SIZE, FORM AND FUNCTION IN THE EARLY LIFE HISTORIES OF THE GASTROPOD GENERA *NUCELLA* AND *LITTORINA*

by

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Many marine invertebrate taxa undergo part or all of development within egg capsules. In most cases this type of development is considered derived from an ancestor with free-swimming feeding larvae ("planktotrophic"). The implications of intracapsular feeding for the ecology and evolution of larvae and juveniles are poorly understood. The velum (the feeding and swimming organ of planktotrophic gastropod larvae) of three *Littorina* species with encapsulated development was shown to be active in endocytotic uptake of protein-rich capsular fluid. Encapsulated *Littorina* larvae lacked the characteristic ciliation patterns of congeneric planktotrophs but retained a large velum in early development. In a variety of other gastropod taxa, in contrast, species with nonplanktotrophic larvae had reduced velums. This suggests that among encapsulated *Littorina* species, a large velum has been retained due to its novel function in the capsule environment.

Larvae of many gastropod species with encapsulated development feed on nondeveloping "nurse eggs," and hatching size varies within and among clutches due to

differential nurse egg provisioning and consumption. Laboratory tests demonstrated that maternal size and food ration did not affect offspring size of *Nucella emarginata*. Large offspring size is assumed to be advantageous in most life-history models, but field tests of this assumption are lacking in benthic marine systems. Hatching size of *Nucella emarginata* was positively correlated with organic content, and large hatchlings grew more and survived longer under laboratory conditions than small siblings.

A method was developed for marking and outplanting hatchling *Nucella* (0.9-2.0 mm) into the field, and hatching size strongly affected growth in the field but did not affect short-term survivorship. Long-term (ca. 1 month) field outplants demonstrated that hatching size could affect survivorship over longer intervals and that the effect of size varied over small spatial scales. Large *Nucella emarginata* hatchlings exhibited higher survivorship than small hatchlings in benign habitat, but not in more severe habitat. The severe habitat varied between extreme and moderate temperatures over a scale of centimeters while the benign habitat was cooler and less variable, suggesting that the relationship between habitat quality and offspring size-dependent performance may vary depending on the microstructure of an organism's habitat. This dissertation includes previously published materials.

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CHAPTER I

GENERAL INTRODUCTION

Many marine invertebrates have microscopic larval forms that must swim and feed in the plankton in order to grow and attain metamorphic competence ("planktotrophic"); others have nonfeeding larvae with yolky eggs or entirely encapsulated development ("lecithotrophic") (Thorson, 1946; Jablonski and Lutz, 1986; Strathmann, 1985). For most taxa, planktotrophy is considered primitive and lecithotrophy, derived. Because it is often accompanied by loss of feeding and swimming characters, the evolutionary switch from planktotrophy to lecithotrophy is thought to be irreversible (Strathmann, 1974, 1985; Hadfield and Iaea, 1989; Emlet, 1991).

Lecithotrophic species whose larvae can no longer obtain the nutrition necessary for development and growth from the plankton must find other means of provisioning offspring. Provisioning is accomplished either by production of large, yolk-rich eggs, or by supplying encapsulated embryos with intracapsular, extraembryonic nutrition such as albumen or nurse eggs. Extraembryonic nutrition, as a food source, is physically very different from the algal food of planktotrophic larvae; therefore, ancestral feeding characters may be altered or new features evolved for feeding on intracapsular food sources. Specialized larval organs have evolved in some gastropods for absorption of

albumen (Rivest 1992, Rivest and Strathmann 1995), and modifications in ciliation have been interpreted as adaptations for feeding on nurse eggs (Hadfield, 1966; Lyons and Spight, 1973; Rivest, 1981). Modifications to the ancestral, planktotrophic larval form can therefore be attributed to either (1) loss of planktotrophic characters due to lack of stabilizing selection (Strathmann 1985), or (2) specialization for feeding on intracapsular nutrition. A third possibility is that planktotrophic feeding characters may be retained for novel functions in the capsule environment. For many encapsulated species, however, mechanisms of feeding and the relationship between morphology and feeding are not well understood.

The presence of larval structures that may be specializations for the uptake and consumption of extraembryonic nutrition strongly suggests that this nutrition in some way benefits the organism, either at the larval or juvenile stage. Embryos of the intertidal gastropod *Nucella emarginata* develop to metamorphosis in benthic egg capsules, and embryos consume up to several hundred nurse eggs during development. Offspring (hatchling) size of nurse egg feeders such as *N. emarginata* is strongly influenced by number of nurse eggs consumed during development (Rivest, 1983; Fioroni, 1988; Baur, 1992). Large hatchlings are therefore likely to represent a higher maternal investment than small hatchlings, but offspring size and organic content are not necessarily correlated in all taxa (McEdward and Carson, 1987; McEdward and Coulter, 1987; McEdward and Chia, 1991).

Large size can confer performance advantages on gastropod hatchlings. In laboratory experiments, large hatchlings grew faster (when fed (Rivest 1981)) and were

less likely to be consumed by some predators (Spight, 1976; Rivest, 1981; Gosselin, 1994) than small hatchlings. Vulnerability to desiccation and heat decreased with increasing shell length as snails aged and grew (Gosselin, 1994), suggesting that hatching size may also influence desiccation and heat tolerance. Starvation, predation and desiccation are only some of many stresses that hatchlings are likely to encounter in the field, and environmental conditions vary greatly both temporally and locally. Therefore, the importance of hatching size in the field cannot be *a priori* assumed, but must be tested under a range of conditions in the field to understand its potential role in shaping life history strategies.

In this dissertation, several experiments are described that explored the relationships among offspring size, morphology, performance, and adult condition in two gastropod genera with nonplanktotrophic larvae that consume intracapsular nutrition during development. Chapter II describes the mechanism of albumen uptake of embryos in the genus *Littorina*, and explores some of the consequences of extraembryonic nutrition for the evolution of larval form in *Littorina* and other gastropods. Chapter III first describes the relationship between hatchling size and organic content in *Nucella emarginata*, then explores the relationship between maternal investment and offspring performance in the field both over time and in contrasting environments.

Before the field experiments in Chapter III could be performed, a durable and nontoxic marker was necessary for labeling gastropod hatchlings. Chapter IV describes 1) a technique that was developed to label *Nucella emarginata* hatchlings with Calcein, and 2) experiments testing the utility of the Calcein mark for long-term labeling and as a

marker for growth. Chapter V describes laboratory experiments that explored the effects of maternal size and food ration on offspring size in a gastropod (*Nucella emarginata*) which relies on nurse eggs for intracapsular nutrition. Nurse egg feeding is a taxonomically widespread mechanism for offspring provisioning, but the impacts of maternal effects on offspring size in nurse egg feeders is poorly understood.

Lastly, Chapter VI provides a brief summary and future directions for research, and three appendices are attached. Appendix A describes the methods used for organic content analysis in Chapter III, and a comparison of the results of two different methods of organic content analysis. Appendix B describes a field experiment that was performed to test the propensity of *Nucella* hatchlings to crawl off of experimental panels such as those used in Chapter III. Appendix C is a photocopy of a published paper that describes the development of *Tegula funebralis*, an abundant and ecologically important species that has been the focus of numerous other ecological and evolutionary studies.

CHAPTER II

INTRACAPSULAR FEEDING BY EMBRYOS OF THE GASTROPOD GENUS *LITTORINA*: IMPLICATIONS FOR THE EVOLUTION OF LARVAL FORM

Introduction

Marine invertebrates exhibit a remarkable variety of reproductive and developmental modes, both within and among taxa. This variation has been of great interest because it provides a unique and comparative means of studying the integration of development, life history and evolution. One of the best-known dichotomies in invertebrate development is between species with larvae that must feed in the plankton in order to grow and attain metamorphic competence (planktotrophic), and species with nonfeeding larvae with yolky eggs or entirely encapsulated development (nonplanktotrophic) (Thorson, 1946; Jablonski and Lutz, 1983; Strathmann, 1985). Embryos of many nonplanktototrophic species develop and metamorphose in benthic egg capsules (encapsulated development) (Thorson, 1946; Pechenik, 1979; Perron, 1981), and both planktotrophic species and species with encapsulated development can occur within

a single genus. Egg capsules are often complex and energetically costly to produce (Pechenik, 1979, 1986), and may function to protect embryos from environmental stresses and predation (Shuto, 1974; Spight, 1977; Pechenik, 1984; Hawkins and Hutchinson, 1988; Rawlings, 1990, 1996) and retain offspring within suitable adult habitat (Wells and Wells, 1962; Chapman, 1965).

In addition to their protective and retentive role, egg capsules may also contain extraembryonic, nutrient-rich materials that provide an energy source for developing embryos. The nature of these materials and their importance for embryonic nutrition vary considerably among gastropods. Extraembryonic nutrition may take the form of nurse eggs (unfertilized eggs or abnormally developing embryos), nutritive yolk, albumen, or a combination. Albumen, a common source of extraembryonic nutrition among gastropods (Fretter and Graham, 1962), is a viscous fluid that surrounds embryos in the egg capsule and is frequently rich in proteins, carbohydrates and/or free amino acids (Horstmann, 1956; De Mahieu *et al.*, 1974; Rivest, 1992; Stöckmann-Bosbach and Althoff, 1989; Penchaszadeh and Rincón, 1996). Among gastropods, albumen is generally consumed endocytotically via receptor-mediated pathways (Elbers and Bluemink, 1960; Fioroni, 1977; Rivest, 1981; Rivest, 1992; Rivest and Strathmann, 1995).

The importance of albumen as a nutrient reserve for developing embryos has been demonstrated in pulmonate gastropods through a decline in nutrient content of the fluid during development and by a correlated increase in lipid and protein content of the embryo (Horstmann, 1956; Morrill, 1964; Raven, 1972; Taylor, 1973; Morrill *et al.*,

1976). Disappearance of albumen is correlated with considerable increases in embryo size during the development of some opisthobranchs (Clark *et al.* 1979, Clark and Jensen 1981) and some prosobranchs (Rasmussen, 1951; Buckland-Nicks *et al.*, 1973; Losse and Greven, 1993). The nutritive importance of albumen probably varies considerably among taxa, depending on egg size, volume of albumen, the nutritive content of albumen fluid, and the presence or absence of additional sources of nutrition such as nurse eggs (Pechenik *et al.*, 1984; Miloslavich, 1996; Penchaszadeh and Rincón, 1996).

Encapsulated, nonplanktototrophic embryos of many taxa possess morphological structures whose functional significance is only apparent in planktotrophic relatives or ancestors. For example, many prosobranch and opisthobranch gastropods with encapsulated development pass through a recognizable veliger stage that retains a bilobed, ciliated velum (e.g., Fretter and Graham, 1962; Buckland-Nicks *et al.*, 1973; Strathmann, 1978; Hadfield and Iaea, 1989), the feeding and swimming organ of planktotrophic gastropod larvae (see Strathmann and Leise, 1979). The presence of planktotrophic larval feeding structures (such as the velum) in encapsulated embryos is commonly accepted as evidence of descent from a planktotrophic ancestor, and is one of numerous independent lines of evidence that support planktotrophy as the ancestral state in caenogastropods (Haszprunar *et al.*, 1995).

However embryos of many encapsulated species, while retaining gross veliger morphology, exhibit some modifications to the ancestral planktotrophic form. One commonly reported feature of encapsulated gastropod embryos is a reduction in size of

the velar lobes (e.g. Jägerston, 1972; Webber, 1977), although the size of the velum varies considerably among encapsulated taxa (Hadfield and Iaea, 1989; this paper). The patterns of ciliation on the velar lobes of nonplanktotrophic species with encapsulated development often differ considerably from the velar ciliation of planktotrophs as well (e.g. Lyons and Spight, 1973; Hadfield and Iaea, 1989). Reduction in the size of velar lobes and alterations to ancestral ciliation patterns have been interpreted variously as the loss of complex, ancestral planktotrophic morphology due to a lack of stabilizing selection and as functional modifications that enhance performance in the intracapsular environment (e.g. respiration and feeding on intracapsular nutrition (Fretter and Graham, 1962; Lyons and Spight, 1973; Hadfield and Iaea, 1989)).

In addition, many gastropods with encapsulated development have transitory, embryonic structures that appear to be specializations for albumen uptake. These structures include the cell surfaces of early cleavage stages and embryonic gut of some pulmonates (Raven, 1972), the podocyst of some pulmonates (Cather and Tompa, 1979), a transitory albumen digestive sac in the embryonic gut of some prosobranchs (Portmann, 1955; Portmann and Sandmeier, 1965), the larval kidneys of many prosobranchs (Rivest, 1992), and the pedal cell complex of neritoidean gastropods (Rivest and Strathmann, 1995). Because these transitory, albumen-absorbing structures are absent in planktonic, feeding larvae, it has been proposed that albumen-absorbing structures have evolved in gastropods with encapsulated embryos to enhance capsular albumen uptake (Rivest and Strathmann, 1995). It is likely that some of these structures serve other functions as well,

such as enhancing intracapsular respiration (Cather and Tompa, 1972; Rivest and Strathmann, 1995).

The mechanisms of consumption of intracapsular nutrition are poorly understood in many taxa. For example, the encapsulated larvae of non-planktotrophic *Littorina* species are surrounded by albuminous fluid in the egg capsule and grow considerably during development (Buckland-Nicks *et al.*, 1973), yet lack obvious albumen-absorbing structures such as larval kidneys (Rivest, 1981). Furthermore, the extent to which ancestral, planktotrophic morphology has been modified in larvae of nonplanktotrophic, intracapsular embryos cannot be addressed fully without making comparisons among closely related taxa with contrasting developmental modes. Such comparisons are unfortunately rare. The objectives of this study were to (1) determine the location and mechanism of albumen uptake by embryos of *Littorina* species with encapsulated development, (2) determine whether congeneric planktotrophs share similar patterns of albumen uptake, and to (3) compare the functional feeding morphology of encapsulated *Littorina* and their planktotrophic congeners.

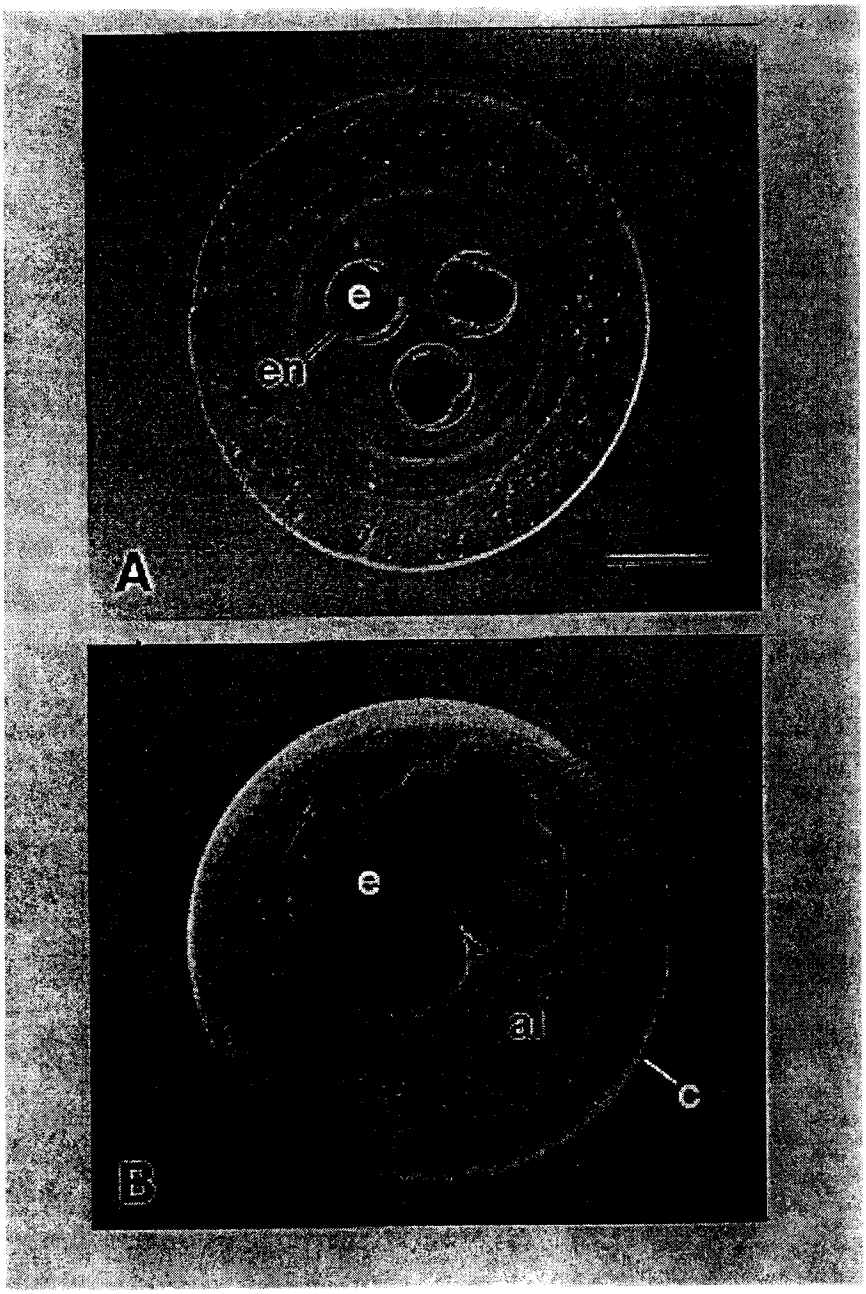
Study organisms

The gastropod genus *Littorina*, the periwinkle snails, contains 19 species found in the high-shore zone throughout the northern Atlantic and Pacific oceans (Reid, 1989; Reid *et al.*, 1996). Two types of development are found in *Littorina*; species that have pelagic egg capsules and planktotrophic larvae, and nonplanktotrophic species whose

larvae are contained within benthic or brooded egg capsules until metamorphosis ("encapsulated") (see Reid, 1989 for review). Planktotrophic species hatch as post-torsional, feeding veligers from planktonic egg capsules, and larvae are contained within individual egg membranes until immediately prior to hatching from the egg capsule (fig. 1A). Embryos of encapsulated species develop to metamorphosis in benthic or brooded egg capsules, in which embryos are surrounded by granular albumen that is consumed during development (Buckland-Nicks *et al.*, 1972) (fig. 1B). Independent phylogenetic evidence supports planktotrophy as the ancestral state (see Rumbak *et al.*, 1994).

Intracapsular, extraembryonic albumen is thought to be of nutritive importance to developing *Littorina* embryos for several reasons. Albumen of one species, *L. saxatilis*, has been found to be protein-rich and dominated by a single protein of molecular weight 80,000 kDa, that disappears from the extraembryonic capsular space during development (Losse and Greven, 1993). Several encapsulated *Littorina* species also grow considerably during development (Buckland-Nicks *et al.*, 1972; Moran, unpub. data), presumably due to the consumption of capsular fluid. The mechanism of capsular albumen uptake has previously been unknown (or assumed to occur via the gut (Buckland-Nicks *et al.*, 1972)); *Littorina* embryos have been reported to lack larval kidneys (Rivest, 1992), and other structures have not been implicated in albumen uptake.

Figure 1. (A) Planktonic egg capsule of *Littorina scutulata*, a species with planktotrophic development, containing three prehatching veliger-stage larvae. (B) Albumen-filled capsule of *Littorina saxatilis*, a species that broods encapsulated embryos until metamorphosis. Capsule was removed from the oviduct of a gravid female. al, albumen; c, capsule; e, embryo; en, egg envelope. Scale bar = 60 μm.


e
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Methods and Materials

Spawning and larval rearing

Seven *Littorina* species were used in these experiments, including four planktotrophs (*L. littorea* (Linnaeus, 1758), *L. keenae* Rosewater, 1978, *L. plena* Gould, 1849, *L. scutulata* Gould, 1849) and three species with encapsulated development (*L. saxatilis* (Olivi, 1792), *L. sitkana* Philippi, 1846, *L. subrotundata* (Carpenter, 1864)) (Table 1). Local species were maintained at the Oregon Institute of Marine Biology (OIMB) in flowing seawater at ambient temperatures in flow-through containers. Non-native species were kept at OIMB in 10 gallon aquaria in vigorously aerated sea water at room temperature or at 12°C. All objects exposed to non-native species were washed with a solution of chlorine bleach to reduce risk of introducing exotic species into the local environment. Egg capsules of planktotrophic species and species with benthic egg masses were obtained by placing live, freshly-collected adult animals into mesh-walled containers (< 1mm diameter mesh size), immersing containers in vigorously aerated sea water, and checking containers regularly. Planktotrophs and nonplanktotrophs generally produced egg capsules after 1 or 2 d and after 1 to 7 d, respectively. To obtain embryos of the nonplanktotrophic brooder *L. saxatilis*, adult animals were cracked with needle-nose pliers and developing embryos were removed from the brood chamber.

Table 1

Species, development and collection information for Littorina *utilized in this study*

Species, Authority	Mode[α]	Collection Locality	Habitat
Littorina littorea (Linneaus, 1758)	P	Woods Hole, MA Mystic, CT	Rocky shoreline
Littorina keenae Rosewater, 1978	P	Monterey, CA	Rocky shoreline
Littorina plena Gould, 1849	P	Charleston, OR	Rocky shoreline
Littorina scutulata Gould, 1849	P	Charleston, OR Monterey, CA	Protected estuary Rocky shoreline
Littorina saxatilis (Olivi, 1792)	NP	Woods Hole, MA Mystic, CT	Rocky shoreline
Littorina sitkana Philippi, 1846	NP	Charleston, OR Friday Harbor, WA	Estuarine marsh Rocky shoreline
Littorina subrotundata (Carpenter, 1864)	NP	Charleston, OR	Estuarine marsh

[α]P = planktotrophic, NP = nonplanktotrophic

Larvae of planktotrophic species were reared at concentrations of ca. one larva/ml in 0.45 μm filtered sea water changed every four d. Planktotrophic larvae were fed ad libitum on a mixture of single-celled algae, Tahitian strain *Isochrysis galbani* and *Dunaliella tertiolecta*. Egg masses of encapsulated *Littorina* species and embryos

dissected out of *L. saxatilis* were maintained in glass dishes of 0.45 μm filtered sea water at 12°C.

Use of fluorescence microscopy to test for albumen uptake in Littorina

Embryos of the three *Littorina* species with encapsulated development (*L. saxatilis*, *L. sitkana*, *L. subrotundata*) were removed from their egg capsules at developmental stages from early cleavage to near hatching. Embryos visibly damaged during removal were discarded. Because most planktotrophic embryos could not be removed from the egg envelope without damage, three of four planktotrophs (*L. littorea*, *L. planaxis*, *L. plena*) were only examined as fully-formed prehatching and hatched veligers. Embryos of the fourth planktotroph (*L. scutulata*) were successfully removed from the egg envelope on one occasion; therefore, *L. scutulata* were examined as pre-shelled embryos as well as prehatching and hatched veligers. All embryos and larvae were placed in solutions of bovine serum albumen labeled with fluoroscein isothiocyanate (FITC-BSA, Sigma #A-9771). FITC-BSA was also made in the laboratory from commercially available BSA and FITC (Sigma #F-7250) using the methods of Rivest (1981). Both purchased and laboratory-made FITC-BSA were used raw or dialyzed for 24 h against several changes of 0.45 μm filtered sea water to remove unbound FITC.

Embryos and larvae were placed in test solutions of 10-1000 μg/ml FITC-BSA in filtered sea water at 12°C for periods ranging from fifteen minutes to 24 h, then rinsed in

filtered sea water for periods ranging from 1 to 48 h. Control embryos received the same treatment but were exposed to test solutions containing filtered sea water only, unlabelled BSA, or unconjugated FITC. Examinations of experimental and control embryos and larvae were made on an Olympus epifluorescence microscope fitted with an FITC filter set (Omega Optics stock number XF23, excitation maximum 485 nm, emission 535 nm).

Preparation of larvae for TEM

Mid-veliger stage embryos of the encapsulated species *L. sitkana* (nonplanktotrophic) were removed from their capsules, rinsed briefly in 0.45 μm filtered sea water, and prepared for transmission electron microscopy using the methods of Rivest and Strathmann (1995) with minor modifications. Embryos were placed for 10 min in a solution of 0.05% osmium tetroxide and 3% glutaraldehyde in 0.1 M (pH 7.35) phosphate buffer, with the osmolarity raised to 990 mOsM with sucrose. Next, embryos were placed in a solution of 3% glutaraldehyde in 0.1 M phosphate buffer with the osmolarity raised to 990 mOsM with sucrose for one h, after which an equal volume of EDTA was added (to dissolve the shell) and embryos were fixed for another h. Embryos were postfixed for one h at room temperature in 2% osmium tetroxide in 1.25% sodium bicarbonate, then dehydrated in an ethanol series. Finally, embryos were exchanged in propylene oxide, embedded in epoxy resin, and thin sections were cut on a Reichert Ultracut E ultramicrotome. Sections were picked up on Butvar films on 200 μm hex grids, stained with uranyl acetate and lead citrate (Reynolds, 1963) and examined with a

Philips CM 12 electron microscope operated at 80 kV. TEM electron micrograph images were recorded on Kodak SO 163 film.

Prior to fixation, some embryos were exposed to a solution of 1 mg/ml ferritin (Sigma catalogue #F4503) in filtered sea water for 12 h. Ferritin is commonly utilized as a marker for receptor-mediated endocytosis (Rivest, 1981).

Measurement of Velar Aspect Ratios

Freshly-laid egg capsules of *L. plena* (planktotrophic) were transferred to filtered sea water and reared at room temperature. When embryos had reached the veliger stage (judged by the appearance of a ciliated velum), one larva was haphazardly chosen from each egg capsule. Brooded embryos of *L. saxatilis* (nonplanktotrophic) were removed from the brood chamber of the adult and several veliger-stage embryos were haphazardly chosen from each female. Measurements of velar width and total larval length were measured on each embryo (fig. 2). Measurements were made by tracing embryos with a camera lucida attached to a compound microscope. Lengths were calculated using a SummaSketch II digitizing pad and the software package SigmaScan for Windows (Jandel Corporation). In order to compare similar stages of development among the two species, only early veligers (lacking well-developed tentacles or propodia, similar in degree of development of larval structures to prehatching *L. plena*) were measured.

To compare the relative size of the velum and to compensate for considerable size differences between embryos of the two species, the "velar aspect ratio" (VAR) was

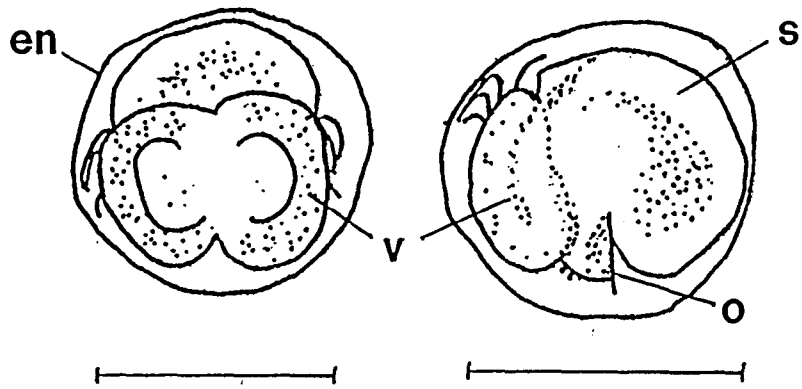


Figure 2. Camera lucida drawing of a prehatching larva of *Littorina plena*, showing measurements of velar width (left, apical view) and larval length (right, lateral view). en, egg envelope; o, operculum; s, shell; v, velum). Larval length bar = 110 μm.

calculated by dividing the width of the velum by the total length of each larva. VARs were calculated for a total of 64 *L. plena* embryos and 60 *L. saxatilis* embryos. To compare VARs of other nonplanktotrophic and nonplanktotrophic prosobranch gastropods, an extensive search of the literature was performed to find drawings or photographs of prosobranch veligers from a variety of taxa. Images were chosen based on two criteria, developmental stage and larval orientation. Because comparisons of planktotrophic and encapsulated *Littorina* were made when both species were fully-developed but young veligers (see above), planktotrophic species were used for this analysis only if larvae were described as prehatching or newly hatched. For nonplanktotrophic species, information regarding the age or developmental stage of

encapsulated larvae was rarely available. However, in the instances in which multiple developmental stages of a nonplanktotroph were depicted, the aspect ratio remained relatively constant until near metamorphosis (Moran, unpub. data). Therefore, images of nonplanktotrophic species were discarded only if embryos were described as mature or near hatching. If multiple stages were available for analysis, the earliest veliger stage was used. The orientation of the veliger was also important: images could be used only if both the full width of the velum and the length of the larva were depicted. However, because the orientation of larvae differed considerably among images, measurements of larval length were necessarily somewhat subjective.

For each selected image the velar aspect ratio was measured with a SummaSketch II digitizing pad and SigmaScan for Windows software as described for *Littorina* (above). If an image contained a scale bar, the scale bar was used to calibrate measurements for that image. If no scale was given, the aspect ratio was measured without units.

Statistical comparisons of the velar width/larval length ratio of planktotrophs and nonplanktotrophs were performed in two ways. Ideally, statistical analyses would take into account the degrees of phylogenetic relatedness among all taxa; to do otherwise may artificially inflate the sample size and hence the degrees of freedom (Martins and Hansen, 1996). However, while there has been much recent interest in higher gastropod phylogeny (e.g. Bieler, 1992; Ponder and Lindberg, 1996; Ponder and Lindberg, 1997; Harasewych *et al.*, in press), there is little consensus regarding relationships at or above the family level (Ponder and Lindberg, 1997). Therefore, for the purposes of this analysis

species were grouped by family and the average VAR was calculated for each family. In six families, VARs were available for both planktotrophic and nonplanktotrophic species (the remaining families were represented by only planktotrophs or nonplanktotrophs, although these families in most instances contained both types of development). To determine whether velar size varied with developmental mode, VARs of planktotrophs and nonplanktotrophs of these six families were compared with a paired Student's t-test.

