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Growth and metamorphosis of *Aplysia oculifera* larvae in laboratory culture

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**Abstract** Eggs of *Aplysia oculifera* (Adams and Reeve, 1850) were incubated in the laboratory. They hatched 8 to 9 d after spawning. Shell length (SL) of the hatched larvae was 102 ± 2 μm. Larvae were fed on the unicellular algae *Isochrysis galbana* in a concentration of $10^4$ cell ml$^{-1}$, and after 45 to 60 d grew to a maximum SL of 385 ± 11 μm. Larvae survived up to 330 d. A total of 12 species of algae from the natural habitat of *A. oculifera* were examined as metamorphosis inducers. Red algae *Dasia* sp., *Jania* sp., *Hypnea* sp. and *Liaogora* sp. induced metamorphosis in 66.7 ± 21.2, 28.3 ± 17.7, 26.0 ± 18.5 and 4.0 ± 8.0% of the larvae, respectively. Green algae *Enteromorpha intestinalis* and *Ulva* sp. induced metamorphosis in 37.0 ± 11.0 and 9.0 ± 10.4% of the larvae, respectively. *Cladophora* sp. and *Codium dichotomum*, and the brown algae *Padina pavonia*, *Colpomenia sinuosa*, *Hydroclathrus clathratus* and *Cystoseira* sp. did not induce metamorphosis. There was no significant difference in the rate of metamorphosis between young (2 to 4 mo) and old (6 to 8 mo) larvae. Postmetamorphic juveniles grew and developed only when fed with *E. intestinalis*. They grew to a body length of >8 mm in 50 d. Postmetamorphic juveniles did not survive on other algae. The longevity of the planktonic *A. oculifera* larvae supports the hypothesis that the larvae can exist in the plankton and survive for several months until the next recruitment. The advantage of non-specificity in metamorphosis induction is discussed.

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**Introduction**

Larvae life history, settlement and metamorphosis are essential in determining the population structure and dynamics of marine benthic invertebrates (Kempf 1981; Pawlik and Hadfield 1990). Two parameters play an important role in these processes. One is the duration of larval stages, and the other is the mechanism of settlement and the induction of metamorphosis.

A short larval stage reduces the distance that the larvae can be swept from the hatching site. This may result in settlement close to the parents’ site, where food is probably available, and may reduce the mortality due to predation in the plankton. On the other hand, a long larval stage may increase the species distribution and allow more time for finding suitable sites for settlement (Kempf 1981; Strathmann and Strathmann 1982; Strathmann et al. 1984).

Metamorphosis induction in marine invertebrate larvae usually involves a chemical cue from the environment (Pawlik and Hadfield 1990). Such a chemical cue may derive from a specific food source (Hadfield 1978, Switzer-Dunlap 1978; Morse and Morse 1984; Hadfield and Scheuer 1985; Chia and Koss 1988; Pawlik 1989) or conspecific aggregation (Burke 1986). There are also larvae that are induced to settle and metamorphose by a microbial film which is usually an indication of the presence of a solid substrate which is required by most sessile organisms (Brancato and Woolacott 1982; Kirchman et al. 1982; Maki and Mitchell 1985).

The chemical signal that induces settlement and metamorphosis may be more or less specific in indicating the settlement time and location. Such a chemical may be non-specific such as in the case of microbial film, or specific, such as the food of some nudibranchs (Crisp 1974; Hadfield and Scheuer 1985; Todd et al. 1991). The level of specificity is an important factor in the strategy of settlement and metamorphosis. A non-specific signal is especially suitable for planktotrophic, sessile filter feeders, while a highly specific signal is necessary for organisms that feed on a specific and rare food source.
Sea hares of the genus *Aplysia* (Gastropoda: Opisthobranchia) possess a planktonic planktotrophic larval stage. Numerous studies have been conducted on the larval life history of different species of *Aplysia* (Kriegstein et al. 1974; Kriegstein 1977; Switzer-Dunlap and Hadfield 1977; Streth and Blankenship 1978; Switzer-Dunlap 1978; Kempf 1981; Usuki et al. 1981; Nadeau et al. 1989; Pawlik 1989). These studies showed that larvae are competent to metamorphose at an age of 30 to 60 d. It was also shown that *A. juliana* larvae survived for as long as 307 d when not induced to metamorphose (Kempf 1981). Regardless of the mechanism of settlement and metamorphosis, these studies suggested that the inducers of metamorphosis are highly specific and originate from algae on which the adults feed (review by Switzer-Dunlap 1978; Carefoot 1987). However, Pawlik (1989) reported that *A. californica* larvae metamorphosed when introduced to 18 different macroalgal species from the adults' habitat. Some of these algae are not eaten by the adults.

