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Calcein as a marker in experimental studies newly-hatched gastropods

Abstract A nontoxic method of marking juvenile animals is a prerequisite for many field studies investigating growth and survivorship in marine invertebrates. This study investigates the effectiveness of low concentrations of calcein in marking hatchling snails (Nucella ostrina), the durability of the calcein mark, and the effects of marking on survivorship and growth. I also describe an inexpensive means of visualizing the calcein mark under a dissecting microscope. Results demonstrate that calcein provides a long-lasting, readily detected fluorescent shell mark that can be used to measure shell growth accurately. In addition, marking with calcein did not affect survivorship or growth, and had no size-dependent effects on growth or survivorship.

Introduction

Growth, mortality and selection during the early juvenile life-history stage are thought to play a major role in shaping the development, population structure, and life-history evolution of benthic marine taxa (Thorson 1946; Keough and Downes 1982; Connell 1985; Osman and Whitlatch 1996; Gossee and Qian 1997). Few experimental field studies with early juveniles have been performed, however, in part due to a lack of methods appropriate to the study of such small and cryptic life-history stages. A method of marking animals is a prerequisite for many types of field experiments, but the small size of newly-settled or hatched juvenile invertebrates can limit the utility of marking techniques used on adults (Southwood 1978); for example, numbered tags may be too large or cumbersome to attach to juvenile shells or carapaces, and glues or paints may be toxic to tiny, thin-shelled animals (Palmer 1990; Gosselin 1993). Hand-labeling of juveniles with tiny identifying marks (e.g. Gosselin 1993) is a powerful tool for tracking individual animals over time, but could be prohibitively time-consuming in studies involving large numbers of animals. Finally, stains or marks that enable investigators to recover small cryptic animals may compromise recovery studies by increasing susceptibility to visual predators (Levin 1990).

Calcein (2,4-bis-[N,N'-di(carboxymethyl)-aminomethy]-fluorescein: Sigma # C 0875) is a fluorescent label that binds to calcium and is incorporated into growing calcium carbonate structures. Immersion in calcein solutions provides a fluorescent mark that may be useful both for identification and measurement of growth of many animals, including fishes (Monaghan 1993; Brooks et al. 1994; Mohler 1997), mammals (Malouvier et al. 1993; Turner 1994), ascidians (Lambert and Lambert 1996), adult molluscs (Day et al. 1995; Kaehler and McQuaid 1999), echinoderms (Stewart 1996; R.B. Emlet and B.A. Miller in preparation) and other taxa (Rowley and MacKinnon 1995). One serious potential drawback with this marking method is that calcein may be toxic to some animals; in larvae of some fish species, survivorship is reduced by marking even at relatively low calcein concentrations (Brooks et al. 1994; Bumguardner and King 1996; Gelsleichter et al. 1997).

Before using this label in experimental studies of growth and survivorship, it is necessary to establish that calcein itself does not negatively affect these life-history parameters. While previous studies have found calcein marking does not lower survivorship of adults or older juveniles of some taxa (e.g. Kaehler and McQuaid 1999; mussels), the reliability of calcein as a label for newly-emerged juveniles and its effects on the growth and survivorship of early life-history stages are poorly known. This study investigates the effectiveness of low concentrations of calcein in marking hatchling Nucella.
ostrina (Gould, 1895) (Prosobranchia: Gastropoda) (recently separated from N. emarginata (Deshayes): see Palmer et al. 1990; Marko 1998), the durability of the calcein mark over time, and the effects of calcein marking on survivorship and growth in this species.

Nucella ostrina is an abundant, intertidal predatory gastropod that develops to metamorphosis in benthic egg capsules. Both adult and juvenile N. ostrina have been the focus of considerable ecological and evolutionary research (e.g. Palmer 1984, 1990; Palmer et al. 1990; Rawlings 1990, 1994a, b, 1996; Gosselin and Chia 1994, 1995; Collins et al. 1996; Gosselin 1997; Marko 1998), but the biotic and abiotic factors affecting the early life history of this species are not well understood. An effective marker for hatching stages is a necessary tool towards understanding the population dynamics and life histories of N. ostrina and other marine benthic taxa.

