Chemoecology
DOI 10.1007/s00049-014-0152-7

CHEMOECOLOGY

SHORT COMMUNICATION

Chemoattraction of the pearlfish *Encheliophis vermicularis* to the sea cucumber *Holothuria leucospilota*

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Received: 29 August 2013 / Accepted: 27 January 2014

Abstract The specificity of the relationship between Indo-Pacific pearlfish (family Carapidae) and their hosts, the sea cucumbers (Holothuridae), was examined in Okinawa, Japan. While some species of pearlfishes lived in several species of sea cucumbers, one species, *Encheliophis vermicularis*, was very specific and was only found in *Holothuria leucospilota*. The role of natural products in this relationship was then examined in maze tests, and highly polar (water soluble) substances in the coelomic fluid, body wall, and Cuvierian tubules of *H. leucospilota* were found to attract the pearlfish. Coelomic extracts were active at low concentrations and holothurins A and B found within these extracts were implicated as the active factors in maze tests. The possible role of these products in other symbiotic relationships between pearlfishes and sea cucumbers, some of which seem species-specific, now needs to be examined.

Keywords Pearlfish · Sea cucumber · Attractant · Holothurins A and B

Introduction

Coral reefs comprise diverse microorganisms, algae, invertebrates, and vertebrates. Some of the interactions between these organisms are mediated through chemicals produced by the organisms themselves. Among the different types of chemical communications, many examples have been demonstrated for allomones (chemicals beneficial to the releasing organism). One example of such communication is defense chemicals such as distasteful metabolites sequestered or produced by nudibranchs (Karuso 1987). However, there have not been many studies examining kairomones and synomones, particularly with respect to chemical characterization. One synomone, amphikuemin, involved in the symbiosis between the anemone fish *Amphiprion perideraion* and the sea anemone *Radianthus kuekenthali* (Murata et al. 1986), could become a representative example in marine organisms, and further studies on such chemicals are needed to more clearly understand their role(s).

Pearlfishes are eel-like fishes belonging to the family Carapidae (order Ophidiiformes). The name might originate from the discovery of a dead fish covered with nacreous material in an oyster shell (Eeckhaut et al. 2004). Although some species are free-living, most pearlfishes are commensal or parasitic to invertebrates—mainly holothuroids (sea cucumbers). Ecomorphological studies have distinguished between commensal and parasitic pearlfish species, as species in the former group have a robust food intake apparatus and belong to the genus *Carapus*, while species in the latter group have a weaker buccal apparatus and belong to the genus *Encheliophis* (Parmentier et al. 2000).

Sea cucumbers host many commensal or parasitic organisms including platyhelminthes, annelids, mollusks, arthropods, and fishes. Among these organisms, pearlfishes are known for their unique life style utilizing the coelomic cavity of sea cucumbers as shelter. The fish enter or exit the cavity through the anus without causing exudation of Cuvierian tubules (Smith et al. 1981), which entangle possible predators in some species of sea cucumbers when they are

Handling Editor: Thomas Schmitt.

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under stress. The pearlfishes may secret mucous material to protect themselves (Trott 1970).

Pearlfishes of the genus *Carapus* leave their host for prey such as small crustaceans and annelids during night, while the pearlfish *E. gracilis* has been reported to feed on the internal tissues of their host (Smith 1964). According to Trott (1981), pearlfishes may find hosts by both visual and chemical cues and can differentiate between host and non-host sea cucumbers by chemical cues alone. van Meter and Ache (1974) also described that the pearlfish *Carapus bermudensis* enters to the host sea cucumber *Actinopyga agassizi* by chemotaxis and by rheotaxis to the anal portion. However, such previous studies have not identified the molecules involved in the chemical cues.

