Palatability and chemical anti-predatory defenses in common ascidians from the Antarctic Peninsula

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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.

KEY WORDS: Ascidian · Antarctica · Palatability · Chemical defense · Benthic ecology

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 nudibranches (Cimino & Ghiselin 2001) as well as several flatworms (de Caralt et al. 2002). Intense predation pressure on sessile marine invertebrates includ-

ing 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* cf. *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 (C. Aumack unpubl. data).

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 pal-

ability 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 and 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 (1997a). 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 × 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 to 16 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 2001). 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 seawater table ($1 \times 2 \times 0.25$ m depth and divided into 3 equal-sized compartments using fine-mesh dividers), or in individual tanks ($0.6 \times 0.6 \times 0.6$ m). Both the water table and tanks were equipped with flowing ambient seawater at 1°C. Initially, fish were maintained on a diet of limpet tissue (whole tissues of 2 *Nacella concinna* with 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 to 14 individuals were offered either a piece of fresh tissue (1 cm³) or a circular alginate food pellet (2 cm diameter $\times$ 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, 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 control 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).

Table 1. Antarctic ascidans 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

Species	Family	Order	Organization	pH	Fresh tissue F	Fresh tissue S	Fresh tissue A	Organic extracts F	Organic extracts S	Organic extracts A
Trididemnum sp.[a]	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 gaimardii	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	+	+	–	+	+	+
Microcosmos sp.	Pyuridae	Stolidobranchia	Solitary	7	+	+	–	+	+	+
Pyura georgiana	Pyuridae	Stolidobranchia	Solitary	7	+	+	–	+	+	+
Pyura setosa	Pyuridae	Stolidobranchia	Solitary	7	+	+	–	+	+	+
Cnemidocarpa verrucosa	Styelidae	Stolidobranchia	Solitary	8	+	+	–	+	+	+

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

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 ≤ 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 *Odon-*
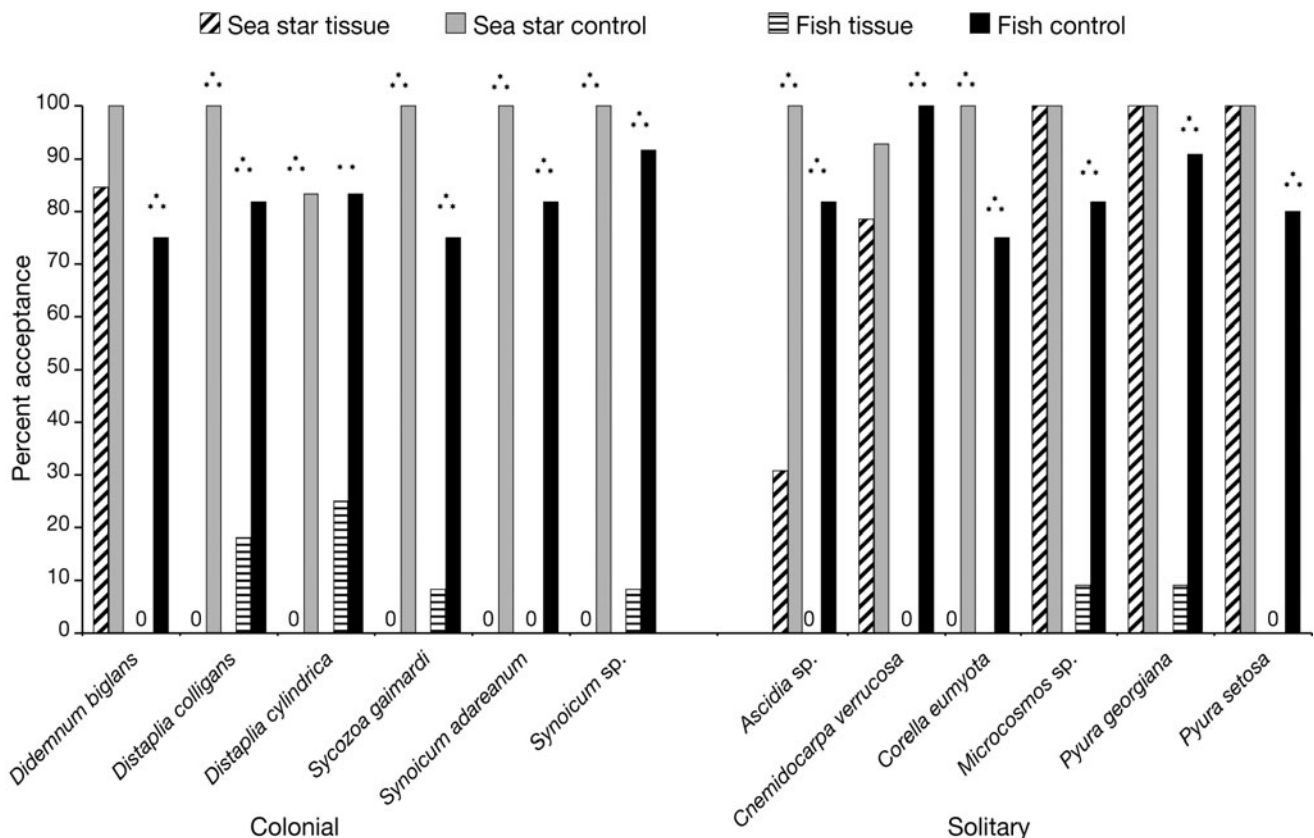

