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Original Article

Metabolite variability in Caribbean sponges of the genus *Aplysina*

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ABSTRACT

Sponges of the genus *Aplysina* are among the most common benthic animals on reefs of the Caribbean, and display a wide diversity of morphologies and colors. Tissues of these sponges lack mineralized skeletal elements, but contain a dense spongin skeleton and an elaborate series of tyrosine-derived brominated alkaloid metabolites that function as chemical defenses against predatory fishes, but do not deter some molluscs. Among the earliest marine natural products to be isolated and identified, these metabolites remain the subject of intense interest for commercial applications because of their activities in various bioassays. In this study, crude organic extracts from 253 sponges from ten morphotypes among the species *Aplysina archeri*, *Aplysina bathyphila*, *Aplysina cauliformis*, *Aplysina fistularis*, *Aplysina fulva*, *A. insularis*, and *Aplysina lacunosa* were analyzed by liquid chromatography–mass spectrometry (LC–MS) to characterize the pattern of intra- and interspecific variabilities of the twelve major secondary metabolites present therein. Patterns across *Aplysina* species ranged from the presence of mostly a single compound, fistularin-3, in *A. cauliformis*, to a mixture of metabolites present in the other species. These patterns did not support the biotransformation hypothesis for conversion of large molecular weight molecules to smaller ones for the purpose of enhanced defense. Discriminant analyses of the metabolite data revealed strong taxonomic patterns that support a close relationship between *A. fistularis*, *A. fulva* and *A. insularis*, while two morphotypes of *A. cauliformis* (lilac creeping vs. brown erect) were very distinct. Two morphotypes of *A. lacunosa*, one with hard tissue consistency, the other soft and thought to belong to a separate genus (*Suberea*), had very similar chemical profiles. Of the twelve metabolites found among samples, variation in fistularin-3, dideoxyfistularin-3 and hydroxyaerothionin provided the most predictive influence in decreasing order. Except for one morphotype, weak relationships were found from within-morphotype analyses of metabolite concentrations as a function of geographic location (Florida, N Bahamas, S Bahamas) and depth (<10 m, 10–20 m, >20 m). Our data suggest that metabolite profiles are strongly influenced by sponge phenotype rather than by the diverse microbiome which many *Aplysina* species share.

Introduction

Sponges now dominate the benthic community of most Caribbean coral reefs, particularly when one considers overall biomass and biodiversity; integrates it across the reef community to deep water, and considers the communities within the reef framework (Diaz and Rützler, 2001; Pawlik, 2011; Villamizar et al., 2013). Sponges are important to the overall ecology of coral reefs for many reasons: they are very efficient filter feeders, providing an important link in benthic-pelagic coupling (Southwell et al., 2008), they appear to be capable of absorbing dissolved organic carbon as a food source (de Goeij et al., 2013), and their bodies provide shelter for large numbers of invertebrates and fishes (Westinga and Hoetjes, 1981; Henkel and Pawlik, 2005). Sponges are aggressive competitors for space (Aerts, 1998) and are primary agents of carbonate bioerosion on coral reefs (Zundelevich et al., 2007).

Of all invertebrates, sponges have yielded the largest number of secondary metabolites that have been isolated and described by marine natural products chemists (Blunt et al., 2013 and previous reviews). Many of these compounds have potent pharmacological activities, including anti-tumor, anti-fungal, anti-viral, and anti-bacterial effects (Laport et al., 2009; Newman and Cragg, 2014). Because these compounds are often structurally complex or are present at high concentrations in sponge tissues, they are likely

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to be metabolically expensive to make and store, and to have some adaptive purpose. By far, the most commonly hypothesized defensive role for these compounds is predator deterrence, but antifouling, anti-overgrowth, antimicrobial and UV-protective functions have also been proposed (Pawlik, 2011).

Our understanding of the ecology of sponges on Caribbean reefs has changed substantially over the past 20 years. Before the mid-1990s, the conventional view of sponge ecology on Caribbean reefs was that there was no top-down (predator) control of sponge populations, because only a few fish species spread their predatory activities over a wide variety of sponge species (Randall and Hartman, 1968). Subsequent research on the chemical defenses of sponges demonstrated the importance of predation, with sponges falling into three categories: preferred species that are grazed completely from the reef and restricted to refugia inside the reef framework, palatable species that are common on the reef and tolerate grazing because they heal, grow or reproduce fast enough to persist, and defended species that are protected from predation by chemical defenses (Pawlik, 2011). A conceptual model of sponge ecology on Caribbean reefs, based on an understanding of sponge chemical defenses and resource trade-offs with growth or reproduction, was validated in cross-Caribbean surveys of sponge communities on heavily overfished and less-fished coral reefs (Loh and Pawlik, 2014). As part of those surveys, it was established that sponges of the genus *Aplysina* are among the most common chemically defended species, with *Aplysina cauliformis* the single most abundant species on Caribbean coral reefs (Loh and Pawlik, 2014).

