AN ABSTRACT OF THE THESIS OF Walter C. Kelly III for the Master of Science in Biology presented March 23, 2004.

Title: Environmental Factors Mediating Juvenile Settlement Behavior in the Vermetid Gastropods from Guam.

Approved: _____
Terry Donaldson Chairman, Thesis Committee

The caenogastropods of the family Vermetidae are ubiquitous members of reef flat and reef front communities on Guam, Mariana Islands (Western Pacific), as well as many other tropical areas. This study sought to determine the factors that mediate settlement behavior for five vermetid species found on Guam: *Dendropoma gregaria*, *D. platypus*, *D. maxima*, *Serpulorbis* n. sp. a new species under taxonomic descriptions, and *Petaloconchus keenae*. Observations of all five species indicated there was a clear settlement preference for the crustose coralline algae *Hydrolithon reinboldii* among all species. *Dendropoma maxima* preferred live coral as well. Bioassays were developed to determine if the settlement inducer was water-soluble or surface bound. A crude methanol extract was tested to determine if a chemical associated with the crustose coralline algae *H. reinboldii* induced settlement. Finally, prematurely hatched larvae were assayed for settlement specificity and survival. The results were inconclusive as to whether *D. gregaria*, *D. platypus,* and *Serpulorbis* n. sp. were attracted by a water-soluble cue and induced to settle on suitable substratum. All five species had a clear settlement preference for the crustose coralline algae *H. reinboldii*. *Dendropoma maxima* also preferred the live coral tissue and exposed skeleton of the hydrocoral *Millepora platyphylla*. Juveniles of *D. gregaria*, *D. platypus, Serpulorbis* n. sp., and *P.*

keenae searched actively for the preferred substratum *H. reinboldii* when placed on a textured substratum with the preferred substratum in direct proximity. The methanol extract of *H. reinboldii* was ineffective in inducing settlement for juveniles of *D. gregaria*, *D. platypus*, *D. maxima*, and *Serpulorbis* n. sp. Prematurely hatched larvae of *D. gregaria*, *D. platypus*, and *D. maxima* did not survive in laboratory assays. The data revealed that vermetid juveniles of all five species were capable of delaying settlement for 2-3 days, followed by non-selective settlement even on non-preferred substrata thereafter.

TO THE OFFICE OF GRADUATE STUDIES

The members of the Committee approve the thesis of Walter C. Kelly III presented March 24, 2004.

Terry J. Donaldson

Barry D. Smith

Peter J. Schupp

Michael G. Hadfield

ACCEPTED

for Mary L. Spencer
Acting Coordinator of Graduate Studies

3/31/04
Date

ENVIRONMENTAL FACTORS MEDIATING JUVENILE SETTLEMENT BEHAVIOR IN THE VERMETID GASTROPODS FROM GUAM

BY

WALTER C. KELLY III

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INTRODUCTION

The marine caenogastropods of the family Vermetidae are a morphologically distinct group characterized by their uncoiled shell that is permanently attached to hard substrata. Sexes are separate, and males release pelagic spermatophores. The female captures spermatophores in her mucous net, and fertilization occurs internally (Hadfield and Hopper 1980). Eggs are brooded in the mantle cavity or attached to the inner shell. These gastropods are capable of locomotion only during the larval and juvenile stages. Although some vermetid species release veliger larvae capable of remaining in the plankton for several weeks, most species release crawling juveniles that have limited dispersal, often resulting in dense adult assemblages.

Adult vermetids are found in a variety of distinctive coastal habitats (Morton 1950, Keen and Morton 1960, Keen 1961, Hadfield et al.1972, Safriel 1975, Hughes 1978, 1979). In Guam these habitats are distributed along the intertidal shoreline, across the reef flat, and down the reef front. These habitats often contain specific substrata, such as crustose coralline algae, live coral, and other hard substrata, such as boulders and pavement. Adult vermetid habitats are associated also with distinct current patterns and various degrees of exposure to wave energy (Safriel 1975). Biochemical and physical factors associated with these different substrata and habitats may influence larval and juvenile dispersal, settlement, and the locations of adult assemblages (Pawlik 1992).

Larvae and juveniles are the dispersal agents for most benthic marine invertebrates. The dispersal phase may be completed in minutes or last for months (Hadfield and Paul 2001). The terms settlement and metamorphosis describe the behavioral and physiological events of the transition from the mobile larvae or juvenile stage to the attached juvenile (Pawlik 1992). The term settlement is used here to describe this overall transition, from contact with the substratum, exploratory behavior, orientation, and metamorphosis which are processes in settlement (Pawlik 1992). Settlement on suitable substrata mark the end of dispersal for sessile marine invertebrates. Having settled, a juvenile is said to have undergone recruitment to the substratum (Keough and Downes 1982).

Detection of suitable substrata may be mediated by many environmental factors, including biochemical and physical cues associated with the habitat (Hadfield and Paul 2001). Settlement and metamorphosis in a wide variety of marine invertebrates are controlled by larval sensory recognition of, and responsiveness to, exogenous biochemical and other environmental stimuli (Morse et al. 1988). This sensory recognition is required for the initiation of the genetically programmed sequence of behavioral and developmental processes that are arrested during the dispersal stage (Morse 1990). For some species, and under certain conditions, settlement specificity contributes to the distribution of recruits in the natural environment (see Morse et al. 1988 for review). Many species are influenced by specific biochemical and or physical cues to settle and metamorphose in appropriate sites (see Crisp 1974, Butman 1987, Pawlik 1992, and Hadfield and Paul 2001, for reviews).

There are two general models proposed to explain the recruitment of sessile marine animals. The lottery, or stochastic, model states that larval settlement and metamorphosis occur randomly as larvae arrive at a given location and locate available substrata, and that subsequent post-metamorphic events shape the final distribution of species (Morse et al. 1988). In contrast, the deterministic model states that organisms respond actively to specific environmental parameters that determine the initial events of settlement and metamorphosis and that these contribute to shape the final population distribution patterns (Morse et al. 1988).

Settlement specificity implies active substratum selection. In almost all instances, the selected substratum is biogenic, and the cues mediating specificity or enhancement of settlement are believed to be chemical in nature (Pawlik 1992). Categories of substratum-specific settlement exhibited by marine invertebrates include gregarious, associative, and hierarchic (Crisp 1974).

Gregarious settlement occurs when larvae settle in response to the presence of adults, juveniles, or recent recruits of the same species (Hadfield and Paul 2001). For example, an unidentified waterborne cue emitted from the adult body of the polychaete *Hydroides dianthus* induces settlement and metamorphosis in their larvae (Toonen and Pawlik 1996). Conspecific adult tubes of *Hydroides ezoensis* contain a biochemical cue involved in larval metamorphosis (Wantanabe et al. 1998). A waterborne chemical associated with adult barnacles, *Balanus amphitrite*, induces settlement of the barnacle cyprid (Rittschof 1985, 1993).

Associative settlement describes the settlement of invertebrate larvae specifically upon other species, both plants and animals (Crisp 1974). Hermaphroditic cyprid larvae of *Conopea galeatus* settle in the axial skeleton of specific gorgonians and cannot survive without access to this substratum. This species also produces specialized male cyprids that settle solely on the external shell of the adult barnacle (Gomez 1973). Phoretic barnacles, those that settle on marine mammals, sea turtles, and sea snakes, are found only on a few host species; this suggests that settlement cues may be involved (Lewis 1978). The predatory nudibranch *Phestilla sibogae* settles on the host prey coral *Porites compressa* in response to waterborne biochemical inducers released from the coral (Hadfield and Pennington 1990). Abalone larvae settle preferentially on red crustose coralline algae (Morse et al. 1980). Crustose coralline algae also promote recruitment of various species of corals (Morse 1990). Heyward and Negri (1999) investigated a variety of natural inducers of coral larval metamorphosis that included crustose coralline algae and coral skeleton, as well as their extracts, all of which induced metamorphic activity. Calvo et al. (1998) observed that the juveniles of *Dendropoma petreaum* settle specifically on the crustose coralline algae *Spongites notarissi*. Juveniles of *Dendropoma corrodens* settle on the crustose coralline algae *Lithothamnion* sp. close to adult female shells (Miloslavich and Penchaszadeh 1992). *Dendropoma corallinaceum* juveniles settle on the crustose coralline algae *Lithothamnion* sp. associated adult colonies (Hughes 1978).

Response to biofilms can also be considered a form of associative settlement. Recruitment in response to factors associated with biofilms has been documented for

many marine invertebrates. The polychaete *Hydroides elegans*, a common component of fouling communities, rapidly forms dense colonies on submerged hard surfaces. Their larvae settle and metamorphose in response to bacterial biofilms on those surfaces. The rate and density of their settlement and metamorphosis is regulated strictly by the density of the biofilm bacteria (Hadfield et al. 1994). The age of the biofilm correlates positively to the density of the biofilm and hence the rate of settlement by sessile marine invertebrates (Keough and Raimondi 1995). The involvement of bacteria and biofilms in induction of settlement and metamorphosis has been documented for many marine species (see Pawlik 1992, and Hadfield and Paul 2001, for extensive reviews).

There may be a hierarchy of cues through which larvae select a general habitat and then a specific site for settlement (Meadows and Campbell 1972). Crisp (1984) illustrated a hypothetical sequence of larval behaviors for barnacle cyprid larvae; at the time of settlement the larva responds sequentially to light, current, presence and proximity of conspecifics, surface texture, and surface hardness. If biological or physical conditions are unacceptable, the larva may return to the previous position in the sequence. This example illustrates the possible complexity of behaviors involved in larval or juvenile settlement and metamorphosis. Evidence for a hierarchy of biochemical signals has been found for larvae of the nudibranch *Onchidoris bilamellata*, a predator of barnacles. Seawater conditioned by the presence of adult barnacles induced competent larvae to cease swimming and begin "exploratory" behavior. Larvae did not metamorphose until they were in contact with adult barnacles. In this case,

independent biochemical cues are thought to govern the onset of "exploratory" behaviors and the induction of metamorphosis (Chia and Koss 1988).

