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Short Communication

Specific free fatty acids induce larval settlement and metamorphosis of the reef-building tube worm *Phragmatopoma californica* (Fewkes)

Joseph R. Pawlik and D. John Faulkner

Scripps Institution of Oceanography, University of California, San Diego, La Jolla, CA 92093, U.S.A.

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Abstract: Planktonic larvae of the Californian reef-building sabellariid polychaete worm, *Phragmatopoma californica*, settle and metamorphose in the presence of specific free fatty acids (FFAs) isolated from the sand matrix of adult tubes. Thirty-seven commercially-available FFAs of increasing acyl chain length and of variable unsaturation were assayed for their capacity to induce larval metamorphosis. At 100 μg FFA/g sand, settlement and metamorphosis peaked in response to palmitoleic acid (16 : 1; 24.5% metamorphosis versus 0.8% metamorphosis in control assays), linolenic acid (18 : 3, 24.9% metamorphosis), eicosapentaenoic acid (20 : 5; 16.9% metamorphosis) and docosahexaenoic acid (22 : 6; 8.7% metamorphosis), and decreased as the acyl chain length or the number of double bonds was changed. Palmitelaidic acid, the *trans* isomer of palmitoleic acid, was ineffective at inducing a larval response. Assays of the monoglycerides and methyl esters of palmitoleic and linoleic acids, *cis*-9-hexadecen-1-ol, *cis*-9,12-octadecadien-1-ol, the corresponding acetates and *cis*-9,12-octadecadiene resulted in little or no larval metamorphosis. Larval response is dependent on (1) the presence of at least one *cis* double bond in the molecule, (2) conservation of molecular shape with increasing acyl chain length by addition of *cis* double bonds, and (3) the presence of a free carboxyl group. This specificity resembles that found in studies of insect chemoreception and suggests that receptors may similarly mediate larval response in this marine invertebrate.

Key words: Settlement; Metamorphosis; Inducer; Free fatty acid; Gregarious; Polychaeta; Sabellariidae

Introduction. Once considered a random process, the larval settlement and metamorphosis of benthic marine invertebrates is now largely regarded as a response to complex, and often highly specific, environmental stimuli, with the larvae of most species prolonging their planktonic existence in the absence of a suitable substratum (for recent reviews, see Chia & Rice, 1978; Burke, 1983; Crisp, 1984). Physical stimuli are important in some cases (Crisp & Ghobashy, 1971), but larval substrate selection appears to be mediated primarily by chemical signals, especially among species which show preferences for microbial films (e.g., Müller, 1973; Kirchman *et al.*, 1982), prey or host plants or animals (e.g., Nishihira, 1968; Morse *et al.*, 1979; Hadfield & Scheuer, 1985), or adult congeners (e.g., Wilson, 1968; Bayne, 1969; Crisp, 1979; Highsmith, 1982). Most chemical inducers of larval settlement are believed to be bound to the substratum, requiring contact for their recognition (Crisp, 1965), although several

exceptions to this generality have been described (references cited in Crisp, 1984; also see Cuomo, 1985; Hadfield & Scheuer, 1985).

Isolation and identification of the naturally-occurring compounds implicated in larval settlement have proven difficult in a majority of the studies undertaken, and consequently, no chemoreceptors responsible for substratum recognition have been unambiguously characterized. Nevertheless, considerable information has been gathered to this end. The substance responsible for the gregarious settlement of the larvae of some barnacle species near conspecific adults was determined to be a group of structural proteins (reviewed in Crisp, 1984). Larval settlement of the spirorbid polychaete *Janua brasiliensis* onto microbial films has been attributed to the specificity of larval lectins for glycoconjugates in the exopolymer produced by film-forming bacteria (Kirchman *et al.*, 1982; Maki & Mitchell, 1985). Neurotransmitter-like molecules induce larval settlement in several molluscan species (gastropods: Bonar, 1976; Morse *et al.*, 1979; Hadfield, 1984; Morse, 1985; bivalves: Cooper, 1982; Coon *et al.*, 1985; Weiner *et al.*, 1985), although these molecules may act upon the larval nervous system rather than on a surface chemoreceptor (Hirata & Hadfield, 1986) and may not be responsible for the natural induction of larval settlement and metamorphosis.

