ases [L. S. Hurley, *Physiol. Rev.* **61**, 249 (1981); R. M. Leach, A. M. Muenster, E. M. Wein, *Arch. Biochem. Biophys.* **133**, 22 (1969).] Within these ranges of manganese and copper intakes, no differences in the severity or onset of scoliosis were observed. Results were identical to those shown in Fig. 1 for chicks (3 to 12 weeks old) given copper at 8 μg/g.

8. Birds were sedated and examined after anterior and posterior roentgenography of the spine (*1*, *11*).
9. J. R. Cobb, "Outline for the study of scoliosis," in *Instructional Course Lectures* (Mosby, St. Louis, 1964), vol. 5, p. 261.
10. The fourth thoracic vertebra was used in the analysis of bone copper. After being cleansed of adhering tissue, the vertebra was dissolved in 70 percent nitric acid for copper analysis by atomic absorption spectrophotometry.
11. A midsection of bone was taken for estimation of the reducible cross-linking amino acids hydroxylysinonorleucine (HLNL) and dihydroxylysinonorleucine (DHLNL). The samples (50 mg) were demineralized in 0.2*M* EDTA and dialyzed. The residue was collected by centrifugation (10000*g* for 60 minutes) and lyophilized. One-milligram portions were then reduced with [^{3}H]$NaBH_3CN$ (96 mCi/mg, New England Nuclear) as described by S. P. Robins and A. J. Bailey [*Biochem. J.* **163**, 339 (1977)]. After hydrolysis, HLNL and DHLNL were identified and separated with the chromatographic system described by R. Stack *et al.* [*Appl. Environ. Microbiol.* **46**, 539 (1983)] and hydroxyproline was assayed as described by J. R. Woessner [*Arch. Biochem. Biophys.* **93**, 440 (1961)].
12. On a dry weight basis, human diets in the United States typically contain 3 to 5 μg of copper per gram, or an amount approaching the lower limit of the copper requirements of most animals [K. E. Mason, *J. Nutr.* **109**, 1979 (1979); L. Klevay, *Biol. Trace Elem. Res.* **5**, 245 (1981)].
13. Supported in part by PHS grant AM 25358.
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13 January 1984; accepted 21 March 1984

Pheromonal Control of Metamorphosis in the Pacific Sand Dollar, *Dendraster excentricus*

Abstract. *Competent larvae are induced to undergo metamorphosis by sand from a sand dollar bed or an aqueous extract of the sand. Gel permeation chromatography and high-performance liquid chromatography of the extract yielded a 980-dalton peptide that will induce metamorphosis between 10^{-6} and 10^{-5} molar. Extracts of whole adults and gonads were also able to induce metamorphosis, and adults can condition substrates to induce metamorphosis. Therefore, the initiation of metamorphosis in* Dendraster excentricus *is controlled by a pheromone released by adult sand dollars.*

Larvae of many benthic marine invertebrates settle and metamorphose after a prolonged planktonic phase. In numerous species the larvae have been shown to metamorphose preferentially in habitats that are suitable for the juvenile or the adult (*1*). Larvae apparently develop in the plankton until they are competent to metamorphose and remain so until they encounter cues associated with preferred benthic habitats. Cues have been shown in several species to be physical or chemical factors that initiate the developmental sequences of metamorphosis (*2*). I now report that larvae of the echinoid *Dendraster excentricus* are rapidly induced to metamorphose by a chemical secreted into sand by adult sand dollars.

