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A. L. Moran

Calcein as a marker in experimental studies newly-hatched gastropods

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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; Gosselin 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(carbomethyl)-aminomethyl]-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*

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A. L. Moran
University of Washington,
Friday Harbor Laboratories, 620 University Road,
Friday Harbor, Washington 98250, USA

Fax: 001 (206) 543-1273
e-mail: moran@fhl.washington.edu

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 hatchling stages is a necessary tool towards understanding the population dynamics and life histories of *N. ostrina* and other marine benthic taxa.

Materials and methods

Hatchling 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 (λ_o 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 (τ_i 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 hatchling *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

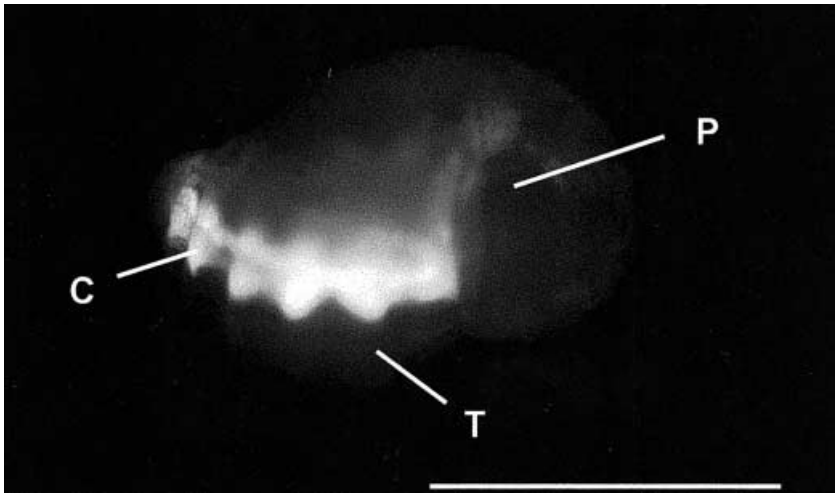

Fig. 1 *Nucella ostrina*. Nine day-old juvenile viewed under blue light with yellow longpass filter. Brightest area is calcein mark (*C*) just beyond protoconch–teloconch boundary; smooth protoconch (*P*) also shows some light labeling. Area of teloconch (*T*) that has grown since marking is entirely unlabelled. Illumination visible in this area is light reflected from calcein mark. Scale bar = 1 mm

the shell was assumed to be negligible. Growth of unmarked snails was measured in the same manner from the protoconch–teloconch (PT) boundary, which corresponded with the calcein mark in marked hatchlings.

An accurate method of estimating initial hatching size is necessary to recapture–based field studies examining the effect of initial size on performance if experimental animals are not individually recognizable. To evaluate the accuracy of the calcein mark as an indicator of original *Nucella ostrina* hatching length, an estimate of the original hatching length of each snail was made by measuring from the shell apex to the siphonal end of the original marked aperture. These estimates were then compared to the hatching length of each snail as measured on Day 0.

Snails were also examined for activity and flesh discoloration and scored as alive or dead.

Hatchlings reared in groups

Hatchlings from each of six ripe clutches were washed through a series of four graded meshes and immediately transferred to seawater. From each clutch, large (1.50 ± 0.08 mm greatest shell length) and small (1.10 ± 0.09 mm) snails (from the largest and smallest meshes, 850 and 600 μm mesh size, respectively) were randomly divided into two groups. One group each of large and small snails was randomly assigned to the marked treatment and marked with calcein, while the other group was exposed to filtered seawater only. After immersion in calcein or seawater, eight hatchlings from each group (marked large, unmarked large, marked small, unmarked small; a total of 32 hatchlings from each clutch) were randomly chosen and were measured for greatest shell length. Each group of eight hatchlings was then pooled with the other members of the same clutch, making six sets of 32 siblings. Each set consisted of 8 large marked, 8 large unmarked, 8 small marked, and 8 small unmarked siblings. Each set of siblings was placed in an individual box sided with 600 μm mesh (for a total of 6 boxes, with 1 clutch per box) and provided with freshly-collected intertidal rocks covered with small barnacles and mussels for food. Boxes were kept in flowing seawater at ambient temperatures.

