EMBRYOLOGY, LARVAL ECOLOGY, AND RECRUITMENT OF *"BATHYMODIOLUS" CHILDRESSI*, A COLD-SEEP MUSSEL FROM THE GULF OF MEXICO

by

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"Bathymodiolus" childressi is a mixotrophic mussel from Gulf of Mexico cold seeps. There is no genetic differentiation of mussels among the seeps, suggesting wide dispersal of their larvae. This dissertation describes larval biology, ecology, and recruitment dynamics for *"B." childressi*. Cleavage is spiral at a rate of one per 3-9 hours, with blastula larvae hatching by 40 hours at 7-8 °C. At 12-14 °C, D-shell veligers developed by day 8 without being fed. Egg size and shell morphology indicate planktotrophy, but feeding was not observed. Embryos developed normally from 7-15 °C and 35-45 ppt. Although survival of larvae declined with temperature, some survived at 25 °C. Larval survivorship was similar at 35 and 45 ppt. Oxygen consumption increased from blastulae to trochophores and was higher for *"B." childressi* than for shallow-water mussel trochophores. Estimated energy content of *"B." childressi* eggs was greater than the energy content of shallow-water mussel eggs. An energetic model predicts that the

eggs provide sufficient energy for *"B." childressi* trochophores to migrate into the euphotic zone. In fact, *"B."childressi* veligers were found in plankton tows of surface waters.

The influence of recruitment on fine-scale distributions of adults at the Brine Pool cold seep was examined through manipulative field experiments. The "*Bathymodiolus*" *childressi* population at this site has a distinct bimodal size structure that shifts across an environmental gradient. New recruits of "*B.*" *childressi* are abundant in the inner zone, where methane and oxygen are high and sulfide is low, leading to the inference that larvae settle preferentially there. Experiments were placed in the inner and outer zones and 2-m away from the bed. The number of larvae collected in traps did not differ among the three zones, nor did settlement density. Juveniles survived and grew in all zones, but more caged than uncaged juveniles survived. Mortality of uncaged juveniles was similar in all zones, suggesting that predation does not cause the bimodal distribution. These results suggest that the bi-modal distribution cannot be attributed to settlement preferences or juvenile mortality, but instead to migration or early post-settlement mortality.

This dissertation includes my co-authored materials.

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This dissertation is dedicated to my parents, who taught me the value of a little hard work and perseverance, and to the memory of grandma and uncle, who were extraordinarily proud of me even before this achievement was completed.

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CHAPTER I

GENERAL INTRODUCTION

Most marine mussels from the family Mytilidae have complex life-cycles that include both benthic and pelagic phases (Fig. 1). Following gametogenesis in these adult mussels, gametes are free-spawned into the water column where fertilization takes place and embryos develop into planktotrophic (feeding) veliger larvae. After feeding and growing in the water column, veligers then develop into pediveligers and discriminately settle onto a benthic substratum. In some cases, after initial settlement the plantigrade may drift by means of a byssus thread before a secondary settlement occurs. Finally, the plantigrades are "recruited" into the adult population.

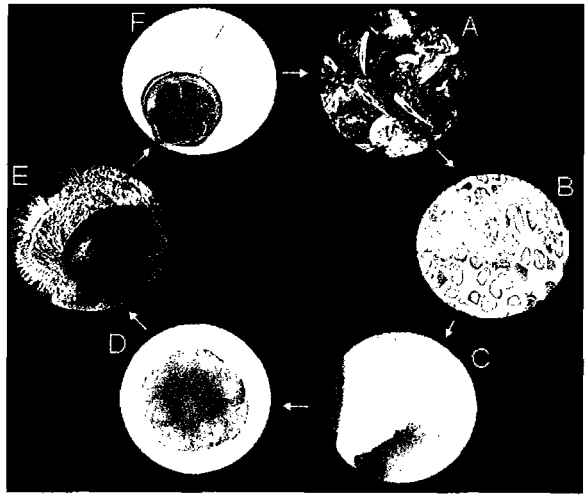

Figure 1. Generalized life cycle of marine mussels showing (A) benthic adults, (B) gametogenesis, (C) free spawning, (D) development in the water column, (E) feeding veligers larva, and (F) the pediveliger probing a settlemenet substratum.

Most of the phases of the life cycles take place in the water column—a very different environment from the benthic adult. Nevertheless, the pelagic phase of life is inextricably linked to the dynamics of the benthic population (Underwood and Keough, 2001; reviewed in Young, 1987; Underwood and Fairweather, 1989). The temporal and spatial variations in production of larvae, their mortality in the plankton and dispersal, the supply of larvae to populations, as well as larval settlement, and survival and growth of new recruits all have important consequences on the temporal and spatial dynamics of adult populations on both ecological and evolutionary time scales.

The importance of the larval phase in determining the spatial and termporal patterns of rocky intertidal mussel populations has been explored for years (reviewed in Lutz and Kennish, 1992; Seed and Suchanek, 1992). The discovery of life at hydrothermal vents in 1977 (Lonsdale, 1977; Corliss and Ballard, 1977) and later at cold methane seeps (Paull, 1984; Hecker, 1985), including dense populations of endemic marine mussels, immediately raised questions about the role of larvae in the connectvity and colonization of these patchy and often ephemeral habitats (Corliss and Ballard, 1977; Corliss *et al.,* 1979; Lutz *et al.*, 1980; Lutz *et al.*, 1984).

Reproductive and larval ecology of bathymodiolin mussels

Mytilid mussels are represented at deep-sea reducing habitats as members of the subfamily Bathymodiolinae (Gustafson *et al.*, 1998). Within this subfamily, the genus *Bathymodiolus* consists entirely of species that live at hydrothermal vents or cold seeps and bear endosymbionts (thiotrophic or methanotrophic) in their gills that serve as their

primary energy source. Little is known of the reproductive and larval ecology of bathymodioloin mussels. Here we summarize the state of the current knowledge.

Gametogenesis

Like most shallow-water mytilids (Strathmann, 1987), the hydrothermal vent mussels *Bathymodiolus azoricus* (Comtet and Desbruyères, 1998; Comtet et al., 1999; Colaço et al., 2006) and *Bathymodiolus puteoserpentis* (Le Pennec and Beninger, 1997) have seasonal gametogenic cycles. Likewise, gametogenesis in *"Bathymodiolus" childressi* is periodic and synchronous among at least three seep sites in the Gulf of Mexico (Tyler *et al.*, 2006).

Development

No descriptions of development for any bathymodiolin mussel are known. However, developmental mode has been inferred in bathymodiolin mussels by examining characteristics such as egg size and the relative size of the larval shells (prodissoconchs I and II). Larvae from species with small eggs typically are planktotrophic (feeding) and those from species with large eggs are usually lecithotrophic (non-feeding) (Thorson, 1950). The relative sizes of the prodissioconch I and II are related to oocyte size. A larger prodissonconch I indicates lecithotrophy, since it is produced from energy reserves in the egg, while a larger prodissoconch II indicates planktotrophy since it is produced during feeding (Ockelmann, 1965). Bathymodiolin mussels are generally considered planktrophic based on the small egg sizes known for the hydrothermal vent mussels *Bathymodiolus azoricus* (Colaço *et al.*, 2006), *Bathymodiolus puteoserpentis* (Hessler *et al.*, 1988), *Bathymodiolus elongata* (LePennec and Beninger, 1997), and *Bathymodiolus*

thermophilus (Berg, 1985), and the cold-seep mussel *"Bathymodiolus" childressi* (see Chapter 2). Likewise, the prodissoconch II's of several species are known to be large (see Table 5 in Chapter 2), indicating planktotrophy.

Symbiosis

Indirect evidence suggests that the hydrothermal vent mussels *Bathymodiolus puteoserpentis* and *Bathymodiolus azoricus* acquire their symbionts from the environment rather than transferring them via the ovum (Won *et al.*, 2003). Transmission electron microscopy reveals the presence of bacterial symbionts in the gill tissue of new recruits and juveniles of the vent mussel *B. azoricus* and the seep mussel *Bathymodiolus heckerae* (Salerno *et al.*, 2005), but it is unknown at what stage these mussels are actually infected. However, isotopic analysis did not suggest that the larval diet of *B. azoricus* or *B. heckerae* consisted of photosynthetically derived organic material (Salerno *et al.*, 2005).

Dispersal

Early reports suggest that hydrothermal-vent larvae probably do not migrate to the surface waters because migration would increase advection of larvae away from suitable habitats (Lutz *et al.*, 1980; Lutz *et al.*, 1984). Hydrothermal vent plumes can entrain and passively transport larvae up and away from vent sites (Kim and Mullineaux, 1998), but near-bottom currents most often transport larvae of hydrothermal vent species (Kim and Mullineaux, 1998; Mullineaux *et al.*, 2005). Because the abundance of bathymodiolin mussel larvae decreased with height above vents along the East Pacific Rise, Mullineaux *et al.* (2005) hypoethesize that larval behavior may maintain these larvae near the bottom.

Scope and objectives of this dissertation

My primary objective in developing this dissertation project was to use manipulative laboratory and field experimentation to examine the linkages between the early life-history and adult populations for the deep-sea benthos endemic to reducing habitats.

Chapters II through IV are focused on using laboratory tests to gain insights into the pelagic phase of life of the mussel *"Bathymodiolus" childressi*. Its unique morphology (Gustafson *et al.*, 1998) and a recent analysis of molecular phylogeny (Jones *et al.*, 2006) raise uncertainty about the taxonomic status of *"B." childressi*. Thus, we follow the recommendation of Jones *et al.* (2006) and place the genus name of "*Bathymodiolus*" *childressi* in quotation marks until taxonomists determine whether a new genus need be erected. "*Bathymodiolus*"*childressi* resides solely at cold seeps and is currently known from the hydrocarbon and brine seeps in the Gulf of Mexico only (Gustafson *et al.*, 1998), where it is often one of the most prominent members of the community. It is known from seep sites in the northern and western Gulf of Mexico ranging in depth from 546 to 2222 m (Gustafson *et al.*, 1998). *"Bathymodiolus" childressi* is a mixotrophic mussel harboring methanotrophic endosymbionts in the gills (Childress *et al.*, 1986) that provide fixed carbon to the host mussel, but can also supplement its dietary needs by filter feeding (Page *et al.*, 1991; Pile and Young, 1999).

The results in Chapters II through IV are discussed in the context of the consequences of development, biology, physiology, and behavior of the larvae on their potential distribution, both vertically and horizontally. In Chapter II, I develop culturing

techniques, describe the embryonic development of *"Bathymodiolus" childressi*, and infer the duration of their planktonic life. Chapter II has been accepted for publication in *The Biological Bulletin* with S.M. Arellano as the primary author and C.M. Young as co-author. The research was funded by NSF grant OCE-0118733 awarded to C.M.Y, who also gave advice on the research and manuscript and edited the final written manuscript. In Chapter III, I examine the physiological tolerances of the larvae to varying temperatures and salinities. In Chapter IV, I utilize an energetic model to estimate the potential for ontogenetic vertical migration of this species and I compare the model's estimates to field observations.

While the opening chapters are focused primarily on the larval life, Chapter V focuses on the recruitment of larvae of *"Bathymodiolus" childressi* into the Brine Pool mussel population. Through a series of manipulative field experiments, we attempt to determine which pre- or post-settlement factors are responsible for a conspicuous bimodal distribution of small and large mussels across the mussel bed.

CHAPTER II

SPAWNING, DEVELOPMENT, AND THE DURATION OF LARVAL LIFE IN A DEEP-SEA COLD-SEEP MUSSEL.

Note

This manuscript has been accepted for publication in *The Biological Bulletin* with S.M. Arellano as the primary author and C.M. Young as co-author. The research was funded by NSF grant OCE-0118733 awarded to C.M.Y. In addition, C.M.Y. gave advice on the research and the manuscript and edited the final written manuscript.

Introduction

Earlier predictions that development in the deep sea should be limited to brooding and direct development (e.g., Thorson, 1950) have now been negated (Pearse, 1994; Young, 1994) and it is well-accepted that virtually all known modes of development are found in the deep sea (reviewed by Young, 2003). Ever since abundant life was discovered at hydrothermal vents (Lonsdale, 1977; Corliss and Ballard, 1977) and later at cold methane seeps (Paull, 1984; Hecker, 1985) it has been recognized that larval dispersal is a central issue in understanding the connectedness and colonization of these patchy and often ephemeral habitats (Corliss and Ballard, 1977; Corliss *et al.,* 1979; Lutz *et al.*, 1980; Lutz *et al.*, 1984). Moreover, it was soon recognized that our traditional understanding of the relationships among developmental mode, dispersal potential, and

geographic range may not always hold true in these chemosynthetic systems (Lutz *et al.*, 1980; Lutz *et al.*, 1984) or even in the deep sea in general (Young et al., 1999). For example, planktotrophic (feeding) larvae are generally presumed to disperse for longer times and greater distances than lecithotrophic larvae or brooded embryos (Thorson, 1950; Wray and Raff, 1991), but developmental and metabolic rates decrease with temperature (Clarke, 1983), and can result in an extended dispersal potential of lecithotrophic larvae in the cold deep sea (Lutz *et al.*, 1984; Turner *et al.*, 1985; Gustafson and Lutz, 1994; Young *et al.* 1997; Le Pennec and Beninger, 2000; reviewed by Young, 2003; O'Connor *et al.*, 2007). This is only one of several reasons why dispersal potential cannot be inferred from egg size alone. Laboratory studies describing developmental modes, developmental rates, and physiological tolerances of larvae as well as current measurements in the field are required to relate developmental mode to dispersal potential and geographic distribution (Lutz *et al*, 1984; Turner *et al.*, 1985).

Culturing larvae of deep-sea species was once thought impossible (e.g., Turner *et al.*, 1985) and is admittedly difficult. Nevertheless, a number of deep-sea invertebrates have been cultured at least through the early larval stages. For example, numerous bathyal echinoderms have been cultured to the four-arm pluteus stage (Mortensen, 1921; Young and Cameron, 1989; Young *et al.*, 1989) and two species have been reared to more advanced stages (Prouho, 1888; Young and George, 2000). It is much more difficult to culture species from abyssal depths, where embryos often require high pressures to develop (Young and Tyler, 1993; Young et al., 1996). Nevertheless, a few hydrothermal-vent and cold-seep organisms have been cultured through the early

embryonic stages, with the abyssal vent organisms requiring the use of pressurization techniques (Young *et al.*, 1996; Eckelbarger *et al.*, 2001; Pradillon *et al.*, 2001, 2005; Marsh *et al.*, 2001).

Mytilid mussels, which are among the most prominent members of many reducing communities, inhabit a wide depth range of cold-seep communities along the upper continental slope in the northern Gulf of Mexico. Five species of mytilid mussels of the subfamily Bathymodiolinae have been described from seeps in this region (Gustafson *et al.*, 1998). Among them, *"Bathymodiolus" childressi* Gustafson *et al.* 1998, a mixotrophic mussel harboring methane-oxidizing endosymbionts in the gills, resides at cold seeps over a depth range from ~540 m to 2200 m (Gustafson *et al.*, 1998). Its unique morphology (Gustafson *et al.*, 1998) and a recent analysis of molecular phylogeny (Jones *et al.*, 2006) raise uncertainty about the taxonomic status of *"B." childressi*. Thus, we follow the recommendation of Jones *et al.* (2006) and place the genus name of "*Bathymodiolus*" *childressi* in quotation marks. There is no evidence for genetic differentiation between "*B.*" *childressi* populations from the shallowest and deepest seep sites, nor is there a relationship between genetic structure and geographic distance across its range, suggesting widespread larval dispersal (Carney *et al.*, 2006). Small egg size, postulated high fecundity, and the shell apices of four vent bathymodiolin mussels (reviewed in Tyler and Young, 1999) and three seep bathymodiolin mussels including *"B." childressi* (Gustafson and Lutz, 1994, Gustafson *et al.*, 1998) all suggest that these species develop planktotrophically and therefore may have high dispersal potential.

Neither the developmental mode nor larval duration of *"Bathymodiolus" childressi* has been determined. Moreover, no descriptions of either early embryology or complete larval development have been published for any deep-sea mollusk (reviewed by Young, 2003; but see Van Gaest, 2006). This study provides the first description of larval culturing techniques and larval development through the early veliger stage for any hydrothermal-vent or cold-seep bivalve and includes a description of the larval shell that can assist in identifying larvae collected from the plankton. We also provide an indirect estimate of larval life span based on a comparison of known spawning times with settlement times estimated from field data on juvenile growth and recruitment.

Materials and Methods

Collection Sites

"Bathymodiolus" childressi adults were collected from two cold-seep sites on the upper continental slope of Louisiana. Mussels were sampled primarily from Brine Pool NR1 (27°43'24" N, 91°16'30" W), a brine-dominated seep located approximately 285 km southwest of the Mississippi Delta at a depth of ~ 650m (MacDonald *et al.*, 1990; 1998). When compared to sites dominated by petroleum seepage (MacDonald, 1998), mussels at the Brine Pool tend to have faster growth and better physiological condition (Nix *et al.*, 1995; Bergquist *et al.*, 2004). Occasionally, mussels were sampled from Bush Hill (27°47'N, 91°30'24"W), an oil-dominated seep at ~540 m depth that is characterized by large aggregations of vestimentiferan tubeworms associated with clumps of *"B." childressi* mussels (McDonald, 1998). Mussels at this site grow relatively slower and are

in poorer physiological condition than those at Brine Pool (Nix *et al.,* 1995; Bergquist *et al.*, 2004).

Collection and Maintenance

The *Johnson-Sea-Link I* and *II* submersibles (Harbor Branch Oceanographic Institution) were used to collect samples. Collections were made with the submersible's hydraulic clam-bucket scoop and placed in a thermally stable acrylic box for transport to the surface. Additionally, mussels were placed in plastic mesh cages at the Brine Pool and recovered later using acoustic releases (see Tyler *et al.*, 2006). Once on deck, the mussels were transferred to clean, cold seawater immediately and maintained in a cold room (7-8° C) until they were transported back the Oregon Institute of Marine Biology (OIMB). When shipboard maintenance time was lengthy (up to two weeks), aquaria containing mussels were bubbled with methane gas periodically and aerated constantly, and seawater was changed regularly.

Aquaria for long-term maintenance at OIMB consisted of a row of six re-circulating tanks (150 L each) connected to a titanium-coil seawater chiller set at 7-8° C. Plastic baskets containing the mussels were transferred daily to a chilled re-circulating feeding tank (200 L) where mussels were "fed" by bubbling methane and air into the tank for at least 30 minutes. Methane levels reach greater than 200 μM at the Brine Pool cold seep (Smith *et al.*, 2000), but only up to 60 μM at Bush Hill (Nix *et al.,* 1995). Because we were unable to measure the methane concentrations in the seawater to determine when the system was saturated with methane, we left mussels in the feeding tank for at least 3 hours with only air bubbling into them, allowing time to oxidize any remaining

methane. Both tank systems were filled with raw seawater from the OIMB running seawater system, which pumps from the inlet of Coos Bay, OR only on incoming tides (salinity 32). Seawater was changed approximately monthly in the feeding tank and once every few months in the maintenance tanks.

Spawning and Culturing

Histological evidence indicates that "*Bathymodiolus" childressi* at the Brine Pool and Bush Hill spawn periodically over an extended period each year that lasts from October to February (Tyler *et al.*, 2006). Mussels collected prior to the known spawning season were maintained for up to several months and used for developmental studies in the late fall and winter.

Spawning and culturing procedures were developed from recommendations reviewed by Strathmann (1987). Spawning was induced in mature mussels (sizes ranging from ~50mm to ~120mm) by a 0.4-0.5 ml injection of 2 mM serotonin into the anterior adductor mussel (Fig. 1a). Prior to injection, mussels were scrubbed and rinsed in fresh water. After injection, approximately 12 mussels were placed in a single 4-L container of 0.22μm filtered seawater and placed in a 7-8° C cold room until they spawned (Fig. 1b,c). After mussels spawned, fertilized eggs were cleaned and placed in clean 0.22 μm filtered seawater. Embryos were maintained in a 7-8° C cold room. One set of cultures in November 2003 (Table 1) was divided after hatching between a 12-14 °C seawater table and the 7-8 °C incubator. For general maintenance, dense cultures (up to 50 embryos per ml) were kept in 2-L glass jars and were not stirred. Larvae were fed *Isochrysis galbana* (approximately 40,000 cells per ml) after hatching, and water was changed daily.

Figure 1. Induction of spawning in *"Bathymodiolus" childressi*. (A) Injection of 2 μM serotonin to the anterior adductor muscle, (B) the release of gametes bound in mucous (scale bar = 50 mm), and (C) negatively buoyant eggs (scale bar = 5 mm).

Table 1. Collection, maintenance time at OIMB, and months spawning was induced in large *"Bathymodiolus" childressi* mussels collected from the Brine Pool and Bush Hill cold seeps. Also indicated is whether induced mussels were maintained together or kept in separate containers, and the maximum developmental stage observed before cultures ceased developing.

Collection Date	Months Maintained	Month Induced	Induced Separately?	Max. Dev. Stage
June & October 2002	2-5	December 2002	Yes	16 cell
June & October 2002	3-6	January 2003	No	Shells forming
February 2003	0.5	March 2003	No	Hatched
November 2003	0.5	December 2003	No	Hatched
November 2003	0.5	December 2003	Yes	No fertilization
November 2003	2	January 2004	Both	D-shell*
November 2003**	2.5	February 2004	Yes	Hatched
July 2004	5	January 2005	Yes	Few Hatched
July 2004	6	February 2005	Yes	No development
August 2006	2-3	Oct. – Nov. 2006	No	Hatched

* D shells developed in a culture that had been raised from an incubation temperature of 7-8 °C to 12 °C approximately 48 hours after hatching.

**some of the mussels induced were maintained at C.R. Fisher's lab at PSU for up to one year.

Microscopy

Light micrographs were taken of each developmental stage on an Olympus BX50 compound microscope with a 40x Nomarski DIC objective, using an Optronics Microfire digital camera. Developing shells were visualized using cross-polarizing filters.

Representative stages were fixed for scanning electron microscopy in 2.5 % glutaraldehyde, washed with Millonig's 0.2 M sodium phosphate buffer wash, and post-fixed in 1% osmium tetroxide buffered in 0.4 M Millonig's buffer and 0.75 M NaCl (1:1:2). Scanning electron micrographs were taken of eggs, embryos, and larvae on a JEOL 6400F Field Emission Scanning Electron Microscope.

Embryological Timing

Because spawning attempts were most successful when multiple mussels were placed in a single container over an extended period of time, our cultures were never synchronous. We drew regular samples of at least 100 embryos and staged them all under 40-x magnification to construct an approximate timetable for development at 7-8 ^{o}C. However, because we could not pinpoint a fertilization time (as can be done with synchronous cultures), our timetable was measured from the time that sperm were removed from the cultures.

Estimation of Planktonic Larval Duration

Scoops of *"Bathymodiolus" childressi* were taken from "inner zone" (Smith et al. 2000, Chapter 5) of the Brine Pool cold seep in March 2002, October 2002, February 2003, September 2003, November 2003, and July 2004. Upon recovery, we counted and measured new recruits (individuals $\leq$ 10 mm) and plotted the size-frequency distributions

of individuals in one-millimeter bins. From the measured lengths, we back-calculated the approximate date of settlement using a settlement size of 0.5 mm long (the size of the prodissoconch in newly settled juveniles) and a mean growth rate of 1.44 $\pm$ 0.30 (S.D) mm 30 d^{-1} as determined by a mark-recapture experiment described elsewhere (Chapter 5). This calculated growth rate also agrees with the growth rate for this size class calculated from Smith *et al.* (2000). We plotted the estimated percentage of individuals settling each month over the entire sampling period.

Shell Description

Veloconcha were collected with larval tube traps placed at the Brine Pool cold seep. Tube traps were 5 cm-wide, 30 cm-tall PVC pipes (aspect ratio = 6) that were mounted on ~2-kg iron discs and had their tops open to capture larvae by horizontal advection across the openings (Yund *et al.*, 1991). Studies suggest that cylindrical traps with aspect ratios above 4 are least susceptible to re-suspension (Yund *et al.*, 1991). In our samples, very few bivalves captured had begun to form a dissoconch. Larval tube traps were filled with 10 % formalin buffered in seawater to prevent escape of larvae (Yund *et al.*, 1991). Post-larvae were collected from settlement racks (Chapter 5) and from plastic "tuffy" scouring pads placed on the "*Bathymodiolus" childressi* mussel bed at the Brine Pool cold seep. Upon recovery, veloconcha and juveniles were transferred to 70% ethanol.

Shells were cleaned in 5% sodium hypochlorite solution, rinsed with distilled water, air-dried, and mounted on adhesive carbon discs for SEM (Rees, 1950; Fuller and Lutz, 1989). Procedures to accurately document the shapes and dimensions of the larval

bivalve shells were modified from those of Fuller *et al.* (1989). The following measurements were taken for ten larval and postlarval shells: height and length of prodissoconch II or dissoconch, shell length and straight hinge length of the prodissoconch I (if possible), provinculum length, and number of teeth. For larval shells, length is the greatest dimension approximately parallel to provinculum and height is the greatest dimension perpendicular to the hinge line. For postlarval shells, dimensions follow convention for adults, with length measured as the greatest antero-posterior dimension and height as the greatest dorso-ventral dimension (Gosling, 1992).

Results

Spawning and Culturing

Vigorous handling, rapid temperature changes, stretching of the adductor muscles, and electrical shock have proved successful in inducing spawning in mussels and other bivalves (Strathmann, 1987), but none of these techniques induced spawning in "*B.* *childressi*. Serotonin injection induced spawning in mature mussels usually within approximately 8-12 hours. Spawning was most successful when several mussels that had been injected with serotonin were placed in a single container. Attempts to spawn mussels in individual containers to keep sperm and eggs separate were mostly unsuccessful, but occasionally produced cultures that developed to hatched blastula larvae (Table 1). Spawning could be induced regularly in mussels that had been maintained in the lab for up to several months (Table 1). Twice, mussels that were maintained in C.R. Fisher's laboratory at The Pennsylvania State University for up to one year were induced to spawn with serotonin injection. However, normal development

proceeded to the D-shell veliger stage only once: when mussels were collected during their spawning season and maintenance time at OIMB was less than 2 months (Table 1).

Natural spawning occurred in the laboratory on two occasions. In both cases, mussels were collected late in the summer. Mussels that were collected in July 2004 and August 2006 spawned in late Aug. – Sept. 2004 and late August 2006, respectively. In both years these cultures developed to hatched blastula larvae, but never began forming shells. Mussels released gametes bound in mucus, but eggs are easily dissociated from the mucus (Fig. 1b,c). Attempts were not made to estimate fecundity, as induction with serotonin can be unreliable and often induce spawning of immature oocytes.

Development

The mean egg diameter was 69.15 $\pm$ 2.36 μm ($\pm$ S.D.; n = 50). Eggs are negatively buoyant and eggs and embryos are dense and optically opaque (Fig. 1, 2). Fertilization was evident by appearance of a thin fertilization envelope (Fig. 2b). In no case was fertilization 100% successful; generally it was less than 50%. Polar bodies appeared within approximately 2.5 hours of fertilization (Fig. 2c, 3a; Table 2). Polar lobes developed prior to first cleavage (Fig. 2c), which results in two unequal blastomeres (AB and CD) (Fig. 2e, 3b). After 7-15 hours, the second cleavage produced 3 equal bastomeres (A, B, C) and a larger D blastomere (Figs. 2f, 3c; Table 2). Successive cleavages followed the typical molluscan spiral cleavage pattern at an average developmental rate (at 7-8 ^{o}C) of one division per 3-9 hours through hatching, with free-swimming blastula larvae hatching by 40 hours (Table 2).

Table 2. Approximate developmental timetable for *"Bathymodiolus" childressi.* Ages are the earliest noted development times at 7-8 °C.

Age (hours)	Developmental Stages present
2.5	Polar body extrusion — 2 cell
7	Polar body extrusion — 4 cell
15	4 – 8 cell
21	8 – 16 cell
30	32 cell – morula
40	32 cell – hatched
~170	Shelled
~150	Shelled*
~185	D shell veligers*

**Culture temperature was raised to 12 °C after hatching.*

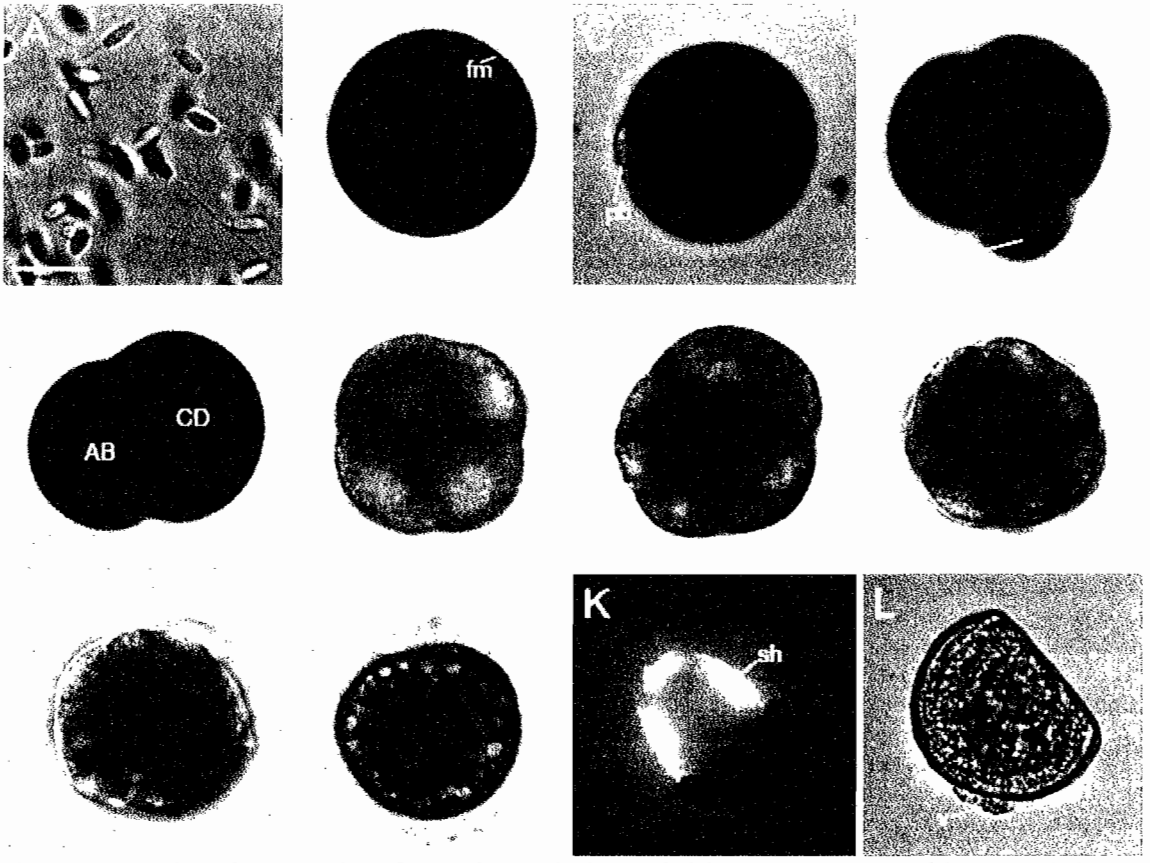

Figure 2. Light micrographs of *"Bathymodiolus" childressi* gametes and larvae. (A) Sperm, with clearly visible acrosomes, scale bar = 30 μm (B) fertilized egg, (C) polar body extrusion, (D) third polar lobe, (E) 2 cell stage with AB and CD cells indicated, (F)-(I) 4-32 cell stages, (J) hatched blastula with long uniform cilia, (K) shell formation with shells illuminated by cross polarized light, and (L) D-shell veliger larva. (B) – (L) Scale bar = 50 μm. fm, fertilization membrane; pb, polar body; pl, polar lobe; c, cilia; sh, shell; v, velum.

