Aspects of the larval biology of the sea anemones

*Anthopleura elegantissima* and *A. artemisia*

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**Abstract.** We investigated several aspects of the larval biology of the anemone *Anthopleura elegantissima*, which harbors algal symbionts from two different taxa, and the non-symbiotic *A. artemisia*. From a 7-year study, we report variable spawning and fertilization success of *A. elegantissima* in the laboratory. We examined the dynamics of symbiosis onset in larvae of *A. elegantissima*. Zoochlorellae, freshly isolated from an adult host, were taken up and retained during the larval feeding process, as has been described previously for zooxanthellae. In addition, larvae infected with zooxanthellae remained more highly infected in high-light conditions, compared to larvae with zoochlorellae, which remained more highly infected in low-light conditions. These results parallel the differential distribution of the algal types observed in adult anemones in the field and their differential tolerances to light and temperature. We report on numerous failed attempts to induce settlement and metamorphosis of larvae of *A. elegantissima*, using a variety of substrates and chemical inducers. We also describe a novel change in morphology of some older planulae, in which large bulges, resembling tentacles, develop around the mouth. Finally, we provide the first description of planulae of *A. artemisia* and report on attempts to infect this non-symbiotic species with zooxanthellae and zoochlorellae.

*Additional key words:* Cnidaria, Anthozoa, Actiniaria, larval settlement, metamorphic inducer, symbiosis, zoochlorellae, zooxanthellae

The 4 species of the sea anemone genus *Anthopleura* found along the western coast of North America—*A. elegantissima* (Brandt 1835), *A. sola* (Pearse & Francis 2000), *A. xanthogrammica* (Brandt 1835), and *A. artemisia* (Dana 1846)—are conspicuous members of rocky intertidal communities. *A. elegantissima* in particular, which is common along the coast from Alaska to Baja California, can form clonal aggregations that occupy large areas within the mid-intertidal zone, to the exclusion of other competitors (Dayton 1971; Sebens 1981a; Fitt et al. 1982). Despite their importance in these communities, examination of the sexual reproductive cycle and life histories of these anemones has been restricted to a handful of studies. Ford (1964), Jennison (1979), and Sebens (1981b) described gametogenesis and an annual reproductive cycle in *A. elegantissima* and *A. xanthogrammica* that started with gonad development (both species are dioecious) in the late fall and ended with spawning in the late summer. Siebert (1974) and Smith (1986) examined laboratory-induced spawning, embryological development, and morphology and behavior of planktotrophic planula larvae of *A. elegantissima* and *A. xanthogrammica*. Finally, Sebens (1981a, 1982) described recruitment of larvae from both species into mussel beds adjacent to sites with adult anemones. No life history studies have focused on either *A. sola* or *A. artemisia*.

*A. elegantissima* and *A. xanthogrammica* form symbioses with two different types of unicellular algae (Muscatine 1971; Muller-Parker & Davy 2001), dinoflagellates from the genus *Symbiodinium* (zooxanthellae, LaJeunesse & Trench 2000) and one or more taxonomically undescribed chlorophytes (zoochlorellae). As in other cnidarian-algal symbioses, the algae provide the anemones with photosynthetically fixed organic carbon and the host anemone in turn supplies the algae with inorganic nutrients, a stable environment, and a refuge from herbivory (reviewed in Muscatine 1990; Muscatine & Weis 1992; Muller-Parker & D’Elia 1997). Anemones of both species containing these different algal types are differentially distributed along a latitudinal and tidal-height gradient, with...
anemones in more northerly and lower intertidal locations containing predominantly zoochlorellae and anemones in more southerly and higher intertidal locations containing predominantly zooxanthellae (Bates 2000; Secord & Augustine 2000).

This distribution of algal types has been attributed to differential physiological tolerance of the algae to temperature and light, with zooxanthellae well adapted to relatively high-light and warm conditions compared to zoochlorellae, which are better adapted to cooler, lower-light environments (Verde & McCluskey 1996a,b, 2001, in press; Saunders & Muller-Parker 1997). A. sola, a newly described, solitary sister species to A. elegantissima (Francis 1979; McFadden et al. 1997; Pearse & Francis 2000), is restricted to latitudes below the likely southern limit of zoochlorellae (Secord & Augustine 2000) and is symbiotic only with zooxanthellae. A. artemisia is a non-symbiotic species (Hand 1955; Pearse & Francis 2000). Recent molecular phylogenetic studies have grouped A. elegantissima, A. sola, and A. xanthogrammica into an eastern Pacific clade, whereas A. artemisia groups with other conspecifics in a distinct western Pacific clade, despite its occurrence in the eastern Pacific (Geller & Walton 2001).