Snails were removed after 15 d and again at Day 34 and measured for new shell length, hatching length (at Day 15 only), and survivorship. Snails were scored as dead if (1) snails were discolored (purple, green or black) and not moving, (2) shells were empty, or (3) snails were missing from the recovered group. All recovered snails were examined with fluorescence microscopy and scored as marked or unmarked based on the presence or absence of a calcein mark. Hatching length was measured as the distance from the shell apex to the position of the original siphonal tip at hatching (at the PT boundary or calcein mark), and hatchlings were identified as coming from the originally large or small categories based on estimated hatching lengths.

Percent survivorship of small marked vs small unmarked snails and large marked vs large unmarked snails were arcsine-transformed and compared among clutches at Day 34 with a paired Student's *t*-test (two tests). To compare growth of marked and unmarked snails, two analyses were performed: growth (measured as new length − original length) of all snails at Day 15 was compared, and size (new length) of all snails at Day 34 was compared using three-factor, mixed-model ANOVAs with initial size (large or small), marked status (marked or unmarked) and box-clutch (1 to 6) as factors, and growth at Day 15 or size at Day 34 as dependent variables. Initial size and marked status were considered fixed factors and box-clutch was a random variable. Size was used as an index of growth at Day 34 because some snails had grown enough to partially wrap over the original, marked aperture, and in these individuals original shell length could only be estimated, not precisely measured.

Results

Calcein mark

Immersion of hatchling *Nucella ostrina* in a 100 ppm solution of calcein in seawater for 12 or 24 h produced a mark at the growing aperture of the shell that was readily visible (Fig. 1) in almost all individuals when viewed with the filter set described above. The mark varied somewhat in brightness among clutches and among individuals, and in a very few (<1 of 50), the mark was dim and difficult to see. Poor marking may have been due to low growth during immersion in the marking solution. The calcein mark was still visible at the original aperture after 34 d growth in the laboratory (Fig. 2), and the mark persisted for at least 50 d in the field (Moran in press). In some older snails, the calcein mark became noticeably fainter, although it was always readily distinguishable. Decreased brightness of the mark appeared to result from erosion of the protoconch, or in some cases from the addition of material on top of the marked portion of shell.

Among individually-reared snails, original hatching length and hatching length as measured from the calcein mark were significantly and tightly correlated (correlation coefficient $r = 0.98$, $n = 44$, $p < 0.001$)

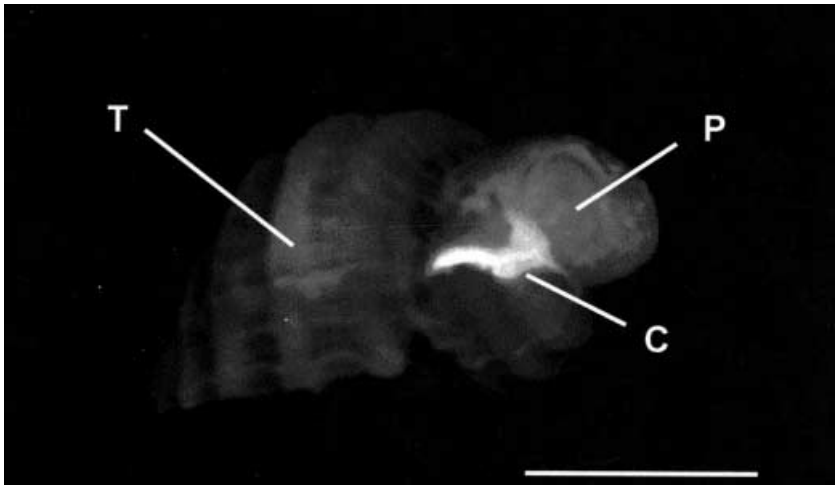

Fig. 2 *Nucella ostrina*. Thirty-four day-old juvenile, viewed on its side with aperture at 90° to substratum. Bright calcein mark (*C*) is visible at protoconch–teloconch boundary (*P* protoconch; *T* teloconch). Teloconch is beginning to wrap around calcein-marked area of original hatchling shell. Scale bar = 2 mm