We studied larval life history and metamorphosis of the sea hare *Aplysia ocellata* (Adams and Reeve, 1850) in the laboratory. *A. ocellata* inhabit rocky coasts of the Indopacific Ocean and the Red Sea. In the Gulf of Elat (Aqaba), adult populations occur from December to July and are absent from August to November, exhibiting a distinct seasonal occurrence (Plaut 1993). Such seasonality of *Aplysia* spp. has been documented for other species from different localities (Carefoot 1967; Usuki 1970; Lederhendler et al. 1975; Sarver 1978; Auedsirk 1979; Gev et al. 1984; Achituv and Susswein 1985; Pennings 1991; Streth and Blankenship 1991). Carefoot (1987) suggested that the disappearance of the populations during part of the year could be related to: (1) the small body size of recruits, which become noticeable after a long growth period; (2) larval settlement derived from other populations which exist out of phase with local populations; (3) the fact that larvae delay their settlement and metamorphosis until they find suitable conditions that will allow successful development of the adult populations; (4) adult migration from the intertidal zone to distant locations in deeper waters.

In the present study, we reared *Aplysia ocellata* larvae in the laboratory and tried to induce metamorphosis with several species of macroalgae. Our objective was to answer two questions: (1) How long can the larvae survive and keep their competency to metamorphose? and (2) What are the conditions required to induce the larvae to settle and metamorphose?

### Materials and methods

#### Eggs source and treatment

Adult *Aplysia ocellata* were collected in the intertidal zone along the northwestern coast of the Gulf of Elat (Aqaba) between February and April of 1989 and 1990. They were kept at the Interuniversity Institute, H. Steinitz Marine Biology Laboratory, Elat, Israel, in 15 to 20 liter aquaria with an open seawater circulation system. Each aquarium contained four to six individuals. The sea hares were fed daily fresh algae (*Ulva* sp.) grown in the laboratory. Newly spawned egg clusters were collected from the aquaria, rinsed, and inserted into 1 liter Erlenmeyer flasks containing filtered seawater (filter holes diameter 0.45 µm) under continuous aeration. At 1 or 2 d before hatching the egg clusters were soaked in polyvinylpyrrolidone iodide complex (PVP-1, 10 mg mL⁻¹) with Trizma pH 8.5 buffer for 30 min to disinfect the eggs. Then, a few drops of sodium thiosulfate (0.1 M) were added to neutralize the PVP-I. Subsequently, 10-mm long portions of egg string were examined by a light microscope. Egg strings in which the embryos failed to show normal rotatory movement were discarded. In each of the healthy strings, the capsules and embryos (per capsule in ten capsules) were counted to calculate the number of embryos per 10 mm of egg string. Portions containing about 2000 embryos were cut off and each of them placed in a 1 liter Erlenmeyer flask containing the culture medium.

The culture medium was made of fresh filtered seawater containing 60 µg mL⁻¹ penicillin-G, 50 µg mL⁻¹ streptomycin sulfate (Hautfield and Scheuer 1985), 0.25 mg mL⁻¹ EDTA (to prevent bacterial infection and reduce heavy metals ions concentration) and unicellular algae (*Isochrysis galbana*) at a density of 10⁴ cells mL⁻¹. After placing the egg string into the culture medium flask, 20% alcohol was sprinkled sparsely on the medium surface to decrease surface tension and to prevent the larvae shells from being trapped at the water surface (Switzer-Dunlap and Hadfield 1977). The culture flasks were kept at 24 ± 1°C and ambient photoperiod.