Caribbean sponges of the genus *Aplysina* are strongly chemically defended against sponge-eating fishes (Pawlik et al., 1995; Loh and Pawlik, 2014). Interestingly, these defenses do not extend to certain nocturnal and cryptic molluscan predators, such as cowries, which leave behind grazing trails and pits on the surfaces of several species, including *Aplysina fistularis* (Pawlik and Deignan, 2015). Ecologically relevant bioassays have also been used to demonstrate that the secondary metabolites of sponges of this genus have potent antimicrobial (Kelly et al., 2005) and allelopathic effects (Pawlik et al., 2007). The metabolites responsible for these biological activities were among the earliest marine natural products described, and they came from *Aplysina* species collected in the Mediterranean (Fattorusso et al., 1972) and the eastern Pacific (Andersen and Faulkner, 1973) in the quest for new drugs from the sea. Since then, over 100 halogenated tyrosine alkaloids have been described from *Aplysina* species worldwide (Lira et al., 2011), with considerable speculation about their importance in ecological interactions and use in human applications (Niemann et al., 2015).

Comparative studies of the inter- and intraspecific variations in secondary metabolites of sponges are uncommon, yet they can provide important information about taxonomic relationships (Erpenbeck and van Soest, 2007) and may yield important clues about the importance of microbial symbionts in metabolite production (Ozturk et al., 2013). In this study, we compared the brominated alkaloid profiles of organic extracts of 253 individual sponges of the genus *Aplysina* (six species and ten morphotypes), a taxon that has been difficult to characterize on the basis of morphology because of the absence of a mineralized skeleton (Zea et al., 2014). Our comparisons were designed to investigate the pattern of compound variation within and between species, between depth zones, and between geographic regions.

Materials and methods

Table 1 provides the relevant information regarding the sites of sponge collection. Samples were collected in the Bahamas Islands during the following months: September 1998, July–August 1999, July–August 2000 and March 2001. Samples were collected in the Florida Keys, USA, in May 2000. Categorization of sponge morphotypes, and subsequent discriminant analysis, was based on the most up-to-date taxonomic determination found in Zea et al. (2014), which includes photographs of the variation in morphologies seen for morphotypes based on location.

Table 1
Sites from which sponges were collected for this study. Sites were subdivided into three regions, Northern Bahamas, Southern Bahamas and Florida Keys.

Site	Region	GPS coordinates
Acklins Island	S Bahamas	22.186474, −74.300231
Andros Island	N Bahamas	24.618540, −77.683952
Bimini Island	N Bahamas	25.667746, −79.319229
Black Rock	S Bahamas	22.662327, −74.023134
Cat Island	S Bahamas	24.123313, −75.513862
Cay Lobos	S Bahamas	22.381946, −77.591482
Cay Santo Domingo	S Bahamas	21.719539, −75.762152
Chub Cay	N Bahamas	25.393290, −77.880825
Conch Reef	Florida Keys	24.946651, −80.456086
Egg Island	N Bahamas	25.498649, −76.898396
Eleuthera Point	S Bahamas	24.606181, −76.154849
Little San Salvador-Pinnacles	S Bahamas	24.588934, −75.974433
Long Cay	S Bahamas	22.562957, −74.393792
North Key Largo Dry Rocks	Florida Keys	25.130833, −80.292017
North North Key Largo Dry Rocks	Florida Keys	25.136778, −80.288621
Pickles Reef	Florida Keys	24.984737, −80.413676
Porpoise Rocks	N Bahamas	25.140337, −77.135227
San Salvador	S Bahamas	24.062287, −74.545403
Stirrup Cay	N Bahamas	25.822565, −77.932398
Sweetings Cay	N Bahamas	26.556550, −77.880492
Three Sisters Reef	Florida Keys	25.021933, −80.396383

Whenever possible, whole sponges were gently removed from the bottom using latex gloves. Larger or thicker sponge individuals were sliced at their base with a sharp knife. Sponge samples were individually placed in sealable plastic bags underwater, keeping track of the site, depth and location of collection. Fresh samples were placed directly into a −20 °C freezer and kept frozen until extraction.

