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Secondary Metabolome Variability and Inducible Chemical Defenses in the Mediterranean Sponge *Aplysina cavernicola*

M. Reverter[1] · **T. Perez**[2] · **A. V. Ereskovsky**[2,3] · **B. Banaigs**[1]

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Abstract Secondary metabolites play a crucial role in marine invertebrate chemical ecology. Thus, it is of great importance to understand factors regulating their production and sources of variability. This work aimed to study the variability of the bromotyrosine derivatives in the Mediterranean sponge *Aplysina cavernicola*, and also to better understand how biotic (reproductive state) and abiotic factors (seawater temperature) could partly explain this variability. Results showed that the *A. cavernicola* reproductive cycle has little effect on the variability of the sponges' secondary metabolism, whereas water temperature has a significant influence on the production level of secondary metabolites. Temporal variability analysis of the sponge methanolic extracts showed that bioactivity variability was related to the presence of the minor secondary metabolite dienone, which accounted for 50 % of the bioactivity observed. Further bioassays coupled to HPLC extract fractionation confirmed that dienone was the only compound from *Aplysina* alkaloids to display a strong bioactivity. Both dienone production and bioactivity showed a notable increase in October 2008, after a late-summer warming episode, indicating that *A. cavernicola* might be able to induce chemical changes to cope with environmental stressors.

Keywords *Aplysina* sponges · Secondary metabolites · Bioactivity · Temporal variation · Intraspecific variations · Reproductive cycle · Dynamic chemical defense · Dienone

Introduction

Among the widespread natural chemical compounds, secondary metabolites play important ecological roles either within organisms or mediating the interaction between them and their environment (Becerro et al. 1997; De Caralt et al. 2013; Engel and Pawlik 2000; Hay 1996; Paul et al. 2007). Several studies indicate that secondary metabolites in marine ecosystems can act as chemical defenses against predators (e.g., Hay and Fenical 1988; Kelman et al. 2000a; Paul and Van Alstyne 1992; Pawlik et al. 1995), pathogens (e.g., Kelman et al. 1998), epibionts (e.g., Plouguerne et al. 2010; Tan et al. 2010), and in space competition (e.g., Porter and Targett 1988).

Production of secondary metabolites is frequently subjected to temporal and spatial variations (Kelman et al. 2000b; Maida et al. 1993; Page et al. 2005), which can be influenced by several biotic (e.g., life cycle, symbiont occurrence, pressure of predation) (e.g., Abdo et al. 2007) and abiotic factors (e.g., temperature regime, environment quality) (e.g., Puglisi et al. 2000; Uriz et al. 1996). Production of these metabolites imposes a considerable cost to the producing organism. Resource allocation to these so-called "secondary" functions is thus optimized with respect to primary biological functions such as growth or reproduction, as it is defined by the optimal defense theory (ODT) (Cronin 2001; López-Legentil et al.

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✉ B. Banaigs
banaigs@univ-perp.fr

[1] CRIOBE, USR 3278 - CNRS/EPHE/UPVD, Université de Perpignan Via Domitia, 52 Avenue Paul Alduy, 66860 Perpignan, France

[2] Institut Méditerranéen de Biodiversité et d'Ecologie marine et continentale (IMBE), CNRS, IRD, Aix Marseille Université, Université Avignon, Station Marine d'Endoume, Rue de la Batterie des Lions, Marseille, France

[3] Biological Faculty, Saint-Petersbourg State University, Universitetskaya nab. 7/9, St. Petersbourg 199034, Russia

2006; Rhoades 1979; Skogsmyr and Fagerström 1992). Abiotic factors can also affect secondary metabolite production either through a direct effect on the organisms' metabolism or indirect effects, such as an increase of epibionts pressure associated with warmer temperatures, or increase of pathogens related to environmental degradation (Sacristan-Soriano et al. 2011, 2012; Turon 2009). Nevertheless, a great part of the intraspecific variability in the production of marine secondary metabolites remains unexplained (Becerro et al. 1998; Matlock et al. 1999; Wright et al. 2000).

Most studies reporting seasonal patterns of secondary metabolism in marine invertebrates have used crude extract bioactivity as an indicator (or proxy) of metabolite biosynthesis (Ferretti et al. 2009; Ivanisevic et al. 2011b; Turon 2009), whereas variability of the level of expression of targeted secondary metabolites has been rarely studied. Firstly, a study on the ascidian *Cystodytes* sp. showed that ascididemnin concentrations displayed cyclic behavior related to the reproductive cycle of the organism (López-Legentil et al. 2007). Ivanisevic et al. (2011c) also reported a seasonal variability for two lysophospholipids produced by the homoscleromorph sponge *Oscarella tuberculata*. More recently, a targeted metabolomic study has revealed deep chemical divergence between the two main morphotypes of the zoanthid *Parazoanthus axinellae*, with one of the morphotypes never producing any parazoanthines, which has supported the hypothesis of a species-complex that might also differ by some ecological traits (Cachet et al. 2015).

Sponges have long received considerable attention from a chemical ecology perspective, and numerous studies have been carried out on their chemical defenses (Abbas et al. 2011; Braekman and Daloze 2004; Thompson 1984). However, only a few studies have documented sponge intraspecific variability in secondary metabolites, and so, there still is poor knowledge of the parameters that affect their production. Leong and Pawlik (2010) and Turon (2009) indicated the existence of trade-offs between sponge secondary metabolite production and growth; while the results of Ivanisevic et al. (2011a) showed that sexual reproduction, gametogenesis, and embryogenesis, were the main factors shaping the secondary metabolism variability in *Oscarella balibaloi*, whereas, biotic (species interaction) and abiotic (temperature) factors had a less pronounced influence.