Among echinoderms, both sea cucumbers and sea stars have been known to be rich in saponins, triterpenoidal and steroidal glycosides (Stonik and Elyakov 1988). The structures are basically composed of two units; a lipophilic aglycone unit and hydrophilic sugar moieties. The former usually consists of a triterpene for sea cucumbers and a steroid for sea stars, while the latter mainly comprises xylose, quinovose, glucose, and their derivatives. As the lipophilic and hydrophilic portions are connected covalently, the total chemical nature of saponins becomes amphiphilic or hydrophilic depending on the number of sugar moieties. A number of physiological activities (i.e., antifungal, antimicrobial, and others) and ecological roles (i.e., defense, repellant) for saponins have been reported (Bordbar et al. 2011; Lucas 1979; Mackie et al. 1968).

In this manuscript we describe field studies that examine the relationship between pearlfishes and sea cucumbers in Okinawa, Japan, as well as laboratory experiments aimed at determining chemical cues involved in the species-specific relationship between the pearlfish *E. vermicularis* and the sea cucumber *Holothuria leucospilota*.

Materials and methods

Study 1: field observation to determine if there is a specific relationship between pearlfishes and their sea cucumber hosts in Okinawa

Live sea cucumbers were collected from shallow (<5 m depths) coral reefs of Komesu (May and December 1987), Maeda (December 1987), Sesoko (December 1987), Sobe (November 1987), Sunabe (July 1987), Yakata (April 1987), and Zampa (April 1987 to January 1988) on Okinawa Island, Japan. The target sea cucumber species were *Holothuria leucospilota*, *Stichopus chloronotus*, *Bohadschia argus*, *Thelenota ananas*, and *H. atra*. The presence or absence of pearlfishes was examined by making a small cut on each specimen of the sea cucumbers. Pearlfishes found were transferred to a container with fresh sea water. After examination, sea cucumbers were released back to the water. The fish specimens were identified by Dr. T. Yoshino, University of the Ryukyus, Okinawa, Japan.

During this field study we examined whether the pearlfish *E. vermicularis* is directly attracted by its sole host *H. leucospilota* or not. After placing a fresh specimen of *E. vermicularis* in a container (35 cm × 40 cm × 4 cm) filled with sea water, a single unexamined *H. leucospilota* was also placed in the container and the behavior of the fish was observed. Figure 1 shows a sequence on how the fish entered its host. This test was repeated five times.

Study 2: an experiment to determine if these symbiotic relationships might be mediated by natural products

A polyvinylchloride (PVC) gutter tube (length 180 cm, diameter 10 cm) having continuous flow of sea water was used as the assay (Fig. 2). The flow rate was controlled at 1.0 L/min. A single *E. vermicularis* pearlfish was placed in a compartment partitioned with a plastic net near the outlet. The net was removed after 5 min. Individual fish that did not move toward the inlet even after an additional 5 min was used for further testing. A sea water-based sample solution of extracts adjusted to 200 ppm was prepared from five portions of the sea cucumber *H. leucospilota* as described below. For lipophilic extracts, a small amount of ethanol was used to dissolve the material. The aqueous solution was dropped into the inlet at the rate of 20 mL/min, and the movement of the fish was observed for 10 min. If the fish stayed around the area where the sample solution dropped, the result was judged as positive. When no apparent movement was observed, the result was judged as negative.

A total of 34 specimens of *H. leucospilota* (18 kg) were divided into five parts: coelomic fluid (4.5 L), body wall (5.3 kg), Cuvierian tubules (0.9 kg), intestines (4.7 kg), and haemal plexus (2.0 kg). Each of these parts except for coelomic fluid was extracted with a sufficient amount of MeOH three times. Then, the methanolic solution was filtered and concentrated under vacuum. For the coelomic fluid, it was filtered and concentrated. Each of the concentrated material was washed with ethyl acetate (EtOAc), then with MeOH. The residues from each part were collected as water-soluble material. After concentration of the solutions and drying, EtOAc (0.3, 0, 0.6, 5, and 0.7 g), MeOH (50, 32, 12, 2, and 38 g), and water-soluble portions (140, 74, 19, 14, and 37 g) were obtained from coelomic fluid, body wall, Cuvierian tubules, intestines, and haemal plexus, respectively. The amount of EtOAc extract from the body wall was very small (almost 0 g) and we could not use it for assay.

