2011

An examination of secondary metabolites and inorganic acids as chemical defenses against predation and fouling in Antarctic and sub-tropical ascidians

Gil Koplovitz
University of Alabama at Birmingham

AN EXAMINATION OF SECONDARY METABOLITES AND INORGANIC ACIDS AS CHEMICAL DEFENSES AGAINST PREDATION AND FOULING IN ANTARCTIC AND SUB-TROPICAL ASCIDIANS

by

GIL KOPLOVITZ

JAMES B. MCCLINTOCK, COMMITTEE CHAIR
CHARLES D. AMSLER
BILL J. BAKER
MICHA ILAN
ROBERT W. THACKER

A DISSERTATION

Submitted to the graduate faculty of The University of Alabama at Birmingham
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

BIRMINGHAM, ALABAMA

2011

AN EXAMINATION OF SECONDARY METABOLITES AND INORGANIC ACIDS AS CHEMICAL DEFENSES AGAINST PREDATION AND FOULING IN ANTARCTIC AND SUB-TROPICAL ASCIDIANS

GIL KOPLOVITZ

DEPARTMENT OF BIOLOGY

ABSTRACT

Palatability of fresh outer tissues of 12 species of ascidians from the Western Antarctic Peninsula was evaluated using the sympatric, omnivorous fish *Notothenia coriiceps* and sea star *Odontaster validus* as model predators. All ascidians were unpalatable to fish, while 58% were unpalatable to sea stars. Lipophilic and hydrophilic extracts of 11 ascidian species were incorporated into food pellets and tested in fish and sea star bioassays. Only the lipophilic extract from *Distaplia colligans* caused feeding deterrence in either predator. Organic extracts were also examined in food pellets using the sympatric, omnivorous amphipod *Gondogeneia antarctica*. Only the lipophilic extract of *Distaplia cylindrica* was deterrent. Five species of ascidians had acidic tunics. Acidified food pellets were deterrent against sea stars but not fish.

The secondary metabolites from a similar suite of Antarctic ascidian species were tested against sympatric marine bacteria and diatoms from the Western Antarctic Peninsula. All ascidians had lipophilic and hydrophilic extracts assayed against twenty bacterial strains and against a sympatric diatom (*Syndroposis* sp.). Only the lipophilic extract of *D. colligans* showed broad-spectrum antimicrobial activity. At least one extract from all but one ascidian taxa caused significant diatom mortality.

The palatability of five species of ascidians commonly found in sub-tropical seagrass habitats were evaluated using the sympatric, omnivorous pinfish *Lagodon rhomboides* as a model predator. Fresh outer tissues of three of the ascidian species were unpalatable to fish. Food pellets containing organic extracts of these species did not deter feeding by the fish. The toughness of the tunic of all five ascidian species was evaluated using a penetrometer. Tunic toughness is likely to explain the lack of palatability of two of the three species. Acidity is unlikely to explain deterrence in the third species as fish consumed acidified food pellets.

Despite their lack of palatability to sea stars and fish, organic chemical defenses against predation are uncommon in both Antarctic and Sub-tropical ascidians. Toughness and inorganic chemicals appear more important. Despite the lack of antibacterial defenses in Antarctic ascidians, secondary metabolites appear to play a potential role in preventing fouling by diatoms.

Keywords: Antarctica seagrass chemical defense ascidian fouling

To
my parents,

David and Shoshana Koplovitz

who made all of this possible.

“We must always remember with gratitude and admiration the first sailors who steered their vessels through storms and mists, and increased our knowledge of the lands of ice in the South.”

Roald Amundsen (1872—1928)

ACKNOWLDGMENTS

I would like to extend sincere thanks to my committee members, Drs. Charles D. Amsler, Bill J. Baker, Micha Ilan and Robert W. Thacker for their professional insight and constructive comments on my research. I am grateful to Dr. Stephen Watts, Dr. Ken Marion and Dr. Bud Fischer for the giving me the opportunity to pursue a graduate degree at UAB and for continued guidance and assistance throughout my graduate career. I would like to thank Margaret Amsler, Dr. Craig Aumack, Philip Bucolo and Dr. Jill Zamzow for their assistance with field collections in Antarctica; Dr. Robert Angus for providing an invaluable assistance with statistics and Dr. Kevin Peters for his friendship and help that was frequently needed. Thanks to Linda Cole at the Smithsonian Institution in Washington DC for her assistance with ascidian taxonomy. I wish to acknowledge the generous logistical support provided by those individuals employed by Raytheon Polar Services Company. I would also like to thank the Birmingham Jewish Federation and the Israel-UAB Medical Exchange Fund for their financial support, and Dr. Eli Capilouto for his moral support and assistance throughout my degree. Finally, I am fully indebted to my advisor and mentor Dr. James B. McClintock for his tireless assistance in every aspect of my research and my development as a scientist. This research was facilitated by National Science Foundation awards to CDA and JBM (OPP-0442769) and to BJB (OPP-0442857). Funding for this study was also provided by support of an Endowed Research Professorship in Polar and Marine Biology given to Dr. James B. McClintock through the University of Alabama at Birmingham.

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INTRODUCTION

Colonial and solitary Ascidians occur in all the world's oceans, and from depths ranging from shallow intertidal zones to the deep sea floor (Tatian et al. 2005). Ascidians represent an important component of food webs in a variety of benthic marine communities (Monteiro et al. 2002, McClintock et al. 2004, Tatian et al. 2005). As sessile, soft-bodied organisms, ascidians are vulnerable to intense predation by generalist predators that include fish (Randall, 1967), sea urchins (Briscoe and Sebens, 1988) and sea stars (Mauzey et al. 1968, McClintock et al. 2004) and by specialist predators that include mollusks – gastropods, lamellarians and nudibranchs (Cimino & Ghiselin 2001) as well as flatworms (de Caralt et al. 2002).

Another significant problem ascidians are faced with is epiphytic and epizootic recruitment (fouling) by settling organisms. In marine environments, an exposed undefended surface is subject to fouling by bacteria, protozoans and macroinvertebrate larvae. For the most part, this interaction results in negative consequences for the fouled organism. Negative effects include competition for nutrients, introduction of pathogens, increased drag, and damage to surface tissues due to mechanical anchoring of the epibionts (Wahl 1989). While not as vulnerable as sponges, heavy fouling on ascidians can result in obstruction of the incurrent and exhalent siphon inhibiting current flow critical to respiration and feeding. Moreover, increased sedimentation and increased drag

resulting from heavy fouling may lead to ascidians being dislodged from sediments (Stoecker 1978).

The problems introduced above have contributed to strong selection for a suite of defenses in response to predation, fouling and interspecific competition for spatial resources. Some marine invertebrates and algae have developed mechanical means of deterring predators and fouling organisms. These physical defense mechanisms may include spicules in sponges (Burns & Ilan 2003) and the sloughing off of surface layer tissues or of mucus that is secreted on the surface (Barthel & Wolfrath 1989, Steinberg et al. 1997). While some solitary and colonial ascidians have evolved a tough outer tunic made of a proteinaceous polysaccharide "tunicin" (in some species this is impregnated with minute spicules that may deter some predators), the primary evolutionary provision of defense appears to be chemical in nature. Secondary metabolites, inorganic acids and heavy metals are the three major classes of chemicals hypothesized to date to play a role in defense against predators, fouling organisms or competitors for spatial resources.

The studies in this dissertation attempt to evaluate the usage of secondary metabolites and inorganic acids as defenses against a host of organisms that come in direct contact with ascidians. The first two chapters concentrate on Antarctic ascidians and examine the chemical defenses they employ against predation (Chapter 1) and fouling (Chapter 2). The third chapter concentrates on ascidians commonly found in

shallow, sub-tropical seagrass habitats and their usage of chemical and physical defenses against a common predator.

In Chapter 1, the palatability of a suite of 12 colonial and solitary ascidians from the Western Antarctic Peninsula is evaluated using 2 sympatric, omnivorous predators: the sea star *Odontaster validus* and the fish *Notothenia coriiceps*. Furthermore, the ability of organic extracts from the ascidians to facilitate unpalatability is tested against the 2 predators mentioned, as well as against a mesograzer – the omnivorous amphipod *Gondogeneia antarctica*. The potential feeding deterrent activity of surface sulfuric acid is also tested against the sea star and fish. The specific hypothesis is: Due to intense predation pressure, Antarctic ascidians will employ chemical defenses, whether organic or inorganic, against the common predators in their habitat

In Chapter 2, the lipophilic and hydrophilic extracts from 14 colonial and solitary ascidians from the Western Antarctic Peninsula are examined for their antimicrobial activity against 20 strains of sympatric marine bacteria. Additionally, the lipophilic and hydrophilic extracts of the ascidians, fractionated to seawater soluble and insoluble fractions, are tested for antifouling activity against the sympatric, chain-forming, pennate diatom *Syndroposis* sp. The specific hypotheses are: (i) Antarctic ascidians will employ organic chemical defenses against marine bacteria, (ii) Antarctic ascidians will employ

organic chemical defenses against fouling diatoms and (iii) chemical defenses against diatom fouling will be more common in colonial ascidians than in solitary ascidians.

In Chapter 3, the palatability of two solitary and three colonial species of ascidians commonly found in sub-tropical seagrass meadows are evaluated using the abundant, sympatric, omnivorous pinfish *Lagodon rhomboides* as a model predator. For any unpalatable species, the lipophilic and hydrophilic extracts are tested against the fish in order to determine whether unpalatability, if present, is chemically mediated. Furthermore, the toughness of the tunic of all five ascidian species is evaluated by measuring the Force (N) required to penetrate the tunic using a penetrometer. The specific hypothesis is: Ascidians associated with seagrass habitat will be defended, either chemically or physically, against the most common generalist predator.

PALATABILITY AND CHEMICAL ANTI-PREDATORY DEFENSES IN COMMON ASCIDIANS FROM THE ANTARCTIC PENINSULA

Gil Koplovitz[1*], James B. McClintock[1], Charles D. Amsler[1] and Bill J. Baker[2]

[1]Department of Biology, University of Alabama at Birmingham, Alabama 35294 USA

[2]Department of Chemistry, University of South Florida, Tampa, 33620, USA

Aquatic Biology 7:81-92

Format adapted and errata corrected for dissertation

ABSTRACT: Palatability of outer tissues of a suite (12 species) of Antarctic ascidians was evaluated using omnivorous fish and sea star predators. Tissues of 100% of those tested were unpalatable to fish, while 58% were unpalatable to sea stars. Lipophilic and hydrophilic extracts of 11 species were incorporated into pellets and tested in fish and sea star bioassays. Only the lipophilic extract from *Distaplia colligans* caused fish feeding deterrence. Organic extracts from 10 ascidian species were also examined in food pellet assays using an omnivorous amphipod. Only the lipophilic extract of *D. cylindrica* was a deterrent. Five of the ascidians possessed acidified outer tunics (pH < 3). We tested the ability of acidified krill pellets (pH 2 to 7) to deter fish and sea star predators and found that, while fish readily ingested acidified food pellets (pH 2), sea stars were deterred at pH 5 or less. Thus either organic or inorganic chemical defenses explain defense in 5 of the 7 ascidian species found unpalatable to sea stars. In contrast, chemical defenses only explain 1 of 12 species found unpalatable to fish, and only 1 of 10 ascidians tested against an amphipod predator. This predator-specific pattern of chemical defense may reflect greater predation pressure on ascidians from Antarctic sea stars. Alternatively, Antarctic ascidians may rely on other factors such as the toughness of their tunic or sequestration of heavy metals such as vanadium to inhibit feeding by Antarctic fish, a taxonomic group known to lack strong jaws.

INTRODUCTION

Ascidians as a group represent an important component of benthic marine communities (Tatian et al. 1998, Monteiro et al. 2002). As suspension feeders, they have a significant influence on bentho-pelagic interactions including impacts on primary production and concentrations of suspended particles (Petersen & Riisgård 1992, Kowalke 1999). As sessile, soft-bodied organisms, ascidians are vulnerable to generalist predators including fish (Randall 1967), sea urchins (Briscoe & Sebens 1988) and sea stars (Mauzey et al. 1968, McClintock et al. 2004) and specialist predators including select species of gastropods, including lamellarians and nudibranchs (Cimino & Ghiselin 2001) as well as several flatworms (de Caralt et al. 2002). Intense predation pressure on sessile marine invertebrates including ascidians has contributed to selection for a suite of defenses. For example, some marine invertebrates have developed mechanical means of deterring predators and fouling organisms. In sponges, physical defenses may include spicules (Chanas & Pawlik 1995, 1996, Burns & Ilan 2003) or sloughing off of surface layer tissues or mucus that is secreted on outer surfaces (Barthel & Wolfrath 1989, Steinberg et al. 1997). Some colonial, and especially solitary, ascidians possess a tough outer tunic comprised of the proteinaceous polysaccharide "tunicin". In some of these species, the tunic is further impregnated with minute spicules that may serve to deter some predators (Lambert & Lambert 1987, López-Legentil et al. 2006). Nonetheless, the primary provision for protection from predators in ascidians appears to be based on chemical defenses (Stoecker 1978, 1980, Davis 1991, McClintock et al. 1991, Lindquist et al. 1992, Pawlik 1993, Pisut & Pawlik 2002, McClintock et al. 2004).

While both organic and inorganic chemicals may play a role in ascidian chemical defenses, many appear to be secondary metabolites. A wide variety of natural products have now been isolated from ascidians, primarily from tropical and temperate latitudes (Pawlik 1993, Faulkner 2002, Paul & Puglisi 2004, Paul et al. 2006, Paul & Ritson-Williams 2008). Only a small subset of ascidians with known secondary metabolites has been subjected to ecological studies. For example, Young & Bingham (1987) tested the palatability of the larvae of the tropical colonial ascidian *Ecteinascidia turbinata* and demonstrated that larvae contained secondary metabolites that were unpalatable to the planktivorous pinfish *Lagodon rhomboides*. Lindquist & Fenical (1991) described an alkaloid in the class tambjamine, (tambjamine C) in both the larvae and adults of the tropical ascidian *Sigillina signifera* that caused feeding deterrence in a suite of sympatric coral reef fish. Alkaloids (eudistomins) were also isolated from the tunic of the tropical colonial ascidian *Eudistoma olivaceum* (Davis & Wright 1989); these alkaloids did not deter fish feeding, but were effective as antifoulants (Davis 1991). Larvae of the Caribbean ascidian *Trididemnum solidum* contain a variety of secondary metabolites which deter feeding in the pinfish *L. rhomboides*, as well as in the sea urchin *Arbacia punctulata* and the sea anemone *Aiptasia pallida*. The ascidian *Sigillina* cf. *signifera* was found to contain an alkaloid (tambjamine E) that deters feeding in 6 coral reef fishes (Lindquist et al. 1992). In a broad survey that encompassed 17 solitary and colonial ascidians from the Western Atlantic, 16 species were found to harbor secondary metabolites that were deterrent against the bluehead wrasse *Thalassoma bifasciatum* (Pisut & Pawlik 2002). Most recently, López-Legentil et al. (2006) found the ascidian *Cystodytes* sp. from the Mediterranean and South Pacific contained an alkaloid

(ascididemin) that deterred feeding in a suite of generalist fish predators but not in a sea urchin.

In addition to organic secondary metabolites, inorganic acids have also been shown to provide chemical defenses in ascidians (Parry 1984, Davis & Wright 1989, Pawlik 1993, Pisut & Pawlik 2002). A number of species sequester sulfuric acid on their tunic surfaces or in special acid-filled surface bladder cells. These acids have been implicated both in the provision of antifoulant activity (Davis & Wright 1989) as well as serving as antifeedants (Pisut & Pawlik 2002). While there has been some suggestion that acids would be rapidly neutralized in seawater (Parry 1984), recent studies have demonstrated that their deterrent properties are retained for a period of time sufficient to deter ecologically relevant predators (Pisut & Pawlik 2002, McClintock et al. 2004).

Perhaps even more so than in temperate and tropical marine environments, ascidians are ecologically dominant members of Antarctic benthic communities (Ramos-Espla et al. 2005). At shallow depths (<30 m), the Antarctic benthos can be greatly influenced by anchor ice and ice scour that produce distinct zonation patterns in community structure (Dayton et al. 1969, 1974, Sahade et al. 1998). Ascidians appear to be able to withstand such physical disturbances better than other sessile marine invertebrates. Moreover, their comparatively high growth rates allow them to effectively colonize recently disturbed habitats (Kowalke et al. 2001). In less physically disturbed areas, ascidians are able to numerically dominate in areas where competitive interactions are the determining factor in shaping the benthic community (Sahade et al. 1998). In shallow benthic communities of the Antarctic Peninsula, ascidians are often the dominant

group of sessile marine invertebrates in terms of biomass, especially on soft sediments below depths of 15 m (Sahade et al. 1998, Tatian et al. 1998). In a study of the ascidian communities in the vicinity of Potter Cove, King George Island, along the western Antarctic Peninsula, Tatian et al. (1998) described 17 species of ascidians, mainly from the genera *Pyura, Molgula* and *Sycozoa*. The majority of the species (11) were solitary while the remaining 6 were colonial. Also noted was that 7 of the solitary species were fouled, while all the colonial species were free of epibionts (Tatian et al. 1998). In a comprehensive comparative study, Ramos-Espla et al. (2005) found 172 species of ascidians occurring at depths ranging between 10 to 600 m along the Antarctic Peninsula, the Scotia Arc, and the Magellan region. Likely predators of Antarctic ascidians include sea stars, fish and meso-crustaceans. Recent studies have indicated that the diverse amphipod communities are important grazers in the nearshore communities of the Antarctic Peninsula (Huang et al. 2007), and may include not only macroalgae but also sessile benthic invertebrates such as sponges and ascidians in their diets (Aumack 2010).