Chemical induction of invertebrate larval settlement and metamorphosis does not appear to be limited to the action of hydrophilic biomolecules, however. Nishihira and coworkers (Kato *et al.*, 1975) isolated and identified diterpenoid chromanols from hexane extracts of the alga *Sargassum tortile* which promoted larval settlement of the epibiotic hydroid *Coryne uchidai*. Keck *et al.* (1971) similarly isolated lipid-soluble substances that enhanced settlement of oyster larvae. Metalloporphyrins fractionated from organic extracts of oil shale promoted the settlement of barnacle larvae (Hill & Holland, 1985). Stimulatory effects have also been noted for inorganic compounds. Sulfide greatly enhanced settlement of the polychaete *Capitella* at concentrations ranging between 0.1 and 1.0 mM (Cuomo, 1985), and sea water artificially augmented with monovalent cations has been shown to induce metamorphosis in a wide range of invertebrate species (Müller, 1973b; Yool *et al.*, 1986), presumably as a result of alterations in cell membrane potentials.

The larvae of gregarious sabellariid polychaetes settle and metamorphose with a high degree of specificity on the sand/cement tube matrix of adult conspecifics over other potential substrata (Wilson, 1968; 1970; Jensen & Morse, 1984). Chemical inducers of larval settlement and metamorphosis in this group have long been believed responsible for this behavior (Wilson, 1968). Pawlik (1986) recently isolated naturally-occurring inducers of larval settlement and metamorphosis from the tube matrix of *Phragmatopoma californica*, a reef-forming sabellariid from California. The inducing molecules were identified as free fatty acids (FFAs); in particular, eicosapentaenoic acid (20 : 5), palmitoleic acid (16 : 1) and linoleic acid (18 : 2) were most abundant. Other FFAs identified as constituents of the active fraction, including 16 : 0, 18 : 0 and 18 : 1, were ineffective at inducing metamorphosis, indicating some specificity of larval response.

Morse *et al.* (1980) described metamorphosis of larval abalone (*Haliotis rufescens*) on exposure to the neurotransmitter γ-aminobutyric acid (GABA) and structurally related analogs. They found larval response dependent on carbon chain length, the presence of terminal carboxylic acid and amine groups and the stereochemistry of the molecules tested. In the present study, 37 FFAs of variable acyl chain length and unsaturation and nine FFA derivatives were assayed to determine the extent to which the metamorphic response of *P. californica* is dependent on FFA molecular structure.

Materials and methods. The larval culture and assay procedures used in this study were identical to those described previously (Pawlik, 1986). Planktotrophic larvae of *Phragmatopoma californica* were reared at 20 °C on a 1 : 1 mixture of *Phaeodactylum tricornutum* Bohlin and *Pavlova* (= *Monochrysis*) *lutheri* Droop at a concentration of 10^5 cells/ml. Thirty larvae, 20 to 30 days old, were transferred to 100 mm-diameter polystyrene Petri dishes filled with 50 ml of sea water. Each dish contained 1 g of Ottawa sand (cement-testing standard, 20–30 mesh, Fisher Scientific, surface area $\simeq 36$ cm^2/g) that had been treated either with a diethyl ether solution of FFA or FFA derivative, or with diethyl ether alone (control), and subsequently placed under vacuum to remove all traces of solvent. After 24 h at 20 °C, larvae were scored as having not metamorphosed (swimming or crawling, no loss of provisional setae or rotation of larval tentacles), metamorphosed (attachment, loss of provisional setae, rotation of larval tentacles, tube construction commenced) or having undergone an abnormal response (loss of provisional setae and rotation of larval tentacles without attachment or tube formation, sometimes death). Unless otherwise indicated in the figures, each experiment was run with five replicates. For ease of comparison, data from assays of nine FFAs (14 : 0, 16 : 0, 16 : 1c, 17 : 0, 18 : 0, 18 : 1, 18 : 2, 20 : 4, 20 : 5) identified from extracts of the sand tubes of adult *Phragmatopoma californica* and presented in Pawlik (1986), were appended to data from assays of the additional 28 FFAs.