For extraction, frozen sponges were cut in 0.5 inch cubes and placed in a graduated cylinder with the extraction solvent mixture to determine the sponge volume. In the case of large specimens, either half or a longitudinal slice of the sponge tube was cut and extracted. Rope-shaped species were sliced. Sponges were exhaustively extracted with a 1:1 DCM:MeCN mixture. This solvent mixture was selected based on its extraction efficiency and the fact that acetonitrile does not favor the formation of artifacts as methanol does. The volume of solvent was always proportional to the volume of sponge extracted, roughly 10 ml of solvent per 1 ml of sponge. Sponges were allowed to extract for several hours in the freezer. Each batch of solvent was decanted off and filtered through diatomaceous earth (Celite). Solvents were removed by rotary evaporation and extracts reduced to an aqueous layer. Initially, the water layer was additionally extracted with butanol to prevent loss of very polar metabolites. The aqueous layer was exhaustively extracted with EtOAc (ethyl acetate). Analysis using thin layer chromatography (TLC) and Diode Array HPLC confirmed that the butanol layer did not contain significant concentrations of secondary metabolites. All the major and minor metabolites were retained in the EtOAc extract.

Individual compounds used as standards for quantification were purified by a combination of standard chromatographic techniques. Crude extracts were subjected to flash column chromatography using reverse phase C-18 Silica as stationary phase and eluted with a gradient of decreasing polarity of water/MeCN to 100% MeCN with final elution with methanol (MeOH) and DCM. Most of the brominated metabolites eluted in one or two fractions, requiring further separation by reversed phase (C-18) HPLC using as mobile phases mixtures of water and MeCN. Pure compounds were stored

under nitrogen in the freezer. Pure compounds were analyzed by LC–MS and ^{1}H and ^{13}C NMR spectroscopy in order to confirm their structures comparing with data reported in the literature.

For each sample, the metabolite composition in the crude extract was analyzed by using Diode Array LC–MS (HP1090 and HP1100). In preparation for LC–MS, crude extracts were passed through a reverse phase C-18 cartridge and eluted with 1:1 MeCN/water in order to remove fats, sterols and pigments in samples. The remaining compounds were resuspended in MS grade MeOH for analysis. Separation was achieved using a Dynamax reverse phase C-18 analytical column (60 Å, 4.6 mm ID × 250 mm L) with a water-MeCN (10–100%) gradient acidified with 0.1% acetic acid at a rate of 0.7 ml/min for 28 min. Compounds were detected at 254 nm and identified by comparing their retention times and UV and MS spectra with those of authentic standards.

The bromine in these compounds gives a very distinctive fragmentation pattern, due to differences in the isotopes giving specific masses of fragment ions and characteristic mass differences between the molecular ion and fragment ions. When bromine is present in the molecule, there are n-1 fragment ions depending on how many bromine atoms are in the molecule. Fragment ions are separated by 2 mass units and the major one (>98%) corresponds to the molecular ion (Pretsch et al., 1989). Quantification of individual compounds in the crude extracts was accomplished by the external standard method, with standard calibration curves for each compound. Once the total amount of a compound in the sample was determined, the total amount (mass) of each compound in the crude extract was estimated and divided by the total volume of sponge extracted to obtain the amount of compound in milligrams per milliliter of sponge tissue extracted (mg/ml).

Differences among *Aplysina* morphotypes, locations, and depths were determined using linear discriminant functions. The sets of potential discriminating variables included concentrations of individual metabolites and the proportion of a given metabolite relative to the total metabolite concentration. Dimension reduction techniques on predictor sets were performed via principal components reductions on each set which employed rotations of the Crawford-Ferguson type (Crawford and Ferguson, 1970). Each set of potential discriminating variables was introduced in a forward selection manner and error rates were compared to determine the quality of the classifier. Error rates were assessed based on the leave-one-out, or jackknife, cross-validation technique (Seber, 2004). All analyses were conducted with SAS/STAT and SAS/GRAPH software (SAS, 2002). Codes used for the morphotypes of *Aplysina* were as follows: ARC = *Aplysina archeri*, BAT = *Aplysina bathyphila*, CAK = *A. cauliformis* – brown erect, CAN = *A. cauliformis* – lilac creeping, FIA = *A. fistularis* – aggregata, FIN = *A. fistularis* – typical tube form, FUL = *Aplysina fulva*, INS = *A. insularis*, LAH = *Aplysina lacunosa* – hard tissue, LAS = *A. lacunosa* – soft tissue.

