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LARVAL INVERTEBRATE WORKSHOP

FACTORS INFLUENCING LARVAL METAMORPHOSIS IN *GOLFINGIA MISAKIANA* (SIPUNCULA)

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ABSTRACT

Experiments on settlement and metamorphosis of oceanic larvae of the sipunculan worm, *Golfingia misakiana,* have demonstrated that under laboratory conditions sea water previously occupied by adults of that species will enhance percentage metamorphosis in the presence of natural substratum. The effective factor associated with the adult is a water-soluble, heat-labile substance of low molecular weight (less than 500) that shows some degree of species-specificity. It is stable at room temperature for at least 8 days and is not dependent on the presence of micro-organisms in the water. Larvae respond to the factor only in combination with untreated substratum into which they burrow at the time of metamorphosis. The exact role of the substratum is not clearly defined by the experiments; possible functions are as an adsorptive surface for inducers, potential source of critical microorganisms or food material, or an essential medium for larval burrowing.

Numerous investigations have demonstrated that settlement and metamorphosis of larvae of gregarious species of marine invertebrates may occur in response to cues emanating from the adults (reviews by Crisp, 1974 and Scheltema, 1974). Most of these studies have focused on invertebrates living on hard substrata such as barnacles (Lewis, 1978), the serpulid polychaetes *Pomatoleios kraussi* (Crisp, 1977) and *Hydroides dianthus* (Scheltema et al., 1981), the oyster *Ostrea edulis* and *Crassostrea virginica* (Knight-Jones, 1951; Crisp, 1967; Bayne, 1969; Hidu, 1969), the holothurian, *Psolus chitinoides* (Young and Chia, 1982), and ascidians (Young and Braithewaite, 1980). A few recent studies have concentrated on larval settlement in gregarious infaunal invertebrates as influenced by stimuli from adults. Highsmith (1982) demonstrated that larvae of the echinoid *Dendraster excentricus* settle and metamorphose in response to the presence of adults and to a low molecular weight substance (<10,000) found in adult-exposed sand. Extracts from adult tissues of this species were shown further by Burke (1984) to contain a peptide (980 daltons) which, in the absence of substratum, elicited larval metamorphosis. Larvae of the echiuran, *Urechis caupo,* have been reported by Suer and Phillips (1983) to settle rapidly in response to a chemical from the skin of adults, but the response occurred only when the substance was adsorbed onto a surface. Preliminary reports on the sipunculan, *Golfingia misakiana,* suggested that adults may release a metamorphosis-inducing factor into sea water which, in combination with substratum, is effective in enhancing larval metamorphosis (Rice, 1978; Rice and Murdoch, 1978).

In the present paper the preliminary reports on *Golfingia misakiana* are substantiated and extended, the properties of the metamorphosis-inducing factor further characterized, and the role of the substratum examined.

MATERIALS AND METHODS

Adult specimens of *Golfingia misakiana* were collected off the central east coast of Florida northeast of Fort Pierce (27°49.5'N, 79°57.3'W) in 75–100 m of water where they inhabit cavities and interstices in oculinid coral rubble. After collection they were extracted from the coral and maintained for experimental use in fine sediment in finger bowls submerged in a recirculating sea water system. For routine preparation of the adult-exposed water used in these experiments, the specimens were sieved

from the sediment and placed in sea water in covered gallon jars (20 adults per 500 ml) and held for 4 days prior to experimentation. The water was then decanted from the adults and used in the experiments described below. Water prepared in this manner will be referred to in this paper as "adult-conditioned water." Control water was kept in jars without animals for a comparable time period preceding the experiments.

Substratum, characterized as fine sand and silt, was collected in a location generally near that of the adult worms or a few km south where the same type of sediment is found (27°27.0'N, 79°53.5'W) at a depth of about 200 m. For experimentation that portion of the sediment was used that would pass through a Nitex (nylon myofilament) screen with mesh openings of 102 μm (Tetko, Inc.). This screen size was selected because it would retain the larvae so that they could be readily recovered for counting and observation at the end of an experiment. An analysis of sediment after sieving showed 84% of the sample to be coarse to medium silt and 16% very fine sand. Organic carbon, as determined by ashing, was 14% by weight. Between the time of collection and use in experiments, the sediment was maintained in aerated sea water.

Larvae were collected in the Florida Current off the east coast of Florida 32 to 40 km east of the Fort Pierce Inlet over bottom depths of 200 to 270 m. Fifteen- to 20-min surface tows were made with a net 0.5 m in diameter, having a mesh of 130 μm. Upon return to the laboratory, the larvae were sorted, placed in glass finger bowls and maintained at room temperature. Larvae used in the experiments reported here were tentatively identified as *Golfingia misakiana* based on features of the adults which were reared from these larvae in the laboratory (Rice, 1978). The larvae were found most abundantly in the plankton from October through April, occurring at times in numbers as great as 1,000 to 2,000 per tow.

For the duration of the experiments, the larvae were placed in covered plastic containers, 11 cm in diameter and 7.5 cm deep with 1 cm of substratum on the bottom and 150 ml of sea water or adult-conditioned water as indicated by the particular experiment. Substratum was present in all treatments, except as noted in Table 3. For each treatment in an experiment, three replicates were used, each with 20 larvae, totaling 60 larvae per treatment. After 4 days the animals were sieved from the substratum for observation and enumeration of metamorphosed specimens. In some treatments a few larvae were not recovered in the sieving process. The missing larvae rarely exceeded 10% of the total number per treatment (see tables) and they were not considered in the calculations of percent metamorphosis.

