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NOTE

Larvae of the sea hare *Aplysia californica* settle and metamorphose on an assortment of macroalgal species

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ABSTRACT: Larvae of the sea hare *Aplysia californica* (Mollusca: Opisthobranchia) spend several weeks feeding in the plankton prior to settlement and metamorphosis. Previous work indicated that metamorphosis was triggered by only one (or at most a few) algal species. However, in the present laboratory study, a mean of 30% or more of the larvae of this sea hare metamorphosed in response to 10 of 18 species of intertidal macroalgae (9 red, 7 brown, 2 green). Metamorphosis was greatest in response to the red algae *Rhodymenia californica, Corallina officinalis, Plocamium cartilagineum* and *Laurencia pacifica*. Juveniles of *A. californica* that had metamorphosed on the last 2 species grazed on them and began to grow, whereas juveniles on the other species tended to crawl off the alga and around the assay dish. Of the 8 algae least preferred, only 1 was red, the remainder brown or green. For larvae of *A. californica*, metamorphosis on a relatively wide spectrum of algal species may be more efficacious than metamorphosis on any one alga, because juvenile sea hares can readily crawl to nearby algal species that they prefer to eat after they have metamorphosed on an alga that is not their preferred food.

Most marine invertebrates pass through a planktonic larval stage prior to settlement and metamorphosis, and the recruitment of their larvae is important in structuring benthic marine communities (Keough & Downes 1982, Keough 1983, Gaines & Roughgarden 1985). Larval settlement is largely regarded as a response to complex, and often highly specific, environmental stimuli (Burke 1983, Crisp 1984), especially chemical cues (reviewed in Hadfield 1986, Pawlik & Faulkner 1986). However, few naturally-occurring chemical inducers of larval settlement and metamorphosis have been isolated and identified (Kato et al. 1975, Cuomo 1985, Pawlik 1986).

Marine molluscs of the genus *Aplysia* are among the most intensively studied animals on earth and have been the subject of research on development, growth and energetics, circadian rhythms, and the neural basis for learning, memory and behavior (reviewed in Kandel 1979, Carefoot 1987). Research interest in one of the best studied sea hares, *A. californica*, from the coast of California, prompted the laboratory cultivation of the planktotrophic larvae of these animals through metamorphosis (Kriegstein et al. 1974). In the field, young recruits of these herbivorous snails were most frequently found eating red algae, primarily of the genus *Laurencia* (Kupfermann & Carew 1974). Moreover, several putatively defensive halogenated natural products isolated from adult *A. californica* were found to be derivatives of metabolites of *L. pacifica* (Stallard & Faulkner 1974), suggesting some dependence of the molluscs on this alga.

Larval settlement of *Aplysia californica* was reported to be highly substrate-specific, and chemical inducers were believed to be responsible for substrate choice (reviewed in Carefoot 1987). Kriegstein et al. (1974) found that laboratory-reared larvae would settle and metamorphose on *Laurencia pacifica*, but not on species of *Plocamium, Polysiphonia, Daysia, Chondrus* or *Ulva*. Subsequently, Capo et al. (1979) reported that 2 red algae from the New England coast, *Neoagardheilla baileyi* and *Gracilaria* sp., would also induce settlement and metamorphosis of *A. californica*. The study described herein was initiated in an effort to isolate and identify compounds from *L. pacifica* that induced metamorphosis of *A. californica*. It was discovered, however, that metamorphosis of *A. californica* was much less specific than had been previously reported.

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Materials and methods. All larvae used in experiments were the progeny of 2 specimens of *Aplysia californica* that had been cultured as larvae in the lab, had metamorphosed upon exposure to branchlets of *Laurencia pacifica*, and had been reared from juveniles to reproductive adults on a diet of *Plocamium cartilagineum* and *L. pacifica*. Small pieces of egg mass were placed in aerated beakers containing 1 µm-filtered, natural seawater (hereafter referred to as 'seawater') until hatching occurred. Larvae were then transferred to 2 l Fernbach flasks filled to the neck with seawater containing 40 mg l^{-1} each of the antibiotics streptomycin sulfate and sodium penicillin G (Sigma Chemical Co., St. Louis, Missouri) and 10^4 cells ml^{-1} of the green flagellate *Pavlova lutheri*. Larval entrapment at the air/water interface was prevented by spreading flakes of cetyl alcohol (1-hexadecanol, Sigma) on the water surface. Flasks were kept at 20 °C in a chamber 35 cm beneath 2 continuously-illuminated 60 W fluorescent lights.

