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Defence mechanisms of adults and larvae of colonial ascidians: patterns of palatability and toxicity

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ABSTRACT: We studied the defence patterns of 6 species of colonial ascidians from the Mediterranean Sea. We tested their palatability to common sympatric predators (fishes and crustaceans). The tests were performed separately for the main compartments (zooids and tunic) of the adults and for larvae. For compartments that proved unpalatable, tests were repeated with chemical extracts. We also tested toxicity with the Microtox method. Total energy content, amount of inorganic material and pH were analysed for all compartments. We looked for patterns of defence among species and compartments, and for relationships of adult/larval palatability, of toxicity/palatability, pH/palatability and food quality/palatability. Overall, we found a high variability among species, compartments and predators, but unpalatability was found in all species in at least 1 test. In general, tunic material was the least and zooid material the most palatable. Only 1 of the species studied had an acidic tunic. Toxicity was in general low and not related to palatability, while energy content was positively related to the latter. The larvae of species with low per zooid fecundity and large larvae (*Cystodytes dellechiajei*, *Polysysncraton lacazei*, *Diplosoma spongiforme* and *Pseudodistoma crucigaster*) were unpalatable to at least 2 of the predators, while larvae of the 2 species with higher fecundity and smaller larvae (*Clavelina lepadiformis* and *Ecteinascidia herdmanni*) were the most palatable. There was no relationship between adult and larval palatability. Tests with extracts substantiated a chemical basis for unpalatability in only a few cases, and there was a pattern of increased palatability of tissues with higher energy content and lower amount of structural material. We conclude that the defence strategies of colonial ascidians are highly variable among species (even of the same family), and that unpalatability may be relatively common and provided by either physical or chemical mechanisms, but that allocation to defence varies between compartments and between ontogenetic states (larvae or adult).

KEY WORDS: Chemical defence · Toxicity · Palatability · pH · Caloric content · Larval defence · Ascidians

INTRODUCTION

Defence mechanisms against predation in adults and larvae of marine invertebrates can be structural, behavioural or chemical (Young & Chia 1987, Pawlik 1993, van Alstyne et al. 1994, Lindquist & Hay 1996). Lack of susceptibility to predation can be due to the production of deterrent substances or to other factors such as structural elements, tissue toughness, and nutritional quality, which have seldom been considered in studies of defence (Harvell & Fenical 1989, van Alstyne et al. 1994, Chanas & Pawlik 1995). Moreover, it has been known for some time that deterrent substances need not be toxic (Schulte & Bakus 1992, Pawlik 1993, Pawlik et al. 1995) and can go undetected in standard toxicity tests. To ascertain the defence strategy of a given species we should, therefore, perform palatability tests (with samples and with their extracts) with ecologically relevant organisms and relate the results to data on the quality as a food of the species.

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Moreover, the within-specimen location of bioactive compounds in marine organisms can provide information on ecological functions. For instance, substances which act in spatial competition are expected to be somehow released into the environment (Uriz et al. 1996a), while antipredatory substances may be concentrated in more vulnerable or valuable parts of the organism (Hay et al. 1988, Paul & van Alstyne 1988). Yet few studies have studied separately the defence mechanisms of the distinct compartments of marine organisms (Hay et al. 1988, Fontana et al. 1993, 1994, van Alstyne 1994, Uriz et al. 1996a,b, Turon et al. 2000).

Adults and larvae of a given species may use distinct physical and chemical defence strategies (Lindquist et al. 1992, Uriz et al. 1996c). Unpalatable larvae do not always come from unpalatable adults (Lindquist & Hay 1996). Larval defence may be associated with the particular reproductive strategy of the species rather than the defence mechanism of the adult. Indeed, larval defence may be a key factor in the evolution of complex life cycles (Roughgarden 1989, Lindquist et al. 1992, Hay 1996, Lindquist 1996). Defence of eggs and/or larvae has been demonstrated or suggested for several groups (e.g. sponges: Uriz et al. 1996c; corals: Sammarco & Coll 1988; echinoderms: McClintock & Vernon 1990; molluscs: Pawlik et al. 1988; ascidians: Young & Bingham 1987, Lindquist et al. 1992, Lindquist & Hay 1996). Low larval and post-metamorphic survival is indeed a bottleneck in the population dynamics of benthic species (Rumrill 1990, Gosselin & Qian 1997, Hunt & Scheibling 1997).

Among benthic invertebrates, ascidians show one of the strongest bioactivity levels, and cytotoxic, antimitotic and antiviral properties have been found in extracts of their tissues (Uriz et al. 1991). Adult ascidians have been reported to present both physical and chemical defences (Stoecker 1978, 1980a,b, Young 1985, Teo & Ryland 1994). However, only rarely has the chemical compound responsible for the defence mechanism been identified (Vervoort et al. 1988, Davis 1991). Evidence of chemical defence mechanisms in the larvae of these marine invertebrates has also been found: larvae of *Ecteinascidia turbinata* were rejected by several fish species in the field (Young & Bingham 1987), and secondary metabolites from larvae of the ascidian *Tridemnum solidum* had deterrent effects on fishes in the laboratory (Lindquist et al. 1992).

In ascidians, besides structural defences and secondary metabolites, high vanadium concentrations and acidic pH in the tunic were suggested to play a role in preventing predation or fouling on some species (Stoecker 1978, 1980a,b, Thompson 1988). The biological significance of vanadium in ascidians remains problematic (Martoja et al. 1994), and a protective function seems unlikely, or perhaps limited to only certain species (Kustin et al. 1983, Parry 1984, Martoja et al. 1994). Several studies also cast doubt on the general effectiveness of pH as a defence mechanism (Parry 1984, Davis & Wright 1989).

The main ascidian predators known in nature are flatworms, crustaceans, gastropods, starfish and fishes (Millar 1971, Monniot et al. 1991). There are even instances of predators sequestering and using secondary metabolites from ascidians for their own defence: e.g. gastropods (Paul et al. 1990) and flatworms (Kubanek et al. 1995). For larvae, fishes and cnidarians (zoanthids and corals) are the common predators (Olson & McPherson 1987, Davis & Butler 1989, Svane & Young 1989, Stoner 1990). Predation on ascidian larvae and adults is a key factor determining population distribution and structure (Young & Chia 1984, Stoner 1990, Osman & Whitlatch 1998). It has been proposed (Lindquist et al. 1992, Lindquist & Hay 1996) that species producing a few large and conspicuous larvae tend to endow them with some kind of defence mechanism that renders them unpalatable. In this way, these species can compensate the higher mortality risk arising from the increased perceptibility and demersal habits of the larvae (Strathmann 1985, Rumrill 1990).

