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Induction of Metamorphosis and Substratum Preference in Four Sympatric and Closely Related Species of Sea Urchins (Genus *Echinometra*) in Okinawa

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M. Aminur Rahman and Tsuyoshi Uehara (2001) Induction of metamorphosis and substratum preference in four sympatric and closely related species of sea urchins (Genus *Echinometra*) in Okinawa. *Zoological Studies* **40**(1): 29-43. Metamorphosis and settlement studies were conducted with 20 to 24-d-old laboratory-reared larvae of 4 closely related and genetically divergent sea urchins of the genus *Echinometra* (*E*. sp. nov. A, *E. mathaei*, *E*. sp. nov. C, and *E. oblonga*) to assess their preferences for various substrata. All the *Echinometra* spp. exhibited a similar high rate of metamorphosis in response to encrusting coralline red algae compared to mixed turfs of coralline algae with: regular brown, green, or mixed fleshy algae, suggesting that potent inducing substances may be sufficiently present in red algae. Lack and/or shortage of inducing materials in brown and green algae may account for the very low rate of metamorphosis and survival. Furthermore, aqueous extracts of coralline red algae induced *Echinometra* spp. larvae to metamorphose, demonstrating that the inducing factor is chemical in nature. These chemicals have been shown by several workers to be proteins which are GABA-mimetic in their interaction with the larval receptors controlling metamorphosis. GABA, which triggers the metamorphosis of several gastropods and strongylocentrotids, also induced it in *Echinometra* spp. larvae at concentrations exceeding 10^{-3} M, indicating that a textural requirement is less likely than chemosensory receptors in inducing metamorphosis. Tests performed by soaking red algal substrata revealed that no inducing substances leak into the surrounding seawater from intact inducing algae. Reduction in the number of live bacteria on the surface of red algae through treatment with antibiotics did not affect the rate of metamorphosis. This is the first attempt to study settlement induction and metamorphosis in 4 closely related but genetically distinct species of the sea urchins of the genus *Echinometra*. The 4 species did not differ in their rates of metamorphosis on each type of substratum, and each species highly preferred a coralline red algal substratum, which is consistent with their close genetic affinity as well as their sympatric existence in nature.

Key words: Settlement, *Echinometra* spp., Genetic affinity, Coralline algae, GABA.

Planktonic larvae of benthic marine invertebrates undergo a process of settlement and metamorphosis to establish the bottom dwelling mode of life of the adult. Settlement may be an important factor determining the distribution and abundance of an organism and often involves substratum selection by the larvae (Underwood and Fairweather 1989, Sutherland 1990, Minchinton and Scheibling 1991, Pawlik 1992, Rodriguez et al. 1993, Bourget and Harvey 1998). Larvae of many species settle and metamorphose selectively on various substrata which have particular physical, chemical, or biological characteristics (Crisp 1984, Butman 1987, Jensen and Morse 1990, Pawlik 1992, Miron et al. 1995, Walters et al. 1997, Beckmann et al. 1999, and many other reviews). In the larvae of many species, settlement/metamorphosis is induced by algal/or microbial (bacterial) films on the substratum (Kirchman et al. 1982, Le Tourneux and Bourget 1988, Bonar et al. 1990, Maki et al. 1990, Hadfield et al. 1994, Johnson and Sutton 1994, Lau and Qian 1997, Qian 1999, Unabia and Hadfield 1999). Some species se-

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lectively settle on algal substrata (Morse et al. 1979b 1980b 1988, Rumrill and Cameron 1983, Eyster and Pechenik 1987, Dirnberger 1990, Hurlbut 1991, and see reviews by Pawlik 1992, Rodriguez et al. 1993, Qian 1999). These include the strongylocentrotid sea urchin (Rowley 1989, Pearce and Scheibling 1990a,b 1991) and the sea stars, *Acanthaster planci* (Henderson and Lucas 1971, Yamaguchi 1973, Lucas and Jones 1976, Moran 1986, Johnson et al. 1991a,b) and *Stichaster australis* (Barker 1977).

The larvae of most benthic invertebrate species are induced to metamorphosis by cue(s), either chemical or physical, that identifies a specific habitat as suitable for juvenile or adult existence (reviewed by Pawlik 1992). This cue or metabolic inducer triggers a radical transformation of the morphology, physiology, ecology, and behavior of the larva to the juvenile stage. In plankton, these larvae typically develop competence to respond to metamorphic inducers, but do not proceed through metamorphosis in the absence of an appropriate stimulus. Often the stimulus is derived from a substratum capable of providing the newly settled juveniles with a source of nutrition and refuge; perception of these stimuli by larvae can involve chemical, tactile, or visual modalities (see reviews by Pawlik 1992, Rodriguez et al. 1993, Hadfield 1998, Qian 1999). In several species, substratum-associated morphogenetic chemical cues have been found to be neurotransmitter-mimetic substances (Morse 1985); in these and other cases, exogenous neurotransmitters can elicit metamorphic responses similar to those induced by natural cues, thus further implicating neuronal receptors in the initial processes (Morse et al. 1979a, Morse and Morse 1984, Morse 1985, and see review by Qian 1999). Chemosensory receptors controlling metamorphosis of larvae of *Haliotis rufescens* (a marine gastropod) in response to exogenous gamma-aminobutyric acid (GABA) and GABA-mimetic compounds purified from a naturally recruiting host alga have been directly labeled and characterized (Trapido-Rosenthal and Morse 1986). Although various substrates induce the settlement and metamorphosis of various species of sea urchins, coralline algae appear to be very effective at this. Recent evidence (Johnson and Sutton 1994) suggests that bacteria on the surface of these algae are also involved in evoking the larval response.

Laboratory-reared larvae of the green sea urchin, *Strongylocentrotus droebachiensis*, were induced to metamorphose upon contact with coralline red algae (Pearce and Scheibling 1988 1990a,b 1991), and the metamorphosis of *S. purpuratus, S. franciscanus, Loxechinus albus, Lytechinus pictus,* and *Arbacia punctulata* larvae was found to be induced by a bacterial film (Cameron and Schroeter 1980, Gonzalez et al. 1987). Field observations also showed that newly settled individuals of sea urchins (*S. purpuratus* and *S. franciscanus*) formed dense populations on rocky areas covered with coralline red algae (Rowley 1989).

