J. Chem. Ecol., 1978, Vol. 4, No. 5, pp. 551–561

TRANSFER OF THE SEA ANEMONE PHEROMONE, ANTHOPLEURINE, BY THE NUDIBRANCH *Aeolidia papillosa*

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(Received November 16, 1977; revised December 30, 1977)

Abstract—After the nudibranch *Aeolidia papillosa* eats the sea anemone *Anthopleura elegantissima*, anthopleurine, an alarm pheromone from the sea anemone, persists in the tissues of the nudibranch. For at least five days following such a meal, nudibranchs are capable of evoking alarm responses in anemones without touching them, presumably by releasing anthopleurine into the water. The anemone's alarm response to anthopleurine is to withdraw the tentacles and oral disk, the preferred sites of attack for *Aeolidia*. This leaves exposed to attack the anemone body regions with the highest anthopleurine concentrations. Specimens of *Aeolidia* collected near sources of *Anthopleura* are more likely to contain detectable amounts of anthopleurine than those more distant; some nudibranchs collected 0.5 m from *Anthopleura* contained enough anthopleurine to evoke alarm responses in anemones they approached. These findings suggest that the predator helps in the transmission of anthopleurine, which may reduce the severity of predation on *Anthopleura*.

Key Words—*Anthopleura elegantissima*, *Aeolidia papillosa*, anthopleurine, alarm pheromone, anemone, nudibranch.

INTRODUCTION

Tissues of the sea anemone *Anthopleura elegantissima* contain large amounts of the betaine, anthopleurine, a compound that may function as an alarm

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pheromone (Howe and Sheikh, 1975; Howe, 1976a). Dilute solutions of anthopleurine activate specific receptors on the tentacles of *A. elegantissima* to evoke a stereotyped alarm response, consisting of rapid bending and shortening of the tentacles and depression of the oral disk, followed by constriction of the upper margin of the oral disk around the tentacles and oral disk (Howe, 1976a,b). The alarm response appears to have been integrated into the behavioral repertoire of *A. elegantissima* in an adaptive way (Howe, 1976c).

Any role for anthopleurine in intraspecific communication is likely to have two characteristics. First, because the anthopleurine in an anemone's tissues appears to be released only upon mechanical injury (Howe, 1976a), such a role probably requires tissue damage. Second, because the rates of diffusion of waterborne pheromones are low (Wilson, 1970) and the habitats of *A. elegantissima* are relatively turbulent (Ricketts and Calvin, 1962), anthopleurine cannot be expected to transmit useful messages more than a few centimeters from its source.

A potential function for anthopleurine in intraspecific communication that meets these two criteria is that anthopleurine released when a predator attacks *Anthopleura* evokes alarm responses in nearby anemones, thereby rendering them less vulnerable to predation. The present study is an experimental evaluation of that hypothesis.

The predator chosen for study was *Aeolidia papillosa*, an anemone-eating nudibranch gastropod that is regularly found in association with *A. elegantissima* and that appears to prefer *A. elegantissima* as prey to other western North American sea anemones (Harris, 1973; Waters, 1973). Two potential routes of anthopleurine release incident to predation by *Aeolidia* were considered: (1) that anthopleurine diffuses from the wounds on anemones that have been attacked, and (2) that anthopleurine diffuses from nudibranchs that have recently eaten *Anthopleura*. When some nudibranchs collected for the initial experiments evoked alarm responses in individual *Anthopleura*, even though all anemone–nudibranch contacts were prevented by manipulating the nudibranchs with a glass rod, the latter of these two alternative routes was chosen for detailed examination.