Sponges of the genus *Aplysina* are widely distributed in subtropical and tropical coastal waters, where they often contribute to the dominant part of the sponge assemblage (Bergquist and Cook 2002). They are considered good models in chemical ecology because they produce a multifaceted class of bromotyrosine-derived metabolites, with multiple bioactivities such as antibiotic, cytotoxic, and fish deterrency) (Azevedo et al. 2008; Betancourt-Lozano et al. 1998; Thoms et al. 2004). These brominated alkaloids, which can reach up to 13 % of the sponge dry weight, have been well described and documented (Carney and Rinehart 1995; Cimincello et al. 1994, 1996a, 1996b, 1999; Lira et al. 2011; Weiss et al. 1996). *Aplysina* alkaloids can be classified into two subfamilies, the bromoisoxazoline-tyrosine derivatives (such as aerophobin-2, aplysinamisin-1 and isofistularin-3) and the lower-molecular-weight bromotyrosine derivatives (such as aeroplysinin-1 and dienone).

Studies have assessed the individual chemical variability of *Aplysina* species in order to better understand the mechanisms that control the chemical production and variability in these sponges. Sacristan-Soriano et al. (2011, 2012) showed that *Aplysina aerophoba* production of brominated alkaloids displayed significant spatial and temporal variation, finding the secondary metabolite concentration in the ectosome positively correlated to water temperature. Another study showed quantitative and qualitative differences in the chemical profiles of healthy and diseased *Aplysina cauliformis* individuals, but in both cases, stress caused by *Aplysina* Red Band Syndrome did not compromise the sponge's ability to chemically protect against putative pathogens and predators (Gochfeld et al. 2012). These studies have given the first insights explaining some of the variability in *Aplysina* secondary metabolites, although most of the parameters regulating this variability remain unknown.

In the present study, the temporal variability of secondary metabolism in *Aplysina cavernicola* was studied through two different approaches: the study of the expression level of the secondary metabolites by using an HPLC-PDA-ELSD system, and an assessment of organic extracts bioactivity (Microtox® bioassay). Complementary antibacterial tests coupled to HPLC fractionation of organic extracts were performed to evaluate different metabolites activities and to relate bioactivity variability with individual *Aplysina* alkaloid expression. Seawater temperature (abiotic factor) and reproductive cycle (biotic factor) were also studied over the same period for their putative contribution to explain secondary metabolism variability.

Methods and Materials

Aplysina cavernicola (Vacelet 1959) is a sciaphilous species found in coralligenous formations or at the entrance of submarine caves generally between 8 to 60 m in the Mediterranean Sea. It presents a typical yellowish color and a similar morphology to the "sister species" *Aplysina aerophoba*, which is found at shallower and well-lit sites.

Sampling All *A. cavernicola* samples required for analyses were collected by scuba diving from September 2007 to January 2009 and at a single site of Maire Island, Marseille (43.2096° N; 5.3353° E), between 14 and 16 m depth in order to avoid spatial variability. At the site, the seawater

temperature was recorded throughout the year at high frequency (one recording every 2 h) with permanent data loggers (Onset Tidbit; N. Bensoussan et al., 2010). Daily mean temperatures were used to study temperature fluctuations during the studied period, whereas mean temperatures from 6 days preceding the sampling were used to correlate the temperature regime with sponge production of secondary metabolites and bioactivity.

Sponges were sampled once a month, and six different individuals were collected randomly. A small piece of each individual (about 1 mm^3) was cut off and fixed for histological analysis; the remaining part was immediately frozen at −20 °C, then freeze-dried and conserved at −20 °C until extraction for chromatographic analysis and bioactivity assays.

