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Chemical and physical defenses against predators in *Cystodytes* (Ascidiacea)

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Abstract

Ascidians utilize both physical (spicules, tunic toughness) and chemical defenses (secondary metabolites, acidity) and suffer relatively little predation by generalist predators. The genus *Cystodytes* (Polycitoridae) is distributed widely in both tropical and temperate waters. Secondary metabolite composition, calcareous spicules and tunic acidity (pH < 1) may act as redundant defense mechanisms against predation in this genus. To assess the relative importance of chemical and physical defenses against predation in ascidians, we studied purple and blue morphs of *Cystodytes* from the western Mediterranean (formerly assigned to *Cystodytes dellechiajei*, but recently shown to belong to two different species), and a purple morph from Guam (USA), identified as *Cystodytes violatinctus*. Crude extracts, spicules, ascididemin (the major alkaloid of the blue morph) and acidity were used in feeding trials to evaluate chemical and physical defense mechanisms in *Cystodytes* spp. We performed feeding experiments in the field with a guild of generalist fish (mostly damselfish), and in the laboratory with a sea urchin and a puffer fish. Our results showed that all crude extracts and ascididemin significantly deterred fish predation, but not sea urchin predation. However, neither acidity alone nor spicules at natural concentrations deterred feeding. These results and other studies on sponges and gorgonians suggest that secondary metabolites are the primary means of defense against fish predators. Spicules and tunic acidity may perform other ecological roles and/or target certain specialist predators.

Keywords: Acidity; Ascidians; Ascididemin; *Cystodytes*; Defense mechanisms; Deterrence; Predation; Toxicity

1. Introduction

Benthic marine invertebrates are under intense competitive pressure for space, light and nutrients. In addition, sessile organisms are easy prey for predators. Many of these organisms, therefore, have developed a range of defense mechanisms, including behavioral (e.g. mimicry), physical (e.g. spicules, tissue toughness) and chemical (e.g. bioactive secondary metabolites, acid pH) strategies, to ensure survival. The relative importance and potential interactions of different defense mechanisms may depend on: the organism examined (Pennings and Paul, 1992; Hay et al., 1994), the inability of a single defense to deter all type of predators and/or competitors (Paul and Hay, 1986; Hay et al., 1987; Schupp and Paul, 1994; Pisut and Pawlik, 2002; Tarjuelo et al., 2002; Burns et al., 2003), the

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organism's life history stage (Uriz et al., 1996; Pisut and Pawlik, 2002), the developmental or physiological constraints that might prevent a particular defense from being used in all parts of the organism, necessitating additional defenses to ensure protection of the whole organism (Harvell and Fenical, 1989), evolutionarily established constraints (Schmitt et al., 1995; Kubanek et al., 2002) and whether structures or compounds are energetically expensive, among other factors. For instance, in some sponges, both large spicules and chemical defenses deter fish feeding (Pawlik et al., 1995; O'Neal and Pawlik, 2002; Burns et al., 2003; Burns and Ilan, 2003). Soft corals and gorgonians produce both chemical defenses and sclerites, which, in some cases, protect them against predators (Pawlik et al., 1987; Harvell et al., 1988; Wylie and Paul, 1989; Pawlik and Fenical, 1992; Puglisi et al., 2000, 2002; O'Neal and Pawlik, 2002). However, the primary function of sclerites may be structural rather than defensive (Koehl, 1982; Lewis and Von Wallis, 1991). In view of these and similar results, Van Alstyne et al. (1994) suggested that, when feeding experiments are designed, all defense mechanisms against predation that an organism might use should be tested in combination, rather than each single defense separately. However, mainly due to experimental limitations, few studies have attempted this (Paul and Van Alstyne, 1992; Schupp and Paul, 1994; O'Neal and Pawlik, 2002; Hill et al., 2005).