Materials and methods

Hatching collection

I obtained newly-hatched juvenile Nucella ostrina by collecting egg capsules at the point of hatching from intertidal rocks at the boathouse dock at the Oregon Institute of Marine Biology (Charleston, Oregon, USA). Hatchlings were gently removed from capsules by washing with a Pasteur pipette. The hatchlings used in these experiments ranged in shell length from 0.9 to 2.0 mm.

Calcein marking solutions and mark visualization

A concentrated stock solution containing 6.25 g l⁻¹ calcein in distilled water was buffered to pH 6 with sodium bicarbonate to enhance the solubility of calcein (after Wilson et al. 1987). This concentrate was added to filtered seawater (calcein otherwise has only limited solubility in seawater) to make a marking solution containing a total calcein concentration of 100 ppm (= 100 mg l⁻¹). Snails were exposed to marking solutions for periods of 12 or 24 h as described in the following subsection.

Nucella ostrina hatchlings are too large to be examined effectively under a compound fluorescent microscope, and fluorescent dissecting microscopes are expensive and often not readily available. In this study, hatchlings were examined for calcein marks under an ordinary dissecting microscope (Wild M5A) equipped with epi-illumination via a blue-light filter (λc center wavelength 460 nm, Corion Corporation Catalog #XM-465) fitted on a fiber-optic light, and a yellow sharp cut-off longpass transmission filter (λc (λ1 max)/2 495 nm, Edmunds Scientific Catalog #A32, 763) fitted over the microscope head. Under this setup the calcein marks were easily visible in a darkened room.

To minimize possible damage from handling, heat, or desiccation, snails were handled with fine-tipped forceps and eyelash-tipped wands and were immersed in seawater except during measurement when hatchlings were emersed but kept damp. Measurement required < 30 s per snail. These techniques very rarely resulted in visible damage to any snail, and no differences were observed in activity of snails before and after measurements.

Tracking individual hatchlings

A ripe clutch was collected from the field, and 46 hatchlings were randomly chosen from the total pool and randomly divided into two groups of 23 that were designated “marked” and “unmarked”. Hatchlings in the marked group were placed in a solution of 100 ppm calcein in filtered seawater for 24 h, and hatchlings in the unmarked group were immersed in filtered seawater for the same period. Each snail was then measured for total length and placed in an individual well of a tissue-culture tray from which the top and bottom had been removed and replaced with 600 µm Nitex mesh. Marked and unmarked snails were placed in alternating wells to eliminate any potential effects of position in the tray on growth or survivorship. Because previous experiments with hatching Nucella ostrina had indicated that hatchlings were very sensitive to flocculent from the flow-through seawater system, tissue-culture trays containing hatchlings were placed in a large (~19 liter) tub of 0.45 µm-filtered seawater. The tub was then covered and partially immersed in flowing seawater (to the water line) to keep the filtered water at ambient temperatures. Hatchlings were not provided with food because the addition of prey items (small barnacles and mussels) to cell culture wells often resulted in anaerobic conditions, morbidity and decay of both hatchlings and prey.

After 6 d, the snails were removed from wells and measured to the nearest 10 µm under a Wild 5A dissecting microscope equipped with blue epifluorescence and a yellow filter (described as above). Snails were scored as marked or unmarked based on the presence or absence of calcein fluorescence. Starved hatchlings did not increase appreciably in length over the 6-d interval, so growth was measured as the quantity of shell added since marking taken as the maximum straight distance from the calcein mark to the new apertural edge along the second spiral rib out from the suture of the body whorl (see rib labeled “T” in Fig. 1). Because the amount of growth was small, error in linear measurements due to coiling of

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