Compared to tropical and temperate ascidians, almost nothing is known about the chemical ecology of polar ascidians (Avila et al. 2008). To date, only 2 Antarctic species have been investigated, the solitary form *Cnemidocarpa verrucosa* (McClintock et al. 1991) and the colonial form *Distaplia cylindrica* (McClintock et al. 2004). Both were found to be unpalatable to various predators and chemically defended. Thus, in the present study our objectives were to evaluate the palatability of a representative suite of Antarctic ascidians to 2 common sympatric omnivorous predators, the fish *Notothenia coriiceps* and the sea star *Odontaster validus.* For those species that proved unpalatable to either predator, we evaluated whether the lack of palability could be attributed to either

organic or inorganic chemicals. We also examined the feeding deterrent properties of organic extracts of outer tissues of this suite of ascidians against the common omnivorous amphipod *Gondogeneia antarctica*.

MATERIALS AND METHODS

Field collections. Ascidians were collected by hand using SCUBA from depths ranging from 2 to 39 m from various locations within a 3.5 km radius of the US Palmer Station, Anvers Island, Western Antarctic Peninsula (64° 46.5' S, 64° 03.3'W). We did not select particular species of ascidians for inclusion in the present study, but rather examined all species we encountered, thus ensuring an unbiased sample of representative species. Collections were made February to June 2007 and March to June 2008. Freshly collected ascidians were subject to volume (by seawater displacement in a graduated cylinder) and wet weight determinations. Those individuals not used for fresh tissue palatability assays or for inorganic acid measurements were immediately frozen at -80 °C for later extraction of secondary metabolites (see below). Individuals of the sea star *Odontaster validus* were collected by hand using SCUBA from the same locations as the ascidians between February to May 2008. The fish *Notothenia coriiceps* were collected using hook and line, fish traps, and occasionally by hand using SCUBA from within 1 km of Palmer Station. Individuals of the amphipod *Gondogeneia antarctica* were collected using SCUBA by first collecting individuals of the alga *Desmarestia menziesii* using fine mesh bags (Huang et al. 2007). When returned to the laboratory, the algae were repeatedly submerged in seawater in buckets, then the bucket contents sieved, and individuals of *G.*

antarctica sorted and held alive in 2 l plastic bottles equipped with holes fitted with fine wire mesh to allow seawater exchange when floated in a flow-through seawater tank.

Inorganic acid (pH) measurements of outer tunic. The presence of inorganic acid (sulfuric acid; Webb 1939, Levine 1961, Stoecker 1980) on the outer tunic surfaces of the targeted suite of solitary and colonial ascidians was determined using analytical pH strips (EM colorpHast). Tunic surface pH measurements were conducted using first a pH strip with a range of 0 to 14 and a resolution of 1 pH unit. Once an initial pH was determined, a more narrow resolution of pH was determined by using the appropriate pH strip for this range of pH values, with more highly refined increments of 0.2 to 0.3 pH units. Because the test was subjective due to pH being determined by matching strip color to a color chart, we chose to be conservative by rounding the pH measurement to the nearest 0.5 unit. All pH strip measurements were made by removing ascidians from seawater and then placing the pH strip against the "dry" outer tunic for a period of 5 min.

Preparation of ascidian organic extracts for feeding bioassays. Organic extracts of ascidians were prepared using whole colonies of colonial ascidians and whole individuals of solitary forms. The general extraction process follows techniques described in McClintock et al. (2004). Several colonies or individuals were weighed, lyophilized and then re-weighed. The freeze-dried tissues were then extracted thrice in dichloromethane/methanol (1:1 ratio) for 24 h. Extracts were combined and filtered through a coarse filter paper and dried down using rotary evaporation to yield a lipophilic extract. A hydrophilic extract was prepared by subsequent extraction of the same freeze-

dried tissue using methanol/water (1:1 ratio) thrice for 24 h. Both lipophilic and hydrophilic extracts were weighed following drying. The natural concentrations of extracts for bioassays were calculated on a volumetric basis as mass of extract per unit volume.

Sea star bioassays: ascidian fresh tissue, organic extracts and inorganic acids. Sea star feeding bioassays were performed using protocols developed by McClintock & Baker (1997b). The sea star *Odontaster validus* was selected because it is an abundant, omnivorous predator (Dayton et al. 1974, McClintock et al. 1988, 2004, McClintock 1994). Moreover, it is frequently seen preying on sessile benthic macroinvertebrates in the vicinity of Palmer Station (Authors' pers. obs.). Sea stars were held in a large, circular, flow-through seawater tank (1.8 m diameter x 0.9 m height) equipped with ambient seawater (1°C). When held in a tank, *O. validus* tend to climb vertically and position themselves at the air-water interface with several of their arms stretched out such that their ambulacral grooves are exposed upwards (McClintock et al. 2004).

Small pieces (approximately 0.5 cm^3) of the outer tissues of ascidians were prepared from freshly collected individuals. During the feeding trials described below, the small pieces of tissue were presented to sea stars such that the outer surface of the tunic was facing the outstretched tube feet within the ambulacral groove of an upward-facing arm.

Artificial food pellets were prepared in a matrix of 2% alginate containing 2% dry weight krill powder as a feeding stimulant (McClintock & Baker 1997a). Extracts were

dissolved in the minimum amount of the solvent used in their initial extraction (see above) and dried onto the krill powder using a rotary evaporator (Hay et al. 1994). Control pellets were prepared using the same amount of solvent dried onto krill powder. A second set of control pellets was prepared with alginate pellets that contained only krill powder but no solvent. Both experimental and control treatment krill powders were placed into 10 cm diameter Petri dishes. Alginate solution was then added to the dishes and thoroughly mixed. A cold solution of 1M $CaCl_2$ was subsequently poured over the mixture causing immediate gelling. Triangular alginate pellets (0.3 cm per side) were then prepared using a single-edged razor blade.

During the sea star feeding trials, 12 to16 individuals (3 to 6 cm radius) were presented with either a piece of fresh ascidian tissue or a food pellet containing the hydrophilic or lipophilic ascidian extract from a species that was rejected in fresh tissue assays. In the case of fresh tissues, each sea star was subsequently presented a non-solvent control food pellet. In the case of the food pellets containing extracts, first a series of feeding assays was performed whereby sea stars were offered a pellet containing extract followed by a non-solvent control food pellet as a satiation control. A second set of sea stars were offered solvent control food pellets followed by a non-solvent satiation control food pellet. In all of the above, the food item was placed in the ambulacral groove in the middle of the arm, equidistant between the arm tip and the mouth. Acceptance (movement of the fresh tissue or pellet towards the mouth and extrusion of the cardiac stomach) or rejection (movement of the fresh tissue or pellet away from the mouth, or displacement from the ambulacral groove) was recorded over a 20 min period. Differences between acceptance of tissue or pellets and the corresponding controls were

determined using a Fisher's exact test (Sokal & Rohlf 1994). For fresh tissue pieces, the controls used in statistical comparisons were the non-solvent food pellets. For pellets containing ascidian extracts, the controls were the corresponding solvent-treated control pellets that were offered to a different set of sea stars (Amsler et al. 2005).

The ability of inorganic acids to deter feeding in the sea star *Odontaster validus* was examined using agar pellets as alginate does not gel under acidic conditions. Agar food pellets were prepared by mixing 1% agar in seawater and 2% dry krill powder as a feeding stimulant. The pellets were then acidified by adding drops of 1.0 N H_2SO_4 to the liquid agar containing the krill powder until the pH was reduced to the desired level. The solution was then poured into 10 cm diameter Petri dishes. Triangular alginate pellets (0.2 cm per side) were then cut using a single-edged razor blade. Acidified agar pellets were prepared at pH values of 2, 3, 4, 5 and 6 (Pisut & Pawlik 2002, McClintock et al. 2004). The pH of the acidified pellets was tested using pH strips as mentioned above. Control pellets consisted of agar pellets prepared at a neutral pH of 7. In order to ensure that pellets retained their acidity over the time-course of the sea star and fish bioassays (see below), 5 pellets acidified to each test pH were submerged in seawater at the temperature used in feeding experiments for a period of 1 min and then their pH levels measured using pH strips. All pellets were found to retain their initial pH level after this time period.

Feeding assays were conducted beginning at the lowest pH, and followed the feeding procedure described above. Briefly, each sea star was offered a control agar food pellet. If accepted, the sea star was then subsequently offered an acidified experimental pellet and acceptance or rejection recorded. Differences between acceptance of control

and treated pellets were analyzed using a Fisher's exact test. In this assay, and in the fresh tissue and organic extract feeding assays, no individual sea star was used more than once in a given treatment.

Fish bioassays: ascidian fresh tissue, organic extracts and inorganic acids. The Antarctic fish *Notothenia coriiceps* was used in ascidian feeding assays. This fish is an abundant, omnivorous predator that occurs along the Antarctic Peninsula (Blankley 1982, Barrera-Oro & Casaux 1990, Casaux et al. 1990, Iken et al. 1997). Individual fish (length approximately 20 to 30 cm) were held in either a sea-water table (1 x 2 x 0.25 m depth) and divided into three equal sized compartments using fine-mesh dividers, or in individual tanks (0.6 x 0.6 x 0.6 m). Both the water table and tanks were equipped with flowing ambient sea water at 1°C. Initially, fish were maintained on a diet of limpet tissue (whole tissues of 2 *Nacella concinna* with a shell length ~3 to 5 cm per fish per day) proffered to them by hand on the tip of 20 - cm forceps. Fish learned within 5 to 7 d to associate the approaching forceps with food, and were then switched to a diet comprised of alginate pellets that contained 3.25% alginate and 5% dried krill powder for a period of 3 d (see pellet preparation techniques above). In all fish feeding trials described below, this level of alginate and krill powder was used to prepare pellets following techniques described above.

In fish bioassays, 9 to14 individuals were offered either a piece of fresh tissue (1 cm^3), or a circular alginate food pellet (2 cm diameter x 2 mm depth) containing either a hydrophilic or lipophilic ascidian extract from a species that was rejected in fresh tissue assays. After being presented with the fresh tissue or alginate food pellet, each fish was

subsequently presented with a control alginate food pellet containing krill powder but no extract. Individual fish were used in no more than one feeding trial for each of the 3 treatments (fresh tissue, hydrophilic extract, lipophilic extract), with at least 6 h allowed between a given feeding trial. An acceptance response was recorded when a fish swallowed the tissue or pellet with no subsequent regurgitation. A rejection response was recorded when a fish mouthed the tissue or pellet and subsequently immediately spat it out. Controls for fresh ascidian tissues consisted of non-solvent alginate food pellets. Similar to sea star feeding assays, controls for the ascidian extract food pellets were the appropriate solvent-treated food pellet. Differences between consumption of fresh ascidian tissues or pellets with ascidian extracts and their corresponding controls were analyzed using Fisher's exact test.

The ability of inorganic acids to deter fish feeding was investigated using similar agar pellet preparation techniques as given for sea stars above, with the exception of preparing pellets at a concentration of 5% dry weight krill powder. However, upon the completion of feeding trials using the lowest pH alginate food pellets tested (pH 2), it became evident that fish were not deterred from consuming pellets at this very low pH. Thus testing for feeding deterrent properties of pellets food acidified at higher pH values was not conducted.

Amphipod bioassays. Artificial food pellets containing hydrophilic and lipophilic organic extracts of 10 of the ascidian species were bioassayed with the common omnivorous amphipod *Gondogeneia antarctica* (Huang et al. 2006, 2007). Measurements of rates of amphipod consumption of fresh tissue were not feasible due to

the lengthy time required for measurable consumption. Similarly, measurements of the consumption of acidified agar food pellets by amphipods exceeded the period of time these pellets retained their acidity.

Amphipod feeding assays using alginate pellets were conducted using a 2-choice feeding assay model (Peterson & Renaud 1989, Amsler et al. 2005). In each assay, an alginate pellet (disc-shaped, 1 cm diameter x 2 mm thick) prepared with 2% alginate and containing a 2% dried krill powder and either a hydrophilic or lipophilic ascidian extract was paired with a control alginate pellet containing solvent-only treated krill powder. These were placed together into a sealed 250 ml bottle and floated in flow-through seawater tanks (1°C). Twenty haphazardly selected adult individuals of the amphipod *G. antarctica* were placed into 10 bottles, while a second set of 10 bottles contained pellets but no amphipods, to serve as an autogenic control for changes in the mass of the pellets (e.g. due to water gain). The feeding experiments ran for a period of 48 to 96 h, until a noticeable change in the mass of the alginate food pellets was observed. Fresh seawater was replaced in each bottle every 24 h. Pellets were then removed from each bottle, blotted gently with a tissue to remove excess seawater and weighed. Feeding deterrence was evaluated by calculating the differences in wet weight pre-and post-experiment of the controls and treatment pellets corrected by the autogenic controls and treatments using a Wilcoxon signed-ranked test.

RESULTS

Outer tunic acidity

pH values for the outer surfaces of the tunic of 6 solitary and 8 colonial species of ascidians ranged from 1.5 to 8.0 (Table 1). High levels of acidity (defined as pH < 4) detected on the outer body surface were more common in colonial ascidians (4 of 8 species) than in solitary ascidians (1 of 6 species).

Fresh ascidian tissue bioassays with sea stars and fish

Twelve of the 14 species of ascidians were assayed in fresh tissue bioassays with sea stars and fish (an insufficient amount of *Aplidium* sp. limited this species to tests of organic extracts only). Seven of these twelve species (64%) were significantly unpalatable ($P < 0.01$) to the sea star *Odontaster validus*, of which 5 species (71%) had colonial organization and 2 species (29%) had solitary organization (Fig. 1). All fresh body tissues from the 12 species of ascidians were significantly ($P < 0.01$) unpalatable to the fish *Notothenia coriiceps* (Fig. 1).

Organic extract bioassays with sea stars, fish and amphipods

Natural concentrations for hydrophilic and lipophilic organic extracts of the 14 species of ascidians ranged from 6.3 to 70.3 and 10.7 to 42.5 mg DW extract per ml wet ascidian tissue, respectively (Table 2). Extracts of the ascidian *Sycozoa gaimardi* were not tested as there was insufficient biomass available for extraction. Of the 7 species that were unpalatable to the sea star *Odontaster validus*, and thus tested as extracts, the

lipophilic extract of the colonial *Distaplia colligans* was a significant deterrent ($P < 0.0001$) (Fig. 2). Similarly, among the 12 species of ascidians that proved to be unpalatable to the fish *Notothenia coriiceps,* the lipophilic extract of *D. colligans* was a significant deterrent ($P = 0.0062$) (Fig. 3).

When hydrophilic and lipophilic extracts of 10 ascidian species were assayed against the amphipod *Gondogeneia antarctica,* only the lipophilic extract of the colonial *Distaplia cylindrica* was a significant deterrent ($P = 0.047$) (Fig. 4). In 8 of the 10 ascidian species, pellets containing extracts were consumed in significantly ($P \leq 0.05$) greater quantities than control pellets (Fig. 4).

Inorganic acids - feeding bioassays

Agar food pellets acidified to pH 2, 3, 4 and 5 were significant deterrents ($P <0.05$) to the sea star *Odontaster validus* in feeding assays (Fig. 5). In contrast, there was no significant deterrence detected when sea stars were presented agar food pellets acidified to pH 6 or neutral pH 7. Acidified pellets (pH 2 to 5) invoked an immediate behavioral response in sea stars (< 1 min) consisting of rapid retraction of the tube feet making contact with the pellet, subsequent removal of the pellet from the ambulacral groove and pellet dropping, and then movement of the entire individual away from their resting position at the air-water interface of the tank.

Agar food pellets acidified to pH 2 were all readily consumed by the fish *Notothenia coriiceps.* As there was no deterrence, feeding assays with pellets acidified to higher pH values were not necessary.

DISCUSSION

The 3 contrasting predators examined in the present study (sea stars, fish, and amphipods) each elicited different patterns of feeding deterrent responses to Antarctic ascidians. *Odontaster validus* is a common omnivorous sea star predator that feeds on a highly diverse assemblage of benthic marine invertebrates (McClintock 1994). Sea stars rejected 7 of the 12 ascidian species that were presented to them as fresh tissue, accepting tissues of the other 5 species. Of the 7 species that lacked palatability, 5 were colonial and 2 had solitary organization. Rejection of 4 of the 7 species that proved unpalatable as fresh tissue was attributable to a chemical defense. The colonial ascidian *Distaplia colligans* was protected from sea star predation by secondary metabolites in its lipophilic extract as well as by inorganic acids sequestered on the outer surface of its tunic. An additional 3 ascidian species (*Distaplia cylindrica, Sycozoa gaimardi* and *Corella eumyota*) had surface acidity at levels unpalatable to *O. validus* via inorganic acids (sulfuric acid) sequestered on the outer surfaces of their tunics. In the present study *O. validus* was deterred from consuming alginate food pellets when pellets had a pH of 5 or less. *Tridemnum* sp. was only tested for acidity and was not used in the bioassays, however it is presumed that its surface pH of 2.5 will also deter predation by *O. validus*. McClintock et al. (2004) similarly examined the response of the sea star *Odontaster validus* to acidified food pellets and demonstrated feeding deterrence at natural pH levels that occur on the outer tunic of *Distaplia cylindrica.* As *O. validus* feeds by extruding its

cardiac stomach and laying it against the outer surface of large sessile prey (e.g. sponges, McClintock 1994), inorganic acids that are sequestered on the outer tunic surface may be particularly effective against the thin, exposed, sensitive tissues of the cardiac stomach (Dayton et al. 1974, McClintock 1994). Moreover, sea star tube-feet are highly chemosensory, allowing them to perceive and evaluate potential prey and their defensive attributes (Sloan 1980a, b, Sloan & Campbell 1982, McClintock et al. 1984, 1994)). When presented acidified krill pellets, the sensory tube feet retract along the ambulacral groove, indicative of a strong deterrent response (McClintock et al. 2000).