Results and discussion

Patterns of metabolite variability among ten morphotypes of Aplysina *spp.*

Analyses, using LC–MS of the crude organic extracts from tissue samples of 253 sponges from ten morphotypes among the species *A. archeri*, *A. bathyphila*, *A. cauliformis*, *A. fistularis*, *A. fulva*, *A. insularis*, and *A. lacunosa*, provided some interesting general patterns of the distribution of the twelve most abundant metabolites (Fig. 1). Total concentration of brominated alkaloids was distinctly highest in *A. archeri* (11.5 ± 1.3 mg ml^{-1}) and lowest in the soft morph of *A. lacunosa* (1.0 ± 0.1 mg ml^{-1}), with the remaining morphotypes ranging 2.4–4.3 mg ml^{-1} (Fig. 2). The relative percentage of total brominated alkaloid metabolites that consisted of the low molecular weight (LMW) compounds (**1**–**3**, Fig. 1) was highly variable across morphotypes, with a high of 44.6% for *A. cauliformis* (brown, erect) and a low of 5.7% for *A. archeri*, with remaining morphotypes ranging from 8.7 to 26.6% (Fig. 2). Among the ten morphotypes, tissue extracts of some yielded no measurable amount of some of the twelve metabolites; in particular, compounds **3**–**8**, and **10**–**12** were consistently absent from some morphotypes, while compound **9** was consistently present (Dataset available at http://people.uncw.edu/pawlikj/2015PuyanaData.xlsx). While tissue extracts of most morphotypes yielded mixtures of some subset of the twelve metabolites, those of *A. cauliformis* (lilac, creeping) consisted primarily of fistularin-3 (**9**).

Discriminant analyses and taxonomic boundaries

A combination of methods was employed to determine the simplest model that could distinguish to the greatest degree among the ten morphotypes of *Aplysina*. Both raw values and proportions of metabolites were considered as potential predictors, in addition to a variety of rotations of principal components reductions of each. Each potential set of predictors was used in construction of linear discriminant functions in a forward selection manner to determine the smallest set of predictors that classified morphotypes with a suitably small error rate (initial targets of 75% correct cross-validated results within each morphotype along with 75% overall). Similar analyses were conducted for depth and location in place of morphotype. Initial results from efforts to classify samples within four morphotypes consisting of *A. fistularis* (FIN), *A. fulva* (FUL), *A. insularis* (INS), and *A. fistularis* forma *aggregata* (FIA) resulted in frequent misclassifications; however, nearly all such errors remained within this set of four morphotypes. Therefore, these were combined into a single category for subsequent analyses (FIS). A similar phenomenon occurred with the hard and soft morphotypes of *A. lacunosa* (LAH and LAS), and these were combined for further analyses as well (LAC).

Discriminant analyses of the six remaining taxonomic categories revealed that the proportions of three compounds, fistularin-3 (**9**), dideoxyfistularin-3 (**12**) and hydroxyaerothionin (**5**), provided the most predictive influence in decreasing order (Table 2, Fig. 3). While FIS has a 71% within-class success rate, this is partly driven by the limited sample size for BAT; if the prior information for the discriminant functions is adjusted to reflect relative sample sizes, the within-class success rate rises to 83% for FIS and 87% for CAK. One primary mode of separation that manifests itself is the relative concentration of dideoxyfistularin-3 (**12**), because three taxonomic groups (*A. archeri*, *A. cauliformis* (lilac, creeping) and *A. lacunosa*) lack this metabolite (Fig. 4). These three taxonomic groups were easily separable on the concentrations of fistularin-3 (**9**) and hydroxyaerothionin (**5**), with the concentration of the former exceeding 50% in nearly all cases for *A. cauliformis* (lilac, creeping) while ranging 10–40% for *A. lacunosa* and below 10% for *A. archeri* (Fig. 4).

Discriminant analyses revealed surprisingly little differentiation within morphotypes as a function of location or depth. One notable exception was for *A. cauliformis* (brown, erect), for which there were greater concentrations of fistularin-3 (**9**) for samples from Florida than for N or S Bahamas combined (Fig. 5). Separating this morphotype into Florida and Bahamas subsets provided clearer separation of them from both *A. fistularis* and *A. bathyphila* (Fig. 5). Aside from this exception, neither location nor depth generated distinctions that were as clear as those that were apparent across morphtypes. For example, when comparing the three depth ranges within morphotypes, error rates below 30% could not be achieved, even when four metabolites were used.

From the standpoint of chemotaxonomic differentiation (Erpenbeck and van Soest, 2007), discriminant analysis of this

