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Defence behind the ramparts: Spicule armament against specialist predators in a subtidal habitat-forming ascidian

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Defence behind the ramparts: Spicule armament against specialist predators in a subtidal habitat-forming ascidian

Abstract

Sessile organisms are reliant on the use of refugia or chemical and physical defences to avoid or reduce attacks from predators. Many solitary ascidians possess a thick leathery outer tunic, sometimes reinforced with inorganic inclusions, that generally acts to deter predators. The genus *Herdmania* is remarkable among ascidians in possessing an abundance of barbed needle-like calcareous spicules in both the external tunic and the internal mantle. Our focus was on a habitat-forming subtidal ascidian - *Herdmania grandis*. We questioned why a large ascidian possessing a leathery test would possess spicules within the mantle - an apparent defence within a defence. We first quantified variation in spicule size and density within animals. Second, we examined the size and density of spicules for individuals of a range of sizes. Third, we incorporated these spicules at natural concentrations into feeding discs and tested their ability to deter gastropod predators that specialise in consuming ascidians and an asteroid that is considered a generalist consumer. Finally, we examined survivorship of lab-reared ascidian recruits when exposed to the starfish predator. Spicules constituted up to 30% dry weight (DW) of the tunic and an astounding 57% DW of the body wall (mantle). Spicules were significantly smaller in the contractile regions of the animal (the siphon and branchial basket) and were also found in lower densities, though not significantly. Mantle spicules were effective feeding deterrents; control feeding discs, lacking spicules, were consumed at three times the rate of discs with spicules ($p < 0.05$) by specialist ascidian predators, the Ranellid gastropods *Cabestana spengleri* and *Ranella australasia*. In contrast, we did not detect a significant difference in the consumption of feeding discs by the seastar *Meridiastra calcar*. In addition, the seastar reduced survivorship of 90 day old lab-reared recruits by four-fold. We contend that the primary defence mechanism of this ascidian is the leathery test and that this is effective against generalist consumers. The second line of defence - spicules in the mantle - is to thwart gastropod predators that bypass the tunic either by boring through it or by inserting their proboscis directly into soft tissues of their prey via the siphons. The presence of both a leathery tunic and spicules in *H. grandis* probably contributes to the dominance of this species on shallow subtidal reefs in southeastern Australia, where it is an important habitat-forming species. Our findings confirm the importance of considering a range of consumer strategies and life history stages in assessing potential control of engineering species by predation.

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Defence behind the ramparts: spicule armament against specialist
predators in a subtidal habitat-forming ascidian

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25 ABSTRACT

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27 defences to avoid or reduce attacks from predators. Many solitary ascidians
28 possess a thick leathery outer tunic, sometimes reinforced with inorganic
29 inclusions, that generally acts to deter predators. The genus *Herdmania* is
30 remarkable among ascidians in possessing an abundance of barbed needle-like
31 calcareous spicules in both the external tunic and the internal mantle. Our focus
32 was on a habitat-forming subtidal ascidian – *Herdmania grandis*. We questioned
33 why a large ascidian possessing a leathery test would possess spicules within the
34 mantle – an apparent defence within a defence. We first quantified variation in
35 spicule size and density within animals. Second, we examined the size and
36 density of spicules for individuals of a range of sizes. Third, we incorporated
37 these spicules at natural concentrations into feeding discs and tested their ability
38 to deter gastropod predators that specialise in consuming ascidians and an
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41 predator. Spicules constituted up to 30% dry weight (DW) of the tunic and an
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46 consumed at three times the rate of discs with spicules ($p < 0.05$) by specialist
47 ascidian predators, the Ranellid gastropods *Cabestana spengleri* and *Ranella*
48 *australasia*. In contrast, we did not detect a significant difference in the
49 consumption of feeding discs by the seastar *Meridiastra calcar*. In addition, the

seastar reduced survivorship of 90 day old lab-reared recruits by four-fold. We contend that the primary defence mechanism of this ascidian is the leathery test and that this is effective against generalist consumers. The second line of defence – spicules in the mantle – is to thwart gastropod predators that bypass the tunic either by boring through it or by inserting their proboscis directly into soft tissues of their prey via the siphons. The presence of both a leathery tunic and spicules in *H. grandis* probably contributes to the dominance of this species on shallow subtidal reefs in southeastern Australia, where it is an important habitat-forming species. Our findings confirm the importance of considering a range of consumer strategies and life history stages in assessing potential control of engineering species by predation.