Based on morphological, ecological, molecular, and biochemical studies, it has been concluded that sympatric *Echinometra mathaei* sensu lato in Okinawa must be recognized as 4 separate but closely related species, tentatively designated as *Echinometra* species A, B, C, and D (Ea, Eb, Ec, and Ed) (Uehara et al. 1990 1991, Arakaki and Uehara 1991, Matsuoka and Hatanaka 1991, Metz et al. 1991, Metz and Palumbi 1996). Although the 4 genetically divergent forms of *Echinometra* discussed here are recognized as 4 distinct species, valid names of these species have been debated (Palumbi et al. 1997). However, recent studies on morphological characters (Arakaki et al. 1998) have suggested that Eb and Ed of Okinawa are the same as *E. mathaei* and *E. oblonga*, respectively. The other 2 Okinawan species, Ea and Ec, will be called *E*. sp. nov. A and *E*. sp. nov. C, respectively (Palumbi et al. 1997, Arakaki et al. 1998). These 4 species live sympatrically in the intertidal zone of shallow seas, but they show slight micro-habitat differences. For instance, *E*. sp. nov. A is abundant in more or less protected, constantly submerged habitat which is very calm and situated below the level of MLWS (Mean Low Water Surface), such as tidepools and shallow reef slopes or other areas protected from strong wave action. *E. mathaei* inhabits burrows which are affected by wave action and are located above the level of MLWS. *E*. sp. nov. C and *E. oblonga* inhabit burrows above the level of MLWS, but their burrows are usually deeper than those of *E. mathaei. E. oblonga* tends to inhabit intertidal areas which are affected by stronger wave action than areas inhabited by *E*. sp. nov. C. Though, the 4 species prefer different microhabitats, in many places the distribution and habitat preferences of these 4 species overlap (Arakaki and Uehara 1991, Nishihira et al. 1991). During our field studies on the shallow subtidal reef flats of the Okinawan islands, we observed that these sympatric species formed much denser populations on coralline red algal-dominated areas than on other algal communities.

The laboratory-raised larvae of *Echinometra mathaei* sensu lato (*E*. sp. nov. A, *E. mathaei*, *E*. sp. nov. C, and *E. oblonga*) can reach a state of competence to undergo metamorphosis at about 20-24 d of age. Complete metamorphosis from feeding larva to

feeding juvenile takes place in about 1 d. This includes the complete development of internal organs as well as the formation of the adult mouth, anus, tube-feet, and spines. Up to now, no published information has been available about the induction of settlement in these urchins. In this study we examine which types of macro-algae will induce settlement in *Echinometra* spp. In a series of laboratory experiments, we characterized the agent responsible for settlement induction. The implications of these results to settlement patterns in the field are discussed.

MATERIALS AND METHODS

Larval rearing

Adults of *Echinometra mathaei* sensu lato were collected at Sunabe Beach 26°07'N; 12°46'E, Okinawa Island during their breeding season (May-Sept. 1998). They were maintained in closed aquaria at the Faculty of Science, Univ. of the Ryukyus. The collected urchins spawned and were fertilized following the methods and techniques previously described by Rahman (1997).

Early stage embryos were reared in standing cultures in small glass beakers. When blastulae were seen swimming at the surface of the water, they were transferred to glass bottles containing 400 ml of sterilized filtered seawater (SFSW), which was stirred constantly by 10-rpm rotating motors. Larval density was maintained at 2-3 individuals/ml of SFSW up to the 4-armed pluteus stage. When the larvae attained the 4-armed pluteus stage, they were cultured in the same system with a larval density of 1 individual/ml. All cultures were maintained in 0.45-µm Millipore-filtered seawater at 26-28 °C, approximating ambient water temperature. About 50%-75% of the culture water was removed by reverse filtration/siphoning every 3 d and replaced with fresh filtered seawater. Larvae were fed with cultured phytoplankton, *Chaetoceros gracilis*, at a concentration of 5000, 10 000, and 15 000 cells per 1 ml of medium at the 4-, 6-, and 8- armed stage periods, respectively, by adjusting the food level every 3 d (Wu et al. 1990).

General experimental protocols

Only competent larvae were used in experiments. Competence was reached at 20-24 d post-fertilization and was indicated by the presence of a large juvenile rudiment and a high percentage of metamorphosis (> 80%) in trial assays with coralline red algal-covered stones. For each experiment, only larvae from a single batch rearing were used. Experiments were conducted in plastic petri dishes (8.2 × 4.0 cm) with 60 ml of SFSW and a test substratum or 60 ml of a test solution. In each experiment 5 replicate petri dishes (each with 20 competent larvae) were used per treatment. Larvae were transferred into experimental petri dishes with a pipette. Experiments were run for 24-30 h in the same environmental conditions as larval cultures.

After 24-30 h, larvae and recently metamorphosed juveniles were located in petri dishes using a binocular microscope and classified as (1) free swimming (larvae only), (2) on test algae (when an algal substratum was used), or (3) on the bottom or sides of the experimental dishes. Three controls were used for each treatment: (1) sterilized filtered seawater, (2) bare carbonate stones with no algal or microbial test substratum to ensure that larvae were not metamorphosing without inducers, and (3) stones covered with encrusting coralline red algae to assess the proportion of larvae capable of metamorphosing, because the rate of metamorphosis is generally maximal in response to these algae.

Experiments with various types of macro-algae

Settlement induction of larval urchins was tested by observing their response to 4 types of algal associations collected from the field. Stones were collected from the intertidal zone of Sesoko Island, Okinawa during low tide. Stones were classified into the following 4 groups: coralline red algae (CRA), regular brown algae (RBA), regular green algae (RGA) and coralline and regular mixed algae (CRMA). Stone sizes were: length 5.5-7.5 cm; width 4.0-5.5 cm; and height 1.5-2.0 cm. The algal-covered stones were bagged separately, transported to the laboratory in coolers, kept in newly established individual glass aquaria, and used within 2-3 d after collection. Mobile animals and epibionts were removed from the stones as far as was possible, and the stones were then thoroughly rinsed with SFSW prior to use in experiments. Algae on all of the stones were identified to the generic level (Table 1). Bare stones (BS), i.e., without visible algae, were collected from intertidal zones, washed in distilled water, dried in a thermostatically controlled oven at 120 °C, and used as a control. We assumed that any bare stone collected from the field had a microbial film on its exposed surface.

