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# Antipredatory Defensive Roles of Natural Products from Marine Invertebrates

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**Abstract**

This chapter provides a broad and critical evaluation of investigations of the antipredatory defenses of marine invertebrates with a target audience of graduate students in ecology or natural products chemistry. After considering important concepts and theoretical issues associated with the research topic, techniques for assessing invertebrate chemical defenses against predators are detailed, with a focus on potential methodological problems. In particular, the importance of determining concentrations of metabolites in invertebrate tissues using a volumetric rather than gravimetric method is explained. Relevant concepts from the recent literature are reviewed and discussed, including the cost of

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chemical defenses, synergistic effects of defenses, optimization of defenses, and structure-activity relationships of deterrent metabolites. Comparisons are made between the life histories and evolutionary environments of terrestrial and marine invertebrates to argue that the highly optimized chemical defense mechanisms and complex systems of color mimicry described for some terrestrial insects are unlikely to be equaled among marine invertebrates.

## 12.1 Introduction and Scope

Marine chemical ecology is a young discipline, having emerged from the collaboration of natural products chemists and ecologists in the 1980s with the goal of examining the ecological functions of the unusual secondary metabolites that were being isolated from the tissues of marine organisms. The result has been a progression of experimental protocols that have increasingly refined the ecological relevance of the research; some would argue, to a greater extent than the much older discipline of terrestrial chemical ecology.

The topic of this chapter is restricted to antipredatory chemical defenses of marine invertebrates, although much of what will be discussed is more broadly applicable to other defensive roles (allelopathic, antifouling, antimicrobial) and to other organisms, plants, and animals, both marine and terrestrial. In point of fact, it is not the author's intention to survey the primary literature for references pertaining to the topic, but only to cite examples that illustrate concepts as they are discussed. This contribution serves as an opportunity to address the subject in a broader, more analytical manner, with special attention to methodological problems, unanswered questions, and new research directions. Moreover, this chapter has been written for a target audience of *beginning graduate students* in ecology, or better, in chemistry, who might be considering marine chemical ecology as their field of study. For more thorough literature reviews, readers are directed to one excellent review of bioassay techniques [1], general reviews of marine chemical ecology [2], and the chemical defenses of marine organisms [3–5].

The author has contributed to the literature on this chapter's topic for over 25 years, and will unabashedly cite the work that he is most familiar with: his own. This might be interpreted as laziness or pretension, but more is now understood about the antipredatory defenses of a larger number of species of Caribbean marine invertebrates, specifically sponges, gorgonian corals, and ascidians, than for invertebrate taxa from any other marine biogeographic region. The Caribbean reef community is dominated by gorgonians and sponges, the species composition is remarkably similar across the entire region, and trophic relationships are well described. Within this framework, research has advanced from autecological characterizations of the defensive metabolites of individual species to community-level investigations that test higher-order ecological theory. As such, studies of Caribbean marine invertebrates provide the best body of work from which to draw examples that illustrate the concepts considered herein.

## 12.2 General and Theoretical Considerations

A series of interrelated research questions provide the framework for this chapter's topic: Do secondary metabolites produced by marine invertebrates defend them from predators? If they do, why? Are they toxic, or do they simply taste bad? Do metabolites require specific structural components to be active as defenses? How do they affect the behavior or physiology of the predator? Do the same metabolites affect all possible predators? Is predation a driving force in the evolution of defensive metabolites? Are defensive metabolites costly to the source invertebrate? If the defended invertebrate has endosymbionts, which of the two makes the metabolites, and how does that affect the cost to the invertebrate? Why don't predators surmount chemical defenses? If a secondary metabolite does not deter predation, does it have some other function? Does it only act in concert with other metabolites to deter predation? Might a secondary metabolite have no function at all?

### 12.2.1 Predator–Prey Interactions, Generalists and Specialists

It is well known that predation is an important force in controlling populations of marine invertebrates and in shaping their evolution. But it is not the only selective force; indeed, a host of abiotic and biotic factors interact to different degrees and at different times in structuring the ecology and evolution of any species (Fig. 12.1) [6, 7]. Because predation is often a dominant factor, organisms have evolved a number of defensive strategies to deter predators, ranging from behavioral mechanisms (nocturnal activity, rapid escape), to physical (spines, armor) and chemical defenses. Some predators have evolved counterstrategies to lesser or greater degrees, and in some cases, an evolutionary "arms race" has resulted in highly specialized predators that are adapted to eat highly defended prey [8]. At its most extreme, specialization can result in specific pairings of predator and prey, as for some nudibranch slugs that eat particular sponges, but more common are diffuse specializations that allow a predator to exploit a range of prey species that have developed a shared defensive trait, such as the jaws and pharyngeal mills of parrot fishes that allow them to eat many species of stony corals as well as calcified algae and sponges [9, 10]. The evolution of specialization has been the subject of considerable theoretical interest [11].

A high degree of prey specialization is comparatively rare, however, and most predators in marine environments are generalists, meaning that they consume many different prey species. So, to cite the example of sponges on Caribbean coral reefs, Randall and Hartman [12] examined the gut contents of the dominant predatory group, fishes, and they found the vast majority were generalist predators that did not eat sponges, a small minority were sponge predators that ate some sponges as part of their diet, and very few species ate sponges as most of their diet. One species appears to eat mostly one sponge species, making it more highly specialized than the rest. As we will see, knowledge of the generalist and specialist predators of

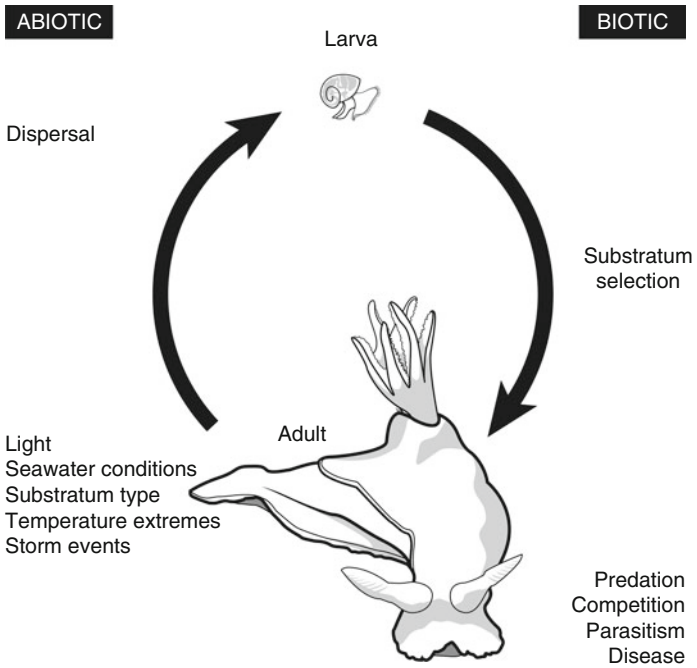


**Fig. 12.1** Some of the abiotic and biotic factors that may affect the distribution and abundance of benthic marine invertebrates. For most species, the dispersive phase of the life cycle occurs during a planktonic (microscopic) larval stage, with the adult phase having limited mobility (the opposite situation is found for terrestrial insects). Note that predation is only one of the many factors that may impact the survival of a given species. Natural selection acts strongly on just a few characteristics of an organism at a time relative to the full range of factors that influence their survival; selective forces do not shape an "optimal" phenotype, nor does evolution permit only the best-adapted organisms to reproduce. Moreover, the focus of selection is likely to change over time as the relative influence of different abiotic and biotic factors change

a target invertebrate species within a community is a prerequisite for designing ecologically relevant experiments for assessing chemical defenses against potential predators. Few marine communities are characterized well enough that this level of understanding of trophic relationships exists [13, 14].

### 12.2.2 Palatability, Toxicity, Learning, and Aposematic Coloration

It is generally supposed that chemically defended prey produce metabolites that are unpalatable to predators (for the purpose of the discussion in this section, we will focus on fish predators, as they are the dominant predators in many marine ecosystems), and further, that this distastefulness is evidence of the toxicity of the metabolites. In the previous sentence, *toxicity* is understood to mean that a metabolite causes physiological damage to the predator that ingests it, while

*unpalatability* (distastefulness, deterrency) means that food offerings containing the metabolite are rejected by potential predators without any necessary subsequent harm to the predator. However, any linkage between palatability and toxicity is far from clear. There are certainly examples of defensive metabolites that are known to be toxic in pharmacological assays [15], but little evidence that other unpalatable metabolites are toxic, and some strongly toxic metabolites may be quite palatable. Indeed, in the limited number of studies that have brought data to bear on the question, no relationship could be found between palatability and toxicity [16, 17].