Larvae of A. elegantissima and A. xanthogrammica are oval planulae, ~150 μm long and 100 μm wide with a conspicuous apical tuft of numerous elongate cilia present at the aboral pole (Siebert 1974). They swim actively in the water column (Siebert 1974), feed by extrusion of a mucous thread that traps food (Siebert 1974; Schwarz et al. 2002), and persist in culture for as long as 3 months (Smith 1986). Despite attempts by many workers to induce settlement by the addition of various natural substrates, such as mussel shells, rocks, and macrophytes, to larval cultures (Siebert 1974; Smith 1986), no larvae have ever been observed to undergo settlement and metamorphosis.

Gametes and larvae of A. elegantissima and A. xanthogrammica lack symbiotic algae (Siebert 1974). These anemones must therefore acquire symbionts from the environment during their development. We were initially interested in larvae of A. elegantissima as a model for the study of the onset of symbiosis between sexually produced cnidarian offspring and their symbiotic zooxanthellae. Symbiosis onset in A. elegantissima can begin in the larval phase during the feeding process, whereby zooxanthellae, freshly isolated from adults, are taken in along with food (Schwarz et al. 2002). Algae are phagocytized by host gastrodermal cells and ultimately persist in the gastrodermal tissue. A similar process of algal acquisition (infection) by feeding during the larval stage has been described in the anemone Aiptasia tagetes (Riggs 1988) and the scleractinian coral Fungia scutaria (Krupp 1983; Schwarz et al. 1999).

What started as the search for an ideal model for the study of cnidarian larval biology and symbiosis onset has instead developed into a 7-year love/hate relationship with larvae of A. elegantissima. Despite our best efforts, answers to key questions surrounding developmental processes, such as spawning and fertilization patterns and settlement and metamorphosis, remain elusive. Here we report results from studies on symbiosis onset, specifically in the examination of infection dynamics of the larvae by both zooxanthellae and zoochlorellae and in the differential infection by zooxanthellae and zoochlorellae as a function of light regime. We describe a morphological change in some mature larvae that may represent the development of tentacles before settlement. Further, we provide the first description of larvae of A. artemisia and the response of this non-symbiotic species to exposure to zooxanthellae and zoochlorellae. We also report on some key failures in our examinations of larval A. elegantissima. We describe variability in spawning and fertilization success through the study period, that has hampered our ability to continue studies of larvae. In addition, we describe our numerous failed attempts to induce settlement and metamorphosis of these larvae, with the aim of preventing repetition of these approaches by other researchers in future studies.

Methods

Animal maintenance, gamete collection, and larval cultures

Adults of Anthopleura elegantissima, to be used as broodstock and as sources of freshly isolated algae, were collected at various times from 1990 to 2001, from the following locations along the Pacific coast of the U.S.: Santa Cruz, California; Strawberry Hill, Neptune Beach, Seal Rock, and Boiler Bay, Oregon; and Swirl Rocks, Washington. Zooxanthellate animals were collected from all locations, but all zoochlorellate animals were collected at Swirl Rocks. Experiments in 1995 and 1996 were performed at Long Marine Laboratory (University of California Santa Cruz) in Santa Cruz, California; those in 1997–2001 were performed at Hatfield Marine Science Center (Oregon State University) in Newport, Oregon; and those in the summer of 2000 were performed at Walla Walla College Marine Station, Anacortes, Washington. All foreign material adhering to the anemones (e.g., fragments of shell and macroalgae) was removed after collection and individual anemones were allowed to attach to bricks (3–10 anemones of one clone per brick). The bricks were submerged in outdoor seawater tanks ex-
posed to ambient sunlight. Animals were fed previously frozen brine shrimp or minced mussel 1–3 times a week.

Techniques modified from Smith (1986) and Schwarz et al. (2002) were used to induce spawning. In the morning, bricks with the anemones were placed into open aquaria containing ambient, static seawater and exposed to ambient sunlight for a period of time without changing the seawater. Water temperature was not controlled and rose during this exposure. On over-cast or foggy days, the seawater in the containers was replaced with cold ambient seawater at dusk. On sunny days, the anemones were exposed for 4–6 h before the water was changed. Within 12 h after a water change, the anemones released gametes.

When spawning occurred, thousands to hundreds of thousands of negatively buoyant eggs settled on the oral disc or on the bottom of the aquaria and were subsequently collected with a large plastic pipette (turkey baster) and placed into a large glass container with seawater. A small amount of sperm suspension was similarly collected and added to the eggs for fertilization. The mixture was maintained in a cold room at 14°C (in California and Oregon), or in 10-liter containers suspended in indoor tanks of flow-through ambient seawater at 12–14°C (in Washington). At 24 h after fertilization, planulae were filtered out of the seawater using a 50-µm mesh screen and placed into fresh seawater in glass or plastic containers. For the California and Oregon experiments, larvae were kept in the incubators mentioned above in a 12-h:12-h light/dark regime at an irradiance of ~40 μmole of quanta/m²/sec. For the Washington experiments, larvae were exposed in outdoor tanks to 55% ambient sunlight using neutral density screens placed over the containers of larvae. In all locations, every 2 days, the seawater was changed throughout the experiments. Larvae kept in unfiltered seawater remained aposymbiotic throughout their ~30-day lifespan unless experimentally infected (see below).