KEYWORDS: Gastropod predators, Feeding discs, *Herdmania*, Physical defence, generalist predators, tunicates

66 INTRODUCTION

67 The important role of predators in structuring benthic assemblages in the
68 marine realm is well established. In addition to modifying the distribution and
69 abundance of their prey (eg Connell 1970), predators can alter the structure,
70 function and dynamics of entire benthic assemblages (eg Paine 1974, Hay 1997).
71 The marine literature also attests to the important role of natural products in
72 mediating species interactions (Davis et al. 1989, Paul 1992, Hay 1996, Puglisi et
73 al. 2014). On the other hand, physical deterrents as mediators of species
74 interactions have received much less attention (Duffy and Hay 2001, Davis
75 2007). The spicules of sponges, cnidarians and ascidians are often seen as
76 playing a role in structural support (Koehl 1982, Palumbi 1986), but evidence for
77 a deterrent role of structural defences such as spicules or spines has now been
78 confirmed in field and laboratory assays for some species within these phyla (eg
79 Young 1986, Van Alstyne and Paul 1992, Van Alstyne et al. 1994, Ferguson and
80 Davis 2008).

81

82 Marine habitats have been a particularly rich environment for examining the
83 effects of generalist vs specialist consumers, with opportunities for direct
84 experimentation under field conditions. There is now overwhelming evidence
85 that generalist consumers, such as sea urchins, can eliminate prey from habitats
86 that are not protected, with the potential for cascading effects through
87 assemblages (eg Hay 1997 and references therein). Specialist consumers are
88 often less susceptible to the defences of their prey, be they structural or
89 chemical, than generalist consumers (Duffy and Hay 2001), with obvious
90 advantages accruing to the specialists (Wylie and Paul 1989). There are

numerous examples of sessile animals exhibiting heightened defences to deter specialist predators (e.g. Wylie and Paul 1989, Becerro et al. 1998, Maldonado et al. 2016).

Ascidians are an important group in benthic communities worldwide at all depths (Lambert 2005, Shenkar & Swalla 2011). They are known producers of secondary metabolites (Davis 1991, Davidson 1993, Molinski 1993, Davis and Bremner 1999, Banaigs et al. 2014) and acidic secretions (Stoecker 1978, Davis and Wright 1989, López-Legentil et al. 2006, Koplovitz et al. 2009) which can protect them from predators. In addition to organic and inorganic chemical defences, ascidians can also possess effective physical defences (Young 1986). The first is the presence of an outer covering, the tunic, that can be tough and leathery in many cases, particularly in solitary forms (Svane 1983). In addition, some ascidians also possess hard structures that can act as physical deterrents such as stiff spines of organic origin (derived from tunic cuticle, Kott 1985), mineral spicules or inorganic inclusions in the tunic (Kott 1985).

All of these physical structures have been interpreted as predator deterrents, although in most instances the evidence is not convincing. Small calcareous spicules from the tunic of colonial ascidians, *Trididemnum solidum* and *Cystodytes* spp. failed to deter generalist consumers when tested in feeding assays in isolation (Lindquist et al. 1992; López-Legentil et al. 2006). If these spicules do play a defensive role, then it is likely that they act in concert with secondary metabolites or acid secretions in these ascidians. Young (1986) used scanning electron micrographs to highlight the presence of a “plush” of sharp

116 tunic spines directed anteriorly in the siphonal inner lining of *Pyura haustor*, a
 117 species which is not consumed by a specialist gastropod predator. He argued
 118 that these structures played a defensive role, although this was not tested
 119 experimentally. Convincing evidence for a defensive role of organic, tunic-
 120 derived spines was obtained for *Halocynthia igaboja*, a species with protruding
 121 stiff spines possessing recurved barbs; on shaving these spines Young (1986)
 122 rendered this ascidian susceptible to predation by the ranellid gastropod
 123 *Fusitriton oregonensis* in laboratory and field trials.

124
 125 Calcareous spicules are found embedded in the tunic of the colonial forms of
 126 ascidians belonging to the families Didemnidae and Polycitoridae (Lambert et al.
 127 1990). These spicules are usually microscopic in size, and of various shapes
 128 (stellate, disc-shaped, spherical). Some solitary ascidians of the family Pyuridae
 129 also have tunic spicules of different sizes and shapes (Kott 1985, Lowenstam
 130 1987, Lambert 1998). However, some pyurid species in the genera *Culeolus*,
 131 *Pyura*, and *Herdmania* also have, in addition to tunic spicules, calcareous spicules
 132 in the mantle tissues which come in antler, rod, or star shapes (Lowenstam 1987,
 133 Lambert 1992, Lambert and Lambert 1997). The mantle spicules of *Herdmania*
 134 *momus* are in the form of barbed shafts, measure 1.5 - 2.5 mm in length and are
 135 found in high densities throughout the mantle (Lambert and Lambert 1987). The
 136 presence of these spicules has been known for more than 140 yrs (Heller 1878,
 137 Herdman 1882) but their ecological role has not been empirically tested,
 138 although it has been suggested that in solitary ascidians tunic spicules are likely
 139 involved in defence whereas mantle spicules may have a supportive function
 140 (Lowenstam 1987, Lambert and Lambert 1987, 1997).