Most vermetid species release benthic juveniles that are capable of crawling and are competent to settle within a day of hatching. These juveniles are also capable of delaying settlement for 4-5 days (Hughes 1979). Given ideal conditions, settlement will take place near the adult. Often conditions are not ideal because waves and currents may cause passive dispersal to occur. Determining the factors that mediate settlement of vermetid juveniles after hatching is the focus of this research. For the juvenile vermetids used in these bioassays, the term settlement is defined as attachment of the protochonch to the substratum followed by the emergence of the teloconch.

My research addressed several basic questions surrounding the settlement behavior of five vermetid species found on Guam: *Dendropoma gregaria*, *Dendropoma platypus*, *Dendropoma maxima*, *Serpulorbis* n. sp., and *Petaloconchus keenae.* Do vermetid juveniles have a settlement preference? If so, are they attracted to it actively, as in the case of a water-soluble biochemical attractant, or is some aspect of the substratum responsible for settlement, such as texture or a surface-bound biochemical inducer? Is there a delay in settlement when only non-preferred substrata are available, and will they settle on non-preferred substratum when preferred substratum is not available? Are prematurely hatched larvae capable of survival and settlement? From an ecological perspective, the answers to these questions will benefit investigation of dispersal, recruitment, and the factors that govern the make up of adult vermetid assemblages, as well as act as a model for other benthic reef dwelling species.

METHODS AND MATERIALS

Species used in bioassays

The observations and laboratory experiments discussed here were conducted from April through August of 2003. They were performed on five of the vermetid species found in Guam: *Dendropoma gregaria*, *D. platypus*, *D. maxima*, *Petaloconchus keenae*, and *Serpulorbis* n. sp.

Collection and maintenance of adults

Adult vermetids were collected from five sites around Guam, Mariana Islands (Western Pacific). At Pago Bay reef flat all five species were collected; at Pago Bay rimmed terraces *Dendropoma gregaria* and *D. platypus*; from the Ylig Point ridges *D. gregaria* was collected; *D. gregaria*, *D. platypus*, *D. maxima*, and *Petalconchus keenae* were collected at Merizo reef flat; and *D. maxima* and *P. keenae* were collected at Luminao reef flat (Figure 1). A chisel and hammer were used to remove individuals and colonies intact on hard substrata, and to remove coral branches that contained embedded adults. Adults embedded in loose rubble were collected by hand. After return to the laboratory, adults were kept intact on their substrata and maintained in 9-to 12-liter plastic tubs containing aerated seawater. The seawater in the tubs was changed daily when larvae and juveniles were being collected. Alternately, adults were maintained in open, flow-through seawater water tables.

Collection of juveniles and larvae

Juveniles and larvae were removed from substrata by applying a stream of water from a large pipet and washing them into the adult holding tub. The wash water from

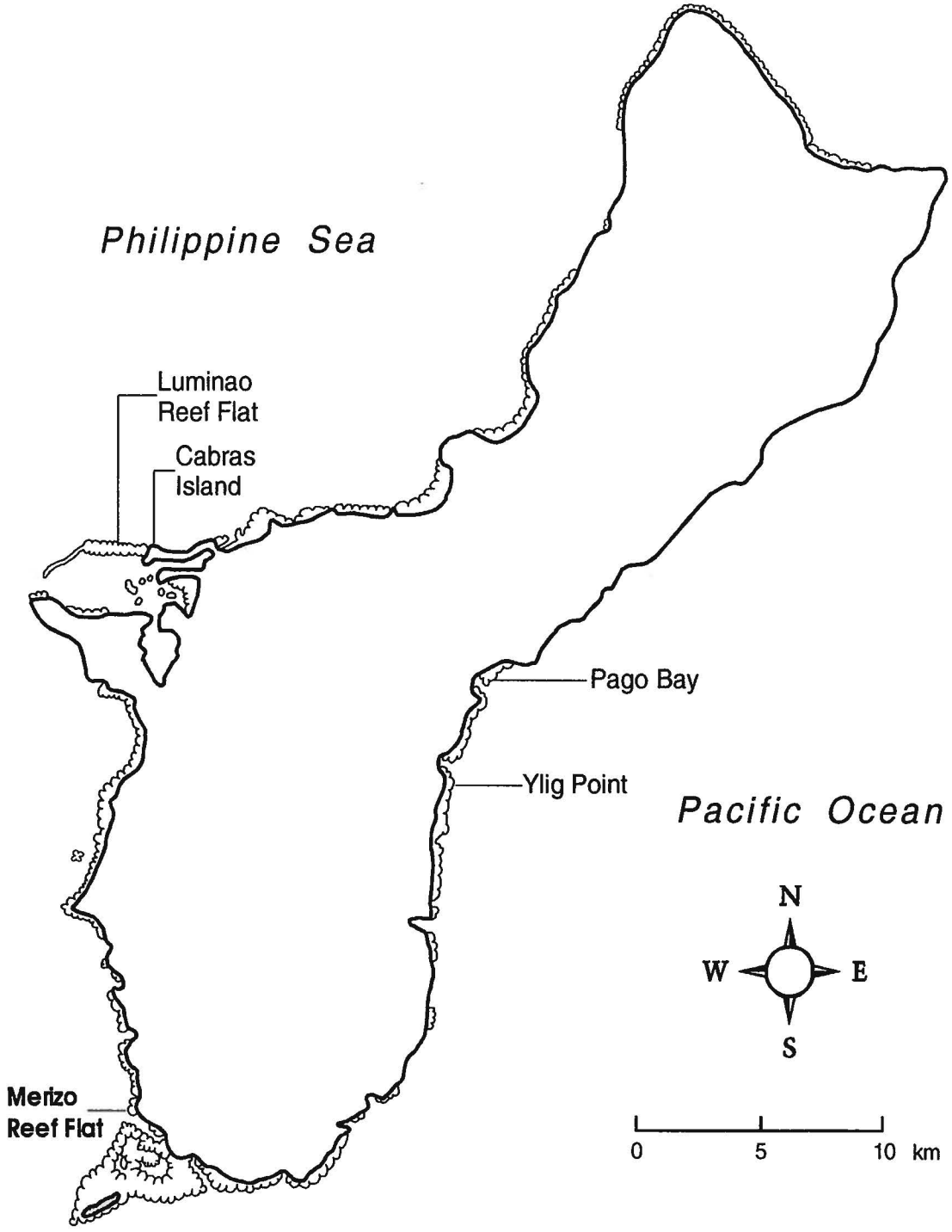


Figure 1. Map of Guam depicting collection sites.

the tubs was then filtered through a 90-μm mesh sieve and the contents of the sieve were rinsed into a 250-ml glass bowl. While viewed through a dissecting microscope, juveniles and larvae were then collected with a glass Pasteur pipette, placed in 10-ml petri dishes, and cleaned of debris in preparation for bioassays.

Bioassay design

Ten juveniles or larvae were added to 250-ml individual glass bowls containing 200 ml of unfiltered seawater and the treatment substratum. Five treatment replicates and five seawater controls were used for each bioassay. Treatment substrata included "reef rock" encrusted with the crustose coralline algae *Hydrolithon reinboldii*, "beach rock" encrusted with the crustose coralline algae *H. reinboldii* skeleton that was sun bleached on the shore of Pago Bay, kiln fired ceramic tile prepared by the University of Guam art department, and for *Dendropoma maxima*, pieces of the living hydrocoral *Millepora platyphylla* that had areas of living tissue as well as exposed skeleton. Seawater in glass bowls was used as a control. Bioassay bowls were maintained on the lanai of the Marine Laboratory at ambient air temperature (27 to 31°C). Bioassays were conducted for 1-3 days without water change. The water was not changed because the mechanical action of changing the water could disturb the settling juveniles. The bioassay bowls were set up in a randomized block design to increase the probability of independence among replicates. Bioassays were checked for settlement of the juveniles every 24 hours. Settlement was considered complete when a juvenile protoconch was cemented to the substratum and teloconch growth became evident. Data were recorded for treatments and controls as the total proportion of larvae settled per day on

substratum or the glass of the bowl out of the total number of larvae in the bioassay bowl. This proportion was reported as a percentage.

Observations. Vermetid adults were collected intact from the field on pieces of their natural substrata. Specimens of each species were put in individual holding tubs for several days to allow the juveniles they released subsequently to settle. Observations were made to determine whether the juveniles settled on the adult substratum, the walls of the holding tubs, or if they settled at all.

Bioassay 1. This bioassay was designed to determine if a water-soluble chemical emitted from the treatment substratum was responsible for attracting juveniles to suitable substrata and inducing settlement. Reef rocks, approximately 4 cm in diameter and 1-2 cm high and encrusted with live *Hydrolithon reinboldii*, were collected from Pago Bay reef flat, cleaned of debris, and put in 250-ml glass bowls with 200 ml of seawater. Sun bleached beach rock encrusted with *H. reinboldii* skeleton, collected from the shore at Pago Bay, and ceramic tiles were used as other settlement choices. Glass bowls containing seawater only were used as controls. One piece of test substratum was put in each of the treatment bowls along the side of the bowl; juveniles were placed on the bottom of the glass bowl on the opposite side not in contact with the substrata. It was thought that a gradient of the water-soluble cue would diffuse in the water and direct the juveniles to the substratum, where they would crawl up on the substratum and settle. Three species were used in this bioassay: *D. gregaria*, *D. platypus*, and *Serpulorbis* n. sp. The duration of each bioassay was three days.

Bioassay 2. This bioassay was designed to determine if the surface texture or a surface- bound biochemical influenced settlement behavior. Juveniles were placed directly on offered substrata: reef rock, beach rock, ceramic tile, or live *Millipora platyphylla* that had areas of exposed skeleton, one piece of substratum per treatment bowl. Glass bowls filled only with seawater were used as a control. Three species were used in this bioassay, *D. gregaria*, *D. platypus*, and *D. maxima*. The duration of this bioassay was three days for *D. gregaria* and *D. platypus* and two days for *D. maxima*.