METHODS AND MATERIALS

Aeolidia between 2 and 3 cm in length were collected in 5–10 m of water from beneath Municipal Wharf #2 at Monterey, California, and were maintained in running sea water without feeding for no more than four days before experiments. The populations from which *Aeolidia* were collected suffered parasitism from a rhabdocoel flatworm; only animals without obvious

parasites were selected for experiments. *Aeolidia* did not survive long in captivity. By the tenth day after capture, about 50% had died. This short laboratory life span provided an upper limit to the duration of experiments. In no case, however, did the particular conditions of an experiment appear to further reduce that life span; experimental animals survived as long, on the average, as animals collected at the same time that were not selected for experiments. Weights of live *Aeolidia* were measured in sea water to the nearest 0.2 mg with a torsion balance. *A. elegantissima* used as nudibranch food were from clones collected at Pacific Grove, California; anemones from the same clone were used to feed *Aeolidia* in any given experiment. Detailed methods for each of the experiments performed are described with the results of those experiments.

RESULTS

Identification of Anthopleurine in Aeolidia

Our initial observation that individual *Aeolidia* could evoke alarm responses in *Anthopleura* at a distance suggested that a substance diffusing from *Aeolidia* was responsible for those alarm responses. That hypothesis was confirmed by the results of the following experiment. A nudibranch (collected, then starved for four days) that consistently evoked alarm responses was homogenized with a motor-driven, Teflon pestle homogenizer in 10 volumes of sea water. The filtered (Whatman GF/C) extract was capable of evoking alarm responses in *Anthopleura* even when diluted $1:5 \times 10^3$ (v/v) with sea water.

The high specificity of the alarm response for anthopleurine (Howe, 1976a) suggested that the alarm substance from *Aeolidia* was anthopleurine. That hypothesis was tested by comparing the alarm substances in *Anthopleura* and *Aeolidia* chromatographically. Whole *Anthopleura* and *Aeolidia* were dried overnight at 90°C and ground in a mortar. Weighed portions of the resulting powders were extracted for 1 hr in 4 parts (v/w) of methanol. The extracts were filtered and spotted (2 μl/spot) onto Whatman No. 1 paper, then developed (ascending) in 1-butanol, acetic acid, and water (4:1:5). Purified anthopleurine (Howe and Sheikh, 1975) was chromatographed separately, next to the tissue extracts. Developed chromatograms were sprayed with Dragendorff reagent or cut into 5-mm vertical sections and assayed for anthopleurine. The center of biological activity (alarm) for both extracts fell between R_f values of 0.14 and 0.17, compared with an R_f of 0.17 for purified anthopleurine on the same chromatogram. This result provides support for the hypothesis that the alarm substance from *Aeolidia* is chemically similar to anthopleurine. Previous structure–activity studies (Howe,

1976a) showed that the alarm response was specific for the anthopleurine structure. In tests with a series of ten commercially available quaternary ammonium anthopleurine analogs, the compound most similar to anthopleurine in structure and most active in the alarm response bioassay, DL-carnitine, was 3×10^3 times less active than anthopleurine. That result suggests that the alarm substance in *Aeolidia* is indeed anthopleurine.

Feeding Experiments

Although anthopleurine had not been reported from any organism except *Anthopleura*, it remained possible that the anthopleurine in *Aeolidia* was synthesized by *Aeolidia*, rather than by *Anthopleura*, especially because the *Aeolidia* used for the preliminary observations were known not to have eaten *Anthopleura* for at least four days. If, on the other hand, predation by *Aeolidia* upon *Anthopleura* was responsible for the presence of anthopleurine in *Aeolidia*, one would predict that the concentration of anthopleurine in the tissues of an *Aeolidia* would decrease with time after consuming *Anthopleura*. To test that prediction, a feeding experiment was performed.

