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A Comparison of Aeration Methods and Diets for Larval Culture of the Edible Sea Urchin, Tripneustes ventricosus (Echinodermata: Echinoidea)

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A COMPARISON OF AERATION METHODS & DIETS FOR LARVAL CULTURE OF THE EDIBLE SEA URCHIN, *TRIPNEUSTES VENTRICOSUS* (ECHINODERMATA: ECHINOIDEA)

BY
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ABSTRACT

Tripneustes ventricosus (Lamarck 1816), a major near-shore herbivore in the Atlantic and Caribbean, has been harvested for human consumption in the Caribbean for centuries (Lawrence 2001a, b), occasionally at rates that exceed sustainability (Smith & Berkes 1991), and is among the species having economic importance (Lawrence & Bazhin 1998). *Tripneustes ventricosus* has recently been observed on the forereef controlling macroalgal growth in the absence of *Diadema antillarum* (Woodley 1999, Aronson & Precht 2000, Haley & Solandt 2001).

Large-scale culturing has the potential to produce *T. ventricosus* in sufficient numbers to be used for: bioremediation of coral reef degradation, restocking of near-shore habitats, and the development of an aquaculture industry for one or more Caribbean islands. Heretofore, *T. ventricosus* has never been cultured from fertilization to exotrophic (feeding) juvenile.

This work presents the results of experiments designed to measure the effectiveness of aeration methods and diets applicable for large-scale larval culture. Airlift aeration, used successfully in larger scale systems, was not effective in the 3.78-L (1-gallon) jars used for my experiments. Success was obtained in cultures reared without mechanical aeration. Fifteen percent of the larvae survived to day 33 and the 6-armed stage was reached. However, paddle aeration, used successfully in many small-scale experimental designs, produced the highest survival rates. *Rhodomonas* sp. produced the most rapid development (23 days to metamorphosis). *Isochrysis* aff. *galbana* (Tahitian strain) supported slower development (36 days to metamorphosis), but produced the highest (48%) survival rate. *Cryptomonas* sp., a mixture of *Dunaliella tertiolecta* and

Isochrysis, and a mixture of *Rhodomonas* and *Isochrysis* were also tested and found to be inferior to the results produced with *Isochrysis*.

Conclusions include: *Tripneustes ventricosus* larvae can be successfully cultured. While more rapid development occurs when the larvae are fed *Rhodomonas*, *Isochrysis* offers a more favorable combination of survival rate and development time.

The perimetamorphic period, from rudiment formation to feeding juvenile, included a life stage heretofore unreported for *T. ventricosus*. Referred to as echinoporculus for its fanciful resemblance to a young pig, this stage was observed as the immediate metamorphic result in nearly half of the metamorphosed animals.

Covering was observed in juveniles as small as 1.5 mm. The adornment remained on the aboral surface regardless of the juvenile's orientation.

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ACKNOWLEDGEMENTS

One always missed the turning, but found, in time
The broken sign that pointed crookedly, loath to
Allow another stranger here.

Only memory will turn down this way
When some old man somewhere recalls his day
On this beach where sea-egg shells once lay.

From Robert Lee's *Skeete's Bay, Barbados*

At age 16, I wanted to be a marine biologist when I grew up. My more practical father directed me toward a nearly half century detour into the business world. The last few years of that detour were spent preparing to follow my youthful dream by taking night school science and math courses that the University of Minnesota didn't require of its business administration undergraduates in the 1960s. Thanks go to Dr. Tom Garrison and professor Dennis Kelly of Orange Coast College, who helped me select the courses needed.

I thank Dr. Phil Sze, the noted phycologist at Georgetown University, for helping me understand that the removal of major herbivores can result in major changes to a marine community and suggesting that man, through bioremediation, may be able to assist nature to reverse those effects.

I thank Dr. Charles Messing, the chair of my committee, who agreed to supervise my efforts to design culturing techniques for one of the major near-shore herbivores of eastern Florida and the Caribbean. Thanks also go to Drs. Bart Baca and Mark Farber, who served on my committee.

I thank Dr. Bernard Riegl, who took an interest in my work, encouraged me and was of great assistance when it came to analyzing my results and finalizing this document. I thank Dr. Andrew Rogerson for showing me little tricks of the trade and for acquiring a larger capacity centrifuge for the algae, and Dr. Shivji for giving me access to an unlimited supply of dH_20.

Thanks go to Richard Hubbard who helped design aeration, filtration and algae culture systems and taught me how to take photographs through the lens of a microscope and preparing them for publication. Thanks also go to all those who willingly gave of their time to dive with me, help collect urchins and seawater and provide other assistance during the research.

I also thank Dr. John Lawrence from the University of South Florida at Tampa, who freely gave of his time and expertise to assist me when the going got tough.

Special thanks go to my wife, Cheri, who encouraged me to follow my dream, did my proofreading and escaped two freezing winters by moving to South Florida.

Perhaps now we have learned how to keep the West Indies sea-egg from being just a memory. And, perhaps soon, I'll be a marine biologist. But, will I ever grow up?

INTRODUCTION

Tripneustes L. Agassiz (1841) is a genus of sea urchin found throughout the tropics and into the sub-tropics. Short white spines cover a dark, often black, test and adults reach a test diameter of 7 to 10 cm. The two extant species are *T. gratilla* (Linnaeus, 1758) from the Pacific and *T. ventricosus* (Lamarck, 1816) in the Atlantic and Caribbean Sea (Lawrence 2001b).

Lamarck initially described and named *Tripneustes ventricosus* in 1816. In 1840, Gray placed it in the genus *Hipponoe,* a name previously assigned to an annelid (Mortensen 1943). In 1872, A. Agassiz thought the species similar to Leske's *Cidaris esculenta* and referred to it as *T. esculentus* (Mortensen 1943). Mortensen pointed out the error and resurrected the senior name, *T. ventricosus*. However, the name *esculentus* for *T. ventricosus* continued to appear in the scientific literature at least until the 1960s (Lewis 1958, Moore & McPherson 1965) and is still used on the French islands of Guadeloupe and Martinique (Internet 1 & 2).

Habitat – Range, Food and Predators. Mortensen (1943) reported that *T. ventricosus* is found in shallow water from Bermuda and south Florida, through the West Indies as far south as Brazil, the Ascension Islands, and along the west coast of Africa. Lewis (1958) also reported that *T. ventricosus* is found along the U. S. coast as far north as the Carolinas. However, it is not found in the Gulf of Mexico (Lawrence 2001b). The poleward distribution of the species appears to be limited by low temperatures. Moore *et al.* (1963) reported that mass mortality occurs when shallow-water temperature is unusually low in Florida but gave no temperatures. At the other extreme, these urchins

become sluggish at 33° C and die within a short period of time at 40° C. (Lawrence 2001b). Also, Cameron *et al.* (1985) reported 100% mortality of *T. ventricosus* larvae below 20° C and above 30° C.

Tripneustes ventricosus occurs in a variety of habitats (Lawrence 2001b), including *Thalassia* beds (Moore *et al.* 1963), coral and coral rubble with algae overgrowth (Scheibling & Mladenov 1987), rocks and rock ledges (McPherson 1965) and inner platform reefs (Cameron 1986). It browses on sea grass and macroalgae (Keller 1983) and appears to be an opportunistic feeder (Scheibling & Mladenov 1987). Species eaten include *Thalassia testudinum* and *Syringodium filiforme* (Tertschnig 1989), fleshy algae and epilithic coralline algae (Scheibling & Mladenov 1987).

Parker & Shulman (1986) stated that *T. ventricosus* has only two predators: the helmet conch, *Cassis tuberosa,* and the porcupine fish, *Diodon hystrix*. However, Lawrence (2001b) included the gastropod, *Charonia variegata*, the cushion seastar, *Oreaster reticulatus*, and several wading birds as additional predators.

Prior to the middle 1980s, *T. ventricosus* had never been found on the forereef of Discovery Bay, Jamaica (Woodley & Gayle 1999). Prior to 1983, the most common forereef urchin in most areas of South Florida and the Caribbean was *Diadema antillarum* (Hughes 1994). However, the epizootic that swept through the Caribbean and South Florida in 1983-1984 reduced the *Diadema* population by 95% (Carpenter 1990). Several studies of Caribbean reef sites reported increased percent cover of algae and shifts from algal turfs and crustose algae to macroalgal species in the months and years following the loss of the *Diadema* (Liddell & Ohlhorst 1986, Carpenter 1990, Foster 1987, Hughes, *et al.* 1987, Levitan 1988).

Since the mid-1980s, *T. ventricosus* has been observed on the forereef in Discovery Bay, Jamaica; by 1996, it reached densities of 1.8 m^{-2} and had greatly reduced abundances of fleshy macroalgae above ~10 m wherever it was present (Woodley & Gayle 1999). Haley & Solandt (2001) reported that, at Discovery Bay, the density of *T. ventricosus* increased dramatically from 1995 to 1998 and then declined as *Diadema* density recovered significantly. Similar successional observations were made at Discovery Bay both by Aronson & Precht (2000) and Edmunds & Carpenter (2001). Based on these results, Haley & Solandt (2001) hypothesized that grazing by *T. ventricosus* may serve a vital role in reef recovery and that biological succession occurs as recovering *Diadema* stocks replace *T. ventricosus* on the forereef.

Human Consumption of *T. ventricosus*. Sea urchin gonads of both sexes, referred to as "roe", have been consumed by humans in the Caribbean since before recorded history (Lawrence 2001a). *Tripneustes ventricosus* is harvested at several of the eastern Caribbean islands, including Barbados, St. Lucia, Martinique (Smith & Berkes 1991) and Guadalupe (Internet 1). The species is subject to periodic population declines resulting from, or at least exacerbated by, over-harvesting (Scheibling & Mladenov 1987).

Barbados was perhaps the first country to regulate any urchin fishery when, in 1879, a law was passed to prohibit harvesting of *T. ventricosus* during the peak of the breeding season from May through August (Scheibling & Mladenov 1987). More than a hundred years later, regulation of *T. ventricosus* harvesting only exists in Barbados, St. Lucia (Smith & Berkes 1991), Guadalupe and Martinique (Internet 1 & 2).

The difficulties of managing a commonly owned resource have existed at least since the days of medieval English grazing commons and continue to plague those who attempt to manage commonly owned resources, including fisheries, today (Hardin 1968). Hardin (1968), who developed a model referred to as the "Tragedy of the Commons", stated that all commonly owned, scarce natural resources are destined to be overexploited unless subjected to effective management and harvest control.

Lewis (1958) and Scheibling & Mladenov (1987) stated that the only extensive consumers of urchins in the Caribbean are the Bajans (citizens of Barbados). The fact that *T. ventricosus* harvesting was forbidden at St. Lucia (A. Smith, pers. comm. 2001), Guadalupe (Internet 1) and Martinique (Internet 2) as a result of over-harvesting suggests either significant local consumption or export of the species. The lack of effective management and harvest control may have caused or contributed to the decline of *T. ventricosus* in the Caribbean during the 1980s and 1990s. Scheibling & Mladenov (1987) reported that, even with the Bajan law regulating harvesting, enforcement was ineffective. They interviewed Bajans who engaged in *T. ventricosus* harvesting and found that 65% admitted to taking them out of season. Smith & Berkes (1991) reported that *T. ventricosus* had recovered from hurricane damage at St. Lucia by 1983; however, uncontrolled harvesting resulted in severely depleted stocks by 1987. As a result, the *T. ventricosus* season was closed again both at Barbados and St. Lucia to permit the stocks to recover (Smith & Berkes 1991). Neither Scheibling & Mladenov nor Smith & Berkes provided statistics regarding the number of urchins harvested or the dollar value of the fishery. Scheibling & Mladenov (1987) did, however, estimate the harvest in the 1970s at Barbados approximated 45 million animals per year. They also reported that the roe

from ~ 15 shelled urchins (approximately 0.1 L) sold for $1 to 2 Bajan dollars in the early 1980s. At the current exchange rate of US$1.00 ~ BD$2.00, the value of the Bajan fishery may have approximated US$1.5 million annually. No data was noted for other areas.

Some recovery did occur in the early 1990s, although the season was closed again at St. Lucia in 1995. Juveniles recruited strongly there in 2000 and harvesting was resumed in 2001 (A. Smith, pers. comm. 2002). However harvesting was still forbidden at Martinique and Guadalupe as of 2001 (Internet 1 & 2).

The rate of recovery of *T. ventricosus* following drastic reductions in populations may be directly related to their method of reproduction. Most echinoids, including *T. ventricosus*, are broadcast spawners (McEdward & Miner 2001). Levitan (1991) reported that the probability of fertilization success decreases with distance between the male and the female according to the following formula:

$$\log \text{ percent fertilization} = 0.72 \log \text{density } (m^{-2}) + 0.49$$

As a result, density is a major factor in reproductive success in free-spawning echinoids. When density drops below 0.2 m^{-2}, the estimated fertilization success rate is only 1% (Levitan 1991). Densities of *T. ventricosus* at St. Lucia in the middle and late 1980s were <0.1 m^{-2} in some areas (Smith & Berkes 1991).

Culturing of *T. ventricosus*. No documentation exists on the successful culturing of *T. ventricosus* from fertilization through metamorphosis to exotrophic (feeding) juvenile. Tennent (1911) reported a failed attempt to culture this species. Pearse & Cameron (1991) reported that both Lewis (1958) and Mortensen (1921) had cultured the larvae through metamorphosis. However, both original publications indicate otherwise.

Mortensen (1943) stated that he reared the larvae to metamorphosis and cited his own (1921) work. However, in that earlier work, Mortensen (1921: page 32) stated, "We have a fully formed larva, which is about to begin its metamorphosis." As his ship was about to depart, Mortensen went on to state, "The metamorphosis of this larva could not be followed, as the sojourn at Tobago ended by this time."

Lewis (1958) documented the growth and development of the *T. ventricosus* larvae obtained in plankton nets. Although he made numerous attempts to rear the larvae, none lived more than 10 days. Cameron (1986) stated that he reared *T. ventricosus* through metamorphosis. However, he cited Lewis' (1958) time to metamorphosis and was unable to provide any specifics regarding settlement, metamorphosis or the early juvenile stage (R.A. Cameron pers. comm. 2002). Woodley (pers. comm. 2001) stated that Mladenov may have cultured *T. ventricosus* in 1985 while working at Barbados. But, Mladenov (pers. comm. 2002) stated that, although he had attempted to culture *T. ventricosus*, he had difficulties with electricity. Scheibling & Mladenov (1987) stated that techniques are being developed and suggest that culturing would be a method of solving the over-harvesting problem. However, neither author mentions any culturing success.