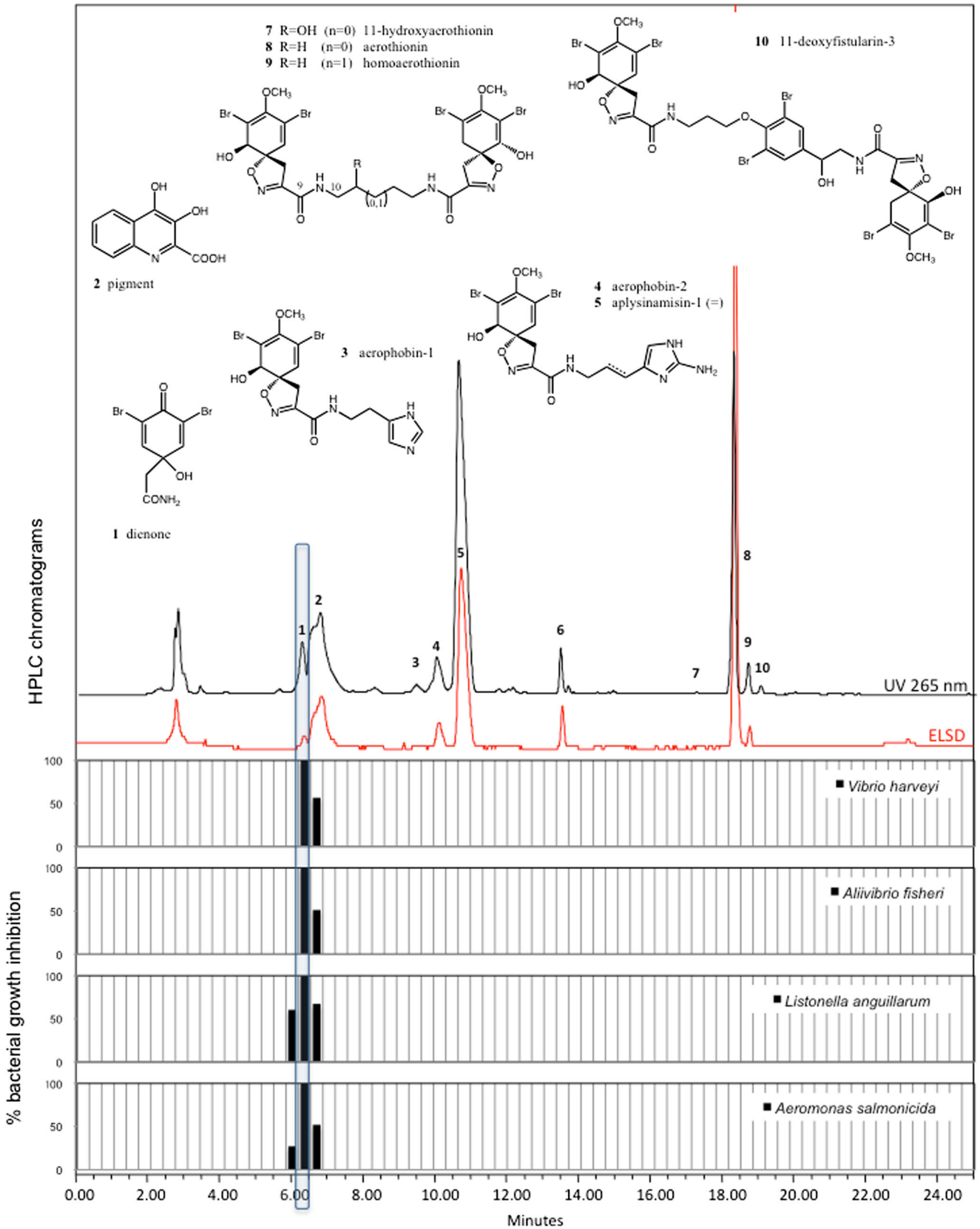
Secondary Metabolites Extraction The freeze-dried samples were ground with a mortar to obtain homogeneous powder. Fifty mg of this powder were weighed and extracted × 3 in 1.5 ml of methanol (MeOH) for 15 min in an ultrasonic bath, and filtered through a 20 μm polytetrafluoroethylene (PTFE) filter. Then, the volume (3 × 1.5 ml) of the methanolic extract was adjusted to 5 ml with MeOH in a volumetric flask. An aliquot of 1 ml was passed through a 0.20 μm PTFE syringe filter prior to high performance liquid chromatography (HPLC). The remaining 4 ml were dried by vacuum rotatory evaporation for bioactivity assays.

HPLC Analysis and Quantification HPLC analyses were performed with a system from Waters including the Alliance separation module 2695, the column heater, the 2998 photodiode array detector, and the 2424 ELS detector (Waters Corp. MA, USA). The equipment was controlled, and the data were handled by the Empower Chromatography Data Software. HPLC elution conditions consisted of two eluants, A (water with 1‰ formic acid) and B (acetonitrile with 1‰ formic acid), an elution profile based on a linear gradient from 15 % to 100 % B in 16 min, a flow rate of 0.5 $ml.min^{-1}$, and an injection volume of 10 μl. Column used was a Synergi Fusion RP (100 × 3 mm) (Phenomenex, CA, USA) with a fixed temperature of 30 °C. HPLC chromatograms were obtained at 245 nm from the data collected across the 210–800 nm wavelength range and compared to that obtained from the ELS detector. Ten compounds were detected, and nine were identified using their UV spectra and complementary mass spectrometry analysis carried out on a LC/DAD/ESI/MS system from Thermo Scientific (MA, USA) equipped with an Accela PDA detector and a LCQ Fleet 2300 mass spectrometer using the same column and the same elution conditions as above. Peak 2 was not well resolved (broad, tailing, and split). As MS and UV spectra taken across the peak do not differ, it clearly indicates that there is one solute present in peak 2. Except for peak 6, every compound has been purified previously and characterized in the laboratory by classic spectrometric techniques (LC/MS, H-NMR C-NMR, and UV profiles) according to published data (Cimincello et al., 1997; Thoms et al., 2004, 2006). The quantification was performed after the calibration procedure described by Sacristan-Soriano et al. (2011). Briefly, several dilutions of pure compounds were analyzed in HPLC and the peak areas were integrated with the Empower Chromatography Data Software to obtain calibration curves.

Precision, Reproducibility, and Stability Two types of variance exist in metabolic variation studies: 1) technical variance due to variation of sample preparation and instrumental analysis, and 2) biological variance existing within a population. To study the biological variance, it was crucial that the technical variance was lower than the biological variance. Method precision was evaluated by five consecutive injections of the same sample solution. Repeatability was assessed by analyzing 5 independently prepared extracts from a mixture of 5 samples from the temporal series of *A. cavernicola* (30 mg per sample, a sample every 3 mo pooled). Variance among the extracts used for the repeatability (technical variance) was compared to variance of the same 5 samples extracted individually (without pooling) (biological variance). Sample stability for metabolic variation studies was critically important, and storage stability of sample solution was evaluated at the time points of 1 h, 24 h, 1 wk., and 1 mo.

Methanolic Extract Bioactivity Assays The standardized Microtox® bioassay (AZUR Environmental, New Castle, DE, USA) (Martí et al. 2003) was used to assess the bioactivity of *A. cavernicola* methanolic extracts. The purpose of this test was to give a rapid assessment of the extract's bioactivity and to measure the temporal pattern of variability. Then, we intended to correlate these measurements of bioactivity with expression level of targeted compounds. The bioactivity analyses were conducted with crude organic extracts (the dried remaining 4 ml) dissolved in artificial seawater with 2 % of acetone. Stock solutions were tested at four diluted concentrations, the initial concentration being set at 2 mg/ml, and a dilution factor of 2 being applied between each following tested concentration. Bioactivity was quantified by measuring the direct effect on the respiration metabolism of the bioluminescent bacterium *Aliivibrio fischeri*, a negative effect being indicated by a decrease in light emitted and expressed as an EC_{50} value. Thus, the lowest EC_{50} value corresponds to the highest methanol extract bioactivity, and the highest EC_{50} corresponds to the lowest bioactivity.

