

INTERACTIONS BETWEEN THE NUDIBRANCH *OKENIA ZOOPOTRYON*  
AND ITS BRYOZOAN PREY *ZOOPOTRYON VERTICILLATUM*

by  
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## ABSTRACT

In the Indian River Lagoon, the nudibranch *Okenia zoobotryon* and its egg masses are found attached to the bryozoan *Zoobotryon verticillatum* throughout the year. *Okenia zoobotryon* is thought to live, feed, and reproduce exclusively on *Z. verticillatum*, which would make this a very specialized predator-prey interaction. The primary goal of my study was to document certain aspects of the ecological and chemical relationships between *Z. verticillatum* and *O. zoobotryon*. Specifically, I wanted to understand the cues used by the nudibranch to locate and remain on *Z. verticillatum*. Population surveys on *Z. verticillatum*, as well as other potential hosts, were performed. From these surveys, I found a small number of *O. zoobotryon* adults and egg masses on the red macroalga *S. filamentosa* mixed with *Z. verticillatum* (0.01 and 0.05 g/wet-weight, respectively), none on the bryozoan *Amathia distans*, and none on the red macroalga *Gracilaria tikvahiae*. To determine if prey identification was associated with an adult cue, I ran paired-choice trials. In these, the adults did not significantly prefer *Z. verticillatum*. Next, laboratory culture of larvae of *O. zoobotryon* was performed. During larval culture some aspects of this previously undocumented life-history were recorded; adults of this species developed from planktotrophic veliger larvae that hatched out of egg masses between 4 and 6 days, the time post-hatching to settlement was 7-8 days, and metamorphosis occurred approximately 24 hours after settlement. The final question addressed in this study was; “Does *O. zoobotryon* feed on and take up chemicals from *Z. verticillatum*?” To address this question, high-performance liquid chromatography was performed on extracts of *Z. verticillatum* and *O. zoobotryon*. Both organisms were compared and found to contain similar compounds, which suggest that the nudibranch is feeding on *Z. verticillatum* and taking up chemicals.

Concentrations of compounds in *Z. verticillatum* varied between populations in the northern and southern regions of the Indian River Lagoon. There are, also, differences between the chemical composition of *Z. verticillatum* in the IRL (northern and southern) and the California species identified by Sato and Fenical (1983).

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## INTRODUCTION

Chemical signals are important to the biology and ecology of organisms in the marine environment (Zimmer-Faust *et al.* 1995; Zimmer and Butman 2000; Stachowicz 2001). Sensory perception of chemical signals by marine organisms can influence predation, competition, and larval settlement and metamorphosis (Zimmer-Faust 1989; Leonard *et al.* 1999; Hadfield and Paul 2001). Some predators use chemical cues to switch among prey items to optimize feeding and thus, fitness and reproduction (Levinton 1995; Todd *et al.* 2001; Trowbridge and Todd 2001). However, other predator-prey relationships are very specific. In specialist predator-prey relationships, a predator consumes only one prey species (e.g. Todd *et al.* 2001). Of the documented specialist relationships in the marine environment, only a limited number involve two invertebrates; most of these relationships involve an opisthobranch mollusk as the predator (Harvell 1984; Hadfield and Scheuer 1985; Lambert and Todd 1994; Avila *et al.* 1999; Todd *et al.* 2001). One such example is the relationship between the dendronotid nudibranch *Marioniopsis fulvicola* and its prey, the soft coral *Parerythropodium fulvum fulvum* (Avila *et al.* 1999). In this relationship, the nudibranch matches the color of its prey and its morphology is similar to that of the coral polyps (Avila *et al.* 1999). The relationship between the dorid nudibranch *Adalaria proxima* and the cheilostome bryozoan *Electra pilosa* is another specialist relationship between two invertebrates (Lambert and Todd 1994). *Adalaria proxima* completes its entire life cycle on a colony of *E. pilosa* (Lambert *et al.* 1997).

In the marine environment, larvae may preferentially settle either gregariously (near conspecifics) or associatively (on a specific organism) (reviews by Chia and Rice 1978; Pawlik 1992; Rodríguez *et al.* 1993). For example, the inductive cues for metamorphosis in sipunculid

larvae were found to be from chemicals released by conspecific adults (Rice 1986). The same is true for some polychaete species (Jensen and Morse 1984; Pawlik 1986), oysters (Crisp 1967), and ascidians (Svane *et al.* 1987). In opisthobranch larvae, these cues are generally associated with adult prey, including bryozoans (Lambert and Todd 1994), sponges (Chia and Koss 1978), and barnacles (Todd 1979). In the study by Lambert and Todd (1994), the inductive cue for settlement of larvae of the nudibranch *Adalaria proxima* was found to be a water-borne chemical cue released from its adult prey, the bryozoan *Electra pilosa*. There have also been many other studies on marine gastropods, especially nudibranchs, which have shown larvae to be attracted to chemical cues from the prey (Bahamondes-Rojas 1988; Chia and Koss 1998; Hadfield 1977; Krug and Manzi 1999; Lambert *et al.* 1994, 1997; Todd *et al.* 1991).

My research examined the potential specialist relationship between the dorid nudibranch *Okenia zoobotryon* and its prey, the bryozoan *Zoobotryon verticillatum*. Specifically, I examined: 1) the distribution and abundance of adult *O. zoobotryon* and its egg masses on *Z. verticillatum*, 2) *O. zoobotryon* preferences for *Z. verticillatum*, 3) the larval cycle of *O. zoobotryon*, 4) settlement and metamorphosis of *O. zoobotryon*, and 5) sequestering of chemicals from *Z. verticillatum* by *O. zoobotryon*.

### ***Biology of Nudibranchs***

Organisms in the phylum Mollusca are a distinctive group without any close resemblance to any other living group (Ruppert and Barnes 1994). There are over 50,000 living species described and nearly that number that are extinct (Ruppert and Barnes 1994). The class Gastropoda is the largest in the phylum (Ruppert and Barnes 1994). Individuals in the superorder Opisthobranchia (nudibranchs) have a shell and mantle cavity that are either reduced or

completely absent (Ruppert and Barnes 1994). The body surface in many nudibranchs is increased by numerous projections called cerata (Ruppert and Barnes 1994). These cerata may be used for defense against predators (Ruppert and Barnes 1994). Aeolid nudibranchs, specialists on cnidarians, can uptake the nematocysts and transfer them to the cerata to be utilized for defense (Rudman 1981, 1991; Todd 1981; Slattery *et al.* 1998). In other nudibranchs, noxious chemicals can be sequestered from the prey and distributed throughout their bodies to deter predators (Thompson *et al.* 1982; Faulkner and Ghiselin 1983; Faulkner 1984; Paul and VanAlstyne 1988a; McPhail *et al.* 1998). This uptake of chemicals from prey may be correlated with shell loss in nudibranchs (Faulkner and Ghiselin 1983; Paul and Van Alstyne 1988).

Gastropods exhibit just about every type of feeding, including herbivory, carnivory, scavenging, deposit feeding, suspension feeding, and parasitism (Ruppert and Barnes 1994). Nudibranchs feed on sessile organisms and each family is usually restricted to one type of prey (Ruppert and Barnes 1994). The feeding methods of nudibranchs are specialized for the prey item being consumed (Purchon 1977). For example, the dorid nudibranch *Jorunna tomentosa* feeds on sponges (Purchon 1977). The outer lips of its mouth are pressed downwards against the sponge, and the radula (composed of transverse rows of teeth) is moved forwards and upwards to tear off sponge fragments that are carried on the teeth to the mouth (Purchon 1977). In contrast, the dorid nudibranch *Adalaria proxima* feeds on three different cheilostome bryozoans (Purchon 1977). The nudibranch breaks open the exoskeleton of the bryozoan, sucks out the soft parts, and then scrapes out the remaining tissue (Thompson 1958; Purchon 1977).

Most nudibranchs are hermaphroditic with internal fertilization (Strathmann 1987). Eggs are encapsulated and are deposited in many different forms of gelatinous masses on various

substrata (Strathmann 1987). The morphology of the egg mass is indicative of each species (Strathmann 1987). Strathmann (1987) describes Type A egg masses as being flat, gelatinous ribbons attached along one edge to the substratum; these are commonly produced by dorid nudibranchs. Type B egg masses are gelatinous cords attached by a gelatinous sheet along one side to the substratum. Type B egg masses are commonly produced by ascoglossans and dendronotid and aeolid nudibranchs (Strathmann 1987). Finally, Type C egg masses are common in cephalaspideans and are gelatinous globules with an elongated end that is anchored into the sand or mud (Strathmann 1987). Most opisthobranchs hatch from egg masses as planktotrophic or lecithotrophic veliger larvae that have a velum, foot, and a shell (Strathmann 1987).

My research focused on *O. zoobotryon*, a dorid nudibranch (order Doridacea). Dorid nudibranchs are so named because their cerata are arranged in a circular pattern on the dorsal end of the organism. Other than taxonomic identification by Valdes and Ortea (1995), no other reports have been published about this genus. Hence, the life cycle of *O. zoobotryon* is not known and larval culture of this species was critical to this study. Lambert and Todd (1994) stated, “The larval biology of nudibranch molluscs has been of increasing interest over the past two decades, but experimental data are presently available for too few species to permit generalizations about larval behavior within the order Nudibranchia”. Results from the larval portion of my study will provide further information on the biology of general nudibranch larvae. I also examined if adult or larvae of *O. zoobotryon* actively selected the bryozoan *Z. verticillatum*.

### ***Biology of Bryozoans and Zoobotryon verticillatum***

The phylum Bryozoa is a widely distributed taxon of sessile organisms in marine and freshwater environments (Christophersen 1985). Bryozoans are sometimes referred to as “moss animals” because they resemble moss-like plants. They are sessile, modular, colonial animals with each colony consisting of a few to millions of individuals or zooids (Barnes *et al.* 1993). Interconnected zooids are polymorphic with each zooid serving a different function (Barnes *et al.* 1993). A single colony is comprised of feeding and non-feeding zooids. Feeding zooids are called autozooids, while non-feeding zooids are collectively called heterozooids that can perform various functions (Ryland 1970). For example, kenozooids make up the stolon from which budding and colony growth takes place (Ryland 1970). Another example is the defensive zooids called avicularia (Ryland 1970). The avicularia are comprised of a “mandible” that can be opened and closed like a jaw when a predator comes in contact with the zooid (Ryland 1970).

*Zoobotryon verticillatum* (class Gymnolaemata), is a stoloniferous bryozoan that is widely distributed in warm temperate and tropical waters in the western Atlantic and the Caribbean (Winston 1995). *Zoobotryon verticillatum* is in the order Ctenostomata, whose members have non-calcified walls and lack opercula (Barnes *et al.* 1993). It is a nuisance species in the solar salt-fields of Australia, growing from early October to mid-June and then dying off for three months each year during winter (Coleman 1999).

Winston (1995) found *Zoobotryon verticillatum* to be the bryozoan with the greatest biomass of any bryozoan in the Indian River Lagoon system (IRL) (Winston 1995). Because of the large biomass, Winston (1995) suggested the species might be an important contributor to the health of the IRL by providing shelter for other species, and having a successful filter-feeding

strategy. Throughout the IRL, *Z. verticillatum* can be found attached to natural and artificial hard surfaces. Settlement trials run from May 2000 to May 2001 showed *Z. verticillatum* settlement to be highly variable in Mosquito Lagoon (L. Walters, unpublished data) (Figure 1). Peaks of the bryozoan were documented May through July, and then tapering off through August with another peak from September to November (L. Walters, unpublished data). From November to May, no settlement was observed (L. Walters, unpublished data). Variable-sized *Z. verticillatum* colonies have been observed drifting, suggesting that fragments are regularly created (pers. obs.). Because *Z. verticillatum* may be an important contributor to the overall health of the Lagoon, it is important to understand the biology of this species in the Lagoon and its association with *O. zoobotryon*.

*Zoobotryon verticillatum* colonies are hermaphroditic and reproduction can either be sexual (through the development of larvae) or asexual (through budding or possibly fragmentation). Asexual reproduction in *Z. verticillatum* is important to the growth of the colony. Sexually produced larvae of *Z. verticillatum* settle on hard substrates (Bullivant 1968). The newly settled larvae of *Z. verticillatum* produce a stolon that forms at its tip an adhesive disc to the substrate (Bullivant 1968; Zimmer and Woollacott 1977). Repeated asexual budding occurs from the stolon to form the colony (Bullivant 1968; Zimmer and Woollacott 1977). Zirpolo (1924) cut and cultured pieces of the stolon and found that one end of the stolon formed attachment structures to anchor the fragment to the substrate (Geiger and Zimmer 2002).

Vegetative fragmentation is a common process in many marine organisms. For example, there are reports of fragmentation in macroalgae (Kilar and McLachlan 1986; Walters *et al.* 2002; Herren *et al. in press*), sponges (Wulff 1991; Wilkinson and Thompson 1997), corals

## Settlement of *Zoobotryon verticillatum* in Mosquito Lagoon

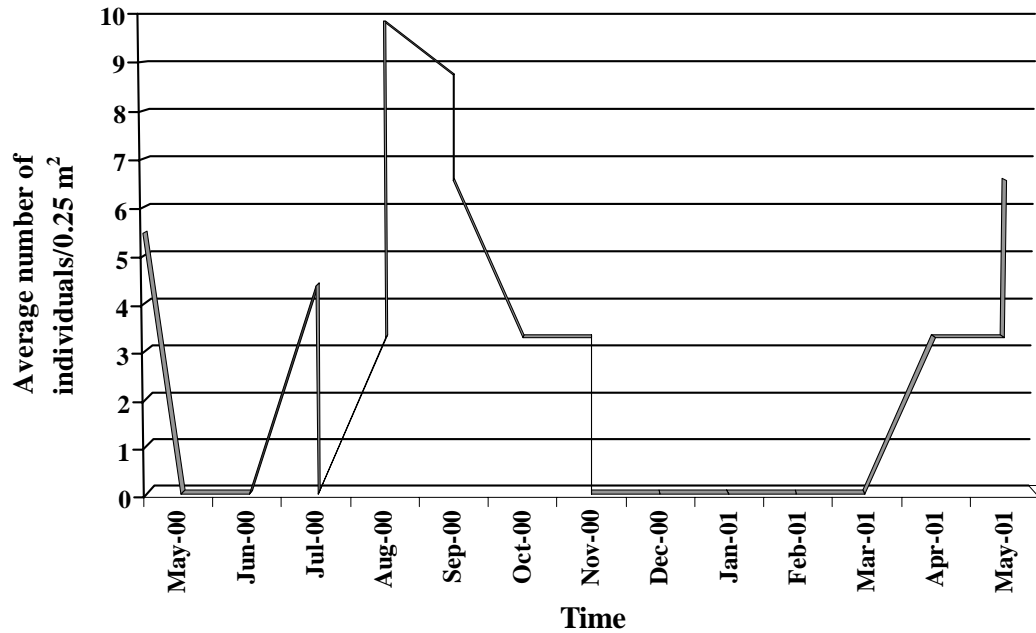


Figure 1: Settlement of *Zoobotryon verticillatum* in Mosquito Lagoon. The graph represents the period of May 2000 through May 2001 (L. Walters, unpublished data). Clean, roughened, plexiglass plates were suspended in the Lagoon for 2 weeks. Numbers of settled organisms were recorded (L. Walters, unpublished data).