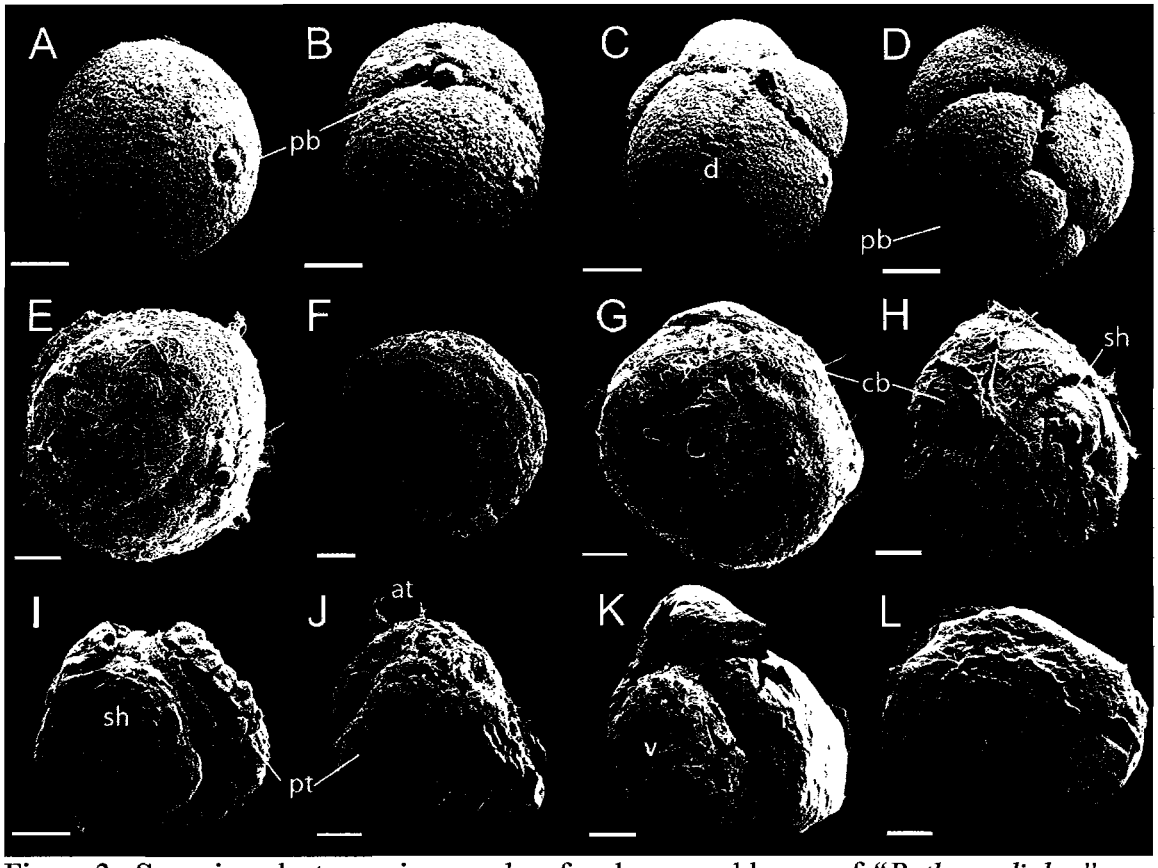

Figure 3. Scanning electron micrographs of embryos and larvae of *"Bathymodiolus" childressi*. (A)-(D) polar body through 8 cell stages, (E)-(H) ciliary band formation, and (I)-(L) early veligers. Scale bars = 10 μm. pb, polar body; d, large D cell; cb, ciliary band; sh, shell; at, apical tuft; v, velum.

At 7-8 °C, shells began forming on day 6. In those cultures that were increased in temperature to 12-14 °C after hatching, more individuals developed shells than in cultures that remained at 7-8 °C. On day 6, 11% had shells in 7-8 °C cultures (n = 101) and 28% had shells in 12-14 °C cultures (n = 103). In some cases, development was delayed, with larval shell formation beginning as late as day 12.

Cultures were maintained for up to two weeks but never metamorphosed in the lab. Although we could not rear larvae to metamorphosis, we did estimate the planktonic larval duration by examining the size distribution of new recruits. Although we generally saw only one clear size peak of individuals ≤10 mm per sampling period (Fig. 4), we saw 5 distinct settlement peaks over a 34 month period—almost two peak settlement periods per year (Fig. 5; Table 3). By comparing those settlement peak dates to the previous spawning periods, we estimate a planktonic larval duration of up to 13 months (Table 3).

Table 3. Estimates of planktonic larval duration of *"Bathymodiolous" childressi* based on major settlement peaks (from Fig. 5) and spawning seasons from October through February (Tyler *et al.*, 2006).

Settlement Peak	Previous spawning period	Approximate larval duration
Nov '01	Oct '00 – Feb '01	9 to 13 months
May '02	Oct '01 – Feb '02	2 to 8 months
Nov '02	Oct '01 – Feb '02	9 to 13 months
Jun '03	Oct '02 – Feb '03	3 to 8 months
Mar '04	Oct '03 – Feb '04	up to 5 months

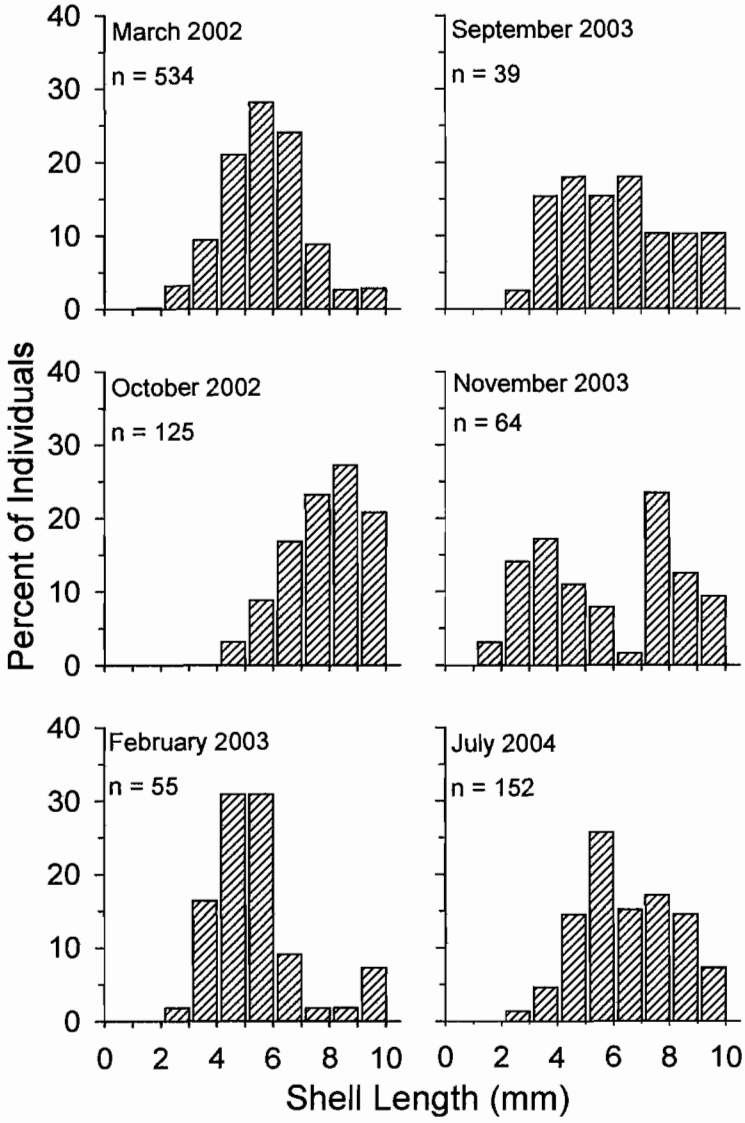

Figure 4. Size-percent frequency for new recruits (≤ 10 mm length) of *"Bathymodiolus" childressi* at the Brine Pool cold seep in March and October 2002, February, September, and November 2003, and July 2004.

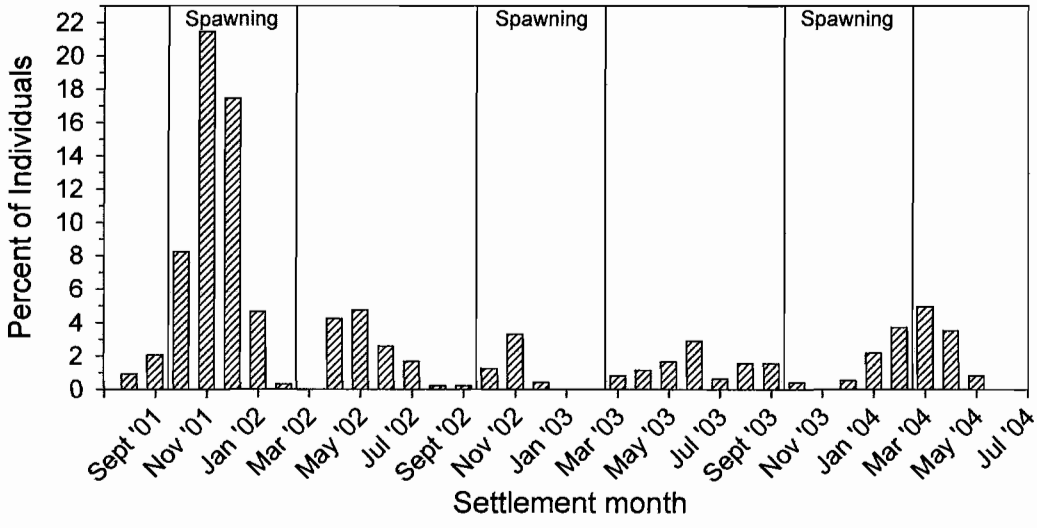

Figure 5. Percent of settlement (n = 969) by month of *"Bathymodiolus" childressi* at the Brine Pool cold seep over a 34 month period. Settlement dates were back-calculated from lengths of recruits (Fig. 4) using a growth rate of 1.44 $\pm$ 0.30 (S.D.) mm 30 d^{-1} and a settlement size of 0.5 mm. Spawning period is indicated between vertical lines.

There was no evidence that larvae fed on *Isochrysis galbana* in the lab. Algae were not observed in the gut either with normal DIC optics or with epifluorescence. Indeed, larvae developed to D-shell veligers by day 8 in those cultures that were increased to 12-14 °C after hatching and these larvae were never fed. At day 10, these D-shell veligers ranged from 86.70 to 103.56 μm long ($\bar{x} \pm$ S.D. = 96.24 ± 8.31; n = 4).

Figure 6 shows larval and post-larval shells of *"Bathymodiolus" childressi* collected in larval tube traps and on settlement surfaces. Prodissoconchs I and II were reddish in color (as previously noted by Gustafson *et al.* 1998), in sharp contrast to the yellowish dissoconchs (Fig. 7). Lengths of larval shells (PII) collected in the tube traps ranged from 432.71 to 453.60 μm ($\bar{x} \pm$ S.D. = 442.56 ± 8.84 μm; n = 5). For these shells, the length of prodissoconch I was 113.35 ± 2.02 μm and the provinculum was 210.15 ± 10.94 μm long, with 31 teeth (Table 4, Fig. 6b). Teeth along the hinge line were numerous and fine and increased in size along the anterior and posterior extensions of the provinculum (Fig. 6b). Larval shells exhibited the "egg shape" (Figs. 5, 6) that is characteristic of mytilids (Chanley, 1970; Le Pennec, 1980).

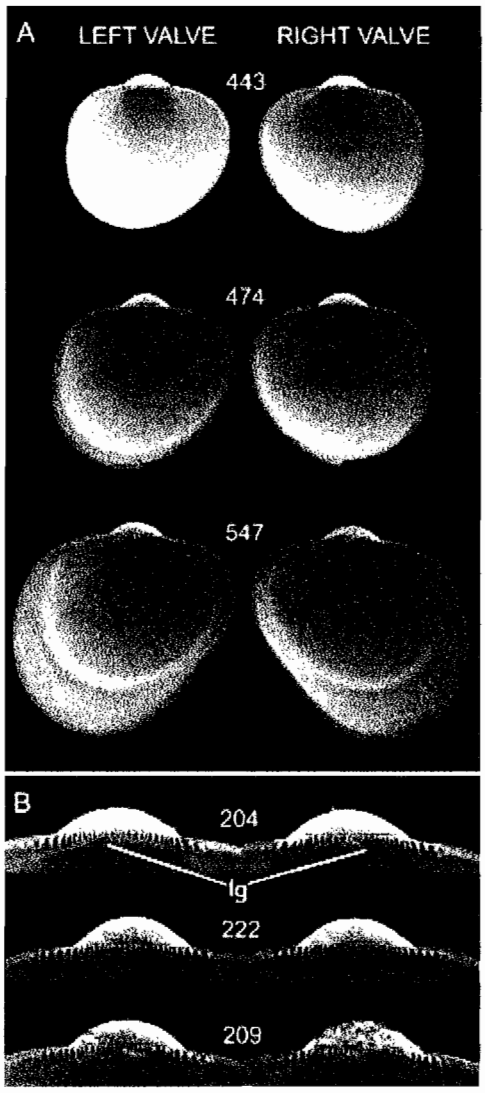

Figure 6. Larval and post-larval shells of "*Bathymodiolus" childressi* collected *in situ* in larval tube traps and on settlement plates. (A) Shows a larval and two post-larval shells with the PII lengths (μm) shown, and the corresponding photos in (B) and the provinculum of each shell, with their lengths (μm) listed. lg marks the ligament.

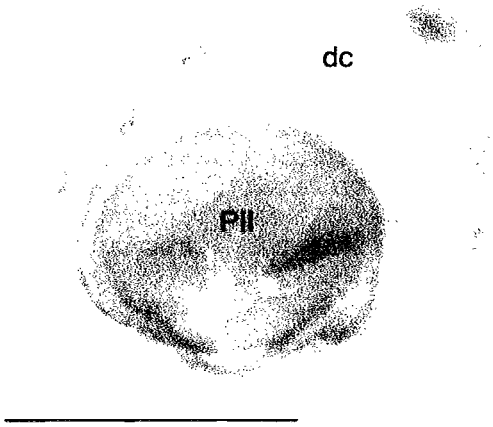

Figure 7. Post-larva of "*Bathymodiolus*" *childressi* collected *in situ* at the Brine Pool cold seep. Note the easy distinction of the reddish prodissoconch II (PII) from the yellowish dissoconch (dc). Scale bar = 500 μm.

Table 4. Larval and post larval shell dimension for *"Bathymodiolus" childressi*. PI and PII are the prodissioconchs I and II and are measured for D-shells and larval shells. PI and dissoconch are measured for post-larval shells. Hinge is the length of the hinge line.

	PI		PII / Dissoconch		Provinculum	
	Hinge	Length	Length	Height	Length	# Teeth
D – shell*	71.98	103.56	n/a	n/a	unknown	unknown
Larval	91.19	--	453.6	428.08	221.21	31
	89.58	115.69	443.23	416.45	204.23	31
	90.46	114.35	434.86	408.54	202.55	31
	89.66	111.45	448.39	408.96	222.78	31
	86.14	111.9	432.71	416.10	199.96	31
Mean	*89.41*	*113.35*	*442.56*	*415.63*	*210.15*	*31*
S.D.	*1.94*	*2.02*	*8.84*	*7.92*	*10.94*	*0*
Post – larval	92.37	115.81	474.24	462.56	222.17	31
	91.87	118.73	546.82	454.17	209.04	31
	92.09	117.42	547.82	460.76	212.87	31
Mean	*92.11*	*117.32*			*214.69*	*31*
S.D.	*0.25*	*1.46*			*6.75*	*0*

* D-shell is an individual cultured in the laboratory.

Discussion

A major hurdle to the study of hydrothermal vent and cold seep organisms is the extreme difficulty of culturing their larvae. To date, only a handful of hydrothermal-vent and cold-seep organisms have been cultured. The vestimentiferan tubeworm *Riftia pachyptila* (Marsh *et al.*, 2001) and the alvinellid polychaete *Alvinella pompejana* (Pradillon *et al.*, 2001; Pradillon *et al.*, 2005), both from the East Pacific Rise, have been cultured using pressurization techniques. More recently, Miyake *et al.* (2006) have cultured the vestimentiferan tubeworm *Lamellibrachia satsuma* that inhabits shallower hydrothermal vents in Kagoshima Bay, Nankai Trough, and along the Izu-Pgasawara Ridge at atmospheric pressure. From cold seeps in the Gulf of Mexico, two vestimentiferan tubeworms, *Lamellibrachia luymesi* and *Seepiophila jonesi*, have been cultured to trochophore larvae (Young *et al.*, 1996), the polychaete "iceworm" *Hesiocaeca methanicola* that resides in methane hydrates at seep sites has been cultured to early ciliated larvae (Eckelbarger *et al.*, 2001), and most recently, encapsulated embryos of the cold-seep neritid gastropod *Bathynerita naticoidea* have been reared in the laboratory to free-swimming, feeding veligers (Van Gaest, 2006). Bathymodiolin mussels, which are dominant members of both vent and seep communities, have not been cultured until now, nor has their larval development been described previously.

Spawning

Evidence suggests that the reproductive patterns of deep-sea invertebrates are often constrained by phylogenetic history (Eckelbarger and Watling, 1995). Like their shallow-water mytilid ancestors (Strathmann, 1987), there is evidence that the

hydrothermal vent mussels *Bathymodiolus azoricus* (Comtet and Desbruyères, 1998; Comtet *et al.*, 1999; Colaço *et al.*, 2006) and *Bathymodiolus puteoserpentis* (Le Pennec and Beninger, 1997) have seasonal gametogenic cycles. Likewise, gametogenesis in *"Bathymodiolus" childressi* is strongly periodic and synchronous among at least three seep sites in the Gulf of Mexico, with the initiation of gametogenesis from December to March, followed by a period of gamete proliferation and spawning from October to February of the following year (Tyler *et al.*, 2006). In the laboratory, *"B." childressi* spawned most readily and cultures developed the furthest when they were collected in November 2003, which is the middle of their spawning season (Tyler *et al.*, 2006), and maintained in the laboratory for only a short period of time (Table 1). In addition, mussels collected in July 2004 and August 2006, near the beginning of their spawning season (Tyler *et al.*, 2006), spawned naturally while being maintained at OIMB. Nevertheless, we found that cultures produced from mussels taken outside of the predicted October to February spawning season (Tyler *et al.* 2006) did not develop as far or regularly as those from mussels taken within the spawning season. Spawning of *"B." childressi* mussels could be consistently induced via serotonin injection by placing multiple individuals of both sexes together in one container after injection; however, using mussels that were collected in November 2003, we successfully produced a small culture that developed to the hatched blastulae stage by inducing mussels individually and obtaining sperm and eggs separately. This suggests that while the presence of gametes of conspecifics may help induce spawning in *"B." childressi,* it may not be

necessary if the adults are collected while they are ripe enough to spawn and maintained in the laboratory for a minimal time period.

Tyler *et al.* (2006) note that the spawning period is 3–4 months in duration, suggesting that individuals could regenerate and spawn repeatedly as seen in *Mytilus edulis* (Myint and Tyler, 1982). Our laboratory studies lend credence to this suggestion; we were able to induce spawning in the same mussels repeatedly over several months, and could even induce spawning in some mussels that had been maintained in the laboratory for up to one year (Table 1).

Larval Development

Early embryonic cleavage patterns in *"Bathymodiolus" childressi* are characteristic of mollusks (Figs. 2, 3). As is typical for embryos developing in the cold waters of the deep-sea (Mullineaux and France, 1995; O'Connor *et al.*, 2007), development of *"B." childressi* is slow, taking 40 hours to reach the hatched blastula stage and approximately one week to begin developing shells at 7-8 °C (Table 1). We cultured larvae of *"B." childressi* to D-shell veligers only when the parent mussels were collected during the peak of their spawning season (Table 1) and the temperature was raised from 7-8 °C to 12-14 °C immediately following hatching. D-shell veligers developed at day 8 (approx. 192 hours) and were maintained through day 10 (max. shell length = 103.56 μm). This growth rate is about half that of shallow-water mytilids. The shallow-water mussel *Mytilus edulis* develops to the D-shell veliger stage by 42 hours at 9 °C (Strathmann, 1987) and Sprung (1984) estimates that larvae of *M. edulis* form a

complete D-shell at 104 hours from fertilization at 6 °C under high food conditions (40 cells ml^{-1}). We were unable to rear larvae beyond the straight-hinge stage.

The mode of development in deep-sea hydrothermal-vent or cold-seep mollusks tends to reflect phylogeny (bivalves: Gustafson and Lutz, 1994; gastropods: Bouchet and Warén, 1994). Because larval culturing techniques had not been developed for deep-sea hydrothermal-vent or cold-seep mollusks until now, developmental mode has been inferred in bathymodiolin mussels by examining characteristics such as egg size and the relative size of the larval shell regions (prodissoconch I and II in bivalves). Larvae from species with small eggs typically are planktotrophic, requiring an extended period in the plankton, and those from species with large eggs are usually lecithotrophic and settle out of the plankton relatively quickly (Thorson, 1950; Wray and Raff, 1991). The relative sizes of the prodissioconch I and II are related to oocyte size. A relatively large prodissonconch I indicates lecithotrophy, since it is produced from energy reserves in the egg, while a relatively large prodissoconch II indicates planktotrophy since it is produced during feeding (Ockelmann, 1965; Lutz *et al.*, 1980).

The egg size of *"Bathymodiolus" childressi* falls within the range of those known for other bathymodiolin mussels and is indicative of planktotrophy. Mean egg diameter for *"B." childressi* is 69.15 $\pm$ 2.36 μm ($\pm$ S.D.; n = 50). The egg diameters of the hydrothermal vent mussels *Bathymodiolus azoricus* (Menez Gwen) range from 70 to 80 μm (Colaço *et al.*, 2006), *Bathymodiolus puteoserpentis* (Snake Pit) range from 50 to 60 μm (Hessler *et al.*, 1988), *Bathymodiolus elongates* (Fiji) range from 50 to 60 μm (LePennec and Beninger, 1997), and *Bathymodiolus thermophilus* is known to have a

small (about 50 μm) egg size (Berg, 1985). Egg sizes of all of the bathymodiolin mussels also fall within the size range (60 to 90 μm) reported for their shallow water relative *Mytilus edulis*, which has planktotrophic larvae (Lutz and Kennish, 1992).

Likewise, the relatively small prodissoconch I and large prodissoconch II of larvae of *"Bathymodiolus" childressi* are indicative of planktotrophy, as previously noted by Gustafson *et al.* (1998). In *"B." childressi,* the prodissoconch I length ranges from 111.45-115.69 μm ($\bar{x} \pm$ S.D. = 113.35 $\pm$ 2.02) (Table 4). Similarly, five other bathymodiolin mussels from hydrothermal vents and cold seeps have prodissoconch I larval shells that range from 95 to 137 μm (Table 5). Prodissoconch I larval shells in shallow-water mytilids range down to 85 μm, as reported for *Brachidontes exustus* (Fuller and Lutz, 1989), and up to 120 μm as Sprung (1984) reported for *Mytilus edulis* (Table 5). The maximum lengths of the prodissoconch II shells in bathymodiolin mussels, up to 600 μm for *Bathymodiolus azoricus* (Salerno *et al.*, 2005), are considerably longer than in shallow-water mytilids, which reportedly range up to only 252 μm for veligers of *M. edulis* (Sprung, 1984) (Table 5). Considering developmental rates are slower for *"B." childressi* than for *M. edulis*, development of bathymodiolin larvae to nearly twice the larval shell size of *M. edulis* would require a lengthy feeding time for bathymodiolin larvae, and, consequently, more time dispersing in the plankton.

Table 5. Larval shell characteristics of hydrocarbon-seep, hydrothermal-vent, and shallow-water mytilid mussels. PI and PII are the prodissioconchs I and II.

Habitat	Species	PI length (μm)	PII length (μm)	Provinculum length (μm)	# teeth	Reference
Hydrocarbon seep	*"Bathymodiolus" childressi*	111.45-115.69 (113.35 ± 2.02)	≤ 453.6	≤ 222.78	≤ 31	this study
		100-110	385-404	--	--	Gustafson and Lutz, 1994 (their Fig. 4.1, 4.2); Gustafson *et al.*, 1998
	Bathymodiolus heckerae	137	468	--	--	Turner and Lutz, 1984 (their "Seep Mytilid Va"); Gustafson and Lutz, 1994
		--	100-600	--	--	Salerno *et al.*, 2005
	Tamu fisheri	--	460	--	--	Gustafson and Lutz, 1994 (their Fig. 4.3); Gustafson *et al.*, 1998
Hydrothermal vent	*Bathymodiolus thermophilus*	95-110	> 400	--	--	Lutz *et al.*, 1980; Turner and Lutz, 1984; Gustafson and Lutz, 1994
		108	470	--	--	Berg, 1985
	Bathymodiolus azoricus	--	100-600	--	--	Salerno *et al.*, 2005
		--	400	--	--	Gustafson and Lutz, 1994 (their "Vent Mytilid III")
Shallow water	*Mytilus edulis*	95-110 (104.1 ± 4.2)	--	70-147	19-32	Fuller and Lutz, 1989
		94	--	--	--	Loosanoff *et al.*, 1966
		95	--	--	--	De Schweinitz and Lutz, 1976
		120 ± 4.0	120 – 252	--	--	Sprung, 1984
		--	--	71-133	19-32	Lutz and Hidu, 1979

Table 4. (continued).

Habitat	Species	PI length (μm)	PII length (μm)	Provinculum length (μm)	# teeth	Reference
Shallow water	*Modiolus modiolus*	100-125	--	95-165	20-29	Fuller and Lutz, 1989
		105	--	--	--	Lutz and Hidu, 1979
	Brachidontes exustus	85-98 (90.2 ± 3.5)	--	67-94	16-26	Fuller and Lutz, 1989
	Brachidontes recurvus	90-165	135-220	--	--	Chanley, 1970
	Ischadium recurvum	95-110 (101.2 ± 4.6)	--	70-93	17-25	Fuller and Lutz, 1989
		90	--	84-98	--	Chanley, 1970
	Geukensia demissa	97-107 (104.9 ± 3.0)	--	69-91	16-22	Fuller and Lutz, 1989
		105	--	--	--	Loosanoff *et al.*, 1966
		105	--	80-90	--	Chanley, 1970
	Amygdalum papyrium	85-98 (92.5 ± 3.4)	--	64-84	14-21	Fuller and Lutz, 1989
		--	--	50-70	--	Chanley, 1970

Although egg size and their larval shells indicate that *"Bathymodiolus" childressi* has a planktotrophic larva, we were unable to confirm this with clear evidence of feeding. We usually fed *Isochrysis galbana* to our cultures after blastulae hatched, but we could not see autofluoresence of the algae in the gut, and cultures developed to similar stages even if they were not fed at all. As a matter of fact, the cultures that developed to D-shell veligers were never fed. There are a number of possible reasons we were unable to confirm feeding in the larvae of *"B." childressi.* First, we began offering food when larvae hatched and began swimming as ciliated blastulae. *Mytilus edulis* larvae develop through the blastula and trochophore stages before fully forming a mouth and gut just hours before beginning to develop their larval shells (Field, 1922). Therefore, it is possible our cultures were simply not ready to feed. We were able to raise larvae to the shelled stages only twice (Table 1). Earlier larval stages were dense and opaque and clearing techniques were not successful, making identification of a mouth or gut difficult.

