Marine Biology 91, 59–68 (1986)

Marine Biology

Chemical induction of larval settlement and metamorphosis in the reef-building tube worm *Phragmatopoma californica* (Polychaeta: Sabellariidae)

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Abstract

Naturally-occurring lipophilic inducers of larval settlement and metamorphosis were isolated and identified for *Phragmatopoma californica*, a gregarious tube worm from southern California. Organic solvent extraction of the sand/organic cement matrix of tubes diminished the inducing capacity of the tube matrix. The inducing capacity was restricted to a single, highly active, HPLC-purified fraction of the organic solvent extract. Chemical analysis of this fraction revealed a mixture of free fatty acids (FFAs), dominated by eicosapentaenoic acid (20:5, ~ 20%), palmitic acid (16:0, ~ 14%) and palmitoleic acid (16:1, ~ 12%). In assays of the nine FFAs that each contributed 3% or more to the active fraction, only 16:1, 18:2, 20:4 and 20:5 induced larval settlement and metamorphosis, while the others were ineffective. The larval response was contact-dependent, highly specific, and concentration-dependent, with a significant response to 16:1 and 20:4 at as low as 10 μg FFA spread onto 1 g of sand (surface area $\cong$ 36 cm^2). Active FFAs were extracted at approximately 14 μg g^{-1} sand from the tube matrix, although the levels encountered by larvae in nature are believed to be higher.

Introduction

The sabellariids constitute a family of polychaetes that builds tubes individually or in small-to-extensive aggregations from a combination of available sediment and a cement secreted by the worm (Vovelle, 1965). Sexes are separate and reproduction is strictly sexual (i.e., there is no asexual budding to produce colonies, as in coral-reef formation). Therefore, the establishment and growth of aggregations relies on the recruitment of feeding larvae which spend several weeks to months in the plankton (Wilson, 1968). Gregarious settlement presents some obvious advantages: proximity of adults allows for synchronization of spawning (especially if a pheromone is involved) and increased fertilization of spawned gametes. Large aggregations have a better chance of surviving physical disturbances, and consequently gain a longer adult life-span and increased overall fecundity.

Reef-forming species are distributed world-wide from the low intertidal to subtidal in subtropical to temperate regions, including the coasts of Oregon (Posey *et al.*, 1984), California (Scholl, 1958), Florida to Brazil (Kirtley and Tanner, 1968), Panamá to Chile (Hartman, 1944), Europe (Wilson, 1971), South Africa (Day, 1967), India (Achari, 1974) and New Zealand (Augener, 1926). Reefs may reach hundreds of meters in width and stretch over tens to hundreds of kilometers of coastline (Dollfus, 1960; Kirtley and Tanner, 1968). Such reefs have been implicated in shipwrecks (Kirtley, 1967), in the stabilization and sorting of beach sand (Kirtley, 1967; Multer and Milliman, 1967; Gram, 1968), in dominating the community structure of intertidal habitats (Taylor and Littler, 1982), and in the fostering of complex communities of invertebrates and fishes within and around their confines (Gruet, 1971; Gore *et al.*, 1978).

In one of the earliest experimental demonstrations of substrate-specificity in a larval marine invertebrate, Wilson (1968), working with the reef-building species *Sabellaria alveolata*, discovered that larval contact with adult sand tubes, tube remnants, or the mucoid tubes of juvenile worms induced rapid settlement and metamorphosis. Factors such as surface contour and roughness, sediment type, water motion and the presence of a microbial film had little or no effect on larval behavior. The metamorphosis-inducing substance of the tubes did not dissolve in water and was unaffected by drying, but was destroyed on treatment with acid. Wilson concluded that a chemical cue in the tube cement induced larval metamorphosis in a fashion similar to that proposed by Knight-Jones (1953) involving the settlement of barnacle larvae on contact with quinone-tanned, cuticular proteins of adult barnacles. Wilson's observations were extended by Jensen and Morse (1984), who

confirmed the contact-dependent, chemical basis for settlement in *Phragmatopoma californica*, a reef-building species from the coast of southern California. They demonstrated that the presence of the anterior portion of adult tubes initiated settlement and metamorphosis, while the presence of various co-occurring algae and invertebrates, sandstone and the posterior portions of adult tubes did not. They also speculated that the chemical cues for settlement were quinone-tanned proteins, precursors or enzymes involved in their synthesis.

This study was undertaken to identify the chemical cues responsible for the induction of larval settlement and metamorphosis in *Phragmatopoma californica*. The approach employed a bioassay-directed isolation procedure that eliminates bias with regard to the chemical nature of the inducer.

Materials and methods

Larval culture

Spawning was initiated in *Phragmatopoma californica* adults by removing worms from their tubes. Females with lavender, egg-filled abdominal setigers and males with sperm-filled, yellow to white abdominal setigers were placed into separate fingerbowls. Spawned eggs adhere to the bottom of the fingerbowl, allowing convenient decantation of spent adult females and debris. Eggs were rinsed with 0.45 μm-filtered seawater, fertilized with a dilute suspension of sperm, and rinsed again 15 min later. After 12 to 18 h at 20 °C, swimming trocophores were transferred to 4-liter wide-mouth glass jars filled with 3 liters of 1 μm-filtered seawater (final concentration of approximately 1 larva ml^{-1}) containing 40 mg l^{-1} each of the antibiotics streptomycin sulfate and sodium penicillin G. Jars were placed in a 20 °C constant-temperature bath, 70 cm beneath two 40 W fluorescent lamps set for a 14 h:10 h light:dark cycle. Larval cultures were continuously agitated by motor-driven, reciprocating paddles (8 × 10 cm lucite paddle face; 3 cycles min^{-1}).