There are good reasons to believe that palatability, and not toxicity, is the important driving force in the evolution of chemical defenses in marine invertebrates, although a defensive metabolite could have both properties. Distasteful secondary metabolites elicit an immediate response by a potential predator that permits the predator to learn to avoid chemically defended prey through the recognition of visual or chemical cues [18]. In aquatic systems, distastefulness is not perceived by predators at a distance, but only when prey mucus or tissue comes in contact with chemosensory structures in or around the mouth of the predator. Because an attack on distasteful prey is not usually fatal for the prey (particularly for clonal marine invertebrates), the prey and others like it will benefit from subsequent avoidance by the predator. If, however, the prey contained toxic metabolites that were not distasteful and had no physiological effect on the predator for many minutes or hours after ingestion, the predator could not learn to avoid the prey, as there would be no direct association between subsequent illness (or death) and the moment of attack on the prey. Moreover, the prey would likely be killed by the predator in the absence of the immediate deterrent effect of distastefulness. So, death of the predator, while seemingly a good strategy for the prey, is not so if it also means the death of the prey.

Why not produce chemical defenses that are both distasteful and highly toxic? The most likely reason is that highly toxic metabolites often have broad-spectrum, negative effects on living cells. Any prey producing a potent toxin would also have to contain it to prevent autotoxicity, and this could come at a high metabolic cost. In addition, if distastefulness alone prevents predatory attack, and confers a survival advantage to the prey, there is no need to bother with the cost of dealing with potent toxicity. But in the absence of toxicity, what would prevent a predator from circumventing distastefulness? Generalist predators (i.e., most predators) have other prey species available to eat, so there is no strong selective pressure to circumvent the distastefulness of one or a few species, as they can simply move on to other prey species. If, however, prey availability is limited to chemically defended species, predators may evolve behavioral and physiological mechanisms to circumvent defenses. Under enhanced predatory pressure, greater toxicity of the chemical defenses of prey may arise, leading to the "arms race" described frequently in the terrestrial chemical ecology literature [19].

So what is the evidence regarding distastefulness, toxicity, and learning among marine invertebrates and their predators? There is abundant confirmation that fish predators, like avian predators in terrestrial systems, use visual cues to discriminate among undefended and defended prey and learn to avoid the latter [18, 20].

Moreover, immediate regurgitation is the usual mode by which naïve predators reject defended prey, although in some cases learning occurs despite a lack of rejection, suggesting that toxic effects may be perceived quickly enough that some predators can learn to avoid prey despite being unable to reject novel defenses at the time of initial consumption [18]. It is conceivable that mildly toxic chemical defenses could have more insidious, long-term effects that alter growth rates, life-span, or fecundity of predators [21], but the selective pressures that would favor a more complex mechanism over the straight-forward path of distastefulness are harder to imagine.

With visually acute predatory fish dominating many marine systems, and with good evidence of learned recognition of chemically defended prey by these predators, one might expect clear evidence of warning coloration (aposematism) and mimicry similar to that described for frogs and butterflies in terrestrial systems. Many sessile benthic invertebrates are brilliantly colored (e.g., sponges), and a clear candidate for mimicry are nudibranch mollusks, brilliantly colored shell-less slugs with potent chemical defenses that they derive from their diet or manufacture themselves, depending on the species [22]. Yet the evidence for these phenomena among marine invertebrates is not nearly as strong as for their terrestrial counterparts (butterflies, beetles, frogs, etc.).

No relationship has been found between bright colors and chemical defenses for Caribbean sponges [16]. Sponge-eating predators, including angelfishes, parrot fishes and hawksbill turtles appear to rely on visual cues that transcend color alone, quickly finding and eating preferred sponge species, whether brightly colored or drab, among similarly colored defended species in experimental arrays [23]. It had been proposed that spongivorous fishes used a "smorgasbord" strategy of alternating colors of sponges in their diets to avoid the accumulation of toxic compounds present in any one species [12, 24], but subsequent experimental work did not support this hypothesis [13, 23]. Indeed, the bright color of sponges may owe more to bacterial symbionts or dietary pigments than to the selective forces of predation [14, 16].

Among opisthobranch mollusks, herbivorous sea hares are mostly cryptically colored, and yet they often bear potent chemical defenses [25–27], although these may be targeted primarily at crustacean predators that rely less on visual cues. Blue and yellow stripes are a common color scheme for chemically defended Caribbean and Mediterranean nudibranchs [28], a pattern that blends to green at a distance, often rendering these slugs cryptically colored, although it has been proposed that the color pattern is indicative of a mimetic circle of species [29]. The large and brightly colored Spanish dancer nudibranch is primarily nocturnal and cryptically concealed during the day, but it reveals dramatically contrasting mantle markings when disturbed or swimming [30]. This species has a mantle that is bright crimson red in shallow water, but it would appear brown or black to potential predators at the range of depths it is most commonly found. Similarly, the alga-eating sacoglossan mollusk *Cyerce nigricans* also strongly chemically defended [31], appears mostly black in contrast to the bright green alga on which it feeds. Two congeneric gastropterid slugs share the same chemically defended host sponge, and both are

similarly unpalatable, but while one has a strongly contrasting pattern on its body, the other is highly cryptic [32]. Even if it is arguable that visual cues help to protect some opisthobranch species, the general level of aposematism among marine slugs does not rise to that found in terrestrial insects, nor does it seem to provide a strong foundation for mimicry [33].

Stronger evidence for aposematism exists for Indo-Pacific nudibranchs of the family Phyllidiidae, but rather than settle the case, it only raises more questions. Natural assemblages of reef fishes consumed less of foods associated with the contrasting color patterns modeled after two of five species of these brightly colored, diurnal nudibranchs, providing evidence of aposematic coloration to the authors of the study [34]. However, the crude organic extract of one of the two nudibranch species having an avoided color pattern was not deterrent at the site in which feeding experiments were conducted, while at least one species with a color pattern that was not avoided by fish predators yielded a deterrent extract. The most strongly contrasting color pattern tested in the study and modeled after *Phyllidia polkadotsa* had no effect on predation.

In a thorough review of aposematic coloration in nudibranchs, Edmunds found the direct evidence inconclusive [33]. As part of his analysis, he noted the absence of extensive examples among nudibranchs of Batesian or Mullerian mimics, both of which are common among butterflies. A similar argument could be made against aposematism in marine flatworms [35] and for polychaetes with brightly colored feeding appendages [36, 37], although some claims have been made for mimicry among flatworms [35, 38]. Overall, the fact that the evidence for aposematism is nowhere near as pervasive for marine invertebrates as it is for their terrestrial counterparts further supports the contention that the relative selective pressures of predation on visual cues related to chemical defenses are not as intense in marine systems and do not result in the levels of optimized defenses, or complex chains of mimicry, that are seen in terrestrial insects.

Why should aposematism and associated mimicry be common among tropical terrestrial invertebrates (particularly butterflies) but rare among their marine counterparts? One possibility is that predation is a much more important selective force on insects than on marine invertebrates (Fig. 12.1) [7]. Butterflies are adults during the dispersive phase of their life history during which time they share the open air with their avian predators and cannot rely on crypsis, while adult benthic marine invertebrates crawl or are sessile. Female butterflies directly deposit their eggs on appropriate food plants for the nondispersive larval stage of their life history, while most marine invertebrates have a pelagic larval stage associated with considerable larval wastage and a very low probability of finding an appropriate settlement substratum [39]; in fact, the prevailing view of marine ecologists is that recruitment processes are a dominant, if not the most important, factor in determining distributions and abundances of marine invertebrates [40]. Many species of butterflies go through multiple generations in a season, greatly accelerating the evolutionary process compared to most marine invertebrates, which have generation times measured in years. Many of these life history differences may also explain why host specialization is often intense for insects, but not so for marine invertebrates.

An intriguing possibility for the limited existence of aposematism and mimicry among some opisthobranch mollusks is that it is evidence of past selection. Predation may have been a much more important factor guiding the evolution of opisthobranch species living in the warmer and more extensive shallow seas of the geological past than it is today.

### 12.2.3 Resource Limitation and Metabolite Function

All organisms have a finite amount of metabolic energy to allocate to the biological functions of maintenance, movement, growth, reproduction, and defense. Greater investment in one of these categories must come at the expense of the others. A marine invertebrate may defend itself in many ways, and it is conceivable that it may not defend itself at all. Mobile animals can hide or flee, while sessile species may exhibit armor, spines, or pincers. While the foregoing defense mechanisms have fairly obvious metabolic costs to the animals that use them, the situation is more ambiguous for chemical defenses. The cost of a chemical defense may be substantial if the invertebrate must synthesize a complex compound from primary metabolic building blocks, move the compound from the site of synthesis to a location where it will be most effective, and then either store the compound or release it. But what if the compound is an effective chemical defense in very small quantities, or it can be stored for long periods of time? What if it is derived directly from the diet of the animal, as for some nudibranch mollusks [30]? What if it is synthesized entirely by symbiotic algae or bacteria living within the tissues of the animal, as may be true for some sponges [41]? It is conceivable that a chemical defense could come at little or no cost to the invertebrate that uses it.