(Highsmith 1982; Coffroth and Lasker 1998; Smith and Hughes 1999; Lirman 2000), ascidians (Stoner 1989), zoanthids (Acosta *et al.* 2001), hydrozoans (Lewis 1991), and other bryozoans (Zirpolo 1924). Fragments may be produced by physical disturbance events, predation, water motion, or as part of the organism's life-history strategy (Cook 1979; Highsmith *et al.* 1980; Highsmith 1982; Fong and Lirman 1995; Kilar and McLachlan 1986; Žuljevic *et al.* 2001; Walters *et al.* 2002). In this study I asked: 1) what is the probability that small fragments of *Z. verticillatum* will attach to hard surfaces after being dislodged from the parent colony, 2) is attachment success dependent on time, and 3) is attachment success dependent on the size of the fragment?

*Zoobotryon verticillatum* contains bromo-alkaloids, specifically 2,5,6-tribromo-*N*-methylgramine (Sato and Fenical 1983; Ortega *et al.* 1993; Kon-ya *et al.* 1994). This chemical is an antifouling substance (Kon-ya *et al.* 1994). In that study, crude extracts of 2,5,6-tribromo-1-methylgramine inhibited settlement of barnacle and mussel larvae. The chemical has been reported to inhibit cell division in fertilized sea urchin eggs (Christophersen 1985). The bromo-alkaloid compounds did not appear to deter *O. zoobotryon* from inhabiting colonies of *Z. verticillatum*. In the IRL adults and egg masses of *O. zoobotryon* were commonly found on colonies of *Z. verticillatum* (pers. obs.). In my study, I examined the possibility that *O. zoobotryon* was feeding on and taking up compounds from *Z. verticillatum*. High-performance liquid chromatography (HPLC) tracings from both organisms were compared for similar compounds.



## METHODS

### *Study sites*

The Indian River Lagoon (IRL) system runs along one-third of central Florida's east coast (Swain *et al.* 1995). This system encompasses three interconnected lagoons: the Indian River Lagoon, the Banana River, and Mosquito Lagoon. There are five ocean inlets along this stretch and more than 10 freshwater tributaries and major canals (Morris *et al.* 1999). Variations in temperature and salinity are found throughout the year (Kennedy Space Center 1997). The Florida Department of Environmental Regulation (1989) deemed the Indian River Lagoon System to be an area of high biological diversity. Mosquito Lagoon is a shallow water area in the northernmost part of the Indian River Lagoon System.

My study was performed for three years beginning in May 2000 and continuing through October 2003. Due to the ephemeral nature of the study organisms, field work occurred when sufficient quantities of *Zoobotryon verticillatum* could be found. The majority of field work was performed in Mosquito Lagoon (Figure 2) and the laboratory work was carried out at Fellers House Field Station in Canaveral National Seashore (25° 54'N, 80° 49'W) (Figure 2). Organisms obtained from Mosquito Lagoon were collected from two sites: Site 1 was in open water adjacent to the field station dock and Site 2 was in a small, protected bay located within walking distance from the field station.

Field work was also performed in the lower portion of the IRL in the vicinity of the Fort Pierce Inlet from July through October 2003 at the Smithsonian Marine Station in Fort Pierce (SMS). Large quantities of the study organisms were found in the areas of Jack Island (27° 29'326"N, 80°W) and Little Jim Island (27° 28'578"N, 80°W) (Figure 2).

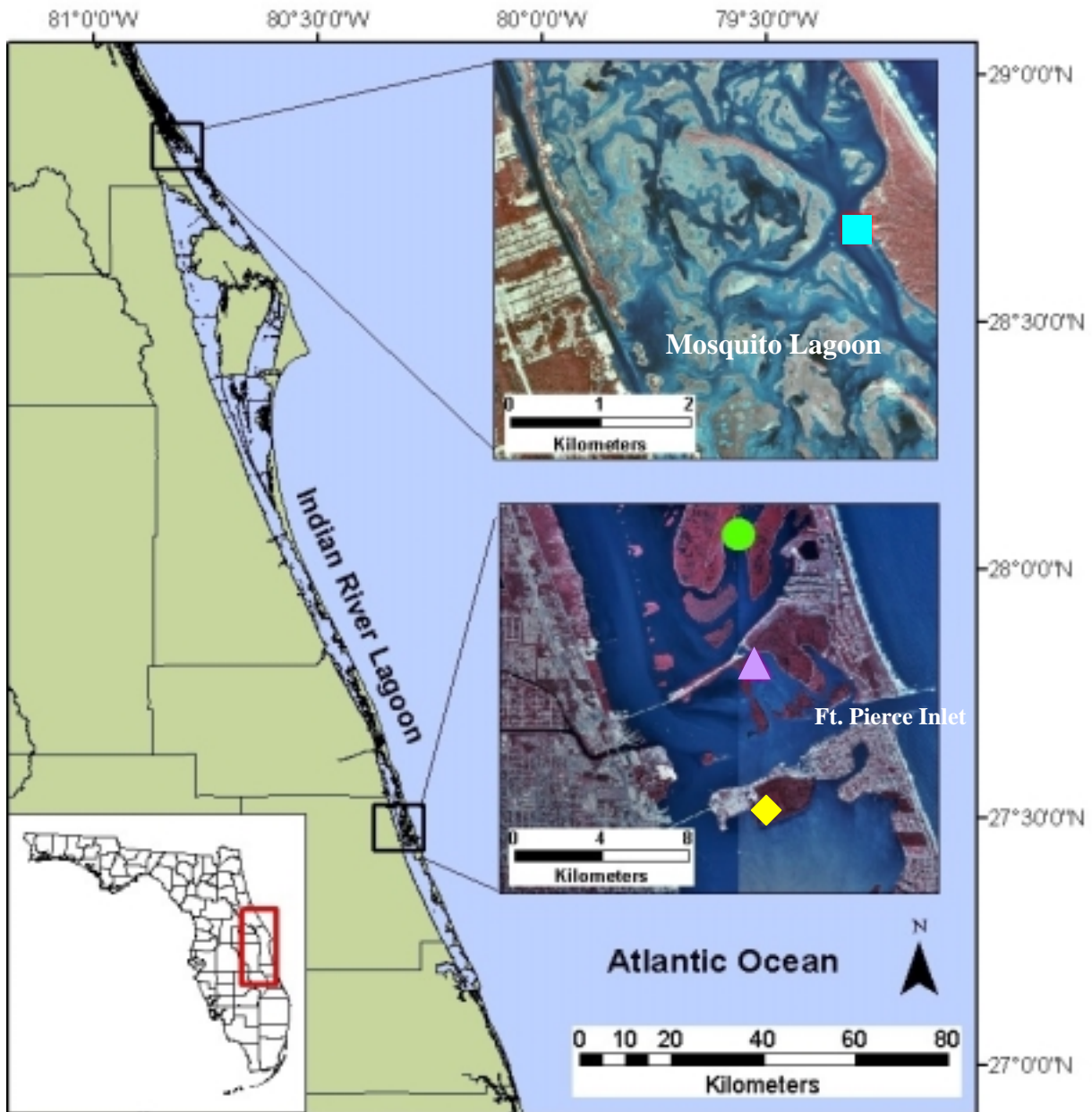


Figure 2: Map of the Indian River Lagoon system and study sites. ■ Fellers House Field Station, Mosquito Lagoon; ● Jack Island, ▲ Little Jim Island, and ◆ Smithsonian Marine Station, Fort Pierce.

### ***Asexual reproduction of Zoobotryon verticillatum via fragmentation***

*Zoobotryon verticillatum* was found at all study sites as unattached variable-sized clumps, suggesting it had undergone fragmentation. Therefore, a series of experiments was run in Mosquito Lagoon to determine if the bryozoan could successfully asexually reproduce via fragmentation. Field collected, attached colonies of *Z. verticillatum* were placed on a wet paper towel, measured, and cut with a razor blade in the laboratory into 10 mm, 20 mm, 30 mm, 40 mm, and 50 mm long fragments (n = 10). Next, the fragments were randomly distributed into compartmentalized plastic boxes (50l x 40w x 40h mm compartments). To ensure water flow, the top and bottom of the boxes were removed and replaced with nylon screen mesh attached with hot-melt glue. Boxes were anchored just below the water's surface with cable-ties to 2 x 2 m PVC frames and left undisturbed for 4, 12, or 24 hours. After the allotted time, fragments were checked and recorded as: 1) attached, 2) not attached, or 3) dead/missing. If no fragment was found in a compartment, the fate of that fragment was unknown and it was recorded as dead/missing. Three trials were performed for each 4, 12, and 24 hour time period. An additional 45 fragments from each size class were blotted dry and wet-weighed on a Mettler Toledo analytical balance (#AB204) to obtain an average weight for each size class. Photographs of the point of attachment were taken under a Leica dissecting microscope with a Pentax 35 mm camera at 40X magnification.

**Data analysis.** Trials were not performed on the same days; thus, the assumptions for parametric statistics (such as an ANOVA) were not fulfilled (Zar 1996). Hence, Fisher's exact tests and Spearman's rho nonparametric statistics were performed on these data. Fragment weights were compared with a one-way ANOVA along with Tukey's *a posteriori* tests (Zar 1996).

***Distribution and abundance of adult Okenia zoobotryon and egg masses of Okenia zoobotryon***

In Mosquito Lagoon, *O. zoobotryon* was observed exclusively associated with *Z. verticillatum*. Therefore, the first step was to document the abundance and sizes of adult *O. zoobotryon* and egg masses found on natural populations of *Z. verticillatum*. The numbers of adult *O. zoobotryon* and its egg masses found on *Z. verticillatum* were recorded weekly when *Z. verticillatum* was abundant for a total of 24 weeks between May 2000 and May 2001. Along with recording actual numbers, adult and egg mass dimensions were recorded.

Six attached colonies of *Z. verticillatum* were randomly collected from the field each week, wet-weighed in the laboratory, and the numbers of adults and egg masses of *O. zoobotryon* were recorded to yield a number per gram wet-weight *Z. verticillatum*. The adults were gently removed from the bryozoan colonies and placed in 1500 ml glass finger bowls with unfiltered seawater to be used in multiple-choice assays. Egg mass lengths were measured with a ruler while attached to *Z. verticillatum*. The egg masses were grouped into three maximum length categories: <10 mm, 10-30 mm, and >30 mm. After removing the adults and counting the egg masses, the *Z. verticillatum* colonies were spun dry using a salad spinner and the wet-weights were recorded.

Adult *O. zoobotryon* dimensions were recorded separately. For this, five *Z. verticillatum* colonies were haphazardly collected and ten *O. zoobotryon* from each *Z. verticillatum* colony were removed, measured with a ruler, blotted dry, and wet-weighed on a Mettler Toledo analytical balance (#AB204).

Because *O. zoobotryon* is considered a specialist predator, it was also important to determine the abundance of *O. zoobotryon* adults and egg masses on other potential hosts in

Mosquito Lagoon. Two red algae, *Spyridia filamentosa* and *Gracilaria tikvahiae*, were found growing in close proximity to *Z. verticillatum* colonies. In fact, *S. filamentosa* was frequently observed growing entangled with *Z. verticillatum* colonies (pers. obs.). Thus, these algae were examined for the presence of adult *O. zoobotryon* and their egg masses. Thirty clumps of each alga, as well as 30 clumps of *S. filamentosa* entangled with *Z. verticillatum*, were haphazardly collected from the field and the numbers of adults and egg masses were recorded in the laboratory. Adults and egg masses found were removed and the algal clumps were wet-weighed as described above. For the mixed clumps of *S. filamentosa* and *Z. verticillatum*, I recorded on which organism the adults and egg masses were found.

In the lower IRL (Fort Pierce area), *O. zoobotryon* adults and egg masses had occasionally been observed on the cheilostome bryozoan *Scrupocellaria regularis*, as well as the ctenostome bryozoan *Amathia distans* (S. Santagata pers. comm.). Hence, it was important to document the abundance of nudibranchs found on these organisms. In the areas of Little Jim and Jack Island, *Amathia distans* and *Scrupocellaria regularis* could be found growing in close proximity to *Z. verticillatum* colonies. Thus, ten *A. distans* colonies were collected and processed in exactly the same manner as *S. filamentosa* and *G. tikvahiae*. Colonies of *S. regularis* were found as small tufts attached to the red alga, *Gracilaria* sp. Intact colonies of *S. regularis* were carefully removed from the alga and processed in the same manner as above. Adults, egg masses, and wet-weights of the *Gracilaria* sp. colonies were also recorded along with the percent cover of *S. regularis*.

### ***Adult Okenia zoobotryon paired-choice preference assays***

Paired-choice assays with adult *O. zoobotryon* were performed to determine if adults were specifically attracted to chemical cues from *Z. verticillatum*. In these assays, one adult *O. zoobotryon* was placed in a random orientation in the center of a 1500 ml glass finger bowl filled with 750 ml of unfiltered seawater. In laboratory trials performed at Fellers House Field Station, the nudibranch was given a choice between similar-sized colonies of *Z. verticillatum* and either the red alga *S. filamentosa* or *G. tikvahiae*, or a *Z. verticillatum* mimic (PVC craft plastic soaked for 24 hours in unfiltered seawater for development of a biofilm). At the end of the 10-minute period, the location of the nudibranch was recorded. Assays were also performed in the same manner to determine if the adults were attracted to conspecific cues from either adults or egg masses. For these assays, the nudibranch was given the choice between two similar-sized colonies of *Z. verticillatum*; one colony had all nudibranchs and egg masses removed while the other colony had either 5-10 adult *O. zoobotryon* or 5-10 egg masses. Chi-square analysis was performed on the 10-minute trials to test for a significant preference of one organism over another.

Similar paired-choice assays were performed at SMS using the bryozoans *Amathia distans* and *Scrupocellaria regularis*. *Amathia distans* and *S. regularis* colonies in the IRL were small compared to *Z. verticillatum* colonies. To treat both organisms equally, assays were performed with colonies displacing a volume of 1 mL for both *Z. verticillatum* and either *A. distans* and *S. regularis*. Observations for these trials were extended to every 10 minutes for one hour. At each 10-minute interval, the position of the nudibranch was recorded.