A 1:1 mixture of the diatom *Phaeodactylum tricornutum* and the flagellate *Pavlova* (= *Monochrysis*) *lutheri* at 10^5 cells ml^{-1} was found to optimize growth of *Phragmatopoma californica* larvae in experiments involving variable densities of six species of phytoplankters routinely used in invertebrate larval culture (M. R. Amieva, unpublished results). Algae were cultured under conditions slightly modified from those outlined in Guillard (1975). At each feeding, cell densities were determined with a Coulter Counter.

Larval cultures were cleaned every other day. Larvae were sieved onto Nitex fabric (Tetko, Inc., Elmsford, New York; mesh openings of 52, 100 and 200 μm used progressively as larvae grew) and resuspended in seawater, while jars were scrubbed in hot freshwater, rinsed in seawater and filled with 1 μm-filtered seawater; thereafter the larvae, food and antibiotics were added. Adoption of these methods yielded large numbers of uniformly-sized larvae, competent for settlement and metamorphosis 18 d after hatching.

Larval assays

Ottawa sand (cement-testing standard, 20 to 30 mesh; Fisher Scientific), baked in a muffle furnace at 550 °C for 6 h to remove organics, was used in all assays. For each assay, 1 g of treated Ottawa sand (surface area $\cong$ 36 cm^2) was weighed out into a disposable, 100 mm diameter, polystyrene petri dish. The dish and sand were rinsed once and then filled with 50 ml of 1 μm-filtered seawater. Into each dish, 30 ($\pm$2) larvae, age 20 to 30 d post-hatching, were transferred. Dishes were placed on a rocking platform set at 28 cycles min^{-1} for 24 h under the same lighting regimen employed in larval culture. Plates were then examined under a stereomicroscope at 15× magnification. As larvae were removed, they were scored as follows: (1) non-metamorphosed – if they swam or crawled normally with no loss of provisional setae or anterior rotation of larval tentacles; (2) metamorphosed – if they shed their provisional setae, formed a primary mucoid tube, rotated their larval tentacles anteriorly, and began sand tube construction; (3) abnormal – (a) if they held their provisional setal bundles forward, rotated larval tentacles anteriorly and behaved sluggishly, or (b) if they shed their provisional setae and rotated larval tentacles anteriorly, but did not form a mucoid tube, and moved sluggishly, or (c) same as (b), but larvae died. Descriptions of normal sabellariid larval behavior at settlement and metamorphosis can be found in Eckelbarger (1978) and Smith and Chia (1985).

Infrequently, larvae would metamorphose on the mucoid tubes of previously metamorphosed juveniles. The resulting clumps of two or three metamorphosed juveniles were only counted as one and the additional juveniles were not included in the total count. The mean percentage of larval response for each assay was determined and the parametric *t*-test was used to ascertain the significance of differences between means in paired comparisons. Data were arcsine-transformed prior to statistical testing when appropriate (Sokal and Rohlf, 1981).

Assay for presence of a lipophilic inducer in the sand/cement matrix

Sand tubes of *Phragmatopoma californica* adults used in experiments were constructed of Ottawa sand in the following fashion: large (approximately 30 × 30 × 15 cm) blocks of living worm reef were sawed-off intertidal boulders during low tides near Ladera Street, Sunset Cliffs, Point Loma, San Diego, California, USA. After adult worms had fully retracted into their tubes, the top 0.5 cm of the block was removed by scraping with a spatula. Smooth-top blocks were then immersed in flowing seawater in laboratory aquaria and covered with Ottawa sand. Worms rebuilt the anterior portions of their tubes with Ot-

tawa sand, which was supplemented at approximately 12 h intervals. Control sand used in these experiments was placed in a shallow dish in the same tank as the reef block, thereby developing a similar surface microflora as the sand incorporated into the tubes. After 2 to 3 d, the anterior portions of the tubes were removed from the block by lightly scraping the block surface with a glass rod. Adult worms withdrew and were uninjured. Anterior tubes and control sand were rinsed in deionized water, frozen and lyophilized. Tubes were broken up by lightly grinding them with a mortar and pestle, and the resulting tube sand and the control sand were separately sieved (retained on mesh sizes 25 and 40) to remove clumps, silt and detritis. Half of the tube sand was successively extracted for approximately 3 h in each of four solvents or solvent mixtures of increasing polarity – hexane; diethyl ether; 1:1 diethyl ether:methanol; and methanol. Extracted sand was then placed under vacuum for 15 to 20 min to remove all traces of organic solvents. Each test of the extracted, unextracted and control sands was run in five replicates.