#### Larval culture

1 or 2 d after preparing the culture, larvae hatched and concentrated in the upper part of the flask. Twice a week the culture medium was filtered through 60-µm plankton mesh and the larvae were placed in 5 ml PVP-I (2–5 mg mL⁻¹) for 10 min. During this time, shell length (SL) of 20 larvae from each flask was measured using an ocular micrometer at 100x. A few drops of sodium thiosulfate (0.1 M) were added to reduce the PVP-I, and the larvae were returned to fresh culture medium.

After gaining a SL of >320 µm, larvae were transferred to 300-ml Pyrex beakers containing 100 ml culture medium. Larval densities were <1 larvae mL⁻¹. The larvae and the culture medium in the beakers were treated in the same manner as described above.

#### Metamorphosis induction experiments

Twelve algae species from the natural habitat of adult sea hares were examined as metamorphosis inducers for *Aplysia ocellata* larvae. The macroalgae used in this experiment represent three major groups: Chlorophyceae, Rhodophyceae and Phaeophyceae. They included *Enteromorpha intestinalis* Link, *Ulva* sp., *Cladophora* sp., *Codium dichotomum* Gray, *Dasia* sp., *Jania* sp., *Hypnea* sp., *Liagora* sp., *Padina pavonina* Gaillon, *Colpomenia sinuosa* Derbes and Solier, *Hydroclathrus clathratus* Howe, and *Cystoseira* sp. The algae were collected in situ 1 to 2 h before an experiment, rinsed in filtered seawater, examined under dissecting microscope, and any attached material was removed.

To induce metamorphosis, ten larvae (SL >320 µm, age >60 d) were placed in a petri dish (50 mm diameter, 15 mm height) which contained 15 ml filtered seawater and 2 cm³ of the tested algae. The control dishes contained only filtered seawater and ten larvae. Each setup (one experiment for one algae and control) was replicated three times. These setups were repeated twice to ten times for each algae species, depending upon its availability. All the experiments began at noon. 4 h later each dish was examined under a dissecting microscope for metamorphosed larvae. Larvae were considered metamorphosed when they lost their larval velum. Such examinations were repeated every 3 to 12 h for the next 3 d. Larvae that did not metamorphose after 3 d were discarded.

In order to determine the effect of larval age on competency for metamorphosis, experiments with *Enteromorpha intestinalis* were
Results

Incubation period, growth and metamorphosis delay

Incubation period of Aplysia oculifera eggs ranged from 8 to 9 d at 24 ± 1°C. At spawning, the color of the egg clusters was bright yellow and became brownish as the embryos developed. After 5 to 6 d the larvae were almost fully developed and rotated inside the capsules, using the velum cilia.

The diameter of the embryos was 60 ± 1 (mean ± SD) μm at spawning. At hatching larval SL averaged 102 ± 2 μm. Larvae grew to 385 ± 11 μm SL in 45 to 60 d (Fig. 1). Growth rates were from 8 μm of SL d⁻¹ immediately after hatching, decreased gradually and stopped at maximal SL (385 ± 11 μm). From this point the larval size remained constant until metamorphosis or death. Development of the larvae was consistent with the description for Aplysia californica (Kriegstein 1977) but no pigment spots appeared on the larval body as indicators of competency for metamorphosis. Maximum longevity for larvae that were not induced to metamorphosis was 330 d.

Settlement and metamorphosis

Metamorphosis percentage on Enteromorpha intestinalis was 40 ± 12% for young larvae and 34 ± 11% for old larvae (n=5 in each experiment). There is no significant difference between these two results (F(8), 921, 0.2 < p < 0.4, Student t-test after arcsin transformation).

Settlement and metamorphosis occurred when larvae were introduced to six out of the 12 tested macroalgae (Fig. 2). All Rhodophyceae species and two out of four Chlorophyceae species induced settlement and metamorphosis. The different algae had significantly different rates of induction [ANOVA, F(5,36) = 13.5, p < 0.001].