Every 4 d, culture vessels were cleaned. Contents of the flask were poured through a series of mesh screens so as to retain the larvae while eliminating larger and smaller particles. The flasks were then scrubbed with hot, fresh water, rinsed in seawater, and reconstituted as before. Cultures were stirred with a jet of 25 ml of seawater once each day. Using these techniques, larvae exhibited growth rates comparable to those observed in other studies (Kriegstein et al. 1974, Paige 1986).

Larvae were competent to metamorphose approximately 35 d after hatching and were used in assays 40 to 60 d after hatching. The development of 4 to 6 red spots on the perivisceral membrane of the larvae did not prove to be a reliable indicator of larval maturity, as detailed by Kriegstein (1977), because larvae lacking perivisceral spots frequently underwent normal settlement and metamorphosis. Twenty larvae were transferred to each glass assay dish (8 cm diam., 4 cm high) containing 60 to 70 ml of seawater coated with flakes of cetyl alcohol. Branchlets of intertidal algae having roughly equivalent surface areas were added to each dish (no alga was added to the control dish). The water surface in each dish was examined under a dissecting microscope, and the larvae trapped in the air/water interface were freed by hitting them with drops of seawater. Dishes were covered with white paper (to decrease light levels and prevent algal branchlets from forming bubbles and floating to the surface) and placed under the same lighting and temperature conditions that had been employed in larval culture. After 2 d, dishes were cleaned in the same manner as larval culture vessels, and the dishes reconstituted as they had been at the start of the assay.

After 4 d, assay dishes, and the algae contained therein, were examined under a dissecting microscope to determine the number of larvae that had undergone metamorphosis. Juveniles had lost their velar cilia and the rudiments of their velar lobes had grown anteriorly to extend beyond the shell when crawling (see Kriegstein 1977, for drawings and details). Unmetamorphosed larvae were distinguished by the presence of velar cilia, whether crawling or not. Larvae and juveniles were removed as they were counted. Metamorphosed larvae were often difficult to locate, particularly on highly branched algae, and data were discarded if less than 15 of 20 larvae or juveniles were recovered per dish.

The intertidal macroalgae used in the assays were collected from Casa Cove and Dike Rock, La Jolla, California, in April and May 1987. Branchlets of algae were added to assay dishes within 24 h of collection. Only branchlets free of epiphytes were used in assays and all associated fauna were removed prior to their transfer to assay dishes. Algal identifications were based on Abbott & Hollenberg (1976). Algae used were: *Codium fragile* and *Ulva* sp. (Chlorophyta); *Colpomenia sinuosa, Dictyopteris undulata, Enderachne binghamiae, Pachydictyon coriaceum, Pelvetia fastigiata, Sargassum muticum* and *Zonaria farlowii* (Phaeophyta); and *Callophyllis violacea, Centroceras clavulatum, Chondria californica, Corallina officinalis, Gigartina canaliculata, Laurencia pacifica, Plocamium cartilagineum, Pterocladia capillacea* and *Rhodymenia californica* (Rhodophyta).

Preliminary experiments were performed in an attempt to characterize the chemical inducers of metamorphosis of *Aplysia californica*. Branchlets of *Plocamium cartilagineum* and *Laurencia pacifica* were separately frozen and freeze-dried, and a portion of the freeze-dried algae was sequentially extracted in distilled organic solvents (hexanes, diethyl ether, ethyl acetate and methanol, in that order). The resulting extracts of each alga were combined and coated onto a disk of filter paper (Whatman, 4 cm diameter). Disks and extracted algae were placed under a vacuum to remove all traces of solvents. Assays were run as before with living, freeze-dried and freeze-dried and extracted algae, and algal extracts on filter paper Freeze-dried and freeze-dried and extracted algae were first rehydrated in seawater under a vacuum.

All assays were run with 3 replicates and the mean percentage of larval response for each assay determined. Differences in mean percentage metamorphosis were tested with 1-way analysis of variance (ANOVA) performed on arcsin-transformed data. The Tukey test was applied a posteriori to determine which treatments resulted in different mean larval responses at the 0.05 level of significance (Zar 1984).