Among benthic invertebrates, ascidians suffer relatively little predation by generalist predators (Millar, 1971; Goodbody and Gibson, 1974; Stoecker, 1980a). These generalist predators are mainly fish (Randall and Hartman, 1968; Myers, 1983), and occasionally urchins (Briscoe and Sebens, 1988). Specialist predators on ascidians include mollusks, such as the lamellarians, cypraeids (Fretter and Graham, 1962; Millar, 1971; Lambert, 1980) and nudibranchs (Millar, 1971; Paul et al., 1990), and polyclad flatworms (Millar, 1971; Morris et al., 1980; Schupp et al., 1999; Caralt et al., 2002).

In addition, ascidians have both physical (spicules, tunic toughness) and chemical defenses (Swinehart et al., 1974; Stoecker, 1978, 1980b; Pisut and Pawlik, 2002; Tarjuelo et al., 2002). Numerous studies support the deterrent role of secondary metabolites (Paul et al., 1990; Davis, 1991; McClintock et al., 1991; Lindquist et al., 1992; Vervoort et al., 1998). High vanadium concentrations (Stoecker, 1980b) and low pH (Webb, 1939; Stoecker, 1978, 1980a,b,c; Pisut and Pawlik, 2002) have also been suggested as anti-predatory defenses. However, other studies indicated that neither the presence of vanadium nor an acidic pH prevented predation (Parry, 1984; Tarjuelo et al., 2002). All in all, little experimental evidence exists regarding the interaction of chemical and physical defense mechanisms in ascidians.

We chose the widely distributed ascidian genus *Cystodytes* (Aplousobranchiata: Polycitoridae) to assess the relative importance of physical and chemical defenses against predation in ascidians. López-Legentil et al. (2005a) identified two chemotypes within Mediterranean specimens of this genus, previously attributed to *Cystodytes dellechiajei* (Della Valle, 1877). Genetic and biological evidence indicates that these two chemotypes correspond to sibling species (López-Legentil and Turon, 2005; López-Legentil et al., 2005b). The first chemotype was characterized by the presence of C_9-unsubstituted pyridoacridines such as ascididemin (Fig. 1; Kobayashi et al., 1988) and 11-hydroxyascididemin (Schmitz et al., 1991), which were present in blue and green morphs from the western Mediterranean. The second chemotype contained the sulfur-containing pyridoacridines kuanoniamine D (Caroll and Scheuer, 1990), shermilamine B (Carroll et al., 1989) and their deacetylated forms (Eder et al., 1998; and López-Legentil et al., 2005a). These pyridoacridines were present in a purple morph from the western Mediterranean, and were also found in a purple morph from Guam, identified as *Cystodytes violatinctus* Monniot, 1988 (López-Legentil, 2005). Some of these substances are highly cytotoxic (Eder et al., 1998; Dassonneville et al., 2000; Bowden, 2000). Ascididemin also has some antibacterial and antifungal action (Lindsay et al., 1995). To our knowledge, apart from a possible antifouling function (Debard et al., 1998), no ecological role has been described for ascididemin. In addition to the bioactive compounds cited above, *Cystodytes* spp. colonies are highly acidic (pH < 2; Parry, 1984; Tarjuelo et al., 2002) and contain calcareous spicules encasing the zooids (Turon, 1987; Kott, 1990). These potential defenses seem to result in an effective defense mecha-

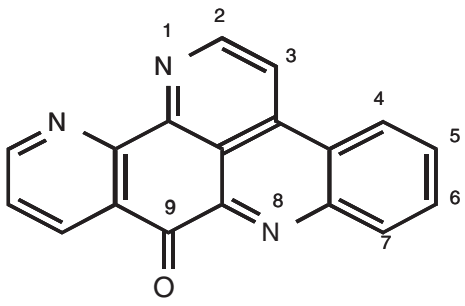

Fig. 1. Chemical structure of ascididemin, the major alkaloid of the Mediterranean blue morph.

nism (although their relative importance and interactions are not known), because *Cystodytes* spp. colonies generally lack epibionts and show scarce signs of predation. As the vanadium concentration in *Cystodytes* (≤10 ppm dry weight; Stoecker, 1980b) is well below the concentration suggested to deter predation in other ascidians (>1000 ppm dry weight; Stoecker, 1980b), it will be ignored in the present study. Tarjuelo et al. (2002) observed a deterrent activity of the crude extract of a blue Mediterranean morph against the hermit crab *Cestopagurus timidus*.