**Field collection of gametes of *A. artemisia***

At Strawberry Hill and Neptune Beach, Oregon during a low tide on 26 June 1998, numerous individuals of *A. artemisia* were observed spawning. Eggs and sperms were collected with a Pasteur pipette and mixed in several 15-ml conical tubes. The tubes were transported back to the laboratory and the developing planulae were maintained in the same fashion as larvae of *A. elegantissima*.

**Preparation of freshly isolated zooxanthellae and zoochlorellae***

Algae were isolated from adults of *A. elegantissima* (see Schwarz et al. 1999, 2002). Adult anemones were cut in half transversely and the basal half was discarded. The oral half was minced with a razor blade, homogenized in filtered seawater (FSW) with a ground-glass tissue grinder, and centrifuged at 4000 g for 5 min to separate the algal cells from the animal tissue. A thin animal pellet fraction, layered above the algal pellet and consisting mostly of nematocysts, was carefully removed and discarded. The algal pellet was re-suspended in FSW and re-pelleted 3 times to partially clean the algae of contaminating animal debris. The final pellet was re-suspended in FSW and this dense suspension was passed through a 150-µm mesh screen to break up large algal clumps. Algal isolates were used within 1 h of preparation.

In numerous preliminary experiments, we attempted to quantify the number of algae that we added to larvae. After the 3 rinses in FSW (described above), both zooxanthellae and zoochlorellae were highly clumped and contaminated with animal debris, making quantification impossible. Additional seawater rinses did not improve results. In an attempt to obtain more uniform suspensions, after the rinses, the algal suspension was placed in a syringe and forced through a metal 50-µm mesh screen. Algae in the resulting suspension were no longer clumped and could be quantified; however, subsequent infection was severely reduced (data not shown), presumably because the algae were compromised during preparation. We therefore decided to provide unquantified, high concentrations of algae to the larvae (see below) and obtained repeatable infection results.

**Infection of *A. elegantissima* and *A. artemisia* with algal isolates***

In order to compare the abilities of the two algal types to infect *A. elegantissima* larvae, we measured the percentage of larvae infected with zooxanthellae and zoochlorellae isolates and the density of algae in larvae through time. For each experiment, ~10,000 4-day-old larvae were divided between two glass or plastic containers. Zooxanthella isolates were added to one container and zoochlorella isolates to the other. Larvae of *A. elegantissima* acquire algae during feeding when algal cells enter through the mouth with food (Schwarz et al. 2002). Therefore, several drops of homogenized brine shrimp (*Artemia* sp.) or mussel tissue (*Mytilus* sp.) were added to the bowls to stimulate a feeding response. After 5–6 hours, algal isolates were still present in large quantities at the bottom of the dish. Larvae were then concentrated with a 50-µm mesh screen, placed into clean filtered seawater, and returned to incubators (in CA and OR, see above) on a 12-h:12-h light/dark regime at 40 μmol quanta/m²/
Table 1. Natural and artificial substrates used in attempts to induce settlement and metamorphosis in larvae of *Anthopleura elegantissima*.

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Age of larvae (days)</th>
<th>Symbiotic state</th>
<th>Location of treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Live mussels, shells, and shell debris (<em>Mytilus</em>)</td>
<td>16, 18</td>
<td>aposymbiotic, zooxanthellate, zoochlorellate</td>
<td>CA, OR</td>
</tr>
<tr>
<td>Miscellaneous rocks collected from intertidal</td>
<td>16, 18</td>
<td>aposymbiotic, zooxanthellate, zoochlorellate</td>
<td>CA, OR</td>
</tr>
<tr>
<td>Adult anemone</td>
<td>16, 18</td>
<td>aposymbiotic, zooxanthellate, zoochlorellate</td>
<td>OR</td>
</tr>
<tr>
<td>Plastic Petri dishes roughened with sandpaper*</td>
<td>13</td>
<td>aposymbiotic</td>
<td>WA</td>
</tr>
<tr>
<td>Plastic Petri dishes scored with scalpel*</td>
<td>13</td>
<td>aposymbiotic</td>
<td>WA</td>
</tr>
<tr>
<td>Plastic Petri dishes with light and dark formica tiles on bottom*</td>
<td>13</td>
<td>aposymbiotic</td>
<td>WA</td>
</tr>
<tr>
<td>Plastic Petri dishes with adult anemone (zooxanthellate and zoochlorellate) in dish*</td>
<td>13</td>
<td>aposymbiotic</td>
<td>WA</td>
</tr>
</tbody>
</table>

* All Petri dishes and tiles were seasoned in unfiltered running seawater for 10 days before use.

...continued...