141
142 Solitary ascidians can dominate rocky intertidal and shallow subtidal
143 assemblages on hard surfaces, particularly in the southern hemisphere (Davis
144 1995, Rius et al. 2017). Many molluscs specialise on consumption of these
145 ascidians; for example, neogastropods in the Ranellidae feed almost exclusively
146 on solitary ascidians (Laxton 1971, Young 1985, 1986, Underwood and
147 Fairweather 1986). Despite the actions of these specialist predators, the tough
148 leathery tunic of solitary ascidians appears to offer defensive advantages and
149 may contribute to their dominance on subtidal reefs (Svane 1983, Young 1986).
150 Here we focus on a large subtidal ascidian, *Herdmania grandis* (Heller 1878),
151 which dominates subtidal rocky reef habitats and imparts considerable biogenic
152 structure to the reefs of southeastern Australia (Fig. 1A). In addition to a very
153 tough test with embedded spicules, the mantle of *H. grandis* possesses large
154 numbers of needle-like spicules in what appears to be a defence behind a
155 defence; an inner mantle with masses of spicules within a leathery tunic. We
156 tested the hypothesis that these spicules play a defensive role against specialist
157 predators that can bypass the tunic and predicted that they would be ineffective
158 against a generalist consumer which was likely deterred by the outer tunic. Our
159 preliminary observations were that specialist predators would readily consume
160 the large co-occurring species *Pyura praeputialis*, also with a thick tunic, in the
161 laboratory, but would not attack *H. grandis*.

162
163 Intra and inter-individual patterns of spicule abundance and size can offer
164 insights on the functionality of these structures. We first examined variation in
165 spicule size and density from several regions within the body including mantle

wall, siphons, and branchial tissues. We predicted that those regions with higher contractibility (siphons, branchial sac) could not accommodate high densities of large spicules. We then looked for size-related variation in length and density of spicules in the tunic and mantle by analysing individuals of different body sizes. Third, we incorporated spicules at natural concentrations into palatable feeding discs to test the ability of spicules to deter two specialist gastropod predators and a generalist predatory seastar in a series of laboratory feeding trials. Our hypothesis was that mantle spicules can act as a defence mechanism against specialist predators that are adapted to bore through the tunic or to insert their proboscis through the siphons of the ascidian in order to eat them. Finally, we sought to explore whether spicules may defend juveniles. We examined the survivorship of 90 day old laboratory-reared *Herdmania grandis* juveniles, which had developed spicules, in the presence of the seastar.

MATERIALS & METHODS

Organisms and study location

Herdmania grandis (Heller 1878) is a stolidobranch ascidian which can reach impressive sizes (ca. 16 cm largest dimension) and dominate shallow subtidal reefs (Fig. 1A). We collected ascidians of this species from two locations on the Illawarra coast (NSW, Australia) by SCUBA: the northern end of Flinders Islet (34.4565° S, 150.9296° E) and Bass Point (34.5960° S, 150.9018° E). Gastropods in the Ranellidae are recognised specialist ascidian predators (Laxton 1971, Young 1985, Underwood and Fairweather 1986). We collected >60 individuals of *Cabestana spengleri* (Perry 1811) and >20 of *Ranella australasia* (Perry 1811) at various locations near the city of Wollongong (34.4278° S, 150.8931° E) from

intertidal-zone rocky reefs. They were then acclimated to laboratory conditions maintaining them in recirculating aquaria at least three weeks prior to doing feeding trials. Periodically these animals were fed live individuals of the large solitary intertidal-zone ascidian *Pyura praeputialis* (Heller, 1878), which is a common prey item for these gastropods (authors' pers. obs.). We also used *P. praeputialis* to prepare artificial food in the feeding trials (see below). A generalist consumer, the starfish *Meridiastra calcar* (Lamarck 1816) was also collected from local reefs (>40 individuals), and was fed pieces of *P. praeputialis* mantle periodically. All consumers were returned to their place of original collection at the conclusion of the laboratory trials.

Intra and inter-individual patterns of spicule abundance and size

Spicule extractions were used to assess the density and length of spicules from several regions within individuals and between individuals of a variety of sizes. We sought to test the prediction that highly contractile regions, such as the inhalant siphon and branchial sac, would not possess large spicules in dense aggregations. To assess variation within individuals we targeted five regions in 6 individuals of large size (9-11cm largest dimension) collected at Bass Point: (i) the buccal siphon region, (ii) the anterior (iii) middle and (iv) posterior mantle (we used the ventral right hand side to avoid the digestive system, located at the left hand side), and (v) the middle of the branchial sac. For (i) we cut the oral siphon from the specimens, for (ii) to (iv) we removed tissue squares of the mantle wall measuring 2x2 cm. Finally, for (v) we removed tissue (2x2 cm) from the central right hand side of the branchial sac.