Measurement of Cilia Length

Freshly-spawned egg capsules of three planktotrophs (*L. keenae*, *L. plena*, *L. scutulata*) and egg masses of one nonplanktotroph (*L. sitkana*) were transferred to filtered sea water and reared at 12°C. The three planktotrophs were initially examined every 2-3 h, and at approximately one d or greater intervals in later development until immediately post-hatching when observations ended. The slower-developing nonplanktotrophs were initially examined at daily intervals, and later at intervals of several days. Cilia were measured in one of two ways; if embryos were moving relatively slowly, the longest prototrochal cilia were drawn using a camera lucida attached to a compound microscope and images were digitized as described for the velar aspect ratios. If embryos were moving too fast to draw cilia with the camera lucida, embryos were crushed under the coverslip and the longest cilia were then drawn.

Results

Fluorescence microscopy

Nonplanktotrophs

Experimental embryos- No experimental eggs or embryos exhibited FITC fluorescence after exposure to FITC-BSA during early cleavage stages, gastrulation, or at any stage prior to the trochophore. In all species with encapsulated development (*Littorina saxatilis*, *L. sitkana*, *L. subrotundata*), FITC-BSA uptake was first seen at the trochophore stage, when larvae had developed the characteristic encircling band of ciliated cells (prototroch). At this stage, fluorescence was confined to the cells of the prototroch (fig. 3A, 3B). Somewhat later in development, at the early veliger stage (before the appearance of eyespots, foot, or the larval shell), FITC fluorescence was seen only in the ciliated cells of developing velum, not in the cells of the head or visceral mass (fig. 4A, 4B). When older embryos (with shell, foot, eyespots) were exposed to FITC-BSA, velar fluorescence was still confined to the cells of the ciliated band and was not evident in cells of the pretrochal region or the developing structures of the head (tentacles, eyespots) (fig. 5A-D). When the ciliated cells of the velum were examined under high magnification, it could be seen that FITC fluorescence was concentrated in small ($< 2\ \mu m$) spheres within individual cells (fig. 6). Fluorescence was seen in these ciliated cells of embryos exposed to FITC-BSA at all stages from trochophore up to

hatching, when the velum was resorbed. Newly-hatched juveniles that had completely resorbed their velums exhibited no fluorescence when exposed to FITC-BSA.

In addition to the ciliated cells of the velum, FITC-BSA fluorescence also appeared in some other regions of the embryo. In older embryos with a well-developed foot, the ciliated cells of the rejection band of the foot fluoresced in a manner qualitatively similar to prototrochal/velar fluorescence. Small, isolated points of fluorescence, similar to the small spherical structures seen in prototrochal/velar cells, were occasionally found in other embryonic regions that did not ordinarily mark. In mature veliger-stage embryos of all nonplanktotrophs, two small areas of FITC fluorescence appeared just posterior to the ciliated band of the velum on either side of the head (fig. 7) ("larval kidneys"). These areas exhibited some autofluorescence in control embryos as well, although the degree of autofluorescence was highly variable among embryos. In mature, fully-formed embryos, FITC fluorescence also appeared in regions of the gut (fig. 8A, 8B). The above patterns of FITC fluorescence were similar in all *Littorina* species with encapsulated development examined in this study (e.g., compare the fluorescence of the ciliated band of the velum in *Littorina sitkana* and *L. saxatilis* in figs. 5B and 5D, respectively).

Controls- With two exceptions, no regions of embryos or larvae of any species exhibited fluorescence in the characteristic blue-green color of the FITC label when animals were exposed to filtered sea water only or to BSA in filtered sea water. The two exceptions were the operculum, which displayed considerable autofluorescence in older

embryos (e.g., fig. 8B), and the dim autofluorescence of the larval kidneys (described above). Embryos exposed to unconjugated FITC displayed considerable FITC fluorescence that appeared in multiple regions including the foot, shell gland, velum and viscera. FITC fluorescence faded in most areas of these embryos when they were rinsed in filtered sea water for > 24 h. FITC fluorescence appeared in the cytoplasm of labeled cells as well as in vesicles, and FITC also adhered to the shell and operculum of mature embryos and larvae. Similar patterns of fluorescence were seen in some embryos exposed to high concentrations of FITC-BSA that had not been dialyzed to remove unconjugated FITC, but these patterns were never seen when FITC-BSA was dialyzed prior to exposure.

Planktotrophs

In control embryos and larvae (exposed to filtered sea water only or BSA in sea water) of *Littorina*, autofluorescence was seen only in the operculum (fig. 9B). Of four planktotrophic species exposed to test solutions containing FITC-BSA, none showed velar uptake of the labeled albumen at later developmental stages when larvae had emerged from the egg envelope but had not hatched from the egg capsule. None of the four species exhibited velar FITC-BSA as hatched, feeding larvae. Embryos of *L. scutulata*, the only species whose larvae were successfully removed from the egg envelope during early development, did not exhibit albumen uptake in the pre-shelled, early veliger stage. Embryos of a second planktotroph, *L. plena*, did not exhibit FITC-BSA uptake at any stage when embryos in the egg envelope were soaked overnight in

FITC-BSA solutions. Hatched larvae of all four planktotrophs exhibited fluorescence in the gut after long (> 4 h) exposure to test solutions, but no fluorescence in the velar cells (fig. 9A, B).

TEM Imaging

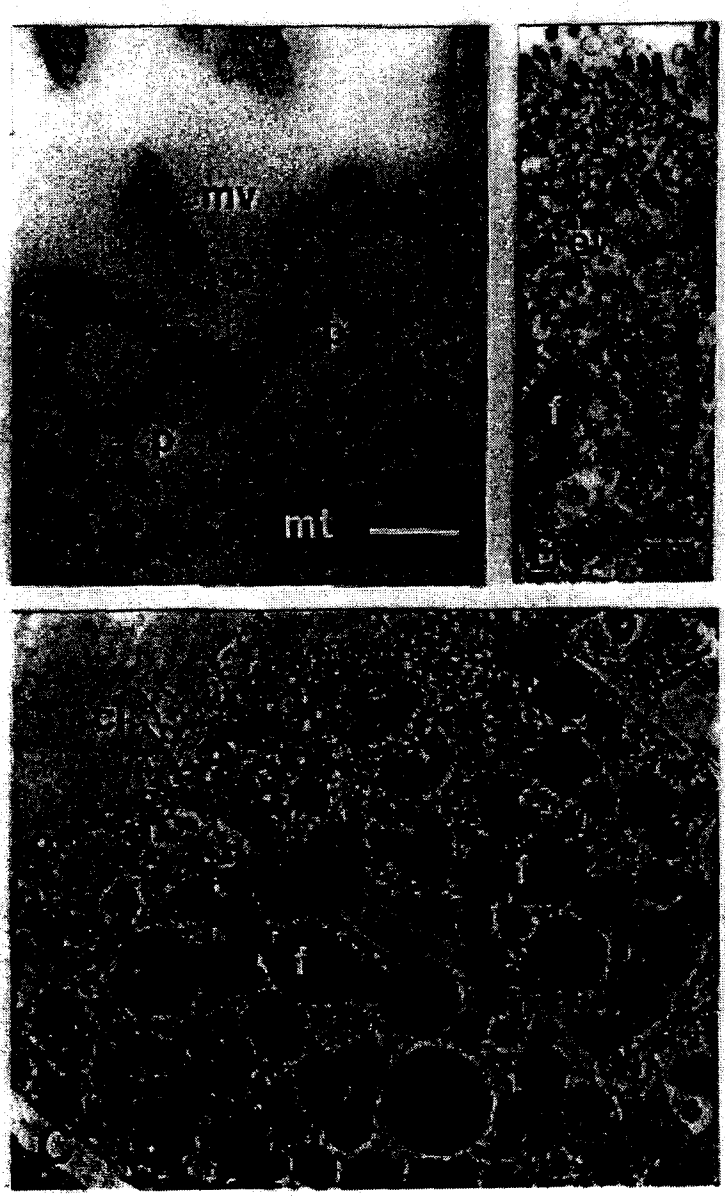
The surface of cells of the ciliated velar band contained numerous invaginations displaying a marked fuzzy thickening on the cytoplasmic surface of the cell membrane (fig. 10A). Numerous pinched-off vesicles were visible immediately inside the cell surface (fig. 10B). Endocytotic vesicles ranged from 0.1 to 0.3 μm in diameter, and smaller vesicles were seen immediately inside the cell surface while larger vesicles tended to appear somewhat further inside (fig. 10B). The surface of these cells also contained numerous microvilli and cilia displaying the typical 9 + 2 arrangement of microtubules. Surface invaginations and vesicles were not seen on non-ciliated epidermal cells.

The cytoplasm of the ciliated cells of the velum of embryos exposed to ferritin contained numerous dark bodies filled with an electron-dense, ferritin-like material (fig. 10C). This material was entirely lacking from the ciliated velar cells of control embryos.

Velar Aspect Ratios

The velar aspect ratios (VARs) of early *Littorina saxatilis* (nonplanktotroph) larvae were not significantly different from the velar aspect ratio of early *L. plena*

Figure 10. Transmission electron micrographs of a ciliated velar cell of a *Littorina sitkana* embryo (nonplanktotroph) exposed to ferritin. (A) High-magnification view of the surface of a cell from the ciliated band of the velum, showing endocytotic pits forming at the cell surface. (B) Slightly lower magnification view, showing numerous endocytotic vesicles immediately inside the cell surface. (C) Lower magnification TEM of two cells of the velar ciliated band, showing large numbers of vesicles filled with a dark, electron-dense, ferritin-like substance. ci, cilium; ev, endocytotic vesicle; f, structures containing ferritin-like electron-dense material; mt, mitochondrion; p, endocytotic pits. Scale bars; A, 0.2 μm; B, 0.85 μm; C, 3 μm.


mV
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f

(planktotroph) larvae (Student's two-sample t-test, $p = 0.77$) (fig. 11A). In many cases the average family VAR of planktotrophs from the literature were greater than nonplanktotrophs, although there was considerable overlap (Table 2; fig. 11B). VARs of planktotrophs were significantly greater than nonplanktotrophs when average VARs were compared among planktotrophs and nonplanktotrophs from six families (fig. 11B; paired Student's t-test, $p = 0.02$).

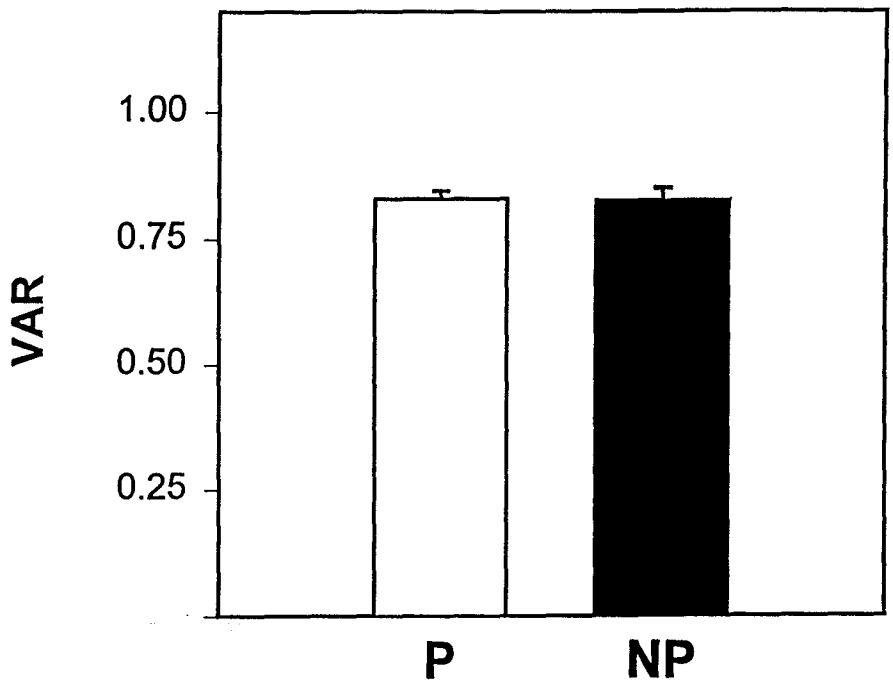
Cilia Length

Velar cilia of planktotrophic *Littorina* larvae grew considerably faster than cilia of the nonplanktotrophic *L. sitkana*, and reached a much greater total length (fig. 12). Observations of cilia length in additional nonplanktotrophic *Littorina* (*L. saxatilis, L. subrotundata*) were consistent with this pattern as well. The velum of nonplanktotrophic *Littorina* bore simple rather than compound cilia and lacked a well-defined prototroch, metatroch or food groove.

Discussion

Many embryonic structures have been associated with the endocytotic uptake of capsular proteins by gastropods. These include the larval kidneys of numerous prosobranch gastropods (Rivest, 1992), the "pedal cell complex" of neritoidean gastropods (Rivest and Strathmann, 1995), the podocyst of some pulmonates (Cather and Tompa, 1972), and ectodermal areas of early embryos of some pulmonates (Fioroni,

Figure 11. (A) Mean velar aspect ratios of 64 early veliger-stage embryos of *Littorina plena* (planktotroph, open bar) and 60 early veliger-stage *Littorina saxatilis* embryos (nonplanktotroph, filled bar). Error bars are 95% confidence intervals. (B) Mean velar aspect ratios of planktotrophs (open circles) and nonplanktotrophs (closed circles) from 21 gastropod families. Error bars are not shown because most families are represented by single species (Table I). The six families to the right of the vertical dotted line are represented by both planktotrophic and nonplanktotrophic species. The horizontal dotted line represents the mean velar aspect ratio of all families combined.


1.00
0.75
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VAR
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NP

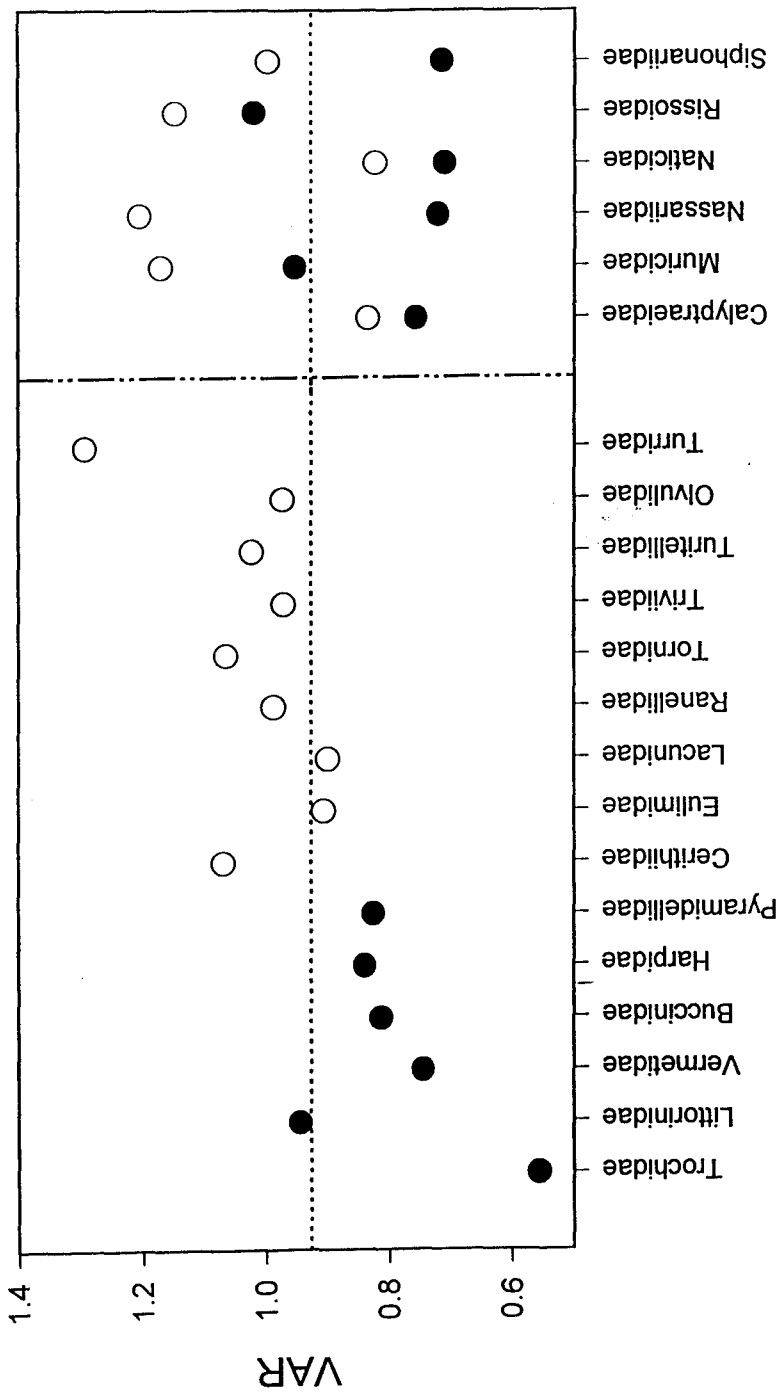


Figure 11. (continued)

Table 2

List of taxa utilized in comparisons of velar aspect ratios among planktotrophs and nonplanktotrophs

Family	Genus/Species	Mode[1]	VAR[2]	References
Buccinidae	*Engoniophos unicinctus*	NP	1.205	Miloslavich and Penchaszadeh, 1994
	Neptunea antiqua	NP	0.418	Pearse and Thorson,1967
Calyptraeidae	*Calyptraea chinensis*	NP	1.074	Lebour, 1936
	Calyptraea trochiformis	NP	0.779	Cañete and Ambler, 1992
	Crepidula adunca	NP	0.47	Moritz, 1939
	Crepidula fornicata	P	0.834	Fretter and Graham, 1962
Cerithiidae	*Cerithiopsis tubercularis*	P	0.924	Lebour, 1933b
	Cerithiopsis barleei	P	1.135	Lebour, 1933b
	Triphora perversa	P	1.144	Lebour, 1933b
Eulimidae	*Eulima distorta*	P	0.829	Thorson, 1946
	Pelseneeria stylifera	P	0.982	Thorson, 1946
Harpidae	*Morum onuscus*	NP	0.839	Hughes, 1990
Lacunidae	*Lacuna vincta*	P	0.900	Lebour, 1937
Littorinidae	*Bembicium vittatum*	NP	0.943	Black et al., 1994
Muricidae	*Chicoreous ramosus*	NP	1.599	Soliman, 1991
	Concholepas concholepas	P	1.169	DiSalvo, 1988
	Nucella lapillus	NP	0.817	Fretter and Graham, 1962
	Nucella lamellosa	NP	0.880	Lyons and Spight, 1973
	Nucella crassilabrum	NP	0.625	Gallardo, 1979
	Nucella canaliculata	NP	0.780	Lyons and Spight, 1974
	Nucella emarginata	NP	0.861	Lyons and Spight, 1974
	Trophon muricatus	NP	1.102	Lebour, 1936
Nassariidae	*Bullia digitalis*	NP	0.720	da Silva and Brown, 1985
	Nassarius incrassatus	P	1.150	Lebour, 1931a
	Nassarius reticulatus	P	1.257	Lebour, 1931a
Naticidae	*Natica catena*	NP	0.574	Thorson, 1946
	Natica (Lunatida) pallida	NP	0.843	Thorson, 1946
	Natica (Lunatida) nitida	P	0.822	Thorson, 1946
Olvulidae	*Simnia barbarensis*	P	0.973	Main, 1974
Pyramidellidae	*Boonea (Odostomia) impressa*	NP	0.825	White et al., 1985
Ranellidae	*Cabestana spengleri*	P	0.987	Riedel, 1992
Rissoidae	*Cingula semicostata*	NP	1.018	Lebour, 1934
	Rissoa sarsii	P	1.134	Lebour, 1934
	Rissoa membranacea	P	1.000	Lebour, 1934
	Rissoa parva	P	1.247	Lebour, 1934
	Rissoa guerini	P	1.202	Lebour, 1934
Tornidae	*Tornus subcarinatus*	P	1.064	Lebour, 1936
Triviidae	*Trivia europa*	P	0.971	Lebour, 1931b
Trochidae	*Calliostoma granulatum*	NP	0.707	Ramon, 1990
	Margarites helicinus	NP	0.393	Holyoak, 1988
	Tegula funebralis	NP	0.566	Moran, 1997
Turitellidae	*Turritella communis*	P	1.022	Kennedy and Keegan, 1992
Turridae	*Haedropleura septangularis*	P	1.205	Lebour, 1936
	Philbertia (Comarmondia) gracilis	P	1.380	Lebour, 1933a
Vermetidae	*Dendropoma corrodens*	NP	0.612	Miloslavich and Penchaszadeh,1992
	Vermetus sp.	NP	0.875	Miloslavich and Penchaszadeh, 1992
Siphonariidae	*Siphonaria serrata*	NP	0.731	Chambers and McQuaid, 1994
	Siphonaria concinna	P	0.996	Chambers and McQuaid, 1994
Trimusculidae	*Trimusculus conica*	NP	0.696	Haven, 1973

[1]P = planktotrophic, NP = nonplanktotrophic

[2]Velar Aspect Ratio

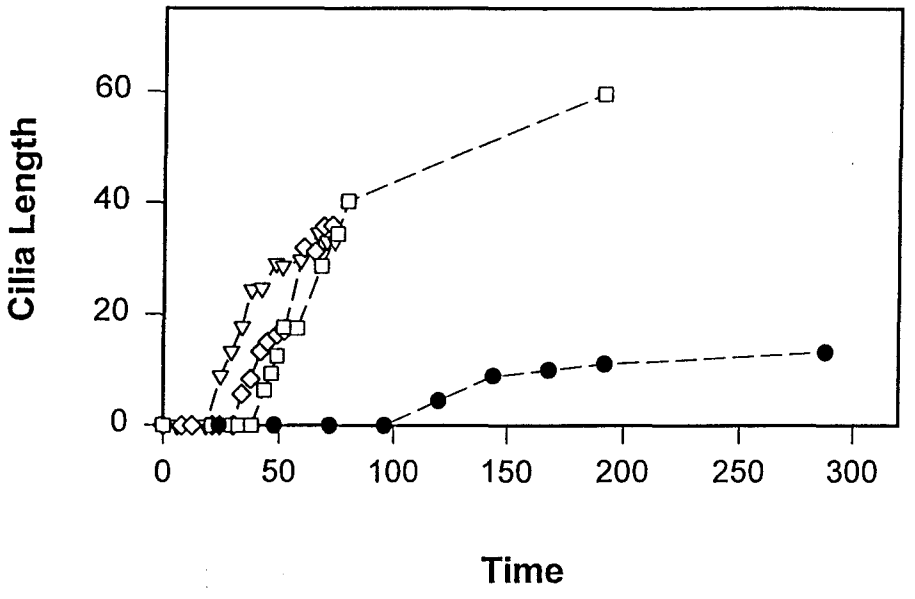


Figure 12. Lengths of the longest velar cilia over partial development of three planktotrophic (open symbols) and one nonplanktotrophic (closed symbols) *Littorina* species. Cilia length units are μm and time units are hours.

1977). In each case, the structures involved in protein uptake are found in encapsulated larvae only; in planktotrophic species whose larvae are encapsulated only during early development, structures such as the pedal cell complex and larval kidneys are resorbed prior to or soon after hatching (Rivest, 1992; Rivest and Strathmann, 1995). Because of their transitory nature, these structures are thought to represent morphological specializations for intracapsular development (Rivest and Strathmann, 1995).

Nonplanktotrophic *Littorina* species utilize a variety of methods for feeding on intracapsular albumen. Two areas of uptake, the larval kidneys and embryonic gut, have been implicated previously in protein uptake in other gastropod species (see Introduction). This study has established that nonplanktotrophic *Littorina* embryos also utilize a third structure for protein uptake, the ciliated cells of the prototroch and velum (hereafter termed "velar uptake"). The velum has not previously been implicated in endocytotic intracapsular nutrition. Furthermore, velar protein uptake differs from protein uptake via larval kidneys or other specialized structures; in *Littorina* the velum, the ancestral planktotrophic larval feeding/swimming structure, has acquired a novel feeding role in the intracapsular environment. To the best of my knowledge, this capacity has not been reported in other gastropod taxa. A comprehensive survey of albumen uptake by gastropod larvae from other taxa is necessary to determine whether prototrochal and velar albumen uptake is unique to nonplanktotrophic members of the genus *Littorina*.

Although I did not make quantitative estimates of protein uptake, several lines of

evidence suggest that albumen is an important source of nutrition in nonplanktotrophic *Littorina*. First, embryos grow considerably during development concurrent with the disappearance of capsular albumen (Buckland-Nicks *et al.*, 1973; Moran, unpub. data), and albumen is the only likely extraembryonic food source in non-brooding species. Second, the capsule fluid of all nonplanktotrophic species examined in this study exhibit schleiren lines when capsules are opened in sea water and electrophoretic evidence indicates that capsular fluid contains various proteins that disappear during development (Losse and Greven, 1993; Moran, unpub. data)). The ciliated cells of the prototroch and velum are likely to be important sites of albumen uptake for the following reasons; 1) The surface area of the velum is a large proportion of the surface area of the total embryo, particularly during early development (e.g. fig. 4); and 2) uptake begins in these cells very early in development (at the trochophore stage) considerably prior to the development of the gut or the appearance of larval kidneys. Gut uptake may be of greater importance later in development when the velum is being resorbed.

In a survey of larval-kidney absorptive cells in gastropods, Rivest (1981, 1992) reported that absorptive larval kidneys were absent in *Littorina sitkana*. In contrast to Rivest's results, all nonplanktotrophic *Littorina* examined in this study possessed two small structures on either side of the larval esophagus that appeared active in albumen uptake. Similarly sized structures in the same location are reported from embryos of the nonplanktotrophic *L. obtusata* (Delsman, 1914; Fretter and Graham, 1967 fig. 202a (as *L. littoralis*)), and have been termed "nephrocysts" (Delsman, 1914) and "larval kidneys"

(Fretter and Graham, 1967). These paired structures are in the same location as the albumen-absorbing larval kidneys reported by Rivest (1992) in numerous other prosobranch gastropods and may be homologous; larval kidneys have been proposed as a synapomorphy of the caenogastropods (Ponder and Lindberg, 1997). The presence of these absorptive structures may have been overlooked in *L. sitkana* because larval kidneys of *Littorina* are considerably smaller in relation to the size of the embryo than in other taxa, in which larval kidneys can comprise 20% of the volume of the developing embryo (Rivest, 1992). In addition, some control *Littorina* embryos exhibited autofluorescence in these cells (considerably dimmer than the fluorescence of experimental embryos) that may have further confounded previous studies. Due to their small size and dim fluorescence after exposure to FITC-BSA (relative to the velum, and to the larval kidneys of other species such as *Nucella emarginata*), the importance of these structures to embryonic nutrition of nonplanktotrophic *Littorina* is likely to be small.

TEM imaging in this study demonstrated that the ciliated cells of the prototroch contain numerous endocytotic vesicles, strongly suggesting that albumen is taken up via pinocytosis across the cell membrane. Endocytotic albumen uptake is further suggested by the presence of large amounts of ferritin-like electron-dense material in the ciliated velar cells of embryos that had been exposed to ferritin, and the absence of this material from control embryos. Velar albumen uptake by *Littorina* is therefore likely to be mechanistically analogous to albumen uptake by other embryonic structures in

gastropods, including the larval kidneys (Rivest, 1981, 1992) and the pedal cell complex (Rivest and Strathmann, 1995).

Larvae of many planktotrophic marine invertebrate taxa have the ability to take up dissolved organic matter (DOM) from sea water. Among molluscs, this ability has been demonstrated in bivalves (Manahan, 1983) and the gastropod *Haliotis rufescens* (Jaeckle and Manahan, 1989). DOM uptake by marine larvae is accomplished via carrier-mediated pathways (Wright and Manahan, 1989), and the primary site of DOM uptake in veliger larvae appears to be the velum (Manahan and Crisp, 1983). Because uptake of DOM is accomplished via carrier-mediated membrane transport, it is unlikely that this process is homologous to receptor-mediated endocytotic protein uptake by the velum of encapsulated *Littorina* species. The endocytotic ability of the velar cells may be newly evolved in encapsulated *Littorina*, or may be homologous to an unknown pinocytotic process in the velum of planktotrophic species.

The velum of nonplanktotrophic *Littorina* may take up other substances in addition to proteins. Losse and Gerven (1993) noted considerable fluorescence in the gut, hepatopancreas and velum of *L. saxatilis* embryos exposed to FITC-labeled dextrans. The intracapsular albumen of some gastropods contains both proteins and additional substances such as carbohydrates and free amino acids (e.g. Horstmann, 1956, for *Lymnaea stagnalis*) that may be of nutritive importance, although their presence has not been established in the capsular fluid of *Littorina*. The mechanism of uptake and the importance of these additional substances to embryonic nutrition are presently not well

understood.