Just as it is likely that a chemical defense comes with a cost to the organism that exhibits it, it also seems reasonable to expect that any complex secondary metabolite found in the tissues of a marine invertebrate must have some sort of function, whether as a chemical defense or some other purpose. Again, this may not be the case. Secondary metabolites may be "biochemical baggage," in that they are produced as by-products of the synthesis of other metabolites, whether primary or secondary, or that they are waste products that accumulate in the tissues of an organism [42]. If selective forces are neutral to the presence of these metabolites, or if they change over time from positive to neutral, they will continue to be produced in the population of organisms that exhibit them.

Marine natural products chemists are familiar with the broad diversity of unusual secondary metabolites present in the tissues of many benthic marine invertebrates, particularly sponges, ascidians, and gorgonian corals [43]. Often a single animal is the source of many compounds. Of this enormous chemical diversity, we can only ascribe ecological function, based on relevant experiments, to a tiny fraction. Bioassay-guided isolation techniques invariably exclude secondary metabolites that are not active in that assay system. It is entirely possible that these excluded metabolites have other important functions, but it is also possible that no particular function exists for these metabolites [42].

One interesting possible example of "biochemical baggage" is prostaglandins in the Caribbean gorgonian coral *Plexaura homomalla* [44]. This common sea whip grows among ten or more other species of gorgonians, including at least one congener, and yet only *P. homomalla* has very high concentrations of prostaglandins in its tissues (1–8% of tissue wet mass, mostly the acetoxymethyl ester of prostaglandin $A_2$($PGA_2$)). Experiments conducted with the hydroxy acids of $PGA_2$ indicated that these compounds were potent antipredatory defenses against fishes [45], but when experiments were performed on the naturally occurring acetoxymethyl esters, they were not deterrent to potential predators [44], even though the crude organic extract of *P. homomalla* deterred predatory fishes, indicating the presence of other defensive metabolites [46]. Experiments also discounted antifouling and allelopathic roles for prostaglandins in *P. homomalla* [47]. Although specialist predatory snails of gorgonians, *Cyphoma* spp., exhibited higher levels of enzymes associated with detoxification when collected from *P. homomalla* [48], these snails are just as likely to graze on *P. homomalla* as any of the other gorgonian species that lack prostaglandins. Why should one common species of gorgonian contain such high concentrations of $PGA_2$ when all of the other species around it, many equally abundant, do not? It appears that the presence of $PGA_2$ in the tissues of *P. homomalla* makes very little difference to the survival of sea whips of this species.

## 12.3 Techniques for Assessing Invertebrate Chemical Defenses Against Predators

### 12.3.1 Historical Development

The field of marine natural products chemistry experienced a "gold rush" in the 1970s and 1980s when organic chemists took advantage of two emerging technologies: SCUBA diving and rapidly advancing spectroscopic methods (mostly NMR). The result was a rapid increase in the number of publications describing novel metabolites from benthic marine invertebrates and algae. Relying upon the assumption that secondary metabolites must serve some purpose (see previous section), many of these publications ascribed ecologically important properties to new compounds without empirical evidence; in point of fact, whole reviews of "chemical ecology" from this period were compiled of references with little or no assay data to support an ecological function for secondary metabolites [49].

At about the same time, ecologists were also taking advantage of the advent of SCUBA diving and describing the distributions and abundances of benthic animals and plants previously known only from much less effective sampling methods, such as dredging. The assumption of these researchers was that anything sessile and soft-bodied must be chemically defended to avoid consumption by abundant and ever-present predators [12]. In an effort to introduce empiricism to what was otherwise descriptive work on species abundances, some ecologists began extrapolating chemical defenses from toxicity assays in which fish (usually goldfish or guppies)

were exposed to aqueous suspensions of crude organic extracts of invertebrate tissues [50]. Looking back, it is surprising that publications in prestigious journals used similar techniques having little or no ecological relevance [51, 52] and that these studies are still widely cited today!

Indeed, not only are past studies that purport to investigate marine invertebrate chemical defenses on the basis of toxicity data cited in the current literature, studies based on toxicity data continue to be published [53–55]. In fact, one of the most up-to-date citations in this chapter is a study that attempts to link greater conspicuousness of nudibranch coloration with chemical defense by drawing entirely on brine shrimp toxicity data [56]! This is not entirely surprising because data from toxicity assays, particularly brine shrimp, Microtox, and fish toxicity experiments, are very easy to generate relative to time-intensive feeding experiments conducted with ecologically relevant predators. But beyond possible (and rather limited) pharmacological significance, toxicity data have no ecological value because these assays have no bearing on the manner in which potential predators perceive prey under natural conditions. In point of fact, studies that have compared data from toxicity and feeding assay experiments have found no relationship between them [16, 17]. Simply put, marine chemical ecologists should dispense with toxicity assays, editors and reviewers should not allow toxicity data to be published in the ecological literature, and past studies consisting of toxicity assays should not be cited as evidence of ecologically meaningful information.

### 12.3.2 Current Approach

In brief, the approach for assessing the antipredatory activity of the tissues of a marine invertebrate can be summarized as follows, and is further detailed in the paragraphs below: (1) determine the appropriate generalist predators for feeding experiments, (2) collect target invertebrates and properly extract secondary metabolites, (3) use an appropriate assay, and (4) employ suitable experimental and statistical methods.

1. *Determine the appropriate generalist predators for experiments*. It seems obvious that an appropriate, co-occurring generalist predator should be chosen for feeding experiments, but many past studies have instead opted for a more convenient "model" predator, which, while certainly better than using toxicity assays (above), nevertheless reduces the ecological relevance of the study. Clearly, a generalist coral reef fish should not be used to investigate defenses of a target invertebrate from a Norwegian fjord; instead, the local population of potential predators, whether vertebrate or invertebrate, should be determined, either from past studies or as part of a survey of the habitat of the target invertebrate. Some examples: fishes are the primary predators of invertebrates on Caribbean coral reefs [12, 23], while sea stars are the primary predators of the Antarctic benthos in McMurdo Sound [14], and crabs and lobsters are the dominant predators on some temperate reefs [57]. Generalist predators, and not specialists, are chosen for feeding experiments because specialists may

have surmounted prey defenses, and the research question addresses defenses against the most common classes of predators for the habitat of the target invertebrate.

2. *Collect organisms and extract secondary metabolites properly*. Under the best of circumstances when collecting target invertebrates, several geographically distant collections of individual specimens are obtained so that independent feeding experiments can be performed on each, thereby revealing potential variability in chemical defenses at the species or population level [58]. However, this is not always practical because multiple collections are not possible, the organism is rare, or the organism is small, and insufficient organic extract is available from individual organisms for assays. Under any of these circumstances, it is better to extract a mass collection of the target invertebrate and assume that the extract reflects a composite mean level of defense for the population of organisms from that collection site [59].

   In preparing crude organic extracts of a target invertebrate, it is best to extract freshly collected tissue to avoid any possibility of sample degradation. As an alternative, fresh collections are quickly frozen and maintained solidly frozen until processed. Some older studies were performed on air-dried invertebrate tissues [60], which necessitated re-evaluation of the experiments performed on extracts from these species with better techniques that resulted in some very different findings [46].

   Prior to extraction, the total volume of the tissue must be determined. This is usually done by adding the tissue samples to a graduated cylinder partially filled with either water or the extraction solvent and recording the displaced volume. After tissue volume has been determined, the tissue may be further chopped or shredded to expedite the extraction process. Each step in the extraction process should use a volume of solvent about twice that of the tissue sample. The solvent mixture of choice for extracting wet tissue is a mixture of equal parts dichloromethane and methanol, which rapidly permeates tissue, solubilizing membranes and dehydrating cellular material. Separation of the dichloromethane phase from the resulting miscible mixture of methanol and water from the tissue occurs very quickly, so it is best to agitate the extraction containers to keep the semi-emulsified extraction liquid in contact with the whole tissue sample. After a minimum of 6 h, preferably under cold and dark conditions, the first extraction solvent mixture is poured off and the tissue squeezed before the same volume of methanol alone is used for the second extraction, which again is best done with agitation for a minimum of 6 h. A third extraction round, also with methanol, is necessary only when the tissue is particularly dense. Evaporation of the solvents should be done to minimize exposure of the extracts to heat and light; for example, the dichloromethane partition of the extract can easily be separated from the aqueous methanol partition in a separatory funnel and evaporated nearly to dryness on low heat by rotary evaporation. Rotary evaporation will also quickly remove solvent from the last methanol extract. The aqueous methanol partition is best evaporated using a vacuum evaporator system. When most of the solvent has been removed

from each, the partitions of the extract are combined into a single vial, completely dried by vacuum evaporation, and stored frozen under nitrogen, if not used immediately for feeding experiments.

