Video Article

A Fish-feeding Laboratory Bioassay to Assess the Antipredatory Activity of Secondary Metabolites from the Tissues of Marine Organisms

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Abstract

Marine chemical ecology is a young discipline, having emerged from the collaboration of natural products chemists and marine ecologists in the 1980s with the goal of examining the ecological functions of secondary metabolites from the tissues of marine organisms. The result has been a progression of protocols that have increasingly refined the ecological relevance of the experimental approach. Here we present the most up-to-date version of a fish-feeding laboratory bioassay that enables investigators to assess the antipredatory activity of secondary metabolites from the tissues of marine organisms. Organic metabolites of all polarities are exhaustively extracted from the tissue of the target organism and reconstituted at natural concentrations in a nutritionally appropriate food matrix. Experimental food pellets are presented to a generalist predator in laboratory feeding assays to assess the antipredatory activity of the extract. The procedure described herein uses the bluehead, *Thalassoma bifasciatum*, to test the palatability of Caribbean marine invertebrates; however, the design may be readily adapted to other systems. Results obtained using this laboratory assay are an important prelude to field experiments that rely on the feeding responses of a full complement of potential predators. Additionally, this bioassay can be used to direct the isolation of feeding-deterrent metabolites through bioassay-guided fractionation. This feeding bioassay has advanced our understanding of the factors that control the distribution and abundance of marine invertebrates on Caribbean coral reefs and may inform investigations in diverse fields of inquiry, including pharmacology, biotechnology, and evolutionary ecology.

Video Link

The video component of this article can be found at http://www.jove.com/video/52429/

Introduction

Chemical ecology developed through the collaboration of chemists and ecologists. While the subdiscipline of terrestrial chemical ecology has been around for some time, that of marine chemical ecology is only a few decades old but has provided important insights into the evolutionary ecology and community structure of marine organisms[1-8]. Taking advantage of the emergent technologies of SCUBA diving and NMR spectroscopy, organic chemists rapidly generated a great number of publications describing novel metabolites from benthic marine invertebrates and algae in the 1970s and 1980s[9]. Assuming that secondary metabolites must serve some purpose, many of these publications ascribed ecologically important properties to new compounds without empirical evidence. 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 from relatively ineffective 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 predators[10]. In an effort to introduce empiricism to what was otherwise descriptive work on species abundances, some ecologists began extrapolating chemical defenses from toxicity assays[11]. Most toxicity assays involved the exposure of whole fish or other organisms to aqueous suspensions of crude organic extracts of invertebrate tissues, with subsequent determination of the dry mass concentrations of extracts responsible for killing half the assay organisms. However, toxicity assays do not emulate the manner in which potential predators perceive prey under natural conditions, and subsequent studies have found no relationship between toxicity and palatability[12-13]. It is surprising that publications in prestigious journals used techniques having little or no ecological relevance[14-15] and that these studies are still widely cited today. It is even more alarming to note that studies based on toxicity data continue to be published[16-18]. The bioassay method described herein was developed in the late 1980s to provide an ecologically relevant approach for marine chemical ecologists to assess antipredatory chemical defenses. The method requires a model predator to sample a crude organic extract from the target organism at a natural concentration in a nutritionally comparable food matrix, providing palatability data that are more ecologically meaningful than toxicity data.

The general approach to assessing the antipredatory activity of the tissues of marine organisms includes four important criteria: (1) an appropriate generalist predator must be used in feeding assays, (2) organic metabolites of all polarities must be exhaustively extracted from the tissue of the target organism, (3) the metabolites must be mixed into a nutritionally appropriate experimental food at the same volumetric concentration as found in the organism from which they were extracted, and (4) the experimental design and statistical approach must provide a meaningful metric to indicate relative distastefulness.