For this experiment, the anthopleurine concentration of *Aeolidia* tissue was monitored by preparing extracts of cerata (finger-like projections of the dorsal surface that contain extensions of the hepatopancreas). Not only were cerata convenient to remove, but their repeated removal from the same animals did not appear to affect the behavior or survival of the nudibranchs. For each test, 1–3 anterior, mid-dorsal cerata were removed and blotted briefly, then placed on glass cover slips and dried to constant weight at 90°C. Cover slips were then weighed to the nearest 0.01 mg before and after scraping the dried cerata into test tubes; the dry tissue weight (mean for all samples = 0.9 mg) was computed by difference. Dried cerata were ground with a blunt glass rod and extracted with 10^4 parts (w/w) of distilled water for 2–4 hr at 2°C, then filtered (Whatman GF/C). Each extract was serially diluted 2-, 4-, and 8-fold, for a total of four concentrations for each extract. Using the same anemones and procedures as for previous bioassays (Howe and Sheikh, 1975), 0.5 ml of each concentration was mixed into a bowl of anemones, and the percentages of anemones giving alarm responses within 30 sec were recorded. Median effective concentrations, estimated graphically from probability plots of the resulting response percentages, were divided into the median effective concentration for pure anthopleurine (Howe and Sheikh, 1975) to yield estimates for the anthopleurine concentrations in the cerata. There was no significant correlation ($r = -0.19$, $P > 0.5$) between the amounts of *Anthopleura* tissue consumed by *Aeolidia*, expressed as a percentage of each nudibranch's weight, and the mean anthopleurine concentration in cerata during the experiment.

Six specimens of *Aeolidia* of approximately equal size (reduced weight range: 34.4–46.0 mg) were starved for four days, then placed in individual containers. Cerata were removed for anthopleurine assays; then these *Aeolidia* were allowed to feed upon pieces of *Anthopleura*. After 24 hr, during which time the *Aeolidia* had increased their weights by an average of 23% (SE = 5%), the pieces of *Anthopleura* were replaced with pieces of *Metridium senile*, an anemone that is a normal component of the diet of *Aeolidia* (Harris, 1973) but does not contain anthopleurine (Howe, unpublished). Cerata were removed for anthopleurine assays at the end of the *Anthopleura* meal and at two-day intervals thereafter. The experiment was terminated seven days after the beginning of the *Anthopleura* meal because of the deaths of three of the *Aeolidia*.

Anthopleurine concentrations in extracts of cerata are plotted (open circles) as a function of time since a meal of *Anthopleura* in Figure 1. Immediately after the *Aeolidia* ate *Anthopleura*, extracts of their cerata became markedly more effective in evoking alarm responses, and that increased effectiveness persisted for the duration of the experiment.

The retention of anthopleurine in the cerata following an *Anthopleura* meal might explain why an *Aeolidia* starved for several days could evoke the alarm response. Results from a bioassay experiment using live caged *Aeolidia* confirm that interpretation. A cage was constructed by replacing the lower wall and bottom of a 2.5 cm diameter plastic vial with plastic window screening. An *Aeolidia* was gently lifted into the cage. Slots cut through the upper wall of the vial accommodated a 2-cm-wide strip of plastic that both prevented the nudibranch's escape and suspended the cage in an anemone bowl. The caged nudibranch was then washed for 30 sec in slowly flowing sea water. The sea water supply to an anemone bowl was stopped; 3 min later the *Aeolidia* cage was introduced, without touching an anemone, and

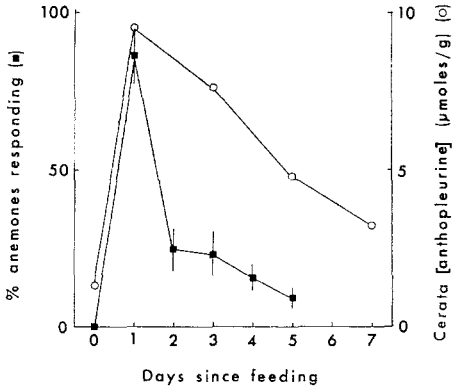

FIG. 1. Effects of an *Anthopleura* meal on anthopleurine concentrations in *Aeolidia* cerata (circles, right ordinate) and on the ability of live *Aeolidia* to evoke alarm responses (squares, left ordinate). Vertical lines through the squares indicate standard errors.

positioned near the center of the bowl, approximately 1 cm from the nearest anemone tentacles. At 30-sec intervals, the cage was gently rinsed with 20 ml of sea water from the bowl. The proportion of anemones giving an alarm response within 3 min was recorded.