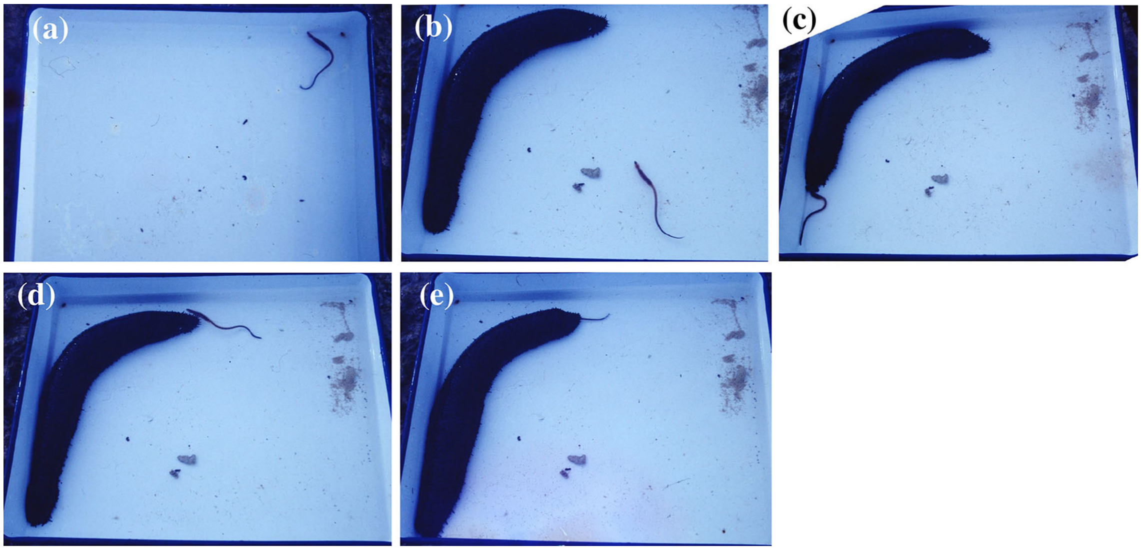

Fig. 1 The behavior of the pearlfish *E. vermicularis* towards the sea cucumber *H. leucospilota*. **a** A single pearlfish is released into the container, **b** a sea cucumber is placed into the container, **c** the pearlfish approaches the mouth portion of the sea cucumber, **d** the pearlfish swims around the body of the sea cucumber and finds the anal portion, and **e** the pearlfish enters headfirst into the sea cucumber through its anus

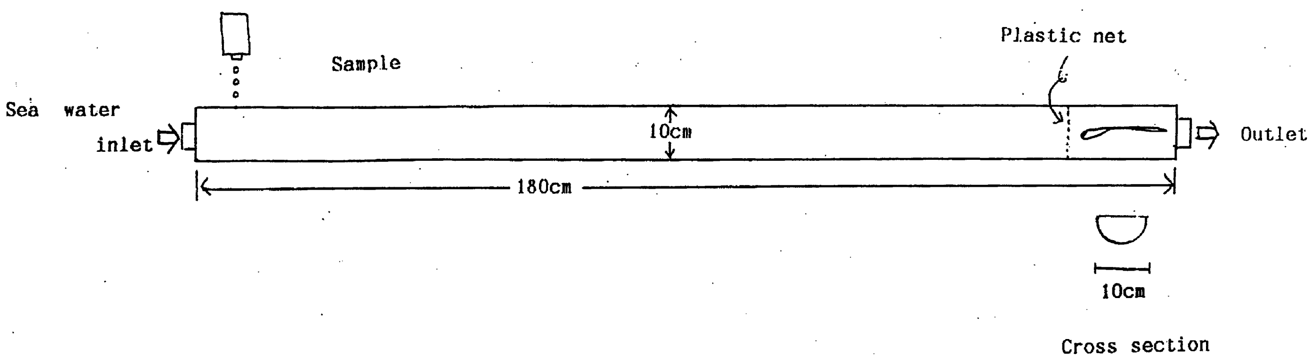

Fig. 2 Schematic drawing of attractant assay apparatus-1

Study 3: a maze experiment to test whether holothurins have role in pearlfish chemoattraction