The objective of our study was to describe patterns of defence in several ascidian species, considering separately different compartments of the adults (zooids, tunic) and the larvae. We also sought to relate palatability levels with toxicity, caloric contents, inorganic contents and the pH of the different compartments.

MATERIALS AND METHODS

Species studied. In the present study, we selected 6 species of colonial ascidians from among the most abundant in sublittoral benthic communities of the northwestern Mediterranean. These species span a wide range of fecundity (number of larvae produced per zooid) and zooid size.

The species belonged to the order Enterogona, and included the Didemnidae *Polysyncraton lacazei* (Giard, 1872) and *Diplosoma spongiforme* (Giard, 1872), the Polycitoridae *Clavelina lepadiformis* (Müller, 1776) and *Cystodytes dellechiajei* (Della Valle, 1877), the Polyclinidae *Pseudodistoma crucigaster* Gaill, 1972 (orange morphotype) and the Perophoridae *Ecteinascidia herdmanni* (Lahille, 1870). All the species were collected during 1998 at Tossa de Mar (41° 43.2' N, 2° 56.4' E, northwestern Mediterranean). Sampling was performed by SCUBA diving along the rocky coast at a depth of between 3 and 12 m. The habitat consisted of vertical and subvertical walls; those facing south were dominated by seaweeds while those facing north had a high percentage of invertebrate cover, mainly

sponges. For a more complete description of the communities see Turon (1990) and Turon et al. (1996).

Colonies of each species were collected at various stages of their reproductive cycle. During samplings, we took care to collect colonies separated by at least 2 to 3 m, in order to avoid collection of clonemates. They were taken to the laboratory and frozen or freeze-dried for the various analyses. Mature larvae were obtained from brooding colonies.

Feeding trials using raw material. For feeding assays, colonies were dissected in the laboratory, and 4 compartments were separated (mature larvae, thorax, abdomen and tunic). For the polyclinid *Pseudodistoma crucigaster*, the abdomen and postabdomen were combined for the tests. For *Ecteinascidia herdmanni*, which lacks a separation between thorax and abdomen, we distinguished only zooid and tunic. The tunic treatment consisted of the tunic of a zooid or, in species with a common embedding tunic, as a fragment approximately the same size of a zooid.

The assays were carried out using 4 predators, each of which was selected because it represented a potential source of predation and because of its abundance in the study area. The damselfish (Pomacentridae) *Chromis chromis* (Linnaeus, 1758) stays in the water column but close to the rocky bottom, so that swimming larvae of benthic organisms represent a large source of potential prey. This fish belongs to the category of microphagic carnivores and its diet includes predominantly organisms from the plankton (Bell & Harmelin-Vivien 1983); pomacentrids are prominent predators of ascidian larvae (Olson & McPherson 1987). The blenniid fish *Aidablennius sphinx* (Valenciennes, 1836) is a bottom feeder, and consumes a variety of prey, mainly small mobile invertebrates (Whitehead et al. 1986, N. Reventos pers. comm.). Larvae swimming close to the bottom are vulnerable to it. The hermit crab *Cestopagurus timidus* (Roux, 1830) and the snapping prawn *Alpheus dentipes* Guérin, 1832, 2 common crustaceans in the study zone, were selected as benthic predators liable to attack both settling larvae and adults. In accordance with their ecological distribution, fishes were used to assay larval palatability, while crustaceans were used to test both larval and adult palatability.

Assays with *Chromis chromis* were carried out in the field at Tossa de Mar. The remaining predators were collected at Tossa de Mar and the nearby locality of Blanes (41° 40.4′ N, 2° 48.2′ E); they were taken to the laboratory, where they were conditioned for some weeks before the assays. In particular, they were adapted to feed on a commercially available fish food in the form of small pellets (Sera granumarin®). Each blenniid (ranging in size between 5 and 8 cm) was placed in a plastic bowl (volume 0.5 l) which was kept at constant temperature (15°C) in a running-water system. The crabs and snapping prawns (ranging between 0.8 and 2 cm length) were kept individually in multiwell plates (5 ml well volume) in filtered seawater at a constant temperature of 17°C.

Field bioassays with the damselfish *Chromis chromis* were carried out for the mature larva of 4 of the 6 species of ascidians (*Polysyncraton lacazei*, *Cystodytes dellechiajei*, *Diplosoma spongiforme*, *Pseudodistoma crucigaster*). The larvae of the other 2 ascidian species were too small to be detected by fishes in the field, even when stained with commercial red food dye. Larvae used in the tests were a mixture of larvae from at least 5 colonies of each species. Larvae and control pellets were released underwater in 100 ml syringes. Fishes were first fed with control pellets to accustom them to feed from the syringes. Within a few minutes, a high concentration of predators would cluster around the diver and readily consume all the pellets released. When the test started, single larva and control pellets were alternately offered to the fishes. The trajectory of each food item (a larva or a control pellet) was visually followed. Larvae were eaten, rejected or ignored by the fishes (in this case the larvae eventually sank to the bottom). Larvae that were ignored were not considered in the analysis, since the fishes probably did not see them. All control pellets were consumed. To minimise the likelihood that the same fish repeatedly ate the food items, and to ensure independency of the replicates, the syringes were discharged in different directions each time. Each single larva plus a control pellet was considered a replicate and between 20 and 30 replicates were run for each species. The results of this test were examined by Fisher's exact test as a 2×2 table representing the number of larvae and control pellets consumed or rejected by fishes.

Laboratory tests with the blenniid fish and the crustaceans were performed under 2 experimental conditions: starved (predators starved for 2 d prior to the experiment) and non-starved (predators fed about 8 h before the test). The treatment consisted of giving each crustacean/fish a single larva, thorax, abdomen or tunic piece, and a positive result (consumption) was scored when the predator ate the whole item. An exception was made with *Clavelina lepadiformis* larvae: because of their small size, 5 of these were given to each predator. In this case, a positive result was scored when the predator ate at least 3 of the 5 larvae. The treatments were added to the wells with the predators, which were left to feed undisturbed for 4 h. A control food pellet was then given and its consumption recorded after a further 4 h period. Replicates in which the predator did not eat any of the 2 food items offered were not considered, as this outcome was attributable to lack of hunger not to any effect of the food items themselves.

True replication was assured by using materials from different colonies for each replicate. Thus only 1 thorax, abdomen, tunic piece or larva per colony was used in each test. Materials from the same colony were used, however, for the different predators and feeding conditions, but each colony was used only once in each test. From 12 to 20 replicates, and hence the same number of different colonies and predators, were used for the crabs and snapping prawns in each feeding condition, while 20 to 25 replicates were used for the blenniid fish.