A thorough review of the literature and personal contact with those who reportedly had success culturing the species indicate that *T. ventricosus* has never been successfully cultured from fertilization to exotrophic juvenile and that *T. ventricosus* is not commercially reared. Its life history, however, suggests that it may be an ideal candidate for aquaculture. In discussing the relationship between life history strategies and potential for aquaculture in sea urchins, Lawrence and Bazhin (1998) concluded that

toxopneustids (the family to which *T. ventricosus* belongs) would be most appropriate for aquaculture because members seem to allocate more energy to production than to protection and maintenance relative to other urchins of commercial importance (Table 1). They cite *T. ventricosus* as most suitable because it grows faster to a larger size and reaches sexual maturity sooner than other toxopneustids.

Table 1. Urchin species considered of economic importance.

Order	**Family**	**Species**
Diadematoida		
	Diadematidae	
		Diadema setosum
Temnopleuroida		
	Toxopneustidae	
		Lytechinus anamesus
		Lytechinus variegatus
		Tripneustes gratilla
		Tripneustes ventricosus
Echinoida		
	Echinidae	
		Echinus esculentus
		Loxechinus albus
		Paracentrotus lividus
	Echinometridae	
		Evechinus chloroticus
		Heliocidaris erythrogramma
	Strongylocentrotidae	
		Strongylocentrotus droebachiensis
		Strongylocentrotus franciscanus
		Strongylocentrotus intermedius
		Strongylocentrotus nudus
		Strongylocentrotus purpuratus

Large-scale culturing of urchin "seed" (juveniles up to approximately 20 mm diameter) is practiced in Japan (Lawrence pers. comm. 2002). Spawning is induced by injection of KCl; larvae are reared in 1000-L containers, and juveniles are reared either in nursery tanks on land or cages suspended in the ocean (Hagen 1996). Juveniles are released once they reach diameters of 7 to 20 mm to mature in the wild and are recaptured at about 4 cm diameter (Hagen 1996). Cultured species include *Strongylocentrotus intermedius, S. nudus, S. pulcherrimus* and *T. gratilla* (Lawrence, pers

comm. 2002). Lawrence also stated that, in 1989, over 30 million juvenile urchins were produced in Japan; the Chinese produced about 13 million *S. intermedius* seed in 2000, and much smaller numbers of *Loxechinus albus* seed are reared in Chile.

At present, no closed-cycle aquaculture facilities culture any sea urchin species from fertilization to harvested adult (Lawrence pers comm. 2002), although full-scale, closed-cycle technology exists in Japan and the French have developed a prototype facility (Hagen 1996). Hagen (1996) also stated that a Norwegian profitability study for a growout (fertilization to mature adult) facility concluded that a large profit potential existed when realistic parameters were used, but no details were provided.

Thesis Question. Large-scale culturing of *Tripneustes ventricosus* appears to be technologically feasible, hypothetically profitable (Hagen 1996) and offers at least three advantages as follows. The species offers an opportunity for bioremediation of coral reefs overgrown by macroalgae following the *Diadema* mass mortality. Woodley & Gayle (1999), Aronson & Precht (2000), Edmunds & Carpenter (2001), Haley & Solandt (2001) reported that *T. ventricosus* were reducing macroalgal cover and producing bare substrate suitable for settlement by coral larvae on the forereef of Discovery Bay. Lawrence (2001b) stated that removal of a major herbivore will likely result in a change in community structure. Scheibling & Mladenov (1987) stated that aquaculture is a means of rehabilitating *T. ventricosus* populations where they have been over-harvested. *Tripneustes ventricosus* also offers potential as a food source and associated industry for several Caribbean nations. If *T. ventricosus* is to be cultured on a large scale, the appropriate method of aeration as well as algal food source, density and frequency of feeding must be determined. Airlift is the preferred aeration method in aquaculture (B.

Baca, pers. comm. 2000), and *Rhodomonas* spp. (Cameron, pers. comm. 2001) is the accepted alga for urchin larvae growth under laboratory conditions. However, it is not known whether these are appropriate under aquaculture conditions for *T. ventricosus* larvae.

The thesis question addressed here is: If *T. ventricosus* can be cultured from fertilization through metamorphosis to exotrophic juveniles, what is the most suitable aeration method and feeding regimen for use in an aquaculture application?

MATERIALS AND METHODS

Seawater Collection and Sterilization. Raw seawater was collected in 20-L Nalgene® carboys ~800 m from the Broward County, Florida, shore and then filtered at the Nova Southeastern University Oceanographic Center (OC) to remove particulate matter and organisms larger than a diameter of 0.5 μm (Figure 1). An Iwaki magnetic drive water pump (model WMD40RLT), purchased from Aquatic Eco-Systems, Inc., Tampa, FL, pumped water from the carboy through 2.54-cm (=1 in) inside diameter (ID) vinyl tubing through three GE SmartWater filters (Home Depot) in series as follows: a particulate filter (model FXWSC); a charcoal filter (model FXWTC) capable of filtering particles, organisms and other items larger than 1.0 μm, and a second charcoal filter (model FWUTC) capable of filtering particles, organisms and other items larger than 0.5 μm.

The filtered seawater left the series of filters through a 1.27-cm (=0.5 in) ID vinyl tube and entered a model EU25 Emperor Aquatics ultraviolet sterilizer (Aquatic Eco-Systems, Inc.) at a flow rate that produced approximately 700,000 $\mu Ws/cm^2$ of UV exposure. Sterile seawater (SSW) left the sterilizer through a 1.27-cm (=0.5 in) ID vinyl

tube and was then stored in separate 20-L Nalgene® carboys used only for SSW storage. SSW storage capacity was limited to 60 L and SSW was never kept for more than a week at a time.

Collection of Adults and Gametes. *Tripneustes ventricosus* is capable of reproducing year round in southeast Florida, although breeding, determined from gonad volume, seems to peak in May and December (McPherson 1965). As McPherson (1965) made no mention of a lunar periodicity, as is the case with *Diadema antillarum* in the same area (Bauer 1976), adults were collected at various times from November 2000 through November 2001 and maintained in a 1200-L circular fiberglass holding tank with a large airstone and open circulation from adjacent Port Everglades. Adults used in culture experiments 3 and a failed attempt preceding 4 were taken at Virginia Key in Miami, FL. All other animals were taken off the Broward County, FL, shore. The animals were fed a variety of algae obtained from the near-shore environment plus romaine lettuce and spinach.

Prior to spawning, the adults were brought into the laboratory, rinsed in SSW to remove as much debris, algae and other organisms as possible and then maintained in a plastic wash basin filled with SSW. To begin the spawning process, each adult was placed, oral side up, on a glass beaker or small glass jar to which SSW had been added to an approximate depth of 3-4 cm. Adults were induced to spawn by intracoelomic injection of 3 ml of 0.55 M KCl administered through Pharmaseal Stylex® disposable 20x1.5-inch syringes. If spawning occurred, it occurred within 5 min for both males and females. Females were allowed to remain on the beaker or jar during the spawning process, and the eggs dropped into the SSW. Males were removed and hand held over

disposable petri dishes, where sperm was collected dry (without added SSW), covered and kept refrigerated prior to activation. After collection, eggs were washed twice in SSW by decanting as much water as possible from the beaker or jar, adding SSW, allowing the eggs to settle and repeating the process (Strathmann 1987).

Five to six drops of sperm were diluted in 200 ml SSW and allowed to stand for 5 to 10 min for activation immediately prior to fertilization. Fertilization was initiated by introducing several drops of diluted sperm to a 500-ml beaker containing the eggs of one female suspended in approximately 200 ml of SSW (modified from Strathmann 1987). Fertilization was confirmed under ocular microscopy by the presence of a fertilization membrane. The fertilized eggs were then placed in a culture dish (13-cm diameter) in a density sufficient to produce a monolayer of embryos covered by approximately 3 cm SSW (Amy 1983). The embryos were monitored under ocular microscopy periodically to measure development time and to identify the blastula stage.

Larval Culture. After reaching the blastula stage, the embryos were transferred to a 1- gal clear glass jar (dense culture jar) containing approximately 3 L of SSW. Embryo density was estimated in a 1-ml Sedgwick-Rafter counting chamber under ocular microscopy. Larvae were also cultured in the same sized jars. Once the blastula stage was reached, culture jars were filled with 3 L SSW plus approximately 1800 larvae (0.6 ml^{-1}). During the initial experiment, the paddle aerated jars only contained 900 larvae. The number of larvae transferred to the culture jars was estimated by volume using a volumetric cylinder. The jars were then placed under an aeration system except for those not aerated during the final experiment. The fluorescent lights in the lab were left on 24 h a day during the larvae culturing, and all jars were covered with a translucent plastic

sheet suspended approximately 10 cm above the tops of the jars to reduce accumulation of dust and other debris.

The SSW in the culture jars was changed every 2-3 days to remove wastes. Approximately 75% (2.25 L) of the SSW was removed by a siphon (Figure 1b) constructed from airline tubing, a 3-cm piece of 1.27 cm (=0.5-in) ID vinyl tubing, the hose adaptor fitting from a carboy spigot, a 1.27-cm (=0.5-in) barb to 1.59-cm (=0.625-in) female-thread adapter, a plastic dispenser tip and 150-μm mesh cloth. The fittings and the mesh cloth reduced the flow rate and prevented the removal of and damage to larvae during the siphoning process. The SSW was replaced by draining it from the storage carboys into 1-gal jars to aerate the water and then adding it to the culture jars. Culture jars were replaced every other time SSW was removed to reduce the build up of film on the sides of the containers. This was performed by pouring the contents of the jar (~0.75 L SSW plus the larvae) into a clean 1-gal jar containing ~2.25 L SSW from the storage carboys. Used jars were scrubbed in seawater, rinsed three times in hot water, three times in cold water, and once in dH_20, inverted to remove excess water and stored for the next use.

Aeration Methods. The first two experiments were aerated by two methods: airlift and paddles. The airlift method produced a stream of air bubbles from the tip of a Pasteur pipette and the paddle method mechanically stirred the culture water. For the airlift method, flow tubes (Figure 2) were constructed using 2.54-cm (=1 in) diameter PVC pipe capped with a 90^o elbow. A 0.476-cm (=0.1875 in) diameter hole was drilled into the elbow through which an airline tube, fitted with a glass pipette, was inserted down through the pipe so that the pipette tip was flush with the bottom of the PVC pipe.

The flow tubes were fastened to the inside of the culture jars and air was provided from the pump used for the algae cultures, but through a separate manifold.

The paddle aeration system (Figure 3), modeled after that of Strathmann (1987), consisted of a frame constructed of 1.27-cm (=0.5-in) PVC pipe, mounted on a 61-cm^2 (=2 ft^2) sheet of 1.27-cm (=0.5-in) plywood. Paddles were fashioned from 5-cm^2 white plastic squares, plastic coat hangers and aquarium sealant. A movable rack, designed to move the paddles, was suspended from the frame by cable ties. A 5-rpm electric instrument motor (Hurst Manufacturing Division of Emerson Electric Company, model PA, purchased from Grainger) was fitted with a 5.08-cm (=2-in) pulley wheel. A small hole was drilled near the outer edge of the outer lip of the pulley wheel. A metal screw, wrapped with Teflon threading tape to smooth the surface, was attached through the hole to make a camshaft. Monofilament line was connected from the camshaft to the movable frame to produce the motion that drove the paddles. The motor assembly was placed between two paddle frames to produce the back and forth motion for both. Each frame was capable of running nine paddles for nine culture jars. Metal washers on the camshaft kept the monofilament lines separated. During the final experiment, four culture jars were not aerated except for that resulting from the thrice weekly water changes, the daily addition of food and the O_2 produced by the photosynthetic algae.

Temperature control. All experiments were conducted in an air-conditioned laboratory and temperature was regulated by thermostat. The initial experiment was conducted at 23±0.5°C after Eckert (1998), the second and third at 23.5±0.5°C the fourth at 24.5±0.5°C and the final experiment at 24.0°±0.5°C Temperature near the culture jars

was recorded using a Hobo temperature recording device set to record the temperature every 12 min.

Algal culture. The algae used during the experiments were *Rhodomonas* sp., *Cryptomonas* sp., both cryptomonads; *Isochrysis* aff. *galbana* (Tahitian strain), a haptophyte, and *Dunaliella tertiolecta* (Butcher), a chlorophyte. *Rhodomonas* sp. has been used to rear other warm water urchin species (Strathmann 1987, Eckert 1998, Cameron & Hinegardner 1974) and had been highly recommended by Eckert (pers. comm. 2000) and Cameron (pers. comm. 2001). *Dunaliella tertiolecta* has also been used in the culture of warm and temperate water urchin larvae (McEdward & Herrera 1999, Hinegardner 1969, Boidron-Metairon 1988). *Isochrysis* has been used successfully, not only for urchin larvae culturing in general (George *et al.* 2000, New Hampshire Sea Grant Program 1997, Metaxas & Young 1998), but in the successful rearing of *T. gratilla* in Japan (Chen & Run 1988) and the Philippines (Juinio-Menez *et al.* 1998). Eckert (pers. comm. 2000) stated that *Rhodomonas* had been very successful in prior echinoderm larvae culturing and suggested that *Cryptomonas* sp. be included as it is also a cryptomonad and might produce favorable results.

