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VARIATION IN SECONDARY METABOLITE CONCENTRATIONS IN YELLOW AND GREY MORPHS OF THE RED SEA SOFT CORAL *Parerythropodium fulvum fulvum*: POSSIBLE ECOLOGICAL IMPLICATIONS

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Abstract—Secondary metabolites in yellow and grey morphs of the soft coral *Parerythropodium fulvum fulvum* were compared between colonies collected from different depths and reef sites along the Red Sea. The concentrations of fulfulvene, the major metabolite in the yellow morph, varied considerably among samples, with significant differences between shallow and deep colonies. The concentrations of 5-hydroxy-8-methoxy-calamenene and 5-hydroxy-8-methoxy-calamenene-6-al, the major metabolites in the grey morph, also exhibited significant differences between shallow and deep colonies. The ecological implications of these variations in secondary metabolites are discussed.

Key Words—Chemical ecology, secondary metabolites, variation, *Parerythropodium fulvum fulvum*, Cnidaria, Octocorallia, Red Sea.

INTRODUCTION

Patterns of intraspecific variation in composition and concentration of secondary metabolites among geographic regions or habitats have been well documented for terrestrial plants (Gershenzon and Croteau, 1991). In the marine environment, however, chemical variation has received less attention. Although several studies have reported the quantification of secondary metabolites of benthic marine organisms, the lack of information on intraspecific variation in the production of chemical defenses and its ecological consequences is notable, and the impor-

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tance and need for such studies have been emphasized (Hay, 1996). A few studies have shown variability in secondary metabolite content within or between individuals of a given species (e.g., Meyer and Paul, 1995; Maida et al., 1993; Harvell et al., 1993; Becerro et al., 1998; Amade and Lemee, 1998). Some of these reports have correlated the variation to spatial variables, such as depth or geography. For example, Maida et al. (1993) found significant differences in the major metabolites, flexibilide and sinulariolide, of the soft coral *Sinularia flexibilis* among sites at Lizard Island (Great Barrier Reef, Australia). Harvell et al. (1993) detected variation in composition and concentration of the major metabolites of the gorgonian *Briareum asbestinum* among depths and geographical sites.

Despite more than three decades of intense interest in secondary metabolites of soft corals, and the isolation of numerous novel compounds with potent biological activity (see review by Faulkner, 1998 and previous reviews by the same author), little is known about within- and between-habitat variability in the levels and types of compounds in these species. Such information is important for our understanding of the factors affecting the production of chemical defenses, as well as to provide insight into the processes that shape the evolution of chemical defenses (Harvell et al., 1993).

Parerythropodium fulvum fulvum (Forskål, 1775) is a common encrusting alcyonacean soft coral on Red Sea reefs (Benayahu and Loya, 1977). Two color morphs exist: yellow–brown and grey, with no taxonomic differences between them (Verseveldt, 1969). The yellow morph is predominantly found at depths of 3–40 m, while the grey one is common usually below 20 m (Benayahu and Loya, 1983). Like many other soft corals, *P. f. fulvum* produces a rich assemblage of secondary metabolites (Green et al., 1992). These are comprised mainly of sesquiterpenes, the most prominent of which is the volatile dye fulfulvene, which gives the living colony its yellow color. These secondary metabolites have been found to play a role in a variety of processes, such as defense against predation by generalist fishes (Kelman et al., 1999), and protection against cooccurring and potentially pathogenic bacteria (Kelman et al., 1998). Green et al. (1992) found significant differences in the composition of secondary metabolites between the two color morphs and between male and female colonies. For the chemically defended *P. f. fulvum*, variability in composition and concentration of secondary metabolites might have implications for the effectiveness of its defense mechanisms. In the present article, we compare the chemical composition of colonies of the two color morphs of *P. f. fulvum* collected at different depths and sites along the Gulf of Eilat and Eritrea, northern and southern Red Sea, respectively. We also measured the concentrations of fulfulvene, the major metabolite in the yellow morph, and 5-hydroxy-8-methoxy-calamenene and 5-hydroxy-8-methoxy-calamenene-6-al, the two major metabolites in the grey morph. The ecological implications of this variation are discussed.