We could not analyse the results as a contingency table: as the same predator was offered a treated and a control pellet, the observations were not independent. We used instead a McNemar test (Sokal & Rohlf 1981), which is the frequency-data equivalent of repeated-measures analyses of continuous variables. The results were assigned to 4 categories for the analyses: number of predators eating both treatment and control pellets; number eating control pellets but not treatment food; number eating treatment food but not control pellets; and number not eating (the latter was set to zero by the experimental procedure of not considering double rejections). As we offered first the treatment item, some control pellets could be rejected because the predator was satiated with the first item. This possibility was minimised by allowing 4 h for the consumption of each item.

In order to summarize the results of the feeding trials and to obtain a palatability value for comparison with other variables, we calculated a palatability index for each compartment assayed. To this end, we combined the percentages of consumption of the diverse predators under the 2 feeding conditions. Only predators that had been assayed with all compartments were included in the calculations. As we assayed the adult materials and the larvae using different predators, 2 indices were calculated, 1 for zooids and the tunic (with the results of the feeding assays with the 2 crustaceans), and the other for larvae (with the results obtained with the crustaceans and the blenniid fish).

Feeding trials using extracts. To ascertain the presumptive chemical nature of the interactions, bioassays with extracts were carried out in the laboratory with the 2 crustaceans (in non-starved condition) for those compartments that were significantly rejected in the raw material bioassays. Compartments, pooling material from at least 5 different colonies, were extracted 3 times (with 1:1 dichloromethane/methanol, dichloromethane and dichloromethane for 1 h each). The same number of food pellets as the larvae, thorax, abdomen or tunic pieces extracted were placed in a small vial, the extracts were added, and the solvent was allowed to evaporate while soaking the pellets. To ensure that no extract remained on the walls of the vials after complete evaporation, the pellets were removed and a few drops of solvent were used to clean the walls of the vial. The solvent was then pipetted out and, drop by drop, again released over the pellets. When the size of the compartment was smaller than the pellets, these were ground and fragments approximately the same size as the test compartment were selected and counted, thereby obtaining a similar volume of food and maintaining the volumetric proportions of the chemical substances within a realistic range. These test pellets were then dried and, at the beginning of the tests, were soaked with filtered seawater. Control food consisted of pellets soaked with solvent and dried.

The bioassays with chemical extracts were performed only with the crustacean predators *Cestopagurus timidus* and *Alpheus dentipes* because these species were more amenable to laboratory conditions than the blenniid fish. The crustaceans were tested in non-starved conditions (see preceding subsection) because this was thought to more closely represent the natural motivation to feed (Lindquist & Hay 1996). Otherwise tests were run and analysed in exactly the same way as those using raw material. From 12 to 20 replicates with each predator species were performed for each compartment.

Toxicity assays and pH measurements. For the toxicity tests, zooids and tunics were freeze-dried, macerated and extracted 3 times (1 h each) with 10 ml dichloromethane for each colony separately. The solvents of the 3 extractions were filtered, pooled, and allowed to evaporate, thus obtaining a dry extract. Six colonies of each species were separately extracted and analysed, except for *Ecteinascidia herdmanni* and *Clavelina lepadiformis*, for which we had only 3 replicates. Given the difficulty in obtaining enough larvae to run replicate tests, larvae were pooled and processed in a single sample for each species.

Toxic activity was tested using the Microtox method described in Ribo & Kaiser (1987). This is an automated procedure based on the reduction of light produced by the marine bacterium *Photobacterium phosphoreum*, and has proven to be sensitive and precise as a quantitative measure of toxicity, and to correlate well with other commonly used assays (Becerro et al. 1995). The extracts were resuspended in artificial seawater (in an ultrasonic bath) to reach the desired concentration. We tested the extracts at a single concentration (500 ppm dry sample weight) and measured the light reduction after a 5 min run. Light reduction is expressed in gamma units (GU) corrected for the spontaneous light reduction in a control, as follows:

$$GU = (Rt \times I_0/I_t) - 1$$

where Rt = correction factor, I_0 = light at time 0 and I_t = light at time t.

Previous studies (M. J. Uriz pers. comm.) showed that, at a concentration of 500 ppm of a given substance, 0.5 GU corresponds to a lethal toxic effect for embryos of the common Mediterranean sea urchin *Paracentrotus lividus* (Lamarck, 1816). This value has therefore been chosen to establish a threshold between toxic and non-toxic treatments in our tests.

To measure the pH of the species studied we followed the method of Davis & Wright (1989). We collected fresh material and carefully separated zooids, tunic and larvae. A sufficient amount of each compartment was placed on Sigma® pH test strips (precision 0.5 units) and pressure was applied until fluids were extruded. We then monitored the resulting colour change of the paper. Three replicates (with different colonies) were obtained per species.

Caloric contents. To examine the potential effect of the energetic value of the treatments and the commercial food pellets, the caloric contents were analysed with a microcalorimeter DSC-30 Mettler Toledo. Analyses were run separately for 3 to 5 colonies of each species. The materials were previously freeze-dried and ground in a mortar. Temperature during the analyses was increased to 600°C at 10°C min^{-1} and was then maintained at 600°C for 1 h. The resulting ash was used to estimate the inorganic structural contents of the different compartments (Chanas & Pawlik 1995). Energy content was related both to total dry mass and to ash-free dry mass. To compute the energy content as a function of wet mass, we established the relationship wet weight/dry weight of all compartments separately (we analysed 3 colonies per species separately and averaged the results). We also performed analyses of the caloric contents of the food pellets used in the palatability tests to ascertain whether they were within the range found for ascidian tissues.

RESULTS

Palatability bioassays with raw material

Significant differences were observed among species and compartments within species. Palatability values were in general equal or lower under the non-starved than starved condition. The percentage of consumed thorax, abdomen, tunic and larvae is shown in Fig. 1 for hermit crabs, snapping prawns, blenniid fish and damselfish. Among the significant treatment effects with adult compartments, *Clavelina lepadiformis* and *Pseudodistoma crucigaster* showed the least palatability (all compartments were significantly rejected), followed by *Polysyncraton lacazei*, *Cystodytes dellechiajei* and *Ecteinascidia herdmanni* (some compartments rejected, and results not always consistent between predators), and only in the case of