3. *Use an appropriate assay*. Feeding experiments can be performed in the laboratory or in the field, with the advantage of the former in simplicity and speed, and of the latter in enhanced ecological relevance. Optimally, feeding experiments will begin in the laboratory and then be duplicated at some level in the field [61]. Feeding experiments are most often behavioral assays in which consumption or rejection of an artificial food that predators do not otherwise recognize is scored. Artificial foods are used because they allow precise control of the nutritional quality of the food as well as the concentration of crude organic extract or metabolites from the invertebrate under investigation. Additionally, the predatory subjects are unfamiliar with artificial foods, have neither learned to avoid nor prefer them, and tend to sample them carefully, resulting in a behavioral assay that is easier for the investigator to observe. For assays in which fish are the predatory subject, artificial foods made from a polysaccharide gelling agent such as agar, carrageen, or Phytagel are commonly employed. These gelling agents add little nutritional value to the artificial food and have the distinct advantage of solubilizing many lipid-soluble metabolites in suspension in the gel matrix, allowing a homogeneous presentation of secondary metabolites in the food. One minor disadvantage of these gelling agents is that they must be heated to boiling after being mixed in water in order for solidification to subsequently occur, and there is always some concern that heat-labile secondary metabolites may be degraded as they are mixed in the molten gel. However, it seems unlikely that wholesale degradation of otherwise stable natural metabolites would occur from such a brief exposure to heat, and the author knows of no example to support this concern. Nevertheless, as an alternative, the sodium salt of alginic acid can be mixed in much the same way as the previously cited gelling agents, but rather than requiring heat, alginic acid forms a solid gel when exposed to a solution of calcium chloride. Proper solidification requires a high surface-to-volume ratio, however, so use of this gelling agent is largely restricted to small volumes of artificial food that are extruded through a syringe housing and into the calcium chloride solution to form long noodles that are then cut to form food pellets [16]. When food pellets are prepared, the feeding assay scores consumption or rejection of individual pellets [16], but when larger food samples are prepared, changes in the mass of treated and control food samples are scored after exposure to predatory fishes [62].

   Whatever the artificial food matrix used to volumetrically reconstitute the secondary metabolites from the tissues of the target invertebrate, the food should match the nutritional quality of the same tissues by addition of a nutritional substitute, such as fish meal, or squid mantle. This is important because it is likely that the same sensory processes that predators use to reject feeding deterrent metabolites also perceive the nutritional quality of foods. Foods with very low nutritional quality may be rejected by potential predators at much lower levels of chemical defense [63], and conversely, secondary metabolites may

only be deterrent at higher-than-natural concentrations if those metabolites are presented in an artificial food that is more nutritious than the tissue from which it was derived. Nutritional quality of tissues is determined using calorimetry (to assess total energy content) as well as specific assays for protein, lipid, and carbohydrate [46, 64]. Of these, it is generally agreed that matching the protein content of the invertebrate tissue in the artificial food is the most important. Powdered, freeze-dried squid mantle is a particularly useful nutritional substitute because it is readily available, easy to measure, and its nutritional characteristics have already been determined [64]. Avoid nutritional substitutes that are excessively oily (e.g., tuna packed in oil) or those that may have high levels of free amino acids (e.g., some commercial fish foods) as the stimulatory effects of these substances may act against potential feeding deterrent metabolites present in the tissues of the target invertebrate.

Feeding experiments involve potential predators making choices between foods treated with secondary metabolites from the target invertebrate and control foods, which lack the secondary metabolites, but may contain the solvents (often methanol) used to dissolve the metabolites for homogeneous addition to the food matrix. Because crude organic extracts of invertebrate tissues are often strongly pigmented while most control food mixtures are not, it may be necessary to color-match the treated and control food samples. This is done to prevent assay fish from learning to reject pigmented food samples when multiple assays of different target invertebrate species are being performed in succession with the same group of assay fish, as when doing a survey for chemical defenses [16]. Color-matching can be done by adding drops of food dye while preparing artificial foods, and it is easier to add dye to both treatment and control mixtures to give the same color (masking the natural pigment of the extract in the treatment mixture) rather than trying to match the color of the treatment mixture by adding dye solely to the control mixture.

Once artificial food has been prepared containing a natural volumetric concentration of secondary metabolites from the target invertebrate and having a nutritional quality that approximates the tissues of the target invertebrate, food samples can be presented to assay predators in laboratory or field assays. A single feeding experiment is made up of multiple replicate assays, each of which must be independently performed. For laboratory feeding experiments with fish, independent replication is achieved by splitting up the population of assay fish into separate cells so that no group of fish is assayed more than once with the same treatment. Independent replication can be more difficult for field assays because one or a few hungry fish may monopolize a SCUBA diver who exposes food samples to fishes in the field, and multiple samples eaten by the same predator would not constitute independent replicates. One way around this problem is to place paired treatment and control food strips at replicate feeding stations that are a sufficient distance apart so that the same fishes are not feeding from more than one station [44, 65]. Paired samples are then removed after predators have had a chance to consume some of the food, with feeding deterrence evident when more of the control has been eaten relative to the

treatment food, as determined by measuring the remaining food strips. The difficulty with this type of field assay is that it works well only with "nibbling" fish predators – those that grab the whole food sample and swim off, only to reject the sample after moving away from the feeding station, will render this field assay method useless.

This review has largely focused on fish feeding assays because fishes are often the dominant predators in marine habitats, but there are many examples of feeding assays that have been designed around invertebrate predators. Invertebrate feeding assays are of two types: those that measure consumed assay foods directly and those that score behavioral differences in response to assay foods. The latter category is often necessary because many invertebrate predators feed slowly, consume little, or feed in such a way that it is difficult to score loss of food material. There are examples of invertebrate assays performed in the laboratory with pelleted assay foods that parallel those described previously for fishes [66, 67]. But the most common method for direct measurement of assay food consumption by invertebrates is the "screen gel assay" adapted from sea urchin assays of algal metabolites [68] and adapted for use with crabs [69], sea stars [70], and other invertebrate predators [71]. For these assays, paired treatment and control gel-based foods (as previously described) are solidified onto fiberglass window screen as a thin coating, and the relative number of squares in the screen within the gel area that are consumed over a certain period of time are recorded. For invertebrates that consume larger amounts of material, cubes or strips of gel-based foods can be incorporated onto the screen and weighed before and after the feeding experiment [72]. An assay designed for the opposite situation, to test whether shrimp feed on small amounts of brightly colored control or treatment foods, scores the color change of the shrimp gut by observing it through its clear carapace [55].

Perhaps the best example of a behavioral assay performed with an invertebrate predator for the purpose of testing for chemical defense is the "tube-foot retraction" assay used to test the responses of sea stars, the dominant benthic predators of McMurdo Sound, Antarctica, to potential prey sponges and mollusks and to the organic extracts from these target invertebrates [73–75]. The assay consists of touching the tube feet along one arm of a replicate sea star with the experimental treatment and then measuring the time it takes for the retracted tube feet to extend again from the ambulacral grove. For assays of extracts or pure compounds, the control used is a glass rod coated with silicone grease, and this elicits a retraction time of about 25 s for tube feet of the sea star *Perknaster fuscus*; the response to grease with fish tissue extract is about 28 s, while the response to grease treated with metabolites from the chemically defended sponge *Latrunculia apicalis* is about twice that amount of time [76].

4. *Employ suitable experimental and statistical methods*. The importance of appropriate experimental methods has already been discussed to some extent in describing collection and assay techniques, particularly regarding replication of sampling and when performing feeding experiments. Additionally, if an

investigator wishes to compare the chemical defenses of one or a group of invertebrates relative to a previous study, it is imperative that the same methodology be used, or any comparative conclusions will be compromised by technical differences between the two studies.

One important concern when isolating the chemical defense of a target invertebrate is in the use of bioassay-guided fractionation. Once the presence of a chemical defense has been ascertained by assaying the crude organic extract of the tissues of the target invertebrate, the same assay is employed as the crude extract is chromatographically fractionated into smaller subsets of compounds that make up the mixture. Again, this should be done on a volumetric basis, using "mL equivalents" of tissue extract rather than mass equivalents. As the separation proceeds, fractions are best assayed as a serial dilution relative to the natural volumetric concentration: 4×, 2×, and 1×. This span of concentrations takes into account the likely reduction in deterrent activity that comes from splitting the active metabolites over two or more chromatographic fractions or from loss of active metabolites through decomposition, reaction, or attachment to chromatographic media. Once the active metabolites have been isolated by bioassay-guided fractionation, the investigator should endeavor to identify them using standard spectroscopic techniques and should also do the same for inactive fractions that may have secondary metabolites. For the reasons explained earlier in this chapter regarding their functional significance, it is equally important to know which secondary metabolites are active in ecologically relevant experiments as to know which metabolites are not.