Nine specimens of *Aeolidia* that had been starved for three days were tested with the cage bioassay. Of those nine, eight failed to evoke any alarm responses. These eight animals were placed in individual containers and offered pieces of *Anthopleura* for 31 hr (mean percent weight gain = 42, SE = 9.5), after which the food was removed, and the specimens of *Aeolidia* were tested for the ability to evoke alarm responses. The cage bioassay was repeated at 24-hr intervals for four more days. As is shown in Figure 1, the proportion of anemones giving an alarm response to live *Aeolidia* (squares) increased from 0 before the meal to 84% (SE = 10%) after the meal. This percentage dropped sharply by the second day of feeding, but still differed significantly from 0 ($t = 2.80$, $P < 0.05$) on the fifth day of testing. This experiment shows that for several days after a meal of *Anthopleura*, an intact *Aeolidia* releases enough anthopleurine to evoke alarm responses in nearby *Anthopleura*.

Site of Attack Preference by Aeolidia

Observations were made on feeding encounters between *Anthopleura* and *Aeolidia* to determine whether *Aeolidia* showed a preference for particular regions of the anemone. In the first experiment, 55 specimens of *Anthopleura* were placed in a shallow pan that had been coated with silicone grease to prevent their attachment and any behavioral responses that required attachment. Fifty nudibranchs 2–3 cm in length were introduced into the pan 24 hr after the anemones. After 5 hr, the nudibranchs were removed, and the anemones were relaxed with $MgCl_2$ and then surveyed for tissue damage. Thirty-three anemones showed tissue damage: 4 (12%) to the column, 11 (33%) to the pedal disk, and 18 (55%) to the oral disk and tentacles. Although the pedal disk would not normally be vulnerable to attack, these results suggest that *Aeolidia* that are allowed to select a site of attack prefer sites other than the surface of the column.

In a second experiment, nudibranchs were allowed to attack attached anemones. Fifty attacks were observed by specimens of *Aeolidia* longer than the column diameter of the attacked anemones. None of these nudibranchs evoked alarm responses at a distance. Thirty-six (72%) attacks were directed to the tentacles and oral disk, and 14 (28%) to the column. Several of the nudibranchs that did eat the column first attempted to reach the tentacles, but the anemones avoided contact between their tentacles and the nudi-

branchs by shortening the tentacles and then extending the column to take the tentacles beyond the reach of the predators. These results show that specimens of *Aeolidia* that do not evoke alarm responses as they approach *Anthopleura* are more likely to attack tentacles and oral disk than the column ($\chi^2 = 9.68$, $P < 0.005$).

Anthopleurine Bioassays on Field-Captured Aeolidia

Twenty specimens of *Aeolidia* from each of four different habitats were collected near Monterey Municipal Wharf #2. For each habitat, the mean distance to the nearest *A. elegantissima* was estimated to the nearest 0.5 m at the time of collection. Cerata were removed from the eight largest animals from each habitat (2–3 cm total length). These cerata were dried, weighed, and extracted in 10^4 parts distilled water as previously described. Each extract was then tested as before for the ability to evoke alarm responses in 14–18 anemones. In Figure 2, responses to cerata extracts for each habitat are expressed as a function of estimated mean distance from *Anthopleura*. For statistical comparisons, the positive responses elicited by the eight specimens of *Aeolidia* collected in each habitat were pooled. The percentage of anemones responding to extracts from *Aeolidia* 0.5 m away (47 of 130) is significantly greater than that from any other habitat ($\chi^2 > 30$, $P < 0.005$), and the percentage of responses to nudibranchs 1.5 m distant (6 of 129) is greater than that from the most distant habitat ($\chi^2 = 4.24$, $P < 0.05$). Although specimens of *Aeolidia* collected nearest those of *Anthopleura* showed considerable variability in their cerata anthopleurine concentrations, two of those nudibranchs had concentrations at least as high as the maximum recorded in laboratory feeding experiments.