Criteria for determination of metamorphosis were loss of metatrochal cilia and inability of the larva to extend its head region. These criteria were not clearly discernible until the third or fourth day after the initiation of metamorphosis. The first change associated with metamorphosis was the behavioral change of burrowing activity in the sediment. However, this could not be used as a reliable criterion for metamorphosis because the time required for the initiation of burrowing varied from a few minutes to 24 h after contact with the stimulating agent and, moreover, buried larvae sometimes emerged from the sediment to swim again in the overlying water. Therefore the experiments were run for 4 days when the most reliable criteria for metamorphosis could be ascertained.

Statistical analyses of the variability among replicates for each treatment and of differences among treatments were carried out with three way *G*-tests, or, as necessary for distinguishing differences between treatments, with a 2-way *G*-test (Sokal and Rohlf, 1969). No significant variability between replicates occurred in any of the experiments ($P < 0.05$). All differences between or among treatments that are reported to be significant in the results section (unless specified) are at the 99% level or greater.

Details of the methodology used for each treatment and any variation from the routine procedures are indicated in the presentation of results which follows.

This study was carried out at the Smithsonian Marine Station at Link Port, located on the central east coast of Florida near Fort Pierce, Florida.

Influence of Adults and Substratum on Larval Metamorphosis

Larval Response to Adult Presence in Substratum.—Dishes containing both substratum and overlying sea water were set up for the following treatments 4 days before introduction of larvae: (1) control, adults absent both before and after introduction of larvae, (2) adults added simultaneously with the larvae, (3) adults present in substratum 4 days before and after introduction of larvae, (4) adults present in substratum 4 days before and removed just prior to introduction of larvae. In the three treatments that included adults, 10 adult *Golfingia misakiana* were used in each replicate. Upon contact with the substratum, adults promptly burrowed into the sediment where they remained throughout the experiment.

Larval metamorphosis in all three treatments in which adults had contact with

Table 1. Effect of adult presence in substratum on larval metamorphosis. In treatments with adults, 10 *Golfingia misakiana* (field-collected) were added to each replicate

Presence of adults in substratum		# Metamorphosed/ # Recovered	% Metamorphosis
Prior to larvae	With larvae		
None	None	11/57	19
None	Present	51/57	89
Present—4 days	Present	49/57	86
Present—4 days	None (removed)	39/57	68

substratum was significantly higher than in the control in which adults were absent (Table 1). Both the simultaneous presence of adults as well as the past presence of adults in substratum enhanced the response of the larvae. However, larval response was significantly higher ($P < 0.05$) when adults were present simultaneously with larvae than when adults were removed prior to the introduction of larvae.

Larval Response to Adult-conditioned Water.—Results from the previous experiment, which suggested that the past-presence of adults in substratum elicited larval response, raised the question of whether the adults release a metamorphosis-inducing factor into the sea water. In this experiment "adult-conditioned water" was prepared by exposure of sea water to adults for periods of 1, 2, 3, and 4 days (20 *Golfingia misakiana* adults per 500 ml sea water). Treatments were as follows: one control with untreated sea water and four with adult-conditioned water including one for each exposure time. Larvae were introduced into dishes containing the control or adult-conditioned water along with substratum. At the end of 4 days the substratum was sieved and the number of metamorphosed larvae enumerated.

Adult-conditioned water, in the presence of untreated substratum, greatly enhanced larval metamorphosis (Table 2). All treatments, regardless of time exposed to adults, significantly increased metamorphosis over the control.

Necessity of Substratum for Larval Metamorphosis.—Substratum was present in all treatments in the two preceding experiments. This experiment examined the influence of substratum on larval metamorphosis by exposing larvae to conditions with and without substratum in the presence of untreated or adult-conditioned water. Four treatments were set up: (1) control water, no substratum; (2) adult-conditioned water, no substratum; (3) control water with substratum; (4) adult-conditioned water with substratum.

Table 2. Effect of adult presence in sea water on larval metamorphosis. Adult-conditioned sea water was prepared by placing adults of *Golfingia misakiana* (field-collected) in sea water (20 animals per 500 ml sea water) for the times indicated. Adults were removed prior to the introduction of larvae. Substratum present in all treatments.

Treatment of water prior to experiment*	# Metamorphosed/# Recovered	% Metamorphosis
Control	1/57	2
Exposed to adults—4 days	29/58	50
Exposed to adults—3 days	29/58	50
Exposed to adults—2 days	43/58	74
Exposed to adults—1 day	25/57	44

* Experiment started at time larvae were introduced.

Table 3. Influence of substratum on larval metamorphosis. Adult-conditioned sea water was prepared by exposing adult *Golfingia misakiana* (20 animals per 500 ml sea water) to the water for 4 days prior to the introduction of larvae

Treatment	# Metamorphosed/# Recovered	% Metamorphosis
Control water		
No substratum	0/59	0
Substratum	4/60	7
Adult-conditioned water		
No substratum	5/60	8
Substratum	53/58	91

Significant metamorphosis occurred only when both adult-conditioned water and substratum were present (Table 3). These three initial experiments formed the basis for further questions that will be considered in the remainder of this paper regarding the nature of the metamorphosis-inducing factor that is found in adult-conditioned water and the role of substratum in enhancing larval metamorphosis.