Pure samples of holothurins A and B, prepared as below, were used in this assay. The PVC gutter tube was partitioned with a 90-cm-long plate as shown in Fig. 3. One inlet was for the test sample in sea water and the other for control. Flow rates for both sample and control were kept at 500 mL/min. A solution of holothurin A or B in sea water was dropped at the inlet at the rate of 20 mL/min. Three minutes later, a single *E. vermicularis* pearlfish was released at the center of an undivided section of the apparatus. We judged the result as positive if the fish moved and stayed at the sample channel for at least 10 min and negative if it moved to the control channel, or if there was no movement to either area. The channels for control and sample were altered for each run of the experiment, and the same test was carried out ten times.

A part of the water-soluble extract of Cuvierian tubules was partitioned between *n*-butanol (*n*-BuOH) and water. A part (50 mg) of the *n*-BuOH extract was separated successively using Sep-Pak (C18, MeOH), HPLC (C18, aq. H_3PO_4:MeCN 3:7), and Sephadex LH20 (MeOH) to furnish holothurin A (4.9 mg). A part from the water-soluble portion of the body wall was similarly treated to furnish 17.1 mg of holothurin B. The presence of holothurins was confirmed by TLC (silica, $CHCl_3$:MeOH:H_2O 7:3:1, lower layer) on comparison with authentic samples provided by Prof. I. Kitagawa, Osaka University (Kitagawa et al. 1981a, b).

Results and discussion

Study 1: field observation

During the period between March 1987 and January 1988, five species of sea cucumbers, *Holothuria leucospilota*, *Stichopus chloronotus*, *Bohadschia argus*, *Thelenota ananas*, and *H. atra,* were examined for the presence or

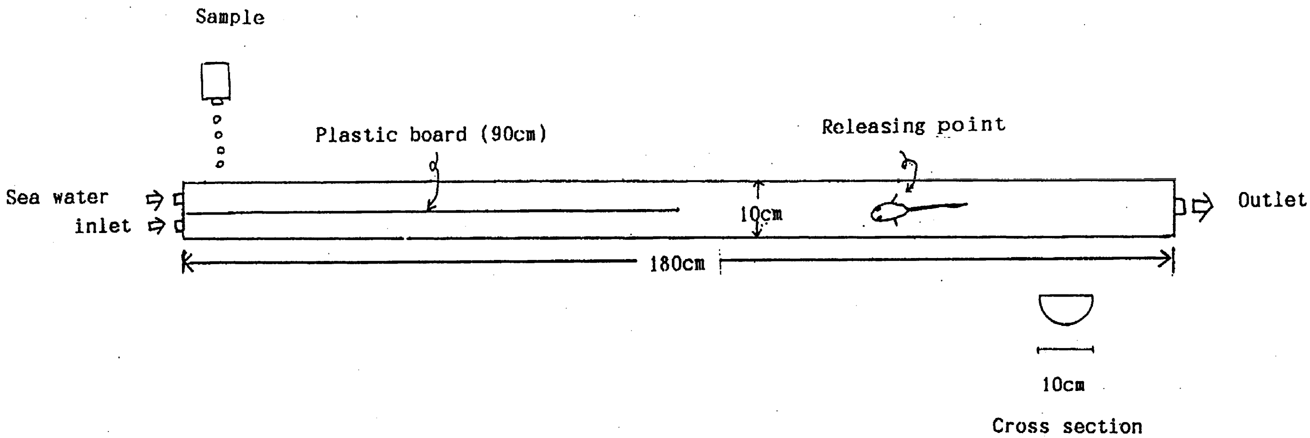

Fig. 3 Schematic drawing of attractant assay apparatus-2

Table 1 Host specificity of pearlfishes in Okinawa

Pearlfish species	Sea cucumber species					
	H. leucospilota 2,201	*S. chloronotus* 1,496	*B. argus* 207	*T. ananas* 4	*H. atra* 48	Total 3,956
E. vermicularis	126	0	0	0	0	126
E. gracilis	0	2	3	1	0	6
C. homei	0	7	6	0	0	13
C. mourlani	0	1	7	0	0	8
Total	126	10	16	1	0	153