Intracapsular albumen is not likely to be of energetic importance during the development of planktotrophic *Littorina* for several reasons. First, early-stage embryos of planktotrophs are contained within individual egg envelopes during most of encapsulated development and would not be directly exposed to intracapsular albumen (if present) until immediately prior to hatching. Embryos contained within the egg envelope did not exhibit FITC fluorescence after overnight soaking in FITC-BSA solutions, suggesting that either 1) the egg envelope is not permeable to proteins of > MW 88,000 kDa, or 2) if the envelope is permeable to proteins, early embryos do not take them up. Second, while egg capsules of planktonic species contain a visible gel-like material external to the egg envelopes of embryos, this material contains no spectrophotometrically detectable protein (Moran, unpub. data) and does not create schlieren lines when capsules are opened in sea water. Finally, mature and post-hatching embryos and larvae of planktotrophic *Littorina* do not appear to utilize the velum or larval kidneys for protein uptake, and for most of early development the gut (which takes up albumen after hatching) is not fully formed.

Uptake by larval kidneys of many gastropod species and by the pedal complex of neritid gastropods have been previously demonstrated using FITC-BSA, and in each case TEM has confirmed protein uptake (Rivest, 1981, 1992; Rivest and Strathmann, 1995; this study). These observations suggest that FITC-BSA is a good indicator of receptor-mediated endocytosis. In this study, however, unconjugated FITC resulted in

fluorescence of the larva, although in a manner visually different from FITC-BSA. Because TEM confirmed that only areas that took up FITC-BSA were endocytotically active, it seems likely that fluorescence in other areas was due to FITC labeling of larval tissues rather than uptake of unconjugated FITC. Therefore, interpretations of FITC-BSA labeling patterns should be made with caution if unconjugated FITC may be present as well.

In marine systems, the evolutionary transition from planktotrophy to nonplanktotrophy is often accompanied by modifications to ancestral planktotrophic feeding characters (Strathmann, 1978). These modifications have been viewed as losses of complex structures that occur concurrently with the loss of planktotrophic larval feeding (Strathmann, 1978), and as novel features that enhance other aspects of nonfeeding larval performance (e.g. swimming, Emlet, 1994). Encapsulation of embryos is often associated with altered ciliary morphology in gastropods (Hadfield and Iaea, 1989). This study documents that nonplanktotrophic *Littorina* veligers have lost the elaborate, ancestral planktotrophic ciliary patterns found in planktotrophic *Littorina*. This loss may be due in part to lack of selection for swimming and particle capture. Alternatively, short cilia may function better than long cilia in the confined and possibly more viscous environment of the egg capsule; functions of cilia in encapsulated *Littorina* might include 1) rotating embryos to enhance oxygen diffusion through benthic, gelatinous egg masses (Hunter and Vogel, 1986; but see Strathmann and Strathmann, 1995) or 2) stirring fluids to enhance feeding on intracapsular albumen.

While numerous authors have remarked that encapsulated gastropod embryos have reduced velar lobes (e.g. Jägerston, 1972; Webber, 1977), possibly because the velum is no longer necessary for swimming and feeding (Jägerston, 1972), very few studies have addressed this issue in a comparative or quantitative context. Results of a literature search described in this study support the hypothesis that during early development, gastropods with encapsulated development have smaller velar lobes than confamilial planktotrophs. Despite this general pattern considerable variation is evident in the degree of velar loss among nonplanktotrophs, ranging from species whose embryos never develop a velum (e.g. Penchaszadeh and Rincón, 1996) to genera such as *Littorina* in which planktotrophs and nonplanktotrophs have velums that are equivalent in size. Retention of the velum may be ascribed to several causes, including developmental constraints (although the reported lack of a velum in some species (e.g. Penchaszadeh and Rincón, 1996) argues that the presence of velar lobes may not be necessary for gastropod morphogenesis) or a recent evolutionary loss of planktotrophy (this second hypothesis has yet to be tested in a comparative, historical context).

A third possibility is that nonplanktotrophs that retain a large velum may do so because the velum serves a novel function in the intracapsular environment. Such suggested functions include feeding on nurse eggs (e.g. Fioroni and Sandmeier, 1964), providing a respiratory surface (Fretter and Graham, 1962), or generating currents that enhance oxygen transport through egg masses (Hunter and Vogel, 1986). I have demonstrated that in *Littorina* the velum takes up intracapsular albumen; this function is

analogous to the velum's ancestral role in free-living, planktotrophic larvae. While the velum of nonplanktotrophic *Littorina* may perform other functions as well, the large size of the encapsulated *Littorina* velum may in part be due to its novel feeding function in the egg capsule.

In summary, the velum (the ancestral planktotrophic feeding organ) of embryos of nonplanktotrophic *Littorina* species is active in endocytotic albumen uptake throughout development. Velar albumen uptake was not seen in planktotrophic *Littorina* species; therefore, endocytotic velar albumen uptake may be newly evolved in nonplanktotrophic *Littorina* species. The feeding morphology of veliger-stage embryos of nonplanktotrophic and planktotrophic *Littorina* differ in that nonplanktotrophic species have shorter cilia and appear to lack an opposed-band pattern of prototrochal and metatrochal cilia. However, the velar apparatus of planktotrophs and nonplanktotrophs is comparable in size during early development. A survey of prosobranch gastropods suggests a general trend towards reduction in the relative size of the velum in nonplanktotrophic species. It is suggested that nonplanktotrophic *Littorina* may retain a large velum in part due to its apparently novel absorptive function.

CHAPTER III

HATCHING SIZE AND HATCHLING PERFORMANCE IN A MARINE SNAIL: EFFECTS OF SPATIAL AND TEMPORAL VARIATION ON THE BENEFITS OF LARGE HATCHING SIZE

INTRODUCTION

A fundamental principle of life-history theory is the presence of a trade-off between the size and number of offspring a female produces (Lack, 1947; Vance, 1973; Smith and Fretwell, 1974; Brockelman, 1975; Kaplan and Cooper, 1984; McGinley *et al.*, 1987; Sinervo, 1990; Levitan, 1993). This trade-off is driven by energetic, physiological and morphological constraints on the total reproductive output of an individual (Drent and Daan, 1980; Godfray, 1987) that preclude increased offspring size without a corresponding decrease in offspring number (Stearns 1992). The number and size of offspring in a given clutch is thought to reflect optimizing action of natural selection balancing advantages of high fecundity with advantages of producing higher-quality offspring (Smith and Fretwell, 1974; Sinervo, 1990). A great deal of evidence supports

the presence of trade-offs in offspring size and number within and among numerous taxa, although exceptions are common (see Stearns, 1992 for review).

Two important assumptions underlying the hypothesized trade-off between size and number of offspring are that (1) fitness (survivorship) increases with offspring size (Smith and Fretwell, 1974); and (2) offspring size and organic content are positively correlated, in that larger offspring represent a greater maternal investment than smaller offspring (McEdward and Carson, 1987). The first assumption, that fitness of individual offspring increases with size, is widely held but not universally supported by empirical evidence (reviewed by Stearns, 1992; Williams, 1994). Some studies have found similar survivorship (an important component of fitness) among large and small offspring (e.g. Wicklund and Karlsson, 1984; Trabanino *et al.*, 1989; Ruohomäki *et al.* 1993), and in some instances small offspring exhibited higher survivorship (e.g. Cowan and Houde, 1990; Litvak and Leggett, 1990; Kaplan, 1992; Marañón and Grubb, 1993; Tejedo, 1993). Deviations from the expected effect of offspring size have been attributed to spatial or temporal environmental variation in environmental quality (Capinera, 1979; Yuma, 1986; Berven and Chadra, 1988; Lyimo *et al.*, 1992), and it has been suggested that the drawbacks of small size are lessened under benign environmental conditions (Spight, 1976; Ferguson and Fox, 1984; Williams, 1994; Fox and Mousseau, 1996).

The second assumption, that larger offspring are more energetically expensive, is not often directly tested but is violated within some taxa in which the relationship between offspring size and organic content is weak or absent (McEdward and Carson, 1987; McEdward and Coulter, 1987; Niciu and McEdward, 1994). Therefore, offspring

size cannot *a priori* be assumed to accurately reflect organic content or maternal investment but must be tested within a given system (McEdward and Carson, 1987; McGinley and Charnov, 1988). If large offspring do not represent higher levels of maternal energy investment than small offspring in a given taxon, the life history of that taxon cannot be predicted to evolve in a context of energetic, size-number trade-offs.

Because natural systems may violate one or both of the above assumptions, interpretations of life history patterns that assume the presence of a size-number trade-off may be incorrect in species or systems in which these assumptions have not been adequately tested. An example of such a system is the rocky intertidal environment, which because of its rich biota and well-defined environmental stress gradients has been the focus of many seminal ecological studies. Marine benthic communities also exhibit a tremendous variety of life-history modes both within and among taxa, and therefore are particularly well suited to studies of life-history evolution (Thorson, 1946; Strathmann, 1985). Despite the large body of research on the ecology of rocky intertidal systems (reviewed partially by Rafaelli and Hawkins, 1996), little is known about the role of the juvenile life-history stage in shaping population or community structure (Keesing and Halford, 1992; Gosselin, 1994). In particular little is known about the potentially important role of offspring size in benthic marine communities, possibly because (1) the minute size of juveniles and the complexity of their natural habitat (Gosselin, 1994) make field manipulations difficult; (2) complex intertidal environmental conditions are difficult to emulate in the laboratory; and (3) substantial intra-population offspring size variation is relatively unusual among intertidal organisms (Spight 1976a).

In this study, I used the intertidal gastropod *Nucella emarginata* ('northern' form; see Palmer *et al.*, 1990) to perform laboratory and field tests of several assumptions of life-history theory. I first determined that hatching size in *N. emarginata* is predictive of hatchling organic content. Next, I used laboratory studies to explore the effects of hatching size on growth and survivorship in the laboratory, both under conditions of starvation and with hatchlings fed *ad libitum*. I then carried out intertidal field outplants of large and small hatchlings to test the effects of hatching size on hatchling growth and survivorship. Finally, I performed outplants in neighboring environments that experienced different heat/desiccation regimes to determine whether the effect of hatching size on survivorship varied under measurably different, but environmentally realistic thermal regimes. Results of these experiments suggest that while increased maternal investment in *N. emarginata* improves offspring performance under many conditions, the relationships among offspring performance, maternal investment and environment may not always meet the predictions of life history theory.

BIOLOGY OF *NUCELLA EMARGINATA* (NORTHERN)

The marine gastropod species *Nucella emarginata* (northern) is a common intertidal snail that ranges from Alaska to Half Moon Bay, California (Palmer, 1990). The ecology and biology of this species is well studied. Both juveniles and adults feed on barnacles and mussels and live in the mid- to high-intertidal (Morris *et al.*, 1973). Female *Nucella emarginata* in Oregon reproduce year-round (Seavy, 1977), laying

multiple clutches of between 4 and 20 benthic egg capsules. Each capsule contains 6 – 23 embryos, and offspring hatch as metamorphosed juveniles ("hatchlings") (Spight 1976a). During prehatching development, embryos feed on nurse eggs (nondeveloping or unfertilized eggs), thereby growing from an egg size of 190 μm to hatching shell lengths of between 0.9 and 2.3 mm (Spight, 1976a; Palmer, 1990). Hatching size is determined by the number of nurse eggs consumed during development, and the maternally-determined ratio of nurse eggs to developing ova varies among capsules within a clutch, among clutches, and among populations (Spight 1976a, Rivest 1983).

Mortality of *Nucella* hatchlings is thought to be very high. Estimates of mortality made from measurements of adult fecundity and size distributions of juveniles and adults suggest that 90 – 99% of *N. emarginata* juveniles die in the first year after hatching (Spight 1976b), and in a congeneric species (*N. lapillus*) only 1 –2 % survive the first two months of life (Feare, 1970). Sources of mortality in the field are largely unknown, but field manipulations have established that microhabitat is very important; juvenile mortality is close to 100% if juveniles are deprived of cover (Gosselin and Chia, 1995). Several potentially important biotic and abiotic sources of mortality include predation, heat/desiccation stress, starvation, salinity and dislodgment by wave action (Underwood, 1979; Pechenik, 1982; Rivest, 1983; Etter, 1989; Gosselin and Chia, 1995), and these pressures may exert considerable selective pressure on early life-history traits. However, the relative importance of these factors to shaping the early life history of *N. emarginata* is not known.

The importance of hatching size to survival in this variable species is also poorly understood. Larger *N. emarginata* hatchlings consume a wider range of prey sizes than small hatchlings (Palmer, 1990), and some predators have been demonstrated to attack smaller (younger) juveniles in the laboratory (Spight, 1976b; Gosselin, 1994). In another intertidal gastropod, larger (older) juveniles are less susceptible to desiccation and predation stress than smaller (younger) hatchlings (Rivest, 1983), but the relative effects of size and age have not been separated.

METHODS

Experiment 1: Relationship of hatching size to organic content and maternal investment

To determine the relationship between shell length (a simple and non-destructive measurement) and organic content, "ripe" clutches were collected from three rocky intertidal sites in Oregon at 43.34° N, 124.38° W (Gregory Point, Cape Arago (GP); Coos Head, Charleston (CH); Boathouse Dock, Oregon Institute of Marine Biology (BHD)). All sites were within 5 km of each other. Ripe clutches were clutches in which all hatchlings had undergone metamorphosis and the hatching plug had dissolved or begun to dissolve, but juveniles had not yet left the egg capsule. Clutches were used only if no or very few juveniles had left the egg capsule, both to ensure that all offspring were represented and because partially-hatched clutches rarely contained enough hatchlings for experimental procedures (see below). Three clutches from BHD and CH were examined in their entirety, and 10 clutches from GP were subsampled. Each hatchling was

measured for total shell length (apex of the shell to the tip of the siphonal canal) to the nearest 10 μm under a Wild dissecting microscope, and then the total organic content of each hatchling was measured using one of two methods.

The first method used to measure hatchlings' total organic content was the potassium dichromate wet oxidation (PDWO) method of Parsons *et al.* (1984), as modified by McEdward and Carson (1987) with some additional modifications (Appendix A). The second method was ash-free dry-weight (AFDW), which was measured by washing each hatchling 5X with distilled water to remove salts, placing hatchlings in individual aluminum pans, and drying at 80°C to a constant weight (> 6 days). The dry weight of each hatchling and pan was recorded, and the pans and snails were then ashed in a muffle furnace at 450°C for 8 hours. To obtain AFDW, the ashed weight was subtracted from the dry weight for each snail and pan. On one occasion, a clutch was randomly divided in half and analyzed with both ADFW and PDWO methods. Because both methods gave equivalent estimates of total organic content (Moran, unpub. data) and AFDW was less time-consuming than PDWO, the AFDW method was utilized in most analyses. AFDW was performed on individual hatchlings from two groups: (1) three entire clutches collected from the field, and (2) 20-24 randomly subsampled hatchlings from each of 12 clutches used in an experimental outplant (see below).

To determine the organic content of growing juveniles in the field, juveniles were collected at Gregory Point, Cape Arago, on April 2 1997. This site was chosen because it displayed consistently high numbers of ripe egg capsules of *Nucella emarginata*. Juveniles were sought by eye, without disturbing the underlying substrata. Searches were

conducted in patches containing numerous adults and egg capsules of *N. emarginata*. A total of 28 juveniles of between 1.7 and 7 mm shell length were collected and AFDW was measured for each juvenile as described above.

Experiment 2: Size-dependent growth and survivorship under both starved and fed conditions in the laboratory

To determine the effect of hatching size on survivorship under starved conditions, two ripe clutches were collected from the field and hatchlings were kept in the laboratory without food. Snails were gently removed from their capsules with dissecting scissors, fine forceps and a Pasteur pipet. Sixty hatchlings from each clutch were randomly chosen (to eliminate possible bias, hatchlings were suspended in filtered sea water, poured into a dish, and the 60 hatchlings closest to a randomly chosen position in the bowl were selected) from the total pool (n = 120, each clutch) and each snail was measured for total shell length under a Wild dissecting microscope to the nearest 10 μm. Each hatchling was placed in an individual well of a 12-well tissue culture tray from which the tops and bottoms of each well had been removed and replaced with 600 μm Nitex mesh. Tissue culture trays were then placed in a large (~ 20 L) tub of 0.45 μm filtered sea water, and this tub was partially immersed (to the water line) in running sea water at ambient sea temperature. Hatchlings were maintained without food and monitored periodically.

Nucella emarginata undergo substantial changes in shell growth pattern and

allometry at metamorphosis, as do many gastropods (Jablonski and Lutz, 1983). In *N. emarginata*, the delineation between the larval shell (protoconch) and the adult shell (teloconch) is very clear (Fig. 1). Therefore, growth could be readily measured in one of two ways; (1) as the amount of shell added since metamorphosis, measured from the protoconch-teloconch boundary (PT boundary) to the new growing aperture at a standardized point (the 2nd shell rib), or (2) as an increase in total shell length, by measuring the length of the embryonic shell at the PT boundary and subtracting this length from the total shell length of a growing juvenile (Fig. 1). Because initial increases in shell length were very small, method (1) was most useful for very young or starved juveniles. Method (2) was useful for older juveniles up to the point of completion of the first body whorl (approx. 1-3 months, depending on initial size and rearing conditions), at which point the adult shell had partially overgrown the PT boundary and it was only possible to estimate hatching size as either Large or Small.

To test the effect of hatching size on growth under starved conditions, hatchlings from both clutches of hatchlings were maintained without food in the laboratory as described above. After 2 weeks, each hatchling was measured for shell growth using measurement method (1) (above). Both clutches were examined for growth after 2 weeks, and after 3 ½ months each snail in the second clutch was individually examined and scored as alive or dead.

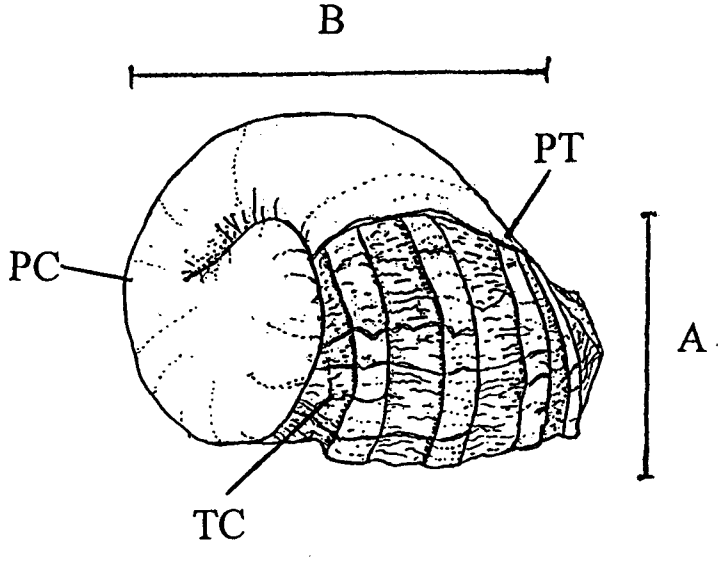


Figure 1. Line drawing of a *Nucella emarginata* hatchling, showing different growth morphologies of the protoconch (PC) and teloconch (TC) and the clear protoconch-teloconch boundary (PT). Line (A) represents type 1 growth measurements from the PT boundary to the new growing aperture of the shell along the 2nd shell rib. Line (B) shows the measurement of hatchling length made from the PT boundary.

Marking and rearing of hatchlings fed ad libitum

To examine the effects of hatching size on fed *N. emarginata* in the laboratory, capsules from six ripe clutches collected at GP were opened with fine dissecting scissors and hatchlings were gently washed out with a Pasteur pipet. The very smallest snails (<

0.9 mm) were discarded because in some instances very small hatchlings exhibited developmental abnormalities such as a poorly calcified shell or a poorly developed foot. Hatchlings from each clutch were then rinsed through series of 4 graded Nitex screens and 16 large and small snails from each clutch (from the largest and smallest mesh screens, respectively) were randomly chosen and measured for greatest shell length. Each group of 32 snails from each of the six clutches was then placed in a 1-pint plastic container whose sides and top had been replaced with 120 μm mesh (one clutch/box), and snails were provided with field-collected rocks covered with small barnacles and mussels.

Snails were removed at approximately 30 d intervals and measured for growth, then replaced in boxes with new barnacle- and mussel- covered rocks. 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. Empty shells were by far the most common, and in no instance was it difficult to distinguish living and dead snails. Hatching length of each snail was measured from the protoconch-teloconch boundary and growth was measured by subtracting hatching length from new length (method 2 as described above). When the first adult whorl had wrapped over the PT boundary (at approximately 30 d, with considerable variation), hatching length was estimated by measuring the exposed portion of the protoconch. This method did not provide a fully accurate measurement of hatching length because a portion of the protoconch was overgrown, but by measuring the exposed portion of the protoconch (> 75% exposed) hatching length of each snail could be clearly distinguished as large or small.

Experiment 3: Size-dependent growth and survivorship in the field

To determine how hatching size affected juvenile performance in the field, large and small hatchlings from several clutches were outplanted to the field and sampled over time. A total of five outplants were performed at BHD, and similar techniques were used in all five outplants. Because pilot studies using entire clutches suggested that the effects of size might be subtle and therefore masked by the inclusion of intermediate-sized hatchlings, for the purposes of this study I separated clutches into 4 size classes (as above) and utilized snails from the largest and smallest size classes only. Hatchlings were removed from their capsules and sorted into size classes as described above. From those clutches, 15 large and 15 small snails were randomly chosen from the largest and smallest screens, respectively, and marked with a solution of Calcein in sea water for 12 hours (see Chapter IV for details on Calcein marking of snails). Calcein is a nontoxic label that creates a permanent mark at the growing edge of hatchlings' shells, and this mark was used to 1) identify experimental animals and 2) measure the original length-at-marking for snails that have undergone considerable growth.

Each group of 30 marked hatchlings was then outplanted to the field on 13 x 13 cm Astroturf™ (Monsanto, Inc.) panels. These panels had been seasoned in the field for ca. 5 months, such that each panel contained abundant cover of small and large barnacles, mussels, algae and numerous other organisms that serve as food and cover for *Nucella* hatchlings. Each panel was surrounded and held down by short segments of PVC pipe, which were bolted to the substrate and coated with Tanglefoot™ (The Tanglefoot

Company, Grand Rapids, MI) to prevent hatchlings from wandering off. Both laboratory and field experiments demonstrated that Tanglefoot™ was an effective barrier to hatchling movement (Appendix B). Tanglefoot™ barriers were refreshed every 2 d in the field or as necessary. Hatchlings were outplanted to the panels in the field by transporting clutches in Eppendorf tubes filled with seawater and gently transferring hatchlings to the panels with a Pasteur pipet. Hatchlings were then gently washed with seawater until all hatchlings had attached to the panel.

Five separate sets of outplant experiments were performed at BHD. The first two experiments, Outplants A and B, consisted of 5 clutches on 5 panels (one panel-clutch, for five panel-clutches) outplanted to the field for 9 d on two separate occasions in the fall of 1995. Because hatchlings' shell length increased relatively little in 9 days, growth was measured by method (1) (shell added from PT boundary) in Outplants A and B. The second two experimental outplants (Outplants C and D) also comprised five panel-clutches, but hatchlings were outplanted for a total of 36 d in the spring (Outplant C) and summer (Outplant D) of 1996 and sampled at 9 d intervals. The fifth outplant, Outplant E, consisted of 12 panel-clutches sampled at 9 d intervals for a total of 54 d in the fall of 1996. Outplants C, D and E were terminated when total recovery was lower than 20% of the original number outplanted. Growth in Outplants C, D and E was measured by method (2) (new length – original length).

After each 9 d experimental interval, panels were sampled in the laboratory by repeated washings with a high-pressure freshwater spray, followed by running the washings through a 600 μm screen and immediately rinsing with sea water. Hatchlings

were never visibly damaged by this procedure and were very active after being returned to sea water. The total shell length of each hatchling was measured, and the hatching length was measured from the Calcein mark (see Chapter IV). The original size class of each individual (large or small) could be readily determined via the Calcein mark. Growth of each individual snail was calculated by subtracting the original hatching length, as measured from the Calcein mark, from the total shell length. "Recovery" was calculated by subtracting the total number recovered at each sampling interval from the original number in each group (15). Laboratory processing of panels was time-consuming and panels and hatchlings were kept in the laboratory for one to three days (depending on number of panels and oceanographic conditions, which sometimes precluded fieldwork). While in the laboratory panels and hatchlings were kept moist and at 4°C to limit growth and metabolism, with no visible negative effects on the hatchlings. After processing, panels were then returned to their original position in the field and hatchlings were replaced as described above. Only time in the field was factored into calculations of hatchling survivorship and growth rate.

Throughout the field experiments, recovery rates were utilized as an estimator of hatchling survivorship. The Tanglefoot ™ barriers placed around panels prevented almost all crawl-away behavior on poorly-seasoned "corral" panels in the field (Appendix B), and were likely an even more effective barrier on the well-seasoned panels utilized in the experiments described here. Hatchlings did not crawl away from seasoned panels in the laboratory even in the absence of Tanglefoot™ barriers (Moran, unpub. data). *Nucella* juveniles do not exhibit the "ballooning" behavior that some gastropods

employ as a strategy for leaving undesirable microhabitat (Martel and Chia, 1991; Gosselin and Chia, 1995), and therefore probably did not purposely leave panels via the water column.

Recovery would not equal survivorship if hatchlings were washed off panels through wave action and successfully reattached themselves elsewhere. However, *Nucella* inhabit a relatively narrow band of the intertidal (Abbott and Haderlie, 1980). Because *Nucella* juveniles deprived of appropriate microhabitat quickly succumb to heat/desiccation stress (Gosselin and Chia, 1995) and because the lower intertidal under and around *Nucella* habitat often contains numerous predators such as anemones and hermit crabs (pers. obs.), a wave powerful enough to dislodge a *Nucella* hatchling would carry the hatchling to an uncertain fate at best (Etter, 1989). Finally, a small number of snails were not dislodged by the freshwater spray in the washing process; this was evident because on two occasions hatchlings were recovered on a later sampling data that were evidently missed in earlier sampling. Pilot studies determined that the washing process generally resulted in very high total recovery, and that hatchling recovery was independent of size. For all the above reasons actual survivorship was probably somewhat higher than recovery rates seen in these experiments. However, there is no evidence to suggest that either crawling, dislodgment/reattachment or limitations of the panel-washing process would act on recovery in a size-dependent manner.

Experiment 4: Size-dependent hatchling growth and survivorship under contrasting field conditions

To determine whether local habitat conditions affected size-dependent offspring performance of *Nucella*, newly-collected hatchlings were outplanted to two sides of a surge channel whose sides differed considerably in degree of sun exposure. Clutches were collected at GP, and hatchlings were removed from their capsules and sorted into size classes as described above. Fifteen randomly-chosen large and 15 small hatchlings from a total of 14 clutches were then marked with Calcein for 12 hours (Chapter IV) and measured for total shell length. The final experimental group consisted of 14 clutches, each clutch containing 15 L and 15 S individuals. This experiment was conducted two times in summer 1997.

The experimental outplant was performed in an intertidal surge channel at Coos Head, Oregon, immediately inside the mouth of Coos Bay. This site experiences considerable wave action in both winter and summer, and summer temperature and salinity conditions are generally close to oceanic (OIMB dock daily records). The surge channel was approximately 1.8 m wide, 2 m deep and 4 m long, and the two long, parallel sides faced ENE and WSW, respectively. The open end of the surge channel faced NNW and the SSE end backed on a ~20 m cliff. Both sides of the surge channel were sandstone and the WSW side (facing ENE) had noticeably lower cover of barnacles, algae and mussels than the ENE, although both sides had populations of *N. emarginata* adults and egg capsules. Preliminary observations suggested that because of shading

from the cliff to the SSE of the surge channel, the WSW side received considerably more sun exposure than the ENE side.

Each of the 14 marked clutches was randomly assigned to one 13 x 13 cm seasoned Astroturf™ panel (similar to those described above), and hatchlings were transferred to the panels with a Pasteur pipet and gently washed with sea water until all hatchlings had attached to the panel. Panels had been previously seasoned in the field for ca. 1 year, such that each panel contained a mature and diverse cover of barnacles, mussels, algae, anemones, etc. among the Astroturf™ tufts. After all hatchlings had attached, panels were randomly assigned to one of two groups of seven (Sun or Shade). After the rock had been locally cleared of barnacles and mussels, the 7 ENE (Shade) and 7 WSW (Sun) panels were bolted to the sandstone at the same tidal height (approx. 2 M above the 0 tide level) on opposite sides of the surge channel, directly across from each other (approx. 1.8 M apart). Panels on each side were approximately 5 – 10 cm apart. Each panel was then surrounded by Tanglefoot™ (see above) to keep hatchlings from wandering off. Tanglefoot™ barriers were refreshed at 2-day intervals or as necessary.

To record temperature on each side of the surge channels during the experimental outplant, two Optic StowAway™ thermistors (Onset Inc.) were bolted to the substrate at the same height as and between panels on each side of the surge channel, where they recorded the ambient temperature at 5-minute intervals for the duration of the experiment. To determine whether temperatures recorded by the thermistors were equivalent to actual panel temperatures and whether temperature varied over the surface of the panels, temperatures were taken at three positions on each panel using a VWR

thermocouple temperature probe on four consecutive days during the 2nd experimental outplant. The temperatures recorded with the probe were then compared to the Optic StowAway™ readings for the same time and date.

Experimental panels and hatchlings remained in the field for a total of 27d and were sampled at 3d, 9d, and 27d for the first 2-site outplant and at 9d and 27d for the second. Panels were sampled in the laboratory and hatchlings were measured for growth and survivorship as described in Experiment 3.