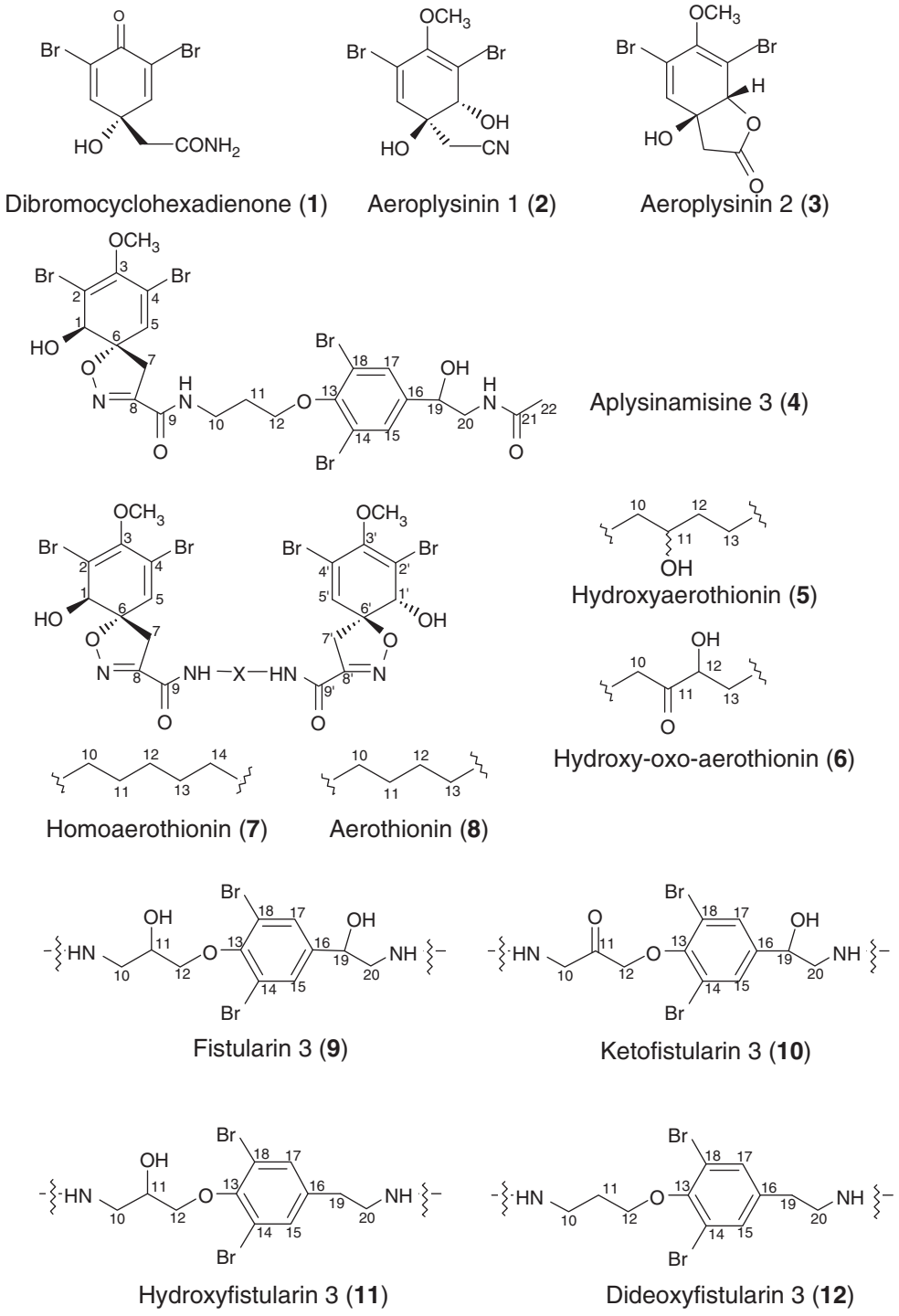

Fig. 1. Structures of the tyrosine-derived brominated alkaloids from Caribbean sponges of the genus *Aplysina*.

metabolite dataset provides some intriguing results. The cross-classification of the five morphotypes in the *A. fistularis* complex supports the general opinion among taxonomists that these morphotypes are very closely related, to the point that two or more growth forms can be seen on the same individual sponge (Zea et al., 2014). Contrarily, there was a clear difference in metabolite pattern between the two morphotypes of *A. cauliformis*, which argues for taxonomic distinction. An even more interesting case is that of *A. lacunosa*, for which taxonomic separation of the hard and soft morphotypes has been proposed. Zea et al. (2014) considered the soft morph to be a different genus (*Suberea*), yet the metabolite profiles of these two morphotypes were not readily separable in the present study. It should be noted, however, that total metabolite concentrations in the tissues of the soft morph were less than half those of the hard morph (Fig. 2), which may be due to the difference in the density (hence, water content) of the tissues of these two morphs.

