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A SIMPLE TECHNIQUE FOR PHYSICAL MARKING OF LARVAE OF MARINE BIVALVES

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ABSTRACT The identification of effective, nontoxic means for physically marking and tracking marine invertebrate larvae is a necessary step towards meeting a major goal of modern marine population biology, the direct measurement of larval dispersal. An inexpensive, rapid and effective means for marking bivalve larvae would be particularly useful because, as a taxonomic group, bivalves contain many commercially important and exploited species. Likewise, bivalves produce large numbers of propagules for experimental procedures and, for many species, methods for rearing larvae have been well established. Calcein has been used as a marker in numerous studies of adults and juveniles of calcium-carbonate-containing marine organisms, but its effects on small and sensitive life history stages such as embryos and larvae can be detrimental. We show that calcein can be used to rapidly and effectively mark large numbers of larvae from two bivalve species, *Argopecten irradians concentricus* (Say, 1822) (the Bay Scallop) and *Mytilus trossulus* Gould, 1850 (the Bay Mussel). Calcein had no detectable negative effects on growth or survivorship of larvae of either species; therefore, this fluorescent mark should serve as a useful tool for directly tracking dispersal of these species in the field. Our marking method is simple and inexpensive and can easily be used to determine the effectiveness and potential toxicity of the calcein mark for other bivalves.

KEY WORDS: *Argopecten irradians, Mytilus trossulus,* calcein, larval dispersal

One of the most important issues in modern marine science is determining the extent to which populations of marine organisms, many of which are increasingly impacted and diminished by human activities, are connected by larval dispersal. The great difficulty in tracking larvae directly, coupled with the complexity of interpretation of population genetic data (Waples 1998, Neigel 2002, Palumbi 2003), has led to an incomplete understanding of the extent to which local populations are either self-recruiting or supplied with new recruits from larvae produced at distant locales. Several methods for physically marking early life history stages of marine invertebrates have been developed (reviewed in Levin 1990), but only a few methods have been tested for efficacy and toxicity in larvae. Development of such physical markers is crucial to directly measuring dispersal of marine larvae, and to testing indirect methods, such as population genetic analyses, of estimating dispersal (Morgan 2000, Thorrold et al. 2002).

Calcein, or 2,4-bis-[N,N′-di(carbomethyl)-aminomethyl1]-flourescein (Sigma # C0875), has been used by a number of researchers to mark adult and larval fish (e.g., Wilson et al. 1987, Monaghan 1993, Brooks et al. 1994, Mohler 1997, Hernaman et al. 2000, Leips et al. 2001), adult ascidians (Lambert & Lambert 1996), juvenile and adult echinoderms (Medeiros-Bergen & Ebert 1995, Stewart 1996, Rogers-Bennett et al. 2003, Russell & Urbaniak in press), brachiopods (Rowley & MacKinnon 1995), cnidarians (Marschal et al. 2004) and larval, juvenile and adult molluscs (Day et al. 1995, Kaehler & McQuaid 1999, Moran 2000, Moran & Emlet 2001, Eads & Layzer 2002, Iyengar 2002, Phillips 2002, Allen & Williams 2003, Clarke et al. 2004). Calcein has many of the characteristics of a good marker for larvae. Calcein is incorporated into growing calcium carbonate structures, and for small organisms such as larvae of molluscs, fish and echinoderms with calcified skeletons, otoliths or statoliths, calcein can be easily and rapidly used to mark large numbers of animals. The mark is visible only under blue light and appropriate filter sets, so it does not increase the vulnerability of larvae to visual predators. The mark is long-lasting in the field (Wilson et al. 1987, Medeiros-Bergen & Ebert 1995, Moran & Emlet 2001, Clarke et al. 2004), and in taxa such as molluscs and fish, in which $CaCO_3$ larval structures are retained on juveniles and adults, later life history stages can potentially be identified from marked larval structures.