Nevertheless, veligers developed to the D-shell stage without being fed algae. Survival and development in the absence of feeding is known in other bivalve larvae. Moran and Manahan (2004) have shown that larvae of the bivalve oyster *Crassostrea gigas* survive in excess of 33 days without being fed after fertilization, far beyond the theoretical supply of energy reserves in the egg. Other nutritional sources are known in bivalve larvae. Dissolved organic matter (DOM) has been suggested as an energy source for larvae (Manahan, 1990), including amino acid uptake by the larvae of *C. gigas* (Manahan and Crisp, 1982; Manahan, 1983, 1989 and uptake of dissolved organic carbon by veligers of the zebra mussel *Dreissena polymorpha* (Barnard *et al.* 2006). Larvae of

the bivalve oyster *Crassostrea virginica* consume heterotrophic ciliates and flagellates (Baldwin and Newell, 1991). Bivalve larvae are also capable of ingesting and assimilating some bacteria (Martin and Mengus, 1977; Baldwin and Newell, 1991; Gallager *et al.,* 1994). For example, live bacterial strains have been used successfully as a food source for *in vivo* culture of veligers of *Mytilus galloprovincialis* (Martin and Mengus, 1977) and veligers of *Mercenaria mercenaria* are capable of using the cyanobacterium *Synechococcus* sp. as a food source (Gallager *et al.* 1994). All of these—DOM, ciliates, and bacteria—are potential food sources for developing larvae of *"Bathymodiolus" childressi* mussels.

Unlike the shallow-water mussels and oysters used in most studies to determine potential larval food sources, adult *"Bathymodiolus" childressi* mussels use methane fixed by methanotrophic endosymbionts as their primary carbon source. Whether endosymbionts contribute energy to larvae is unclear because the stage at which *"B." childressi* mussels are infected with their symbionts is unknown. Indirect evidence suggests that the hydrothermal vent mussels *Bathymodiolus puteoserpentis* and *Bathymodiolus azoricus* acquire their symbionts from the environment rather than transferring them via the ovum (Won *et al.*, 2003). The presence of bacterial symbionts in the gill tissue of post-larvae (shell length 0.6-1.2 mm) and juveniles of the vent mussel *B. azoricus* and the seep mussel *Bathymodiolus heckerae* is inferred from transmission electron microscopy (Salerno *et al.*, 2005), but still it is unknown at what stage these mussels are actually infected. Nevertheless, Salerno *et al.* (2005) found no convincing

evidence (by isotopic analysis of post-larvae) that the larval diet of *B. azoricus* or *B. heckerae* consisted of photosynthetically derived organic material.

Implications of Development on Dispersal Potential

Although morphological characteristics may be linked to developmental mode, it is recognized that linkages of developmental type to dispersal potential probably cannot be generalized to organisms that develop in the deep sea (Lutz *et al.*, 1984; Turner *et al.*, 1985; Gustafson and Lutz, 1994; Young *et al.* 1997; Le Pennec and Beninger, 2000; reviewed by Young, 2003). In the Gulf of Mexico, there is no evidence for genetic differentiation between "*Bathymodiolus" childressi* populations in the shallowest and deepest seep sites, nor is there a relationship between genetic and geographic distance, suggesting widespread larval dispersal of this species (Carney *et al.*, 2006). Here we have developed culturing techniques for the first time for any deep-sea bivalve with the expectation that information on the developmental mode and length of larval period might explain the wide geographic range of *"B." childressi.*

Egg and prodissoconch sizes indicate planktotrophy in bathymodiolin mussels including *"Bathymodiolus" childressi*, although we were unable to positively identify a food source for the larvae of *"B." childressi.* Cultures of *"B." childressi* to the D-shell veligers stage show a definitive larval life of at least 8 days, although we kept trochophore larvae in the laboratory for more than 12 days without shell development or metamorphosis. However, it is clear from the capture of much larger late-stage larvae that our laboratory cultures do not give a good estimate of total larval duration. We obtained indirect estimates of larval life spans by examining the size distribution of new

recruits. By back-calculating the settlement dates from the length of new recruits, we saw settlement peaks in November 2001, May 2002, November 2002, June 2003, and March 2004 (Table 3). We calculated larval durations by comparing the settlement date to the most previous spawning period. The two spring peaks (May 2002 and June 2003) indicate planktonic durations of up to 8 months. Two of the settlement peaks occurred in November (2001 and 2002), which is during the spawning season. Because larval development rates are very slow, it is extremely unlikely that these settlers came from the concurrent spawning season; if they did, their entire larval growth to 500μm would have to take place in less than one month. Even larvae of *Mytilus edulis* developing at 6 °C with much higher food concentrations than found in the deep sea would not reach the 500 μm settlement size until after approximately 4 months of development (see Fig. 2 in Sprung, 1984). We reason, therefore, that November settlers must have been drifting for at least 9 months (if they came from the end of the previous spawning season) and perhaps as long as 13 months (if from the beginning of the previous spawning season). Similarly, we estimate that settlers in March 2004 must have come either from the beginning of the October 2003-February 2004 spawning season (about 5 months larval duration) or from individuals spawned the previous year, in which case they might have been swimming for as little as 13 months or as long as 17 months.

Another mollusk from the cold seeps in the Gulf of Mexico also has a lengthy developmental period. The snail *Bathynerita naticoidea* develops within egg capsules for approximately four months before it hatches as a planktotrohic larva (Van Gaest 2006). Subsequent to hatching the veliger has been maintained in the laboratory for more than

90 days (A. Van Gaest, pers. comm.) and it has been captured in the plankton nearly one year after the previous hatching period (Van Gaest, 2006). Long delay of metamorphosis is not uncommon in mollusks. Indeed, veligers of the cymatiid snail *Fusitriton oregonensis* have been maintained in the laboratory for up to 4.5 years before metamorphosis (Strathman and Strathman 2007)! Embryos of some hydrothermal-vent polychaetes arrest development in cold water until warmer temperatures are encountered (Pradillon *et al.*, 2001) and metamorphosis in *Mytilus edulis* can be delayed if an appropriate settlement cue is not available (Bayne, 1965). Thus, a planktonic larval duration of up to one year for *"Bathymodiolus" childressi* is entirely within reason.

The planktotrophic mode of development and lengthy duration of larval development in the plankton suggest a biological explanation for the widespread dispersal of the larvae of *"Bathymodiolus" childressi*. Still unknown is the interaction between behavior and dispersal potential *"B." childressi* larvae. Position in the water column probably influences larval dispersal potential; as larvae ascend through the water column, passive advection by currents should increase. Early hypotheses were that hydrothermal vent larvae probably do not migrate to the surface waters, since migration increases advection of larvae away from suitable habitats (Lutz *et al.*, 1980; Lutz *et al.*, 1984; Turner *et al.*, 1985). Although hydrothermal vent plumes can entrain and passively transport larvae up and away from vent sites (Kim *et al.*, 1994; Mullineaux *et al.*, 1995; Kim and Mullineaux, 1998), near bottom currents most often transport larvae of hydrothermal vent species (Kim and Mullineaux, 1998; Mullineaux *et al.*, 2005). Even planktotrophic bathymodiolin mussel larvae showed decreasing abundance with height

above vents along the East Pacific Rise (Mullineaux *et al.*, 2005), suggesting that larval behavior may maintain these larvae near the bottom. This sort of behavior is not unknown in larval bivalves; for example, Shanks and Brink (2005) provide evidence that certain weak swimming bivalve larvae behave in a manner to maintain their position nearshore during periods of upwelling and downwelling.

Nevertheless, ontogenetic vertical migration is known for other deep-sea mollusks (Killingly and Rex, 1982; Bouchet and Warén, 2004) and negative geotaxis is a common behavior in mytilid mussel veligers (Bayne, 1964). Vertical distribution of the larvae of bathymodiolin mussels from shallower cold seep sites, including *"Bathymodiolus" childressi*, is previously uninvestigated. Genetic studies suggest that larvae of *"B." childressi* disperse widely in the Gulf of Mexico is (Carney *et al.*, 2006) and minimal genetic differentiation between *"B." childressi* and congeners from western and eastern Atlantic seeps has further ignited questions about dispersal capabilities of this species (Olu Leroy *et al.*, 2007). Ontogenetic vertical migration of these larvae combined with their lengthy development period would suggest that these larvae may be teleplanic (long-distance dispersing) which would provide an explanation for dispersal throughout the Gulf of Mexico and across the Atlantic Ocean. Scheltema (1966, 1971a, b, 1988) has demonstrated transatlantic dispersal in several families of gastropods and at least two families of bivalves (Scheltema, 1971c; Scheltema and Williams, 1983). Thus, whether vertical swimming behavior plays a role in the dispersal of these larvae warrants further research. Here we have provided a description of the shell of competent (pediveliger) larvae of *"B." childressi* (Fig. 6, Table 4). Larval shell characters are useful for

identification of bivalve larvae (Rees, 1950; Fuller and Lutz, 1989) and we have used these characters to help identify *"B." childressi* larvae taken from the plankton above the cold seeps in the Gulf of Mexico (see Chapter 4).

Bridge

In Chapter 2 we described culturing techniques and development of *"Bathymodiolus" childressi.* In Chapter 3 we will use these culturing techniques to experimentally determine the tolerances of developing embryos and larvae of *"B." childressi* to variations in temperature and salinity.

CHAPTER III

TEMPERATURE AND SALINITY TOLERANCE OF LARVAE OF THE DEEP-SEA MYTILID *"BATHYMODIOLUS" CHILDRESSI* WITH COMPARISON TO THE SHALLOW-WATER MYTILID *MYTILUS TROSSULUS*

Introduction

The strategies that permit marine invertebrates to successfully occupy varying marine environments have been studied extensively in adult organisms (Charmantier and Wolcott 2001). Such strategies include adaptations to temperature, pressure, density, currents, oxygen availability, food availability, light, salinity, and pH, and can be manifested through molecular, biochemical, physiological, morphological, and behavioral changes. As Batholomew (1987, p. 18) states, "an organism must not only be adapted to meet major environmental challenges, it must also be capable of carrying out essential functions during its entire life." Marine invertebrates with complex life cycles often have stages (embryos and larvae) that occupy habitats drastically different from those of the adults. Thus, it is necessary to consider strategies by which early life-history stages survive the various environmental conditions they encounter.

In species with complex life histories, physiological tolerances of the dispersive stages may influence adult population distributions, genetic exchange, colonization of new habitats, and, on evolutionary time scales, speciation. Thus, the environmental

conditions that larvae tolerate may reflect the environmental conditions characteristic of the geographic range of the adults. In particular, temperature (O'Connor et al. 2007) and salinity (Qiu et al. 2002) have been implicated as key factors affecting distribution and dispersal of early life history phases and subsequent distribution of the adult populations. For example, adult *Mytilus* mussels have wide temperature and salinity tolerances, with variations in tolerance among populations or species depending on their ecological or evolutionary history (Braby and Somero 2006a, b). Within a species of *Mytilus* mussels, physiological tolerances by larvae to temperature and salinity have been shown to vary depending on the history of their parents (e.g., Bayne 1965). In addition, larval thermal (Bayne 1965) or salinity tolerances (Qui et al. 2002) varies between species and may be major determinants of geographical ranges of *Mytilus* mussels.

In addition to their widespread geographic distribution, mytilid mussels are represented in deep-sea habitats as well. Molecular evidence supports the hypothesis of progressive evolution of mytilid mussels from shallow- to deep-water reducing habitats (Craddock et al. 1995; Jones et al. 2006), where they are represented as members of the subfamily Bathymodiolinae (Gustafson et al. 1998; Distel et al. 2000). Reproductive (Eckelbarger and Watling 1995) and developmental strategies of deep-sea mollusks (Gustafson and Lutz 1994; Bouchet and Warén 1994) may be constrained by phylogeny, but other adaptations to the deep-sea and to chemosynthetic environments are necessary. In particular, the early life-history stages of bathymodiolin mussels face a suite of environmental conditions that shallow-water mytilid mussels are unlikely to encounter. Although extensive work has been focused on the physiological ecology of adult

bathymodiolin mussels, little is known about the physiological ecology of their larvae. Whether ranges of environmental tolerances of embryos and larvae are similar between deep-sea bathymodiolin mussels and shallow water mytilids is unknown. In addition, whether tolerances by larvae to environmental conditions such as salinity and temperature play a role in shaping the geographical or depth distribution of bathymodiolin mussel populations also remains unexplored.

Within the Bathymodiolinae, the genus *Bathymodiolus* consists entirely of species that are endemic to hydrothermal vents or cold seeps (Distel et al. 2000). The cold-seep mussel "*Bathymodiolus*" *childressi* has been described from brine- and oil-dominated seeps at depths from ~540 m to 2200 m along the upper continental slope in the Gulf of Mexico (Gustafson et al. 1998). (We place the genus name in quotation marks following recommendations due to uncertainty raised by its morphology (Gustafson et al. 1998) and molecular phylogeny (Jones et al. 2006)). Eggs and developing embryos of *"B." childressi* are negatively buoyant (Chapter 2), exposing them to stresses associated with these cold-seep environments, including elevated salinities, hypoxia, and high concentrations of hydrogen sulfide and methane. As embryos of *"B." childressi* develop into free-swimming, feeding (planktotrophic) larvae (Gustafson et al. 1998; Chapter 2), they face a different suite of environmental conditions depending on their vertical swimming behaviors. It is unknown whether bathymodiolin mussel larvae undergo ontogentic vertical migration as do some shallow-water mytilid mussels (Bayne 1976; Gosling 2003). It has been suggested, however, that physiological tolerances to the environmental conditions of the euphotic zone may be useful indicators of the potential

for larvae of benthic deep-sea organisms to migrate to surface waters (Young and Tyler 1993; Young et al. 1996a, b).

Until recently, it has been impossible to test the physiological tolerances of bathymodiolin embryos and larvae. Using culturing methods that have been developed for the cold-seep mussel *"Bathymodiolus" childressi* (Chapter 2), we provide the first steps in understanding their larval physiology by testing the physiological tolerances of *"B." childressi* embryos to some of the physical conditions associated with cold seeps and the tolerances of the early larvae to the physical conditions associated with the water column above the cold seeps in the Gulf of Mexico. Specifically, we compare temperature and salinity tolerances for embryos and larvae of *"B." childressi* to those of the embryos and larvae of their shallow-water relative *Mytilus trossulus*. We test the hypotheses that (1) tolerances of both species widen with age, and (2) embryos and larvae of *"B." childressi* have narrower temperature and salinity tolerance ranges than do those of *M. trossulus*. Finally, because embryos and larvae of *"B." childressi* are deep-water and should not be exposed to low salinities and because they often inhabit brine-dominated seeps, we also hypothesize that (3) they are more tolerant of higher salinities than of lower salinities

Materials and methods

Temperature and salinity profiles

Temperature and salinity profiles were taken in the Gulf of Mexico over the Louisiana slope cold-seep sites in March 2002, May 2002, February 2003, November 2003, December 2004, and August 2006 using a conductivity/temperature/depth (CTD)

profiler (Sea-Bird electronics, Inc.). Dates and exact locations of the CTD casts are given in Table 1.

Table 1. Dates and locations of CTD casts. All casts were taken above the Brine Pool cold seep (27°43'24" N, 91°16'30" W).

Date	Latitude	Longitude
March 11, 2002	27°43.51 N	91°16.78 W
May 10, 2002	27°43.52 N	91°16.86 W
July 30, 2002	27°43 N	91°16 W
February 9, 2003	27°43.21 N	91°15.31 W
November 10, 2003	27°43 N	91°16 W
July 19, 2004	27°43 N	91°16 W
December 11, 2004	27°43.606 N	91°16.93 W
August 13, 2006	27°43.44 N	91°16.65 W

Collection, spawning, and culturing

"Bathymodiolus" childressi

The *Johnson-Sea-Link I* and *II* submersibles (Harbor Branch Oceanographic Institution, Ft. Pierce, FL) were used to collect samples of *"Bathymodiolus" childressi* from cold seep sites in the Gulf of Mexico. Most collections were made from the "Brine Pool" cold seep (27°43'24" N, 91°16'30" W), but some were from the "Bush Hill" cold seep (27°47'N, 91°30'24"W) (MacDonald 1998). Collections were made with the submersible's hydraulic clam-bucket scoop and placed in a thermally stable acrylic box for transport to the surface. Once on deck, the mussels were transferred to clean seawater and maintained shipboard in aquaria at 7 °C until they were transported back the Oregon Institute of Marine Biology (OIMB). Shipboard maintenance included periodic bubbling with methane gas, constant aeration, and regular changes of seawater. Procedures for maintaining mussels in the laboratory are detailed in Chapter 2. Briefly, mussels were

held in chilled (7-8 °C), recirculating tanks (150 L each) that were periodically bubbled with methane. Water was changed monthly.

Spawning was induced in a batch of mussels (50 to 120 mm long) that consisted on both males and females by a 0.4-0.5 ml injection of 2 mM serotonin into the anterior adductor mussel (Chapter 2). After mussels spawned, fertilized eggs were filtered from the batch, rinsed, and placed in clean 0.22 μm filtered seawater (FSW). Because we were unable to spawn males and females separately, development time = 0 was set when the sperm were removed, even though fertilization time may not have been synchronous. The embryology and larval development of *"Bathymodiolus" childressi* is described in Chapter 2. Unless otherwise noted, dense cultures (up to 50 embryos per ml) were kept unstirred in 2- L glass jars until they hatched. After hatching, larvae were moved to clean FSW and placed in experiments as described below. All experiments were conducted with blastulae (within 24 hours of hatching) and trochophore larvae (4 days post-hatching) of *"Bathymodiolus" childressi.* Cultures were maintained in a 7-8° C cold room unless noted in sections below.

Mytilus trossulus

Mytilus trossulus, a native to the North Pacific and a member of the blue mussel species complex, was collected during its spawning season (December-January; Strathmann 1987) from the floating docks of the inner boat basin in Charleston, Oregon. Although hybridization with the invasive blue mussel *Mytilus galloprovincialis* occurs along the west coast of the United States, a recent genetic analysis indicates that blue mussel populations in Charleston, OR remain purely *M. trossulus* (Braby and Somero

2006a). Mussels were maintained in a flow-through seawater system at the OIMB. Spawning was induced by placing ripe mussels in individual containers of seawater and slowly warming from an ambient temperature of 10-12 °C to 20-25 °C (Strathmann 1987). Next, 500 to 1000 eggs per ml of 0.22μm FSW were inseminated with 0.1 ml concentrated sperm for each 100 ml egg suspension and then diluted at least 40 times to the desired volume (Strathmann 1987). Unless otherwise noted, cultures were maintained at approximately 7 embryos ml^{-1} FSW and were fed 30-50,000 cells *Isochrysis galbana* ml^{-1} (Loosanoff and Davis 1963; Bayne 1965; Strathmann 1987). Because larvae of *M. trossulus* develop faster than do those of *"Bathymodiolus" childressi,* all experiments with *M. trossulus* were conducted with trochophore larvae only (about 24 hours after fertilization).

Salinity tolerance experiments

Developmental effects

To assess the effects of salinity on early development of *"Bathymodiolus" childressi* and *Mytilus trossulus*, we cultured developing embryos in four salinity treatments: 25, 35, 45, 50. A control salinity of 33 was also included in the *M. trossulus* experiment. Preliminary experiments showed no survival of *"B." childressi* at or above 60. Salinities were measured with a hand-held refractometer and are expressed without units (UNESCO 1985). We mixed seawater with brine or deionized water to make the experimental salinities. Brine was made by freezing seawater and siphoning off the high salinity melt-water. Experiments were held at the mussels' ambient temperatures: in a 7-

8 °C incubator for *"B." childressi* and an 11-12 °C seawater table for *M. trossulus*. Replicate 250-ml cultures were made at densities of 10 fertilized eggs per ml for each treatment (for *"B." childressi* n = 3; for *M. trossulus* n = 4). One 22-ml sample was taken from each replicate at 2, 6, 10, 15, 20, 30, 40 hours for *"B." childressi* and 6, 10, 20 and 40 hours for *M. trossulus*. The 22-ml samples were fixed in 2% formalin until approximately 100 in each subsample were counted and staged under 40 x magnifications. Next, we examined the distributions of mean frequencies of each developmental stage (showing only 2-cell through shelled larva) at each time interval. We then assigned a single stage to each replicate and plotted stage of each replicate against sampling time. For *"B." childressi* we used the furthest developmental stage and for *M. trossulus* we used the stage with the highest percentage of embryos, since there was a stage with an overwhelming peak frequency in each replicate.

Larval survival

For each species, 40 larvae were placed in each of four replicate 20 ml scintillation vials filled with FSW altered to salinities of 25, 35, 45, or 50. Before experimentation, larvae were acclimated incrementally (salinity increments = 5) from ambient salinity for approximately 30 minutes at each increment. The scintillation vials were fitted with plastic lids and incubated at 7-8 °C for 24 hours before the surviving larvae were counted. Larvae were not fed for the duration of the experiment. Percent survival data were arcsine transformed and the effect of salinity on survival was analyzed. Because nearly all *Mytilus trossulus* trochophores survived in all salinities, the

effect of salinity on their survival was analyzed in a separate single-factor ANOVA, with salinity as a fixed factor (Sokal and Rohlf 1981). The effect of salinity on the survival of *"Bathymodiolus" childressi* blastulae and trochophores (stage) were compared using a two-way, fixed factor ANOVA with salinity X stage as the factors (Sokal and Rohlf 1981).

Temperature tolerance experiments

Developmental effects

The effect of temperature on early development of *"Bathymodiolus" childressi* and *Mytilus trossulus* embryos was determined through controlled temperature experiments. Three replicate cultures from a single spawning event (density: approx. 10 embryos ml^{-1}) were placed in four incubators set to 7, 15, 20, and 25 ^{o}C. Subsamples of approximately 100 embryos (22 ml) were taken from each replicate culture at 2, 10, 15, 20, 30, and 40 hours for *"B." childressi* and 2, 6, 10, 20, 30, and 40 hours for *M. trossulus*. Subsamples were fixed with 2% formaldehyde until they were counted and staged under 40 x magnification. As in the salinity experiment, we examined the distributions of the mean frequency of each embryological stage (2-cell through shelled larva) at each time interval, then assigned a single stage to each replicate and plotted stage against time for each replicate.

Larval survival

For each species, 40 larvae were placed in each of four replicate 20 ml scintillation vials filled with FSW of ambient salinity (33 for *Mytilus trossulus*, 35 for

"Bathymodiolus" childressi) at approximately 7-8 °C. The scintillation vials were capped and placed in an aluminum temperature gradient block adjusted to create the following temperature treatments: 2, 7, 15, 20, and 25 °C. Larvae were not fed for the duration of the experiment. After 24 hours the number of surviving *M. trossulus* or *"B." childressi* larvae at each temperature was counted and percent survival was arcsine transformed and analyzed in separate ANOVA's. The effect of temperature on survival of *M. trossulus* trochophores was analyzed in a single-factor ANOVA, with salinity as a fixed factor (Sokal and Rohlf 1981). The effect of temperature on the survival of *"B." childressi* blastulae and trochophores (stage) was compared using a two-way, fixed factor ANOVA with temperature X stage as the factors (Sokal and Rohlf 1981).

Results

Temperature and salinity profiles

Sea surface temperature over the continental slope in the Gulf of Mexico varies widely throughout the year (Fig. 1). We measured a minimum sea surface temperature in February 2003 (= 20.3 °C; Fig. 1c) and a maximum in August 2006 (= 30.3 °C; Fig. 1h). At the maximum depths recorded (~630 m), temperature remained approximately 7-8 °C. Salinity generally varied between approximately 35 and 36 throughout the water column (Fig. 1), but was more variable at the surface. For example, in July 2004 (Fig. 1g), salinity reached a low of 28 in the surface waters (< 10 m depth).

Salinity tolerance

"Bathymodiolus" childressi embryos did not develop beyond the 4-cell stage at a salinity of 25 or beyond the 8-cell stage at salinity 50 (Fig. 2a, 3). Likewise, *Mytilus trossulus* embryos did not develop beyond the 4 cell stage at 50 (Fig. 2b, 3), but nearly 100% of embryos in the salinity 25 treatments advanced to the morula stage by 20 hours (Fig. 2b). Embryos of both mussels developed to advanced stages at the intermediate salinities (33, 35, 45) (Figs. 2, 3). Although some embryos of *"B." childressi* reached the morula stage in 45 before they did in 35 (Fig. 3), more larvae developed through advanced stages by 20-40 hours in 35 than in 45 (Fig. 2a). For *M. trossulus,* larvae in the 25, 33, 35 salinity treatments reached the morula stage by 10 hours, with more than 80% in 33 and more than 50% in 35 at the morula stage, but only 5% at the morula stage in salinity 25 (Fig. 2b). The majority of *M. trossulus* embryos at 25 and 45 did not reach the morula stage until 20 hours (Fig. 2b).

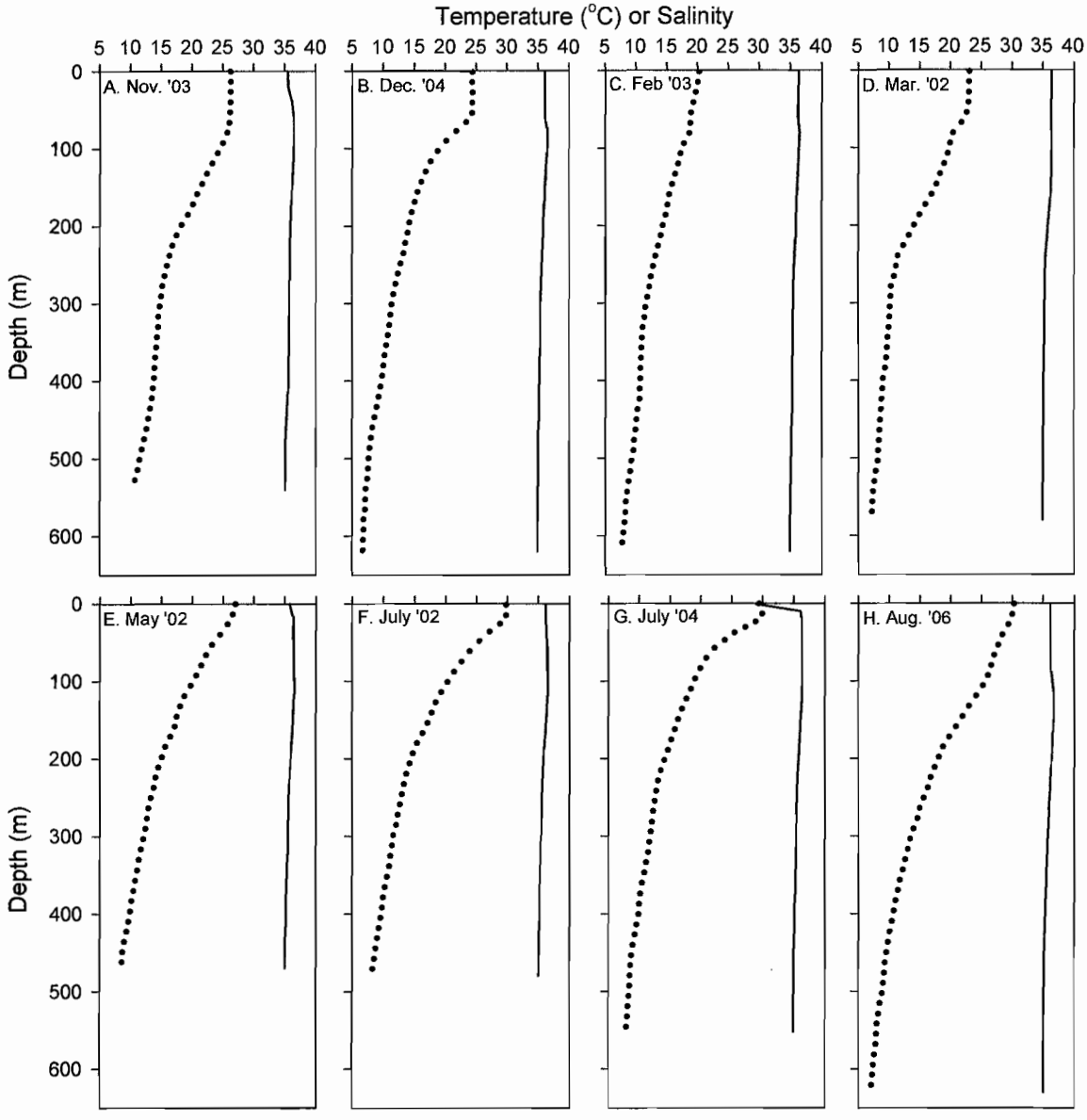

Figure 1 Temperature (dotted lines) and salinity (solid lines) depth profiles over the Louisiana continental slope in the Gulf of Mexico in (a) November 2003, (b) December 2004, (c) February 2003, (d) March 2002, (e) May 2002, (f) July 2002, (g) July 2004, and (h) August 2006. See Table 1 for exact dates and locations.