Experiments with algal extracts

To examine the settlement of urchin larvae in

response to a water-soluble extract of coralline red algae, fragments of red macro-algae were chiseled off coralline stones, scrubbed with a sterile brush, and washed with SFSW. All detectable epibionts were then removed through microscopic examinations. Two hundred grams of algal fragments was then ground in 400 ml of SFSW. The homogenete was centrifuged at 24 000 g for 10 min at 2-3 °C to remove particulates. The resulting supernatant was then decanted and refrigerated overnight at 4 °C. The following day, this algal extract was recentrifuged at 24 000 g for 5 min at 2-3 °C for clarification, and then the supernatant was filtered through a sterile 0.22-µm filter. Samples were processed rapidly from centrifugation through filtration to prevent small and highly motile bacteria from passing through the filter. The resulting supernatant crude extract was then serially diluted to 1:5, 1:10, 1:100, 1:1000, and 1:10 000 with SFSW. For every replicate, 60 ml of this solution was added into each petri dish along with larvae.

Experiments with algae-conditioned seawater

This experiment was designed to test whether red algae release a chemical into the water column which is sensed by urchin larvae without having to make physical contact with algae. Twelve stones encrusted with coralline red algae were cleaned with SFSW and placed in 1000 ml of SFSW in a beaker for 24 h. The algal stones were then removed and the red alga-conditioned FSW was centrifuged at 24 000 g for 5 min at 2-3 °C to remove particulates. The supernatant was then filtered through a sterile 0.22-µm filter and used for experiments.

Experiments with algal spores

To examine whether algal spores induce metamorphosis of larvae, red alga-encrusted reef stones were placed in petri dishes with SFSW for 4 d. Red algae occasionally released minute spores (mean diameter ± sd: 104 ± 12 µm, n = 125) that attached to the bottom of the petri dishes. The 20 petri dishes with spores were rinsed 2-3 times with SFSW (spores remained attached to the bottom of the petri dishes) and were allocated for experiments.

Experiments with GABA

The amino acid neurotransmitter, gamma-aminobutyric acid (GABA) has been shown to induce settlement and metamorphosis of some marine invertebrate larvae (Morse et al. 1979a, Rumrill and Cameron 1983, Morse 1984 1985, Pearce and Scheibling 1988 1990a). To determine the effects of GABA on the metamorphosis of urchin larvae, experiments were carried out with competent larvae that had been cultured from a single fertilization. GABA (obtained from Sigma Chemical Company, St. Louis, Missouri, USA) was diluted in SFSW, and its ability to induce metamorphosis of larvae of

Table 1. Identification of coralline macro-algae to generic level on various natural algal substrata

Substratum	Macro-algae (generic level)	Algal division	Algal group
Coralline red algae (CRA)	*Amphiroa* sp., *Jania* sp., *Liagoria* sp., *Lithophyllum* sp., *Laurencea* sp., *Hypnea* sp.	Rhodophyta	red
Coralline & regular mixed algae (CRMA)	*Amphiroa* sp., *Jania* sp., *Liagoria* sp., *Lithophyllum* sp., *Laurencea* sp., *Hypnea* sp.	Rhodophyta	red
	Sargassum sp., *Hizikia* sp.	Phaeophyta	brown
	Ulva sp., *Monostroma* sp., *Codium* sp., *Enteromorpha* sp., *Chaetomorpha* sp., *Acetabularia* sp.	Chlorophyta	green
Regular brown algae (RBA)	*Sargassum* sp., *Hizikia* sp.	Phaeophyta	brown
Regular green algae (RGA)	*Ulva* sp., *Monostroma* sp., *Codium* sp., *Enteromorpha* sp., *Chaetomorpha* sp., *Acetabularia* sp., *Velonia* sp., *Cladomorpha* sp.	Chlorophyta	green
Bare stone (BS)	No visible algae observed		

Echinometra spp. was tested over the concentration range of 10^{-7} to 10^{-2} M.

Experiments with antibiotic-treated algal substrates

This experiment was designed to test whether a reduction in live bacteria on the surface of coralline red algae (CRA) would influence the rate of metamorphosis of urchin larvae. Red algal-encrusted stones were scrubbed with a sterile brush and rinsed with SFSW. The stones were then put into each of 2 glass beakers. One beaker received 1000 ml of SFSW, the other 1000 ml of SFSW which was treated with a mixture of penicillin and streptomycin (1000 units/ml), following the methods described by Pearce and Scheibling (1990a,b). This concentration was a compromise chosen to maximize the effect of the antibiotics on the bacteria but to avoid possible deleterious effects to the alga. The 2 beakers were then stirred several times to facilitate mixing of the antibiotics with the algal stones. After 42 h of treatment with antibiotics, the experimental algal stones in each bowl were rinsed 7-8 times with SFSW to remove the antibiotics and dead bacteria from the antibiotic-treated stones, following the methods described by Pearce and Scheibling (1990a,b), Johnson et al. (1991a), and Johnson and Sutton (1994). The antibiotic-treated and untreated pieces of stones were then allocated to separate experimental petri dishes.

Population density of *Echinometra* spp.

A field study was conducted to determine the distribution of *Echinometra* spp. in the Okinawan reef flats and the relationship between the distribution of these urchins and algal distribution. Population densities of *Echinometra* spp. were estimated during day time at low tides in summer months (Apr. to Sept. 1998). Samples were taken on 5 categories of substratum (i.e., coralline red algal stone, coralline and regular-mixed algal stone, regular brown algal stone, regular green algal stone, and bare stone). Urchins of various test sizes (range: 3.0-58.6 mm) were enumerated in 1-m^2 quadrates. A total of 5 quadrates was sampled on each substratum monthly over a period of 6 mo.

Data analysis

Data were expressed as the percentage of metamorphosed individuals over the total number of larvae tested per petri dish. All statistical analyses were carried out on arcsine-transformed data. This transformation helped to normalize the data and reduce heteroscedasticity. Bartlett's test was used to analyze the homogeneity of variances. Any replicate in which no larvae metamorphosed was given a value of 1/4 n to improve the arcsine transformation (Bartlett 1937). When variances were not significantly heteroscedastic and no major departures from normality were observed, two-way ANOVA (completely randomized design) was used followed by Tukey's multiple comparisons test.