The procedure outlined below is designed specifically to assess antipredatory chemical defenses in Caribbean marine invertebrates. We employ the bluehead wrasse, *Thalassoma bifasciatum*, as a model predatory fish because this species is common on Caribbean coral reefs and is known to sample a wide assortment of benthic invertebrates[19]. Tissue from the target organism is first extracted, then combined with a food mixture, and finally offered to groups of *T. bifasciatum* to observe whether they reject the extract-treated foods. Assay data using this method have provided important insights into the defensive chemistry of marine organisms[12,20-21], life history trade-offs[22-24], and community ecology[25-26].

Protocol

NOTE: Step 3 of this protocol involves vertebrate animal subjects. The procedure has been designed so that animals receive the most humane treatment possible and has been approved by the Institutional Animal Care and Use Committee (IACUC) at the University of North Carolina Wilmington.

1) Tissue Extraction

1. Use tissue that is in its natural state of hydration and not squeezed, dried-out or overly wet as this will alter the volumetric concentration of secondary metabolites. Cut or chop tissue to pieces or slices that can be inserted into a 50 ml centrifuge tube. Note: Fresh tissue can be used in some cases, but it is often better to cut or chop frozen tissue, which is not subject to squeezing when cut.
2. Add tissue pieces to 30 ml of a 1:1 mixture of dichloromethane (DCM) and methanol (MeOH) in a graduated centrifuge tube until a final volume of 40 ml is reached. Be sure to conduct all steps involving the transfer of solvent in a fume hood with adequate ventilation.
3. Cap the tube and invert it several times, then agitate repeatedly during a 4 hr extraction period. Note: During this period, water combines with the MeOH and the resulting MeOH:water phase separates from the DCM phase. The tissue is alternately exposed to DCM and MeOH:water as an emulsion as the tubes are agitated.
4. Transfer the DCM extract to a round-bottom flask and evaporate to dryness on a rotary evaporator using low heat (<40 °C). Using minimal solvent, transfer the dried extract to a 20 ml scintillation vial. Fit the vial with a rotary evaporator adapter and again evaporate to dryness on a rotary evaporator using low heat (<40 °C).
   Note: The next step requires the use of a homemade compression instrument that may be assembled by screwing the following items in sequential order onto the end of a threaded rod: (1) nut, (2) washer, and (3) acorn nut. The washer must either be perforated or fitted so that it is less than the internal diameter of a 50 ml centrifuge tube.
5. Returning to the graduated centrifuge tube that contains tissue and the MeOH:water extract, squeeze the extraction medium out of the tissue through compression. Transfer the MeOH:water extract to the same round-bottom flask and store chilled (<10 °C).
6. Add MeOH to the graduated centrifuge tube until the now dehydrated tissue is submerged for a second extraction of 2 to 6 hr duration, then transfer the new MeOH extract to the chilled round-bottom flask containing the MeOH:water extract. If there is any concern that the tissue has not been fully extracted, repeat the 2 to 6 hr MeOH extraction.
7. Dry off the MeOH on a rotary evaporator using low heat (<40 °C). Transfer the remaining aqueous extract from the round-bottom flask to the scintillation vial containing the dried nonpolar extract, using a minimal volume of MeOH to rinse the round-bottom flask.
8. Evaporate the aqueous extract to dryness using low heat (<40 °C) on a vacuum concentrator. The scintillation vial now contains the total dry crude organic extract of 10 ml of tissue. Evacuate the head space of the vial with N_2 gas to prevent oxidation, seal tightly, and store frozen (-20 °C).

2) Food Preparation

1. Prepare freeze-dried squid mantle powder.
   Note: Squid mantle provides a source of nutrition that is comparable to that of other benthic invertebrates, and will be used as an ingredient in the substeps of 2.2.
   1. Thaw frozen rings of squid mantle in warm deionized (DI) water, then puree them in a high-speed blender.
   2. Pour a thin layer of pureed squid mantle onto a shallow cookie sheet and freeze (-20 °C), then break the sheet of frozen squid puree into small pieces to be lyophilized.
   3. Lyophilize the frozen squid mantle puree following the operating procedures of the freeze-drier.
   4. Pulverize the lyophilized pieces of squid mantle puree in a high-speed blender to form a powder.
   5. In a fume hood, pour the powdered squid mantle into a rotary flour sifter and sift to separate large chunks of tissue from the fine powder.
   6. Transfer the fine powdered squid mantle to a sealable container. Evacuate the container head space with N_2 gas to prevent oxidation and store frozen (-20 °C).