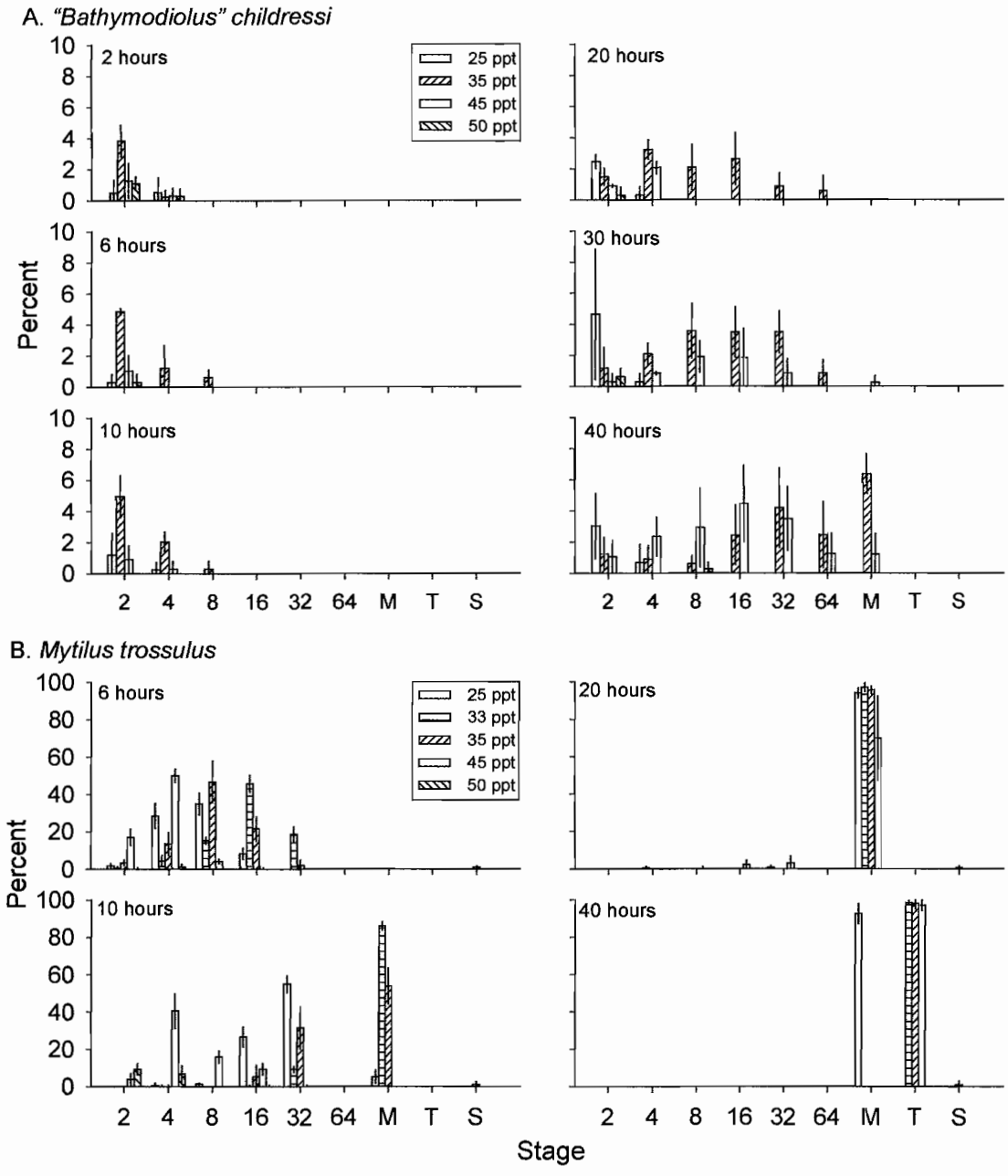

Figure 2. Percent frequency of embryos developing to each developmental stage in cultures of salinities in 25, 35, 45, and 50 for (a) *"B." childressi* embryos (n = 3) after 2, 6, 10, 20, 30, and 40 hours and (b) of *M. edulis* embryos (n = 4) after 6, 10, 20, and 40 hours. Developmental stages are 2-cell through 64 cell, morula (M), Trochophores (T), and larvae with shells forming (S); irregular or non-developing embryos are not presented here. Error bars are $\pm$ 1. S.D..

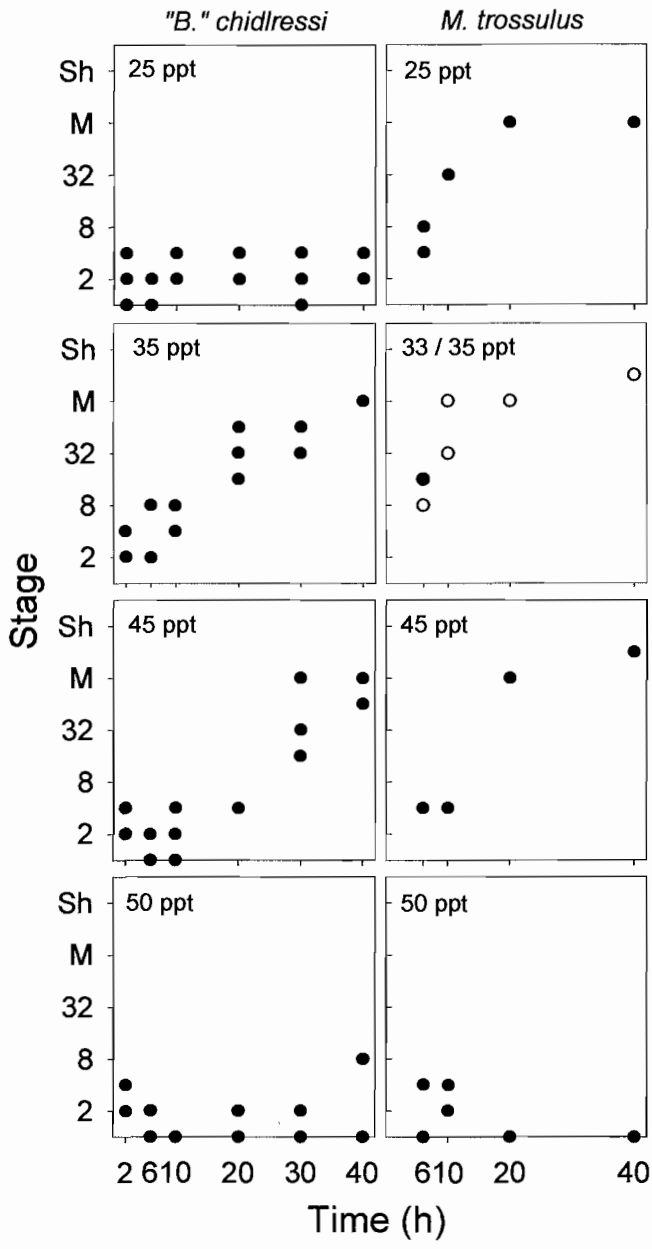

Figure 3. Maximum developmental stage for *"Bathymodiolus" childressi* and modal developmental stage for *M. trossulus* reached in replicate cultures (n = 3 and 4, respectively) at different salinities. For *M. trossulus* stages at 33 and 35 ppt are represented together. Developmental stages are 2-cell through 64 cell, morula (M), Trochophores (between M and Sh), and larvae with shells forming (Sh).

Survival of *Mytilus trossulus* trochophores was high (mean > 88 %) in all salinities and there was no significant difference among them ($F = 1.601$, $p = 0.241$; Fig. 4). Conversely, both stage and salinity significantly affected percent survival of larval *"Bathymodiolus" childressi* (Table 2, Fig. 4). There were no significant differences in percent survival at 35, 45, or 50, but survival of larvae exposed to the lowest salinity treatment (25) was significantly lower than in the others (Tukey HSD: $p < 0.05$ for each). Likewise, survival of trochophore larvae was significantly lower than survival of blastulae at all salinities ($F = 7.836$, $p = 0.01$; Table 2, Fig. 4).

Table 2. Two-factor ANOVA table showing the effect of stage (blastulae or trochophore) and salinity (25, 35, 45, 50) on arcsine transformed survival ratios of larvae of *"Bathymodiolus" childressi.*

Source	df	Type III SS	MS	F	Sig.
Stage	1	0.075	0.075	7.836	0.010
Salinity	3	0.234	0.078	8.178	0.001
Stage*Salinity	3	0.032	0.011	1.107	0.366
Error	24	0.229	0.010		

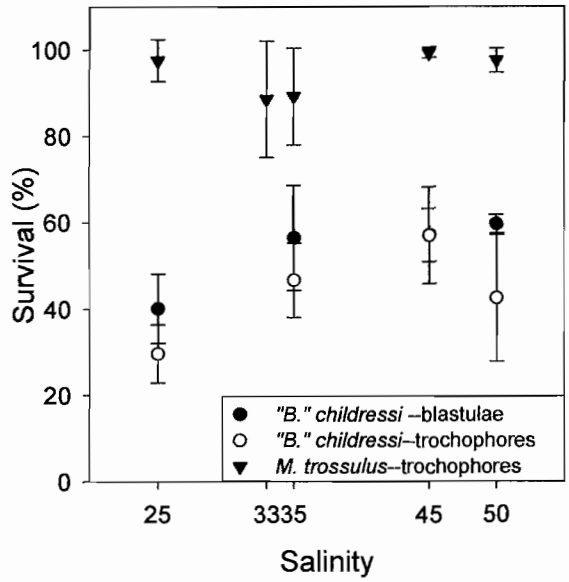

Figure 4 Mean percent survival of *"Bathymodiolus" childressi* and *Mytilus trossulus* larvae incubated in varying salinities of FSW for 24 hours (n = 4). Error bars are $\pm$ 1. S.D..

Temperature tolerance

As in the salinity experiments, a higher percentage of *Mytilus trossulus* embryos developed at all temperatures than did *"Bathymodiolus" childressi* embryos (Fig. 5). Likewise, strong peak frequencies of certain developmental stages were obvious for *M. trossulus* at each temperature and time, but multiple stages were represented in *"B." childressi* cultures (Fig. 5). In particular, 2-cell embryos were present in high frequencies through 20 hours, and were still present at 30 and 40 hours (Fig. 5a). Embryos of both mussels developed more rapidly with increasing temperature, but embryos of *"B." childressi* develop to the latest stages in temperatures up to 20 ^{o}C only, never advancing beyond the 4-cell stage at 25 ^{o}C (Fig. 6). Although *"B." childressi* embryos that developed in the 20 ^{o}C culture reached the advanced stages sooner than those in the 7 ^{o}C

culture (Fig. 6), the percent developing in 20 °C cultures was considerably lower (Fig. 5a). *Mytilus trossulus* embryos, on the other hand, developed to the most advanced stages in high percentages at all temperatures (Fig. 5b, 6).

Percent survival was high (>83%) for *Mytilus trossulus* trochophores at all temperatures (Fig. 7). However, temperature did significantly affect *M. trossulus* larval survival ($F = 11.55$; $p < 0.001$), such that survival was higher in 2 °C than in 25 °C (Tukey HSD: $p = 0.001$) and higher in 7 °C than in 15-25 °C (Tukey HSD = $p < 0.05$ for each). Temperature affected survival of *"Bathymodilus" childressi*, but stage did not have a significant effect (Table 3). Survival decreased with temperature (Fig. 7) and was significantly lower at 25°C than at all other temperatures (Tukey HSD: $p < 0.02$ for each), and significantly higher at 2 °C than at either 20 °C or 25 °C (Tukey HSD: $p < 0.01$ for each). For *"B." childressi*, mean survival ranged from 28.5 ± 15.2 % ($\bar{x}$ ± S.D.) for trochophores at 20 °C to 62.7 ± 21.5 % ($\bar{x}$ ± S.D.) for trochophores at 7 °C. At 25 °C, only 5.6 ± 2.4 % ($\bar{x}$ ± S.D.) of blastulae and 14.6 ± 11.7 % ($\bar{x}$ ± S.D.) of trochophores survived.

Table 3. Two-factor ANOVA table showing the effect of stage (blastulae or trochophores) and temperature (2, 7, 15, 20, 25 °C) on arcsine transformed survival ratios of larvae of *"Bathymodiolus" childressi.*

Source	df	Type III SS	MS	F	Sig.
Stage	1	0.028	0.028	1.047	0.314
Temperature	4	1.599	0.400	14.89	0.000
Stage*Temperature	4	0.200	0.050	1.866	0.142
Error	30	0.805	0.027		

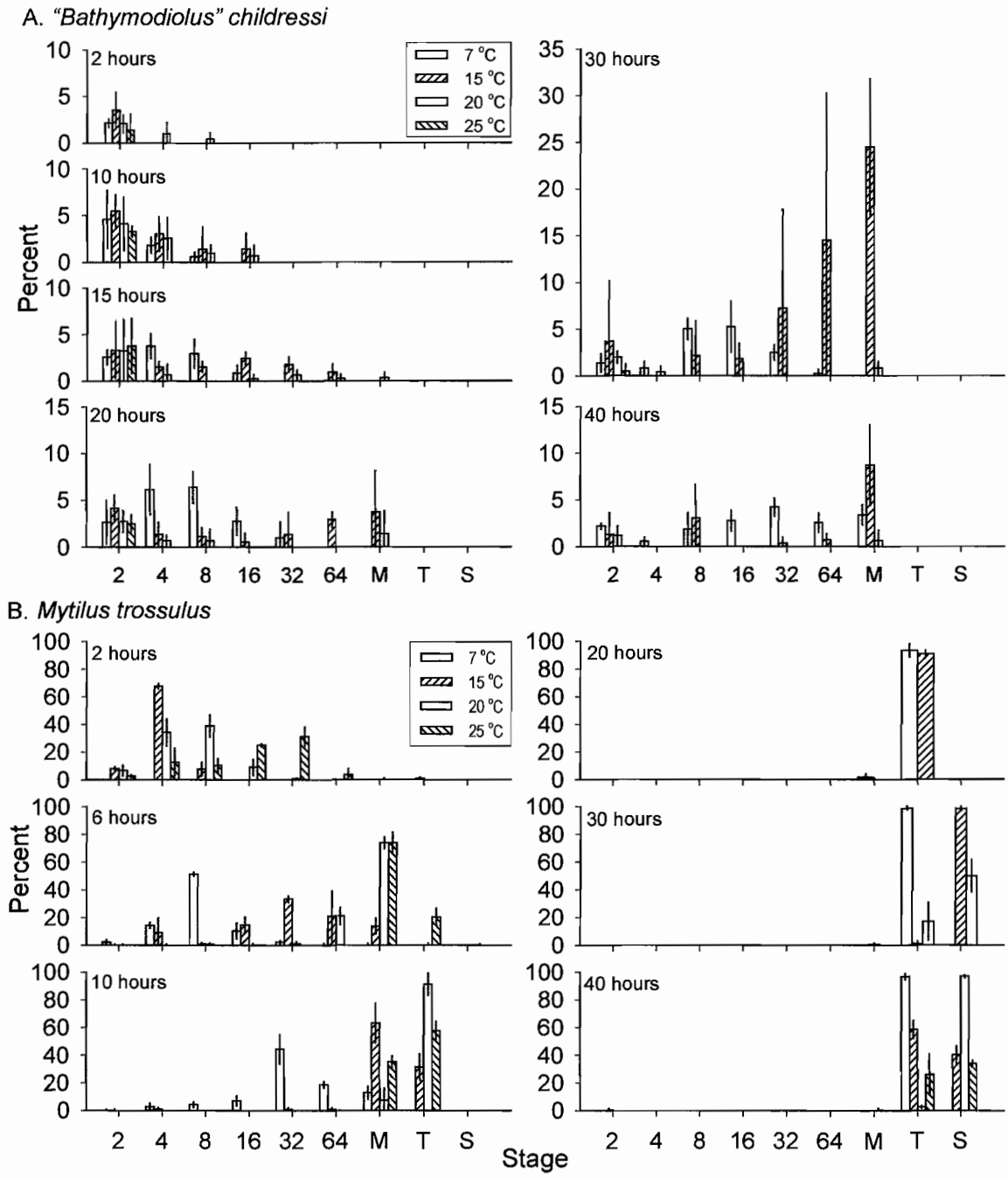

Figure 5 Percent frequency of embryos developing to each developmental stage in cultures at 7, 15, 20, and 25 °C for (a) *"Bathymodiolus" childressi* embryos (n = 3) after 2, 10, 15, 20, 30, and 40 hours, and (b) *Mytilus edulis* embryos (n = 3) after 2, 6, 10, 20, 30, and 40 hours. Developmental stages are 2-cell through 64 cell, morula (M), Trochophores (T), and larvae with shells forming (S); irregular or non-developing embryos are not presented here. Error bars are ± 1. S.D.

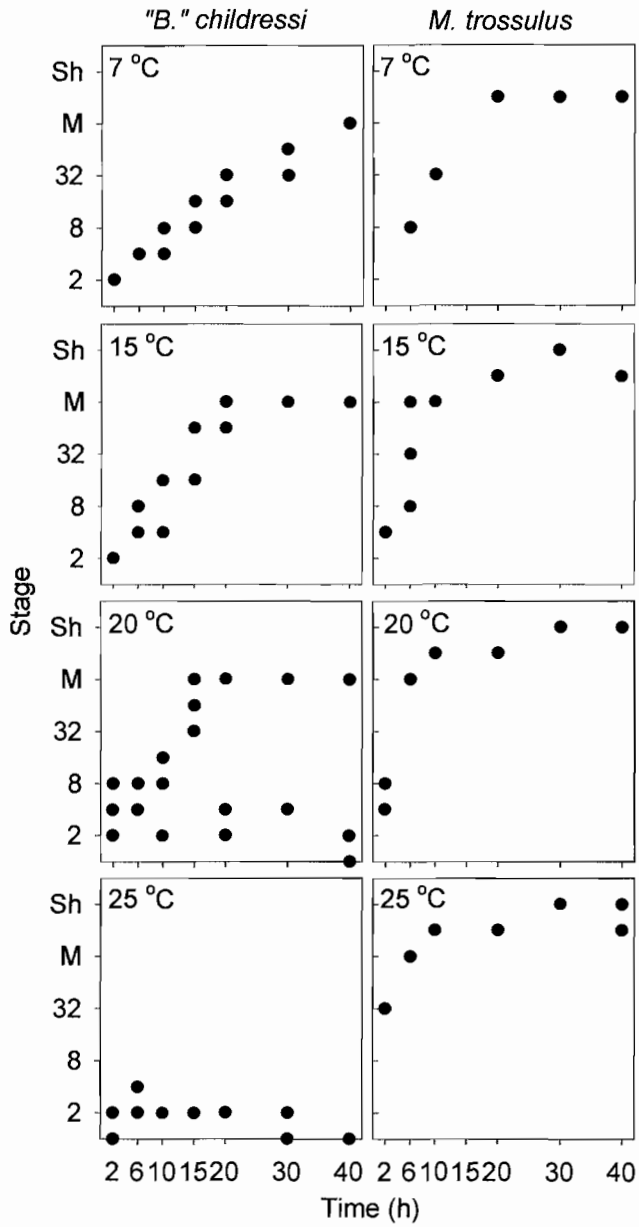

Figure 6. Maximum developmental stage for *"Bathymodiolus" childressi* and modal developmental stage for *Mytilus trossulus* reached in replicate cultures (n = 3 each) after being incubated at 7, 15, 20, and 25 °C for 2, 10, 15, 20, 30, and 40 hours Developmental stages are 2-cell through 64 cell, morula (M), Trochophores (between M and Sh), and larvae with shells forming (Sh).

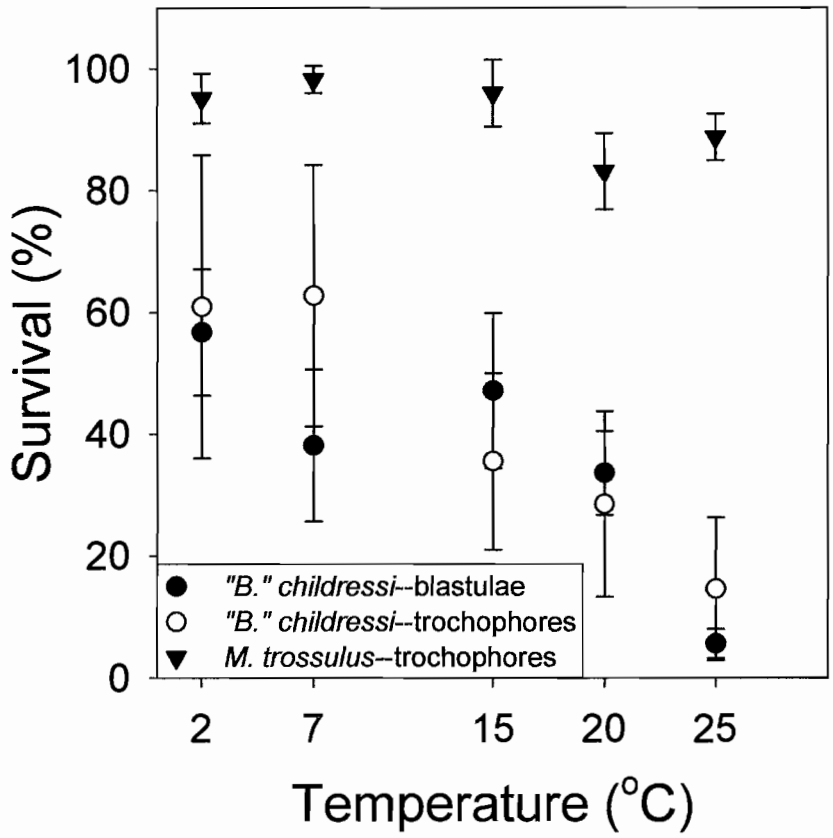

Figure 7. Mean percent survival of larvae of *"Bathymodiolus" childressi* and *Mytilus trossulus* at 2, 7, 15, 20, and 25 °C for 24 hours (n = 4). Error bars are ± 1. S.D.

Discussion and conclusions

Overall, a much higher percentage of *Mytilus trossulus* embryos developed under the experimental conditions than did embryos of *"Bathymodiolus" childressi.* We do not attribute the differences in total percent development to meaningful differences between the species. Rather, this probably reflects differences in our abilities to successfully culture shallow verses deep-sea larvae. In Chapter 2 we note that fertilization rates in *"B." childressi* cultures were consistently low. Nevertheless, comparisons between the developmental responses of these two species to alterations in salinities and temperatures outside their ambient ranges can be made.

Development of *"Bathymodiolus" childressi* was about 4 times slower than that of *Mytilus trossulus* at comparable temperatures and salinities. For example, most *"B." childressi* were at the 32-cell stage at 7 °C and a salinity of 35 at 40 hours, while most *M. trossulus* reached the 32 cells at the same temperature and salinity by 10 hours (Fig. 5).

Our results indicate a wide tolerance to salinity (25-45) for developing *Mytilus trossulus* embryos. This range is similar to other estimates for *M. trossulus* and other *Mytilus* mussels (Table 4). Qiu et al. (2002) tested tolerance of *M. trossulus* to hyposaline conditions and showed that embryos can develop down to a salinity of 15, but not below. However, Yaroslavtseva and Sergeeva (2006) report a high percentage of development of *M. trossulus* embryos after limited (24 hour) exposure to seawater of salinity 8. Taken together, these data suggest that *M. trossulus* may develop under euryhaline conditions anywhere from 8 to 45. *Mytilus edulis* may also develop under a wide range of salinities, but data on tolerances of developing embryos of this species to

varying salinities are equivocal. Successful fertilization has been shown for *Mytilus edulis* at salinities from 15 to 40 (Bayne 1965; Saranchova and Flyachinskaya 2001). However, while Bayne (1965) shows that development in *M. edulis* occurs at salinities from only 30 to 40, Saranchova and Flyachinskaya (2001) show that 100% of *M. edulis* embryos develop at salinities from 22 to 26, but not higher. These data suggest the possibility of local adaptation.

Unlike *Mytilus trossulus*, which can tolerate low salinities, development of "*Bathymodiolus" childressi* embryos is restricted to higher salinities. We showed that by 40 hours most embryos of "*B." childressi* at a salinity of 35 are morulas and are 16-cells at salinity 45. We did not continue sampling beyond the 40-hour time point so it is unclear whether embryos at salinity 45 would have continued to develop, but at a slower rate than at 35 (Fig. 2, 3). Similarly, salinity increased to 45 appeared to cause a decrease in the rate of development of embryos of *M. trossulus*; while most embryos at ambient salinities are morulas by 10 hours, embryos in 45 are only at the 4-cell stage (Fig. 2, 3). Decreased developmental rate may be a result of salinity stress in general; Qiu et al. (2002) showed that development is slowed in hyposaline conditions.

Table 4. Temperature and salinity tolerance ranges reported for various life-history stages of blue mussels. Ranges are defined by more than 50% survival. Stages are defined as follows: *embryo*—fertilized eggs developing to swimming trochophores, *trochophore*—swimming trochophores to D-shell, and *veliger*—D-shell to settlement. In parenthesis are the temperature and salinity ranges tested. For adults temperatures and salinities are from the conditions of their habitats.

Species	Stage	Temperature (°C)	Salinity	Source
Mytilus trossulus	embryo	—	15-25 (5-25)	Qiu et al. 2002
		10-20 (4-20)	—	Yaroslavtseva and Sergeeva 2006
	trochophore	—	15-25 (5-25)	Qiu et al. 2002
	veliger	—	15-25 (5-25)	Qiu et al. 2002
		4-20 (4-20)	—	Yaroslavtseva and Sergeeva 2006
		14-22 (14-22)	—	Limbeck 2003
	juvenile	—	10-25 (5-25)	Qiu et al. 2002
	adult	—	10-25 (5-25)	Qiu et al. 2002
		—	8-20	Yaroslavtseva and Sergeeva 2006
		7-21 (7-21)	22-28 (22-34)	Braby and Somero 2006b[a]
Mytilus edulis	embryos	—	20-25 (5-25)	Qiu et al. 2002
		8-18 (5-22)	30-40 (15-40)	Bayne 1965[b]
		6-18 (6-18)	—	Sprung 1984
		—	22-26 (5-45)	Saranchova and Flyachinskaya 2001
	trochophore	—	20-25 (5-25)	Qiu et al. 2002
		—	20-26 (5-45)	Saranchova and Flyachinskaya 2001
	veliger	—	20-25 (5-25)	Qiu et al. 2002
		10-22 (5-22)	19-32 (15-40)	Bayne 1965[b]
		—	14-30 (10.9-30.5)	Bayne 1965[b]
		6-18 (6-18)	—	Sprung 1984
		—	5-45 (5-45)	Saranchova and Flyachinskaya 2001
		5-20 (5-30)	15-40 (15-40)	Hrs-Brenko and Calabrese 1969
		14-22 (14-22)	—	Limbeck 2003

Table 4. Continued.

Species	Stage	Temperature (°C)	Salinity	Source
Mytilus edulis	juvenile	—	10-25 (5-25)	Qiu et al. 2002
		—	10-45 (5-45)	Saranchova and Flyachinskaya 2001
	adult	—	10-25 (5-25)	Qiu et al. 2002
		8-18	13-34	Bayne 1965[b]
		7-14 (7-21)	22 (22-34)	Braby and Somero 2006b[a]
Mytilus galloprovincialis	veliger	15-30 (15-30)	20-35 (20-35)	His et al. 1984
	adult	2-25	20-34	His et al. 1984
		7-21 (7-21)	22-34 (22-34)	Braby and Somero 2006b[a]

[a]Data given here are based on Braby and Somero (2006b) Figure 5 in which they tested mortality at combinations of salinity and temperature. Temperature ranges given here are at salinity 28 and salinity ranges are at a temperature of 21 °C.
[b]Bayne (1965) examines two populations of mussels. The first salinity ranges given here are for the intertidal population, while the second ranges are for the subtidal population. Ranges of embryos are from only the intertidal populations and adult ranges are a composite of the two populations.

Developmental and metabolic rates tend to decrease with temperature (Clarke 1983) and this trend is evident for the embryological development of both *Mytilus trossulus* and *"Bathymodiolus" childressi*. However, even with slower rates, embryos of *M. trossulus* developed normally over a wider temperature range than did those of *"B." childressi*. High percentages of *M. trossulus* embryos developed to the most advanced stages at all temperatures (7-25 °C). These results are similar to those of Yaroslavtseva & Sergeeva (2006), who show 100% development of *M. trossulus* embryos to veligers from 10-20 °C. The temperature tolerance for *M. trossulus* embryos is wider than that published for *Mytilus edulis* (Table 4). Bayne (1965) shows that, although fertilization of *M. edulis* eggs occurs between 5 and 22 °C, cleavage is normal from only 8 to 18 °C (or 6-18 °C in Sprung 1984), with no cleavage at lower temperatures and abnormal cleavages at higher temperatures. The temperature range Bayne (1965) and Sprung (1984) show for developing embryos of *M. edulis* is similar to the temperature range over which we found *"B." childressi* embryos to develop normally. The percent development of embryos of *"B." childressi* was highest at 7 and 15 °C, with development faster at 15 than 7 °C, and a very low percentage of embryos developing at 20 °C as well.

The salinity and temperature ranges to which embryos of *"Bathymodiolus" childressi* may be exposed at brine-dominated seep sites are within the ranges over which they develop normally. Eggs of *"Bathymodiolus" childressi* are spawned bound in mucus and are negatively buoyant (Chapter 2). As a result, embryos are likely to develop under the influence of the physical parameters characteristic of the environments the adult mussels inhabit, including elevated salinities up to about 45 just beneath the

mussels at the Brine Pool (Smith et al. 2000) and an average temperature of 7 – 8 °C at the Brine Pool and Bush Hill cold seeps. In addition, deeper populations of *"B." childressi* are known where temperatures can reach as low as 2 °C. Malfunctioning equipment prevented rigorous testing of development at 2 °C, but preliminary experiments showed that development of *"B." childressi* embryos occurred at 2 °C but was slower than at higher temperatures.

Do tolerances change with developmental stage?

The range of tolerance to environmental conditions often varies during ontogeny, with earlier developmental stages of most invertebrates exhibiting narrower tolerance ranges than later stages or adults (Kinne 1970, 1971). A review of the literature shows that this generalization holds true for many shallow-water mytilid mussels; both temperature and salinity tolerance ranges tend to widen as embryos develop into veligers and then into juveniles and adults (Table 4).

We found a wide tolerance to salinity for *Mytilus trossulus* trochophores, with survival >88 % from 25 to 50, compared to a normal developmental range of only 25 to 45. Tolerance to temperature remained wide for *M. trossulus* embryos and larvae. *Mytilus trossulus* embryos developed to the most advanced stages at all temperatures tested, and, likewise, most trochophores (> 83%) survived in all temperatures. These ranges are reflected in the adults; compared to other blue mussels, *M. trossulus* are more tolerant of both low temperature and salinity, consistent with the physical parameters of the environment in which they evolved (Table 4; Braby and Somero 2006a, b).

Survival of *Mytilus trossulus* trochophores exposed to a range of salinities and temperatures was greater than for *"Bathymodiolus" childressi* blastulae and trochophores. Like other mytilids though, salinity and temperature tolerance ranges for *"B." childressi* widened with age. While development was highest at 35 and 45, both blastulae and trochophores survived at all salinities. However, more blastulae than trochophores survived in all salinities. Stress in parents can lead to limited or abnormal larval development and in vitro culture of deep-sea invertebrates to advanced stages is notoriously difficult (see Chapter 2). Thus, because there was not a significant interaction between stage and salinity, we are led to believe that the higher survival of blastulae than trochophores is due to physiological condition of the parents, rather than differential responses to salinity by the larvae. Although significantly fewer larvae survived at the lowest salinity tested (25), the percent survival at this salinity was still more than 50% of the maximum.