**Attempts to induce settlement and metamorphosis of *A. elegantissima* using substrates and metamorphic inducers**

Several substrates (Table 1) and compounds (Table 2) were added to cultures of both symbiotic and aposymbiotic larvae of *A. elegantissima* in an attempt to initiate settlement and metamorphosis. For the experiments with natural substrates in California and Oregon, hundreds of larvae were added to glass bowls containing one of the substrates listed in Table 1. The bowls were incubated as described above for larvae cultures and were monitored daily under a dissecting microscope for evidence of settlement. For the experiments in Washington, ~25–30 larvae were added to plastic Petri dishes. All Petri dishes were conditioned in unfiltered running seawater for 10 days before larvae were added. Groups of larvae for each treatment were subdivided into 8 replicates; 4 replicates were placed in a refrigerated incubator and 4 in an outdoor flow-through seawater tank at 55% ambient sunlight. The dishes were examined daily for ~15 days under a dissecting microscope for evidence of larval settlement and metamorphosis.

Larvae were exposed to several compounds known to elicit settlement and metamorphosis in various marine invertebrates, including some cnidarian species (see references in Table 2). Approximately 15 larvae were placed in each well of 24-well culture plates in FSW (5 ml per well) with one of the compounds for the duration of the monitoring period (n = 8 wells for each compound at each concentration). Controls were set up under identical conditions without a chemical inducer. Plates were incubated as described for larval cultures. The plates were examined daily during the monitoring period (see Table 2), under a dissecting microscope, for evidence of larval settlement and metamorphosis.

**Statistical analyses**

All data were checked for normality and homogeneity of variances (Cochran's method, Winer 1971) before conducting statistical analysis. If data were non-normal or heterogeneous, the data were log-transformed before analysis. All percentage data were square-root arcsine transformed before analysis. Analysis of variance (2-way ANOVA), post-hoc Tukey (HSD) test, and linear regression were performed using the statistical package STATISTICA® (Statsoft, Inc.).
Table 2. Metamorphic inducers used in attempts to induce settlement and metamorphosis in larvae of *Anthopleura elegansissima*.

<table>
<thead>
<tr>
<th>Metamorphic inducer</th>
<th>Concentration of inducer</th>
<th>Age of larvae (days)</th>
<th>Duration of monitoring (days)</th>
<th>Symbiotic state*</th>
<th>Location of treatment</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metamorphosin A</td>
<td>1, 2, 5, 10, 20 mM</td>
<td>14</td>
<td>5</td>
<td>no record</td>
<td>CA</td>
<td>Leitz et al. 1994</td>
</tr>
<tr>
<td>(peptide: DNPGGW)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12-O-tetradecanoyl-</td>
<td>10^-6, 10^-7, 10^-8,</td>
<td>11</td>
<td>3</td>
<td>APO, ZX</td>
<td>OR</td>
<td>Henning et al. 1996, 1998</td>
</tr>
<tr>
<td>phorbol-13-acetate</td>
<td>10^-9, 10^-10, 10^-11, 10^-12, 10^-13, 10^-14, 10^-17 M</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(TPA)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phorbol 12,13-</td>
<td>10^-6, 10^-7, 10^-8,</td>
<td>11</td>
<td>3</td>
<td>APO, ZX</td>
<td>OR</td>
<td>Henning et al. 1996, 1998</td>
</tr>
<tr>
<td>dibutyrate (PDBu)</td>
<td>10^-9, 10^-10, 10^-11, 10^-12, 10^-13, 10^-15, 10^-17 M</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>10^-2, 10^-3, 10^-4,</td>
<td>15</td>
<td>15</td>
<td>APO, ZX, ZC</td>
<td>WA</td>
<td>Coon et al. 1990</td>
</tr>
<tr>
<td></td>
<td>10^-5, 10^-6, 10^-7, 10^-8 M</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KCl</td>
<td>10, 20, 30, 40 mM</td>
<td>15</td>
<td>15</td>
<td>APO, ZX, ZC</td>
<td>WA</td>
<td>e.g., Pechenik &amp; Gee 1993; Bryan et al. 1997; Biggers &amp; Lauf 1999</td>
</tr>
<tr>
<td>γ-aminobutyric acid</td>
<td>10^-2, 10^-3, 10^-4,</td>
<td>15</td>
<td>15</td>
<td>APO, ZX, ZC</td>
<td>WA</td>
<td>e.g., Morse 1985; Bryan et al. 1997</td>
</tr>
<tr>
<td>(GABA)</td>
<td>10^-5, 10^-6, 10^-7, 10^-8 M</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3,4-dihydroxy-</td>
<td>10^-5, 10^-6, 10^-7, 10^-8 M</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>e.g., Morse 1985; Bryan et al. 1997</td>
</tr>
<tr>
<td>phenylalanine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(DOPA)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* APO = aposymbiotic, ZX = zooxanthellate, ZC = zoochlorellate.