### ***Larval observations of Okenia zoobotryon***

The life cycle of *O. zoobotryon* may play an important role in the relationship between the nudibranch and its prey. Because the life cycle of this species has not been previously documented, larval culture was conducted in the laboratory and the major stages were recorded. Large individuals of *O. zoobotryon* were collected, brought back to the laboratory, and placed in 1500 ml glass finger bowls along with several colonies of *Z. verticillatum* and unfiltered seawater (salinity = 36 ppt). The individuals were maintained in the laboratory at room temperature (~24 °C) until the production of egg masses was observed. Egg masses laid directly on *Z. verticillatum* were difficult to remove without rupturing and, therefore, were not used. Egg masses laid on the bowl were gently removed and transferred by pipette to watch glasses with 0.22 µm antibiotic filtered seawater (AFSW). The antibiotics Penicillin G (90 µg ml<sup>-1</sup>) and Streptomycin sulfate (75 µg ml<sup>-1</sup>) were used to prevent bacterial growth (Ritson-Williams *et al.* 2003). The water was changed every other day to further reduce the possibility of contamination.

Egg masses were observed daily for ruptures and subsequent swimming larvae. Upon hatching, swimming larvae were transferred with a glass pipette to Nalgene 500 ml bowls and maintained at densities of 10 larvae/1 ml of 0.22 µm AFSW. A few flakes of Cetyl alcohol were added to prevent a surface-air interface in which the larvae could get trapped (Hurst 1967; Chia and Koss 1978). Cultures were fed a monoculture of *Isochrysis galbana* at a density of 10<sup>5</sup> cells/ml. Fifteen larvae were preserved immediately after hatching in 3.7% formalin and measurements were taken under 10X magnification with a Leica DMLB 100S microscope.

Seven separate egg masses were used to determine the number of egg capsules in an egg mass. The egg masses were measured for length and a 3 mm section was removed with a razor

blade from the middle of the egg mass. The 3 mm section was opened using a dissecting pin and the number of egg capsules was counted.

### ***Settlement and metamorphosis of Okenia zoobotryon larvae***

Once larval culture had been completed, larvae of *O. zoobotryon* could be used in trials to determine what, if any, cues stimulated settlement and subsequent metamorphosis. Settlement of larvae in response to *Z. verticillatum*, as well as the bryozoans *A. distans* and *Bowerbankia maxima*, was tested simultaneously. The following procedures are similar to assays used by Ritson-Williams *et al.* (2003). For each assay, ten actively swimming larvae were transferred with a glass pipette into 5 ml wells on Costar® media culture well-plates {Polystyrene (non-Pyrogenic) tissue culture treated}. A 10 mm piece of *Z. verticillatum*, *A. distans*, or *B. maxima* and 5 mL of 0.22  $\mu$ m FSW were added to individual wells with the larvae. A control was also run which consisted of 0.22  $\mu$ m FSW and larvae only. Because the exact number of post-hatching days required for metamorphic competence was not known for this species, metamorphosis was tested on days 6, 7, 8, and 9 post-hatching (400 total larvae). Settlement was determined by non-swimming larvae adhered to the treatment organism until metamorphosis. The number of larvae settled was scored for each treatment. Metamorphosis was determined from settled larvae only upon finding empty shells in the well and subsequently viewing newly metamorphosed individuals on the substrate provided.



### ***Chemistry of Zoobotryon verticillatum and adult Okenia zoobotryon***

To examine whether *O. zoobotryon* was feeding on and taking up chemicals from *Z. verticillatum* chemical studies using HPLC were performed. *Zoobotryon verticillatum* colonies were placed in a graduated cylinder with unfiltered seawater and the volume was measured. The colonies were then spun dry with a salad spinner and wet-weighed. Adult *O. zoobotryon* were collected on *Z. verticillatum*. The nudibranchs were then removed from their prey, blotted dry, and wet-weighed. Because of their small size, nudibranchs were pooled together and separated by site to have sufficient material for chemical analysis. Both the bryozoan colonies and the nudibranchs were freeze-dried in an Econotherm LabConco Freezone 6 freeze drier at -48° C overnight. After freeze-drying, the specimens were weighed again and stored in a freezer until needed.

I attempted to isolate and identify the same compound already isolated and identified by Sato and Fenical (1983). Once I identified this compound in *Z. verticillatum*, I could then repeat the procedure with *O. zoobotryon*. For this, multiple extractions were performed on *Z. verticillatum* colonies collected from Mosquito Lagoon. Freeze-dried colonies of *Z. verticillatum* were soaked in 100% methanol (MeOH) for 48 hours. The MeOH was decanted and replaced after 24 hours. The extracts obtained were pooled together and concentrated by rotary evaporation (Büchi Rotavapor R-200) and speed vacuuming (Thermo Savant SPD 121P). The extract was then weighed in a glass scintillation vial on an analytical balance to obtain the maximum crude extract yield (recorded as grams of extract per dry weight). The crude extract was next fractionated by silica gel column chromatography (Varian, Mega Bond Elut SI). For this procedure, the crude extract was dissolved in MeOH, applied to the column and eluted with

200 ml of each solvent mixture using a solvent scheme of 1:0, 9:1, 1:19, 2:1, 1:1, and 0:1 ethyl acetate (EtOAc) to MeOH to yield seven crude fractions. Solvents were then removed by speed vacuuming. Each fraction was examined by thin layer chromatography (TLC) on a 20 x 20 cm 250  $\mu$ m Whatman PE SiLG/UV TLC plate in 80% EtOAc/20% MeOH. Since 2,5,6-tribromo-1-methylgramine has been documented to fluoresce when exposed to UV light (Sato and Fenical 1983), the extracts were observed under a 254/366-nm UV light (Entela Mineralight Lamp Multiband UV, model #UVGL-58). The fraction that appeared to have the highest amount of UV activity (fraction 5: 20% MeOH: 80% EtOAc) was refractionated using a reverse-phase silica gel column chromatography (Varian Mega Bond Elut C<sup>18</sup> column). The crude extract from fraction five was dissolved in MeOH, applied to the column with 200 ml of each solvent mixture using a solvent scheme of 2:1, 1:1, 1:3, 0:1 water (H<sub>2</sub>O) to MeOH to yield eight fractions (each 100 ml of solvent was divided into 2 fractions). These fractions were again run through TLC and observed for UV activity. The compounds with the highest amount of UV activity (fractions 1 and 2: 80% H<sub>2</sub>O/ 20% MeOH) were combined and used to isolate the bromo-alkaloid compound by HPLC. HPLC was performed using a 250 x 10 mm Econosil C<sup>18</sup> column (Lot# 2344) with a pore size of 10 $\mu$ . Compounds were detected using a Waters 2487 Dual  $\lambda$  Absorbance Detector at wavelengths of 232 and 298 (based on Sato and Fenical 1983). The first HPLC method used was a gradient of 20% MeOH: 80% H<sub>2</sub>O to 100% MeOH for 55 minutes with an injection loop of 200 ml. To perform HPLC the dried extract was dissolved in the 20% MeOH:80% H<sub>2</sub>O. To remove any particles, the extract was filtered through an Alltech 0.45  $\mu$ m 13 mm syringe filter. After filtering, 200 mL of the extract was injected into HPLC. To obtain better elucidation of chemical compounds, a second method was employed using a gradient of 100% H<sub>2</sub>O to 80%

MeOH: 20% H<sub>2</sub>O for 55 minutes. During HPLC, yields from each peak were isolated into separate vials and labeled. Nuclear magnetic resonance (NMR) spectroscopy was performed on yields of interest (determined from prior information about the chemicals by Sato and Fenical 1983). Unfortunately, the NMR results were not comparable to those of Sato and Fenical (1983). Thus, identification and isolation of 2,5,6-tribromo-*N*-methylgramine was not achieved.

It was, therefore, decided to compare the entire chemical composition of *O. zoobotryon* and *Z. verticillatum* to determine if the nudibranch was taking up compounds from *Z. verticillatum*. For this, HPLC was performed on crude extracts obtained from *Z. verticillatum* and *O. zoobotryon* collected from Little Jim Island. This HPLC method incorporated both gradients previously used. Thus, the gradient was 100% H<sub>2</sub>O to 100% MeOH over a 95 minute period. Three replicate HPLC trials were performed with crude *O. zoobotryon* extract and one replicate with crude *Z. verticillatum* extract from Little Jim were performed. The peaks from the HPLC tracings for the two species were compared. Additionally, one replicate HPLC tracing of crude *Z. verticillatum* from Mosquito Lagoon was run and compared with *Z. verticillatum* from Little Jim Island.

## RESULTS

### *Asexual reproduction via fragmentation of Zoobotryon verticillatum*

Photographic evidence supports that vegetative fragments do attach after being separated from the parent colony (Figures 3a-d). Attachment success was dependent on time and size (Table 1 and Figure 4). No fragments were attached at 4 hours, but attachment did occur at 12 and 24 hours (23% and 77%, respectively) with a significantly higher percentage attaching at 24 hours (Fisher's Exact test,  $p < 0.001$ ) (Table 1). Percent attachments were combined from 12 and 24 our trials to test for an effect of size on attachment. Spearman's rho ( $p < 0.001$ ) showed that attachment success was dependent on fragment size ( $p < 0.001$ ) (Figure 4). Mean percent attachment of 10 mm fragments was 24%, while mean percent attachment of 50 mm fragments was 41%.

Fragment weight increased with fragment length (Figure 5). The shortest fragments (10 mm) had a mean weight of 5.22 mg ( $\pm$ SE), while the longest fragment size (50 mm) had a mean weight of 93.69 mg ( $\pm$ SE). All weights were significantly different when compared with a one-way ANOVA ( $p < 0.001$ ) and Tukey's *a posteriori* test (Figure 5).

### *Distribution and abundance of adult Okenia zoobotryon and egg masses*

The numbers of *O. zoobotryon* adults and egg masses found on *Z. verticillatum* fluctuated throughout the year (Figure 6). The largest numbers were recorded in June 2000 at 0.69 adults and 1.44 egg masses per gram wet-weight *Z. verticillatum*. In March and April 2001, no adults or egg masses were observed (Figure 6). Figure 7 demonstrates that the numbers of adults and egg masses rose and fell simultaneously (except in November 2000). Regression analysis showed

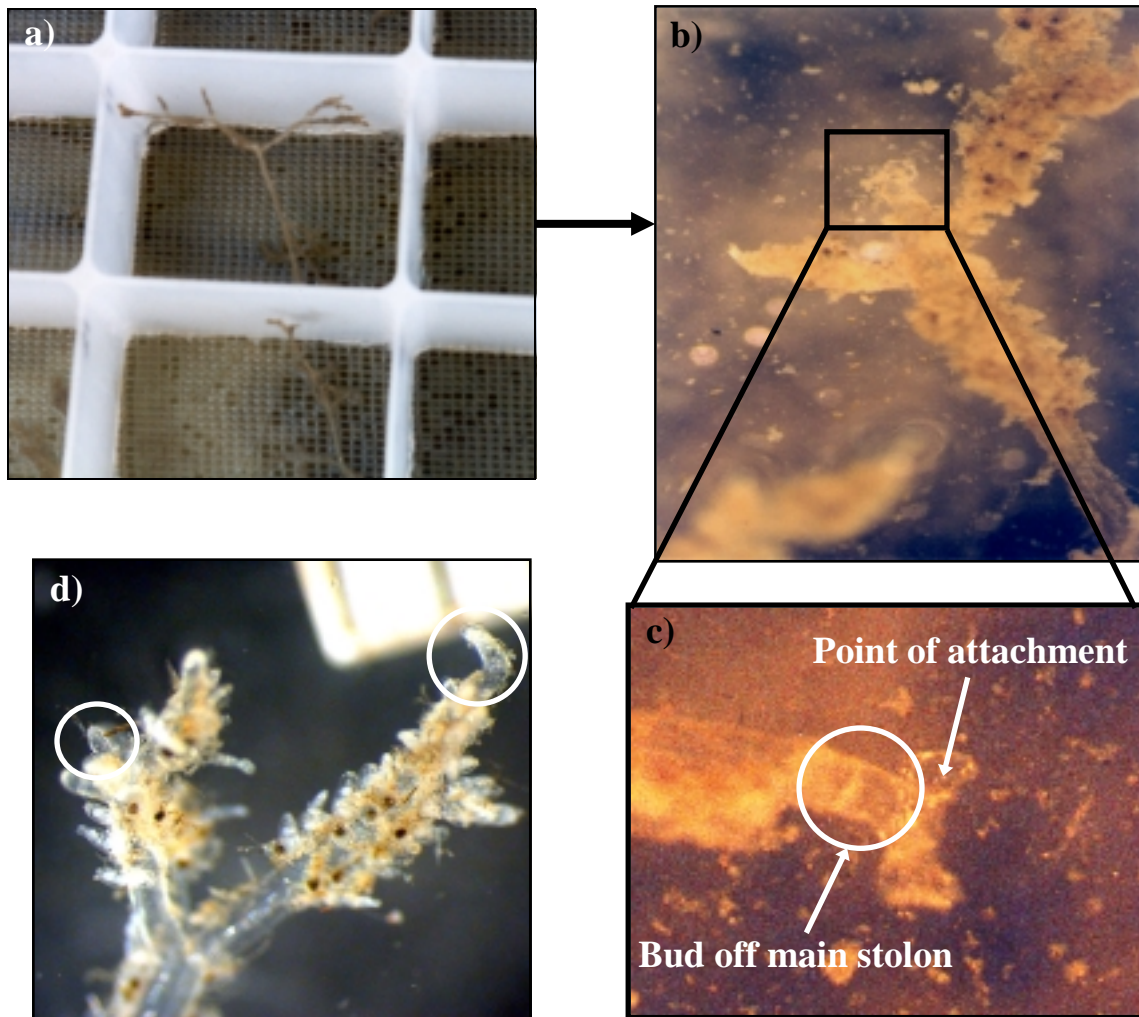


Figure 3: Photographs of *Zoobotryon verticillatum* attachment structure. a) Close-up of fragment in compartment after attachment (10X magnification). b) Close-up of attachment point (40X magnification). c) Budding tip off of main stolon (circled) with point of attachment (shown attached to a piece of plexiglass). d) Budding tips.

Table 1: Fragment trials were performed at 4, 12, and 24 hours to determine if attachment success were dependent upon time (n = 30). Any compartment where a fragment was not found was termed “dead/missing.” This term was given on the basis of not being able to determine the fate of the fragment. Attachment success at 12 and 24 hours was significantly different (Fisher’s exact test,  $p < 0.001$ ).