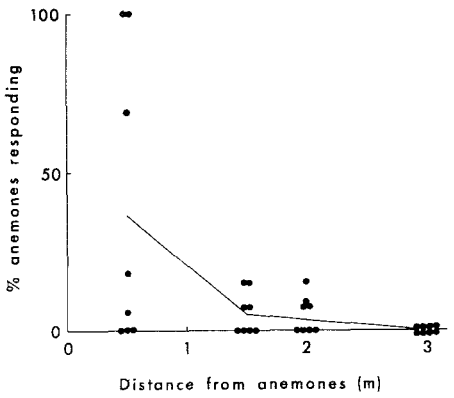

FIG. 2. Alarm responses to cerata extracts from four samples of eight specimens of *Aeolidia* collected at increasing mean distances from the nearest specimens of *Anthopleura*. Each point represents the percentage of 14–18 anemones that gave alarm responses to a cerata extract (0.2 mg/liter) from a single nudibranch. The line connects the pooled response percentages for each of the four collections.

TABLE 1. ANTHOPLEURINE CONCENTRATIONS IN DIFFERENT ANEMONE BODY REGIONS

Body region	Anthopleurine (μmol/g ± 95% CI)
Tentacles	23 ± 5
Oral disk and pharynx	58 ± 10
Upper column margin	63 ± 16
Upper column wall	66 ± 17
Lower column wall	106 ± 21
Pedal disk and lower column margin	121 ± 28
Mesenteries	29 ± 6

Location of Anthopleurine in Anemones

Identification of the anatomical sites where a pheromone is stored may provide useful information about the biological role of that pheromone (see Wilson and Pavan, 1959, for example). To locate anthopleurine in anemone bodies, a large, solitary specimen of *A. elegantissima* was dissected into the seven pieces described in Table 1. Pieces were dried to constant weight at 90°C, ground in a mortar, and extracted with 50 parts (w/w) distilled water at 20°C for 10 hr. Extracts were centrifuged, and the supernatant liquids were biologically assayed for anthopleurine. The results (Table 1) show that the apparent anthopleurine concentration is significantly higher in lower column margin/pedal disk and lower column wall than in other body regions and lower in the tentacles and mesenteries than in other body regions ($P < 0.05$). The results of two additional experiments suggest that these apparent differences in the anthopleurine activity of different tissues represent real differences in anthopleurine concentration.

To assess the possibility that enzymatic degradation of anthopleurine at different rates in different body regions contributed to the apparent differences in pheromone concentration, a second experiment was performed. Tentacles and lower column margin/pedal disk were removed from a single, live, clonal anemone, blotted briefly, then weighed. Each tissue type was homogenized in 50 parts (w/w) water and assayed for anthopleurine as before. In this experiment, the apparent anthopleurine concentration of lower column margin extract (0.24 μmol/ml) was about eight times that of the tentacle extract (0.03 μmol/ml). Three micro-moles of purified anthopleurine (Howe and Sheikh, 1975) in 0.3 ml water were added to 5 ml of each extract. Each extract was incubated 11 hr at 14°C, then assayed for anthopleurine. The anthopleurine concentration in each extract had increased by the expected 0.6 μmol/ml, suggesting that the tentacle extract could not degrade the added anthopleurine appreciably.

A final experiment was performed to examine the possibility that the low apparent anthopleurine concentration in tentacles could be due to binding of anthopleurine to specific anthopleurine receptors, previously shown to be located in tentacles (Howe, 1976b). Portions (10 ml) of the two extracts prepared for the preceding experiment were dialyzed (Spectrapore 3 membrane) against many changes of distilled water for 12 hr at 4°C. Volumes of the retentates were adjusted to 11 ml and each was assayed for anthopleurine. Neither had detectable levels (<2 nmol/ml). Purified anthopleurine (1 μmol) was added to each extract, and the extracts were incubated for 2 hr at 20°C, then assayed for anthopleurine. If anthopleurine receptors in the dialyzed tentacle extract bound some of the added anthopleurine, one might have expected it to have a lower apparent concentration than the lower column margin/pedal disk extract. Instead, the anthopleurine concentrations of the extracts did not differ significantly.