It is intriguing that 80% (4 of 5 species) of the Antarctic ascidians that may employ inorganic chemical defenses against sea star predation were colonial rather than solitary forms. Moreover, qualitative observations indicated that solitary Antarctic ascidians appeared to have tougher, more protective, outer tunics than colonial forms, and thus colonial forms may be under greater selective pressure to employ chemical defenses. Similarly, Pisut and Pawlik (2002) examined 17 species of common ascidians in the Caribbean and found that the majority (6 of 9 species) that employed sulfuric acid as a chemical defense against fish were colonial, rather than solitary, forms. McClintock et al (2004) found that inorganic acid feeding deterrent properties of *Distaplia cylindrica* were complemented by lipophilic secondary metabolite(s) against the sea star *O. validus.* Similar bioactivity was not detected against *Odontaster validus* in the lipophilic extract of this ascidian in the present study. This suggests that *D. cylindrica* may have the capacity to display temporal or local geographic variation in the production of secondary metabolite defenses, as seen in soft corals (Harvell et al. 1993).

The common benthic fish *Notothenia coriiceps* exhibited strong and consistent rejection of fresh tissues of all 12 species of ascidians. This lack of palatability, however, was in large part not chemically mediated, as alginate pellets containing tissue level concentrations of organic extracts were, with the exception of the lipophilic extract of the colonial ascidian *Distaplia colligans*, readily consumed by the fish. Moreover, in contrast to sea stars, acidified agar krill pellets were readily consumed by fish, even at a highly acidic pH of 2, with no apparent ill effects post-consumption.

The diet of *Notothenia coriiceps* includes a wide variety of foods including macroalgae, as well as gastropods, amphipods, polychaetes and krill (Barrera-Oro & Casaux 1990, Iken et al. 1997, Iken et al. 1999). Although not a common food item, this fish species has been known to occasionally include ascidians in its diet (Barrera-Oro & Casaux 1990). In the present study, the general lack of palatability of pieces of outer fresh tissues of ascidians for this fish might be attributed to several factors. Kühne (1997) and Kowalke et al (2001) suggest that low nutritional value of some solitary Antarctic ascidians may contribute to their unattractiveness as prey. However, it can be argued that Antarctic ascidians, both solitary and colonial, possess a nutritional value that does not differ significantly from other benthic sessile prey (e.g. sponges) readily consumed by predators such as sea stars (McClintock et al. 1991, 2004). Certainly macroalgae, one of the common food items of *N. coriiceps*, are likely to possess a lower nutritional value than ascidian prey. The solitary Antarctic ascidian, *Cnemidocarpa verrucosa*, whose tissues proved to be unpalatable to *N. coriiceps* in the present study, and to 2 additional Antarctic fish, *Patagothenia borchgrevinki* and *Trematomus bernacchii,* in a previous study (McClintock et al. 1991), has outer tissues (tunic) that

possesses a relatively high energy content (McClintock et al. 1991). That fresh outer tissues of *C. verrucosa* were accepted as prey by the extraoral feeding seastar *Odontaster validus*, suggests that the lack of palatability of the outer tissue of this ascidian to fish, a biting predator, is likely related to the toughness of the tunic.

The amphipod *Gondogeneia antarctica* is an omnivorous mesograzer that includes both plant and animal foods in its diet (approximately 30% of its diet is comprised of invertebrate prey, C. Aumack, unpubl. data). This amphipod occurs in extremely high densities (estimated densities of up to 1660 ind. m^{-2} benthos, based on calculations using estimates from Huang et al. 2007 and Amsler et al. 2008) along the central western Antarctic Peninsula. This amphipod has been used as a model mesograzer in studies of the chemical feeding deterrent properties of Antarctic macroalgae (Amsler et al. 2005). The preference of this amphipod for pellets containing organic extracts of marine algae or sessile invertebrates is a phenomenon commonly observed in this species (Amsler et al. 2005, Amsler et al. 2009), and attributed to extracts being phagostimulatory.

Despite the methodological limitations that prevented us from testing fresh tissues or acidified food pellets against this common mesograzer, it is noteworthy that, similar to our findings for sea stars and fish, it is the lipophilic extract of the ascidian genus *Distaplia* that is the feeding deterrent to amphipods. This not only reflects broad spectrum of secondary metabolites in this genus in Antarctica, but indicates that secondary metabolite defenses are relatively rare among Antarctic ascidians. In contrast, the literature on tropical and temperate ascidians suggests secondary metabolite feeding

deterrents are more common (Young & Bingham 1987, Lindquist & Fenical 1991, Lindquist et al. 1992, Pisut & Pawlik 2002, López-Legentil et al. 2006).

The present study provides additional information relevant to the long standing debate about the role of inorganic acids in the provision of defenses against predation in ascidians (Parry 1984, Davis & Wright 1989, Pawlik 1993, Pisut & Pawlik 2002). Stoecker (1978, 1980) concluded that sulfuric acid in the tunics of Aplousobranch and Phlebobranch ascidians deterred potential predators and inhibited fouling. Hirose (1999, 2001) examined the tunics of several solitary and colonial ascidians and reported that upon injury, acid-filled bladder cells burst, releasing their contents. He proposed this facilitated predator deterrence as well as disinfecting the injured area. Nonetheless, Parry (1984) argued that inorganic acids in ascidians cannot be anti-predatory, as acids would be rapidly neutralized by the calcareous spicules in the tunic or, in species lacking spicules, by neutralization in seawater. In contrast, several subsequent investigations have demonstrated an anti-predatory role for inorganic acids in ascidians. Pisut & Pawlik (2002) demonstrated that acids at levels detected on the surfaces of ascidians were effective in deterring the generalist predatory fish *Thalassoma bifasciatum*. Moreover, McClintock et al. (2004) and the present study demonstrate that acids at levels found in outer tissues of some Antarctic ascidians can be effective deterrents against sea star predators. Contrary to Parry's (1984) claims that sulfuric acid is quickly neutralized in sea water, sulfuric acid-treated food pellets were not neutralized by seawater and retain their deterrent properties over the time course of sea star (McClintock et al. 2004) and fish feeding assays (Pisut & Pawlik 2002, McClintock et al. 2004). More recently, Odate & Pawlik (2007) proposed that the effects of low pH on the oxidation state of vanadium,

a heavy metal common to ascidians, might increase the effectiveness of this metal as a feeding deterrent.

Over the past 2 decades it has become increasingly apparent that chemical defenses in Antarctic benthic macroalgae and sessile and sluggish marine invertebrates are not uncommon (reviewed in McClintock & Baker 1997b, Amsler et al. 2001a, 2001b, McClintock et al. 2005, Amsler et al. 2008). Amsler et al. (2005) conducted an exhaustive survey of the palatability and chemical defenses of common Antarctic macroalgae and found a high incidence of chemical defenses against sea star, fish and amphipod predators. More recently, we conducted a similar survey of palatability and chemical defenses in common Antarctic peninsular sponges and found a high incidence of chemical defenses effective against both sea stars and fish predators (Peters et al. 2009). The present study demonstrates that chemical defenses against sea stars and fish that are attributable to secondary metabolites are not as prevalent in Antarctic solitary and colonial ascidians as they are in Antarctic macroalgae and sponges (Amsler et al. 2005, Peters et al. 2009). Nonetheless, inorganic chemical defenses (acids) are not uncommon in Antarctic ascidians, and while ineffective against fish may deter sea star predation.

Acknowledgements. We thank M. Amsler, C. Aumack, and J. Zamzow for their assistance with field collections. We also thank M. Amsler and J. Zamzow for their assistance with running the fish bioassays. We are very grateful to L. Cole at the Smithsonian Institution in Washington DC for her assistance with ascidian taxonomy. We wish to acknowledge the generous logistical support provided by those individuals employed by Raytheon Polar Services Company. This research was facilitated by NSF

awards to CDA and JBM (OPP-0442769) and to BJB (OPP-0442857). JBM acknowledges the support of an Endowed Research Professorship in Polar and Marine Biology through the University of Alabama at Birmingham.

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Table 1. Antarctic ascidians examined including their taxonomic classification, body organization, surface pH of the tunic and the specific feeding deterrent bioassays (fresh tissue or organic extracts) performed against sea stars (S), fish (F) or amphipods (A). +/–: bioassay performed/not performed

					Bioassays Conducted					
					FT			OE		
Species	Family	Order	Organization	Surface pH	F	S	A	F	S	A
* *Trididemnum sp.*	Didemnidae	Aplousobranchia	Colonial	2.5	-	-	-	-	-	-
Didemnum biglans	Didemnidae	Aplousobranchia	Colonial	7	+	+	-	+	+	+
Distaplia cylindrica	Holozoidae	Aplousobranchia	Colonial	2	+	+	-	+	+	+
Distaplia colligans	Holozoidae	Aplousobranchia	Colonial	1.5	+	+	-	+	+	+
Sycozoa gaimardi	Holozoidae	Aplousobranchia	Colonial	1.5-2.0	+	+	-	-	-	-
Aplidium sp.	Polyclinidae	Aplousobranchia	Colonial	7	-	-	-	+	+	+
Synoicum adareanum	Polyclinidae	Aplousobranchia	Colonial	7.5	+	+	-	+	+	+
Synoicum sp.	Polyclinidae	Aplousobranchia	Colonial	7	+	+	-	+	+	+
Ascidia sp.	Ascidiidae	Phlebobranchia	Solitary	7	+	+	-	+	+	+
Corella eumyota	Corellidae	Phlebobranchia	Solitary	2	+	+	-	+	+	+
Microcosmus sp.	Pyuridae	Stolidobranchia	Solitary	7	+	+	-	+	+	+
Pyura georgiana	Pyuridae	Stolidobranchia	Solitary	7	+	+	-	+	+	+
Pyura setosa	Pyuridae	Stolidobranchia	Solitary	7	+	+	-	+	+	+
Cnemidocarpa verrucosa	Styelidae	Stolidobranchia	Solitary	8	+	+	-	+	+	+

+ = bioassay performed; - = no bioassay performed

FT=Fresh Tissue; OE=Organic Extracts; F=Fish; S=Sea stars; A=Amphipods

* *Trididemnum sp.* was not collected in large enough quantities for bioassays and was only tested for pH.

Table 2. Volumetric extract yields (the natural concentration of an extract) expressed as mg dry extract per ml wet ascidian tissue. WW: wet weight; DW: dry weight; Vol: volume; n/a: not available

Species	Volumetric extract yields		Ratios	
	Lipophilic	Hydrophilic	ww:dw	ww:vol
Tridemnum sp.	20.4	39.8	8.72	1.72
Didemnum biglans	19.9	28.2	11.36	1.02
Distaplia cylindrica	18.3	18.5	23.09	1.04
Distaplia colligans	42.5	70.3	N/A	1.11
Sycozoa gaimardi	26.2	6.3	17.63	1.17
Aplidium sp.	14.6	25.4	9.92	1.02
Synoicum adareanum	10.7	24.2	16.86	1.03
Synoicum sp.	12.5	21.9	12.74	1.00
Ascidia sp.	11.2	24.7	13.37	1.04
Corella eumyota	26.0	8.0	20.35	1.02
Microcosmus sp.	11.2	19.3	14.17	0.98
Pyura georgiana	23.2	19.3	10.17	1.04
Pyura setosa	14.8	19.3	7.13	0.95
Cnemidocarpa verrucosa	14.5	20.5	16.30	0.98

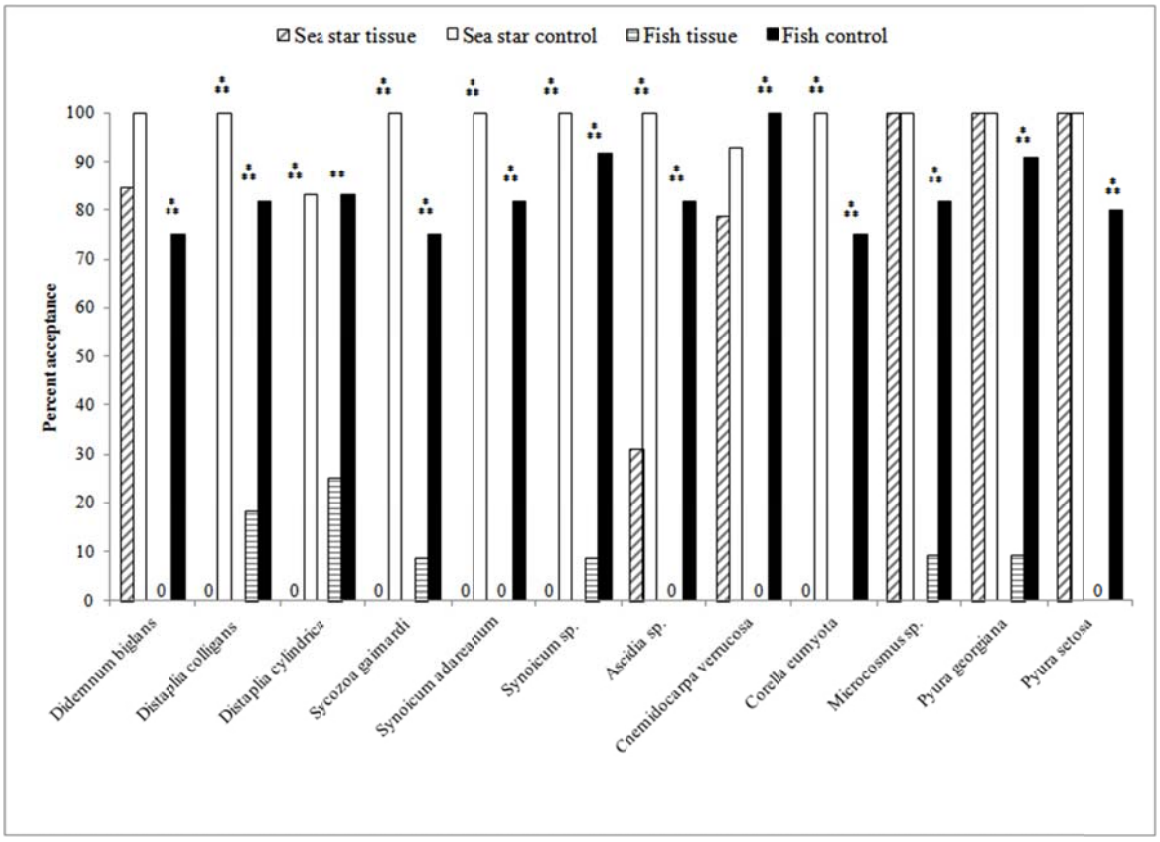

Fig. 1. *Odontaster validus* and *Notothenia coriiceps*. Results of bioassays offering pieces of ascidian tunic tissue to the sea star *O. validus* and the fish *N. coriiceps*. Asterisks indicate significant difference between tissue and control (Fisher's extact test); **: $P \leq 0.01$; ***: $P \leq 0.005$, Ascidians are listed in alphabetic order from left to right with the first group representing colonial and the group solitary species (same pattern shown in Figs 2-4).

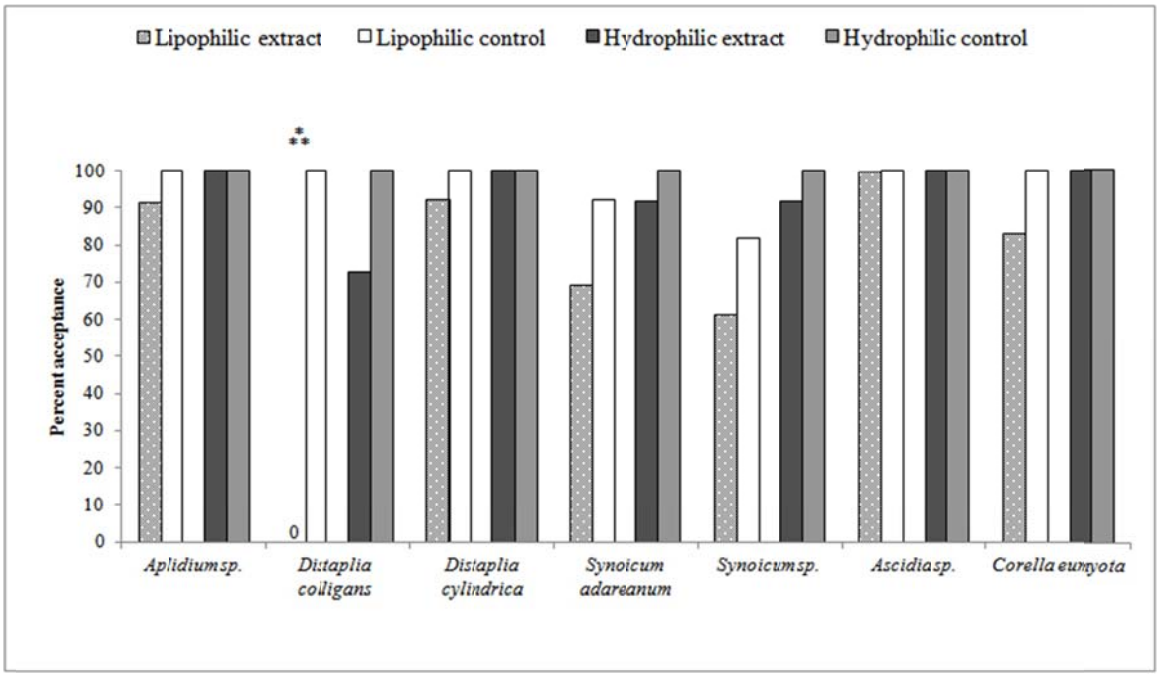

Fig. 2. *Odontaster validus*. Results of bioassays offering artificial foods containing lipophilic or hydrophilic ascidian extracts to sea stars. Asterisks indicate significant difference between tissue and control (Fisher's exact test). *: $P \leq 0.05$; **: $P \leq 0.01$; ***: $P \leq 0.005$