To assess the relative importance of chemical and physical defenses against predation in ascidians, we studied a purple and a blue morph from the western Mediterranean, corresponding to the two chemotypes explained above, and a purple morph assigned to *C. violatinctus* (Lambert, 2003) from Guam (USA). Crude extracts, asciddemin (the major alkaloid of the blue morph), spicules, and tunic acidity, as well as combinations of these, were tested against a naturally occurring guild of generalist fish (mainly the damselfish, *Abudefduf vaigiensis* and *A. sexfasciatus*) in the field, and the urchin *Diadema savignyi* and the puffer fish *Canthigaster solandri*, both in the laboratory.

2. Materials and methods

2.1. Ascidian samples

Specimens of the purple and blue morph of the ascidian *Cystodytes* were sampled by scuba diving in Catalonia (NE Spain, western Mediterranean) in 2002 and 2003. For a morphological description, see López-Legentil and Turon (2005). The purple *C. violatinctus* was collected in Togcha Channel on Guam (NW Pacific, USA) in 2003 and identified according to Monniot (1988) and Lambert (2003). All forms had disc-shaped calcareous spicules, ca. 1 mm in diameter. The purple Mediterranean morph had, in addition to discoidal spicules, small spherical spicules ca. 70 μm in diameter (López-Legentil and Turon, 2005). The spicules added to the artificial food were the discoidal ones or the discoidal plus the spherical ones, as indicated in each test.

2.2. Chemical extraction, spicule separation and concentrations assayed

Biologically active compounds of *Cystodytes* are restricted to the least polar fraction (Becerro et al., 1997), and are mainly pyridoacridine alkaloids (Bontemps, 1996; López-Legentil et al., 2005a). Colonies were weighed and freeze-dried. They were then extracted three times in a 1:1 (v/v) mixture of dichloromethane and methanol and the combined extracts were concentrated under vacuum to leave a powdery organic residue. The mean natural concentration of crude extract found was 2.01% (±0.21 S.E.M., $N=5$ for each color morph) per wet mass of the ascidian. We used this concentration, relative to hydrated artificial food, in the assays. We also tested asciddemin (the major alkaloid of the blue morph) synthesized in the University of Perpignan, following Bracher (1989). The asciddemin concentration used in feeding assays was 0.019% wet weight, which was in the lower half of concentrations found by López-Legentil (2005) after monitoring a natural population over 2 years, so our results would be, if any, more conservative.

Spicular weight per wet mass was calculated by averaging the concentration found in 4 independent colonies per morphotype. A piece of each colony was weighed, freeze-dried and then boiled in commercial bleach for several minutes until the tissue dissolved completely. Then, spicules were washed several times in water to remove organic remains, dehydrated in absolute ethanol and finally dried in an oven at 60 °C. The average spicular concentration found and used in our assays was 0.64% (±0.01 S.E.M.) of the wet mass of the ascidian. Several colonies of each morphotype were processed to obtain enough spicules to conduct the experiments.

It has been recognized that performing anti-predatory assays using dry mass ratios to determine concentrations of metabolites (or spicules) may be misleading, as predators consume hydrated material (Harvell et al., 1988; O'Neal and Pawlik, 2002) and differences in dry weight/wet weight ratios may translate into important differences in the effective concentrations assayed when using dry weights. Volumetric concentrations and wet weight concentrations do not suffer from this problem. The two coincide when the density of the source organism and the artificial food is the same. We chose to work with wet weight concentrations because, assuming that a predator consumes a given weight of hydrated material per day, wet weight ratios may accurately reflect the amount of metabolites/spicules that a predator ingests. We performed comparative assays with asciddemin using volumetric and wet weight ratios and found similar outcomes (see Results). In any case, as the densities of our artificial foods were slightly lower (cubes for field assay = 1.06 g ml^{-1}; sea urchin food = 1.16 g ml^{-1}; puffer fish food = 1.15 g ml^{-1}) than that of the ascidian itself (1.23 g ml^{-1}), our results are conservative as we would be adding