FFAs and FFA derivatives were purchased from Sigma Chemical Co. (St. Louis, MO) and Nu Chek Prep Inc. (Elysian, MN) at the highest available purity. Double bond placement of the unsaturated FFAs was as follows (the number of carbon atoms precedes the colon, the number of double bonds follows; c = *cis*, t = *trans*; carbon number for double bond placement counted from the carboxylic acid end): 13 : 1 c12; 14 : 1 c9; 15 : 1 c10; 16 : 1 t9; 16 : 1 c9; 17 : 1 c10; 18 : 1 c6; 18 : 1 c9; 18 : 1 c11; 18 : 2 c9, 12; 18 : 3 c9, 12, 15; 18 : 3g c6, 9, 12; 18 : 4 c4, 8, 12, 15; 19 : 1 c10; 20 : 1 c11; 20 : 2 c11, 14; 20 : 3 c11, 14, 17; 20 : 4 c5, 8, 11, 14; 20 : 5 c5, 8, 11, 14, 17; 22 : 1 t13; 22 : 1 c13; 22 : 2 c13, 16, 19; 22 : 4 c7, 10, 13, 16; 22 : 6 c4, 7, 10, 13, 16, 19; 23 : 1 c14; 24 : 1 c15. FFA derivatives used in assays were the monoglyceride, methyl ester, alcohol and alcohol acetate ester of 16 : 1 and these four derivatives plus the n-alkene of the 18 : 2 series.

Results. The responses of *Phragmatopoma californica* larvae to FFAs of variable chain length and unsaturation at three concentrations are presented in Fig. 1. At 1 mg FFA/g