Larvae grown in laboratory culture are competent to metamorphose after 4 to 6 weeks (*3*). When exposed to sand from a sand dollar bed, 82.5 ± 9.6 percent (mean ± standard deviation; four trials) metamorphosed within 24 hours; when exposed to sand collected from a similar, adjacent area without sand dollars, 2.5 ± 5.0 percent (four trials) metamorphosed. Competent larvae not exposed to sand but maintained under culture conditions have been kept competent for over 5 months with less than 5 percent metamorphosing spontaneously. Lyophilized, aqueous extracts of sand from a sand dollar bed will also rapidly induce metamorphosis. Of larvae treated with 5 mg of extract per milliliter, 92.5 ± 9.5 percent (four trials) metamorphosed within 1 hour (*4*). The same concentration of an extract of sand from outside the sand dollar bed induced 5.0 ± 5.7 percent (four trials) to metamorphose. These results indicate that an extractable component of the sand from sand dollar beds will induce metamorphosis.

Gel permeation chromatographs of extracts indicated that the activity eluted in a single peak (Fig. 1A). When compared with the standards (tryptophan and vitamin B_{12}), the active peak had a molecular size of 980 ± 110 daltons. By reversed-phase high-performance liquid chromatography (HPLC), the active peak from gel permeation chromatographs was shown to contain several components (Fig. 1B); however, activity was only detected in a single peak. Spectrophotometric scans of active fractions showed absorption maxima at 206 and 266 nm. Active fractions also reacted positively with the Lowry (biuret/folin–phenol) method for protein determination (Fig. 1A). Activity in fractions purified by gel permeation was significantly reduced by treatments with insoluble protease E (16.7 ± 9.8 percent; three trials), trypsin (46.7 ± 11.5 percent; two trials), or heat (0; two trials) (*5*). These findings indicate that the active substance is probably a peptide (*6*).