absence of pearlfishes along the coast of Okinawa Island. A total of 3,956 specimens, comprising 2,201 (56 %) *H. leucospilota*, 1,496 (38 %) *S. chloronotus*, 207 (5 %) *B. argus*, 4 *T. ananas*, and 48 (1 %) *H. atra* were examined in this study (Table 1). A total of 126 specimens of the pearlfish *Encheliophis vermicularis* were found only in *H. leucospilota*, while three species of pearlfishes (*E. gracilis*, *Carapus homei* and *C. mourlani*) were found in two sea cucumber species, *S. chloronotus* and *B. argus*. One additional specimen of *E. gracilis* was found from *T. ananas*.

The results indicate that the chance of finding *E. vermicularis* in *H. leucospilota* was small (5.7 %) and that the relation of this pair of species is species-specific in the Okinawa area. *Encheliophis vermicularis* has been previously reported to associate with *H. leucospilota*, *H. atra,* and *T. ananas,* in addition to three other sea cucumber species which may not inhabit Okinawa (Eeckhaut et al. 2004), however, we could not find any *E. vermicularis* from *H. atra* and *T. ananas* in this study. The numbers of examined specimens of *H. atra* and *T. ananas* were much smaller than *H. leucospilota*, and further field studies are required to confirm specificity. The three other pearlfishes found in this study, *E. gracilis*, *C. homei*, and *C. mourlani,* apparently do not have a one-to-one relationship with sea cucumbers. *T. ananas* could be host to *C. homei* and *C. mourlani* as well as *E. gracilis*, however, we could not collect many specimens of *T. ananas* compared to other species, and this species is more likely to inhabit deeper coral reefs. All the species pairs found for the four pearlfishes and four sea cucumbers in this study have already been observed in previous studies (Eeckhaut et al. 2004).

During the field study, we also examined whether the pearlfish *E. vermicularis* was directly attracted to its sole host sea cucumber *H. leucospilota* or not (Fig. 1). The fish swam around (1b), found its host, tried to enter from the wrong entrance (mouth) (1c), and then moved to the anal portion (1d). The fish entered headfirst into the coelomic cavity of the sea cucumber through the anus (1e). In a total of five tests, the fish entered the sea cucumber via the anal portion four times.

Study 2: chemoattraction by sea cucumber extracts

To check whether the chemical constituents of *H. leucospilota* attract *E. vermicularis*, first we dissected sea cucumber specimens into five different portions (coelomic fluid, body wall, Cuvierian tubules, intestines, and haemal plexus), and prepared EtOAc, MeOH, and water-soluble extracts from each portion.

Then, these extracts were dissolved in sea water at 200 ppm and subjected to attractant assay using an apparatus (apparatus-1, Fig. 2). The results summarized in Table 2 indicated that all extracts from the intestine and haemal plexus did not give any positive results, while MeOH extract of coelomic fluid and water extracts from the body wall, Cuvierian tubules and coelomic fluid showed (partially) positive results.

Study 3: holothurins A and B as chemoattractants

Holothurins A and B were separated from *n*-BuOH extracts of the body wall and Cuvierian tubules by chromatography. Both compounds were dissolved in sea water at 5 ppm and tested for attractant property using a two-choice apparatus (apparatus-2) shown in Fig. 3. The results are summarized in Table 3. Holothurin A attracted the fish to the sample channel in all the tests, while holothurin B attracted the fish in most cases. These results may indicate that holothurin A has stronger properties than holothurin B. As the saponin solution (20 mL/min) was diluted with the flow (500 mL/min) of sea water in the experiments, the fish appear to detect the molecules far below 5 ppm.