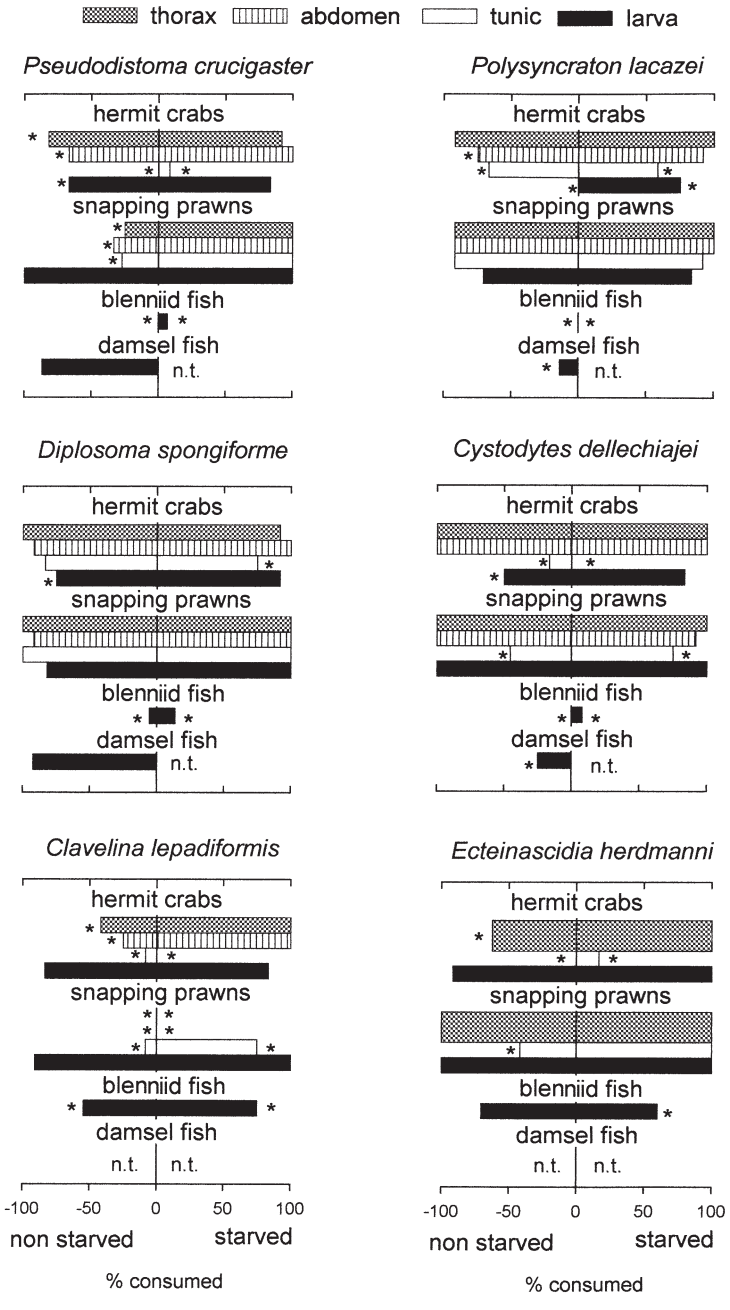

Fig. 1. Percentage of thorax, abdomen, tunic and mature larvae of 6 ascidian species consumed by 4 predators. Left graphs: non-starved conditions; right graphs: fasting conditions. For *Ecteinascidia herdmanni*, the whole zooid with no separation of thorax and abdomen was tested. n.t.: not tested with damselfish; *treatments significantly rejected (McNemar test)

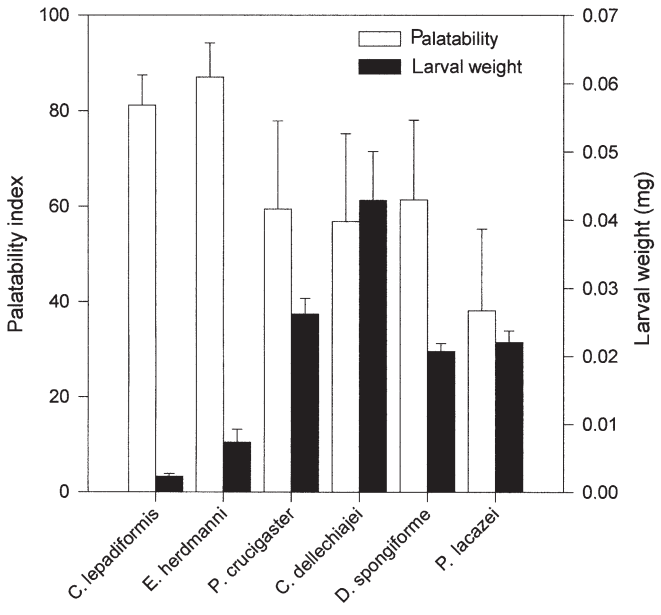

Fig. 2. Larval palatability index (palatability to hermit crabs, snapping prawns and blenniid fish) and larval dry weight of the ascidian species studied. Bars are +SE. Full specific names as in Fig. 1

Diplosoma spongiforme were all adult compartments readily consumed by predators.

However, the results for larvae differ. Some species that were highly unpalatable as adults had palatable larvae (e.g. *Clavelina lepadiformis*; cf. also adult and larval palatability of *Pseudodistoma crucigaster* fed to snapping prawns), while some species that were palatable as adults had unpalatable larvae (e.g. *Diplosoma spongiforme* fed to hermit crabs and blenids). For the 4 species whose larvae were tested in the field with the demersal predator *Chromis chromis*, only *Polysyncraton lacazei* and *Cystodytes dellechiajei* larvae had a significant deterrent effect.

For both starved and non-starved conditions, there was a pattern of larval consumption among predators. Snapping prawns tended to consume more larvae (averaging the data for the 6 species, mean percentage of consumed larvae was 90.55% in non-starved, and 97.22% in starved condition). Hermit crabs consumed fewer larvae (non-starved: 61.11%, starved: 86.11%), while blenniid fish were the most selective of all (non-starved: 21.90%, starved: 27.17%, with no consumption for 3 of the species under non-starved conditions). Damselfish consumed an average of 54.44% of the larvae of the 4 species tested with this predator.

A composite index of palatability of the larvae of the 6 ascidian species was calculated by averaging their consumption under both experimental conditions (starved and non-starved) by the 3 predators (crustaceans and blenids) for which data covering all 6 species was available. Fig. 2 shows the results together with average larval size (mg dry weight, obtained by weighing separately the larvae of at least 5 colonies). The error bars in this Fig. 2 reflect variation among predators, not within the larvae compartment. The palatability index for larvae showed that *Polysyncraton lacazei* was the most unpalatable (38.15 ± 17.14% consumed, mean ± SE), while the larvae of *Clavelina lepadiformis* (81.19 ± 6.34%) and *Ecteinascidia herdmanni* (87.04 ± 7.14%) were the most palatable. The 4 most deterrent species (*P. lacazei*, *Cystodytes dellechiajei*, *Pseudodistoma crucigaster* and *Diplosoma spongiforme*) were also those with the largest larvae (>0.02 mg dry weight). They significantly deterred at least 2 of the 3 predators tested in non-starved conditions. They also correspond to species with low per zooid fecundity, as estimated by the number of larvae incubated by zooids (1 for the Didemnidae and *C. dellechiajei*, 3 for *P. crucigaster*). In contrast, the 2 species with the most palatable larvae: *C. lepadiformis*, which significantly deterred only 1 predator, and *E. herdmanni*, which deterred none, were also the species with the smallest larvae (<0.01 mg dry wt) and higher per zooid fecundity (mean of 7.58 for *E. herdmanni* and 66.7 for *C. lepadiformis*: Tarjuelo 2001).

