Marine Biology 115, 387–392 (1993)

Marine Biology
International Journal on Life in Oceans and Coastal Waters

The induction of larval settlement and metamorphosis of two sea urchins, *Pseudocentrotus depressus* and *Anthocidaris crassispina*, by free fatty acids extracted from the coralline red alga *Corallina pilulifera*

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Received: 27 August 1992 / Accepted: 19 October 1992

Abstract. Lipophilic inducers of larval settlement and metamorphosis of *Pseudocentrotus depressus* and *Anthocidaris crassispina*, two commercially important sea urchin species in Japan, were isolated from the foliose coralline red alga *Corallina pilulifera* (collected in 1990 near Saga, Japan) and identified. Larval assays of the fractions obtained by silica gel column chromatography of the total lipids showed that non-polar groups of lipids were effective at inducing larval settlement and metamorphosis. The effective fractions were further subjected to gel filtration (Sephadex LH-20) and also to silica gel column chromatography, and the effective components isolated as single spots by thin-layer chromatography. The components at a concentration of ca. 0.4 mg paper^{-1} (sample was adsorbed on a paper with 20 cm^2) induced high rates of larval settlement of both *P. depressus* and *A. crassispina*. Chemical analyses of the components revealed a mixture of free fatty acids (FFAs), dominated by eicosapentaenoic acid (20:5, 41 to 50%), palmitic acid (16:0, 11 to 17%), arachidonic acid (20:4, 9 to 15%), and palmitoleic acid (16:1, 4 to 5%). In assays with the four standard FFAs, only 20:4 and 20:5 induced larval settlement and metamorphosis of the two species, while 16:0 and 16:1 were ineffective. The larvae underwent significant rates of settlement and metamorphosis in response to the two former FFAs at levels as low as 0.18 mg paper^{-1}. Amongst the free fatty acid components of the alga, 20:5 was isolated as the chemical inducer of larval settlement and metamorphosis of the sea urchins in the laboratory.

Introduction

The sea urchins *Pseudocentrotus depressus*, *Anthocidaris crassispina*, *Hemicentrotus pulcherrimus*, *Strongylocentrotus intermedius*, and *S. nudus* are important commercial species in Japan. The former three species and the latter two inhabit the western and northern parts of Japan, respectively. Marketing of the gonads of these species was worth some $ 200 million per year during the period 1979 to 1989.

For the promotion of starting stock of these sea urchins in the holding areas, fish farming centers prepare juvenile seeds using artificial fertilization. For example, in western Japan the fish farming centers in Nagasaki and Saga prefectures produce ca. two million seeds of *Pseudocentrotus depressus* (3 to 28 mm in test diameter) and ca. one million seeds of *Hemicentrotus pulcherrimus* (3 to 12 mm) every year. *Anthocidaris crassispina* is also popular, but only a small number of seeds are produced in the above centers. In the northern part of Japan, a total of some 30 million seeds of *Strongylocentrotus intermedius* and *S. nudus* are produced artificially every year.

Fully developed, 8-armed larvae of *Pseudocentrotus depressus* and *Anthocidaris crassispina* do not metamorphose in a clean beaker, but show high rates of metamorphosis upon contact with benthic diatoms and algae such as the brown algae *Hizikia fusiformis* and *Sargassum thunbergii* (Tani and Ito 1979, Ito 1984, Ito et al. 1991). In the fish farming centers in Saga prefecture and Nagasaki City, the benthic diatoms *Navicula, Achnanthes, Amphora,* and *Nitzschia* spp. are cultured on "Nami-ita" (waved, transparent, poly-vinyl-chloride plates) in order to induce metamorphosis of the sea urchin larvae. These diatoms are also suitable as primary foods for juvenile sea urchins (Ito et al. 1987).

Laboratory-reared larvae of the green sea urchin, *Strongylocentrotus droebachiensis*, were induced to metamorphose upon contact with coralline red algae (Pearce and Scheibling 1990, 1991) and 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 Hinegardner 1974, Cameron and Schroeter 1980, Gonzalez et al. 1987). Field observations showed that newly settled individuals of sea urchins (*S. purpuratus* and *S. franciscanus*) formed dense populations on rocky areas covered with crustose, coralline red algae (Rowley 1989).

