Lipophilic Inducers Extracted from *Corallina pilulifera* for
Larval Settlement and Metamorphosis of Two Sea
Urchins *Pseudocentrotus depressus* and
Anthocardis crassispina^{*1,2}

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(Received April 15, 1991)

Lipophilic extracts from the coralline alga *Corallina pilulifera* which induced settlement and metamorphosis of fully developed 8-armed larvae of *Pseudocentrotus depressus* and *Anthocardis crassispina*, were found by a simple bioassay method. Benzene, ether, and methanol extracts from the alga also had an inducing effect. Column chromatography showed that simple lipids such as hydrocarbons, triglycerides, and free fatty acids were the inducers. Glycolipid and phospholipid did not induce settlement or metamorphosis.

The sea urchin "Akauni" *Pseudocentrotus depressus* is an important species for the fisheries industry in the west part of Japan. For example, the fish farming centers in Saga and Nagasaki Prefectures prepare by means of artificial fertilization, about one million seeds of *P. depressus* (5-10 mm in test diameter) every year, for promoting starting stock in the holding area. "Murasakiuni" *Anthocardis crassispina* is also popular, but only a small number of seeds are produced in the above centers.

Fully developed, 8-armed larvae of *P. depressus* and *A. crassispina* do not metamorphose in a clean beaker, but show high rates of metamorphosis upon contact with benthic diatoms, algae such as "Hiziki" *Hizikia fusiformis* and "Umitorano" *Sargassum thunbergii*, and both crustose and foliose coralline algae.¹⁻³⁾

Field observations showed that newly settled individuals of sea urchins *Strongylocentrotus purpuratus* and *S. franciscanus* form dense populations on rocky areas covered with crustose coralline algae.⁴⁾ Metamorphosis of these larvae and *Loxechinus*, *Lytechinus*, and *Arbacia* larvae can also be induced by a bacterial film on a solid substrate.⁵⁻⁷⁾

These studies led us to examine the assumption that diatoms, bacterial film and some kinds of algae possess chemical inducers of settlement and metamorphosis of sea urchin larvae. How-

ever, there are only two reports on such chemical inducers. Cameron and Schroeter⁸⁾ studied a chemical inducer from bacteria, and Pearce and Scheibling⁹⁾ indicated that aqueous extracts of coralline algae can induce metamorphosis of *S. droebachiensis* larvae. In neither study were the chemical characteristics of these inducers made clear.

We have studied lipophilic and water soluble substances extracted from the foliose coralline alga *Corallina pilulifera* to clarify the chemical inducers of larval settlement and metamorphosis of *P. depressus* and *A. crassispina*. In this paper we describe the results obtained for lipophilic inducers.

Materials and Methods

Test Larvae

P. depressus and *A. crassispina* larvae, cultured to the 8-armed stage with a fully developed urchin rudiment, were used in the experiments. After fertilization, *P. depressus* larvae were cultured for about three weeks at 17~22°C in the laboratory. 8-armed larvae were also obtained from the fish farming centers in Saga Prefecture and Nagasaki city. *A. crassispina* larvae were cultured for about two weeks at 22~25°C in the laboratory. The diatom *Chaetoceros gracilis* was used as food for these larvae.

*1 This work was supported by grants from the Ministry of Education, Science, and Culture of Japan.

*2 The outline of this report was presented at the autumn meeting of Japan. Soc. Sci. Fish., Nara, 1990.

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Extraction and Fractionation of Lipophilic Substances

The foliose coralline alga *Corallina pilulifera* was collected from the rocky shore near the Fish Farming Center in Saga. The alga (5 g, wet-weight) was air-dried for one day, and soaked in 100 ml of benzene at room temperature for a similar period. The solvent was decanted and evaporated to dryness. The ether and methanol extracts were also obtained in the same manner.

Alga (800 g, wet-weight) was air-dried for one day and soaked in chloroform-methanol (2:1, 1,000 ml) for two days. The solvent was decanted and soaking repeated three times. The combined extracts were shaken with water (about 600 ml) to obtain both lipophilic and water-soluble substances. The organic layer was evaporated to dryness, to yield about 3,600 mg (about 4.5 mg per 1 g of wet-weight).

A portion of the crude lipid (30 mg) was subjected to silica gel column chromatography (Mallinckrodt 100 mesh, (ϕ 2 $\times$ 18 cm), packed with chloroform). The elution was made as follows; chloroform (200 ml, Fraction-1, F-2), acetone (100 ml, F-3), methanol (100 ml, F-4), and 10% aq. methanol (100 ml, F-5). Each elute was evaporated to dryness at 36°C and redissolved in less than 3 ml of chloroform. Thin-layer chromatography (TLC) of these five fractions was performed on silica gel plates (Whatman, 60A, 0.25 mm thickness 20 $\times$ 20 cm) with chloroform-acetone ($\approx$ 9:1 or 1:1). Lipids on the plates were visualized by spraying with 50% H₂SO₄, anthrone, and Dittmer reagents.

Bioassay Method

The three organic solvents extracted, the lipid extracted with chloroform-methanol and the fractions, F-1~F-5, were bioassayed for the effect on settlement and metamorphosis of the two species of sea urchin larvae.