Characteristics of the Metamorphosis-inducing Factor Associated with Adults

Possible Influence of Microorganisms.—Microorganisms have often been implicated in inducing larval metamorphosis (Wilson, 1955; Gray, 1967; Scheltema, 1974), therefore their possible influence on the metamorphosis-inducing factor in sipunculans was investigated. Two procedures were utilized for eliminating microorganisms from adult-conditioned water: autoclaving for 10 min at 120°C and filtering through a Millipore filter of 0.22-μm pore size. All dishes contained substratum with overlying sea water prepared as follows: (1) control, untreated; (2) control, autoclaved; (3) control, Millipore-filtered; (4) adult-conditioned, otherwise untreated; (5) adult-conditioned, autoclaved; (6) adult-conditioned, Millipore-filtered.

Adult-conditioned water that had received no further treatment or that had been Millipore-filtered induced significantly higher metamorphosis than any of the controls (Table 4). However, adult-conditioned water that had been autoclaved effected no significantly greater metamorphosis than the control water. This ex-

Table 4. Response of larvae to presence or absence of microorganisms in adult-conditioned water. Water was autoclaved for 10 min at 120°C. Filtered water was passed through a millipore filter of 0.22 μm pore size

Treatment	# Metamorphosed/# Recovered	% Metamorphosis
Control water		
No treatment	1/58	2
Autoclaved	2/60	3
Millipore-filtered	6/56	11
Adult-conditioned water		
No treatment	37/58	64
Autoclaved	5/59	8
Millipore-filtered	44/60	73

Table 5. Specificity of larval response to adult-conditioned water from various species. Laboratory-reared *G. misakiana* had been in the laboratory for 44 months and field-collected *G. misakiana* for 1 week before preparation of adult-conditioned water

Treatment	# Metamorphosed/ # Recovered	% Metamorphosis
Control: No treatment of water	1/34	3
Adult-conditioned water from:		
Laboratory-reared *G. misakiana*	12/55	22
Field-collected *G. misakiana*	34/57	60
Golfingia pellucida	2/56	4
Paraspidosiphon fischeri	4/57	7

periment does not rule out the possibility of toxic or inhibiting compounds resulting from autoclaving, nor does it eliminate possible contamination of bacterial products during preparation of the adult-conditioned water. The results suggest that autoclaving destroys the inducing factor.

Species-specificity.—Larval response to adult-conditioned water prepared from two sipunculan species commonly found in association with *Golfingia misakiana* in the field was compared with response to water from field-collected *G. misakiana* and laboratory-reared *G. misakiana.* The latter animals were reared from oceanic larvae (the same larval species used throughout this paper) and have been tentatively identified as *G. misakiana* despite some minor taxonomic variations (Rice, 1978). *Golfingia pellucida* and *Paraspidosiphon fischeri* are both found in association with *Oculina* coral rubble in the habitat of *G. misakiana. Paraspidosiphon fischeri* falls within the same size range as *G. misakiana* (trunk length about 0.5 cm) whereas *G. pellucida* is somewhat larger (trunk length about 1 cm). The field-collected specimens of *G. misakiana* had been in the laboratory for 1 week prior to the experiments and the laboratory-reared specimens for 44 months. Adult-conditioned water for all species was prepared as stated for *G. misakiana* in Materials and Methods.

Larval response to water from *Golfingia pellucida* and *Paraspidosiphon fischeri* was not significantly different from the reponse to untreated control water (Table 5). Water from field-collected *Golfingia misakiana* yielded the highest larval metamorphosis and from laboratory-reared *G. misakiana* the next highest, although significantly less than the former.

The discrepancy between larval response to water from field-collected adults and response to laboratory-reared adults of *G. misakiana* suggested that the environment of the laboratory might influence the effectiveness of the metamorphosis-inducing factor. Subsequently the effect of long-term maintenance in the

Table 6. Influence of length of time of adults in laboratory on the effectiveness of adult-conditioned water on larval metamorphosis

Treatment	# Metamorphosed/ # Recovered	% Metamorphosis
Adult-conditioned water (*G. misakiana* 2 years in laboratory)	25/58	43
Adult-conditioned water (*G. misakiana* 18 days in laboratory)	27/60	45