These studies led us to examine the assumption that benthic diatoms, bacterial films, and or some kinds of

algae possess chemical inducers of larval settlement and metamorphosis in sea urchins. However, there are only two reports on such chemical inducers. Cameron and Hinegardner (1974) studied a chemical inducer from bacteria, and Pearce and Scheibling (1990) indicated that aqueous extracts of coralline algae could induce metamorphosis of *Strongylocentrotus 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 red alga *Corallina pilulifera* in order to clarify the chemical inducers of larval settlement and metamorphosis of *Pseudocentrotus depressus* and *Anthocidaris crassispina*. A previous report (Kitamura et al. 1992) showed that larvae of these two species were induced to settle and metamorphose by lipophilic substances extracted from the alga, the inducer supposedly belonging to non-polar groups of lipids. For the present report, such lipophilic inducers were identified as free fatty acids.

Materials and methods

Larval culture

Pseudocentrotus depressus and *Anthocidaris crassispina* larvae, cultured to the 8-armed stage with a fully developed urchin rudiment, were used in the experiments. Methods of fertilization and larval culture were as follows.

Spawning of the sea urchins was initiated by 0.5 *M* KCl solution. Eggs were rinsed with filtered seawater (Whatman glass fiber filter, GF/C; 1.2 μm), fertilized with a dilute suspension of sperm, and rinsed several times with filtered seawater. After ca. 20 h, swimming, pyramidal-shaped larvae were transferred to two or three 30-liter tanks filled with 25 liters of filtered seawater (final concentration ca. 0.8 larva ml^{-1}). The culture tanks were continuously aerated (200 ml min^{-1}) in a dark room. Half of the seawater in the culture tanks was renewed every day. Polyethylene nets, with mesh openings of 111 and 225 μm, were used progressively as the larvae grew to filter off the larvae. Food for the larvae was added after refilling the tanks.

The diatom *Chaetoceros gracilis* was used as food for larvae at concentrations of 0.8×10^4 to 3.0×10^4 cells ml^{-1} in the culture tank; the concentration was increased as the larvae grew. *C. gracilis* was propagated under unialgal conditions in 1-liter flat-bottom flasks with 1 liter of modified Provasoli ES medium (Ito et al. 1985) at 25 °C. The flasks were illuminated at ca. 10 000 Lux and aerated (10 liter min^{-1}) continuously. The diatoms increased in number from an initial density of 5.0×10^4 cells ml^{-1} to more than 1.0×10^7 cells ml^{-1} within 1 wk.

After fertilization, *Pseudocentrotus depressus* larvae were cultured in the laboratory at 17 to 22 °C for ca. 3 wk from October to April. In addition, 8-armed competent larvae were obtained from the fish farming centers in Saga prefecture and Nagasaki City. *Anthocidaris crassispina* larvae were cultured in the laboratory at 22 to 25 °C for ca. 2 wk from May to September.

Larval assays

Whatman chromatography paper (46 × 57 cm), which was cut into circles of 5-cm diameter (ca. 20 cm^2), was used in all assays. Each sample in organic solvent was adsorbed on the paper and the solvent 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 was added. Subsequently, 30 8-armed competent larvae of *Pseudocentrotus depressus* or *Anthocidaris crassispina* were placed into the beaker. Paper with adsorbed organic solvent only was used as a control. The beakers were kept in a dark room at 18 and 22 °C for *P. depressus* and *A. crassispina*, respectively. After 24 h, the state of each larva was checked with a stereoscopic microscope. Larval assays were performed two to six times with each sample.

Assessment of the larval response was made on the basis of settlement (%) and metamorphosis (%). The former was expressed as the percentage of individuals with extruded feet, but which retained larval cilia. These larvae could swim away from their substrata, so some larvae continued swimming and the others attached on the paper. Most of the 8-armed competent larvae had no feet without inducers. Therefore, we consider individuals with feet to be partially metamorphosed larvae. The latter, metamorphosis (%), was the percentage of individuals which had undergone full metamorphosis to the juvenile sea urchin form. At the end of the assay period, 30 individuals were easily found, and these two percentages were based on this number.