Table 1
Artificial food recipes for damselfish, puffer fish and sea urchins

Recipes	Damselfish	Puffer fish	Urchins
Agar (g)	1.25	0.15	0.25
Carageenan (g)	1.25	0.15	0.25
Catfish (g)	5	2	2
Enteromorpha sp.	–	–	1
Water (ml)	80	18	25

Agar (Sigma no. A-6924), carageenan (Sigma no. C-1013), Kruse's Perfection Brand catfish and freeze-dried *Enteromorpha* sp.

16%, 6%, and 7% more metabolites/spicules, respectively, had we worked with volumetric ratios.

2.3. Field assays – generalist predators

Field bioassays were conducted at a depth of 8 m at Gun Beach (Guam, USA). We conducted a feeding deterrence experiment using the field method of Schupp and Paul (1994). The following components were added to an artificial diet at the wet weight concentrations mentioned above (see Table 1): crude extract, ascididemin, disc-shaped spicules alone or with spherical spicules, and combinations thereof. For comparison, the test with ascididemin was run at volumetric and wet weight concentrations. In addition, to test the role of low pH, 800 μl of sulfuric acid were added to obtain pH<1. Preliminary trials were run to monitor the changes in acidity over time in acid-treated food. When solvents were required to dissolve crude extracts or ascididemin in the treated food, the same amount of solvent was added to the control. The mixture was poured into 1-cm^3 molds containing a rubber O-ring, which allowed the use of safety pins to attach cubes to ropes. Each rope contained either 4 controls or 4 treated food cubes. We placed a total of 24 pairs of control and treated ropes on the reef, one pair at a time. In all treatments, each pair was removed when approximately half the cubes had been eaten. We used Wilcoxon's signed-rank test for paired comparisons to test for significant differences in the number of cubes eaten between control and treated food. For the acid treatments, the whole experiment was limited to 35 min and a mean of 20 pairs of ropes because the acidity of the cubes changed from pH 1.5 at time 0 to pH 3.5 after 40 min in seawater (Fig. 2). To measure pH of the cubes, we followed the method of Davis and Wright (1989). Each cube measured was placed on Sigma® pH test strips (precision=0.5 units) and pressure was applied until fluids were extruded. We then monitored the resulting color change of the paper. When acid and spicules were combined, a slight bubbling in the cubes was observed under the stereomicroscope. However, although some calcium carbonate was probably dissolved with the acid, no change in the spicular shape was observed and the pH increased only slightly at time 0 (~0.5). The addition of crude extract or ascididemin to the artificial food did not have any obvious effect on the original color of the food.

2.4. Laboratory feeding assays – benthic predators

The sea urchin *D. savignyi* is very common on coral reefs around Guam and is normally found in shallow waters (0.5 m to 3 m). Sea urchins are mainly grazers that feed on algae, but are usually referred to as generalist predators that also feed on a variety of invertebrates (Barnes, 1987; Briscoe and Sebens, 1988). *D. savignyi*

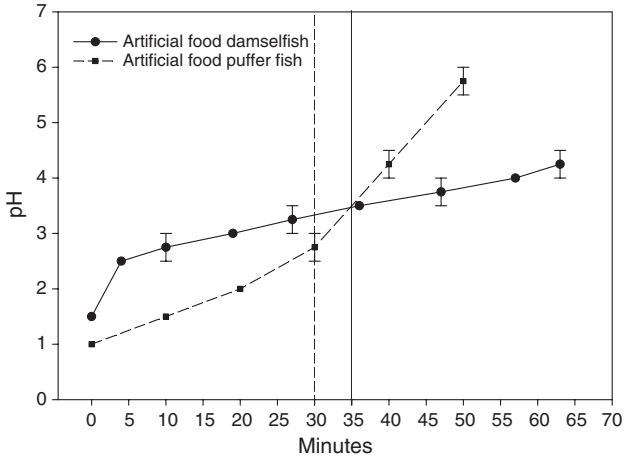

Fig. 2. pH variation of artificial food over time. Vertical straight line indicates the ending time of the in situ acid tests with damselfish. Vertical dashed line shows the end time of puffer fish tests. Bars indicate standard errors.