Statistical analysis of differences among the means (Tukey test, after arcsin transformation, p < 0.05, Fig. 2) shows that four groups of macroalgae induced significantly different rates of metamorphosis. Dasia sp. induced significantly higher rate of metamorphosis (66.7 ± 21.2%). Enteromorpha intestinalis, Jania sp., Hypnea sp. and Ulva sp. induced metamorphosis at a median rate (37.0 ± 11.0, 28.3 ± 17.7, 26.0 ± 18.5 and 9.0 ± 10.4%, respectively). Littorina sp. induced low rate of metamorphosis (4.0 ± 8.0%, not significantly different from Jania sp., Hypnea sp. and Ulva sp.). Cladophora sp., Codium dichotomum and all of the Phaeophycean algae failed to induce metamorphosis.

Discussion

Eggs of Aplysia oculifera hatched 8 to 9 d after spawning. within the range of other Aplysiids (Carefoot 1987). In the intertidal zone the eggs may be vulnerable to predation, and a short incubation period. together with chemical defense (Yamazaki et al. 1985; Pawlik et al. 1988) may decrease the hazards of predation and/or infection by bacteria or fungi. After hatching, the larvae swim toward the water surface, presumably a negative geotactic behavior made possible by the statocyst that exists in the hatching
**Fig. 2** *Aplysia oculifera*. Metamorphosis induction in larvae (% ± SD) by 12 macroalgal species collected from the adults’ habitat (no. of experiments in parentheses). Experiment made for each macroalgal species with 30 larvae divided into three dishes. Vertical bars join algal species that do not differ significantly in induction of metamorphosis (Tukey test, p > 0.05 after arcsin transformation).

**Fig. 3** *Aplysia oculifera*. Growth of the sea hare after metamorphosis in the laboratory, fed on *Enteromorpha intestinalis*

larvae (Hadfield and Switzer-Dunlap 1984). The behavior of swimming upward helps the larvae to be caught in horizontal currents and swept offshore. Carefoot (1987) suggested that the larvae concentrate near the water surface because higher light intensity there makes high densities of phytoplankton possible. This suggestion does not hold for the Gulf of Eilat where nutrient concentrations increase with depth. Thus the phytoplankton, which the larvae feed on, concentrates at a depth of 40 to 100 m (Reiss and Hottinger 1984; Lazar and Erez 1981).

*Aplysia oculifera* has two major developmental stages during larval life. In the first stage larvae grow rapidly to maximum size. In the second stage growth stops and the larvae remain constant in size. Growth in the first stage is affected by several factors, e.g. temperature, nutrition, salinity etc. (Carefoot 1987). In our study, at 24°C, ambient salinity (4.08%), and fed on *Isochrysis galbana* (10⁴ cells ml⁻¹), larvae grew from a SL of 102 to 385 µm in 44 d. This growth rate is similar to those reported for other *Aplysia* spp. reared in the laboratory (Kriegstein et al. 1974; Switzer-Dunlap and Hadfield 1977; Kempf 1981).

Larvae of *Aplysia oculifera* survived up to 330 d in our study. This period is similar to that of *A. juliana* in laboratory culture, which survived up to 307 d (Kempf 1981). Ability to survive for such a long period is important for several species of *Aplysia*. Adult populations of *A. oculifera* are present in the northern Gulf of Eilat during only 4 to 6 mo each year (Plaut 1993), and some other populations of *Aplysia* spp. appear also in a seasonal pattern (Usuki 1970; Sarver 1978; Audesirk 1979; Gev et al. 1984; Susswein et al. 1987; Streth and Blankenship 1991). The *Aplysia* spp. larvae must have the ability to survive a long time without losing their competency to metamorphose in order to be able to outlive seasons when the environmental conditions are not suitable for establishing a reproductive adult population. This also enables them to extend their distribution to new suitable habitats.