**Results**

**Spawning and fertilization**

Observations of spawning and fertilization of gametes of *Anthopleura elegansissima* were recorded through the spring and summer of 1995–2001 (Table 3). Spawning and fertilization occurred as early as April 1 in one year to as late as September 4 in another. There was a high variability in both spawning and fertilization success throughout the monitoring period. Between 1995 and 1998, 82% of the attempts to induce spawning were successful (does not include spontaneous spawning). In contrast, from 1999 through 2001, just 69% of attempts yielded gametes, and 4 of these 9 events resulted in release by only one gender, usually males (6 of 7 such instances). Similarly, in 1995–1998, when both sexes spawned (both spontaneous or induced), gametes were successfully fertilized 100% of the time. In contrast, gametes generated from 1999–2001 were successfully fertilized in only 20% (1 of 5) of events in which both sexes released gametes.

**Infection of *A. elegansissima* larvae with zooxanthellae and zoochlorellae**

In 1999 in Oregon and in 2000 in Washington, we examined the dynamics of infection by zooxanthellae and zoochlorellae in larvae of *A. elegansissima*, in numerous trials using larvae of different ages and from different parents. When unquantified algae were provided at saturating concentrations, 60–100% of larvae acquired algae on day 0 (selected data shown in Fig. 1). In most experiments, the percentage of infected larvae decreased over time in both zooxanthellate and zoochlorellate populations to as low as 25% by 14–16 days post-infection. In all infection experiments, zoochlorellae infected a higher percentage of larvae (usually >90%) on day 0 than did zooxanthellae (60–90%).

Algal infection was strongly influenced by different light regimes (Fig. 1). Zoochlorellae appear to persist in larvae incubated in relatively low-light conditions and zooxanthellae persist in those incubated in relatively high-light conditions. In a low-light environment of 40 μmol quanta/m²/sec (OR, June 1998), lar-
Table 3. Spawning and fertilization of Anthopleura elegantissima. Gametes from adults maintained in outdoor seawater tanks.

<table>
<thead>
<tr>
<th>Date of attempt</th>
<th>Location</th>
<th>Induced or spontaneous</th>
<th>Spawning Males</th>
<th>Spawning Females</th>
<th>Fertilization</th>
</tr>
</thead>
<tbody>
<tr>
<td>1995 June 21</td>
<td>CA</td>
<td>Induced</td>
<td>Y</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>1995 July 3</td>
<td>CA</td>
<td>Spontaneous</td>
<td>Y</td>
<td>Y</td>
<td>Yes</td>
</tr>
<tr>
<td>1995 July 18</td>
<td>CA</td>
<td>Spontaneous</td>
<td>Y</td>
<td>Y</td>
<td>Yes</td>
</tr>
<tr>
<td>1995 August 24</td>
<td>CA</td>
<td>Induced</td>
<td>N</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>1995 August 27</td>
<td>CA</td>
<td>Induced</td>
<td>N</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>1996 April 1</td>
<td>CA</td>
<td>Spontaneous</td>
<td>Y</td>
<td>Y</td>
<td>Yes</td>
</tr>
<tr>
<td>1996 April 2</td>
<td>CA</td>
<td>Spontaneous</td>
<td>Y</td>
<td>Y</td>
<td>Yes</td>
</tr>
<tr>
<td>1996 June 24</td>
<td>CA</td>
<td>Induced</td>
<td>Y</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>1996 July 2</td>
<td>CA</td>
<td>Spontaneous</td>
<td>Y</td>
<td>Y</td>
<td>Yes</td>
</tr>
<tr>
<td>1996 July 22</td>
<td>CA</td>
<td>Induced</td>
<td>Y</td>
<td>Y</td>
<td>Yes</td>
</tr>
<tr>
<td>1997 May 28</td>
<td>OR</td>
<td>Induced</td>
<td>Y</td>
<td>Y</td>
<td>Yes</td>
</tr>
<tr>
<td>1997 September 4</td>
<td>OR</td>
<td>Induced</td>
<td>Y</td>
<td>Y</td>
<td>Yes</td>
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<tr>
<td>1998 June 5</td>
<td>OR</td>
<td>Induced</td>
<td>Y</td>
<td>Y</td>
<td>Yes</td>
</tr>
<tr>
<td>1998 June 24</td>
<td>OR</td>
<td>Induced</td>
<td>Y</td>
<td>N</td>
<td></td>
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<tr>
<td>1998 July 7</td>
<td>OR</td>
<td>Induced</td>
<td>Y</td>
<td>Y</td>
<td>Yes</td>
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<tr>
<td>1998 August 12</td>
<td>OR</td>
<td>Induced</td>
<td>Y</td>
<td>Y</td>
<td>Yes</td>
</tr>
<tr>
<td>1999 June 17</td>
<td>OR</td>
<td>Induced</td>
<td>N</td>
<td>N</td>
<td></td>
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<tr>
<td>1999 June 24</td>
<td>OR</td>
<td>Induced</td>
<td>Y</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>1999 August 3</td>
<td>OR</td>
<td>Induced</td>
<td>Y</td>
<td>Y</td>
<td>No</td>
</tr>
<tr>
<td>2000 August 20</td>
<td>OR</td>
<td>Induced</td>
<td>Y</td>
<td>Y</td>
<td>No</td>
</tr>
<tr>
<td>2001 August 3</td>
<td>OR</td>
<td>Induced</td>
<td>Y</td>
<td>Y</td>
<td>No</td>
</tr>
</tbody>
</table>