ANALYSES

All data used in analyses were first tested for fit to the assumptions of normal distribution with a one-sample Kolmogorov-Smirnov test with Lilliefors option (Systat, 1996) (paired tests and multiple-sample tests) and Cochran's test for homogeneity of variance (Winer, 1971) (ANOVA). If data did not meet the assumptions of equal variance and normal distribution, the appropriate nonparametric test was utilized and is described below.

Organic Content-The relationship between hatchling length and hatchling organic content was estimated with a linear, ordinary least-squares (OLS) regression of organic content in μg on shell length using the program SigmaPlot 3.0 for Windows (Jandel Scientific). Because of the probable curvilinear relationship between length and organic content, Y data (organic content) were log-transformed prior to analysis (Zar, 1984). Although a reduced major axis (RMA) regression is generally more appropriate than OLS regression for estimating the relationship between two sets of measurements that contain

error (McArdle, 1987), data for these experiments were analyzed with OLS because I was interested in the predictive power of the relationship and I know of no method for calculating prediction intervals (see below) around an RMA regression. In addition, given the high r values in this study (see Table 1) the difference between slopes calculated by OLS and RMA regression analyses would in most cases be small (Seim and Sather, 1983). To estimate the value of hatching length as a predictor of organic content, 95% prediction intervals were calculated around each regression using the formula from Zar, 1984 (page 276), which calculates the 95% confidence intervals around a single value of Y at a given X value. If the prediction intervals did not overlap for the entire length of the regression, hatching size was considered to have predictive value for that clutch (McEdward and Carson, 1987).

Laboratory experiments - To determine the effect of hatching size on growth under starved conditions, a correlation analysis was performed of 2-week growth (measured as the distance from the PT boundary to the new apertural edge along the 2nd rib, as above) and initial hatching length of hatchlings from 2 clutches. To determine size-dependent survivorship of starved snails, I first tested for an effect of tray. Because there was no significant interaction between tray and survival (Pearson Chi-square = 1.808, $p = 0.771$), tray was discarded as a variable. The hatching length of hatchlings that were alive at 3 ½ months were then compared to the hatching length of snails that did not survive this interval of starvation with a 2-sample Student's t-test.

Growth of fed laboratory hatchlings was analyzed at d 65 with a two-way model III ANOVA using initial size (L or S) as a fixed factor, box-clutch (1 – 6) as a random

factor, and shell length at d 65 as the dependent variable. Shell length was used as the dependent variable rather than total growth because fed laboratory-reared snails rapidly overgrew the PT boundary, making accurate, nondestructive measurements of hatching length difficult. In addition, these snails grew so much during the experimental interval that initial hatching length was a relatively low percentage of total length. The percent survivorship of large and small fed hatchlings in each box was arcsin transformed to normalize the distribution of percentage data (Zar, 1987) and compared among clutch/boxes with a paired Student's t-test.

Field outplants - Percent recovery on the final day of each single-site field outplant was arcsin transformed and compared among large and small hatchlings using a paired Student's t-test, except for the 12-clutch, 54 d outplant: due to the low recovery at d 54, recovery data for this experiment were compared on d 36 and growth was compared on d 27. Growth of large and small hatchlings was compared using a two-factor, model III ANOVA with initial hatching size (L or S) as a fixed factor, panel-clutch as a random factor, and growth as the dependent variable.

In both two-site field experiments percent recovery of large and small hatchlings was paired within plate and therefore nonreplicated within treatment, obviating the use of ANOVA or similar analyses to test for a site-by-size interaction. Therefore, recovery of large and small hatchlings was compared only within each of the two sites. On the Shade side, recovery of large and small hatchlings was compared within panels using a paired Student's t-test. On the Sun side, arcsin - transformed paired differences in recovery did not meet the assumption of normal distribution, so recovery of the two groups were

compared with a Wilcoxin's signed ranks test (Sokal and Rohlf, 1987). Because it is difficult to compare results of data analyses performed with parametric and nonparametric analyses, data for the Sun side were also analyzed with a paired Student's t-test, which is quite robust to departures from normality particularly when tests are two-tailed and sample sizes are equal (Zar, 1984; Underwood, 1997).

RESULTS

Experiment 1: Relationship of hatching size to organic content and maternal investment

All three field-collected clutches from which all hatchlings were measured exhibited a positive and significant relationship between organic content and shell length (Fig. 2a - c; Table 1a-c). For each of the three clutches 95% prediction intervals for the largest and smallest snails were nonoverlapping, indicating that a single snail in the largest size class could be predicted, at the 95% confidence level, to have a higher organic content than a single snail in the smallest size category for these clutches (Fig. 2a - c). There was also a significant and positive relationship between organic content and shell length when data from all three clutches were combined and analyzed with an OLS regression (Table 1d), and 95% prediction intervals indicated that this relationship had predictive value as well (Fig. 2d). Ten of ten subsampled clutches (subsampled from siblings of snails used in the 12-clutch experimental outplant) also exhibited a significant and positive correlation between organic content and shell length (Table 1, e-m).

Figure 2. Log_{10} organic content plotted against shell length for individual hatchlings from three field-collected ripe clutches (a, b, c) and for combined hatchlings from all three clutches (d). Symbols in (d) are consistent with a, b and c, and regression equations are shown in Table 1. Solid lines are least-squares regressions; dotted lines are 95% prediction intervals around a single value of y at a given x value (Zar, 1984). Note that in each case, the 95% prediction intervals for the largest and smallest hatchlings are nonoverlapping, indicating that shell length can be used to predict at the 95% confidence level that individual large and small hatchlings differ in organic content.

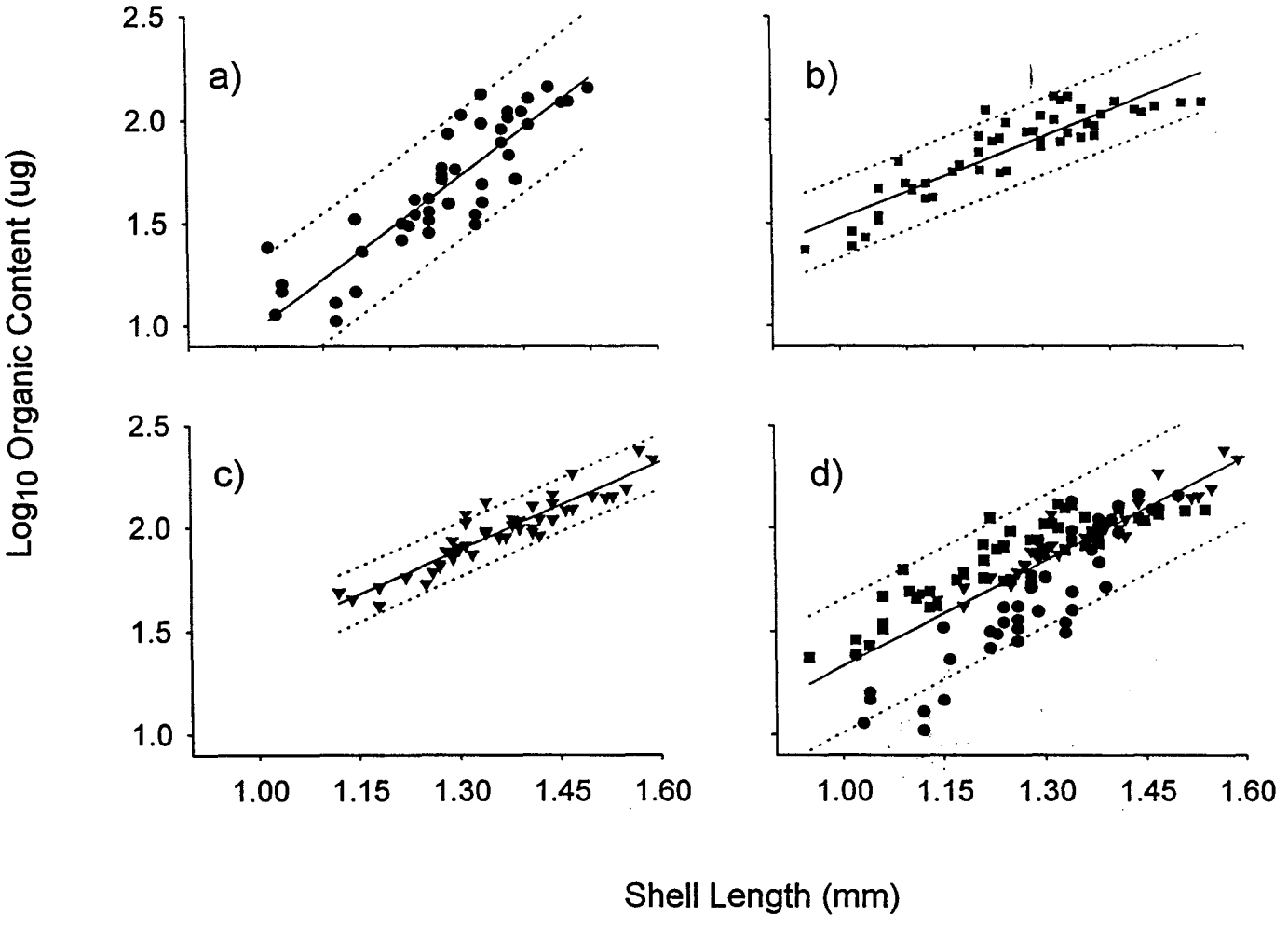

a)
b)
c)
d)
2.5
2.0
1.5
1.0
1.00
1.15
1.30
1.45
1.60
Log10 Organic Content (ug)
Shell Length (mm)

Table 1. Regression equations, squared r and n for the relationship between organic content (μg) and shell length for three complete field-collected clutches (A, B, C), these three clutches combined (D), hatchlings subsampled from ten additional field-collected ripe clutches (E – N) and older juveniles collected in the field.

Clutch	Regression Equation	r^2	n
A	log y = 1.43 x + 0.03	0.88***	45
B	log y = 2.44 x – 1.46	0.80***	47
C	log y = 1.31 x + 0.21	0.80***	51
D (A + B + C)	log y = 1.69 x – 0.39	0.68***	143
E	log y = 1.56 x – 0.11	0.82***	24
F	log y = 0.78 x + 0.83	0.41***	24
G	log y = 1.45 x – 0.07	0.57***	24
H	log y = 1.61 x – 0.08	0.86***	23
I	log y = 1.47 x – 0.01	0.66***	22
J	log y = 1.82 x – 0.42	0.86***	24
K	log y = 1.12 x + 0.37	0.69***	24
L	log y = 1.53 x – 0.18	0.88***	24
M	log y = 0.89 x + 0.63	0.76***	22
N	log y = 1.12 x + 0.43	0.84***	24
Wild juveniles	log y = 0.29x + 1.81	0.96***	28

*** Indicates the correlation coefficient (r) is significant at the $p < 0.001$ level.

Very small field-collected, post-hatching juveniles were similar in organic content to prehatching juveniles in the same size range. However larger (and older) juveniles of greater than 2 mm shell length had relatively less organic content per unit shell than did recently-metamorphosed hatchlings from ripe clutches, and the slope of the regression line of organic content on shell length was significantly different from the slopes of

calculated for hatchlings from ripe clutches (Fig. 3) (ANCOVA, $F = 25.71$, $df = 169$, $p < 0.001$).

Experiment 2: Size-dependent growth and survivorship under both starved and fed conditions in the laboratory

Growth at 2 weeks was positively and significantly correlated with hatching length for both clutches maintained in the laboratory without food (Clutch 1: $r = 0.75$, $df = 55$, $p < 0.001$. Clutch 2: $r = 0.67$, $df = 33$, $p < 0.001$) (Fig. 4). The hatching size of snails that survived to 3 ½ months under starved conditions (total survivorship at 3 months = 48.3%) in the laboratory was significantly greater than the hatching size of snails that did not survive to this age (Student's two-sample t-test, pooled variance $t = 4.61$, $df = 58$, $p < 0.001$). Of six clutches of snails kept in the laboratory and fed *ad libitum*, survivorship of large snails was significantly greater than survivorship of small snails on d 65 (Student's paired t-test, $t = 3.26$, $p = 0.022$). When the growth of the two size classes was compared, snails from the initially large group were significantly larger on d 65 than snails from the small group (Fig. 5, Table 2). There was also a significant effect of box-clutch and a significant interaction between size class and box-clutch on d 65 (Table 2), indicating that (1) clutch-box significantly affected growth, and (2) the effect of initial size on growth varied significantly among box-clutches.

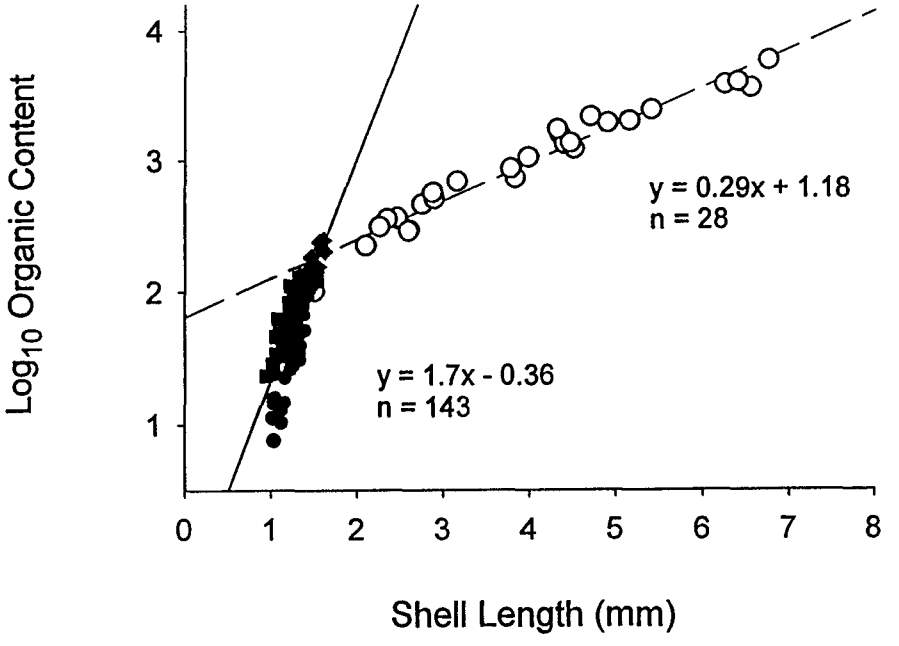


Figure 3. Log_{10} organic content plotted against shell length for individual, post-hatching juvenile Nucella emarginata of a range of sizes (1.5 – 7mm) collected in the field (open circles; dashed line). For comparison, all data from individual hatchlings from 3 field-collected ripe clutches are also plotted (closed symbols; solid line). Lines are least-squares regressions.

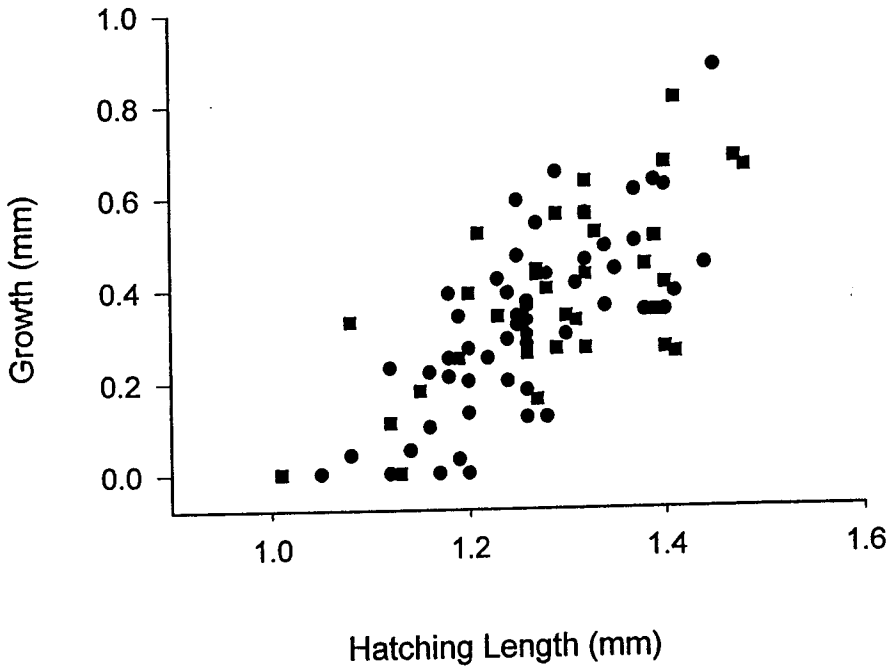


Figure 4. 2-week growth (measured as amount of shell added from the PT boundary to the new, growing aperture) plotted against original hatchling length for two clutches of starved, laboratory-reared *N. emarginata* clutches. Squares = clutch 1 (n = 36), circles = clutch 2 (n = 56).

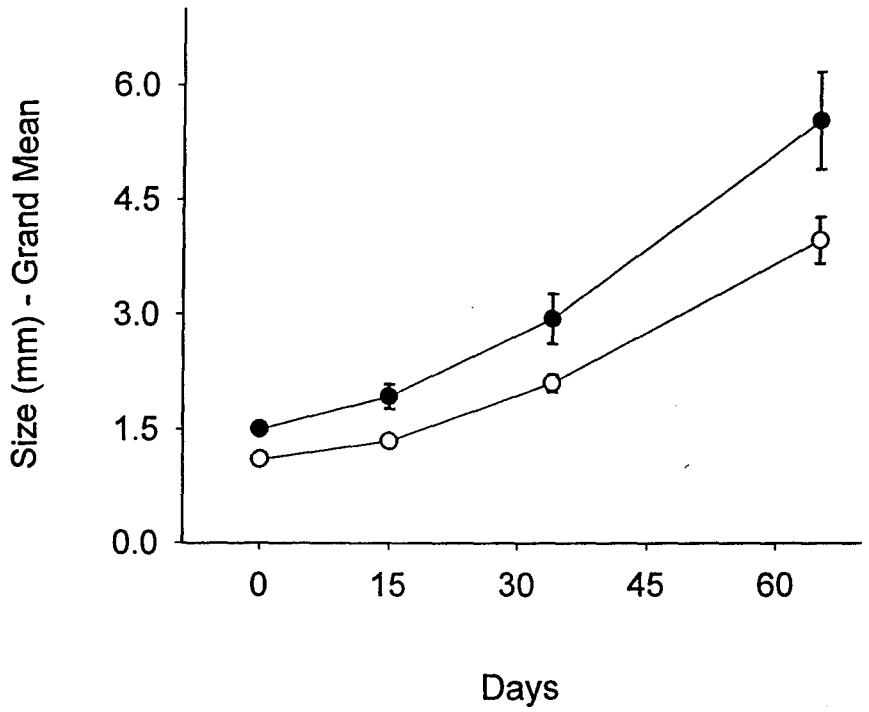


Figure 5. Size of large and small laboratory N. emarginata hatchlings fed ad libitum in the laboratory over 65 days. Snails were measured at d 0, 15, 34 and 65. Closed circles represent the grand mean of large snails from 6 clutches, and open circles represent grand means of small snails from the same 6 clutches. Error bars are 95% confidence intervals.

Table 2. Model III ANOVA results for the effects of hatching size and box-clutch affinity on size when laboratory-reared hatchlings were fed *ad libitum*.

Source	df	SS	F	*P*
Clutch	5	93.519	33.861	< 0.001
Size Class	1	42.180	29.120	0.003
Clutch x Size Class	5	7.242	2.622	0.029
Error	89	49.161		

Experiment 3: Size-dependent survivorship and growth in the field

The effect of hatching size on recovery varied among five experimental outplants. Large and small hatchlings did not exhibit significantly different recovery in either of the two 9-day outplants (Outplants A and B) (paired Student's t-test, A: $t = 1.32$, $p = 0.26$, B: $t = 1.48$, $p = 0.21$) (Fig. 6). In the first of two 36-day outplants (Outplant C), large hatchlings exhibited significantly higher recovery than small hatchlings on the last day of the experiment (paired Student's t-test, $p = 0.002$) (Fig. 7a). In the second 36-d outplant (Outplant D), recovery of large and small hatchlings did not differ significantly (paired Student's t-test, $p = 0.16$) (Fig. 7b). In the 54-day outplant (Outplant E), large hatchlings exhibited significantly higher recovery than small hatchlings on d 36 of the experiment (paired Student's t-test, $p < 0.0001$) (Fig. 7c).

Hatching size positively and significantly affected growth in all experimental outplants as tested on d 9 of experiments A and B, d 18 of outplants C and D and d 27 of

Figure 6. Percent recovery of large (closed bars) and small (open bars) hatchlings over two 9 d experimental outplants, each comprising 5 clutches. a = Outplant A; b = Outplant B. Neither experiment found significant differences in recovery between large and small hatchlings.

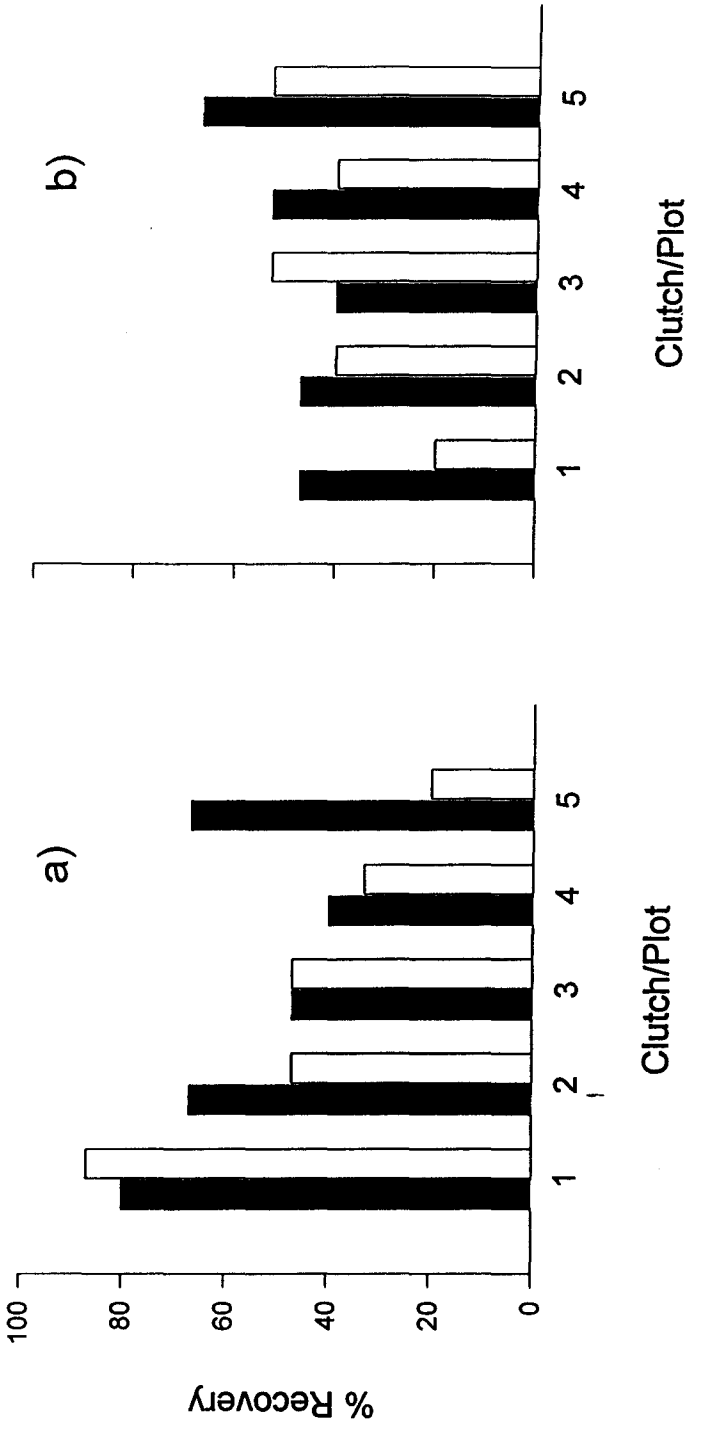
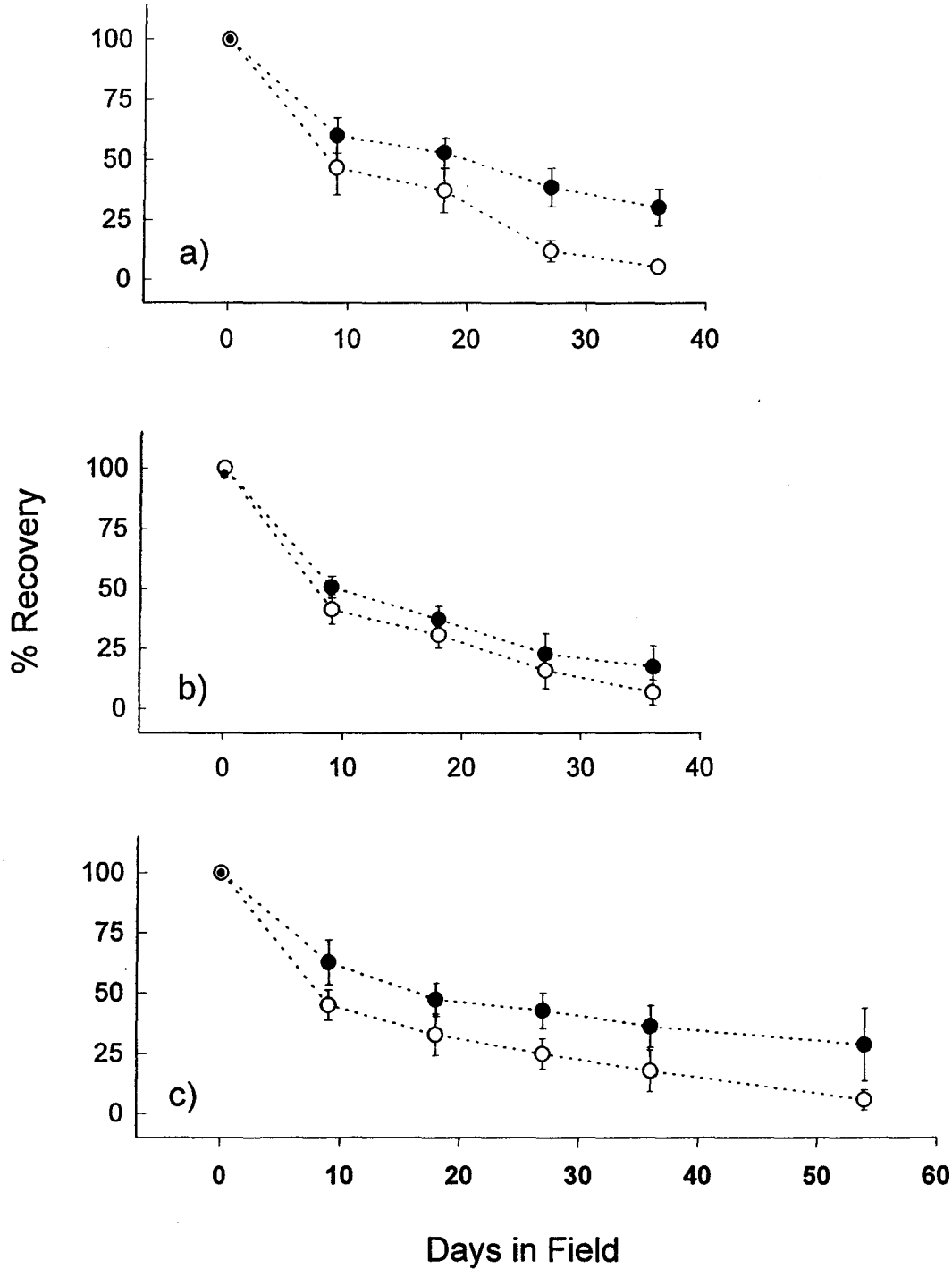

a)
b)
% Recovery
0
20
40
60
80
100
1
2
3
4
5
Clutch/Plot

Figure 7. Mean percent recovery of large (closed circles) and small (open circles) hatchlings over time in three field outplants. a = Outplant C (5 clutches, spring 1996, 36 days duration); b = Outplant D (5 clutches, summer 1996, 36 days duration); c = Outplant E (12 clutches, fall 1996, 54 days duration). Error bars are standard errors.