Thus, as shown above, both holothurins A and B were identified as chemical attractants of the pearlfish *E. vermicularis* from the sea cucumber *H. leucospilota*, though EC_{50} values on the attractive effect of holothurins were not obtained due to the limited number of pearlfish *E. vermicularis* available for the experiments. This work may be the first proof that pure saponins or triterpenoidal glycosides of a sea cucumber attract a pearlfish.

Table 2 Activity of extracts from five portions at 200 ppm (number of positives/number of tests)

	Coelomic fluid	Body wall	Cuvierian tubules	Intestines	Haemal plexus
EtOAc extract	0/2	nt	0/2	0/2	0/2
MeOH extract	1/2	0/2	0/2	0/2	0/2
Water extract	2/2	1/2	1/2	0/2	0/2

nt Not tested

Table 3 Attractant assay results of holothurins at 5 ppm (number of positives/number of tests)

	Sample	Control	No movement
Holothurin A	10/10	0/10	0/10
Holothurin B	8/10	0/10	2/10

Saponins or triterpenoid glycosides are ubiquitous in sea cucumbers among echinoderms, with structural variation from species to species. It has been discussed and also confirmed that pearlfishes detect host echinoderms with chemical cues (Trott 1970; van Meter and Ache 1974), however, Trott (1970) also described that sea cucumbers release holothurins as irritants that repel pearlfishes.

All the major saponins reported from the five sea cucumbers in this study share the same structural component, a triterpenoidal aglycon of a lanosterol skeleton with 18(20)-lactone and a polysaccharide chain of 2–6 sugar units linked to C-3 (Kobayashi et al. 1991; Stonik 1986; Stonik and Elyakov 1988). Characteristic structural features found for holothurins A and B include a tetrahydrofuran moiety on side chain, 17-α-hydroxyl group on lanostane skeleton, and a sulfate group at C-4′ of xylose. Any of these moieties may contribute to the species-specificity of the *E. vermicularis*–*H. leucospilota* relationship. Interestingly, *H. atra* (Kobayashi et al 1991; Stonik et al. 1979; Kuznetsova et al. 1982), from which both we and a previous study in Guam (Smith 1964) could not find any pearlfish, contained holothurins A and B together with similar molecules named echinosides A and B, with structures that do not retain a tetrahydrofuran moiety. As Eeckhaut et al. (2004) reported that *H. atra* is one of the host sea cucumbers of *E. vermicularis*, there may be a reason (i.e., morphology of the sea cucumber or the presence of echinosides A and B) why the pearlfish prefer to inhabit in *H. leucospilota* instead of *H. atra* in Okinawa.

The three other pearlfish species in this study, *E. gracilis*, *C. homei*, and *C. mourlani*, were found in a three other sea cucumber species, *S. chloronotus*, *B. argus*, and *T. ananas*, and in these species saponins share the same structural features as a lanosterol-type aglycon with variation of oxidation position and a hexaglycoside chain with almost the same sugar units. As a future research subject, it would be of interest to test how each saponin attracts these pearlfishes.

Conclusion

The species-specificity of pearlfishes and their host sea cucumbers in Okinawa was revealed with field investigation. Among the relationships, the pearlfish *E. vermicularis* was chemically attracted to holothurins A and B, the major saponins of *H. leucospilota*.

Acknowledgments This manuscript is based on an undergraduate thesis by the first author, who passed away 2 years after this work was completed. Although the corresponding author felt that accumulation of more data on this work was needed, the study did not move forward over the following years. As there have been no other reports on the identification of saponins as chemoattractants in pearlfish, we believe that these data are still worthy of publication 25 years after the original work. The authors thank the journal for publishing this work and for their understanding with this long overdue report.

The authors thank Dr. T. Yoshino, for the identification of the pearlfishes, Prof. I. Kitagawa and M. Kobayashi, Osaka University, for the gift of authentic holothurins A and B, Prof. T. Higa, University of the Ryukyus, for discussion and revision of the manuscript, and confidential reviewers who gave very positive and helpful comments.

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