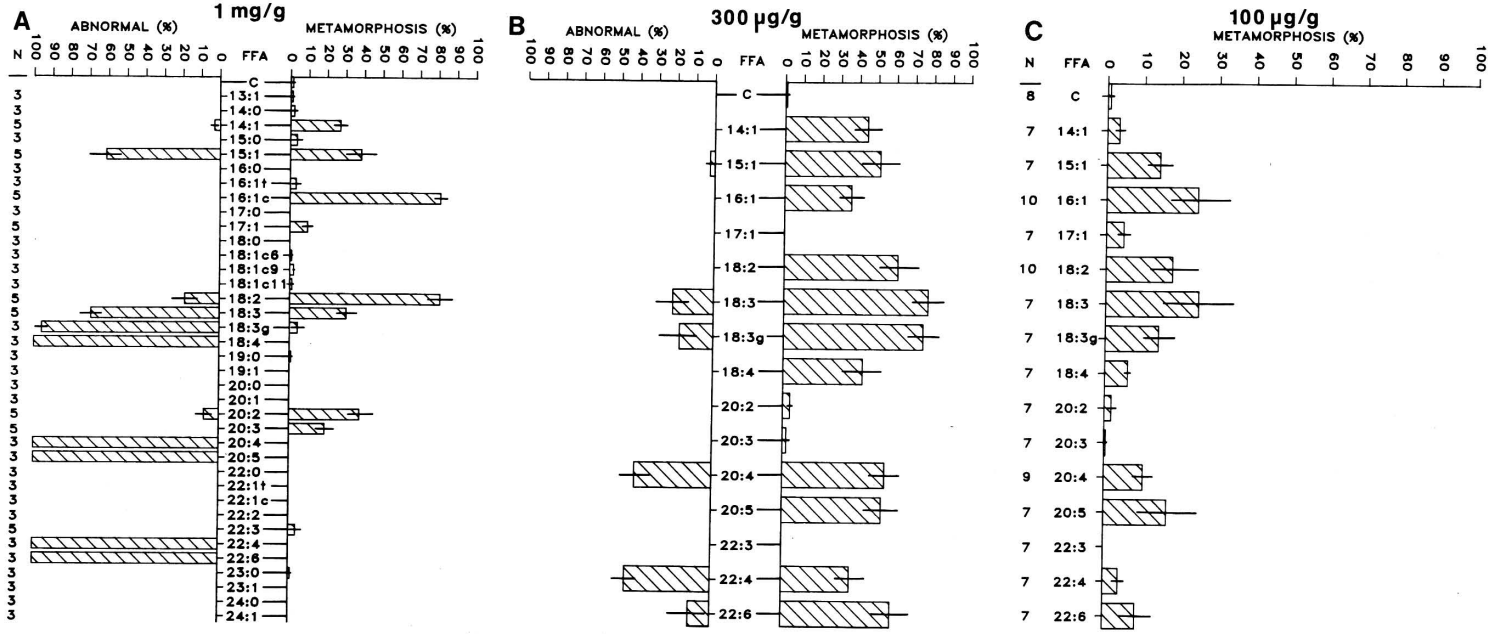

Fig. 1. Mean percentage response (± SE) of *Phragmatopoma californica* larvae to control sand (c) and sand treated with free fatty acids (FFAs) at (A) 1 mg/g sand, (B) 300 µg/g sand, (C) 100 µg/g sand: note change in scale for (C); thirty larvae assayed per replicate; $n = 5$ replicates unless otherwise indicated.

sand, there was little or no response to fully saturated FFAs ranging from 14 to 24 carbon atoms in length. The *trans* isomer of 16 : 1 (palmitelaidic acid) was ineffective at inducing larval metamorphosis, while the *cis* isomer (palmitoleic acid) induced 81% metamorphosis at the same concentration. The level of larval response decreased as the acyl chain was increased in length: 17 : 1 induced an average of 10% larval metamorphosis, 18 : 1 induced a maximum of 2% metamorphosis (placement of the *cis* double bond at the 6, 9 and 11 position gave no significant difference; $P > 0.05$; paired *t*-test of arcsine transformed data; Sokal & Rohlf, 1981), 19 : 1, 20 : 1, 22 : 1, 23 : 1 and 24 : 1 were completely ineffective at stimulating a larval response. At 100 μg/g sand, larval metamorphosis similarly peaked upon exposure to 18 : 3, 20 : 5, and 22 : 6 (Fig. 1C). The abnormal metamorphic response also varied in a structure-dependent fashion at 1 mg/g sand (cf. 18 : 2, 18 : 3, 18 : 3 g and 18 : 4, Fig. 1A) and was replaced by normal metamorphosis at lower concentrations (Figs. 1B, 1C). Exposure of larvae to the polyenoic acids 18 : 4, 20 : 4, 20 : 5, 22 : 4 and 22 : 6 at 1 mg/g sand resulted in 100% larval mortality. In all other cases, larvae which responded abnormally had shed their provisional setae and rotated their larval tentacles forward, as in normal metamorphosis, but had failed to form a primary mucoid tube and appeared lethargic.

Modification of the carboxylic acid portion of the inducer molecules 16 : 1 and 18 : 2 (Fig. 2) resulted in the loss of metamorphosis-inducing capacity (Fig. 3). Assays of the monoglyceride, methyl ester, alcohol and alcohol acetate ester of 16 : 1 and these four derivatives plus the *n*-alkene of the 18 : 2 series yielded little or no larval response.

Discussion. The response of *Phragmatopoma californica* larvae to the assayed FFAs and FFA derivatives was highly specific with regards to the length and conformation

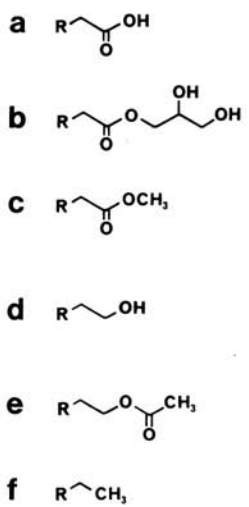

Fig. 2. End-group structures of a FFA (a) and of FFA derivatives (b–f): b: monoglyceride: c: methyl ester; d: alcohol; e: acetate; f: alkene.

