

## Morphology and biological roles of spicule networks in *Cadlina luteomarginata* (Nudibranchia, Doridina)

Brian K. Penney<sup>a</sup>

Department of Biological Sciences, University of Alberta, Edmonton, AB, Canada T6G 2E9  
and Bamfield Marine Station, Bamfield, BC, Canada V0R 1B0

**Abstract.** Many cryptobranch dorid nudibranchs contain innumerable calcareous spicules, yet the function of these elements is unknown. Two possible roles are defense against predators and structural support. In one dorid, *Cadlina luteomarginata*, whole-mount and thin-section staining revealed an intricate network of spicule tracts and connective tissue ramifying throughout the body, with muscle fibers associated with this spicule/connective tissue matrix and inserting into it. Spicules were present in high concentrations in all areas of the body, but highest in exterior mantle tissue. Relative investment increased isometrically with body size for most body regions, in contrast to the positively allometric investment seen in prosobranch shells. Bioassays with artificial food indicated that spicules alone did not deter generalist crabs and anemones, and only weakly increased the deterrence of secondary chemicals to anemones. Thus, while nudibranch spicules may serve as a defense against other predators, their primary role may be in body support.

*Additional key words:* antipredator defense, musculature, skeletal elements

Dorid nudibranchs and several other opisthobranch taxa such as notaspideans are unusual for gastropods in possessing calcareous spicules (Hyman 1967). These are often present as a large percentage of total dry weight (Cattaneo-Vietti et al. 1993), yet their function has not been explicitly tested. Spicules have often been assumed to be defensive, perhaps because of the bright colors and shell-less form of nudibranchs, but no study has directly tested the effect of nudibranch spicules against predators independent of antipredator chemicals (Todd 1981; Foale & Willan 1987). The defensive value of spicules in other organisms varies widely, from effective as deterrents alone (Harvell et al. 1988; Van Alstyne et al. 1992), to only effective in combination with chemical defense (Gerhart et al. 1988), to being sometimes altogether ineffective (Wylie & Paul 1989; Lindquist et al. 1992; Chanas & Pawlik 1996). Clearly, putative defensive roles of nudibranch spicules require direct testing.

Nudibranch spicules could also serve as skeletal support as they do in gorgonians and many echino-

derms (Brusca & Brusca 2003), and they certainly support external structures such as papillae (García et al. 1986; Valdés & Gosliner 2001). We know little about spicule arrangement inside sea slug bodies, as most authors have only examined surface conformations (Kress 1981; Foale & Willan 1987), but they form complex networks in at least one dorid nudibranch, *Doriopsilla areolata* BEGRH 1880 (García et al. 1986). Characterizing these networks is crucial for understanding their functional roles and may also prove useful as a phylogenetic character (Wägele & Willan 2000).

I investigated potential defensive and structural roles of spicules in *Cadlina luteomarginata* MACFARLAND 1966 (Nudibranchia: Doridina), a nudibranch common in the Northeastern Pacific that has been the focus of numerous investigations of chemical defense (Avila 1995). First, I used whole-mount and thin-section staining to describe the internal arrangements of spicules and their relations with other tissues. Second, I measured spicule investment as a function of body size, to compare investment to that seen in shelled gastropods, and of specific body regions, to investigate their relevance for structural and defensive roles. Finally, I tested direct deterrence of spicules to local generalist predators, alone and in combination with chemical defense, using artificial food in laboratory assays.

<sup>a</sup>Biology Department, Box 1742, Saint Anselm College, Manchester, New Hampshire 03102, USA. E-mail: bpenney@anselm.edu

## Methods

### Histology

Five individuals of *Cadlina luteomarginata* were collected by hand using SCUBA from Barkley Sound, Vancouver Island, BC. They were anesthetized in isotonic  $MgCl_2$  and preserved in buffered formalin in seawater for 1–2 weeks. For whole-mount staining, three individuals were transferred to 95% EtOH, and then cleared and stained using a standard ichthyology protocol modified as follows. Specimens were first dissected into foot, mantle, rhinophores, and gill to allow better visualization. Viscera were not further treated, but were kept as vouchers. Samples were cleared in phenol over 2 d until translucent and then further digested in a stock alcohol–hydroxide solution (70% EtOH and 1% NaOH, mixed 15:1). Cleared samples were stained with Alizarin red mixed at 0.03% by weight in the above alcohol–hydroxide solution, and slight overstaining was removed through 24 h treatment in the stock alcohol–hydroxide solution alone. Samples were mounted in glycerol to further clear tissues and observed through a Wild stereomicroscope and digitally photographed (Nikon DMX, Act 1 software, Nikon, Melville, NY).

Samples of mantle tissue from near the anterior/posterior midline of the remaining two specimens were sectioned to observe the relationship between spicule networks and other tissues. Specimens were rinsed in tap water, decalcified in RDO (Apex Engineering Products Corporation, Aurora, IL) for 5 h, rinsed in tap water, dehydrated via an ascending ethanol series (70%, 95%, and 100%), and then embedded in paraffin. Sections were cut to 5  $\mu m$  using a manual rotary microtome, adhered to glass slides using 3% glycerol/albumin solution (Humason 1972), stained using a Masson's Trichrome recipe optimized for marine molluscs, and mounted in DPX (Electron Microscopy Sciences, Fort Washington, PA). Stained sections were observed through a light microscope and digitally photographed (Leica DM IRBE [Leica, Bannockburn, IL] with Nikon DMX 1200 or Olympus BX60 [Olympus, Melville, NY] with Nikon Coolpix 995 and Scopetronix mount [Scopetronix, Cape Coral, FL]).

### Investment by size and body region

Fifteen individuals of *C. luteomarginata* of 1.1–5.3 g wet weight were collected by hand, intertidally or subtidally using snorkeling or SCUBA, from Barkley Sound, BC. Animals were anesthetized in a 1:1 mix of 7%  $MgCl_2$  to seawater (Smith & Carlton 1975), dabbed dry with a paper towel, weighed, and then dissected into four external parts (rhinophores, gill, man-

tle, and foot) with the viscera removed. Each part was placed in an individual, pre-ashed, pre-weighed aluminum tray and dried at 56°C for ~24 h to stable weight, and weighed twice to obtain an average weight.