Likewise, we calculated a palatability index for the remaining compartments by averaging their consumption by the 2 predators (crustaceans) for which data covering all 6 ascidian species were available under both feeding conditions. Thorax and abdomen values were averaged to give a single measure of palatability for the zooid because there was no clear pattern of

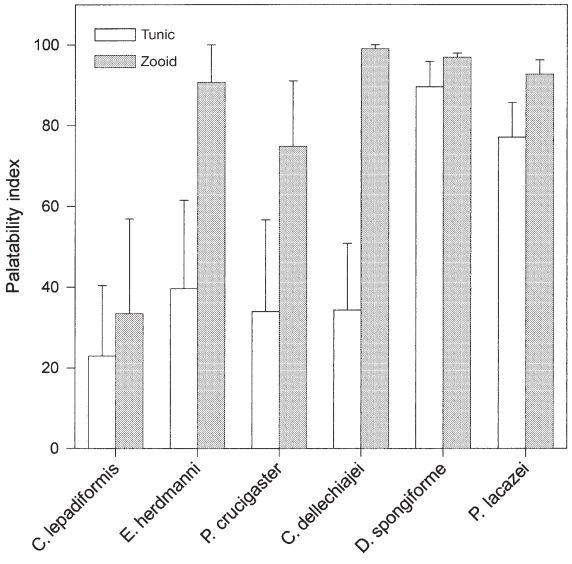

Fig. 3. Palatability index (palatability to hermit crabs and snapping prawns) of the zooids and tunic of the ascidian species studied. Bars are +SE. Full specific names as in Fig. 1

deterrence for thorax and abdomen (Fig. 1). In 20 comparisons (5 species × 2 predators × 2 feeding states), the abdomen was least consumed in 8 cases, the thorax in 3, and there were 9 ties. Fig. 3 shows the values of this palatability index for the tunic and zooids. High variances reflect the differential behaviour of both predators: consumption was in general higher for the snapping prawns than for the hermit crabs (except for *Clavelina lepadiformis*). Tunic material was, on average, less palatable than zooids in the 6 species studied (Fig. 3). In 24 comparisons between zooids and the tunic (6 species × 2 predators × 2 feeding states), the tunic was less palatable than in 17 zooids, there was a tie in 4, and zooids were least palatable in 3. The lowest zooid palatability value was for *C. lepadiformis* (33.3 ± 9.8% consumed zooids, mean ± SE), while the highest was for *Cystodytes dellechiajei* (98 ± 1.1%). The species with the lowest tunic palatability was again *C. lepadiformis* (22.9 ± 14.6%), and *Diplosoma spongiforme* presented the highest index (89.6 ± 2.1%). There was no relationship between the palatability of larvae and zooids for the 6 species ($r = 0.469$, $p = 0.346$, $n = 6$) or between larvae and tunic material ($r = -0.552$, $p = 0.256$, $n = 6$), neither was there a significant relationship between the palatability of zooids and the tunic ($r = 0.58$, $p = 0.228$, $n = 6$).

Palatability bioassays with extracts

Fig. 4 shows the results of the tests carried out with the crustaceans under non-starved conditions using the extracts of those treatments that were significantly rejected in the raw material assays. The consumption of commercial food pellets with an extract at the original volumetric concentration was in general higher than that of raw material. Controls were eaten in 95% of the trials for both predators. Only the extract of *Polysyncraton lacazei* tunic was significantly deterrent for both benthic predators, while extracts of *Cystodytes dellechiajei* tunic (for hermit crabs) and the thorax of *Clavelina lepadiformis* and *Pseudodistoma crucigaster* (for snapping prawns) were significantly rejected by 1 of the predators. In contrast, none of the 4 species whose larvae were tested showed significant extract deterrence.

Toxicity and pH

Toxicity levels at a concentration of 500 ppm (measured as light reduction in GU) were measured for zooids, tunic and larvae of all 6 species (Fig. 5). The results showed remarkable differences among species and among compartments for any given species. As for inter-individual differences, the coefficient of variation was in general below 50%, except for the tunic of *Cystodytes dellechiajei* (SD higher than the mean). Higher mean toxicity was detected in zooid than in tunic material, except for *C. dellechiajei*. Larvae were less toxic on average than zooids in 5 species, and only in *Ecteinascidia herdmanni* were larvae more toxic than the other compartments.