The SSW for culturing algae was first Pasteurized by bringing it to a temperature of at least 78^{o}C and again after 24 h to further assure that no exotic organisms were added to the cultures. Stock solutions of culture medium were prepared to industry specifications (Guillard & Ryther 1962, Guillard 1975). The major nutrient stock solution consisted of 75.0 g $NaNO_3$ and 5.0 g $NaH_2PO_4.H_20$ added to enough dH_2O to make 1.0 L total volume. Two stock solutions of minor nutrients consisted of (1) 9.8 g $CuSO_4.5H_2O$, 6.3 g $Na_2MoO_4.2H_2O$, 22.0 g $ZnSO_4.7H_2O$, 10.0 g $CoCl_2.6H_2O$ and 180.0

g $MnCl_2.4H_2O$ added to enough dH_2O to make 1.0 L total volume, and (2) 1.0 ml of the first solution plus 3.15 g $FeCl_3.6H_2O$ and 4.36 g $Na_2EDTA.2H_2O$ added to enough dH_2O to make 1.0 L total volume. Vitamin stock solution consisted of 0.001 g vitamin B12, 0.01 g Biotin and 200 g Thiamine HCl added to enough dH_2O to make 1.0 L total volume. Vitamins were obtained from a local pharmacy. For non-cryptomonads, 1.0 ml of the major nutrient solution, 1.0 ml of the second stock solution of minor nutrients and 0.5 ml of the vitamin stock solution were added to each liter of algal culture water. The same amounts were initially used for the cryptomonads. However, a higher concentration of the major nutrients and vitamins was required to develop the normal red color in the cryptomonads. As a result, 2.0 ml of the major nutrient solution and 1.0 ml of the vitamins were added to water per L for the cryptomonads.

The aeration system, designed to provide CO_2 for photosynthesis as well as O_2, consisted of a WhiteWater model LT15 air pump (Aquatic Eco-Systems), 0.47625 cm (=0.1875 in) ID vinyl tubing, eight-valve manifolds with plastic valves and 14.605-cm (5.75 in) long Pasteur capillary pipettes (Figure 4). A cotton plug, approximately 2 cm in length, was placed in the vinyl tubing to serve as an air filter.

As it may take several weeks to grow an adequate volume of algal cells to support the larvae, cultures were started approximately one month prior to the initial urchin spawn and maintained during the remainder of the project. Algae starter cultures in 15-ml screw-cap tubes were obtained from the Algae Collection at the University of Texas, Austin, and delivered by Federal Express. The initial culturing process involved placing the 15 ml of culture and approximately 30 ml of algal culture water in an empty 125-ml flask, which had been heat sterilized with boiling dH_2O. A Pasteur pipette and tubing

assembly were inserted and the flask was capped with cotton balls. As the cell density increased in the flask, additional culture water was added to bring the total volume to 60 ml and eventually to 100 ml. When the culture became dense at the 100 ml volume, it was transferred to a 250-ml flask that had been heat sterilized with boiling dH_2O. The volume of the 250-ml flask was gradually increased as cell density increased. When the volume of dense culture reached 200 ml, the culture was poured into a new plastic 2-L club soda bottle. Club soda bottles were selected because, as the contents were bottled for human consumption, they should be relatively sterile and need no preparation after removing the club soda. Any remaining residue was carbonated water, easily utilized by the algae. A hole was drilled into each bottle cap and the tubing assembly was inserted through the hole to eliminate the need for cotton balls.

Two or three culture bottles of each algal species were maintained at all times to assure an adequate supply of larval food in the event a culture became contaminated or crashed. To keep the algae cultures growing in log phase, approximately ½ of the culture was discarded each week and replaced with fresh culture water. During larval culturing, removal of algae for larval feeding eliminated the need to periodically discard a portion of the algae culture.

Preparation of algae & Feeding of larvae. Prior to each feeding, algae were poured from the algal culture bottles into 50-ml plastic centrifuge tubes and centrifuged at 2000 rpm to separate the algae from the culture medium (Figure 4). *Rhodomonas* and *Cryptomonas* required 40 sec, *Dunaliella* 60 sec and *Isochrysis* 180 sec to separate the algae from the medium. The culture medium was poured off and the algae resuspended in SSW. For the fifth culture experiment only, resuspended algae were poured through a

50-µm mesh cloth to remove dead cells and other detritus that had accumulated in the culture bottles. Cell density was determined by ocular microscopy using a hemocytometer. The calculated volume of algae in SSW, to approximate the needed cell concentration in the culture jars, was measured using a volumetric cylinder. When water and/or jars were changed, algae were added following the change. The density and frequency of feeding are summarized in Table 2 below:

Table 2. Algal cell density and frequency of feeding of *T. ventricosus* larvae.

Experiment	Frequency	Cell Density
1	At Water Change	10 μl^{-1}
2	At Water Change	10 μl^{-1}
3	Daily	10 μl^{-1} once, then 7.5 μl^{-1}
4	Daily	10 μl^{-1} once, then 7.5 μl^{-1}
5	Daily	10 μl^{-1} once, then 7.5 μl^{-1}

Feeding frequency was increased for the final three cultures to maintain more consistent algal density in the culture jars. During the last three experiments, the cell density of each feeding following the first was reduced to 7.5 μl^{-1} to avoid overfeeding.

Amino Acids. Fenaux (1982) reported that echinoderm larvae absorb dissolved organic matter from seawater and that glutamic acid and glycine have been added into water of other cultured echinoid larvae at concentrations of 0.1 to 51.0 μgL^{-1}. To determine the effect of these amino acids on *T. ventricosus* larvae, glutamic acid and glycine were obtained from the Health Professions Division at NSU and a single stock solution was prepared at 25 mgL^{-1} each in deionized water. Vials containing 3 ml of the stock solution were then frozen. At each water and/or jar change, 3 ml of the stock

solution was introduced to each of four culture jars during experiments 3 and 4 to produce a concentration of approximately 25 μgL^{-1}.

Growth and Development Measurement. At several water and/or jar changes during each experiment, 3-6 larvae were removed from each culture jar, placed in small plastic petri dishes, preserved in Lugol's iodine (5 gm iodine, 10 gm KI per 100 ml dH_2O), sealed with paraffin film and refrigerated until a later date. Larval development was observed visually using a compound microscope and their growth measured using an ocular micrometer. Complete mortality in a culture jar was confirmed by ocular microscopy after siphoning all but ~100 ml of a jar's contents and viewing that volume using a compound microscope.

Statistics. Paired comparison *t*-tests were used to test differences between aeration methods and algal diets for experiments 1, 2 and 5. One-way Analysis of Variance (ANOVA) (Sokal & Rohlf 1998) was used to test for differences in developmental stages at day 20 and numbers of larvae surviving to settlement time in experiment 5 among the three feeding regimens and between the two aeration methods. Experiments 3 and 4 produced no results requiring statistical analysis.

RESULTS

A description of variations in early larval development precedes results of the five larval culture experiments. Following these results, observations made during the perimetamorphic stage and documentation of early covering are presented.

Larval Culture: Early Development. Fertilization, as indicated by the presence of the fertilization membrane around the egg and confirmed at the first division, exceeded 95% in each of the five culture experiments as estimated by use of the Sedgwick-Rafter

counting chamber. Embryo and early larval development time varied among the cultures for which early development time was measured (Table 3). In the later stages of early development it was not unusual to have animals at three stages present at the same time. The times recorded (Table 3) are those at which different stages were first seen during each culture.

Table 3. Development time table for *T. ventricosus*. Times given are in minutes following insemination. Data presented for Amy (1983) are mean times for embryos to reach the stages listed.

Development Stage	**Amy 1983 26±2º C**	**Culture1 23±0.5º C**	**Culture 2 23.5±0.5º C**	**Culture 5 24±0.5º C**
2-Cell	64	95	79	75
4-Cell	98	125	124	
8-Cell	142	153	168	
16-Cell	190	217	195	207
32-Cell	235	237	265	
64-Cell	285	278	310	
Blastula	375	375	410	
Gastrula	1002	1565	1620	1410
Prism	1700	2540		
Early Pluteus	2100		3140	2705

In the early pluteus stage, the first two arms are extended, but the larva is not feeding. Once larvae begin to feed, developmental stages are defined by the number of arms present: 2-arm, 4-arm, 6-arm and 8-arm. Eight-armed larvae have two additional developmental stages, the first marked by the presence of a rudiment and the second by three larval pedicellaria.

Development times for the culture experiments were slower than that reported by Amy (1983). For example, the mean time, reported by Amy, to reach the gastrula stage

was 1002 minutes following fertilization. In the results reported here, the time to gastrulation was minimally 1410 min and varied by 210 min among experiments.

Culture Experiment 1. The initial experiment consisted of six treatments with four replicates each (Table 4). Two treatments were airlift-aerated; one received a diet of 50% *Isochrysis* and 50% *Rhodomonas* (*Iso/Rho*), the other a mixture of *Isochrysis, Rhodomonas, Dunaliella* and *Cryptomonas* (Mixture). Diets for paddle-aerated treatments were: (1) *Iso/Rho*, (2) 100% *Rhodomonas* (*Rho)*, (3) 50% *Isochrysis*, 50% *Dunaliella* (*Iso/Dun*) (4) and 100% *Cryptomonas (Cry).* No larvae survived beyond day 12. However, different aeration methods produced significant differences in survival time (Table 4). No larvae in airlift aerated jars survived to day 6, while larvae in 12 of the 16 paddle-aerated jars survived to at least day 10. Survival data for the two aeration methods were compared for the jars in which the larvae were fed *Iso/Rho*.

Using the *t*-test for paired comparisons, the differences in survival time between the two aeration methods are significant at the 0.05 level ($p = 0.02$), with the paddle method superior. Larval survival among the paddle-aerated treatments varied by diet. Larvae in all four jars receiving *Cry* survived at least until day 10, while larvae in only three of the four jars receiving *Rho* and *Iso/Rho*, and those in only two of the four jars receiving *Iso/Dun* survived that long. A few misshapen larvae (perhaps less than 10%), typically without developed arms and/or similar in shape to degenerating larvae (Figures 6 & 9), were noted and were not uncommon in all five experiments.

Table 4. Culture experiment 1. Age at death of *Tripneustes ventricosus* by aeration method and diet. Time is given in days following fertilization when no animals remained alive. Diets are as follows: *Isochrysis* and *Rhodomonas* mix (*Iso/Rho), Rhodomonas (Rho), Isochrysis/Dunaliella* mix (*Iso/Dun), Cryptomonas (Cry)*, and Mixture (a combination of all the algae).

Aeration method	Algal Diet	Replicate 1	Replicate 2	Replicate 3	Replicate 4
Airlift	*Iso/Rho*	6	6	6	6
Airlift	Mixture	6	6	6	6
Paddle	*Rho*	12	12	12	6
Paddle	*Iso/Rho*	12	12	12	6
Paddle	*Iso/Dun*	12	12	6	6
Paddle	*Cry*	12	12	12	12

Culture Experiment 2. This experiment consisted of five treatments with four replicates each (Table 5). One treatment was airlift-aerated and fed *Iso/Rho*. Diets for paddle-aerated treatments were: *Rho, Iso/Rho, Iso/Dun* and *Cry*. Survival time for both the aeration and diet variables improved relative to the initial experiment. Aeration rate (bubbles per time unit) was increased over the rate in experiment one to prevent algae from settling on the bottom of the culture jars. All larvae in the airlift-aerated culture jars died by day 8. Microscopic examination of dead larvae indicated that they had been physically damaged. Survival data for the two aeration methods were again compared for the jars in which the larvae were fed *Iso/Rho*.

A *t*-test for paired comparisons found no significant differences in survival time between the two aeration methods at the 0.05 level ($p = 0.21$). Larval survival among the paddle-aerated treatments again varied by diet. No larvae receiving either *Iso/Dun* or *Cry* survived to day 11. All larvae in three of the four jars receiving *Iso/Rho* died by day 11; all larvae in one jar receiving *Rho* died by day 21, and all in another jar died by day 31.

However, larvae in the two remaining jars receiving *Rho* and those in one of the *Iso/Rho* jars survived to day 39, but had all died by day 42. A *t*-test for paired comparisons found no significant differences in survival time among the paddle-aerated cultures fed *Rho* at the 0.05 level ($p = 0.14$).

By day 12, flocculent material had appeared in the water column and on the bottom of culture jars receiving *Rho* and *Iso/Rho*. Floc density increased over time and was accompanied by unidentified ciliates that attacked dead and dying larvae.

Photographs (Figure 5) of larvae at day 18 confirm that a rudiment had developed on at least one animal fed *Rho*. However, the rudiment was not identified until the photographs were examined several weeks later.

Table 5. Culture experiment 2. Age at death of *Tripneustes ventricosus* by aeration method and diet. Time is given in days following fertilization when no animals remained alive. Abbreviations as in Table 4.

Aeration method	Algal Diet	Replicate 1	Replicate 2	Replicate 3	Replicate 4
Airlift	*Iso/Rho*	8	8	8	8
Paddle	*Rho*	42	42	32	21
Paddle	*Iso/Rho*	42	11	11	11
Paddle	*Iso/Dun*	11	11	11	11
Paddle	*Cry*	11	11	11	11

Culture Experiment 3. This experiment consisted of four diet treatments with four replicates each: *Rho, Iso/Rho, Iso/Dun* and *Rhodomonas* plus amino acids (*Rho* + AA). Adults for this experiment were collected at Virginia Key, Miami, FL. All treatments were paddle-aerated (Table 6). The cultures in which the diet consisted of *Rho* or the *Iso/Rho* mix developed the dense floc again, and, by day 14, no live larvae could be found in three of the *Rho* culture jars and one of the *Rho* + AA jars. The density

of the flocculent material was not as great in the cultures fed *Iso/Rho* or in three of the cultures receiving *Rho* + AA. The ciliates were also noticed again in all culture jars except those receiving the *Iso/Dun* mix. Their density appeared to be greater in cultures fed *Rho* and *Rho* + AA.

Excrement accumulated in the water column and on the bottom of *Iso/Dun* cultures but neither to the degree nor the consistency of the floc in the other cultures. All larvae fed *Iso/Dun* died by day 22 (Table 6). Although this survival time approached the metamorphosis time recorded by Lewis (1958) (28 days) and observed in experiments four and five (see below), the larval development was not as advanced as in the cultures receiving the other diets. *T. ventricosus* larvae develop eight arms prior to the development of the rudiment and three pedicellaria (Lewis 1958 and personal observations). None of the larvae fed *Iso/Dun* had advanced to the 8-arm stage by day 20. However, on day 20 in the four *Iso/Rho* culture jars and the only *Rho* jar to survive day 14, approximately 40% of the larvae had reached the eight-arm stage and about 30% had begun to develop a rudiment.