For some infection experiments, the density of algae per larva was monitored through time, in addition to infection percentage. Although the average densities varied between experiments, the density pattern was always the same and is illustrated (Fig. 2) for one experiment (OR, August 1998; same larvae as in Fig. 1B). The average number of algae per larva immediately after feeding was highly variable for both zoochlorellae and zooxanthellae, but zoochlorellae were always present at a higher density than were zooxanthellae. For example, for the experiment shown in Fig. 2, the average number of zoochlorellae per larva on day 0 was 70 (40% of larvae contained over 100 algal cells), significantly higher than the average of just 20 algae per larva for zooxanthellate larvae (Tukey, p < .001; Fig. 2A,B). Average densities of both algal types decreased dramatically starting 1 day after infection, such that by day 3, most larvae contained <10 algal cells per larva regardless of algal type (significantly lower than day 0 for both algal types, Tukey, p < .001; Fig. 2A,C). Algal numbers continued to decrease gradually (but not significantly) throughout the 14-day experiment to values of 1 or 2 algal cells per larva for both algal types (Fig. 2A).
Infection of larvae of *Anthopleura elegantissima* with zooxanthellae or zoochlorellae in 3 different larval cohorts exposed to different light regimes. Larvae from: (A) Oregon in June 1998; (B) Oregon in August 1998; and (C) Washington in July 2000. Percentage of infection decreases through time regardless of symbiont type; however, zoochlorellae persist in larvae in low light and zooxanthellae persist in larvae in high light. Larvae were 4 days old when infected with algal isolates. Each point represents counts of 25–30 larvae.

**A. artemisia** larvae and infection with zooxanthellae and zoochlorellae

Larval development in *A. artemisia* was essentially identical to that of *A. elegantissima*. Within one day of fertilization, barrel-shaped planulae, with an apical

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**Fig. 1.** Infection of larvae of *Anthopleura elegantissima* with zooxanthellae or zoochlorellae in 3 different larval cohorts exposed to different light regimes. Larvae from: (A) Oregon in June 1998; (B) Oregon in August 1998; and (C) Washington in July 2000. Percentage of infection decreases through time regardless of symbiont type; however, zoochlorellae persist in larvae in low light and zooxanthellae persist in larvae in high light. Larvae were 4 days old when infected with algal isolates. Each point represents counts of 25–30 larvae.

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Larval biology of *Anthopleura elegantissima*

Fig. 3. Larvae of *Anthopleura elegantissima* displaying a lobed morphology. This morphology was observed in only a few groups of larvae, 2–3 weeks old, and occurred regardless of symbiotic state (see text for details). (A) Planula (aposymbiotic) beginning to change shape, still barrel-shaped but small bulges at oral end around mouth are evident. (B) Larva (zoochlorellate) with more distinct oral bulges and a shortened oral-aboral axis, causing the larva to take on a rounded appearance. (C) Larva (aposymbiotic) with fully-developed lobed morphology, with highly pronounced bulges around the mouth. Open block arrows in all three panels indicate bulges emerging from oral end around mouth. Apical tuft (AT) is evident in larvae in A and C. ZC denotes zoochlorellae. Scale bars, 50 μm.

tuft like that of *A. elegantissima*, were actively swimming in the water column. By day 4, larvae had developed a mouth. In order to determine if this non-symbiotic species was capable of becoming infected in the larval stage with zooxanthellae and/or zoochlorellae, we conducted infection experiments like those described for *A. elegantissima*, providing saturating concentrations of zooxanthellae or zoochlorellae isolated from adults of *A. elegantissima*. We monitored the larvae on days 0, 2, and 4 only (n = 25 larvae counted daily for each algal type). On day 0, 64% of larvae incubated with zooxanthellae acquired algae whereas only 20% incubated with zoochlorellae acquired algae. By day 2, no larvae incubated with zooxanthellae contained algal cells and only 12% of those exposed to zoochlorellae still contained any algal cells. By day 4, larvae containing zoochlorellae decreased again to 4%. Regardless of algal type or day, the number of algal cells per larva always remained <10 (data not shown). We did not determine whether algal cells were phagocytized by gastrodermal cells or merely remained inside the gastric cavity without entering host cells.