The dose-response curve of fractions purified by gel permeation showed that

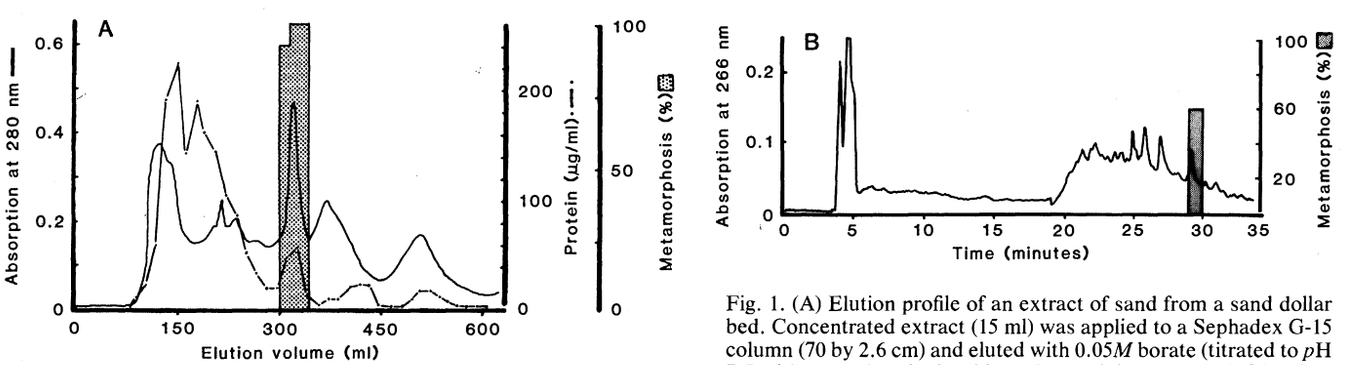

Fig. 1. (A) Elution profile of an extract of sand from a sand dollar bed. Concentrated extract (15 ml) was applied to a Sephadex G-15 column (70 by 2.6 cm) and eluted with 0.05*M* borate (titrated to *p*H 7.5 with potassium hydroxide and containing 0.03*M* NaCl). Flow rate was 1 ml per minute at 4°C, and 15-ml fractions were collected. Protein concentration of collected fractions was determined by the Lowry method (see text). For the bioassay, 0.5 ml of each fraction was mixed with 0.5 ml of twofold concentrated artificial seawater (ASW; Marine Biological Laboratories formula). Ten competent larvae were added, and the number metamorphosed was counted after 1 hour. (B) Separation by reversed-phase HPLC of the components of the active peak from a single gel permeation chromatography run. A Supelcocil LC-18 column (250 by 4.6 mm) with an LC-18 guard column was used with a Varian 5000 instrument and Vari-chrome detector. The sample was concentrated with a C18 Sep Pac (Waters), and a 0.8-ml sample was injected. The mobile phase was 10 percent acetonitrile (AN) and 0.25*M* formic acid [buffered to *p*H 6.5 with triethylamine (TEAF)] for the first 10 minutes. A 1 percent ascending gradient to 60 percent AN:TEAF was used to elute components. Flow rate was 1.0 ml per minute, and maximum pressure was 140 atmospheres. One-milliliter fractions were collected, concentrated with a vacuum centrifuge, and dissolved in 0.2 ml of ASW before being assayed for activity. The base line from a blank run, in which 0.8 ml of TEAF was injected, has been subtracted from the data presented.

activity reached a maximum at about 10 μg of protein per milliliter and was reduced to less than 50 percent at 1 μg of protein per milliliter (Fig. 2). Thus, for a peptide with a molecular size of 980 daltons, maximum response was reached between 10^{-6} and $10^{-5}M$. The purified substance induced metamorphosis simultaneously within 3 to 5 minutes, and the transformation to a benthic juvenile was essentially completed in 45 minutes (Fig. 3). The sequence of events of peptide-induced metamorphosis was identical to that observed with other forms of induction, and the juveniles appeared normal. Extracts of sand obtained from intertidal populations of *D. excentricus* (Esquimalt Lagoon, Victoria, British Columbia) and subtidal populations (Breaker Beach, Bamfield, British Columbia) had the same gel permeation elution characteristics and acted equally well on larvae derived from adults of the intertidal population.

To show that adults are the source of the peptide, I transferred six adult sand dollars to a bowl of fine marble sand and six others to a bowl of beach sand. After 6 weeks the marble sand induced metamorphosis in 62.5 ± 12.6 percent (mean ± standard deviation; four trials) of the larvae tested, whereas marble sand from a bowl containing no sand dollars induced 7.5 ± 9.5 percent metamorphosis. Beach sand that had been inhabited by sand dollars for 6 weeks induced metamorphosis in 67.5 ± 12.5 percent of the larvae, and control beach sand induced metamorphosis in 5.0 ± 10.0 percent. Lyophilized aqueous extracts of whole adults or gut tissues of adults induced metamorphosis in significantly higher percentages than did controls or extracts of body wall or coelomic fluid (Fig. 4). Extracts of gonad-induced metamorphosis in about the same proportion of the larvae tested as did extracts of sand from sand dollar beds. Gel permeation chromatographs of gonad extracts had a small peak of activity that eluted at the same volume as the active peak from extracts of sand. Thus, the peptide appears to be derived directly from adult tissues and to be released into the sand.

Pheromones are defined as chemicals released into the environment that evoke behavioral, physiological, or developmental responses in conspecifics (*7*). The peptide described fits this definition well. Pheromones controlling behavioral or physiological responses have been described or inferred in several groups of marine invertebrates (*8*), but pheromones controlling developmental processes are common only in insects in terrestrial environments (*7*). The phenomenon of gregarious recruitment in marine invertebrates is widespread, and the presence of adults has been shown to play a role in inducing metamorphosis in barnacles (*9*), oysters (*10*), clams (*11*), ascidians (*12*), sipunculans (*13*), holothuroids (*14*), crinoids (*15*), and polychaetes (*16*). In some species, chemical cues associated with adults are thought to mediate (*9–11*), although they are not commonly referred to as pheromones (*1, 2*). Because the induction of metamorphosis in some marine invertebrates fits the concept of pheromonal communication, analysis with reference to this means of chemical communication in animals may benefit our understanding of the underlying mechanisms and their evolution.