Antibacterial Assays Coupled to HPLC Extract Fractionation Complementary antibacterial tests were done on 96-well plates previously coupled to HPLC fractionation to evaluate bioactivities of individual secondary metabolites. Antibacterial activity was evaluated on 4 g-negative marine bacteria, *Aliivibrio fisheri*, also used in the Microtox® bioassay and also


7 R=OH (n=0) 11-hydroxyaerothionin
8 R=H (n=0) aerothionin
9 R=H (n=1) homoaerothionin
10 11-deoxyfistularin-3
2 pigment
3 aerophobin-1
4 aerophobin-2
5 aplysinamisin-1 (=)
1 dienone
HPLC chromatograms
UV 265 nm
ELSD
% bacterial growth inhibition
Vibrio harveyi
Aliivibrio fisheri
Listonella anguillarum
Aeromonas salmonicida
Minutes
0.00
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◀ **Fig. 1** Representative HPLC metabolite profile of *Aplysina cavernicola* organic extract (sample from October 2007). The 9 compounds were identified by HPLC-UV and LC-MS comparison with the previously purified compounds. Antibacterial activity was evaluated directly in 96-well fraction collection plates represented below the chromatogram. Maximum bacterial growth inhibition (100 %) corresponds to the well 19th (black bars), which corresponds to dienone (**1**)

on three opportunistic pathogenic bacteria, *Vibrio harveyi*, *Vibrio anguillarum*, and *Aeromonas salmonicida*. These tests provide an estimate of the antifouling and antimicrobial potential of *Aplysina* secondary metabolites. The experiment was carried out on the Waters HPLC-PDA-ELSD system described above with the same conditions (column, elution), completed with a Fraction Collector III (WFC III) (Waters, MA, USA), equipped with a rack supporting 96 deep well microtiter plates. *Aplysina cavernicola* sample from October 2007 was chosen to be extracted (presence of all identified compounds), separated through HPLC-PDA-ELSD analysis and recovered on a 96-well plate. HPLC elution conditions consisted of two eluants, A (water with 1‰ formic acid) and B (acetonitrile with 1‰ formic acid), an elution profile based on a linear gradient from 15 % to 100 % B in 20 min, a flow rate of 1 $ml.min^{-1}$, and an injection volume of 30 μl of an extract of 30 mg/ml. Routine detection was set at 245 nm, and the column used was XTerra RP_{18} 5 μm (4,6 × 250 mm, Waters). From the column outlet, the eluate was divided, 0.3 ml/min were analyzed by PDA and ELS detectors, and 0.7 ml/min were collected continuously at uniform volume intervals (262 μl/well) in 96 deep well microplates.

Bacterial strains *V. harveyi* and *A. fischeri* were grown on Marine Agar 2216 (Difco) and Marine Broth 2216 (Difco). *Vibrio anguillarum* and *A. salmonicida* were grown on LB agar (Simca) supplemented with 15 mg/l NaCL and LB Broth (5 g/l yeast extract (Simca), 10 g/l peptone (Simca) and 20 g/l NaCl). Bacteria were grown on culture broths at 28 °C overnight before use for antibacterial tests. Five μl of DMSO (dissolve the secondary metabolites) and 195 μl of 1/25 diluted bacterial solution (1 mDO) were added to freeze-dried microplates. Six positive controls were done by microplate. Microplates were incubated at 28 °C for 24 h. Bacterial growth was estimated reading optical absorbance at 600 nm with a microplate reader Aviso Sirius HT (Ebersberg, Germany), after inoculation and 24 h later, % of bacteria growth inhibition were calculated.

Reproductive Cycle Description Histological subsamples were used to characterize the reproductive cycle and assess the reproductive effort. They were fixed in Bouin fixative. Tissue fragments were dehydrated through an ethanol series and then embedded in paraffin. Serial sections of 6 μm in thickness were mounted on glass slides, stained with Trichrome of Masson and Goldner hematoxylin, and then observed under a light microscope. Digital pictures were captured in order to quantify the reproductive elements (spermatocyste, oocyte) using the ImageJ Software (http://rsb.info.nih.gov/ij/index.html). For each reproductive sponge, the reproductive effort (RE) was expressed as a percentage of reproductive tissue, as a ratio between the surface covered by each reproductive elements and the overall surface of the sponge section.

Data Analysis Normality of data distribution (Shapiro-Wilk test) and homogeneity of variances (Levene tests) were tested and not satisfied, thus non-parametric tests were used. *Kruskal-Wallis* test and multiple comparison *Kruskal-Wallis post-hoc tests* were performed to test variability of variables (metabolites, bioactivity and reproductive effort) in time (season and month) and in relation to temperature fluctuation.

The *Spearman Rank correlation* and logarithmic correlation were used in order to test for a correlation between RE and temperature, secondary metabolites content and temperature, secondary metabolite content and bioactivity, and bioactivity and temperature. *Principal Component Analysis* (PCA) was employed to analyze the relationship between the variables, notably between bioactivity and the different secondary metabolites, in order to establish what was the contribution of each metabolite to the overall bioactivity. Correlation matrix was used to analyze correlation between secondary metabolite concentrations.