METHODS AND MATERIALS

Collection. Colonies of the two color morphs of *Parerythropodium fulvum fulvum* were sampled from several sites along the Red Sea (Figure 1) between December 1995 and May 1997. At Eilat, samples were collected from both shallow (3–10 m) and deep (25–30 m) reefs, across from the Inter University Institute of Marine Biology (five colonies of each morph at each depth). In addition, colonies were obtained from the shallow reefs of Ras-Burka, El-Hibek, and Dahab (five colonies of the yellow morph from each site). An additional sample of the yellow morph was collected from 10 m at Kundibilu Island, Dahlak archipelago, Eritrea, southern Red Sea (Figure 1). In order to avoid collection of clone mates, samples were obtained from patches located at least 5 m apart. The colonies were cleaned of algal epiphytes and frozen in separate plastic bags

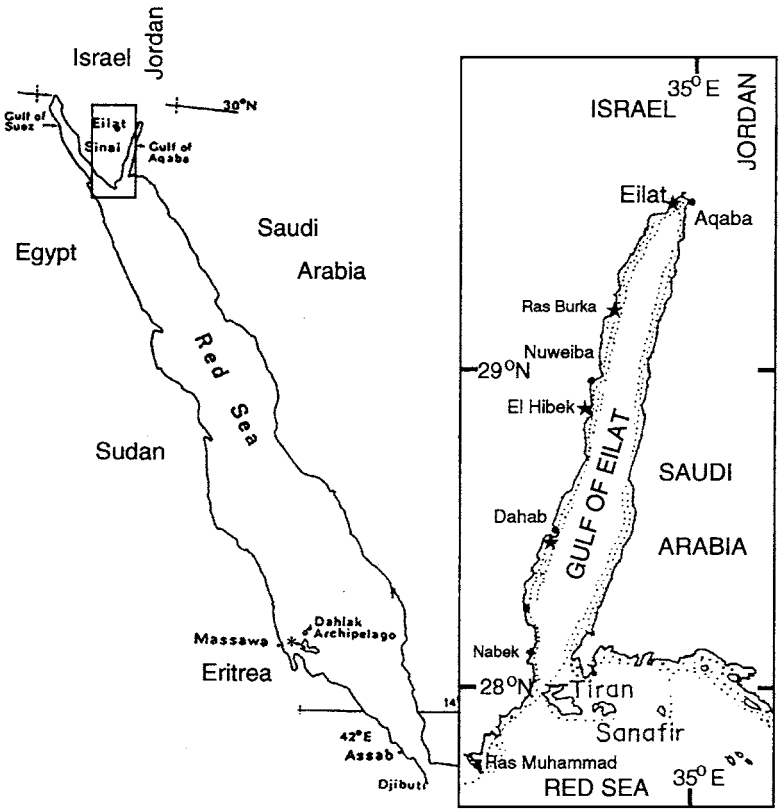


FIG. 1. Collection sites along the Red Sea (marked with stars).

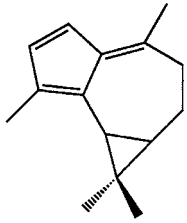
at −70°C before being transferred on ice to Tel-Aviv, and kept there at −20°C for further analysis.

Sample Extraction and Preanalysis Preparation. Each colony (3–40 g wet weight) was cut into small pieces and extracted in dichloromethane (~50 ml) for 24 hr at room temperature. The organic extracts were separated from the water phase by using a 100-ml separation funnel, filtered, and the solvent removed by rotary evaporator under vacuum at 10–15°C. Care was taken to maintain cold evaporation conditions, and freeze-drying was avoided prior to extraction in order to prevent possible evaporation of volatile compounds, such as the sesquiterpene fulfulvene (see Green et al., 1992). The extracts were weighed and kept at −20°C until use. The remaining coral tissue after extraction was freeze-dried and weighed. The total coral dry weight was calculated by combining the dry tissue weight (after extraction) with the crude extract weight. All samples were prepared in the same manner to allow sufficient comparison.

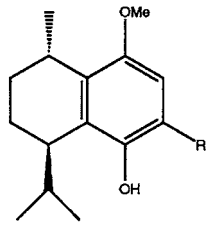
Qualitative Analysis. Analysis of the secondary metabolite composition of the samples was performed by electrospray ionization mass spectrometry (ESI-MS). This ionization procedure provides a spectrum of peaks with no fragmentation, so that each peak represents only one compound. Samples of ca. 1 mg of crude extract were analyzed by using a low-resolution Quattro ESI-MS at the Mass Spectrometry Lab, University of Illinois at Urbana-Champaign, Illinois.