Appropriate statistical analyses of data are as important for behavioral assays as for any other scientific research that involves determining the significance of differences in experimental outcomes. Fortunately, these analyses are usually simple and routine. The significance of most laboratory feeding experiments, in which consumption of control and treated foods is compared, is usually determined with some form of contingency table analysis of which Fisher's exact test is commonly employed [16]. The assumption for these experiments is that all of the control food offerings will be consumed because the investigator would not be using experimental predators that were not feeding on control foods. In one example, a useful boundary in food pellet consumption was designated that separates palatable from defended treatment foods, based on Fisher's exact test, in which a treatment is considered deterrent if six or less of ten food pellets are eaten [16].

To analyze data from field experiments, paired, nonparametric statistical tests are usually employed, such as the Wilcoxon paired-sample test [63]. Nonparametric, paired tests are necessary because the variance in the amount of food consumed between replicate pairs of samples positioned in different places in the field is often greater than the difference in consumption between the control and treatment food sample within a pair, a reflection of the "patchiness" of predators in field situations. These nonparametric tests analyze the directionality of the results rather than comparing the mean consumption of control and treated foods.

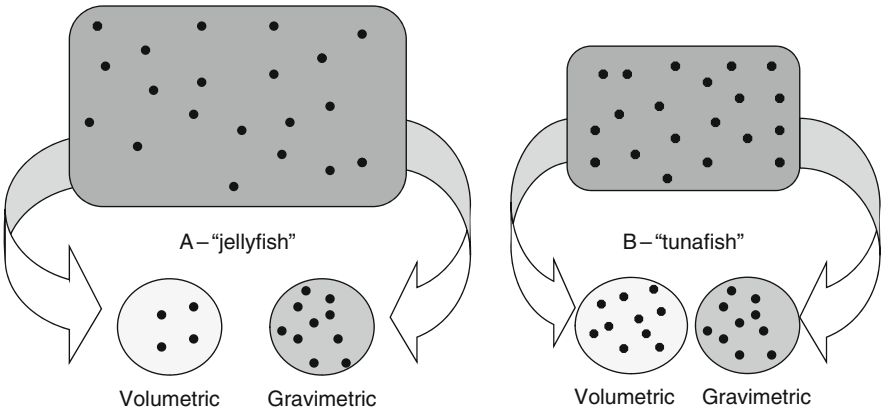


**Fig. 12.2** Comparison of volumetric and gravimetric methods for reproducing the concentration of a defensive component in invertebrate tissues. In these diagrams, the *black dots* represent the components of interest (secondary metabolites, spicules, sclerites, etc.) that are assumed to be homogenously dispersed in the freshly collected (wet) tissue. In example A, the components are sparsely distributed in highly hydrated tissue (jellyfish), while in B, the same amount of components are distributed in about half the volume (tuna fish). Any predator taking an equal volume bite out of A would experience the defensive components at half the concentration of the same bite of B. If the components are reconstituted in an artificial food matrix as a function of wet tissue volume (volumetric), the concentration of the components are identical to the original tissue concentration for both A and B, but if the components are reconstituted as a function of dry mass (gravimetric), they are likely to be more highly concentrated than the original tissue for A (depending on the dry mass of the artificial food used)

### 12.3.3 Technical Problems

*Gravimetric vs. volumetric concentration determination*. One long-standing technical problem plaguing the marine chemical ecology literature is with the determination of extract or metabolite concentration in an organism and duplication of the natural metabolite concentration in artificial assay foods. Natural products chemists are accustomed to reporting metabolite concentrations as a function of dry tissue mass, but predators eat wet tissue, and marine invertebrate tissues vary widely in the amount of water contained in their tissues. From the perspective of a predator, a bite of a jellyfish or sea anemone would contain substantially more water per unit dry mass than the same sized bite of a squid or sea slug. For highly hydrated tissues, the concentration of metabolite per unit dry mass would be much higher than per unit volume, but volume (bites) is the measure that is ecologically relevant (Fig. 12.2).

In addition to differences in water content, tissues of marine invertebrates may have very different densities because of skeletal inclusions. For example, some sponges have tissues that are perfused with glass spicules, which have a very high density, but are not part of the living organism (similarly dense limestone inclusions are found in soft corals and tunicates). Dry mass calculations of metabolite concentration would include the heavy mass of these spicules, which are not part of the

living tissue of the sponge, driving down the relative mass of any metabolite found in the tissue. Because the concentration of skeletal inclusions can vary greatly between organisms of the same species, and sometimes between parts of the same organism, perceived differences in metabolite concentration based on dry mass could be accountable entirely to differences in skeletal element concentrations.

The problem of gravimetric concentration determination particularly affects any study aimed at testing for differences in levels of defense between parts of the same organism or between individuals in a population. For example, investigators wishing to test for differential allocation of chemical defenses in one part of the body of a study organism are faced with potential differences in hydration or skeletal inclusions that could confound any differences in metabolite concentration on a dry mass basis. Similarly, investigations of seasonal cycles in metabolite concentration may be confounded by changes in hydration, tissue quality associated with changes in diet (e.g., presence of more lipids in well fed organisms), changes in skeletal inclusions due the changes in flow or wounding, or changes in the abundance or development of gametes.

Determination of metabolite concentration by volume solves both the problems of differences in tissue hydration and density variation from skeletal and tissue inclusions, and it is the most relevant measure from the standpoint of consumption of tissue by a potential predator. It requires that tissue volume be measured at the onset of an extraction protocol, by displacement of water or solvent in a graduated cylinder, and that the resulting extract be treated as a *volume-equivalent* (e.g., "mL equivalents") throughout the bioassay-guided metabolite isolation process. Dry mass determinations should also be performed during the isolation process because these comparative data are necessary for the marine natural products literature should a novel metabolite be isolated and reported.

The importance of using volumetric methods for determination of metabolite concentration is illustrated in surveys of the chemical defenses of Caribbean gorgonian corals against the generalist predatory bluehead wrasse, *Thalassoma bifasciatum*. The initial survey of 1987, one of the earliest to systematically examine chemical defenses of a broad range of species from a biogeographic community, used a gravimetric approach to estimate natural concentrations of metabolites in experimental foods and documented considerable variability in chemical defenses among common Caribbean gorgonian corals [60]. Subsequent studies introduced the technical superiority of volumetric concentration determination [77]. The gorgonian survey was repeated 15 years later, using volumetric concentration determination and improved techniques for sample processing, and this time, all 32 gorgonian species yielded deterrent crude organic extracts [46]. As an example from the technically improved study, the dry masses and volumes of two tissue samples from the common Caribbean sea fan, *Gorgonia ventalina* were 19.72 g and 27 mL and 22.86 g and 57 mL, respectively (i.e., the mass of the second was 16% greater than that of the first but occupied 111% more volume). In a gravimetric assay, the extracts of these two samples would be applied to similar masses of food of unknown volume because the assay food is also measured and prepared on the basis of mass, not volume. In a volumetric assay, however,

the extract of the second sample would be applied to more than twice the volume of food as the first. The relative concentrations of each extract would be very different in the gravimetric and volumetric assays, as likely would be the feeding responses by the assay organisms.

As was also pointed out in the foregoing technically improved survey of Caribbean gorgonian chemical defenses [46], gravimetric feeding assays fail to control for the nutritional quality of the target organisms. Invertebrate tissues contain nutritionally valuable (e.g., protein) and inert components (e.g., mineralized skeletal elements). When assay foods are prepared gravimetrically, the mass of the nutritionally poor portion is replaced almost entirely by an equivalent mass of nutritionally valuable matrix. As a result, a gravimetrically prepared assay food cannot replicate the nutritional quality of tissue. When assay foods are prepared volumetrically, however, organic extract from a volume of tissue is added to a volume of assay food that can be modified to have a similar nutritional quality (more or less powdered freeze-dried squid mantle). The proportion of the nutritional components is controlled for in a volumetric assay. Thus, volumetric assays are more ecologically relevant than their gravimetric counterparts because the assay food more closely models hydrated tissue in both nutritional quality and extract concentration. A note of caution, however: the foregoing assumes that tissue constituents (secondary metabolites, skeletal elements, etc.) are homogeneously distributed in the tissue under investigation. If it is suspected that the tissue constituent of interest is, for example, concentrated in the surface of the target organism, then a more careful, separate extraction of inner and outer layers of tissue will be required.

*Extraction protocol*. Experiments designed to assess chemical defenses of marine invertebrates require that secondary metabolites be extracted and assayed separately from any structural defenses that may be present in their tissues. To this end, the full range of potential defensive metabolites, from nonpolar (e.g., terpenes) to polar (e.g., glycosides), must be extracted from tissues and recovered in nearly 100% yield with minimal degradation prior to reconstituting the metabolites in assay foods and performing feeding experiments with potential predators.