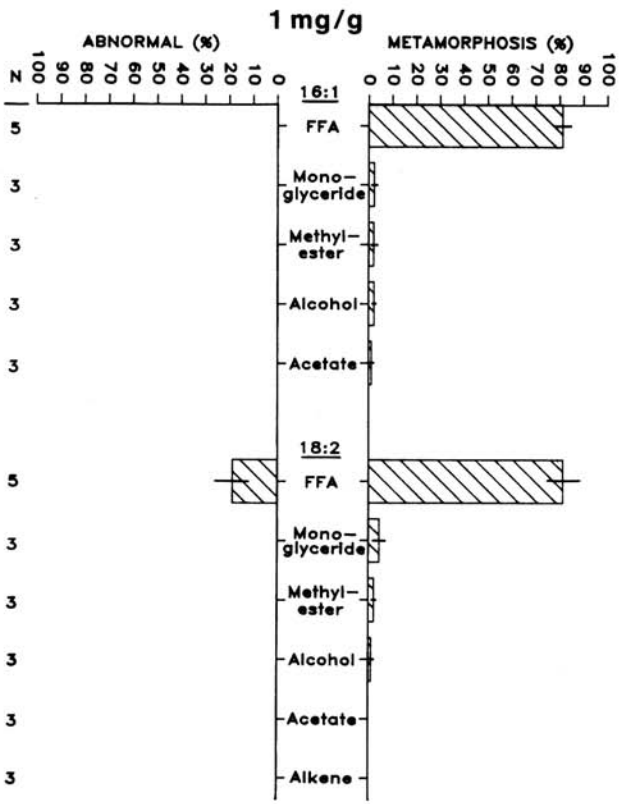

Fig. 3. Mean percentage response (± SE) of *Phragmatopoma californica* larvae to sand treated with the FFA, monoglyceride, methyl ester, alcohol and alcohol acetate ester of 16 : 1 and these five plus the *n*-alkene of 18 : 2 at 1 mg/g sand (refer to Fig. 2).

of the acyl chain and the presence of the carboxylic acid functional group. Larval response was stereospecific for the *cis* isomer of 16 : 1, and a *cis* double bond was required for inductive activity among the FFAs ranging from 14 to 17 carbon atoms in length. The greater acyl chain length of 18 : 3, 20 : 5, and 22 : 6 appeared to be offset by additional double bonds which both shorten inter-carbon bond distances and twist the FFA molecule, preserving a similar overall molecular geometry and metamorphosis-inducing activity (Fig. 4). In addition to the importance of chain length, geometry and stereochemistry, the carboxylic acid functional group was also necessary for induction of larval metamorphosis, with larval response effectively attenuated upon alteration of the terminal carboxylic acid functional group.

Abnormal larval metamorphosis in response to high concentrations of some FFAs was marked by many of the behavioral components of a normal metamorphic response, including rotation of larval tentacles and loss of provisional setae, but without larval attachment or mucoid tube formation. Exposure to some polyenoic FFAs (e.g. 20 : 4,