Quantitative Analysis. Comparison of concentrations of pure compounds was performed by the ^{1}H NMR method after Maida et al. (1993) on the major secondary metabolites of *P. f. fulvum*. The sesquiterpene fulfulvene (Figure 2) was chosen for the yellow morph since it has a separate peak that does not overlap with the other compounds. For the grey morph, we analyzed 5-hydroxy-8-methoxy-calamenene and 5-hydroxy-8-methoxy-calamenene-6-al (Figure 2). The crude extract of each sample was evaporated to dryness, and a carefully weighed aliquot of at least 10 mg was transferred to a 5-ml vial. A predetermined amount of 2,4-dinitrobenzene (~1 mg) was added as internal standard, and deutero chloroform (~0.5 ml) was used as a solvent. The ^{1}H NMR spectrum of each sample was recorded on a Bruker AM360 NMR spectrometer. Signals measured for 2,4-dinitrobenzene resonated at δ7.82 and for fulfulvene at δ6.26 (two vinyl protons); the calamenene derivatives were estimated on the basis of their *O*-methoxy signal at δ3.79. This *O*-methoxy signal was affiliated only with the calamenene derivatives. Since the vinyl protons of fulfulvene represent two protons and the *O*-methoxy signal represents three protons, the value was divided by 2 and 3, respectively. The absolute amounts of each compound were calculated in milligrams by using the relation between the heights of each proton signal to the height of the proton of the standard signal, leading to the number of moles of each secondary metabolite and standard.

Statistical Analyses. One-way ANOVA tests were used for the comparison of concentrations of compounds and extract yields (Sokal and Rohlf, 1995).



Fulfulvene



5-hydroxy-8-methoxy-calamenene (R=CH_3)

5-hydroxy-8-methoxy-calamenene-6-al (R=CHO)

FIG. 2. Molecular structures of secondary metabolites of *Parerythropodium fulvum fulvum* used for the comparative analyses in this study.

Since the data were in proportions, arcsine transformation was performed in order to comply with ANOVA assumptions (normality and homogeneity of variances).

RESULTS

Qualitative Variation of Secondary Metabolites Between Depths. The ESI-MS analysis of the crude extracts of shallow and deep colonies of the two color morphs revealed qualitative differences in their secondary metabolites composition. At least two compounds present in the shallow yellow morph did not appear in the deep colonies, and at least five compounds were present solely in the deep colonies and not in the shallow ones. The shallow grey morph colonies contained at least nine additional compounds, which were not detected in the deep ones.

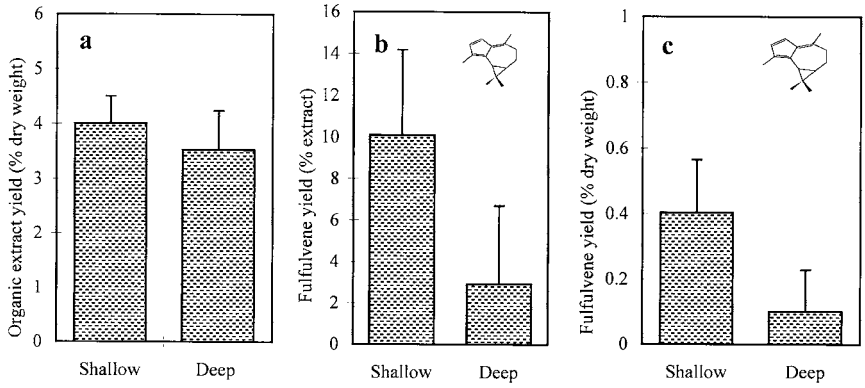


FIG. 3. Depth variability in the yellow morph of *Parerythropodium fulvum fulvum:* (a) organic extract yields in shallow and deep colonies (+ 1 SD); (b) fulfulvene yields in shallow and deep colonies presented as percentage of crude organic extract (+ 1 SD); (c) fulfulvene yields in shallow and deep colonies presented as percentages of dry weight (+ 1 SD). For each depth $N = 5$.

Quantitative Variation of Secondary Metabolites Between Depths. The organic extract yields were similar in both shallow and deep yellow-morph colonies, with an average yield of 3.8 ± 0.7% of dry weight (one-way ANOVA, $P = 0.3$; Figure 3a). The concentration of fulfulvene in the yellow morph colonies showed variation between depths (one-way ANOVA, $P = 0.034$). This compound occurred at an average concentration of 10.1 ± 4.1 and 2.9 ± 3.8% of crude organic extract in the shallow and deep colonies, respectively (Figure 3b). The differences in fulfulvene concentration were consistent when we calculated the overall yields of fulfulvene between shallow and deep colonies, which were 0.4 ± 0.16 and 0.1 ± 0.13% of dry weight, respectively (one-way ANOVA; $P = 0.019$; Figure 3c).