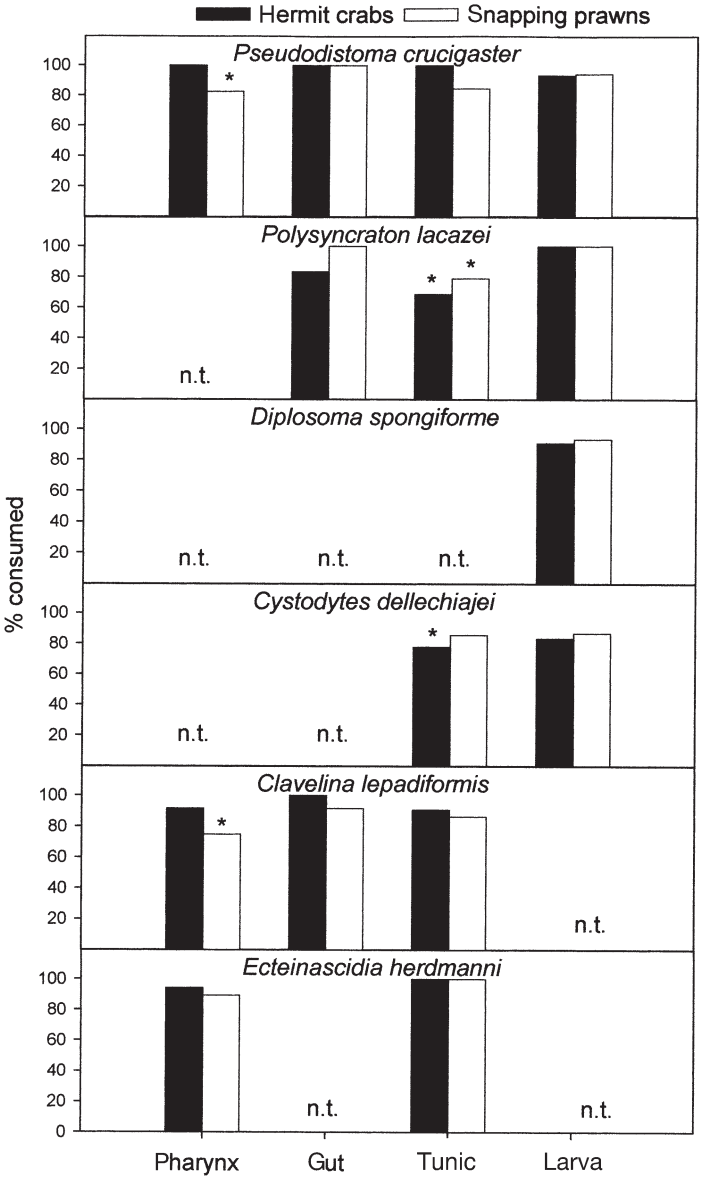

Fig. 4. Percentage of consumed pellets with extracts of compartments (thorax, abdomen, zooid, tunic and mature larvae) that proved significantly deterrent in the raw material tests. n.t.: not tested; * treatment significantly rejected (McNemar test)

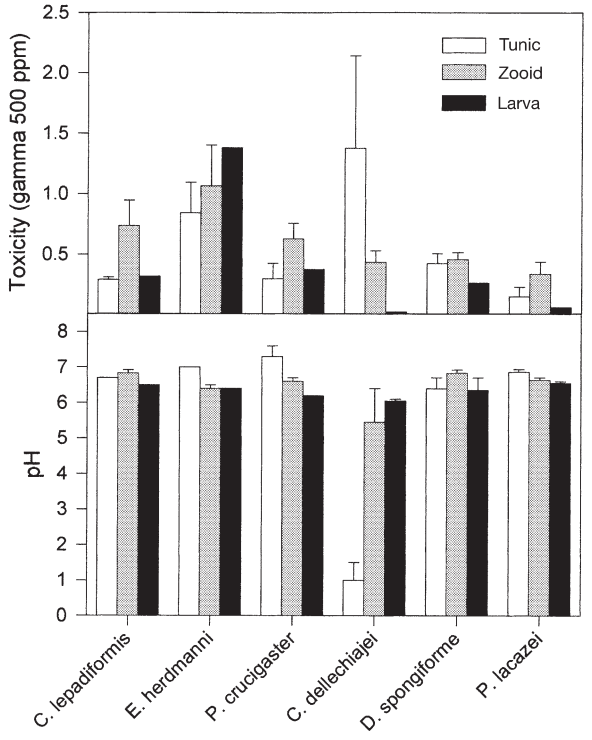

Fig. 5. Toxicity (in gamma units at 500 ppm) and pH values of the zooids, tunic and larvae of the 6 ascidian species studied. Bars are +SE. Full specific names as in Fig. 4

If we consider a material as toxic when a threshold of 0.5 GU is reached, then toxic levels were found only in the tunic of *Cystodytes dellechiajei* (1.37 GU), the zooids of *Pseudodistoma crucigaster* and *Clavelina lepadiformis* (0.62 and 0.73 GU, respectively), and the tunic, zooids and larvae of *Ecteinascidia herdmanni* (0.84, 1.06, and 1.38 GU, respectively).

Values of pH are also shown in Fig. 5. The pH of most compartments was neutral or nearly so (mean pH values between 6 and 7), with the exception of the tunic of *Cystodytes dellechiajei*, which was strongly acidic (pH values ca. 1). However, zooids (mean pH = 5.45) or larvae (mean pH = 6.05) of this species did not feature this acidity.

To reveal any relationships between these parameters and palatability, the values of toxicity and palatability variables for zooids, tunic, and larvae of the 6 ascidian species are plotted in Fig. 6. No significant pattern emerged ($r = 0.0467$, $p = 0.85$, $n = 18$). Neither did correlations between palatability and toxicity of the different compartments calculated separately show any significant pattern for the 6 species (larvae: $r = 0.748$, $p = 0.087$; zooids: $r = -0.318$, $p = 0.539$; and tunic: $r = -0.331$, $p = 0.521$; $n = 6$ in all cases). The same holds true for the relationship between pH and palatability. The resulting correlation was not significant ($r = 0.02$, $p = 0.536$, $n = 18$), neither were the correlations for each compartment separately (larvae: $r = 0.206$, $p = 0.696$; zooids: $r = -0.452$, $p = 0.369$; and tunic: $r = 0.083$, $p = 0.875$; $n = 6$ in all cases). In fact, we did obtain low palatability values for the acidic compartment (tunic of *Cystodytes dellechiajei*), but these were in the same order as those of other, nearly neutral, compartments.

Caloric contents

Fig. 7 shows the caloric content of the compartments studied for the 6 ascidian species, as well as the corresponding ash contents. Caloric values were lower whenever ash content was high (both variables showed a clear negative relationship: $r = -0.747$, $p < 0.001$, $n = 23$). This occurred particularly in the tunic compartment, notably in the 2 species with calcareous spicules embedded in the tunic (*Cystodytes dellechiajei* and *Polysyncraton lacazei*). However, differences in relative caloric content cannot be explained solely on the basis of differences in ash content. When caloric content is expressed relative to ash-free matter, a significant positive relationship between energy g^{-1} and energy $g(\text{ash-free})^{-1}$ ($r = 0.70$, $p < 0.001$, $n = 23$) is observed, indicating that compartments with a higher caloric content relative to total weight also tend to have a higher caloric content relative to ash-free weight. The analyses of the caloric content of the control

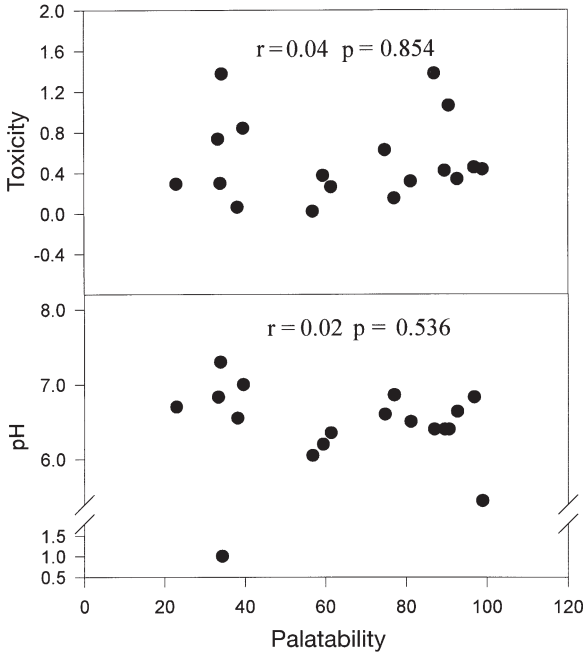

Fig. 6. Correlations of toxicity and pH with palatability of ascidian zooids, tunic and larvae