Fig. 1. *Odontaster validus* and *Notothenia coriiceps*. Results of bioassays offering pieces of ascidian tunic tissue to the sea star *O. validus* and the fish *N. coriiceps*. Asterisks indicate significant difference between tissue and control (Fisher's exact test). **p ≤ 0.01; ***p ≤ 0.005. Ascidians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

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 | | ——— Ratio ——— | |
	Lipophilic	Hydrophilic	WW:DW	WW:Vol
Trididemnum 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 gaimardii	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
Microcosmos 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

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. *Trididemnum* 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

taster 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.
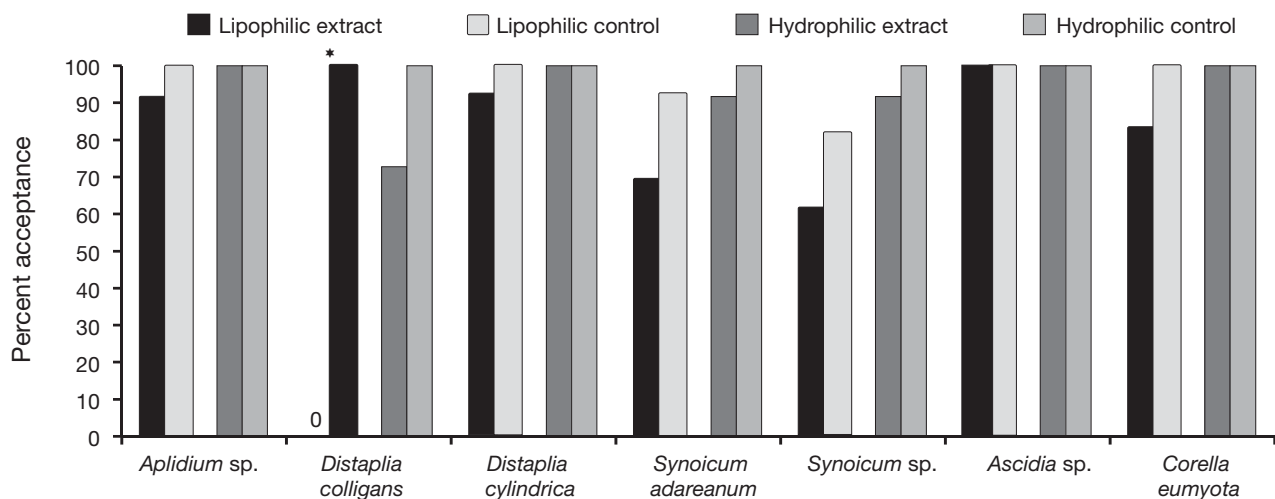