Along with these characteristics, a useful larval marker for dispersal studies must be nontoxic and must not adversely affect growth or survivorship. Whereas calcein in many cases does not appear to have detrimental effects (e.g., Kaehler & McQuaid 1999, Moran 2000, Eads & Layzer 2002, Bernhard et al. 2004), early life-history stages (larvae and juveniles) have in many cases been shown to be sensitive to calcein through a reduction in survivorship and/or growth even at low marking concentrations (Brooks et al. 1994, Bumguardner & King 1996, Gelsleichter et al. 1997, Russell & Urbaniak in press). Though an increasing number of studies in recent years have used calcein as a marker (reviewed earlier), few have directly tested whether calcein negatively affects growth or survivorship of early life history stages. This study tests the efficacy of calcein for marking veligers of two commercially important species of bivalve, the Bay Scallop (*Argopecten irradians*) and the Bay Mussel (*Mytilus trossulus*), and the effect of marking on larval survivorship and growth. The results show calcein to be an effective, long-lasting marker that does not negatively impact performance either before or after metamorphosis; this suggests that calcein is a useful tool for field experiments to estimate bivalve larval dispersal.

METHODS

Calcein Marking Solution

A 6.25 g l^{-1} solution of calcein in distilled water was buffered to pH 6 with sodium bicarbonate (to enhance the solubility of calcein, after Wilson et al. 1987). This stock solution was added to Instant Ocean (Aquarium Systems, Inc.) (mixed to a concentration of 32 ppt) for a final concentration of 100 ppm calcein (after Moran & Emlet 2001). Veligers of two species, the Bay Scallop (*Argopecten irradians*) and Bay Mussel (*Mytilus trossulus*), were maintained in marking solution for 72 h. To determine whether lower concentrations of calcein would be effective, some larvae of *A. irradians* were also marked with 50 ppm calcein for 48 h. During and after marking, larvae were fed *Isochrysis galbana* at

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20,000 cells mL^{-1} and water was changed on a daily basis. New calcein stock was added to cultures at each water change for the 2- or 3-day marking period. To assess the effectiveness of calcein as a marking substance, animals were examined under FITC filter sets with either a Zeiss Axiophot compound fluorescence microscope equipped with an Axiovision 4.1 digital camera, or an Olympus BX51 microscope equipped with an Olympus Q-Fire color digital camera. Larvae were examined after 9 days of feeding (*A. irradians*) or 8 days of feeding (*M. trossulus*). To determine whether the mark was long-lasting, a separate experiment was performed in which juveniles of *A. irradians* that were marked as larvae were grown well past metamorphosis and examined for a calcein mark 63 days after fertilization, when larvae had metamorphosed and grown to a size at which they were readily visible to the naked eye. On all larval and juvenile specimens, the presence or absence of blue-green calcein fluorescence was noted, as was the location and relative visibility of the mark.

Growth and Survivorship of Marked and Unmarked Larvae

Argopecten irradians

Larvae were purchased from Bay Shellfish Co., Palmetto, Florida, at 3 days of age. After visually determining that larvae were healthy and swimming, larvae were transferred into 35 mL of Instant Ocean at 32 ppm in 50 mL Falcon tubes that were capped, placed on their sides and constantly and gently agitated on a rotary shake table. Larvae were kept in 12 replicate Falcon tubes (= "containers") at a density of 10 larvae mL^{-1}. Six containers were randomly assigned to the experimental group for calcein marking (= "Marked" containers). Larvae in these six containers were marked with calcein at 100 ppm for 72 h. The other six containers received identical treatment except that an equivalent volume of tap water was added to cultures in place of calcein marking solution (= "Unmarked" containers). Water was changed and larvae were fed daily.

Larvae were sampled for growth and survivorship after 9 days of feeding (when larvae were 12 days old). To compare growth of marked and unmarked larvae, larval sizes were measured and compared between the two treatment groups. The number of larvae measured from each container at each treatment was 30, except for one container in which only 18 surviving larvae were recovered because of an error during sampling. Larvae were measured under an Olympus BX-41 compound microscope equipped with an ocular micrometer accurate to ±2 μm. Size was measured as the greatest length across the larval shell. Sizes of larvae were compared using 1-factor nested ANOVAs with container as a random factor nested within treatment (fixed factor = unmarked or marked). To assess differences in survivorship, the total proportion of "live" (= intact larval tissue in shell) relative to "dead" (decayed tissue in shell, or empty shell) animals was calculated for each marked and unmarked container. Proportions were arcsine-transformed and compared between "Marked" and "Unmarked" treatments using Student unpaired *t*-tests at each sampling date.

Mytilus trossulus

Methods for rearing, marking and testing the effect of the calcein mark on growth and survivorship were identical to methods described earlier for *A. irradians*, with the following exceptions. Larvae were obtained at 4 days of age from Coast Seafoods Company, Bellevue, WA. Eight rearing containers were used, for a total of four "Marked" and four "Unmarked" replicates. Larvae were fed for 7 days (including marking time), then cultures were broken down and the size and survivorship of marked and unmarked larvae were measured and compared as earlier.