When fresh *Corallina pilulifera* (5 g wet wt) was put into 100 ml of 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 alga contained ca. 23 mg (4.5 mg g^{-1} wet wt of alga) of total lipids as mentioned later. For the present report, 30 mg $paper^{-1}$ of total lipids were usually assayed to evaluate larval response.

Extraction and isolation of the lipophilic inducer

The coralline alga *Corallina pilulifera* was collected in 1990 from a rocky shore near the fish farming center in Saga. The fresh alga (1500 g wet wt) was air-dried for 1 d and soaked in chloroform-methanol (2:1, 2000 ml) for 2 d to extract the total lipids, according to the method of Folch et al. (1957). Such extraction was repeated three times, resulting in ca. 6750 mg of total lipids. The lipid extracts were stored at −25 °C with butylated hydroxytoluene (BHT) (0.1%) pending further experimental procedures.

Total lipids (700 mg) were subjected to silica gel column chromatography (Mallinckrodt 100 mesh, ϕ 2 × 18 cm, packed with chloroform). The elution was made as follows: chloroform [200 ml, Fraction (F) 1 and F2], acetone (100 ml, F3), and methanol (100 ml, F4). The fractionation was performed eight times (i.e., 700 mg × 8) following the same procedure. Each similar fraction was combined, evaporated to dryness at 36 °C and redissolved in chloroform. The four component fractions (F1 to F4) comprised 37, 10, 27, and 20% of the total lipids, respectively.

Kitamura et al. (1992) reported that fraction F2 induced high larval settlement and metamorphosis in *Pseudocentrotus depressus* and *Anthocidaris crassispina*. Therefore, this fraction was further fractionated by Sephadex LH-20 column chromatography (fine, ϕ 4 × 60 cm), eluted with chloroform-methanol (1:100, 1 ml min^{-1}, 10 ml $tube^{-1}$). Two separate fractionations were performed, resulting in six fractions (F2/1 to 6) for *P. depressus* and five fractions (F2/1 to 5) for *A. crassispina* being obtained.

Each effective fraction (F2/3 for *Pseudocentrotus depressus* and F2/5 for *Anthocidaris crassispina*) was also subjected to silica gel column chromatography, packed with hexane-ether (7:3), resulting in six further fractions (F2/3/① to ⑥ and F2/5/① to ⑥). The elution was made as follows: hexane-ether (7:3, 50 ml), hexane-ether (3:7, 50 ml), ether (50 ml), ether-ethylacetate (7:3, 50 ml), ether-ethylacetate (3:7, 50 ml), ethylacetate (50 ml), ethylacetate-methanol (1:1, 100 ml), and methanol (100 ml).

Identification of effective components

The constituents of the effective fractions for larval settlement and metamorphosis in the two sea urchin species were analysed by

thin-layer chromatography (TLC) with standard lipids (Sigma and Wako) and by Proton nuclear-magnetic-resonance spectroscopy (NMR, JEOL, JNM-GX400). TLC was performed on silica gel plates (Whatman 60 A, 0.25-mm thickness, 20 × 20 cm) with chloroform-acetone (9:1). Lipids on the plates were visualized by spraying with 50% H_2SO_4, anthrone, and Dittmer reagents. Such chemical analyses revealed a mixture of free fatty acids (FFAs). Consequently, fatty acid contents of the fractions were analysed by gas chromatography (GC) on a Shimazu G-14 A gas chromatograph equipped with a flame ionization detector on the capillary column (ULBON HR-20M).

Results

Larval responses to total lipids and fractions

Larval responses of *Pseudocentrotus depressus* to total lipids and the four fractions (F1 to F4) are shown in Fig. 1. More than 80% of larvae responded to total lipids at 10 and 30 mg paper^{-1}, and ca. 50% metamorphosed at 30 mg paper^{-1}. Of the four fractions, F2 showed the highest metamorphosis (%), 62% at 3 mg paper^{-1}, which was equivalent to 30 mg of total lipids. The metamorphosis (%) of the other fractions were lower than 15%. Throughout all the experiments, the settlement (%) and metamorphosis (%) of the two species in control tests were always lower than 15 and 0%, respectively.