Each sample was incinerated in a muffle furnace at 500°C for 24 h to burn off organic tissue, and the remaining inorganic material was weighed twice and averaged. Overall ash weight was determined to be a more accurate and precise measure than chemical digestion of tissue and subsequent isolation of spicules from solution for weighing (unpubl. data), and I therefore use this as a reasonable proxy for spicule weight. However, this method has two disadvantages. First, although inorganic salts comprise an almost constant 3.5% of wet mass for most sea slugs (Penney 2002) and should be subtracted from the ash weight to obtain the weight of spicules alone, this was not possible for this study as wet weights of individual parts proved impossible to obtain precisely. Second, because visual inspection indicated residual organic material after lesser intervals, ashing times were longer than those recommended in the literature. Although the loss of  $CaCO_3$  after 8 h at temperatures > 600°C is high (Paine 1971), loss over 8 h at 500°C is minimal and has a linear slope (A.R. Palmer, pers. comm.); extrapolation indicated that loss at 500°C over 24 h was less than the extra weight presented by remaining physiological salts. I therefore have presented the ash weights as the best measure of spicule weight per body region, but these data may slightly overestimate the true values. Data were compared in three ways: (a) as a regression of log total spicule weight versus log total slug dry weight, (b) as a regression of log spicule weight versus log dry weight for individual body regions, and (c) as a one-factor ANOVA comparing the average ratio of spicule content to dry weight among body regions.

### Bioassays against predators

To test whether spicules are potentially deterrent to consumers, I used feeding assays of local generalist predators. The crabs *Cancer productus* RANDALL 1839 and *C. gracilis* DANA 1852 are generalist predators on molluscs and other organisms (Orensanz & Gallucci 1988). Both are large enough to attack adult dorids and overlap the habitat of *C. luteomarginata* in geography and depth (low intertidal to ~100 m from Alaska to California), although *C. gracilis* is found primarily on softer bottoms (Gotshall 1994) and might not often encounter individuals of *C. luteomarginata*. Crabs were collected either by baited trap or by hand, using SCUBA, from Bamfield and Grappler Inlets, Bamfield, BC. The aggregating

anemone *Anthopleura elegantissima* BRANDT 1835, a sit-and-wait predator that consumes crustaceans and molluscs that have been swept off rocks (Kozloff 1983) found from the intertidal to 20-m depth on rocks and other structures from Alaska to California (Gotshall 1994), was extremely common at nudibranch collection sites. Individuals were removed from rocks by hand at Scott's Bay, Bamfield, BC, and allowed to acclimatize to the laboratory. All predators were maintained in individual containers with flowing natural seawater at Bamfield Marine Station and fed every other day, initially with *Mytilus* spp. (blue mussels) and then with pieces of squid mantle for 1 week before the experiment.

To test the effects of spicules alone or in combination with chemical defense, both spicules and extracts were added at natural concentrations to an artificial food recipe. Chemical extracts were made from 17 individuals of *C. luteomarginata* collected using SCUBA from Scott's Bay, Bamfield, BC, with viscera removed after anesthetization so that only compounds contained in the body wall (and not those present only from undigested prey in the gut) would be in extracts. The remaining 117 g wet weight of flesh was thrice extracted with  $3 \times$  volume MeOH:CHCl<sub>3</sub> (2:1) for 24 h each time. This extract was reduced to a small volume of methanol under vacuum using a rotary evaporator. The base artificial food was made from squid flesh and water (1:1) mixed with sodium alginate (2% of final wet weight) in a Waring blender (Waring Laboratory, Torrington, CT) (Lindquist & Hay 1996). To this base was added (a) either spicules at natural concentration (4.6% of wet weight) or not or (b) either methanolic extracts at 1 g nudibranch equivalent per 1 g of food or methanol alone. This formed four treatments: control (no additives), spicules alone, extracts alone, or spicules plus extracts. After ingredients were mixed thoroughly, the food was then solidified to approximately the consistency of cooked pasta in a solution of 0.25 mol L<sup>-1</sup> CaCl<sub>2</sub>. Because nutritional content can affect the acceptability of food to predators (Penney 2002), I ensured that this recipe produced food at the natural nutritional quality of dorid nudibranch tissue, ~12% organic content per unit wet mass.

Individual predators received each treatment once, at the rate of one treatment per day. The order in which treatments were presented to each individual was determined with a random number table; the number of individuals receiving each treatment on a given day was similar. Before bioassays, each individual predator was tested with a small piece of squid flesh (~1 cm<sup>2</sup>) to ensure it was hungry before receiving experimental food. Food pellets were delivered directly to predator "mouthparts" (i.e., tentacles or chelae) and, if neces-

sary, left in tanks for 2 h (crabs) or overnight (anemones). I recorded acceptance if visual inspection of the tank indicated more than three-fourths of the food was consumed, or rejection otherwise. For anemones, I also recorded rejection for any piece ejected whole from the enteron within the next 24 h, making this a conservative estimate of food acceptability. Each individual predator was used only once in each bioassay to avoid pseudoreplication, and the results of bioassays were analyzed using Cochran's Q-test (Zar 1984).