Post-metamorphic Growth and Survivorship of *A. irradians*

To determine whether marking with calcein had long-term effects on growth and survivorship, and to determine how visible the calcein mark is on postmetamorphic juveniles, we marked additional larval cultures of *A. irradians* and raised them for several weeks postmetamorphosis. Adult *A. irradians* for these experiments were obtained from the wild in North Carolina and induced to spawn with temperature shock. Larvae obtained from these crosses were reared to 48 h of age (the D-veliger stage), then transferred to 12 1-L culture vessels containing Instant Ocean at 32 ppt. Initial larval concentrations in the cultures were one larva mL^{-1}. Six of the 12 beakers were randomly chosen for the "Marked" treatment and calcein stock was added as stated earlier, for a final concentration of 50 ppm. The other six beakers served as an unmarked control and received an equal volume of tap water. Marked larvae were maintained in the marking solution for 48 h. All cultures were then transferred to Instant Ocean at 32 ppt and reared through to metamorphosis (beginning at approximately 16 days) and beyond. During larval and postmetamorphic development, scallops were fed *Isochrysis galbana* at 20,000 cells mL^{-1} and water was changed every other day. To minimize damage or loss through handling, larvae were only checked irregularly for metamorphosis. After 63 days of culturing (including larval and postmetamorphic stages), when juveniles were easily visible to the naked eye, all beakers were sampled for surviving juveniles. Each juvenile was measured for total shell width under an Olympus SZX9 microscope equipped with an Olympus Q-Fire color digital camera and BioSuite image analysis software. Juveniles were also examined under a fluorescence microscope (as mentioned earlier) for the presence or absence of a fluorescent calcein mark. Sizes of marked and unmarked juveniles were compared with a Student *t*-test on the grand mean of juvenile length in each beaker (*t*-tests were used instead of ANOVAs because several beakers had only one, or no survivors). Survival was compared between the "Marked" and "Unmarked" treatments by counting the total number of living juveniles in each beaker and testing for differences between treatments with a Student *t*-test.

RESULTS

Calcein Marking

All Marked larvae examined under fluorescence microscopy had clearly visible, bright, yellow-green fluorescent FITC bands that ran parallel to (and in line with) the concentric growth rings of the larval shell (Fig. 1a, 1b). These marks were not visible under transmitted light microscopy (Fig. 1a). Unmarked larvae had no such mark and displayed very little or no autofluorescence under the FITC filter set. The visual appearance of the mark was very similar in *M. trossulus* and *A. irradians*. Larvae treated with 50-ppm calcein for 48 h had marks of approximately equal brightness to those treated at 100-ppm calcein for 3 days.

Growth and Survival of Marked and Unmarked Larvae

Argopecten irradians

After 9 days of feeding (12 days old and 6 days after marking), survivorship in the "Unmarked" containers averaged 61 ± 11%