DISCUSSION

We have examined the hypothesis that an alarm pheromone transferred by a predator among members of a prey species may reduce the severity of damage by the predator. Our evidence supports that hypothesis in two major respects.

First, transfer of anthopleurine by *Aeolidia papillosa* can occur in the laboratory and probably does occur under natural conditions. Upon eating *Anthopleura*, *Aeolidia papillosa* accumulates anthopleurine in its cerata. The initial concentration (about 100 μmol/g) drops by about 10 μmol/g per day after feeding (Figure 1, circles), suggesting that *Aeolidia* can neither digest nor excrete anthopleurine rapidly. For at least five days after a meal of *Anthopleura*, *Aeolidia* is capable of evoking alarm responses in *Anthopleura* at a distance of at least 1 cm under experimental conditions (Figure 1, squares). Some nudibranchs collected near specimens of *Anthopleura* had concentrations of anthopleurine comparable to the levels observed in the early stages of the laboratory feeding experiments, a result that suggests that individual *Aeolidia* evoke alarm responses as they approach specimens of *Anthopleura* under natural conditions as well as in the laboratory.

Although the results from the two experiments summarized in Figure 1 are similar, a critical comparison reveals one difference: after the post-feeding peaks at day 1, the ability of *Aeolidia* to evoke alarm responses at a distance declines more rapidly than the anthopleurine concentration in the cerata. A possible explanation for this difference is that cerata may become saturated with anthopleurine after a meal. Any anthopleurine consumed in excess of the amount required to saturate the cerata might then be rapidly

lost to the medium, after which the anthopleurine concentration in the cerata might decline more slowly. That the concentration of anthopleurine in the cerata after a meal does not depend on the size of the meal (see results of first feeding experiment) supports this hypothesis.

Second, both the behavioral response to anthopleurine and the location of the pheromone in the body of an anemone suggest that *Anthopleura* benefits by responding to predator-borne anthopleurine. By enclosing the tentacles and oral disk, important but relatively delicate structures, within an envelope of column wall, the alarm response ensures that the preferred sites of attack for *Aeolidia* are less available for predation. Further, the regions of the anemone body that remain exposed to attack after an alarm response are those in which the anthopleurine concentration is highest (Table 1), ensuring that predation upon an alarmed anemone will result in maximal transfer of alarm pheromone to the predator. It seems especially advantageous that the lower column has the highest anthopleurine content, because this body region would be contacted first by predators, such as *Aeolidia,* that feed close to the substratum. Because *Aeolidia* apparently prefers to attack parts of the anemone body that are relatively deficient in anthopleurine, it is tempting to speculate that anthopleurine may be functioning both as an alarm pheromone and as a chemical feeding deterrent. No additional evidence supports that speculation at present.

In intertidal habitats, *A. elegantissima* undergoes longitudinal fission to form clones of genetically identical individuals (Francis, 1973), a reproductive pattern that should favor the evolution of an alarm pheromone communication system (Hamilton, 1963). We have considered only one set of circumstances under which such a system might operate. Other possible roles for anthopleurine in intraspecific communication remain to be investigated. For example, anthopleurine released when wave-borne logs (Dayton, 1971) or rocks damage anemones may cause alarm responses in nearby clonemates, making them smaller, less vulnerable targets. Anthopleurine released from a damaged, detached anemone could reduce the risk of being cannibalized by other anemones by evoking alarm responses instead. Apart from any other messages that may be conveyed by anthopleurine, it appears likely that anthopleurine transmission by eolid nudibranchs may help protect *Anthopleura.* Like the fabled mice who belled the cat to be warned of her approach, *Anthopleura* may have a means to ensure that it is forewarned of the presence of one of its major predators.

Acknowledgments—We wish to thank the National Science Foundation and the ARCS Foundation for graduate fellowships to NRH, the staff of Hopkins Marine Station for technical assistance, and Drs. D.P. Abbott, L.J. Lester, and R.K. Josephson for critical comments on the manuscript.

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