Fraction F2 was further fractionated into six fractions (F2/1 to 6) by Sephadex LH-20 column chromatography, but only fraction F2/3 induced larval settlement and metamorphosis. This fraction was again fractionated into six further fractions (F2/3/① to ⑥) by silica gel column chromatography. Fig. 2 represents the larval responses to fractions F2/3 and F2/3/② to ⑥. The fraction F2/3/① showed no response. Larval response to fraction F2/3 was ca. 100% at 0.6 and 1.2 mg paper^{-1} (equivalent to 30 and 60 mg of total lipids, respectively), with more than 70% of larvae metamorphosing at 1.2 mg paper^{-1}. Fractions F2/3/② to ⑥, each equivalent to 60 mg of total lipids, showed less response compared to fraction F2/3. Although fractions F2/3/③ to ⑤ had higher response than that of the fraction F2/3/②, the latter fraction showed only a single spot following TLC. We can easily analyse consitituents of a fraction having a single spot. The other fractions (③ to ⑥) had several spots, but the main spot in these fractions was the same as that of fraction F2/3/②. When the fraction ② was assayed at 0.36 mg paper^{-1}, larval response increased to ca. 100% (Fig. 2).

Larval responses of *Anthocidaris crassispina* to total lipids and the four fractions are shown in Fig. 3. More than 94% of larvae responded to total lipids at 10 and 30 mg paper^{-1}, with 90% of larvae metamorphosing at 30 mg paper^{-1}. Fraction F2 induced the highest settlement and metamorphosis of the four fractions.

Further fractionation of fraction F2 was conducted, as for the experiments with *Pseudocentrotus depressus*, with six final fractions (F2/5/① to ⑥) being obtained. Fig. 4 shows the larval responses to fractions F2/5 and F2/5/① to ④. The other fractions, F2/1 to 4 and F2/5/⑤ and ⑥, were not effective. Fraction F2/5 induced higher than 90% of larval response at 1.8 and 3.6 mg paper^{-1}

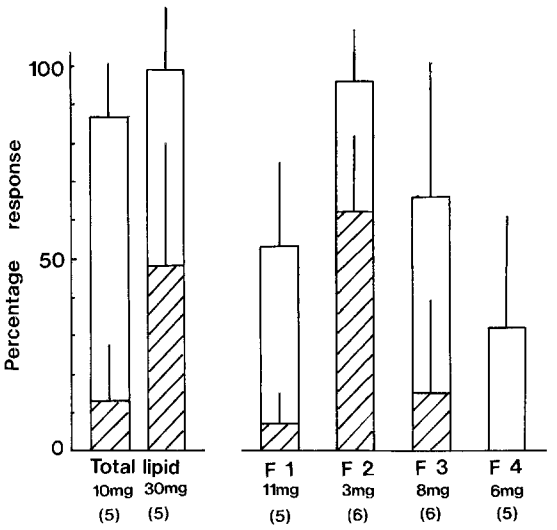

Fig. 1. *Pseudocentrotus depressus.* Mean percentages of larval response to total lipids (10 and 30 mg paper^{-1}) and fractions F1 to F4 (each equivalent to 30 mg of total lipids) obtained by silica gel column chromatography of total lipids. (Error bars and parentheses denote standard deviation and number of experiments, respectively). □: settlement (%); ▨: metamorphosis (%)

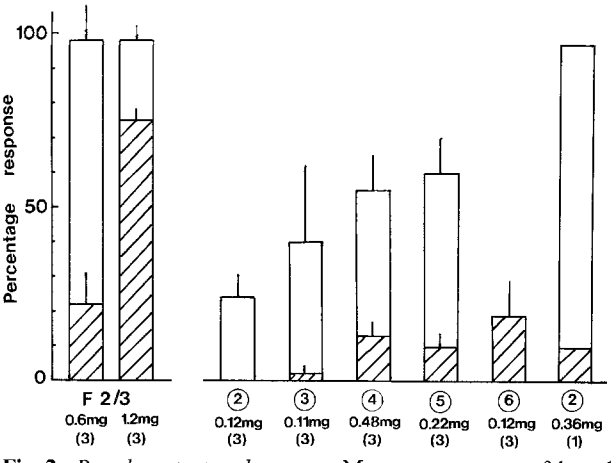

Fig. 2. *Pseudocentrotus depressus.* Mean percentages of larval response to fraction F2/3 (equivalent to 30 and 60 mg of total lipids) obtained by Sephadex LH-20 column chromatography of F2 and fractions F2/3/② to ⑥ (each equivalent to 60 mg of total lipids, fraction F2/3/① showed no response) obtained by silica gel column chromatography of F2/3. F2/3/② also assayed at a concentration equivalent to 180 mg of total lipids. Error bars and parentheses denote standard deviation and number of experiments, respectively). □: settlement (%); ▨: metamorphosis (%)