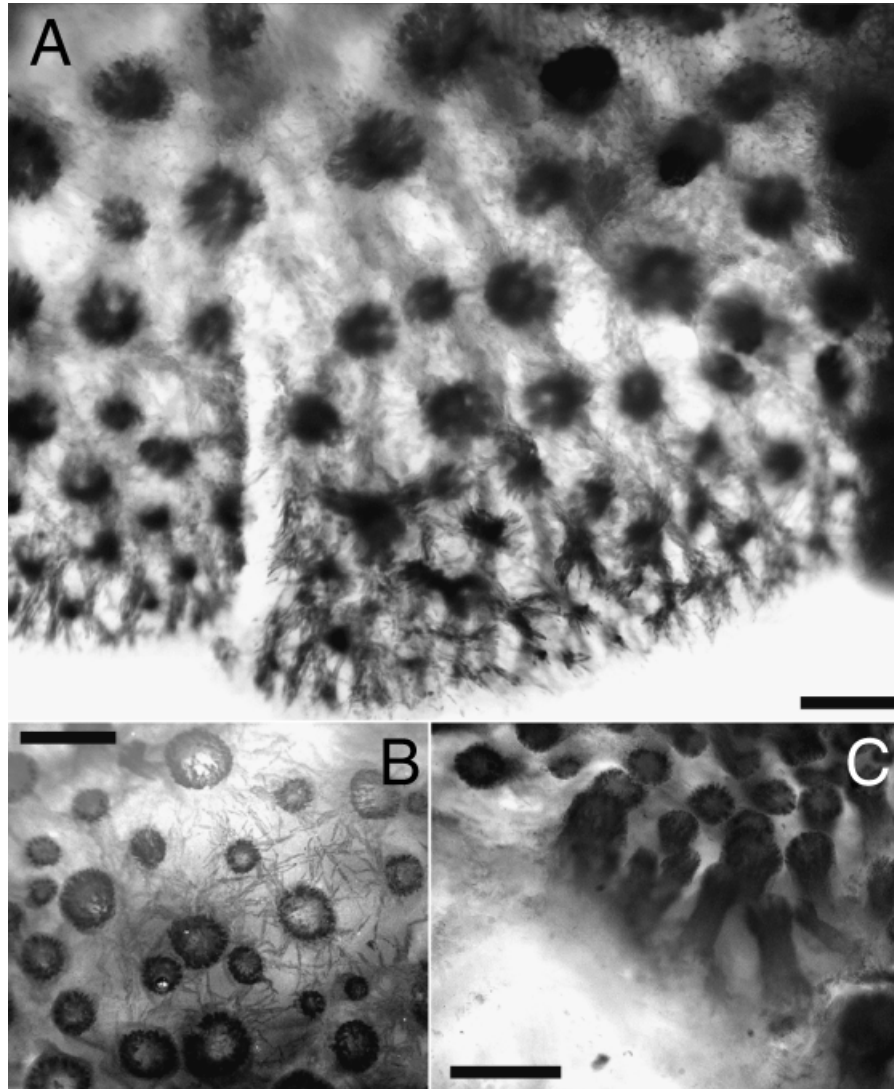
## Results

### Histology

Whole-body staining indicated that spicules were organized into tracts, often five or more spicules wide, with individual spicules mostly parallel to the long axis of the tracts (Fig. 1A). These fibers formed an underlying reticulated network, from which issued thick vertical tracts leading to loosely organized rosettes of spicules supporting the papillae (Fig. 1C). Spicule tracts were more finely reticulated toward the edge of the mantle. Horizontal spicule tracts were not all in the same plane, and this network was generally less dense through the central notum over the viscera (Fig. 1B). Magnifications  $\leq \times 500$  revealed only fusiform spicules (sensu García et al. 1986); stellate spicules were absent. Spicule networks in the foot were organized as in the mantle, but lacked the vertical tracts and papillae. The foot also had a more definite border of spicules (Fig. 2A) that was finer and more highly reticulated near its edge. Tracts were less dense near the midline of the foot, but continued uninterrupted all the way across its breadth (Fig. 2B). Unlike in the mantle, these tracts appeared to be mostly in the same horizontal plane.

Gill branches were supported by a fine axial skeleton, clearly organized into two rows of spicules up each main branch (Fig. 2C). Most spicules seemed oriented perpendicular to the axis of the branch. The mantle around the gills was not significantly more densely spiculated than the rest of the mantle, but this ring was still supported by numerous vertical tracts leading to the surrounding papillae. Rhinophore lamellae were supported by dense rows of parallel spicules (Fig. 2D). The surrounding sheath was densely lined with spicules, but not clearly in ramified networks as in the rest of the mantle. Spicules did not extend through the shaft below the level of lamellae.

Mantle cross sections indicated that spicules are embedded in a connective tissue matrix in discrete frameworks (Fig. 3A). Horizontal tracts paralleled the shape of the mantle, and vertical tracts extended



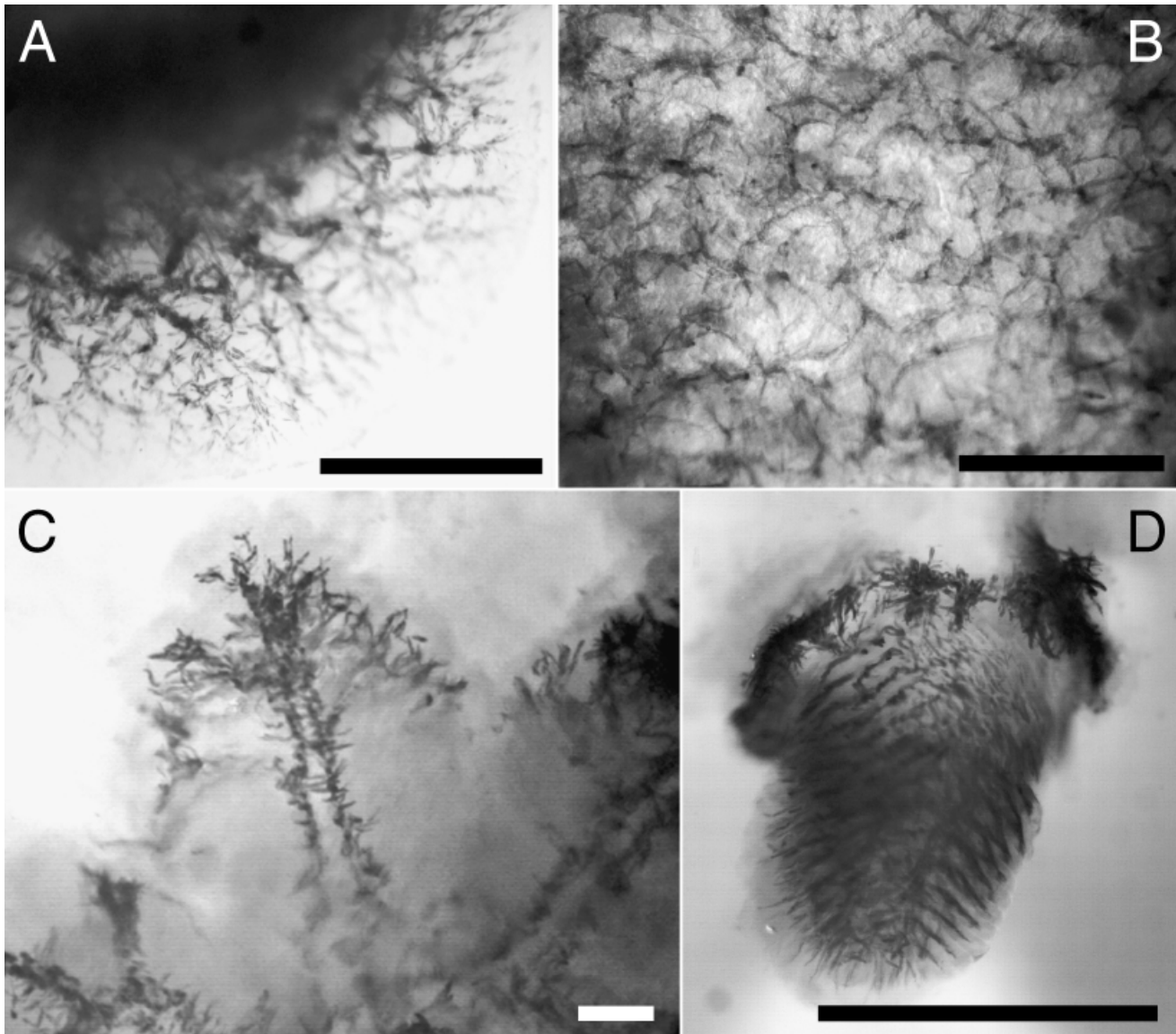
**Fig. 1.** Light micrograph of mantle, cleared and stained for visualizing spicules. **A.** Montage of mantle edge, dorsal view. The anterior–posterior axis is approximately horizontal (scale bar, 500  $\mu\text{m}$ ). **B.** Central mantle, dorsal view, showing vertical tracts and spicule rosettes supporting the papillae. The anterior–posterior axis is approximately vertical (scale bar, 500  $\mu\text{m}$ ). **C.** Vertical tracts supporting papillae surrounding the gill ring, dorsal view. Anterior is to the right of the image (scale bar, 100  $\mu\text{m}$ ).

into disorganized rosettes supporting papillae. Thin bands of muscle were often closely aligned with the spicule tracts and surrounding connective tissue. Close connections between muscle and spicules at various points included thicker plates of connective tissue (Fig. 3B,C). Muscle bands and apparent connections to the spicule network were larger and most common within the mantle edge, i.e., the mantle region distal to its connection to the main body, and largely missing from the region overlying the viscera. The bottom edge of the mantle had a structure different from that of overlying areas due to the short interspersed bands of muscle and connective tissue.