<b>Mean (%)</b>	<b>Time (hours)</b>		
	<b>4</b>	<b>12</b>	<b>24</b>
Attached	0	23	77
Not Attached	87	70	20
Dead/Missing	13	7	3

### Percent Fragment Attachment

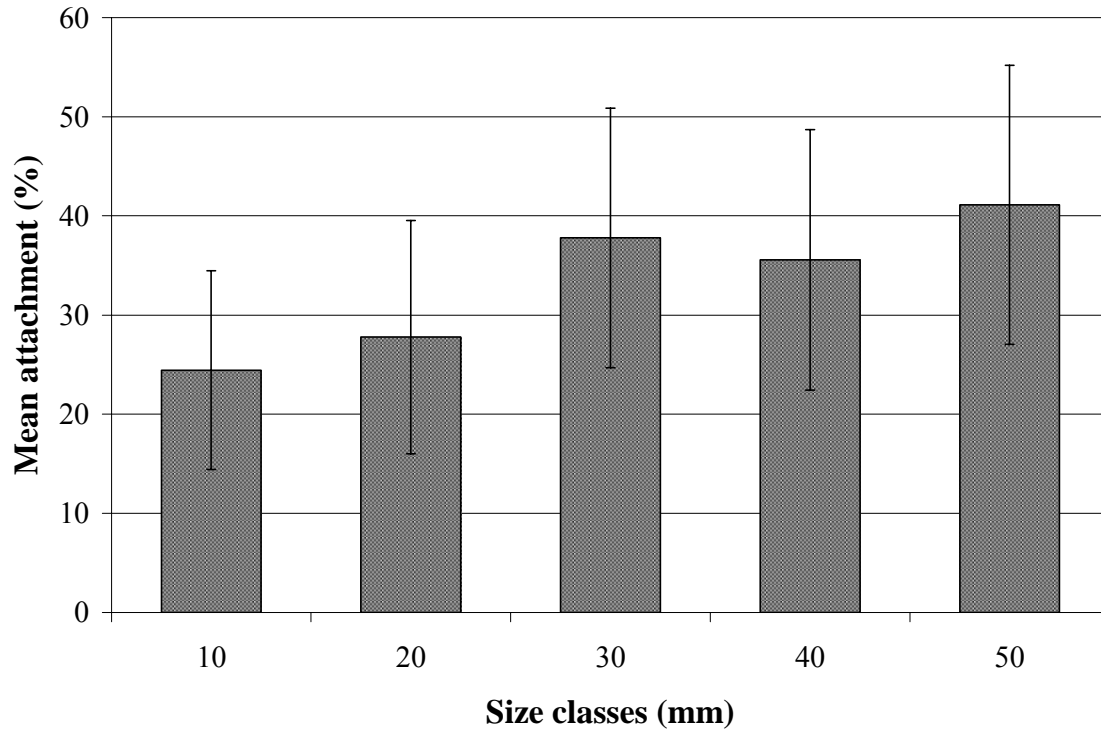


Figure 4: Mean percentage attachment among size classes ( $\pm$ SE). Attached fragments from 12 and 24-hour trials were combined together to determine if the size of the fragment was important for attachment success ( $n = 30$ ). Spearman's rho test demonstrated a significant difference in attachment success among fragment size classes ( $p < 0.001$ ).

### Fragment Weights of *Zoobotryon verticillatum*

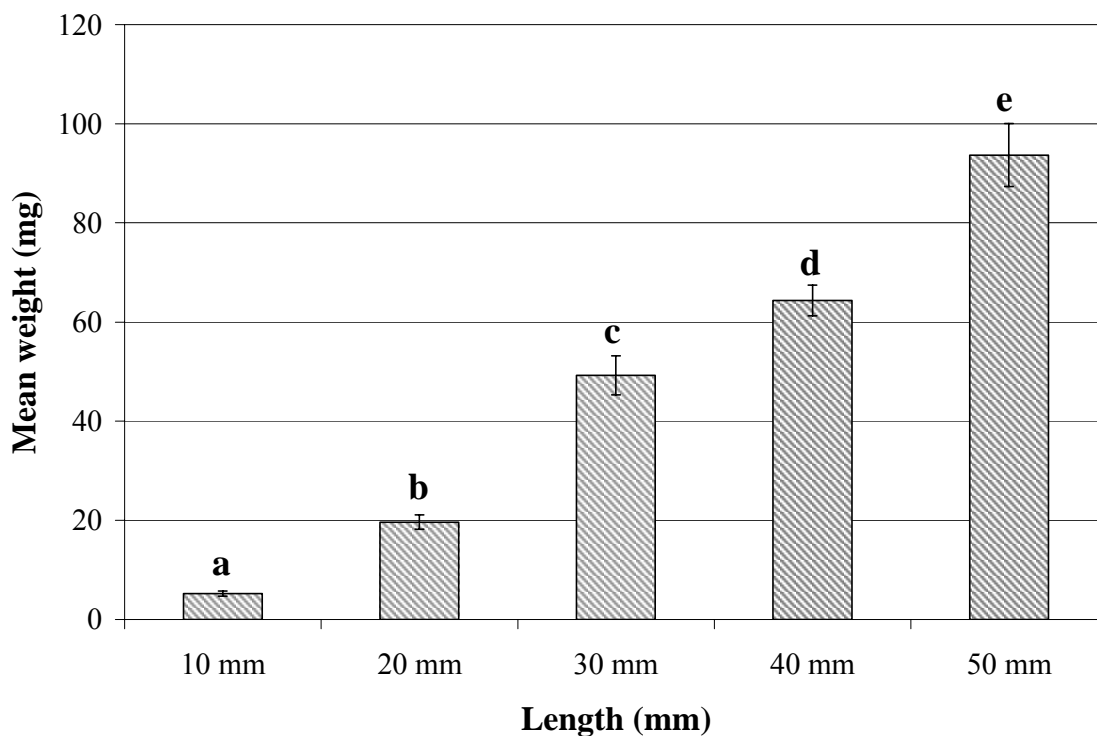


Figure 5: Mean blotted dry wet-weights of fragments from each of the size classes ( $\pm$ SE). Forty-five fragments were weighed for each fragment size. Fragment weight increased with fragment length. All weights were significantly different when compared with a one-way ANOVA ( $p < 0.0001$ ) and confirmed with Tukey's *a posteriori* test (different letters indicate means that are significantly different at  $\alpha = 0.05$ ).



### Population surveys of *Okenia zoobotryon*

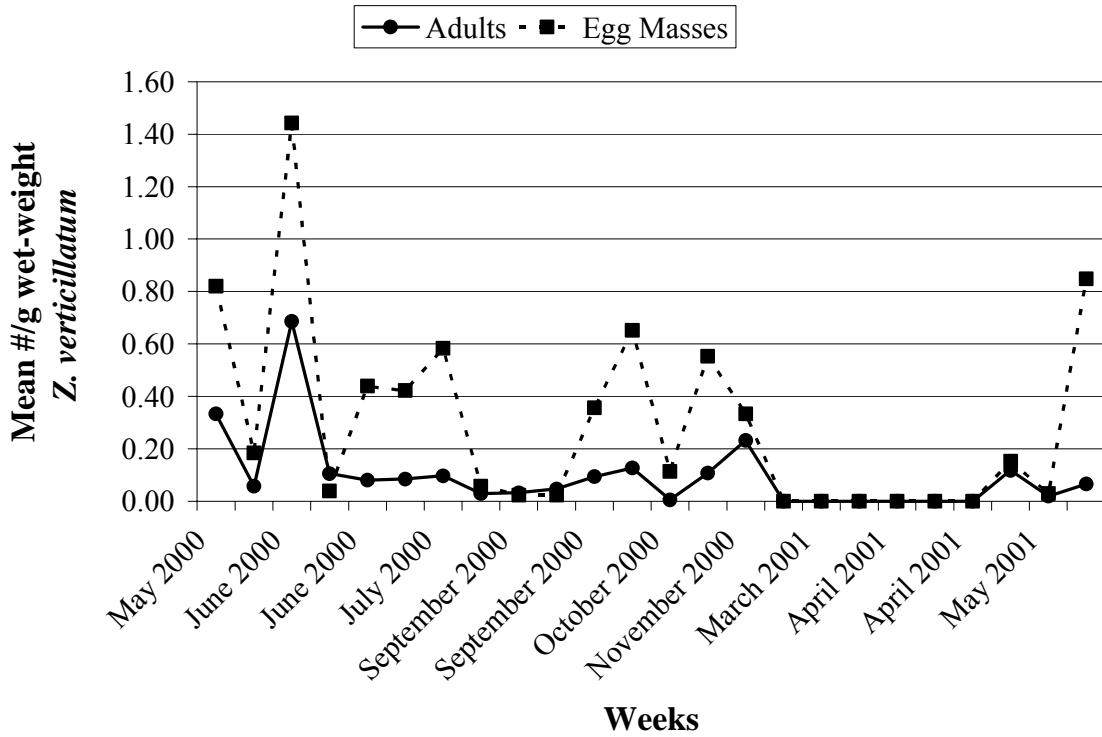


Figure 6: Mean number of individual *Okenia zoobotryon* adult and egg masses found on colonies of *Zoobotryon verticillatum* per gram wet-weight (n = 6). Results are from 24 weeks of population surveys in Mosquito Lagoon between May 2000 and May 2001. Colonies of *Zoobotryon verticillatum* were collected and the adult and egg masses of *Okenia zoobotryon* removed before the wet-weights were taken. Egg mass means include all egg mass size classes (<10, 10-30, and >30 mm). There was a significant correlation between the adult and egg masses numbers ( $R^2 = 0.6941$ ).

there to be a significant correlation between adult and egg mass numbers ( $R^2 = 0.6941$ ).

Lengths of *O. zoobotryon* (n=50) ranged from 2 to 8 mm, with the majority (82%) measuring between 3 and 6 mm. Weight increased with length ( $R^2 = 0.9136$ ) (Figure 7). Nudibranchs measuring 3 mm in length had a mean weight of 5.10 mg while nudibranchs measuring 6 mm in length had a mean weight of 7.9 mg. *Okenia zoobotryon* egg masses were divided into 3 categories: <10 mm, 10-30 mm, and >30 mm (Figure 8). A total of 616 egg masses were observed; 303 (49%) fit into the 10-30 mm category.

Small numbers of *O. zoobotryon* adults and egg masses were found during population observations of potential hosts other than *Z. verticillatum* (Figure 9). Egg masses were only observed on the mixed clumps of *S. filamentosa* and *Z. verticillatum* (Figure 9). Within those clumps the numbers of adults observed on *S. filamentosa* were 0.05 g/ wet-weight, while 0.22/g wet-weight were found on *Z. verticillatum* (Figure 9). A small number of adults (0.01/g wet-weight) were also found on *S. filamentosa* (Figure 9). Adults were also observed in small numbers (0.06g/wet-weight) on *Gracilaria* sp. in mixed clumps with *S. regularis* (Figure 9). Paired t-tests showed significant differences between all potential hosts and *Z. verticillatum* (Table 2).

#### ***Adult Okenia zoobotryon multiple-choice preference assays***

Adult *O. zoobotryon* multiple-choice assays were performed to determine if prey preference was related to a conspecific adult cue. Multiple-choice assays showed there to

## Nudibranch Sizes

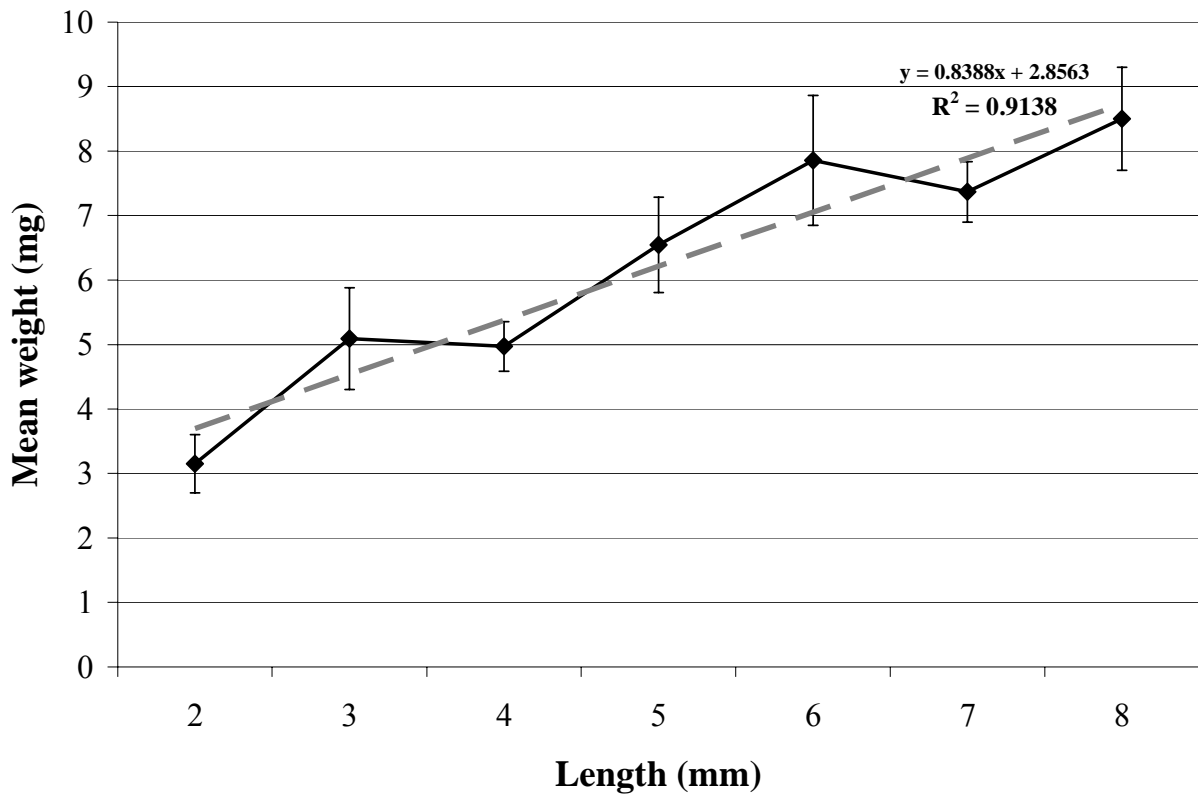


Figure 7: Lengths and mean weights of adult *Okenia zoobotryon* ( $\pm$ SE) (n = 50). Most of the nudibranchs collected measured between 3 and 6 mm (82%).

### Egg Mass Size Classes

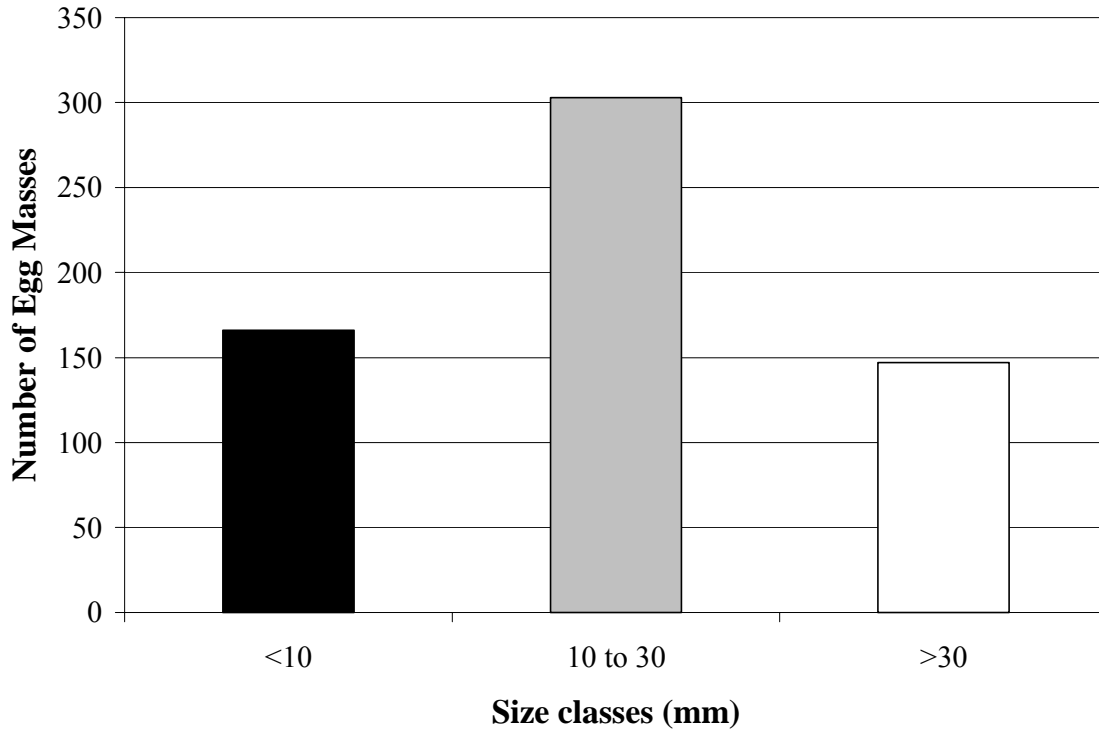


Figure 8: Total number of egg masses observed for each size class. Numbers were taken during the 24 weeks of population observations from May 2000 to May 2001.