100
75
50
25
0
a)
0
10
20
30
40
% Recovery
b)
c)
60
Days in Field

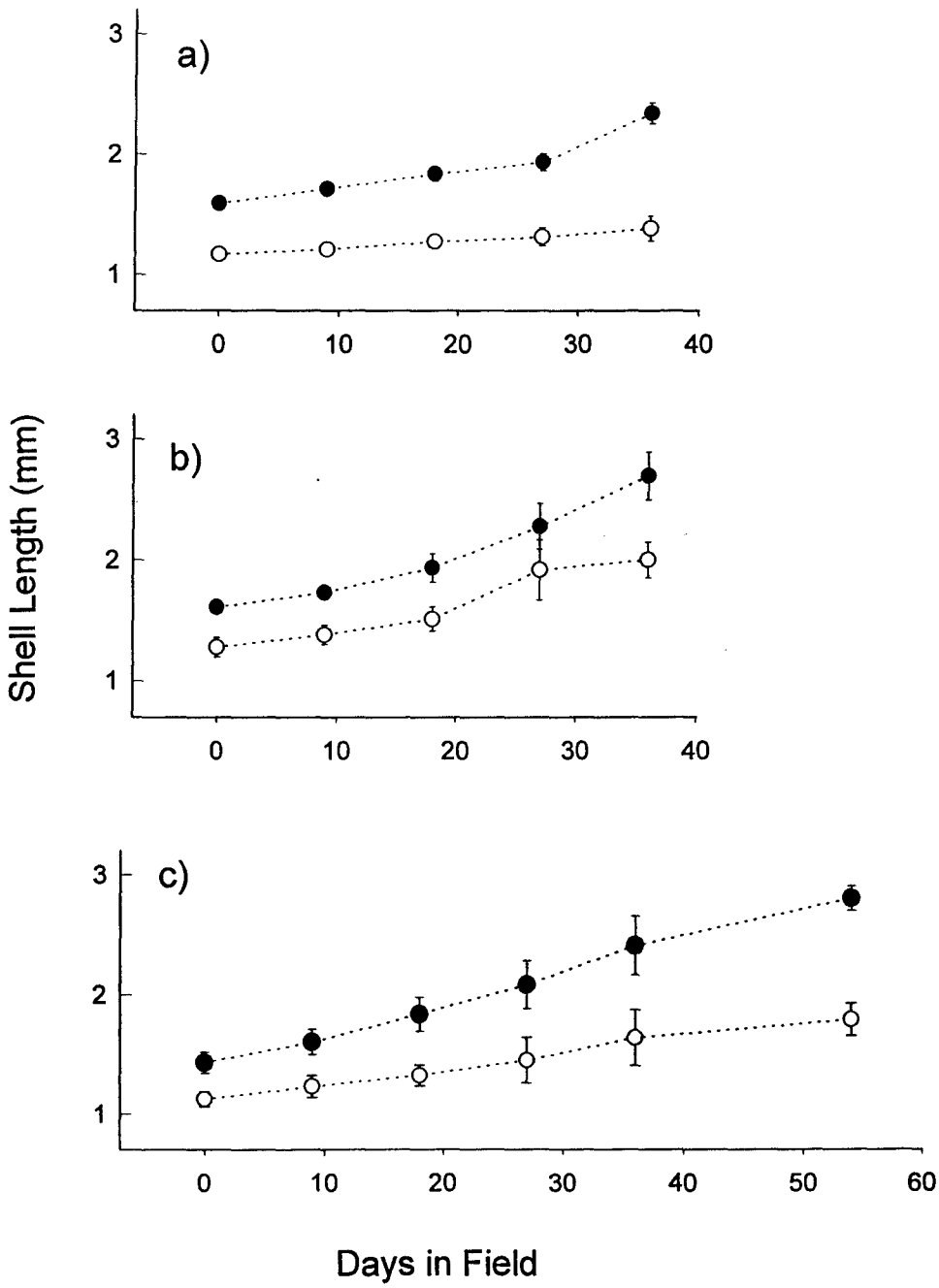
outplant E. The mean of panel-clutch mean size at each sampling day in experiments C, D and E are shown in Fig. 8; results of model III ANOVAs (fixed factor = size, random factor = panel-clutch) for each panel are presented together for convenience in Table 3. Panel-clutch significantly affected growth in experiments D and E, and there was a

Table 3. Results of model III ANOVAs for large and small hatchlings' growth during five experimental field outplants. *P* values were calculated independently for each experiment.

Experiment	Source	df	SS	*F*	*P*
Outplant A (9 days)	Panel-Clutch	4	1.57[a]	0.854	0.496
	Size	1	10.62[a]	7.967	0.048
	PC X SC	4	5.33[a]	1.030	0.029
	Error	68	30.41[a]		
Outplant B (9 days)	Panel-Clutch	4	2.77[a]	1.159	0.337
	Size	1	4.79[a]	7.787	0.049
	PC X SC	4	2.46[a]	1.030	0.398
	Error	66	40.61[a]		
Outplant C (on d 18)	Panel-Clutch	4	0.826	1.098	0.366
	Size Class	1	9.242	85.075	0.001
	PC x SC	4	0.435	0.578	0.680
	Error	59	11.094		
Outplant D (on d 18)	Panel-Clutch	4	0.866	14.436	0.000
	Size Class	1	0.433	51.131	0.002
	PC x SC	4	0.034	0.564	0.690
	Error	41	0.615		
Outplant E (on d 27)	Panel-Clutch	11	0.965	1.906	0.047
	Size Class	1	2.470	25.808	0.000
	PC x SC	11	1.053	2.081	0.029
	Error	98	4.509		

[a] Actual values divided by 10^5 for convenience

Figure 8. Grand mean of sizes of large (closed circles) and small (open circles) *Nucella emarginata* hatchlings over time in three field outplants. a = Outplant C (5 clutches, spring 1996, 36 days duration); b = Outplant D (5 clutches, summer 1996, 36 days duration); c = Outplant E (12 clutches, fall 1996, 54 days duration). Error bars are standard errors.

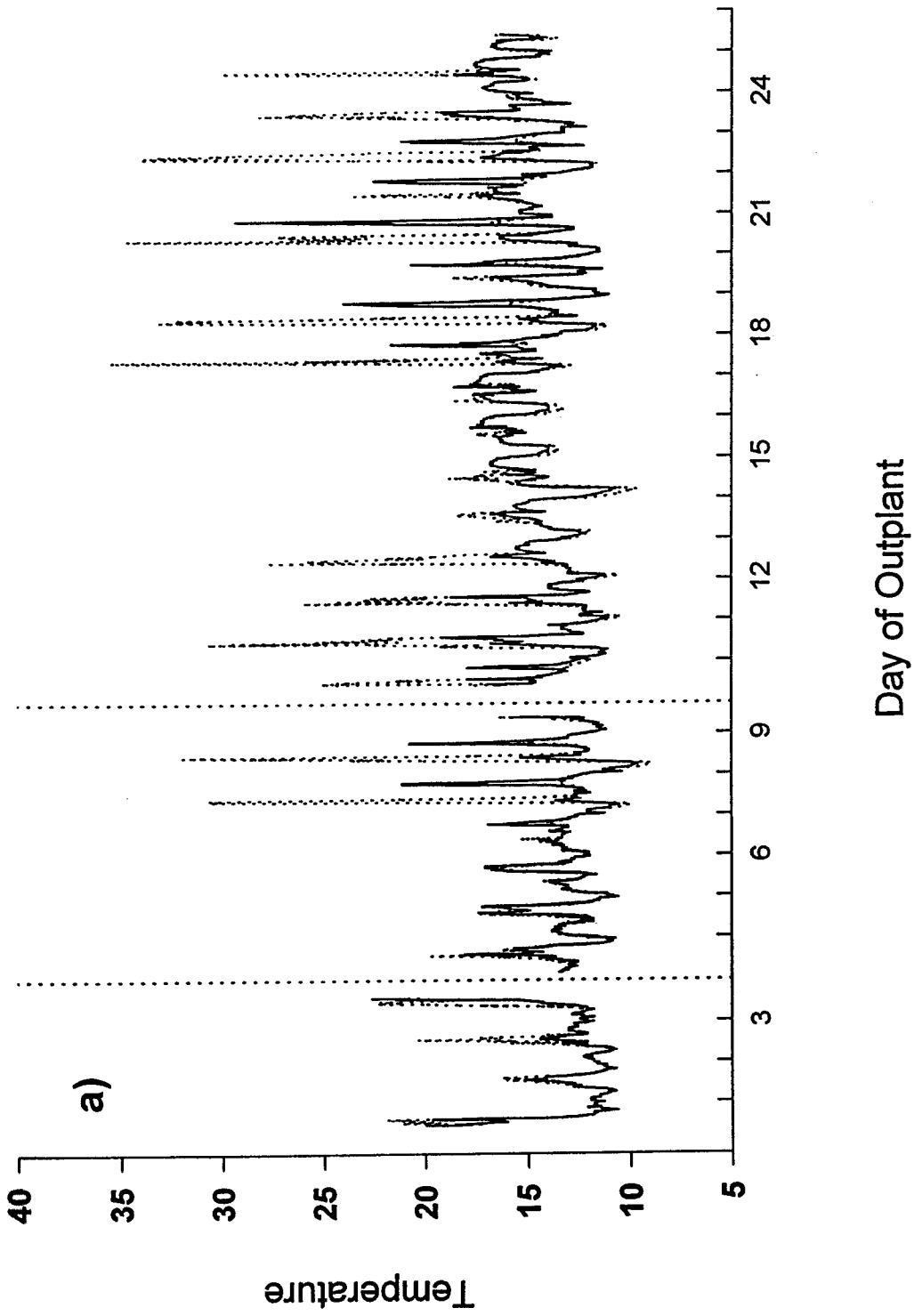

a)
b)
c)
Shell Length (mm)
Days in Field
1
2
3
0
10
20
30
40
50
60

significant interaction between panel-clutch and size class in experiment E (indicating that the effect of size class on growth varied among clutches in this experiment) (Table 3). Growth data in experiment E violated the assumption of homogeneity of variances (Cochran C statistic = 0.1539, p = 0.019), and data transformations were not successful at removing variance heterogeneity. The p value of the size effect was considerably lower than that of the Cochran C statistic (0.019; Table 3) and is therefore the effect of size on growth is robust (Underwood, 1981). However, because the p value for the interaction term in experiment E was > 0.019 (Table 3), this result may be subject to Type I error and the significance of the interaction term should be viewed with caution.

Experiment 4: Size-dependent survivorship under contrasting field conditions

Temperature data recorded by the Optic StowAways™ showed that the Sun side (WSW) reached considerably higher temperatures than the Shade side (ENE) in both experiments, and the greatest differences between sides occurred during AM low tides (Fig. 9a, b). The mean of 3 temperatures recorded manually by the thermocouple were generally very similar within and among panels (1 degree or less variation), and were generally very close to the Optic StowAway™ values recorded at the same time. The one exception was that within-panel temperature varied by as much as 6°C when panels

Figure 9. Temperature as recorded during two outplants to an intertidal surge channel. a = Experiment 1; b = Experiment 2. Each graph shows temperatures measured every five minutes by a StowAway datalogger on the Sun (WSW) (dotted lines) and Shade (ENE) (solid lines) sides. Tick marks on the X axis are situated at 12:00 noon on each day; note that on the Sun side, the highest temperatures occurred during morning low tides. Vertical dotted lines indicate breaks in the data when panels and dataloggers were brought into the lab for sampling.


a)
Temperature
40
35
30
25
20
15
10
5
3
6
9
12
15
18
21
24
Day of Outplant

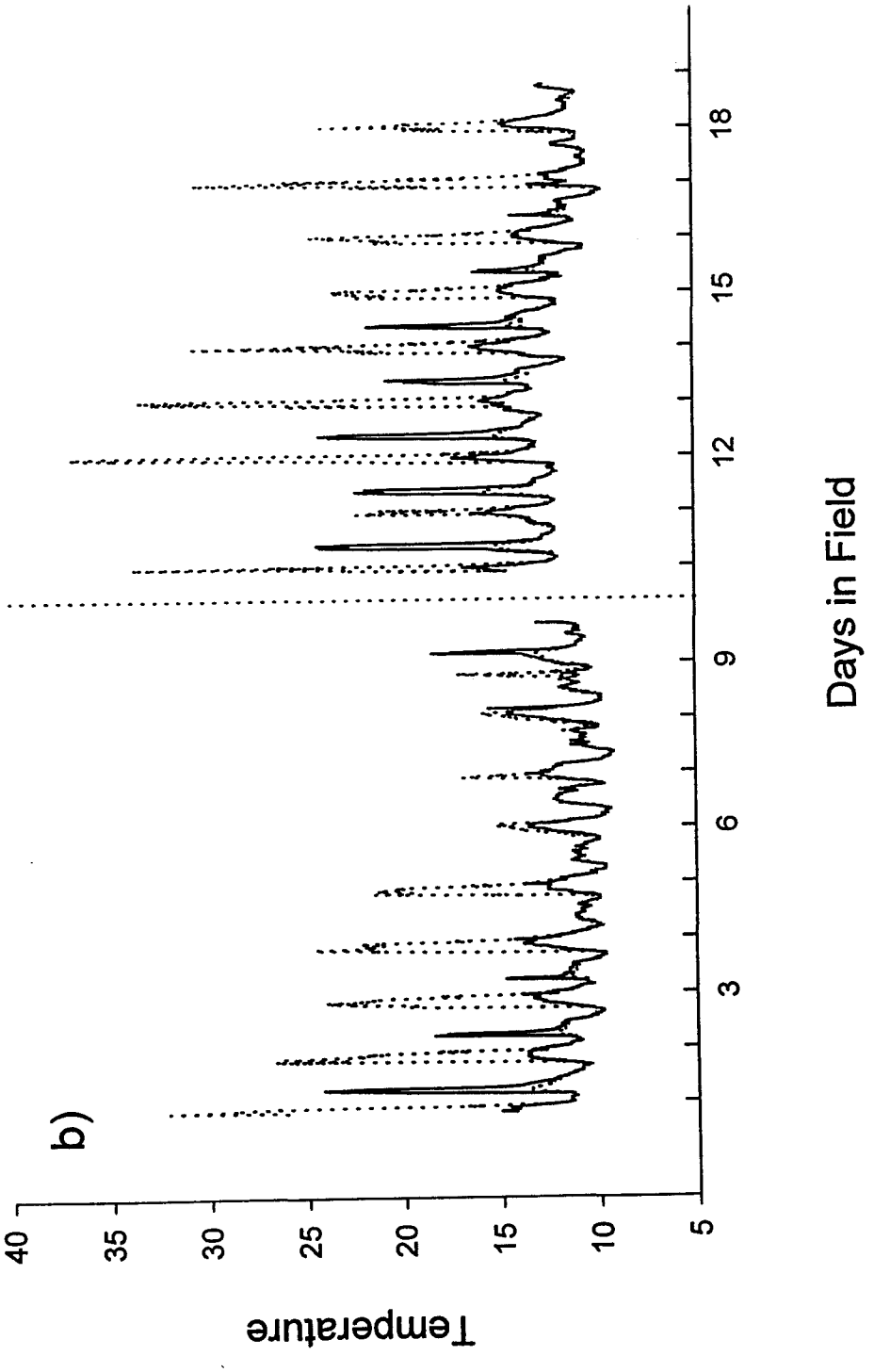


Figure 11. (continued)

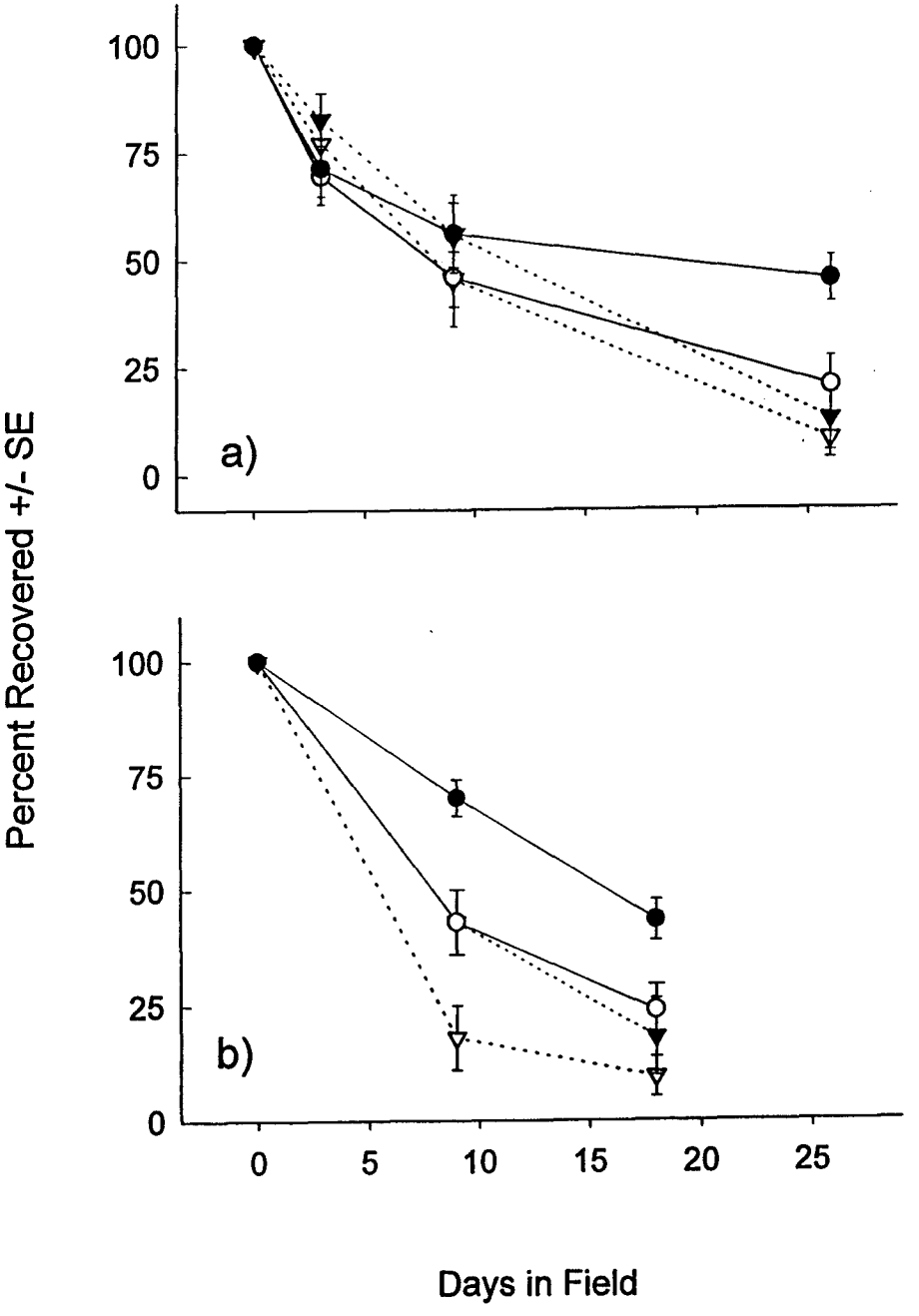
were in the sun, and mean panel temperatures were generally 1-2 degrees cooler than Optic StowAway™ temperatures when temperatures were recorded in full sunlight. In both 2-site outplants, overall per-panel recovery at d 27 (Exp. 1) and d 18 (Exp. 2) (the final day of each experiment) was significantly higher on the Shade side than on the Sun side (Exp. 1: Kruskal-Wallis test, Mann-Whitney U test statistic = 139.00, $p = 0.004$. Exp. 2: Student's two-sample t-test, $t = 3.60$, $p = 0.01$). Large hatchlings exhibited significantly higher recovery than small hatchlings on the Shade side (Exp. 1: paired Student's t-test, $t = 3.77$, $p = 0.013$. Exp. 2: $t = 2.58$, $p = 0.042$), but not on the Sun side (Exp. 1: Wilcoxin's signed ranks test, $p = 0.36$, paired Student's t-test, $t = 1.00$, $p = 0.36$. Exp. 2: paired Student's t-test, $t = 0.94$, $p = 3.38$) (Fig.10a, b).

DISCUSSION

Size and Organic Content

A primary assumption underlying many life-history models is that offspring size reflects offspring organic content and maternal investment per offspring (McEdward and Carson, 1987). In gastropods, shell length is a convenient, simple and non-destructive measure of size. Shell length is tightly correlated with organic content in offspring of *Nucella emarginata*, as demonstrated by significant and predictive relationships between hatching length and organic content both within and among clutches (Fig. 2). The strength of this relationship is much greater than that reported for egg volume and egg organic content in several species of starfish (McEdward and Carson, 1987; McEdward

Figure 10. Percent recovery of large (closed symbols) and small (open symbols) *Nucella emarginata* hatchlings on two sides of an intertidal surge channel, for two separate experiments. a = Experiment 1: b = Experiment 2. Hatchlings from the Sun side are represented by triangles and dotted lines; hatchlings from the Shade side are represented by circles and solid lines. Error bars are standard errors.


Percent Recovered +/- SE
100
75
50
25
0
a)
100
75
50
25
0
b)
0
5
10
15
20
25
Days in Field

and Coulter, 1987; r^2 between 0.02 and 0.39) and a marine fish (Niciu and McEdward, 1994). Therefore, while offspring organic or energetic content clearly cannot be assumed *a priori* to scale with offspring size in every taxon, the strength of the relationship between these two variables in *N. emarginata* confirms that this species can be used as a model system to test the effect of offspring size on offspring performance.

The variations in slopes of the regression line between organic content and shell length among clutches (Table 1, Fig. 2) might be caused by several factors. First, the scaling of organic content and shell length might differ among clutches if hatching size were not entirely dependent on the number of nurse eggs consumed by a given hatchling. However, because hatching size is very closely tied to nurse egg ration in *N. emarginata* and several other gastropod taxa (Rivest, 1983; Fioroni, 1988), this is an unlikely cause of variation in slopes among clutches. A second possibility is that different slopes may represent natural within-population variation in scaling of size and organic composition among clutches. For example, if nurse eggs varied in quality within some clutches, large offspring might be more organically dense than small offspring if large embryos in these clutches selectively consumed high-quality nurse eggs. While somewhat elaborate, this possibility cannot be ruled out because very little information is available on either variation in nurse egg quality or intracapsular feeding behavior in *Nucella* or other gastropods.

A third explanation for interclutch variation in slopes between size and organic content is that embryonic shell growth allometry may vary among clutches, thereby altering the scaling of size (= shell length) and organic content without corresponding

changes in organic density. There were noticeable differences in coiling allometry in different clutches, in that snails in some clutches were qualitatively more rotund than others (shell coiling allometry was not quantified). Qualitative differences were also noticed within clutches (pers. obs.), and may have accounted for some of the unexplained variance in the relationship between organic content and length within clutches. The dramatic shift in scaling of organic content and length observed after hatching and concurrent with the shift from embryonic to adult shell growth patterns (see Fig. 3) also supports the hypothesis that interclutch variation in embryonic shell coiling allometry may account for differences in the scaling of hatchling organic content and shell length, rather than differences in organic density.

If offspring performance is affected by organic content rather than by size *per se*, and the relationship between size and organic content varies between clutches or populations, then results of experiments testing the relationship between offspring size and performance may vary depending on the clutches or populations examined. It is unlikely that the outcome of these experiments with *Nucella* was greatly affected because a) shell length was a very good predictor of organic content in all clutches and b) size-dependent performance was in all cases compared *within clutch.* However, the degree of intrapopulation variation among clutches in this study suggests that (1) if the degree of intra- or interclutch variation in scaling of size and organic content is very large, as seen in several marine organisms (e.g. McEdward and Coulter, 1988; George, 1994; McEdward and Niciu, 1994), and (2) if the population or clutch affinity of experimental

offspring is not controlled, then tests of the relationship between offspring size and performance should be interpreted with caution.

Growth and Survivorship of Lab-reared Hatchlings

Shell growth is an energy-requiring process (Geller, 1990; Palmer, 1992), and the significant correlation between hatchling length and growth of starved, laboratory-maintained snails suggests that snails that are large at hatching have greater nutrient resources to apply towards growth than small hatchlings. Greater growth under starved conditions might provide a survival advantage to large hatchlings because larger shells provide greater predator resistance to certain predators (Spight, 1976; Rivest, 1983; Gosselin, 1994).

Large hatching size conferred a survival advantage to *Nucella emarginata* hatchlings under starved conditions, in conformity with patterns of size-dependent starvation resistance in numerous other taxa (e.g. Tessier and Consolatti, 1989). Size-dependent starvation resistance is expected, because of the inverse relationship between mass and mass-specific metabolic rate (Peters, 1983) and because large hatching size is correlated with higher nutrient reserves. In the field, large hatching size might be advantageous to *N. emarginata* hatchlings if they were washed away from food-bearing substrate by wave action, or if time of hatching corresponded with periods of low prey recruitment or local prey extinction. While approximately ½ of the hatchlings in this experiment were capable of surviving 3 ½ months without food in the laboratory, under field conditions starvation may act on shorter time scales by reducing hatchlings'

resistance to environmental stresses or by limiting hatchlings' ability to feed if food becomes available.

Hatching size also strongly affected both growth and survivorship in the laboratory when hatchlings were fed *ad libitum*. Because hatchlings were offered rocks bearing a natural assemblage of prey items, which ranged from small and newly-settled to fully-grown barnacles and mussels, differential growth rates of large and small hatchlings may have been due to size-dependent feeding success. Palmer (1990) found no relationship between the size of a hatchling and the size of prey it would attack; however, attacks by small hatchlings on large prey items were often unsuccessful, suggesting that hatching-size dependent differences in growth rates may be due to less efficient feeding by small hatchlings. It is also possible that large hatchlings could competitively exclude small hatchlings from food resources, but because hatchlings were fed *ad libitum* and reports of interference competition are rare in gastropods, this scenario is unlikely.

One consequence of small offspring size may be that smaller individuals spend more time as juveniles before reaching sexual maturity (Stearns, 1992). Although *N. emarginata* hatchlings were not followed through to adulthood, small hatchlings grow more slowly and are likely to reach reproductive size more slowly (or mature at a smaller size). In the experiments performed in this study small hatchlings did not appear to exhibit compensatory higher growth rates to catch up with larger siblings as has been demonstrated in other taxa (Ruohomäki *et al.* 1993), nor did small hatchlings appear to exhibit lower relative growth rates (relative growth rate was not calculated because the

relationship between shell length and mass of growing *N. emarginata* was not known for each clutch). Therefore, greater growth of large hatchlings may have been caused by a corollary of size (such as size-dependent attack success) rather than to differences in quality between large and small hatchlings (such as developmental abnormalities or compositional differences). Small hatchlings are likely slowed from reaching reproductive maturity by approximately the amount of time required to grow to the initial hatching size of larger siblings. In the laboratory, small hatchlings reached the initial hatching size of large hatchlings in approximately 20 days, while this point was reached much more slowly in the field. Because the juvenile period is probably more vulnerable to environmental stresses than the adult stage (Vermeij, 1987), small hatching size might have negative impacts on future survival and reproduction that would occur beyond the time range of these field studies.

Large hatchlings exhibited higher survivorship than small hatchlings in the laboratory, but the underlying cause of this difference is not clear. Hatchlings in laboratory cages were not exposed to predation, desiccation, temperature or salinity stresses, which may in part explain the overall higher survivorship in the laboratory compared to the field. Intraclutch cannibalism was not a source of mortality, because empty shells of dead snails did not bear drill holes characteristic of *Nucella* predation. One likely cause of mortality in the laboratory was anoxia (and/or the resultant buildup of hydrogen sulfide), which was occasionally seen in localized areas of laboratory cages. Other possible causes of mortality in the laboratory include disease, parasites, or a possible negative effect of constant submergence, any of which might have a size-

dependent effect on mortality. Higher laboratory mortality of small hatchlings suggests that small hatchlings may be more susceptible overall; however, because laboratory sources of mortality are not known, it is unclear whether patterns of size-dependent mortality in the laboratory are relevant to interpretations of field mortality patterns.

Survivorship and Growth in the Field

In field outplants of *Nucella emarginata*, hatching size influenced both hatchling growth and survivorship. Large hatchlings exhibited higher growth and larger final size than small hatchlings in all experimental outplants, probably because of greater feeding efficiency of larger hatchlings (see above). Overall growth differences between large and small hatchlings suggest that under natural conditions small hatchlings are set back one month or more relative to large hatchlings (Fig. 8), a considerable fraction of the 1-2 year lifespan of *Nucella emarginata* in the field (Spight, 1975). Therefore, different growth rates of large and small *N. emarginata* offspring may have important consequences for time to sexual maturity and may cause hatchlings from the same clutch to reach reproductive maturity at different times (Tessier and Consolatti, 1989), which in turn may increase survivorship of large hatchlings by reducing time spent in the smallest and potentially more vulnerable size classes (Vermeij, 1978, 1987).

Field experiments also indicated that panel-clutch significantly affected growth in two out of five experimental outplants (Outplants D and E), and there were also significant panel-clutch x size interaction in two of five outplants (Outplants A and E) (the significance of both effects in Outplant E should be viewed with caution, see

Methods). Potential sources of variation in the panel-clutch factor and the interaction term within experiments include effects of clutch identity, panel identity, or panel location. Panel quality and location are an unlikely cause of variation because all panels were seasoned equivalently and there were no obvious differences in biota, orientation or exposure of panel sites. In addition, Outplants A and B used the same the same panels and panel positions (but different clutches, and at different times) yet exhibited no consistent differences in mortality or growth on particular panel/positions between the two experiments. While panel identity, location and stochastic events cannot be ruled out at present as sources of variation in growth on *N. emarginata* hatchlings, clutch identity is likely an important factor in determining both hatchling growth and the relationship between hatchling size and growth. Interclutch differences in performance and size-dependent performance in the field might be due to maternal effects, genetic differences, and environmental differences during early development among clutches.

Single-site field outplants performed in this study strongly suggest that hatching size influences survival of *N. emarginata* hatchlings, consistent with the relationship between offspring size and survivorship found in many other taxa (Stearns, 1992). However, the advantage of large hatching size in *Nucella* also varied temporally. For example, hatching size did not significantly affect recovery in two short (9 d) experiments (Outplants A and B) and one long-term outplant (Outplant D), while a significant effect of hatching size was seen in two out of three longer-term experiments performed at the same site (Outplants C and E) and in Shade treatments of both 2-site outplants. This suggests that environmental factors that favor large hatching size, such as

heat/desiccation stress, predation, or wave action, were not always present. The intertidal environment exhibits a great deal of temporal variation in each of the above parameters (Nybakken, 1996); consequently, variability in the effect of hatching size on growth and recovery in these experiments may have been due in part to the variable nature of the intertidal habitat.