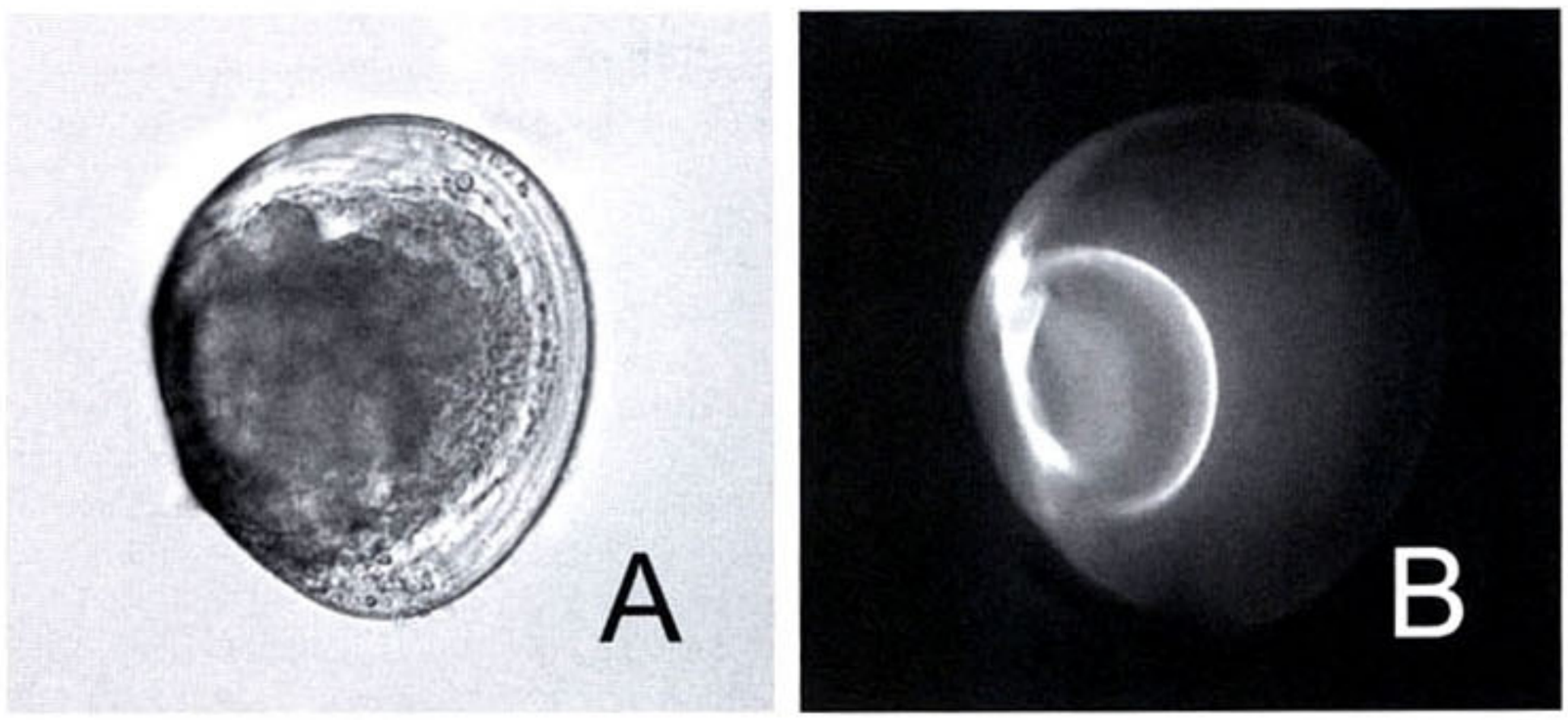

Figure 1. Micrograph of a calcein-marked larva of *Argopecten irradians* viewed under transmitted light (A) and under fluorescence microscopy with an FITC filter set (B). Larva is 12 d old and 140 μm in greatest shell height.

(SD), whereas survivorship in the "Marked" containers averaged 59 ± 11%; this difference was not statistically significant (T = 0.21, DF = 10, P = 0.84). The average size of marked larvae, estimated as the grand mean of shell lengths measured on all containers, was 123.6 ± 4.7 μm. The average size of unmarked larvae was 126.1 ± 5.4 μm. When sizes of marked and unmarked larvae were compared, they were not significantly different (MS effect = 484.2, DF = 1, MS error = 723.7, P = 0.354). There was a highly significant container effect on growth (MS effect = 723.7, DF = 10, MS error = 301.6, $P < 0.01$).

Mytilus trossulus

After 7 days of feeding (4 days after marking, 11-day-old), survivorship in the "Marked" containers averaged 38 ± 3% (SD), whereas survivorship in the "Unmarked" containers averaged 21 ± 4%; this difference was statistically significant (T value = 6.6, $P < 0.001$, DF = 6) with larvae from "Marked" containers showing higher survivorship than "Unmarked" containers. The average size of unmarked larvae, estimated as the grand mean of shell lengths measured on all containers after 7 days of growth, was 182.6 ± 0.9 μm. The average size of marked larvae was 191.2 ± 10.4 μm after the same growth interval. Sizes of unmarked and marked mussel larvae were not significantly different (MS effect = 4411.8, DF = 1, MS error = 1635.0, F = 2.7, P = 0.15). There was a significant effect of container on growth (MS effect = 1635.0, DF = 6, MS error = 426.6, F = 3.8, $P < 0.005$).