Bioassay 3. This bioassay was conducted to determine if juveniles would actively search for preferred substratum. Beach rock, determined previously as non-preferred substrata in Bioassay 2, was placed on the bottom of treatment bowls, and reef rock, a preferred substratum, was placed on top in direct contact with the beach rock. Then the juveniles were placed directly on the beach rock. Beach rock and glass bowls with seawater alone were used as controls as they had proved to be non-preferred substrata. Four species were used in this bioassay: *D. gregaria*, *D. platypus*, *Serpulorbis* n. sp., and *P. keenae*. The duration of this bioassay was three days for *D. gregaria* and *P. keenae* and two days for *D. platypus* and *Serpulorbis* n. sp.

Bioassay 4. To determine if a surface-bound biochemical substance was responsible for the induction of settlement, a crude methanol extraction of *Hydrolithon reinboldii* was prepared. Pieces of glass encrusted with the crustose coralline algae *H. reinboldii* were collected at Pago Bay. Thirty grams of the crustose coralline algae were separated from the glass with a hammer, and then made into a paste with a mortar and pestle and 0.2-μm-filtered seawater. The paste was washed in 0.2-μm-filtered seawater,

centrifuged into a pellet, and the supernatant discarded. Three washes were conducted. The final pellets were then added to 500 ml of methanol and stirred for 24 hours at 20°C. The slurry was filtered through fiberglass filters, and the filtrate saved. The filtrate was dried partially with a Rotovap, and a Speed Vac was then used to finish the drying process. The residue was weighed and re-suspended in 10 ml of methanol. The final stock solution concentration was 8.38 mg/ml. From the stock solution, 500 µl were adsorbed onto beach rock, dried, and offered as a treatment in glass bowls filled with 200 ml of seawater. Reef rock was used as a positive control, and beach rock and seawater in glass bowls as negative controls. The juveniles were placed directly on the substratum. Four species were used in this bioassay, *D. gregaria*, *D. platypus*, *D. maxima*, and *Serpulorbis* n. sp. The duration of this bioassay was 24 hours.

Bioassay 5. This bioassay was designed to determine if encapsulated larvae could survive when removed mechanically from their egg capsule prior to natural hatching and if they had a settlement preference. From observation, the larvae were in the late veliger stage, that is, with velum, apex full of yolk, and an underdeveloped foot. The larvae were placed directly on the offered reef rock or beach rock substratum, or placed in glass bowls filled with seawater alone. Three species were used in this bioassay, *D. gregaria*, *D. platypus*, and *D. maxima*. The duration of this bioassay was two days.

Statistical analysis. A Bartlett's Test and an F-test were conducted on all bioassays to confirm homogeneity of variance among groups. Data were arcsine transformed and two-way analysis of variance (ANOVA) tests were performed with the software program Statview to compare the effect of treatments and days and their interaction.

RESULTS

Observations. Juveniles were found on specific types of substrata where adults occurred. Juvenile *Dendropoma gregaria* (Figure 2A) were found attached to the shells of adult conspecifics, live *Hydrolithon reinboldii*, or coralline algae skeleton (Figure 2B, 2C, and 2D). Juvenile *D. platypus* (Figure 3A) were also observed attached to *H. reinboldii* or occasionally the operculum of a conspecific (Figure 3B and 3C). Juveniles of *D. maxima* (Figure 4A) were consistently found attached to the coral skeleton of *Millepora platyphylla* or *Porities cylindrica* close to the living portion of the colony. Most juveniles were attached within centimeters of an adult vermetid on the coral skeleton (Figure 4B). Juveniles were also observed attached to the living coral, and in some cases they settled gregariously in groups consisting of two or three individuals (Figure 4C). Settlement in *D. maxima* also occurred on *H. reinboldii* when it was encrusted on a section of the adult substratum (Figure 4D). Juveniles of *Petaloconchus keenae* (Figure 5A) attached to live *H. reinboldii* encrusted on the coral skeleton of *P. cylindrica* within millimeters of the conspecific adults or on reef rock encrusted with *H. reinboldii* (Figure 5B and 5C). *Serpulorbis* n. sp. juveniles (Figure 5D) also settled within close proximity of the conspecific adults on coral skeleton or reef rock encrusted with live *H. reinboldii* (Figure 5E and 5F).

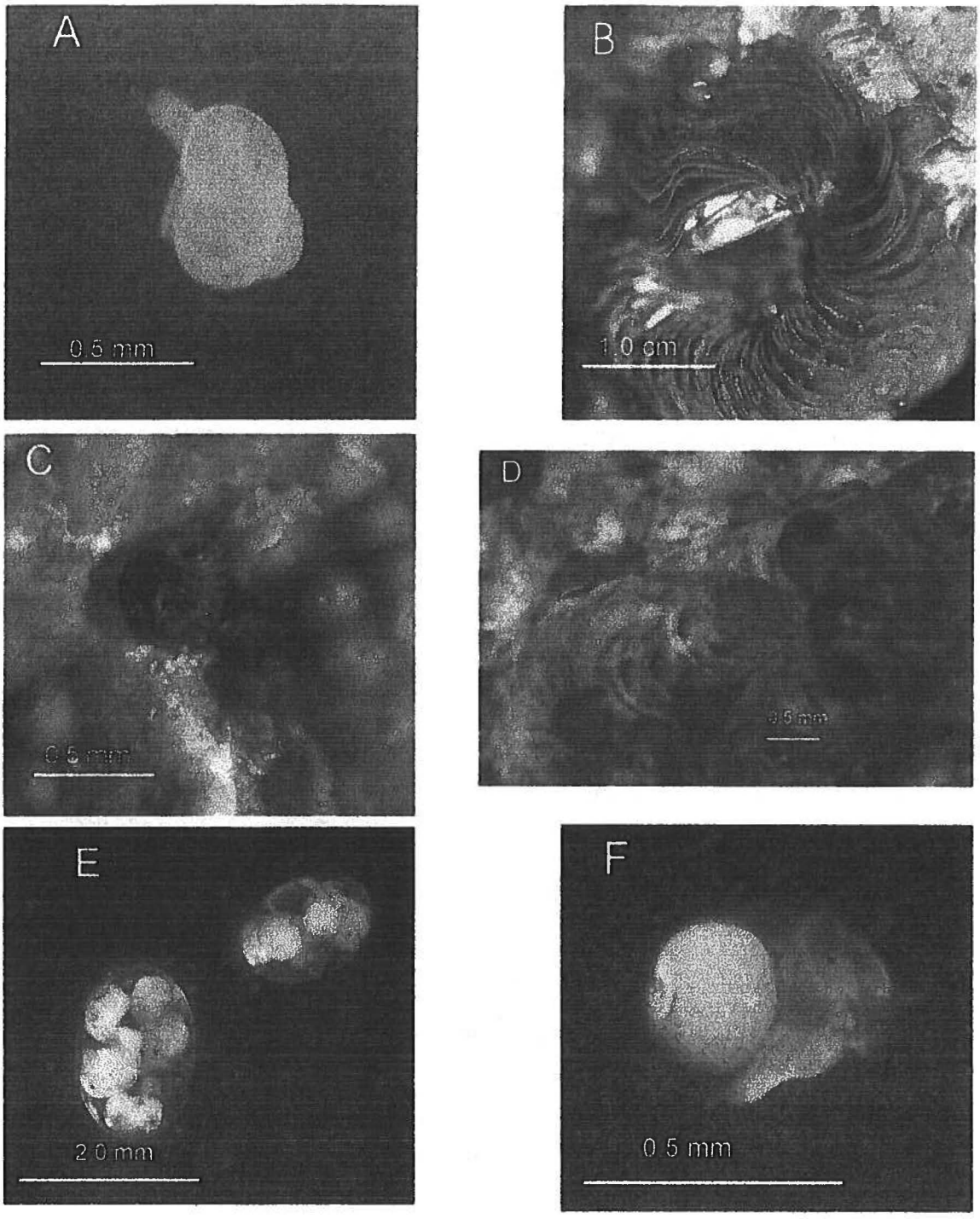


Figure 2. *Dendropoma gregaria* juveniles and larvae. (A) Juvenile, (B) juvenile settled on conspecific adult tube, (C) juvenile settled on *Hydrolithon reindboldii.* (D) juveniles settled gregariously. (E) encapsulated larvae, and (F) larva.