On day 28, all remaining larvae were placed in a 13-cm diameter dissecting dish containing SSW to an approximate depth of 5cm and *Rho* at a density of approximately 10 μl^{-1}. The larvae spread out in the dish; some remained in the water column, but others settled to the bottom. Two glass microscope slides, which had been sitting in the brood stock holding tank under running seawater to collect a biofilm (bacteria, diatoms, etc.), were introduced to the dish to provide a settlement cue. After the slides were added, the majority of the larvae that had settled to the bottom of the dish arranged themselves along the edges of the slides. By day 32 all but 17 of the larvae had died. However, on that day

several were observed to have changed shape dramatically (Figure 6). Over a 5-hour period, the larval tissue slowly resorbed and a small, hemispherical, immobile form (diameter <0.5 mm) remained on the bottom of the dish. After day 32, no larvae remained alive in the dish.

Table 6. Culture Experiment 3. Age at death of *Tripneustes ventricosus* by diet (aeration method for all cultures was paddles). Time is given in days following fertilization when no animals remained alive. Abbreviations: *Rhodomonas* plus amino acids (*Rho* + AA); all others as in Table 4. * indicates that larvae developed a rudiment and at day 28 were placed in a settlement dish.

Algal Diet	Replicate 1	Replicate 2	Replicate 3	Replicate 4
Iso/Rho	28*	28*	28*	28*
Rho	14	14	14	28*
Iso/Dun	22	22	22	22
Rho+AA	14	22	22	22

Culture Experiment 4. An attempt to fertilize *T. ventricosus* eggs was made on September 6, 2001. Adults were collected in shallow water at Virginia Key where the water temperature had risen to approximately 33^{o} C. The adults were taken to the lab where sperm and eggs were collected within four hours of removal from their natural habitat. All procedures were performed as described in the Methods and Materials section and the laboratory temperature was 24.5^{o} C. Four and one half hours following the introduction of sperm to the eggs, no cell cleavage had occurred. It was concluded, by microscopic examination, that none of the eggs had been fertilized. Experiment 4 commenced two weeks later using adults collected from Broward County in cooler water.

This experiment consisted of four diet treatments with four replicates each: *Rho, Iso/Rho, Cry* and *Rho* + AA. The floc and ciliates appeared again in the cultures fed *Rho,*

Cry, Rho + AA and, to a lesser extent, *Iso/Rho*. While the material was initially suspended in the culture water following a water change, most of it tended to collect on the bottom of the jars and remain there despite the water movement generated by the paddles. On day 12, 5 ml of water containing the material was pipetted from the bottom of one of the *Rho* jars to a small petri dish and examined under a compound microscope. The culture water contained live *Rho*, an unidentified ciliate, live and dead larvae and the flocculent material, which blanketed both the larvae and the bottom of the petri dish. On day 13, three of the four cultures fed *Rho,* one of the four cultures receiving *Rho* + AA and two of the four cultures fed *Cry* contained no living larvae. All larvae in the remaining *Rho* + AA and *Cry* cultures were dead by day 25 and all had substantial amounts of the flocculent material in the culture water. On day 18, specimens taken from all remaining cultures had reached the 8-arm stage and appeared to have a developing rudiment (the Lugol's iodine changed the color of the preserved specimens so that rudiment confirmation was difficult).

On day 18, approximately 30% of the remaining plutei in the *Iso/Rho* cultures had developed rudiments. Twelve, with more advanced rudiment development, were placed in a 7-cm diameter dissecting dish containing a biofilm-covered microscope slide. These larvae were continued on the *Iso/Rho* diet and placed under a constant slow drip of SSW to provide aeration and maintain water quality. The first live product of metamorphosis, identified on day 23, did not resemble a typical juvenile urchin. Under magnification, it appeared as a ball of tissue with five tube feet and one or a few small protrusions extending upward from the tissue (Figure 7). Due to its fanciful resemblance to a small pig, the stage will be referred to as echinoporculus.

Four or five days later, spines appeared and the animal took on the appearance of a juvenile urchin (Figure 7). On day 25, water in the remaining 4 culture jars, three receiving *Iso/Rho* and one fed *Rho,* was reduced to approximately 100 ml and siphoned into petri dishes 10 ml at a time. The larvae remaining alive were counted, placed in dissecting dishes and aerated with the drip system as described above.

By day 27, six echinoporculi and two normal juveniles had been identified. By day 34, no more post-larvae had appeared, but four were now juveniles and two remained in the echinoporculus stage (two had died). On day 35, test diameters of the juveniles and echinoporculi (measured using an ocular micrometer) were 0.46 - 0.58 mm and 0.39 - 0.47 mm, respectively. The two remaining echinoporculi became juveniles by day 40. One juvenile died on day 47. On day 54, test diameters of the remaining 5 juveniles ranged from 0.46 to 0.53 mm. Although no test growth was observed during the prior 19 days, development of a functioning jaw was confirmed by ocular microscopy on day 54 in three juveniles (Figure 8).

Table 7. Culture Experiment 4. Age at death of *Tripneustes ventricosus* by diet (aeration method for all cultures was paddles). Time is given in days following fertilization when no animals remained alive. Figures in () are numbers of live larvae removed at day 25. All abbreviations as in Tables 4 and 6.

Algal Diet	Replicate 1	Replicate 2	Replicate 3	Replicate 4
Iso/Rho	25(~325)	25 (60)	25 (~325)	13
Rho	13	13	25(~75)	13
Rho + AA	25	25	25	13
Cry	13	25	13	25

Culture Experiment 5. This final experiment consisted of four treatments with four replicates each. Three were paddle-aerated: one received a diet of 100% *Iso*, one a

diet of *Iso/Rho* and the third was fed 100% *Rho*. The fourth treatment was fed 100% *Iso*, but received no mechanical aeration. The floc appeared again in cultures fed *Rho*, and its density was greatest in the 100% *Rho* cultures. Ciliate concentrations were not as dense as in experiments 3 and 4. Although ciliates were present in the *Iso* fed cultures, their density appeared to be much lower compared with that in other cultures. Misshapen larvae, typically without developed arms and/or similar in shape to degenerating larvae (Figures 6 & 9) and noted in all experiments, were present in samples taken on day 20 from paddle-aerated cultures fed *Iso* and *Iso/Rho* mix (Table 8).

Table 8. Culture experiment 5. Larval development at day 20 following fertilization by aeration method and diet. Data presented are the percentages of larvae identified at each stage. Figures in () are actual total numbers of larvae at each stage from all four jars for each treatment.

	Aeration Method and Algal Diet			
Development Stage	None ***Isochrysis***	Paddle ***Isochrysis***	Paddle *Iso/Rho*	Paddle *Rho*
2-arm	4.2 (1)		2.6 (1)	
4-arm	70.8 (17)	20.5 (15)		
6-arm	25.0 (6)	31.5 (23)	2.6 (1)	
8-arm		34.2 (25)	35.9 (14)	
Rudiment[1]		1.4 (1)	41.0 (16)	100 (2)
Rudiment and Pedicellaria[1]		8.2 (6)	10.3 (4)	
Misshapen		4.1 (3)	7.7 (3)	
Total	(24)	(73)	(39)	(2)

[1] 8-armed larvae develop a rudiment; larval pedicellariae appear prior to settlement.

Larval survival and development differed by diet and by aeration (Table 8). At day 20 culture water in each jar was reduced to approximately 750 ml and a 10-ml sample was taken from each, totaling 40 ml for each treatment. The live larvae in each

sample were counted and the developmental stages noted (Table 8). Twenty-four live larvae were found in the four samples (40 ml total) taken from cultures fed *Iso* and maintained in jars without aeration; none had reached the 8-armed stage. The samples of *Iso*-fed larvae taken from the paddle-aerated jars contained approximately three times the number of live larvae (73) and 43.8% had reached the 8-armed stage, including those that had also developed pedicellaria and/or rudiments. Thirty-nine live larvae were found in the samples taken from the cultures fed *Iso/Rho*; 87.2% had reached the 8-arm stage, including 41.0% that had developed a rudiment and 10.3% that had developed a rudiment and pedicellaria. Only two live larvae were found in the four *Rho*-fed samples; both had reached the 8-armed stage and had developed rudiments.

For the first time, at least one larva in each paddle-aerated culture jar developed a viable rudiment and survived to settlement. Settlement refers to the larvae's settling to the substrate prior to the rapid change from the larval stage. Metamorphosis refers to the rapid change from the mature larvae stage to the endotrophic juvenile or echinoporculus stage. Metamorphosis began on day 23 in experiment 4. Anticipating similar timing for experiment 5, larvae in the paddle-aerated, *Rho*-fed jars were removed from the jars, counted and placed in settlement dishes containing biofilm-covered microscope slides on day 20 (Table 9). Slower development was anticipated for the paddle-aerated, *Iso/Rho*-fed and *Iso*-fed cultures; they were removed from the culture jars, counted and placed in biofilm-containing settlement dishes on day 24 and 28, respectively (Table 9). The non-aerated larvae were counted and placed in biofilm-containing dishes on day 33 to encourage metamorphosis prior to the experiment's termination on day 37 (Table 9).

Table 9. Culture experiment 5. Number of animals (including larvae, echinoporculi and juveniles) surviving at approximate settlement date. Measurement date is in days following fertilization. All replicates (culture jars) initially contained 1,800 larvae. [1] includes 61 that reached the echinoporculus stage. [2] includes 8 that reached the echinoporculus stage and 4 juveniles.

		Aeration Method and Algal Diet			
Development Stage	**Replicate**	**None *Isochrysis***	**Paddle *Isochrysis***	**Paddle *Iso/Rho***	**Paddle *Rho***
Day 33	1	312		2.6 (1)	
	2	246			
	3	274			
	4	257			
Day 28	1		1008		
	2		886		
	3		821		
	4		758		
Day 24	1			377[1]	
	2			176[2]	
	3			9	
	4			1	
Day 20	1				96
	2				13
	3				4
	4				1

A one-way ANOVA found significant differences in percent survival ($p<0.001$). A Student-Newman-Keuls multiple range post-hoc test with a significance level of 0.05 indicated that significantly greater numbers of larvae survived to settlement in paddle-aerated cultures fed *Iso* relative to the other three treatments.

The date metamorphosis began differed by treatment method: day 23 for *Rho*- and *Iso/Rho*-fed cultures and day 36 for paddle-aerated, *Iso*-fed cultures. Metamorphosis

was not observed in the non-aerated cultures because all remaining larvae, juveniles and echinoporculi from all cultures were transferred to aquaria on day 37.

On days 24-26; a total of thirty echinoporculi were placed in a dissecting dish to monitor their subsequent development. Over an eight-day period, twelve of the 30 eventually produced visible spines and developed the typical juvenile form. In the *Rho* and *Iso/Rho* cultures, the frequency of new echinoporculi decreased as metamorphosis progressed among the larvae. No echinoporculi were noted among the *Iso*-fed metamorphosed larvae.

Covering Response.

By day 60, juveniles from experiment 5 were observed on rocks in the aquaria and climbing the aquaria walls. Some held pieces of *Halimeda*, grains of sand and/or other items on their aboral side with tube feet. At this size (approximately 1.5 mm test diameter) the juveniles did not change the orientation of adornment as they moved from a horizontal surface to a vertical one (aquarium wall, algae or *Thalassia*). One sand-grain-adorned juvenile was removed from the aquarium and placed in a small petri dish containing a few grains of sand and a small stone. The sand grains were removed from the urchin with a pipette. The juvenile responded by placing three sand grains on its aboral surface and moving under the stone.

DISCUSSION

These experiments produced the first exotrophic juveniles from cultures that started with the fertilization of *T. ventricosus* eggs. The early, endotrophic, development of *T. ventricosus* larvae was well documented by Amy (1983). Lewis (1958) documented the exotrophic larval stages, though he never cultured the larvae from fertilization to

metamorphosis and failed to recognize the echinoporculus as a viable stage in the perimetamorphic process. As a result, development stages prior to settlement are not discussed in any detail here.

Culture Temperature. *Tripneustes ventricosus* embryonic development appears to be temperature sensitive. Mortality during the early embryonic period increases as the temperature deviates from 25° C and reaches 100% at 20° C and 30° C (Cameron *et al.* 1985). The initial experiment was conducted at 23° C, the ambient temperature in the laboratory. As warmer weather arrived, the temperature near the culture jars increased to 23.5° ± 0.5 C. The final two experiments were run at 24 – 24.5° C, the upper comfort limit in the laboratory.

The failed fertilization attempt on September 6, 2001, was performed using adults collected from water at approximately 33° C, suggesting that temperature may also impact the viability of eggs and/or sperm. McPherson (1965) observed *T. ventricosus* sperm and eggs in Florida during each month of the year and concluded that spawning occurs throughout the year. The spawning results obtained during the 12 months of this project support McPherson's (1965) conclusions. However, viability of the spawn product may be temperature limited.

The time to reach the several early development stages varied among the cultures for which data were maintained, but not always inversely relative to temperature. All recorded times were slower than those reported by Amy (1983), who performed his work at 26° C. The fact that metamorphosis was observed only in the final two experiments may be attributed, perhaps at least in part, to the higher temperature for those cultures.

However, no other data exists on success of metamorphosis relative to temperature for *T. ventricosus.*

Aeration Methods. Echinoderm larvae have been cultured successfully without aeration. Onoda (1936) successfully cultured several species in 200-ml containers; Chen & Run (1998) cultured *T. gratilla* at 25^{o} C in 500-ml beakers without aeration and obtained metamorphosis at day 30 with a bio-film stimulus and at day 47 spontaneously. Juinio-Menez *et al.* (1998) also cultured *T. gratilla* at 25-26^{o} C in 3-L glass jars without aeration and obtained settlement in 42 days. Chen & Run (1998) stated that photosynthesis by *Isochrysis* in the culture containers added O_2 to the culture water. Oxygenation also must have occurred at the culture water-air interface and when oxygenated water was added with water changes every 4 to 5 days. As a result, it is not technically correct to state that the cultures received no aeration. Rather the cultures received no constant mechanical aeration.

The scientific culture of echinoderm larvae is usually performed in 100-ml to 4000-ml containers with aeration via paddles or similar mechanical devices (Eckert 1998, Hinegardner 1969, Cameron & Hinegardner 1974, Strathmann 1987). In August 2001, I could find no one at the North American Echinoderm conference who recommended an aeration method other than paddles for the culture of echinoderm larvae.

Airlift, however, is a standard aquaculture aeration method (Bart Baca, pers comm. 2000). In Japan, the largest producer of cultured sea urchin "seed" (Lawrence pers. comm. 2002), larvae are typically cultured in 1000-L tanks with continuous water flow. Aeration is provided by a gentle airflow from one airstone at the bottom of the tank and one near the surface (Hagen 1996). Grosjean *et al.* (1998) described a land-based,

closed-cycle system used in France, in which echinoderm larvae are cultured in 200-L tanks with aeration provided by a central bubbler.