RESULTS

The first 3 events in the metamorphosis of urchins (extension of the tube feet, bending of the arms, and attachment of the tube feet to the substratum) are reversible. A larva may bend its arm, and then later return to its original larval shape. Sometimes the larva attaches its tube feet only to let go and swim away. However, collapse and retraction of tissue are irreversible, and, if begun, metamorphosis follows.

Coralline and regular macro-algae

Competent larvae of each of the *Echinometra* spp. responded in similar ways to 4 different algal substrata (Table 2). The highest mean percentage of metamorphosis was displayed by each of the 4 species in response to coralline red algae (CRA) compared to those on coralline and regular mixed algae (CRMA), regular brown algae (RBA), and regular green algae (RGA). The mean values among species within each algal substratum did not significantly differ (Tukey's test, $p > 0.05$). A very low percentage of metamorphosis was observed on brown and green algae. Mean percentage metamorphosis of all 4 species was the lowest on bare stone (BS), and no larvae metamorphosed in sterilized filtered seawater (SFSW) (not included in the ANOVA) indicating the requirement for an external inducing cue (Table 2).

This experiment was continued for up to 7 d following metamorphosis. During this time, the mean survival of *Echinometra* spp. juveniles was found to be significantly higher (Tukey's test, $p < 0.05$) on CRA than on CRMA (Table 3). In the mixed algal treatments, juveniles were always found to be attached on coralline red algae but not on other algae. The very small survival percentage on brown algae (2%-3%) and green algae (1%-2%) differed significantly (Tukey's test, $p > 0.05$) across all species

groups.

Coralline red algae and their surface materials and extracts

Echinometra spp. showed high rates of settlement and metamorphosis upon contact with various coralline red algae, while brown and green macroalgae were less likely to induce settlement. Experiments were undertaken to investigate what component of these red algae was responsible for inducing high rates of metamorphosis in *Echinometra* spp.

Percentage of metamorphosing larvae of *Echinometra* spp. on red algae treated with antibiotics did not differ significantly (Tukey's test, $p > 0.05$) from that on fresh intact red algae (Table 4) indicating that bacteria on the surface of the algae might not be responsible for induction of metamorphosis.

Induction of larval settlement and metamorphosis in 4 *Echinometra* spp. by a crude extract of red algae in SFSW was concentration dependent. The rate of metamorphosis was minimal (≤ 19%) at higher dilutions (Fig. 1). The higher percentages of metamorphosis were observed at 1:5 and 1:10 dilutions (Fig. 1), and these values did not differ significantly (Tukey's test, $p > 0.05$) from those on CRA (Table 4).

Spores of red algae adhering to the bottom of petri dishes triggered metamorphosis of the larvae of *Echinometra* spp., although the mean values were significantly lower than those on intact red algae (Table 4). This may be explained by the surface area covered by spores which was only a small fraction of that covered by the algae. Settlement and metamorphosis in response to spores may have accounted for some of the recently metamorphosed juveniles found on the bottom and sides of experimental petri dishes after treatment with intact red algae (pooled

Table 2. Percentage of metamorphosis of competent larvae of *Echinometra* spp. (*E*. sp. nov. A, *E. mathaei*, *E*. sp. nov. C, and *E. oblonga*) in response to various groups of macro-algae on stone substrata. Each treatment consists of 5 replicates with 20 larvae per replicate. All values represent the mean ± sd with ranges in parentheses. Mean arcsine transformation values for each algal group within each experiment with common superscripts do not significantly differ (Tukey's test, $p > 0.05$)

Treatments/algal groups	*E*. sp. nov. A	*E. mathaei*	*E*. sp. nov. C	*E. oblonga*	Mean arcsine transformation value across all species
Coralline red algae (CRA)	87.0 ± 2.7 (85.0-90.0)	89.0 ± 4.2 (85.0-95.0)	88.0 ± 2.7 (85.0-90.0)	86.0 ± 4.2 (80.0-90.0)	1.070[a]
Coralline & regular mixed algae (CRMA)	53.0 ± 4.5 (50.0-60.0)	54.0 ± 5.5 (50.0-60.0)	51.0 ± 2.2 (50.0-55.0)	50.0 ± 5.0 (45.0-55.0)	0.528[b]
Regular brown algae (RBA)	11.0 ± 2.2 (10.0-15.0)	12.0 ± 2.7 (10.0-15.0)	11.0 ± 4.2 (5.0-15.0)	13.0 ± 2.7 (10.0-15.0)	0.118[c]
Regular green algae (RGA)	9.0 ± 2.2 (5.0-10.0)	8.0 ± 2.7 (5.0-10.0)	10.0 ± 3.5 (5.0-15.0)	10.0 ± 5.0 (5.0-15.0)	0.093[c]
Bare stone (BS)	3.0 ± 2.7 (0.0-5.0)	2.0 ± 2.7 (0.0-5.0)	3.0 ± 2.7 (0.0-5.0)	2.0 ± 2.7 (0.0-5.0)	0.025[d]
Sterile filtered sea water (SFSW)	0	0	0	0	

ANOVA Table: *Echinometra* spp. Summary of the analysis of variance of larval settlement and metamorphosis on various macro-algae in the laboratory. Statistical analyses were carried out on arcsine-transformed data, and treatment means were tested by completely randomized design. [s] = significant, [ns] = non-significant; SS: sum of squares, DF: degrees of freedom, MS: mean square.