The temperature tolerance range was wider for both hatched blastulae and trochophores of *"Bathymodiolus" childressi* than for developing embryos and there was no difference in the tolerances of the two stages. While the highest percent of embryos of *"B." childressi* developed at only 7 and 15 ^{o}C, between ~ 30 and 60 % of blastulae and trochophores survived from 2 and 20^{o}C and, although survival was significantly lower, about 5 -14 % of blastulae and trochophores survived at even the highest temperature treatment (25 ^{o}C). While nothing is known of their salinity tolerances, *"B." childressi* adults have a higher temperature tolerance than we found for developing embryos or larvae. Although *"B." childressi* is adapted to the cold temperatures characteristic of the

deep-sea, adults have not lost the ability to respond to elevated temperature and can tolerate up to 27 °C before suffering 50% mortality (Berger and Young 2006).

Wide salinity and temperature tolerance has been noted for the larvae of another endemic cold-seep mollusk; 90 to 100% of larvae of the snail *Bathynerita naticoidea* survived when exposed to salinities from 15 to 60 (Van Gaest 2006), a much wider tolerance range that includes tolerance to higher salinities than we exhibit for *"B." childressi.* Although salinity tolerance is not as high for larvae of *"B." childressi* as for larvae of *B. naticoidea*, the free-swimming larvae of *"B." childressi* are not as likely to encounter the high salinities that encapsulated larvae of *B. naticoidea* may at brine-dominated cold seeps. Additionally, *B. naticoidea* veligers had much higher survivorship at high temperatures. Van Gaest (2006) showed that 100% of *B. naticoidea* veligers survived for 3 days at temperatures up to 29 °C, with only 15% mortality at 32 °C. However, the *B. naticoides* veligers in the experiments conducted by Van Gaest (2006) were considerably older than the mussel larvae in these experiments and we have shown here that temperature tolerance of *"B." childressi* widens with age.

Influence of larval tolerances on ontogenetic vertical migration potential

Whether hydrothermal-vent and cold-seep organisms migrate to surface waters, has been a subject of debate since the communities were first discovered (e.g., Lutz et al. 1980, 1984). Lutz et al. (1980, 1984) contended that bathymodiolin mussels from hydrothermal vents probably do not vertically migrate. Nevertheless, genetic connectivity of populations of *"Bathymodiolus" childressi* throughout the Gulf of

Mexico is high, suggesting widespread dispersal of their larvae in the Gulf of Mexico (Carney et al. 2006). Vertical migration into the higher current speeds above the benthic boundary layer is a potential mechanism to increase larval dispersal potential for *"B." childressi*. In addition, both field and laboratory studies report diel migration patterns in the planktotrophic larvae of shallow-water bivalves (Gosling 2003), and in particular, mytilid mussel larvae are capable of actively controlling their vertical distribution (Bayne 1976). Physiological tolerances to the environmental conditions such as salinity and temperature of the euphotic zone may be among the most useful indicators of ontogenetic vertical migration potential of larvae of benthic deep-sea organisms (Young and Tyler, 1993; Young et al. 1996a, b).

Temperature is a more likely limiting factor to vertical distribution of the dispersive larval stages of *"Bathymodiolus" childressi* than salinity because salinity does not vary significantly through the water column. Although tolerance to increased salinity by early developmental stages and by juveniles and adults may be beneficial for life at brine seeps, larvae of *"B." childressi* are less tolerant to low salinities than are blue mussel larvae. However, while shallow-water mytilids often inhabit brackish waters necessitating tolerance to lower salinities by larvae, tolerance to low salinities by *"B." childressi* larvae may not be necessary. Although the coastal waters in the Gulf of Mexico may reach low salinities seasonally (Li et al. 1997) and a low-salinity lens produced by the Mississippi River penetrates the upper water column far offshore (Müller-Karger and Walsh 1991), the surface waters in the Gulf of Mexico above the

cold seeps inhabited by *"B." childressi* remain above 30 (Li et al. 1997). In fact, the lowest sea surface salinity we measured above the continental slope cold seeps was 28.

Conversely, sea surface temperatures in the Gulf of Mexico can surpass the tolerance limits *"Bathymodiolus" childressi* trochophore larvae. Above the cold seeps on the Louisiana slope, sea surface temperature can reach approximately 30 °C in the summertime (Li et al. 1997) and we measured temperatures > 30 °C in the uppermost 20 m of the water column in August 2006. However, the likelihood that *"B." childressi* larvae would be found at the surface in the summer months is low though. Tyler et al. (2006) show that gametogenesis initiates from December to March followed by a spawning season from October to February of the following year. If larvae of *"B." childressi* vertically migrate through the water column, assuming a mean swimming speed 0.11 cm s^{-1} (Chia et al. 1984), larvae could potentially be found in the surface waters within one week of spawning, or October through February. Our records during those months ranged from 20.5 °C in the coldest winter month measured (February 2003) to 26.0 °C November 2003 (Fig. 1). Additionally, even in November 2003 the temperature falls to approximately 20 °C by ~175 m depth. Survival of trochophores is not significantly different at 20 °C than it is at 7 °C, which is the ambient temperature of the adults (Table 3; Fig. 7). Moreover, some (5-14 %) *"Bathymodiolus" childressi* blastulae and trochophores survived at even 25 °C. Since survivorship with temperature depends on age, tolerance may increase at the veliger stage. Indeed, veligers of other mussels (*Mytilus galloprovincialis*) have been shown to tolerate 30 °C for at least 8 days (His et al. 1984). Thus, it does not appear that temperature would limit ontogenetic

vertical migration of *"B." childressi* larvae up to at least 175 m depth during the warmest periods of their spawning season, and some trochophore larvae may even survive the temperatures of the uppermost water column of the Gulf of Mexico throughout the year.

Conclusions

The goal of our experiments was to test the hypotheses that (1) the temperature and salinity tolerance of both *"Bathymodiolus" childressi* and *Mytilus trossulus* widen with age, (2) embryos and larvae of *"B." childressi* have narrower temperature and salinity tolerance ranges than do those of *M. trossulus*, and (3) embryos and larvae of *"B." childressi* are more tolerant of higher salinities than of lower salinities. Indeed, our experiments show that tolerances do increase with developmental stage, but that tolerance ranges are not as high for the larvae of "*B." childressi* as for the larvae of *M. trossulus*. *"Bathymodiolus" childressi* embryos and larvae are more tolerant of high than low salinities, which may aid development of negatively buoyant early embryos at brine seeps. Finally, although temperature increases significantly near the sea surface over seeps in the Gulf of Mexico, some trochophore larvae of *"B." childressi* can survive the temperatures in even the uppermost water column during the months we expect to find larvae there.

Bridge

Chapter 3 examined the temperature and salinity tolerances of the embryos and larvae of *"Bathymodilus" childressi* and related those tolerances to their potential for ontogenetic vertical migration. Chapter 4 will examine whether a long-distance ontogenetical vertical migration from the deep sea to the photic zone is energetically feasible.

CHAPTER IV

VERTICAL MIGRATION OF THE DEEP-SEA MUSSEL *"BATHYMODIOLUS" CHILDRESSI*: EMPIRICAL TEST OF AN ENERGETIC MODEL

1. Introduction

An understanding of larval dispersal gives insights into the connectivity, colonization, and dynamics of the benthic populations, including those at deep-sea hydrothermal vents and hydrocarbon seeps. Many factors including gametogenic cycle, developmental mode, planktonic larval duration, larval behavior, and ocean circulation influence the spatial and temporal patterns of dispersal (reviewed in Levin, 2006). Planktotrophic (feeding) larvae are generally presumed to remain in the plankton for longer periods and to disperse farther than lecithotrophic larvae or brooded embryos (Thorson, 1950; Wray and Raff, 1991). However, this pattern may not hold true in the deep sea because low metabolic rates at cold temperatures tend to increase planktonic durations of even lecithotrophs (Lutz et al., 1984; Turner et al., 1985; Gustafson and Lutz, 1994; Young et al. 1997; reviewed by Young, 2003). Vertical migration behavior further complicates the predicted dispersal patterns by interacting with hydrodynamics to increase dispersal or retain larvae (Cowen et al., 2000; Kingsford et al., 2002). Thus, the genetic linkage of metapopulations also depends upon the migration behavior of larvae.

Planktotrophic larval development is fairly common among bathyal and even abyssal animals (reviewed by Young, 2003), but deep-water food sources for these larvae are unknown. Moseley (1880) suggested that larvae of deep-sea animals might migrate into the euphotic zone to feed and develop. His hypothesis, though, was overshadowed by Thorson's (1950) assertion that such a migration would be energetically impossible. Recently though, planktotrophic larvae of several deep-sea taxa found in surface plankton tows has provided direct evidence of ontogenetic vertical migration of larvae originating in the deep sea (reviewed in Bouchet and Warén, 1994; Pawson et al., 2003; Van Gaest, 2006). Direct evidence is rare, however, so in its absence other methods have been employed to demonstrate the migration potential of larvae of benthic, deep-sea organisms. For example, evidence based on the morphology and isotopic ratios of larval shells indicates that larvae of deep-sea gastropods may migrate to the euphotic zone (Killingley and Rex, 1985; Bouchet and Warén, 1994). Additionally, some researchers suggest that larval physiological tolerances to the environmental conditions of the euphotic zone may be useful indicators of ontogenetic vertical migration potential of larvae of benthic deep-sea organisms (Young and Tyler, 1993; Young et al., 1996a,b). Finally, an energetic model suggests that energy stores of the planktotrophic larvae of bathyal echinoids probably do not limit their ability to vertically migrate (Young et al., 1996a).

Due to the difficulty in culturing deep-sea larvae, indirect evidence of migration potential that combines physiological tolerances, shell morphology or chemistry, and larval energetics is rarely obtained. Likewise, the direct detection of larvae in plankton

samples is often difficult due to high dilution rates and difficulty in identifying larvae to the species level. However, advances in techniques to culture deep-sea larvae and to identify larvae from the plankton may allow us to bypass these difficulties.

We have developed techniques for culturing the deep-sea cold-seep mytilid *"Bathymodiolus" childressi* (Chapter 2). *"Bathymodiolus" childressi* is a mixotrophic mussel that harbors methanotrophic endosymbionts in the gills (Childress et al., 1986) and is known from hydrocarbon seeps in the northern and western Gulf of Mexico, ranging in depth from 546 to 2222 m (Gustafson et al., 1998). A member of the subfamily Bathymodiolinae, the genus *Bathymodiolus* consists entirely of species that are endemic to hydrothermal vents or cold seeps (Distel et al., 2000). However, its unique morphology (Gustafson et al., 1998) and a recent analysis of molecular phylogeny (Jones et al., 2006) raise uncertainty about the taxonomic status of *"B." childressi*; thus, we follow the recommendation of Jones et al. (2006) and place the genus name of *"Bathymodiolus" childressi* in quotation marks. In Chapter 3 we have shown that trochophore larvae of *"B." childressi* can withstand both the temperatures and salinities present in the upper water column of the Gulf of Mexico. Additionally, shell morphology suggests the larvae are planktotrophic (Gustafson and Lutz, 1994; Gustafson et al., 1998; Chapter 2). Both lines of evidence suggest that larvae could migrate to the euphotic zone to feed.

Yet unknown is whether migration to surface waters would be energetically advantageous to the larvae of *"Bathymodiolus" childressi*. Although Young et al. (1996a) have suggested that the ability of some plaktotrophic larvae of bathyal

invertebrates to vertically migrate is not limited by energy stores, energetic data for the larvae of deep-sea organisms is rare; indeed, Young et al.'s (1996a) original test of their energetic model utilized energetic data from shallow-water echinoderms.

Here we test the energetic model provided by Young et al (1996a):

$$E_t = \int_o^t Q_o k e^{-rmvt} dt, \quad (1)$$

where E_t = cumulative energy consumption, k = a factor converting oxygen consumption to energy units, Q_o = rate of oxygen consumption, r = rate of change in oxygen consumption with temperature, m = rate of temperature change per meter, v = swimming velocity of larva, and t = time since the larva began swimming. Utilizing these parameters, the model predicts the cumulative energy the larva would consume over time (t), which can be compared the initial energy available in the egg as well as to the vertical distance the larva could swim at swimming velocity (v) in time (t). Using *"Bathymodiolus" childressi* larvae cultured *in vitro*, we empirically derive each parameter of the model and, examine the change in the energetics of *"B." childressi* larvae with age. Finally, we determine the accuracy of the model's prediction of migration potential by comparing it to direct evidence from plankton samples.

2. Methods

2.1 Egg composition—

Mean diameters, volumes, dry weights, ash-free dry weights (AFDW), lipid and protein content, and total energy were calculated for eggs that were collected from mussels induced to spawn. *"Bathymodiolus" childressi* adults were collected from cold-seeps ("Brine Pool": 27°43'24" N, 91°16'30" W and "Bush Hill": 27°47'N, 91°30'24"W)

on the upper Louisiana continental slope in the Gulf of Mexico using the *Johnson-Sea-Link I* and *II* submersibles (Harbor Branch Oceanographic Institute, Ft. Pierce, FL), returned to the Oregon Institute of Marine Biology (OIMB), and maintained in the laboratory as detailed in Chapter 2. Spawning was induced in ripe mussels (50 to 120 mm long) by a 0.4-0.5 ml injection of 2 mM serotonin into the anterior adductor mussel (Chapter 2).

We used a local mytild mussel as a control for all of our energetic measurements. *Mytilus trossulus* adults were collected during their spawning season (December-January; Strathmann, 1987) from the floating docks of the inner boat basin in Charleston, Oregon. Although hybridization with *Mytilus galloprovincialis* occurs along the west coast of the United States, a recent genetic analysis assures us that blue mussel populations in Charleston, OR are purely *M. trossulus* (Braby and Somero, 2006a). *Mytilus trossulus* mussels were maintained in a flow-through seawater system at the OIMB until spawning was induced by placing ripe mussels in individual containers of seawater and slowly warming from an ambient temperature of 10-12 °C to 20-25 °C (Strathmann, 1987).

Egg diameters were measured from light micrographs of eggs (*"Bathymodiolus" childressi*, n = 50; *Mytilus trossulus*, n = 14) using UTHSCSA ImageTool 3.0 image analysis software and volumes were calculated using the formula of a sphere ($1/6\pi d^3$). For *Mytilus trossulus*, egg collections were from individual females, but for *"Bathymodiolus" childressi* the data came from several females whose eggs were combined. Eggs were washed and re-suspended in isosmotic ammonium formate (3% w/v) to remove salt. Then, eggs in multiple samples of a known volume of egg

suspension were counted until the coefficient of variation of the counts was less than 10%. To obtain dry weights, known volumes of egg suspension (estimated number of eggs for *"B." childressi* = 7500 eggs; *M. trossulus* = 9600 eggs) were placed in pre-ashed and pre-weighed aluminum boats, dried in a 60 °C oven for two days, and then weighed. Next, eggs were ashed in a 550 °C muffle furnace for 6 hours and weighed again. AFDW is the difference between the dry weight and the ash weight.

Egg samples used to determine lipid and protein content were rinsed in fresh water, counted, and stored at -80 °C until analysis. Lipid content was determined for 12 replicate samples of 2513 eggs for *"Bathymodiolus" childressi* and 6 replicate samples of 1114 eggs for *Mytilus trossulus*. Lipids were extracted following the methods of Bligh and Dyer (1959), modified for microextraction by Reisenbichler and Bailey (1991). Total lipid content of eggs was determined following methods by Barnes and Blackstock (1973). Total protein content of eggs was estimated using the micro-assay procedure outlined in the Bio-Rad Protein Assay Kit II (Bio-Rad, USA), based on the methods of Bradford (1976). To assure that we used the appropriate number of eggs (ie, the total protein content fell within the standard curve), we analyzed 3 replicates each of 600, 465, and 300 eggs for *"B." childressi*, and 978, 960, 540, and 240 eggs for *M. trossulus*. All values fell within the standard curve so we used the mean protein content per egg calculated from the 9 replicates for or *"B." childressi* and 12 replicates for *M. trossulus*.

Energy in the eggs was estimated using combustion enthalpies of 39.5 kJ g^{-1} for lipid and 24.0 kJ g^{-1} for protein (Gnaiger, 1983). The energy in the "remainder fraction," or the difference between AFDW and the lipid, protein, and carbohydrate content

(Jaeckle and Manahan, 1989), was estimated using an average combustion enthalpy of 27.0 kJ g^{-1} (Jaeckle, 1995). Since we did not measure the content of carbohydrate, which is generally low and has the lowest combustion enthalpy (17.5 kJ g^{-1}; Gnaiger, 1983), it is included in our "remainder fraction."

2.2 Oxygen consumption—

After spawning was induced in *"Bathymodiolus" childressi* and *Mytilus trossulus* as described in section 2.1, cultures were made for each. Culturing procedures followed those outlined in Chapter 2 for *"B." childressi* and Chapter 3 for *Mytilus trossulus*. Cultures were reared in 0.22 mm filtered seawater (FSW) and *"B." childressi* cultures were reared in a 7-8° C cold room while those of *M. trossulus* were incubated in a 10-12 °C flow-through seawater table. All experiments were conducted with blastulae (within 24 hours of hatching) and trochophore larvae (4 days post-hatching) of *"B." childressi.* Because larvae of *M. trossulus* develop faster than do those of *"B." childressi,* all experiments with *M. trossulus* were conducted with trochophore larvae only (about 24 hours after fertilization).

Oxygen consumption of the mussel larvae was measured at 2, 7, and 15 °C using the end-point method described in Marsh and Manahan (1999). Although sea surface temperatures reach more than 30 °C in the summer in the Gulf of Mexico, we did not take respiration measurements at higher temperatures to avoid confounding effects of significant mortality at temperatures above 15 °C (Chapter 3). We used 1-ml Wheaton Brand V-vials overfilled to 1.6 ml as micro-scale Biological Oxygen Demand (μBOD) vials. Prior to experimentation, we tested the effectiveness of the μBOD vials by filling

them with de-oxygenated water and incubating them as in our experimental protocol, then measuring the oxygen concentration after incubation; oxygen content did not increase, indicating that they were airtight. We ran several preliminary tests to determine the optimal number of larvae and incubation time for the oxygen sensors to read a change. Between 120 and 250 larvae were counted into four replicate V-vials under the microscope then overfilled with sterile FSW (at culturing temperature) and capped carefully to ensure that no air bubbles clung to the glass or remained under the lid. Vials were then incubated horizontally to prevent accumulation of larvae in the "v" or at the surface, for approximately 12 hours at each test temperature. Four replicate "blanks" filled with only sterile FSW were incubated alongside the treatments. At the end of each incubation period, the lid of each replicate was removed and approximately 500 µl of the water was immediately withdrawn with a gas-tight syringe while viewing through a horizontally mounted stereoscope, allowing us to count any larvae inadvertently withdrawn with the water sample. The samples were injected into a MC100 Microflow cell (volume: 70 µl) equipped with a polargraphic oxygen sensor (Strathkelvin Model 1302) connected to an oxygen meter (Strathkelvin Model 781) that was calibrated at the appropriate temperature. Total oxygen consumed was the difference between the oxygen content remaining in vials incubated with larvae and the average oxygen concentration of the four blanks at each temperature.

The exact number of larvae incubated in each replicate was counted after all measurements were taken. An average spherical volume of the larvae (n = 10) in each replicate was determined by converting their areas, measured using UTHSCSA

ImageTool 3.0 image analysis software. However, to conform to convention, we expressed our data as oxygen consumed per larva, adjusting the number of larvae per replicate to a mean volume (across all three types of larvae) of 0.187 $\pm$ 0.020 nl ($\pm$ 1 S.D.). The range of final adjusted total number of larvae was no more than 100 for any one treatment. The total oxygen consumption (pmol h^{-1}) was plotted against the total adjusted number of larvae for each larvae type. An exponential model was fitted to the individual rate of oxygen consumption (pmol oxygen consumed $larva^{-1}$ h^{-1}) across the three temperatures for blastulae and trochophores of *"Bathymodiolus" childressi* and trochophores of *Mytilus trossulus*. Rates of oxygen consumption were converted to energy utilization rates using an oxyenthalpic conversion factor of 480 kJ mol^{-1}—the average of the oxyenthalpic equivalents for lipid, protein, and carbohydrate (Gnaiger 1983).

2.3 Vertical swimming velocity—

The vertical swimming velocities of trochophore larvae were empirically measured for both *"Bathymodiolus" childressi* and *Mytilus trossulus*. Swimming velocities measured were not instantaneous linear velocities (Cragg, 1980), but rather velocities of vertical displacement. Vertical swimming velocities of trochophore larvae (4 days post-hatching for *"B." childressi*, 24-48 post-fertilization for *M. trossulus*) were measured at 3 temperatures: 7, 15, and 20 °C in a temperature insulated swim chamber. The swim chamber was a polystyrene tissue culture vial (15 mm x 35 mm x 60 mm) filled with FSW that was suspended in a stirred, glass bath filled with de-ionized water with its temperature regulated by water from a re-circulating water bath set to the

appropriate temperature running through an aluminum coil immersed in the glass water bath (Fig. 1). Temperature within the swim chamber was measured with an ethanol thermometer mounted inside one corner. We were assured that convection cells were not created within the swim chamber by introducing colored FSW into the bottom of the chamber and observing that it did not circulate. Horizontal lines were etched into the swim chamber at 0.5 cm increments. Trochophore larvae were acclimated to the appropriate temperature for at least thirty minutes prior to experimentation and then many of them (20-50) were carefully introduced to the bottom of the swim chamber with a glass Pasteur pipette. Larvae began slowly swimming upward immediately. When larvae reached the middle of the chamber, we measured the speed of vertical swimming through a 0.5 cm increment using a horizontally mounted stereoscope and a stopwatch.

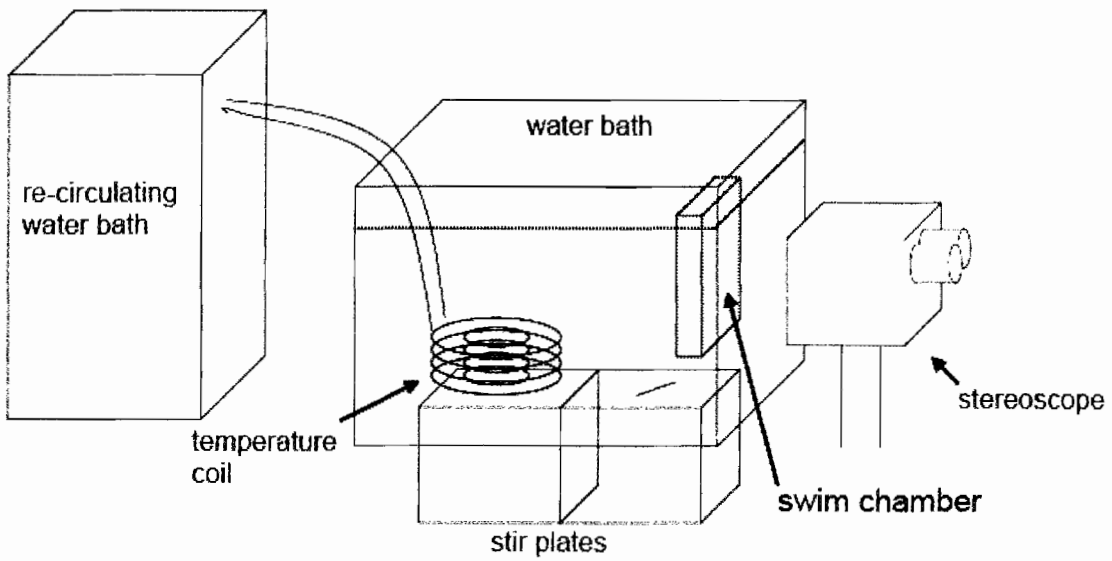

Figure 1. Schematic of swim chamber and water bath used for measuring vertical swimming velocities of trochophore larvae.

To reduce variation caused by wall-induced drag (Winet, 1973), we measured the swimming velocity of only those trochophores that were in the plane of focus at the center of the chamber. We measured the velocity of ten individuals for each temperature, except at 15 °C we measured the velocity of 12 trochophores of *M. trossulus*. Additionally, we conducted two trials at 7 °C for *"B." childressi* trochophores. Because the velocities were not significantly different among these trials, we pooled them for a total sample size of 20. We analyzed the data with separate single factor ANOVAs for each species, with temperature as a fixed factor (Sokal and Rohlf, 1981).

2.4 Temperature profile—

Temperature profiles were taken in the Gulf of Mexico over the Louisiana slope cold-seep sites in March 2002, May 2002, February 2003, November 2003, December 2004, and August 2006 using a conductivity/temperature/depth (CTD) profiler (Sea-Bird electronics, Inc.). Locations of the CTD casts are given in Table 1.

Table 1. Dates and locations of CTD casts. All casts were taken above the Brine Pool cold seep (27°43'24" N, 91°16'30" W).

Date	Latitude	Longitude
March 11, 2002	27°43.51 N	91°16.78 W
May 10, 2002	27°43.52 N	91°16.86 W
February 9, 2003	27°43.21 N	91°15.31 W
November 10, 2003	27°43 N	91°16 W
December 11, 2004	27°43.606 N	91°16.93 W
August 13, 2006	27°43.44 N	91°16.65 W

2.5 Predicting migration potential—

We chose to use the depth of the Brine Pool cold seep as the initial depth and temperature for the model because (1) it was our study site for a series of other studies, (2) it was our primary adult mussel sampling site, and, most importantly, (3) it supports a large (540 m^2) and healthy bed of *"Bathymodiolus" childressi* mussels (MacDonald et al., 1990; Nix et al., 1995). The depth at the Brine Pool is approximately 650 m and the temperature remains at 7-8 °C throughout the year.

Equation 1 can be integrated into the following (Young et al. 1996a):

$$E_t = -(kQ_o/rmv)\, e^{-rmvt} \qquad (2).$$

Parameters for Equation 2 were estimated from the results of the experiments outlined in the previous sections. The conversion factor k, which converts oxygen consumption to energy units, is the average of the oxyenthalpic equivalencies for protein, lipid, and carbohydrates and equals 480 kJ mol^{-1} oxygen (Gnaiger, 1983). The rate of oxygen consumption (Q_o) is calculated from the equation for the change in rate of oxygen consumption with temperature. For *"Bathymodiolus" childressi* trochophores, this rate follows an exponential model (see section 3.2; Young et al., 1996a):

$$Q_t = Q_o e^{r\Delta T} \qquad (3)$$

and was calculated herein (section 3.2) as $y = 3.0706e^{0.1492x}$. Accordingly, $Q_o = 3.0706$ pmol h^{-1} and $r = 0.1492$. Based on gametogenic cycles, spawning times (Tyler et al., 2006; Chapter 2) and mean mytilid veliger swimming velocities of 3.96 m h^{-1} (Konstantinova, 1966), we expect larvae of *"B." childressi* to be in the water column from mid-October through at least February, but not in the summer months. Thus, we

calculated the rate of temperature change per meter (m) based on our temperature profile over the Brine Pool cold seep during the coldest winter month (February 2003) that we measured (m = -0.0202 ^{o}C m^{-1}; see Fig. 6 in section 3.4). We also wanted to predict E_t when the sea surface temperature is highest within the period in which we expect to find larvae in the water column. Thus, we used the temperature profile during the warmest fall month that we measured (November 2003) to calculate m as well. Due to the strong thermocline in November 2003, a linear regression predicts too low a temperature at our starting depth of 650m. To alleviate this problem, we divided the November 2003 temperature profile into 3 parts and ran the model over each part. From 650 to 400 m depth we use m = -0.0275, from 400 to 200 m we use m = -0.022, and from 200 to 0 m we use m = -0.0392 (Fig. 2).

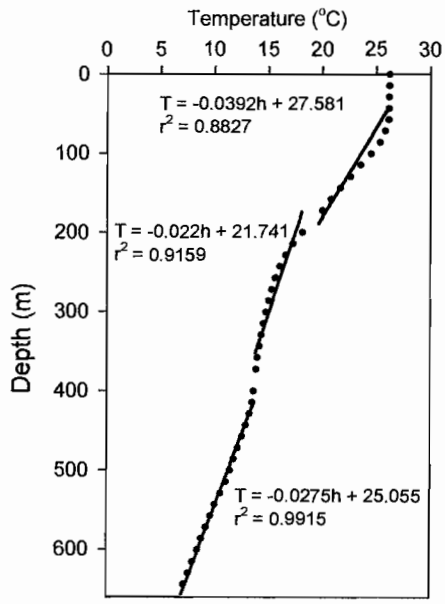

Figure 2. Thermal profile of the water column above the cold seeps in the Gulf of Mexico in November 2003. The profile was divided into three parts and regressions were used separately in the ontogenetic vertical migration model.

We used five swimming velocities (v). First, we used our minimum (= 0.105 m h^{-1}; Table 4, section 3.3), maximum (= 1.06 m h^{-1}; Table 4, section 3.3), and mean (= 0.510 m h^{-1}; Table 4, section 3.3) velocities for *"B." childressi* trochophores at 15 °C. We calculated E_t at 24 hour intervals (t). We set $E_t = 0$ at a ΔT (= $-mvt$) of 7 °C and a depth of ~650 m by subtracting the E_t where $\Delta T \leq 7$ °C and plotted E_t against depth (vt). Next, we used published minimum (= 3.96 m h^{-1}; Table 5, section 3.3) and maximum (= 7.20 m h^{-1}; Table 5, section 3.3) velocities for *Mytilus edulis* at 12 °C. For these, we set $E_t = 0$ at the depth and temperature where the model predicts a trochophore to be after 6 days of swimming, which is the time at which *"B." childressi* trochophores develop into D-shelled veligers *in vitro* (Chapter 2).