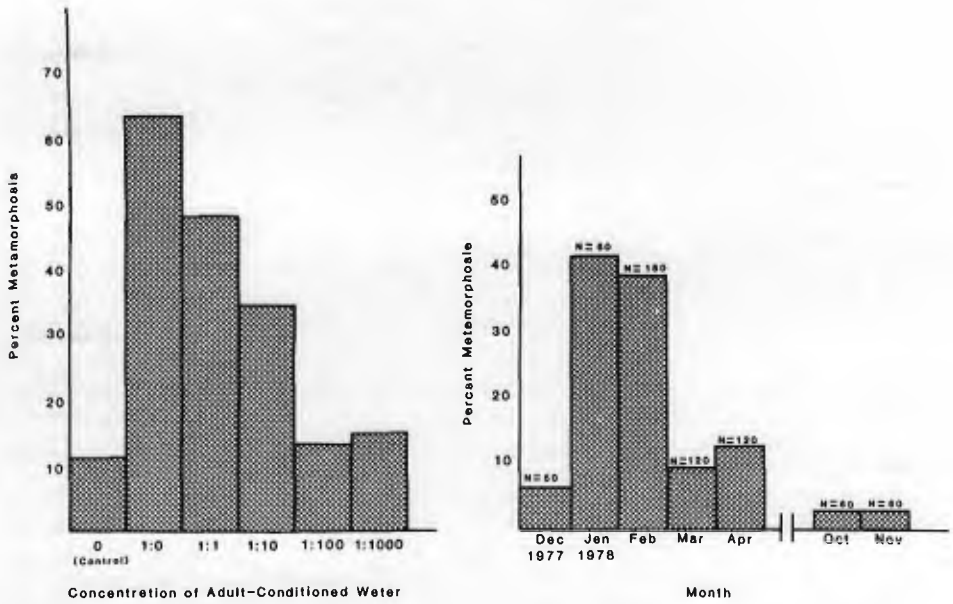

Figure 1. (Left) Effect of concentration of adult-conditioned water on larval metamorphosis.

Figure 2. (Rigbt) Metamorphosis of control larvae in the laboratory. Larvae were in untreated sea water with untreated, natural substratum. Time in laboratory before exposure to substratum ranged from 1 to 16 days, and averaged 5 days. Metamorphosis was recorded 4 days after exposure to substratum. No larvae were tested or collected May through September.

laboratory on the ability of adults to produce an effective factor for inducing metamorphosis was tested. Larval response was compared between adult-conditioned water prepared from field-collected *Golfingia misakiana* that had been in the laboratory for 18 days with water from *G. misakiana* that had been maintained in the laboratory for two years. The adult-conditioned water from the two groups of animals did not yield significantly different results in larval response (Table 6).

Effective Concentration.—Concentrations of adult-conditioned water were tested for their effectiveness in inducing metamorphosis in dilutions up to 1:1,000. Adult-conditioned water was prepared according to the usual procedure and diluted with sea water to the desired concentrations just prior to the experiment. Larvae were subjected to concentrations of 1:0, 1:1, 1:10, 1:100, and 1:1,000 as well as to control sea water. Substratum was present in all tests.

Undiluted adult-conditioned water gave the highest percent metamorphosis whereas a 1:100 dilution was not significantly different from the control (Fig. 1).

Molecular Weight.—Molecular weight of the metamorphosis-inducing factor was approximated by processing adult-conditioned water through an Amicon-filtration apparatus (Amicon Corporation, Lexington, Massachusetts) which concentrated various molecular weight fractions. These fractions were then exposed to larvae for a test of metamorphosis-inducing capacity. Adult-conditioned water was prepared as usual and frozen until the time of filtration. The entire process of Amicon-filtration was carried out at 4°C over a period of 8 days. Each con-

Table 7. Response of larvae to different molecular weight fractions of adult-conditioned water. Fractions were prepared by the Amicon ultrafiltration system (4°C). Adult-conditioned water was passed through each of the following filters in the sequence indicated: XM-100, XM-50, PM-30, UM-10, UM-05. Filtration was begun with the filter having the highest molecular weight cutoff. An aliquot of the filtrate was taken for testing and the remainder passed through the filter with the next highest molecular weight cutoff. The procedure was repeated for each filter and aliquots were also taken for testing from the initial retentate and the final effluent. Each filter concentrated the solutes of molecular weights higher than the cutoff while retaining some solutes of lower molecular weights. All aliquots therefore contained the lowest molecular weight components. There were two replicates per treatment with 30 larvae per replicate

Concentrates of adult-conditioned water	# Metamorphosed/# Recovered	% Metamorphosis
≥100,000 MW	31/59	53
50,000–100,000 MW	23/59	39
30,000–50,000 MW	32/59	54
10,000–30,000 MW	35/58	60
500–10,000 MW	34/57	60
<500 MW	31/56	55
Control water, no treatment	11/58	19

centrate was frozen as collected and remained frozen until tested for metamorphosis activity. The filters used for each concentrate and the corresponding molecular weight of the material concentrated were: XM-100, molecular weight greater than 100,000; XM-50, molecular weight greater than 50,000; PM-30, molecular weight greater than 30,000; UM-10, molecular weight greater than 10,000; UM-05, molecular weight greater than 500. Each filter concentrated solutes of molecular weight higher than the cutoff, while retaining some solutes of lower molecular weight. In addition to the above five concentrates, the effluent (molecular weight less than 500) and control sea water were tested for larval response with a total of seven treatments. It is assumed that each concentrate also included all lower molecular weight fractions, but did not include those higher molecular weight compounds removed by the preceding filter. Therefore, of the six tests which included some fraction of the adult-conditioned water, all contained the fraction with the lowest molecular weight, i.e., less than 500. The volume of each concentrate was sufficient only for duplicate dishes for each treatment, rather than the three replicates used in all other experiments. Sixty larvae were tested per treatment, with 30 larvae per dish. Substratum was present in all treatments.

Larval responses to the six fractions of adult-conditioned water were not significantly different, but response to each of the fractions was significantly greater ($P < 0.05$) than to the control water (Table 7).

A second procedure utilized for examining molecular weight was dialysis. Two types of dialysis tubing were used: one with a molecular weight cut-off of 50,000 and another with a cut-off of 1,000. Adult-conditioned water, prepared with twice the usual number of adults, (40 per 500 ml) was dialyzed overnight at 23°C in an equal volume of Millipore-filtered (0.22 μm) sea water. Four treatments, all with substratum, were set up as follows: (1) control, untreated water; (2) adult-conditioned, diluted 1:1 with sea water; (3) dialysate of adult-conditioned water, less than 1,000 molecular weight; (4) dialysate of adult-conditioned water, less than 50,000 molecular weight.