Very little comparative work has been published on extraction protocols, but the choice of wet or freeze-dried tissue and extraction solvent can substantially alter extraction efficiency [78]. Minor variations on the aforementioned extraction scheme are the norm for most studies of marine invertebrate chemical defenses [16, 46], particularly when natural products chemists are involved as collaborators. Substitution of one extraction solvent for another of the same polarity is unlikely to have much effect on the extraction outcome, particularly if the extract is prepared from freshly distilled solvents and processed quickly. Tissue extraction may be incomplete, however, if an inappropriate solvent is used. Extraction with methanol alone, for example, may not liberate all the nonpolar metabolites from the tissue. Freeze-drying tissue before extraction, which is often done to speed and simplify the process by removing water, may also be problematic because very polar metabolites may not be extracted from freeze-dried tissue with methanol unless the tissue is first rehydrated. Experience in the author's laboratory has

shown that yields of some metabolites from sponges are higher when using wet tissue than freeze-dried tissue, as has been determined for similar extractions of macroalgae [78].

A combination of the aforementioned technical pitfalls is illustrated in a recent set of studies that claims to demonstrate latitudinal variation in sponge chemical defenses as a function of relative predation pressure, comparing species that are found off the coast of the southeastern United States, where fish predators are scarce, and in the Caribbean, where predators are common [79, 80]. Sponge tissue samples were freeze-dried and then extracted only in a mixture of equal parts dichloromethane and methanol, a protocol that would likely not liberate the most polar secondary metabolites from the tissues of some sponge species. Data from feeding assays performed using sponge extracts prepared in this manner were compared to those done on wet-extracted tissue using the protocol described previously [16], and the authors found lower levels of chemical defense in the sponges from the coast of the southeastern US than had previously been documented from the Caribbean [79]. Unfortunately, incomplete extraction of sponge tissue is an alternative and more parsimonious explanation for the difference in feeding assay outcomes for the more recent study, and a less equivocal test of the latitudinal variation hypothesis will require complete and identical extraction protocols. Moreover, the preceding example provides a cautionary tale in support of using identical methodologies when seeking to compare data from two studies.

*Nutritional quality of assay foods and the feeding state of assay predators*. When conducting feeding experiments to determine whether organic extracts of marine invertebrates contain chemical defenses, it is important that other variables that affect assay food quality not confound or interact with the effects of the metabolites being tested. In particular, the nutritional quality of assay foods is important [63, 81]. Metabolites that are mildly deterrent are more likely to induce predators to reject them when they are incorporated into foods with little or no nutritional quality than when they are incorporated into high-quality foods. High food quality is usually linked with protein content, and many associated amino acids and peptides are strong feeding stimulants [82], which may interact with feeding deterrents. Indeed, the converse may also be true – assay foods with very high nutritional quality may mask the deterrent effects of minimally effective defensive metabolites.

It is incumbent on the investigator to match the nutritional quality of the artificial assay food with that of the marine invertebrate from which the crude organic extract or metabolites have been derived. Sometimes this is relatively simple; for example, the mantle tissue of a nudibranch and a squid are likely to be fairly similar in food value, so an artificial food prepared from the latter should be a good stand-in for the tissue of the former. But the tissues of sponges, ascidians, and soft corals may contain high concentrations of both water and skeletal elements that make their interspecific nutritional quality highly variable, particularly on a volumetric basis (see above, Fig. 12.2) [46, 64, 83]. Therefore, replicate tissue samples should minimally be subjected to a combination of bomb calorimetry for total caloric content and a suitable assay of soluble protein to determine reasonable nutritional

parameters for artificial assay foods [46]. Because the gelling agent used in most assay foods (agar, carrageenan, alginic acid, or similar) has little nutritional value, a nutritional component can be added to the gelling agent to approximate the food value of the invertebrate tissue. Performing the same analyses on the selected nutritional component, such as powdered, freeze-dried squid mantle or a commercial flake food for aquarium fish, will provide the comparative basis for determining the recipe to match the invertebrate tissue, which, again, should be calculated on a volumetric basis.

Along the same lines, the feeding state of the assay predator can have an important effect on the outcome of feeding experiments. Predators that have been starved are likely to consume artificial food offerings despite the presence of a chemical defense that would otherwise deter them. This problem is well known to experimentalists who regularly run feeding experiments, but few studies have quantified the importance of the feeding state of consumers [84]. It is likely that predators of marine invertebrates are well fed; observations of fishes on coral reefs certainly support this contention [10, 23]. Therefore, if experiments are to be performed in the laboratory, assay predators should be maintained on a healthy diet to observe normal feeding behavior. However, it is not uncommon to deny assay predators food for a short period of time prior to a feeding experiment in order to boost the speed at which a feeding choice is made.

## 12.4 Additivity and Synergism

Complicating any exploration of chemical defenses of marine invertebrates is the possibility that a single metabolite does not act in isolation but in association with other metabolites or in association with physical defenses. The interaction of the individual component defenses may be *additive*, that is, their combined deterrent effect is equal to the sum of their individual effects, or it may be *synergistic*, wherein their combined deterrent effect is greater than the sum of their individual effects. A third case, in which the interactive effect of components leads to a loss of activity, is termed *antagonistic* and is exemplified in the previously cited interaction between compounds that enhance feeding (e.g., amino acids) and chemical defenses.

Marine invertebrates are likely candidates for interactive effects of potential defenses. Many species of sponges, soft corals, and ascidians are known to contain in their tissues: (1) mixtures of secondary metabolites of the same structural class, (2) multiple secondary metabolites of different structural classes, and (3) skeletal elements, such as glass spicules (sponges) or calcitic inclusions (soft corals, ascidians). The possibilities for exploring the interactions among these components quickly become numerically astronomical, but what remains ecologically relevant is whether their sum is deterrent to potential predators, and this is an experimentally approachable question using the techniques already described. However, when components are separated and are no longer deterrent at natural concentrations, two questions arise: (1) Did degradation or loss of metabolites during isolation or

purification result in activity falling below some threshold level? (2) Did separation of multiple active components from each other result in activity falling below threshold? Additionally, the effectiveness of any combination of chemical or physical defenses may depend on the nutritional quality of the prey [85].

Experimental examinations of additivity or synergism are rare in chemical ecology [86], and the terms may be used improperly. The complexity of evaluating additive vs. synergistic effects is illustrated in an exchange of publications on the chemical and physical defenses of calcified marine algae against herbivorous fishes [68, 87, 88].

In only one case has a systematic statistical approach for identifying additivity or synergism been undertaken: Jones et al. [89] used an isobolographic analysis and logit model to examine the interaction between chemical defenses and spicules in the tissues of Caribbean sponges. For each sponge species examined, multiple assays of each defensive component (crude organic extract or isolated siliceous spicules) in artificial foods were performed using the bluehead wrasse fish as the experimental predator. Assays of serial dilutions of each defensive component were performed to determine the 50% effective dose ($ED_{50}$) of each, and then a series of combination assays were carried out at concentrations equal to or less than the $ED_{50}$ of each component in isolation. Synergism between chemical and physical defenses at natural component concentrations was observed for three of seven sponge species that exhibited intermediate levels of chemical defenses, suggesting that spicules may synergistically enhance chemical defenses for some sponge species, but the authors suggested that this effect was more likely to be an exaptation of the primary function of spicules in sponges, which is to provide structural support to the organism. While Jones et al. [89] provided a novel analytical technique and an important proof of concept, their procedure has yet to be applied to defensive variability within a single species.

## 12.5 Optimization, Differential Allocation, Induction, and Activation

It is generally supposed that organisms are "optimized" in all respects for their environment by natural selection, but as discussed previously, this is certainly not true: The physical and biological environment changes through time, and evolution acts on the sum of the phenotypic characteristics that make up an individual organism – some will be more important at any one time, some less, some perhaps not at all. If defense is particularly important to the survival of an organism because predation is intense, and a coevolutionary "arms race" develops in which the prey must develop ever more sophisticated methods of combating predatory attack while somehow keeping the cost of those methods to a minimum, we might expect optimization of chemical defenses. In fact, optimization strategies have been described in the terrestrial chemical ecology literature, particularly for insects, and include mechanisms like differential allocation or induction of chemical defenses, as well as other protective measures, such as mimicry.

Chemical defenses that are *differentially allocated* are found at higher concentrations in the parts of an organism that are more susceptible to attack, thereby saving the organism the cost required to make and store defensive metabolites throughout its body. *Induction* describes the initiation of metabolite synthesis in response to tissue damage caused by predatory attack. An *induced chemical defense* is very economical because metabolites are produced only when needed, but the process would not provide immediate protection because metabolite synthesis would require some time. Another strategy often confused with induction is termed activation. An *activated chemical defense* is stored by the organism in a less bioactive form but is converted to a more potent form either when tissue is damaged or the defense is released in response to attack. The advantage of an activated defense to the prey is one of storage, in that it is potentially less costly to maintain relatively nontoxic precursor metabolites than to deal with autotoxicity from storing the product metabolite.