Isolation of the lipophilic inducer

Large quantities of extract were required for isolation and identification of inducers (Fig. 1a). Blocks of worm reef were collected as previously described and adult worms were removed, either by forcing each adult out of its tube with a forceps, or by crushing the tubes and rinsing adult worms away with repetitive washings of seawater. The resulting 2 to 3 kg of worm-free tube sand was rinsed in deionized water, frozen, lyophilized and sequentially extracted as previously described. After extraction with methanol, tube sand was additionally extracted with double-distilled water. Thin-layer chromatography revealed that the hexane, diethyl ether and 1:1 diethyl ether:methanol extracts shared most of the same compounds and preliminary assays indicated each had some capacity to induce larval settlement and metamorphosis. These three extracts were combined and passed through 30 ml of silica gel by "flash" chromatography (Still *et al.*, 1978) with 1:1 hexane:diethyl ether as the eluant, prior to separation by high-performance liquid-chromatography (HPLC) on Partisil with the same solvent system (Fig. 1b). In all, eight extracts or fractions were assayed: the methanol and water extracts of the tubes, the adsorbed material on the flash column subsequently eluted with a more polar solvent system, the waste material not collected during HPLC fractionation, and the four HPLC fractions (Fig. 1a; five replicates each). Extracts and HPLC fractions were solubilized in known volumes of water (for the water extract), methanol (for the methanol extract) or diethyl ether (for the remaining extracts and fractions). All solutions were kept tightly capped at −15 °C until use. For each assay, 150 μl of the solvent solution containing 1 mg of each extract or fraction was spread onto 1 g of baked Ottawa sand on glassine weighing paper. Control sand was treated with solvent alone. After the solvent had evaporated, control and extract-treated

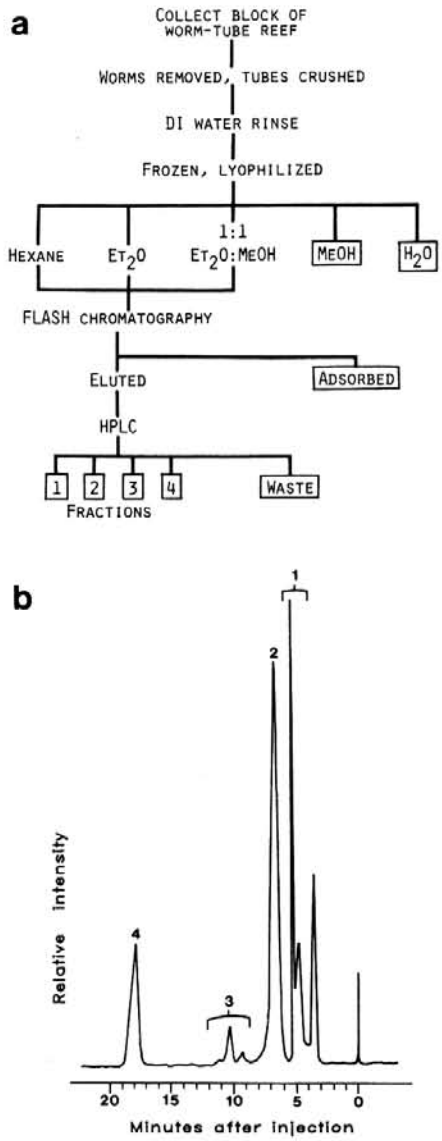

Fig. 1. *Phragmatopoma californica.* Extraction and isolation of lipophilic inducers of larval settlement and metamorphosis. (a) Extraction and separation scheme; extracts and fractions enclosed in blocks were subjected to assay experiments (see "Materials and methods – Isolation of lipophilic inducer"). DI: deionized. (b) High-performance liquid chromatography (HPLC) trace (refractive index) of non-polar components of organic extraction; fractions referred to in (a); Partisil column, 1:1 hexane:diethyl-ether eluant

sands were separately added to disposable polystyrene petri dishes which were then placed in a chamber under vacuum for 10 to 15 min to remove any traces of solvent. Assays were run as previously described.

Identification of active components

Four identically prepared, active FFA fractions (HPLC-Fraction 2) were esterified by reaction with diazomethane in distilled diethyl ether to obtain the corresponding methyl esters. The composition of the methyl-ester mix-

tures was determined on a gas chromatograph (Hewlett-Packard Model 5840-A; flame ionization detector; 3% OV-1 column) by comparison with retention times of known standards. Fatty acid methyl ester identities were confirmed on an additional active fraction with a gas chromatograph-mass spectrometer (Finnigan model 4021; 3% Dexsil column) and comparison with known mass spectra.

Assays of FFA standards

Free fatty acid (FFA) standards used in assays were purchased from Sigma Chemical Co. (St. Louis, Missouri) and Nu Chek Prep Inc. (Elysian, Minnesota) at the highest available purity (> 99% except for 20:5, approximately 90% purity; all double bonds in *cis* configuration). Standards were treated and assayed in the same manner as extracts and HPLC fractions at concentrations of 1 000, 300, 100, 50, and 10 $\mu g\ g^{-1}$. The number of replicates for each assay are indicated in Fig. 4.