The three treatments in which adult-conditioned water was present were not significantly different from one another in their ability to induce larval metamorphosis (Table 8). All were significantly different from the control.

Table 8. Response of larvae to dialysates of adult-conditioned water. Water, prepared by exposure to twice the usual number of adults (40/500 ml), was dialyzed overnight at 23°C in an equal volume of sea water (Undialyzed adult-conditioned water was diluted 1:1 for the experiment)

Treatment	# Metamorphosed/ # Recovered	% Metamorphosis
Control sea water	1/60	2
Adult-conditioned water, diluted 1:1	25/58	43
Adult-conditioned dialysate, mol. wt. cutoff = 1,000	23/45	51
Adult-conditioned dialysate, mol. wt. cutoff = 50,000	25/57	44

Heating and Freezing.—The effect of heating and freezing on the metamorphosis-inducing capacity of adult-conditioned water was examined in the following experiment. Larval response was tested in the presence of adult-conditioned water and control water, both of which were subject to the following conditions: heating to 90°C for 15 min, freezing overnight, or no treatment. In all tests, water was brought to room temperature (23–24°C) at the time of the experiment and substratum was present.

Percent metamorphosis of larvae exposed to heat-treated, adult-conditioned water was similar to that in controls and significantly less than that in frozen or untreated adult-conditioned water (Table 9). The effect of freezing adult-conditioned water, when compared with untreated water, was not significant.

Stability of Metamorphosis-inducing Factor with Time.—Adult-conditioned water was prepared in the usual manner of exposing adult *Golfingia misakiana* to sea water for a period of 4 days. After removal of the animals, the water was kept in the laboratory at room temperature (24°C) and aliquots removed and frozen at 0 time, 4 days, and 8 days. Immediately before the experiment the aliquots were thawed and allowed to reach room temperature. In each treatment, including one with control sea water, substratum was present.

There was no significant difference in the metamorphosis effected by adult-conditioned water, whether 0 time, 4 days, or 8 days had elapsed after preparation (Table 10).

Properties of Substratum which Influence Larval Metamorphosis

Particle Size of Substratum.—The influence of particle size of substratum on larval response was tested with sizes up to a maximum of 250 μm. Three experimental categories were established: less than 63 μm, 63–125 μm, 125–250 μm. Larger particle sizes were not practical for use in experimentation as larvae could not be recovered by sieving. Except for sieving, the substratum used was untreated. Adult-conditioned water was present in all categories.

Within the range tested, particle size was not a significant factor in influencing larval metamorphosis (Table 11). This information confirmed the suitability of the 102-μm sieve for routine use in preparing sediment for metamorphosis experiments. A large fraction of the sediment from the offshore collection site will pass through this pore-size readily and, at the same time, the larvae and juveniles are retained.

Microorganisms in Substratum.—The possible influence of microorganisms on

Table 9. Effect of temperature on metamorphosis-inducing factor

Treatment	# Metamorphosed/# Recovered	% Metamorphosis
Control water		
Untreated	2/56	4
Heated 90°C, 15 min	2/57	4
Frozen overnight	0/60	0
Adult-conditioned water		
Untreated	20/57	35
Heated 90°C, 15 min	5/60	8
Frozen overnight	14/59	24

the effect of substratum in larval metamorphosis was examined in three repetitive experiments in which larval response was compared in the presence of autoclaved and untreated substratum. After autoclaving at 120°C for 10 min, the substratum was cooled to room temperature and rinsed with Millipore-filtered (0.22-μm filter) sea water. In all experiments adult-conditioned water was used.

The results (Table 12), consistent in the three experiments, demonstrated that autoclaving substratum decreases percent metamorphosis of larvae.

Organic Matter in Substratum.—In an experimental analysis of the importance of organic matter in the substratum as a factor in larval metamorphosis, substratum was treated by (1) ashing, (2) exposing to sodium hypochlorite and (3) air drying. Ashing was accomplished at 500°C for 4 h. Substratum treated with sodium hypochlorite was soaked overnight in full strength commercial Chlorox (=5.25% sodium hypochlorite) and the solutions changed several times until the color was light tan—an indication that the organic matter was reduced. For air drying, substratum was spread out in large plastic trays and dried at approximately 24° C for 3 days. Dried substratum, whether ashed or dried at room temperature, was in the form of a hardened mass which was crushed with mortar and pestle and re-sieved through a 102-μm mesh screen before experimental use. After sieving, it was resuspended in millipore-filtered sea water and rinsed several times until salinity and pH of sea water were normal. All tests with treated substratum were carried out in the presence of adult-conditioned water. The experiment also included a control with untreated substratum and adult-conditioned water and a control with untreated substratum and untreated water.