Results

Chemical Diversity and Methodology Validation Nine peaks were identified and purified, dienone (2-(3,5-dibromo-1-hydroxy-4-oxocyclohexa-2,5-dien-1-yl)acetamide) **(1)**, pigment (3,4-dihydroxyquinoline-2-carboxylic acid) **(2)**, aerophobin-1 **(3)**, aerophobin-2 **(4)**, aplysinamisin-1 **(5)**, 11-OH-aerothionin **(7)**, aerothionin **(8)**, homoaerothionin **(9)** and 11-deoxyfistularin-3 **(10)**, by comparing their retention times, mass and UV profiles to those purified and characterized previously in the laboratory (Fig 1). These compounds represent over 95 % of the detected chemical diversity (based upon integration of all detected peaks in PDA (λ = 245 nm) and ELS detector) exhibited by the *A. cavernicola* methanolic extract. *Major* compounds (pigment, aerophobin-1, aerophobin-2, aplysinamisin-1, aerothionin, and homoaerothionin) were observed in all samples, whereas dienone could not be detected in 39 % of the samples, 11-OH-aerothionin in 96 %, and 11-deoxyfistularin-3 in 97 % of the samples. In 62 % of the samples an unknown peak named Peak 6 **(6)** was observed (1.3 % ± 0.3 % of the chemical compounds observed). Peak 6 was not purified, thus we did not include it in our analyses, and it currently is being characterized in the laboratory.

Precision, repeatability, and stability were evaluated to ensure the validity of the analytical HPLC method. All validations

were expressed by calculating their relative standard deviations (RSD) of the major compounds concentrations. Concerning method precision and repeatability, the RSD (Relative Standard Deviation) values were lower than 3 % and 15 %, respectively. Chemical stability of crude extracts also was demonstrated, as no degradation could be observed in any of the metabolites tested (Table in Supplementary Material).

Additionally, comparison of the variances from the extracts prepared from the 5 different samples (a sample every 3 months) of *A. cavernicola* (biological variability) with those of the 5 independently prepared extracts from a mixture of 5 samples (technical variability) showed that biological variability was 18 times greater than technical variability, which indicates that the analytical method was adequate to study intraspecific chemical variability in *A. cavernicola*. Therefore, all RSDs values indicated that the fingerprinting analysis is valid and satisfactory (Table in Supplementary Material).

Variation of the Secondary Metabolites over Time The total amount of secondary metabolites varied significantly throughout the monitored period (Kruskal-Wallis (month), $H = 53.71$, $P < 0.001$; Kruskal-Wallis (season), $H = 30.55$, $P < 0.001$) (Fig 2). The highest metabolite production occurred during summer (Kruskal-Wallis multiple comparison test, $P < 0.001$), while the lowest was registered in the winter months. All compounds except aerophobin-2 displayed significant differences in their concentrations between summer and other seasons ($P < 0.01$). Dienone concentration did not vary between summer and fall, but there was a significantly higher production in summer and fall than in winter and spring ($P < 0.01$).

Temporal Variability of the Sponge Bioactivity The average bioactivity of *Aplysina cavernicola* during the monitored period indicated by the standardized Microtox® assay was 79.3 ± 46.0 μg/ml (EC_{50} ± SD). The highest bioactivity was observed in October 2008 (EC_{50} = 16.9 ± 11.6 μg/ml), while the lowest bioactivity was recorded in December 2007 (EC_{50} = 185.9 ± 46.0 μg/ml) (Fig 2). We observed significant bioactivity variation throughout time (month and season) (Kruskal-Wallis (month), $H = 55.41$, $P < 0.001$; Kruskal-Wallis (season), $H = 28.00$, $P < 0.001$). *Post-hoc* tests indicated that the bioactivity was significantly lower in winter than in fall and spring ($P < 0.01$).

Relationship Between Expression Levels of Secondary Metabolites and Bioactivity Assays Correlation analysis showed that bioactivity was not correlated with the total amount of metabolites. However, there was a negative correlation in the case of dienone ($P < 0.05$, $R_s = -0.48$). Logarithmic transformation showed that 50 % of bioactivity could be explained by dienone. The PCA analysis performed is in accordance with these results, highlighting dienone as the main compound responsible for bioactivity, with the pigment the second one. All the other products do not seem to be much related (Fig 3). Complementary antibacterial assays coupled to HPLC separation were pursued to evaluate individual secondary metabolites bioactivities. Under the conditions used, 100 % inhibition of bacterial growth was observed for the well corresponding to dienone in the 4 marine bacterial strains tested (*V. harveyi*, *A. fischeri*, *V. anguillarum*, and *A. salmonicida*), whereas none of the other compounds inhibited any bacterial growth. These results show the strong antibacterial power and toxicity of dienone, confirming this bromotyrosine derivative as responsible for the bioactivity observed in *A. cavernicola* extracts (Fig 1).

All secondary metabolite concentrations were positively correlated with each other, except for dienone that was negatively correlated with all other compounds (Fig 4). This negative correlation was especially important in October 2008 when all compounds decreased in production while dienone was substantially overexpressed.

Relationship Between Concentrations of Secondary Metabolites and Temperature The average seawater temperature during the monitored period was 16.4 ± 2.4 °C (T^a ± SD). The highest mean temperature was recorded in September 2008 (21.12 ± 2.4 °C). The lowest mean temperature was recorded in March 2008 (13.17 ± 0.08 °C) (Fig 5). Maximal temperatures occurred during summer 2008 (T_{max} = 23.51 °C), whereas maximal temperature in summer 2007 was 22.27 °C. The total amount of secondary metabolites was positively correlated with temperature variations, the highest production being recorded during the warmest periods (Fig 6). This trend was followed by all compounds except for the concentration of aerophobin-2, which did not significantly correlate with temperature variations. Spearman's rank correlation indicated that 11.3 % of the variance in the total amount of metabolites can be explained by temperature fluctuations. Dienone was the compound with the strongest temperature correlation ($P < 0.05$, $R_s = 0.50$).