Airlift is thus a viable aeration method in larger containers. The failures of airlift during this project may be related to the size of the culture jars. Researchers continuing work with *T. ventricosus* larval culturing and seeking to increase the container size may wish to reexamine the airlift method.

The results of Experiment 5 indicate that *T. ventricosus* will develop, at least to the 6-armed stage, without aeration. However, when larvae are paddle-aerated, the survival rate is higher and development is more rapid compared with larvae receiving no aeration. Results of microscopic examination of larval development showed that none of the larvae cultured without mechanical aeration and fed *Iso* had reached the 8-armed stage by day 20 while 43.8% of those subjected to paddle aeration and fed *Iso* had reached that stage. A paired comparison *t*-test of the survival rates between the two aeration methods (where all larvae were fed *Iso*) showed the difference to be significant at the 0.001 level ($p < 0.001$). The results of the first two experiments indicate that the airlift aeration method was unsatisfactory for cultures in 1-gal jars. None of the larvae aerated with this method lived beyond eight days while some from experiment 1 and 2, which were reared in culture jars aerated with paddles and fed *Rho* or *Iso/Rho*, survived at least 10 and 39 days, respectively.

Algal Diet. After exhausting the maternally provided yolk reserve, the young larvae must become planktotrophic in order to survive until the development of a functioning jaw following metamorphosis. Larvae use the cilia along their bodies to capture food and move it toward the mouth (Hart & Strathmann 1995). If the density of

the algal food becomes too great, the cilia seem to become confused and feeding stops (Strathmann 1987). On the other hand, Boidron-Metairon (1988) demonstrated that intentionally starved larvae resorb tissue from their arms, but develop normally once feeding is resumed. In the first two experiments, tissue was resorbed from the arms of larvae fed *Iso/Dun*, suggesting that the larvae were not receiving enough nourishment. As a result, the frequency of feeding was increased from thrice weekly to every day. To keep the algal density from getting too great, the cell concentrations were reduced from 10 μml^{-1} to 7 μml^{-1} following the initial feeding. Tissue resorption was not noticed in subsequent experiments and the larvae continued to feed until metamorphosis.

Urchin larvae are also osmotrophs, deriving some of their nutrition from dissolved organic matter (DOM) in seawater (Levin & Bridges 1995). Because of the care taken to maintain axenic algal monocultures in these experiments, which involved filtration to 0.5 μm and ultraviolet sterilization of culture seawater, the magnitude of organic material available to the larval cultures was reduced. As a result, larval growth and development rates may have been slowed. No effort, other than the introduction of amino acids in experiments 3 and 4, was made to replace the material.

Survival and development rates during the five experiments varied among feeding regimens, indicating that the nutritional content of the algal species were not equal. The biochemical composition of several microalgae used in aquaculture has been studied intensively, and protein, carbohydrate and lipid concentrations vary both by species (Table 10) and by culture technique (Brown *et al.* 1998). However, no consensus appears to have been reached regarding the relative importance to echinoderm larvae of the various macromolecules, as well as monomers, mineral and vitamins identified (Boidron-

Metairon 1995, Lawrence pers. comm. 2002). George *et al.* (1990) reported that lipids affect the nutritional value of microalgae and that lipid content of the diet correlates positively with faster development and better survival of echinoderm larvae. Further, Enright *et al.* (1986) and Volkman *et al.* (1989) contended that specific lipid classes are important factors, and Brown (1991) suggested that sugar composition of the polysaccharides in microalgae may contribute to differences in nutritional value among species.

Table 10. Nutritional content of algae used as food for larval *T. ventricosus* and other echinoderm cultures. Sources: [1]Brown *et al.* 1998, [2]Brown 1991.

	Percentage of dry weight		
Algae Species	**Protein**	**Carbohydrates**	**Lipid**
Dunaliella tertiolecta	20	12.2	15
Isochrysis aff. *galbana*[2]	23	6	20
Rhodomonas salina[1]	48	22	22

With respect to the species used here, no information regarding the nutritional composition of *Rhodomonas* sp. could be obtained. However, Brown *et al.* (1998) reported data for *R. salina* (Table 10) and Enright *et al.* (1986) reported that a *Rhodomonas* identified only to genus had high levels of protein, carbohydrates and lipids. The paper did not provide the percentage composition data, but did report that the *Rhodomonas*-fed larval development and growth rates were more rapid compared with

other diets. The rapid development of the *Rhodomonas*-fed larvae in the current experiments is consistent with the findings of Enright *et al.* (1986).

The fatty acid composition of lipids is also important to larval growth and development (Jeffrey *et al.* 1994). Enright *et al.* (1986) reported that diets deficient in fatty acids 20:5(n3) and 22:6(n3) result in poor larval growth. Brown (1991) stated that green algae, such as *Dunaliella*, are deficient in both, perhaps explaining the relative poor performance of larvae fed this alga. Cryptomonads, including *Rhodomonas* and *Cryptomonas*, contain a relatively high total lipid level and have a very high proportion of 22:6(n3) (Enright *et al.* 1986). *Isochrysis* eff. g*albana* has a lipid content similar to the cryptophytes and has a high concentration of 22:6(n3), although the percentage appears to differ by species strain and culture technique (Jeffrey *et al.* 1994). Jeffrey *et al.* (1994) concluded that: "Species of *Isochrysis* … are favored [as a feed for larvae in aquaculture] because of their small size, fast growth rates, wide temperature ranges, absence of tough cell wall, and absence of toxins that could affect either the animal or the human consumer."

Cell wall structure may also play a role in survival of larvae fed *Rhodomonas* and *Cryptomonas*. While *Isochrysis* lacks a tough cell wall, the crytomonads not only have a double layered periplast, a proteinaceous subcellular layer, covering the cell (Leadbeater & Green 1993), but the cell also contains ejectosomes, ejectile organelles similar to nematocysts in coral (Bold & Wynne 1978). To the extent that all or a portion of the periplast is not digestible by the *T. ventricosus* larvae, the flocculent material that accumulated in the culture jars may have consisted of an aggregation of millions of these cell coverings that blanketed the larvae and prevent them from swimming freely. The

ejectosomes may also have irritated the external and digestive surfaces of the larvae, also contributing to their death. However, this second hypothesis is not consistent with the findings of Eckert (1998) on *Diadema antillarum*, Cameron (pers. comm. 2001) on echinoderms in general, or Hubbard & Wolcott (unpublished) on *Lytechinus variegatus*). In all of these cases, *Rhodomonas* performed well as a larval food. In the results reported here, the *Rho* and *Iso/Rho* diets resulted in more rapid growth and development compared with *Iso*. However, significantly higher survival rates resulted with *Iso* compared with the other two diets. *Isochrysis* is thus a suitable feed for culturing *T. ventricosus;* there is little value in continuing to experiment with cryptomonads.

Perimetamorphic Period. Confusion over the definition of the term metamorphosis appears to exist in the literature. While agreement exists that the term describes the change in structure from the larval to the juvenile form, identification of the commencement and conclusion of the period appears to have changed over time. Mortensen (1943) stated: "The larva was reared to metamorphosis by Mortensen, Op. cit. 1921." (p. 495) and Mortensen (1921) stated that his experiment was terminated when the larvae appeared competent (rudiment present). Lewis (1958) considered larvae with a rudiment and swimming in the water column to be metamorphosing larvae. He observed that the "power [of swimming] appears to be increased over that of a mature [8-armed] pluteus" (p. 617). Cameron (1986) equated metamorphosis with the end of the larval period, and later stated (pers. comm. 2001) that the classical definition of metamorphosis, used by Mortensen and Lewis, implied a more lengthy period and that, in the 1980s, he had redefined and limited the term to mean the rapid change in form from larva to juvenile after the larva had settled.

This seems inconsistent because Cameron (1986) quoted Lewis (1958) when stating that *T. ventricosus* metamorphose in about 28 days. Yet, Lewis clearly referred to larvae swimming in the water column. In Experiments 4 and 5, paddle-aerated larvae fed *Iso/Rho* and *Rho*, Cameron's preferred diet, became juveniles in 23 days.

Gosselin & Jangoux (1998) used Cameron's new definition when discussing the onset of metamorphosis in *Paracentrotus lividus*. However, to accommodate the many changes that occur at this stage, they coined the term perimetamorphic period to begin at the time the rudiment appears and end when the juvenile becomes exotrophic. The term may be more appropriate for describing the process whereby *T. ventricosus* reaches the exotrophic juvenile stage, as it eliminates the need for an immediate transition from larval to juvenile status.

During experiments 4 and 5, the form referred to as the echinoporculus frequently appeared following metamorphosis and preceding the juvenile form. Lewis (1958) observed the same stage, but called it a "degenerate metamorphosing larva" (p. 617). That several in experiments 4 and 5 became juveniles indicates that the echinoporculus is not necessarily a degenerate form. Echinoporculi bear short spines visible under light microscopy, indicating that at least part of the juvenile structure is present. Gosselin & Jangoux (1998) described a post-metamorphic structure similar to the echinoporculus, and Hubbard & Wolcott (unpublished) observed the form in *Lytechinus veriagatus.* However, no other references to this life history stage have been found. Neither Cameron (1986 and pers. comm. 2002) nor Mladenov (pers. comm. 2002) reported observing it.

Covering By Juveniles. The practice by sea urchins of holding particles with their spines and tube feet is referred to as covering. Dambach & Hentschel (1970)

suggest that the activity may be reflexive in response to a stimulus, such as light. Lawrence (1976) listed protection from wave action, light, and predation as possible reasons for the behavior, and suggested that the animal may also absorb nutrients from these items. He suggested that an experiment in which urchins, held in the inverted position, moved the covering material from the aboral surface to the oral surface demonstrates that the action is similar to the reflexive action of righting themselves.

During the covering experiment, *T. ventricosus* as small as 1.5 mm covered themselves with foreign material on the aboral surface regardless of the animal's orientation, and one juvenile urchin methodically placed three grains of sand on its aboral surface and crawled under a stone after sand grains the animal had been carrying were removed. These observations, at least in the very young *T. ventricosus*, indicate that light appears not to be the stimulus for covering.

CONCLUSIONS AND SUGGESTIONS FOR FUTURE RESEARCH

- *Tripneustes ventricosus* can be successfully cultured from fertilization to exotrophic juvenile.
- Aeration with paddles increased the survival rate and shortened the time between fertilization and metamorphosis.
- Amino acids, at least those used as a supplement and at the concentrations used, had no observable effect on the survival or development of *T. ventricosus* larvae.
- An alga with a soft cell covering and high in long-chain polyunsaturated fatty acids, such as *Isochrysis,* may delay metamorphosis. However, it results in low mortality when compared to cryptomonads. Algae deficient in long chain fatty acids appear to be a poor diet for the larvae.

- Cryptomonads, while promoting rapid development, seem to cause a high level of larval mortality due to the accumulation of flocculent material.
- The echinoporculus stage is a viable life form and not necessarily a degenerate one as concluded by Lewis (1958).
- Thus, when fed an appropriate diet and aerated using a method that does not damage tissues, *T. ventricosus* is a promising candidate for large-scale aquaculture.

The following are suggestions for future work with *T. ventricosus* larvae:

- Initial culture densities were limited to approximately 0.6 L^{-1}. Other researchers (Eckert 1995, Cameron pers. comm. 2001) have been successful with initial densities of 1.0 L^{-1}, suggesting that the higher density will be effective with *T. ventricosus* and should be attempted.
- Nothing in the protocols developed for these cultures precludes the use of a larger culture container. However, care should be taken to avoid container materials that may be toxic to the larvae (Strathmann 1987).
- Paddle aeration was effective and could be employed for larger containers. Airlift has been reported to be effective for larger containers and should be tried. Another circulation-aeration method worth consideration would be to place an airstone and tubing in a sheath of very small-meshed material. This may reduce the size of the bubbles to a point where they do not damage the larvae.
- Haptophytes, such as *Isochrysis*, seem to be high in long-chain fatty acids. Another such alga, used successfully in other countries, is *Pavlova*. Consideration should be given to comparing the two as larval food.

- Prepared feed has also been used successfully with juveniles (Lawrence *et al.* 2001) and should be tried with larvae.
- The amino acid experiments were inconclusive. However, larvae do absorb DOM (Levin & Bridges 1995). As a result, further experimentation with DOM, including amino acids and vitamins, seems appropriate.
- The culture water was filtered to 0.5 μm, subjected to UV sterilization and changed thrice weekly. This may be excessive and experimental results with less filtering and unsterilized water, which is changed less often, should be considered. Chen & Run (1998) successfully cultured *T. gratilla* in non-UV sterilized, filtered seawater, which was changed every 4 or 5 days. They did not report the degree of filtration.

- Algal culture water was removed centrifugally and density was determined by counting cells on a hemocytometer. Removal of the culture water may not be necessary if the f/2 formula (Guillard & Ryther 1962 and Guillard 1975) is used (the nutrient level used for all but the cryptomonads). Chen & Run (1998) demonstrated that *T. gratilla* larvae are not negatively impacted by this level of nutrients in algal culture water. Cell counts can be estimated by light absorption. Experiments should be conducted to see if the larvae can tolerate the small amount of nutrient contained in the algal culture water and if reasonable estimates of cell density can be made without counting cells.
- When culturing in larger containers, it may be necessary to check algal density in larval culture containers on a daily basis. This was not necessary for my work. However, with a larger water volume, it may become more important.

- Further research will be needed, including examination under SEM, to gain a better understanding of the developmental processes that occur in the echinoporculus stage.