#### Investment by size and body region

Spicule weight (log ash weight) increased in direct proportion to slug size (log total dry weight) for the

whole animals and for the mantle and foot (Table 1). Therefore, spicules comprise a fairly constant proportion by mass of these body regions over the range of sizes investigated. However, both rhinophores and gills showed small but statistically significant departures from isometry (Table 1). Investment in spicules was heavy in some areas, e.g., up to 45% of dry weight in the mantle (Fig. 4). Distribution of values for ash weight/dry weight was not statistically different from normal ( $p = 0.633$ , Shapiro–Wilk  $W$ -test), and variances were homogenous (Levene test,  $F$ -ratio = 1.90,  $p = 0.139$ ). Group means were significantly different (ANOVA,  $p < 0.0001$ ), with significant differences between all pairwise comparisons (Scheffe's test,  $p < 0.0001$ ) except foot versus rhinophore ( $p = 0.9980$ ). Because the  $y$ -intercepts for each regression on untransformed ash weights were close to zero, a factorial ANOVA is acceptable to compare



**Fig. 2.** Light micrograph of external structures, cleared and stained for visualizing spicules. **A.** Edge of foot, ventral view. The posterior of the specimen is to the top of the image (scale bar, 250  $\mu$ m). **B.** Foot center, ventral view. The anterior–posterior axis is approximately vertical (scale bar, 100  $\mu$ m). **C.** Montage of the gill (scale bar, 250  $\mu$ m). **D.** Rhinophores (scale bar, 1 mm).

these ratios rather than an ANCOVA (Packard & Boardman 1988).

### Bioassays against predators

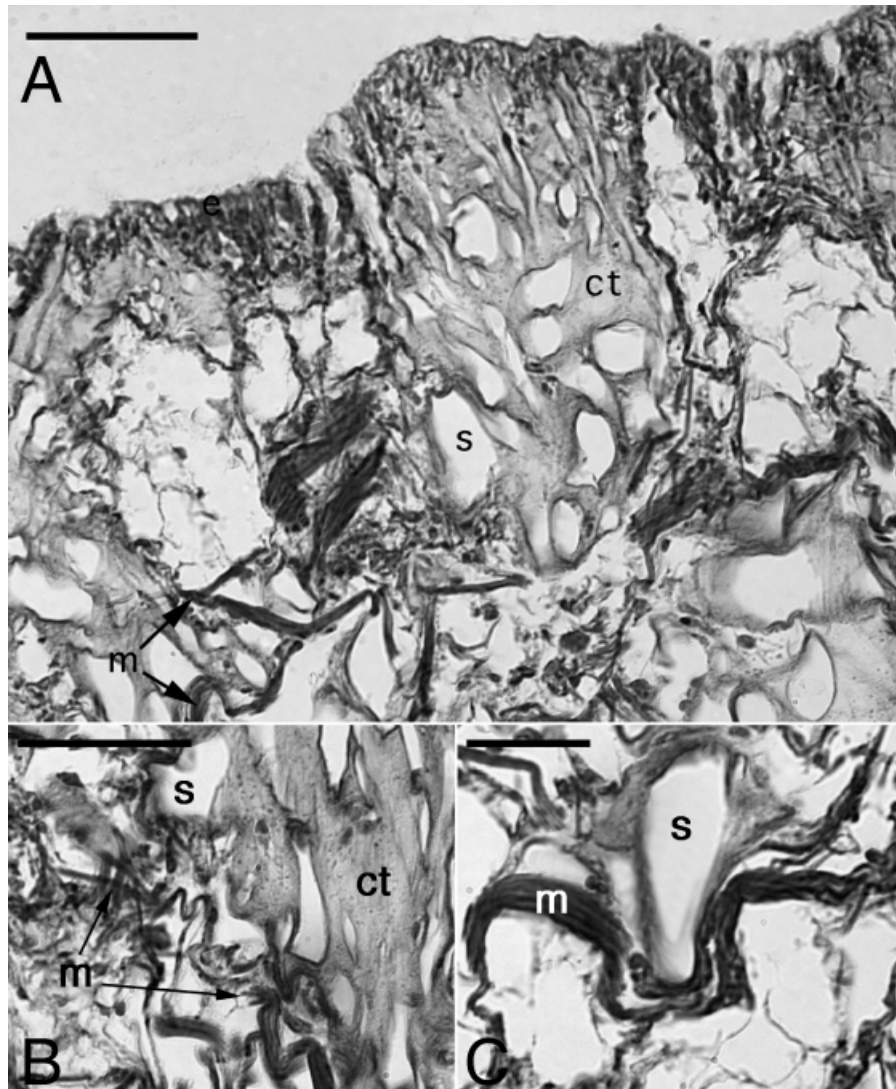
Crabs were significantly deterred by nudibranch extracts (Cochran's  $Q$ ,  $p < 0.05$ ) but not spicules. The latter alone had little deterrent effect, and did not seem to increase the effectiveness of chemical extracts (Fig. 5A,B). Crabs of both *Cancer productus* and *C. gracilis* responded similarly. Anemones were not deterred by spicules or extracts alone, but the com-

bination of spicules and chemical extracts significantly reduced feeding with respect to control food (Cochran's  $Q$ ,  $p < 0.05$ ; Fig. 5C).