Fig. 2. *Odontaster validus*. Results of bioassays offering artificial foods containing lipophilic or hydrophilic ascidian extract to sea stars. Asterisks indicate significant difference between tissue and control (Fisher's exact test). *p ≤ 0.05
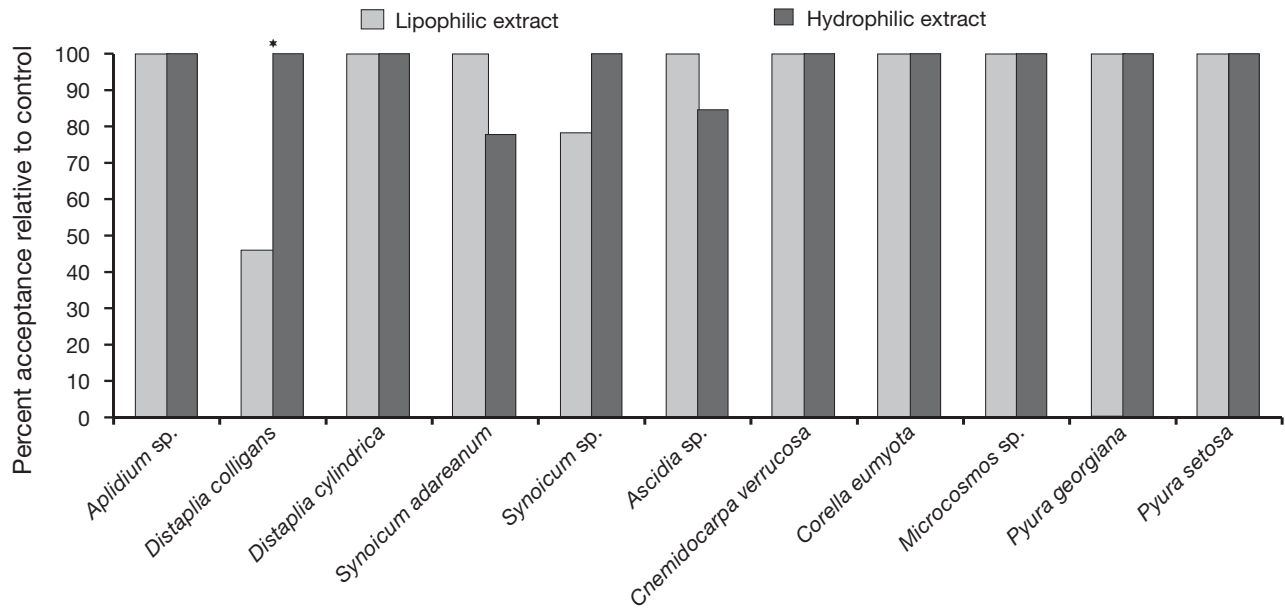
Fig. 3. *Notothenia coriiceps*. Results of bioassays offering artificial foods containing lipophilic or hydrophilic ascidian extract to fish. Asterisks indicate significant difference between tissue and control (Fisher's exact test). *p ≤ 0.05

