascidians (*Eudistoma olivaceum* and *E. capsulatum*): is surface acidity an effective defense? *Mar. Biol.* 102:491–497.

DINGLEY, A. L., KUSTIN, K., MACARA, I. G., MCLEOD, G. C., and ROBERTS, M. F. 1982. Vanadium-containing tunicate blood cells are not highly acidic. *Biochim. Biophys. Acta* 720:384–389.

DWORJANYN, S. A., DE NYS, R., and STEINBERG, P. D. 1999. Localisation and surface quantification of secondary metabolites in the red alga *Delisea pulchra*. *Mar. Biol.* 102:491–497.

FRANK, P., CARLSON, R. M. K., CARLSON, E. J., and HODGSON, K. O. 2003. Medium-dependence of vanadium K-edge X-ray absorption spectra with application to blood cells from phlebobranch tunicates. *Coord. Chem. Rev.* 237(1–2):31–39.

GOODBODY, I. 1962. A biology of *Ascidia nigra* (Savigny). I. Survival and mortality in an adult population. *Biol. Bull.* 122:40–51.

HERNÁNDEZ-ZANUY, A. C. and CARBALLO, J. L. 2001. Distribution and abundance of ascidian assemblages in Caribbean reef zones of the Golfo de Batabanó (Cuba). *Coral Reefs* 20:159–162.

HIROSE, E. 1999. Pigmentation and acid storage in the tunic: protective functions of the tunic cells in the tropical ascidian *Phallusia nigra*. *Invertebr. Biol.* 118(4):414–422.

HIROSE, E., YAMASHIRO, H., and MORI, Y. 2001. Properties of tunic acid in the ascidian *Phallusia nigra* (Ascidiidae, Phlebobranchia). *Zool. Sci.* 18:309–314.

KELLY, S. R., JENSEN, P. R., HENKEL, T. P., FENICAL, W., and PAWLIK, J. R. 2003. Effects of Caribbean sponge extracts on bacterial attachment. *Aquat. Microb. Ecol.* 31:175–182.

KUSTIN, K., LEVINE, D. S., MCLEOD, G. C., and CURBY, W. A. 1976. The blood of *Ascidia nigra*: blood cell frequency distribution, morphology, and the distribution and valence of vanadium in living blood cells. *Biol. Bull.* 150:426–441.

LAMBERT, G. and LAMBERT, C. C. 1987. Spicule formation in the solitary ascidian, *Herdmania momus*. *J. Morph.* 192:145–159.

LÓPEZ-LEGENTIL, S., TURON, X., and SCHUPP, P. 2006. Chemical and physical defenses against predators in *Cystodytes* (Ascidiacea). *J. Exp. Mar. Biol. Ecol.* 332(1):27–36.

LINDQUIST, N., HAY, M. E., and FENICAL, W. 1992. Defense of ascidians and their conspicuous larvae: adult vs. larval chemical defenses. *Ecol. Monogr.* 62(4):547–568.

MICHIBATA, H., YAMAGUCHI, N., UYAMA, T., and UEKI, T. 2003. Molecular approaches to the accumulation and reduction of vanadium by ascidians. *Coord. Chem. Rev.* 237:41–51.

NEWBOLD, R. W., JENSEN, P. R., FENICAL, W., and PAWLIK, J. R. 1999. Antimicrobial activity of Caribbean sponge extracts. *Aquat. Microb. Ecol.* 19:279–284.

PARRY, D. L. 1984. Chemical properties of the test of ascidians in relation to predation. *Mar. Ecol. Prog. Ser.* 17:279–282.

PAWLIK, J. R. 1993. Marine invertebrate chemical defenses. *Chem. Rev.* 93:1911–1922.

PAWLIK, J. R., CHANAS, B., TOONEN, R. J., and FENICAL, W. 1995. Defenses of Caribbean sponges against predatory reef fish: I. Chemical deterrency. *Mar. Ecol. Prog. Ser.* 127:183–194.

PISUT, D. P. and PAWLIK, J. R. 2002. Anti-predatory chemical defenses of ascidians: secondary metabolites or inorganic acids? *J. Exp. Mar. Biol. Ecol.* 207:203–214.

REHDER, D. 1999. The coordination chemistry of vanadium as related to its biological functions. *Coord. Chem. Rev.* 182:297–322.

SELBIN, J. 1966. Oxovanadium(IV) complexes. *Coord. Chem. Rev.* 1(3):293–314.

SAHADE, R., TATIAN, M., KOWALKE, J., KUEHNE, S., and ESNAL, G. B. 1998. Benthic faunal association of soft substrates at Potter Cove, King George Island, Antarctica. *Polar Biol.* 19(2):85–91.

STOECKER, D. 1978. Resistance of a tunicate to fouling. *Biol. Bull.* 155:615–626.

STOECKER, D. 1980a. Relationships between chemical defense and ecology in benthic ascidians. *Mar. Ecol. Prog. Ser.* 3:257–265.

STOECKER, D. 1980b. Chemical defenses of ascidians against predators. *Ecology* 61(6):1327–1334.

SWINEHART, J. H., BIGGS, W. R., HALKO, D. J., and SCHROEDER, N. C. 1974. The vanadium and selected metal contents of some ascidians. *Biol. Bull.* 146:302–312.

TARJUELO, I., LÓPEZ-LEGENTIL, S., CODINA, M., and TURON, X. 2002. Defence mechanisms of adults and larvae of marine invertebrates: patterns of toxicity and palatability in colonial ascidians. *Mar. Ecol. Prog. Ser.* 235:103–115.

THOMPSON, T. E. 1960. Defensive acid-secretion in marine gastropods. *J. Mar. Biol. Assoc. U.K.* 39:499–517.

VAN ALSTYNE, K. L., MCCARTHY, J. J., HUSTEAD, C. L., and KEARNS, L. J. 1999. Phlorotannin allocation among tissues of Northeastern Pacific kelps and rockweeds. *J. Phycol.* 35:483–492.

WAHL, M., JENSEN, P. R., and FENICAL, W. 1994. Chemical control of bacterial epibiosis on ascidians. *Mar. Ecol. Prog. Ser.* 110:45–57.

WEBB, D. A. 1939. Observations on the blood of certain ascidians, with special reference to the biochemistry of vanadium. *J. Exp. Biol.* 16:499–523.

ZANGERL, A. R. and RUTLEDGE, C. E. 1996. The probability of attack and patterns of constitutive and induced defense: a test of optimal defense theory. *Am. Nat.* 147:599–607.