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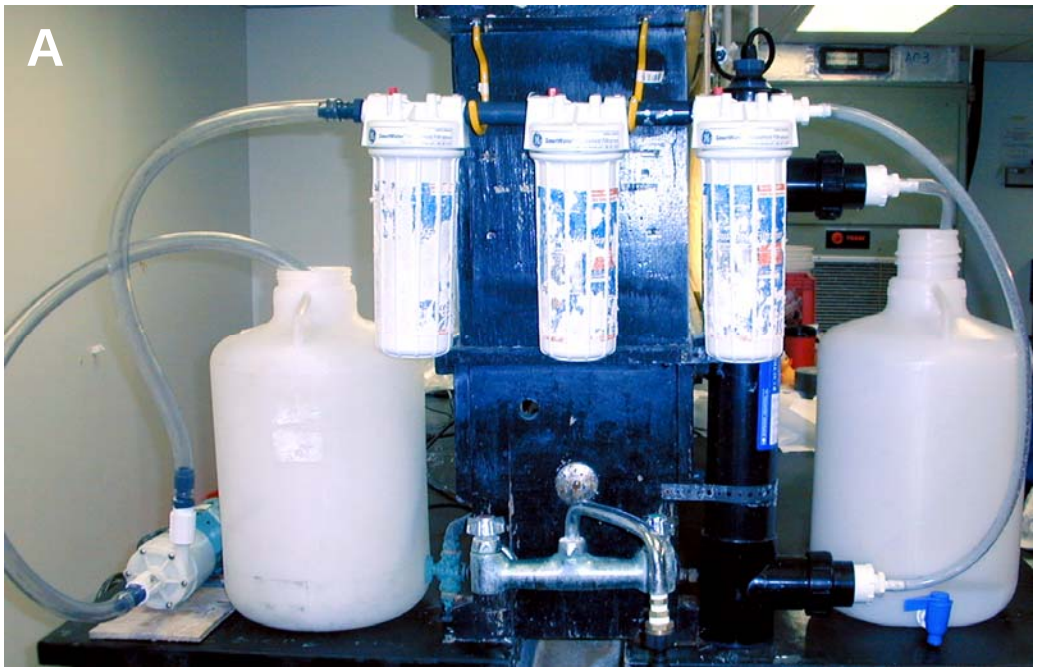

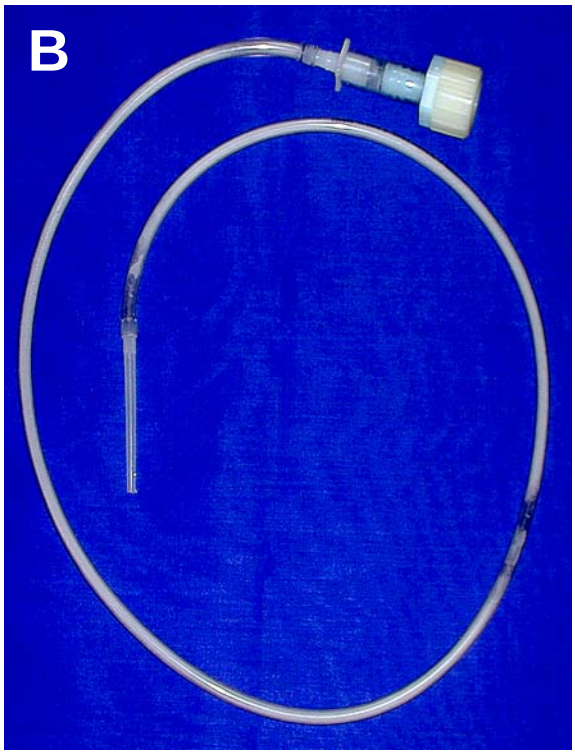

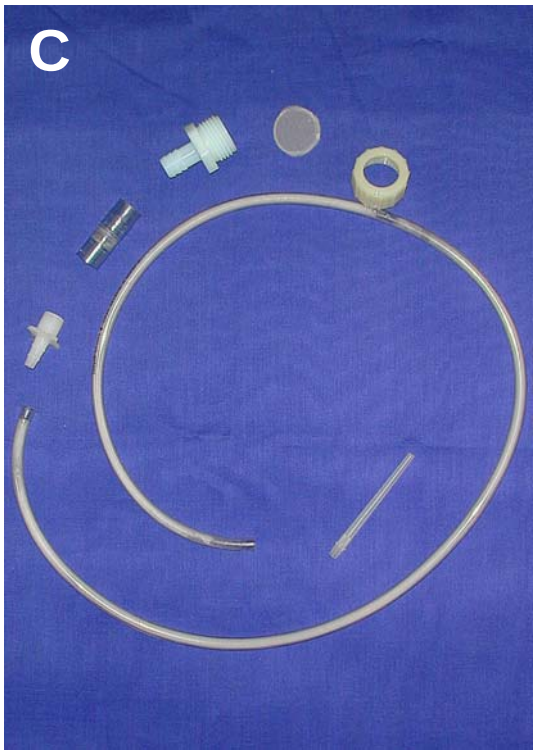

Figure 1. Seawater filtration and sterilization system and siphon. A. Seawater filtration and sterilization system. Water is pumped from the 20-L carboy at left, up through the three filtration cartridges (center), through the UV sterilizer (behind the third filtration cartridge) and into the storage carboy (right). B. Siphon. C. Siphon components.

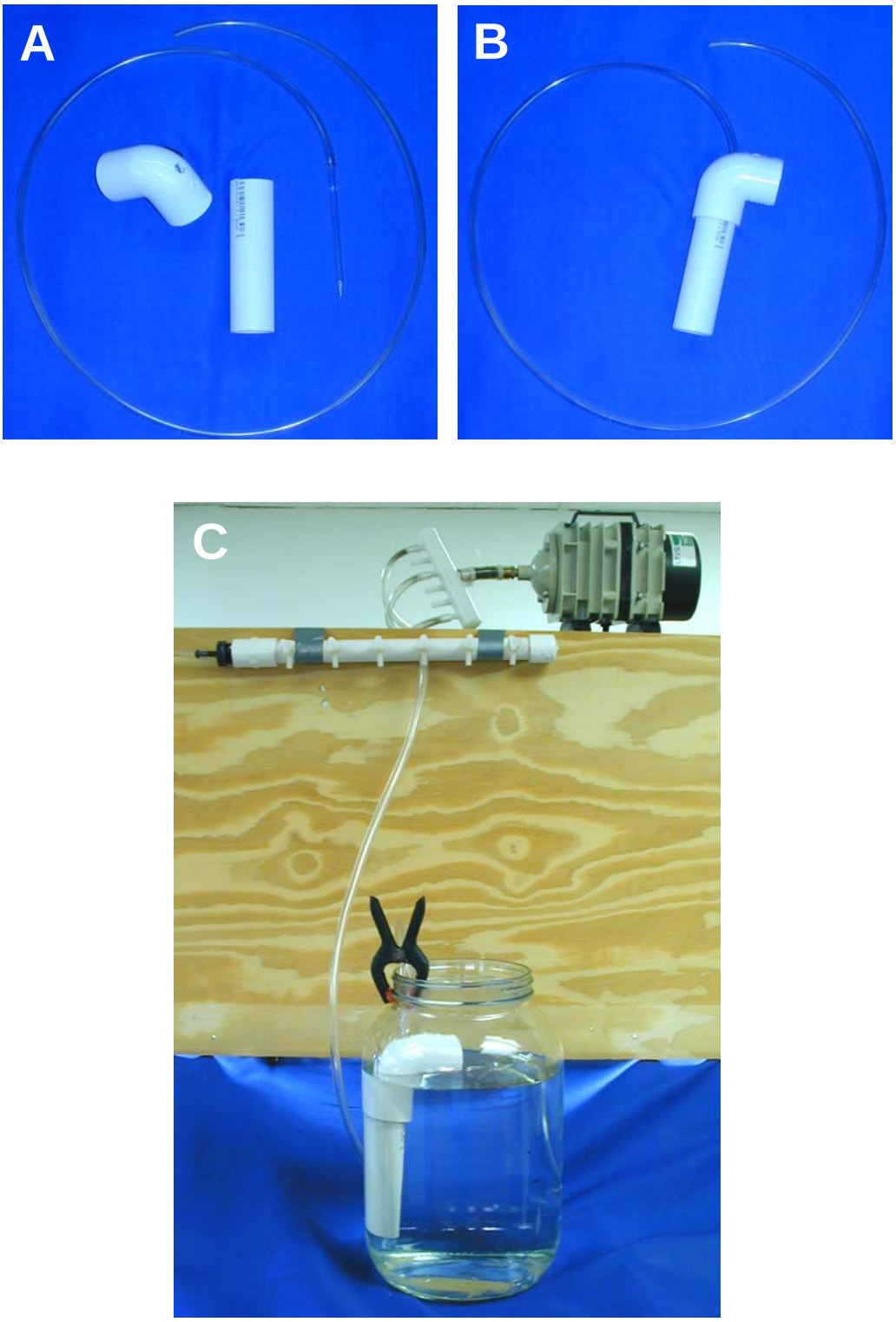

Figure 2. Airlift system. A. Airlift pipe and tubing components. B. Airlift pipe and tubing assembly. C. Airlift system in operation. Note that a clamp holds the pipe against the inside of the culture jar.

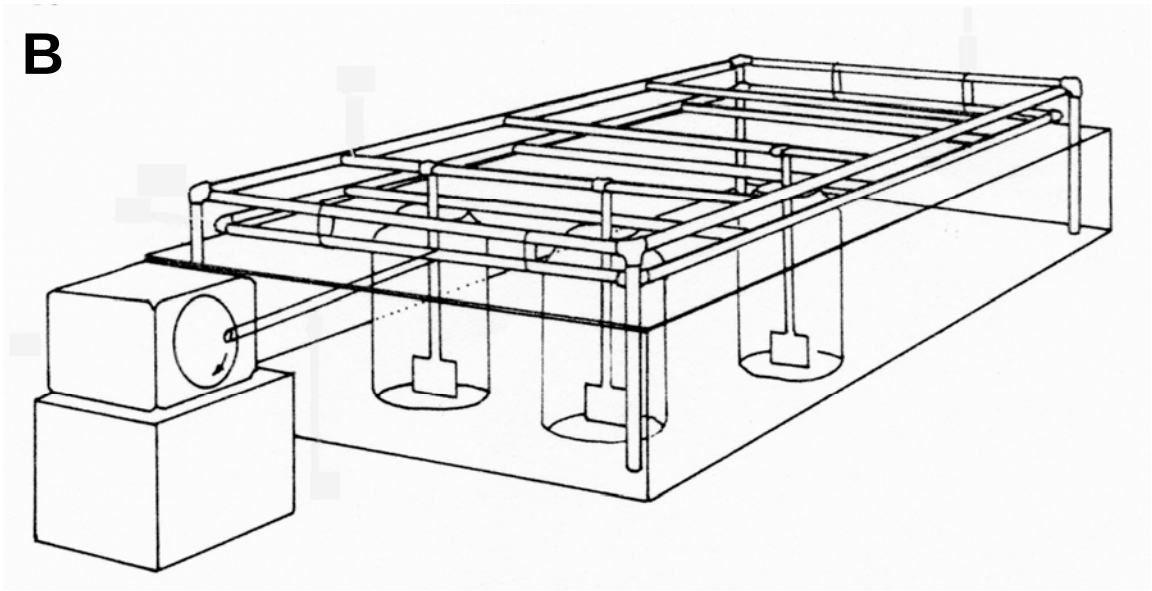

Figure 3. Paddle aeration system. A. Paddle-aeration system as used in culture experiments 1 – 5. B. Paddle-aeration system as drawn by Strathmann (1987, page 16).

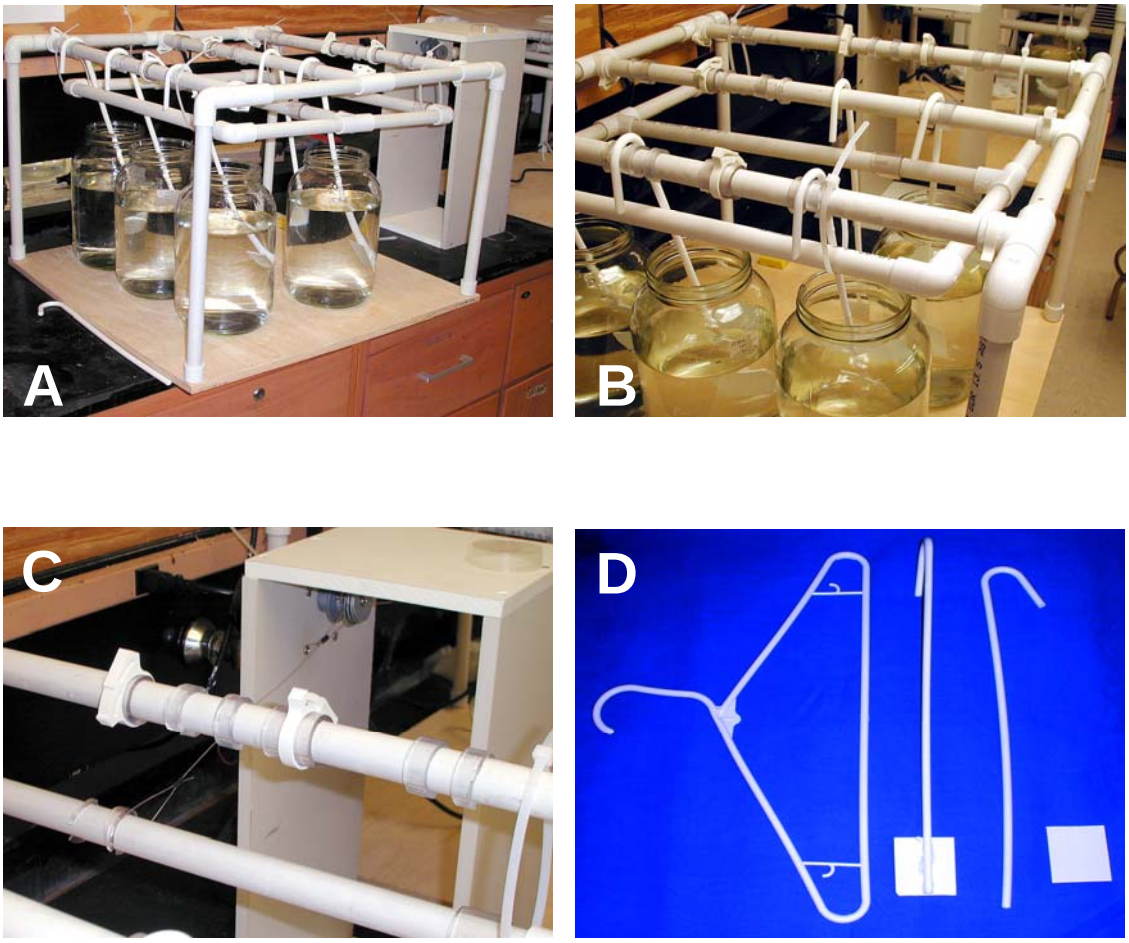

Figure 4. Paddle-aeration system. A. & B. Close up views of one side of the system showing suspended rack that moved the paddles. C. Close up of motor stand. D. Paddles were constructed from plastic coat hangers and pieces of white plastic, glued together with aquarium sealant.