2. Prepare the food mixture.
   Note: When running multiple consecutive assays, it is practical to prepare ~100 ml of food mixture, however this recipe may be scaled to smaller volumes if necessary.
   1. Combine a mixture of 3 g alginic acid and 5 g freeze-dried squid mantle powder with 100 ml of DI water in a 150 ml beaker. Stir vigorously with a microspatula for a few minutes until the powder is fully hydrated and the mixture is homogeneous.
      Note: If desired, food coloring may be added at this step: it is easier to add dye to the food mixture that will generate both treated and control mixtures (masking the natural pigment of the extract in the extract-treated mixture) rather than trying to match the color of the extract-treated mixture by adding dye to the control mixture. A greenish or brownish food color is often desirable to mask any pigments in the crude extract.
   2. Load exactly 10 ml of food mixture into a graduated syringe. Take care to avoid the inclusion of air bubbles during this process.
   3. Remove the 20 ml scintillation vial with dry crude organic extract from the freezer. Add a drop or two of MeOH, then stir the extract into a homogeneous mixture with a microspatula.
   4. Eject the loaded 10 ml syringe of food matrix into the 20 ml scintillation vial and stir with a microspatula to homogenize the extract-treated food mixture.

Note: It may help to eject the syringe in smaller increments (*i.e.*, eject 2 ml and homogenize, then repeat until all 10 ml have been homogenized).

3. Prepare the assay pellets.
    1. Load a very small volume of the extract mixture (~1 ml) into a syringe, and submerge the syringe tip in a solution of 0.25 M $CaCl_2$. Eject the contents of the syringe to form a long, spaghetti-like strand.
    2. After a few minutes, remove the hardened strand, chop it into 4 mm long pellets on a glass cutting board with a razor blade, then rinse in seawater.
    3. Repeat steps 2.3.1 and 2.3.2 without including tissue extract to make control pellets. Be sure to treat control pellets with an equivalent volume of solvent (see addition of MeOH to treated mixture in step 2.2.3) to control for solvent addition. If a negative control is desired to confirm that assay fish can be deterred from feeding, add denatonium benzoate at a concentration of 2 mg ml^{-1} to the raw food mixture[27].

3) Palatability Bioassays

1. Perform feeding assays with wild-caught yellow-phase bluehead wrasse, *Thalassoma bifasciatum,* kept in groups of three in opaque-sided compartments of laboratory aquaria.
2. Deliver food pellets from a beaker of seawater using a glass pipette with a rubber bulb. Note: It may take a few days to train fish to receive food in this manner. A conditioning stimulus (*e.g.* a few taps of the pipette on the aquarium glass) that precedes the delivery of food may be helpful to train the fish to expect the addition of food pellets.
3. Scoring pellets. Consider a pellet accepted if readily consumed by the fish. Consider a pellet rejected if not eaten after a minimum of three attempts by one or more fish to take it into their mouth cavity, or if the pellet is approached and ignored after one such attempt.
4. Scoring samples. Note: The assay procedure is depicted as a flowchart in **Figure 1**. Groups of fish that refuse to eat control pellets at any step in the protocol are not considered further. There are two potential outcomes of a single run of the assay: the sample is either accepted or rejected.
    1. Begin with a control pellet to confirm that the group of fish is cooperative. Offer a treated pellet. If the fish accept the treated pellet, score the sample as accepted. If the fish reject the treated pellet, offer a subsequent control pellet to determine whether the fish have ceased feeding. If the fish accept the subsequent control pellet, score the sample as rejected.
5. Replication. Repeat the assay procedure with ten independent groups of fish for each extract.