## Discussion

### Histology

Stained whole mounts and mantle cross sections revealed that spicules were organized into tracts bound by connective tissue that formed a ramifying network, with almost rhomboidal lumens in



**Fig. 3.** Light micrograph of connective tissue and musculature in mantle cross sections. **A.** Top of mantle, approximately halfway between the body wall and mantle edge, showing papillae and underlying structure. **B, C.** Muscle insertion into spicule/connective tissue matrix. ct, connective tissue; e, epidermis; m, muscle bundle; p, papilla; s, spicule. Scale bars, 50  $\mu\text{m}$ .

between, throughout the mantle and foot (Figs. 1A,B, 2A). Tracts were thinner near the edges of both body regions, and also possessed fewer and thinner tracts toward the midline of the body (Figs. 1A,B, 2A,B). Previous observations have also indicated connective tissue sheaths surrounding spicule tracts in dorid nudibranchs (Hyman 1967; García et al. 1986). The spicule network is faintly visible in living individuals of *C. luteomarginata* as reticulating, less translucent lines on the ventral surface of the mantle edge (MacFarland 1966). In mantle cross sections, only one horizontal tract is visible per section, indicating that the multiple levels seen in whole-body mounts are actually horizontal tracts extending at different heights. In the mantle, vertical tracts extend from this network and lead dorsally to disorganized rosettes of spicules supporting papillae (Figs. 1C, 3A). Rosettes in cross sections are not noticeably

wider than the tracts supporting them, unlike those seen in whole-body staining. This may indicate some slight shrinkage during processing, or slight variation among individuals.

The network also branches into smaller structures supporting the rhinophores and gill (Fig. 2C,D). Chemosensation and gas exchange both require large surface area (Brusca & Brusca 2003), which in dorids is accomplished by highly folded or branched exterior structures supported by a fluid-filled or muscular hydrostat. Strong external flow past such a structure would tend to push the lamellae or branches together like leaves on a tree in wind and reduce the active surface area, a problem likely exacerbated by the formation of a boundary layer around the condensed group of structures, further reducing exchange with the medium (Vogel 1988, 1994). In such situations, endoskeletal elements that keep exchange structures

**Table 1.** Spicule investment in *Cadlina luteomarginata*.  $N = 15$  for all comparisons. Least-squares regression equations for whole body dry weight or log (whole body dry weight) versus various body regions. Adj.  $r^2$ , adjusted  $r^2$ ; p, exact probability; RMA, reduced major axis; SE, standard error.  $p_{\text{slope}}$  indicates the probability that the regression slope equals zero, while  $p_{\text{allom}}$  indicates the probability that the slopes conform to an isometric relationship (i.e., one-sample t-test against 1.0).

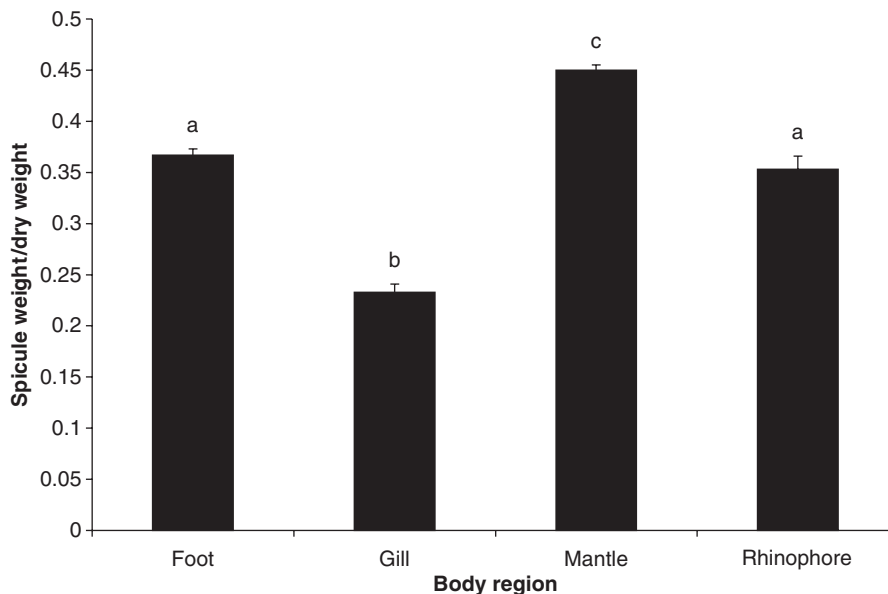
Body region	Units	Slope ( $\pm$ SE)	Intercept	Adj. $r^2$	$p_{\text{slope}}$	Slope <sub>RMA</sub>	$p_{\text{allom}}$
Whole body	Ash	$0.418 \pm 0.011$	0.006	0.991	0.0001		
	Log ash	$0.959 \pm 0.030$	-0.038	0.986	0.0001	0.965	0.2600
Mantle	Ash	$0.353 \pm 0.011$	0.006	0.987	0.0001		
	Log ash	$0.949 \pm 0.035$	-0.452	0.982	0.0001	0.958	0.2500
Foot	Ash	$0.060 \pm 0.003$	-0.001	0.963	0.0001		
	Log ash	$1.017 \pm 0.052$	-1.231	0.965	0.0001	1.035	0.5119
Gill	Ash	$0.003 \pm 0.000$	0.000	0.931	0.0001		
	Log ash	$1.320 \pm 0.111$	-2.466	0.909	0.0001	1.379	0.0042
Rhinophore	Ash	$0.001 \pm 0.000$	0.000	0.798	0.0001		
	Log ash	$0.713 \pm 0.101$	-2.861	0.778	0.0001	0.800	0.0131

separate may be useful in maintaining physiological function.

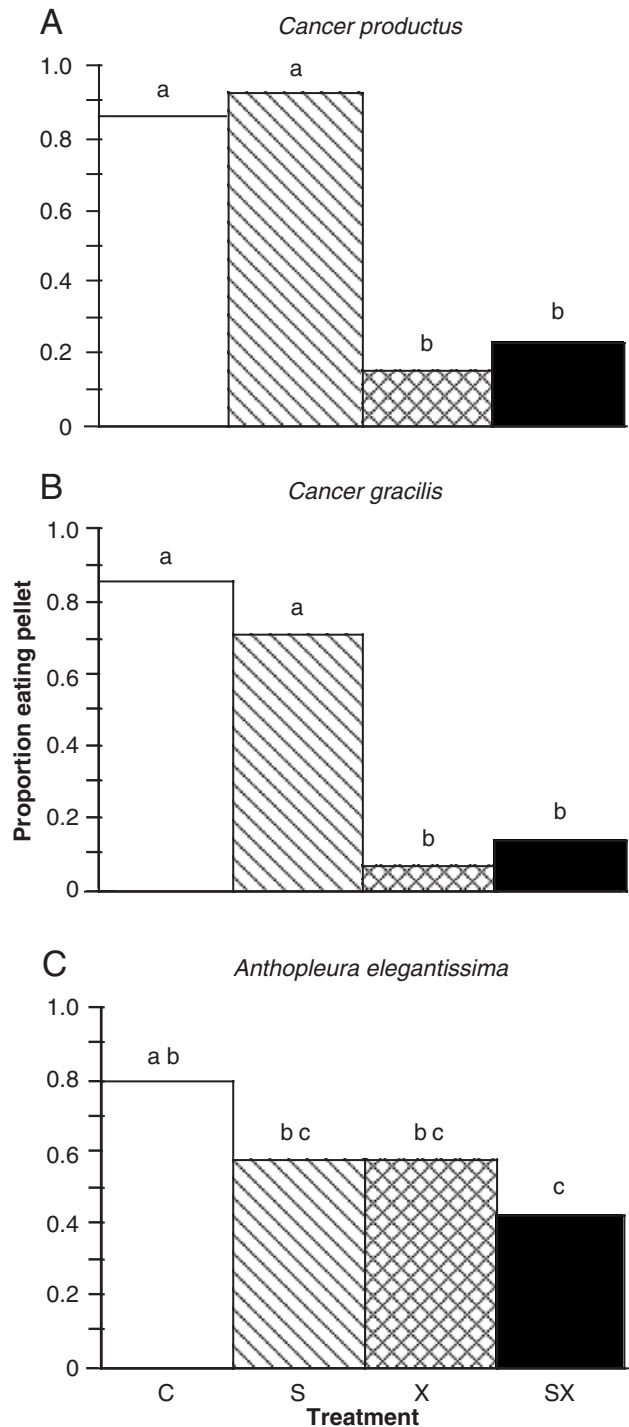
The only other detailed study of spicule networks in a dorid nudibranch, *Doriopsilla areolata* (García et al. 1986), described a network very similar to that found in the present study, even though the two species are not closely related (Valdés & Gosliner 1999; Valdés 2002a). The networks in the mantle and foot are very similar, and *C. luteomarginata* varies primarily in that (a) foot tracts are not as clearly perpendicular to the longitudinal body axis, (b) foot tracts become thinner centrally but continue all the way across the foot, instead of disappearing near the midline, (c) the gill is more heavily spiculated, has