(equivalent to 30 and 60 mg of total lipids, respectively). Of the four fractions (① to ④), fraction F2/5/② showed the highest settlement and metamorphosis at 0.40 mg paper^{-1}. This fraction had a single spot following TLC, which was almost the same as that of F2/3/② for *P. depressus*.

Although some metamorphosed individuals showed deformities, most of the juvenile sea urchins appeared normal. Subsequent growth of metamorphosed juveniles continued normally for more than 2 wk; the food provided was the diatom *Navicula ramosissima*, which is a suitable primary food for these sea urchins (Ito et al. 1987).

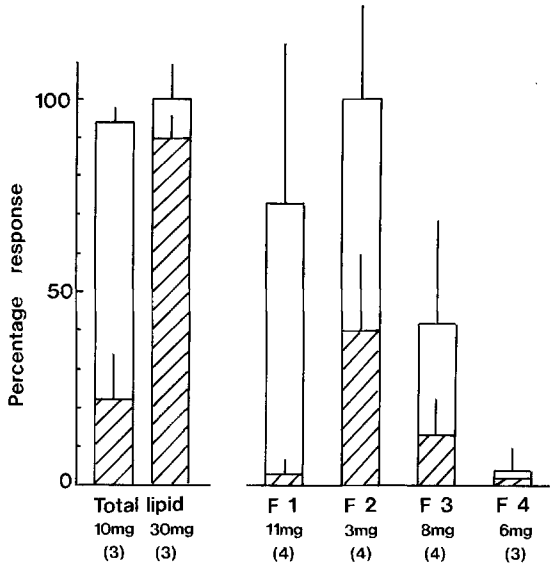

Fig. 3. *Anthocidaris crassispina.* Mean percentages of larval response to total lipids (10 and 30 mg paper^{-1}) and fractions F1 to F4 (each equivalent to 30 mg of total lipids). (Error bars and parentheses denote standard deviation and number of experiments, respectively). □: settlement (%); ▨: metamorphosis (%)

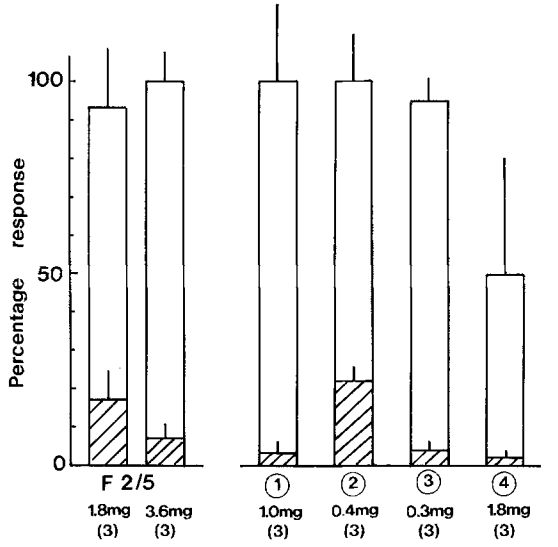

Fig. 4. *Anthocidaris crassispina.* Mean percentages of larval response to fractions F2/5 (equivalent to 30 and 60 mg of total lipids) and F2/5/① to ④ (each equivalent to 60 mg of total lipids, fraction F2/5/⑤ and ⑥ showed no response). (Error bars and parentheses denote standard deviation and number of experiments, respectively). □: settlement (%); ▨: metamorphosis (%)