Results. Contrary to previous reports (Kriegstein et

al. 1974, Kriegstein 1977), larvae of *Aplysia californica* did not settle and metamorphose specifically on *Laurencia pacifica* (Fig. 1). Although there were significant differences in mean metamorphosis among treatments (ANOVA, $F_{18,38} = 4.22$, $p < 0.001$), analysis of the differences between means (Turkey test, $\alpha = 0.05$) revealed no difference between larval response to *L. pacifica* and any of the other algae tested. There was significantly greater metamorphosis in dishes containing one of the 4 species of red algae, *Rhodymenia californica, Corallina officinalis, Plocamium cartilagineum* or *L. pacifica,* than in control dishes. A mean of 30% or more larvae metamorphosed in response to 10 of the 18 algae tested, of which 8 species were red algae and 2 species were brown. Of the 8 algae least preferred, only 1 was red, the remainder were brown and green.

Some larvae (3.4%) metamorphosed in control dishes containing no algae at all. This result was corroborated by the frequent discovery of juvenile sea hares on the walls of culture vessels containing larvae older than 50 d. Larvae in culture vessels would likely have come in contact with a film of *Pavlova lutheri* that colonized the bottom and sides of the culture vessel. Larvae in the control dishes, however, were exposed only to clean glass surfaces in seawater containing no phytoplankton. Therefore, it appears that a small percentage of the larvae of *Aplysia californica* will metamorphose in response to nothing more than a hard substrate.

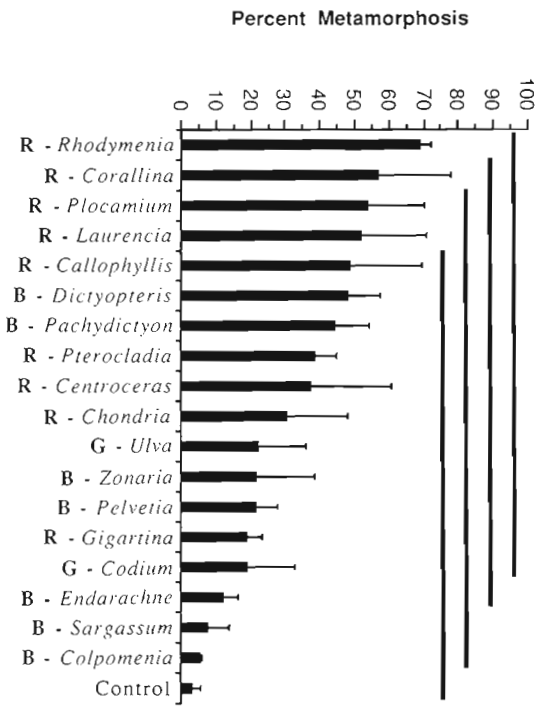

Fig. 1. *Aplysia californica*. Percentage metamorphosis of larvae in laboratory assay dishes containing branchlets of intertidal macroalgae. Horizontal line extending beyond each histogram indicates 1 standard deviation above the mean ($N = 3$). Vertical bars to the right of the histograms overlap algal species that do not significantly differ in their induction of larval metamorphosis (Tukey test, $\alpha = 0.05$). The letter preceding each genus name indicates whether the alga is red (R), brown (B) or green (G). Species names of algae are listed in text

In the course of scoring assay dishes, it was noted that juveniles of *Aplysia californica* in dishes containing *Laurencia pacifica* and *Plocamium cartilagineum* were most often found grazing on the algae, but juveniles in dishes containing any of the other algae were most often found crawling on the bottom or sides of the dish. Recently-metamorphosed juveniles that were given only *Ulva* sp. to eat consumed small amounts of the alga, and showed some growth, but spent most of their time crawling around their container and eventually died. Although juveniles of *A. californica* seem to require a diet of *L. pacifica* or *P. cartilagineum,* this did not prevent them from initially metamorphosing on other species of algae.

There was no significant difference between mean larval response to branchlets of *Plocamium cartilagineum* that were living, freeze-dried or freeze-dried and extracted, or to extracts spread onto filter paper (Fig. 2). There was a significant difference in mean metamorphosis among treatments ($F_{4,10} = 4.61$, $p < 0.05$), but this difference was solely between dishes containing living *P. cartilagineum* and control dishes. Freeze-drying and organic extraction did not remove the capacity of *P. cartilagineum* to induce metamorphosis. Branchlets of *Laurencia pacifica* that had been freeze-dried, freeze-dried and extracted, and the resultant extracts on filter paper, all proved toxic to a percentage of larvae to which they were exposed (Fig. 2). Surprisingly, the toxic component did not entirely partition into the organic extract, but was equally present in the fully extracted alga.