Source	DF	SS	MS	F value	*p* value
Species	3	0.006	0.002	0.4554[ns]	
Algae	4	15.492	3.873	866.6140[s]	0.0000
Species × algae	12	0.059	0.005	1.1045[ns]	0.3683
Error	80	0.358	0.004		
Total	99	15.195			

Table 3. Percentage survival of juvenile urchins of *Echinometra* spp. (*E.* sp. nov. A, *E. mathaei*, *E.* sp. nov. C and *E. oblonga*) 1 wk following metamorphosis on various groups of macro-algae-encrusted stones. Each treatment consists of 5 replicates with 20 larvae per replicate. All values represent the mean ± sd with ranges in parentheses. Mean arcsine transformation values for each group within each experiment with common superscripts do not significantly differ (Tukey's test, $p > 0.05$)

Treatments/algal groups	*E.* sp. nov. A	*E. mathaei*	*E.* sp. nov. C	*E. oblonga*	Mean arcsine transformation value across all species
Coralline red algae (CRA)	85.0 ± 3.5 (80.0-90.0)	84.0 ± 4.2 (80.0-90.0)	86.0 ± 5.5 (80.0-95.0)	85.0 ± 3.5 (80.0-90.0)	1.021[a]
Coralline & regular mixed algae (CRMA)	44.0 ± 4.2 (40.0-50.0)	43.0 ± 4.5 (40.0-50.0)	43.0 ± 2.7 (40.0-45.0)	42.0 ± 5.7 (35.0-50.0)	0.445[b]
Regular brown algae (RBA)	2.0 ± 2.7 (0.0-5.0)	3.0 ± 4.5 (0.0-10.0)	3.0 ± 4.5 (0.0-10.0)	3.0 ± 4.5 (0.0-10.0)	0.028[c]
Regular green algae (RGA)	1.0 ± 2.2 (0.0-5.0)	2.0 ± 2.7 (0.0-5.0)	1.0 ± 2.7 (0.0-15.0)	2.0 ± 2.7 (0.0-5.0)	0.015[d]
Bare stone (BS)	0	0	0	0	

ANOVA Table: *Echinometra* spp. Summary of the analysis of variance of juvenile survival on various macro-algae 1 wk following metamorphosis in the laboratory. Statistical analyses were carried out on arcsine-transformed data, and treatment means were tested with a completely randomized design. [s] = significant, [ns] = non-significant; SS: sum of squares, DF: degrees of freedom, MS: mean square.

Source	DF	SS	MS	F value	*p* value
Species	3	0.001	0.000	0.0903[ns]	
Algae	3	13.455	4.485	1,451.856[s]	0.0000
Species × algae	9	0.006	0.001	0.2253[ns]	
Error	64	0.198	0.003		
Total	79	13.660			

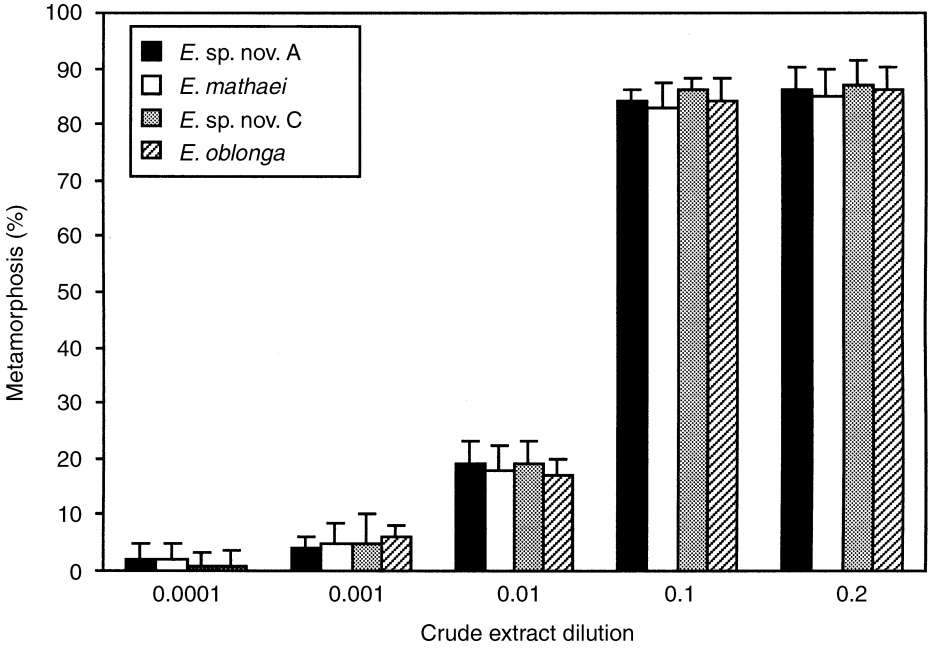

Fig. 1. *Echinometra* spp. Mean percent metamorphosis of larvae in response to serial dilution of an extract of CRA. Each treatment consists of 5 replicates with 20 larvae per replicate. Error bars indicate standard deviation.

from 15 experiments); 22.3% ± 4.2% (mean ± sd) of all individuals were juveniles and were located on the bottom or sides of petri dishes, whereas, 65% ± 3.5% were on the algal surface.

Induction of larval settlement and metamorphosis was negligible in the presence of red algae-conditioned SFSW compared to the very high rate of metamorphosis on CRA (Table 4) indicating that inducers are not leaking into surrounding SFSW (at least at concentrations that larvae can detect). Thus metamorphosis of urchin larvae appears to require contact with the algae. This result indicates the "surface matrix effect" on metamorphosis.

GABA, a neurotransmitter amino acids, triggers metamorphosis of the larvae of various marine invertebrates. As can be seen from figure 2, GABA induced settlement and metamorphosis in all *Echinometra* spp. larvae tested in a concentration-dependent manner. Significantly (Tukey's test, $p < 0.05$) lower rates of metamorphosis (≤ 24%) were observed at lower concentrations of GABA (10^{-5}-10^{-7} M) (Fig. 2). A higher percentage of metamorphosis was induced in all 4 *Echinometra* spp. larvae by higher concentrations of GABA at 10^{-3} M and at 10^{-2} M (Fig. 2), which did not differ significantly (Tukey's test, $p > 0.05$) from that in response to CRA (Table 4). All the newly metamorphosed juveniles, during an assay period of 24-30 h, appeared to be healthy,

Table 4. *Echinometra* spp. Induction of larval settlement and metamorphosis (%) by using coralline red algae (CRA), their surface materials, and extracts. Each treatment consists of 5 replicates with 20 larvae per replication. All values represent the mean ± sd with ranges in parentheses. Mean arcsine transformation values for each group within each experiment with common superscripts do not significantly differ (Tukey's test, $p > 0.05$)