We examined spicule size and density for 29 individuals of a broad spectrum of sizes collected at the northern end of Flinders Islet. Individuals were measured (antero-posterior longest dimension) with callipers, and the tunic and mantle were then dissected. We extracted tunic and mantle spicules from squares of tissue measuring 2x2 cm. Tunic was taken from the right ventral side and carefully cleaned of any epibionts. Mantle was taken from the right ventral side halfway between siphons. The remaining tunic and mantle were oven dried at 80° for 24 hrs and weighed. Total dry weight (DW) of the individuals was obtained by adding the weights of all fragments removed from each individual (see below).

To obtain spicules we digested the organic mantle material from the calcareous spicules using strong chlorine bleach. Squares of fresh tissue cut from *Herdmania grandis* mantle were oven dried at 80° for 24 h and weighed to obtain a DW, then placed in bleach in an 80°C water bath until complete dissolution of organic matter (usually within 10 hrs). The tunic was too refractory for direct dissolution in bleach, so to isolate tunic spicules we added an extra step after oven drying; we charred the pieces of tunic in a muffle furnace at 500 °C for 5 hours. These tunic fragments were then placed in bleach as for the mantle fragments. Spicules were rinsed twice with distilled water and three times with absolute ethanol to remove all traces of bleach and were then oven dried at 50°C.

We measured the length of tunic and mantle spicules for the intra- and inter-individual assessments with a dissecting microscope (Zeiss). We selected haphazardly four visual fields and measured the first 5 spicules seen in each of

241 them (n=20). We also examined the spicules with scanning electron microscopy
242 (Fig. 1C-F). Dry spicules were sputter-coated with gold and viewed with a Hitachi
243 H2300 Scanning Electron Microscope at 15 Kv.

245 *Feeding assays*

246 We used mantle tissue from *Pyura praeputialis* to prepare palatable agar discs,
247 reasoning that it was common prey of the specialist consumers in the field.
248 Animals were separated from their tunic, the mantle was removed and dissected
249 to eliminate internal organs. The remaining mantle (mostly body wall) was
250 frozen, then freeze dried and ground to a powder for inclusion in feeding discs.
251 Control discs contained only freeze-dried *P. praeputialis* tissue, while *Herdmania*
252 *grandis* spicules were added to treatment discs. We estimated tissue densities by
253 immersing 5 pieces of ventral mantle wall of *P. praeputialis* and *H. grandis* in a
254 container with distilled water placed on a scale and noting the change in weight
255 that corresponds to the volume of water displaced. From these density estimates
256 and the appropriate dry weight/wet weight ratios we determined the correct
257 quantities of freeze-dried *P. praeputialis* mantle and *H. grandis* spicules to
258 incorporate into feeding discs so that the amount of food and spicules equals
259 natural values on a volumetric basis (López-Legentil et al. 2006).

261 We made agar feeding discs by slightly modifying the methodology of Ferguson
262 and Davis (2008). Agar (Oxoid LP0013, Thermo Scientific ®) was dissolved in
263 seawater at 6.5% w/v and boiled in a microwave for 2 min until it was clear.
264 When the agar had cooled to ~45°C we mixed 25 ml of agar solution, 4.08g of
265 freeze-dried *Pyura stolonifera* mantle and 15 ml of seawater. The mixture was

poured into small (25 mm diameter) glass petri dishes to prepare 5 replicate control discs of ca. 8 ml volume each. Treatment discs used the same recipe, but had 5.322g of *Herdmania grandis* spicules added (according to the spicule/wet weight ratio calculated from the mid-ventral pieces of the inter-individual study, see above). Once the discs had cooled they were removed from the petri dishes and weighed. They were then used immediately in feeding trials. Densities of tissues were 1.022g/ml for *P. praeputialis* and 1.188g/ml for *H. grandis* as measured from 5 ventral pieces of mantle. Densities of discs were 1.064g/ml for the control and 1.197g/ml for the treatment, thus very close to natural densities.

Feeding trials ran for 48 hours with a precautionary complete water change after 24 hours. Predators were housed in 2l containers each with a treatment disc (with spicules) and a control disc lacking them. Two animals per container were used for *Cabestana spengleri* and *Meridiastra calcar*, and only one for the larger *Ranella australasia*. The discs were removed after 48 hours, blotted dry and weighed to establish percent consumed. We ran 15 replicates each of treatment and control for *C. spengleri* and 10 for *R. australasia* and *M. calcar*. Autogenic change in treatment and control discs was assessed with 3 replicates for each species and although slight (ranging from 1.7% to 5.9 %) was factored into the estimates of percent consumption.