When compared with the control in which adult-conditioned water and untreated substratum were used, the 3 experimental treatments of the substratum

Table 10. Effect of time on metamorphosis-inducing factor (Adult-conditioned water frozen at 0 time, 4 days, and 8 days after preparation; immediately prior to the experiment, water was thawed and brought to room temperature)

Treatment	# Metamorphosed/# Recovered	% Metamorphosis
Control water	8/41	20
Adult-conditioned water		
0 time	49/50	98
4 days	45/51	88
8 days	48/54	89

Table 11. Response of larvae to particle size of substratum (Adult-conditioned water was present in each treatment)

Treatment	# Metamorphosed/# Recovered	% Metamorphosis
Substratum <63 μm	59/60	98
Substratum 63–125 μm	53/60	88
Substratum 125–250 μm	52/56	93

resulted in significantly reduced larval metamorphosis (Table 13). However, the reduction was not as great in the presence of air-dried or hypochlorite-treated substratum as in the presence of ashed substratum. When compared with the control in which the water was not treated (i.e., not adult-conditioned), the ashed substratum showed no difference in larval response whereas the air-dried and hypochlorite-treated substrata yielded a significantly greater response. Therefore, the two latter methods reduced larval metamorphosis, whereas ashing entirely eliminated the larval response.

Response of Larvae to Inert Substrata.—Several unsuccessful attempts were made in preliminary experiments to employ artificial substrata in testing larval metamorphosis. Larvae exposed to glass beads (Cataphote No. 1420, diameter 74–105 μm, Class IVA, regular) in adult-conditioned water did not burrow or metamorphose. When adults were added to the beads, they too remained on the surface, only occasionally extending the introvert down into the beads, but then withdrawing it. When placed on cryolite (Ward's Natural Science Establishment), that had been crushed and sieved through a 102-μm screen, larvae sometimes burrowed initially, but did not remain burrowed and showed no signs of metamorphosis. If left on the cryolite for one week, most larvae died. Both glass beads and cryolite were soaked for several days in untreated sea water before use in experiments.

Competency of Larvae

Seasonal Change in Competency of Untreated Controls.—During the course of the experiments reported here and in other similar experiments, it was noted that a certain percentage of larvae metamorphosed under control conditions of untreated sea water and untreated substratum. This percentage varied considerably

Table 12. Response of larvae to presence or absence of microorganisms in substratum (Adult-conditioned water in all treatments)

Substratum treatment	# Metamorphosed/ # Recovered	% Metamorphosis
Experiment #1		
None	38/56	68
Autoclaved, 10 min, 120°C	14/58	24
Experiment #2		
None	44/56	79
Autoclaved, 10 min, 120°C	16/58	28
Experiment #3		
None	37/57	65
Autoclaved, 10 min, 120°C	21/57	37

Table 13. Response of larvae to organic matter in substratum (Substratum was treated by ashing at 500°C for 4 h, soaking in 5.25% sodium hypochlorite overnight or drying (24°C) for 3 days)

Treatment		# Metamorphosed/ # Recovered	% Metamorphosis
Substratum	Water		
Untreated	Untreated	3/59	5
Untreated	Adult-conditioned	43/58	74
Ashed 500°C	Adult-conditioned	4/59	7
Na Hypochlorite	Adult-conditioned	25/56	45
Dried	Adult-conditioned	19/56	34

in different experiments (0–50%) in which different collections of larvae were used, although in all cases, the percentage increase in metamorphosis was considerably greater in the presence of adult-treated water. The data were collated sequentially, according to the month collected, for a period of 1 year. The year selected for analysis, 1977–1978, included numerous collections throughout the season of larval availability (October through April). A summary of the data, presented in Figure 2, indicates that the highest percentage metamorphosis, under control conditions, occurred during the months of January and February.

Competency with Time in the Laboratory.—Whether competency to metamorphose, in the absence of added stimuli of adults, increased with time of larvae in the laboratory was examined over a period of 4 weeks after collection. Larvae were maintained in dishes with untreated sea water and untreated substratum. Observations were made during the month of February, 1983. No significant increase or decrease in metamorphosis occurred at 2-, 3-, and 4-week intervals after collection (Table 14).

Discussion and Conclusions

The experimental results have demonstrated the presence of a metamorphosis-inducing factor associated with adults in the sipunculan, *Golfingia misakiana.* The factor is a water-soluble, heat-labile substance of low molecular weight (<500), that shows some degree of species specificity. Its effectiveness is not reduced by holding at room temperature for at least 8 days nor by freezing. It is not dependent on living microorganisms for its action, as indicated by the negative effect of filtration through a 0.22-μm Millipore filter; however, its activity is altered by autoclaving and by heating to 90°C. The experiments do not eliminate the possibility that products of bacteria, associated with the adult or with the substratum, may contribute in some way to the inducing factor.

The characteristics of the compound, are consistent with those of several other

Table 14. Effect of time in laboratory on competency of larvae to metamorphose (Untreated substratum and adult-conditioned water present in all treatments)

Time in laboratory (weeks)	# Metamorphosed/ # Recovered	% Metamorphosis
2	25/59	42
3	29/58	50
4	26/59	44

known chemical inducers of larval metamorphosis. For example, Hadfield (1977; 1978a) found that for the opisthobranch mollusc, *Phestilla sibogae,* a water-soluble compound of less than 500 molecular weight from the prey coral *Porites compressa,* induces larval metamorphosis. Also, a number of simple compounds containing choline induce metamorphosis in this opisthobranch. In a series of experiments by Morse et al. (1979; 1980) on larvae of the abalone, *Haliotis rufescens,* settlement was induced by a specific crustose red alga and by several simple, homologous chemical inducers contained within the alga, among the most active of which is gamma-amino-butyric acid (GABA). Cameron and Hinegardner (1974) found a non-particulate organic chemical (molecular weight less than 5,000), of bacterial origin, that initiates metamorphosis in laboratory-reared sea urchins in the presence of a surface. A metamorphosis-inducing chemical of less than 10,000 molecular weight was found by Highsmith (1982) in sand associated with adults of the echinoid, *Dendraster excentricus,* a species which inhabits soft substrata. Burke (1984) further defined the metamorphosis-inducing compound of *Dendraster excentricus,* as a peptide of 980 daltons which he extracted from adult-associated sand as well as from tissues of adults. For echiurans, Suer and Phillips (1983) reported a heat-labile, water-soluble compound or "scent" (3,500–14,000 daltons) derived from the skin of the adult, which induces larval metamorphosis when adsorbed onto a surface.