2.6 Plankton sampling—

To determine the distribution of *"Bathymodiolus" childressi* larvae in the water column, plankton samples were taken with a Multiple Opening/Closing Net Environmental Sampling System (MOCNESS) above the Brine Pool several times in November 2003. The MOCNESS is a sophisticated net sampling system consisting of nine nets that can be opened and closed at desired depths. On November 11, 2003 and November 15, 2003 eight nets (150 μm mesh size) were opened and closed every 50 vertical meters starting at 600 meters depth and ending at 200 meters depth. On November 10, 2003, the starting depth was 700 m and ended at 300 m. The nets were towed at the maximum depth for 10 minutes and then pulled through a 50-meter column of water at a 45-degree angle at about 20-25 m per minute. Samples were sorted into large (> 500 μm) and small (< 500 μm) portions. The small portions were swirled and

the heavy shelled plankton removed and preserved in 95% ethanol until the bivalve larvae could be identified with molecular techniques. The rest of the samples were fixed in 10% formalin buffered in seawater, then stored in 70% ethanol until processing. In addition, a MOCNESS sample from 0-100 m was taken on February 11, 2003, split into large and small portions, fixed in 10% formalin buffered in seawater, then stored in 70% ethanol until processing.

All ethanol-preserved samples were sorted for bivalves, but we sorted the formalin-fixed samples from only November 15, 2003 and February 11, 2003 (0-100m) because we had already removed the heavy plankton (i.e., bivalves and gastropods) from the November samples. After bivalves were removed from the samples, each was photographed under 10-20 x magnification and assigned a grouped based on morphology (size, shape, and color). After visual sorting, samples were processed for either molecular or SEM identification.

2.6.1 Molecular identifications

DNA of individual larvae was extracted following the methods of Li and Hedgecock (1998) and Johnson and Geller (2006). An approximately 500-bp fragment of the 18S rRNA gene was amplified with the universal eukaryote 18s rDNA primers U519 and U915. Total reaction volume was 10 μl, with 2 μl template DNA, 1x bovine serum albumin, 1x PCR buffer, 2.5 mM $MgCL_2$, 2mM dNTP's, 10 μM each primers, 0.1μl *AmpliTaq* Gold DNA polymerase (Applied Biosystems, Foster City, CA), and sterile water to final volume. Thermocycling occurred in a Cetus 9600™ DNA Thermal Cycler (Perkin-Elmer Corp. CT) as follows: 95 °C for 10 minutes, 35 cycles of 94°C/1',

55°C/1', and 72°C/1', followed by a final extension period for 7 minutes and held at 4 °C. The PCR products were electrophoresed on a 1% agarose gel using 1x TAE buffer (Montage©) and purified by gel excision and Montage filter units (Millipore Corp. Billerica, MA). Products were sequenced bi-directionally with the same primers used in PCR on an ABI 3100 capillary sequencer using BigDye™ terminator chemistry (V3.1, Applied Biosystems, Foster City, CA). Using Sequencher (v4.7, Gene Codes Corp, Ann Arbor, MI), DNA sequence alignments were constructed from 52 individual bivalve larvae collected in the plankton, and 4 recent settlers (presumably *"Bathymodiolus" childressi*) and an adult from the Brine Pool. A neighbor joining tree was created with PAUP 4.0b10 (Swofford, 1998) using reference sequences from Gen Bank of representative bivalves from families found in the Gulf of Mexico (Table 2).

Table 2. Reference bivalve species and GenBank accession numbers used in neighbor-joining analysis.

Species	Accession #
Mytilus edulis	L33448
Bathymodiolus brooksi	AY649826
Tamu fisheri	AF221642
"Bathymodiolus" childressi	AF221641
Idas macdonaldi	AF221647
Modiolus americanus	AF229624
Leiosolenus lithurus	AB201236
Limaria hians	AF120534
Glycymeris insubrica	AF207647
Notocorbula coxi	AY192684
Bankia carinata	AF120564
Calyptogena magnifica	AF120556
Galeomma turtoni	AF120547
Abra prismatica	AF120554
Crassostrea gigas	AB064942
Mytrea spinifera	AY70115

2.6.2 SEM identifications

Scanning electron micrographs of larval shells were taken on a JEOL 6400F Field Emission Scanning Electron Microscope. Shells were cleaned in 5% sodium hypochlorite solution, rinsed with distilled water, air-dried, and mounted on adhesive carbon discs for SEM (Rees, 1950; Fuller and Lutz, 1989). Procedures to accurately document the shapes and dimensions of the larval bivalve shells were modified from those of Fuller et al. (1989). For each larval shell the following measurements were taken: height and length of prodissoconch II, shell length and straight hinge length of the prodissoconch I (if possible), provinculum length, and number of teeth. Length is the greatest dimension approximately parallel to the provinculum and height is the greatest dimension starting from and perpendicular to the hinge line. SEMs are presented of only individuals with characters similar to *"Bathymodiolus" childressi.*

3. Results

3.1 Egg composition—

The diameters measured for *"Bathymodiolus" childressi* and *Mytilus trossulus* eggs were within the ranges of those published for *Mytilus edulis* and *Mytilus galloprovincialis* (Table 3). Egg diameters of *"B." childressi* ($\bar{x} \pm$ S.D. = 69.15±2.36 μm; n = 50) were only slightly larger than those of *M. trossulus* ($\bar{x} \pm$ S.D. = 66.93±1.30 μm; n = 14), but the volumes of *"B." childressi* eggs were significantly greater (Table 3; F = 12.98, p = 0.001). The percentage of AFDW made up of lipids was 44.8 % for *"B." childressi* eggs and 35.2 % for *M. trossulus* eggs, while proteins made up 12.2 % of the AFDW of *"B." childressi* eggs and 15.5 % of the AFDW of *M.*

trossulus eggs (Table 3). Likewise estimated total energy content was greater for eggs of *"B." childressi* ($\bar{x} \pm$ S.D. = 2.95±0.082 mJ egg^{-1}) than for eggs of *M. trossulus* ($\bar{x} \pm$ S.D. = 1.85±0.95 mJ egg^{-1}) (Table 2). Our estimates of the energy in the eggs of *M. trossulus* are higher than estimates determined by other methods published for the eggs of shallow-water congeneric mussels (Table 3).

3.2 Oxygen consumption—

The number of larvae used in each treatment varied from 120 to 250 for all treatments. However, there were no significant linear relationships between the number of larvae in the μBOD containers and the total oxygen consumed by *"B." childressi* blastulae and trochophores and *M. trossulus* trochophores at any of the temperatures tested (Fig. 3). The variance of our readings was higher than the effect of a small change in the number of larvae. Thus, we did not regress oxygen consumption against the number of larvae in order to obtain the respiration rate as suggested by Marsh and Manahan (1999). Instead, we simply divided the total oxygen consumed by the number of larvae in each μBOD vial to obtain an average oxygen consumption rate per larva for each temperature.

Table 3. Biochemical content of eggs from *"Bathymodiolus" childressi* and *Mytilus trossulus*. Diameters and volumes are means of 50 eggs for *"B." childressi* and 10 eggs for *M. trossulus*. For weights, n = 7 for *"B." childressi* and n = 4 for *M. trossulus*. Also given are published values for *Mytilus edulis* and *Mytilus galloprovincialis*. Errors are ± 1standard deviation.

Species	Diameter (μm)	Volume (nl)	Dry Weight (ng)	AFDW (ng)	Lipid (ng) *(mJ)*	Protein (ng) *(mJ)*	Total energy[a] *(mJ)*
"Bathymodiolus" childressi	69.15±2.36	0.174±0.016	123.73±30.50	91.38±27.12	40.95±13.06 *1.62±0.52*	11.12±2.01 *0.267±0.048*	*2.95±0.082*
Mytilus trossulus	66.93±1.30	0.157±0.009	184.89±49.14	59.96±31.72	21.10±13.96 *0.833±0.552*	9.27±3.11 *0.223±0.075*	*1.85±0.95*
Mytilus edulis[b]	--	--	52.5[b]	--	8.36±0.319 *0.330±0.013*	17.98±2.99 *0.431±0.072*	*1.207*[c]
Mytilus edulis[d]	73.2	0.205	--	80.0	16.0 *0.632*	-- --	*1.78*
Mytilus galloprovincialis[e]	61.0±1.0	0.119±0.006	53.0±1.9	47.33±1.04	11.7±1.17 *0.462±0.046*	24.1±1.09 *0.579±0.026*	*1.33±0.05*

[a]Energy of "remainder fraction" (including carbohydrates) calculated using a combustion enthalpy of 27.0 kJ/g (Jaeckle 1995).
[b]Data from Bayne et al. (1975). Lipid and protein content are means from eggs spawned from mussels with either high or low food rations at 8 °C and 16 °C.
[c]Unpublished data cited in Bayne et al. (1975).
[d]Data from Honkoop et al. (1999).
[e]Data from Sedano et al. (1995).

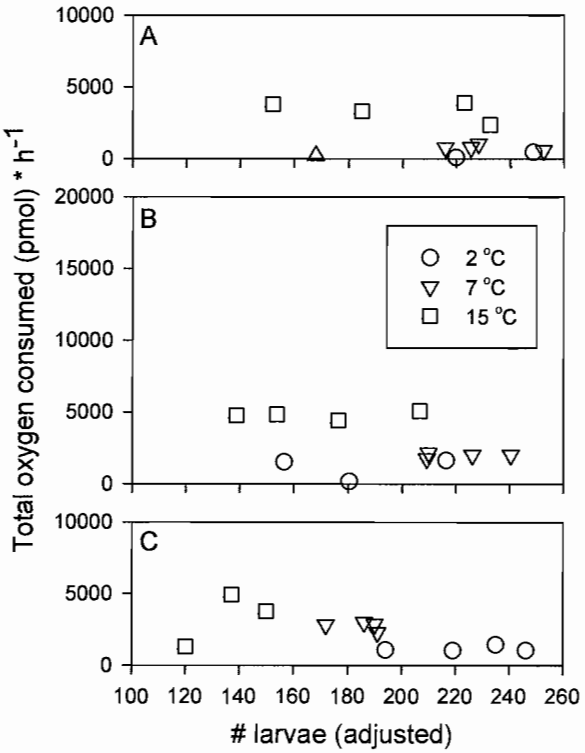

Figure 3. Total oxygen consumed per hour in individual μBOD chambers plotted against the total number of (A) *"Bathymodiolus" childressi* blastulae per chamber, (B) *"B." childressi* trochophores per chamber, and (C) *Mytilus trossulus* trochophores per chamber. Measurements are at 3 different temperatures and there was no significant linear relationship between the number of larvae and the oxygen consumed for any larval type or temperature.

Rate of oxygen consumption (pmol larva^{-1} h^{-1}) increased exponentially with temperature for all *"Bathymodiolus" childressi* blastulae and trochophores and were within the range of oxygen consumption measurements for *Mytilus trossulus* trochophores. The relationship between oxygen consumption and temperature fit the following exponential models significantly: $y = 0.6322e^{0.2216x}$, $r^2 = 0.865$ ($F = 51.27$, $p < 0.001$) for *"B." childressi* blastulae; $y = 3.0706e^{0.1492x}$, $r^2 = 0.6653$ ($F = 17.89$, $p = 0.002$) for *"B." childressi* trochophores; and $y = 4.8129e^{0.124x}$, $r^2 = 0.7169$ ($F = 22.79$, $p = 0.001$) for *M. trossulus* trochophores (Fig. 4). The oxygen consumption rates across temperature ranged from 0.39 to 18.0 pmol larva^{-1} h^{-1} for *"B." childressi* blastulae, 0.9 to 34.4 pmol larva^{-1} h^{-1} for *"B." childressi* trochophores, and from 4.4 to 52.6 pmol larva^{-1} h^{-1} for *M. trossulus* trochophores (Fig. 5). Oxygen consumption rates across temperature correspond to energy utilization rates ranging from 0.19×10^{-3} to 8.64×10^{-3} mJ larva^{-1} h^{-1} for *"B." childressi* blastulae, 0.45×10^{-3} to 16.51×10^{-3} mJ larva^{-1} h^{-1} for *"B." childressi* trochophores, and from 2.11×10^{-3} to 25.26×10^{-3} mJ larva^{-1} h^{-1} for *M. trossulus* trochophores (Fig. 4).

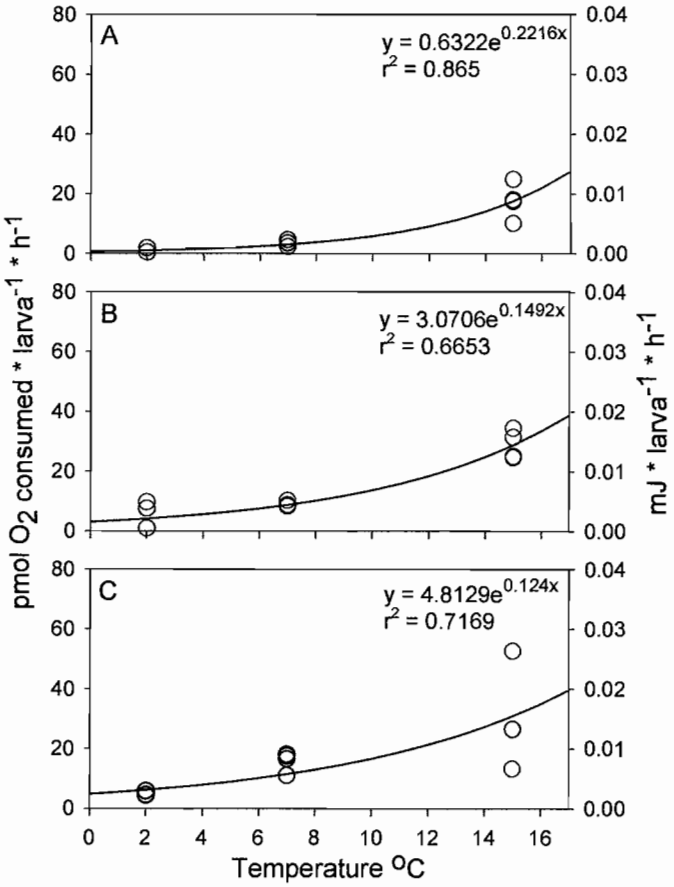

Figure 4. Oxygen (left axis) and energy (right axis) consumption rates per larva across 3 temperatures for (A) *"Bathymodiolus" childressi* blastulae (B) *"B." childressi* trochophores, and (C) *Mytilus trossulus* trochophores. The equations for the relationships between oxygen consumption and temperature are shown. Energy consumption rates were calculated using the average of the oxyenthalpic equivalencies for protein, lipid, and carbohydrates (480 kJ mol^{-1} oxygen; Gnaiger, 1983).

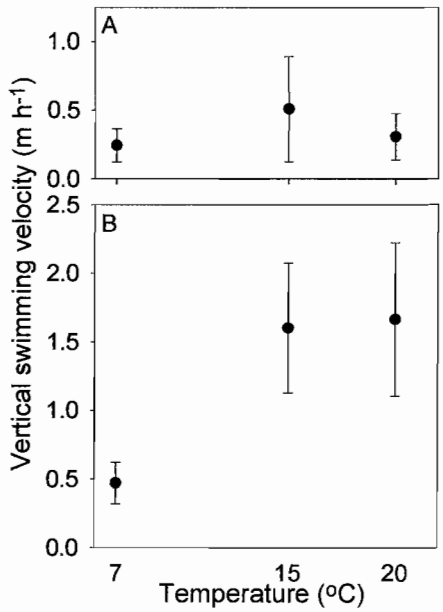

Figure 5. Vertical swimming velocities (m h^{-1}) for trochophores of (A) *"Bathymodiolus" childressi* and (B) *Mytilus trossulus* at 3 temperatures. Plotted are means ± S.D.

3.3 Vertical swimming velocity—

Vertical swimming velocities varied widely for both species (Table 4; Fig. 5). Temperature had a significant effect on vertical swimming velocity of *"Bathymodiolus" childressi* trochophores (Fig. 5a; $F = 4.698$, $p = 0.015$), such that velocities were significantly faster in 15 °C than in 7 °C (Tukey HSD: $p = 0.011$), but not different between 15 and 20 °C (Tukey HSD: $p = 0.126$) or 7 °C and 20 °C (Tukey HSD: $p = 0.740$). Likewise, temperature significantly affected vertical swimming velocities of *Mytilus trossulus* trochophores (Fig. 5b; $F = 24.54$, $p < 0.001$). Velocities at 15 °C and 20 °C did not vary significantly from each other (Tukey HSD: $p = 0.940$), but both were significantly faster than in 7 °C (Tukey HSD: $p < 0.001$). Compared to published swimming velocities for *Mytilus edulis* veligers (Table 5), our velocities for trochophores of *"B." childressi* and *M. trossulus* are an order of magnitude slower.

Table 4. Maximum, minimum, and mean vertical swimming velocities measured for trochophores of *"Bathymodiolus" childressi* and *Mytilus trossulus* at several temperatures.

Species	Temp. (°C)	Vertical velocity (cm s^{-1}) Minimum	Maximum	$\bar{x} \pm$ S.D.	n
"B." childressi	7	0.002503	0.010473	0.006779 ± 0.003461	20
	15	0.002921	0.029464	0.014156 ± 0.010639	10
	20	0.004421	0.018096	0.008573 ± 0.004661	10
M. trossulus	7	0.008959	0.022789	0.013064 ± 0.004182	10
	15	0.017662	0.060386	0.044513 ± 0.013151	12
	20	0.014189	0.063694	0.046252 ± 0.015535	10

Table 5. Published maximum swimming velocities for *Mytilus edulis* veligers.

Temperature (°C)	Size (μm ±S.E.)	Velocity (cm s^{-1})	Reference
6	261±23	0.125	Sprung 1984a
12	226±27	0.200	Sprung 1984a
18	245±29	0.330	Sprung 1984a
—	veliger	0.110	Konstantinova 1966[a]

[a]As reported in Chia et al. (1984).

3.4 Temperature profile—

Sea surface temperature over the continental slope of the Gulf of Mexico varies widely throughout the year (Fig. 6). We measured a minimum sea surface temperature in February 2003 (= 20.3 °C; Fig. 6c) and a maximum in August 2006 (= 30.3 °C; Fig. 6f). At the maximum depths recorded (~630 m), temperature remained about 7-8 °C. Although a thermocline was evident between 200 and 100 m depth in some months, a straight line was a good fit for each profile (r^2 was $\geq$ 0.89 for each).

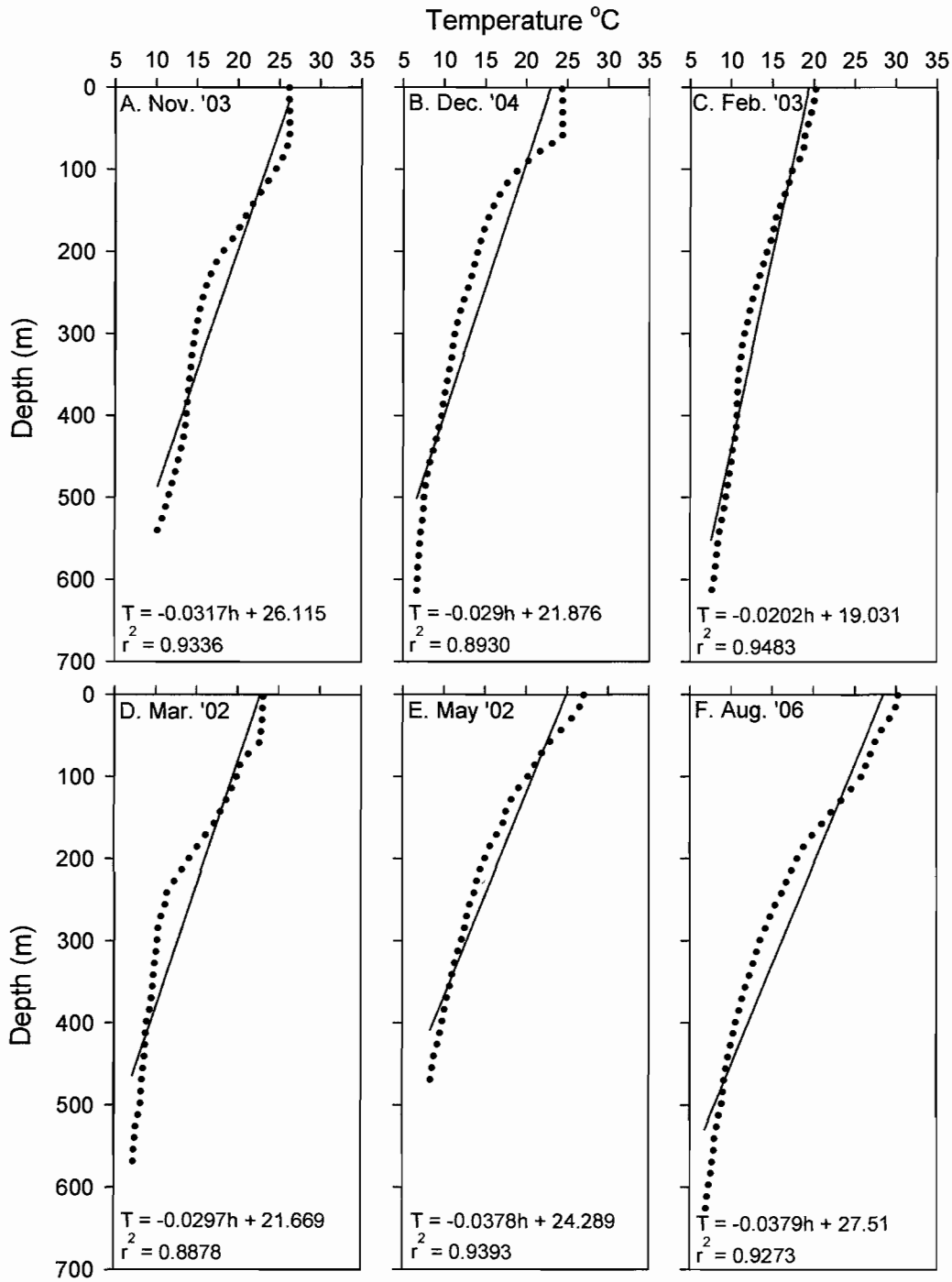

Figure 6. Temperature-depth profiles in the Gulf of Mexico. Exact dates and locations are given in Table 1. (A) – (C) are within the spawning season of *"B." childressi* and larvae are expected in the water column through at least March (D). Larvae are not expected in the water column in May through August (E)-(F). Regressions of temperature with depth are given on each profile. The model parameter *m* is the slope of the regression.

3.5 Predicting migration potential—

Using the temperature profile for February 2003, the total energy consumed by a larva would equal the energy reserves in its egg ($\bar{x} \pm$ S.D. = 2.95 $\pm$ 0.082 mJ; Table 3) by 31 days at a depth of 574 m at a trochophore's slowest swimming velocity (0.105 m h^{-1}), after 22 days and a depth of 379 m at a trochophore's mean swimming velocity (0.510 m h^{-1}), and by 17 days at a depth of 217 m at a trochophore's maximum swimming velocity (1.06 m h^{-1}; Fig. 7a). In November 2003, the total energy consumed by a larva would equal 2.95 mJ at 25 days and a depth of 587 m at a trochophore's slowest swimming velocity (0.105 m h^{-1}), by 18 days and a depth of 429 m at a trochophore's mean swimming velocity (0.510 m h^{-1}), and by 14 days at a depth of 294 m at a trochophore's maximum swimming velocity (1.06 m h^{-1}; Fig. 7b). Assuming that hatched larvae swim for 6 days before developing into D-shell veligers (Chapter 2), a trochophore swimming at average speed would reach a depth of 575 m and use a total of 0.576 mJ of energy in February 2003 and would reach a depth of 576.5 m using a total of 0.723 mJ of energy in November 2003 (Fig. 7a,b). If a larva then begin swimming at veliger swimming velocity in February 2003 it could reach the surface in less than 7 more days at 3.96 m h^{-1} and less than 4 more days at 7.20 m h^{-1} before using up all the energy reserves in the egg (Fig. 7c). However, at the higher sea surface temperatures in November 2003, a veliger would use up all the energy reserves in the egg by 100 m and within 5 more days at 3.96 m h^{-1}, but would reach the surface within 4 more days at 7.20 m h^{-1} (Fig. 7d).

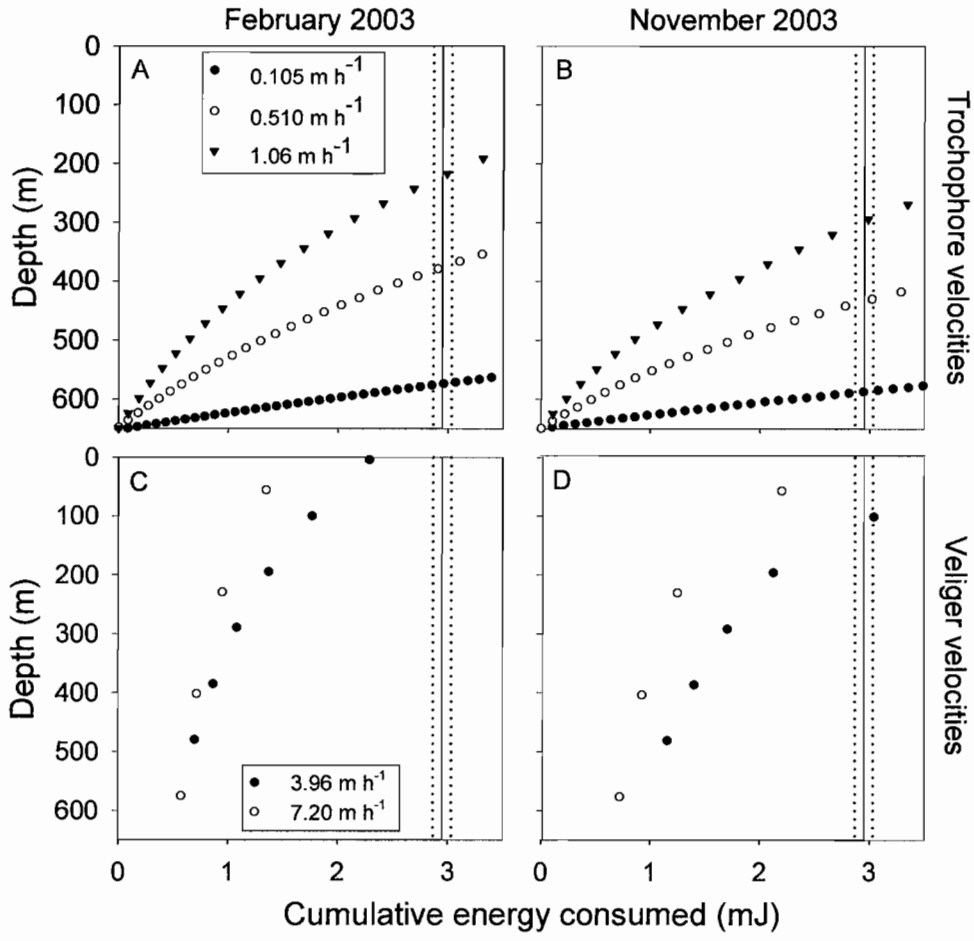

Figure 7. Predicted cumulative energy consumption (mJ) as a function of depth for *"Bathymodiolus" childressi* using a starting depth of 650 m and temperature of 7 °C at multiple swimming speeds for trochophores (top) and veligers (bottom) and two sea surface temperatures (low-February 2003 and high-November 2003). For veligers (bottom), we used a starting depth of ~ 575 m (the depth after 6 days of swimming as trochophores). Points on all curves are 24 hours apart. The solid vertical lines represent the mean total energy in the egg with S.D. represented by dotted lines.

3.6 Plankton sampling—

3.6.1 SEM identifications

On the basis of larval shell size and other shell characteristics revealed by SEM, we identified 2 veligers from the February 11, 2003 surface sample (0 to 100 m depth) as *"Bathymodiolus" childressi* (Fig. 8). Like the larval shells of *"B." childressi*, the two veligers presented here had the "egg shape" characteristic of the family Mytilidae (Chanley 1970). PII's were smaller than those published for *"B." childressi*, which are maximum lengths (Table 6; Chatper 2), but were larger than other described shallow-water mytilids (reviewed in Chapter 2; Fuller and Lutz, 1989). Larval shell characteristics resembled those from a published description of the larval shell of *"B." childressi* (Table 6; Chapter 2). Dentition is taxodont, with shorter central teeth and larger teeth at the anterior and posterior ends of the provinculum (Fig. 8). In mytilids, the addition of larger teeth at the anterior and posterior extremes of the provinculum continues through the larval life (Fuller and Lutz, 1989 and references therein), which explains the variance in tooth number and the smaller provinculum lengths in our samples. Similarly, a primary ligament pit (as shown in Fig. 8a) does not form until metamorphosis of settlement (Lutz and Hidu, 1979; Fuller and Lutz, 1989). No veliger shells resembling the larval shells of *"B." childressi* were found in the formalin-fixed samples from November 15, 2003.

Table 6. Larval shell dimensions for veligers found in plankton tows taken from 0-100 m depth on February 11, 2003. PI and PII are the prodissioconchs I and II. Hinge is the length of the hinge line. Means (n = 5) given for *"Bathymodiolus" childressi* with S.D. in italics are from Chapter 2.