Experiments with each predator were run on successive days over the same week. All consumers were starved in the laboratory for a period of two weeks prior to initiating the feeding trials. Large quantities of spicules were observed in the faeces of some gastropods that consumed treatment discs at the conclusion

of the feeding trials (Supplemental Fig. 1A) and so these animals were held in the laboratory for an additional two weeks to assess their survivorship.

We examined the survivorship of laboratory-reared recruits of *Herdmania grandis* when housed with the generalist starfish consumer, *Meridiastra calcar*. Mature adults were collected at Bass Point in March and reproductive products were obtained by dissection from a minimum of four individuals and combined in filtered seawater (0.45 μ m). Fertilised ova were washed five times with filtered seawater (FSW) in the first hour and then left to develop for the next 24 hours at 18°C in small glass fingerbowls. Larvae were allowed to settle onto 8 perspex Petri dishes and were then held in aquaria. After maintaining recruits in the laboratory for 90 days with water changes of unfiltered seawater every three days, we distributed the Petri dishes (with ca. 15 juveniles each) into four, 60l aquaria. In two of these aquaria we placed 8 starfish and the remainder were predator-free. We measured mortality by counting losses after 1, 4 and 6 days. Recruits that did not contract in response to a prod with a blunt probe were considered dead.

Statistical analyses

Spicule densities and sizes within individuals were analysed with repeated-measures ANOVA; the individual measured (6 individuals) was the repeated factor and the within individual factor the position (6 levels: tunic, siphon, branchial sac, and anterior, middle and posterior mantle wall). We checked normality of the data with the Kolmogorov-Smirnov test, and the circularity assumption for repeated measures analysis was assessed with Mauchly's

sphericity test (von Ende 2001); no correction was necessary. *Post hoc* tests were made using the Student-Newman-Keuls (SNK) procedure. Relationships between individuals of spicule length and density with individual size (as measured by total DW) were examined with the Pearson correlation coefficient.

In the feeding trials we used t-tests to compare among treatments (with and without spicules). We assessed normality with the Kolmogorov-Smirnov test and homogeneity of variances with Levene's test. No correction was necessary. All statistical analyses were done in SigmaStat v 3.5 or Statistica v. 6.

RESULTS

Spicule description and intra-individual patterns

Spicules were in all cases elongated shafts with small barbs. There was a clear distinction between mantle and tunic spicules; spicules in the mantle wall were long (usually ca. 2 mm, but reaching up to 3 mm) with the surface thoroughly covered by fine barbs arranged in circles and pointing posteriorly (Fig. 1 B-D). In the branchial sac the spicules have the same overall appearance but were smaller (usually less than 1 mm) and more irregular, with an abundance of curved shapes (Fig 1E). On the other hand, tunic spicules were very much shorter (up to 0.1 mm) with only 12-18 whorls of posteriorly directed spines and a small spherical knob at the proximal end (Fig 1F).

The abundance of spicular material (ratio spicule/dry weight of tunic or mantle) was significantly different among compartments (repeated-measures analysis,

F=10.56, df= 5,25, $p<0.001$) (Fig. 2A). This was due to a significantly lower density of spicules in the tunic (mean $\pm$ SE = 0.291 ± 0.03) than in the mantle (SNK pairwise tests). No significant differences in spicule density were detected among mantle compartments (SNK tests), albeit they were slightly lower in the siphonal area (0.476 ± 0.08) and branchial sac tissue (0.482 ± 0.03) than in the mantle wall, where spicular ratios ranged between 0.510 ± 0.11 and 0.577 ± 0.103 depending on the position (Fig. 2A).

The length of spicules was also significantly different among compartments (repeated-measures analysis, F=300.35, df= 5,25, $p<0.001$) (Fig 2B). Aside from an order of magnitude difference in the tunic spicule lengths (0.069 ± 0.01 mm, mean $\pm$ SE), those in the mantle also showed significant differences in a post-hoc SNK test. The branchial spicules (0.776 ± 0.17 mm) were significantly shorter than all others, and the siphonal spicules (1.653 ± 0.14 mm) were significantly shorter than the ones in the mantle wall, which showed no significant differences along the body (overall 1.964 ± 0.20 mm) (Fig. 2B).