Results

Larval settlement and metamorphosis after organic extraction of tube sand

The percentage of *Phragmatopoma californica* larvae that settled and underwent metamorphosis on the three kinds of sand is shown in Fig. 2. Average percentage settlement and metamorphosis was significantly reduced ($p < 0.001$) from 72.3% for unextracted tube sand to 13.0% for extracted tube sand. Inductive capacity was significantly greater ($p < 0.001$) for unextracted tube sand than for microbially-filmed control sand, which averaged 1.9% metamorphosis. Extracted sand induced a greater percentage of metamorphosis than did control sand ($p < 0.01$).

Isolation of the lipophilic inducer

Fig. 3 presents results of larval assays of the methanol and water extracts, the adsorbed material on the flash column, the four HPLC fractions and the waste material from HPLC fractionation (see Fig. 1). HPLC-Fraction 2 induced a significantly greater level ($p < 0.001$) of larval settlement and metamorphosis, an average of 77.8%, than did any other fraction or extract. Waste from HPLC fractionation and the water extract of tubes induced an average of 4.4 and 4.0% metamorphosis, respectively; however, neither was significantly different ($p > 0.05$) than the control (1.9% metamorphosis).

Identification of active components

Proton nuclear-magnetic-resonance spectroscopy of HPLC-Fraction 2 indicated the presence of acyl groups containing some degree of unsaturation. Analysis by gas chromatography (HP-GC; four runs) and GC-mass spectroscopy (one run) revealed the presence of a mixture of free fatty acids (FFAs; Table 1) dominated by eicosapentadienoic acid (20:5; by convention, the number of carbon atoms in the molecule precedes the colon, the number of double bonds in the molecule follows), palmitic acid (16:0) and palmitoleic acid (16:1). The same suite of FFAs was identified from extracts of both natural tubes and tubes constructed of Ottawa sand by worms in the laboratory. The FFAs making up 3% or more of either the averaged HP-GC runs or the GC-MS run were purchased in purified form for further assay.

Assays of FFA standards

The nine most common FFAs in HPLC-Fraction 2 were assayed for induction of larval settlement and metamorphosis at 1 mg FFA g^{-1} sand. The results are shown in Fig. 4a. Linoleic acid (18:2) and palmitoleic acid (16:1) induced significantly greater levels ($p < 0.001$) of normal larval metamorphosis than any other FFA, at 81.3 and 81.2%, respectively, but at levels not significantly different ($p > 0.05$) from HPLC-Fraction 2, at 91.5% metamorphosis. Exposure of larvae to 20:4 and 20:5 at this level invariably resulted in shedding of provisional setae, anterior rotation of larval tentacles, and death within the 24 h assay period. An aver-

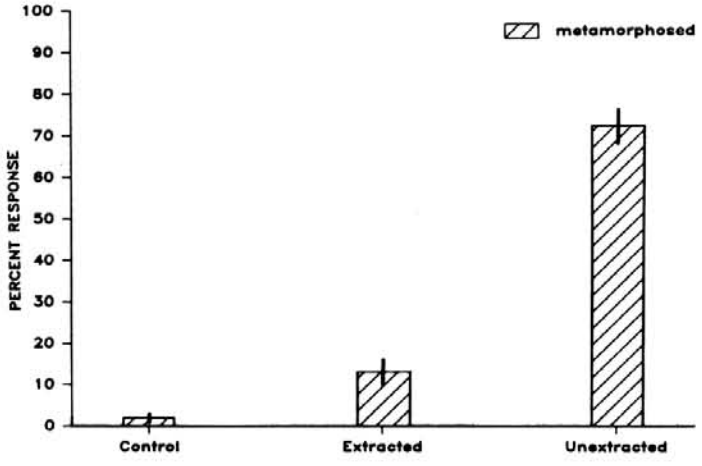

Fig. 2. *Phragmatopoma californica.* Mean percentage response of larvae to microbially-filmed control sand, organic solvent extracted tube sand and unextracted tube sand (± SE; $N = 5$). Non-metamorphosis made up the balance of the percentage response for each treatment

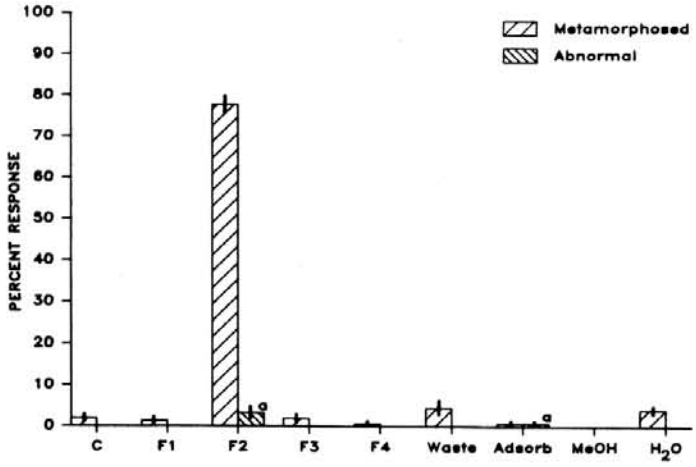

Fig. 3. *Phragmatopoma californica.* Mean percentage response of larvae to control sand (C), and sand treated with 1 mg g^{-1} of each of HPLC-Fractions 1–4 (F1–F4), waste from HPLC fractionation (Waste), material adsorbed onto FLASH column (Adsorb), methanol extract (MeOH) and water extract (H_2O) (see Fig. 1a) (± SE; $N=5$). Non-metamorphosis made up the balance of the percentage response for each treatment. "a" adjacent to bars indicates abnormal response as defined in "Materials and methods – Larval assays"