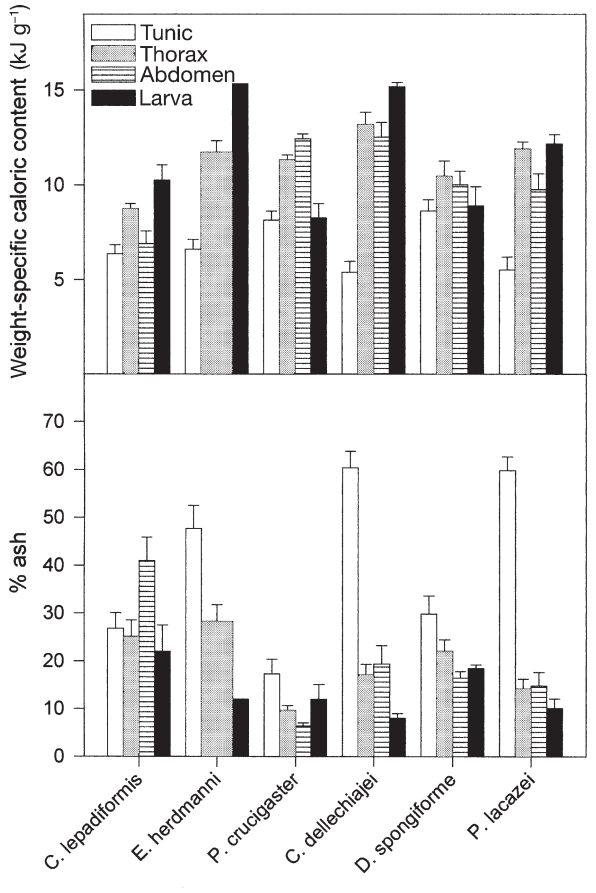

Fig. 7. Weight-specific caloric content and ash content of the compartments analysed in the 6 ascidian species studied. For *Ecteinascidia herdmanni*, the whole zooid with no separation of thorax and abdomen was tested. Bars are +SE. Full specific names as in Fig. 4

pellets (12.5 ± 0.3 kJ g^{-1}; mean ± SE) showed that it was relatively high but within the range of that of the treatments.

The palatability index was positively related to the caloric content (g dry wt^{-1}) of the items consumed ($r = 0.640$, $p < 0.01$, $n = 23$: Fig. 8). A more meaningful relationship, however, would involve caloric content wet wt^{-1}, as predators do not eat dry matter. We repeated the correlation considering the caloric content relative to wet weight, and this was significant ($r = 0.471$, $p < 0.02$, $n = 23$; note that a significant relationship was still found after removing the 2 outstanding values with higher palatability and caloric contents: Fig. 8). The relationship of palatability and food caloric content may be due to the unpalatability of structural, inorganic material, which is higher in low-energy foods. But, again, ash content alone does not explain the feeding preference, as the positive relationship still holds when palatability is plotted against energy content relative to ash-free weight ($r = 0.540$, $p < 0.01$, $n = 23$, Fig. 8).

To take into account the possible interactions among variables, we performed a multiple regression of palatability with all other variables analysed (toxicity, pH, caloric contents and ash contents) in the different compartments studied (values for thorax and abdomen were combined into a single zooid value for caloric contents and ash contents). The results confirm those obtained through simple correlation between pairs of variables: the multiple correlation coefficient, R, was 0.604 ($n = 18$) with the 4 variables included. However, only the partial correlation coefficient for caloric contents was significant ($p = 0.032$), while the remaining were not. In a forward stepwise addition of variables, entering the caloric contents results in an R of 0.527, and no significant improvement of the correlation was obtained by adding any other of the variables.

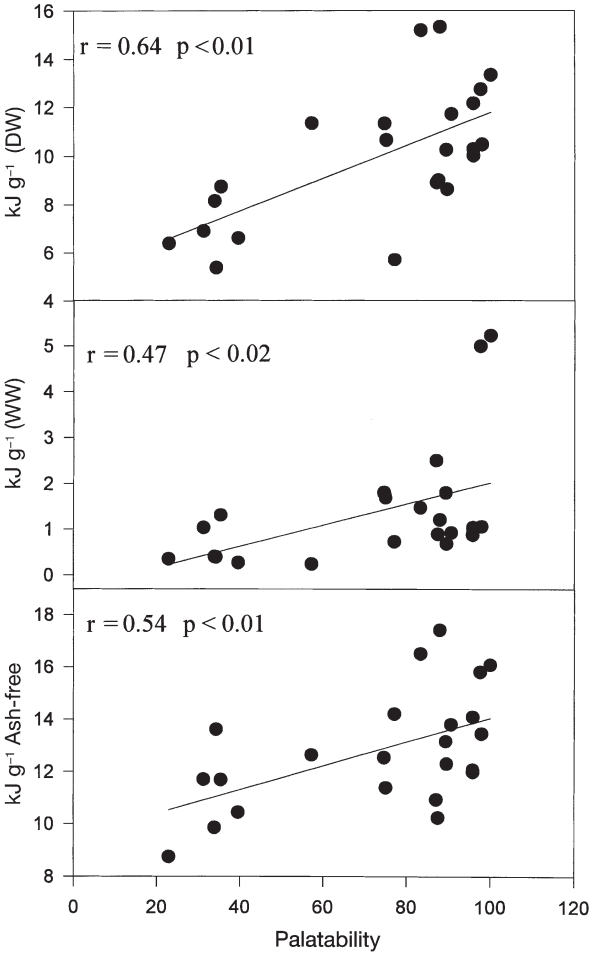

Fig. 8. Correlations between palatability and weight-specific caloric content as a function of total dry wt, total wet weight and ash-free dry wt of zooids, tunic and larvae of the 6 ascidian species studied

DISCUSSION

Our study shows that protection against predation is relatively common among colonial ascidians: all 6 species proved unpalatable for one or other of the predators and one or other of the compartments analysed. However, some species are clearly more highly defended than others, and a high variability has been observed both among species (even of the same family) and among compartments within species. In general, toxicity was low and was not related to the palatability of the compartments tested. The lack of relationship between toxicity and feeding deterrence has been repeatedly noted for marine invertebrates (Coll 1992, Pawlik 1993, Pawlik et al. 1995). Our results also confirm previous findings (e.g. Parry 1984) that acidic defences are not as important in ascidians as previously suggested. Only one compartment of the species assayed (the tunic of *Cystodytes dellechiajei*) was strongly acidic, but the extracts of the tunic of this species were toxic and deterred feeding in one of the predators, indicating that pH alone is not responsible for the low palatability of this compartment. With only one instance of acidic pH, we cannot reliably assess the relationship between pH and palatability.