adapts poorly to laboratory conditions and has to be kept in separate tanks to avoid mortality (up to 75%, if they are kept together). Therefore, each sea urchin was placed in a 30-l flow-through tank and repeatedly offered artificial food until it was used to feeding on it. The tanks were regularly cleaned to prevent algal growth. Artificial food was prepared, as indicated in Table 1. Treatments were prepared by addition of one of the following: crude extract, ascididemin and disc-shaped spicules, at the indicated wet weight concentrations. The mixture was heated and poured into a mold backed with aluminum window screening to form a strip that covered a mean of 325 squares of the window screening. Prior to experiments, the urchins were not fed for 24 h. Although it is known that some animals change their feeding preferences when they are starved, we used this fasting period to homogenize the state of the sea urchins. A deterrent food (note that we are performing deterrence assays, not preference ones in which test animals have food choice) should not be consumed, even if the test organism is starved. If there were any discrepancy, the results using slightly starved urchins would be more conservative. Experimental sets consisted of 14 urchins that were offered either treatment or control screens (7 replicates each). Urchins were allowed to feed for one day and two nights. We used either *t*-tests or Mann–Whitney analyses (if the assumptions of normality or heteroscedasticity failed) to identify significant differences in the number of squares eaten between control and treated food.

Due to the duration of this experiment, assays with acid were precluded. Prior to experiments, we performed some preliminary tests to ensure (via TLC) that crude extract and ascididemin were retained in the artificial food for the entire experiment.

The puffer fish *Canthigaster solandri* is a benthic predator that feeds on benthic algae, ascidians, and other invertebrates (Amesbury and Myers, 1982). It adapts well to laboratory conditions and has been previously used for deterrence tests (Rogers and Paul, 1991; Becerro et al., 1998). Artificial food was prepared as indicated in Table 1. Treatments were prepared by addition of crude extract, ascididemin, disc-shaped spicules and some combinations thereof (each at wet weight concentrations). For treatments involving acidification, 150 μl sulfuric acid was added to obtain $pH < 1$. The changes in acidity over time in acid-treated food were monitored in preliminary assays. The mixture was heated for 30 s in a microwave, and after addition of the defense mechanism tested, the whole was poured into a mold backed with fiberglass window screening to form a strip that covered a mean of 277 squares of the window screening. Puffer fish were placed separately in 30-l flow-through tanks and normally started feeding on control food within one to two weeks. The tanks were cleaned regularly to prevent algal growth. Prior to the experiment, the fish were starved for 48 h to homogenize their state. Experimental sets were composed of 14 fish, and were offered either treatment(s) or control screens (7 replicates each). The fish were allowed to feed for 1 h, in which time approximately half the squares were eaten in any of the treatments. If the treatments contained sulfuric acid, the fish were allowed to eat for 30 min, as pH reached values of ~ 3 after that time (the pH of 2 extra screens submerged in empty tanks was recorded at the end of each test; Fig. 2). We used either *t*-tests or Mann–Whitney analysis (if the assumptions of normality or heteroscedasticity failed) to identify significant differences between the number of squares eaten in the control and in the treatment.