(Fig. 3), and original hatching length explained 95% of the variance in estimated hatching length. A paired student's *t*-test comparing individuals' original hatching length to hatching length as measured from the calcein mark found no significant difference between the two ($t = -0.39$; $p = 0.70$; $df = 44$). The average difference between Day 0 and Day 6 hatching length measurements was 7.6 μm (0.6% of mean hatchling length) with a standard deviation of 8.5 μm.

The calcein mark of snails fed ad libitum was still readily visible after Days 15 and 34. At Day 15, hatching length was readily measured on all snails via the PT boundary (unmarked snails) or the calcein mark (marked snails). At 34 d, however, the PT boundary and calcein mark could only be utilized to estimate initial marked length and distinguish between originally large and small hatchlings, because the teloconch of many snails was wrapped over the marked area. Because the body whorl does not completely wrap over previous whorls, the calcein mark was still readily apparent (Fig. 2).

Hatchlings reared individually

Overall mortality was very low among starved snails over the 6 d experimental interval; 22 of 23 snails survived in both the marked and unmarked treatments. There was no significant difference in growth between marked and unmarked snails at Day 6 (one-factor ANOVA, $F = 0.126$, $p = 0.72$), nor did the relationship between hatching size and growth differ significantly among marked and unmarked snails (ANCOVA: slope, $F = 3.021$, $p = 0.090$: intercept, $F = 0.234$, $p = 0.631$) (Fig. 4).

Hatchlings reared in groups

No significant difference was found in survivorship at Day 34 between large marked and large unmarked snails (paired Student's *t*-test, $t = 0.052$, $p = 0.961$, $df = 5$) or between small marked and small unmarked snails (paired Student's *t*-test, $t = -1.102$, $p = 0.358$, $df = 5$) from the six clutches (Table 1). In the first three-way ANOVA, both initial size and box-clutch factors had significant effects on growth at Day 15. In the second three-way ANOVA, both initial size and box-clutch had significant effects, and there was a significant interaction between box-clutch and initial size at Day 34 (Table 2). Size is known to effect *Nucella ostrina* growth in both the laboratory and field (Moran 1999; Moran in press), and the box-clutch effect and significant interaction term is like due to differences in environments among boxes, or genetic or maternal effects on growth that differ between clutches. Notably, no factors containing the marking variable were significant: there was no significant effect of calcein marking on growth, no significant interaction between marking and initial size, no significant interaction between box-clutch and marking, and no significant three-way interaction among marking, initial size and box-clutch (Table 2).

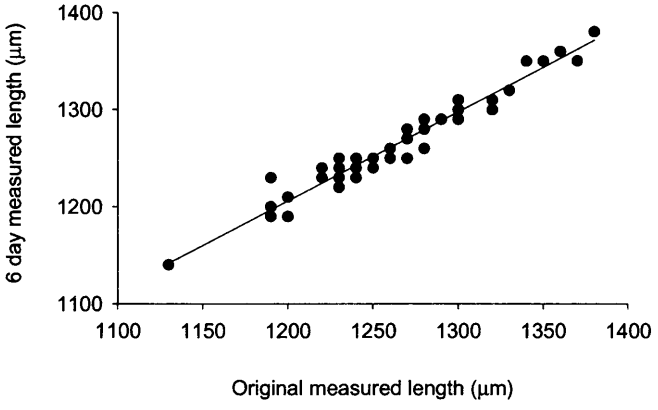

Fig. 3 *Nucella ostrina*. Correlation between estimates of hatchling length made from calcein mark (*Y* axis) and original measured hatchling length (*X* axis) after 6 d starvation in laboratory. Data points represent individual hatchlings