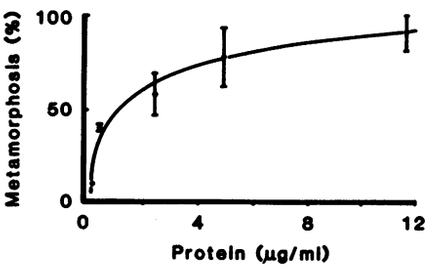

Fig. 2. Dose-response curve of an extract of sand purified by gel permeation. Protein concentrations were determined by the Lowry method (see text), and samples were diluted with distilled water to obtain ranges of concentrations. The samples were mixed with an equal volume of twofold concentrated ASW, and competent larvae were added. Metamorphosis was scored after 1 hour. Data presented are mean ± 1 standard deviation of 20 larvae (two trials).

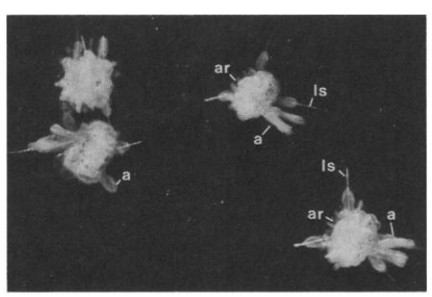

Fig. 3. Larvae of *Dendraster excentricus* that are simultaneously metamorphosing 11 minutes after treatment with an extract of sand (10 μg/ml) purified by gel permeation. Adult rudiments (*ar*) have been everted, and the larval arms (*a*) have begun retraction from the larval skeleton (*ls*).

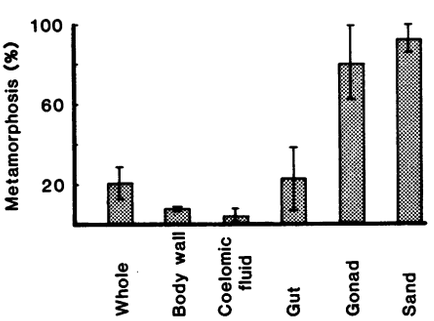

Fig. 4. Bar graphs showing the percentage of metamorphosis achieved after 1 hour when competent larvae were treated with lyophlized aqueous extracts (1 mg/ml). Results are mean ± 1 standard deviation of 50 larvae (four trials).

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2. R. D. Burke, *Can. J. Zool.* **61**, 1701 (1983).
3. Larval culture procedures outlined in R. D. Burke, *Biol. Bull.* **164**, 176 (1983).
4. Extract was prepared by removing salt water from sand, freezing, and then heating to 70°C in an equal volume of distilled water. Acetic acid was added to a final concentration of 0.1*M*, and the slurry was stirred for 20 minutes. Sand was removed, and the filtrate was frozen and lyophilized.
5. Insoluble protease E (10 mg/ml) (Sigma, 0.6 to 0.8 U/ml) was stirred constantly with partially purified extract (1 mg/ml) in phosphate-buffered saline for 2 hours. Controls contained no protease E. Trypsin (1 mg/ml) (Sigma) was incubated in salt water containing partially purified extract (6 mg/ml) for 1 hour. Soybean trypsin inhibitor (2 mg/ml) (Sigma) was added 20 minutes before activity was assayed. Partially purified extract (6 mg/ml) was heated in salt water to 100°C for 30 minutes, cooled, restored to initial volume with distilled water, and assayed.
6. This conclusion is similar to that reported by R. C. Highsmith [*Ecology* **63**, 329 (1982)] who treated sand with various proteases.
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17. Supported by a Natural Sciences and Engineering Research Council of Canada University Research Fellowship and operating grant and a University of Victoria Faculty Research Award. I thank S. K. Chaudhary, J. N. Owens, and R. G. B. Reid for the use of equipment; N. J. Sherwood for advice on HPLC; and R. A. Cloney, J. Purcell, N. J. Sherwood, G. D. Caine, and A. W. Gibson for comments on the manuscript.

9 January 1984; accepted 24 April 1984