4) Evaluating Significance

1. Evaluate the significance of differences in the consumption of control vs treated pellets with a modified version of Fisher's exact test[26]. Modify the test so that the marginal totals for control and treated pellets are fixed, treating them both as random samples. Note: This provides p = 0.057 when 7 pellets are eaten; hence, any extract is considered deterrent if 6 or fewer pellets are eaten, and palatable if 7 or more pellets are eaten.
2. To compare the relative palatability among groups of extracts, calculate a mean number of pellets eaten within each group. Keep the threshold at 6 pellets so a group of replicate extracts are considered deterrent if the mean number of pellets eaten + standard error (SE) ≤6. Note: In the representative results, the group assignment is species, so replicate extracts come from distinct individuals and the relative palatability may be compared among species.

Representative Results

Here we report results of this bioassay for six species of common Caribbean sponges (**Figure 2**). These data were initially published in 1995 by Pawlik *et al.*[12] and demonstrate the power of this approach to survey differences in chemical defense strategies among co-occurring taxa. Results were reported as a mean number of food pellets eaten + standard error (SE) for each species. Almost no pellets were eaten in assays with crude organic extracts from *Agelas clathrodes*, *Amphimedon compressa*, and *Aplysina cauliformis*. In contrast, pellets made with extracts from *Callyspongia vaginalis*, *Geodia gibberosa*, and *Mycale laevis* were readily consumed in the assay[12]. Fewer than six pellets were eaten for the first three species, so they were considered significantly deterrent. In contrast, the second three species were not significantly different from the controls, and were considered palatable.

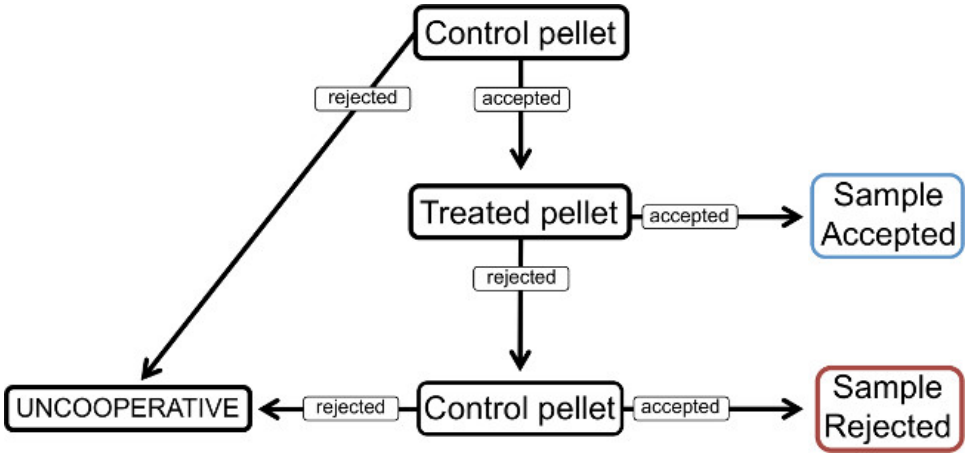

Figure 1: Schematic of the assay procedure. At all stages, the rejection of a control pellet indicates that this set of assay fish are uncooperative or satiated and cannot be used further. The protocol begins by offering each set of fish a control pellet followed by a treated pellet. Next, if the treated pellet is accepted the sample is scored as accepted. If the treated pellet is rejected but the subsequent control pellet is accepted, the sample is scored as rejected.