Spicule size and density between individuals

There was a highly significant correlation between size of the individuals, measured as the longest antero-posterior dimension, and total DW ($r=0.843$, $p<0.001$), we therefore used the latter for correlation analyses. The density of spicules in the tunic and the body wall, represented as a proportion of dry weight (DW) of those tissues, was highly variable (Fig. 3A,B). We recorded body wall spicule densities of ca. 0.3 to 0.85 of mantle DW (mean $\pm$ SE = 0.571 ± 0.11), whilst values for the tunic varied between ca. 0.1 and 0.4 of tunic DW (mean $\pm$ SE =

0.226±0.04). There was no relationship between spicule density and animal size, represented as DW ($r = 0.190$, $p = 0.323$ for mantle spicules, $r = -0.160$, $p = 0.406$ for tunic spicules, Fig 3AB).

There was a significant positive relationship between average length of mantle spicules and size of the individuals ($r = 0.482$, $p = 0.008$) (Fig. 4A), and this relationship is stronger if we consider maximal, instead of average, spicule length ($r = 0.520$, $p = 0.003$). On the other hand, there were no significant relationships between size of the ascidians and average or maximal tunic spicule length ($r = 0.043$, $p = 0.830$ and $r = -0.035$, $p = 0.860$, respectively) (Fig. 4B).

The mean sizes of spicules in the mantle and the tunic were significantly and positively correlated ($r = 0.543$, $p = 0.003$), but there was no relationship between tunic and mantle spicular densities ($r = 0.082$, $p = 0.673$).

Deterrent effects of spicules

Spicules incorporated into palatable agar discs were highly effective at deterring the specialist predators *Cabestana spengleri* and *Ranella australasia* from feeding. Consumption rates on control discs, that is those lacking spicules, were three times that of treatment discs (Fig. 5). These differences were significant in the two species (*C. spengleri*, $t = 2.201$, $df = 28$, $p = 0.038$; *R. australasia*, $t = 2.485$, $df = 18$, $p = 0.023$). The overall percentage of discs consumed was quite low, around 10-15% in the controls, but this almost certainly reflects the way in which these animals feed. Consumed discs showed clear evidence of the removal of furrows by the proboscis of animals (Supplemental Fig. 1B). Gastropods that

consumed treatment discs produced faeces with evident spicule material (Supplemental Fig 1A), but no harmful effect could be detected after keeping these individuals in aquaria for two further weeks.

The generalist consumer *Meridiastra calcar*, was not deterred from feeding by the presence of spicules in discs ($t = 0.004$, $df = 18$, $p = 0.997$, Fig. 5). The asteroid feeds quite differently than the gastropods as it everts its stomach over the feeding discs, resulting in consumed discs being uniformly worn, rather than having distinct marks (Supplemental Fig. 1C).

Juvenile mortality

Mortality of laboratory-reared recruits of *Herdmania grandis* increased four-fold in the presence of *Meridiastra calcar* (Fig. 6). Spicules were apparent in the juveniles' tunic, albeit at very low densities (Supplemental Figure 1D).

DISCUSSION

We provide evidence that the large, numerous, needle-like spicules of *Herdmania grandis* are effective at deterring specialist predators. Rates of predation by gastropods on feeding discs with spicules were just a third of that seen in controls. In contrast, the generalist consumer, the asteroid *Meridiastra calcar*, was undeterred by the presence of spicules, at least in agar discs. Generalist predators would have to contend with a tough leathery tunic and are unlikely to pose a threat unless the tunic of the ascidian has already been breached. Several studies have emphasised the important role of the tunic in enhancing

415 survivorship and contributing to the dominance of solitary ascidians in benthic
 416 habitats (Svane 1983; Young 1985; 1986).

417

418 Why then should *Herdmania grandis* apparently invest so heavily in defensive
 419 spicules in the body wall, which lies inside an already formidable leathery tunic?

420 We contend that the leathery tunic does not constitute an effective defence
 421 against gastropod predators which specialise in consuming ascidians.

422 Gastropods can drill through the tunic, as is commonly observed with *Cabestana*
 423 *spengeleri* attacking *Pyura praeputialis* (Underwood and Fairweather 1986;

424 author's pers. obs.). Alternately, gastropod predators may avoid the tunic

425 altogether and gain access to the internal organs of the ascidian via the siphons.

426 We note that the genus *Herdmania* lacks the organic spines present in the
 427 siphonal lining of other pyurids (Kott 1985), which may act to prevent

428 gastropods accessing the internal organs of ascidians via the siphon (Young
 429 1986).

430

431 Besides their defensive role, another potential function of the spicules is
 432 structural support. Lambert and Lambert (1987), for instance, studying another
 433 species (*Herdmania momus*) suggested a defensive role for tunic spicules, as they
 434 were protruding from the tunic and caused skin irritation during manipulation.