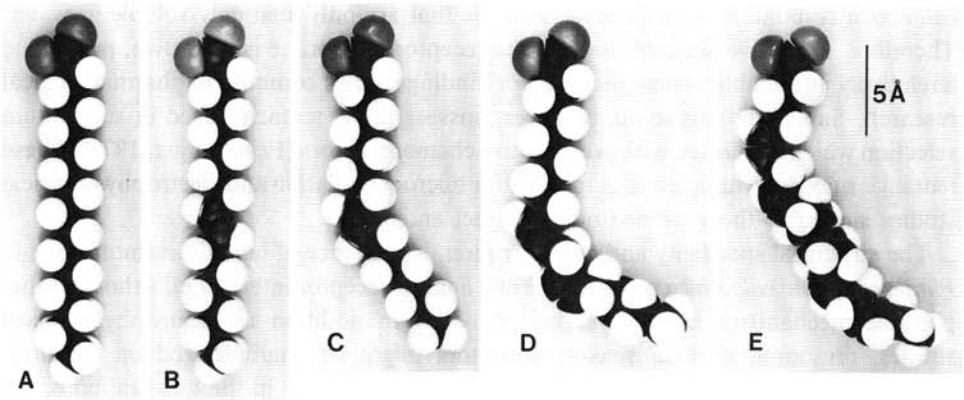

Fig. 4. Space-filling molecular models of the most active inducers of larval metamorphosis (C, D, E) compared with inactive FFAs (A, B): A: palmitic acid (16 : 0); B: palitelaidic acid (16 : 1 – *trans* 9); C: palmitoleic acid (16 : 1 – *cis* 9); D: linolenic acid (18 : 3 – *cis* 9, 12, 15); E: eicosapentaenoic acid (20 : 5 – *cis* 5, 8, 11, 14, 17); FFAs shown in anionic forms.

20 : 5, 22 : 4, 22 : 6) at 1 mg/g sand further resulted in larval death within the 24-h assay period. The FFA concentrations responsible for abnormal effects were nearly two orders of magnitude greater than those extracted from natural tube sand (Pawlik, 1986), and, as extraction of FFAs was believed to be incomplete, at least one order of magnitude greater than those encountered by larvae under natural conditions. Polyenoic FFAs appear to have toxic effects at these elevated concentrations. Similar toxicity has been observed in studies of molluscan metamorphosis in response to neuroactive inducers at high concentrations (Morse *et al.*, 1979; Coon *et al.*, 1985).

The specificity of *Phragmatopoma californica* larval response to FFAs bears a striking similarity to responses found in studies of insect chemoreception, in which the carbon skeleton, functionality and stereochemistry of the stimulatory molecules are particularly important (Hansen, 1978; Prestwich, 1985). Unlike olfactory chemoreception, in which large and complex antennae may perceive volatile pheromones on a per molecule basis, contact chemoreception in insects involves the tactile perception of nonvolatile chemical cues at considerably higher concentrations (Städler, 1984). Shimada (1978), in a study of the labellar contact chemoreceptor of the fleshfly, *Boettcherisca peregrina*, demonstrated that receptor response was dependent on the chain length, shape and functionality of various short-chain fatty acids tested as 10 mM solutions. FFA chain length and saturation were important in eliciting an olfactory response in *Necrophorus* beetles (Boeckh, 1961). Exposure to FFAs of four to nine carbon atoms in length induced depolarization of the receptor nerve, FFAs of 12 to 18 carbon atoms in length were non-stimulatory, but exposure to 18 : 1 (oleic acid) resulted in nervous depolarization.

As in the case with chemoreception in many insects, larvae of *Phragmatopoma*

californica respond to stimulatory chemicals that are only sparingly soluble in water. Therefore, the active concentration at the receptor membrane is unknown, presenting a relatively intractable system for receptor binding studies common to pharmacological research. Sabellariid larvae do, however, possess larval tentacles used in substratum selection which are dotted with presumptive chemoreceptors (Eckelbarger, 1978). These tentacles may be of sufficient size to allow for microcannulation and electrophysiological studies similar to those carried out on insect antennae.

The structural specificity and concentration dependence of larval metamorphosis in *Phragmatopoma californica* is suggestive of a ligand–receptor interaction, although other possible mechanisms cannot yet be excluded. In addition to electrophysiological studies, presumptive chemosensory receptors might be characterized on an ultrastructural level, with radiolabelled FFA molecules employed in the localization of the site of inducer–receptor interaction.

The phenomenon of gregarious larval settlement is widespread among benthic marine invertebrates and offers distinct advantages to members of the resulting aggregations (Crisp, 1979). The polychaete family Sabellariidae includes species which build solitary tubes, others that form massive aggregations throughout their range and some that form aggregations only at specific localities within a broader range where they are otherwise non-gregarious (Pawlik & Faulkner, 1986). The diversity of settlement strategies within this group affords an opportunity to decouple the relative importance of chemical inducers from other factors that might influence larval settlement, including physical cues (e.g. surface texture, water flow) or developmental constraints (e.g. ability to delay metamorphosis) by comparing the larval development and settlement responses of representative gregarious and non-gregarious species.

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