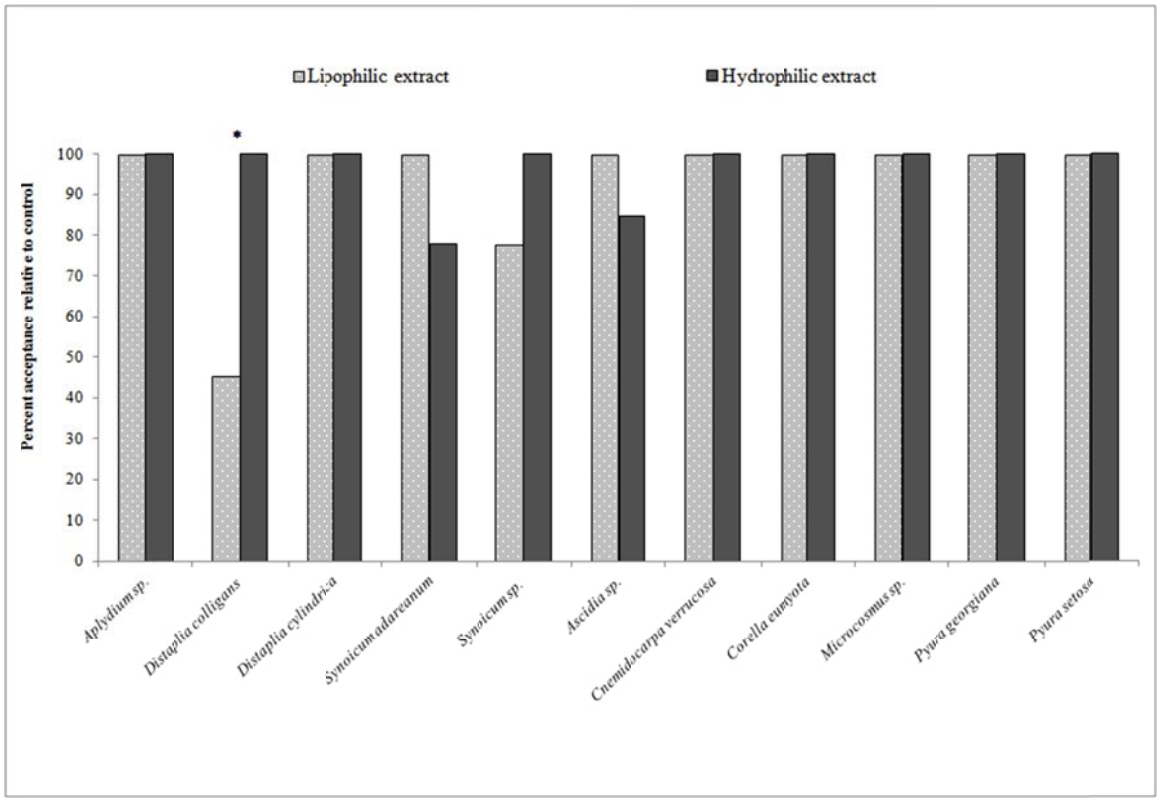

Fig. 3. *Notothenia coriiceps*. Results of bioassays offering artificial foods containing lipophilic or hydrophilic ascidian extract to fish. Asterisks indicate significant difference between tissue and control (Fisher's exact test). *: $P \leq 0.05$; **: $P \leq 0.01$; ***: $P \leq 0.005$

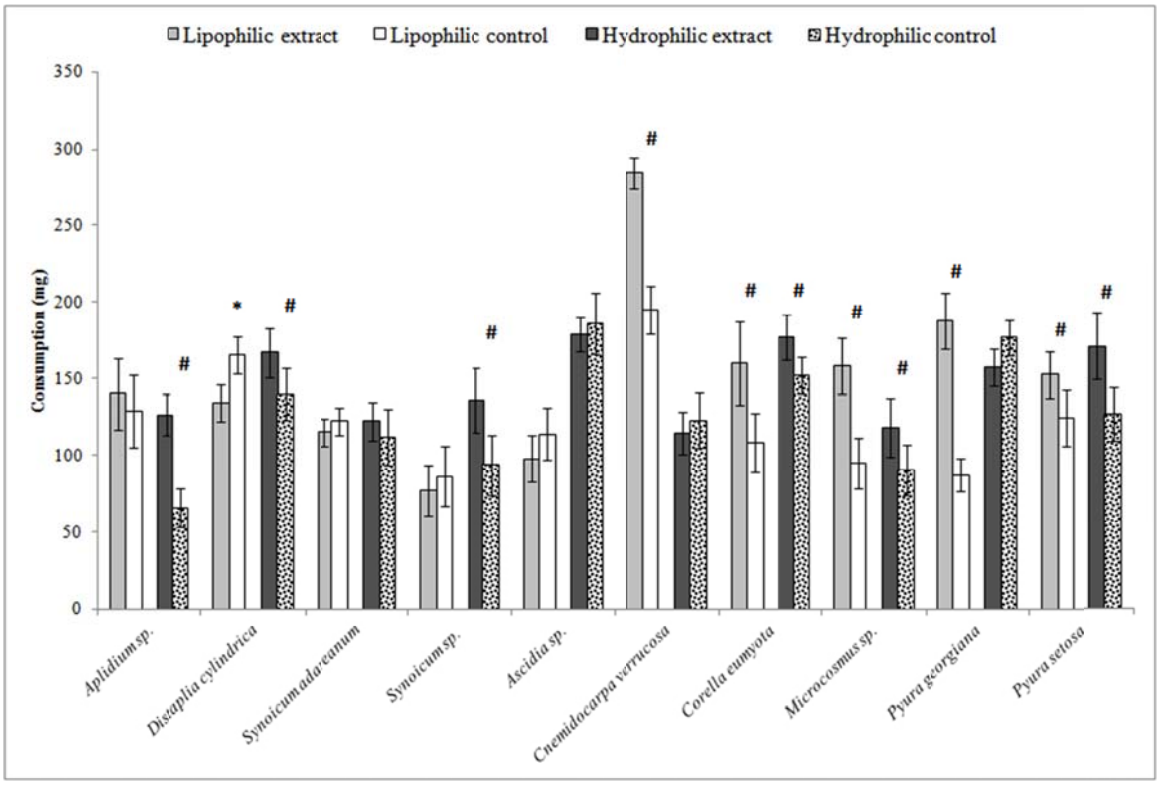

Fig. 4. *Gondogeneia antarctica*. Results of bioassays offering artificial food containing lipophilic or hydrophilic ascidian extract to amphipods. Values are means ± SEM. Asterisks indicate significant difference between extract and control (Wilcoxon signed ranks test) with controls significantly preferred to extract. *: $P \leq 0.05$; **: $P \leq 0.01$; ***: $P \leq 0.005$. Pound symbols (#) indicate significant differences ($P \leq 0.05$) between extract and control (Wilcoxon signed-rank test) with extract significantly preferred to controls.

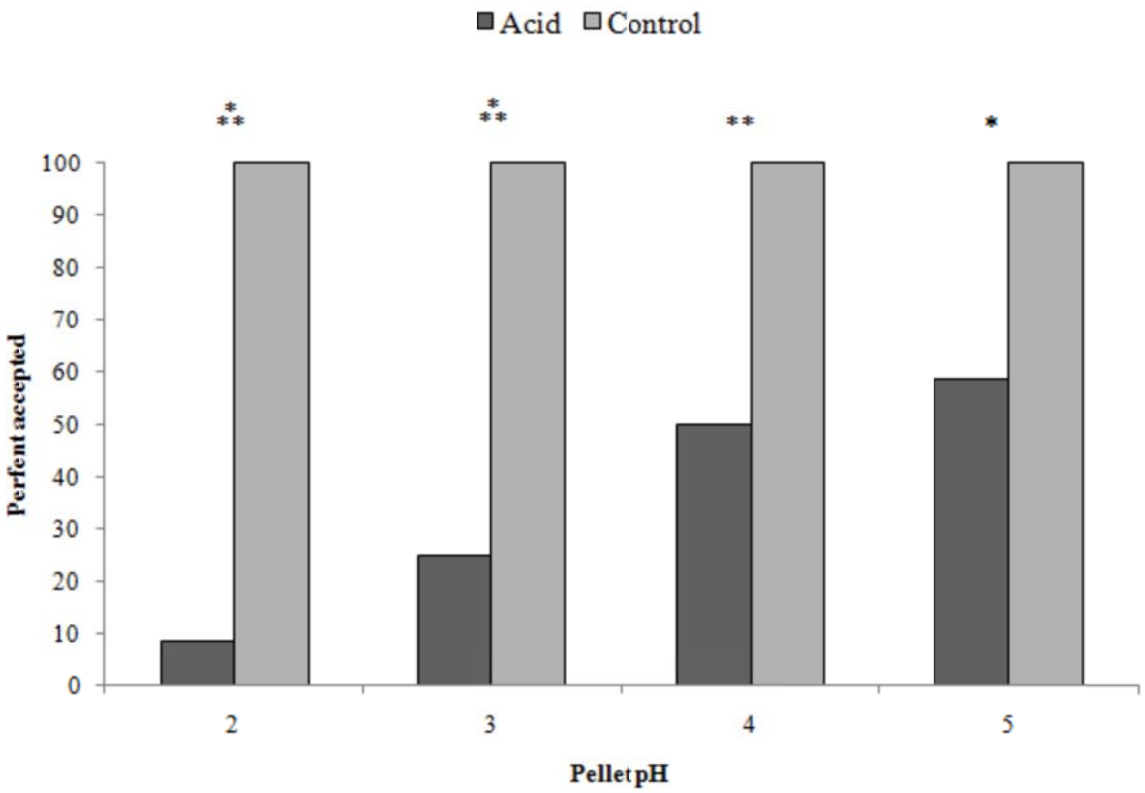

Fig. 5. *Odontaster validus*. Results of bioassays offering agar food pellets containing a 2% krill powder and acidified with sulfuric acid. Asterisks indicate significant difference between tissue and control (Fisher's extact test). *: $P \leq 0.05$; **: $P \leq 0.01$; ***: $P \leq 0.005$

A COMPREHENSIVE EVALUATION OF THE POTENTIAL CHEMICAL DEFENSES OF ANTARCTIC ASCIDIANS AGAINST SYMPATRIC FOULING MICROORGANISMS

Gil Koplovitz[1*], James B. McClintock[1], Charles D. Amsler[1] and Bill J. Baker[2]

[1]Department of Biology, University of Alabama at Birmingham, Birmingham, AL 35294 USA

[2]Department of Chemistry, University of South Florida, Tampa, FL, 33620, USA

Submitted to Marine Biology

Format adapted for dissertation

Abstract

The present study analyzed the bioactivity of whole body extracts from six solitary and eight colonial ascidian taxa against twenty sympatric bacterial isolates and one sympatric diatom species from nearshore waters of the Western Antarctic Peninsula. Ascidians had crude lipophilic and hydrophilic extracts assayed against twenty bacterial strains. The lipophilic extract of one ascidian caused growth inhibition in all bacterial isolates at 3X tissue-level concentrations and in one bacterial isolate at 1X concentration. The lipophilic and hydrophilic extracts were fractionated into seawater soluble and insoluble fractions and assayed at three concentrations against a sympatric diatom species. Significant diatom mortality was detected at 3X and 1X concentrations in all but one ascidian taxon. Lipophilic fractions caused higher diatom mortality than hydrophilic extracts. The specificity of secondary metabolites against diatom fouling and the lack of activity against bacteria suggest a higher selective pressure for chemical defenses against diatom fouling in Antarctic ascidians.

Introduction

In marine benthic environments, exposed undefended surfaces are subject to fouling by bacteria, algae and macroinvertebrate larvae. Sessile marine invertebrates are particularly susceptible to epiphytic and epizootic recruitment by a variety of settling organisms (Wahl 1989). Although the fouling organisms can sometimes be beneficial to their hosts by supplying them with vitamins and nitrogenous compounds, for the most part this interaction is harmful. Negative effects include increased hydrodynamic drag, competition for nutrients, and damage to surface tissues due to mechanical anchoring of epibionts (Wahl 1989).

Ascidians are sessile, soft bodied, filter feeding organisms that occur in solitary and colonial forms in all the world's oceans. They represent important components of food webs in a variety of benthic marine communities (Monteiro et al. 2002; McClintock et al. 2004; Tatian et al. 2005). While not as vulnerable to fouling as marine sponges, fouled ascidians can be compromised by obstruction of the incurrent and exhalent siphon or clogging of the branchial basket, both critical to respiration and feeding (Lambert 1968). Moreover, increased sedimentation and increased drag caused by heavy fouling may lead to ascidians being dislodged from sediments (Stoecker 1978). A large diversity of fouling organisms commonly occur on outer surfaces of ascidians, particularly solitary forms (Tatian et al. 1998). These include diatoms (McClintock et al. 2004), bryozoans and hydroids (McClintock and Baker 1997), as well as bacteria, algae and other macroinvertebrates (Bryan et al. 2003).

Chemical defenses against fouling are common in ascidians and have been examined in a variety of species (Stoecker 1978; Davis and Wright 1989; Wahl and

Banaigs 1991; Teo and Ryland 1995; Bryan et al. 2003; Murugan and Ramasamy 2003; Ramasamy and Murugan 2003; McClintock et al. 2004). Additionally, antimicrobial compounds have been reported in ascidians (Azumi et al. 1990b; Tsukamoto et al. 1994; Wahl et al. 1994; Findlay and Smith 1995; Wahl 1995; Murugan and Ramasamy 2003; Ramasamy and Murugan 2003). Ascidians have also been shown to harbor bacterial communities on the surface of their tunic with epibacterial densities varying between species (Wahl et al. 1994; Wahl 1995; Schuett et al. 2005). Moreover, some ascidians host intracellular bacteria in a presumptive symbiotic relationship (Moss et al. 2003). For example, a symbiotic relationship has been described in several tropical colonial ascidians that host the cyanobacterium *Prochloron* sp. (Cox 1986; Hirose 2005; Yokobori et al. 2006). Some bioactive secondary metabolites that have been isolated from ascidians are thought to have a bacterial origin (Bernan 2001; Schmidt et al. 2005; Donia et al. 2006; Simmons et al. 2008). In several of these cases, specific bacteria that occur on the outer surfaces of ascidians have been shown to produce antibacterial and antifouling compounds (Holmström et al. 2002; Franks et al. 2005; Dobretsov et al. 2006).

Ascidians are ecologically important members of Antarctic benthic communites (Ramos-Espla et al. 2005) that often dominate biomass in shallow benthic communities along the Antarctic Peninsula (Sahade et al. 1998; Tatian et al. 1998). Marine prokaryotic communities in Antarctic Peninsular waters occur in high abundance year-round (Murray et al. 1998; Ducklow et al. 2001), while microalgae dominate during seasonal spring and summer blooms (Cerrano et al. 2004). Diatoms are one of the primary fouling organisms in Antarctic benthos (El-Sayed and Fryxell 1993). For example, Antarctic sponges have been shown to be heavily fouled seasonally by benthic

diatoms (Amsler et al. 2000; Cerrano et al. 2000), sometimes with deleterious effects on the host sponge (Bavestrello et al. 2000; Cerrano et al. 2000).

The present study evaluates the potential antifouling bioactivity of secondary metabolites in tissue extracts of a suite of fourteen taxa of solitary and colonial ascidians from the Western Antarctic Peninsula against a suite of twenty sympatric bacterial isolates and the diatom *Syndroposis* sp. An evaluation of the presence of potential antifouling compounds facilitates further studies to determine whether Antarctic ascidians possess ecologically relevant concentrations of these compounds on the outer tunic or in the surface boundary layers.

Materials and Methods

Field collections.

Ascidians were collected by hand using SCUBA from depths ranging from 2-39 m from various locations within a 3.5 km radius of the U.S. Palmer Station, located on Anvers Island along the Western Antarctic Peninsula (64° 46.5' S, 64° 03.3'W). In order to ensure an unbiased sample of representative species, we examined all ascidian species we encountered. Collections were made during two consecutive field seasons (Feb-June 2007 and Mar-June 2008). Freshly collected solitary and colonial ascidians were subject to volumetric (by seawater displacement in a graduated cylinder) and wet weight determinations. Ascidians were then placed in zip-lock bags with identification tags and frozen at -80°C for later preparation of crude organic extracts.

Preparation of organic extracts for antibacterial and antifouling assays

Organic extracts of ascidians were prepared using whole individuals of solitary taxa and whole colonies of colonial taxa. Extraction techniques are described in McClintock et al. (2004). Briefly, several individuals or colonies were weighed, lyophilized, and then re-weighed. The freeze-dried tissues were then extracted thrice in dichloromethane/methanol (1:1 ratio) for 24 h. Extracts were combined and filtered through a coarse filter paper and dried down under reduced pressure to yield a lipophilic extract. A hydrophilic extract was prepared by subsequent extraction of the same freeze-dried tissue using methanol/water (1:1 ratio) thrice for 24 h. Both lipophilic and hydrophilic extracts were weighed following drying.

Antibacterial assays

The marine bacteria used in the present study were collected and identified in a previous study using techniques described in Peters et al. (2009). In brief, bacteria were collected from surfaces of sympatric benthic marine invertebrates by scraping their surfaces with a sterile scalpel and transferring the collected material into both 100% Difco marine broth 2216 (Difco Laboratories, Sparks, MD, USA) and 50% glycerol in marine broth using aspetic technique. The samples were then frozen at no greater than -70ºC and shipped back to the United States. Frozen samples were thawed, emulsified and incubated on marine agar to facilitate the isolation of individual bacterial colonies. The isolated colonies were grown on Difco marine agar 2217 at 4ºC. Isolates were identified by sequencing their 16S rRNA gene using an ABI Prism® 3100 DNA Sequencer (AME Bioscience, Toroed, Norway). Marine broth was then inoculated with pure cultures and

incubated on a shaker at 4ºC until the bacteria reached the stationary phase. A 100 µl suspension of each bacterial culture was spread on marine agar plates and allowed to soak in for five min before adding each extract.