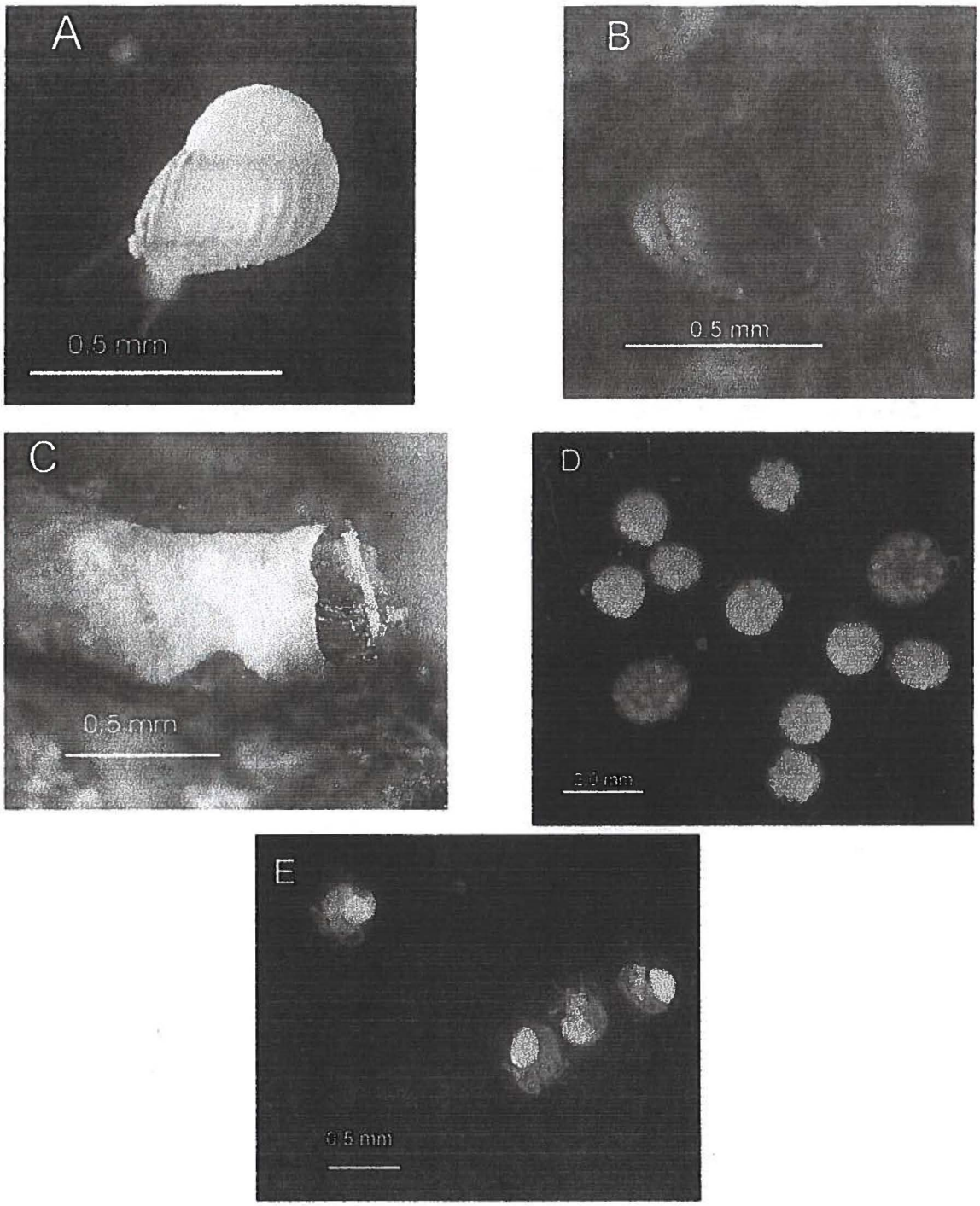


Figure 3. *Dendropoma platypus* juveniles and larvae. (A) Juvenile, (B) juvenile settled on live *Hydrolithon reinboldii*,(C) juvenile settled on adult operculum, (D) encapsulated larvae, and (E) larvae.

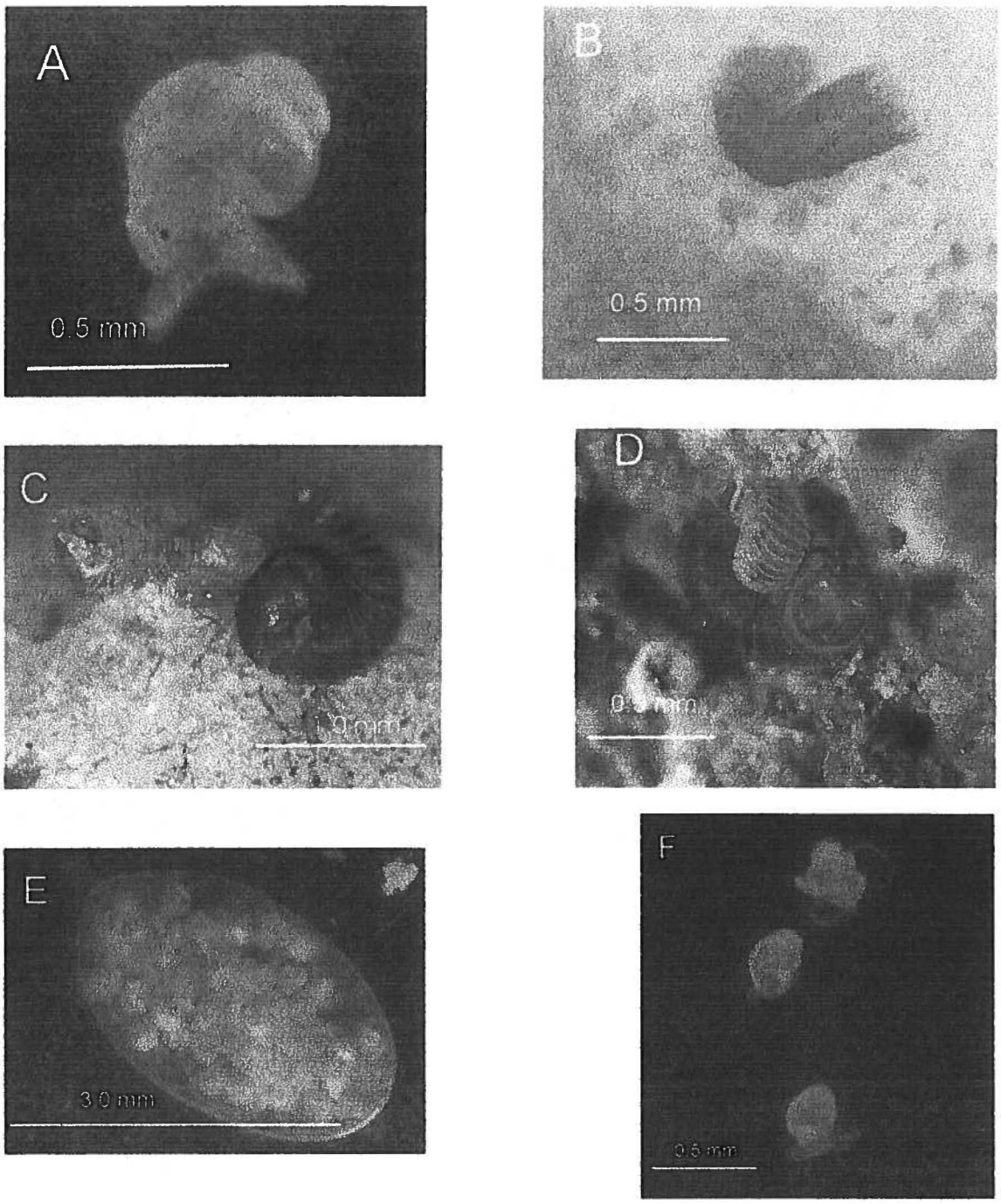


Figure 4. *Dendropoma maxima* juveniles and larvae. (A) Juvenile, (B) juvenile settled on coral skeleton near live coral, (C) juveniles settled gregariously on live coral, (D) juvenile settled on live *Hydrolithon reinboldii*, (E) encapsulated larvae, and (F) larvae.

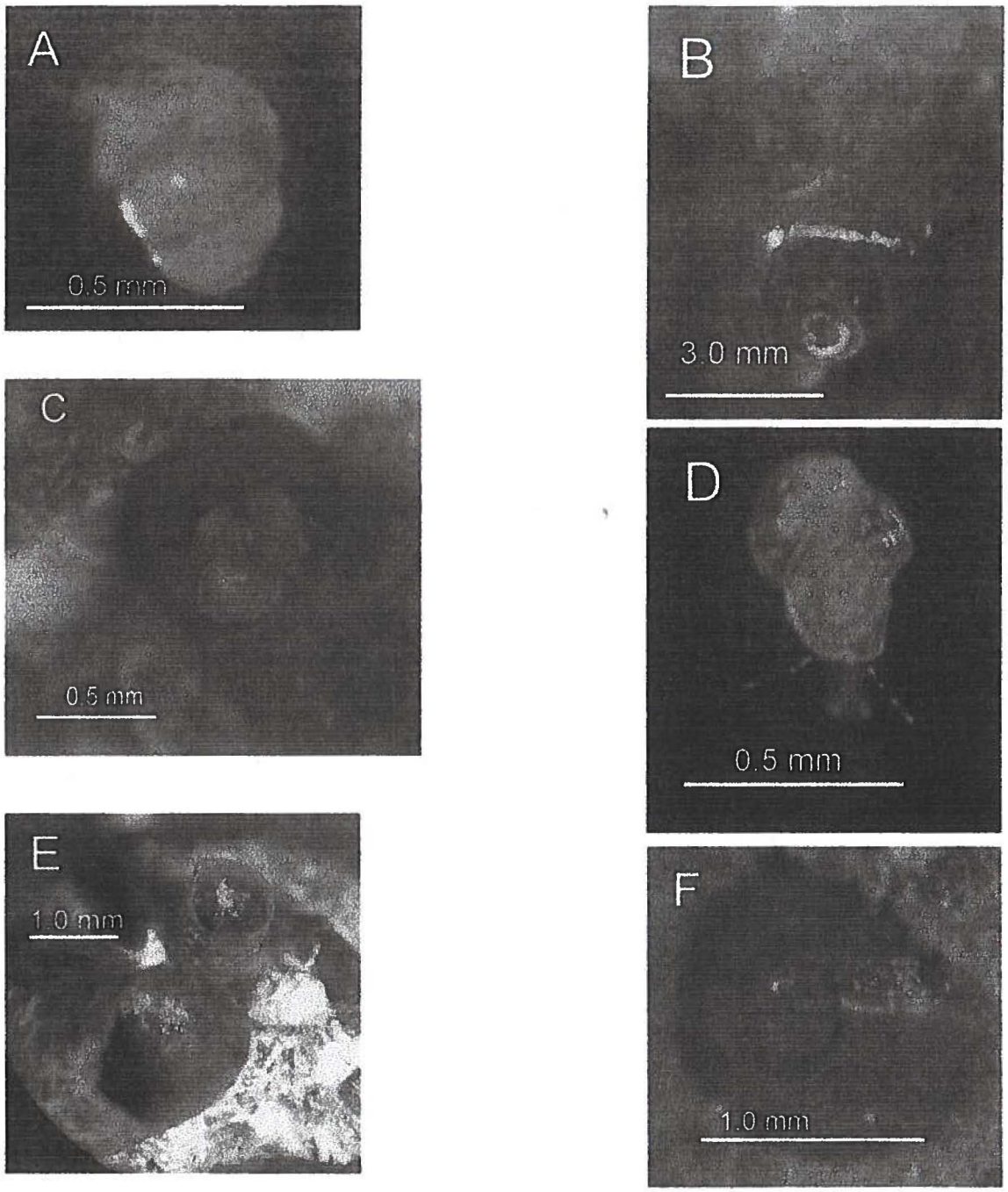


Figure 5. *Petaloconchus keenae* and *Serpulorbis* n. sp. juveniles. (A) *P. keenae* juvenile, (B) *P. keenae* juvenile settled on adult shell, (C) *P. keenae* juvenile settled on live *Hydrolithon reinboldii*, (D) *Serpulorbis* n. sp. juvenile,. (E) *Serpulorbis* n. sp. juvenile settled on coral skeleton next to adult tube, and (F) *Serpulorbis* n. sp. juvenile settled on *H. reinboldii*.