Bioactivity and Expression Levels of Secondary Metabolites in Relation to the Sponge Reproductive Cycle *Aplysina cavernicola* is an oviparous and gonochoric species (sex ratio male/female =4/1) with a very short period of reproduction occurring between June and early July. Spermatogenesis starts in early June and lasts until early July, while oogenesis starts in mid-June until early July (Fig 5). Over the studied period, both the male and female RE reached their maximal values in early July, male's reproductive effort (33.7 %) being two times higher than female reproductive effort (16.6 %).

No correlation was found between RE and bioactivity of crude extracts. There was a moderate positive correlation ($R_s = 0.32$) between RE and the total amount of metabolites, which was due mainly to pigment, aerophobin-1, aplysinamisin-1, and aerothionin. This result is likely to be a consequence of the higher secondary metabolites production in summer months coinciding with the reproductive period.

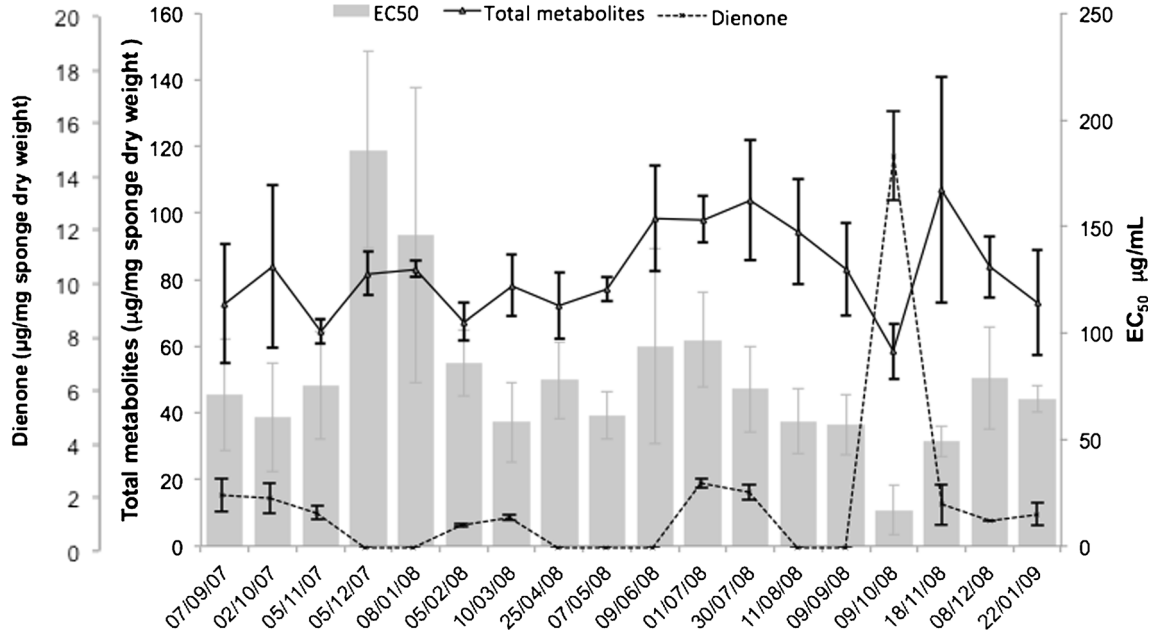

Fig. 2 Temporal variability of total metabolites amount (mean ± SD), dienone (mean ± SD), and crude extracts bioactivity (EC_{50} mean ± SD) for the temporal series of *Aplysina cavernicola*

Discussion

Factors explaining intraspecific variations of marine invertebrate secondary metabolism are poorly understood. Here, temporal variations of the secondary metabolites of *A. cavernicola* were studied through analysis of the concentrations of 9 different metabolites and organic extract bioactivities. These two indicators of *A. cavernicola*'s secondary metabolism were then related to the variations of the seawater temperature and the sponge reproductive cycle, the objective being to clarify the putative influence of these abiotic and biotic factors.

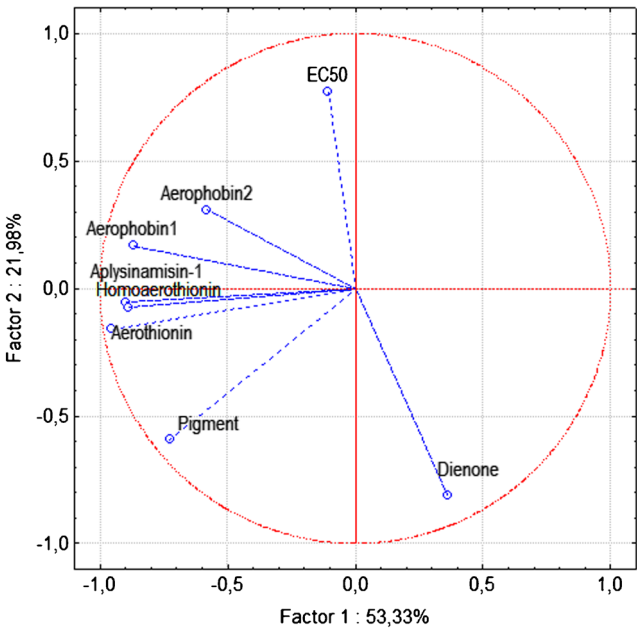

Fig. 3 PCA secondary metabolome characteristics. Circle of correlation of bioactivity (EC^{50}) and secondary metabolites from *Aplysina cavernicola*

Few previous studies have shown that the expression level of secondary metabolites may vary under the influence of environmental changes or cyclic physiological features such as reproduction. Our results show that the production of secondary metabolites in *A. cavernicola* is positively correlated with water temperature fluctuations, with the highest production in summer months (June, July, August) and lowest in winter months (December, January, February). These results are in accordance with the results obtained by Sacristán-Soriano et al. (2012) who showed that major compounds of *A. aerophoba* (isofistularin-3 and aplysinamisin-1) were more abundant during the warmest season. However, it remains unclear whether temperature plays a direct role in affecting sponge secondary metabolism or if it is rather a consequence of indirect processes associated with warmer temperatures.