Extracts from the 14 ascidian taxa were resuspended in either methanol (for lipophilic extract) or 1:1 methanol:water (for hydrophilic extracts) at 1 ml per 3 g wet tissue originally extracted (representing 3X natural concentration). Paper antimicrobial assay disks, 6 mm diameter (BBL Microbiology Systems 31039, Cockesville, MD, USA), were prepared by adding 20 µl (10 µl per side) of the extract solutions or of solvent only to the disks. Once the solvent has evaporated from the disks, they were placed onto the inoculated marine agar plates and incubated at 4ºC for several days until bacterial growth was visible and zones of growth inhibition could be measured. If activity was detected at 3X concentration, the assay was performed again with the affected strain with disks containing 1X natural concentration. Antibacterial activity was defined as zones of inhibition around disks containing extracts when compared to solvent control disks. Each assay was replicated on three separate days with three separate bacterial cultures of each strain for a total of 3 replicates per extract concentration.

Anti-diatom assay

In order to estimate the natural concentrations of secondary metabolites found on the surface of the ascidians where diatoms would be present, 1 cm x 2 cm surface squares of dried ascidians were cut with a single-edge razor blade down to a depth of approximately 0.5 mm and then weighed, approximating the dry weight of the outermost 0.5 mm of a 1 cm^2 ascidian surface. An average of the weights of available ascidian was used for all

ascidians (mean = 0.032 g dry wt, range = 0.012-0.092 g dry wt). This representative weight was then used to calculate the estimated yield of lipophilic extract per surface area of an ascidian on a wet weight basis. As there is no known method of measuring the exact concentration of the organic compounds on the outer surface, we assumed that extracts were evenly distributed throughout the ascidian. In order to account for variation in the distribution of organic compounds on the surfaces of different ascidians, we bracketed the concentrations or extracts tested (see below).

The diatom that was used in the antifoulant assays was *Syndroposis* sp., a sympatric chain-forming pennate diatom previously isolated from the intertidal green alga *Cladophora repens* near Palmer Station. Diatoms were maintained in f/2 media (McLachlan 1973) at the University of Alabama at Birmingham, and designated Pal D1.2, as in previous publications (McClintock et al. 2004; Amsler et al. 2005).

In order to determine which compounds (in terms of polarity) are more likely to serve as antifoulants, the lipophilic and hydrophilic extracts of the 14 ascidian taxa were further fractionated. Lipophilic extracts were resuspended in 8:2 MeOH:seawater and centrifuged (in glass vials at 1500 x g) to produce a soluble and an insoluble fraction. The soluble fraction was then dried and resuspended in seawater and centrifuged again to separate remaining seawater-insoluble compounds. Both insoluble fractions were combined, dried and resuspended in 1:1 CH_2Cl_2:MeOH. The hydrophilic extracts were resuspended in seawater and centrifuged (in plastic Eppendorf tubes at 16000 x g) to produce a soluble and an insoluble fraction. The insoluble fraction was dried and then resuspended in MeOH.

Due to the high reactivity of CH_2Cl_2 with plastic, experiments using the insoluble fraction of the lipophilic extracts were conducted in a borosilicate glass flat-bottomed 96-well tissue culture plate (Zinsser Analytic, Frankfurt, Germany). The rest of the fractions were tested in a standard Falcon® 96-well tissue culture plate (Becton Dickinson, Franklin Lakes, NJ, USA). For the insoluble fractions (from the lipophilic and hydrophilic extracts), the yield of extract per surface area of the ascidian was used to determine the amount of extract needed for each 6 mm diameter well (for hydrophilic extract) and 7.5 mm well (glass plate, for lipophilic extract) to approximate the natural surface concentration found in each ascidian. This concentration, as well as 30% and three times the natural concentration were determined and used in the bioassays. The solubilized extract fractions were then transferred to the wells. In order to ensure the extracts coated only the bottom surface of each well, the extracts were transferred in aliquots that covered only the bottom of the well. The plates with the extracts were then dried under reduced pressure and subsequent aliquots were added until the appropriate amount of crude extract coated the surface of each well. Solvent control wells were made using the same method using only the appropriate solvents (1:1 CH_2Cl_2:MeOH for the lipophilic extract and MeOH for the hydrophilic extract). For the insoluble fraction of the hydrophilic extract, we initially used both solvent and non-solvent controls. After several assays, we saw no difference between controls and subsequently used only non-solvent controls. The plate was then chilled and 40 µl of seawater was added to each well, along with 40 µl of concentrated diatoms in f/2 media. Non-solvent control wells contained only 40 µl of seawater and 40 µl of concentrated diatoms in f/2 media. The yields of the seawater-soluble fractions of the lipophilic and hydrophilic extracts were calculated on a

volumetric basis as mass of extract per unit volume. Solubilized fractions were then diluted with seawater, 40 µl of the solutions were added to each well, followed by 40 µl of concentrated diatoms in f/2 media for a total concentration of 80 µl. This resulted in final concentrations of 0.3X, 1X and 3X natural concentration on a volumetric basis.

The plates were then incubated at 1.5ºC (±0.5ºC) for 3 days with a 12:12 hour light:dark photoperiod at an irradiance of 25µmol photons m^{-2} s^{-1}. After incubation, the diatoms were stained with fluorescein diacetate and Evan Blue following the procedures described in Amsler et al. (2000). Thereafter, the cells were observed at 400x magnification under epiflourescence and brightfield illumination on a compound microscope. Under blue epi-illumination, live cells appeared green due to the fluorescein diacetate and under brightfield illumination dead cells appeared blue due to the Evans blue staining. To calculate percent dead, a minimum of 100 cells from each replicate well were haphazardly chosen and recorded as live or dead.

Statistical analyses were performed using SigmaPlot 11.0 (SPSS Incorporated, Chicago, IL). Percentages of dead cells were arsine (square-root) transformed. The data were then subjected to Leven's Test of Equality of Variance. All the variances were equal, therefore the data were subjected to one way ANOVA followed by Tukey's post hoc tests. In some cases, where all three replicates in all three treatments were 100% dead and all controls were 0% dead, post hoc analysis was inappropriate. In these instances, treatments were designated as not significantly different from one another. Mean percent mortality for each concentration of each fraction was compared between solitary and colonial ascidian taxa using a Mann-Whitney U-test (Zar 2009).

Results

Antibacterial assays

Fourteen ascidian taxa had lipophilic and hydrophilic extract assayed against 16 strains of Gamma Proteobacteria, 1 Flavobacterium and 3 unidentified species of bacteria isolated from sympatric invertebrates in the study area. The bacterial species, strains and number of isolates used are available in Peters et al. (2009). Of the 14 taxa of ascidians that were tested against the 20 strains of sympatric bacteria, only the lipophilic extract of one ascidian species – the colonial ascidian *Distaplia colligans*, showed broad spectrum anti-microbial activity against all bacterial strains at three times the natural concentrations. (Fig. 1).

At natural concentration, the lipophilic extract of *D. colligans* caused growth inhibition (average: 0.3mm, range: 0.2-0.4mm) in one strain of Gamma Proteobacterium (P22 – *Psychrobacter fozii*). The hydrophilic extract of *D. colligans* caused partial growth inhibition (0.5 mm zone of inhibition with sporadic colonies) in 3 bacterial strains (p22 - *Psychrobacter glacinola*, p29 - *Psychrobacter glacinola* and p34 - *Psychrobacter fozii*) in one replicate only.

Antifouling assays

Fourteen ascidian taxa had seawater-soluble and insoluble fractions of both lipophilic and hydrophilic extracts assayed at three concentrations (0.3X, 1X and 3X estimated natural concentrations) against the sympatric chain-forming pennate diatom *Syndroposis* sp. The seawater-insoluble fraction of the natural concentration of crude lipophilic extract caused significant (ANOVA, $P<0.05$) diatom mortality in 11 out of 14 ascidian taxa when compared to controls (Fig. 2). At 3X natural concentration, significant diatom

mortality was detected in 13 out of 14 taxa. Even at 0.3X natural concentration, eight taxa had fractions that caused significantly higher diatom mortality when compared to controls. The seawater-soluble fraction of the crude lipophilic extract caused significant (ANOVA, $P<0.05$) diatom mortality in 10 out of 14 taxa (Fig. 3). At 3X natural concentration, significant diatom mortality was detected in 13 out of 14 taxa. At 0.3X natural concentration, seven out of fourteen taxa had fractions with significantly higher diatom mortality compared to control.

The seawater-insoluble fraction of the crude hydrophilic extract caused significant diatom mortality in five taxa (Fig. 4). However, mean diatom mortality was generally low. At 3X natural concentration, significant diatom mortality was detected in four taxa. At 0.3X natural concentration only the extracts of 2 ascidian taxa caused significantly higher diatom mortality when compared to controls. The seawater-soluble fraction of the crude hydrophilic extract caused significant (ANOVA, $P<0.05$) diatom mortality in extracts of only 2 species of *Distaplia* (Fig 5). At 3X natural concentration significant diatom mortality was detected in nine out of fourteen taxa, whereas at 0.3X natural concentration, significant diatom mortality was only detected in a single species – the colonial ascidian *Distaplia colligans*.

The seawater-soluble fraction of the hydrophilic extract of *Distaplia colligans* proved to be highly acidic, with pH values of 1.9, 3.0 and 4.2 (measured with standard analytical pH strips - EM ColorpHast) at the 3X, 1X and 0.3X concentrations, respectively. The low pH interfered with the live-dead staining, mainly with the Evans Blue, which appeared yellow, but due to appearance of the diatoms compared to the controls, all three concentrations were deemed to cause 100% diatom mortality.

The comparison of mean percent diatom mortality between extracts from the six solitary and eight colonial ascidian taxa yielded no significant differences in bioactivity among all concentrations and all fractions.

Discussion

Chemical defenses against biofouling in marine organisms have been reported from a wide variety of taxa including bacteria, algae, and a wide variety of invertebrates (e.g. Davis et al. 1989; Wahl 1989; Steinberg et al. 2002 for review). The control of epibiosis may be mediated by non-polar compounds that are present on the outermost layer of an organism's surface or in the boundary layer, or more polar compounds that spread beyond the boundary layer (Steinberg and de Nys 2002; Steinberg et al. 2002). Whereas the more polar compounds that leach into and beyond the boundary layer might serve in repelling or preventing the settlement of fouling organisms (Walters et al. 1996; Bryan et al. 2003), in the present study we chose to concentrate on the growth inhibition of bacteria and the antifouling activity in post-settlement diatoms. There are several limitations that arise when trying to evaluate ecologically relevant concentrations of bioactive organic compounds that inhibit fouling microorganisms (see Amsler et al. 2000). The localization of secondary metabolites varies between ascidians (Pisut and Pawlik 2002; Salomon and Faulkner 2002; Seleghim et al. 2007), and it is unknown whether antifouling compounds are present throughout the tunic or concentrated in only the outmost layer. The Optimal Defense Theory predicts organisms will allocate defenses to areas that are most vital in terms of fitness (Rhoades 1979). Therefore, in ascidians, antifoulant metabolites are unlikely to be localized within internal tissues such as the

viscera or the gonads. As the non-polar compounds will only be encountered on the surface, for the anti-diatom assays we attempted to estimate the natural concentration based on surface area, while assuming such antifouling compounds will be concentrated on the topmost layer. For the polar compounds, as well as for the antibacterial assays, we used volumetric concentrations. In order to account for variability in the distribution of the compounds on the ascidian body, we also assayed using 30% (0.3x) and 300% (3X) of the estimated natural concentrations.

For the antibacterial assays we initially assayed the lipophilic and hydrophilic extracts at 3X estimated natural concentration. Even so, out of 14 ascidian taxa tested, bacterial growth inhibition was detected only with the lipophilic extract of the colonial *Distaplia colligans*. This growth inhibition activity was broad-spectrum affecting all 20 microbial isolates. At the estimated natural concentration there was minor growth inhibition detected in only one of the bacterial isolates. The marine bacteria employed in the present study were isolated from a variety of sympatric benthic marine invertebrates and it is unknown whether any are pathogens to ascidians. Peters et al. (2009) assayed lipophilic and hydrophilic extracts from 25 Antarctic sponges collected near Palmer station against the same bacterial strains and found only one strain (*Alteromonas elyakovii* – P37) was inhibited by extracts from all 25 sponges, and four additional bacterial strains displayed sporadic grown inhibition in response to some sponge extracts. The lack of bacterial growth inhibition to extracts of 13 out of 14 ascidian taxa, including those that showed sensitivity to all 25 sponge extracts (Peters et al. 2009) suggests that Antarctic ascidians may not be prone to bacterial pathogens. Alternatively, Antarctic ascidians may be susceptible to bacterial pathogens, but have not evolved antibacterial

compounds. A number of studies have examined the effects of ascidian secondary metabolites on human pathogenic bacteria (Azumi et al. 1990a; Azumi et al. 1990b; Raub et al. 1992; Tsukamoto et al. 1994; Findlay and Smith 1995). More recently, the ecological roles of antibacterial compounds have received attention (Engel et al. 2002; Bryan et al. 2003; Murugan and Ramasamy 2003; Ramasamy and Murugan 2003). Wahl et al. (1994) examined the anti-fouling and antimicrobial activities of secondary metabolites from a suite of temperate ascidians and found that while settlement inhibition was positively correlated with epibacterial abundance, antimicrobial activity was not. This suggests ascidians use a variety of mechanisms to control bacterial epibiosis. As epibacterial abundances on ascidians were not examined in the present study, we are unable to evaluate whether ascidians along the Western Antarctic Peninsula use non-chemical mechanisms to control bacterial epibiosis, or simply allow bacteria to settle upon the outer surfaces, whether as commensal or mutualistic symbionts.

The present study shows that the bioactivity of ascidian secondary metabolites against diatom fouling is substantial compared to that of antimicrobial activity. Unlike sponges, whose ostia can become clogged during diatom blooms (Barthel and Wolfrath 1989; Peters et al. 2009), ascidians are able to prevent clogging by actively closing the inhalant aperture or by squirting water and particles from the branchial sac through the apertures (Hoyle 1953). However, similar behaviors have not yet been recorded in Antarctic ascidians (Kowalke 1999) which may suggest Antarctic ascidians use means other than the physical expulsion of diatoms to prevent clogging. There has been at least one report of ascidians harboring diatoms and other eukaryotic algae within the tunic, with no apparent detrimental effects (Lambert et al. 1996). Heavy diatom spring blooms

have been observed to obstruct the siphons and clog the branchial basket in the solitary ascidian *Corella willmeriana*, potentially contributing to mortality (Lambert 1968).

Unlike the antimicrobial assays, which were performed on agar plates, the anti-diatom assays were performed in seawater, more closely representing the environment the diatoms encounter in nature. We therefore fractionated the lipophilic and hydrophilic compound into seawater-soluble and seawater-insoluble fractions in order to narrow down and target the more bioactive fractions. At both 0.3X and 1X natural concentrations, antifoulant activity against the diatom *Syndroposis* sp. was observed in at least one fraction, in twelve out of fourteen ascidian taxa, and in thirteen out of fourteen ascidians at 3X natural concentration. In the latter, complete diatom mortality was observed in all solitary ascidians and in six out of eight taxa of colonial ascidians.

The colonial ascidian *Synoicum adareanum* occurs in two different morphs near Palmer station (Authors' personal observation). One morph occurs as a large, bulbous colony (*Synoicum adareanum* 1) and the other morph occurs as several lobes attached at the base (*Synoicum adareanum* 2). Whereas all four fractions of *S. adareanum* 1 showed no bioactivity against diatoms, *S. adareanum* 2 caused significant diatom mortality at 3X natural concentration in both lipophilic fractions as well as the most polar hydrophilic fraction. This suggests a need for further taxonomic resolution in this species.

Extracts of the colonial ascidian *Distaplia colligans* displayed consistently high bioactivity with complete diatom mortality even at 0.3X natural concentration in three of four fractions. The most polar fraction also exhibited a low pH which may have contributed to diatom mortality. A similar high acidity in the organic extract of this species was observed in a previous study (Koplovitz et al. 2009). In this study *D.*

colligans was found to be the only ascidian among the same suite of taxa examined in the present study to have an organic extract that deterred feeding in the sympatric omnivorous sea star *Odontaster validus* and fish *Notothenia coriiceps*. In the present study, *D. colligans* was the only taxon to exhibit antimicrobial activity. Therefore, the organic compounds from Antarctic ascidians appear to have broad bioactivity against diatom fouling, but little to no antimicrobial or anti-predatory activity. Bandurraga and Fenical (1985) isolated secondary metabolites from the pacific coral *Muricea fruticosa* and also found activity against only diatoms, with no measurable cytotoxic, ichthyotoxic, or antimicrobial activity. Evidence of anti-diatom specificity was also seen in organic extracts of sponges from McMurdo Sound, Antarctica (Amsler et al. 2000).

Overall, anti-diatom bioactivity in both the seawater-soluble and seawater-insoluble fractions of lipophilic extracts was higher than in the hydrophilic fractions. These fractions contain the most non-polar compounds which are likely to remain in, or close to, the boundary layer or on the surface of the tunic where they are effective as anti-foulants (Steinberg and de Nys 2002). A similar pattern is seen in Antarctic macroalgae (Amsler et al. 2005) and Antarctic sponges (Peters et al. 2009). The seawater-soluble fraction of the hydrophilic extract (i.e. containing the most polar compounds), caused significant diatom mortality in nine out of fourteen ascidian taxa but only at 3X natural concentration. The exceptions were in *Distaplia colligans*, where 100% mortality occurred at all concentrations tested, and in *Distaplia cylindrica* where 100% mortality occurred at 1X natural concentration. The percentages of the hydrophilic extracts that were insoluble in seawater was generally very low (mean 3.58%, range 1.66-8.31%) and

the bioactivity of these fractions generally low as well. One exception was in *Distaplia colligans* where 100% diatom mortality occurred at 3X natural concentration.