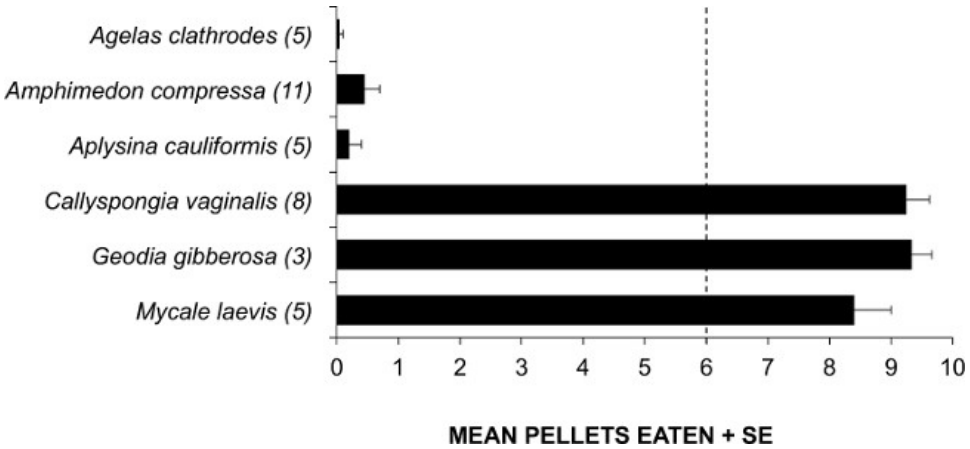

Figure 2: Consumption by *Thalassoma bifasciatum* of food pellets (mean + SE) containing crude organic extracts of sponges at natural concentrations, first reported in 1995 by Pawlik *et al.*[12] Fish consumed all 10 control pellets in all cases. After each species name, the number of replicate samples is specified (each replicate from the separate extraction of a geographically distinct sample of sponge tissue). For any individual assay, extracts were considered deterrent if the number of pellets eaten was less than or equal to 6 (p = 0.057, modified Fisher's exact test), as indicated by the dotted line on the graph.

Discussion

The procedure described herein provides a relatively simple, ecologically relevant laboratory protocol for assessing antipredatory chemical defenses in marine organisms. Here we review the important criteria that are satisfied by this set of methods:

(1) Appropriate predator. This feeding assay employs the bluehead wrasse, *Thalassoma bifasciatum*, one of the most abundant fishes on coral reefs throughout the Caribbean. The bluehead is a generalist carnivore known to sample a wide assortment of benthic invertebrates[19]. Generalist predators are the best choice for these initial assays because the majority of predatory fish on reefs are generalists, and it would be expected that antipredatory defenses would be broadly directed against them, as opposed to specialist predators that may have evolved mechanisms to circumvent defenses. Laboratory surveys of chemical defenses using a single potential predator are often followed by more time-consuming and complicated field experiments that rely on the responses of a full complement of potential predators under field conditions[28-33].

(2) Extraction procedure. The first tissue extraction step, which uses a solvent mixture of equal parts dichloromethane (DCM) and methanol (MeOH), rapidly permeates tissue, solubilizing membranes and dehydrating cellular material. The tissue is dehydrated after this step, so the subsequent steps extract remaining metabolites of all polarities in MeOH. Repeating the extraction in MeOH until the tissue is fully extracted constitutes an exhaustive extraction procedure. Minor variations on this extraction scheme are acceptable, such as substitution of one extraction solvent for another of the same polarity, but tissue extraction may be incomplete if an inappropriate solvent is used. Potential pitfalls of improper tissue extraction procedures are discussed in detail elsewhere[8].

(3) Preparation of experimental food. The artificial food matrix must simulate the tissue of the target organism in both the nutritional quality and concentration of secondary metabolites. It is likely that the same sensory processes that predators use to reject feeding-deterrent metabolites are also involved in the perception of the nutritional quality of foods. Foods with low nutritional quality may be rejected at much lower levels of chemical defense, 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. Powdered, freeze-dried squid mantle is a useful nutritional substitute because it is readily available, easy to measure, and its nutritional characteristics have already been determined[34].

The second consideration in preparing the experimental food concerns the determination of the concentration of the extract, which must be done on the basis of volume, not mass. Predators eat wet tissue, and the tissues of marine organisms vary widely in water content. 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. Furthermore, tissues of marine organisms may have very different densities because of mineral skeletal elements. Determination of metabolite concentration by volume solves both problems and is the most relevant measure from the standpoint of consumption of tissue by a potential predator. This topic, including examples from the literature, is discussed in detail elsewhere[8].