Organic extract yields (percent dry weight) were also similar in shallow and deep grey morph colonies (one-way ANOVA, $P = 0.147$), with an average yield of 4.88 ± 0.63% dry coral weight (Figure 4a). Differences were found in the concentration of the calamenene derivatives between shallow and deep colonies (one-way ANOVA, $P = 0.011$), occurring at a concentration of 41.07 ± 4.38 and 68.53 ± 7.13% of crude organic extract, respectively (Figure 4b). A comparison of the overall yields of calamenene derivatives between shallow and deep colonies showed differences (one-way ANOVA, $P = 0.024$) occurring at a concentration of 1.8 ± 0.34 and 3.5 ± 0.71% of dry weight, respectively (Figure 4c).

Site Variation of Secondary Metabolites. The crude organic extract yields of shallow yellow-morph colonies from different reef sites along the Gulf of Eilat

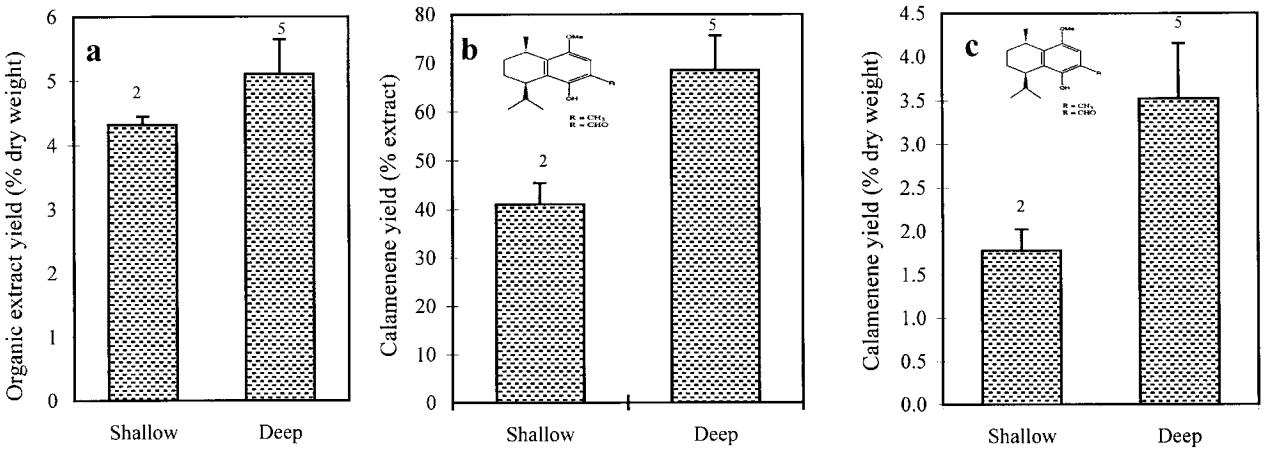


FIG. 4. Depth variability in the grey morph of *Parerythropodium fluvum fulvum*: (a) organic extract yeilds in shallow and deep colonies (+ 1 SD); (b) calamenene derivatives yields in shallow and deep colonies presented as percentages of crude organic extract (+ 1 SD); (c) calamenene derivatives yields in shallow and deep colonies presented as percentage of dry weight (+ 1 SD). Number of examined colonies is indicated for each depth.

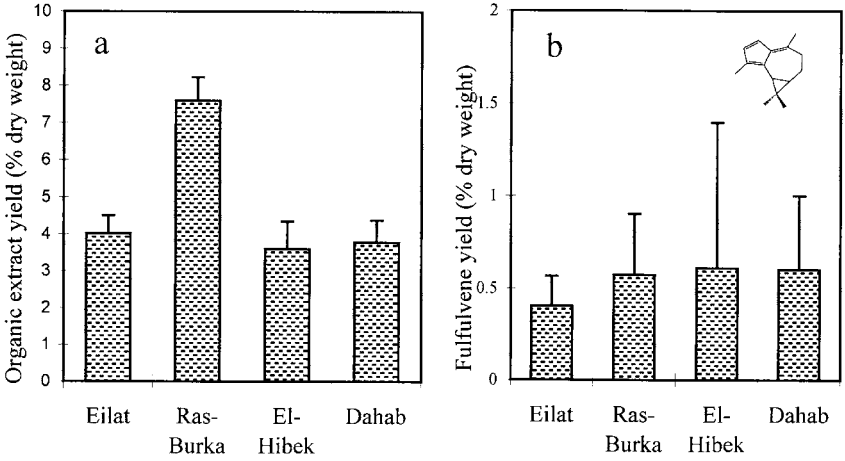


FIG. 5. Geographical variability of *Parerythropodium fulvum fulvum* yellow morph: (a) organic extract yields (+ 1 SD); (b) Fulfulvene yields presented as percentage of dry weight (+ 1 SD). For each site $N = 5$.