**Experiments using substrates and compounds to induce settlement and metamorphosis in *A. elegantissima* larvae**

No definitive settlement or metamorphosis was observed by larvae placed in any of the treatments tried (Tables 1, 2). Some larvae placed in containers with mussels and intertidal rocks (Table 1) did display some early signs of settlement behavior. Larvae were seen attaching to rocks and shells. They would remain for minutes to hours but would be missing later when checked again. Larvae placed with these natural substrates were extremely difficult to follow, because of their small size (<200 μm) and the complexity of the substrate. In all cases, within 1 to 2 days of placing the larvae in these containers, they disappeared.

Larvae placed with artificial substrates (Table 1) or in metamorphic inducers (Table 2) never displayed any settlement behavior and remained actively swimming in the water column for the duration of the exposures.

**Changes in morphology in older larvae**

Larvae of *A. elegantissima* from three different spawns (CA, April 1996; OR, September 1997; and OR, June 1998) displayed marked changes in morphology at 2–3 weeks of age. Multiple bulges were observed emerging from the oral end (Fig. 3A,B). Over time, these bulges became pronounced and took on the look of developing tentacles (Fig. 3C). At the same time, in many larvae, the oral-aboral axis became
larvae assumed this morphology. However not all lar-

val groups that reached 2-3 weeks of age underwent a change to this morphology. Some groups persisted as barrel-shaped planulae for the duration of their lives (−1 month).

Discussion

Spawning and fertilization

Spawning in Anthopleura elegantissima was ob-

This study demonstrates the ability of larvae of A. elegantissima to acquire zoochlorellae, freshly isolated from an adult, during feeding, in the same manner as larvae acquire zooxanthellae (Schwarz et al. 2002). The proportion of larvae infected with either algal type declined over time in most experiments (Fig. 1). No comparable data have been reported for other symbiotic cnidarian planulae. These declines in percent infection may be due to differential mortality of symbiotic compared to non-symbiotic larvae or, alternatively, to expulsion or digestion of algae in some symbiotic larvae over time.

Zooxanthellate larval cultures remained relatively stable in high-light treatments compared to zoochlorellate cultures, which were stable in low-light treatments (Fig. 1). These data are consistent with the variety of studies that have shown differential habitat distribution of A. elegantissima and A. xanthogrammica harboring the two algal types (Bates 2000; Secord & Augustine 2000) and differential tolerance of the two algal types to temperature and light in adults of A. elegantissima (Verde & McCloskey 1996a,b, 2001, in press; Saunders & Muller-Parker 1997). Zooxanthellae have been shown to out-perform zoochlorellae, as measured by growth rates and photosynthetic productivity, in warmer, higher-light environments, and have been hypothesized to outcompete zoochlorellae in anemones found in these habitats. The opposite has been found in anemones in cooler, lower-light environments where zoochlorellae perform better than zooxanthellae. Our study suggests that this differential environmental tol-
erance of the symbionts may be manifest as early as the larval stage of these anemones.

The temporal profiles of algal density in *A. elegantissima* larvae were very similar between algal types and between different experiments. The high numbers of algae in larvae at day 0 are likely a count of algae in the gastric cavity, taken in during feeding and not phagocytized by the cells of the larval gastroderm (Fig. 2). The much lower numbers of algae on subsequent days likely represent algae that have been phagocytized by gastrodermal cells. This interpretation is supported by previous work in which transmission electron micrographs of *A. elegantissima* larvae 4 days after infection showed zooxanthellae only in gastrodermal cells and none in the gastric cavity, although it is possible that algal cells remaining in the gastric cavity could be washed away during fixation (Schwarz et al. 2002). Zoochlorellae average 9.6 µm in diameter compared to an average of 12.5 µm for zooxanthellae in *A. elegantissima* (Verde & McCloskey 1996b). Therefore, the significantly higher densities of zoochlorellae compared to zooxanthellae in larvae at day 0 could be due to the gastric cavity being able to accommodate a greater number of smaller zoochlorellae than larger zooxanthellae. By several days after infection, algal density stabilized at ~1–2 algal cells per larva (Fig. 2) and was consistent between experiments and across all light regimes (data not shown). In contrast, when experimentally infected with zooxanthellae, larvae of the scleractinian coral *Fungia scutaria*, larvae of the anemone *Anthopleura elegantissima*, harbored an average of 20–23 algal cells per larva (Weis et al. 2001). Whether this discrepancy in algal density between *F. scutaria* and *A. elegantissima* is a real biological difference or reflects sub-optimal culturing conditions of *A. elegantissima* is not known. Veggs (1988) did not quantify algal density in experimentally infected larvae of *Aiptasia tagetes*. To our knowledge, no other infection studies have been performed on larvae from any other species of cnidarian.

There is no information on the mode of acquisition of symbionts by larvae of *A. elegantissima* or any other cnidian, in the field, therefore it is difficult to know how relevant the artificial conditions created in this study are to the biology of symbiosis onset. In the laboratory, larvae of *A. elegantissima* can acquire zooxanthellae from "black egesta," which contain high concentrations of zooxanthellae, released from adult anemones (Schwarz et al. 2002). Another possible source of high quantities of algae is the feces of the nudibranch *Aeolidia papillosa*, which feeds on adults of *A. elegantissima*, passes the symbionts unharmed through its digestive tract, and releases them in very high concentrations in the fecal pellets (Seavy & Muller-Parker 2002). If either of these sources of algal cells are used by larvae, the high concentrations of cells used in this study could be mimicking these infection modes.