Bioassay 1. Juveniles placed on the bottom of glass bowls not in contact with offered substratum.

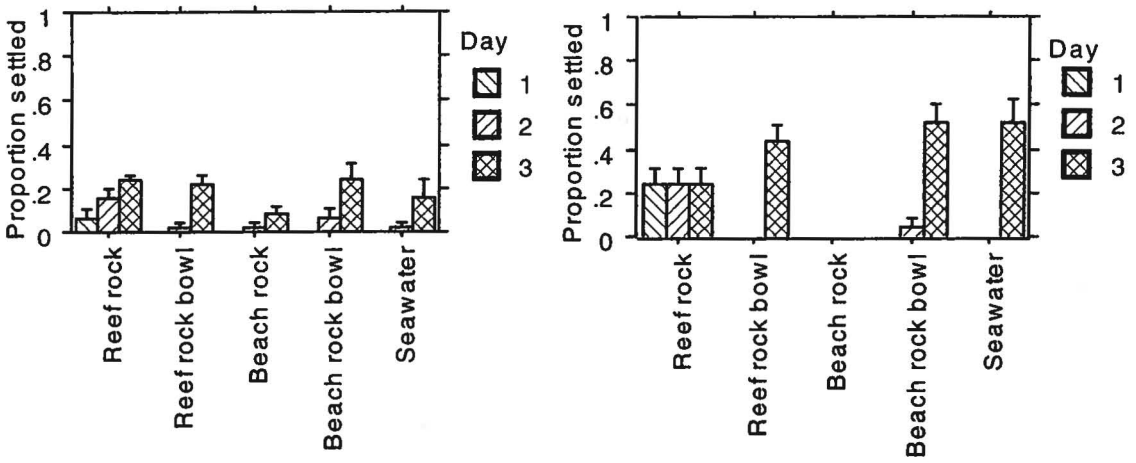
Dendropoma gregaria. When juveniles of *D. gregaria* juveniles were offered reef rock, 6 % settled on Day 1, 22 % settled on the reef rock by Day 3, and 22 % settled on the glass by Day 3 . When offered substratum was beach rock, 8 % settled on beach rock and 24 % settled on the glass by Day 3. In seawater alone, 16 % settled on the glass by Day 3 (Table 1 & Figure 6). There was a significant difference among treatments ($P = 0.0035$) and over the days ($P<0.0001$). However, there was no significant interaction between treatment and day ($P = 0.3914$).

Dendropoma platypus. When juveniles of *D. platypus* were offered reef rock, 24 % of settled by Day 1. There was no further settlement on the reef rock, and by Day 3, 44 % had settled on the glass. There was no settlement on the beach rock, and 52 % settled on the glass by Day 3. In the bowls with seawater alone, 52 % had settled on the glass by Day 3 (Table 1 & Figure 6). There was a significant difference among treatments, days, and interaction between treatment and day ($P<0.0001$).

***Serpulorbis* n. sp.** Eight percent of the *Serpulorbis* n. sp. juveniles settled on reef rock on Day 1, and 40 % settled by day 3. By Day 3, 48% settled on the glass of the bowls. No juveniles settled on the beach rock and by Day 3, 64 % had settled on the glass of the bowls. By Day 3, 80 % settled on the glass of the bowls with seawater alone (Table 1 & Figure 6). There was a significant difference among treatments, days, and interaction between treatment and day ($P<0.0001$).

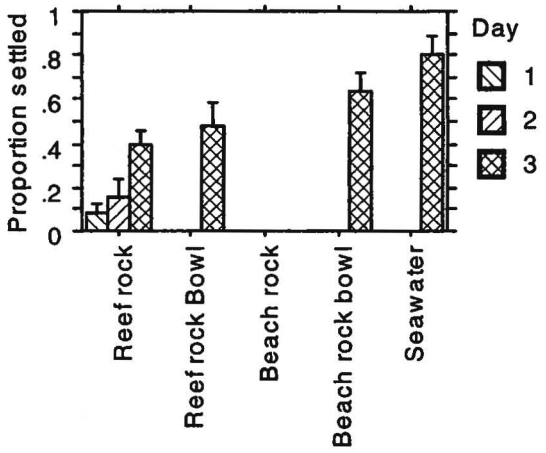
Table 1. Mean proportion and standard error of settlement for Bioassay1.

Species and day	Reef rock	Reef rock bowl	Beach rock	Beach rock bowl	Seawater
Dendropoma gregaria d1	0.06±0.04	0±0	0±0	0±0	0±0
Dendropoma gregaria d2	0.14±0.04	0.02±0.02	0.02±0.02	0.06±0.04	0.02±0.02
Dendropoma gregaria d3	0.22±0.037	0.22±0.037	0.08±0.037	0.24±0.068	0.16±0.081
Dendropoma platypus d1	0.240±0.075	0±0	0±0	0±0	0±0
Dendropoma platypus d2	0.240±0.075	0±0	0±0	0.04±0.04	0±0
Dendropoma platypus d3	0.240±0.075	0.44±0.75	0±0	0.52±0.08	0.52±0.102
Serpulorbis n. sp. d1	0.08±0.49	0±0	0±0	0±0	0±0
Serpulorbis n. sp. d2	0.16±0.075	0±0	0±0	0±0	0±0
Serpulorbis n. sp. d3	0.4±0.063	0.48±0.102	0±0	0.640±0.075	0.8±0.089



A. *Dendropoma gregaria*

B. *Dendropoma platypus*



C. *Serpulorbis* n. sp.

Figure 6. Bioassay 1: Proportion of vermetid juveniles settled when placed on the bottom of glass bowls not in contact with the offered substrata reef rock, beach rock, or seawater in glass bowls. Proportion of settlement is cumulative over days. (A) *Dendropoma gregaria*. (B) *Dendropoma platypus*. (C) *Serpulorbis* n. sp. Error bars depict standard error.

Bioassay 2. Juveniles placed directly on offered substrata.

Dendropoma gregaria. One-hundred percent of the *D. gregaria* juveniles settled on the reef rock by Day 1. No juveniles settled on the ceramic tile, and 56 % settled on the glass by Day 3. By Day 3, 60 % settled on the glass with seawater alone (Table 2 & Figure 7). There was a significant difference among treatments, day, and interaction between treatment and day ($P<0.0001$).

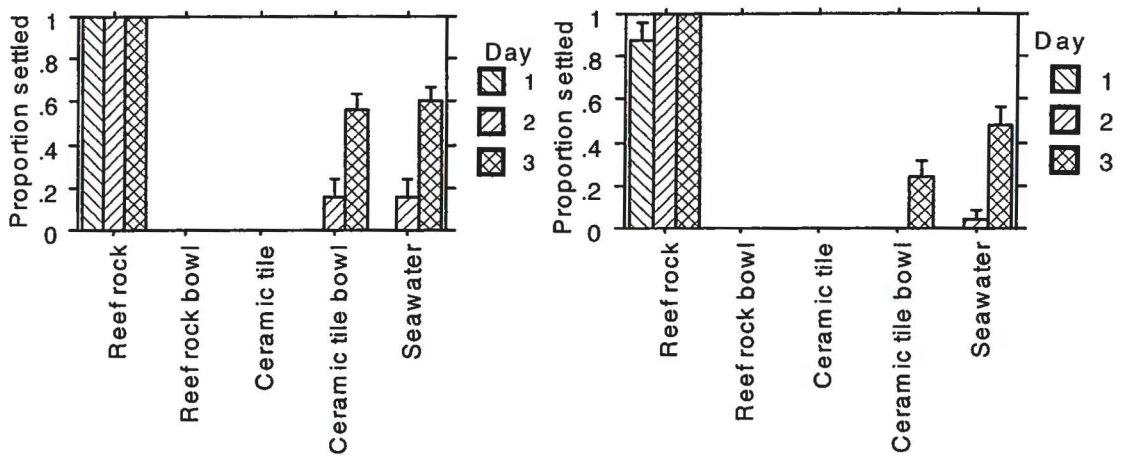
Dendropoma platypus. Eighty-eight percent of the *D. platypus* juveniles settled on reef rock on Day 1, and the remainder by Day 2. No juveniles settled on the ceramic tile, and 24 % settled on the glass by Day 3. Forty-eight percent settled on the glass with the seawater treatment by Day 3 (Table 2 & Figure 7). There was a significant difference between treatment, day, and interaction between treatment and day ($P<0.0001$).

Dendropoma maxima. Twelve percent of *D. maxima* juveniles settled on the live coral on Day 1, and 14% settled by Day 2. Eighty-six percent of the juveniles settled on the skeleton portion of the coral by Day 1, and none settled on the glass. No juveniles settled on the ceramic tile or the glass. Two percent settled on the beach rock on Day 2, and none settled on the glass. No juveniles settled on glass in the seawater treatment (Table 2 & Figure 7). There was a significant difference among treatments ($P<0.001$). There was no significant difference among days or interaction between treatment and day ($P = 0.8768$ and $P > 0.999$, respectfully).

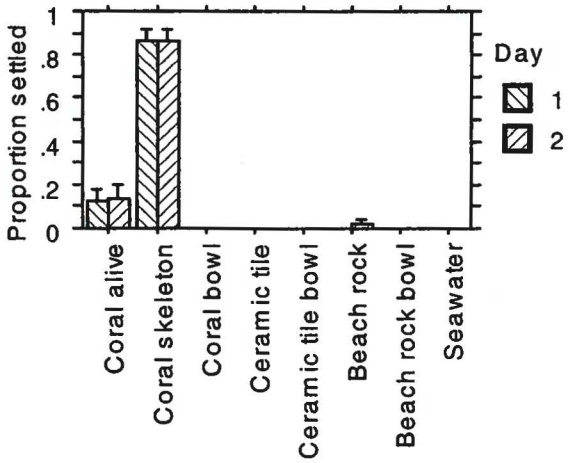
Table 2. Mean proportion and standard error of settlement for Bioassay 2.