Five of the ascidian taxa examined in the present study exhibited low pH on the surfaces of their tunics (Koplovitz et al. 2009). Parry (1984) suggested that tunic surface acidity may serve to prevent fouling. However, he also observed several species of non-acid producing stolidobranch ascidians that were free of epibionts and speculated that surface acidity could not be solely responsible for ascidian antifoulant defenses. Stoecker (1978) suggested that high vanadium concentration, as well as outer surface acids enable the solitary ascidian *Ascidia nigra* to remain epibiont-free. In a survey of 35 solitary and colonial ascidians from Bermuda, Stoecker (1980) found that twelve colonial ascidians and one solitary ascidian, with high surface acidity (pH<2) all lacked macroscopic epibionts on the tunic surface. Davis and Wright (1989) examined two colonial ascidians from Florida and found that *Eudistoma capsulatum* which had high surface acidity (pH=1-2) was heavily fouled by marine invertebrates, while its non-acidic (pH=6) congener *Eudistoma olivaceum* was almost epibiont free. They concluded that surface acidity was an ineffective defense against fouling or overgrowth by sessile invertebrates in the field. While we did not examine the effects of surface acidity or the concentration of vanadium on fouling in the present study, it is worth noting that the extracts of *Distaplia colligans* contained organic acids with a low pH. Moreover, Lebar et al. (2011) found that *D. cylindrica* had high vanadium concentrations in the body tissues.

The high incidence of bioactivity of secondary metabolites against diatom fouling in Antarctic ascidians is indicative of their possible role as antifoulants. However, the

ecological relevance of such bioactivity does not always correlate with in situ observations of fouling patterns. For example, both colonial *Distaplia* species are free of fouling organisms, yet the solitary *Pyura setosa*, whose tissue extracts exhibited high diatom mortality in bioassays, are heavily fouled (Authors' personal observations). As such, it is likely that in select species, surface concentrations of bioactive compounds are insufficient to control fouling and/or are produced for other purposes.

Acknowledgements

We thank Margaret Amsler, Craig Aumack, Jill Zamzow, and Philip Bucolo for their assistance with field collections. We are very grateful to Linda Cole at the Smithsonian Institution in Washington DC for her assistance with ascidian taxonomy. We wish to acknowledge the generous logistical support provided by those individuals employed by Raytheon Polar Services Company. This research was facilitated by NSF awards to CDA and JBM (OPP-0442769) and to BJB (OPP-0442857). JBM acknowledges the support of an Endowed Research Professorship in Polar and Marine Biology through the University of Alabama at Birmingham.

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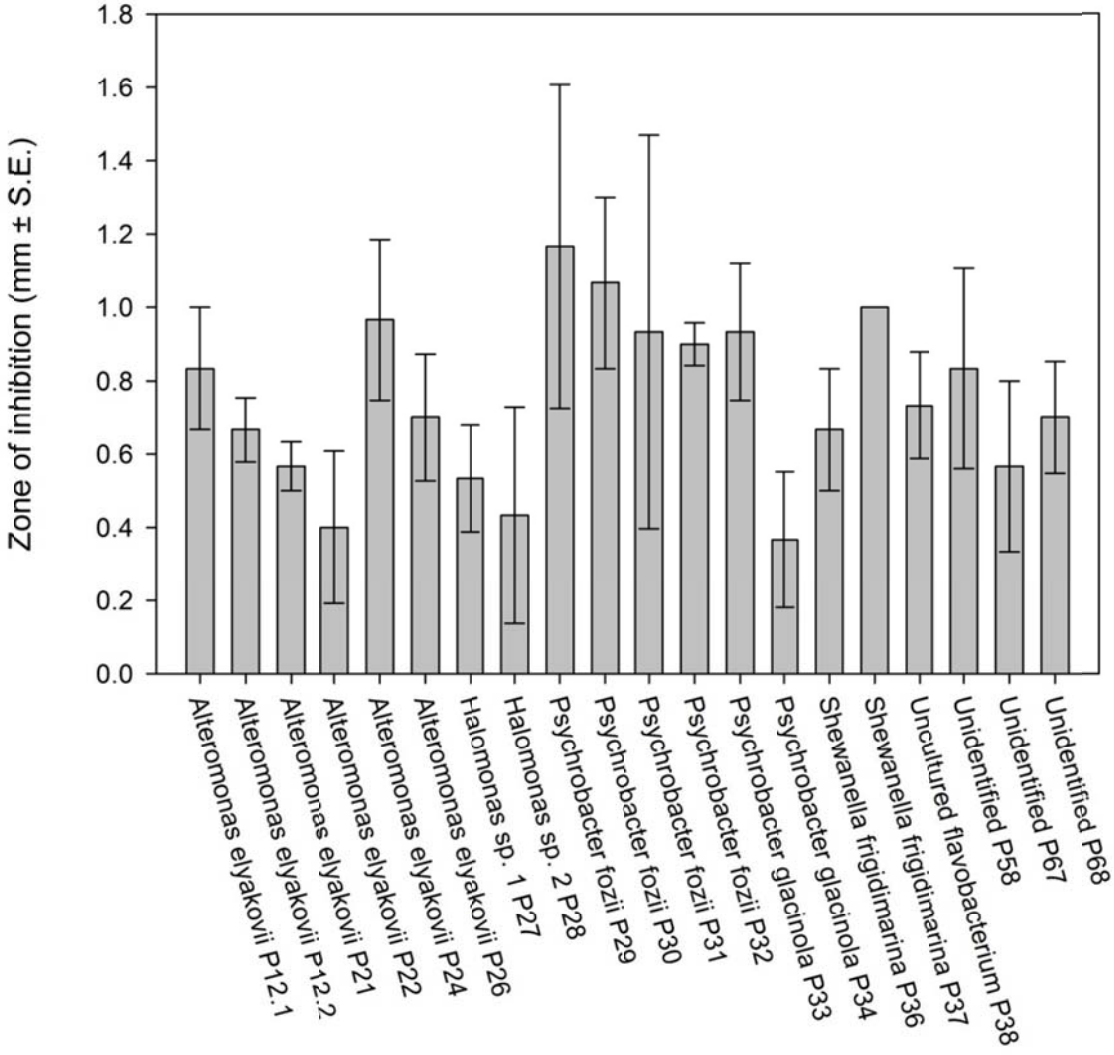

Fig. 1 *Distaplia colligans*. Results of antibacterial bioassay with lipophilic extract at 3X estimated natural concentration. There was no activity in the control disks

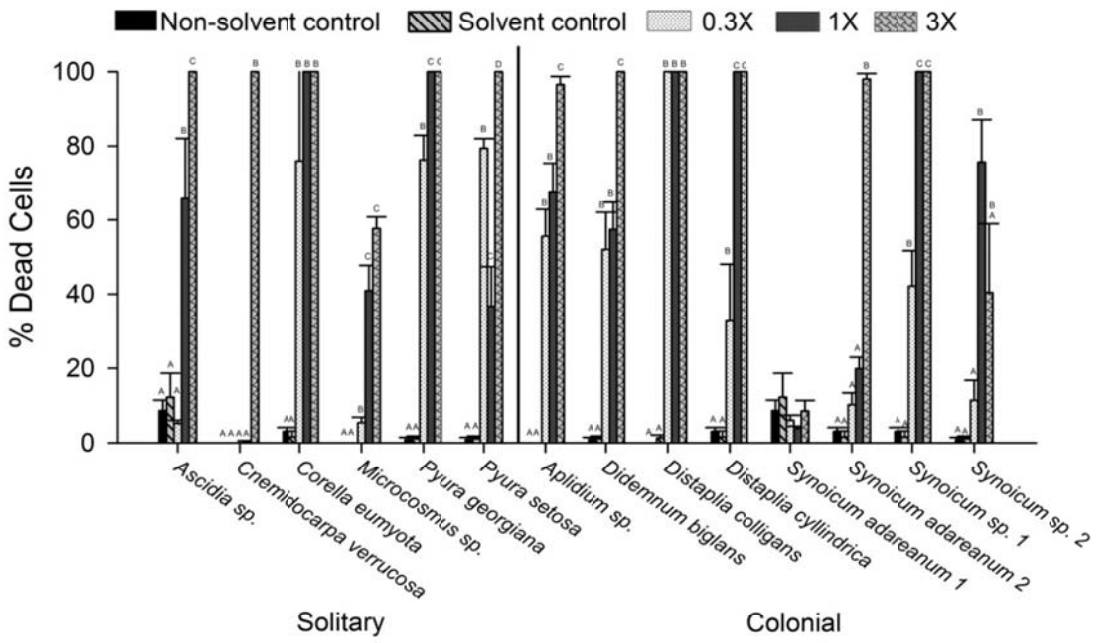

Fig. 2 Percent dead diatoms using three concentrations of seawater-insoluble fraction of lipophilic crude extracts. Shown are mean (± 1 SE) percentage of dead cells (n=3). Non-solvent control consisted of diatoms incubated in seawater and F/2 medium. Solvent control consisted of 1:1 dichloromethane:methanol which was dispensed on bottom of well in aliquots and allowed to evaporate before dispensing seawater and diatoms in F/2 medium. One-way Analysis of Variance followed by Tukey's test were used to compare the different treatments from each species to both controls. Bars with different letters are significantly different from one another ($P \leq 0.05$)

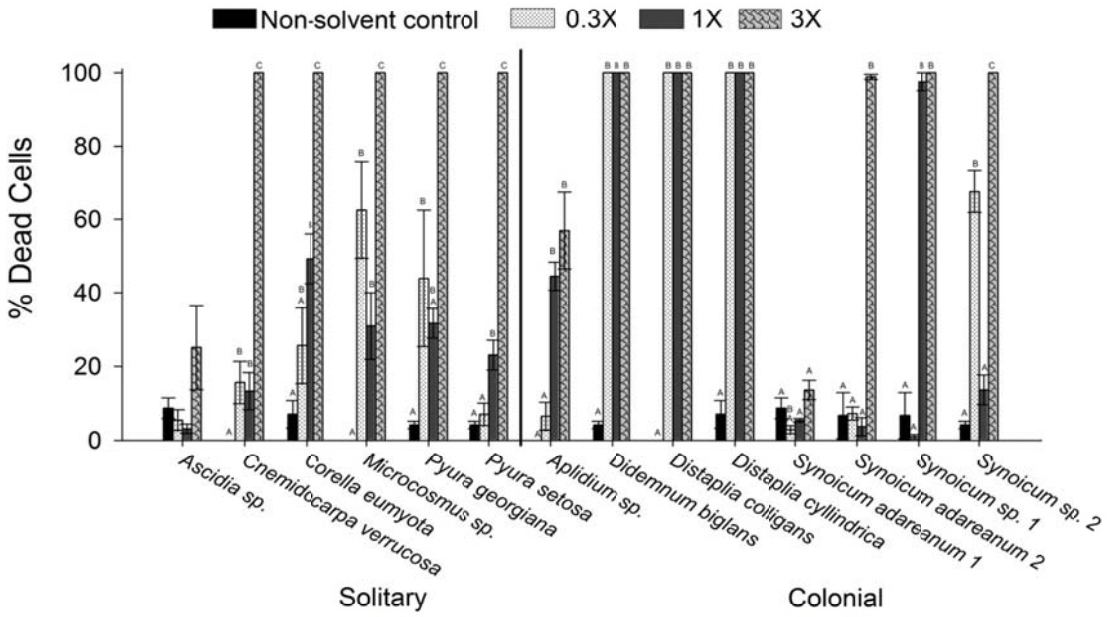

Fig. 3 Percent dead diatoms using three concentrations of seawater-soluble fraction of lipophilic crude extracts. Shown are mean (± 1 SE) percentage of dead cells (n=3). Non-solvent control consisted of diatoms incubated in seawater and F/2 medium. Extracts were re-suspended in seawater, therefore no solvent was used. One-way Analysis of Variance followed by Tukey's test were used to compare the different treatments from each species to both controls. Bars with different letters are significantly different from one another ($P \leq 0.05$)

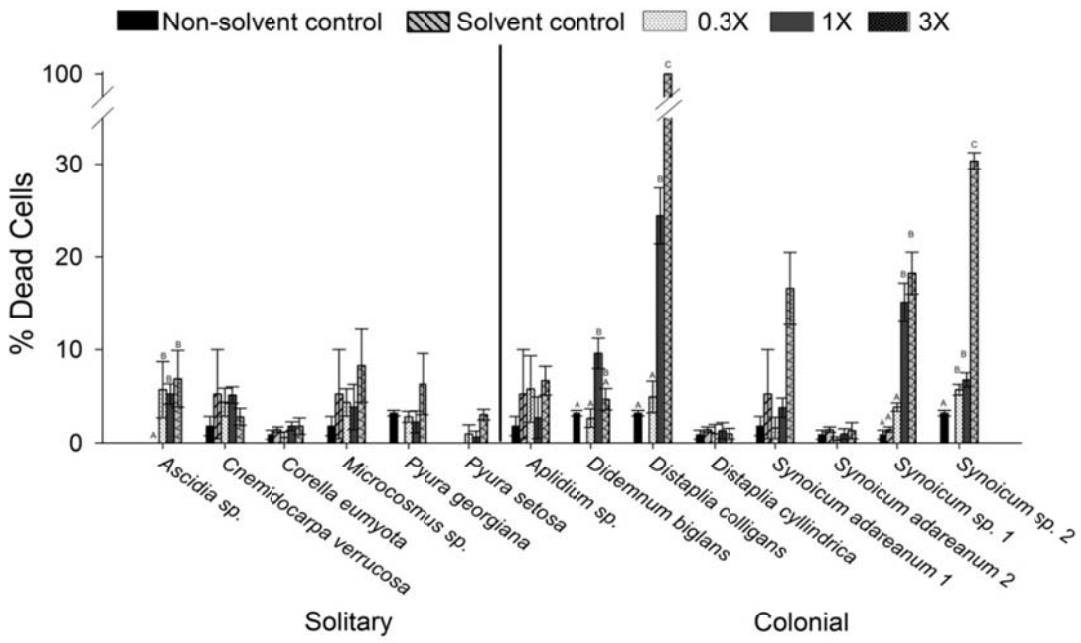

Fig. 4 Percent dead diatoms using three concentrations of seawater-insoluble fraction of hydrophilic crude extracts. Shown are mean (± 1 SE) percentage of dead cells (n=3). Non-solvent control consisted of diatoms incubated in seawater and F/2 medium. Solvent control consisted of methanol which was dispensed on bottom of well in aliquots and allowed to evaporate before dispensing seawater and diatoms in F/2 medium. One-way Analysis of Variance followed by Tukey's test were used to compare the different treatments from each species to both controls. Bars with different letters are significantly different from one another ($P \leq 0.05$)

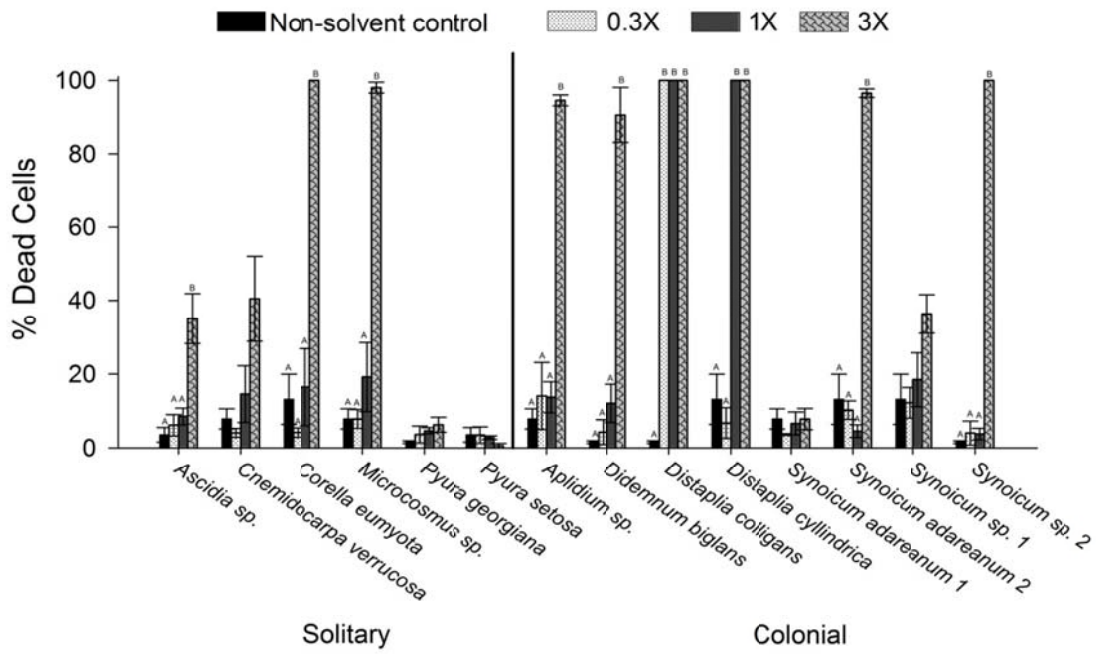

Fig. 5 Percent dead diatoms using three concentrations of seawater-soluble fraction of hydrophilic crude extracts. Shown are mean (± 1 SE) percentage of dead cells (n=3). Non-solvent control consisted of diatoms incubated in seawater and F/2 medium. Extracts were re-suspended in seawater, therefore no solvent was used. One-way Analysis of Variance followed by Tukey's test were used to compare the different treatments from each species to both controls. Bars with different letters are significantly different from one another ($P \leq 0.05$)

AN EVALUATION OF CHEMICAL AND PHYSICAL DEFENSES AGAINST FISH PREDATION IN A SUITE OF SEAGRASS-ASSOCIATED ASCIDIANS

Gil Koplovitz and James B. McClintock

Department of Biology, University of Alabama at Birmingham, Birmingham, Alabama

35294-1170, USA

Accepted for publication in:
Journal of Experimental Marine Biology and Ecology

Format adapted for dissertation

ABSTRACT

The palatability of two solitary and three colonial species of ascidians commonly found in sub-tropical seagrass meadows were evaluated using the abundant, sympatric, omnivorous pinfish *Lagodon rhomboides* as a model predator. Bite-sized pieces of fresh tissues of both solitary and one of the three colonial ascidian species were unpalatable to fish. Lipophilic and hydrophilic extracts of the three unpalatable species did not cause feeding deterrence indicating that secondary metabolites are not responsible for the lack of palatability. *Distaplia bermudensis*, the one colonial ascidian that was unpalatable to fish, had a highly acidified outer tunic (pH = 1.5). We tested the ability of acidified agar food pellets (pH = 1.5) to deter pinfish and found that the fish readily ingested acidified pellets. The toughness of the tunic of all five ascidian species was evaluated by measuring the Force (N) required to penetrate the tunic using a penetrometer. Tunic toughness is likely to explain the lack of palatability of the solitary ascidians Style *plicata* and *Molgula occidentalis* as their tunics required a force of > 34 N to penetrate. Tunic toughness may be a particularly effective adaptation for ascidian defense in seagrass habitats where fish with strong crushing jaws, such as those that commonly occur in coral reef systems, are rare.