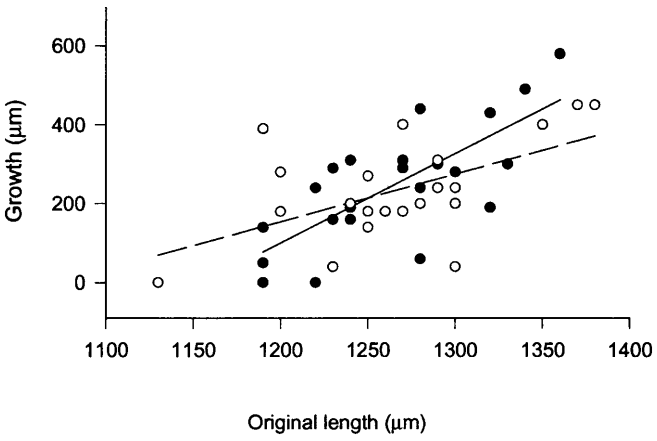

Fig. 4 *Nucella ostrina*. Growth of calcein-marked (●, solid line) and control (○, dashed line) hatchlings after 6 d starvation in laboratory, plotted against original hatchling length. Lines are least-squares regressions

Discussion

The advantages of calcein marking in *Nucella ostrina* are as follows. First, calcein produces a mark that can be

Table 1 *Nucella ostrina*. Percent survivorship of hatchlings over 34 d experimental interval, grouped by clutch, size and mark (*M* marked; *U* unmarked)

Clutch	Large M	Large U	Small M	Small U
1	100.0	75.0	75.0	50.0
2	87.5	37.5	25.0	50.0
3	75.0	87.5	37.5	50.0
4	87.5	100.0	75.0	87.5
5	62.5	75.0	46.0	62.5
6	87.5	100.0	50.0	37.5
Mean	83.3	79.2	50.0	56.25

Table 2 *Nucella ostrina*. Results of three-factor, mixed-model ANOVA testing the effect of calcein marking on growth of large and small hatchlings after feeding ad libitum for 15 and 34 d in laboratory. Size (large or small) and mark (marked or unmarked) = fixed factors; box-clutch = random factor

Effect	*df*	MS	*F*	*P*
Fed 15 d				
Box-clutch (BC)	5	0.092	3.770	<0.050
Size (S)	1	2.593	144.574	<0.050
Mark (M)	1	0.007	0.421	0.545
BC × S	5	0.018	0.738	0.596
BC × M	5	0.016	0.679	0.640
S × M	1	0.265	0.822	0.406
BC × S × M	5	0.032	1.327	0.257
Error	135	0.024		
Fed 34 d				
Box-clutch (BC)	5	0.387	2.627	<0.050
Size (S)	1	17.341	44.514	<0.005
Mark (M)	1	0.001	0.007	0.936
BC × S	5	0.390	2.644	<0.050
BC × M	5	0.141	0.956	0.448
S × M	1	0.070	1.698	0.249
BC × S × M	5	0.041	0.279	0.923
Error	130	0.147		

viewed under a dissecting scope using the inexpensive, readily available filter sets described here. Second, because calcein fluoresces under visible (blue) wavelengths, animals can be examined without the tissue damage that occurs under UV light (Moran unpublished data). Third, because the calcein mark is only visible with special filter sets, this marking technique does not increase the vulnerability of animals to visual predators. Fourth, large numbers of animals can be rapidly, easily and inexpensively marked, and the calcein mark can be used to accurately estimate original (marked) size and growth after time in the laboratory or field. Finally, calcein does not inhibit growth or cause mortality of hatchlings, which are very small and potentially vulnerable to toxic effects of many standard marking techniques; nor does it differentially affect small or large individuals, an issue of importance in field experiments examining the effects of offspring size on performance.