Survivorship as an Index of Habitat Severity

Small-scale spatial variation (centimetres to metres) variation in microhabitat quality is well-documented in the rocky intertidal (Underwood and Chapman, 1996). In replicated tests of size-dependent performance in two contrasting habitats in this study, hatchling recovery was consistently different in two adjacent and contrasting habitats: one habitat (Sun) exhibited significantly lower recovery rates than the other habitat (Shade). These differences in recovery strongly suggest that the Sun side was a quantitatively harsher environment for *Nucella* hatchlings than the Shade side during the timeframe of these experiments. Heat/desiccation stress was probably the environmental variable that varied the most between the two sides and may have caused differences in recovery for a number of reasons. First, the two sides were very similar in many ways; they were initially chosen because of their similarity in wave exposure, height, and angle of orientation. Likewise, because panels on the two sides were in close proximity (< 2 m apart) and were physically connected, hatchlings on the two sides were probably exposed to the same population of potential predators.

Second, when temperature differences between the two sides were minimal, for example in the first 3 d interval of the first 2-site outplant, differences in mortality between the two sides were minimal as well. Third, of three long-term (> 9 d) experimental outplants (Outplants C, D and E) carried out during spring, summer and fall, recovery was lowest in the summer. This may be due to greater heat/desiccation in the summer than fall or spring (however, temperatures were not measured near panels in Outplants C, D or E). Other experiments indicate that heat/desiccation stress is one of the most important parameters shaping community structure in the intertidal (Raffaelli and Hawkins, 1996), and is probably one of the most important causes of mortality for *Nucella* hatchlings in many habitats (Spight, 1976; Etter, 1989; Gosselin, 1994). Therefore, it is likely that measured differences in temperature, caused by differences in sun exposure, were the cause of the quantitatively greater harshness of the Sun side and the resultant differences in recovery of *Nucella* hatchlings over a small spatial scale.

Size-dependent Survivorship in Habitats that Vary in Severity

Habitats have been categorized based on the relationship between offspring size and fitness as either offspring size-sensitive (OSS) or offspring size-insensitive (OSI) (Begon, 1985; Begon *et al.*, 1996). In OSS habitats, size affects individual offspring fitness because either a) the habitat contains sources of mortality to which small offspring are particularly vulnerable, or b) competitive interactions among offspring favor large size. In OSI habitats, in contrast, fitness of individual offspring is not related to size because a) resources are superabundant and all offspring do well, b) some environmental

factors favor small offspring size, or c) mortality is random with respect to offspring size (Begon, 1985; Begon *et al.*, 1995). The return on different offspring size/number maternal investment strategies will vary depending on the type of habitat (OSS or OSI), which may affect many aspects of life-history evolution.

Several models of life-history evolution contain the assumption that "harsh" environments will fit the OSS habitat type, in that fitness of parents producing large offspring will be higher under conditions of physiological or competitive stress (e.g. Kaplan and Cooper, 1984) or larger offspring will do better in all environments, but this difference will be more pronounced in severe environments (e.g. McGinley *et al.*, 1987; Fig. 3C). Under more "benign" conditions, the effects of offspring size on performance will not be as pronounced. Benign conditions at the time of an experiment have been invoked to explain the absence of an experimentally-determined survival advantage of large offspring in some experiments (Smith *et al*, 1995; Fox and Mousseau, 1996). Likewise, several studies testing the effects of offspring size under different resource regimes have borne out the prediction that large offspring exhibit a performance advantage under adverse conditions. For example, in the seed beetle *Stator limbatus*, offspring from large eggs perform better on a poor-quality host plant but egg size does not affect performance on a high-quality host (Fox and Mousseau, 1996). This hypothesis is also supported by other studies comparing the effect of offspring size under different regimes of resource availability (e.g. Ferguson and Fox, 1984; Tessier and Consolatti, 1989; Hutchings, 1991), and it has been suggested that offspring size

variation has implications for offspring performance only under adverse conditions (Fox and Mousseau, 1996).

Similar predictions have been made about the relationship between offspring size and habitat type in intertidal gastropods. Spight (1976) predicted that selection should favor large hatching size of *Nucella* under more severe environmental conditions, because large hatchlings exhibit superior feeding abilities and lower susceptibility to environmental stresses (predation, starvation and heat and desiccation). Etter (1989) found that hatching size in *N. lapillus* was greater on wave-protected shores than on wave-exposed shores, and argued that this pattern was consistent with Spight's (1976) hypothesis because hatchlings on wave-sheltered shores suffer greater levels of predation (Menge, 1983) and greater physiological stresses (Etter, 1989) than hatchlings on exposed shores.

In contrast to these predictions, of two habitats that varied in harshness (judged by differences in overall recovery) in this study, size positively and significantly affected survivorship of *Nucella* hatchlings in superior habitat (Shade) in two separate outplant experiments. In poorer habitat (Sun), however, while there was a trend towards higher survivorship of large hatchlings, this difference was not significant in either experiment. The majority of laboratory studies support the hypothesis that large hatching size provides an advantage to intertidal gastropod hatchlings in resisting both predatory and specific physiological stresses. Why, then, was the advantage of large hatching size greater in the "benign" habitat than the "harsh" habitat in the experiments described in this study?

The results of experiments testing size-dependent mortality under contrasting conditions may depend in part on the nature of environmental stress to which organisms are exposed. In experiments described above, large offspring size conferred a performance advantage when quality or quantity of resources was low; performance differences under these conditions could well be attributed to the higher nutrient stores of large juveniles buffering the effects of a poor-food habitat (Bagenal, 1969; Williams, 1994), or the competitive or functional superiority of large offspring in acquiring resources (Salthe, 1979; Parker and Begon, 1986; Parker *et al.*, 1989; Lyimo *et al.*, 1992). In contrast, to the best of my knowledge *Nucella* hatchlings on both Sun and Shade sides experienced very similar habitat in terms of food quality and supply, but the two sides differed in sun exposure and consequent levels of physiological (heat/desiccation) stress. In the intertidal habitat and the juvenile period of *Nucella* hatchlings, factors in the physical environment and resource-related factors may act on size-dependent performance in very different ways.

The lack of a significant effect of hatching size on recovery on the Sun side, which experienced overall higher mortality, may have been due to interactions between physical factors and microhabitat structure. One striking difference between Shade and Sun sides of the surge channel (in addition to overall differences in maximum daily temperature) was due to differences in insolation. Surface temperatures within single panels varied much more in sunlight than in shade, and Sun panels were in direct sunlight far more often than Shade panels. As a result, Sun panels were a more spatially complex temperature environment than Shade panels; within-panel temperature ranges on the

Shade side were rarely greater than 1 – 1.5°C, and while spots on any given panel often reached temperatures of 30°C on the Sun side (and frequently higher), at the same time temperatures elsewhere on the panel could be > 6°C cooler.

Therefore, during periods of high temperature stress, each Sun-side panel represented a complex array of microhabitats that varied in degree of physiological (heat/desiccation) stress on the order of centimetres. In this type of environment, survival may depend more on location than size; in many microhabitats on a sun-exposed panel, heat/desiccation stress may have been so severe as to be lethal to large and small hatchlings alike. *Nucella* hatchlings are motile (Gosselin, 1994; pers. obs.) and probably distributed themselves around each panel in search of food and protective microhabitat. If survival depended largely on a hatchling dispersing to non-lethal microhabitat, and microhabitat selection was random with respect to hatchling size, then in such a patchwork habitat would be OSI because survivorship would be largely random with respect to size. The pattern of hatchling recovery on the Sun side of the surge channel, a nonsignificant trend towards higher recovery of large hatchlings, suggests that seasoned panels on the Sun side may have simulated such a complex, patchwork habitat.

Significantly higher recovery of large hatchlings on Shade panels may have occurred because panels on the Shade side rarely if ever resembled the haphazard patchwork of severe and moderate localized temperature stress described above. The Shade side was a quantitatively better habitat, and probably was equivalent to good natural *Nucella* habitat. Average number of snails lost on the Shade side in the two outplants was 2.6% (Outplant 1) and 3.7% (Outplant 2) per day, well within the 1.7 –

30% range of *Nucella* hatchling mortality found by Gosselin (1994) in different natural microhabitats over a single low tide. Rates of mortality (= nonrecovery) on the Sun side, though higher (3.2 % and 4.8 % per day), were still easily within this range; therefore, even the Sun side was probably not unnaturally severe. Even though the Shade side was protected from severe heat stress, hatchlings were still exposed to a full range of natural and potentially size-dependent sources of mortality including wave force, predation, and even moderate levels of heat/desiccation stress. The "benign" habitat in this study may have been offspring-size-sensitive (OSS) because, although the habitat was relatively good, it was not benign in the sense that all hatchlings performed well regardless of size (as in the first OSI environment type described by Begon (1986); and Begon *et al.* (1995).

Environments may be offspring-size-insensitive in several ways (see above; Begon *et al.*, 1995). Most models and research comparing the relationship between offspring size and performance have involved the first type of OSI environment, one in which all offspring do well. From these models and research, some authors (e.g. Williams, 1994; Ferguson and Fox, 1996) predict that large offspring size will be advantageous in poor environments but less so in benign environments. Because offspring size affects many other aspects of life history biology (Stearns, 1992), this prediction has major implications for understanding how life histories evolve in different environments.

The research described in this paper suggests that the relationship between offspring size and offspring performance in different environments may not always

resemble an inverse relationship between habitat quality and optimal offspring size, particularly for *N. emarginata* and other organisms that inhabit complex juvenile environments. The intertidal environment varies in a wide range of biotic and abiotic stresses, many of which may or may not favor large *Nucella* hatching size depending on their duration and severity. Temporal and spatial variation over both small and large scales is likely to be high (Underwood and Chapman, 1996), and it is possible that in nature, *Nucella* hatchlings very rarely experience long-term conditions of the benign OSI type in which offspring of all sizes perform well. Support for the hypothesis comes from significant size-dependent hatchling mortality at two separate locations and in 2 of 3 long-term experimental outplants performed at different times of the year; one exception was the long-term outplant performed in summer (Outplant D), in which recovery of large and small hatchlings was not significantly different. However, overall recovery in Outplant D was low relative to C and E, suggesting that summer panels did not represent a benign-type OSI environment. In fact, the outcome of Outplant D may have been due to stressful desiccation conditions similar to the Sun side of the 2-site outplants.

If an organisms' environment varies in one or only a few stresses that act in a size-dependent manner (e.g. resource availability), certain benign habitat types may be OSI when these stresses are absent and all offspring sizes perform well; this is the OSI habitat type that has received the most attention. However, some habitats may be OSI because mortality is random with respect to offspring size. In these experiments, the harsher environment (Sun) was OSI because mortality, while high, was indiscriminate with respect to offspring size. In contrast the more benign (Shade) side was OSS,

possibly because it experienced moderate and spatially homogeneous levels of stress that acted in a size-dependent manner. Therefore, in environments such as the intertidal that are spatially and temporally variable in a number of factors that may act and interact in both size-dependent and size-independent manners, the relationship between offspring size and performance is likely to be highly variable as well. Likewise, estimates of overall habitat quality may not always be useful in predicting either the importance of offspring size or optimal maternal investment strategies in a given habitat.

CONCLUSIONS

This study demonstrated that the organic content of *Nucella emarginata* hatchlings scales closely with size, and that hatching size can therefore be used as a proxy for maternal investment. The scaling coefficient varied between clutches, and therefore within-clutch comparisons will be most powerful. Hatching size positively affected both survivorship and growth under starved conditions and when hatchlings were fed *ad libitum* in the laboratory. In the field, hatching size always has a positive effect on growth, but not recovery; hatching size does not affect recovery in short (9 d) outplants but does affect recovery in two out of three long (36 or 54 d) outplants. In replicated experiments testing the relationship between hatching size and survivorship in two environments that differed in degree of heat and desiccation stress, size significantly and positively affected recovery in the more benign habitat (Shade) but not in the harsh environment (Sun). These data suggest that the advantage of large offspring size is not always negatively correlated with environmental quality.

CHAPTER IV

EFFECTIVENESS OF THE FLUORESCENT MARKER CALCEIN AS A LABEL FOR JUVENILE *NUCELLA EMARGINATA* (GASTROPODA: PROSOBRANCHIA)

Growth, mortality and selection during the 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, 1966; Denley and Underwood, 1979; Connell, 1985; Hurlbut, 1991; Keesing, 1994; Osman and Whitlatch, 1996; Gosselin and Quian, 1997), but the ecology of juveniles in benthic marine systems is poorly understood. A method of marking juvenile animals is a prerequisite for many types of field experiments. This study investigates the effectiveness of low concentrations of Calcein (2,4-bis- [N, N'-di (carbomethyl)-aminomethyl] fluorescein) in marking juvenile *Nucella emarginata* (Deshayes) (Prosobranchia: Gastropoda), the durability of the Calcein mark over time, and the effects of marking on survivorship and growth in this species.

Comparatively few studies have measured mortality and growth of juvenile stages in benthic marine systems, in part because such studies may be complicated by several factors that affect studies of adults to a lesser degree. One such factor is size; the small size of newly released or newly settled juveniles may hinder collection, identification and

recovery. In addition, recovery may be difficult because tiny juveniles of motile species may utilize cryptic microhabitats (Feare, 1970; Hernnkind and Butler, 1986; Gosselin and Chia, 1995). Small size is also a complicating factor in marking juveniles for experimental field outplants. Mechanical difficulties or toxicity of marking materials limits the utility of many 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 small, thin-shelled animals (Palmer, 1990; Gosselin, 1993). Labeling of individual animals with unique identifying marks (Gosselin, 1993), while potentially a powerful tool, could be prohibitively time-consuming in studies involving large numbers of animals. In addition, stains or marks which enable investigators to recover marked animals may increase the animal's susceptibility to visual predators (Levin, 1990).

The marker examined in this study, Calcein, is a fluorescent label that binds to calcium and is incorporated into growing calcium carbonate structures. Calcein has been used with considerable success to mark both adults and juveniles in many taxa including fish (Monaghan, 1993; Brooks *et al.*, 1994), mammals (Malouvier *et al.*, 1993; Turner, 1994), and echinoderms (Stewart, 1996). Calcein has also been proposed as a good marker for field studies of marine invertebrates because many such taxa contain calcium carbonate structures which are marked effectively by Calcein; likewise, in many groups Calcein provides a mark in the growing calcium carbonate structures that can be used both for identification and measurement of some aspects of growth (Rowley and

MacKinnon, 1995). However, some studies in vertebrates have found toxic effects even at relatively low concentrations (Brooks *et al.*, 1994; Bumguardner and King, 1996), and the effects of Calcein on growth have not been investigated for most invertebrates. Therefore, before Calcein can be utilized to mark early life-history stages of a given taxon, its toxicity, effectiveness as a marker, and its effect on growth must first be investigated.

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

Methods

Hatchling Collection

Snails used in this study were newly hatched juvenile *Nucella emarginata* ranging in shell length from 0.9 to 2.0 mm. Ripe clutches of egg capsules were collected at the boathouse dock, Oregon Institute of Marine Biology (ripe capsules were identified as at or near the point of hatching).[i]

Calcein Marking Technique

A concentrated stock solution containing 6.25 g/L Calcein in distilled water was buffered to a pH 6 with sodium bicarbonate to enhance the solubility of Calcein (after Wilson et al., 1987). This concentrate was added to filtered seawater to make a desired volume of marking solution containing a total Calcein concentration of 100 ppm. Snails were exposed to marking solutions for periods of 12 or 24 hours as described below.

Measurement of Marked Snails

Snails were examined for Calcein marks under a Wild M5A dissecting microscope equipped with epi-illumination via a blue-light filter (λ_o center wavelength 460nm, Corion Corporation catalog #XM-465) fitted on a fiberoptic light, and a yellow sharp cut-off longpass transmission filter (λc (τ_i max/2) 495nm, Edmund Scientific catalog #A32,763) fitted over the microscope head. To minimize possible damage from handling, heat, or desiccation, snails were handled with fine-tipped forceps and eyelash-tipped wands and were immersed in sea water except during measurement. Measurement required < 30 seconds per snail. These techniques very rarely resulted in visible damage to any snail, and there were no observed differences in activity of snails before and after measurements were made.

Snails were measured to the nearest 10 μm with a Wild M5A dissecting microscope. Growth of starved, marked snails was measured as the quantity of shell

added since marking, from the mark to the new growing aperture along the 2nd shell rib (fig. 1, chapter 2). Because hatchlings utilized in this study had added very little teloconch when the study was initiated, the Calcein mark was at the protoconch-teloconch (PT) boundary in almost all snails. Therefore, growth of starved, unmarked snails was measured from the PT boundary to the new growing aperture along the 2nd shell rib and these measurements were considered equivalent to measurements from the Calcein mark in marked snails.

The hatching length of older, fed, marked snails was calculated as the increase in total shell length since marking (new length (at day 34) – marked shell length). Shell length of marked snails was measured from the shell apex to the marked original siphonal tip. Growth of unmarked snails was measured by subtracting protoconch length at the protoconch-teloconch boundary (fig. 1, Chapter III) from shell length at day 34. Where possible, measurements were performed blind (without knowing the marked/unmarked status or original measured length of each hatchling).

Rearing of Starved Hatchlings

A ripe clutch was collected from the field (as above) 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 sea water for 24 hours, and hatchlings in the unmarked group were placed in filtered sea water for the same period. Each snail was

then placed in an individual well of a tissue culture tray, from which the tops and bottoms 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 starved hatchling *N. emarginata* indicated that hatchlings were very sensitive to flocculant from the flow-through sea water system, tissue culture trays containing hatchlings were placed in a large (~ 5 gal.) tub of 0.45 μm filtered sea water. The tub was then covered and partially immersed in flowing sea water (to the water line) to keep the filtered sea water at ambient temperatures.

After 6 days, snails were removed from wells and examined under a dissecting microscope equipped with blue epifluorescence and a yellow filter (as above). Snails were scored as marked or unmarked based on the presence or absence of Calcein fluorescence and measured for total length. Snails were also examined for activity and flesh discoloration and scored as alive or dead. The 6-day growth of each marked snail was calculated by measuring the distance from the Calcein mark to the new apertural edge along the 2nd rib from the body whorl (fig. 1, Chapter III). Growth of unmarked snails was measured from the PT boundary. To evaluate the accuracy of the Calcein mark as an indicator of original hatching length, an estimate of the original hatching length of each snail was made by measuring from the shell apex to the original, marked, siphonal end of the aperture (fig. 1, Chapter III). These estimates were then compared to the hatching length of each snail as measured on day 0.

Rearing of Hatchlings Fed *Ad Libitum*

Hatchlings from each of six ripe clutches were washed through a series of 4 graded meshes and immediately transferred to sea water. Approximately 20 large and 20 small snails (from the largest and smallest meshes) from each clutch were then randomly divided into two groups for a total of four groups per clutch, and one group each of large and small snails was randomly assigned to a marked treatment. Large and small marked snails from each clutch were placed in a solution of 100 ppm Calcein in filtered sea water for 12 hours, and unmarked snails from each clutch were placed in filtered sea water for the same interval. After 12 hours, all snails were rinsed 8x with sea water and any dead or inactive snails were removed (inactive/dead snails occurred in only one clutch and were evenly distributed among marked and unmarked snails). Eight snails from each group were then randomly chosen and a total of 32 snails from each clutch (8 large marked, 8 large unmarked, 8 small marked, 8 small unmarked) were measured for greatest shell length. Each set of 32 snails from each of the six clutches was then placed in an individual box sided with 600 μm mesh (for a total of six boxes) and provided with freshly-collected intertidal rocks covered with small barnacles and mussels.

Snails were removed after 34 days and measured for new size, hatching/marked size 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. Empty shells were by far the most common, and in no instance was it

difficult to distinguish living and dead snails. All recovered snails were examined with fluorescence microscopy (as above) and scored as marked or unmarked based on the presence or absence of a Calcein mark. Snails were scored as large or small based on shell length at the PT boundary (unmarked snails) or at the Calcein mark (marked snails).

Survivorship of two groups, small marked vs. small unmarked snails and large marked vs. large unmarked snails, were compared among clutches at d 34 with a paired Student's t-test (two tests). To compare growth of marked and unmarked snails, size of all snails at d 34 was compared using a three-factor, mixed-model ANOVA with initial size (large or small), marked status (marked or unmarked) and box-clutch (1-6) factors and size at d 34 as the dependent variable. Initial size and marked status were considered fixed factors and box-clutch was a random variable.

Results

Calcein Mark

Immersion of hatchlings in a 100 ppm solution of Calcein in sea water for either 12 or 24 hours produced a mark at the growing aperture of the shell of hatchling *Nucella* that was readily visible when snails were viewed with the filter set described above (fig. 1). This mark varied considerably in brightness among clutches and among individuals, but was always present. The Calcein mark was still visible at the original aperture after 34 days of growth in the laboratory (fig. 2), and the mark persisted for at least 50 days in

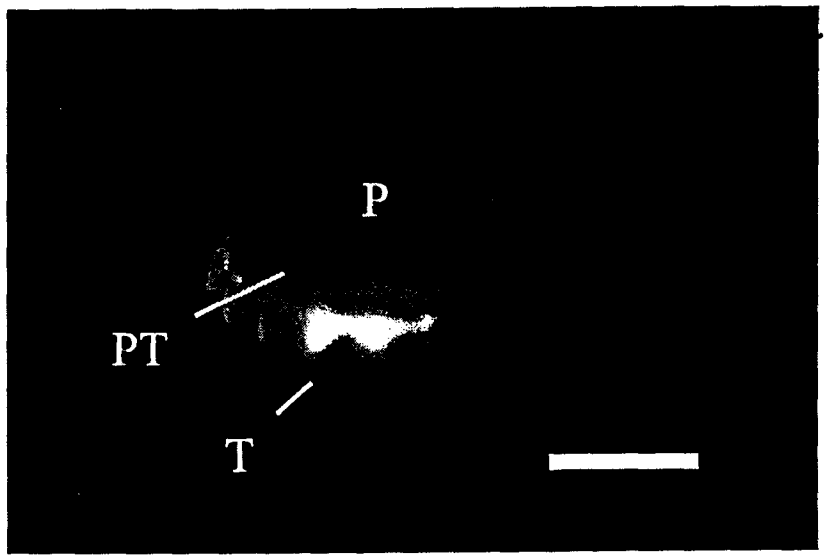


Figure 1. Photograph of a 9 day old *Nucella emarginata* juvenile, viewed under blue light with a yellow longpass filter. The brightest area is the Calcein mark just past the protoconch-teloconch boundary (PT), and the smooth protoconch (P) shows some areas of labeling as well. The area of the teloconch (T) that has grown since marking is entirely unlabelled. Illumination seen in this area is light reflected from the Calcein mark. Scale bar = 0.5 mm.

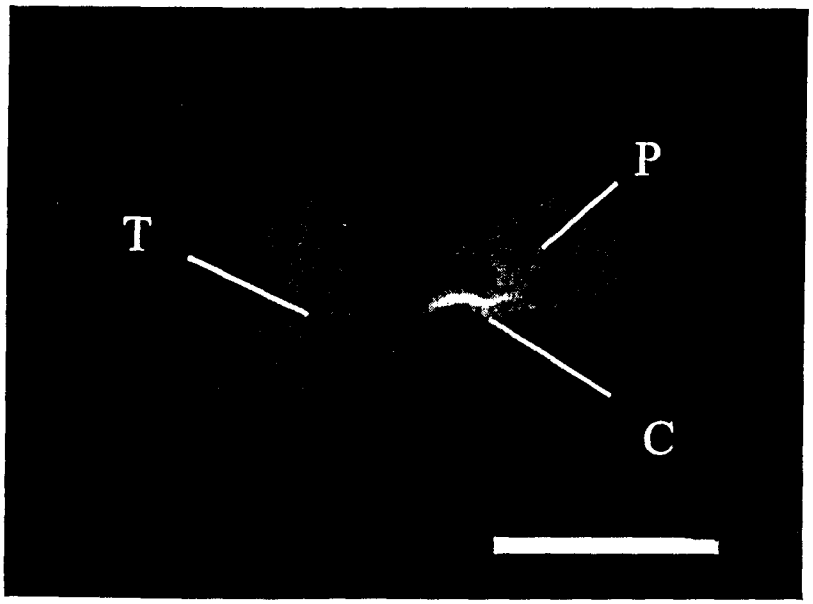


Figure 2. Photograph of a 34 day old *Nucella emarginata* juvenile viewed on its side with the aperture at 90° to the substratum. A bright Calcein mark (C) can be seen at the protoconch-teloconch boundary (P = protoconch, T = teloconch). The teloconch is just beginning to wrap around the Calcein-marked area of the original hatchling shell. Scale bar = 1 mm.

the field (see Chapter III). In some older snails the Calcein mark became noticeably fainter, though it was always readily distinguishable. Decreased brightness of the mark appeared to result from the addition of new shell layers on top of the marked portion of shell, or in some cases erosion of the protoconch.

Among starved snails, original hatching length and hatching length as measured from the Calcein mark were significantly and tightly correlated ($r = 0.98$, $n = 44$, $p < 0.001$) (fig. 3) and original hatching length explained 95 % of the variance in estimated hatching length. The mean difference between original and 6-day measurements (absolute value) 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 34 days and could be utilized to measure initial marked length and growth. However, at day 34 the teloconch of many snails was on the point of wrapping over the marked area, after which snails would be identifiable as marked or unmarked but original length could no longer be nondestructively measured.

Starved Hatchlings

There was very low mortality among starved snails over the 6 d experimental interval; 22 out of 23 snails survived in each group. There was no significant difference between growth of marked and unmarked snails at 6 d (one-factor ANOVA, $F = 0.126$, $p = 0.72$), nor did the relationship between hatching size and growth differ significantly

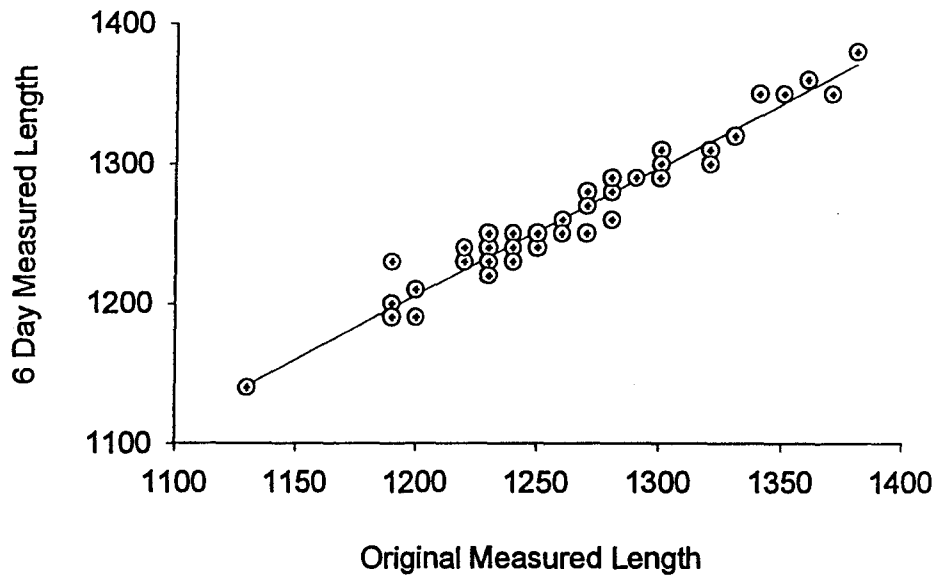


Figure 3. Correlation between estimates of hatchling length made from the Calcein mark (Y axis) and the original measured hatchling length (X axis) after 6 days of starvation in the laboratory. Points represent individual hatchlings.

among marked and unmarked snails (ANCOVA: slope, $F = 3.021$, $p = 0.090$: intercept, $F = 0.234$, $p = 0.631$) (fig. 4).

Fed hatchlings

There was no significant difference in survivorship at day 34 between large marked and large unmarked snails (paired Student's t-test, $t = 0.052$, $p = 0.961$) or between small marked and small unmarked snails (paired Student's t-test, $t = -1.102$, $p =$

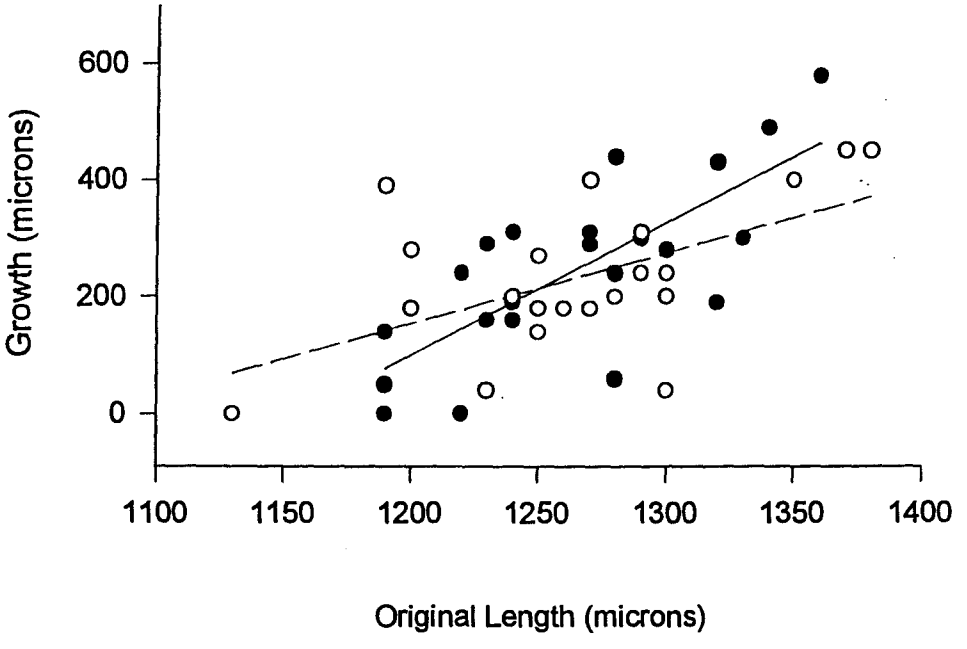


Figure 4. Growth of Calcein-marked (closed circles, solid lines) and control (open circles, dotted line) *Nucella emarginata* hatchlings after 6 days of starvation in the laboratory, plotted against original hatchling length. Lines are least-squares regressions.