The dependence of the larval response of the sipunculan, *Golfingia misakiana,* on the presence of natural substratum in combination with the metamorphosis-inducing factor of the adult suggests the probable complexity of the response and raises questions regarding the mechanisms of larval recognition of stimuli. The properties of the substratum which are important to the larval response are not clearly delineated by the experimental results. However, observations do suggest several possible roles for substratum either as independent factors or synergistic contributions to the effectiveness of the metamorphosis-inducing factor. For example, the substratum could be providing a surface for adsorption of the chemical inducer, or for microorganismal films. Or microbial products of substratum might facilitate response to the adult factor. Also, the potential of the substratum as a food source could be a contributing influence on larval response as could its potential as a medium for burrowing.

Crisp (1974), and Crisp and Meadows (1963), based on studies of barnacle larvae that settle on hard substrata, proposed that the larvae only sense chemical inducers that are adsorbed to a surface. An exception has been reported by Hadfield (1977) who showed that larvae of the epifaunal opisthobranch, *Phestilla sibogae,* metamorphosed after exposure to chemical inducers in a swirling suspension. A study of an infaunal species, in which the adsorption of chemical inducers was considered as a factor in larval response, was made by Suer and Phillips (1983) for the echiuran, *Urechis caupo.* The echiuran larvae responded to the "scent" of the adult only in the presence of sediment, glass beads, or the surfaces of glass beakers to which the "scent" had been adsorbed. It was assumed that the larvae sensed the sediment by chemo-tactile receptors. The behavior of the sipunculan larva of *Golfingia misakiana,* when placed on a substratum of fine sand and silt, involves exploratory movements over the surface which can be interpreted as testing or sensing the sediment (Rice, 1978). They move over the substratum in the manner of an inchworm with ventral groove of the head applied downward. In this position sand grains are passed by ciliary action through the ventral groove of the head where they are adhered together by secretions. The larvae thus leave strands of adhered sand grains as trails, marking their passage over the surface of

the sediment. As there is no evidence that the ingestion of sand occurs as the strands are formed, one explanation of the behavior is that it involves the detection of adsorbed chemical inducers on the sand grains by means of chemo-tactile receptors in the ventral groove of the head. If, on the other hand, the substratum serves simply as a surface for adsorption, one might expect that inert or artificial substrata would provide the necessary surface as well as natural sediment. However, when glass beads of the same size range as the sediment were substituted for sediment, the larvae did not respond to the inducer.

Bacterial films have been demonstrated to be important for settlement of larvae of many different invertebrate larvae (review by Scheltema, 1974). The most detailed studies for infaunal animals are those by Wilson (1955) on *Ophelia bicornis* and Gray (1967) on *Protodilus rubropharyngeus.* These studies conclude that the most important factor in inducing metamorphosis in these species is the presence of living microorganisms on sand grains. In the experiments reported here on the sipunculan *Golfingia misakiana,* both autoclaving and drying of the sediment reduced larval metamorphosis in the presence of the adult-associated inducer. Thus, the possibility exists that bacterial films interact with the chemical inducer or in some other way serve as a stimulus for larval metamorphosis.

When organic matter is removed from substratum, a decrease in settlement response has been shown to follow for larvae of several deposit-feeding marine invertebrates (Scheltema, 1961; Gray, 1974). A similar decrease was found for the larvae of *Golfingia misakiana,* which responded less favorably to the metamorphosis-inducing factor when organic matter of the substratum was reduced experimentally. As organic matter serves as a source of nutrition for juvenile and adult deposit feeders, larval selection of an organic-rich substratum for settlement would have obvious advantages for the juveniles. However, the procedures by which organic matter was removed from the substratum also eliminated microorganisms. Thus, from the results obtained in these experiments, it is not possible to discriminate between the biological and chemical stimuli provided by the substratum.

Most larvae that have been studied in settlement-response experiments do not burrow prior to metamorphosis. An exception is *Ptychodera flava,* reported to burrow as a partially metamorphosed larva, completing metamorphosis within the sand (Hadfield, 1978b). The larvae of *Golfingia misakiana,* under the conditions reported here, most commonly burrow in substratum before undergoing any of the morphological changes of metamorphosis. Juveniles and adults of *Golfingia misakiana* and other species of sipunculans—particularly sand-burrowers—do not survive for prolonged periods out of the substratum. Eventually the cuticle develops blebs, the body wall ruptures, and the animal dies. Thus, an important attribute of the substratum as a stimulus for metamorphosis could be its suitability as a medium for burrowing.