3. Results

3.1. Field assays – generalist predators

At Gun Beach (Guam, USA) the damselfish *Abudefduf vaigiensis* and *Abudefduf sexfasciatus* were the main consumers during experiments, although there was occasional consumption by the wrasse *Thalassoma lutescens* and the triggerfish *Balistapus undulatus*. When a new treatment set was placed, fish nibbled randomly the cubes presented to them; therefore, the final choice did not depend upon which cube was tasted first. Ascididemin, which was tested in both volume and wet mass ratios, and ascididemin combined with spicules deterred feeding by reef damselfish ($p < 0.001$ in all cases; Fig. 3). The consumption of treated vs. control food in ascididemin treatments at volume and wet weight ratios was virtually the same (0.60 ± 0.07 and 0.59 ± 0.10, respectively; mean ± S.E.M.). The three crude extracts deterred fish feeding significantly more than controls ($p < 0.001$ in all cases; Fig. 3). The disc-shaped spicules, which are typical of the genus, alone or combined with the spherical spicules found in the purple Mediterranean morph, did not deter predation ($p = 0.48$ and $p = 0.56$, respectively). Cubes with sulfuric acid or a combination of sulfuric acid with spicules did not deter fish feeding. In addition, when we added sulfuric acid to ascididemin, or to ascididemin and spicules, no deterrent effect was found ($p = 0.25$ and $p = 0.07$, respectively). However, when cubes were treated with sulfuric acid, the fish displayed flushing behavior in

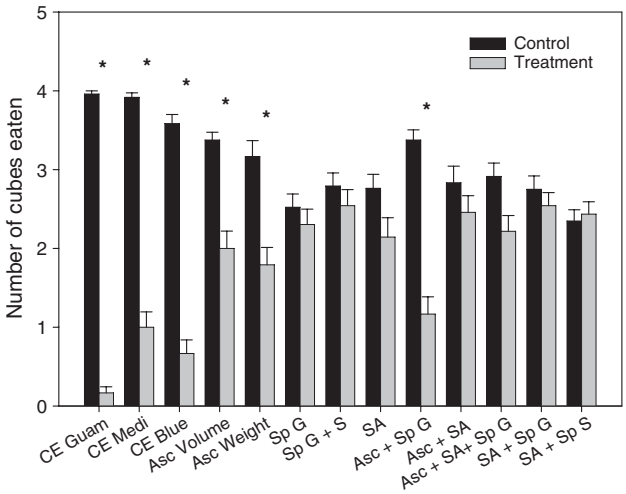

Fig. 3. Number of cubes eaten for each treatment by the damselfish *Abudefduf vaigiensis* and *Abudefduf sexfasciatus* during the non-choice assays. Codes for treatments: CE Guam, crude extract of the purple Guam morph; CE Medi, crude extract of the purple Mediterranean morph; CE Blue, crude extract of the blue Mediterranean morph; Asc Volume, ascididemin in a volume ratio; Asc Weight, ascididemin in a mass ratio; Sp G, disc-shaped spicules typical of the genus; Sp S, spherical spicules found in addition to disc-shaped ones in the purple Mediterranean morph; SA, acidified with sulfuric acid. Asterisks show significant differences. Bars indicate standard errors.

which they repeatedly swallowed and regurgitated the food cubes.

3.2. Laboratory feeding assays – benthic predators

D. savignyi showed no significant differences in consumption of control food or in any of the treatments offered (Fig. 4). Indeed, the urchins appeared to move randomly in their tanks at night and eat whenever they found a screen, regardless of its content: aluminum screens were sometimes damaged by urchin bites. A period of two nights was needed for at least 90% of the sea urchins to encounter the screen.

All crude extracts tested, whether alone or in combination with acid or spicules, significantly deterred predation by the puffer fish *Canthigaster solandri* (Fig. 5). As all fish sampled the offered food, in no case was any negative result (zero consumption) recorded. Ascididemin, alone or with spicules, also significantly deterred predation ($p=0.023$ in both cases), although, as happened during field experiments with the damselfish, the addition of sulfuric acid seemed to counteract somehow the deterrent effect of the alkaloid plus spicules, yielding no significant deterrent effect ($p=0.073$). No significant differences were found between consumption of control and treated food