Treatments/ additions	*E.* sp. nov. A	*E. mathaei*	*E.* sp. nov. C	*E. oblonga*	Mean arcsine transformation value across all species
None (SFSW only)	0	0	0	0	
GABA concentrations					
a) 10^{-2} M	83.0 ± 2.7 (80.0-85.0)	82.0 ± 2.7 (80.0-85.0)	82.0 ± 5.7 (75.0-90.0)	84.0 ± 4.2 (80.0-90.0)	0.988[a]
b) 10^{-3} M	83.0 ± 4.5 (80.0-90.0)	81.0 ± 4.2 (75.0-85.0)	82.0 ± 6.7 (75.0-90.0)	82.0 ± 2.7 (80.0-85.0)	0.979[a]
Coralline red algae (intact CRA)	89.0 ± 4.2 (85.0-90.0)	86.0 ± 4.2 (80.0-90.0)	88.0 ± 4.2 (85.0-95.0)	87.0 ± 2.7 (85.0-90.0)	1.070[a]
Antibiotic-treated (intact CRA)	85.0 ± 5.0 (80.0-90.0)	84.0 ± 4.2 (80.0-90.0)	86.0 ± 5.5 (80.0-90.0)	84.0 ± 4.2 (5.0-15.0)	1.017[a]
Crude extract					
a) 1:5 dilution	86.0 ± 4.2 (85.0-90.0)	85.0 ± 5.0 (80.0-90.0)	87.0 ± 4.5 (80.0-90.0)	86.0 ± 4.2 (0.0-5.0)	1.041[a]
b) 1:10 dilution	84.0 ± 2.2 (80.0-85.0)	83.0 ± 4.5 (80.0-90.0)	86.0 ± 2.2 (85.0-90.0)	84.0 ± 4.2 (80.0-90.0)	1.005[a]
Spores	17.0 ± 2.7 (15.0-20.0)	16.0 ± 4.2 (15.0-20.0)	17.0 ± 2.7 (15.0-20.0)	16.0 ± 2.2 (15.0-20.0)	0.166[b]
Algae-conditioned (supernants)	2.0 ± 2.7 (0.0-5.0)	1.0 ± 2.2 (0.0-5.0)	2.0 ± 2.7 (0.0-5.0)	1.0 ± 2.2 (0.0-5.0)	0.015[c]

ANOVA Table: Statistical analyses were carried out on arcsine-transformed data, and treatment means were tested by completely randomized design. [s] = significant, [ns] =non-significant; SS: sum of squares, DF: degrees of freedom, MS: mean square.

Source	DF	SS	MS	F value	*p* value
Species	3	0.018	0.006	1.1728[ns]	0.3228
Algae	7	25.957	3.708	741.823[s]	0.0000
Species × algae	21	0.020	0.001	0.1932[ns]	
Error	128	0.640	0.005		
Total	159	26.635			

and there was no evidence of deleterious effects or toxicity even at higher concentrations of GABA. Similarly, Pearce and Scheibling (1988 1990a) and Johnson et al. (1991a) in their studies observed no toxic effects of higher concentrations of GABA on larvae of *S. droebachiensis* and *A. planci*, respectively.

Substratum preference by *Echinometra* spp. in the field

Significantly higher (Tukey's test, $p < 0.05$) densities of *Echinometra* spp. (considering all 4 species together) were observed on CRA (Fig. 3) (range: 31%-57%) compared to those on CRMA (range: 18%-35%), RBA (range: 0%-4%), and RGA (range: 0%-3%). No urchin was found on bare stones during field observations.

DISCUSSION

Under laboratory conditions, the larvae of all 4 species of sea urchins in the genus *Echinometra* (*E.* sp. nov. A, *E. mathaei*, *E.* sp. nov. C, and *E. oblonga*) showed similar rates of metamorphosis and substratum selectivity when offered a variety of algal substrata. All *Echinometra* spp. exhibited a high rate of metamorphosis in response to coralline red algae and a very low percentage on green and brown algae among the various algal substrata tested. In the presence of brown and green algae, most larvae continued to swim, while a few settled either on the algal surfaces or on the bottom/sides of the petri dishes used in these experiments. The relatively high percentage of metamorphosis in treatments with red algae is consistent with Rowley's (1989) finding that coralline red algae from barren ground and red algal turf from a kelp bed both induced a high percentage of larvae of *Strongylocentrotus purpuratus* to metamorphose in the laboratory (89% and 95%, respectively) and harbored high densities of recently settled juveniles of *S. purpuratus* and *S. franciscanus* in the field. Passive entrapment of *Echinometra* spp. larvae may result in higher settlement on the more structurally branched and rugose coralline red algae than on the relatively smooth crusts of brown and green algae.

Algae release extracellular organic matter which may increase with stress (e.g., Sieburth 1969, Kroes 1970). Brown and green algae generally release larger amounts of polyphenols than do red algae (Sieburth 1969), which may account for the relatively much lower percent of metamorphosis and survival of juveniles (survival was checked 7 d after metamorphosis) in treatment with these algae than on red algae (compare Tables 2 and 3). This contrasts with the observations of Pearce and Scheibling (1991), who found that metamorphosed juveniles of *S. droebachiensis* on blades of *Fucus disticus* (31%)

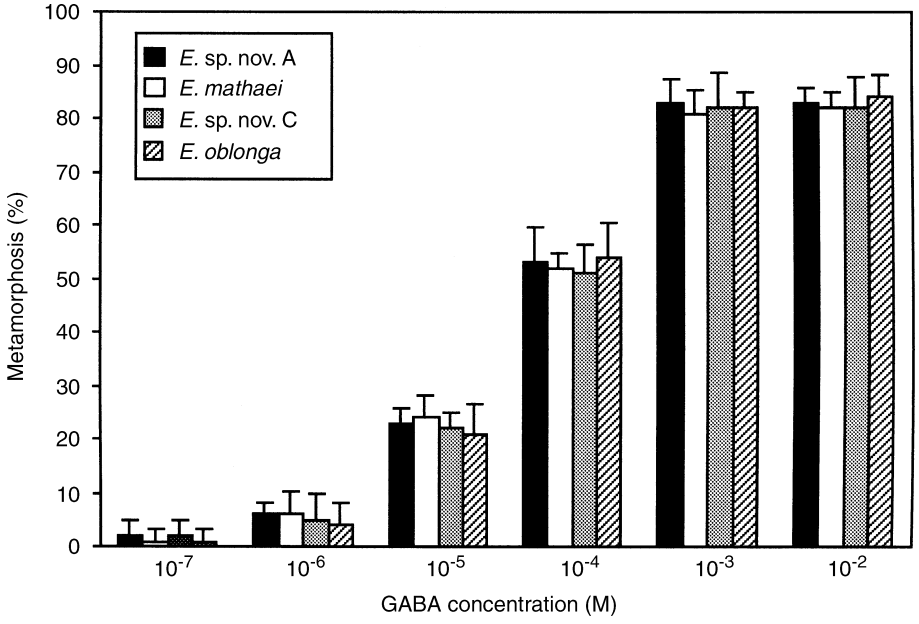

Fig. 2. *Echinometra* spp. Mean percentage of larvae that metamorphosed in response to various concentrations of gamma-aminobutyric acid (GABA). Each treatment consists of 5 replicates with 20 larvae per replicate. Error bars indicate standard deviation.

appeared healthy, indicating that polyphenols (at the level reached during the experiment) did not appear to be toxic to the larvae.