Verongida life cycle and reproductive biology have been documented in some older works on *A. aerophoba* (Scalera et al. 1971; Vacelet 1959), but they have never been studied in relation to their secondary metabolism. In the Homoscleromorph sponge *Oscarella balibaloi*, Ivanisevic et al. (2011a) studied the life cycle and bioactivity, and found negative correlations, thus indicating a possible trade-off between resources allocated to secondary chemistry and towards reproduction. Our results did not show a significant correlation between bioactivity and RE. There was a moderate positive correlation between concentrations of some secondary metabolites and RE, but this most likely is a secondary result from the higher secondary metabolites production in summer months and not a direct relationship between

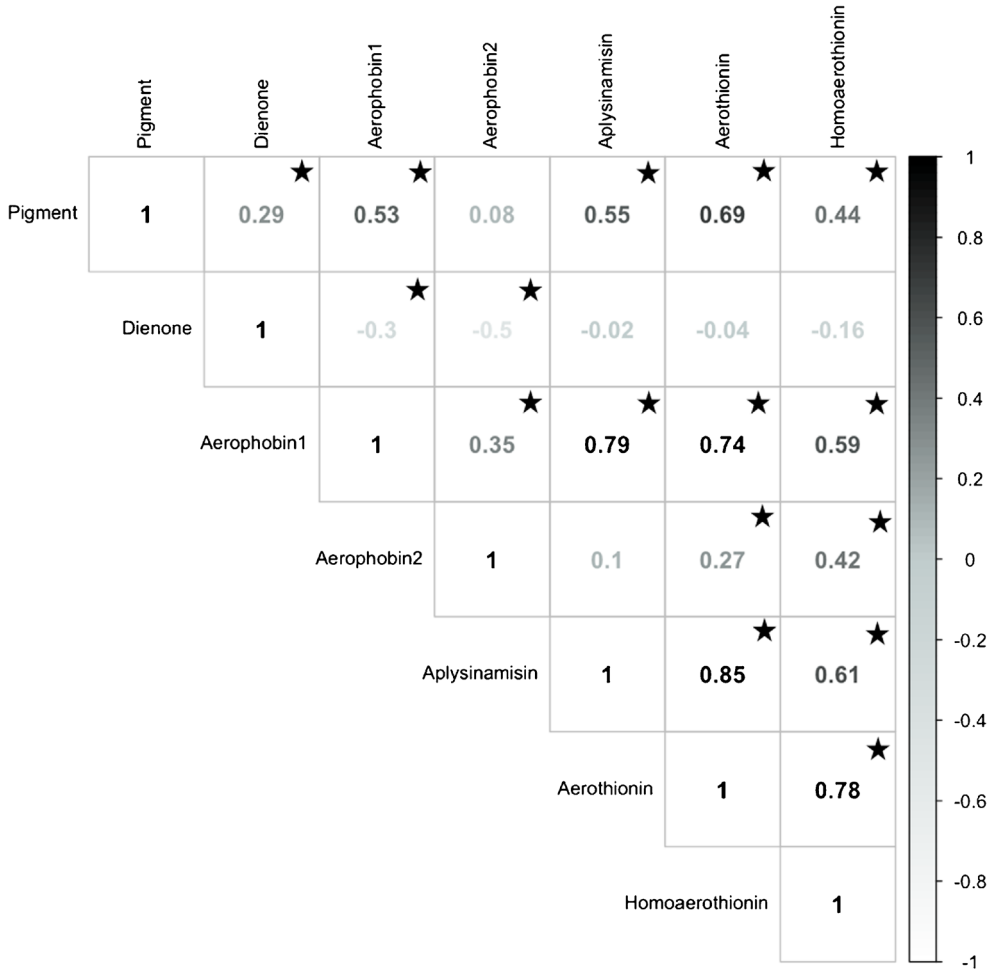

Fig. 4 Correlation graph representing the correlation between major metabolites and dienone. Stars represent significant differences (*P*-value <0.05)

secondary metabolites and RE. *Oscarella balibaloi* is ovoviviparous with a reproductive cycle that lasts almost all year long, whereas, *A. cavernicola* is an oviparous sponge whose reproduction period lasts barely one month. We hypothesize that energy investment in reproduction of oviparous sponges with short reproduction periods does not have a significant effect on the energy budget allocated to secondary metabolism. Hence, environmental parameters like temperature might have a greater influence on secondary metabolism of oviparous sponges like *A. cavernicola* than on their reproductive cycle.