Species and day	Reef rock	Reef rock bowl	Beach rock	Beach rock bowl	Ceramic tile	Ceramic tile bowl	Coral live	Coral skeleton	Coral bowl	Seawater
Dendropoma gregaria d1	1.0±0	0±0	NA*	NA	0±0	0±0	NA	NA	NA	0±0
Dendropoma gregaria d2	1.0±0	0±0	NA	NA	0±0	0.16±0.075	NA	NA	NA	0.16±0.075
Dendropoma gregaria d3	1.0±0	0±0	NA	NA	0±0	0.56±0.075	NA	NA	NA	0.60±0.063
Dendropoma platypus d1	0.88±0.08	0±0	NA	NA	0±0	0±0	NA	NA	NA	0±0
Dendropoma platypus d2	1.0±0	0±0	NA	NA	0±0	0±0	NA	NA	NA	0.04±0.04
Dendropoma platypus d3	1.0±0	0±0	NA	NA	0±0	0.24±0.075	NA	NA	NA	0.48±0.08
Dendropoma maxima d1	NA	NA	0±0	0±0	0±0	0±0	0.12±0.058	0.86±0.06	0±0	0±0
Dendropoma maxima d2	NA	NA	0.02±0.02	0±0	0±0	0±0	0.14±0.06	0.86±0.06	0±0	0±0

*NA: Not Assayed



A. *Dendropoma gregaria* B. *Dendropoma platypus*



C. *Dendropoma maxima*

Figure 7. Bioassay 2: Proportion of vermetid juveniles settled when place directly on offered substrata, reef rock, ceramic tile, beach rock, *Millepora platyphylla*, or seawater in glass bowls. Proportion of settlement is cumulative over days.. (A) *Dendropoma gregaria*. (B) *Dendropoma platypus*. (C) *Dendropoma maxima.* Error bars depict standard error.

Bioassay 3. Juveniles placed directly on beach rock with reef rock placed on top of it.

Dendropoma gregaria. On Day 1, 96 % of the *D. gregaria* juveniles crawled up on the reef rock and settled, and by the second day all were settled on the reef rock. Four percent of the juveniles settled on the beach rock alone, and 64 % of them settled on the glass Day 3. By Day 3, 60 % of the juveniles settled on glass in the seawater treatment (Table 3 & Figure 8). There was a significant difference between treatment, day, and interaction between treatment and day ($P<0.0001$).

Dendropoma platypus. By the first day, 96 % of the *D. platypus* juveniles crawled up to the reef rock and settled. No more juveniles settled on the reef rock or the glass by the following day. By Day 2, no juveniles settled on the beach rock alone, and 4 % settled on the glass. By Day 2, 4 % settled on glass in the seawater treatment (Table 3 & Figure 8). There was a significant difference for treatment ($P<0.0001$). There was no significant difference for day or interaction between treatment and day, ($P = 0.6747$ and $P = 0.9962$, respectively).

***Serpulorbis* n. sp.** One-hundred percent of the *Serpulorbis* n. sp. juveniles crawled up to the reef rock and settled by the first day. No juveniles settled on the beach rock alone, and 8 % settled on the glass by Day 2. Four percent settled on glass in the seawater treatment by the second day (Table 3 & Figure 8). There was a significant difference for treatment ($P<0.0001$). There was not a significant difference for day or interaction between treatment and day ($P = 0.0638$ and $P = 0.1575$, respectively).

Petaloconchus keenae. Ninety-eight percent of the *P. keenae* juveniles crawled up and settled on the reef rock on the first day, and none settled on the glass. No juveniles settled on the ceramic tile, and 6 % settled on glass by Day 3. By the third day, four percent settled on the beach rock alone, and 4 % settled on the glass. Four percent settled on glass in the seawater treatment by the third day (Table 3 & Figure 8). There was a significant difference for treatment ($P<0.0001$). There was no significant difference for interaction for day and between treatment and day ($P = 0.3575$ and $P = 0.9996$, respectively).

Table 3. Mean proportion and standard error of settlement for Bioassay 3.

Species and day	Reef rock	Reef rock bowl	Beach rock	Beach rock bowl	Ceramic tile	Ceramic tile bowl	Seawater
Dendropoma gregaria d1	0.96±0.04	0±0	0±0	0±0	NA*	NA	0±0
Dendropoma gregaria d2	1.0±0	0±0	0.04±0.04	0.16±0.075	NA	NA	0.06±0.049
Dendropoma gregaria d3	1.0±0	0±0	0.04±0.04	0.64±0.075	NA	NA	0.60±0.11
Dendropoma platypus d1	0.96±0.04	0±0	0±0	0±0	NA	NA	0±0
Dendropoma platypus d2	0.96±0.04	0±0	0±0	0.04±0.04	NA	NA	0.04±0.04
Serpulorbis n. sp. d1	1.0±0	0±0	0±0	0±0	NA	NA	0±0
Serpulorbis n. sp. d2	1.0±0	0±0	0±0	0.08±0.049	NA	NA	0.04±0.04
Petaloconchus keenae d1	0.98±0.02	0±0	0±0	0±0	0±0	0±0	0±0
Petaloconchus keenae d2	0.98±0.02	0±0	0.02±0.02	0±0	0±0	0±0	0±0
Petaloconchus keenae d3	0.98±0.02	0±0	0.04±0024	0.04±0.024	0±0	0.06±0.04	0.04±0.024

*NA: Not Assayed

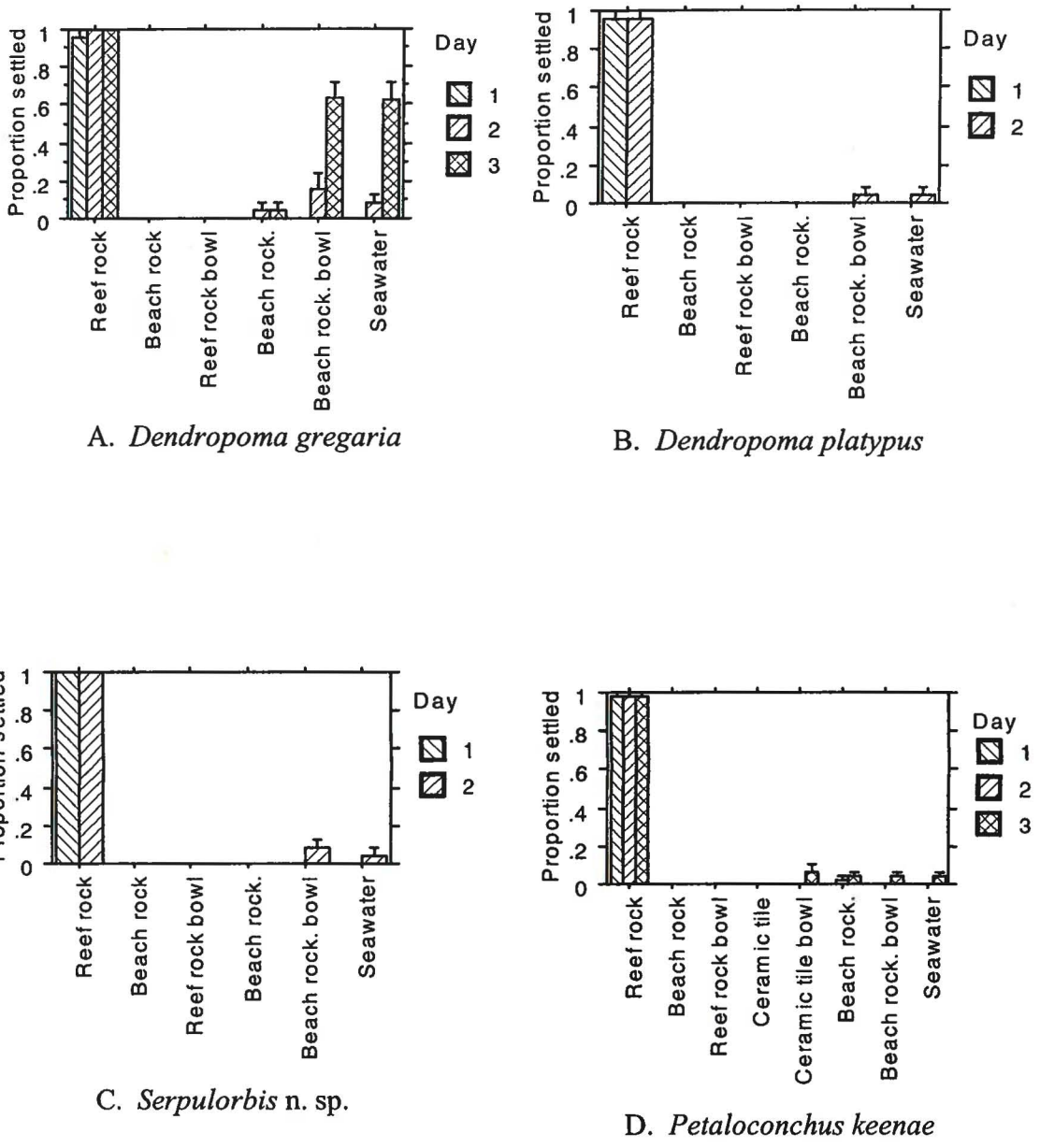


Figure 8. Bioassay 3: Proportion of vermetid juveniles settled when placed on beach rock with reef rock on top, beach rock, or seawater in glass bowls. *Petaloconchus keenae* was also offered ceramic tile. Proportion of settlement is cumulative over days. (A) *Dendropoma gregaria*. (B) *Dendropoma platypus*. (C) *Serpulorbis* n. sp. (D) *Petaloconchus keenae*. Error bars depict standard error.