Evidence for optimized chemical defenses of marine invertebrates has been relatively scarce. This is interesting, because marine chemical ecologists often look for examples of optimized defenses in the system they are studying that parallel those described in terrestrial systems, not only to provide additional examples of the phenomena but also because they are intrinsically interesting, "just so" evolutionary narratives. When optimized defenses are found in a system, it is often interpreted that they can be generalized to other organisms, but when optimized defenses are not found, the research results are generally not published. Therefore, the paucity of reports of optimized chemical defenses among marine invertebrates more likely reflects scarceness rather than insufficient effort in uncovering the phenomena.

Differential allocation is certainly present in nudibranch mollusks, which have higher concentrations of deterrent metabolites in their dorsal surface and eggs [30], or associated with specialized glands on the dorsum and sides of the slugs [90]. For sessile benthic marine invertebrates, it has been claimed that some gorgonian corals invest higher levels of chemical defenses in their polyps than in the coenenchyme tissue that surrounds the axial skeleton of the coral [91], but the comparative data used to support this conclusion were based on gravimetric determinations of metabolite concentration (dry mass basis) rather than on a volumetric determination. Because the polyps of most gorgonians are free of calcitic sclerites, while the coenenchyme is infused with them to varying degrees, the high density of sclerites would result in much lower concentrations of metabolites on a dry mass basis in coenenchyme than on a volumetric basis, as explained previously [46]. Therefore, claims of differential allocation of chemical defenses in gorgonian corals await confirmation using a volumetric approach.

For sponges, differential allocation of defenses has been investigated for several species, with no evidence of the phenomenon in some [92] and some evidence in others [76, 93–95]. Here, the issue may be complicated by the method by which a predator feeds or whether the metabolite plays multiple roles. Fish predators of sponges take large bites of sponge tissue [23], which would make differential allocation ineffective [92]. But sea stars, the dominant invertebrate predators in

Antarctic benthic communities, feed by everting their guts on the surface of prey and appear to have driven the evolution of differential allocation of defenses to the surface tissues in some co-occurring sponge species [76]. The same may be true for sponges that are grazed by nudibranch mollusks [94] or have chemical defenses against fish predators that also serve as antifouling agents [93].

Differential allocation has been invoked for one species of morphologically distinct sponge from the Indo-Pacific, *Oceanapia* sp., which grows buried in the substratum but produces a stalk and cap structure that sticks up into the water column, where it is ostensibly subject to greater predatory attack [95]. As with the previous gorgonian example, the claim that the protruding structures contain higher concentrations of deterrent metabolites is confounded by the gravimetric (dry mass) method of determining metabolite concentration [95] because the concentration of glass spicules in the sponge tissue is highly variable, much greater in the sponge base and decreasing to the tip of the protruding structure. While the growth form of *Oceanapia* sp. certainly makes it a likely candidate for differential allocation of chemical defenses, volumetric measurements of metabolite concentrations will be needed to confirm this.

Induced defenses of marine invertebrates have been the subject of far fewer published studies than differential allocation of defenses, and in no case is there unequivocal evidence of the phenomenon. The chicken liver sponge *Chondrilla nucula* presents an excellent candidate for induced defenses: it has a high degree of intraspecific variability in chemical defense [16] and is abundant in a variety of habitats in the Caribbean, from reefs where fish predators are common to mangrove and grass bed habitats where they are rare. A more in-depth study of variation in chemical defenses of this species failed to resolve site-specific patterns in chemical defenses or induced increases in defense as a function of simulated predation [92].

Following up on preliminary data that suggested an induced defense [96], a transcriptome profiling approach was taken in a study of an Indo-Pacific soft coral *Sinularia polydactyla* that tracked changes in mRNA pool complexity as well as metabolite concentration for corals reciprocally transplanted to areas of high and low predation [97]. While there were clear differences in transcriptome complexity between transplanted corals, perhaps representing induced changes in metabolite production, the metabolite profiles in transplanted corals did not necessarily support induced defenses [97].

One of the more interesting and controversial areas of research into marine invertebrate antipredatory defenses has been that of activated defenses. It is important to remember that activated defenses are not optimized in the same way that differentially allocated or induced defenses are because these last two mechanisms reduce the overall expenditure of the organism on defensive metabolite production: They are cost-saving measures that optimize the use of defenses. An activated defense requires the synthesis of a full complement of precursor molecules, in addition to whatever enzyme or catalyst is required for conversion of the precursors into the defensive metabolite after the organism is attacked. As such, an activated defense could conceivably be *more expensive* to maintain than a constitutive defense. The potential advantage of an activated defense is one of

potency: A nonreactive precursor can be stored easily until attack, at which time tissue disruption and interaction of precursor and catalyst results in the nearly instantaneous formation of a highly deterrent (and possibly autotoxic) product metabolite. The foregoing is important because although it is easy to understand the evolution of resource-saving optimization schemes like differentially allocated or induced defenses (despite the paucity of evidence for their general existence among marine invertebrates), the evolution of activated defenses is more difficult to envision. After all, if the organism has to go to all the trouble of making the full complement of a chemical defense, why not make it constitutive?

An activated antipredatory defense was originally proposed for the Mediterranean sponge *Aplysina aerophoba* on the basis of laboratory experiments with freeze-dried sponge tissue [98]. Mechanistically, high molecular weight brominated tyrosine derivatives were thought to be rapidly converted by a putative enzyme to form smaller, more active chemical defenses after predatory damage to the sponge tissue. Because *Aplysina* spp. are found worldwide and all contain very similar secondary metabolites, it was proposed that this "biotransformation" was a common feature of the chemical defense of the genus. However, no evidence of activation was found in a combination of rigorous laboratory and field experiments with living specimens of two species of *Aplysina* in the Caribbean [99]. Without going into excessive detail, well over a decade after the initial report [98] and despite the rigorous field experiments performed in the Caribbean [99], there are now more than ten publications that directly relate to the putative activated defenses of *Aplysina* spp. [100]. The putative enzyme responsible for this activation defies isolation and characterization, and it is now suggested that the smaller brominated metabolites are important as antimicrobial agents that defend the wounded sponge tissue but not as antipredatory defenses [100, 101].

Another curious example of an activated defense has been described for the hydroid *Tridentata marginata*. Like most hydroids, *T. marginata* has stinging cells (cnides), but in addition it has a chemical defense against generalist fish predators that is effective even after the cnides have been discharged [102]. Further research suggested that the metabolites responsible for the chemical defense were stored in a nondeterrent form in the cnides, and that the crushing action associated with predation resulted in release and conversion to the deterrent metabolites, tridentatols A-C [103]. At one level, it is unclear why one species of hydroid should develop chemical defenses along with cnides while other co-occurring species have cnides alone, although the authors suggest that cnides offer a relatively ineffective physical defense in this species [102]. But in this example, the justification for activated defenses based on autotoxicity seems to be obviated by the isolation of the defensive precursors in cnide capsules.

The most recent example of an activation scheme has been proposed for the Indo-Pacific reef sponge *Aplysinella rhax*, a close relative of sponges of the genus *Aplysina* [104]. Maceration or wounding of the sponge tissue caused the rapid formation of psammaplin A from its sulfate salt, a reaction that was suggested to be enzyme catalyzed. The activated metabolite was claimed to be a more deterrent defense on the basis of feeding assays using fractions of extracts of the sponge, but

when the purified metabolite and the sulfate salt were assayed in the field, there was no difference in consumption of the two, casting doubt on the importance of the conversion as a requirement for an enhanced chemical defense. The authors of this study [104] suggest that evidence of activated chemical defenses have been underreported in the literature on marine chemical ecology, but an alternative explanation is that activated defenses are very rare (if they exist at all) for the simple reason that they do not provide an evolutionary advantage over constitutive defenses. Past observations of activated defenses in sponges may be attributable to differential tissue extraction efficiency, hydrolysis from less soluble precursors, or the heterogeneous distribution of metabolites in sponge tissue [99].

In summary, research to date warrants that claims of optimized or activated defenses among marine invertebrates be assessed with skepticism and with a higher standard of evidence. While the evolutionary narrative inspired by studies of terrestrial plants and insects is highly appealing, marine invertebrates in general have not been under the same selective predatory pressures that result in more complicated optimized defense strategies (Fig. 12.1). Comparing the basic aspects of the biology and ecology of marine invertebrates to terrestrial invertebrates (insects) may give us clues that explain why induction, differential allocation, and activation, as well as complex levels of aposematism and mimicry are common to the latter but not to the former: Marine invertebrates are fairly long-lived and exhibit indeterminate growth (some sponges may live thousands of years [105]), and while predation is an important part of their ecology, so too are other aspects, particularly recruitment. Terrestrial insects, on the other hand, may complete multiple generations in a single summer and exhibit determinate growth, and their populations are under intense and directed predatory pressure. Therefore, it is not surprising that many of the interesting phenomena found among butterflies and beetles are not evident among sponges, corals, and nudibranchs.