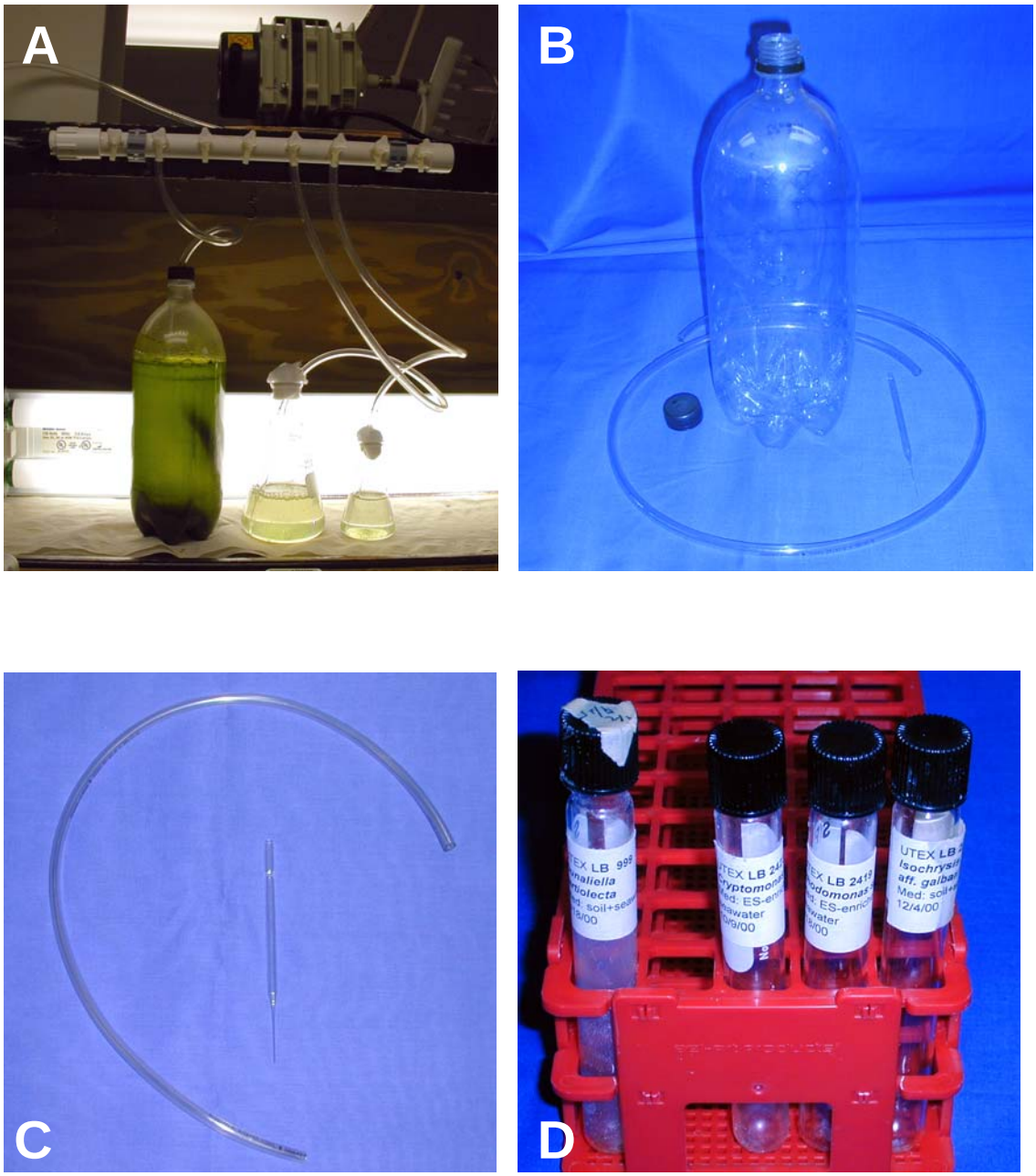

Figure 5. Algal Culture. A. Algal cultures in various sized containers. B. Pipette, tubing and 2-L bottle. C. Pipette and tubing for flasks. D. Starter culture in 15-ml screw-top tubes.

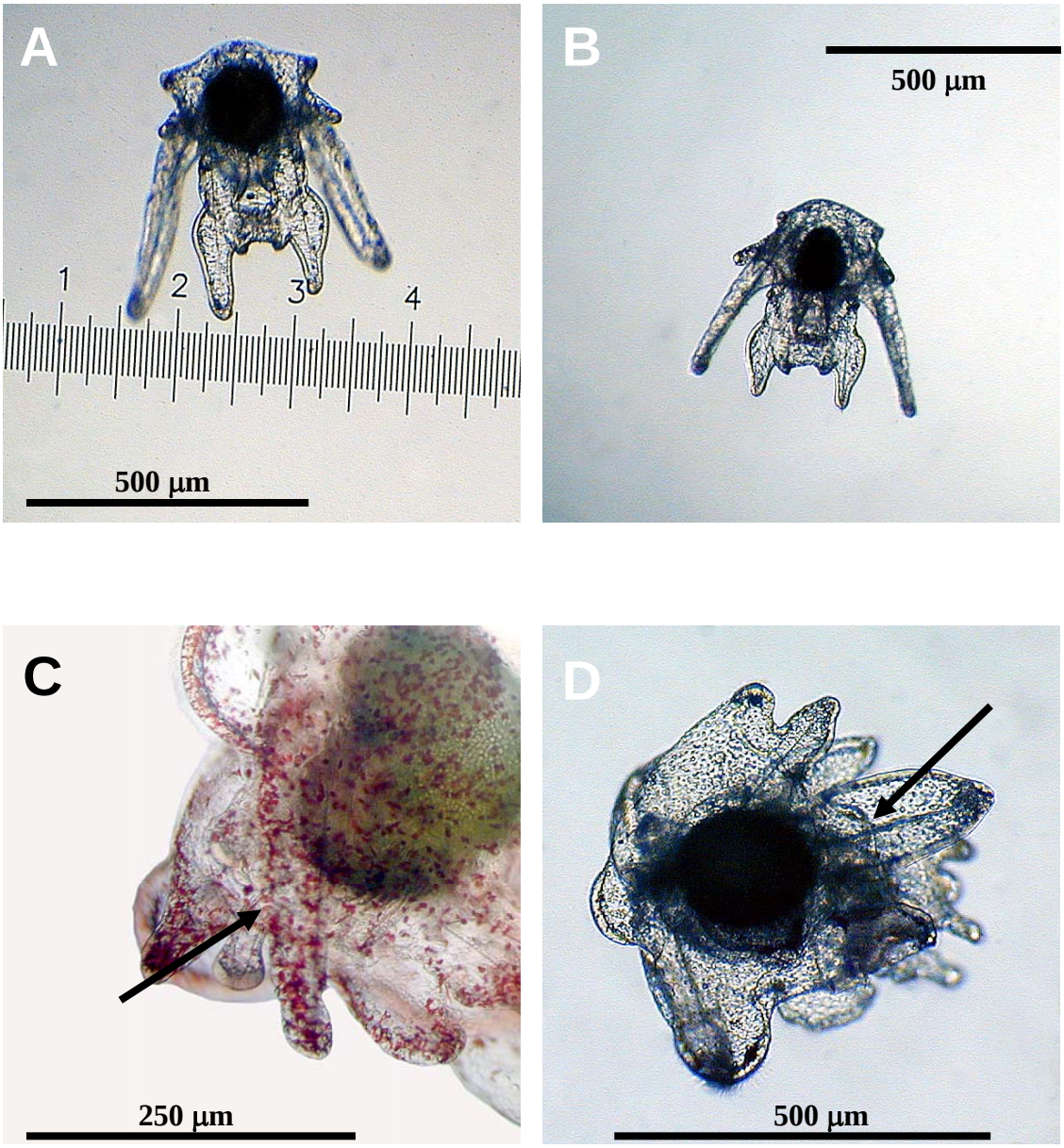

Figure 6. Mature larvae. A & B. Mature larvae without rudiment. C & D. Mature larvae (experiment 2) with rudiment (arrow).

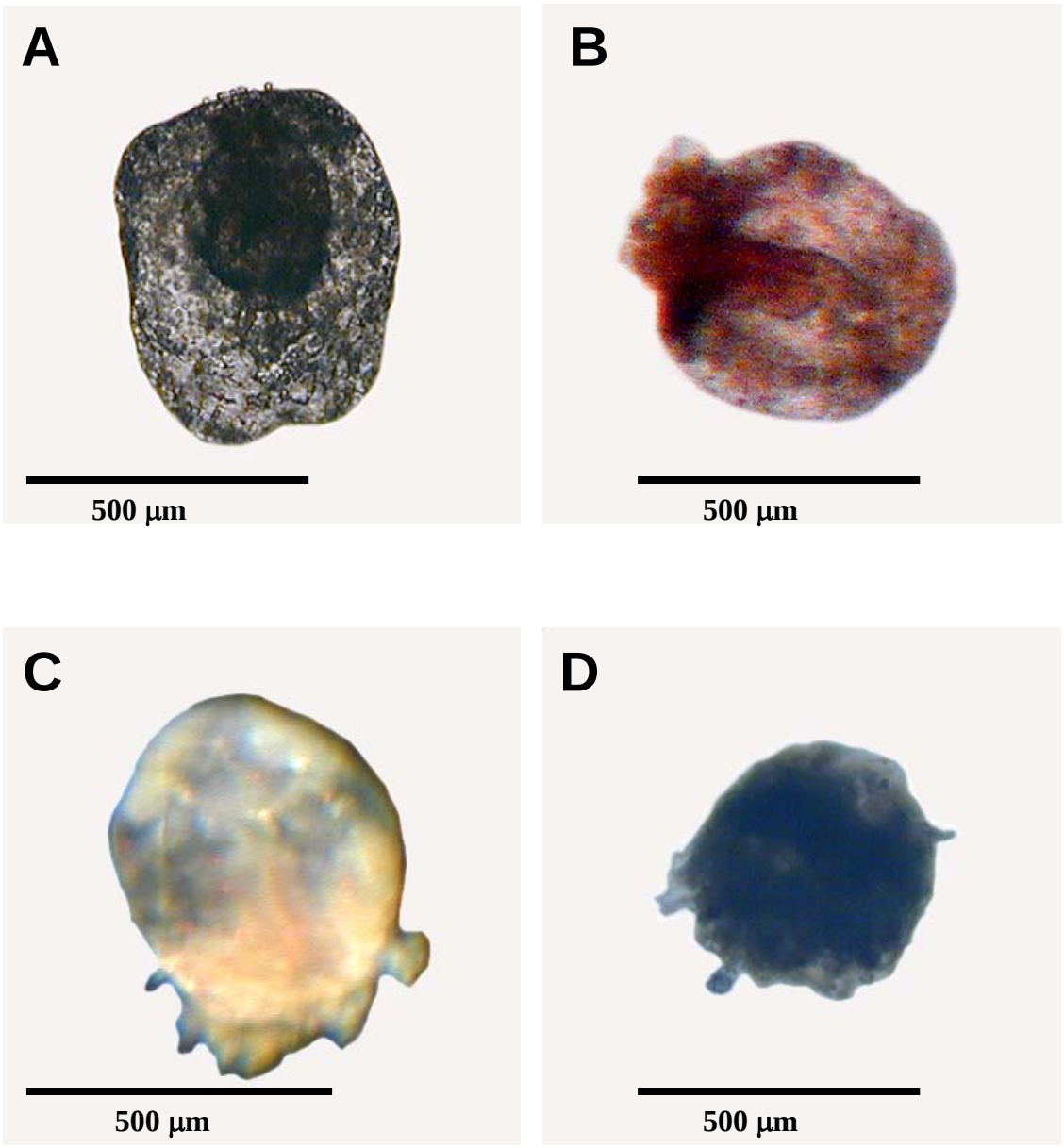

Figure 7. Degenerating larvae (experiment 3). A. Prior to settlement. Note lack of arms, globular shape and lack of rudiment. B & C. Settled degenerating larva. D. The remains of a degenerate larva. Note similarity in shape to echinoporculus stage (Figure 8).