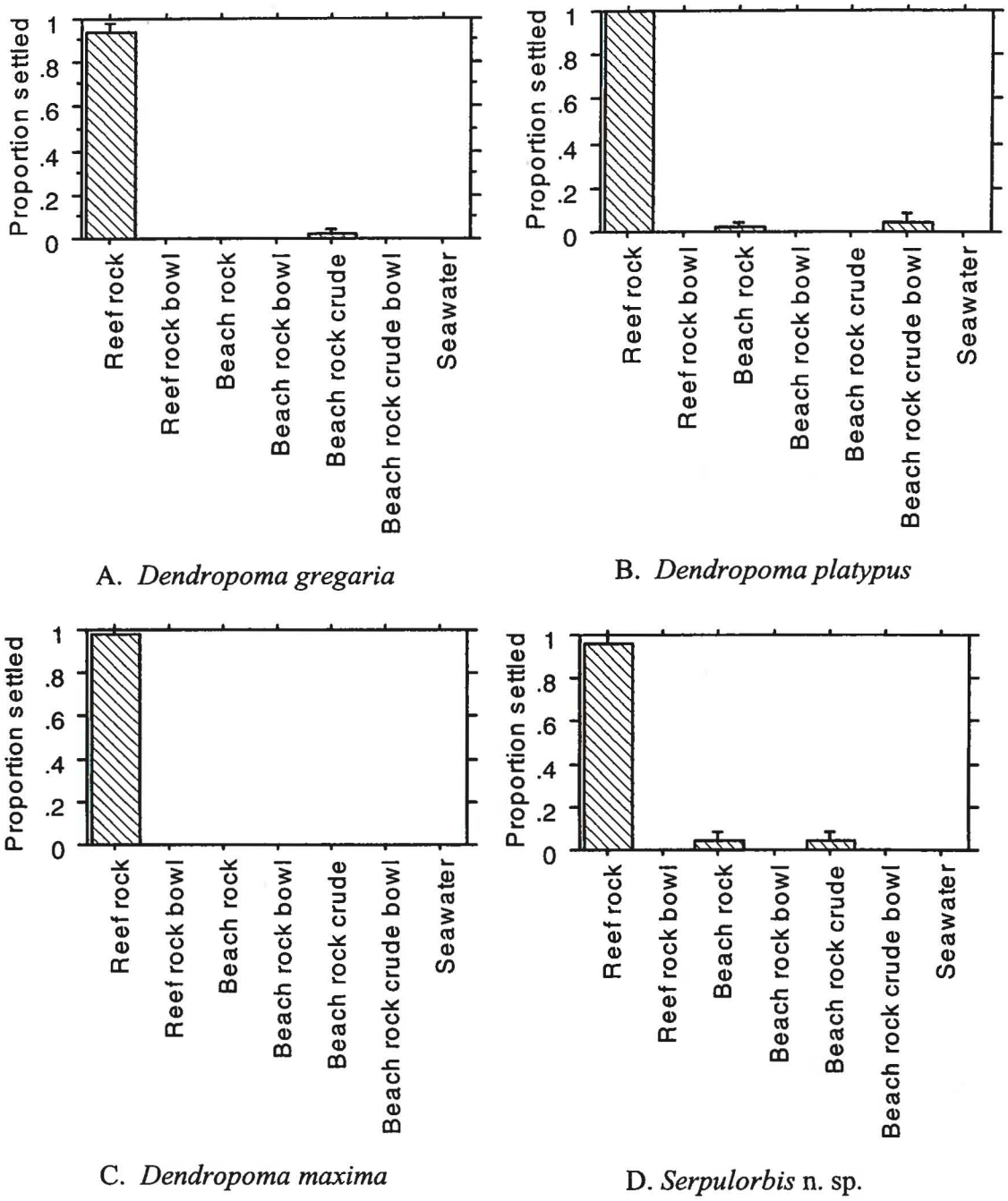
Bioassay 4. *Hydrolithon reinboldii* crude extract.

Dendropoma gregaria. Two percent of the *D. gregaria* juveniles settled on the beach rock with the crude extract adsorbed to it, while 94 % settled on the reef rock ($P < 0.0001$). None of the *D. gregaria* juveniles settled in the seawater treatment (Figure 9).

Dendropoma platypus. Four percent of the *D. platypus* juveniles settled on the beach rock with the adsorbed crude extract, and 100 % settled on the reef rock ($P < 0.0001$). Two percent settled on the beach rock. There was no settlement on the glass in the seawater treatment (Figure 9).

Dendropoma maxima. None of the *D. maxima* juveniles settled on the beach rock with the crude extract adsorbed to it, and 98 % of the juveniles settled on the reef rock ($P < 0.0001$). None of the juveniles settled on the other substrata (Figure 9).

***Serpulorbis* n. sp.** Four percent of the *Serpulorbis* n. sp. juveniles settled on the beach rock with the adsorbed crude extract, and 96 % settled on the reef rock ($P < 0.0001$). Four percent settled on the beach rock alone, and none settled on the glass in the seawater treatments (Figure 9).



A. *Dendropoma gregaria*

B. *Dendropoma platypus*

C. *Dendropoma maxima*

D. *Serpulorbis* n. sp.

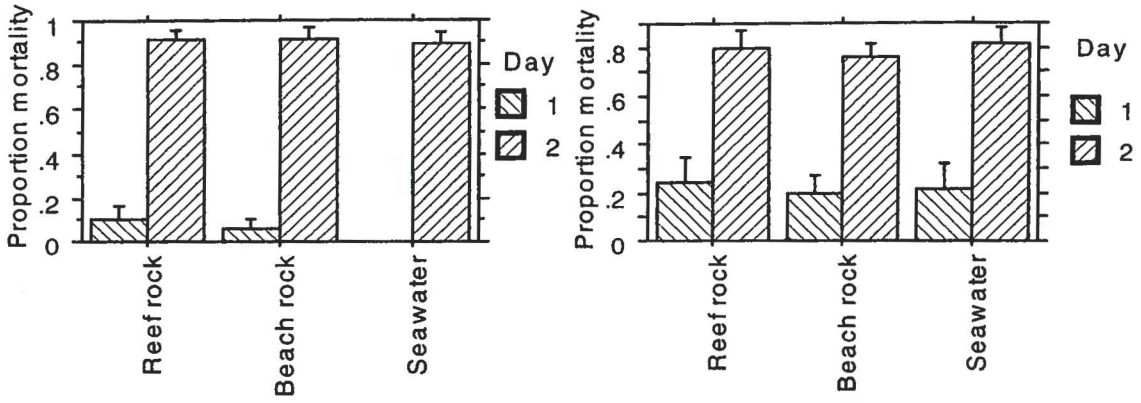
Figure 9. Bioassay 4: Proportion of vermetid juveniles that settled when offered beach rock with 500 µl of *Hydrolithon reinboldii* methanol extract adsorbed to its surface, reef rock, beach rock alone, or seawater in glass bowls. Proportion of settlement is cumulative over days. (A) *Dendropoma gregaria*. (B) *Dendropoma platypus*. (C) *Dendropoma maxima*. (D) *Serpulorbis* n. sp. Error bars depict standard error.

Bioassay 5. Mechanically release pre-hatched larvae.

Dendropoma gregaria. Ten percent of the *D. gregaria* larvae (Figure 2e and 2f) died the first day, and 90 % died by Day 2 (Figure 10). There was a significant difference over days ($P < 0.0001$). There was no significant difference in treatment or interaction ($P = 0.9476$ and $P = 0.9836$, respectively).

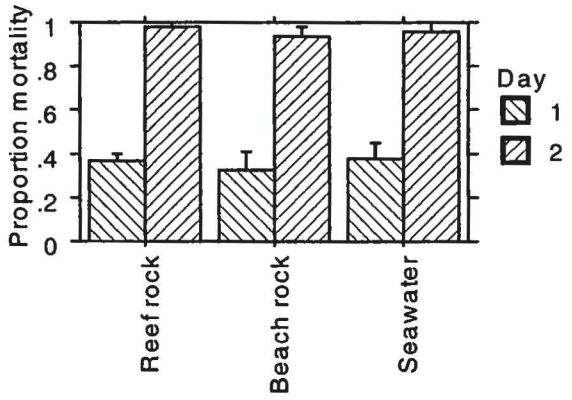
Dendropoma platypus. Twenty percent of the *D. platypus* larvae (Figure 3d and 3e) died the first day, and 80 % died by Day 2 (Figure 10). There was a significant difference over days ($P < 0.0001$). There was no significant difference in treatment or interaction ($P = 0.6909$ and $P = 0.8544$, respectively).

Dendropoma maxima. Thirty-five percent of the *D. maxima* larvae (Figure 4e and 4f) died the first day, and 95 % died by Day 2 (Figure 10). There was a significant difference over days ($P < 0.0001$). There was no significant difference in treatment or interaction ($P = 0.6565$ and $P = 0.8976$, respectively).



A. *Dendropoma gregaria*

B. *Dendropoma platypus*



C. *Dendropoma maxima*

Figure 10. Bioassay 5: Proportion of vermetid larvae settled when offered reef rock, beach rock or seawater in glass bowls. Proportion of mortality is cumulative over days. (A) *Dendropoma gregaria*. (B) *Dendropoma platypus*. (C) *Dendropoma maxima*. Error bars depict standard error.