Identification of liphophilic inducers

Effective fractions, F2/3/② for *Pseudocentrotus depressus* and F2/5/② for *Anthocidaris crassispina*, showed a single spot following TLC. The R_f (*rate of flow*) values of these spots were 0.25 to 0.40, as shown in Table 1. Because the results of TLC (with the mixture of standard) and the NMR analysis indicated that these single spots of effective fractions were mixtures of FFAs, GC analysis was performed (Table 1). In fraction F2/3/② for *P. depressus*, eicosapentaenoic acid (20:5; by convention, the number of carbon atoms in the molecule precedes the colon, with the number of double bonds in the molecule following) was the major component at 41%, palmitic acid (16:0) was 17%, and arachidonic acid (20:4) was 15%. In fraction F2/5/② for *A. crassispina*, 20:5 was again highest (50%), with 16:0 and 20:4 being 11 and 9%, respectively. Other FFAs, such as 16:1, 18:1, 18:2, and 18:3 comprised less than 5% in these two fractions.

Table 1. *Pseudocentrotus depressus* and *Anthocidaris crassispina.* R_f (rate of flow) values (chloroform:acetone = 9:1) and fatty acid contents of the effective fractions

Fraction	R_f	Fatty acid contents (%)						
		16:0	16:1	18:1	18:2	18:3	20:4	20:5
F2/3/②	0.25–0.35	17	4	3	2	3	15	41
F2/5/②	0.30–0.40	11	5	3	2	4	9	50

Table 2. *Pseudocentrotus depressus* and *Anthocidaris crassispina.* Mean larval settlement and metamorphosis induced by free fatty acids (FFAs)

Sea urchin	FFA (mg paper^{-1})	Larval response		Number of experiments
		Settlement (%)	Metamorphosis (%)	
Pseudocentrotus depressus	16:0 (0.32)	0	0	2
	16:1 (0.32)	7	0	2
	(0.56)	17	0	2
	20:4 (0.10)	41	0	5
	(0.18)	80	10	5
	(0.32)	53	47	5
	(0.56)	70	30	5
	20:5 (0.10)	40	0	5
	(0.18)	55	36	5
	(0.32)	21	79	5
	(0.56)	14	85	5
Anthocidaris crassispina	16:0 (0.32)	0	0	3
	(0.56)	0	0	2
	16:1 (0.32)	0	2	2
	(0.56)	0	0	2
	20:4 (0.10)	10	0	5
	(0.18)	57	23	5
	(0.32)	48	45	5
	(0.56)	69	28	5
	20:5 (0.10)	17	0	5
	(0.18)	41	13	5
	(0.32)	56	24	5
	(0.56)	32	65	5

Larval assays of standard FFAs

The four FFAs (20:5, 20:4, 16:0, and 16:1) common in the effective fractions were further examined for larval response (Table 2) and showed similar effects for both species. Palmitic acid (16:0) and palmitoleic acid (16:1) had little or no inducing effect, but eicosapentaenoic acid (20:5) and arachidonic acid (20:4) induced high levels of larval settlement and metamorphosis. At more than

0.18 mg paper^{-1} each, except at 0.18 mg paper^{-1} for *Anthocidaris crassispina*, 20:5 and 20:4 induced more than 80% of larvae to settle and metamorphose.

Discussion and conclusions

Larval responses to total lipids and fractions

Pseudocentrotus depressus and *Anthocidaris crassispina* larvae settled and metamorphosed in response to total lipids at 10 and 30 mg paper^{-1} (Figs. 1, 3), which were equivalent to ca. 2 and 7 g of fresh *Corallina pilulifera*, respectively. Such amounts of this alga in a 200-ml beaker are similar to those occurring naturally. These results suggested that the alga had some lipophilic inducers of the larval settlement and metamorphosis of these sea urchin species.

Of the fractions (F1 to F4) obtained by silica gel column chromatography, each fraction tested being equivalent to 30 mg paper^{-1} of total lipids, fraction F2 induced the highest larval settlement and metamorphosis in both species. Larval responses were similar to those observed for the total lipids (Figs. 1, 3). TLC showed fraction F2 to contain hydrocarbons, triglycerides, and free fatty acids. These results confirmed that the inducers for *Pseudocentrotus depressus* and *Anthocidaris crassispina* larvae belonged to non-polar groups of lipids (Kitamura et al. 1992).