Biotransformation hypothesis

The pattern of brominated alkaloids across morphotypes of *Aplysina* from Caribbean coral reefs has a bearing on the hypothesis that these metabolites are part of an activated chemical

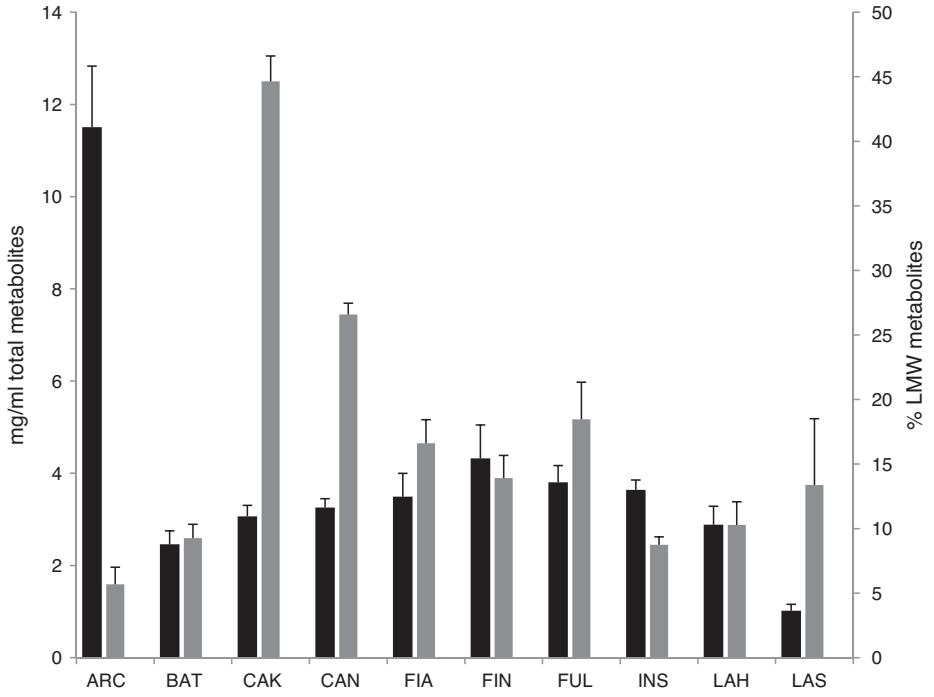

Fig. 2. Total metabolite concentration (black bars) and percentage of low molecular weight compounds (**1**–**3**; gray bars) in crude organic extracts of all samples for each of the 10 morphotypes. ARC = *A. archeri*, BAT = *A. bathyphila*, CAK = *A. cauliformis* – brown erect, CAN = *A. cauliformis* – lilac creeping, FIA = *A. fistularis* – aggregata, FIN = *A. fistularis* – typical tube form, FUL = *A. fulva*, INS = *A. insularis*, LAH = *A. lacunosa* – hard tissue, LAS = *A. lacunosa* – soft tissue.

defense system for this genus of sponges. Originally proposed for the Mediterranean species *Aplysina aerophoba* on the basis of experiments done with freeze-dried sponge samples and cell-free extracts, the biotransformation hypothesis posits that damage to sponge tissues results in the rapid, enzyme-catalyzed transformation of large molecular weight metabolites (*e.g.*, **4**–**12**) to smaller molecules, primarily dibromocycloheadienone (**1**) and aeroplysinin 1 (**2**), which were suggested to have greater defensive capacities against pathogens or predators (Teeyapant and Proksch, 1993). This optimized defense concept appears to have been modeled on established activated defense systems known for cyanogenic and cruciferous terrestrial plants, and several subsequent studies by the same research group have supported the hypothesis (Niemann et al., 2015). However, the hypothesis was rigorously tested in the field (Bahamas) on living specimens of *A. archeri* and *A. insularis*, and no evidence of conversion of large to small molecules was observed (Puyana et al., 2003).

The present study similarly does not support the biotransformation hypothesis for Caribbean species of *Aplysina*. Tissue samples across all ten morphotypes were prepared using the same methods, yet the percentage of low molecular weight molecules present in extracts of these samples varied significantly, but was

Table 2
Cross-validation summary of linear discriminant analysis of the concentration of the 3 most predictive metabolites, fistularin-3 (**9**), dideoxyfistularin-3 (**12**) and hydroxyaerothionin (**5**) in the most cohesive morphotype categories of *Aplysina*: ARC = *A. archeri*, BAT = *A. bathyphila*, CAK = *A. cauliformis* (brown erect), CAN = *A. cauliformis* (lilac creeping), FIS = *A. fistularis* (includes *aggregata, fulva, insularis*), LAC = *A. lacunosa* (includes hard and soft morph). In this analysis, 100% of samples of ARC, BAT and CAN are classified correctly, while only 71% of FIS samples are classified correctly.