	PI		PII		Provinculum	
	Hinge	Length	Length	Height	Length	# Teeth
"Bathymodiolus" childressi	89.41	113.35	442.56	391.92	210.15	29-31
	1.94	*2.02*	*8.84*	*7.39*	*10.94*	*0*
Figure 8B	88.82	115.48	437.76	382.63	191.85	31
Figure 8C	—	—	425.81	378.17	177.26	29

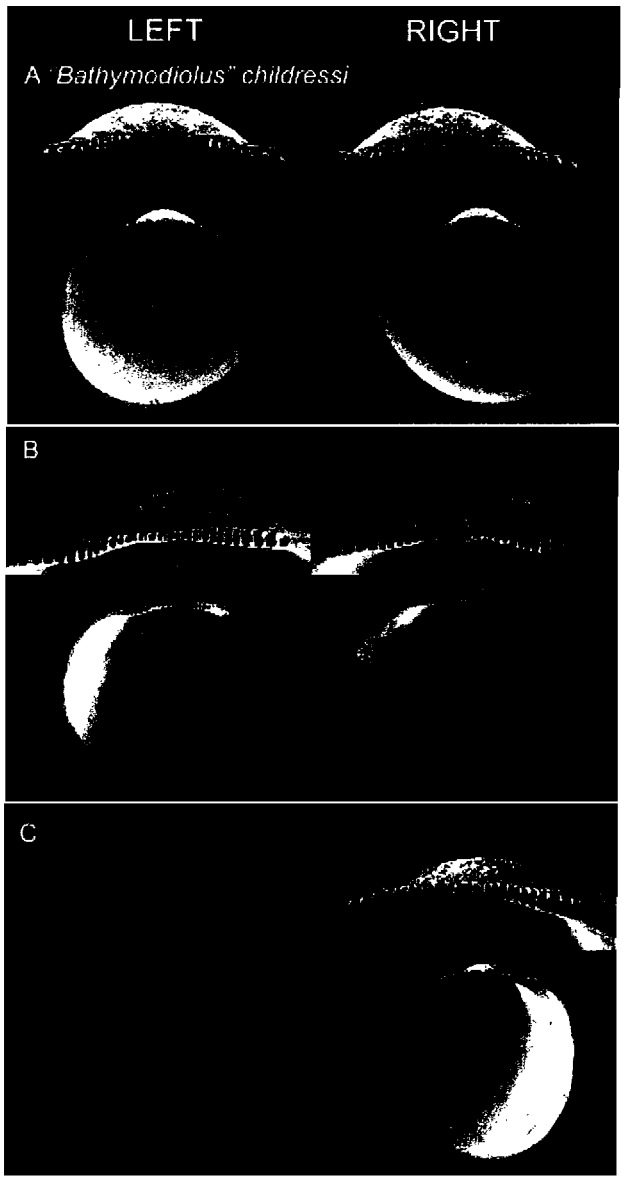

Figure 8. Larval shells of (A) *"Bathymodiolus" childressi* taken from the Brine Pool cold seep, and (B) and (C) two mytilid samples found in a plankton tow from 0 to 100 m depth from above the Brine Pool cold seep in February 2003. Shell morphometrics are given in Table 6.

3.6.2 Molecular identifications

Neighbor-joining analysis discriminated 32 groups of bivalves in at least 10 families from the November 2003 ethanol-preserved samples (Fig. 9). Of these, four individuals were identified as mytilids both through molecular techniques (Fig. 9) as well as through assessment of morphology under light microscopy (Fig. 10). Lengths, collection dates, and depths for these mytilids are given in Table 7. Of the mytilids, only one was initially placed in a separate group based on its shape (Fig. 10b). Neighbor-joining analysis grouped this individual with *Leisolenus lithurus* (Fig. 9). In addition, of the three veligers that we could not discriminate with light microscopy (Fig. 10a,c,d), individuals C and D also grouped with *L. lithurus* (Fig. 9). Only individual A was positively identified as *"Bathymodiolus" childressi* based on its alignment with our adult sample and four recently settled juveniles (S1, S2, S3, and S4) collected from the Brine Pool, as well as adult *"B." childressi* sequences from GenBank (Fig. 9). This individual was collected from 300 to 350 m depth on November 15, 2003 and was 278.4 μm long.

Table 7. Collection data and sizes of mytilid larvae shown in Figure 10.

Sample	Collection date	Depth (m)	Length (μm)
Figure 10A	November 15, 2003	300-350	274.8
Figure 10B	November 11, 2003	200-250	385.1
Figure 10C	November 15, 2003	400-450	236.6
Figure 10D	November 10, 2003	300-351	323.7

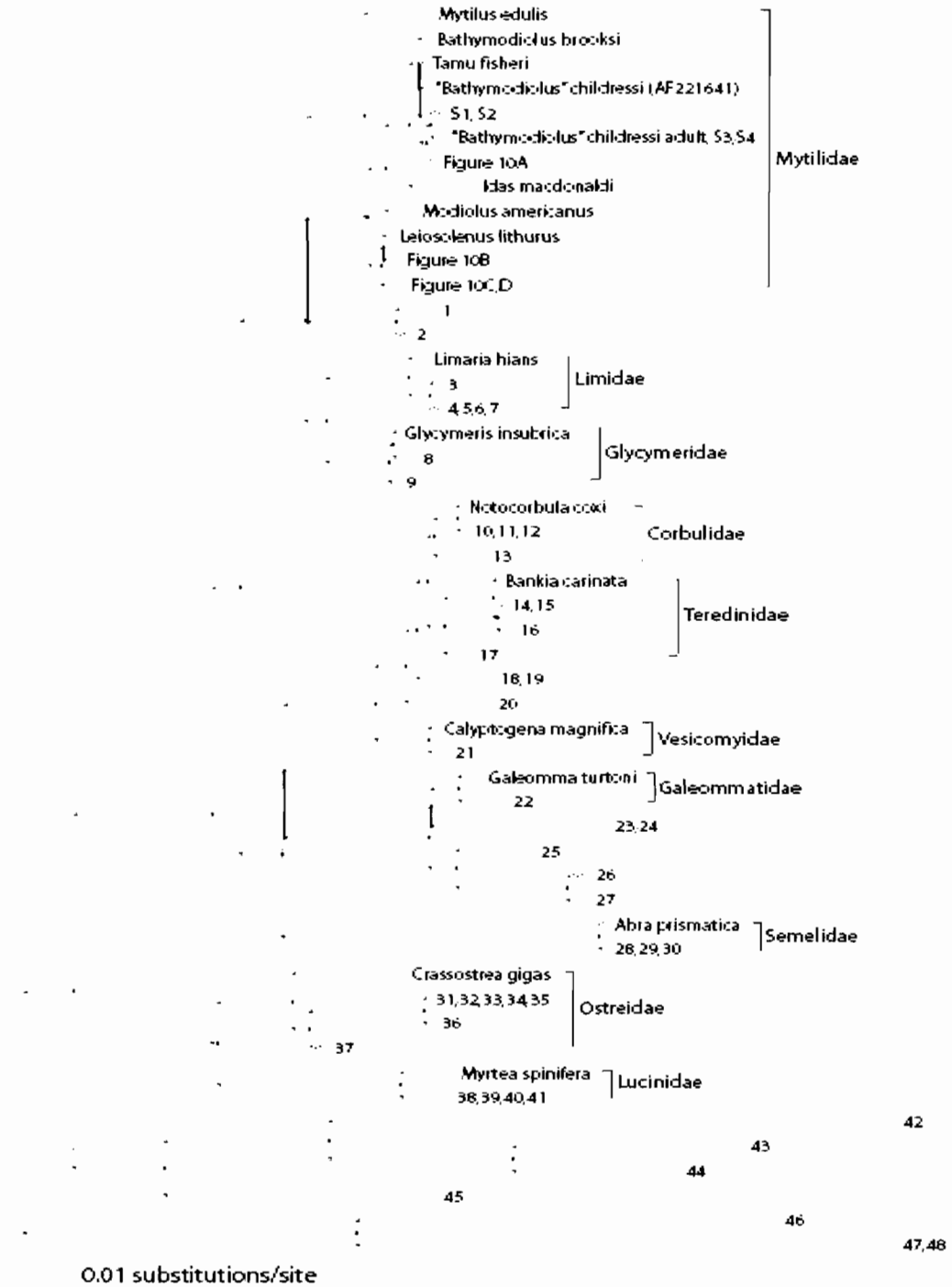

Figure 9. Neighbor-joining tree based on 18S molecular sequence data of bivalve larvae found in plankton tows above the Louisiana Slope cold seeps in November 2003.

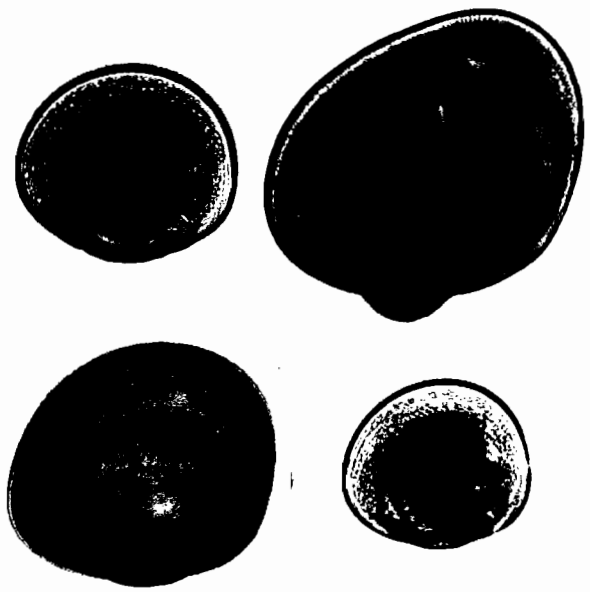

Figure 10. Light micrographs of mytild larvae found in plankton tows above the above the Louisiana Slope cold seeps in November 2003. Individuals correspond to those mytilids given in Figure 9. (A) is *"Bathymodiolus" childressi* and (B)-(D) group with *Leiosolenus lithurus*. Collection data and sizes are given in Table 7. Scale bar = 100 μm.

4. Discussion

Whether the planktotrophic larvae of deep-sea invertebrates migrate to the euphotic zone to feed has remained an important question to deep-sea ecologists because, although planktotrophic larval development is common among bathyal and abyssal benthic organisms, deep-water food sources for them are mostly unknown (reviewed by Young, 2003). Moreover, vertical migration behavior of larvae originating in the deep-sea will affect their survival, their dispersal distance and trajectories, and ultimately the dynamics of deep-sea benthic populations.

We tested whether the migration model of Young et al. (1996a) predicts the ontogenetic vertical migration potential for larvae of the deep-sea, cold-seep mussel

"Bathymodiolus" childressi, as evidenced by the vertical distribution of larvae found in plankton tows. In doing so, we address several questions. First, is an ontogenetic vertical migration from 650 m depth to the surface waters energetically feasible? If so, can *"B." childressi* larvae tolerate the environmental conditions of the upper water column? Does direct evidence of larvae in the water column correspond with predictions based on larval energetics and physiological tolerances? Finally, what may be the consequences of ontogenetic vertical migration by larvae of *"B." childressi*?

4.1 Is a vertical migration to surface waters energetically feasible?

Using mean swimming speeds for trochophore larvae of *"Bathymodiolus" childressi* from days 0-6, which is the time from hatching to D-shell veligers (Chapter 2), and assuming typical mytilid veliger swimming speeds thereafter, we calculate that the larvae of *"B." childressi* are provisioned with enough energy in the egg to migrate to the surface from 650 m depth (the depth of the Brine Pool cold seep) within 10-13 days during our coldest recorded winter conditions. However, there are several simplifying assumptions built into the model by Young et al. (1996a) that should be addressed. In the following sections, we discuss our assumptions that throughout a larva's migration 1) swimming remains upward and velocity is constant, 2) metabolic rate does not change, and finally 3) the larva does not feed.

4.1.1. Assumption 1: Swimming is upward and velocity is constant

We have made several overly simple assumptions concerning larval swimming in *"Bathymodiolus" childressi*. First, we have assumed constant upward swimming from hatching until reaching the surface. However, in reality, little is known of the swimming

behaviors of these larvae. Laboratory studies show that larvae of *Mytilus edulis* respond to light, gravity, and pressure, becoming photopositive as veligers and remaining negatively geotactic through most of their larval lives (Bayne 1963, 1964). In culture, we observed *Mytilus trossulus* larvae accumulating at the surface once they begin to swim. Conversely, the trochophore larvae of *"B." childressi* did not accumulate at the surface of our cultures, but instead were distributed throughout the culture. Likewise, we have observed that the *"B." childressi* trochophores did not swim unidirectionally upward, but when left in culture tend to meander upward and then sink back downward. Because we did not culture the larvae to more advanced veliger stages, we do not know whether *"B." childressi* veligers are photopositive or negatively geotactic as are *Mytilus* veligers.

In addition, we assumed that swimming velocity did not change during the larva's migration. In reality, swimming speed should change both as the larva ages and as the temperature of the water column increases with decreasing depth. Ontogenetic changes in larval swimming behaviors are known for mytilid larvae (Bayne 1963, 1964), but less is known of changes in swimming velocities. However, bivalve larvae swim by ciliary locomotion and as the ciliary band changes with development into a veliger, swimming trajectories and velocities will be altered as well. We compensated for this stage change by using our mean swimming velocity for *"B." childressi* trochophores until the time they reach D-shell veligers (6 days) followed by utilizing published veliger swimming velocities in the energetic model, recognizing that in reality the shift from trochophore to veliger is not sudden and that velocities may continue to change as the veliger grows.

Like other biological rates, swimming speed changes as a function of temperature (reviewed in Young 1995) due to both physiological responses to temperature and the physical changes in water viscosity with temperature (Podolsky and Emlet, 1993). Sprung (1984a) reports that swimming velocities of veligers of *Mytilus edulis* increase with temperature, with a Q_{10} of 2.25 between 6 and 18 °C (Table 5). However, the variance of the swimming velocities we measured for trochophores of *"B." childressi* was high, and, although velocity in one temperature treatment (15 °C) was significantly higher than in the ambient treatment (7 °C), we did not observe a positive trend with increasing temperature (Fig. 5). Because we do not know the relationship between swimming velocity and temperature for *"B." childressi* veligers, we did not modify the model by Young et al. (1996a) to include the effect of temperature on velocity. Instead, we predicted migration potential over a realistic range of swimming velocities.

4.1.2. Assumption 2: Metabolic rate does not change

Although eggs of *"Bathymodiolus" childressi* are within the size range of other bathymodiolin mussels (reviewed in Chapter 2) and within the size range of eggs of shallow-water mytilids (Table 3), the total energy content in *"B." childressi* eggs is approximately twice that of eggs of shallow-water mytilid mussels (Table 3). This is primarily due to the higher lipid content in *"B." childressi* eggs than in *Mytilus* mussel eggs (Table 3). We expected higher energy content in the eggs of bathymodiollin mussels because the larvae grow to larger sizes before settlement than do most shallow-water mytilids (reviewed in Chapter 2). Because few data on the energetics of eggs of deep-sea organisms are available, Young et al. (1996a) were forced to use the

relationship between egg size and total energy in the egg derived from energetic data in shallow-water echinoderms and to assume the total energy in the egg was available for metabolism. However, comparing the total energy consumed during a vertical migration to the total energy in the egg is an overestimate for two reasons. First, development alone requires energy. For example, Marsh et al. (1999) estimated that an unfed Antarctic echinoderm larva at -1.5 °C would use 0.86% of its initial energy reserves in the egg per day for development. If we assume a similar rate of metabolism for developing embryos of *"B." childressi*, on average 0.05 mJ of the egg's energy would be utilized by the time the larva developed to a swimming stage. Second, not all the energy in the egg is available for metabolism; as a matter of fact, in estimations of metabolic lifespan for the hydrothermal-vent siboglinid worm *Riftia pachyptila*, Marsh et al. (2001) assume that only 50% of an egg's protein mass and 90% of its wax ester lipid mass could be metabolized.

In addition, Young et al.'s (1996a) model does not account for changes in respiration rate throughout the migration. Respiration rate increases steadily with age and temporal variations occur with major developmental events (Marsh et al. 1999). Few data are available on metabolism throughout development for molluscan larvae though, and those that are available use a variety of respirometry methods, which may be less accurate than the end-point method we use here (Marsh and Manahan 1999; Marsh et al. 1999). Nonetheless, respiration rate data via the gradient diver method are available for the larvae of *Mytilus edulis* across several temperatures and with age (Sprung 1984a,b). We used the respiration data available in Sprung (1984b), converted energy units back to

respiration rates (using their conversion factor of 20.1 J ml^{-1} O_2; Crisp 1971), and plotted respiration rate (pmol O_2 h^{-1} $larva^{-1}$) against temperature for veligers of shell length 120, 150, 200, 250 μm (Fig. 11). As *M. edulis* veligers grow from 120 μm to 250 μm long, the rate of oxygen consumption (Q_o) changes from 1.0 to 10.8 and the rate of change with temperature (r) changes from 0.15 to 0.09, resulting in higher cumulative energy consumption (E_t) estimates for larger individuals at mean swimming velocities (Fig. 12). Combined with rising temperatures as growing larvae migrate vertically through the water column, the effect of growth on respiration rate could drastically increase estimates of cumulative energy consumption and reduce the estimated vertical distance a larva could travel on energy reserves alone. Data on the effect of growth on oxygen consumption by larvae at multiple temperatures is unavailable for *"B." childressi*, any other deep-water organism, or other shallow-water bivalves. In the future, steps should be taken to incorporate the effect of growth on metabolism into the model.

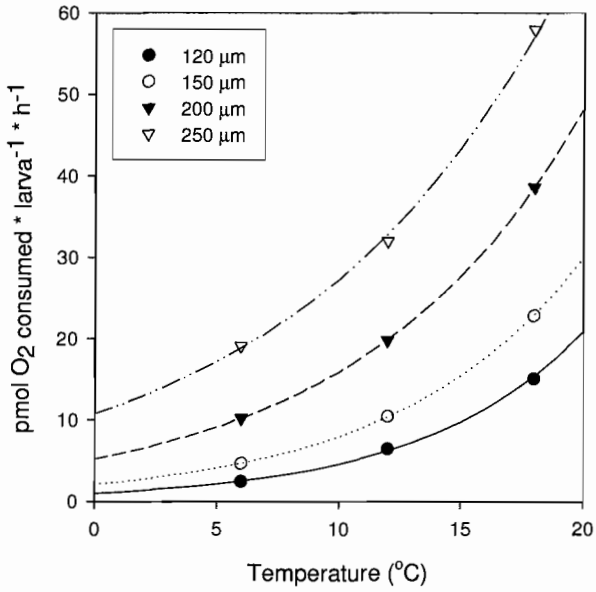

Figure 11. Oxygen consumption rates of *Mytilus edulis* veligers at 4 shell lengths as a function of temperature. Data are from Sprung (1984a, b). 120 µm: $y = 1.0013e^{0.1518x}$, $r^2 = 0.9986$; 150 µm: $y = 2.1119e^{0.1325x}$, $r^2 = 0.9999$; 200 µm: $y = 5.2402e^{0.1109x}$, $r^2 = 1$; 250 µm: $y = 10.813e^{0.0925x}$, $r^2 = 0.9983$.

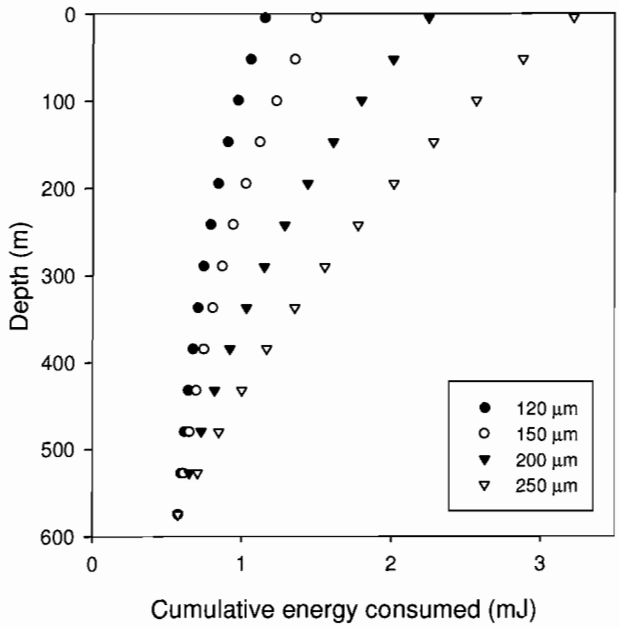

Figure 12. Predicted cumulative energy consumption (mJ) at 3.96 m h^{-1} in November 2003 as a function of depth for *Mytilus edulis* larvae showing the change between 4 different sizes of larvae. Starting depths were at ~ 575 m and we assumed larvae had already used 0.576 mJ energy swimming to the starting depth.

4.1.3. Assumption 3: Larvae do not feed

Egg size and larval shells of *"Bathymodiolus" childressi* are indicative of planktotrophy (Gustafson et al., 1998; Chapter 2), but, although larvae grew to veligers in culture, food sources for *in vitro* cultures are unknown (Chapter 2). Young et al. (1996a) assumed larvae utilize only the energy reserves in the egg through their migration and tested the energetic capability of larvae to reach in the euphotic zone before they feed. However, non-algal nutritional sources are known in bivalve larvae. As a matter of fact, isotopic analysis of post-larvae of *Bathymodiolus azoricus* or *Bathymodiolus heckerae* did not indicate that the larval diet consisted of photosynthetically derived organic material (Salerno et al., 2005). Manahan (1990) has suggested that dissolved organic matter may be a significant energy source for bivalve larvae and several examples are

known. Oyster larvae (*Crassostrea gigas*) uptake amino acids (Manahan and Crisp, 1982; Manahan, 1983, 1989) and veligers of the zebra mussel *Dreissena polymorpha* are known to uptake dissolved organic carbon (Barnard et al., 2006). In addition, larvae of the oyster *Crassostrea virginica* consume heterotrophic ciliates and flagellates (Baldwin and Newell, 1991) and some bivalve larvae are capable of ingesting and assimilating bacteria (Martin and Mengus, 1977; Baldwin and Newell, 1991; Gallager et al., 1994). Live bacterial strains have been used as a food source for *in vitro* culture of veligers of *Mytilus galloprovincialis* (Martin and Mengus, 1977) and veligers of *Mercenaria mercenaria* can consume the cyanobacterium *Synechococcus sp.* (Gallager et al., 1994). Dissolved organic matter, ciliates, and bacteria are all potential food sources for developing larvae of *"Bathymodiolus" childressi* mussels as they migrate through the water column and more work should be conducted to discover the nutritional sources of bathymodiolin larvae.

Alternatively, *"Bathymodiolus" childressi* adults host methanotrophic endosymbiotic bacteria in their gills and are capable of growth using methane as a sole source of energy and carbon (Childress et al., 1986; Cary et al., 1988; Kochevar et al., 1992). Although it is suggested that other bathymodiolin mussels acquire their symbionts from the environment rather than transferring them via the ovum (Won et al., 2003), the stage at which mussels become infected is unknown. However, because methane is necessary for energy metabolism by the endosymbionts, the use of endosymbiosis as an energy source for larval mussels would not support the hypothesis of ontogenetic vertical

migration. Rather, larvae would need to be retained near cold seeps—the sources of methane.

4.2 Can larvae tolerate the environmental conditions of the upper water column?

Physiological tolerances to the environmental conditions of the euphotic zone may be useful indicators of vertical migration potential of larvae of benthic deep-sea organisms (Young and Tyler, 1993; Young et al., 1996a,b). In Chapter 3 we conducted experiments examining the survival capabilities of trochophore larvae of *"Bathymodiolus" childressi* when exposed to a range of salinities and temperatures. They suggested that salinity may not play a significant role in the vertical distribution of larvae because survival rates were not significantly different over the salinity range found throughout the water column (Chapter 3). Instead, temperature may be a more important factor affecting larval distribution. Survival of trochophores was similar at the ambient temperature at the Brine Pool cold seep (7 °C) up to 20 °C. Our temperature profiles show that during the spawning season of *"B." childressi* (October through February) sea surface temperatures range up to approximately 25 °C, but are near 20 °C in the winter (February 2003). However, Chapter 3 suggest that since some (5-14 %) *"B." childressi* blastulae and trochophores survived at the highest temperature measured (25 °C), and since temperature tolerance tends to increase with age, *"B." childressi* veligers may be tolerant of the temperatures in the upper water column of the Gulf of Mexico in even the warmest months of the year.

4.3 Does direct evidence of larvae in the water column correspond with the model's predictions and the larva's physiological tolerances?

Our surface plankton tows (0-100 m) provide direct evidence that larvae of *"Bathymodiolus" childressi* do indeed have the energetic capability of a migration into the euphotic zone. The model we use here predicts that a larva swimming upward from 650 m depth in February 2003, could reach the surface within 10 to 13 days, assuming *"B." childressi* veligers swim at mean swimming velocity for *Mytilus edulis* veligers. We found two *"B." childressi* veligers (length = 425-438 μm) in our surface plankton tows (0-100 m depth) in Febraury 2003 when the sea surface temperature was 20 °C. However, at the higher sea surface temperatures in November 2003, veligers would use up all the energy reserves in the egg by 100 m depth (25 °C) within 11 days at average veliger swimming speeds, but would reach the surface (26 °C) within 10 days at fast veliger swimming speeds. We did not find any *"B." childressi* veligers in the November 2003 surface tows, but we found one veliger (274.8 μm long) at a depth of 300-350 m (14-15 °C). However, the spawning season of *"B." childressi* begins in October and mussels continue to spawn through February (Tyler et al., 2006; Chapter 2). Thus, the veliger we found at 300-350 m depth in November may have been from an egg that was only recently spawned, not allowing time for a complete migration. The energetics model predicts that in November 2003 a larva would reach 300-350 m within 8 to 9 days of swimming, or 10 to 11 days from fertilization (Chapter 2). Similarly, to reach 100 m depth in February 2003, a larva would have to swim for 11 days and would be about 13 days old (Chapter 2). The shallow-water mussel *Mytilus edulis* forms a complete D-shell

by 42 hours (1.75 days) at 12 °C and by 104 hours (4.33 days) from fertilization at 6 °C under high food conditions (40 cells μl^{-1}; Sprung, 1984c). Growth rates of *"B." childressi* are less than half the rates of *M. edulis* at similar temperatures. In Chapter 3 we suggest that growth developmental rates for *"B." childressi* are four times slower than for *Mytilus trossulus* at similar temperatures and salinities and Chapter 2 shows that unfed *in vitro* cultures at 12-14 °C developed to D-shell veligers by day 8 and by 10 days post-fertilization D-shell veligers ranged in size from 86.70 to 103.56 μm long ($\bar{x} \pm$ S.D. = 96.24 ± 8.31; n = 4). The slow growth rates measured for *in vitro* cultures of *"B." childressi* suggest that 11 days (November sample) and 13 days (February sample) would not be sufficient to grow to 275 μm or 438 μm long respectively. If so, this suggests that larvae probably do not swim immediately or continuously upward upon developing to a swimming stage.

In addition, we did not find as many larvae of *"Bathymodiolus" childressi* as we expected, even in the deeper plankton tows. This is probably due to high dilution. Without strong, continuous upward swimming, larvae may be horizontally transported throughout their long vertical migration, thereby increasing dilution. In addition, although spawning is periodic, it is not episodic. In other words, populations of mussels at the Brine Pool and Bush Hill cold seep spawn continuously over an extended (5 month) period (Tyler et al, 2006). Thus, although fecundity of any one mussel may be high and population densities are high, if only part of the population is spawning at a time, dilution will be an even bigger factor.

4.4. What may be the consequences of vertical migration by larvae of "Bathymodiolus" childressi?

Population genetics reveal no differentiation throughout the Gulf of Mexico, suggesting widespread larval dispersal in this region (Carney, 2005). In addition, the connection between the hydrocarbon seep communities in the Gulf of Mexico and the remarkably similar Nigerian cold-seep assemblages across the Atlantic off the coast of West Africa has recently garnered much attention (Cordes et al., 2007; Olu Leroy et al., 2007). Some evidence of historical exchange between the Gulf of Mexico seeps and the West African seeps has been presented for the *"Bathymodiolus" childressi* species complex (Cordes et al., 2007; Olu-Leroy et al., 2007). However, although dispersal of these species seems to be widespread, dispersal patterns and the strategies utilized to effect widespread dispersal are unclear. In shallow-water systems, it is well known that vertical migration behavior can interact with flow regime to transport or retain larvae (eg, Cowen et al. 2000, Kingsford et al. 2002). In addition, by modeling dispersal potential based on season, development time, and currents, Kelly et al. (1982) showed that the dispersal range of a deep-sea red crab increases when it migrates to surface waters. Vertical migration by larvae of *"B." childressi* may allow them to take advantage of surface currents, providing a long-distance dispersal mechanism.

Alternatively, larvae may remain at deeper, colder temperatures before beginning to swim upward or some may remain in deep water for their entire larval lives. Metabolic efficiency of cold-water organisms can be high. Larvae of Antarctic echinoderms at -1.5 $^{\circ}$C have metabolic capability of surviving for upwards of one year

using only the energy reserves in their eggs (Marsh et al., 1999). Similarly, Marsh et al. (2001) predict that the larvae of hydrothermal vent siboglinid tubeworm *Riftia pachyptila* have a mean metabolic lifespan of 38 days at 2 °C. Using mean total energy in the egg ($\bar{x} \pm$ S.D. = 2.95 $\pm$ 0.082 mJ) and respiration rates for blastulae of *"Bathymodiolus" childressi* at 7 °C ($\bar{x} \pm$ S.D. = 0.00166 $\pm$ 0.000442 mJ) larvae would have a mean metabolic lifespan of about 74 days. Using respiration rates for trochophores ($\bar{x} \pm$ S.D. = 0.004285 $\pm$ 0.000442 mJ), the metabolic lifespan decreases to about 29 days without feeding.

Additionally, the total planktonic larval duration for *"Bathymodiolus" childressi* is thought to be high. By comparing settlement times (calculated from lengths of new recruits and known growth rates) to known spawning seasons, in Chapter 2 we predict that larvae of *"B." childressi* may remain in the plankton from 2-3 months to more than one year before settling. Here, we found larvae in the plankton in February and November 2003 that were 425-438 µm and 274.8 µm long, respectively. If these larvae developed from the eggs spawned at the beginning of their spawning seasons (the October immediately prior to the month of collection), they would be 4.5 or 1.5 months old, respectively. In other words, larvae would have grown ~275 µm in 1.5 months, or 438 µm in 4.5 months. While growth rate estimates based on the time it would take larvae to swim to the depths at which larvae were found are probably high (see section 4.3), growth rate estimates based on spawning season are more realistic, as they are similar to growth rates of veligers of *Mytilus edulis* at cold temperatures. In comparison, using the linear growth equation (y = 3.46x + 92.7) from Sprung (1984c) for larvae of

Mytilus edulis developing at 6 °C with food rations of 40 cells μl^{-1}, we can calculate that *M. edulis* larvae would reach 275 μm in 53 days (~1.75 months) and 438 μm in 100 days (~3.33 months). Extended periods in the plankton in combination with ontogenetic vertical migration into waters with higher current velocities could lead to high dispersal distances.