Whatman chromatography paper (46 $\times$ 57 cm) was used for the bioassay substrate. The paper was cut into circles of 5 cm diameter (about 20cm²). Each extract or fraction in chloroform was adsorbed on the paper, and the organic solvent was evaporated off in a draft. The paper with adsorbed lipophilic substances was set on the bottom of a 200 ml Pyrex beaker, and 100 ml of filtered seawater (Whatman glass fiber filter, GF/C) was poured into the beaker. After that, thirty 8-armed larvae of *P. depressus* or *A. crassispina* were placed into the beaker. Paper with adsorbed organic solvent only was used as a control. The

beakers were kept at 22°C in a dark room. After 24 h, the state of each larva was checked by stereoscopic microscope.

Assessment of the effect on the settlement and metamorphosis was made on the basis of settlement rate and metamorphosis rate. The former was expressed as the percentage consisting of individuals which are partially metamorphosed larvae (with extruded feet, but which still had larval cilia) and juvenile sea urchin. The latter was the percentage of individuals which had undergone full metamorphosis to the juvenile sea urchin form.

Results

When the fresh, foliose coralline alga (5 g, wet-weight) was placed into 100 ml filtered seawater in a 200 ml beaker, more than 90% of the 8-armed larvae of both sea urchin species metamorphosed within 24 h.

The settlement and metamorphosis rates in the benzene, ether, and methanol extracts are shown in Table 1. More than 80% of both species larvae settled in response to the presence of these extracts. Although the metamorphosis rate in benzene extract was 4%, the ether and methanol extracts induced metamorphosis in about 90% of *P. depressus* larvae, and about 70% of *A. crassispina* larvae. Throughout all of the experiments, the settlement and metamorphosis rates of the control tests were lower than 15% and 0%, respectively.

Table 2 shows the effect of the lipids extracted with chloroform-methanol (2:1). All *P. depressus* larvae settled with a dose of more than 10 mg lipid/paper, whereas 87% metamorphosed with

Table 1. Induction of settlement and metamorphosis of *P. depressus* and *A. crassispina* larvae by extracts from 5 g (wet weight) of the foliose coralline alga *Corallina pilulifera*

		(%)	
Sea urchin	Extract	Settlement Rate	Metamorphosis Rate
<i>Pseudocentrotus depressus</i>	Benzene	80	4
	Ether	100	93
	Methanol	100	87
	(Control)	<15	0
<i>Anthocidaris crassispina</i>	Benzene	100	67
	Ether	100	67
	Methanol	100	70
	(Control)	<15	0

Table 2. Induction of settlement and metamorphosis of *P. depressus* and *A. crassispina* larvae by the lipid extracted with chloroform-methanol (2:1) from the foliose coralline alga *Corallina pilulifera*

Sea urchin	Lipid (mg/paper)	Settlement Rate (%)	Metamorphosis Rate (%)
<i>Pseudocentrotus depressus</i>	0*	<15	0
	5.6	83	7
	10	100	17
	19	100	37
	30	100	87
<i>Anthocardia crassispina</i>	0*	<15	0
	10	97	33
	30	100	83

* Control test.

Fig. 1. The settlement rate of *Pseudocentrotus depressus* larvae induced by fractions from silica gel column chromatography of the lipid extract (30 mg). Weights of F-1~F-5 were 9.0, 2.4, 8.1, 7.5, and 2.6 mg lipid/paper, respectively. The figure in parentheses denotes the metamorphosis rate. The settlement and metamorphosis rates in control tests were always lower than 15% and 0%, respectively.

30 mg/paper. Most *A. crassispina* larvae settled at 10 and 30 mg/paper, the metamorphosis rate at 30 mg/paper being 83%. The effects of the lipid on both larvae grew more pronounced as the amount of lipid increased.

The five fractions obtained by silica gel column chromatography showed similar effects on the settlement and metamorphosis of *P. depressus*

and *A. crassispina* larvae. As shown in Fig. 1, more than 90% of *P. depressus* larvae settled in response to the F-1 and F-2 fractions, whereas there was little or on response to fractions F-3~F-5. The metamorphosis rate for F-2 was 73%, but only 17% for F-1.

Although some metamorphosed individuals showed deformities, most of the juvenile sea urchins appeared normal. Subsequent growth of metamorphosed juveniles continued normally for more than two weeks, with the diatom *Navicula ramosissima*, which is one of the suitable primary foods for these sea urchins.⁹⁾

Discussion

Although the metamorphosis rate in benzene extract was 4%, the ether and methanol extracts from the alga (5 g) induced high levels of both settlement and metamorphosis in both species (Table 1). All larvae settled at more than 10 mg lipid/paper (Table 2). These results clearly suggested that the alga had some lipophilic substances acting as inducers for settlement and metamorphosis of these sea urchin larvae.

The fractions F-1 and F-2 obtained by silica gel column chromatography induced high percentages of larval settlement and metamorphosis in the two species (Fig. 1). TLC showed fractions F-1 and F-2 to contain simple lipids, such as hydrocarbons, triglycerides, and free fatty acids. After consideration of these factors, we concluded that the inducer belongs to the above groups of lipids, because of the inefficiency of settlement and metamorphosis in glycolipid and phospholipid extracted as fractions F-3~F-5.

For further studies, this simple bioassay method is very useful for detection of chemical inducers for larval settlement and metamorphosis of sea urchins.

Acknowledgements

We wish to express our gratitude to the staff of the fish farming centers in Saga Prefecture and Nagasaki City for providing us with sea urchin larvae. This study was supported in part by a grant from the Research Institute of Marine Invertebrates.

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