## Population Surveys of Potential Hosts

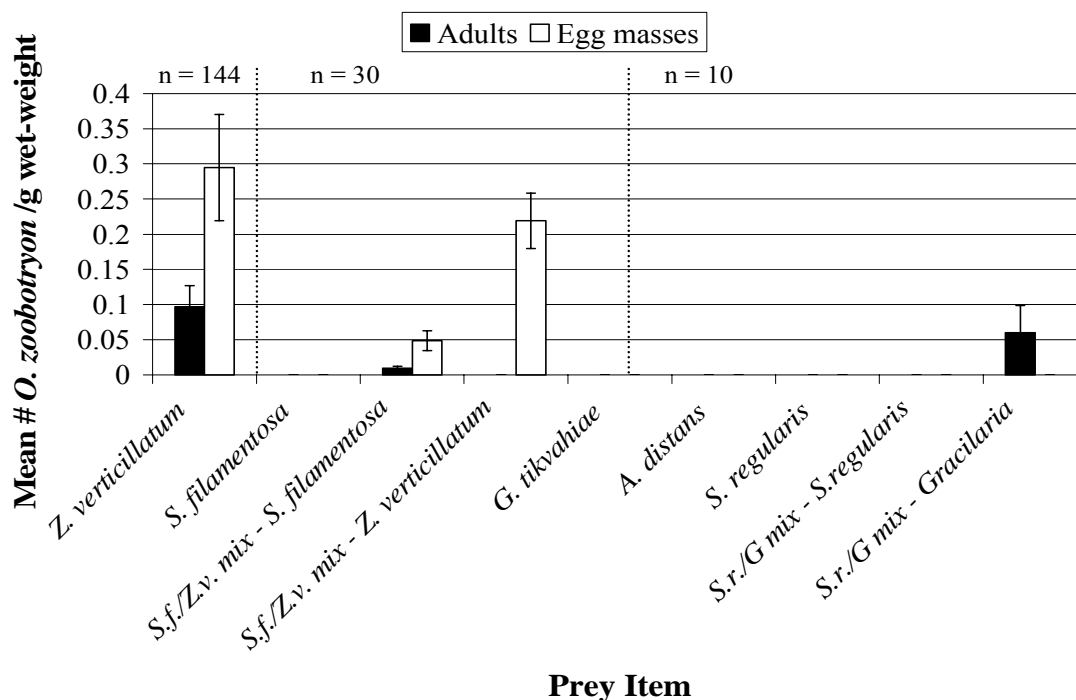


Figure 9: Mean number ( $\pm$ SE) of *Okenia zoobotryon* adults and egg masses on *Zoobotryon verticillatum* (per gram wet-weight) compared to other potential host items. Note: N is different for several of the potential host items from the *Zoobotryon verticillatum*. (*S.f./Z.v.mix* = *S. filamentosa/Z. verticillatum* mix; *S.r./G mix* = *S. regularis/Gracilaria* spp. mix)

Table 2: Results from paired t-tests show all survey numbers were significantly different when compared with paired t-tests ( $\alpha = 0.05$ ).

Paired t-Test (one-tail)			Paired t-Test (one-tail)		
Adults - <i>Zoobotryon verticillatum</i> vs.:	t	p	Egg Masses - <i>Zoobotryon verticillatum</i> vs.:	t	p
<i>S.f./Z.v. mix</i> – <i>S. filamentosa</i>	3.058995	0.002781	<i>S.f./Z.v. mix</i> – <i>S. filamentosa</i>	4.047812	0.00025
<i>S.r./G mix - Gracilaria</i>	2.027437	0.027179	<i>S.f./Z.v. mix</i> – <i>Z. verticillatum</i>	2.510249	0.009773

be a significant preference for *Z. verticillatum* versus the mimic, with *Z. verticillatum* being chosen 70.5% of the time ( $\chi^2 = 10.76$ ) (Table 3). In assays where the red algae *S. filamentosa* and *G. tikvahiae* were tested with *Z. verticillatum*, the nudibranch showed no significant preference for either alga (Table 3). Multiple-choice assays were also performed to test for a conspecific cue using colonies of *Z. verticillatum* containing either adults or egg masses of *O. zoobotryon*. The results of these assays also found no significant preference by the nudibranch (Table 3). In both assays, *Z. verticillatum* void of any adults or egg masses was chosen 43% of the time. When given a choice between *Z. verticillatum* and either *A. distans* or *S. regularis*, the nudibranch was not observed on either of the organisms after 10 minutes. Instead, 80% of the time *O. zoobotryon* was located on the side or bottom of the bowl or on the surface of the water (significant at  $\chi^2 = 5.4$  and 3.6, respectively). Table 4 shows the results from the hour-long observations. In 1-hour choice assays with *A. distans*, nudibranchs were observed on *Z. verticillatum* in 8 out of 15 replicates (53%) (Table 4). In assays with *S. regularis*, nudibranchs were observed on *Z. verticillatum* in only 1 out of 10 replicates (Table 4).

#### ***Larval observations of Okenia zoobotryon***

Egg masses of *O. zoobotryon* were Type A (Strathmann 1987). Egg masses of *O. zoobotryon* were white, gelatinous ribbons and 49% were between 10 and 30 mm in length (n = 616). Egg capsules were observed embedded within the egg masses. Figure 10 is a close-up of a portion of an egg mass 2 hours after deposition. The number of egg capsules within an egg mass varied considerably (Table 5). Regression analysis showed a significant correlation between egg mass length and number of egg capsules ( $R^2 = 0.90$ ).

Table 3: Ten-minute paired-choice assays. Table represents the preferences of adult *Okenia zoobotryon* when given a choice. \*Other = bottom or side of the glass bowl and at an air-water interface. *Zoobotryon verticillatum* clean = *Zoobotryon verticillatum* in which all egg masses and adults were removed plus all other epizooians.

<b>Trials</b>	<b>N</b>	<b>Zoobotryon (%)</b>	<b>Test organism (%)</b>	<b>Other* (%)</b>	<b><math>\chi^2</math></b>	<b>Preference</b>
<i>Zoobotryon verticillatum</i> vs. mimic	44	70.5	22.7	6.8	0.001	<b>Zoobotryon</b>
<i>Zoobotryon verticillatum</i> vs. <i>Spyridia filamentosa</i>	34	58.8	32.4	8.8	0.106	None
<i>Zoobotryon verticillatum</i> vs. <i>Gracilaria tikvahiae</i>	29	34.5	31	34.5	0.819	None
<i>Zoobotryon verticillatum</i> vs. <i>Amathia distans</i>	15	20	0	80	0.020	<b>Other</b>
<i>Zoobotryon verticillatum</i> vs. <i>Scrupocellaria regularis</i>	10	0	20	80	0.058	<b>Other</b>
<i>Zoobotryon verticillatum</i> clean vs. <i>Zoobotryon verticillatum</i> with egg masses	30	43.3	40	16.7	0.841	None
<i>Zoobotryon verticillatum</i> clean vs. <i>Zoobotryon verticillatum</i> with adult <i>Okenia zoobotryon</i>	30	43.3	43.3	13.3	0	None

Table 4: Results from paired-choice assays performed with *Amathia distans* and *Scrupocellaria regularis*. Observations were made every 10 minutes for one hour. A = *Amathia distans*, Z = *Zoobotryon verticillatum*, S = *Scrupocellaria regularis*, O = other.

<i>Amathia distans</i>							<i>Scrupocellaria regularis</i>						
Rep.	10	20	30	40	50	1 hr	Rep.	10	20	30	40	50	1 hr
1	O	O	A	A	A	A	1	O	O	O	O	O	O
2	O	O	O	O	O	O	2	O	O	O	O	O	O
3	Z	Z	Z	Z	Z	Z	3	S	O	O	O	O	O
4	O	O	A	A	A	O	4	O	S	S	S	S	S
5	O	O	O	O	O	O	5	O	O	O	O	O	O
6	O	Z	Z	Z	Z	Z	6	O	O	O	O	O	O
7	Z	Z	Z	Z	Z	Z	7	O	O	O	O	O	O
8	O	Z	Z	O	Z	Z	8	S	S	S	S	S	S
9	O	O	O	Z	Z	Z	9	O	O	O	O	O	Z
10	O	Z	Z	Z	O	O	10	O	O	O	O	O	O
11	O	O	O	O	Z	Z							
12	O	O	O	O	O	O							
13	Z	Z	Z	Z	Z	Z							
14	O	O	O	O	Z	O							
15	O	O	O	O	Z	Z							





Figure 10: Egg mass under 40X magnification within 2 hours after being produced.

Table 5: Number of egg capsules in egg masses. One 3 mm section of each egg mass was cut and broken open. Egg capsules were counted with a dissecting microscope (Wild M5 #105971).

<b>Replicate</b>	<b>Egg Mass Length (mm)</b>	<b># of egg capsules/mm</b>	<b>Total # of egg capsules</b>
1	18	125	2250
2	13	86	1118
3	15	163	2445
4	18	142	2556
5	20	143	2860
6	9	133	1197
7	29	140	4060

Development of *O. zoobotryon* within the egg capsule proceeded for 4 to 6 days before hatching (Figure 11). The pattern of developmental stages up to hatching appeared to be the same as described for other dorid nudibranchs (Thompson 1958; Chia and Koss 1978). The time between each developmental stage was not documented for *O. zoobotryon*. Just prior to hatching, the walls of the egg masses became distended due to the spinning and moving of the developing veligers. This is similar to reports on the dorid nudibranch *Doridella obscura* (Perron and Turner 1977). Once an egg mass ruptured, veligers rapidly swam out of the egg mass. The veligers demonstrated no movement either toward or away from a fiber optic light placed on the side of a plastic petri dish.

As with *D. obscura*, mortality of *O. zoobotryon* embryos was low with approximately 95% hatching (Perron and Turner 1977). The majority of the *O. zoobotryon* veligers hatched at days 4 and 5 post-deposition (43 and 40%, respectively) (Figure 12). The mean dimensions of larvae immediately post-hatching were 94.4 x 113  $\mu\text{m}$  (n = 15) (Figure 13). As documented by Perron and Turner (1977), newly hatched veligers lacked eyes and were unpigmented (Figure 14). When available, larvae immediately began feeding on *Isochrysis galbana* (Figure 14). The stomachs of fed larvae turned green within hours (Figure 14). The larvae continued to grow and develop until 5 days post-hatching. At this point, mortality became substantial and daily losses of approximately 25% continued until no larvae remained in the cultures. Due to this high mortality rate, other crucial developmental parameters including larval growth, size before settlement, and exact age of metamorphic competency were not documented.

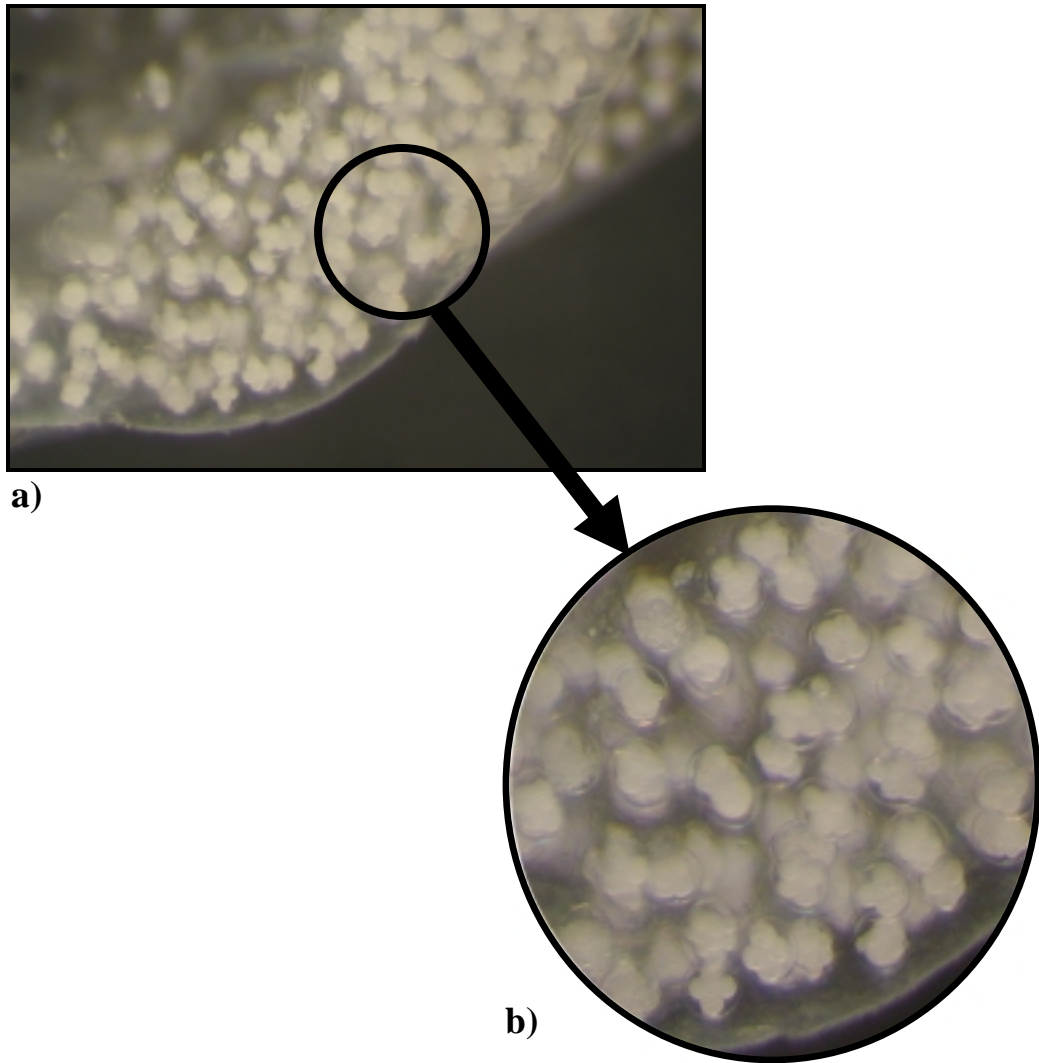


Figure 11: Egg masses with egg capsules undergoing development. a) Portion of an egg mass with egg capsules at different cleavage stages (observed under 10X magnification). b) Magnified view of a.