spicules further up the gill branches, and spicules are more clearly organized into tracts up each branch, and (d) the basal third of the rhinophore shaft lacks spicules. Individuals of *C. luteomarginata* also apparently lack stellate spicules. These should have been visible at the magnification used; however, I did not specifically digest tissue to look for them. This absence may be due to variation among individuals or species, as stellate spicules have been found in *C. flavomaculata* (MacFarland 1966).

Despite the similarity between these two species, spicule presence and arrangement vary noticeably among dorid taxa. Spicules are present in most basal species and likely represent a plesiomorphic



**Fig. 4.** Spicule content of individuals by body region. Bars sharing letters were not significantly different ( $n = 15$ ; ANOVA, Sheffe's test,  $p < 0.0001$ ).



**Fig. 5.** Bioassay results of spicules and chemical extracts with generalist predators. **A.** *Cancer productus* ( $n = 13$ ). **B.** *Cancer gracilis* ( $n = 14$ ). **C.** *Anthopleura elegantissima* ( $n = 14$ ). Treatments: c, control; s, spicules; x, extract, sx, spicules plus extract. Bars sharing letters within each panel were not significantly different (Cochran's Q-test,  $p < 0.05$ ).

character (Valdés 2002a). However, they are absent both in species derived from near the origin of the dorids, e.g., *Hexabranchnus sanguineus* RUPPELL & LEUCKHART 1828 and *Bathydoris spiralis* VALDÉS 2002, and in a number of more recently derived taxa such as *Hypselodoris* (Valdés 2002a,b), and therefore may represent an evolutionary labile character. Recent evidence also indicates that dorid spicule networks can take several forms other than the ramifying network described in the present study (unpubl. data). The reason for this variation is unclear without a better understanding of the physiological function of these networks. However, spicule presence and network form may prove useful to resolve the phylogeny of this group once variation among taxa is better understood (Wägele & Willan 2000).

Bands of muscles within the mantle often, but not exclusively, run close to the spicule network (Fig. 3A). The mantle musculature of opisthobranchs is not well understood (Hyman 1967; Gosliner 1994) except for *Cylichna* (Lemche 1956), which has a shell and consequently a much thinner mantle, precluding direct comparisons. However, the muscle bands seen in *C. luteomarginata* may be homologous to the mantle and infrapallial retractors seen in other gastropods (Fretter & Graham 1962). Mantle cross sections also revealed areas characterized by close connections of muscles and spicules, with thick plates of connective tissue (Fig. 3B,C), which I interpret to be muscle insertion points. Similar muscular attachments to spicules are seen in caryophyllidia of *Rostanga arbutus* ANGUS 1864 and other species, and may allow retraction of these structures, although this has not yet been observed in living specimens (Kress 1981; Foale & Willan 1987). These muscle insertions suggest the intriguing possibility that the role of this spicule network is partly for muscle antagonism. The specific biological function of such an arrangement is unclear. Further insights may come from comparing the strength and frequency of muscle insertion among body regions, as well as by comparing the musculature of spiculate dorids with members of some large-bodied genera such as *Hypselodoris* and *Hexabranchnus* that have a thick mantle yet lack spicules (Valdés 2002a).

### Investment by size and body region

Over the range of body sizes tested, investment in spicules increased isometrically overall and for both the mantle and foot in *C. luteomarginata* (Table 1), in contrast to the slightly negatively allometric investment in spicules seen in another cryptobranch, *Discodoris atromaculata* BERGH 1880 (Cattaneo-Vietti

et al. 1993). Rhinophores showed a slightly negative allometry, whereas the gill showed a small positively allometric spicule investment (Table 1). Although the small size of these organs and resulting difficulty in precise measurement possibly account for the slightly lower  $r^2$  values, the highly significant values for  $p_{\text{slope}}$  in each case suggest a real departure from isometry. Both organs are more structurally complex than the mantle or foot (Figs. 1, 2), and the changing requirements for support material versus soft tissue with size are likely explainable by physiological function. The number of rhinophore leaves and gill branches is sometimes constant enough within a species to be used for species identification (Behrens 1991, 2004) and therefore may not increase proportionally with body size. If the rhinophore base—which has few to no spicules (Fig. 2D)—is the only part of the organ growing bigger with body size, then negative allometry is expected as the base becomes proportionally larger than the leaves and remaining shaft. The gill comprises a series of branching tubes held into flow, and the increased diameter of such tubes requires disproportionately greater wall cross-sectional area or additional support material to resist buckling (Vogel 1988).

The overall isometric investment in spicules contrasts with the very strong positive allometry of shell mass often seen in prosobranchs (Palmer 1981, and references therein) and bivalves (Dame 1972; Hickman 1979), whereby adults produce less porous or more massive shells than juveniles. This difference seems most simply explained by the differences in skeletal shape and function. Most mollusc shells are exoskeletons, which resist puncturing and compression stresses (biting, crushing). In order to resist the same compressive forces, a shell with larger diameter must be disproportionately thicker (Vogel 1988), creating a positive allometric relationship. However, structures such as nudibranch spicule networks—innumerable small hard elements in a sheath of connective tissue—are better suited to resist tensile (tearing or pulling) or torsional (twisting) stresses (Vogel 1988). The stiffness imparted by spicules depends on spicule shape (surface area to volume), the orientation of the long axis relative to the direction of stress, and their volume fraction (Koehl 1982). The former two factors do not appear to change with nudibranch size (unpubl. data); therefore, the same stiffness can be attained at any size with the same volume fraction. Spicule weight should therefore increase isometrically with body mass.