were highly variable (Figure 5a; one-way ANOVA, $P < 0.0001$). A comparison of the extract yields from Eilat, El-Hibek, and Dahab showed no differences, with an average yield of 3.8 ± 0.7% of coral dry weight (one-way ANOVA, $P = 0.6536$). The average yield of extracts of colonies from Ras-Burka was 7.6 ± 0.8% of dry weight, differing from those of the other sites. The average fulfulvene concentration of colonies from the four sites were similar but highly variable within each site (Figure 5b), with an average concentration of 0.55 ± 0.52% of coral dry weight. The extract yield of the colony from Kundibilu (Dahlak archipelago, Eritrea; Figure 1) was 4.1% of coral dry weight, and the fulfulvene concentration was 0.55% of coral dry weight. These concentrations were within the same range as the colonies from the northern Red Sea.

DISCUSSION

This study demonstrates intraspecific variation in composition of secondary metabolites of *Parerythropodium fulvum fulvum*, as well as in concentrations of its major metabolites. The qualitative differences were between the two color morphs and depths and were consistent with the results of Green et al. (1992). Quantitative variation in concentration of fulfulvene, one of the major yellow morph secondary metabolites, showed major variability among colonies collected from various depths and reef sites. The grey morph colonies are known to primarily colonize reefs deeper than 20 m (Benayahu and Loya, 1983), although

some colonies are occasionally found in shallower waters. Apart from the major qualitative differences between shallow and deep colonies, significant quantitative differences were found in the calamenene derivatives between depths. These metabolites were present in the extract at a high concentration of about 40% of the shallow dichloromethane crude extract, and about 70% of the deep one. Although there were significant differences in concentrations of calamenene derivatives, the organic extract yields were similar among the colonies examined. The fact that variation in major secondary metabolites existed, while the total extract concentrations remained the same, indicates that changes in concentrations occurred at the expense of other lipid-soluble components present in the extract.

The function of fulfulvene and calamenene derivatives in *P. f. fulvum* is still unknown. One possible cause of the qualitative variation in this species in general, and the quantitative variation in fulfulvene in the yellow morph and the calamenenes in the grey morph in particular, might be a response to selection pressure by pathogens and predators. The crude organic extract of this species has shown effective biological activity against cooccurring and potentially pathogenic marine bacteria (Kelman et al., 1998), as well as potent feeding deterrence against generalist predatory reef fish (Kelman et al., 1999). The sclerites of this species, however, did not deter predation, and are, thus, considered to possess a structural function only. Furthermore, the sclerite content of colonies from the different morphs, depths, and reef sites was around 80% of the dry weight and did not show any variation among the samples (Kelman, 1998). Selection pressure by predators may have induced production of secondary metabolites in *P. f. fulvum* rather than in structural defense.

The compound 5-hydroxy-8-methoxy-calamenene has been previously reported from the gorgonian *Subergorgia hicksoni* (see Kashman, 1979). Recently, this compound has been isolated in *Subergorgia* sp. from Guam (M. Puglisi, personal communication) and shown to exhibit feeding deterrence against a natural assemblage of reef fishes. This finding may suggest that the compound could be responsible for the deterrent activity found in the grey morph colonies of *P. f. fulvum* (see Kelman et al., 1999). Extracts from both shallow and deep grey morph colonies exhibited feeding deterrence, but they also showed variation in calamenene concentrations. These findings may have resulted from a differential selection pressure by predators. However, since predation is considered to be higher in shallow reef habitats, it is predicted that higher deterrent activity should also occur in shallow colonies. Nonetheless, our data are not consistent with this prediction, as higher levels of calamenenes were found in deeper colonies. Similarly, a deviation from this prediction was shown in the octocoral *Briareum asbestinum* by Harvell et al. (1993), who found higher levels of diterpenes in deeper colonies. This was explained by their possibly slower growth rate, which generates a surplus of carbohydrates for production of natural

products. We, therefore, suggest that a similar situation may occur in the deeper grey morph colonies of *P. f. fulvum*, whose higher levels of calamenenes provide a greater defense, compensating for the potentially high cost of replacing lost tissues due to predation when the growth rate is low.

In summary, the present study reveals a qualitative variation in the secondary metabolite content of *P. f. fulvum* between habitats and a quantitative variation in the possibly defensive calamenenes in the grey morph colonies between depths. Although the potential adaptive nature of such variation is still unknown, it could well be in response to selection pressure by pathogens and/or predators. The ability of *P. f. fulvum* to defend itself from such harmful agents could effectively explain its success in occupying a wide range of depths and habitats. Furthermore, one should take intraspecific variation into account whenever investigating the biological activity of natural products, especially when presenting a chemical fingerprint of a given species.

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