(4) Experimental design and statistical approach. Appropriate experimental design and 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. The analysis described herein is simple: differences are determined with a modified contingency table. The method requires that all control food offerings be consumed because the investigator would not be using experimental predators that were not feeding on control foods[8]. Although the use of Fisher's exact test has been modified from its initial use by Pawlik *et al.*[12], the threshold value of 6 treated pellets eaten remains unchanged. Over the years, other statistical tests have been suggested as substitutes, but discarded after consultation with collaborator James E. Blum (UNCW Dept. of Mathematics and Statistics). For example, McNemar's test has been suggested, but is inappropriate, both because it lacks a matched set of data, and because one row of the contingency table is fixed at 10 control pellets eaten.

Despite our experience that this assay method provides remarkably clear results, it nevertheless relies on a behavioral response. If fish are starved for a period of time before the assay, they may eat more treated pellets than they would if fish were well-fed, particularly if a defensive metabolite is present in the treated food pellets at a near-threshold concentration of activity. For these reasons, results of feeding assays should not be over-interpreted. For example, a difference between two tissue samples of 1/10 vs 9/10 pellets eaten indicates the first sample is deterrent and the second is not, but a difference of 3/10 vs 5/10 pellets eaten may be due to behavioral variation between assays, and the first sample is not necessarily more deterrent than the second.

A key application of this bioassay is its use in bioassay-guided fractionation, whereby successive partitions of the crude extract are tested on fish to isolate the chemical compounds responsible for the feeding-deterrent activity[29,32-33,35-38]. Once the presence of a chemical defense has been ascertained, the crude organic extract is chromatographically fractionated into smaller subsets of compounds that make up the mixture, and these subsets are fed to fish in the same feeding assay. 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 may identify them using standard spectroscopic techniques and should also do the same for inactive fractions that may have secondary metabolites. It is equally important to know which secondary metabolites are active in ecologically relevant experiments as to know which metabolites are not[8].

Elements of this procedure may also be used to design new experimental techniques. For example, this bioassay was adapted for invertebrate predators (*e.g.* crabs[39] and seastars[40]), for other geographic regions[41], and even to address other research questions (*e.g.* structural defenses[31,34,42] and aposematism[27]). The four criteria should serve as a guide to future adaptations of this method. In summary, this bioassay procedure provides a more ecologically relevant method for assessing the antipredatory chemical defenses from tissues of marine organisms. Studies using this procedure have advanced our understanding of the factors that control the distribution and abundance of marine invertebrates on Caribbean coral reefs (*e.g.* most recently, Loh and Pawlik[26]) and may inform investigations in diverse fields of inquiry, including pharmacology, biotechnology, and evolutionary ecology.

Disclosures

The authors declare that they have no competing financial interests.

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References

1. Paul, V. J., ed. *Ecological roles of marine natural products.* Comstock Publishing Associates: Ithaca, N.Y. (1992).
2. Pawlik, J. R. Marine invertebrate chemical defenses. *Chemical Reviews.* **93** (5), 1911—1922, doi:10.1021/cr00021a012 (1993).
3. Hay, M. E. Marine chemical ecology: what's known and what's next? *Journal of Experimental Marine Biology and Ecology.* **44** (5), 476—476 (1996).
4. McClintock, J. B., & Baker, B. J. *Marine Chemical Ecology.* CRC Press, Boca Raton, Fla (2001).
5. Amsler, C. D. *Algal Chemical Ecology.* Springer: New York (2008).
6. Hay, M. E. Marine chemical ecology: Chemical signals and cues structure marine populations, communities, and ecosystems. *Annual Review of Marine Science*. **1**, 193—212 (2009).