The mantle edge had the highest concentration of spicules of any body region,  $\leq 45\%$  of dry weight (Fig. 4). This distribution may be useful defensively,

as the mantle edge is the region first encountered by predators. A qualitatively similar pattern has been found in *D. atromaculata* (Cattaneo-Vietti et al. 1993), but *D. atromaculata* is unusual among dorids in that it can autotomize its mantle edge in response to attacks (Edmunds 1974). In contrast, juvenile *D. areolata* apparently have little spiculation in their mantle edges, possibly related to a behavior of undulating the mantle edge only seen in this stage of the life cycle (García et al. 1986).

### Bioassays against predators

Nudibranch spicules did not seem an important defense against the generalist predators tested. Spicules did not significantly deter crabs, either alone or when added to chemical defense (Fig. 5A,B). Interestingly, the crabs *C. gracilis* responded the same as *C. productus* in assays, even though they would not often encounter slugs of *C. luteomarginata* in their natural habitat, suggesting that the deterrent effect of *C. luteomarginata* compounds may be independent of the predator's previous experience. Anemones were not deterred by spicules alone, but the combination of spicules and chemical extracts significantly reduced feeding relative to control food (Fig. 5C). Unfortunately, this experimental design precludes specific testing for the synergistic effects suggested by these results. The lack of extract deterrence compared with that of whole slugs (Penney 2004) may indicate that some deterrent compounds were lacking in the final mixture. Anemones posed an extra complication because they sometimes ejected food pieces several days after tests. However, because a whole nudibranch would often be dead after such time (Penney 2004), these pieces were still scored as "consumed."

Given the variable effectiveness seen for spicules of other taxa such as gorgonians (Wylie & Paul 1989; Lindquist et al. 1992; Chanas & Pawlik 1996; Koh et al. 2000), spicules may still be effective against other generalist predators of nudibranchs. Fish are among the more important potential predators of nudibranchs (Todd 1981; Penney 2004), and preliminary results indicate that tidepool sculpins (*Oligocottus maculosus* GIRARD 1856) are only deterred by a combination of spicules and chemical extracts (G. Lichota, pers. comm.), similar to the anemone data presented here. Members of *Navanax inermis* COOPER 1863, a specialist opisthobranch predator on other opisthobranchs, seem to avoid nudibranchs with spicules (Paine 1963), although the reason for this avoidance is unclear.

Spicules may also be important defensively for reasons other than direct deterrence of predators. Three general possibilities are lowered nutritional quality,

increased toughness, or reduced deformability (Koehl 1982; Hay et al. 1994). Lowered nutritional quality can be effective, but likely only functions in combination with other defenses (Penney 2002; but see Lippert & Iken 2003). For spiculated nudibranchs, toughness is likely more important than reduced deformability, as the arrangement of short, incompressible elements in a connective tissue sheath would be more resistant to tearing while allowing deformation (Vogel 1988). Increased tissue toughness and the associated reduced damage from exploratory attacks by generalist predators may have been an important prerequisite for individual selection to favor chemical defense in nudibranchs (Penney 2004). Interestingly, the phylogenetic pattern of spicule presence in dorid nudibranchs suggests that it is a primitive character for the group that is subsequently lost in taxa with the most effective chemical defenses (Faulkner & Ghiselin 1983; Cimino & Ghiselin 1999). The reason for this pattern is unclear without evidence for direct deterrent effects of spicules against predators. An increased understanding of spicule networks in nudibranchs may better explain the advantages and trade-offs inherent in such structures.

**Acknowledgments.** Shane Servant, Jennifer Sunday, and the BMS divers helped with collections, and Kristin Howard helped process additional histological specimens. Genaro Hernandez Castillo provided crucial Spanish translation. Ron Koss and Randy Mandryk provided photographic expertise and histological protocols modified for optimal use on slugs. Comments from Ángel Valdés and an anonymous reviewer greatly improved this manuscript. David J. Arsenault aided with collections, statistics, and bioassay design, while A. Richard Palmer provided invaluable encouragement, advice, and editing. Most of this work is part of a PhD dissertation in Biological Sciences at the University of Alberta, with resources provided by A.R. Palmer (National Science and Engineering Research Council of Canada operating grant 06395) and the director and staff of the Bamfield Marine Station. The Biology Department at Saint Anselm College provided additional resources and funding. To all I am extremely grateful.

## References

- Avila C 1995. Natural products of opisthobranch molluscs: a biological review. *Oceanogr. Mar. Biol. Annu. Rev.* 33: 487–559.
- Behrens D 1991. *Pacific Coast Nudibranchs*, 2nd ed. Sea Challengers, Monterey, CA. 107 pp.
- Behrens DW 2004. Pacific coast nudibranchs, supplement II: new species to the Pacific coast and new information on the oldies. *Proc. Cal. Acad. Sci.* 55: 11–54.
- Brusca RC & Brusca GJ 2003. *Invertebrates*, 2nd ed. Sinauer Associates, Sunderland, MA. 936 pp.
- Cattaneo-Vietti R, Angelini S, & Bavestrello G 1993. Skin and gut spicules in *Discodoris atromaculata* (Bergh, 1880) (Mollusca: Nudibranchia). *Boll. Malacol.* 28: 173–180.
- Chanas B & Pawlik JR 1996. Does the skeleton of a sponge provide a defense against predatory reef fish? *Oecologia* 107: 225–231.
- Cimino G & Ghiselin MT 1999. Chemical defense and evolutionary trends in biosynthetic capacity among dorid nudibranchs (Mollusca: Gastropoda: Opisthobranchia). *Chemoeology* 9: 187–207.
- Dame RF 1972. Comparison of various allometric relationships in intertidal and subtidal American oysters. *Fish Bull. US* 70: 1121–1126.
- Edmunds M 1974. *Defence in Animals: A Survey of Anti-Predator Defences*. Longman Group Ltd, Harlow, Essex, UK. 357 pp.
- Faulkner DJ & Ghiselin MT 1983. Chemical defense and evolutionary ecology of dorid nudibranchs and some other opisthobranch gastropods. *Mar. Ecol. Prog. Ser.* 13: 295–301.
- Foale SJ & Willan RC 1987. Scanning and transmission electron microscope study of specialized mantle structures in dorid nudibranchs (Gastropoda: Opisthobranchia: Anthobranchia). *Mar. Biol.* 95: 547–557.
- Fretter V & Graham A 1962. *British Prosobranch Molluscs*. The Ray Society, London, UK. 755 pp.
- García FJ, García JC, & Cervera JL 1986. Estudio morfológico de las espículas de *Doriopsilla areolata* (Gastropoda: Nudibranchia). *Malacologia* 27: 83–96.
- Gerhart DJ, Rittschof D, & Mayo SW 1988. Chemical ecology and the search for marine antifoulants. *J. Chem. Ecol.* 14: 1905–1917.
- Gosliner TM 1994. Gastropoda: Opisthobranchia. In: *Microscopic Anatomy of Invertebrates*. Harrison R, ed., pp. 253–355. Wiley-Liss Inc., New York.
- Gotshall DW 1994. *Guide to Marine Invertebrates: Alaska to California*. Sea Challengers, Monterey, CA. 105 pp.
- Harvell CD, Fenical W, & Green CH 1988. Chemical and structural defenses of Caribbean gorgonians. *Mar. Ecol. Prog. Ser.* 49: 287–294.
- Hay ME, Kappel QE, & Fenical W 1994. Synergisms in plant defenses against herbivores: interactions of chemistry, calcification, and plant quality. *Ecology* 75: 1714–1726.
- Hickman RW 1979. Allometry and growth of the green-lipped mussel *Perna canaliculus* in New Zealand. *Mar. Biol.* 51: 311–327.
- Humason GL 1972. *Animal Tissue Techniques*, 3rd ed. W.H. Freeman & Associates, San Francisco. 641 pp.
- Hyman LH 1967. *The Mollusca*, Vol. I. McGraw-Hill Book Company, San Francisco. 792 pp.
- Koehl MAR 1982. Mechanical design of spicule reinforced connective tissue: stiffness. *J. Exp. Biol.* 98: 239–267.
- Koh LL, Goh NKC, Chou LM, & Tan YW 2000. Chemical and physical defenses of Singapore gorgonians