Post-metamorphic Growth and Survivorship of *Argopecten irradians*

Sixty-three days after spawning, an average of 4.3 ± 1.0 (SD) juveniles were alive in the six marked cultures and an average of 1.2 ± 1.2 (SD) were alive in the unmarked cultures. These numbers represented an average of 0.4% and 0.1% survival, respectively, from cultures started with 1,000 larvae each. Survivorship was significantly higher in the marked cultures (T = 4.97, DF = 10, $P < 0.001$). The average size of marked juveniles, estimated as the grand mean of shell lengths measured on all containers, was 1278.5 ± 149.5 μm, and the mean size of unmarked juveniles was 1270.5 ± 189.2 μm. When sizes of marked and unmarked juveniles were compared, they were not significantly different (T value = 0.98, P = 0.36, DF = 8). All marked juveniles retained a clear, bright band of calcein fluorescence on the prodissoconch that was readily visible under the fluorescence microscope (Fig. 2a, 2b). Unmarked juveniles uniformly lacked any blue-green autofluorescence.

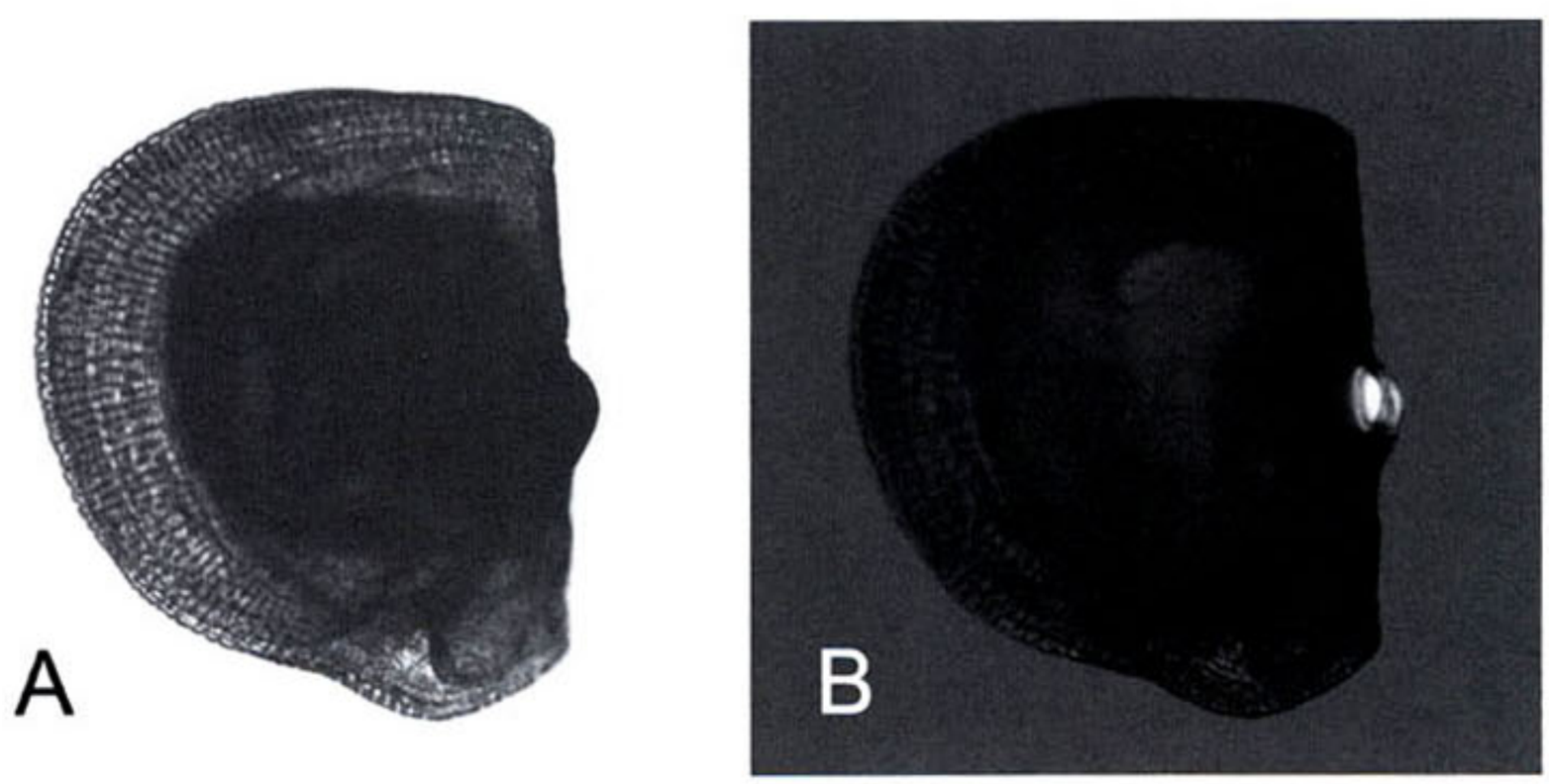

Figure 2. Micrograph of a juvenile of *Argopecten irradians* marked with calcein during the larval phase and grown in the laboratory to 1 mm shell length. A, Specimen viewed under transmitted light. B, Specimen viewed under blue light with an FITC filter set and a small amount of transmitted light, showing calcein fluorescence of the prodissoconch. Juvenile is 57 d old (postfertilization).