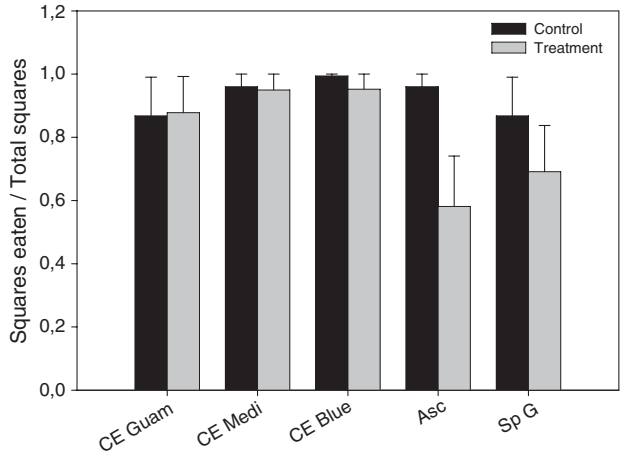

Fig. 4. Number of squares eaten by the sea urchin *Diadema savignyi* per total number of squares during the non-choice assays. *X*-axis labels as in Fig. 3. Bars indicate standard errors.

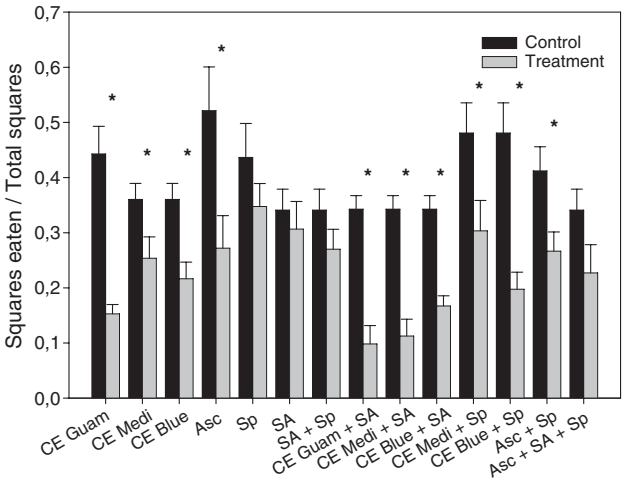

Fig. 5. Number of squares eaten by the puffer fish *Canthigaster solandri* per total number of squares during the non-choice assays. Sp indicates disc-shaped spicules characteristic of the genus; the other *X*-axis labels are as in Fig. 3. Asterisks show significant differences. Bars indicate standard errors.

containing spicules, acid or a combination of the two ($p > 0.1$ in all cases).

4. Discussion

Our study showed that crude extracts of the purple and blue Mediterranean morphs of *Cystodytes*, and of the purple *C. violatinctus* from Guam, as well as ascididemin, the major alkaloid of the blue morph, significantly deter fish but not sea urchin predation. In contrast, acidity and spicules by themselves deterred neither fish nor urchin feeding. All crude extracts gave similar results, although the alkaloid composition of the purple forms (from Guam and the Mediterranean) and the blue Mediterranean morph were qualitatively different in their pyridoacridine composition (see Introduction). Our results suggest, therefore, that both chemotypes have evolved into an effective defense mechanism against predation.

Crude extracts alone significantly deterred damselfish feeding in field assays and puffer fish feeding in the laboratory. However, while damselfish left many cubes nearly untouched, puffer fish repeatedly sampled the offered food. Puffer fish are benthic predators and occasionally feed on ascidians (Amesbury and Myers, 1982). Therefore, they may be more used or tolerant to amino acid-derived metabolites such as alkaloids, the main defensive compounds in ascidians (Davidson, 1993; Molinski, 1993). However, the addition of acid to the combination of ascididemin and spicules made the mixture palatable. This loss of deterrence might be due to the nitrogen atoms of ascididemin becoming protonated at low pH. Indeed, in the living ascidian, acidic vacuoles are located in bladder cells (Webb, 1939; Hirose, 1992), metabolites seem to be stored in pigment cells (Turon et al., 2005), and spicules are found in the tunic matrix encasing the zooids (Turon, 1987; Kott, 1990). Thus, all the potential defense mechanisms are located in separate compartments. In addition, acidity by itself or in combination with spicules did not deter fish feeding, although fish were observed to repeatedly regurgitate and swallow the food before they finally consumed it. When present, calcareous spicules may cause an extremely rapid neutralization of acid and, even without spicules, seawater may be a sufficient buffer to quickly neutralize the acid (Parry, 1984) and thus allow fish to feed.