Larvae of *Aplysia oculifera* metamorphosed in different percentages when introduced to red and green macroalgae collected in the adult’s natural habitat. No spontaneous metamorphosis occurred. Former studies showed that *Aplysia* spp. larvae settled and metamorphosed in the presence of specific macroalgae on which the adults feed (Kriegstein et al. 1977; Streth and Blankenship 1978; Otuska et al. 1981). However, similar to our results, Pawlik (1989) reported that *A. californica* larvae settled and metamorphosed on 18 different algae species found in the adult’s natural habitat, including algae which are not used as food by the adults. Our results, like those of Pawlik (1989), suggest that the inducer may be a common chemical, and that the induction is not as specific as it was thought to be.
Low specificity of metamorphosis induction seems to be beneficial for *Aplysia* spp. They have the ability to move after metamorphosis, and their food is relatively common. Thus, they do not have to settle exactly at the most favorable site for the adult’s development. Mobility at the postmetamorphic stage allows them to settle and metamorphose in the vicinity of the optimal site and to move toward it later. In such cases, there is no need for a very specific inducer to designate the exact location of the optimal substrate. An inducer that indicates the surrounding of the optimal site will be efficient.

A highly specific inducer is essential for marine invertebrates that specifically feed on uncommon food (Hadfield and Scheuer 1985; Todd et al. 1991). This will directly affect the adult’s chances for survival. However, such specificity decreases the probability that the planktonic larvae will find a suitable substrate for settlement and increases the mortality of larvae in the plankton due to predation or being swept to unsuitable habitats with no food source (Pawlik 1989).

*Aplysia oculifera* postmetamorphic juveniles move and can subsequently find the macroalgae *Ulva* sp. or *Enteromorpha intestinalis* in order to feed and continue their development toward the adult stage. Thus, a signal produced by algae which are members of the natural community of *Ulva* sp. or *E. intestinalis* indicates for *A. oculifera* larvae a high probability of finding these species, and it can function as an inducer for settlement and metamorphosis. This mechanism increases the probability of larval settlement. Highly specific inducers have been suggested for induction of settlement and metamorphosis in *Aplysia* spp. (Switzer-Dunlap and Hadfield 1977; Carefoot 1987) probably because recruited juveniles were found mostly on the algae on which the adults feed (Server 1978; Audesirk 1979; Gev et al. 1984). However, our results and those of Pawlik (1989) disprove this hypothesis regarding high specificity in the induction for metamorphosis.

Rates of metamorphosis in our experiments showed high variabilities among algae and among replications. Field observations (Strehn and Blankenship 1991; Plaut 1993) showed that recruitment of *Aplysia* spp. in nature is highly variable and occurs only when edible algae are present. However, the presence of these algae is not sufficient to cause *Aplysia* spp. recruitment (Plaut 1993). The variability of the results and the observations in the field suggest that the inducer is released from the algae in certain stages and in different amounts throughout the algal life cycle. Additional field observations demonstrate peaks in recruitment (Audesirk 1979; Plaut 1993) and suggest that the inducer is released mostly when the algae are at the beginning of their blooming period.

Induction of metamorphosis at this stage guarantees *Aplysia* spp. recruits the longest period of food supply. It also prevents settlement at the end of the algal season, when the recruits will not be able to develop into reproductive adults due to extinction of the algae or due to competition with adult sea hares that recruited previously.

Larvae that metamorphosed on algae not suitable as food survived up to 22 days without eating and crawled continuously around the experimental dish wall. The ability to survive for a long period after metamorphosis without feeding supports our suggestion that the inducer is not as specific as it was thought to be. It enables the postmetamorphic larvae to search until suitable food is found.

Growth rate of *Aplysia oculifera* postmetamorphic larvae was slower than that reported for other *Aplysia* spp. Postmetamorphic larvae of *A. californica*, *A. juliana* and the Aplysiaid *Bursatella leachii* grew to 8 mm (about 10 mg body mass) at about 25 days after metamorphosis (Kriegstein 1977; Server 1978; Paige 1988). *A. oculifera* juveniles grew to 8 mm (about 10 mg body mass) at 40 to 50 days after metamorphosis. These growth rates are slower than in nature (Plaut 1993) and may be caused by sub-optimal conditions in the postmetamorphic juveniles cultures.

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