Table 1. *Phragmatopoma californica.* Percentage composition of HPLC-Fraction 2. For free fatty acids (FFAs), the number of carbon atoms in the molecule precedes the colon, the number of double bonds follows (br = branch- and straight-chain FFAs present). Data from a total of five separate extraction and isolation procedures. GC-MS: One run by Finnigan gas chromatography-mass spectrometry; HP-GC: normalized averages of four runs by gas chromatography alone

Detected compounds	GC-MS	HP-GC
FFAs		
14:0[a]	2.1	4.9
15:0 br	1.8	2.0
16:2	0.2	15.6
16:1[a]	10.4	
16:0[a]	12.0	16.6
17:0 br	4.9	3.0
18:3	1.8	12.1
18:2[a]	3.1	
18:1[a]	9.5	
18:0[a]	4.7	6.4
20:5[a]	19.2	23.5
20:4[a]	4.9	4.7
20:3	1.7	
20:2	0.8	
20:1	2.0	
22:4	2.5	6.4
22:3	1.3	
22:2	2.9	
Phthalates	12.0	4.9

[a] FFA constituting more than 3% of the sample in either column

age of 18.7% of the larvae exposed to 18:2 at 1 mg g^{-1} appeared to be at various stages of abnormal metamorphosis and moved very little. The remaining 81.3% metamorphosed normally and appeared healthy.

The FFAs 16:1, 18:2, 20:4 and 20:5 were further assayed at 300, 100, 50 and 10 μg g^{-1} sand; 16:0, 18:0, untreated sand and HPLC-Fraction 2 were included for comparative purposes. Results of these assays are shown in Fig. 4b–e.

At the 300 μg g^{-1} concentration level (Fig. 4b), 16:1, 18:2, 20:4, 20:5 and HPLC-Fraction 2 all induced significantly greater levels ($p<0.001$) of larval settlement and metamorphosis than controls. However, the active FFA standards induced greater levels ($p<0.05$) of metamorphosis than HPLC-Fraction 2. Abnormal metamorphosis was limited to 41.7% of larvae exposed to 20:4, the remaining larvae metamorphosed normally.

At the 100 μg g^{-1} level (Fig. 4c), 16:1, 18:2, 20:4, 20:5 and HPLC-Fraction 2 all induced significantly greater levels ($p<0.05$) of larval settlement and metamorphosis than controls. At this level, HPLC-Fraction 2 was not significantly different ($p>0.05$) from the FFAs in its inductive capacity. No abnormal metamorphosis was observed.

At 50 μg g^{-1} (Fig. 4d), 16:1, 20:4 and 20:5 induced a significantly greater percentage ($p<0.05$) of larval settlement and metamorphosis than controls. At the 10 μg g^{-1} sand level (Fig. 4e), only 16:1 and 20:4 induced a significant larval response over controls ($p<0.05$).

Discussion

Natural concentration of free fatty acids in the tube sand of *Phragmatopoma californica*

In six separate extraction and purification procedures, the yield of HPLC-Fraction 2 made up an average of 35 μg g^{-1} tube sand. FFAs that are active at inducing larval metamorphosis constitute approximately 40% of HPLC-Fraction 2, hence an average of 14 μg active FFAs g^{-1} natural tube sand. The significance of such a low concentration requires examination, because the 16:1 standard induced an average of only 2.6% larval settlement and metamorphosis at 10 μg g^{-1} sand.

Incomplete extraction could lead to an underestimation of the levels of active FFAs in the tube matrix. Thin-layer chromatography revealed the presence of FFAs in each of the solvent extracts of the series, indicating only partial extraction at each step. Incomplete extraction is also reflected in the significant response of larvae to extracted tube sand over control sand (13% as compared to 1.9%). FFAs may be trapped or bound in some way within the organic cement matrix, a situation analogous to that found by Hoer-