Several lines of evidence have indicated that direct contact by the larvae with the inducing algal surface is necessary for induction (Morse et al. 1979a 1980b 1988). Experiments with abalone larvae have shown that no visual or light-dependent process is required (Morse et al. 1979a, Morse and Morse 1984). Tests performed with coralline red algal stone in petri dishes with SFSW have demonstrated that recently metamorphosed juveniles of *Echinometra* spp. do not always locate on algal surface. This is because some larvae of *Echinometra* spp. can be induced to metamorphose by contact with spores, which were released by red algae and which spread over the bottom of experimental petri dishes. Alternatively, some larvae may land on the algal surface and then swim or crawl to adjacent areas, before or shortly after metamorphosis. The latter phenomenon was observed with the larvae of the coral, *Agaricia tenuifolia* (Morse et al. 1988), and a sea urchin, *Strongylocentrotus droebachiensis* (Pearce and Scheibling 1990a). This contrasts with observations of the larvae of the abalone, *Haliotis rufescens*, which settle and metamorphose exclusively on coralline red algae and not on adjacent non-algal surfaces (Morse et al. 1980a).

Tests performed by soaking red algal substrata in petri dishes containing SFSW demonstrated that no inducing substances leak into the surrounding seawater from the intact inducing algae; therefore any role of chemotaxis in the mechanism of metamorphosis was ruled out. Similarly, Pearce and Scheibling (1990a) observed that the surface contour of host alga has no role in initiating metamorphosis in *S. droebachiensis*. This contrasted with some other findings that settling larvae do respond to surface contour (the term, contour, is used when the scale of roughness is larger than the larvae itself) (Crisp 1976, Yule and Walker 1984, Carleton and Sammarco 1987, Le Tourneux and Bourget 1988, Bourget et al. 1994, Nellis and Bourget 1996, Bourget and Harvey, 1998).

Aqueous extracts (1:10-1:5 dilutions) of coralline red algae induced levels of metamorphosis of *Echinometra* spp. similar to those observed on intact algae, indicating that these algal extracts contain potent chemical inducers which mimic the properties of chemicals on the algal surface. Pearce and Scheibling (1990a) demonstrated that an algal extract (1:5 dilution) which contained 60 µg/ml protein induced larvae of *S. droebachiensis* to metamorphose at a rate similar to that on intact algae. Larvae of the sea urchin, *S. purpuratus* (Rowley 1989), are induced to settle and metamorphose in response to the same

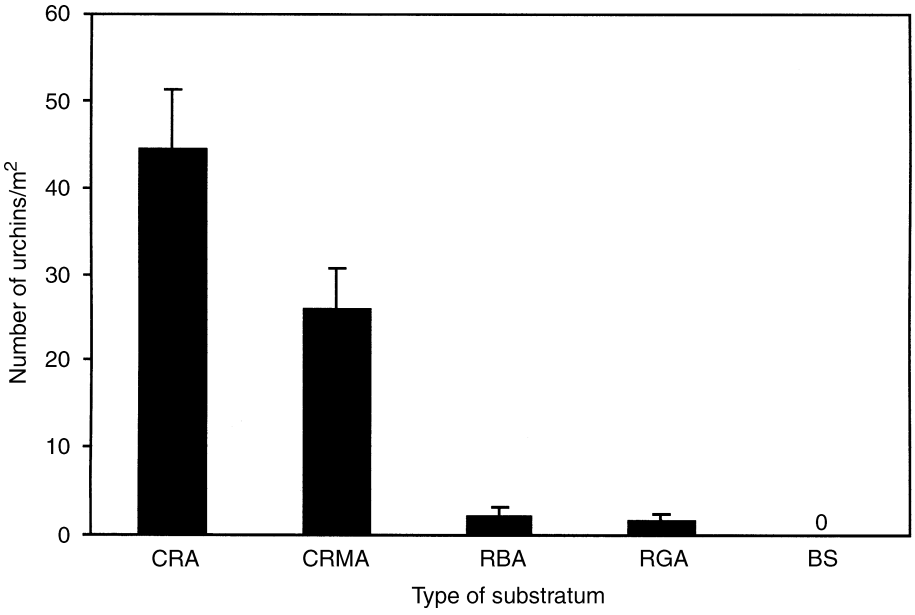

Fig. 3. Preferences for various algal substrata by *Echinometra* spp. in the field. Sea urchin densities were calculated as the total number of all 4 species of *Echinometra* (*E.* sp. nov. A, *E. mathaei*, *E.* sp. nov. C and *E. oblonga*) per square meter on each substratum. Sampling was carried out during a period of 6 mo (Apr.-Sept. 1998). In total, 30 samplings were done for each substratum with 5 per month. Vertical bars indicate means and standard deviations calculated for the 30 values. See text for abbreviations of substratum.

small peptide inducer, purified from extracts of coralline red algae (*Lithothamnion californicum*), which induces the larvae of *H. rufescens* to metamorphose (Morse and Morse 1984). These surface protein-linked oligopeptides have been demonstrated to be GABA-mimetic in their interaction with the larval receptors controlling the metamorphosis of *H. rufescens* (Trapido-Rosenthal and Morse 1986).