Keywords: Ascidian, Chemical defense, Palatability, Seagrass, Benthic Ecology

1. Introduction

Seagrass meadows host a wide variety of marine invertebrates and vertebrates (Orth et al., 1984; MacArthur and Hyndes, 2007). Ascidians are conspicuous members in seagrass communities and play an important role in carbon cycles by filtering plankton (Lemmens et al., 1996). They also serve as prey to gastropods (Young, 1989b), sea urchins (Hiratsuka and Uehara, 2007), fish (Lemmens et al., 1996; MacArthur and Hyndes, 2007), sea turtles (Witzell and Schmid, 2005), dugongs (Preen, 1995) and manatees (O'Shea et al., 1991; Courbis and Worthy, 2003). As generally soft-bodied sessile organisms, few ascidians have been considered to rely on physical defenses against predation (Lambert and Lambert, 1987; López-Legentil et al., 2006). However, many ascidians are known to utilize chemical defenses to deter predators. Modes of chemical defenses in ascidians vary and may encompass secondary metabolites, high acidity due to sequestered sulfuric acid in outer tunic tissues, or high tissue concentrations of the heavy metal vanadium (Stoecker, 1980a; Lindquist et al., 1992; Pisut and Pawlik, 2002; McClintock et al., 2004; Koplovitz et al., 2009).

Historically, secondary metabolites in ascidians have been primarily investigated within the context of marine natural products chemistry (Faulkner, 2002; Paul and Ritson-Williams, 2008). Nonetheless, in recent years an increasing number of ascidian secondary metabolites and organic tissue extracts have been manipulated experimentally to evaluate the ecological role of secondary metabolites as feeding deterrents (Young and Bingham, 1987; Davis and Wright, 1989; Davis, 1991; Lindquist and Fenical, 1991; Lindquist et al., 1992; Pisut and Pawlik, 2002; McClintock et al., 2004; López-Legentil et al., 2006; Koplovitz et al., 2009; Núñez-Pons et al., 2010), and in some cases as anti-

foulants (Stoecker, 1978; Davis and Wright, 1989; Teo and Ryland, 1995; Bryan et al., 2003; McClintock et al., 2004). To date, most feeding deterrent studies of ascidians have focused on sub-tropical and tropical species. For example, the tropical solitary ascidian *Sigillina* cf. *signifera* possess alkaloids in the tambjamine class that deter feeding by sympatric coral reef fish (Lindquist and Fenical, 1991; Lindquist et al., 1992). The larvae of the tropical colonial ascidian *Ecteinascidia turbinata* contains secondary metabolites that are unpalatable to the omnivorous pinfish *Lagodon rhomboides* (Young and Bingham, 1987). Similarly, the larvae of the Caribbean colonial ascidian *Trididemnum solidum* contain metabolites that render their larvae unpalatable to pinfish, as well as to the sea anemone *Aiptasia pallida* and the sea urchin *Arbacia punctulata* (Lindquist et al., 1992). In a broad survey of feeding deterrence, Pisut and Pawlik (2002) found that sixteen of seventeen species of ascidians from the Western Atlantic have secondary metabolites that are deterrent to the bluehead wrasse *Thalassoma bifasciatum*.

The role of inorganic acids has also been investigated in relation to feeding deterrence in ascidians. Some ascidians produce and sequester sulfuric acid in specialized bladder cells on the surface of the tunic (Webb, 1939; Swinehart et al., 1974; Parry, 1984; Hirose, 2001). Whereas in an earlier ascidian study acid was thought to be quickly neutralized by seawater (Parry, 1984), more recent studies have demonstrated that sulfuric acid is retained in food pellets (and presumably the tunic) a sufficient period of time to serve as a potential feeding deterrent (Pisut and Pawlik, 2002; McClintock et al., 2004). In a recent study, we demonstrated that the acidity of the tunic of five species of Antarctic ascidians deterred feeding in an omnivorous sympatric sea star, but not in an omnivorous sympatric fish (Koplovitz et al., 2009).

The purpose of the present study was to examine the palatability of five species of seagrass-associated ascidians using the sympatric, omnivorous, pinfish *Lagodon rhomboides* as a model predator. For those ascidians that were unpalatable, we conducted experiments to evaluate whether the lack of palatability was attributable to chemical defenses (secondary metabolites or inorganic acid) and/or physical defenses (tunic toughness). We chose not to evaluate ascidian spicules as a physical defense because previous studies have demonstrated ascidian spicules are too small to serve as fish deterrents.

2. MATERIALS AND METHODS

2.1 Field collections.

Ascidians were collected from shallow (1-2 m depth) seagrass habitats of Saint Joseph Bay, Florida in the northern Gulf of Mexico (29°N, 85.5°W) (Table 1). Saint Joseph Bay has dense beds of the seagrasses *Thallasia testudinum* and *Halodule wrightii* which provide habitat for a variety of ascidians (Beddingfield and McClintock, 2000). The most common ascidians that occur in seagrass beds in this region are the solitary ascidians *Molgula occidentalis* and *Styela plicata*, and the colonial ascidian *Aplidium stellatum* (Young, 1989b; Bryan et al., 2003). Individual ascidians were collected by hand and placed in zip-lock bags in coolers on ice and transported to the University of Alabama at Birmingham. Upon return to the laboratory, each individual was cleaned by hand of any associated plant or animal material. Total wet wt was then determined using a top-loading balance and whole animal volume measured by seawater displacement using a volumetric flask. Individuals used in fresh tissue feeding assays were prepared

using the techniques described below. Ascidians that were used to prepare organic extracts were frozen in a -20ºC freezer until later processed.

2.2 Preparation of organic extracts

Ascidian tissue extractions were based on methods given in McClintock et al. (2004) and in Koplovitz et al. (2009). Frozen tissues were transferred to a -80ºC freezer for several hours, then lyophilized and re-weighed. The lyophilized tissues were then extracted in 1:1 dichloromethane/methanol thrice for 24 hours to produce a lipophilic extract. Extracts were filtered through coarse filter paper and the solvent was removed using rotary evaporation to yield a dry lipophilic extract. The same tissue was then subsequently extracted using 1:1 methanol/dH_2O thrice for 24 hours. Solvent was removed using rotary evaporation to yield a dry hydrophilic extract. Both lipophilic and hydrophilic extracts were weighed. Natural tissue level concentrations of extracts for preparation of artificial food pellets (see below) were calculated on a volumetric basis as the mass of dried extract divided by the volume of each individual ascidian. In order to control for the possibility of intraspecific variation in the levels of chemical defenses (organic extracts) within a given species, three individuals of each species were extracted to yield three separate lipophilic and hydrophilic extracts.

2.3 Fish maintenance and preparation of fresh tissues and alginate food pellets

The pinfish *Lagodon rhomboides* was selected as a model fish predator because it is a sympatric, omnivorous, generalist predator and one of the most common predatory fish in seagrass habitats of the Gulf of Mexico (Hansen, 1969; Stoner, 1979; Livingston, 1982; Luczkovich et al., 1995; Heck et al., 2000). Furthermore, ascidians are known to comprise a significant portion of the diet of *L. rhomboides* (Luczkovich et al., 1995; Motta et al., 1995). *Lagodon rhomboides* has been used successfully in previous studies examining the feeding deterrent properties of ascidians (Young and Bingham, 1987; Lindquist et al., 1992).

Fourteen pinfish (*Lagodon rhomboides)* of similar size (mean length = 10 cm) were procured from Gulf Specimen Marine Laboratories (Panacea, FL, USA). Fish were maintained in the laboratory in aquaria at 25ºC at densities of two fish per 34L aquarium. Fish were held individually by separating each aquarium into two equal sections using a plastic tank divider. Aquaria were provisioned with 31 ppt artificial seawater (Instant Ocean, Aquarium Systems, Mentor, OH) and fed a maintenance diet of 0.16 dry g commercial fish food flakes (TetraColor)/fish/day.

For the fresh tissue assays, small, bite-size pieces of fresh tissue from ascidians that measured approximately 0.5 x 0.5 cm length and width were cut using a scalpel. The depth of tissue pieces varied with species; depth was approximately 0.5 cm for all three colonial species, while 0.1 cm for the solitary *Molgula occidentalis* and 0.4 cm for the solitary *Styela plicata*.

Artificial food pellets were prepared following procedures given in Burns et al. (2003).

Briefly, pellets were prepared so as to consist of a matrix of 3% cold-hardened alginate containing a concentration of 5% dry wt squid mantle powder as a fish feeding stimulant. A mass of crude lipophilic or hydrophilic extract equivalent to the mass extracted from a 10 ml volume of tissue from each ascidian species was added to the mixture of alginate and powdered krill mantle in distilled water to yield a final volume of 10-ml. One drop of red food color was added to make the food pellets visible to fish. The mixture was stirred vigorously until the extract was distributed homogenously and then loaded into a 10 ml syringe. The syringe tip was then submerged into a 1M solution of cold calcium chloride and slowly injected into the solution to form a long string of cold-hardened alginate. After a few minutes, the alginate string was removed from the solution and cut with a razor blade into 3 mm length food pellets. Control pellets were made in a similar fashion but without the addition of extracts.

2.4 Fish feeding assays

With fish held in their individual aquarium sections, each individual was presented fish either a piece of fresh ascidian tissue or an alginate food pellet containing either a lipophilic or hydrophilic ascidian extract. Each fish was subsequently presented a control alginate food pellet containing only squid powder but no extract. Fish feeding responses were recorded either as acceptance or rejection. Acceptance was defined as a fish taking the tissue or pellet into its mouth and subsequently swallowing it. Rejection was defined as a fish ignoring the tissue or pellet for a period of 30 seconds or as a fish

taking the tissue or pellet into its mouth and subsequently spitting it out. Fish were allowed to repeatedly spit out the tissue or pellet up to five times before the assay was deemed a rejection. No fish was used more than once in a given experimental trial. Differences between the frequency of acceptance of tissue pieces or pellets and the corresponding controls were determined using a Fisher's exact test (Zar, 2009).

2.5 Tunic toughness measurements

In order to determine whether the acceptance or rejection of fresh tissues was potentially influenced by the toughness of the tunic, a penetrometer (pin = 3 mm diameter) was used to measured the amount of Force (N) necessary to penetrate the tunic of each ascidian species. For solitary species, the tunic was considered as a discrete unit. For colonial species there was no way to separate the tunic from the imbedded zooids. Here, we measured the force necessary to penetrate the colony (both tunic and zooids) but for ease of description refer to this also as the "tunic" throughout the manuscript. For the solitary species *Styela plicata*, the weight needed for the penetrometry pin to penetrate the tunic was higher than the safe operating weight for the penetromenter (5 kg). As such, a weight of 5 kg was used as an estimate of the Force for tunic penetration. For each individual, the mean force required to penetrate the tunic was measured as an average of penetration force at five different locations. The Force required to penetrate the tunics of all five species were statistically compared with one another using One Way Analysis of Variance (Zar, 2009). The analysis was followed by a Tukey's multiple comparison post-hoc test.

2.6 Tunic acidity measurements

The acidity of the outer surfaces of the ascidian tunics was measured using standard analytical pH strips (EM ColorpHast). Tunic surface pH measurements were first conducted by gently laying a pH strip with a broad sensitivity range of 0 to14 whole pH units on to the outer tunic surface. Subsequently, higher resolution was obtained using pH strips capable of resolving pH to an accuracy of 0.3 pH units. Following measurements of the tunic surface, the tunic was sliced with a razor blade and the pH measured once again by inserting of pH strips into the slice. This allowed a measurement of the internal pH of the tunic. Because the pH test was based on color visual discrimination we considered this a qualitative measurement and chose to round each pH measurement off at 0.5 unit increments so as to be conservative.

To test the effects of acidity on the palatability of ascidians to the pinfish *Lagodon rhomboides*, agar food discs were prepared by mixing a 1% agar solution made up in seawater with a 5% dry squid mantle powder as a feeding stimulant. The mixture was heated to boiling on a hot plate. Once the mixture had cooled it was acidified to 1.5 pH units by adding several drops of 2.0 N H_2SO_4 to bring it to a given pH as determined using analytical pH strips. The cooled acidified mixture of agar and squid food was then poured into 10-cm diameter Petri dishes and hardened in a refrigerator. Once hardened, 6 mm diameter discs were cut using a cork borer. Control food discs were prepared using the same protocols except no sulfuric acid was added. The control food discs had a slightly basic pH of 8.5, probably due to the squid powder. Fish feeding bioassays were

conducted for the acidified and control food discs following the feeding bioassay protocols given above.

3. Results

3.1 Fish feeding bioassays

Bite-size pieces of tunic from both species of solitary ascidian (*Styela plicata* and *Molgula occidentalis*) and one of the three colonial ascidians (*Distaplia bermudensis*) were significantly ($P<0.01$) unpalatable to the pinfish *Lagodon rhomboides* in feeding bioassays (Fig. 1). Control food pellets were readily consumed by pinfish. The lack of palatability did not appear to be attributable to defensive secondary metabolites. Fish feeding bioassays with alginate food pellets containing natural tissue concentrations of lipophilic and hydrophilic extracts from all three unpalatable species did not deter pinfish. Acceptance rates of food pellets containing organic extracts were consistently high, ranging from 71 - 100%, and were not significantly different from acceptance rates for control pellets. For unpalatable species, no significant intra-specific variation was detected when comparing feeding deterrence in alginate pellets containing tissue-level concentrations of organic extracts from individuals of the same species. The pinfish *Lagodon rhomboides* readily consumed both control and pH 1.5 agar pellets.

3.2 Tissue toughness

The mean ± 1 SE force (N) required to penetrate the tunic of the solitary species *Molgula occidentalis* and *Styela plicata* was 34.65 ± 1.08 (n=3) and 49.02 ± 0 (n=3) (see section 2.5), respectively. The mean ± 1 SE force (N) required to penetrate the tunic of the colonial species *Aplidium stellatum*, *Botrylloides nigrum* and *Distaplia bermudensis* was 11.83± 1.41 (n=3), 5.80 (n=1) and 3.26 ± 0.41 (n=3), respectively. ANOVA results were significant ($F_{3,11}$=507.410; $P \leq 0.001$). Pairwise comparisons using Tukey test indicated that the mean Force (N) required to penetrate the tunic of the solitary species *Styela plicata* and *Molgula occidentalis* was significantly greater ($P<0.001$) than that of all three colonial ascidians (Fig. 2). The solitary *Styela plicata* also required significantly greater Force to penetrate than the solitary *Molgula occidentalis*. Amongst the colonial species, *Aplidium stellatum* required significantly greater Force to penetrate than *Distaplia bermudensis*, but not significantly different than *Botrylloides nigrum*.

3.3 Tunic acidity

A high level of acidity (pH = 1.5) was measured on the outer surface of the tunic of the colonial ascidian, *Distaplia bermudensis*. The remaining four species of ascidians had outer tunic pH values that ranged from 6 to 7.0 (Table 1).

4. Discussion

Seagrass meadows are important in nearshore habitats as they provide refuge and food for a variety of life stages of marine invertebrates and fish while also contributing significantly to coastal carbon flow (Livingston, 1982; Orth et al., 1984; Pollard, 1984; Motta et al., 1995; Lemmens et al., 1996; Williams and Heck, 2001; Boström et al., 2006). All five species of ascidians examined in the present study have a wide geographical distribution, ranging from tropical to temperate habitats (Van Name, 1945); nonetheless they generally occur in high abundance in seagrass meadows (Young, 1989b; Bryan et al., 2003). As such, it is important to consider the trophic relationship of these common seagrass-associated ascidians with the most abundant predatory fish in nearshore seagrass habitats of the Gulf of Mexico; the pinfish *Lagodon rhomboides*. Importantly, pinfish are a well-documented predator of ascidians (Luczkovich et al., 1995; Motta et al., 1995)

Three of the five ascidian species examined in the present study proved to be unpalatable to pinfish. This lack of palatability did not appear to be mediated by secondary metabolites. Neither lipophilic nor hydrophilic extracts of the three unpalatable species imbedded in alginate pellets at tissue-level concentration were effective in deterring pinfish predation. Rather, the lack of palatability in two of the three species is likely attributed to physical defenses. Both *Molgula occidentalis* and *Styela plicata*, the two solitary ascidians known to occur in seagrass meadows in Saint Joseph Bay (Koplovitz, personal observation), are also the most common ascidians in the shallow subtidal zone of the northern Gulf of Mexico (Young, 1989a; 1989b). Despite

adult *Lagodon rhomboides* being a biting fish predator that is known to feed on colonial ascidians, their mouth morphology may limit their ability to cut through the tissue of tough prey (Luczkovich et al., 1995). Both *M. occidentalis* and *S. plicata* have the body design of a typical solitary ascidian with a discrete, outer, tunic that is both thick and tough. Their tunics are likely to provide an effective refuge against the biting force exerted by the small jaws of *L. rhomboides*. The toughness of the tunic of the *M. occidentalis* may be enhanced by a frequent coverage of sand grains. Young (1989b) demonstrated that this layer of sand coating the tunic of *M. occidentalis* enhanced the deterrent properties against the predatory gastropod *Fasciolaria hunteria*.