0.358) from the six clutches. There was no overall significant effect of marking on size at day 34, no significant interaction between marking and initial size, no significant interaction between box-clutch and marking, and no significant 3-way interaction among marking, initial size and box-clutch (Table 1).

Table 1. Results of three-factor, mixed-model ANOVA testing effects of Calcein marking on large and small *N. emarginata* hatchlings after 34 days fed *ad libitum* in the laboratory. Size (large or small) and Mark (marked or unmarked) = fixed factors; box-clutch = random factor.

Effect	df	MS	*F*	*P*
Box-Clutch	5	0.387	2.627	0.028
Size	1	17.341	44.514	0.001
Mark	1	0.001	0.007	0.936
B-C * S	5	0.390	2.644	0.027
B-C * M	5	0.141	0.956	0.448
S * M	1	0.070	1.698	0.249
B-C * S * M	5	0.041	0.279	0.923
Error	130	0.147		

Discussion

The advantages of Calcein marking in *N. emarginata* are as follows. First, Calcein produces a mark that can be viewed under a dissecting scope using the inexpensive filter sets described above. Second, because Calcein fluoresces under visible (blue) wavelengths, animals can be examined without the tissue damage that occurs under

UV light (Moran, unpub. 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 marked rapidly, easily and inexpensively, and the Calcein mark provides an accurate estimate of original (marked) size and growth after time in the laboratory or field. Finally, Calcein does not inhibit growth or cause mortality of *Nucella emarginata* hatchlings, nor does it differentially affect small or large individuals.

Although Calcein at low concentrations is nontoxic to *N. emarginata* 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 higher concentrations (toxic effects were reported at 160 mg/l) (Bumguardner and King, 1996), and the relative sensitivity of different taxa to Calcein has not been thoroughly examined. Therefore, prior to using Calcein for the first time in a selected taxon, pilot experiments should be performed to establish nontoxic concentrations and immersion times that result in an effective mark. Likewise, a low number of *N. emarginata* hatchlings (< 1 in 50) failed to developed a visible 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. The techniques in this study also cannot be used to identify individuals; techniques such as those described in Gosselin (1993) would be more applicable to experiments requiring recognition of individual animals.

Results of this study demonstrate that Calcein provides a long-lasting, readily detected fluorescent shell mark in *Nucella emarginata* hatchlings that can be used to both identify experimental snails and to accurately measure marked length of hatchlings up to the completion of the first teloconch whorl. Therefore, Calcein can be utilized as a marker in laboratory or field studies using *N. emarginata* (and potentially in other molluscan 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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[i] Data from a later date in this same experiment are reported in Chapter III to describe the relationship between hatchling size and growth and survivorship of fed laboratory hatchlings. Data were pooled because there was no difference in survivorship between marked and unmarked snails, and in Chapter III data were analyzed at a later date (d 60) when survivorship was considerably lower.

CHAPTER V

EFFECTS OF MATERNAL SIZE AND FOOD RATION ON REPRODUCTIVE OUTPUT AND OFFSPRING SIZE IN THE MARINE GASTROPOD *NUCELLA EMARGINATA*

INTRODUCTION

Marine organisms exhibit a remarkable variety of life-history strategies both within and among taxa (Thorson, 1946; Strathmann, 1985), and this variety provides a unique, comparative means of studying the evolution of development, morphology and life history. One important component of life history theory is offspring size. Offspring size is correlated with and may be causally related to many other aspects of life history including offspring number (reviewed in Stearns, 1992), length of larval period (Perron, 1981; Perron and Kohn, 1985; Strathmann, 1985; Sinervo and McEdward, 1988), juvenile growth rate (Ferguson and Fox, 1984; Emlet and HoeghGuldberg, 1997; chapter III, this thesis), and adult environment (Spight, 1976; Rivest, 1983; George *et al.*, 1990), size (Tanaka, 1995; George, 1996; Kaplan and King, 1997), and condition (Ebert, 1993; Reznik *et al.*, 1996). Despite the evident importance of offspring size to many aspects of the life history of marine organisms, little is known about the specific physical,

environmental and physiological factors that regulate offspring size in benthic marine systems.

The offspring size of a single female may be affected by a number of factors, including local environmental conditions, female size, nutritive condition, and genotype. Environmental variation in offspring size has been reported in a number of species and has been attributed to physiological effects of geographic clines in temperature (Hadfield, 1989), differing selective regimes (Spight, 1976; Etter, 1989), and phenotypic plasticity (Ebert, 1993; Guisande *et al.*, 1996; Foeger and Pegoraro, 1996). Female size is positively correlated with offspring size in some taxa (Sargent *et al.*, 1987; Landa, 1992; Fox, 1993; Tanaka, 1995; George, 1996; Kaplan and King, 1997) but not others (Yusa, 1994; Iguchi and Yamaguchi, 1994; Bridges and Heppell, 1996; Odinetz Collart and Rabelo, 1996; Corkum *et al.*, 1997; Roosenburg and Dunham, 1997). The effect of maternal size on offspring size can also vary among closely related taxa (Kirk, 1997).

Maternal nutritional conditions can also strongly affect offspring size, and in some groups offspring size can change as a phenotypic response to food availability in the environment. Females of some taxa produce large offspring in low-food environments, presumably because high offspring nutrient reserves improve offspring performance under low-food conditions (Ebert, 1993; Guisande *et al.*, 1996; Reznik *et al.*, 1996). In contrast, in other groups offspring size may be negatively correlated with maternal food ration (Foeger and Pegoraro, 1996; Reznik *et al.*, 1996).

Lastly, in some taxa offspring or maternal genotype regulates offspring size (Boag and Grant, 1978; Ebert, 1993; Fox, 1994; Sinervo and Doughty, 1996). Because the effects of environment, maternal size and maternal nutritive state are often very strong and can vary greatly within and among taxa (Kaplan and King, 1997), the assumption that offspring size is genetically based (and therefore subject to natural selection) may be seriously flawed if maternal effects are ignored (Bernardo, 1996).

The gastropod *Nucella emarginata* is a predatory, intertidal whelk that ranges from Alaska to central California (Palmer *et al.*, 1990). *Nucella emarginata* has nonplanktonic development, and embryos develop to metamorphosis inside of benthic egg capsules. During development embryos consume nurse eggs (unfertilized or nondeveloping eggs), and hatching size is determined by the ratio of nurse eggs to embryos in a capsule (Spight, 1976). Females are reproductive throughout most of the year (Seavy, 1977) and lay between 4 and 20 capsules per clutch. Each capsule contains 6-23 embryos, and hatchlings can vary in shell length from 0.9 to > 2 mm (Spight, 1976; Moran, unpub. data). Hatching size varies among hatchlings from a single capsule, among capsules in a single clutch, between clutches at a single site, and between populations at different sites (Spight, 1976). Variability in offspring size in *Nucella* may reflect local adaptive or phenotypic responses to contrasting environmental conditions, such as varying levels of predation, sun or wave exposure (Spight, 1976; Etter, 1989; Gosselin, 1994).

In the present study, laboratory experiments were performed to test the hypothesis that maternal size and nutritive state affect offspring (hatchling) size in *Nucella emarginata*. *N. emarginata* displays a high degree of offspring size variation and is easily maintained in the laboratory, making it an excellent subject for testing the effects of maternal size and condition on offspring size in the marine benthos. A large component of hatchling size variation is due to variance in nurse egg provisioning and consumption; very little is known about the maternal effects or genetic mechanisms that regulate nurse egg ration and offspring size in *N. emarginata*, or other taxa that consume nurse eggs during development.

METHODS

Nucella emarginata adults were collected from the Boat House dock at the Oregon Institute of Marine Biology in Charleston, OR in fall of 1995. Snails were sexed in the laboratory and females were randomly assigned to two groups of 22 snails each, high-food (HF) and low-food (LF). Calipers were used to measure snails across the greatest length of the shell, from the tip of the siphonal notch to the shell apex. Female snails were placed individually in 1-pint plastic boxes on which 2 sides had been replaced with window screening (1 mm mesh size) attached with hot glue. A single male was placed in each box along with the female as a source of sperm, and a small piece of intertidal rock was placed in each box to weigh boxes down, keeping them under water.

The boxes were then submerged in flowing seawater in a flow-through sea table, with boxes from the two groups (HF and LF) in alternating positions.

Each cage was cleaned and checked at approximately 50d intervals. Snails were fed freshly collected, small (< 2 cm shell length) *Mytilus californianus* and *Mytilus trossulus* from the OIMB Boat House or a similar site. Snails were given a large supply of mussels (30-50) when fed, such that snails only very rarely consumed all mussels between cage cleanings. HF snails were fed at each cleaning interval, and LF snails were fed at every other feeding interval. During intervals in which LF snails were not fed, all mussels were removed from LF cages.

To compensate for potential effects of differences in maternal condition at the time of collection, measurements of capsule production and hatchling size were not initiated until adult snails had been on HF or LF rations for 8 months. After this period, snail cages were examined at approximately 50 d intervals and the total number of capsules in each cage was counted. Capsule counts made over long intervals were possible because capsules did not dissolve over time, nor did snails consume or remove them. Total per-snail capsule production during a given interval was calculated by subtracting the number of capsules present in a box at the beginning of the interval from the number present at the next sampling date, then dividing by the total number of days in the interval. Capsule production was measured on 10 dates. To compare capsule production and parental size, total per-day capsule production was calculated for the first four sampling intervals and compared to initial measurements of parental size for each

snail. Only the first four sampling intervals were utilized because snails grew very little during this period, and to maximize the statistical power of the analysis (21/22 snails survived to the fourth sampling interval in both groups).

ANALYSES

Prior to statistical comparisons, all data were tested for normality and homogeneity of variances. Deviations from these parametric assumptions, where present, are discussed below.

Capsule production

The relationship between adult size and mean number of capsules produced per day for each snail was examined separately for HF and LF snails using a Pearson's correlation matrix. Total capsule production of HF and LF snails was compared using both univariate and multivariate repeated-measures analysis of variance. Greenhouse-Geiger and Huyhn-Feldt epsilons were calculated to adjust the univariate results for violations of the compound symmetry assumption. Mauchly's sphericity test was utilized to test for violations of the assumption of sphericity.

Hatchling size

The relationship between adult size and mean offspring size for each snail was examined separately among HF and LF snails, using a correlation analysis. Because

many capsules did not develop normally, there were insufficient data to examine all within-snail hatchling sizes across dates. Therefore, hatchling measurements from all sampling dates were pooled within snail (Parent), and hatching size of offspring of HF and LF snails were compared with a mixed-model nested ANOVA with parent as a random factor and food as a fixed factor. This model also tested for the presence of a parent*food interaction. To test whether parental food ration affected variance in offspring size, variances around the mean hatchling size of each parent were compared with a Student's t-test.

To test for the presence of consistent differences in hatchling size among parents, comparisons were made among six females that produced hatchling broods on each of the same 3 sampling dates. Hatchling sizes of these 6 females were compared using a repeated measures ANOVA with parent (6 levels) as a random factor. Because hatchling number varied among broods, hatchling number was equalized among dates for each parent by using all data from the date with the smallest number of hatchlings, and randomly subsampling an equal number of hatchlings from the other two dates.

RESULTS

Capsule production

Mean per-day capsule production was not significantly correlated with maternal size among either HF or LF snails (HF; $r = 0.14$, $p > 0.50$. LF; $r = 0.26$, $p > 0.20$) (fig. 1).

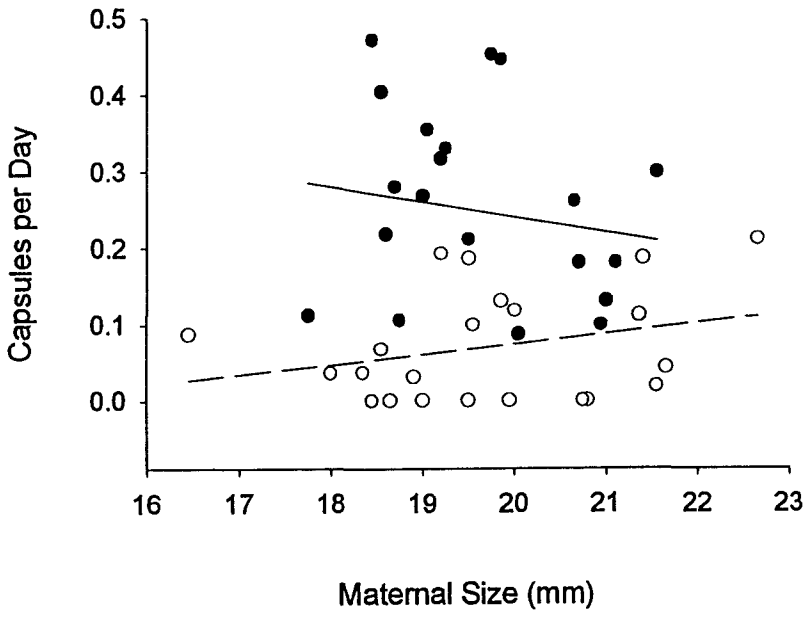


Figure 1. Mean number of capsules produced per day by individual high-food (closed circles, solid line) and low-food (open circles, dotted line) Nucella emarginata females. Lines are least-squares regressions. High-food: $y = -0.02x + 0.64$. Low-food; $y = 0.01x - 0.02$.

HF parents produced more egg capsules than LF parents on all sampling dates, and results of a repeated measures ANOVA comparing food ration and capsule production rate indicated that these differences were highly significant (fig. 2, Table 1). The distribution of within- date capsule production was non-normal on 6 out of 8 sampling dates, due to varying levels of kurtosis (> 0 in 6 of 8 cases) and skew (> 0 in 8 of 8 cases).

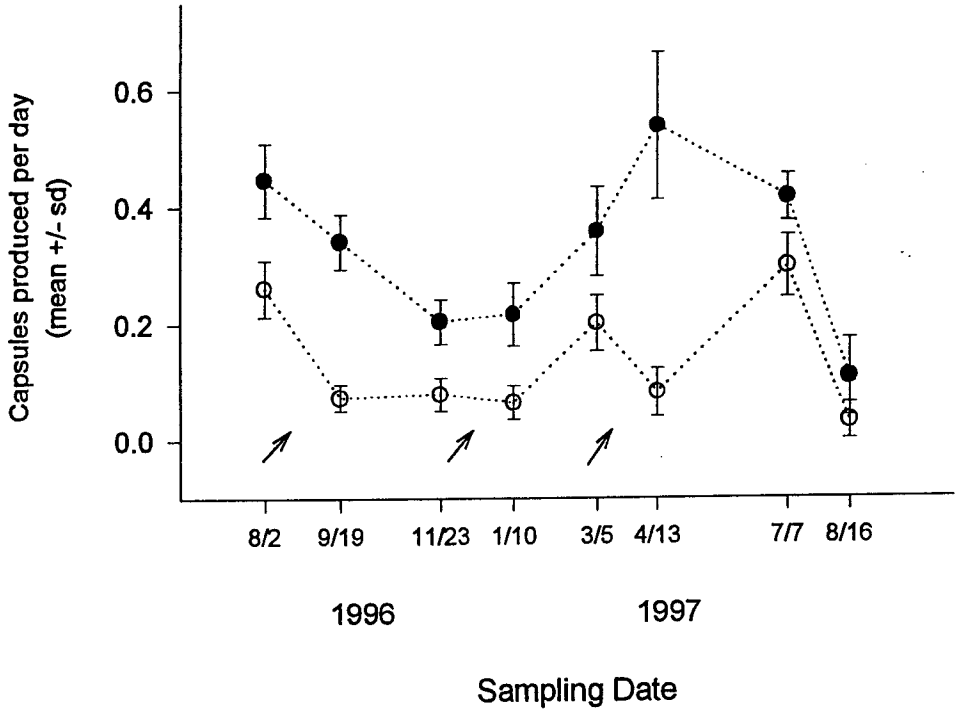


Figure 2. Mean number of capsules produced per day by all high-food (closed circles) and all low-food (open circles) *Nucella emarginata* females combined on each of 8 sampling dates. Error bars are standard errors. Arrows indicate dates on which low-food females were fed. Note the rise in capsule production that occurred after the second and third feedings of low-food females.

Table 1. Univariate and multivariate repeated-measures ANOVAs examining the effects of parental food ration on capsule production of adult *Nucella emarginata*.

Univariate Analysis of Variance (ANOVA)

Between Subjects

Source	SS	df	MS	*F*	*P*
Date	1.386	1	1.386	14.150	0.001
Error	2.645	27	0.098		

Within Subjects

Source	SS	df	MS	*F*	*P*	H-F
Date	2.276	7	0.325	7.893	0.000	0.000
Date x Food	0.732	7	0.105	2.583	0.016	0.025
Error	2.645	27	0.098			

Huynh-Feldt Epsilon: 0.8065

Multivariate Analysis of Variance (MANOVA)

Hypothesis	Wilks' lambda	df	Error df	*F*	*P*
Test of: Date	0.219	7	21	10.706	0.000
Test of: Date x Food	0.557	7	21	2.388	0.058

The F test is fairly robust to skew, and kurtosis of > 0 will increase the probability of incorrectly accepting the null hypothesis (Type II error) but not incorrectly rejecting the null hypotheses (Type I error) (Lindman, 1974). Because these results indicate that null

hypothesis can be rejected, violations of the assumption of normality in these data probably do not affect interpretation of the results.

Egg capsule production changed considerably over time for both the HF and LF snails, and was significantly affected by date (fig. 2, Table 1). Changes in egg capsule production were most noticeable at the end of the experiment, when both HF and LF capsule production fell sharply. LF capsule production appeared to be affected by feeding date, with increases in capsule production occurring during intervals in which snails were fed (fig. 2). Univariate results also suggested a significant date*food interaction (Table 1), indicating that the effect of food treatment on capsule production may vary across time.

Because capsule data violated the assumption of sphericity (Mauchly's sphericity test, $p < 0.001$), date and date*food effects from the multivariate analysis (MANOVA), which does not assume sphericity, are reported as well (Table 2). In the MANOVA design, capsule production was significantly affected by date; however, the date*food interaction was not significant (Table 1). Because results of the univariate and multivariate tests vary in this respect, the interaction between date and food, if present, is probably not strong.

Hatchling size

Mean hatchling size was not significantly correlated with maternal size in either HF or LF snails (HF: $r = 0.10$, $p > 0.50$. LF: $r = 0.04$, $p > 0.50$) (fig. 3). Results of a

Table 2. ANOVA table showing the effects of food ration on mean size of offspring (hatchlings) of *Nucella emarginata*.

Source	SS	df	MS	Model III *F*	*P*
Food	0.150	1	0.150	0.376	0.544
Parent (Food)	13.946	35	0.398	17.741	0.000
Error	84.107	3745	0.022		

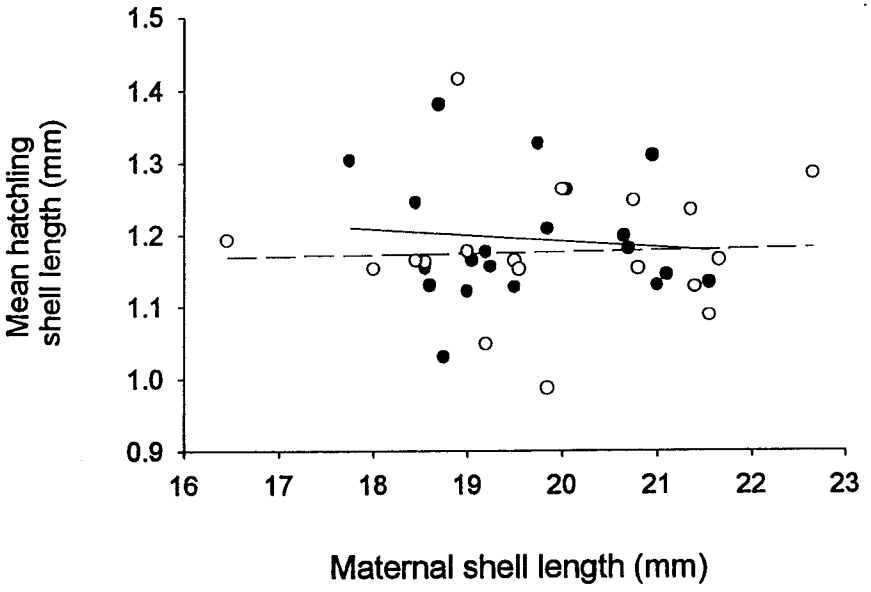


Figure 3. Mean hatching length of all offspring from individual high-food (closed circles, solid line) and low-food (open circles, dotted line) *Nucella emarginata* females plotted against maternal shell lengths. High food; y = -0.008x + 1.36. Low-food; y = - 0.002x + 1.1.

nested ANOVA indicated that mean hatchling size was not significantly affected by parental food ration (fig. 4; Table 2), and there was no difference between the variance in hatchling size of HF and LF parents (Student's two-sample t-test, pooled variance $t = 0.334$, $df = 36$, $p = 0.740$). Mean hatchling size was significantly affected by parent (fig. 4; Table 2), indicating that parents produced hatchlings of significantly different sizes when hatchlings from all broods were pooled. When 6 parents were considered separately over 3 dates on which all 6 sets of parents produced hatchlings, a significant effect of parent on mean hatchling size was found (Table 3, fig. 5). There was no significant effect of date on size, and a significant parent*date interaction indicated that hatchling sizes produced by single parents varied significantly among dates.

DISCUSSION

The size of female *Nucella emarginata* did not affect total capsule production or offspring size in this study, in contrast to some other taxa in which maternal size can be positively correlated with total reproductive output, offspring size, or both (see Introduction). Brood or offspring size might be expected to increase with maternal body size because a) larger females may be more able to acquire resources or have higher nutrient reserves to apply to reproduction (Salthe, 1969), and b) limiting factors such as ovary or brood chamber size may scale with female size (Leutinegger, 1979; Ebert, 1993). Larger females may also be able to care for greater numbers of offspring (Stearns,

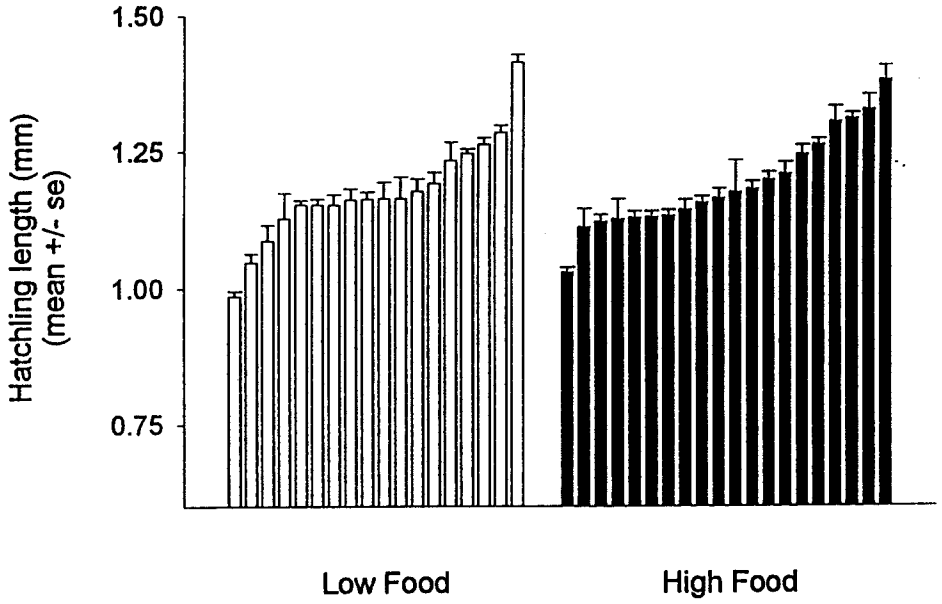


Figure 4. Mean hatching sizes of all offspring from individual high-food (closed bars) and low-food (open bars) *Nucella emarginata* females, showing differences among females. Females are ordered according to mean hatchling size (lowest to highest). Error bars are standard errors.

Table 3. Univariate and multivariate repeated-measures ANOVAs examining the effects of date and parent on offspring size, of 6 female *N. emarginata* that produced hatchlings on each of 3 sampling dates.

Univariate Analysis of Variance (ANOVA)

Between Subjects

Source	SS	df	MS	*F*	*P*
Parent	0.619	5	0.124	7.773	< 0.001
Error	1.639	103	0.016		

Within Subjects

Source	SS	df	MS	*F*	*P*	H-F
Date	0.076	2	0.038	0.332	0.725	0.725
Date x Parent	1.139	10	0.114	6.654	< 0.001	< 0.001
Error	3.528	206	0.017			

Huynh-Feldt Epsilon: 1.000

Multivariate Analysis of Variance (MANOVA)

Hypothesis	Wilks' lambda	df	Error df	*F*	*P*
Test of: Date	0.956	2	102	2.332	0.102
Test of: Date x Parent	0.611	10	204	5.703	< 0.001

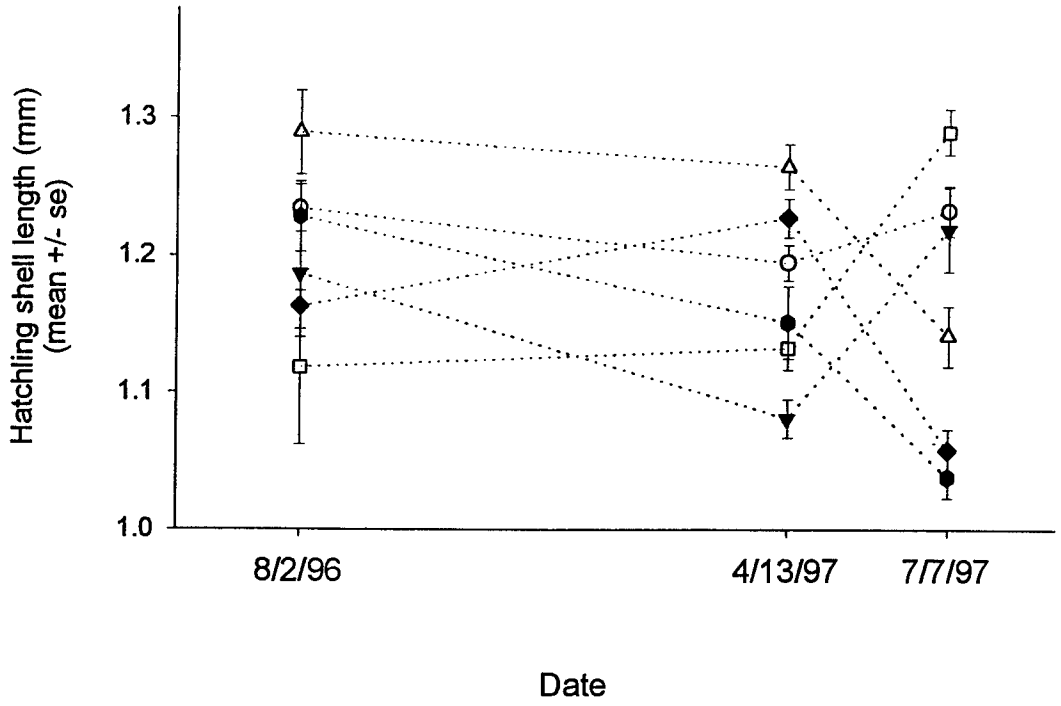


Figure 5. Mean offspring hatching size of 6 *Nucella emarginata* females that produced hatchlings on each of 3 sampling dates. Each snail is represented by the same symbol on each date. Open symbols = low-food females; closed symbols = high-food females. Dotted lines connect broods from each female. Error bars are standard errors. X axis is days.

1992). However, this relationship is not present or always found in all groups (e.g. Oberhauser, 1997). Among gastropods a positive correlation between female size and fecundity has been observed in some species including *Turbonilla* sp. (Cumming, 1993) and several muricids (Spight *et al.*, 1974).

In the experiments described in this study, maternal size did not affect capsule production and this may have occurred for a number of reasons. First, because the size

range of females used in these experiments was limited, size-dependent differences in reproductive output may not have been apparent. Second, egg capsule size was not measured. Capsule size increases with female size in some marine gastropods (e.g. Hadfield, 1989; T. Baker, unpublished student report), but not others (e.g. Bieler and Hadfield, 1990; Miloslavich and Defresne, 1994); in *N. emarginata*, Spight and Emlen (1976) found that capsule size was positively correlated with maternal size in a Washington population. If this were the case in the Oregon population used in this study, then reproductive output may have increased with female size even if rate of capsule production did not.

A third possible explanation is that snails used in this study were > 1.5 years old and grew little during the experimental period, and may have passed the point at which age or size is correlated with capsule production. This possibility is supported by a study in which Spight and Emlen (1976) found that while large female *N. emarginata* produce more egg capsules than small females overall, fecundity dropped off sharply after snails had reached a size of 24-26 mm. Size of adult *Nucella* varies between populations (Etter, 1989) and it is possible that while snails used in this study were smaller (18 - 23 mm), some may have reached or past the point at which fecundity scales positively with adult size. Many factors, including the small range of female sizes, possible maternal size-dependent differences in capsule size, maternal age, or a combination of factors including random variation in the quality of laboratory conditions (food, microhabitat) may have

obscured any underlying parental-size dependent differences in total reproductive output in the experiments described in this study.