DISCUSSION

The identification of effective, nontoxic means for physically marking and tracking marine invertebrate larvae can provide an important tool for directly measuring larval dispersal, which is currently a major goal of marine population biology (Thorrold et al. 2002). An inexpensive, rapid and effective means for marking bivalve larvae is particularly valuable because bivalve adults produce large numbers of gametes, methods for rearing bivalve larvae have been well-established for many species, and, as a taxonomic group, bivalves contain many commercially important and exploited species. Calcein has been used as a marker in numerous studies of adults and juveniles of calcium-carbonate-containing marine organisms (reviewed in Introduction), but its effects on small and sensitive life history stages such as embryos and larvae are in some cases detrimental (Brooks et al. 1994, Bumguardner & King 1996, Gelsleichter et al. 1997) but more often are unknown. We show that calcein can be used to rapidly and effectively mark large numbers of larvae from two bivalve species. The mark had no detectable negative effects on growth or survivorship of larvae of either species, and was long lasting and readily visible on shells of metamorphosed juveniles even after considerable growth (up to >1.4 mm shell length).

Because patterns of shell wear may differ between the laboratory and field and an intact prodissoconch is necessary to identify settled juveniles that were marked as larvae, we collected 14 juveniles between 3.3 and 6.3 mm in shell length from seagrass beds in Bogue Sound, North Carolina to determine whether the prodissoconch was retained on juveniles that settle and grow in natural habitats. We examined the prodissoconch regions of these shells under scanning electron microscopy with a Cambridge/Leica Stereoscan 440 Scanning Electron Microscope operated at 15 kV. We found that the prodissoconch was clearly visible (Fig. 3) on 13/14, or 93%, of these shells. Because the larval shell appears to be retained on the majority of juvenile scallop specimens in the field and the calcein mark is visible on laboratory-reared juveniles that are large enough to be readily visible to the naked eye (>1.4 mm in shell width), the mark should serve as a useful tool for directly tracking dispersal of bivalve larvae in the field.