### Larval Hatching Time

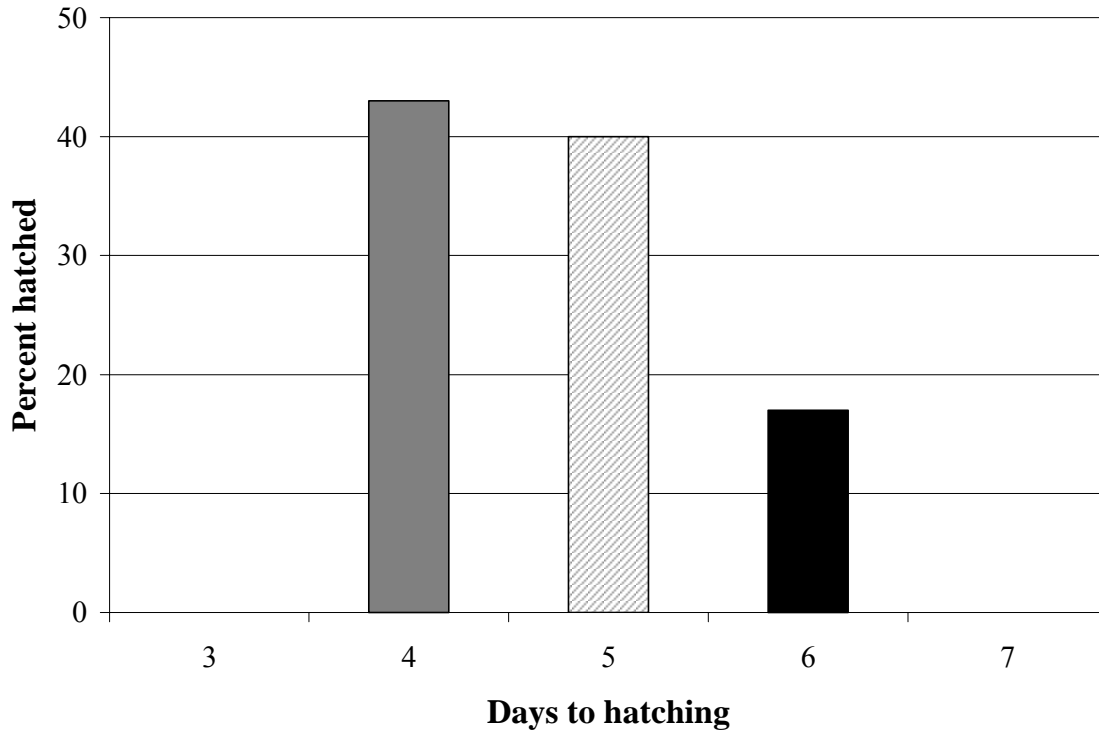


Figure 12: Larval hatching time. Egg masses ( $n = 30$ ) were checked every 24 hr for the presence of swimming larvae. All observed egg masses hatched between 4 and 6 days.

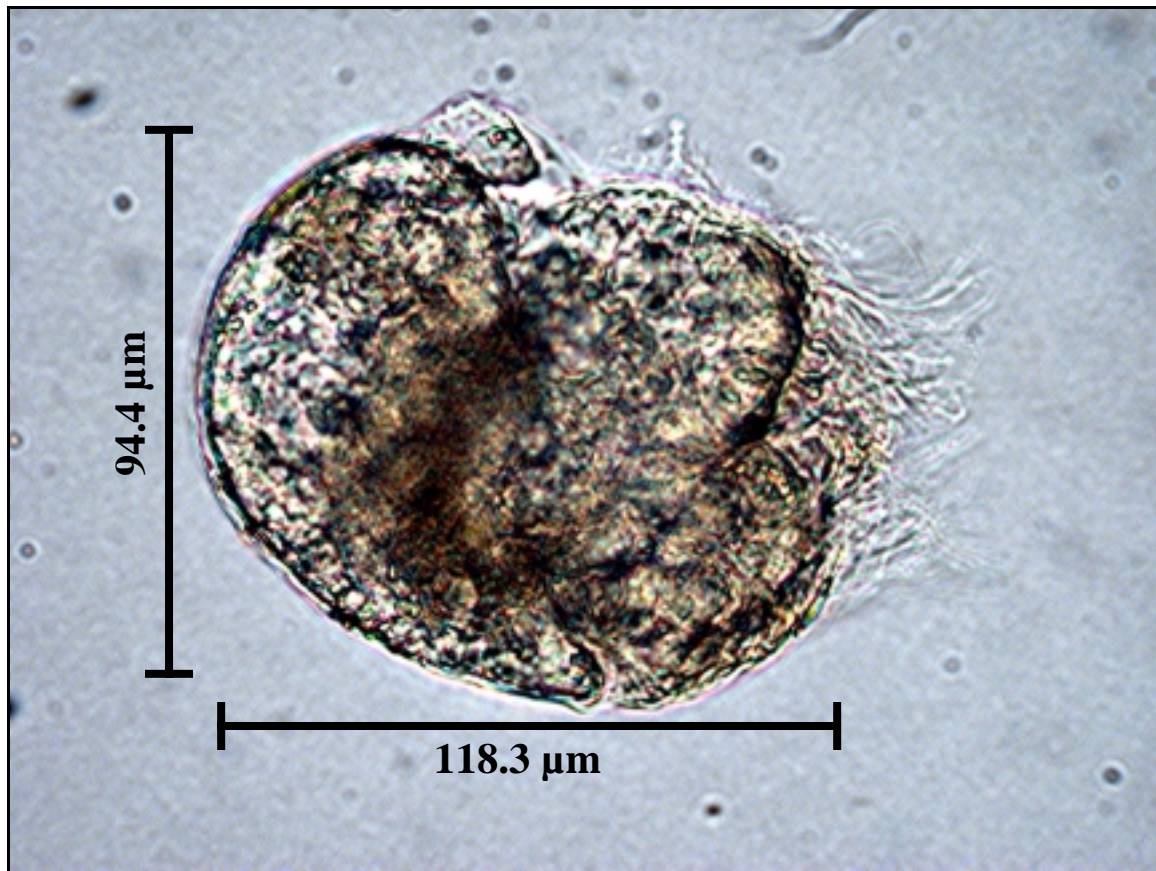


Figure 13: Photograph of a larva of *Okenia zoobotryon* immediately after hatching taken under a Leica DMLB 100S microscope with a Nikon Coolpix 4500 digital camera (10X magnification). The mean dimensions were taken from 15 preserved larvae. Note the presence of cilia attached to the velum of the larva.

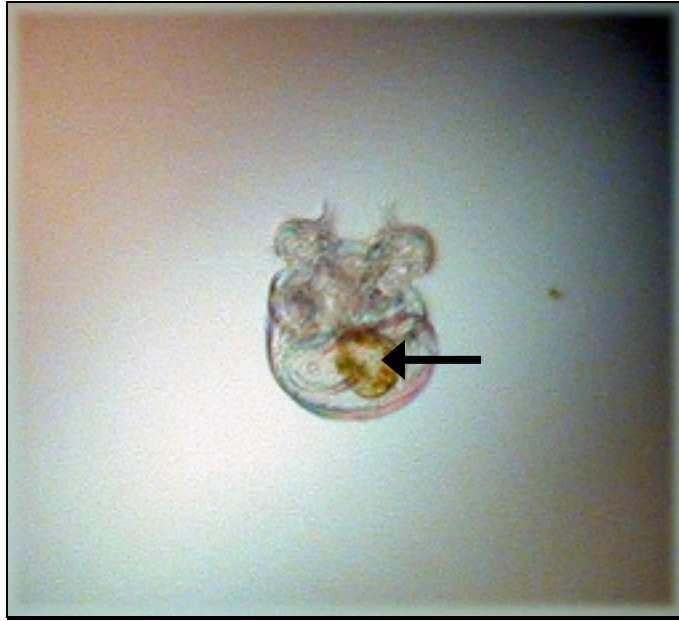


Figure 14: Light microscopy photo (Leica DMLB 100S microscope, Nikon Coolpix 4500 digital camera) of a fed *Okenia zoobotryon* veliger larva. A fed larva is distinguished by a pigmented, filled gut (marked with arrow).

### ***Settlement and metamorphosis of Okenia zoobotryon larvae***

Mortality of larvae was high and only a few larvae were reared to metamorphosis. In a series of trials, 600 (200 unfed and 400 fed) larvae were tested for settlement and metamorphosis. None of the unfed larvae settled by eight days post-hatching and all were dead at nine days post-hatching. Of the 400 fed larvae, 14 settled (Table 6). Settlement occurred only when in contact with *Z. verticillatum* at seven and eight days post-hatching. No settlement occurred in the control (0.22  $\mu\text{m}$  FSW), with *Amathia distans*, or *Bowerbankia maxima*. Five of the 14 settled larvae underwent metamorphosis within 24 hours (8 and 9 days post-hatching).

### ***Chemistry of Zoobotryon verticillatum and adult Okenia zoobotryon***

The chemical compositions of *Z. verticillatum* and adult *O. zoobotryon* were compared through HPLC tracings to look for similar natural products. HPLC was performed on *Z. verticillatum* collected from Mosquito Lagoon and Little Jim Island. Freeze-dried specimens of *Z. verticillatum* weighed 2.90 g (Little Jim Island) and 10.39 g (Mosquito Lagoon). After MeOH extraction, crude yields were 0.986 g and 4.48 g, which constituted 43% and 34% of the freeze-dried specimens, respectively. Only a portion of the crude extract was utilized for HPLC injection. The portions used were kept consistent to produce comparable HPLC tracings (83.9 mg and 82.3 mg, respectively). Freeze-dried specimens of *O. zoobotryon* weighed 24 mg, 75.8 mg, and 12.1 mg. MeOH extraction yielded crude extracts of 22% (5.3 mg), 32% (24 mg), and 16% (1.9 mg), respectively of the freeze-dried specimens. Total crude extract was utilized for HPLC injection in all *O. zoobotryon* specimens.



Table 6: Results from settlement and metamorphosis experiments with *Okenia zoobotryon* in the presence of *Zoobotryon verticillatum* with larvae fed *Isochrysis galbana*. Ten larvae were used in each trial.

<b>Trial</b>	<b>Number settled on <i>Zoobotryon verticillatum</i></b>	<b>Days post-hatching to settlement</b>	<b>Number metamorphosed</b>	<b>Days post-hatching to metamorphosis</b>
1	0	-	0	-
2	0	-	0	-
3	4	7	1	8
4	1	8	1	9
5	1	7	0	-
6	1	8	0	-
7	0	-	0	-
8	2	8	2	9
9	2	8	0	-
10	3	8	1	9

HPLC tracings were consistent among all three extracts of *O. zoobotryon* (Figure 15). Similar peaks are highlighted in Figure 16. Comparison of HPLC tracings between *Z. verticillatum* from Mosquito Lagoon and Little Jim Island showed no similarities between sites (Figure 16). When the HPLC tracing from *O. zoobotryon* was compared with *Z. verticillatum* from Little Jim Island, similar peaks were identified (Figure 17).

## *Okenia zoobotryon* HPLC comparisons

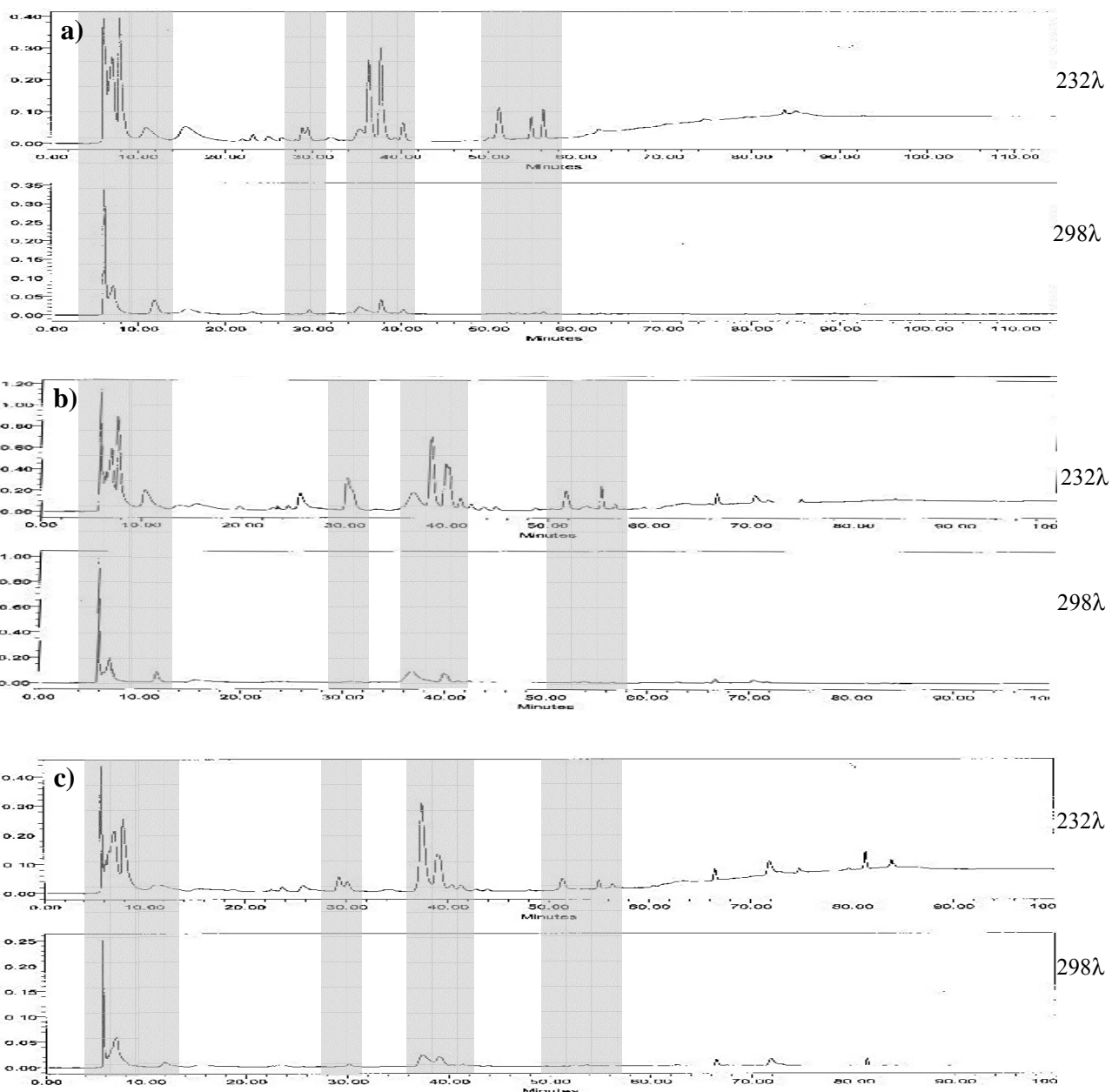


Figure 15: HPLC tracings from three crude *Okenia zoobotryon* extract (from Little Jim Island) replicates (a, b, and c represent 3 individual samples). HPLC detection was run at two different wavelengths (232λ and 298λ). All 3 replicates were similar. The highlighted areas represent major compounds that are identical in all 3 replicates, but relative concentrations varied among replicates.