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 (Day-

ton 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).
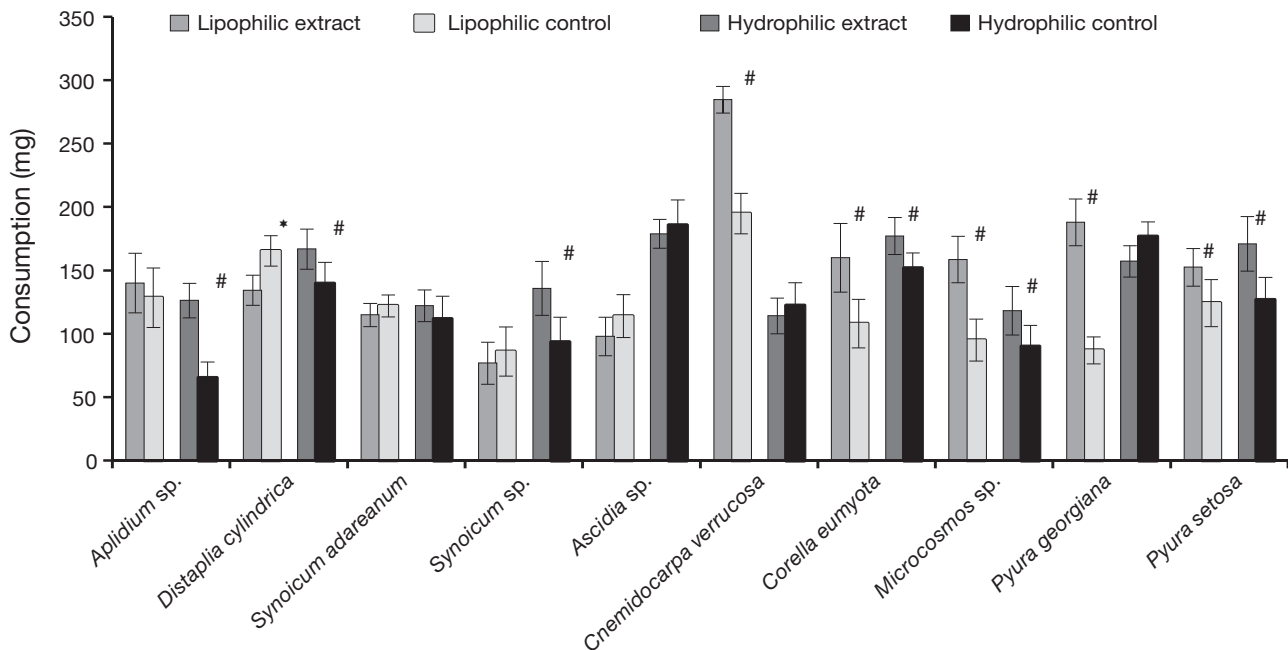

Fig. 4. *Gondogeneia antarctica*. Results of bioassays offering artificial food containing lipophilic or hydrophilic ascidian extract to amphipods. Values are means ± SEM. Asterisks indicate significant difference between extract and control (Wilcoxon signed-rank test) with controls significantly preferred to extract. *p ≤ 0.05. #: Significant differences (p ≤ 0.05) between extract and control (Wilcoxon signed-rank test), with extract significantly preferred to controls
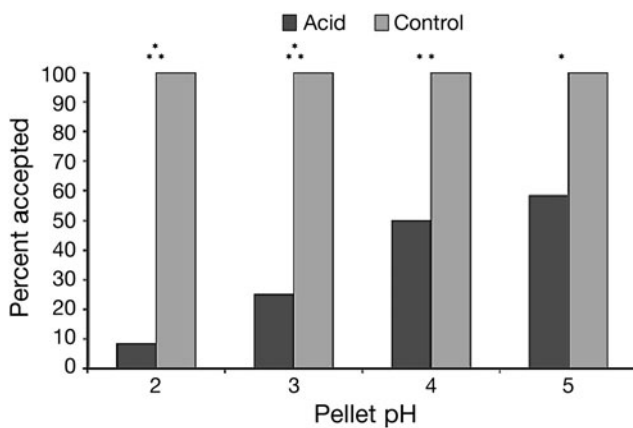
Fig. 5. *Odontaster validus*. Results of bioassays offering agar food pellets containing a 2% krill powder and acidified with sulfuric acid. Asterisks indicate significant difference between tissue and control (Fisher's extact test). *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.005

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 & 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 *Odontaster validus*. Similar bioactivity was not detected against *O. 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, 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 tissue 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 sea star *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) and sponges (Amsler et al. in press). 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, in press) 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 seawater, sulfuric acid-treated food pellets were not neutralized by seawater and retained 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,b, 2008, McClintock et al. 2005). 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

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