The solitary ascidian *Styela plicata* is a common species that is an invasive in a variety of marine habitats (Yamaguchi, 1975; David et al., 2010). In contrast to the present study, Pisut and Pawlik (2002) observed that fresh pieces of its tunic are readily consumed by the bluehead wrasse *Thalassoma bifasciatum*. This difference could be attributable to wrasse having more powerful jaws than pinfish. Pisut and Pawlik (2002) found that bluehead wrasse rejected the gonads of *S. plicata* suggesting that chemical defenses may be sequestered in the gonads. In the present study, pinfish were not presented gonads of *S. plicata*, so it is not possible to evaluate whether they also find them distasteful. Population regulation in *S. plicata* has been hypothesized to be controlled by fish predation on juveniles (Sutherland, 1974; Fisher, 1977; Mook, 1983). Mook (1983) hypothesized that adult individuals may have a refuge in size as they are relatively free of fish predation. The present study suggests that the basis of this size refuge may be tunic toughness; pinfish rejected fresh tunic even when rendered as small

bite-size pieces suggesting that adult *S. plicata* are well protected by the toughness of the tunic alone.

The results from our penetromenty measurements support earlier qualitative observations about the toughness of the tunic of the solitary *Styela plicata* and *Molgula occidentalis*. We found that the tunic of both species was three to twelve-fold tougher to penetrate that that of the tunic (includes both tunic and zooids) of the three colonial species examined. A number of studies have examined the potential role of physical defense in ascidians (Lambert and Lambert, 1987; Lindquist et al., 1992; Tarjuelo et al., 2002; López-Legentil et al., 2006). However, all of these studies have focused on the potential role of the calcareous spicules that are embedded in the tunic. These studies determined that spicules have no deterrent effects against fish, probably because they are very small, even when compared with sponge spicules. One exception may be that noted by Lowenstam (1989) who hypothesized that spicules projecting from the siphonal opening of the solitary ascidian *Bathypera* sp. may prevent amphipods from gaining access to vulnerable inner tissues. Importantly, our study reports the first quantitative assessment of ascidian tunic toughness. While our sample size of species is small, our findings that solitary species possess much tougher tunics than colonial species suggests further studies are warranted to evaluate this trend. Moreover, tunic toughness may prove to be a particularly effective defense in seagrass habitats where durophagous (strong jawed) fish are rare compared to coral reef systems.

Defenses in ascidians have also been evaluated in the context of food value with some investigators suggesting ascidian tissues are low in nutrients and energy (Kühne,

1997; Kowalke et al., 2001). However, a number of studies have demonstrated that the nutritional value of ascidians is at or above that of other sessile marine benthic invertebrates such as sponges commonly consumed by predators (Steimle and Terranova, 1985; McClintock et al., 1991; Tarjuelo et al., 2002; McClintock et al., 2004). These studies indicate that ascidians are generally high in protein and carbohydrate and have tissue energy values ranging from 6.0 to 22.4 kJ per gram dry weight. As such, low nutritional value is an unlikely factor responsible for the lack of palatability in the present study.

For the three colonial ascidians that were assayed as fresh tissue, only the Aplonsobranch *Distaplia bermudensis* was unpalatable to pinfish. Despite this lack of palatability, alginate food pellets containing tissue concentrations of lipophilic and hydrophilic extracts were readily consumed by fish. Nonetheless, we detected a high tunic- surface level of acidity (pH = 1.5). While it is possible that the acidic tunic of *D. bermudensis* may deter other sympatric marine invertebrate and fish, there was no feeding deterrence in pinfish (*L. rhomboides*) offered agar food pellets acidified with sulfuric acid to a pH of 1.5.

Fresh pieces of the tunic of the colonial ascidians *Aplidium stellatum* and *Botrylloides nigrum* were readily consumed by *L. rhomboides.* Pisut and Pawlik (2002) also found that bluehead wrasse (*T. bifasciatum)* readily consumed the latter species either as fresh tissue or in food pellets with hydrophilic extract, but in contrast to our study they found that the lipophilic extracts of *A. stellatum* deterred feeding in bluehead wrasse. They also reported that when the outer tunic of *A. stellatum* was abraded by a

dissecting probe it had a pH of 1.5 and an even lower pH of 1.0 when the tunic was cut open with a scalpel. We found that the tunic of *A. stellatum* had a pH of 7.0, both when measured on its outer surface or internally. The difference between the tunic pH suggests that pH may vary temporally or spatially or the need for a further resolution of taxonomy may be appropriate for this species.

The role of inorganic acids as a chemical defense against predation has been the topic of a long-standing debate (Parry, 1984; Davis and Wright, 1989; Pawlik, 1993; Pisut and Pawlik, 2002; McClintock et al., 2004; Koplovitz et al., 2009). Hirose (1999; 2001) suggested that sulfuric acid that is released from ascidian bladder cells upon injury might aid in predator deterrence and in disinfecting the injured area. In contrasts, Parry (1984) claimed that the inorganic acid in ascidians would be neutralized by seawater too rapidly to serve as effective feeding deterrents. Subsequently, Pisut and Pawlik (2002) demonstrated that sulfuric acid is effective in deterring feeding by the generalist fish *Thalasoma bifasciatum*. Additional studies of ascidians have further demonstrated that ascidian acids can be effective in deterring predation in the omnivorous Antarctic sea star *Odontaster validus* (McClintock et al., 2004; Koplovitz et al., 2009). In the present study, only the colonial ascidian *Distaplia bermudensis* exhibited high acidity on the outer surface of its tunic. However, acidity alone was insufficient to deter feeding by the pinfish *L. rhomboides*. Odate and Pawlik (2007) hypothesized that high acidity, coupled with a high concentration of vanadium may alter the oxidation state of vanadium and increase its effectiveness as a feeding deterrent. We did not address the sequestration of vanadium in the present study. However, it is noteworthy that Stoecker (1980b) found that *D. bermudensis* had the highest concentration of vanadium among a suite of 35

ascidians examined (3211 ± 1278 ppm). High concentrations of vanadium have been postulated to serve as a chemical defense against predation, but there remains insufficient experimental evidence to evaluate this hypothesis (Stoecker, 1980a; b; Parry, 1984; Pawlik, 1993; Odate and Pawlik, 2007). A high concentration of vanadium, coupled with an acidic tunic in *D. bermudensis*, could well explain its lack of palatability to pinfish.

Acknowledgements

We are grateful to Linda Cole at the Smithsonian Institution in Washington DC for her assistance with ascidian taxonomy. JBM acknowledges the support of an Endowed Research Professorship in Polar and Marine Biology through the University of Alabama at Birmingham.

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Table 1. Ascidians examined in the present study including their taxonomic classification, body organization, surface pH of the tunic, pH after the tunic was sliced with a razor, and the specific feeding deterrent bioassays performed. FT=fresh tissue, OE=organic extract. + = bioassay performed; - = no bioassay performed

				pH	pH	Bioassays	
Species	Family	Order	Organization	Surface	Cut	FT	OE
Aplidium stellatum	Polyclinidae	Aplousobranchia	Colonial	7	7	+	-
Botrylloides nigrum	Styelidae	Stolidobranchia	Colonial	7	7	+	-
Distaplia bermudensis	Holozoidae	Aplousobranchia	Colonial	1.5	1.5	+	+
Molgula occidentalis	Molgulidae	Stolidobranchia	Solitary	6.5	6.5	+	+
Styela plicata	Styelidae	Stolidobranchia	Solitary	7	7	+	+

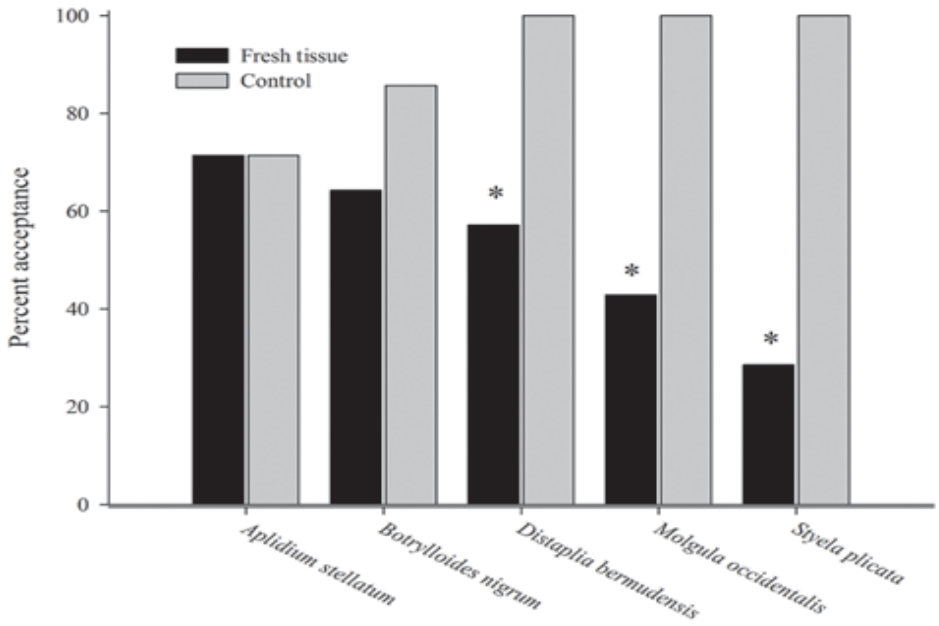

Figure 1. *Lagodon rhomboides*. Results of bioassays offering pieces of tunic tissue to the fish *L. rhomboides*. Asterisks indicate significant difference between tissue and control ($p<0.01$). In both experimental and control bioassays n = 14.

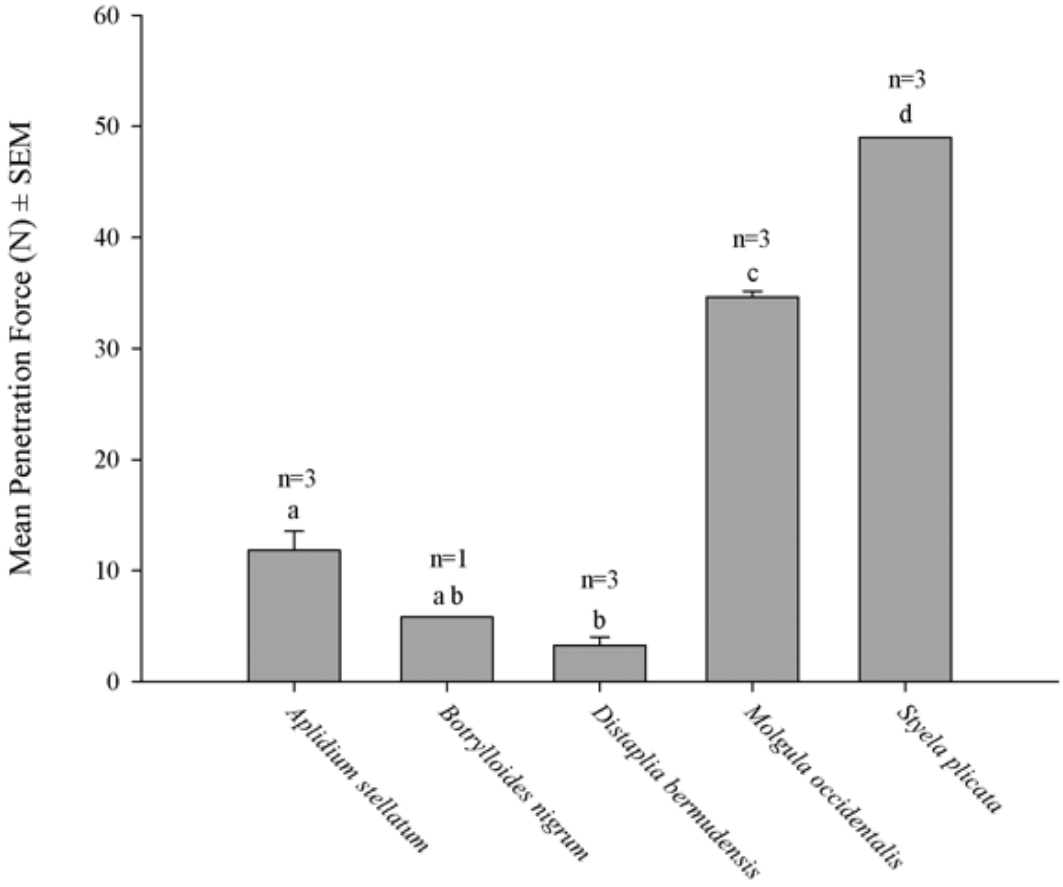

Figure 2. Mean + 1 SE of force required to penetrate the tunic of five species of ascidians. Bars with different letters are significantly different from one another (ANOVA; $p \leq 0.001$). n is given above each bar.

CONCLUSION

During our analysis of the overall palatability of the ascidians from the Western Antarctic Peninsula, we found that all of the ascidians collected in the shallow coastal waters of the Western Antarctic Peninsula were unpalatable to the omnivorous fish *Notothenia coriiceps*, and that 7 out of the 12 ascidians species were unpalatable to the omnivorous fish *Odontaster validus*. With the exception of the colonial ascidian *Distaplia colligans*, this unpalatablity was not found to be mediated by organic chemical defenses. A similar trend was observed in the ascidians found in the seagrass habitats of the Northern Gulf of Mexico, where 3 out of 5 species were unpalatable to the omnivorous pinfish *Lagodon rhomboides*, but the unpalatability was not mediated by organic chemical defenses. Whereas we were unable to test the palatability of the tissues of the Antarctic ascidians against the omnivorous amphipod *Gondogeneia antarctica*, only the organic extracts of the colonial ascidian *Distaplia cylindrica* caused feeding deterrence. These results show that the majority of the Antarctic and Sub-tropical ascidians examined do not rely on organic chemical defenses to deter predation by the most common generalist predators in their respective habitats.

The results of the inorganic acid experiments add some information to the long-standing debate on the effectiveness of sulfuric acid as a defense against predation. The feeding assays with the Antarctic and sub-topical generalist fish predators showed that these fish are unaffected by low pH. The sea star O. *validus*, on the other hand, exhibited a strong rejection response to the acidified pellets. As *O. validus* feeds by extruding its cardiac stomach and laying it against the outer surface of large sessile prey, inorganic acids that are sequestered on the outer tunic surface may be particularly effective against

the thin, exposed, sensitive tissues of the cardiac stomach. Moreover, sea star tube-feet are highly chemosensory, allowing them to perceive and evaluate potential prey and their defensive attributes. This suggests that inorganic acids are an effective defense mechanism against sea stars, an important predator group in the Western Antarctic Peninsula.

Although we did not measure the direct effects of tissue toughness on the ability to withstand the biting force exerted by the jaws of the pinfish *Lagodon rhomboides*, the results of the penetrometry analysis suggest that the tough outer tunic of some solitary ascidians can provide refuge from certain fish predators. Unlike tropical coral reefs, strong-jawed fish are not common in seagrass habitats, where there is an abundance of plant material, small crustaceans and other small invertebrate food. Similarly, durophagous fish are absent from Antarctic coastal waters. The tough outer tunic of certain ascidians, especially solitary forms, likely provides an effective defense against fish predation in these habitats. The unpalatability of the softer ascidians could be a result of low nutritional value, the presence of high concentrations of heavy metals, or a combination of 2 or more of the aforementioned defense mechanisms.

The role of the organic extracts against potential harmful microorganisms was also examined and several conclusions can be drawn. Bacteria isolated from the water column and internal tissues of organisms near the ascidians from the Western Antarctic Peninsula were mostly unaffected by the organic extracts isolated from the ascidians. This leads us to believe that although our sample size was not very large (20 bacterial isolates), bacterial pathogens do not appear to be a prevalent problem for the ascidians in this system, or alternatively, Antarctic ascidians did not evolve defenses against bacterial

pathogens. However, the generally high bioactivity against diatoms detected in the organic extracts (especially the lipophilic) from most of the ascidians suggests that potential detrimental effects of surface colonization by diatoms did drive the evolution of antifouling compounds in ascidians in this system. This study sets the groundwork for future studies that will try to identify the individual compounds responsible for the antifoulant activity. Developing new methods to measure the surface concentrations of these compounds, as well as performing in-situ experiments, will help us understand the actual ecological chemically mediated interactions as they occur in the field.

Overall, this study provides new insights into the prospective roles of organic and inorganic compounds and physical properties of the tunic in shaping benthic community structure in ascidian-mesograzer-macrograzer-epibiont complexes of nearshore environments of the Western Antarctic Peninsula, as well as ascidian-fish predator-prey interactions in coastal seagrass habitats of the Northern Gulf of Mexico.

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APPENDIX

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE APPROVAL FORM

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

NOTICE OF APPROVAL

DATE: August 22, 2008

TO: James B. McClintock, Ph.D.
CH-368 1170
FAX: 975-6097

FROM: Judith A. Kapp
Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee

SUBJECT: Title: An Examination of Secondary Metabolites and Inorganic Acids as Chemical Defenses Against Predation and Fouling in Antarctic and Sub-Tropical Ascidians.
Sponsor: Internal
Animal Project Number: 080808211

On August 22, 2008, the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) reviewed the animal use proposed in the above referenced application. It approved the use of the following species and numbers of animals:

Species	Use Category	Number in Category
Fish	A	20
Invertebrates	A	60

Animal use is scheduled for review one year from August 2008. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 080808211 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at 934-7692.

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

NOTICE OF APPROVAL

DATE: August 25, 2009

TO: McClintock, James B.
CH-368 1170
975-2525

FROM: Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee

SUBJECT: Title: An Examination of Secondary Metabolites and Inorganic Acids as Chemical Defenses Against Predation and Fouling in Antarctic and Sub-Tropical Ascidians.
Sponsor: Internal
Animal Project Number: 090808211

On August 25, 2009, the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) reviewed the animal use proposed in the above referenced application. It approved the use of the following species and numbers of animals:

Species	Use Category	Number in Category
Fish	A	20
Invertebrates	A	60

Animal use is scheduled for review one year from August 2009. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

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