Although calcein at low concentrations had no detectable deleterious effects on *Nucella ostrina* juveniles, caution should be used in applying this result to all taxa. Calcein has been reported to have some toxic effects on fish when used at slightly higher concentrations (toxic effects were reported at 160 mg l^{-1}) (Bumguardner and King 1996), and the relative sensitivity to calcein of different taxa and differently-sized individuals within species has not been thoroughly examined. Therefore, prior to using calcein for the first time in a selected taxon, investigators should perform pilot experiments to establish the lowest concentrations that will result in an effective mark. Likewise, a low number of *N. ostrina* hatchlings (< 1 in 50) failed to develop a useful mark during immersion in the calcein solution. If total number of experimental animals is of importance, animals that have been immersed in calcein solutions should be examined individually before experiments are begun in order to ensure that each individual is properly marked. If a substantial number of animals fail to take up the mark, perhaps due to differences in vitality among juveniles or episodic growth, then researchers should consider whether excluding these individuals might bias experimental results. The techniques in this study also cannot be used easily to identify individuals; techniques such as those described in Gosselin (1993) are more applicable to experiments requiring tracking of individual animals.

The results of this study demonstrate that calcein provides a long-lasting, readily detected fluorescent shell mark in *Nucella ostrina* hatchlings that can be used both to identify experimental snails and to accurately measure marked length of hatchlings up to the completion of the first teloconch whorl (in the laboratory, at approx. Day 34). Therefore, calcein can be utilized as a marker in laboratory or field studies using *N. ostrina* hatchlings (and potentially early life-history stages of other taxa as well) to explore mortality, growth, and size-dependent effects. Such a marker is a necessary and potentially valuable tool for understanding the early life-history ecology of benthic marine invertebrates.

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References

Brooks RC, Heidinger RC, Kohler CC (1994) Mass-marking otoliths of larval and juvenile walleyes by immersion in oxytetracycline, calcein, or calcein blue. N Am J Fish Mgmt 14: 143–150

Bumguardner BW, King TL (1996) Toxicity of oxytetracycline and calcein to juvenile striped bass. Trans Am Fish Soc 125: 143–145

Collins TM, Frazer K, Palmer AR, Vermeij GJ, Brown WM (1996) Evolutionary history of northern hemisphere *Nucella* (Gastropoda, Muricidae): molecular, morphological, ecological, and paleontological evidence. Evolution 50: 2287–2304

Connell JH (1985) The consequences of variation in initial settlement vs. post-settlement mortality in rocky intertidal communities. J exp mar Biol Ecol 93: 11–45

Day RW, Williams MC, Hawkes GP (1995) A comparison of fluorochromes for marking abalone shells. Mar Freshwat Res 46: 599–605

Gelsleichter J, Cortes E, Manire CA, Hueter RE, Musick JA (1997) Use of calcein as a fluorescent marker for elasmobranch vertebral cartilage. Trans Am Fish Soc 126: 862–865

Gosselin LA (1993) A method for marking small juvenile gastropods. J mar biol Ass UK 73: 963–966

Gosselin LA, Chia F-S (1994) Feeding habits of newly hatched juveniles of an intertidal predatory gastropod, *Nucella emarginata* (Deshayes). J exp mar Biol Ecol 176: 1–13

Gosselin LA, Chia F-S (1995) Distribution and dispersal of early juvenile snails: effectiveness of intertidal microhabitats as refuges and food sources. Mar Ecol Prog Ser 128: 213–223

Gosselin LA (1997) An ecological transition during juvenile life in a marine snail. Mar Ecol Prog Ser 157: 185–194

Gosselin LA, Qian P-Y (1997) Juvenile mortality in benthic marine invertebrates. Mar Ecol Prog Ser 146: 265–282