DISCUSSION

Observations of juvenile settlement on adult substrata in the laboratory revealed a clear pattern of settlement preference that corresponded to observations of field habitat for each species. In the field, *Dendropoma gregaria* adults often form dense, monospecific intertidal aggregations that are generally on substrata encrusted with *H. reinboldii* (pers. obs.). In laboratory observations, *D. gregaria* juveniles were observed attached to the adult tubes and to *Hydrolithon reinboldii* near the adult. *D. platypus* adults are found singly on a variety of substrata on the reef flat or in low-density colonies that are usually encrusted with *H. reinboldii* (pers. obs.). In laboratory observations, *D. platypus* juveniles were found attached to *H. reinboldii* near adults, as well as on *H. reinboldii* skeleton, and occasionally on an adult operculum, which is often encrusted with *H. reinboldii*. In the field, *D. maxima* adults were often found embedded in live coral and less often on *H. reinboldii* encrusted pavements (pers. obs.). In laboratory observations, *D. maxima* juveniles settled near or on the live coral of the adult substratum or on *H. reinboldii*. *Petaloconchus keenae* adults are often found embedded in coral and on *H. reinboldii*-encrusted boulders and hard pavement on the reef flat and along the reef crest and reef front, although not in dense colonies (pers. obs.). In laboratory observations, *P. keenae* juveniles were found attached to *H. reinboldii* within centimeters of the adult and the living coral. *Serpulorbis* n. sp. adults are found in the field in low-density assemblages embedded in corals or on *H. reinboldii*-encrusted pavement or rubble (pers. obs.). In laboratory observations, *Serpulorbis* n. sp. juveniles

were found attached to coral skeleton on live coral colonies and reef rocks encrusted with *H. reinboldii*.

The results from Bioassay 1 indicate that crawling juveniles do not appear to be able to locate suitable substratum from a distance. By placing the juveniles on the smooth bottom of the glass bowls, without a detectable water-soluble cue to find suitable substratum, they would randomly encounter the offered substratum. If the encountered substratum was suitable for settlement, the juvenile would settle. For each species, the greatest amount of settlement on offered substratum was on the reef rock, although low in percentage when compared to the other bioassays. Settlement on the reef rock was 24 % on the first day for *D. platypus* and did not change; settlement increased over the days for *D. gregaria* to 22 % by the third day and to 40 % by the third day for *Serpulorbis* n. sp., indicating successful random encounters with suitable substratum. If the encountered substrata were not suitable, the juveniles would not settle, but they would continue random movement in the bowls for a period before settling on non-preferred substrata. In this bioassay most of the settlement occurred on the glass by the third day (see Table 1), and only 8 % of the *D. gregaria* juveniles settled on the beach rock. Here, the apparent factors regulating the rate of settlement were 1) the initial substratum the juveniles were placed on, 2) available suitable substratum, and 3) internal yolk reserves. Calvo et al. (1998) observed that the juveniles of *Dendropoma petraeum* metamorphose when the embryonic yolk is totally consumed. From observations of the bioassays presented here, juveniles that settled within 1-2 days after release had reserve yolk, as seen as the white colored apex of the juvenile protoconch. Hughes (1978) noted

that emergent juveniles of *Dendropoma corallinaceum* and *Serpulorbis natalensis* can delay settlement for 4 or 5 days by surviving on in the embryonic yolk. The results of this bioassay suggest that two modes of settlement induction may be occurring, an external cue that is associated with the reef rock and an internal cue that involves consumption of embryonic yolk. Hughes (1978) noted that *D. corallinaceum* juveniles delayed settlement for 4 to 5 days only when unfavorable surfaces, such as glass, were present. Chia and Koss (1985) observed that when larvae of *Onchidoris bilamellata* were placed in glass bowls with no treatment substratum available, the larvae would crawl without metamorphosing. However, when suitable substratum, such as living or dead barnacles, was available and the larvae made physical contact with it, crawling was restricted to the barnacle shell, and metamorphosis began within an hour after contact. The use of "Y" maze experiments may solve the question of whether a water-soluble chemical attracts juveniles to suitable substratum and induces settlement. The use of a "Y" maze has been effective in determining food preferences in the opisthobranch *Aplysia californica* (Audesirk 1975, Teyke et al. 1992).

When the vermetid juveniles were placed directly on the offered substratum, reef rock, or coral for *D. maxima*, juveniles of all the species used in this bioassay chose overwhelmingly these live biogenic substrata. This indicates that reef rock and coral are preferred suitable substrata. The texture of a suitable substratum may induce crawling behavior and a surface-bound chemical may induce settlement There was no settlement on the ceramic tile and little settlement on the beach rock after one day of treatment, indicating they are not preferred substrata. The texture of these substrata may have

induced crawling, but there was no apparent cue to settle. Settlement did increase by the third day on the glass and beach rock, indicating that the juveniles can delay settlement and do settle on non-preferred substrata. When the internal yolk is consumed settlement may become obligatory no matter what the substratum.

When the juveniles were placed directly on the non-preferred substratum beach rock with the preferred substratum reef rock sitting on top, settlement on the reef rock was high and approached 100 % within one day for juveniles of all three species used in this bioassay. This indicates that vermetid juveniles actively search for preferred substrata. In contrast to Bioassay 1, where juveniles were placed on glass, here juveniles were placed on a textured substratum, the beach rock, which was in direct contact to the reef rock. The texture of the beach rock may have induced crawling , and because of its proximity, the reef rock may have been found more readily by random encounter or there may have been a water-soluble cue that only is detectable at close range. Observations of direction and crawling rates on different substrata, such as glass, beach rock, and reef rock as well as different combinations of these substrata, may reveal behavioral searching patterns.

The results for Bioassay 4 were inconclusive. Settlement was very low or not at all on the beach rock that had the crude *Hydrolithon reinboldii* extract adsorbed into it. Settlement was high on the untreated reef rock after one day, however. The effectiveness of the crude extract may be in question. No dose response assays were run, and only a methanol extraction was performed. If there is an inductive chemical responsible for settlement, then a higher or lower dose may induce this settlement.

Calvo et al. (1998) cited several instances in laboratory settings where females released intact egg capsules, and suggested this may be a method of dispersal in the field. From Bioassay 5, it may be concluded that prematurely hatched larvae do not survive in laboratory assays. Miloslavich and Penchaszadeh (1992) observed that encapsulated embryos of *Vermetus* sp. died within hours of removal from the female mantle cavity. Kemph and Hadfield (1985), however, noted that when the facultative planktotrophic larvae of the nudibranch *Phestilla sibogae* were fed, they could survive up to 42 days and retain metamorphic competence. The planktotrophic larvae of the vermetid *Serpulorbis variabilis* feed for several weeks before becoming competent to settle, indicating that larval feeding is present in this family. In this bioassay the direct-developing, lecithotrophic larvae were deprived of inter-capsular yolk, and they were not fed. Determination if vermetid larvae of direct-developing species can feed should be an area of further investigation. In early bioassays juveniles of all five species did feed on the microalgae *Nanochloris* sp. (see below). If egg capsules were to remain intact or the larvae were capable of feeding this might be a means of dispersal.

All species of vermetid juveniles used in these bioassays demonstrated the ability to delay settlement when only non-preferred substrata were available. During early bioassays, juveniles were treated in10-ml petri dishes and fed single-cell microalgae (*Nanochloris* sp.). In a number of bioassays, juveniles survived for more than two weeks without settling, their intestines were green in color, and they released green fecal pellets. Apparently, the conditions in petri dishes in some of the assays were not suitable for settlement, but the juveniles did appear to feed by using ciliary filtering.

These early bioassays also suggested that biofilm was not an inducer of settlement. During the early bioassays, 0.2-μm-filtered seawater was used to fill the dishes, and the water was partially changed each day. The petri dishes were not cleaned or changed, however, giving ample time for biofilm to develop. Also, during these bioassays, pieces of substratum seasoned in the seawater tables and allowed to develop a biofilm were not chosen by the vermetid juveniles as preferred substrata. Again, the conditions in the petri dishes were apparently not suitable for bioassays, and this fact may have affected the results. Further investigation into the role of bacteria and microbial biofilms is needed.

Vermetid juveniles do appear to recognize suitable substrate, but how they do so remains unclear. It is apparent, however, that the surface texture or a surface-bound biochemical, or both, are involved in settlement. Juveniles do actively search the substratum for preferred settlement sites. What is unclear is the sensory method used. The juvenile propodium is a likely site of sensory receptors. Observations of juveniles during locomotion indicate that, as they move, they first touch the substratum with the propodium, then progress outward. Calvo et al. (1998) observed similar behavior with *Dendropoma petraeum*. Most authors note crawling behavior in their descriptions (see Hadfield et al. 1972, Hughes 1978, Miloslavich and Penchaszadeh 1992, and Calvo et al. 1998). In investigating the embryonic and juvenile nervous system of the prosobranch *Crepidila fornicata*, Dickinson et al. (1999) noted that the innervation of the larval foot consisted in part of catecholaminergic cells and their processes that extend to the epithelial surface of the foot. This innervation of the foot originated in the apical

sensory cells. The function of these putative sensory cells is uncertain, but they may be involved in metamorphosis. Dickinson et al. (2000) identified catecholaminergic neural cells in the foot of juvenile *Aplysia californica*, and Voronezhskaya et al. (1999) found similar catecholaminergic cells in the foot of the pond snail *Lymnaea stagnalis*. In each case these subepithelial somata sent projections that penetrated the epithelium and terminated on the surface of the foot. Although details about the role of these sensory cells and their relationship to metamorphosis were not given, a sensory role for these cells in the juvenile foot was indicated. Chia and Koss (1988) showed that for the larvae of the nudibranch *Onchidoris bilamellata* it was necessary for the larval foot to contact suitable substratum before metamorphosis occurred. Similar sensory innervation is likely to be found in the vermetid juvenile propodium, and may function in the location of suitable substratum, as well as induction of settlement.

From these experiments, it is clear that vermetid juveniles have settlement preferences, that they are capable of delaying settlement, and that settlement preferences change during their free-living juvenile life. When the adult habitat is in a stable area, it would be beneficial for juveniles to settle near the adults on suitable substratum (Hughes 1979). Benefits would include a stable food supply, close proximity to others for reproduction, and when dense colonies are formed, the exclusion of competition from other species (Hughes 1979). When the adult colony is in a marginal or poor area, the ability to delay settlement and change settlement preferences may be beneficial for dispersal to a better environment (Hughes 1979). Vermetids are adapted for both of these modes of recruitment, thus partially explaining their evolutionary success.

CONCLUSIONS

Vermetid juveniles appear to have settlement preferences for preferred substratum; they can delay settlement, and they will settle on non-preferred substratum when preferred substratum is not available. The component of the substratum that induces settlement remains unclear. How they locate preferred suitable substratum also remains unclear. Prematurely hatched veliger larvae did not survive in the laboratory setting.

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