It is interesting to note that delta-aminolevulinic acid, an analog of GABA, has been implicated as a precursor in the synthesis of phycobiliproteins, which are characteristic of red algae (Bogoard 1975, Troxler 1977). GABA at a concentration of 10^{-3} M induced a level of metamorphosis of *Echinometra* spp. which was similar to that brought about by an intact red algal substratum (Table 4). Similar trends were also observed in *S. droebachiensis* (Pearce and Scheibling 1990a). Neurotransmitter amino acid compounds have been isolated from homogenized extracts of *Lithothamnion* sp. and *Lithophyllum* sp. (Morse et al. 1979b). GABA, phycoerythrobilin, and other protein-conjugated GABA analogs are potent inducers of settlement in several *Haliotis* spp. and some other gastropods (Morse 1981). A concentration of GABA between 10^{-5} and 10^{-7} M was most effective in initiating the settlement response of abalone larvae (Morse et al. 1980b); a much higher concentration of GABA is required to induce a high proportion of larvae of *Echinometra* spp. to metamorphose. A number of neurotransmitters and/or derivatives can affect larval settlement of marine invertebrates. Although the mechanisms remain unclear, it has been suggested that the neurotransmitters enhance or inhibit larval settlement by acting directly on the nervous systems of larvae rather than on the chemoreceptors involved in settlement and normal development (Morse et al. 1979a,b, Morse 1985 1990, Jensen and Morse 1990, Pawlik 1990, Pearce and Schibling 1990a, Bryan et al. 1997a, Pechenik and Qian 1998, and see reviews by Pawlik 1992, Qian 1999).

In situations where a coralline red alga induces settlement and metamorphosis of invertebrate larvae, the source of the morphogenic compounds may be epiphytic bacteria on the algal surface and not the alga itself (Johnson et al. 1991a,b); but this idea has not received critical attention. Although initial experiments showed that treating CCA (crustose coralline algae) with antibiotics reduced metamorphosis (Johnson et al. 1991a), the possibility could not be ruled out that the antibiotics might be affecting the activity or production of inducing substances independently of its effects on bacteria (Johnson 1992, Johnson and Sutton 1994, Lau and Qian 1997, Beckmann et al. 1999, Unabia and Hadfield 1999). In the present experiment, treating CRA with antibiotics did not reduce the rate of metamorphosis of *Echinometra* spp. However, some residual bacteria or other microbes (such as diatoms or protozoa) unaffected by the antibiotics may be responsible for the production of an inducing factor. A reduction of bacterial concentrations by 2 orders of magnitude using the same antibiotics employed in this study did not reduce the rate of metamorphosis in *S. droebachiensis* (Pearce and Scheibling 1990a). In contrast, brachiolaria larvae of *Acanthaster planci* settled and metamorphosed in lower numbers on the antibiotic-treated shards of red algae that were not reinfected by bacteria, suggesting that induction of metamorphosis by the alga, *L. pseudosorum*, is mediated by bacteria on the surface of the algae (Johnson and Sutton 1994).

The aggregated distribution of juvenile and adult urchins of *Echinometra* spp. on coralline red algal substrata (Fig. 3) could be the result of preferential settlement on their principal food. There would be an advantage for urchins to settle preferentially on the food they predominantly consume rather than having to search for it. Similar patterns of settlement have been reported for other invertebrates (Steneck 1982, Morse and Morse 1984, Shepherd and Turner 1985, Hadfield and Miller 1987, Todd 1991, and see review by Pawlik 1992). In the shallow rocky intertidal zone of the subtropical Okinawan islands, *Echinometra* spp. generally aggregate on coralline red algal-dominated communities rather than on other types of algae (pers. observ.). A similar phenomenon has been well documented for *S. droebachiensis* in eastern Canada, where selective recruitment of strongylocentrotid urchins occurs on various coralline red algal communities (Miller 1985, Scheibling 1986, Raymond and Scheibling 1987). Our results suggest that differential habitat-specific settlement may determine the relative densities of sea urchin populations on different algal substrata.

This is the first successful attempt to study settlement induction and metamorphosis in 4 closely related but genetically distinct species of sea urchins of the genus *Echinometra*. The 4 species showing similar trends of undergoing metamorphosis and selecting substrata may be due to their close genetic affinity as well as their sympatric existence in nature. Although all species did not show particular differences in metamorphosis in each type of substratum, each species highly prefers coralline red algal substratum for their higher metamorphosis, and better growth, development, and recruitment over other algal (brown and green) substrata. Further studies are

needed to identify, purify, and biochemically characterize the inducer from these coralline algae.

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琉球長海膽屬中四種共域且親緣關係相近的海膽幼生之誘引變態與底質偏好

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在長海膽屬(*Echinometra*)內有四種海膽(*E.* sp. nov. A, *E. mathaei*, *E.* sp. nov. C, and *E. oblonga*)，它們之間親緣關係相近但是遺傳組成歧異度卻很大，本次研究以實驗室飼養 20 ～ 24 天的四種幼生進行變態與著床實驗，比較牠們對不同底質的偏好。這四種長海膽屬幼生在殼狀珊瑚紅藻中的變態率較高，而在混合有褐色、綠色的帶狀珊瑚藻或是其他混合的球狀藻中變態率相對較低，顯示出能夠誘發變態的物質可能在紅藻中，而在綠藻或褐藻中可能沒有這種誘導物質或是含量較少，所以海膽在此條件下才會有較低的變態率與生存率。而殼狀珊瑚紅藻的萃取液亦能誘導幼生變態，更能進一步說明誘導物質是天然化學物質。

這些化學物質已經被多人確定是蛋白質，會和控制幼生變態的接收器相結合，作用機制類似 γ- 胺基丁酸。γ- 胺基丁酸是一種誘導變態的物質，作用在一些腹足類與海膽 (strongylocentrotids) ，長海膽屬的幼生在超過 10^{-3}M 的濃度中也會發生變態，顯示化學感應接收器在誘導變態上可能比組織本身的改變來得重要。將　底質以紅藻浸泡的測試結果，可以確定沒有任何物質從完整的誘導性藻類外露至海水中。利用抗生素減少紅藻表面細菌的測試，結果也沒有影響到幼生的變態率。

這次的誘導著床與變態實驗是首度在長海膽屬中這四種海膽進行，它們之間親緣關係相近但是遺傳組成歧異度很大。這四種海膽在不同底質上的變態率相同，也都偏好珊瑚紅藻的底質，這個結果和它們相近的遺傳屬性以及共域的特徵一致。

關鍵詞：著床，長海膽屬，遺傳屬性，珊瑚藻，γ- 胺基丁酸。

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