Kaehler S, McQuaid CD (1999) Use of the fluorochrome calcein as an in situ growth marker in the brown mussel *Perna perna*. Mar Biol 133: 455–460

Keough MJ, Downes FJ (1982) Recruitment of marine invertebrates: the role of active larval choices and early mortality. Oecologia 54: 348–352

Lambert G, Lambert CC (1996) Spicule formation in the New Zealand ascidean *Pyura pachydermatina*. Connect Tissue Res 34: 263–269

Levin LA (1990) A review of methods for labeling and tracking marine invertebrate larvae. Ophelia 32: 115–144

Malouvier A, Martin F, Orus L, de Pollak C, Marie PJ, Holy X, Zerath E (1993) Comparative use of calcein and oxytetracycline for the analysis of bone mineralisation in rhesus monkeys. Med Sci Res 21: 423–425

Marko PB (1998) Historical allopatry and the biogeography of speciation in the prosobranch snail genus *Nucella*. Evolution 52: 757–774

Mohler JW (1997) Immersion of larval Atlantic salmon in calcein solutions to induce a non-lethally detectable mark. N Am J Fish Mgmt 17: 751–756

Monaghan JP Jr (1993) Comparison of calcein and tetracycline as chemical markers in summer flounder. Trans Am Fish Soc 122: 298–301

Moran AL (1999) Size and performance of juvenile marine invertebrates: potential contrasts between intertidal and subtidal benthic habitats. Am Zool 39: 304–312

Osman RW, Whitlatch RB (1996) Processes affecting newly-settled juveniles and the consequences to subsequent community development. Invert Reprod Dev 30: 217–225

Palmer AR (1984) Species cohesiveness and genetic control of shell colour and form in *Thais emarginata* (Prosobranchia, Muricacea); preliminary results. Malacologia 25: 477–491

Palmer AR (1990) Predator size, prey size, and scaling of vulnerability: hatchling gastropods vs. barnacles. Ecology 71: 759–775

Palmer AR, Graydon SD, Woodruff DS (1990) Reproductive, morphological, and genetic evidence for two cryptic species of northeastern Pacific *Nucella*. Veliger 33: 325–338

Rawlings TA (1990) Associations between egg capsule morphology and predation among populations of the marine gastropod, *Nucella emarginata*. Biol Bull mar biol Lab, Woods Hole 179: 312–325

Rawlings TA (1994a) Effect of elevated predation risk on the metabolic rate and spawning intensity of a rocky shore marine gastropod. J exp mar Biol Ecol 181: 67–79

Rawlings TA (1994b) Encapsulation of eggs and embryos by marine gastropods: effect of variation in capsule form on the vulnerability of embryos to predation. Evolution 48: 1301–1313

Rawlings TA (1996) Shields against ultraviolet radiation: an additional protective role for the egg capsules of benthic marine gastropods. Mar Ecol Prog Ser 136: 81–95

Rowley RJ, MacKinnon DI (1995) Use of the fluorescent marker calcein in biomineralisation studies of brachiopods and other marine organisms. Bull Inst Océanogr, Monaco 14: 111–120

Southwood TRE (1978) Marking invertebrates. In: Stonehouse B (ed) Animal marking. Recognition marking of animals in research. MacMillan Press, London, pp 102–106

Stewart B (1996) Growth dynamics of the radial shields of the euryalid snake star *Astrobrachion constrictum* (Echinodermata: Ophiuroidea). Invert Biol 115: 321–330

Thorson G (1946) Reproduction and larval development of Danish marine bottom invertebrates. Medds Kommn Danm Fisk-og Havunders (Ser Plankton) 4: 1–52

Turner RT (1994) Cancellous bone turnover in growing rats: time-dependent changes in association between calcein label and osteoblasts. J Bone Miner Res 9: 1419–1424

Wilson CA, Beckman DW, Dean JM (1987) calcein as a fluorescent marker of otoliths of larval and juvenile fish. Trans Am Fish Soc 116: 668–670