Results are also dependent on the predator assayed. In our case, we used 1 demersal and 1 benthic fish for larvae, and 2 crustaceans for adults and larvae. In general, in assays with larvae, fishes were more selective than crustaceans. Among the latter, snapping prawns consumed more treatments than hermit crabs. Lindquist (1996) and Lindquist & Hay (1996) also found that fishes tended to reject more species of larvae than benthic invertebrate predators (anemones and corals). These results highlight the need to choose a representative range of predators that covers the distinct life stages of the prey species.

To our knowledge, this is the first study that considers separately the defence characteristics of the zooids (thorax and abdomen), tunic and larvae of ascidians. Toxicity does not show a consistent pattern, and the distribution of toxicity among compartments varies from species to species. As regards palatability, in all cases tunic material was less palatable on average than zooids, while there was no clear trend for the thorax and abdomen. The tunic may be consumed less because of its lower caloric content, which, in turn, is partly related to a higher proportion of inorganic, structural material. The organic tunic material itself is refractory and difficult to digest, a factor that is not addressed in this study. However, 'soft' tunics are sometimes less palatable than harder, spiculated ones (compare *Clavelina lepadiformis* and *Polysyncraton lacazei*: Fig. 3), therefore it seems that a combination of factors (energy content, digestibility, chemicals, pH) determines tunic palatability, and that generalizations based only on texture are unreliable.

We found that larvae of species with the largest larvae and lower per zooid fecundity (*Polysyncraton lacazei*, *Pseudodistoma crucigaster*, *Diplosoma spongiforme* and *Cystodytes dellechiajei*) are better defended against predation, while those of the 2 species with the highest fecundity and smallest larval size (*Clavelina lepadiformis* and *Ecteinascidia herdmanni*) offer little or no deterrence. For the damselfish, only the largest larvae could be tested, and of these *P. lacazei* and *C. dellechiajei* showed strong deterrent properties. The pattern found confirms the hypothesis of higher protection against larval predation in species which have low fecundity, and produce large and costly larvae that settled locally (Lindquist et al. 1992, Lindquist 1996, Lindquist & Hay 1996). These larvae are able to settle immediately not far from the adults, which often results in enhanced fitness (Strathmann 1980, Jackson 1986), but protection is required to compensate their higher perceptibility and the higher predator abundance near the bottom. This pattern of variation in larval perceptibility and degree of protection has been associated with the dichotomy between feeding and non-feeding larvae (Strathmann 1985) or the related one between broadcast-spawning and brooding species (Lindquist & Hay 1996). Here we show that there is also variation within brooding forms producing lecitotrophic larvae: most colonial ascidians are brooders, yet those with low fecundity and larger larvae have reduced larval palatability. On the other hand, in species with higher numbers of small, palatable larvae another defence mechanism may counteract their palatability: they seem to be too small to be detected by even small fishes such as damselfishes. A note of caution is necessary in this respect, however, as we offered non-mobile larvae to the fishes, a fact that may affect their prey perception. In our study, there was no relationship between deterrence of adults and that of their larvae, which suggests ontogenetic shifts in defence strategy, as observed in other groups (e.g. Uriz et al. 1996c).

Although we found low levels of palatability in many of the compartments studied, we could not substantiate a chemical basis for this in most of our tests with extracts. Only in the zooids and tunic of some species (i.e. the thorax of *Clavelina lepadiformis* and *Pseudodistoma crucigaster* and the tunic of *Cystodytes dellechiajei* and *Polysyncraton lacazei*) could a chemical mechanism be demonstrated. This result contrasts with findings for other groups such as gorgonians (Pawlik et al. 1987) or sponges (Pawlik et al. 1995), in which a high percentage of species with unpalatable extracts are recorded. Our data, on the other hand, are in agreement with those of Teo & Ryland (1994), who

found little deterrent effect of extracts from 9 temperate-water ascidians on shore crabs and fishes. However, these authors found no deterrent effect of *C. lepadiformis*, while we found significant deterrence with both its raw material and extracts. Remarkably, no evidence of a chemical mechanism was found in our study in the tests with extracts of larvae of any of the species that were significantly rejected in the tests with raw material. This seems difficult to explain, as they lack any obvious physical defence mechanism aside from the tunic itself. Moreover, the amount of structural material in larvae (as estimated by ash content) is in general lower than in other compartments. Young & Bingham (1987) and Lindquist et al. (1992) demonstrated a chemical basis for the unpalatability of larvae of *Ecteinascidia turbinata* and *Trididemnum solidum*, respectively. Other instances of unpalatable ascidian larvae can be found in the literature (e.g. Olson 1983, Davis & Butler 1989), but in other cases palatable larvae have been reported (Olson & McPherson 1987, Young & Bingham 1987, Lindquist 1996, Lindquist & Hay 1996). It is not clear if tunic toughness alone can protect ascidian larvae from predation. Our failure to find a chemical effect may have been due to extraction artefacts, such as lack of extraction of the particular metabolite implicated in deterrence or a chemical change in such a substance. Furthermore, the effects of the texture and digestibility of the commercial food pellets used should be taken into account. More chemical studies and, in particular, the identification and quantification of active metabolites in the larvae, should be carried out to determine the nature of the unpalatability of the larvae studied.

The energy content and the quality of the food (amount of inorganic material) were related to the palatability of the distinct compartments. Chemical and physical defence mechanisms interact in complex ways in marine organisms (Harvell & Fenical 1989, Pennings & Paul 1992, Hay et al. 1994, Pennings et al. 1996, Becerro et al. 1998), and they are not necessarily negatively related (Chanas & Pawlik 1995, Becerro et al. 1997). Moreover, the physical texture of the prey and, particularly, toughness may be key factors (Pennings & Paul 1992, Chanas & Pawlik 1995), and they have not been considered in this study, although toughness of the soft parts of zooids is small and is not a likely protection. Overall, we found that energetically rich tissues are consumed more, and that the differences cannot be explained merely by variations in the amount of inorganic, structural material.

In conclusion, it seems that the defence strategy of colonial ascidians is highly variable among species (even of the same family), and that unpalatability may be common and assured by either physical or chemical mechanisms, but that allocation to defence varies among compartments and among ontogenetic states (larvae or adult). Joint studies on defence strategy together with research on other biological traits such as reproductive effort, growth rates and mortality in these and other species may shed light on the role of defence in the evolutionary shaping of complex life cycles in invertebrates.

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