	Observations/percent classified into category						
Actual category	ARC	BAT	CAK	CAN	FIS	LAC	Total
ARC	24	0	0	0	0	0	24
	100.00	0.00	0.00	0.00	0.00	0.00	100.00
BAT	0	6	0	0	0	0	6
	0.00	100.00	0.00	0.00	0.00	0.00	100.00
CAK	0	3	38	0	6	0	47
	0.00	6.38	80.85	0.00	12.77	0.00	100.00
CAN	0	0	0	57	0	0	57
	0.00	0.00	0.00	100.00	0.00	0.00	100.00
FIS	1	7	5	0	66	14	93
	1.08	7.53	5.38	0.00	70.97	15.05	100.00
LAC	3	0	2	0	0	21	26
	11.54	0.00	7.69	0.00	0.00	80.77	100.00
Total	28	16	45	57	72	35	253
	11.07	6.32	17.79	22.53	28.46	13.83	100.00

	Error rates						
	ARC	BAT	CAK	CAN	FIS	LAC	Total
Rate	0.0000	0.0000	0.1915	0.0000	0.2903	0.1923	0.1124

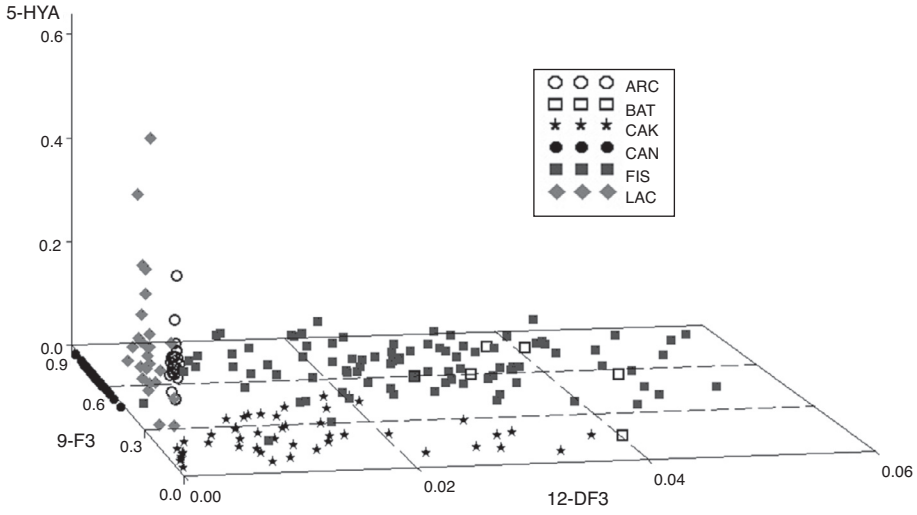

Fig. 3. Three-dimensional graph of proportional metabolite concentration of fistularin-3 (**9**), dideoxyfistularin-3 (**12**) and hydroxyaerothionin (**5**) for the six remaining morphotypes ARC = *A. archeri*, BAT = *A. bathyphila*, CAK = *A. cauliformis* – brown erect, CAN = *A. cauliformis* – lilac creeping, FIS = *A. fistularis* (combined FIA, FIN, FUL, INS), LAC = *A. lacunosa* (combined LAH, LAS). See legend of Fig. 2 for other morphotype codes.

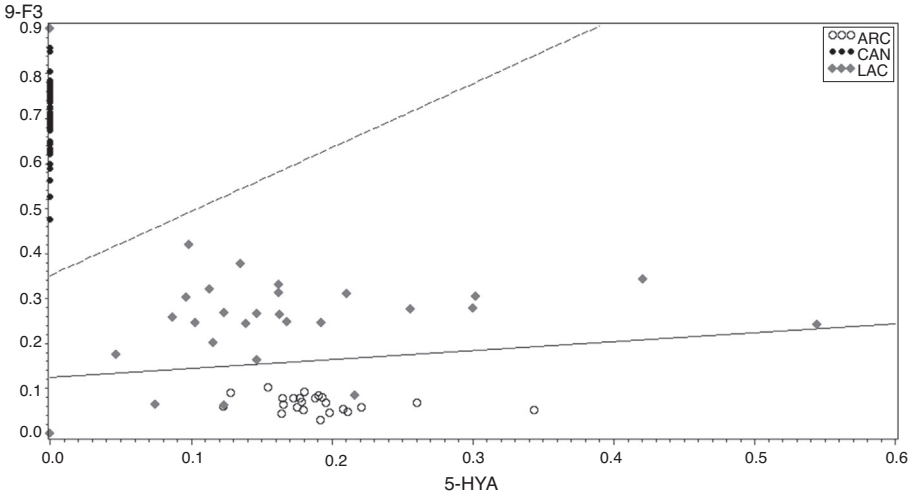

Fig. 4. Two-dimensional graph of proportional metabolite concentration of fistularin-3 (**9**) and hydroxyaerothionin (**5**) for ARC, CAN and LAC with boundaries indicated for dideoxyfistularin-3 (**12**) set to zero. ARC/LAC boundary, solid line; LAC/CAN boundary, dashed line. See legend of Fig. 3 for morphotype codes.