- (Octocorallia: Gorgonacea). *J. Exp. Mar. Biol. Ecol.* 251: 103–115.
- Kozloff EN 1983. *Seashore Life of the Northern Pacific Coast: An Illustrated Guide to Northern California, Oregon, Washington, and British Columbia*. University of Washington Press, Seattle, WA. 370 pp.
- Kress A 1981. A scanning electron microscope study of notum structures in some dorid nudibranchs (Gastropoda: Opisthobranchia). *J. Mar. Biol. Assoc. UK* 61: 177–191.
- Lemche H 1956. The anatomy and histology of *Cylinchna* (Gastropoda: Tectibranchia). *Skrift. Udg. Univ. Zool. Mus.* 16: 1–278.
- Lindquist N & Hay ME 1996. Palatability and chemical defense of marine invertebrate larvae. *Ecol. Monogr.* 66: 431–450.
- Lindquist N, Hay ME, & Fenical W 1992. Defense of ascidians and their conspicuous larvae: adult versus larval chemical defenses. *Ecol. Monogr.* 62: 547–568.
- Lippert H & Iken K 2003. Palatability and nutritional quality of marine invertebrates in a sub-Arctic fjord. *J. Mar. Biol. Assoc. UK* 83: 1215–1219.
- MacFarland FM 1966. *Studies of Opisthobranch Mollusks of the Pacific Coast of North America*. California Academy of Sciences, San Francisco. 546 pp.
- Orensanz JM & Gallucci VF 1988. Comparative study of postlarval life history in four sympatric species of *Cancer* (Decapoda: Brachyura: Cancridae). *J. Crustacean. Biol.* 8: 187–220.
- Packard GC & Boardman TJ 1988. The misuse of ratios, indices and percentages in ecophysiological research. *Physiol. Zool.* 61: 1–9.
- Paine RT 1963. Food recognition and predation on opisthobranchs by *Navanax inermis* (Gastropoda: Opisthobranchia). *Veliger* 6: 1–9.
- 1971. The measurement and application of the calorie to ecological problems. *Annu. Rev. Ecol. Syst.* 2: 145–164.
- Palmer AR 1981. Do carbonate skeletons limit the rate of body growth? *Nature* 292: 150–152.
- Penney BK 2002. Lowered nutritional quality supplements nudibranch chemical defense. *Oecologia* 132: 411–418.
- 2004. Individual selection and the evolution of chemical defence in nudibranchs: experiments with whole *Cadlina luteomarginata* (Nudibranchia: Doridina). *J. Moll. Stud.* 70: 399–400.
- Smith RI, & Carlton JT, eds. 1975. *Light's Manual: Intertidal Invertebrates of the Central California Coast*, 3rd ed. University of California Press, Berkeley, CA. 761 pp.
- Todd CD 1981. The ecology of nudibranch molluscs. *Oceanogr. Mar. Biol. Annu. Rev.* 19: 141–234.
- Valdés A 2002a. A phylogenetic analysis and systematic revision of the cryptobranch dorids (Mollusca, Nudibranchia, Anthobranchia). *Zool. J. Linn. Soc.* 136: 535–636.
- 2002b. Phylogenetic systematics of “*Bathydoris*” s.l. Bergh, 1884 (Mollusca, Nudibranchia), with the description of a new species from New Caledonian deep waters. *Can. J. Zool.* 80: 1084–1099.
- Valdés A & Gosliner TM 1999. Phylogeny of the radula-less dorids (Mollusca, Nudibranchia) with the description of a new genus and a new family. *Zool. Scr.* 28: 315–360.
- 2001. Systematics and phylogeny of the car-yophyllidia-bearing dorids (Mollusca: Nudibranchia), with a description of a new genus and four new species from Indo-Pacific deep waters. *Zool. J. Linn. Soc.* 133: 103–198.
- Van Alstyne KL, Wylie C, Paul VJ, & Meyer K 1992. Antipredator defenses in tropical Pacific soft corals (Coelenterata: Alcyonacea). I. Sclerites as defenses against generalist carnivorous fishes. *Biol. Bull.* 182: 231–240.
- Vogel S 1988. *Life's Devices*. Princeton University Press, Princeton, NJ. 367 pp.
- 1994. *Life in Moving Fluids*, 2nd ed. Princeton University Press, Princeton, NJ. 467 pp.
- Wägele H & Willan RC 2000. Phylogeny of the Nudibranchia. *Zool. J. Linn. Soc.* 130: 83–181.
- Wylie CR & Paul VJ 1989. Chemical defenses in three species of *Simularia* (Coelenterata, Alcyonacea)—Effects against generalist predators and the butterflyfish *Chaetodon unimaculatus* Bloch. *J. Exp. Mar. Biol. Ecol.* 129: 141–160.
- Zar JH 1984. *Biostatistical Analysis*, 2nd ed. Prentice-Hall, Inc., Englewood Cliffs, NJ. 718 pp.