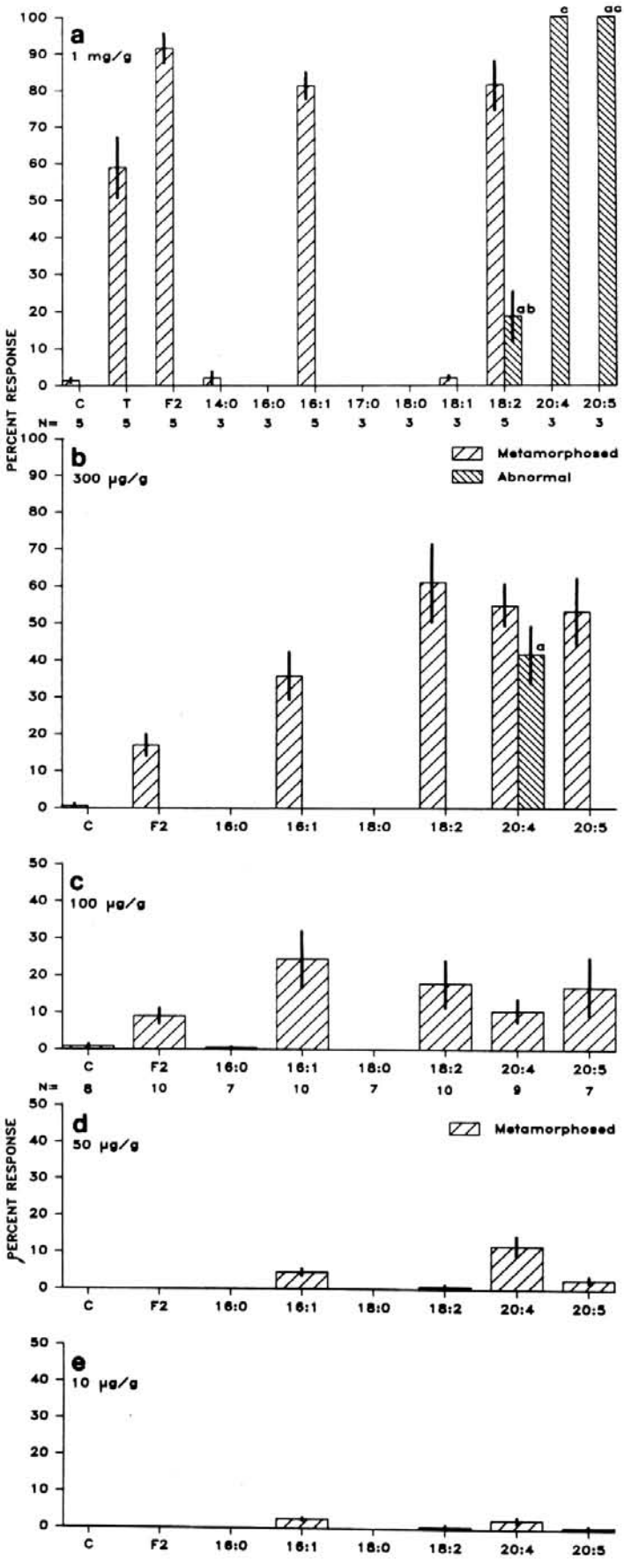

Fig. 4. *Phragmatopoma californica.* Mean percentage response of larvae to sand treated with free fatty acids (FFAs) (± SE; $N=5$ unless otherwise indicated). (a) 1 mg FFA g^{-1} sand (responses to control sand, C, unextracted tube sand, T, and HPLC-Fraction 2, F2, at same concentration are shown for comparison); (b) 300 μg g^{-1} sand; (c) 100 μg g^{-1} sand; (d) 50 μg g^{-1} sand; (e) 10 μg g^{-1} sand

ing and Abelson (1965) for fatty acids bound by the kerogen polymer matrix of sedimentary rocks.

Inductive FFAs may be aggregated on natural tubes so as to lead to an underestimation of their activity or concentration. In the assays undertaken in this study, extracts, fractions and FFA standards were uniformly spread over the surface of the sand grains. On natural tube sand, larvae may be encountering discrete patches of FFAs, possibly resulting in higher levels of larval metamorphosis than if the FFAs were evenly spread. It is known that the organic component of the tube matrix is secreted by adult sabellariids in the form of discrete globules (Vovelle, 1965; see photograph in Jensen and Morse, 1984). In addition, the low FFA-concentration estimate arrived at by organic solvent extraction may reflect localized concentrations of FFAs in the most anterior portions of tubes, where the cement matrix has been freshly secreted and can be contacted by planktonic larvae. Wilson (1970) reported a loss of inductive capacity as the sand tubes of *Sabellaria alveolata* aged. Jensen and Morse (1984) found that the posterior, older sections of tubes (5 cm or more from the tube opening) of *Phragmatopoma californica* induced very little larval settlement and metamorphosis. Reef blocks extracted in this study were anywhere from 10 to 20 cm in thickness, thus one-half to four-fifths of the reef volume was represented by older, posterior tubes. Unsaturated FFAs, especially polyunsaturates like 20:5 and 20:4, are subject to oxidative degradation over time (Parker, 1969; DeBarr *et al.*, 1983). Investigations are underway to determine whether one or a combination of these factors may be responsible for the low estimate of active FFAs in the natural tube matrix.

Possible source of FFAs in the tube matrix

Fatty acids are ubiquitous in both marine and terrestrial environments as important components of acylglycerides and phosphoglycerides in the lipids of prokaryotes and eukaryotes (Sargent and Whittle, 1981). Free fatty acids, however, are quite rare. Reports of the presence of more than trace amounts in extracts of living cells are thought to reflect the activities of lipases during extraction (Wood, 1974). In extracts of the worm-free sand/cement matrix of the tubes of *Phragmatopoma californica*, FFAs made up fully 10% of the extractable lipids. Slow enzymatic degradation of phospholipids is known to occur in frozen samples during storage (Sasaki and Capuzzo, 1984); in this study, tube sand was frozen immediately and lyophilized within 48 h of preparation. Bacterial lipids, in general, contain only saturated and monounsaturated fatty acids (Shaw, 1974; Volkman *et al.*, 1980). The presence of branch-chain C15 and C17 FFAs may imply some bacterial input, however. The relative composition of FFAs observed in HPLC-Fraction 2 is similar to that known for the esterified component fatty acids isolated from phytoplankton, particularly diatoms (DeMort *et al.*, 1972; Volkman *et al.*, 1980). However, given the paucity of FFAs in living cells, it seems unlikely that epiphytes associated with the tube sand are directly involved in the production of the FFAs responsible for larval induction of settlement and metamorphosis.