Burns and Ilan (2003) found that deterrence in sponges was linked to spicule size, and only those spicules larger than 250 μm deterred predation. Chanas and Pawlik (1995) found no significant deterrence caused by siliceous spicules in sponges and suggested a deterrent role of sclerites of calcium carbonate related to an alteration of pH in the acidic gut of putative predators. A similar defense mechanism was previously suggested by Schupp and Paul (1994) for calcified algae. The disc-shaped spicules, up to 1 mm in diameter, characteristic of the genus *Cystodytes*, represent 0.64% of the wet mass of the ascidian. However, as observed by Lindquist et al. (1992) for other ascidian

species, spicular composition and concentration did not significantly deter fish or sea urchin predation. In artificial food, spicules were distributed at random, whilst in the living ascidian they encase each zooid. In fact, spicules form a compact and relatively hard capsule that lodges around the abdomen and into which the thorax can be retracted as a response to any disturbance. Therefore, although the disc-shaped spicules did not act as a deterrent in artificial food, they may help protect the zooid in living colonies. In contrast, no role could be attributed to the spherical spicules found in the Mediterranean purple morph, as they measured only ~70 μm in diameter, are scarce and are randomly distributed throughout the tunic (López-Legentil and Turon, 2005). Calcareous spicules may also function as a shield to protect zooids from the acid release that may occur during cell rupture. This suggestion was supported by stereomicroscope observation of bubbling in broken colonies, presumably caused by a neutralization of acid as a result of calcium carbonate (spicules) dissolution.

Previous studies found that chemical defenses deterred feeding by some, but not all, predators assayed (Paul and Hay, 1986; Hay et al., 1987; Schupp and Paul, 1994; Pisut and Pawlik, 2002; Tarjuelo et al., 2002; Burns et al., 2003). Similarly, our results show that all crude extracts of *Cystodytes* deter fish feeding, but not sea urchin grazing. Indeed, urchin feeding seemed to be based on how likely they were to find the screen, rather than on any deterrence associated with the crude extracts, ascididemin or the spicules. Acidity is commonly found in combination with other defense mechanisms, and Stoecker (1980a) and Pelletreau and Muller-Parker (2002) suggested that acidity had a major deterrent function against crawling predators, because of the rupture of acid vesicles when they contacted the ascidian surface. As we could not test acidity with sea urchins, a role for acidity in deterring sea urchin predation cannot be ruled out. However, we have occasionally observed urchin bites in the purple and blue morphs of *Cystodytes* from the Mediterranean. Consumption by Mediterranean sea urchins was confirmed by the observation of disc-shaped spicules in the gut of urchins close to grazed *Cystodytes* spp. colonies.

As well as deterring predators, defense mechanisms often act at other levels, such as fouling avoidance or space competition (Stoecker, 1980a; Schmitt et al., 1995; Becerro et al., 1997). Furthermore, they may act at different life history stages, as suggested by Uriz et al. (1996) and Pisut and Pawlik (2002). At the same time, their multiple functions may constrain their evolution (Schmitt et al., 1995; Kubanek et al., 2002), meaning that there is always scope for the evolution of specialist predators able to circumvent the defense mechanisms of a given species.

In conclusion, secondary metabolites seemed the most effective defense mechanism of *Cystodytes* spp. against fish predators but did not deter sea urchins. Although spicules and tunic acidity may perform other ecological roles and/or target certain specialist predators, our inability to keep low pH for more than 1 h and to arrange the spicules in capsules inside artificial food prevented us from drawing definite conclusions as to their ecological role. Our results and other studies of sponges and gorgonians (Pawlik et al., 1995; Koh et al., 2000; O'Neal and Pawlik, 2002; Puglisi et al., 2002) indicate that secondary metabolites are the primary means of defense against fish predators. In addition, our study highlights the importance of considering all possible defenses of an organism against as many potential predators as possible, in order to reliably assess their ecological roles.

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