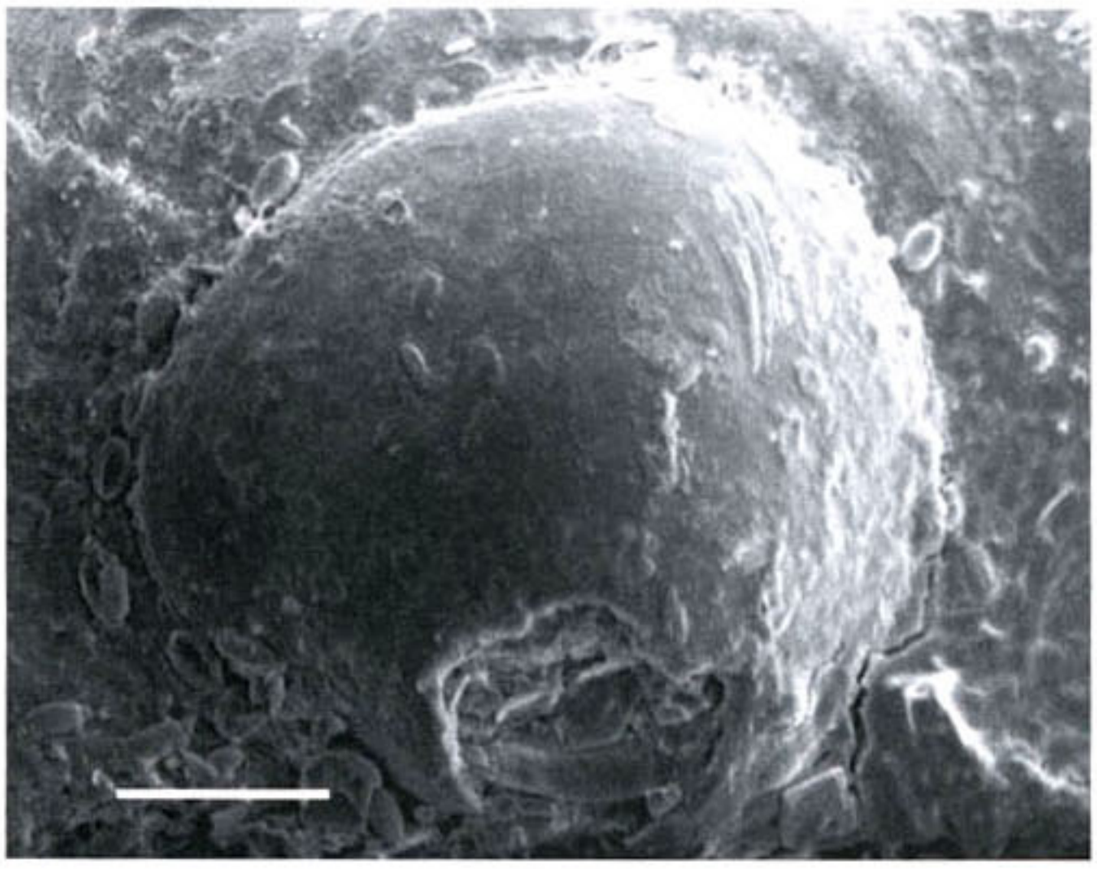

Figure 3. Scanning electron micrograph of the prodissoconch region of a field-collected juvenile of *Argopecten irradians*, showing an almost-intact larval shell. The juvenile was 5.5 mm in shell length. Scale bar is 50 μm.

The only significant treatment effects noted in this study were positive effects of calcein marking on survival of larvae of *Mytilus trossulus* and survival of *Argopecten irradians* reared for several weeks after metamorphosis. Other studies that exposed larvae or juveniles to calcein found either a negative effect (e.g., Brooks et al. 1994, Bumguardner & King 1996, Russell & Urbaniak in press) or no effect (e.g., Moran 2000, Leips et al. 2001, Bashey 2004), and the underlying mechanism for a positive effect of calcein on survival is not known. A major source of mortality in invertebrate larval culture is infection or competition from microorganisms (bacteria, viruses, protists, fungi). We did not quantify microorganism concentrations in our cultures, but if calcein had antibiotic, antiviral or antifungal effects this might enhance larval survivorship.

Such an effect would not alter the effectiveness of calcein in field studies of larval transport, because the effect would presumably be lost once larvae were released into the field. Although enhanced survivorship in the presence of calcein is currently difficult to explain, the absence of a negative effect on *M. trossulus* or *A. irradians* larvae in the laboratory suggests that calcein will not negatively impact growth or survivorship in the field. As with any physical or chemical marker, if field studies are performed using a mark that negatively affects growth or survivorship of larvae, this will artificially inflate the perceived mortality rate in the field. Therefore, because larvae of some species may be harmed by calcein whereas others are not, it is crucial to test each species of interest prior to initiating field studies.

A method such as the one described in this study is particularly amenable to testing the effects of calcein or other markers on larvae of numerous species because it is simple, inexpensive and requires little space, allowing researchers to rear larvae in large numbers of small-volume containers. Large numbers of containers are important because "container effects" on growth, such as those seen in *A. irradians* and *M. trossulus* in this study, are very common in larval rearing (particularly in small volumes). The noise introduced by container effects might mask a small deleterious treatment effect if only a few containers were used per treatment. The relative simplicity of this method also facilitates troubleshooting, because researchers could readily test the effects of calcein at different concentrations or over varying periods of immersion in the marking solution.

The final advantage of this technique is related to the fact that the release and subsequent recovery of marked larvae in the field is a daunting task, because of the tiny size of most larvae, their high mortality and generally high rates of advection and diffusion from the release site (reviewed in Morgan 1995). Bivalves that produce planktonic larvae are highly fecund (reviewed in Strathmann 1987), and many bivalve species are commercially important and have been the focus of aquaculture studies that have developed techniques for conditioning adults, spawning adults, rearing larvae and collecting spat in the field. Therefore, the production of large numbers of calcein-marked bivalve larvae is feasible and should provide a valuable opportunity to directly measure the dispersal shadow of free-spawning marine invertebrates where retention of larvae may be already suspected.

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