435 On the other hand, the spicules in the mantle, contained in protective fibrous
 436 sheaths, were postulated to have a supportive function. For *H. grandis*, we found
 437 the opposite pattern, the spicules in the tunic were about half the size (and with
 438 half the complement of spine whorls) reported for *H. momus*, and there was no
 439 harm when we were manipulating the tunic. On the other hand, the long (up to 3

mm) mantle spicules, even if they were also embedded in sheaths, broke free from the tissues easily. Any manipulation of the mantle had to be done with gloves to prevent spicules lodging into the skin, which were very hard to dislodge.

Although mantle spicules can have a supportive role, there are arguments against it for *Herdmania grandis*. First, other pyurids reach similar sizes than *H. grandis*, with similarly elaborated branchial sacs, and yet they do not require any internal skeleton. Second, the acicular shape of the spicules, with low surface to volume ratio, does not seem adequate for support (Koehl 1982). Other species of pyurids have antler-shaped mantle spicules (Lowenstam et al 1987); these are more likely to be structural, as they have high surface to volume ratios and can interlock to provide support to soft tissues (Lambert and Lambert 1997). Indeed, they are found in abundance in delicate tissues such as the branchial sac of some species (Lowenstam 1987, Lambert and Lambert 1997).

The intra-individual patterns of spicule distribution can also provide insights about their function. We note that spicules were significantly smaller and in lower densities in contractile tissues such as the siphon and branchial basket. It is likely that strong contraction of a spicule-loaded tissue will lead to breakage of the containing sheaths and tissue damage, as observed with even the slightest manipulation of mantle in the lab. The highest spicule content and the longest spicules were found in the muscular mantle wall, which argues against a supportive role, in which case we would expect high abundance, for instance, on the complex folded structures of the branchial sac. Further, of all the pyurids we

have worked with, living *H. grandis* show the least capacity to contract and close siphons. All of this points to a trade-off between the potential advantages of spicules and the need to keep the functionality and mobility of tissues. Mantle spicule densities did not increase with size of the animals, so older ascidians did not seem to accumulate more spicules. In contrast, there was a significant association between mantle spicule length (both average and maximal length) with size, and hence age, of the ascidians. A similar relationship was noted by Lambert and Lambert (1987) in *H. momus*. The tunic spicules, however, showed no relationship of density or length with size of organisms. On the other hand, there was a significant correlation between average length of spicules in the mantle and in the tunic, but none between spicule density in these compartments, indicating that they may respond to different pressures.

Colonial ascidians also invest in spicule production. Many didemnid ascidians possess small (20-50 μm) stellate spicules (Lindquist et al. 1992) and they can be present in extremely high densities. For example, Lindquist et al. (1992) reported volumetric spicule densities of 500mg/ml for the Caribbean *Trididemnum solidum*. This figure is three times the spicular density we observed in the body wall of *Herdmania grandis* (≈ 133 mg/ml). Several tropical reef sponge species also possess spicular densities higher than those we observed for *H. grandis* (summarised in Ferguson and Davis 2008). Densities of calcified sclerites among soft corals from the tropical Pacific can exceed 50% of the tissue dry weight at colony tips and up to almost 90% of dry weight at the base of colonies (Van Alstyne et al. 1992, 1994). It would appear then that the density of spicules in *H. grandis* is not exceptional among soft-bodied organisms but, unlike

ascidian tunic, sponge mesohyl, or cnidarian cenosarc, the mantle spicules in *H. grandis* are found in highly mobile tissues such as the musculature, branchial sac or siphonal areas.

Previous experimental examination of the deterrent properties of ascidian spicules at natural concentrations have concluded that they are ineffective as predator deterrents (Lindquist et al. 1992; López -Legentil et al. 2006), although they may act in concert with secondary metabolites. Rather than a direct deterrent role for spicules, Duffy and Paul (1992), have argued that the presence of spicules modifies the nutritional quality of tissues and therefore their attractiveness to predators. In contrast, Lindquist et al. (1992) observed that very high concentrations of spicules did not deter predators. Olson (1986) suggested an alternate role for the spicules of tropical colonial ascidians; providing shade to photosynthetic symbionts in high light environments. For *Herdmania grandis*, the barbed nature of the spicules is consistent with a role as a direct deterrent rather than simply a means of indirectly modifying nutritional quality. In this regard, we were surprised to see the faeces of *Ranella australasia* that had fed on treatment discs, bristling with spicules. We found no evidence of elevated mortality as a result of animals ingesting these spicules, but anticipate sublethal effects of these barbed structures passing through the gut

It has been argued that as spicules in feeding discs do not lie in natural tracts, as in the animal, they likely underestimate the defensive capacity of these structures (Hill and Hill 2002). This may indeed be the case, but it would mean feeding trials should then be a conservative test of the antifeedant hypothesis.