Activation of chemical defenses in terrestrial and marine organisms after being injured has been documented several times (e.g., Paul and Van Alstyne 1992). However, chemical activation in *Aplysina* sponges has been a subject of controversy among several research groups (Puyana et al. 2003). Some studies have shown that after mechanical damage, some

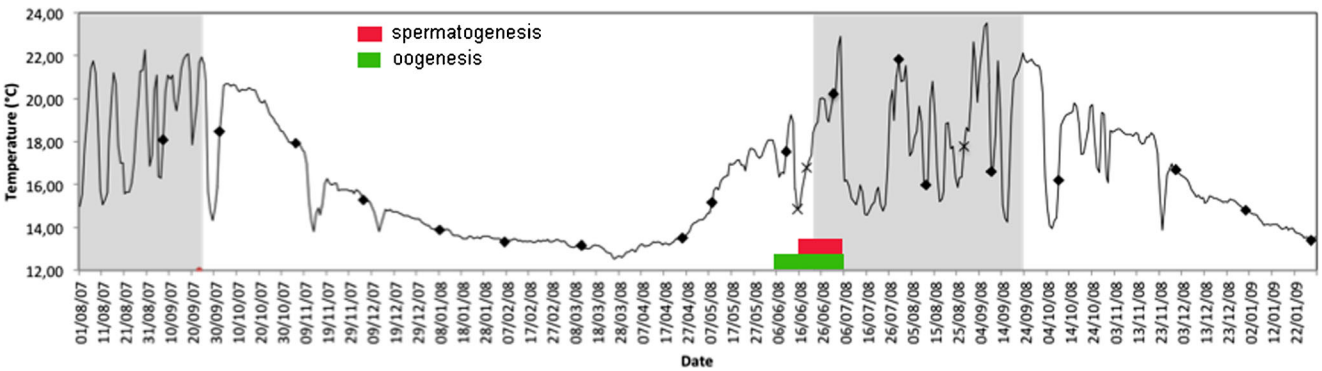

Fig. 5 Daily mean seawater temperature between August 2007 and January 2009 at 15 m depth in Grotte à Corail (Marseille). Grey zones present summer seasons. Black ribbons present *Aplysina cavernicola* sampling events, while black crosses represent sampling events used only in the reproduction study. Reproductive periods are represented in red (spermatogenesis) and green (oogenesis)

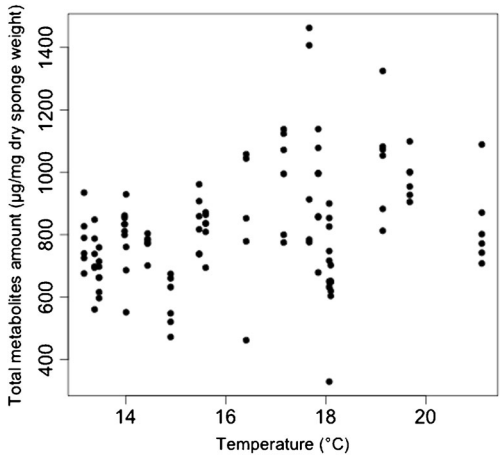

Fig. 6 Correlation between total metabolites amount (µg/mg dry sponge weight) and temperature (°C). Rs: Spearman's coefficient. R: Linear regression

bromoisoxazoline-tyrosine alkaloids are in contact with biotransformation enzymes and are transformed by an enzyme reaction into lower-molecular-weight bromotyrosine alkaloids such as aeroplysinin-1. Then, sea-water alkalinity, transforms aeroplysinin-1 into dienone and semi-quinone radicals (Ebel et al. 1997; Thoms et al. 2004, 2006). A recent study has elucidated the presence of nitrile hydratase in *A. cavernicola* that is responsible for the bioconversion of dienone (Lipowicz et al. 2013). In our experiments, the sampling and manipulation method were done in such a way to minimize effects on living tissues and by using methanol to obtain crude extracts, thus, avoiding degradation or biotransformation of secondary metabolites after sample collection. Consequently, the presence of dienone in some of our samples is interpreted as a secondary metabolite biosynthesized by the sponge itself and not as an artifact of the experimental procedure.

In October 2008, we observed an increase in the production of dienone and a decrease in production of the major metabolites (aerophobin-1, aerophobin-2, aplysinamisin-1, aerothionin, and homoaerothionin) together with a considerable increase of bioactivity. Complementary antibacterial tests coupled to HPLC fractionation pointed out dienone as the only compound from *Aplysina* alkaloids that displayed a strong antibacterial activity, and thus confirming the relationship between the increase of dienone and the increase of organic extract bioactivity observed. These results give some insight to the current controversy, and are consistent with the results of Ebel et al. (1997) and Teeyapant et al. (1993) that showed that secondary metabolites originating from bioconversion (dienone) display higher bioactivity than their precursors. They also suggest that this bioconversion could act as an activated chemical defense of the sponge against external danger.

In the Mediterranean Sea, sponges suffer regularly from disease outbreaks related to climatic anomalies. These events are often related to positive temperature anomalies that occur in late summer, either through metabolic stress related to high temperatures or thermo-dependent pathogen outbreaks (for review see Lejeusne et al. 2010 or Pérez and Vacelet 2014). We speculate that higher seawater temperatures in summer 2008 might have triggered bioconversion of *A. cavernicola* major alkaloids into the more bioactive metabolite dienone, as an inducible defense strategy useful against the putative proliferation of thermo-dependent pathogens. Whether the bioconversion is triggered directly by the temperature rise or by the pathogens remains unknown. Therefore, more studies should be conducted in order to follow the secondary chemistry of Aplysinidae sponges under thermal stress, to clarify the mechanisms of dienone bioconversion and its role as a dynamic chemical defense in warm temperature episodes.

In this study, temporal variations in the production of secondary metabolites in *A. cavernicola* and the bioactivity of crude extracts were studied in order to better understand parameters that regulate secondary metabolism. Energy investment in reproduction does not seem to compromise energy budget allocated to secondary metabolites production. However, secondary metabolism seems to be influenced by water temperature, producing higher metabolite concentrations in warmer temperatures. Bioactivity studies confirm that the minor secondary metabolite dienone accounts for most of the bioactivity observed. A peak of bioactivity coinciding with a peak of dienone in October 2008, indicates a possible dynamic protection via secondary metabolite biotransformation against environmental stressors such as higher late-summer water temperatures and associated processes.

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