Histochemical studies of the organic matrix of tubes and the adult secretory glands of *Sabellaria alveolata* (Vovelle, 1965) indicated the presence of several osmophilic substances in the tube cement and in the glands associated with the building organ. It is hypothesized that the high levels of FFAs present in the tube matrix of *Phragmatopoma californica* are exuded upon lipolytic cleavage by the adult worms. The FFA composition may reflect the phytoplankton diet of adult worms, biosynthesis in the worm (see Sargent and Whittle, 1981), or a combination of these factors.

Concentration-dependence and specificity of induction of larval settlement and metamorphosis

Two results of the present work suggest receptor mediation of larval response. First, the response is concentration-dependent, as indicated by Fig. 5. Second, larval response appears highly dependent on the structure of the FFA. Saturated FFAs are ineffective at inducing settlement and metamorphosis; 16:1 is effective, but 18:1 is not (Fig. 4a). The progression of effective inducers from 16:1 to 18:2 to 20:4 and 20:5 indicates stereospecificity of larval response (Fig. 6). A double bond appears necessary in order for the FFA to be an effective inducer; greater levels of unsaturation at longer carbon chain lengths appear to preserve molecular length relative to 16:1, because double bonds both shorten and twist the fatty acid molecule (compare 16:1 and 20:5, Fig. 6).

Stereospecific responses to chemical cues are well characterized in some invertebrates, particularly in insects (Hansen, 1978). Among marine invertebrate larvae, Morse *et al.* (1980) revealed the stereospecific induction of metamorphosis in the larvae of red abalone by gamma-aminobutyric acid, a potent vertebrate neurotransmitter, and a limited number of structural analogs. In addition to stereochemical specificity and concentration-dependence, criteria of importance in defining pharmacologic receptors, usually by means of binding assays, include reversibility, saturability and tissue-specificity (Hollenberg and Cuatrecasas, 1979). Studies of invertebrate gustatory and olfactory chemoreception have not reached the same level of complexity (Hansen, 1978), and receptors in these systems are not as rigidly defined. Further work is required to assess the extent of the stereochemical specificity of larval response in *Phragmatopoma californica* and to determine whether additional receptor criteria are satisfied.

The abnormal response of larvae to high concentrations of polyunsaturated FFAs, especially 20:4 and 20:5, is intriguing. The abnormal response is partly similar to normal metamorphosis. Moreover, the abnormal response is replaced with a normal metamorphic response at lower FFA concentrations, and the two responses may occur in the

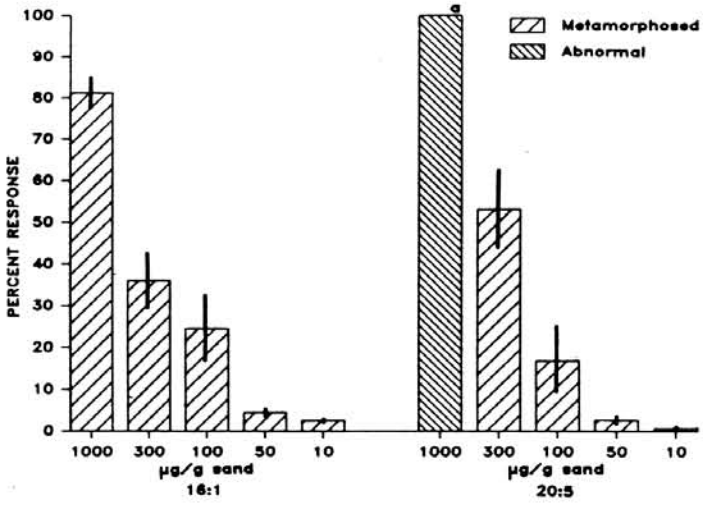

Fig. 5. *Phragmatopoma californica.* Concentration-dependence of response of larvae to FFAs. Data show mean percentage response to palmitoleic acid (16:1) and eicosapentaenoic acid (20:5) (from Fig. 4)