7. Pawlik, J. R. The chemical ecology of sponges on Caribbean reefs: Natural products shape natural systems. *BioScience.* **61** (11), 888—898, doi:10.1525/bio.2011.61.11.8 (2011).
8. Pawlik, J. R. Antipredatory Defensive Roles of Natural Products from Marine Invertebrates. *Handbook of Marine Natural Products.*, 677—710 (2012).
9. Pawlik, J. R., Amsler, C. D., Ritson-Williams, R., McClintock, J. B., Baker, B. J., & Paul, V. J. Marine Chemical Ecology: A Science Born of Scuba. *Research and Discoveries: The Revolution of Science through Scuba.* **39**, 53—69 (2013).
10. Randall, J. E., & Hartman, W. D. Sponge-feeding fishes of the West Indies. *Marine Biology.* **1**, 216—225 (1968).
11. Bakus, G. J., & Green, G. Toxicity in sponges and holothurians — geographic pattern. *Science.* **185**, 951—953 (1974).
12. Pawlik, J. R., Chanas, B., Toonen, R. J., & Fenical, W. Defenses of Caribbean sponges against predatory reef fish. 1. Chemical deterrency. *Marine Ecology Progress Series.* **127**, 183—194 (1995).
13. Schulte, B. A., & Bakus, G. J. Predation deterrence in marine sponges — laboratory versus field studies. *Bulletin of Marine Science.* **50**, 205—211 (1992).
14. Jackson, J. B. C., & Buss, L. Allelopathy and spatial competition among coral reef invertebrates. *Proceedings of the National Academy of Sciences.* **72**, 5160—5163 (1975).
15. Bakus, G. J. Chemical defense mechanisms on the great barrier reef, Australia. *Science.* **211**, 497—499 (1981).
16. Gemballa, S., & Schermutzki, F. Cytotoxic haplosclerid sponges preferred: a field study on the diet of the dotted sea slug *Peltodoris atromaculata* (doridoidea: nudibranchia). *Marine Biology.* **144**, 1213—1222 (2004).
17. Voogd, N. J., & Cleary, D. F. R. Relating species traits to environmental variables in Indonesian coral reef sponge assemblages. *Marine and Freshwater Research.* **58**, 240—249 (2007).
18. Mollo, E., *et al*. Factors promoting marine invasions: a chemolecological approach. *Proceedings of the National Academy of Sciences.* **105**, 4582—4586 (2008).
19. Randall, J. E. Food habits of reef fishes of the West Indies. *Studies in Tropical Oceanography.* **5**, 665—847 (1967).
20. O'Neal, W., & Pawlik, J. R. A reappraisal of the chemical and physical defenses of Caribbean gorgonian corals against predatory fishes. *Marine Ecology Progress Series.* **240**, 117—126 (2002).
21. Hines, D. E., & Pawlik, J. R. Assessing the antipredatory defensive strategies of Caribbean non-scleractinian zoantharians (Cnidaria): is the sting the only thing? *Marine Biology.* **159** (2), 389—398, doi:10.1007/s00227-011-1816-2 (2012).
22. Walters, K. D., & Pawlik, J. R. Is there a trade-off between wound-healing and chemical defenses among Caribbean reef sponges? *Integrative and Comparative Biology.* **45** (2), 352—358 (2005).
23. Leong, W., & Pawlik, J.R. Evidence of a resource trade-off between growth and chemical defenses among Caribbean coral reef sponges. *Marine Ecology Progress Series.* **406**, 71—78, doi:10.3354/meps08541 (2010).
24. Leong, W., & Pawlik, J. R. Comparison of reproductive patterns among 7 Caribbean sponge species does not reveal a resource trade-off with chemical defenses. *Journal of Experimental Marine Biology and Ecology.* **401** (1—2), 80—84, doi:10.1016/j.jembe.2011.02.043 (2011).
25. Pawlik, J. R., Loh, T.-L., McMurray, S. E., & Finelli, C. M. Sponge Communities on Caribbean Coral Reefs Are Structured by Factors That Are Top-Down, Not Bottom-Up. *PLoS ONE.* **8** (5), e62573, doi:10.1371/journal.pone.0062573 (2013).