### *Zoobotryon verticillatum* HPLC comparisons

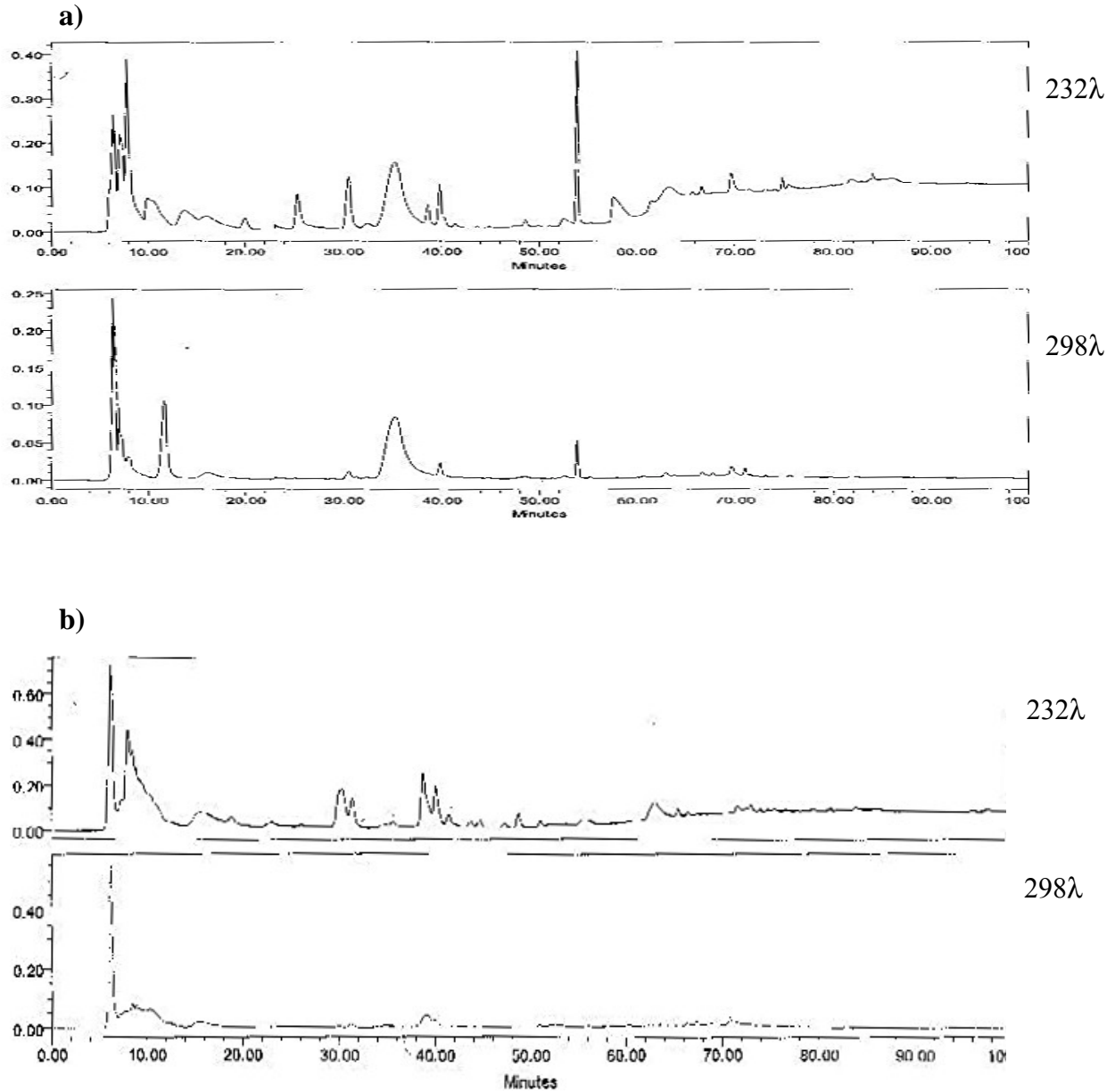


Figure 16: HPLC tracings from crude *Zoobotryon verticillatum* extracts from a) Mosquito Lagoon and b) Little Jim Island. HPLC detection was run at two different wavelengths 232λ and 298λ. Tracings represent different chemical compositions for *Zoobotryon verticillatum* from different areas of the Indian River Lagoon.

## *Zoobotryon verticillatum* and *Okenia zoobotryon* HPLC comparisons

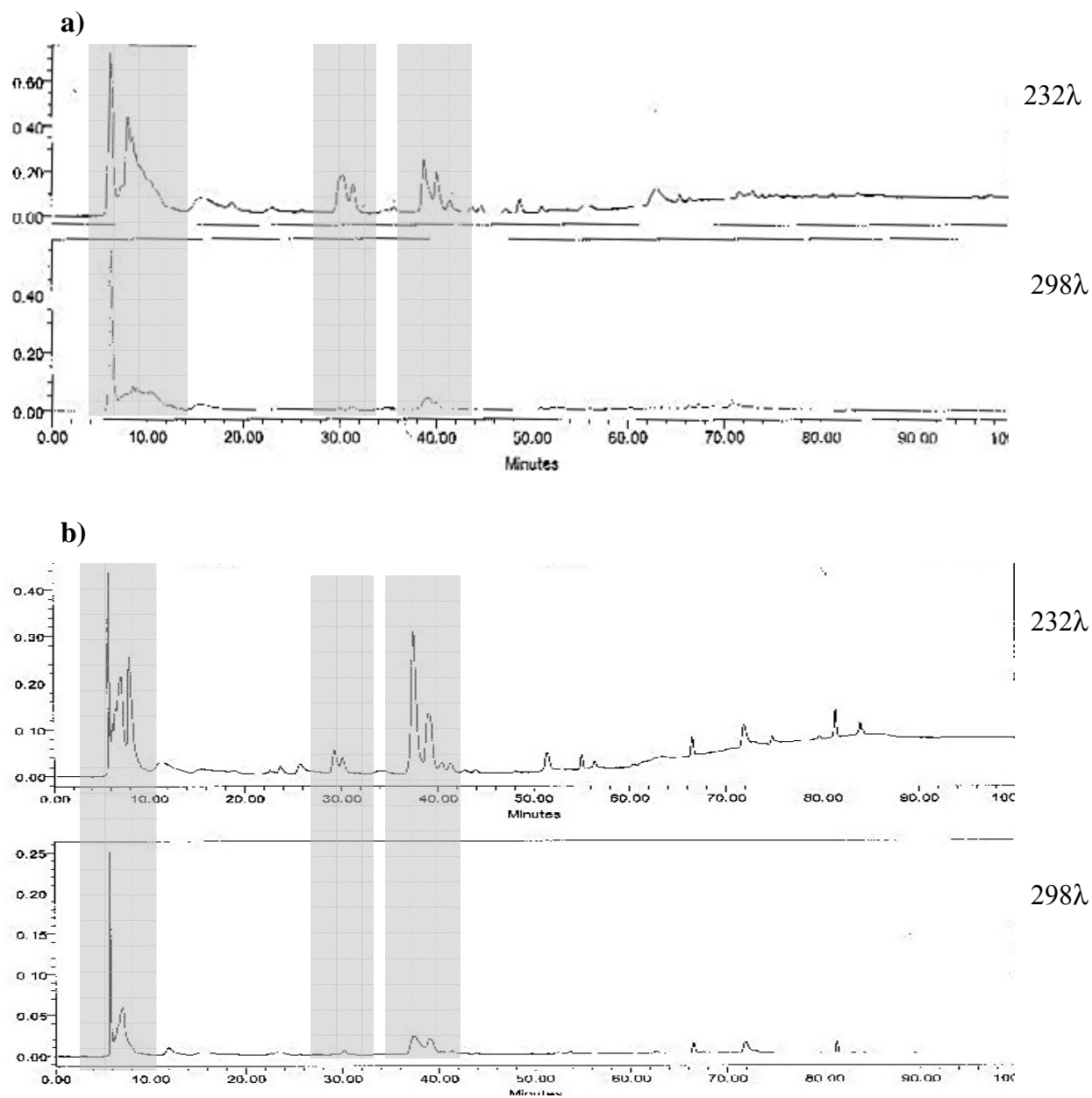


Figure 17: HPLC tracings from crude a) *Zoobotryon verticillatum* and b) *Okenia zoobotryon* extracts from Little Jim Island. HPLC was run at two different wavelengths 232λ and 298λ. Tracings indicate similar results between *Zoobotryon verticillatum* and *Okenia zoobotryon*. Highlighted areas represent major compounds that are similar in both samples, although relative concentrations vary.

## DISCUSSION

### *Asexual reproduction via fragmentation of Zoobotryon verticillatum*

From the results of my study and a study by Zirpolo (1924), vegetative fragmentation is thought to be an important reproductive strategy for *Z. verticillatum*. Fragments were able to survive and attach to substrates after being separated from the parent colony (Table 1 and Figure 4). I determined that attachment was dependent on time and fragment size (Table 1 and Figure 4). Larger fragments had greater attachment success than did smaller fragments (Figure 4). A larger fragment should be more functionally prepared to handle separation from the parent colony. Studies of other invertebrates have shown that larger fragments have greater success of attachment and subsequent survival. For example, larger coral fragments have a greater chance of survival and growth than small fragments (Loya 1976, Highsmith *et al.* 1980, Hughes 1989; Smith and Hughes 1999; Lirman 2000). Larger fragments likely had a higher potential for contact with the available substrate. The compartments of the fragment boxes were 40 x 40 x 40 mm. The largest fragment sizes were 50 mm in length, and thus, had more surface area and attachment points available for contact with the available substrate than the smallest fragments (10 mm). In a natural setting, large, heavy fragments would have a greater chance to become entangled with other organisms. This entanglement could create a suitable place where the fragments could reattach.

Fragments survived in my trials (Table 1 and Figure 4). This does not necessarily mean that they still have the capacity to continue to grow. During an additional experimental run in the field for seven days all fragments of various sizes (10 through 50 mm) had at least doubled in size and had feeding zooids (pers. obs.). Also, in the study by Zirpolo (1924), cut pieces of *Z.*

*verticillatum* survived and produced attachment structures that fastened the new colony to the substrate. In another study, Bullivant (1968) documented that growth in *Z. verticillatum* occurred primarily through a budding process. The ancestrula (larva after undergoing metamorphosis) attaches via the tip of a produced stolon (Bullivant 1968). The tip spreads out as a disc. A zooid then develops off the stolon and subsequently severs the original attachment point (Bullivant 1968). After this, buds develop on the stolon (Bullivant 1968). Budding produced more zooids, thus creating a newly developed colony. This appears to be exactly the same process taking place after the fragmentation event. From the pictures taken under microscopy in the current study (Figure 3) and from drawings in Zirpolo's (1924) study, a distinct bud can be identified off the main stolon. In the current study, a distinctly spread out disc was also noted arising from this budding point off the main stolon. Although it was not directly tested, it is likely a new stolon will be created from this point in which a zooid will arise. The budding process described above will continue, thereby creating a new colony.

The creation of fragments is highly likely given that *Z. verticillatum* colonies can become quite large. Colonies can grow to fist-size in weeks and may become a meter or more in length in 6-8 months (Winston 1995). Morphologically, *Z. verticillatum* is fragile and prone to breakage. These large, attached colonies are extremely vulnerable to fragmentation. Additionally, from the attachment process described above, *Z. verticillatum* colonies may not be tightly secured to the substrate and any disturbance could dislodge the colony, which is then free to be dispersed by water currents. Large colonies of *Z. verticillatum* are commonly found throughout the IRL, especially entangled with other drift algae or in seagrass communities (Winston 1995). When found, these colonies were usually healthy and feeding zooids could be observed (pers. obs.).

Bullivant (1968) maintained colonies of *Z. verticillatum* at two different temperatures (20 and 25-26° C) for four months before the release of larvae. The colonies maintained at the higher temperatures were the only ones to produce larvae. Thus, sexual reproduction in *Z. verticillatum* may occur very infrequently and the environment appears to be a major influence on larval production. Water temperatures in the IRL are high throughout the summer months, but can decrease dramatically in the winter months. Thus, *Z. verticillatum* is limited in the time frame to produce larvae. During the winter months, adult colonies were rarely found at my collection sites in the IRL. Geiger and Zimmer (2002) report on a study by Jebram (1973) which found that *Z. verticillatum* developed an overwintering strategy by which fragments of the stolon filled with a yolk-like substance and formed resting structures. These structures could then attach to a substrate when the conditions are favorable to form a new colony (Jebram 1973; Geiger and Zimmer 2002). For this reason, asexual reproduction is thought to be extremely important to the survival of *Z. verticillatum*.

#### ***Distribution and preference of Okenia zoobotryon***

Population surveys of adult *O. zoobotryon* and egg masses and adult multiple-choice assays were performed to provide information on any predator-prey relationship between *O. zoobotryon* and *Z. verticillatum*. Population surveys demonstrated that adult *O. zoobotryon* and its egg masses were primarily found on *Z. verticillatum*. A small number of adults were found on mixed colonies of *S. regularis* and *Gracilaria* sp. In the field, these organisms grow in close proximity. It is possible that the *S. regularis* and *Gracilaria* sp. colonies had contacted sessile or drift *Z. verticillatum*. In order to definitely state that *O. zoobotryon* is only found on *Z.*



*verticillatum* in the IRL, more colonies should be examined. This is especially true because adult *O. zoobotryon* have been observed on another bryozoan, *Bowerbankia maxima*, in the southern IRL (S. Santagata pers. comm.). All of the population studies in the current project were performed in the IRL when *Z. verticillatum* was abundant. Surveys should also be undertaken during the months when *Z. verticillatum* is rare or absent.