Further, we were struck by the similarity of the orientation of spicules in the body wall of *Herdmania grandis* and those in our feeding discs (Supplementary Fig 1E, F). That is, both appeared to be largely oriented at random. A defensive strategy that we consider unlikely was proposed by Kott (2002). She noted the well-developed body musculature of *H. grandis* and suggested that contraction of these muscles would form a tough interwoven matt of spicules to “form a hard layer over the whole of the body wall” (Kott 2002). We question this supposition, as muscular contraction with such sharp structures would seem likely to lead to significant self-harm.

We did not test the role of the tunic in thwarting predator attacks directly, but our experiments suggest that unless the leathery tunic is already breached generalist consumers are unlikely to successfully attack adult *Herdmania grandis*. However, as our focus was on a single species of generalist consumer it may be premature to draw conclusions more broadly, as has been cautioned for terrestrial systems (Ali and Agrawal 2012). Nevertheless, early life stages of *H. grandis* are clearly susceptible to mortality by the generalist consumer *Meridiastra calcar* and probably other echinoderm species. The activities of predators affecting early life history phases of ascidians while adults remain unharmed has been observed before (Davis 1988). These findings confirm the importance of considering a range of predator strategies and of life history stages in assessing threats posed by specialist and generalist consumers.

CONCLUSIONS

Taken together our findings support the notion that the leathery tunic is the primary defence of *Herdmania grandis*, while the mantle spicules - a second line

of defence behind the ramparts - are deterrent against specialist predators,
capable of breaching the tough tunic. It is not uncommon for sessile organisms to
invest in an array of defensive strategies to cope with a variety of consumers
(Hay 1987). For *H. grandis* it appears that this includes multiple physical
defences. The resistance of this ascidian to abundant specialist predators may
explain its dominance in the shallow subtidal reefs of southeastern Australia.

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Figure Captions:

Fig. 1. *Herdmania grandis* in-situ and calcareous spicules at varying degrees of magnification. A) Biogenic structure provided by *H. grandis* on a shallow subtidal reef. All of the siphons visible are those of *H. grandis*. B) Light micrograph of spicules extracted from the mantle. C, D) Scanning electron micrographs (SEM) of mantle spicules. E) SEM micrograph of branchial sac spicules. F) SEM of tunic spicules. Scale bars: A, 50 mm; B, 1.5 mm; C, 0.5 mm; D, 50 μ m; E, 100 μ m; F, 50 μ m.

Fig. 2. Intra-individual variation in (A) spicule abundance (presented as ratios of spicule weight/DW of the corresponding compartment, tunic or mantle) and (B) spicule length of *Herdmania grandis*. Mean and SE of the values of 6 individuals (20 spicules measured in each compartment for each individual). Anterior, middle, posterior part refers to the corresponding parts of the right ventral zone of the mantle. Horizontal bars join compartments not significantly different in a *post hoc* SNK test. See text for details.

Fig 3. Weight ratios of mantle and tunic spicules as a function of the overall size (expressed as total dry weight) of *Herdmania grandis*.

Fig 4. Length of mantle (A) and tunic (B) spicules as a function of the overall size (expressed as total dry weight) of *Herdmania grandis*. Note different Y-axes. Mean and SE of 20 spicule measured per individual.

Fig. 5. Mean percent ($\pm$ SE) consumption of treatment (containing mantle spicules of *Herdmania grandis*) and control agar discs by specialist ascidian consumers, *Cabestana spengleri* and *Ranella australasia* (Gastropoda, Ranellidae) and by the generalist consumer *Meridiastra calcar* (Echinodermata: Asteroidea,). Replication: n = 30 (15 control, 15 treatment) for *C. spengleri*, n = 20 (10 control, 10 treatment) for *R. australasia* and *M. calcar*.

Fig. 6. Outcome of feeding trials with *Meridiastra calcar* (Asteroidea). Mortality of 90 day old juveniles of *Herdmania grandis* in the presence or absence of the generalist consumer.

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Supplemental Figure 1

Supplemental Figure 1: Images relating to laboratory feeding trials of specialist and generalist ascidian consumers of *Herdmania grandis*. A) Faeces of gastropod *Ranella australasia*, with spicules visible, after consuming treatment discs. Consumption of palatable feeding discs B) by the gastropod *Ranella australasia* and C) the generalist consumer *Meridiastra calcar* (Asteroidea). D) image of a laboratory-reared juvenile of *Herdmania grandis*, white specks in the tunic are spicules (magnified in inset). E) Natural spicule tracts in the bodywall of *Herdmania grandis*. F) Surface of treatment feeding disc, spicules are clearly apparent with orientations similar to those in the bodywall. Scale bars: A, 2 mm; B, C, 10 mm; D, 2 mm (inset, 15 μ m); E,F, 1.5 mm.