In order to isolate the inducer for *Pseudocentrotus depressus* larvae, effective fractions were further fractionated, and the fractions F2/3/② to ⑥ were obtained (Fig. 2). Although the fraction F2/3/② showed lower response at 0.12 mg paper^{-1} than the other fractions (③, ④, and ⑤), only this fraction had a single spot after TLC. The other fractions had several spots, and each main spot was the same as for fraction F2/3/②. Based on these results, the inducer was considered to be in the main spot. When fraction F2/3/② was tested at a higher concentration (0.36 mg paper^{-1}), the response increased from 24 to ca. 100% (Fig. 2). The low larval response at the lower concentration (0.12 mg paper^{-1}) may have been due to the dispersal of the effective component into the other fractions.

For *Anthocidaris crassispina* larvae, the inducers of larval settlement and metamorphosis were also isolated in the same manner (Figs. 3, 4) as for *Pseudocentrotus depressus*. The effective fraction F2/5/② showed a single spot after TLC, this spot being almost the same as that of fraction F2/3/②. The inducer was effective at ca. 0.4 mg paper^{-1} for *A. crassispina* larvae, approximately the same as for *P. depressus*.

Identification of the lipophilic inducer

The chemical analyses (TLC and NMR) of the fractions, F2/3/② for *Pseudocentrotus depressus* and F2/5/② for *Anthocidaris crassispina*, showed the components to include a mixture of free fatty acids (FFAs), four of which (20:5, 20:4, 16:0, and 16:1) were common in the fractions according to GC analysis (Table 1).

In assays of the four standard FFAs, only 20:5 and 20:4 induced larval settlement and metamorphosis in the two sea urchin species at concentrations as low as 0.18 mg paper^{-1} (Table 2). Since the fractions induced larval responses at ca. 0.4 mg paper^{-1} (Figs. 2, 4), the concentration of 20:5 was determined to be ca. 0.2 mg paper^{-1} (see Table 1). Such a concentration of 20:5 was sufficient to induce larval settlement and metamorphosis of the two species of sea urchin. Because the level of 20:4 was low, 20:5 was believed to be the lipophilic inducer present in *Corallina pilulifera*.

Relationship to other work and conclusions

Although there are no reports available on lipophilic inducers of sea urchin larvae, Cameron and Hinegardner (1974), and Pearce and Scheibling (1990) have studied water-soluble inducers. The former indicated that the chemical inducer involved was a non-volatile compound of bacterial film, with a molecular weight less than 5000. The latter showed that aqueous extracts of coralline red algae could induce metamorphosis, the inducer being heat-labile. In our other studies, water-soluble substances from algae have also induced larval settlement and metamorphosis in sea urchins. However, the chemical characteristics of these inducers have not been made clear.

There have been some accounts of induction of larval settlement and metamorphosis by lipophilic substances in oysters (Keck et al. 1971), hydroids (Kato et al. 1975), and polychaetes (Pawlik 1986). Pawlik studied lipophilic inducers for *Phragmatopoma californica* and reported that larval response to FFAs extracted from a tube matrix of the polychaeta was concentration-dependent with a significant response to 16:1 and 20:4. In the present study, 20:4 induced larval settlement and metamorphosis, but 16:1 did not. The reason for the differing response to 16:1 is not clear.

There are two reasons for studying the chemical inducer. One is to improve mass production of sea urchin seeds in fish farming centers. An emulsion of the effective FFAs with alcohol may be a useful method for controlling the metamorphosis of sea urchin larvae. The other is to understand the inducers of larval settlement and metamorphosis under natural conditions. In the present study, FFA (20:5) was isolated as the chemical inducer of larval settlement and metamorphosis of sea urchins in the laboratory. Whether or not the FFA binds to some receptor in the larvae or stimulates a response following contact with the membrane of a larval cell is still unknown. In order to further clarify these mechanisms of the natural inducer, it is necessary to isolate the water-soluble inducers and to study in detail the chemosensory organs and receptor-response systems of the sea urchin larvae.

Acknowledgements. This work was supported in part by a grant from the Research Institute of Marine Invertebrates and the Ministry of Education, Science, and Culture of Japan. We express our gratitude to the staff of the fish farming centers in Saga and Nagasaki City for providing us with sea urchin larvae.

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Communicated by T. Ikeda, Niigata