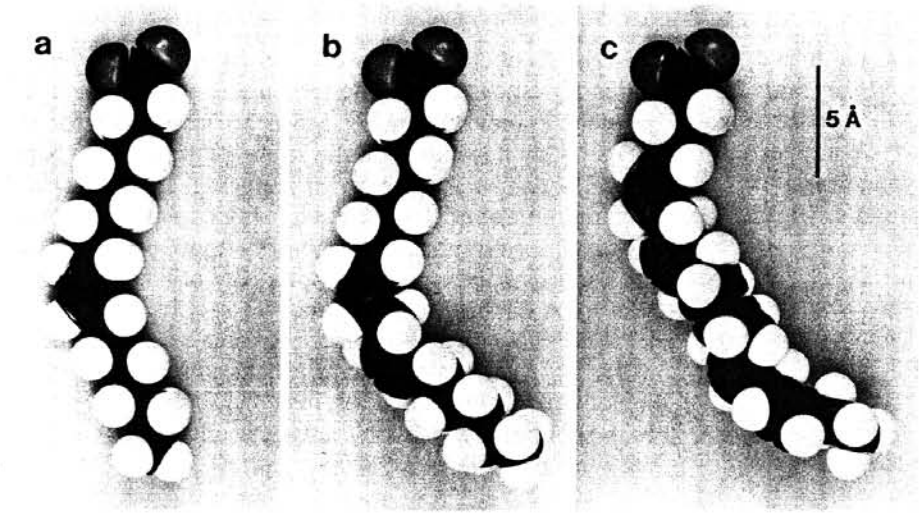

Fig. 6. *Phragmatopoma californica.* Space-filling models of the three most abundant inducers of larval settlement and metamorphosis found in purified extracts of tube sand (HPLC-Fraction 2). (a) Palmitoleic acid (16:1 – *cis* 9); (b) linoleic acid (18:2 – *cis* 9, 12); (c) eicosapentaenoic acid (20:5 – *cis* 5, 8, 11, 14, 17)

same assay dish (e.g., 18:2 at 1 mg g^{-1}). These facts suggest that the abnormal response is a FFA concentration-dependent aberration of the normal metamorphic response, possibly resulting from an overstimulation of the larval chemoreceptor. Such receptors may be located in the putative chemosensory organs described from the body surfaces of larval sabellariids by Eckelbarger (1978).

Phthalates in HPLC-Fraction 2

The presence of 4 to 12% phthalates in HPLC-Fraction 2 was, at first, perplexing. *Phragmatopoma californica* adults construct tube reefs out of suspended particles, including small amounts of man-made flotsam such as bits of plastic and styrofoam. *Sabellaria alveolata* has been reported to similarly incorporate plastics in building worm reefs in the Severn Estuary (Horne, 1982). Phthalates are common plasticizing agents in plastics and were probably extracted from the flotsam integrated into the tube matrix. A phthalate fraction purified from HPLC-Fraction 2 did not induce larval settlement and metamorphosis. This result was not surprising if one considers the structural dissimilarity between phthalates and the active FFAs.

Relationship to other work and conclusions

Studies of the chemical induction of larval settlement and metamorphosis have recently focused on hydrophilic biomolecules as chemical cues: proteins for barnacle cyprid larvae (e.g. Larman, 1984), proteins, amino acids, choline derivatives and related compounds for molluscs (e.g. Veitch and Hidu, 1971; Morse *et al.*, 1979; Hadfield, 1984; Morse *et al.*, 1984) and sugar moieties for spirorbid polychaetes (Kirchman *et al.*, 1982). In contrast, there have been some accounts of induction of larval settlement and metamorphosis by lipophilic substances in oysters by Keck *et al.* (1971), and in hydroids by Kato *et al.* (1975).

In their study of *Phragmatopoma californica* larval settlement and metamorphosis, Jensen and Morse (1984) found that the inductive capacity of worm tubes was de-

stroyed by boiling in distilled water for 15 min. This result is entirely consistent with the present study on *P. californica;* in boiling water, FFAs would be removed from the tube matrix and oxidative degradation of active, unsaturated FFAs would occur rapidly. Jensen and Morse report having obtained preliminary evidence that dihydroxyphenylalanine(DOPA)-related compounds were found at high concentrations in acid hydrolysates of tube cement and that DOPA analogs show varying degrees of activity at inducing larval settlement and metamorphosis. While the current study appears sufficient to explain chemical induction of larval settlement and metamorphosis in *P. californica,* additional chemical cues involving hydrophilic biomolecules cannot be ruled out.

Wilson (1968) demonstrated that the chemical cue for metamorphosis in *Sabellaria alveolata* was water-insoluble, stable to drying and unstable to acid, also consistent with the results reported herein. It remains to be seen whether other species of gregarious sabellariids respond to lipophilic inducers of settlement and metamorphosis.

Lipophilic substances such as FFAs are virtually insoluble in seawater, and, concordant with the ideas of Crisp (1981), remain adsorbed on the substrate, providing the advantage of relative permanence for larvae of the sort that rely on tactile stimulation to initiate settlement. Lipophilic cues may be found to play an important role in the initiation of larval settlement in other site-specific invertebrates as well.

Acknowledgements. This work was supported by NSF Grant CHE 81-21471 to D. J. Faulkner, and an NSF predoctoral fellowship to the author. I thank M. R. Amieva for assisting with larval culture and data acquisition, R. A. Lewin for use of algal culture facilities, L. Sweetman for operating the GC-mass spectrometer, N. D. Holland, R. G. Ackman, J. Nevenzel and E. F. DeLong for advice and comments, and D. J. Faulkner for spectral interpretation and for providing chemical insight.

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Date of final manuscript acceptance: December 6, 1985.
Communicated by R. S. Carney, Moss Landing