26. Loh, T.-L., & Pawlik, J. R. Chemical defenses and resource trade-offs structure sponge communities on Caribbean coral reefs. *Proceedings of the National Academy of Science.* **111**, 4151-4156 (2014).
27. Miller, A. M., & Pawlik, J. R. Do coral reef fish learn to avoid unpalatable prey using visual cues? *Animal Behaviour.* **85**, 339-347 (2013).
28. Pawlik, J. R., & Fenical, W. A re-evaluation of the ichthyodeterrent role of prostaglandins in the Caribbean gorgonian coral, *Plexaura homomalla*. *Marine Ecology Progress Series.* **52**, 95-98 (1989).
29. Fenical, W., & Pawlik, J. R. Defensive properties of secondary metabolites from the Caribbean gorgonian coral *Erythropodium caribaeorum*. *Marine Ecology Progress Series.* **75**, 1-8 (1991).
30. Pawlik, J. R., & Fenical, W. Chemical defense of *Pterogorgia anceps*, a Caribbean gorgonian coral. *Marine Ecology Progress Series.* **87**, 183-188 (1992).
31. Chanas, B., & Pawlik, J. R. Does the skeleton of a sponge provide a defense against predatory reef fish? *Oecologia.* **107** (2), 225—231 (1996).
32. Chanas, B., Pawlik, J. R., Lindel, T., & Fenical, W. Chemical defense of the Caribbean sponge *Agelas clathrodes* (Schmidt). *Journal of Experimental Marine Biology and Ecology.* **208** (1—2), 185—196, doi:10.1016/S0022-0981(96)02653-6 (1997).
33. Wilson, D. M., Puyana, M., Fenical, W., & Pawlik, J. R. Chemical defense of the Caribbean reef sponge *Axinella corrugata* against predatory fishes. *Journal of Chemical Ecology.* **25** (12), 2811—2823 (1999).
34. Chanas, B., & Pawlik, J. R. Defenses of Caribbean sponges against predatory reef fish. II. Spicules, tissue toughness, and nutritional quality. *Marine Ecology Progress Series.* **127** (1), 195—211 (1995).
35. Albrizio, S., Ciminiello, P., Fattorusso, E., Magno, S., & Pawlik, J. R. Amphitoxin, a new high molecular weight antifeedant pyridinium salt from the Caribbean sponge *Amphimedon compressa*. *Journal of Natural Products.* **58** (5), 647—652, doi:10.1021/np50119a002 (1995).
36. Assmann, M., Lichte, E., Pawlik, J. R., & Köck, M. Chemical defenses of the Caribbean sponges *Agelas wiedenmayeri* and *Agelas conifera*. *Marine Ecology Progress Series.* **207**, 255—262 (2000).
37. Kubanek, J., Fenical, W., & Pawlik, J. R. New antifeedant triterpene glycosides from the Caribbean sponge *Erylus Formosus*. *Natural Product Letters.* **15** (4), 275—285, doi:10.1080/10575630108041292 (2001).
38. Pawlik, J. R., McFall, G., & Zea, S. Does the odor from sponges of the genus *Ircinia* protect them from fish predators? *Journal of Chemical Ecology.* **28** (6), 1103—1115 (2002).
39. Waddell, B., & Pawlik, J. R. Defenses of Caribbean sponges against invertebrate predators. I. Assays with hermit crabs. *Marine Ecology Progress Series.* **195**, 125—132 (2000).
40. Waddell, B., & Pawlik, J. R. Defense of Caribbean sponges against invertebrate predators. II. Assays with sea stars. *Marine Ecology Progress Series.* **195**, 133—144 (2000).
41. Burns, E., Ifrach, I., Carmeli, S., Pawlik, J. R., & Ilan, M. Comparison of anti-predatory defenses of Red Sea and Caribbean sponges. I. Chemical defense. *Marine Ecology Progress Series.* **252**, 105—114 (2003).
42. Jones, A. C., Blum, J. E., & Pawlik, J. R. Testing for defensive synergy in Caribbean sponges: Bad taste or glass spicules? *Journal of Experimental Marine Biology and Ecology.* **322** (1), 67—81, doi:10.1016/j.jembe.2005.02.009 (2005).