## 12.6 Resource Trade-Offs and the Cost of Chemical Defense

While terrestrial chemical ecologists have addressed costs in some systems [106–108], few studies in marine ecology have attempted to demonstrate that chemical defenses have a cost to the marine invertebrates that produce them. Perhaps the easiest way to address the cost of defense is to compare resource parameters within a species that has defended and undefended individuals, whether by constitutive (genotypic) or facultative (induced) means. As we have seen above, there are few examples of intraspecific variability in defenses among marine invertebrates, and none in which costs have been examined.

An alternative method for investigating the cost of chemical defenses is to quantify relative rates of growth or reproduction among co-occurring species that exhibit different levels of defense. Assuming that all life functions (respiration, growth, reproduction) have a cost, the production of chemical defenses should result in a trade-off, such that defended species exhibit lower rates of growth or reproduction than undefended species. Making this comparison can be difficult,

either because relative levels of defense among species in the community are unknown or because all the species in the community are similarly well defended, as in Caribbean gorgonian corals [46]. Moreover, different species may invest in mechanisms other than chemical defenses, which are also likely to have costs. Also, if endosymbionts of a chemically defended invertebrate (e.g., zooxanthellae in hard and soft corals) are responsible for the production of the chemical defense, it may come at little or no cost to the host invertebrate.

The Caribbean coral reef sponge community has provided an interesting system in which trade-offs between chemical defense and life functions have been evident. The most common sponges on Caribbean reefs are either chemically defended or undefended [16], and the latter group is consumed by a suite of sponge-eating fishes [10, 23]. When the rate of wound healing was compared between the two groups, undefended species were found to heal much faster than defended species [109]. More indicative of resource trade-offs between the two groups, however, were the results of surveys of an artificial reef shipwreck that provided new substratum for sponge recruitment and growth [110]. If undefended sponge species are able to divert metabolic resources to growth and reproduction, they would be expected to colonize new substrata faster than defended species that use their resources to synthesize and store chemical defenses. And in fact, undefended sponge species dominated an artificial reef shipwreck off the Florida Keys 4 years after it was sunk (96.0% of sponge cover was made up of undefended species vs. 15.2% on adjacent reefs), with initial recruits of defended species observed 18 months later [110], corroborating a resource trade-off between chemical defense and reproduction or growth.

## 12.7 Structure-Activity Relationships and the Commonality of Chemical Defenses

In pursuit of developing more effective insecticides, plant–insect chemical ecologists have systematically investigated the relationships between metabolite structure and feeding deterrent activity for many insect species [111]. Similar efforts have been rare in marine chemical ecology. When multiple pure compounds have been assayed together for feeding deterrent activity, it is not uncommon for comparisons to be made and preliminary conclusions drawn about relationships between structure and activity [59, 112], but these have not addressed the issue in a systematic way.

One study has examined the relationship between metabolite structure and feeding-deterrent activity for a series of analogs having minor modifications to determine the importance of metabolite size, shape, and functionality [113]. Using a fish feeding assay, 21 compounds, including pyrrole-imidazole alkaloids isolated from sponges of the genus *Agelas* and synthetic analogs, were tested at a range of concentrations. Additional observations of structure-activity relationships for metabolites from *Agelas* sp. were reported in a companion publication [114]. The pyrrole moiety was required for feeding deterrent activity, while the imidazole

group enhanced activity. Imidazole metabolites lacking the pyrrole were not active, while feeding-deterrent activity was enhanced by dimerization, increased polarity, or the addition of bromine.

Interestingly, there do not yet appear to be any common structural themes among secondary metabolites that have been isolated and identified as chemical defenses of marine invertebrates. Compounds of several classes and of very different polarities are represented among feeding deterrents, from nonpolar terpenoids [115] to polar glycosides [116]. This variability suggests that compound polarity, and therefore solubility in water, is not a critical factor in the evolution of marine invertebrate chemical defenses. Bad-smelling volatile compounds present in some sponges do not appear to act as chemical defenses [61]. While acidity is known to deter feeding, and is used by some ascidians as a chemical defense [83], this mechanism of defense is surprisingly uncommon, and many ascidians also have secondary metabolites as chemical defenses [59], perhaps suggesting that the metabolic demands of containing inorganic acids within invertebrate tissues are greater than their advantages relative to organic compounds as chemical defenses.

Recent technical developments in molecular genetics may permit structure-activity relationships to be addressed at the cellular level. Metabolites from the sponges *Ectyoplasia ferox* and *Erylus formosus* that had previously been identified as feeding deterrents using co-occurring fish predators [116, 117] also deterred feeding of the freshwater zebra fish [118]. Transcripts made from a zebra fish cDNA library were expressed in the oocytes of the frog *Xenopus laevis* and tested for chemoreceptor activation using electrophysiological techniques. Oocytes expressed gene sequences from the library and exhibited electrophysiological responses when exposed to the deterrent metabolites formoside and ectyoplasides A and B, indicating that the chemical defense-activated signaling pathway was reconstituted in *Xenopus* oocytes [118].

There is some evidence that chemical defenses may be broadly effective against predators, not only against different species (such as fishes) [79, 119], but also against different predatory taxa (crabs, seastars) [69, 70]. This commonality in response suggests that chemoreceptive responses of diverse taxa of predators are similar at the molecular level. In at least one case, for triterpenoid glycosides from two Caribbean sponges, multiple defensive roles have been proposed that extend beyond antipredatory effects to allelopathic and antifouling functions [93]. In terms of resource allocation, the advantages of multifunctional defenses are clear, and this is an area that is ripe for further investigation.

## 12.8 Study Questions

1. An eminent Ivy League bryozoan taxonomist finishes her survey of Indonesian coastal waters and sends 410 dried bryozoan samples representing 53 species ($N > 2$ for each) to her first-year graduate student, with instructions to investigate their chemical defenses. Following the advice of the elderly entomologist in the lab next-door, the graduate student decides to use diethyl ether as the sole

extraction solvent and to run Microtox toxicity assays on serial dilutions of each extract as a function of extract dry mass. He is delighted to discover very clear differences in toxicity between mangrove and coral reef species and runs a complex statistical analysis to support his conclusions. He submits their work on "The chemical defenses of Indonesian bryozoans" to a top-rated ecology journal.

Q1: What problems are reviewers of this manuscript likely to cite in recommending that it not be published?

Q2: What should the graduate student do after receiving the negative decision?

2. A graduate student in a natural products laboratory is studying the variability of secondary metabolites in a species of soft coral that produces a potential anti-cancer drug, xenopterolide. She determines that there is a significant difference in the mean concentration of xenopterolide, which is also a well-known fish feeding deterrent, in replicate samples (N = 20) from high-flow reef crest environments (30 μg xenopterolide/mg dry tissue mass) vs. low-flow patch reef environments (100 μg/mg). She concludes that fish predation on this soft coral is more intense in patch reef environments.

   Q3: What is an alternative explanation? Hint: Soft corals increase the density of their skeletal elements (limestone sclerites) as a function of increasing flow regime.

   Q4: How would you re-design the methodology for this project?

3. A postdoctoral researcher is studying seasonal variation in secondary metabolite concentration in a species of sponge by removing a tissue sample from each of 20 sponges every month for a year (no sponge is sampled more than once). The sponge is hermaphroditic, producing sperm and eggs in the spring, brooding large numbers of large embryos throughout its tissue for several months, and then releasing them in late summer. The researcher determines metabolite concentrations as a function of both volume and mass.

   Q5: What pattern of metabolite concentration might the researcher expect to see, on a gravimetric vs. volumetric basis, if the sponge tissue is full of glass spicules, but the embryos are free of spicules?

   Q6: What if neither sponge nor embryos have spicules?

   Q7: What if the sponge differentially provisions embryos with high levels of chemical defenses?

   Q8: For a seasonal study like this, why might the investigator also want to measure the mass of the crude organic extract for each tissue sample?

4. An MS graduate student completes his 2-year dissertation research on the ecological functions of a suite of previously characterized secondary metabolites in a temperate colonial ascidian. He uses all the proper extraction and assay techniques to explore possible defenses against several co-occurring predators, as well as meticulously investigating antifouling, allelopathic, and antimicrobial functions. His data show no defensive effect of the metabolites in any of his assays. His advisor is sympathetic but tells him that his work is not publishable because "nobody should publish negative results" and that "all secondary

metabolites must have ecological functions, otherwise the organism wouldn't make or store them."

Q9: Why is the advisor wrong about each of the statements in quotes?

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