Results from the paired-choice assays demonstrated that *O. zoobotryon* adults were not attracted by a chemical cue associated with *Z. verticillatum*, conspecific adults or conspecific egg masses. In all assays, adults did not preferentially choose *Z. verticillatum* over the other organism offered (Table 3). The nudibranch only preferentially chose *Z. verticillatum* over a plastic (PVC) mimic which could have been due leaching of a chemical (Table 3). In assays when given a choice between *Z. verticillatum* and a macroalga, the nudibranch showed no preferential selection for *Z. verticillatum* (Table 3). Since adult *O. zoobotryon* are thought to be carnivorous, these findings were not unexpected. When provided the choice between *S. regularis* or *A. distans*, the nudibranch did not choose either of the organisms offered within the 10-minute period. This could be because of the amount of free space in the bowl. *Scrupocellaria regularis* and *A. distans* form small, tufted colonies, while *Z. verticillatum* forms large, branching colonies. Thus, for the two organisms being compared to be similar in size, volume (not mass) of the organisms was used. The bryozoan colonies were, thus, small and did not occupy much space in the bowl. When the nudibranch was placed with the small colonies, it was not in close contact with either organism. For these trials, the 10-minute observation period may not have been long enough to allow the nudibranch time to locate the organisms. These trials were continued for an hour with observations every ten minutes. In assays with *A. distans*, nudibranchs were observed

on either organism 60% of the time, which indicated that given more time, the nudibranch located a host (Table 4). In assays with *S. regularis*, this was not the case. In these assays, the nudibranchs were only observed on a host 30% of the time (Table 4). In both *A. distans* and *S. regularis* assays, once the nudibranch located a host, it remained there until the end of the trial. This suggests that adults of *O. zoobotryon* are not preferentially seeking out one host over another.

Results from the field population surveys and paired-choice lab assays were contradictory. According to the population surveys, *O. zoobotryon* is a specialist using *Z. verticillatum* as its prey; yet, based on the paired-choice assays it is a generalist. In order to determine which alternative is correct, more intensive studies need to be performed. As mentioned previously, one important component to the population surveys was that they were all performed while there was an abundance of *Z. verticillatum* in the areas of collection. The time when *O. zoobotryon* was observed on other species (*Bowerbankia* sp.) in the IRL was when *Z. verticillatum* colonies were not abundant in those areas (S. Santagata pers. comm.).

Thus, it is possible that *O. zoobotryon* adults are specialist feeders that can exploit other organisms for nutrition if necessary. It has been well documented that many specialist nudibranchs can exploit several prey species (Todd *et al.* 2001). McDonald and Nybakken (1997) recorded the diets of nudibranchs throughout the world. They reported that 50% of the nudibranch species preyed upon a single species and 75% were associated with one to three species (McDonald and Nybakken 1997). Although, many specialist nudibranchs can exploit several prey species, they tend to prefer only one species (Todd *et al.* 2001). For example, *Adalaria proxima* is a specialist predator of the cheilostome bryozoan *Electra pilosa* (Todd *et al.*

2001). However, in field and laboratory observations, it occasionally fed on another cheilostome (*Callopora lineate*) and rarely on three ctenostome bryozoans (*Alcyonidium gelatinosum*, *Alcyonidium birsutum*, and *Flustrellidra hispida*) (Todd *et al.* 2001). Organisms that can prey on one to three species are termed “stenophagous” (Todd *et al.* 2001). This stenophagous feeding strategy would be advantageous for *O. zoobotryon* in the IRL because of the ephemeral nature of *Z. verticillatum* (Figure 1). When there is no *Z. verticillatum* around, the nudibranch could feed on other bryozoans and survive until the return of its preferred prey *Z. verticillatum*.

The previous set of results also provides questions as to whether prey preference is an adult choice or a larval choice. Because of this, settlement and metamorphosis experiments were performed to determine if there was a larval preference.

#### ***Larval observations of Okenia zoobotryon***

In the present study, several important aspects of the larval cycle for *O. zoobotryon* were documented for the first time. The egg mass type (Type A) was consistent with information provided by Strathmann (1987). The egg masses ranged in length from 10-30 mm, and there was no correlation between egg mass length and the number of egg capsules.

Swimming veliger larvae hatched out of the egg masses four to six days after deposition. This is consistent with the hatching time for the temperate dorid nudibranch *Doridella obscura* (Perron and Turner 1977). This hatching time, however, is in stark contrast to that described for the tropical dorid nudibranch *Rostanga pulchra* in which veligers hatch 15-16 days after oviposition (Chia and Koss 1978). These results provide evidence that generalizations regarding nudibranch development should not be made.

Feeding began immediately after hatching and the stomachs of fed larvae turned green within a few hours of being fed (Figure 14). This is similar to findings by Perron and Turner (1977). The larvae of *O. zoobotryon* were determined to be planktotrophic because: 1) there was no evidence of a yolk-filled sac in the newly hatched larvae, and 2) feeding began immediately upon addition of microalgae. As the number of days post-hatching increased, the activity level of the larvae began to slow. Starting around day two post-hatching, large numbers of larvae were noted crawling on the bottoms of the bowls. This phase of larval life was termed the “searching” phase by Thompson (1958). This “searching” phase is said to indicate that the larvae are looking for suitable substrate for settlement. Perron and Turner (1977) found that the “searching” phase occurred when the larvae of *D. obscura* had reached metamorphic competence. It was only at this point that larvae of *D. obscura* would undergo settlement when exposed to its prey, *Electra crustulenta* (Perron and Turner 1977). This was not the case for *Rostanga pulchra* (Chia and Koss 1978); larvae of *R. pulchra* remained in an active “search” phase for 14 days before competent to settle (Chia and Koss 1978). Larvae of *O. zoobotryon* also did not show any signs of metamorphic competence at the onset of the “searching” phase and made no attempt to settle on *Z. verticillatum* until seven to eight days post-hatching (Table 6). However, larvae continued to feed and grow during this time period.

Larval mortality was low (5-10% total) for four days post-hatching. However, at five days post-hatching, mortality rates became substantial with an approximate 25% daily loss of larvae. This mortality rate continued until there were no larvae remaining in culture. Because of these high mortality rates, other larval stages were not documented. Also, settlement and metamorphosis experiments were hindered. Replicate trials were not performed and adequate

data is not available to determine settlement and metamorphosis cues of *O. zoobotryon*. Of the cultured larvae, only 14 settled on *Z. verticillatum*, and of those, seven metamorphosed. From these data, it can be only be inferred that a chemical cue from the prey *Z. verticillatum* is required for settlement and metamorphosis of *O. zoobotryon*.

Larval culture can be difficult, especially in planktotrophic larvae that require long periods of time before reaching metamorphic competence. Therefore, any number of factors could have accounted for the high mortality (i.e., water temperature, bacterial infection, density of larvae to antibiotic filtered seawater, density of food, aeration vs. non-aeration, etc.). Instead of maintaining cultures in the laboratory, cultures should be maintained in an outside flow system to simulate the natural temperature fluctuations or in an incubation chamber where the temperature and flow would have been kept constant. The food source could also have been problematic for larvae of *O. zoobotryon*. The larvae of *O. zoobotryon* were fed a monoculture of *Isochrysis galbana* at a density of  $10^5$  cells/mL. This density could have been too concentrated and larvae may have become satiated. Some larvae cannot exist on a monoculture diet; they require additional nutrients provided by mixed cultures of microalgae. This could have been the case for larvae of *O. zoobotryon*. In previous studies, larvae of *Doridella obscura* developed and metamorphosed adequately to a monoculture of *Isochrysis galbana* (Perron and Turner 1977). In contrast, larvae of *R. pulchra* only developed and metamorphosed to a mixed culture of *Monochrysis lutheri*, *Isochrysis galbana*, *Platymonas tetrahele* and *Phaeodactylum tricorutum* (Chia and Koss 1978). These data, again, provide further information that generalizations cannot be made for development of dorid nudibranchs.

Laboratory culture of *O. zoobotryon* should be followed-up. Because the life-history of this dorid nudibranch has not been documented, quantitative data should be obtained at every stage of the life cycle. I believe that with more controlled conditions, culture of *O. zoobotryon* larvae can be successfully performed.

### ***Chemistry of Zoobotryon verticillatum and adult Okenia zoobotryon***

“Chemistry is the foundation of all life” (Harper *et al.* 2001). Organisms produce chemicals that are essential for the survival and health of the organism (primary metabolites) (Harper *et al.* 2001). However, there are also a suite of chemicals produced by organisms that are not classic primary metabolites, and are, therefore, considered secondary metabolites or natural products (Harper *et al.* 2001). Secondary metabolites are increasing in scientific interest due to their many valuable functions, including treatment of human diseases, antifouling products, and mediators of predator-prey relationships (Sennett 2001; Rittschoff 2001; Stachowicz 2001).

Our understanding of the chemistry of bryozoans is limited and only a few secondary metabolites have been identified for this taxa (Christophersen 1985). One-hundred and one compounds have been isolated from this group, with 27 of these originating from the order Ctenostomata and the other 74 from the order Cheilostomata (Harper *et al.* 2001). *Zoobotryon verticillatum* collected from California has been reported to contain the bromogramine derived alkaloids 2,5,6-tribromo-*N*-methylgramine and the corresponding side chain *N*-oxide (Sato and Fenical 1983) and *Z. verticillatum* from Spain is reported to contain 2,5,6-tribromo-*N*-methylindole-3-carbaldehyde (Ortega *et al.* 1993). Also, antifouling properties associated with the compound 2,5,6-tribromo-*N*-methylgramine were isolated from a species in Japan (Kon-ya *et*

al. 1994). In the current study, I examined the chemical structure of *Z. verticillatum* from the Indian River Lagoon, Florida. The chemical composition of this organism was found to be more complex than originally thought. Many compounds found in the IRL species were not mentioned in the previous reports. In the current study, I used similar techniques to Sato and Fenical (1983) to isolate and identify 2,5,6-tribromo-*N*-methylgramine. NMR spectroscopy was performed on HPLC fractions that were assumed to contain this compound (Sato and Fenical 1983). However, the NMR spectral data was not comparable to that of Sato and Fenical (1983). Therefore, none of the compounds from *Z. verticillatum* in Florida could be identified as 2,5,6-tribromo-*N*-methylgramine. Because actual HPLC tracings of the previously reported compounds from California, Japan, and Spain were not available, direct comparison of entire chemical composition of *Z. verticillatum* was not performed. These findings could indicate that the chemical composition of *Z. verticillatum* from Florida is different from that of the other species. This does not mean that the compounds are entirely different. *Zoobotryon verticillatum* from Florida may, in fact, contain the same bromogramine derived compounds, but they may be structurally different from 2,5,6-tribromo-*N*-methylgramine and 2,5,6-tribromo-*N*-methylindole-3-carbaldehyde. Climatic zones may have an impact on the geographical variation of *Z. verticillatum*. California, Spain, and Japan are temperate climates whereas Florida is a subtropical climate. HPLC was also performed to compare the chemical structure of *Z. verticillatum* from the northern IRL (Mosquito Lagoon) to the southern portion of the IRL (Little Jim Island, Fort Pierce). HPLC tracings of bryozoan colonies from these two areas were distinctly different from one another, suggesting they contain different natural products (Figure 16).

Geographical variation in secondary metabolites has been reported in terrestrial plants, marine algae, gorgonians, and other bryozoans (Sturgeon 1979, Paul and Van Alstyne 1988b; Harvell *et al.* 1993; McGovern and Hellberg 2003). The findings from my study could represent three likely possibilities: 1) local adaptation, 2) limited gene flow among populations, or 3) cryptic speciation.

Organisms in estuaries are in discrete habitats separated by geographical distance (Bilton *et al.* 2002). Asexual reproduction via vegetative fragmentation of *Z. verticillatum* could create new populations in various areas of the IRL. The resulting colonies would be genetically the same but living under different environmental conditions where the food source could be different. If, in fact, bryozoans receive their chemicals from the food they ingest, this would account for the chemical variation. In a study by Harvell *et al.* (1993), they found that the gorgonian *Briareum asbestinum* showed significant chemical variation amongst habitats. This variation, they determined, was due to localized genetic adaptation (Harvell *et al.* 1993).

According to Keenan (1994), “estuaries are likely to conform more closely to a one-dimensional model of gene flow, promoting subpopulation differentiation.” Genetic differentiation can be considerable between estuarine populations (Bilton *et al.* 2002). This differentiation is likely due to intense natural selection under estuarine conditions (Blaber 1991) and barriers to gene flow (Bilton *et al.* 2002). These conditions could lead to morphologically identical, but reproductively isolated, cryptic species (McGovern and Hellberg 2003). A number of studies have demonstrated the presence of cryptic species in estuarine organisms (Muus 1967; Cognetti and Maltagliati 2000; Lee 2000; Staton *et al.* 2000; McGovern and Hellberg 2003). Specifically, the bryozoan *B. neritina* contains two cryptic species along its Pacific range in



California (Davidson and Haygood 1999) as well as a complex of cryptic species along its Atlantic range (McGovern and Hellberg 2003). In addition, both studies found *B. neritina* contained different strains of the endosymbiont *E. sertula* (Davidson and Haygood 1999; McGovern and Hellberg 2003). Although *Z. verticillatum* has never been documented as having endosymbionts, there is a possibility of this type of endosymbiotic relationship. Further research should be undertaken to examine this possibility.

Comparison between *Z. verticillatum* and *O. zoobotryon* revealed similarities in their chemical composition (Figure 17). HPLC tracings showed similar peaks in both *Z. verticillatum* and *O. zoobotryon* crude extracts (Figure 17). These data suggest that *O. zoobotryon* feeds on *Z. verticillatum*. The compounds in *Z. verticillatum* were not isolated or identified. Therefore, it is not known if the chemicals the nudibranchs are taking up are toxic in nature or if they are being used for defense by the nudibranch. However, these data do provide the basis for future studies into this dynamic relationship. Future studies should be performed to isolate and identify the major natural products in *Z. verticillatum*. Because previous reports have focused on the bromogamine-derived alkaloids, there is no information regarding the nature of the other compounds of the bryozoan. Because the chemical structures appear to be different for the species from different locations in Florida, all unique compounds need to be identified and tested for anti-predatory or anti-fouling properties. Once the compounds are identified in the bryozoan, the nudibranch compounds should be isolated and identified to be certain that they are the same. After isolating the compounds, deterrence feeding assays could be performed to determine if the nudibranch is using these compounds as an anti-predatory defense. Other opisthobranch molluscs have been documented to take up chemicals from their hosts (Paul and VanAlstyne 1988a;

Faulkner 1992; Pennings 1994; McPhail *et al.* 1998; Becerro *et al.* 2001; Ginsburg and Paul 2001).

### *Conclusions*

Predator-prey relationships vary dramatically from one relationship to the next. The results of the current research have provided some insight into several important aspects of the relationship between the nudibranch *O. zoobotryon* and its prey the bryozoan *Z. verticillatum*. This study suggests that *O. zoobotryon* is a specialist feeder on *Z. verticillatum*, with the ability to switch to generalist feeding in the absence of *Z. verticillatum*. Due to the ephemeral nature of the bryozoan, this would be an effective life-history strategy for the nudibranch. No adult prey preference was documented in this study and because settlement and metamorphosis only occurred in response to *Z. verticillatum*, it is suggested that prey preference is related to a larval cue. Several stages of the life cycle of *O. zoobotryon* were documented and did not fit neatly into the published life cycle strategies of other dorid nudibranchs. More studies on this and other predator-prey relationships are important to furthering our knowledge and understanding of these types of interactions.

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