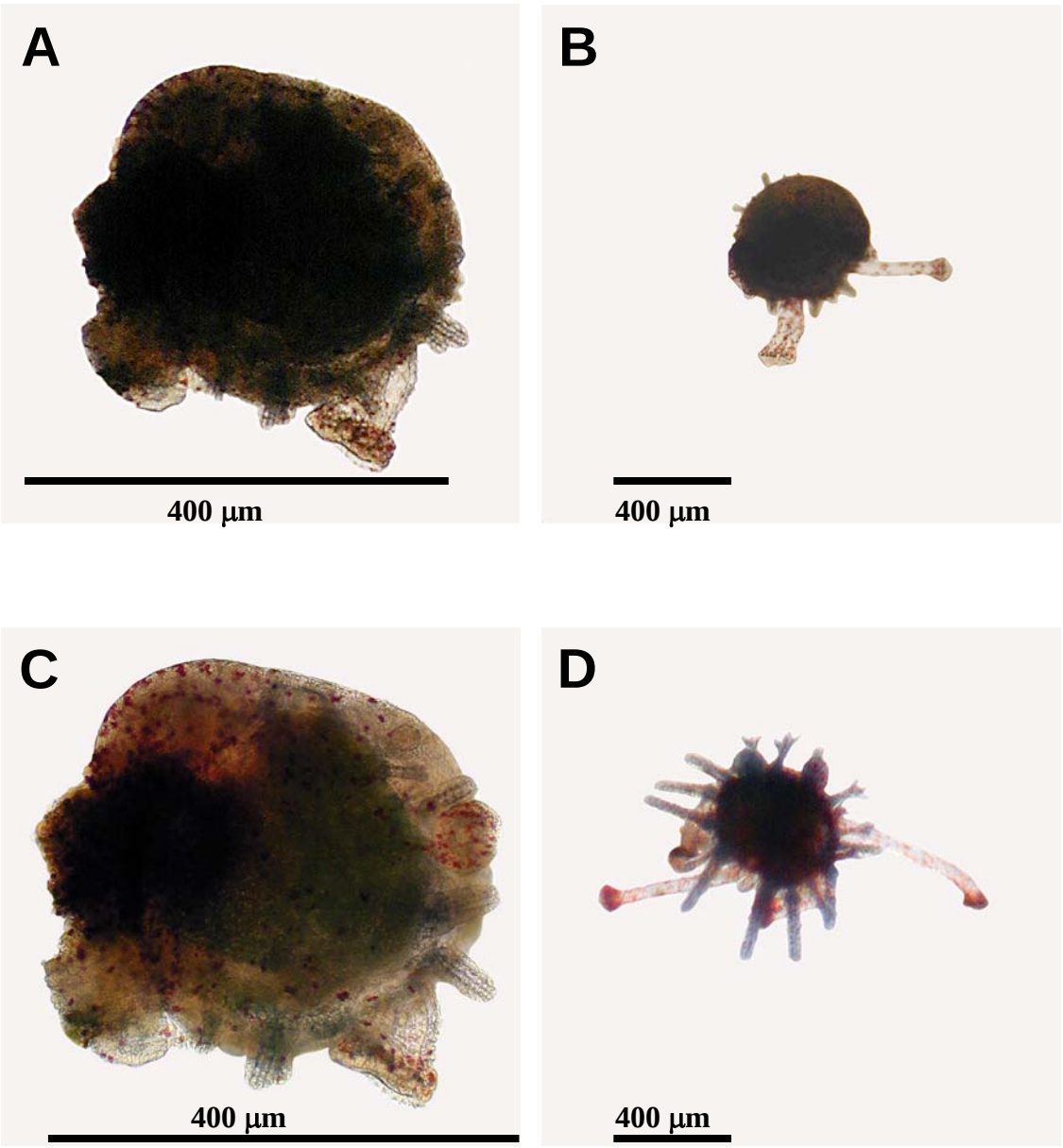

Figure 8. Echinoporculus stage (experiments 4 and 5). A. Newly metamorphosed echinoporculus. Note that form is similar to the remains of a degenerate larva (Figure 7D) but with tube feet. B. Day old echinoporculus extending tube feet. C. Close up of echinoporculus showing spines within the soft, apparently unresorbed, larval tissue. D. Juvenile derived from echinoporculus.

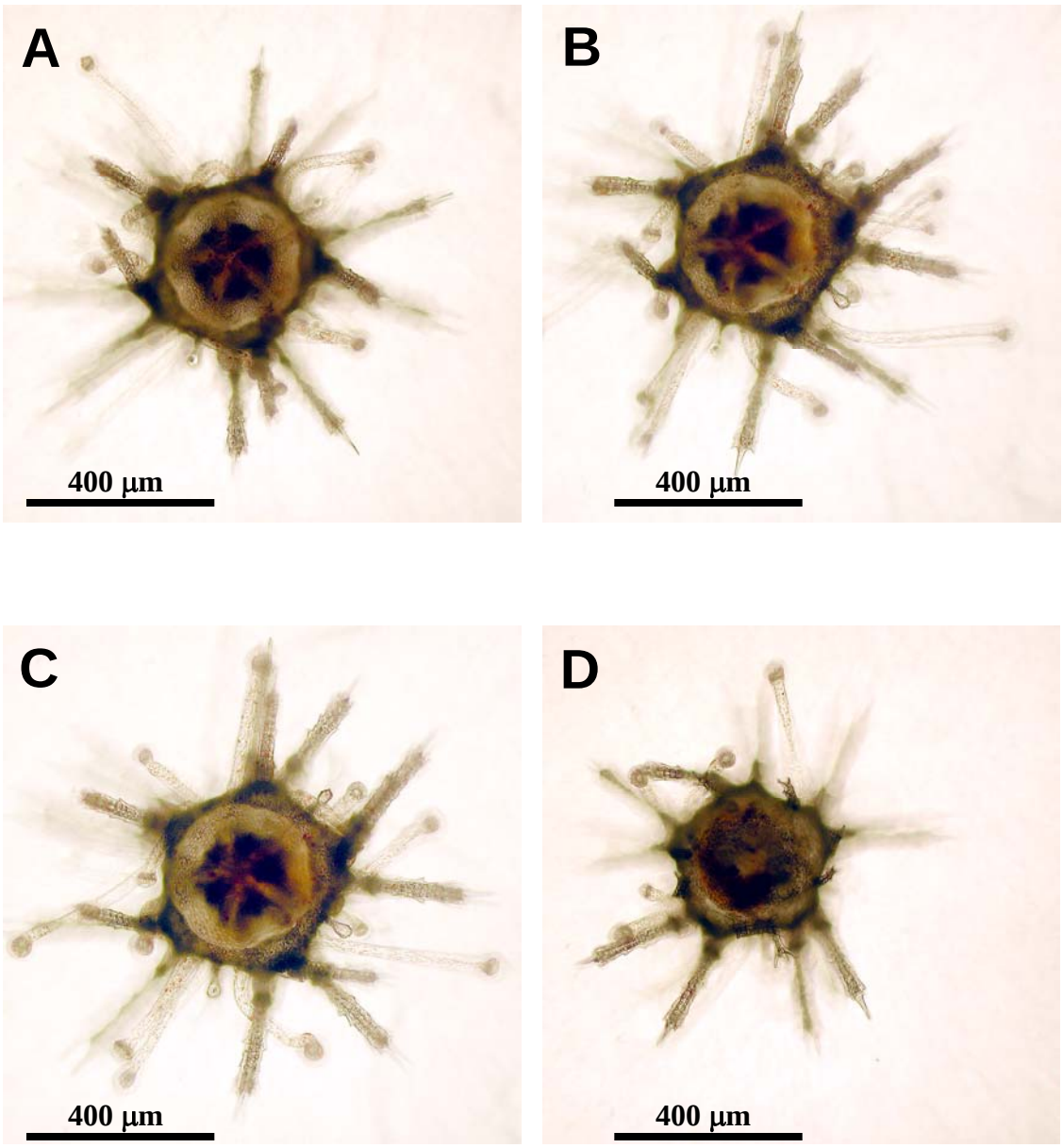

Figure 9. Juvenile urchins. A.-C. Functional jaw present at day 54 (experiment 4). D. Juvenile lacking functional jaw at day 54 (experiment 4).

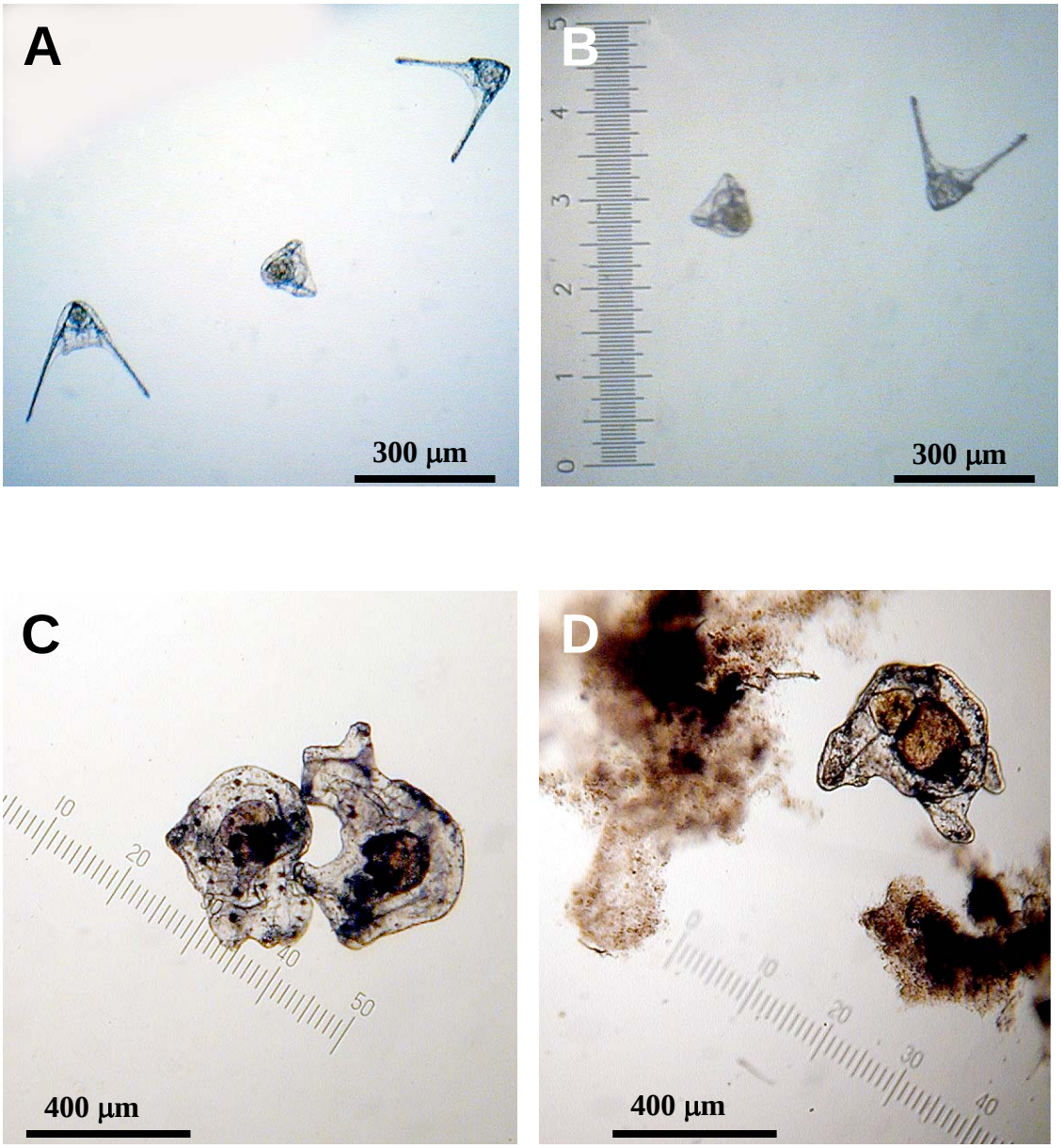

Figure 10. Deformed larvae. A. Larva in center lacks extended arms. B. Larva at left has deformed arms. C & D. Deformed larvae settling (experiment 3).

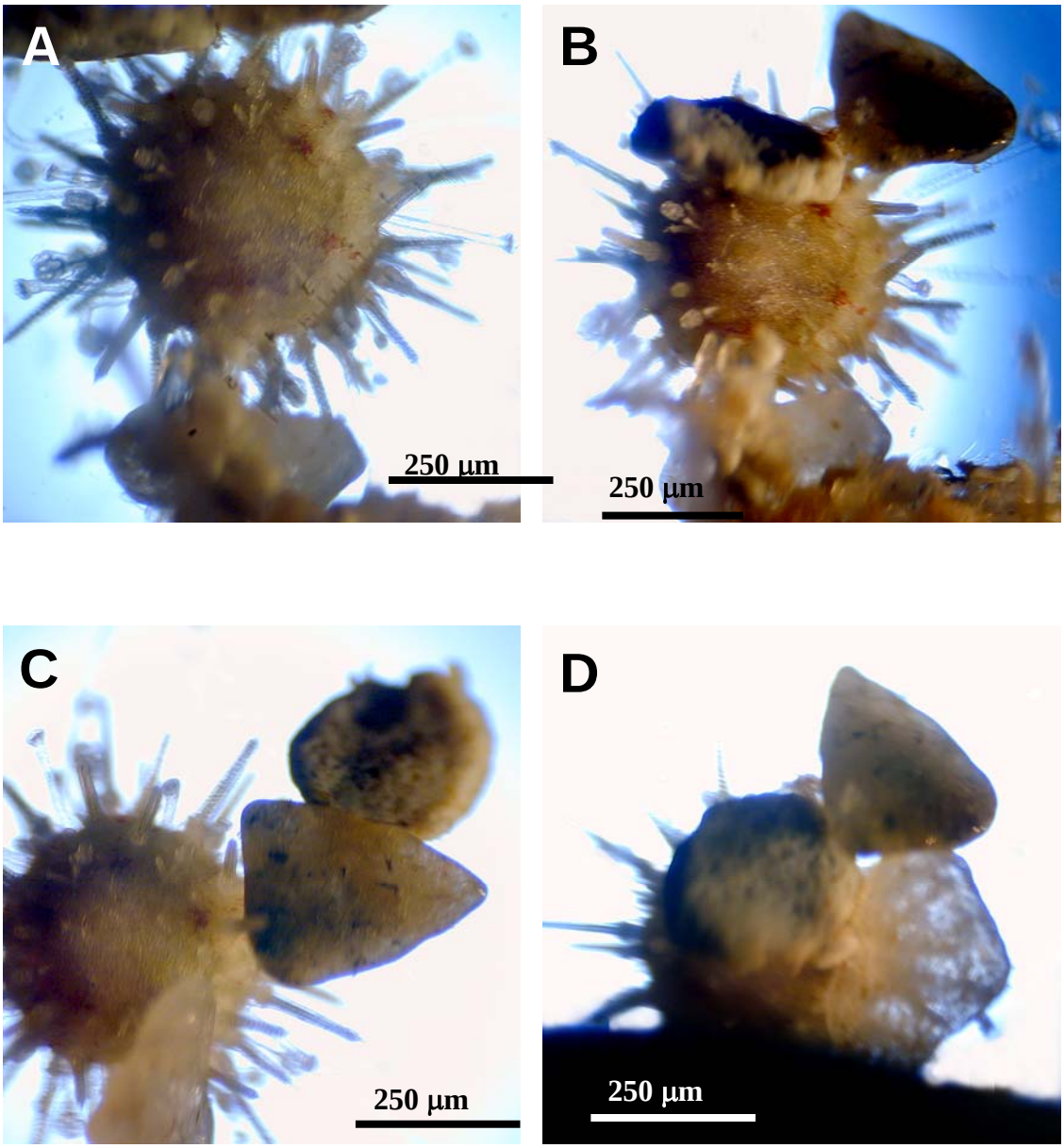

Figure 11. Juvenile covering. A. Juvenile with sand grains removed. B. & C. Juvenile placing sand grains on aboral surface. D. Juvenile crawling under stone. All viewed from above.