Reproductive output (capsule production) was strongly affected by food ration in *N. emarginata*, as has been found in numerous other taxa (see Introduction) and as has been suggested by field correlations between *N. emarginata* capsule frequency and food availability (Spight and Emlen, 1976). In addition to the overall pattern of lower capsule production, an apparent relationship between feeding times and reproductive output was also evident among low-food *Nucella* females; LF females appeared to produce more egg capsules during intervals in which snails were fed than during intervals of starvation (fig. 2). This suggests that females of *N. emarginata*, which are reproductive year-round, can respond rapidly to increases in food resources by increasing reproductive output. This is in contrast to reproductive patterns of the congeneric *N. lamellosa*, which has one reproductive event per year. In *N. lamellosa*, food ration acts on reproductive output through its cumulative effects on female size (and possibly condition) at time of spawning (Spight and Emlen, 1976).

Total egg capsule production of *N. emarginata* also varied significantly with sampling date. Although *N. emarginata* are reproductive year-round (Seavy, 1977), peaks in reproductive activity have been reported in late summer, spring and fall (Spight and Emlen, 1976). In this study, laboratory spawning was lowest in the summer and peaked in spring; however, because laboratory populations were followed for only one year, this cannot be established as an intrinsic seasonal pattern. Other potential causes of

variation in reproductive output include seasonal variation in the food value of field-collected mussels, or seasonal variation in water temperature (which was not measured). In addition, it is possible that declines in reproductive activity at the end of the experiment may have been related to female age; in many taxa reproductive output declines with age (Oberhauser, 1997), and this pattern has been suggested but not clearly documented in *N. emarginata* (Spight and Emlen, 1976).

Mean hatchling size of *N. emarginata* offspring was not correlated with maternal size in these experiments. Because offspring size in marine gastropods is achieved through a variety of mechanisms, the relationship between maternal and offspring size is likely to vary depending on how offspring size is regulated. For example, in the nurse-egg feeder *Petaloconchus montereyensis*, both capsule size and total number of eggs/capsule are positively correlated with female size. Because each capsule contains only one developing embryo and all other eggs are nurse eggs, embryos from larger capsules consume more nurse eggs and hatch at a greater size than embryos from small capsules; therefore, larger females produce larger offspring (Hadfield, 1989).

In contrast, while both capsule size and number of eggs per capsule may be positively correlated with female size of *N. emarginata* (Spight and Emlen, 1976), *N. emarginata* place multiple developing embryos in each capsule. At present, there is little reason to believe that nurse egg/embryo ratio changes with capsule size (Spight, 1976). Therefore, large *N. emarginata* offspring may produce larger capsules, but if nurse egg/embryo ratio does not change with capsule size, maternal size and offspring size are

unlikely to be correlated (as shown in this study). This suggests that among species whose larvae feed on nurse eggs during development, the strength of maternal effects (e.g. size) may vary considerably depending on how a given species allocates embryos and nurse eggs among capsules.

The experiments described in this study also did not find a significant relationship between parental food supply and mean offspring size. The effect of parental food ration on offspring size can be highly variable both within and among closely-related taxa (see Introduction); low food rations can cause offspring to be smaller or larger than offspring of high-food parents, or may have no effect on offspring size. In the first instance, the production of large offspring in the face of low-food conditions is often considered an adaptive response, because large offspring may be more tolerant of low-food conditions (Hutchinson, 1951; Jamieson and Burns, 1988; Ebert, 1993). In *N. emarginata*, large hatchling size provides increased starvation resistance (chapter 2, this thesis). However, because egg capsules do not hatch for several months in the field (Spight, 1975), and because the intertidal environment is highly variable, hatchlings that emerge from the capsule are likely to experience different conditions than the mother at time of laying. Therefore, unless females could "predict" environmental quality 2 – 3 months after time of laying, phenotypic shifts in offspring size in response to food conditions would likely be ineffective.

The significant effect of parent on offspring size demonstrated that female *N. emarginata* in this experiment produced hatchlings of different mean sizes. Because of

the highly significant degree of variance heterogeneity in hatchling size data (Cochran's $C = 0.051$, $n > 3000$, $p < 0.0001$), the significance of the parent effect on offspring size must be viewed with caution. However, results of a repeated measures ANOVA comparing the hatchling sizes of 6 parents across 3 dates found both significant effects of parent and parent*date. These data suggest that some females do produce consistently different brood sizes from other females; however, there was also significant offspring size variation among sequential clutches of single females over the 3 dates examined.

Results of the experiments performed in this study indicate that female *Nucella emarginata* respond to low food availability by reducing reproductive effort but not by changing offspring size, and female size does not appear to affect offspring size in this species. These results suggest that differences in hatchling size among populations of *N. emarginata* (e.g. Spight, 1976) and other *Nucella* (e.g. *N. canaliculata*, Rivest, 1981; *N. lapillus*, Etter, 1989) are not likely to be related to differences in food resources or adult size among sites. Rather, these data support the suggestions by several authors that hatchling size differences may in fact represent either phenotypic responses to varying environmental conditions or underlying genetic differences between populations (Spight, 1976; Rivest, 1981; Gosselin, 1994; Etter, 1989). Because there were significant differences in hatchling size among females in this experiment even though females were maintained for long (> 1 yr.) periods in apparently identical circumstances, it seems unlikely that interfemale variation is entirely regulated by phenotypic responses to environmental factors. This raises the possibility that genotype plays a role in regulating

hatchling size; however, studies specifically addressing the heritability of offspring size are needed to fully separate the effects of environment and genotype.

CHAPTER VI

GENERAL SUMMARY

The research described in this dissertation explored the relationships among offspring size, offspring performance, larval morphology, and adult condition in two gastropod genera with nonplanktotrophic larvae that rely on intracapsular nutrition during development. Chapter II described a novel feeding mechanism in intracapsular *Littorina* larvae, the direct uptake of capsular albumen across the cells of the velum. This research also compared relative velar sizes among numerous other gastropod taxa with feeding or nonfeeding development, and suggested that this novel mechanism may have led to the retention of some aspects of ancestral planktotrophic feeding morphology. This in turn suggests that the evolution of larval form in nonplanktotrophic species may not result in alterations to gross larval morphology if, as in *Littorina*, ancestral characters are coopted to serve novel functions in the capsule environment.

Very few studies have addressed this question in other taxa; the cooption of planktotrophic characters for novel functions in nonplanktotrophic species may be widespread. Intracapsular nutrition is found in many invertebrate groups including the gastropods, and many groups share similar forms of intracapsular nutrition (e.g. nurse

eggs). If larval morphology of encapsulated species is shaped by the physical requirements of feeding on intracapsular nutrition, then taxonomically distant groups whose encapsulated larvae share the same type of intracapsular nutrition may show convergent feeding morphologies. Other characters such as capsule shape or ancestral larval form may constrain convergence in some comparisons. The functional morphology of feeding on extraembryonic, intracapsular nutrition such as nurse eggs, and the degree to which type of intracapsular nutrition is correlated with similar feeding morphologies among taxa, is poorly understood and may be a productive area for future research.

Because many taxa with encapsulated development have modified larval morphology that may be adaptations to the functional requirements of feeding on intracapsular nutrition, it seems likely that intracapsular nutrition serves an important function in promoting larval or juvenile performance. Chapter III explored the relationship between hatching size (a corollary of number of nurse eggs consumed) and hatchling performance in *Nucella emarginata*, and Chapter IV described a technique for marking *Nucella* hatchlings for field experiments. This research demonstrated that maternal investment, or the number of nurse eggs consumed by a larva during development, can have strong effects on post-hatching performance. However, size-dependent performance varied temporally and spatially and variability may be related to habitat quality. Therefore, at the single site examined in this research, directional selection for large or small hatching size may not occur at the level of female or

population. Studies exploring the effects of hatching size on hatchling performance in strongly contrasting environments, such as areas of very different wave exposure, may in the future shed light on the interactions among habitat, offspring size and selection that act at the population level in marine intertidal systems.

Chapter V found that two potentially important maternal effects, maternal size and food ration, had no detectable effect on offspring size of *N. emarginata* females from a single population. This suggests a possible genetic basis for observed offspring size differences between females. However, this genetic effect may not be strong; extensive variation in offspring sizes among both single and sequential clutches from single females suggests that even if nurse egg to embryo ratio is genetically determined, the uneven distribution of nurse eggs among capsules and unequal acquisition of nurse eggs by embryos may mask underlying differences among females. This variation is another factor that may dampen any effects of directional selection for offspring size in a given habitat. Therefore, comparing females from contrasting environments (such as different wave exposures, as above) might determine whether interfemale or interpopulation differences in offspring size are detectable. Likewise, breeding experiments testing the heritability of hatching size (a character must be heritable to evolve in response to selection) should be conducted between populations from contrasting environments, if these environments show population-level differences in nurse egg/embryo ratio and offspring size.

APPENDIX A

METHODS FOR POTASSIUM DICHROMATE WET OXIDATION METHOD OF ORGANIC CONTENT ANALYSIS AND COMPARISON WITH ASH-FREE DRY WEIGHT

Potassium dichromate wet oxidation method

Hatchlings were first measured for total shell length to the nearest 10 μm with a Wild 5A dissecting microscope. Each snail was placed in an individual Eppendorf tube, rinsed 10 x with distilled water to remove salts, and lyophilized for 48 hours. Each hatchling was then placed in an individually-labeled 10 ml Pyrex tube (all tubes were cleaned at 450°C for 6 hours in a muffle furnace to remove trace organics) and run through the procedure below. Glucose standards of 50, 100, 200 and 300 μg were mixed up in 70% phosporic acid and run simultaneously.

Procedures

1. To each tube, add 250 μl 70% phosporic acid
2. Heat tubes at 110°C in block heater or oven for 15 minutes
3. Add 500 μl 0.484% potassium dichromate in concentrated sulfuric acid to each tube
4. Vortex thoroughly
5. Heat at 110°C for 10 minutes, allow to cool ca. 10 minutes

6. Add 850 µl distilled water
7. Vortex thoroughly
8. Read absorbance at 440 nm

Comparison of PDWO and AFDW

A single ripe clutch was collected in the field at Gregory Point, Cape Arago (ripe clutches contain entirely metamorphosed juveniles and capsules whose plugs are dissolved or beginning to dissolve) and randomly divided into two groups of 44 hatchlings each. The two groups were randomly designated as "AFDW" or "PDWO" and analyzed for organic content. AFDW hatchlings were measured for total shell length, rinsed 10 x with distilled water to remove salts, dried at 60°C to a constant weight (ca. 5 days), weighed in small aluminum pans (pans had been previously ashed at 450°C for 6 hours to remove trace organics) on a Mettler ME30 microbalance accurate to 1 µg, ashed at 450°C for 6 hours, then reweighed. Hatchling AFDW was calculated by subtracting ashed weight of pan + snail from the previously measured dry weight of the same pan + snail. Organic content of the PDWO snails was measured with PDWO methods described above.

To test whether PDWO and AFDW gave similar measurements of total organic content, the weight of each snail (µg) in both groups was regressed on shell length (mm) and plotted (fig. 1). Because these two lines were not significantly different (ANCOVA, slope: $F = 1.103$, $p = 0.297$. intercept, $F = 0.145$, $p = 0.145$), AFDW and PDWO results were combined for analyses in Chapter III.

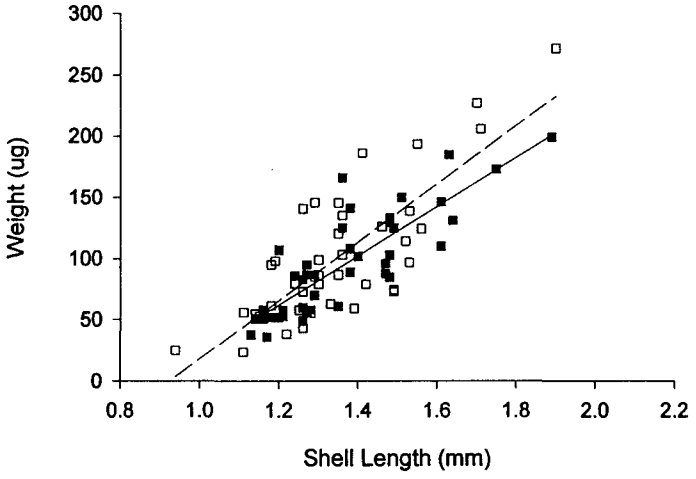


Figure 1. Weight in µg plotted against shell length in mm for hatchlings from a single *Nucella emarginata* clutch, measured with either the ash-free dry weight method (closed squares, solid line) or the potassium dichromate wet oxidation method (open squares, dotted line).

APPENDIX B

USE OF CORRAL PANELS TO TEST FOR CRAWL-AWAY BEHAVIOR IN EXPERIMENTALLY OUTPLANTED *NUCELLA EMARGINATA* HATCHLINGS

In August 1996, field tests were performed to test the propensity of *Nucella emarginata* hatchlings to crawl off Astroturf ™ panels surrounded by Tanglefoot™, such as those described in Chapter III. Although hatchlings would not cross intact Tanglefoot™ barriers in the laboratory, field barriers of Tanglefoot™ could only be replaced once a day at the most. Corral panel tests were performed because Tanglefoot™ becomes encrusted with sand and other intertidal detritus over time, and I suspected that hatchlings might be able/willing to cross compromised Tanglefoot™ barriers in the field.

Six 13 x 13 cm Astroturf™ panels were cut and surrounded by four slotted pieces of PVC pipe (as described in Chapter III) to serve as the Inner panels. Six larger (26 x 26 cm) Astroturf™ panels were cut and surrounded by 16 slotted pieces of PVC pipe (one per side) to serve as the Outer panels (fig. 1). 16 short pieces were utilized as borders for the Outer panels rather than 4 longer PVC lengths to allow panels to flex and be flush to the uneven substrate. All Astroturf™ tufts were shaved from a central, 13 x 13 cm square in the outer panels, and the inner 13 x 13 cm panels were placed in these shaved

areas (fig. 1). Holes were then drilled through each piece of PVC pipe and each of the six sets of panels (a set = one "corral" panel) were bolted to flat intertidal rocky areas that had been locally cleared of barnacles and mussels. Duct or electrical tape was used to seal the junctions between PVC pipe segments. Corral panels were allowed to season in the field for one month prior to experimental outplants. During this time, numerous small barnacles settled on and among the Astroturf™ tufts on each panel.

Ten ripe clutches of *N. emarginata* hatchlings were collected from the field, hatchlings were removed from their capsules, pooled, and passed through a series of 4 graded meshes. All hatchlings from the largest and smallest screens were marked for 12 hours with Calcein (methods in chapter 3), and hatchlings from the large and small groups were randomly chosen to form 6 groups of 15 large and 6 groups of 15 small hatchlings. One set of large and small hatchlings (30 snails total) was randomly assigned to each of the 6 corral panels.

Hatchlings were gently transferred to the inner panels (in the field, on a low tide) with a Pasteur pipet and washed with seawater until each hatchling had attached. Both inner and outer PVC borders were coated with a thin layer of Tanglefoot™. Panels were left in the field for a total of 9 days, and Tanglefoot™ barriers were refreshed at days 4 and 7. After 9 days in the field, corral panels were brought into the laboratory, separated into inner and outer panels, and the inner and outer panels were sampled separately with a high-pressure freshwater spray (as described in Chapter III). All recovered hatchlings were then examined for a Calcein mark (as described in Chapter IV) and remeasured.

Of a total of 180 hatchlings outplanted onto the inner panels, 35 were recovered (19.4%). Recovery data is summarized in Table 1. The relatively low overall recovery was probably due in part to the fact that corral panels were seasoned for only one month in the field and contained less structurally complex habitat than panels used in experiments described in Chapter III. Therefore, hatchlings on the corral panels may have been particularly vulnerable to desiccation or wave stress. Of the 35 recovered marked snails, 34 were on inner panels and one was on an outer panel.

These data suggest that very little (2.9%, or less than 1 in 30 over the course of a nine-day outplant) of the hatchling nonrecovery described in Chapter III is attributable to hatchlings' crawling off experimental panels. Estimates of crawling-off rates from corral panels are likely to be high relative to crawling-off rates in experimental field outplants (Chapter III), because Tanglefoot™ barriers were refreshed every 2 days in experimental field outplants but were refreshed only twice in 9 days during corral experiments.

Hatchling recovery rates might also be an artifact of snail wandering behavior if small hatchlings, which exhibited overall lower recovery in experimental outplants, were more likely to wander off panels. However, because the only hatchling recovered from an outside panel in corral experiments was from the large size class, there is no reason to believe that small hatchlings crawl away from panels at higher rates than large hatchlings. For the reasons listed above, corral panel experiments support the hypothesis that recovery rates on Astroturf™ panels utilized in Chapter III were little affected by snail crawl-away behavior, and that crawl-away behavior, if present, is not a likely cause of lower recovery of small *Nucella emarginata* hatchlings in these experiments.

Table 1. Recovery of Calcein-marked *N. emarginata* hatchlings from inner and outer corrals panels.

Number recovered (from 15 original)				
	Inside		Outside	
Panel	Large	Small	Large	Small
one	1	2	0	0
two	5	1	1	0
three	4	2	0	0
four	4	0	0	0
five	2	3	0	0
six	8	2	0	0
Total	24	10	1	0

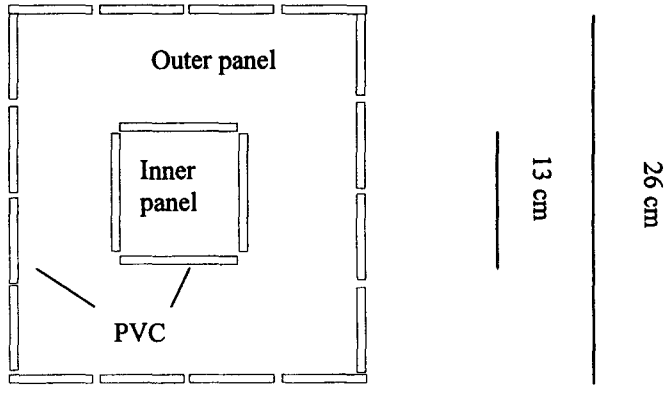


Figure 1. Astroturf™ panels used in corral experiments. During outplants, PVC pipe was coated with Tanglefoot™ and joints between pipe segments were sealed with electrical or duct tape.

APPENDIX C

CHAPTER II PHOTGRAPHIC PLATES

Legends

Figure 3. (A) Excapsulated trochophore larva of *Littorina saxatilis*, viewed obliquely from the apical end under transmitted light (dark field). Arrow indicates position of prototroch (cilia are not visible). (B) Same embryo viewed with epifluorescence microscopy and FITC filter set. Only the prototroch (arrow) is fluorescent, indicating localized uptake of FITC-labeled albumen by the cells of the prototroch. Scale bar = 70 μ m.

Figure 4. (A) Excapsulated early veliger-stage embryo of *Littorina saxatilis* (nonplanktotroph) viewed from the side under transmitted light (bright field). (B) Same embryo viewed with epifluorescence microscopy and FITC filter set. Only the prototroch is fluorescent; no fluorescence appears in the pretrochal region or visceral mass. m, visceral mass; pr, pretrochal region; v, velum (v is positioned on ciliated band). Scale bar (on fig. 3) = 70 μm.

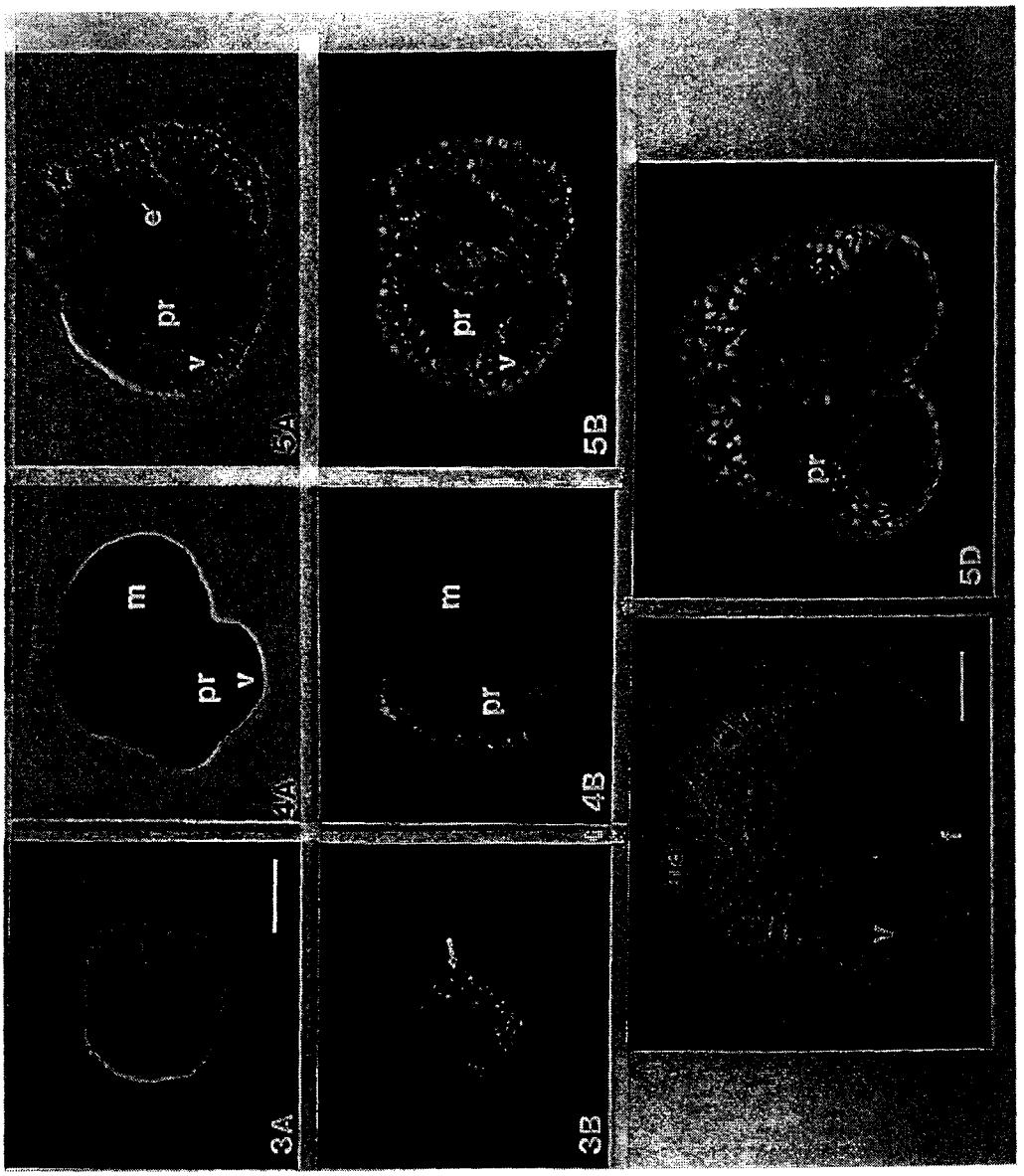
Figure 5. (A) Velum of excapsulated, mature embryo of *L. saxatilis* (viscera and foot have been removed), viewed under transmitted light (bright field). (B) Same partial embryo, viewed with epifluorescence microscopy and an FITC filter set. The ciliated velar cells are brightly fluorescent while the pretrochal region exhibits no fluorescence. The fluorescent area in the center of the velum is continuous with the ciliated band of velar cells and is probably an extension of this band. (C) Excapsulated, mature veliger-stage embryo of *Littorina sitkana*, viewed head-on under transmitted light and epi-illumination. (D) Same embryo, viewed with epifluorescence microscopy and FITC filter set. Ciliated velar cells are brightly fluorescent, and some fluorescence can be seen (out of focus) in the ciliated rejection band of the foot. No fluorescence is seen in the pretrochal region or mantle. E, eyespot; m, visceral mass; ma, mantle; pr, pretrochal region; v, velum (v is positioned on ciliated band). Scale bars; A - B (on fig. 3A) = 70 μm, C-D (on fig. 5C) = 50 μm.

Figure 6. High-magnification view of velum of mature *Littorina saxatilis* embryo viewed under epifluorescence microscopy (FITC filter set). Fluorescence appears in the ciliated band of velar cells but not the pretrochal region. Fluorescence is concentrated in small (< 2 mm) vacuoles within the prototrochal cells. pr, pretrochal region; v, velum (positioned on ciliated band). Scale bar = 20 μm.

Figure 7. Veliger-stage embryo of *Littorina saxatilis*, exposed to FITC-BSA and viewed laterally under fluorescent light with FITC filter set. The orientation of this embryo is similar to that of the embryo in figure 3. Immediately anterior to the ciliated band is one of two symmetrically-positioned fluorescent regions (larval kidneys) that were apparent in mature veliger-stage embryos. k, larval kidney; v, velum (positioned on ciliated band). Scale bar = 75 μm.

Figure 8. (A) Mature veliger-stage embryo of *Littorina saxatilis*, close to hatching, viewed under transmitted light. (B) Same embryo, viewed under fluorescent light with FITC filter set. Fluorescence can be seen in the ciliated cells of the velum, the autofluorescent operculum, and in a short region of the gut. The dim illumination of the foot is due to light reflected from the operculum. f, foot; g, gut; o, operculum; s, shell; u, umbilicus; v, velum. Scale bar = 150 μm.

Figure 9. (A) Newly hatched veliger of *Littorina plena*, viewed under transmitted light. (B) Same larva viewed under fluorescent light with FITC filter set, showing strong FITC fluorescence in the gut. The operculum is autofluorescent, and the foot is dimly illuminated by light reflected from the operculum. Note the complete absence of fluorescence in the velum. f, foot; g, gut; o, operculum; pc, prototrochal cilia; s, shell; v, velum. Scale bar = 25 μm.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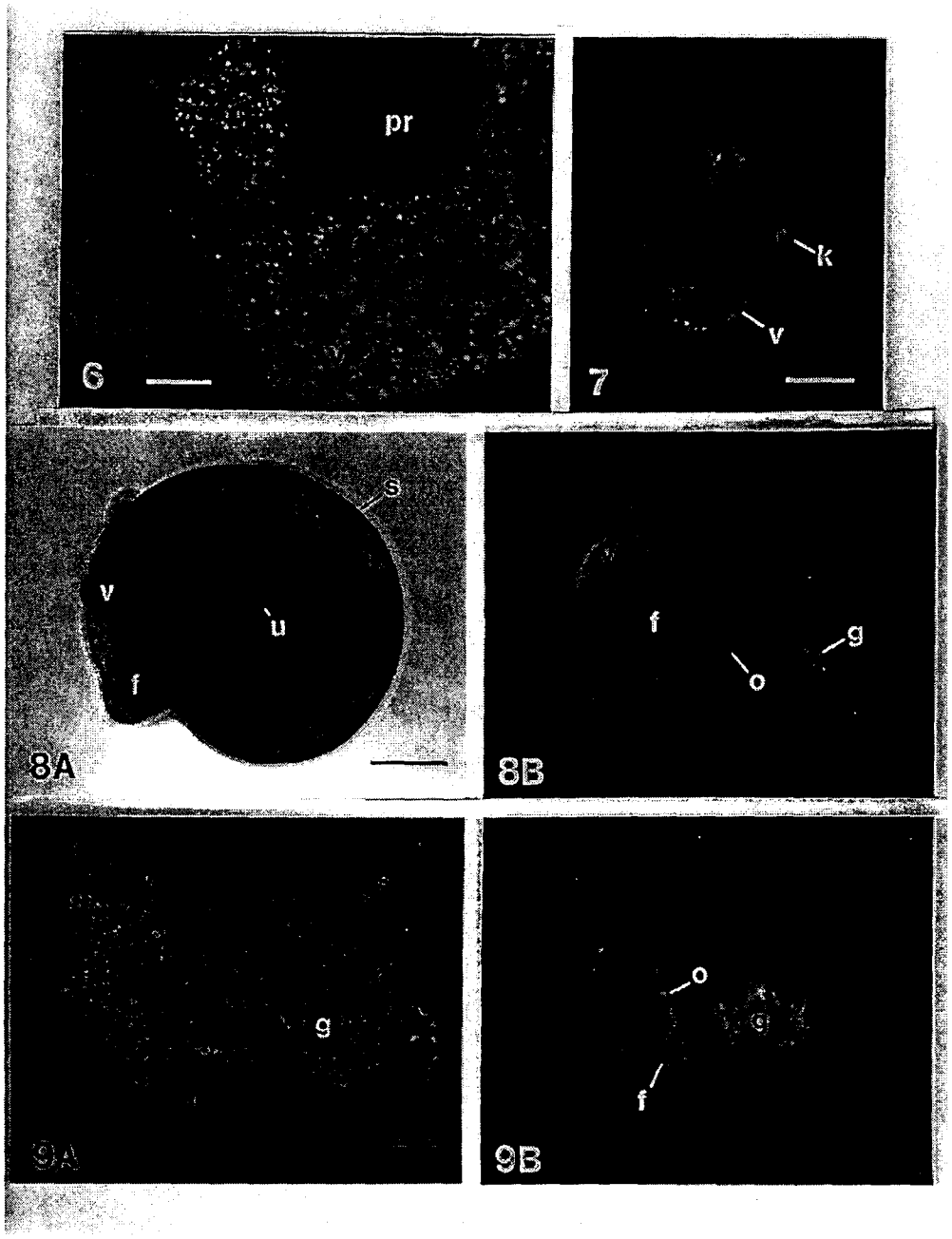
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