Discussion. Considerable research on chemical induction of larval settlement and metamorphosis has been carried out on molluscs (reviewed in Morse 1985, Hadfield 1986). Larvae of the nudibranch *Phestilla sibogae* metamorphose specifically in response to a water-soluble substance emanating from the tissue or mucus of the coral on which adult animals feed (Hadfield & Scheuer 1985). Larval metamorphosis of the abalone *Haliotis rufescens* may be specific on some species of encrusting red algae (Morse & Morse 1984; however, compare Slattery 1987, Leighton 1988). The naturally-occurring inducers in each of these systems remain to be identified.

The specificity of larval settlement and metamorphosis of aplysiid gastropods has been reviewed previously (Switzer-Dunlap 1978, Carefoot 1987). With the

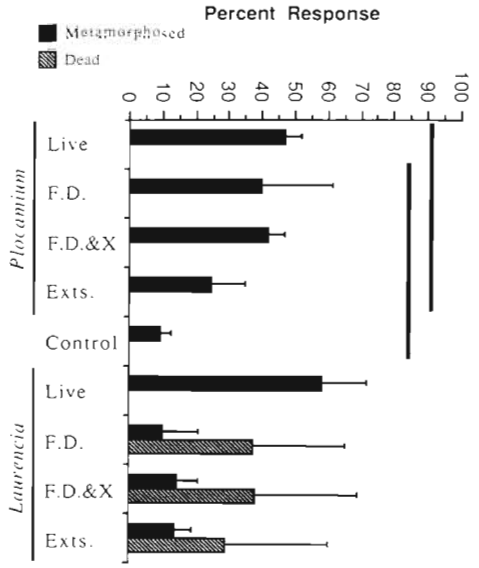

Fig. 2. *Aplysia californica*. Percentage response of larvae in laboratory assay dishes containing living branchlets of algae (Live), freeze-dried branchlets (F.D.), branchlets that were freeze-dried and extracted with organic solvents (F. D. & X), and filter paper impregnated with the organic extraxts (Exts.), of the algae *Plocamium cartilagineum* and *Laurencia pacifica*. Symbols are the same as in Fig. 1

exception of *Aplysia juliana*, sea hares of the genus *Aplysia* metamorphose preferentially on red algae, but in no previous study have these preferences been rigorously assessed. Most frequently, only one or a few species of algae were tested for their ability to induce metamorphosis (e.g. Strenth & Blankenship 1978, Otsuka et al. 1981). In the case of *A. californica*, the previously reported specificity of larval metamorphosis on *Laurencia pacifica* was partially inferred from the distribution of young recruits in the field (Kriegstein et al. 1974).

Before a juvenile invertebrate is said to have 'recruited' into its chosen habitat, it must settle, metamorphose, and grow large enough to be counted by an ecologist (Keough & Downes 1982). Between the time of metamorphosis and recruitment, mortality and (in the case of invertebrates with mobile juveniles) movement can take place. In order to reduce post-metamorphic mortality, many invertebrate larvae appear to exercise considerable selectivity in choosing a substrate on which to settle and metamorphose.

Gregarious settlement is particularly common among species with sessile juveniles and adults (e.g. barnacles, mussels, oysters, tube worms); the presence of adults at the site of settlement is a good indicator that conditions are proper for post-metamorphic survival and growth. In the case of the reef-building tube worm *Phragmatopoma lapidosa californica* 60 to 90% of the larvae settle on the tube sand of adult worms, but only about 1% settle on alternative substrates (Pawlik 1986, 1988). Such substrate specificity must have a cost, however. Larvae that 'hold out' in the plankton for the proper settlement cue risk increased mortality resulting from predation and advection to unsuitable habitats.

Unlike sessile species, juveniles of *Aplysia californica* are mobile immediately after metamorphosis, when they are still <0.5 mm in shell length. At the time of recruitment (i.e. detection by the field ecologist), the juveniles have increased in size some 20-fold and are associated solely with *Laurencia pacifica* and *Plocamium cartilagineum* (Kriegstein et al. 1974, Pennings 1988). The observations of the present laboratory study suggest that juvenile *A. californica* move onto *L. pacifica* and *P. cartilagineum* from other macroalgae after metamorphosis. Larvae of *A. californica* may decrease the risks associated with 'holding out' for a specific alga by metamorphosing on a variety of macroalgae. Juveniles that have successfully found a macroalgal assemblage are then only required to locate *L. pacifica* and *P. cartilagineum*, both relatively common. For settlement and metamorphosis of *A. californica*, then, larvae appear to be selective enough to increase the likelihood of juvenile survival, but sufficiently broad in their response so as to minimize larval mortality.

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