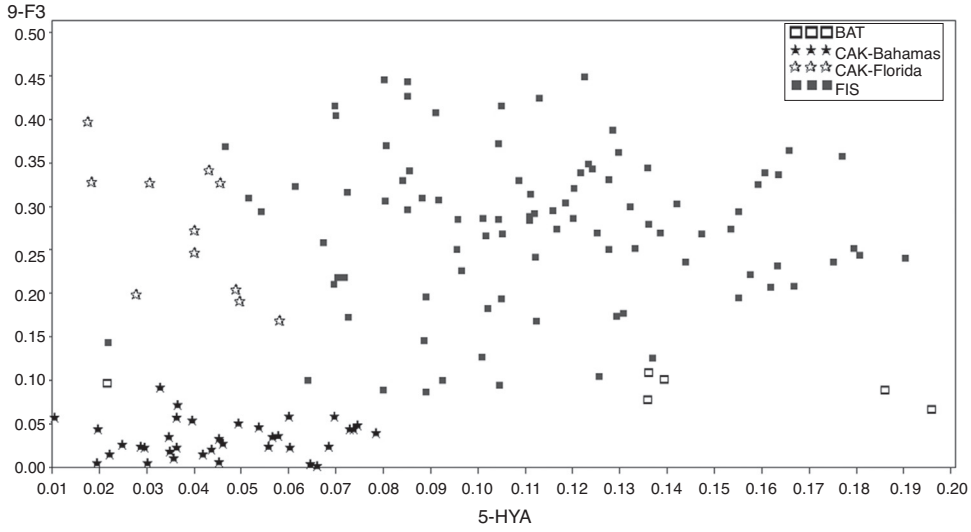

Fig. 5. Two-dimensional graph of proportional metabolite concentration of fistularin-3 (**9**) and hydroxyaerothionin (**5**) for BAT, CAK and FIS with data for CAK separated into two sets by location; samples from Florida and samples from the Bahamas (N and S combined). See legend of Fig. 3 for morphotype codes.

highly consistent by morphotype (Fig. 2). If the proposed activated defense system was common to species across the genus, one would expect very low levels of low molecular weight metabolites in rapidly prepared extracts of sponge tissue regardless of morphotype, but this was not the case, with nearly 50% of metabolites in extracts of *A. cauliformis* (brown, erect) present in the low molecular weight category (Fig. 2). It seems unlikely that the complicated defense mechanism proposed for *A. aerophoba* (Niemann et al., 2015) would have evolved independently for that species and not for several others within the larger genus.

Microbial contribution to secondary metabolite production

Like all verongid sponges, species in the genus *Aplysina* have tissues that are perfused with symbiotic microorganisms, placing them in the high microbial abundance (HMA) sponge category (Gloeckner et al., 2014). Whether or not the sponge microbiome is responsible for the production or alteration of secondary metabolites found in the sponge tissue has been an issue of great interest to natural products chemists and sponge biologists alike (Unson et al., 1994). Metabolite synthesis and alteration has been a particular subject of study for the Mediterranean species *A. aerophoba*, with X-ray microanalysis evidence suggesting that the sponge is mostly or entirely responsible for metabolite production (Turon et al., 2000). Subsequent studies of *A. aerophoba* have linked individual brominated alkaloids with specific microbial phylotypes (Sacristán-Soriano et al., 2011), but the issue remains unresolved for this species (Sacristán-Soriano et al., 2015).

The cross-species survey of metabolites from the tissues of Caribbean *Aplysina* species provided in this study tends to support sponge-derived, rather than microbiome-derived brominated alkaloids. If the microbiome was substantially involved with either the production or alternation of brominated alkaloids, the predicted outcome of this survey would have been considerably more metabolite variation within morphotypes, particularly as a function of location and depth, yet this level of variation was not observed. Depth is a particularly important factor, because *Aplysina* species are known to contain cyanobacteria that are variably important in providing sponge nutrition (Erwin and Thacker, 2008), and it would be expected that the influence of photosymbionts would change with light intensity as a function of depth, but this was not the case. Instead, metabolite profiles were highly correlated to morphotype, which suggests that the sponge cells are primarily responsible for the production of the chemical defenses present in sponge tissue. Nevertheless, it remains to be determined whether some metabolite changes are subject to the activities of highly conserved components of the microbiome (Sacristán-Soriano et al., 2015).

Authors' contributions

MP, JRP and WF designed the study. MP performed the field collections and laboratory analyses. JEB devised and performed the statistical analyses. JRP wrote the first draft, and all authors contributed to the critical reading and editing of the manuscript. All authors read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

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