**Larvae of *A. artemisia***

We witnessed a mass spawning of *A. artemisia* during a low tide in July of 1998 at Strawberry Hill and Neptune Beach, Oregon. Virtually all observed individuals were spawning at both locations we visited. Similar events of single species spawning have been observed for *A. elegantissima* (E. Sanford, Stanford University, pers. comm.) and for *A. xanthogrammica* (J. Schwarz, unpubl. obs.).

*A. artemisia*, a nonsymbiotic anemone species (Hand 1955; Pearce & Francis 2000) residing in a separate west Pacific clade from *A. elegantissima*, *A. sola*, and *A. xanthogrammica* (Geller & Walton 2001), did acquire zooxanthellae and zoochlorellae, but algal cells occurred at low densities and at very low percentages. We did not determine if any algal cells entered host gastrodermal cells or if they remained in the gastric cavity. The initial numbers and densities of algae in the larvae at day 0 were much lower than those of *A. elegantissima*. We have hypothesized that algae are initially taken into the gastric cavity in *A. elegantissima* and the coral *F. scutaria* as a result of a nonspecific feeding response (Schwarz et al. 1999, 2002). If this were true, then one would predict that larvae of *A. artemisia* would respond in the same way to algae in the presence of food; initially they would acquire large numbers of algal cells that would later be either expelled or digested. Instead, far fewer larvae of *A. artemisia* took up algae and far fewer algal cells were acquired per larva than in *A. elegantissima*. This suggests that some specificity is involved in the early stages of symbiont-host contact in *A. elegantissima*. Alternatively, reduced uptake rates in *A. artemisia* could be explained by the use of freshly isolated algae that had been passed through a 50-µm mesh screen, a technique that sometimes reduced infection rates in *A. elegantissima* larvae (see Methods).

**Attempts to induce settlement and metamorphosis in *A. elegantissima***

There is a rich literature on inducers of marine invertebrate larval settlement (see Table 2 for examples of references). Much is understood in some species of invertebrates, including some cnidarians, about the cues and signals, both natural and artificial, that induce settlement. Borrowing from these studies, we tried to induce larvae of *A. elegantissima* to settle using numerous substrates and chemicals, and Tables 1 and 2
document our failed attempts. Other researchers have attempted settlement studies on *A. elegantissima*, using various natural substrates and larvae of approximately the same age, also without success (Siebert 1974; Smith 1986). Settlement is a complex signaling process that can vary dramatically between groups of invertebrates (Hofmann et al. 1996). It is possible that we did not try the right concentration, duration, or combination of cues that the larvae need to initiate settlement. Furthermore we did not consider environmental parameters, such as light, temperature and even emersion that could be required for settlement. Another possible explanation for the inability to induce settlement is that the larvae used in the studies were too young and not yet competent to settle. It has been hypothesized that planulae of *A. elegantissima* are long-lived planktotrophic larvae capable of long-distance dispersal (Siebens 1981a,b, 1982). Larvae have been kept alive in culture for as long as 3 months (Smith 1986), in our hands, ~6 weeks. To date we have been unable to keep large cultures healthy and axenic for long enough to attempt settlement experiments on larval older than 3 weeks.

**Changes in morphology of older *A. elegantissima* larvae**

Some, but not all, groups of larvae over 2 weeks of age, developed a morphology that consisted of several large protuberances situated around the mouth (Fig. 3). Although these structures resemble tentacles in their location and shape, histological studies are needed to confirm the presence of two cell layers and the gastric cavity. These structures were much larger than the “collar” described by Siebert (1974) and observed in our cultures, which consists of a small circular ridge surrounding the mouth of larvae of all ages. Smith (1986) does not report any comparable morphological changes in his study of larval of *A. elegantissima*, and to our knowledge, no similar accounts of this multilobed morphology have been described in other cnidarian planulae. We initially hypothesized that the appearance of the morphology signaled competence to settle; however larvae placed in bowls with natural substrates and the phorbol esters TPA and PDBu (see Table 2) did not settle.

**Conclusions**

Our ongoing struggles—to obtain *A. elegantissima* planulae predictably, to infect larvae with a quantified number of algal cells, and to induce larvae to settle—illustrate just some of the difficulties encountered in examining the early life history stages of cnidarians and the onset of their symbioses with algae. This explains in part why progress in this area of cnidarian biology and symbiosis lags behind studies on adults. Despite the limitations we have encountered during the course of our 7 years of work on *A. elegantissima*, this study produced some interesting insights into dynamics of infection by both zooxanthellae and zoochlorellae, and changes in morphology of older planulae.

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