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Literature Cited

Bayne, B. L. 1969. The gregarious behaviour of the larvae of *Ostrea edulis* L. at settlement. J. Mar. Biol. Ass. U.K. 49: 327–356.

Burke, R. D. 1984. Pheromonal control of metamorphosis in the Pacific sand dollar, *Dendraster excentricus*. Science 225(4460): 442–443.
Cameron, R. A. and R. T. Hinegardner. 1974. Initiation of metamorphosis in laboratory cultured sea urchins. Biol. Bull. 146: 335–342.
Crisp, D. J. 1967. Chemical factors influencing settlement in *Crassostrea virginica* (Gmelin). J. Anim. Ecol. 36: 329–335.
———. 1974. Factors influencing the settlement of marine invertebrate larvae. Pages 177–265 *in* P. T. Grant and A. M. Mackie, eds. Chemoreception in marine organisms. Academic Press, New York.
———. 1977. The development of the serpulid *Pomatoleios kraussii* (Annelida, Polychaeta). J. Zool. Lond. 183: 147–160.
——— and P. S. Meadows. 1963. Absorbed layers: the stimulus to settlement in barnacles. Proc. Roy. Soc., B 158: 364–387.
Gray, J. S. 1967. Substratum selection by the Archiannelid *Protodrilus rubropharyngeus*. Helgo. Wiss. Meersunters. 15: 253–269.
———. 1974. Animal-sediment relationships. Oceanogr. Mar. Biol. Ann. Rev. 12: 223–261.
Hadfield, M. G. 1977. Chemical interactions in larval settling of a marine gastropod. Pages 403–413 *in* D. J. Faulkner and W. H. Fenical, eds. Marine natural products chemistry. Plenum Publishing Co., New York.
———. 1978a. Metamorphosis in marine molluscan larvae: an analysis of stimulus and response. Pages 165–175 *in* F. S. Chia and M. E. Rice, eds. Settlement and metamorphosis of marine invertebrate larvae. Elsevier, New York.
———. 1978b. Growth and metamorphosis of planktonic larvae of *Ptychodera flava* (Hemichordata: Enteropneusta). Pages 247–254 *in* F. S. Chia and M. E. Rice, eds. Settlement and metamorphosis of marine invertebrate larvae. Elsevier, New York.
Hidu, H. 1969. Gregarious setting in the American oyster *Crassostrea virginica* Gmelin. Chesapeake Sci. 10: 85–92.
Highsmith, R. C. 1982. Induced settlement and metamorphosis of sand dollar (*Denraster excentricus*) larvae in predator-free sites: adult sand dollar beds. Ecology 63: 329–337.
Knight-Jones, E. W. 1951. Gregariousness and some other aspects of the settling behaviour of *Spirorbis*. J. Mar. Biol. Ass. U.K. 30: 201–222.
Lewis, C. A. 1978. A review of substratum selection in free-living and symbiotic cirripeds. Pages 207–218 *in* F. S. Chia and M. E. Rice, eds. Settlement and metamorphosis of marine invertebrate larvae. Elsevier, New York.
Morse, D. E., N. Hooker, H. Duncan and L. Jensen. 1979. Gamma-aminobutyric acid, a neurotransmitter, induces planktonic abalone larvae to settle and begin metamorphosis. Science 204: 407–410.
———, H. Duncan, N. Hooker, A. Baloun and G. Young. 1980. GABA induces behavioral and developmental metamorphosis in planktonic molluscan larvae. Fed. Proc. 39(14):3237–3241.
Rice, M. E. 1978. Morphological and behavioral changes at metamorphosis in the Sipuncula. Pages 83–102 *in* F. S. Chia and M. E. Rice, eds. Settlement and metamorphosis of marine invertebrate larvae. Elsevier, New York.
——— and J. D. Murdoch. 1978. Influence of adults on metamorphosis of oceanic sipunculan larvae. Amer. Zool. 18(3): 664.
Scheltema, R. S. 1961. Metamorphosis of the veliger larvae of *Nassarius obsoletus* (Gastropoda) in response to bottom sediment. Biol. Bull. 120: 92–109.
———. 1974. Biological interactions determining larval settlement of marine invertebrates. Thal. Jugos. 10(1/2): 263–296.
———, I. P. Williams, M. A. Shaw and C. Loudon. 1981. Gregarious settlement by the larvae of *Hydroides dianthus* (Polychaeta: Serpulidae). Mar. Ecol. Prog. Ser. 5: 69–74.
Sokal, R. R. and F. J. Rohlf. 1969. Biometry. The principles and practice of statistics in biological research. W. H. Freeman, San Francisco. 776 pp.
Suer, A. L. and D. W. Phillips. 1983. Rapid, gregarious settlement of the marine echiuran *Urechis caupo* Fisher and MacGinitie 1928. J. Exp. Mar. Biol. Ecol. 67: 243–259.
Wilson, D. P. 1955. The role of microorganisms in the settlement of *Ophelia bicornis* Savigny. J. Mar. Biol. Ass. U.K. 34: 531–543.
Young, C. M. and L. F. Braithwaite. 1980. Larval behavior and post-settling morphology in the ascidian, *Chelyosoma productum* Stimpson. J. Exp. Mar. Biol. Ecol. 42: 157–169.
——— and F.-S. Chia. 1982. Factors controlling spatial distribution of the sea cucumber, *Psolus chitinoides*: settling and post-settling behavior. Mar. Biol. 69: 195–205.

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