5. Conclusions

Initial hypotheses were that the larvae of hydrothermal-vent bathymodiolin mussels probably do not migrate to the surface waters, since migration increases advection of larvae away from suitable habitats (Lutz et al., 1980; Lutz et al., 1984; Turner et al., 1985). Evidence suggests that near-bottom currents most often transport larvae of hydrothermal-vent species (Kim and Mullineaux, 1998; Mullineaux et al., 2005). In particular, Mullineaux et al. (2005) show that the abundance of planktotrophic bathymodiolin mussel larvae decreases with height above hydrothermal vents along the East Pacific Rise and they nypothesize that larval behavior may maintain these larvae within bottom currents.

Here we have shown that the larvae of the cold-seep bathymodiolin mussel *"Bathymodiolius" childressi* are energetically capable of migrating to the surface from 650 m depth within 10-13 days (assuming typical veliger swimming speeds) and without feeding. Moreover, molecular and SEM identifications of veligers found in the plankton provide direct evidence that *"B." childressi* larvae can migrate to at least 100 m depth. However, the large size of these larvae suggests a slow migration to the surface of up to 4 months and the swimming behavior of these larvae is yet unknown. Calculated metabolic

lifespans of up to 74 days indicate that larvae could live at depth for extended periods without feeding. Carney et al. (2006) show no relationship between genetic and geographic distance of populations of *"B." childressi* within the Gulf of Mexico, and Olu Leroy et al. (2007) show minimal genetic differentiation amongst the *"B." childressi* species complex from western and eastern Atlantic seeps. Both pieces of evidence suggest widespread dispersal of the larvae. We suggest larvae of *"B." childressi* utilize a bet-hedging strategy of remaining at depth for a period of time before slowly swimming upward, with some reaching the surface. This strategy would allow larvae to take advantage of currents throughout the water column, retaining some larvae near local seeps within the Gulf of Mexico while dispersing others further, possibly all the way across the Atlantic. It is possible, therefore, that increasing the sampling efforts across the Atlantic equatorial belt, as well as continuing exploration of these areas could reveal previously unknown populations of *"B." childressi* outside of the Gulf of Mexico.

6. Bridge

Chapters 2 through 4 have explored the larval characteristics that contribute to the large scale geographic distribution of *"Bathymodilus" childressi* in the Gulf of Mexioc. Chapter 5 will focus on factors of larval ecolgy that contribute to fine-scale variations in the distributions of adult mussels, using the Brine Pool cold seep as a case study.

CHAPTER V

PRE- AND POST-SETTLEMENT EFFECTS ON THE SPATIAL DISTRIBUTION OF THE MUSSEL *"BATHYMODIOLUS" CHILDRESSI* AT THE BRINE POOL COLD SEEP, GULF OF MEXICO

INTRODUCTION

For species with dispersive larvae, recruitment links the larval and adult stages and variation in recruitment often controls the distribution and abundance of adult populations and communities (Young 1987, Underwood & Fairweather 1989, Underwood & Keough 2001). The importance of recruitment processes in determining spatial patterns of populations has been described in fouling (e.g. Hurlbut 1991), mangrove (e.g. Bingham 1992), coral reef (e.g. Gutiérrez 1998), soft-sediment (e.g. Butman 1987), subtidal (e.g. Osman & Whitlatch 1998), and rocky intertidal (e.g. Connell 1985) communities.

Recruitment processes also influence patterns of distribution and abundance in some deep-sea chemosynthetic communities. In these isolated and often ephemeral habitats such as hydrothermal vents (Van Dover et al. 1988) and cold methane seeps (Sibuet & Olu 1998), recruitment processes control initial colonization and subsequent succession. Many investigations at hydrothermal vents include observations of recruitment and succession (e.g. Hessler et al. 1985, Hessler et al. 1987, Van Dover et al.

1988, Tunnicliffe et al. 1997, Comtet & Desbruyères 1998, Mullineaux et al. 1998, Shank et al. 1998, Thiébaut et al. 2002), and some recent vent studies include experimental tests of hypotheses about recruitment processes and biological interactions (e.g. Mullineaux et al. 2000, 2003, Micheli et al. 2002, Hunt et al. 2004). Ecological studies at cold seeps, however, have remained largely observational and tend to focus on correlations between physico-chemical gradients and established distribution patterns but lack studies on factors that control recruitment (Sibuet & Olu 1998 for review). Research on physiological condition, demography, and growth rates of seep organisms have led to speculation about recruitment dynamics (e.g. Nix et al. 1995, Smith et al. 2000, Bergquist et al. 2002, 2003, 2004). However, we know of only one *in situ* experiment explaining links between larval and adult populations in cold-seep communities (Levin et al. 2006).

The Brine Pool NR 1 (BP) cold seep on the upper continental slope is an ideal site to test the role of recruitment in establishing adult distributions because its mussel population has a distinct bimodal size structure that shifts predictably across a short environmental gradient. The BP is a collapsed salt diapir filled with methane-saturated brine and located ~120 km south of the Louisiana coast (27°43'24" N, 91°16'30" W) at a depth of ~650 m (MacDonald & Fisher 1996). A bed of *"Bathymodiolus" childressi* mussels encircles the brine pool. *"Bathymodiolus" childressi* is a mixotrophic mussel that harbors methanotrophic endosymbionts in the gills (Childress et al. 1986) and is known from seep sites in the northern and western Gulf of Mexico ranging in depth from 546 to 2222 m (Gustafson et al. 1998). The endosymbionts provide fixed carbon to the host mussel, which is capable of growth using methane as a sole source of energy and

carbon (Cary et al. 1988, Kochevar et al. 1992). In addition, the dietary needs of *"B." childressi* mussels are supplemented by filter feeding (Page et al. 1991, Pile & Young 1999).

Because of its unique morphology (Gustafson et al. 1998) and recent analysis of molecular phylogeny (Jones et al. 2006), there is uncertainty about the taxonomy of *"B." childressi*. Thus, we follow the recommendation of Jones et al. (2006) and place the genus name of "*Bathymodiolus*" *childressi* in quotation marks.

The fine-scale population distribution of *"Bathymodiolus" childressi* at the BP has been characterized well (MacDonald et al. 1990c, Smith et al. 2000). The mussel bed can be divided into three zones: the inner, middle, and outer zones (Smith et al. 2000, Bergquist et al. 2005). The inner zone is characterized by high methane and oxygen concentrations (>200 μM & ≤160 μM, respectively) and non-detectable hydrogen sulfide (Smith et al. 2000). The outer zone of the mussel bed has similar methane concentrations, lower average oxygen levels (sometimes <50 μM), and very high hydrogen sulfide (>1000 μM) (Smith et al. 2000). Large mussels and empty mussel shells are observed on the fringes of the mussel bed (outer zone) and mostly living mussels of all sizes are found adjacent to the pool (inner zone).

New recruits of *"Bathymodiolus" childressi* are abundant at only the innermost edge of the BP, leading to the inference that larvae settle preferentially at the pool's edge (MacDonald et al. 1990a, b, c, MacDonald & Fisher 1996, Smith et al. 2000). Because MacDonald et al. (1989) showed a significant positive correlation between methane concentrations and location of *"B." childressi* at the "Mussel Beach" seep site in the Gulf

of Mexico, methane has been implicated as a potential settlement cue or cause of recruitment to the pool edge (MacDonald et al. 1990c, MacDonald & Fisher 1996). However, the underlying cause of this correlation remains untested. Methane is not likely to be the sole cause of the observed recruitment pattern because concentrations of methane across the BP mussel bed: (1) are highly variable and not significantly different among zones (Smith et al. 2002), (2) meet the minimum requirements necessary for mussel growth in all zones (Cary et al. 1988, Kochevar et al. 1992), and (3) are typically within the range or higher than methane concentrations at other seep sites on the continental slope of Louisiana that also host very small mussels (Bush Hill: MacDonald et al. 1989; Mussel Beach: MacDonald et al. 1990a; GC 272, GC234, & Bush Hill: Nix et al. 1995).

Alternatively, initial settlement by *"Bathymodiolus" childressi* veligers may be widespread, with post-settlement factors creating the bimodal distribution maintained at the BP mussel bed. It is important here to differentiate between settlement and recruitment. Settlement is the initial establishment of larvae onto a substratum and is affected by pre-settlement factors such as larval dispersal, larval supply, settlement cues, and habitat selection. Recruitment is the first record of the settled larvae by an observer (*sensu* Keough & Downes 1982). Thus, recruitment is influenced by pre-settlement as well as post-settlement processes such as juvenile mortality, predation, facilitation, competition, and migration. Applying these definitions, larval processes determine any patterns of distribution described shortly after settlement, whereas established juvenile and adult distributions are affected by post-settlement processes as well (Gutiérrez 1998).

The mussel distribution patterns evident at the BP could be explained by a number of different pre-settlement, settlement, and post-settlement events. For example, the availability of a suitable physical substratum for settlement has been implicated in the successful recruitment of seep tubeworms (Bergquist et al. 2002) and mussels (Nix et al 1995) and in the lack of mussel recruitment to other brine pools in the Gulf of Mexico (MacDonald et al. 1990c, MacDonald 1992). Appropriate physical substrata for settlement may be associated with the abundant living mussels at the pool's edge rather than the mostly dead mussels in the outer zone. Although the environment throughout the BP mussel bed is suitable for survival and growth of adult *"Bathymodiolus" childressi* (Smith et al. 2000), we do not know if the outer fringes of the BP mussel bed can support settlement and the maintenance of new recruits. Differential predation on recruits, which can influence the structure of hydrothermal vent communities (Micheli et al. 2002), could also influence distribution of mussels across the bed.

In this study we compare the initial settlement distribution of *"Bathymodiolus" childressi* at the BP cold seep with established juvenile and adult distributions in order to determine whether pre- or post-settlement processes establish the distinct bimodal size distribution at the BP. Specifically, we test whether the bimodal size distribution is structured by the following pre-settlement factors: (1) differential larval supply across the BP mussel bed and (2) substratum selection by settling larvae; or by the following post-settlement factors: (1) juvenile mortality due to environmental conditions, (2) differential growth across the BP mussel bed, and (3) differential predation across the BP mussel bed.

MATERIALS AND METHODS

Zone definitions

The mussel bed ringing the pool can be divided into three zones (inner, middle, and outer) made up of concentric rings of equal widths (Smith et al. 2000, Bergquist et al. 2005). The inner zone is one-third of the bed directly adjacent to the brine pool, and the outer zone is one-third of the bed adjacent to the bare sediment. The middle zone is transitional between the inner and outer zones and is not considered a distinct zone because it is patchy in mussel composition and water chemistry, resembling the inner zone in some places and the outer zone in others (Smith et al. 2000). We characterized mussel size distributions and community composition in the middle zone, but because this zone is variable, all field experiments took place at the inner and outer zones and at a site approximately 2 meters away from the outer periphery of the mussel bed in bare sediment. We refer to this location, which is presumably not influenced by brine seepage, as the "away" zone.

Size distribution

To verify the persistence of the reported bimodal size distribution and to monitor recruitment events across the BP mussel bed throughout the year, samples of *"Bathymodiolus" childressi* were taken from the inner, middle, and outer zones on the west side of the BP in March 2002 (inner and outer zones only), October 2002 (inner zone only), February 2003, September 2003, November 2003, July 2004, and August 2006. To investigate potential biases associated with sampling location around the pool, additional samples from the three zones were collected at the northern and eastern sides

of the BP in July 2004. Samples were not taken at the southern side of the BP, since the pool overflows its southern rim, killing most mussels found there. Mussel collections were made with the scoop of *Johnson-Sea-Link I & II* (J-S-L) submersibles (Harbor Branch Oceanographic Institution) and were transported to the surface in either a hydraulically-sealed acrylic box or one of the covered acrylic buckets located on the sub's lower work platform. Upon recovery, we counted the mussels in each zone and measured their lengths (mm). Preliminary measurements (October 2002) showed that length is correlated tightly with both width and height; thus, only length was used as a measure of size throughout this study (see also Gustafson et al. 1998).

Length data are presented as percent-frequency histograms. Length data were not normally distributed, so size distributions between zones within each month were compared using pairwise Kolmogorov-Smirnov tests. When two zones did not have significantly different size distributions at $\alpha = 0.05$, they were combined for comparison to the third zone. To explain a skewed pattern in the July 2004 west side sample, we compared the mean lengths (square-root transformed) of the mussels in the inner zones around the pool (west, north, or east) using a single-factor ANOVA, with location around pool as a fixed factor.

We compared the density of juvenile mussels across the BP mussel bed through quantitative sampling. In November 2003 and August 2006, density (number m^{-2}) of small mussels (<10 mm long) was quantified using 3 replicate quantitative samples taken for analyses of predator distribution (see below). The samples taken from the inner and outer zones were from the most extreme edges of the zones still containing living

mussels. The collections from the middle were approximately halfway between the two edges of the mussel bed. In November 2003, the scoop and suction apparatus on the J-S-L submersible were used to collect all organisms within a 2500 cm^2 PVC quadrat. These samples were collected into the biobox and were identified and counted upon recovery to the surface. However, due to space and dive constraints, only one 2500 cm^2 quadrat was sampled in each zone in 2003. Smaller samples from each zone were collected on subsequent dives with the sub's scoop alone; these were treated as replicates of the 2500 cm^2 quadrat collections in November 2003 (for a total of three replicate samples). In August 2006, all samples were collected with the sub's scoop alone. For the scoop samples, the J-S-L pilot was instructed to collect two adjacent scoops that were combined from each zone, after first using suction to collect associated fauna and any small mussels within the area. The scoop's gape encloses an area of approximately 345 cm^2, so the total area collected in each of these samples was ~690 cm^2. Each of the three replicate samples was standardized to area for analysis and a fourth root transformation was applied to normalize the data. Transformed density (m^{-2}) of small mussels was compared across the three zones using a mixed-model two factor ANOVA with trial (collection date) as a random factor. Because trial was not significant at the $\alpha = 0.25$ level (Quinn & Keough 2002), trials were pooled and the data were reanalyzed as a single factor ANOVA with zone as a fixed factor.

Larval supply

To determine if supply of *"Bathymodiolus" childressi* veligers is equal at the inner, outer, and away zones of the BP mussel bed, three replicate larval tube traps were

placed in each zone alongside settlement experiments (see below). Tube traps were 30-cm tall PVC pipes with a 5 cm diameter opening (aspect ratio = 6:1) that were mounted to five-pound iron discs as bases. Empirical studies have shown that cylindrical traps with sufficiently high aspect ratios (Yund et al. 1991 use aspect ratios 4 and 12) are least susceptible to re-suspension (Lau 1979, Hargrave & Burns 1979, Butman 1986, Hawley 1988, Yund et al. 1991). Larval tube traps were filled with 10 % formalin buffered in seawater to prevent escape of larvae (Yund et al. 1991). Because capture rate can vary as a function of horizontal advection at the mouth of the larval tube trap (Yund et al. 1991), six integrated current readings were taken with a Marsh McBirney Model 2000 Flowmeter at the mouth of one tube trap in each zone in February 2003 only. Current velocity data were analyzed with a single factor ANOVA with zone as a fixed factor. Tube traps were deployed for 271 days from February 12 to November 10, 2003 and again for 247 days from November 11, 2003 to July 15, 2004. Upon recovery, contents were transferred to 70 % ethanol for storage until all bivalves could be classified and counted. Larvae of *"B." childressi* were identified visually. The conspicuous reddish coloration of the larval shell (Chapter 2) and lack of a dissoconch (adult shell), which forms upon metamorphosis (Bayne 1976), implied that all individuals counted had not settled previously. Based on the density of juveniles across the mussel bed calculated here and the differential density of adult mussels across the bed as shown in Smith et al. (2000), we expected the mean number larvae captured per day to decrease from the inner to the outer to the away zones. Thus, we analyzed each trial separately with a

Jonckheere-Terpstra Test for ordered alternatives to test the hypothesis that $M_{away} \leq M_{outer} \leq M_{inner}$ (Daniel 1989).

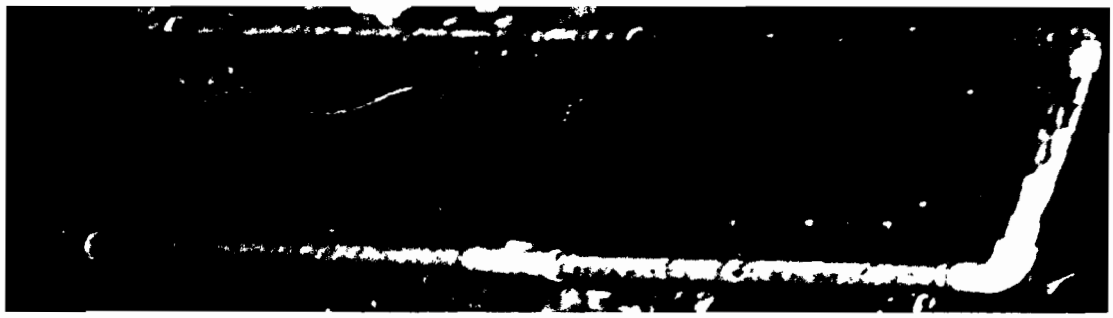

Figure 1. Photograph of a settlement racks placed in the outer zone of the BP mussel bed. Each rack contained four substratum treatments: large living mussels (LM), small mussel shells (SS), large mussel shells (LS), and small living mussels (SM).

Settlement and substratum selection

Three replicate settlement racks were placed in the inner, outer, and away zones of the BP mussel bed on October 10, 2002 for a total of nine racks. Each settlement rack consisted of four settlement substrata placed in random order within caged (0.5 cm Vexar mesh) PVC racks (90 cm x 23 cm x 4 cm) (Fig. 1). Although the total surface area of each settlement rack (= cage plus plate substratum treatments) was calculated individually, the racks in the inner and outer zones offer an average total surface area of 0.39 m^2 available for settlement. Because the racks in the away zone were placed on bare sediment, we assumed the bottom surface would not be available for settlement, leaving an average total surface area of 0.30 m^2. The caged racks were necessary to exclude potential predators and grazers and to hold settlement substrata, which included living mussels. Settlement substrata within the racks were large mussel shells (LS); large, living mussels (LM); small mussel shells (SS); and small, living mussels (SM) (Fig.e 1).

Large mussels or shells were > 90 mm long and small mussels and shells were from 30 to 50 mm long. The surface areas of the various substratum treatments were calculated from mussel shell length using the following equation (Bergquist, pers. comm., Bergquist et al. 2005): $SA = 0.5794*L^{2.06}$, where SA = surface area (cm^2) and L = length (cm) The average total surface area of the settlement substrata in each treatment was 110 ± 14 cm^2 ($\bar{x} \pm$ S.D.).

Settlement racks were recovered July 9-15, 2004. Settlers on the racks themselves and on the substratum treatments were counted and measured. Individuals up to 0.5 mm in length were defined as "settlers". We justify this definition because the larval shell of *"Bathymodiolus" childressi* reaches ~0.5mm in length (Gustafson et al. 1998, Chapter 2) before the adult shell begins forming immediately upon settlement and metamorphosis (Bayne 1976). This definition of settlers is necessary to eliminate the inclusion of individuals that may have been subjected to post-settlement factors or may have migrated onto the PVC rack after settlement. Treatments were analyzed in two ways. First, to determine if gross settlement differs across the mussel bed, all settlers on the racks (cages plus shells or mussels) were summed to become three replicate settlement racks in each zone. Settlement density (settlers m^{-2}) in each zone was analyzed with a single-factor ANOVA, with zone as a fixed factor. In addition, to determine if the numbers of settlers reflect adult *"B." childressi* density in each zone, we used a continuity-corrected, single-classification goodness-of-fit test (Sokal & Rohlf 1981). Based on juvenile density data presented here and the fact that adult mussels in the inner zone outnumber those at the outer zone by a conservative ratio of approximately

six to one (Smith et al. 2000), we expected a settlement ratio of 6:1 if settlement alone explains the distribution patterns of *"B." childressi* adults. However, we expected zero settlement in the away zone. Thus, we used the 6:1 ratio to generate expected values for the numbers of settlers in the inner and outer zones, after scaling the total observed numbers of settlers in the inner and outer zones to the available settlement surface area in the away zone. A Williams' adjustment was applied to the *G*-statistic.

Second, to determine whether substratum selection is an important determinant of distribution, recruit density was determined for each substratum treatment and analyzed by a two-factor ANOVA, with shell size (large or small) x state (live mussel or mussel shell) and all factors fixed (Sokal & Rohlf 1981). Because recruitment did not occur on all treatments in the outer and away zones, we analyzed the data from only the inner zone. We included all individuals up to 14 days old in the analysis. We calculated the size of 14-day-old recruits using our calculated mean growth rate of 1.44 ± 0.30 mm $30d^{-1}$ (see *Survival and growth* section) and assuming individuals are 0.5 mm at settlement (Table 1). The data were not normally distributed because many treatments had no settlers. We chose to analyze these data with ANOVA nevertheless because the analysis is robust to violations of the normality assumption (Underwood 1981).

Table 1. Maximum size of 14-day-old juveniles based on empirically derived growth rates and an initial settlement size of 0.5mm.

Zone	Length (mm)
Inner	1.298
Outer	0.817
Away	0.668

In situ caging experiments

Two caging experiments were used to test for variations in 1) predation pressure across the mussel bed, 2) survival of juveniles in each zone in the absence of predation, and 3) growth of juveniles in each zone. In caging experiment 1, to determine predation pressure and survival in the absence of predators across the bed, fifteen young juveniles (<10 mm long) collected from the inner zone of the BP mussel bed were attached to sheets of 1-mm plastic mesh with cyanoacrylate adhesive ("super glue") and placed in cages (13 x 13 x 8 cm^3), partial cages, and no cages. The glue was necessary to keep small mussels from escaping the cages and as a means for transplanting small, uncaged mussels. Our preliminary experiments showed that gluing did not affect survival; glued individuals survived in the lab and began laying down byssal threads almost immediately. On October 10, 2002, three replicates of each caging treatment were placed in the inner, outer, and away zones. Fluorescein injection around the cages at the time of deployment indicated that the cages did not alter flow notably. The caging experiments were recovered in February 11, 2003. Another run of the experiment was deployed February 11, 2003 and recovered in September 15, 2003. However, due to limited collections of small juvenile mussels for the second deployment, individuals <15 mm were used and only two replicates were placed in each zone.

A second set of caging experiments (caging experiment 2) was used to assess variance in survival in the absence of predators, growth, and predation pressure associated with both zone and height above the bed (e.g., predation by benthic vs. pelagic predators). We glued ten juveniles (<20mm long) that were collected from the inner zone

of the BP mussel bed to a small piece of 1 mm mesh and fastened them within cages and outside of cages (treatment). Treatments were placed in the inner, outer, and away zones during November 12, 2003. Half of all the treatments were benthic (sitting on the mussels or sediment) and the others were floating 30 cm above the benthic treatment. Due to limitations in the availability of mussels within the size class, only one set of treatments was placed in each zone. Cages were recovered on July 9, 2004 and the number of survivors was counted in each treatment. Statistical analyses for survival, growth, and predation will be described in the following sections.

Survival and growth

To determine whether young juveniles can survive in the absence of predators in all zones across the bed, we calculated percent survival of individuals placed within the caged treatments of caging experiment 1, which included inner, outer, and away zone treatments. If survival explained the differential density of juvenile and adult mussels across the mussel bed (this study, Smith et al. 2000), we expect survival of transplanted juveniles to decrease from the inner to the outer to the away zones. Thus, we analyzed each trial separately with a Jonckheere-Terpstra Test for ordered alternatives to test the hypothesis that $M_{away} \leq M_{outer} \leq M_{inner}$ (Daniel 1989).

In addition, twenty juvenile mussels (<25 mm long) were collected from the inner zone of the BP mussel bed and transplanted in 1-mm mesh bags to the inner and outer zones in March 12, 2002. Three bags were transplanted to the inner zone and two bags were transplanted to the outer zone. They were recovered on October 9-11, 2002 and the percent survival in each bag was calculated. Because only a large difference in survival

between juveniles in the inner and outer zones would create the observed size distribution pattern, we used a bioequivalence test to test the null hypothesis that the difference in the mean survivals was greater than 50% (Garrett 1997, Hoenig & Heisey 2001).

To examine survival across the three zones in the absence of predators, we used a two-factor *G*-test for independence on counts of survivors within the caged treatments only of caging experiment 2. Both benthic and floating cages were included. A Williams' adjustment was applied to the *G*-statistic.

We examined growth in these same transplants by measuring each individual prior to the experiment and gluing them in a known order to mesh pieces fastened within the cages. Upon recovery on July 9, 2004, the surviving individuals were measured and the change in length was noted for each. For growth comparisons, individuals from only the caged treatments were treated as replicates and changes in individual lengths were analyzed with a two-factor ANOVA, with zone (inner, outer, and away) and height (benthic or floating) as fixed factors.

Predation

To determine if predation differed with the zones across the mussel bed, the percent juvenile mussel survival within the caged, uncaged, and partially caged treatments (caging experiment 1) was calculated and arcsine transformed for initial analysis with a three-factor, mixed model ANOVA (zone x treatment x trial), using trial as a random factor (Sokal & Rohlf 1995). Because trial was not significant at the $\alpha = 0.25$ level (Quinn & Keough 2002), trials were pooled and the data were re-analyzed with a two-factor ANOVA. To determine if patterns of survival were related to a factor

associated with height (e.g., predation by benthic vs. pelagic predators), we compared survival in caged and uncaged treatments across the three zones and between the benthic and floating treatments (caging experiment 2). Survival was analyzed with a three-factor *G*-test for goodness of fit to a log-linear model (Sokal & Rohlf 1981).

Distribution of potential predators

To determine the distribution of potential predators at the BP, quantitative samples were collected in November 2003 and August 2006 from the inner, middle, and outer zones of the BP mussel bed as described previously (see "Size Distribution" section). The mean densities (m^{-2}) for each of three potential predators, including unidentified polyclad flatworms, *Eosipho canetae* (a buccinid snail), and small galatheid crabs, and one potential "bulldozer" (*Bathynerita naticoides*, a seep-endemic neritid snail) were calculated within each zone and a square root transformation was applied. The transformed densities of the polyclad flatworms, galatheid crabs, and *B. naticoides* in the zones were analyzed in separate two-factor, mixed model ANOVA's, with collection month as a random factor. When month was not significant at the $\alpha = 0.25$ level, months were pooled (Quinn & Keough 2002), then the data were re-analyzed as single factor ANOVA's. Because we did not collect any *E. canetae* in August 2006, only the November 2003 data were analyzed as a single factor ANOVA. Scoop collections cannot sample large, mobile fauna, so observations on the distributions of large invertebrates and fishes were noted during multiple dives and while viewing video footage from dives.

Shipboard predation experiments

Because we had no indication from field observations that any consumers in the mussel bed actually prey upon juvenile mussels, we conducted several shipboard experiments to determine if the common carnivores consume young *"Bathymodiolus" childressi.* In November 2003, the following potential predators were used: unidentified polyclad flatworms, *Eosipho canetae* (a large buccinid snail), *Rochina crassa* (a large spider crab), and galatheid crabs of the genus *Munidopsis*. Three replicate flatworms were each placed in ~300 ml seawater with 10 small (<5mm long) mussels, five replicate *E. canetae* (each 10 cm long) were each placed in 2 L seawater with 10 small mussels (~10 mm long), two replicate *R. crassa* of similar size were each placed in ~35 L seawater with 20 small mussels (~10 mm long), and three replicate galatheid crabs of similar size were each placed in ~300 ml of seawater with 10 small mussels (~5 mm long). Each replicate was left undisturbed in a 7-8° C cold room on the ship for 48 hours and then were scored for percent mortality.

In July 2004, three more shipboard predation experiments were conducted using *Eosipho canetae*, galatheid crabs, and *Sclerasterias tanneri* (a large starfish) as potential predators. Three replicate *E. canetae* (4-5 cm long) were each placed in 2 L seawater with 5 mussels from 2-3 cm long and 5 mussels less than 1 cm long. Two replicate *S. tanneri* of equal sizes were each placed in ~35 L seawater with 5 mussels from 2-3 cm long and 5 mussels less than 1 cm long. Three replicate galatheid crabs of similar sizes were each placed with 10 juvenile mussels (<5 mm long) in 1 L of seawater. Each replicate was left undisturbed in a 7-8° C cold room on the ship for 10 days. The water

was changed in each treatment on days 4 and 7 and they were scored for percent mortality on day 10.

RESULTS

Size distribution

A distinct bimodal size distribution across the three zones of the BP mussel bed is evident throughout the sampling period (Figs. 2, 3). Although the size distributions of mussels in the middle and outer zones were not always significantly different, the size distributions of mussels in the inner zone samples were significantly different from the distributions in the other two zones in all samples except August 2006 (Table 2).

Table 2. Results of pairwise Kolmogorov-Smirnov tests comparing the distributions of the inner, middle, and outer zones within each sampling period. When they did not have significantly different size distributions at the $\alpha = 0.05$, the middle and outer zones were pooled (Mid/Out) for comparison to the inner zone. In March 2002, mussels were not collected from the middle zone.

Sampling period	Zone 1	Zone 2	Z	Sig.
March 2002	Inner	Outer	3.993	0.000
September 2003	Inner	Mid/Out	4.508	0.000
February 2003	Inner	Mid/Out	5.345	0.000
November 2003	Inner	Mid/Out	7.103	0.000
July 2004-West	Inner	Mid/Out	1.555	0.016
July 2004-North	Inner	Mid/Out	7.140	0.000
July 2004-East	Inner	Mid/Out	6.102	0.000
August 2006	Inner	Middle	3.471	0.000
	Inner	Outer	1.024	0.245
	Middle	Outer	3.714	0.000

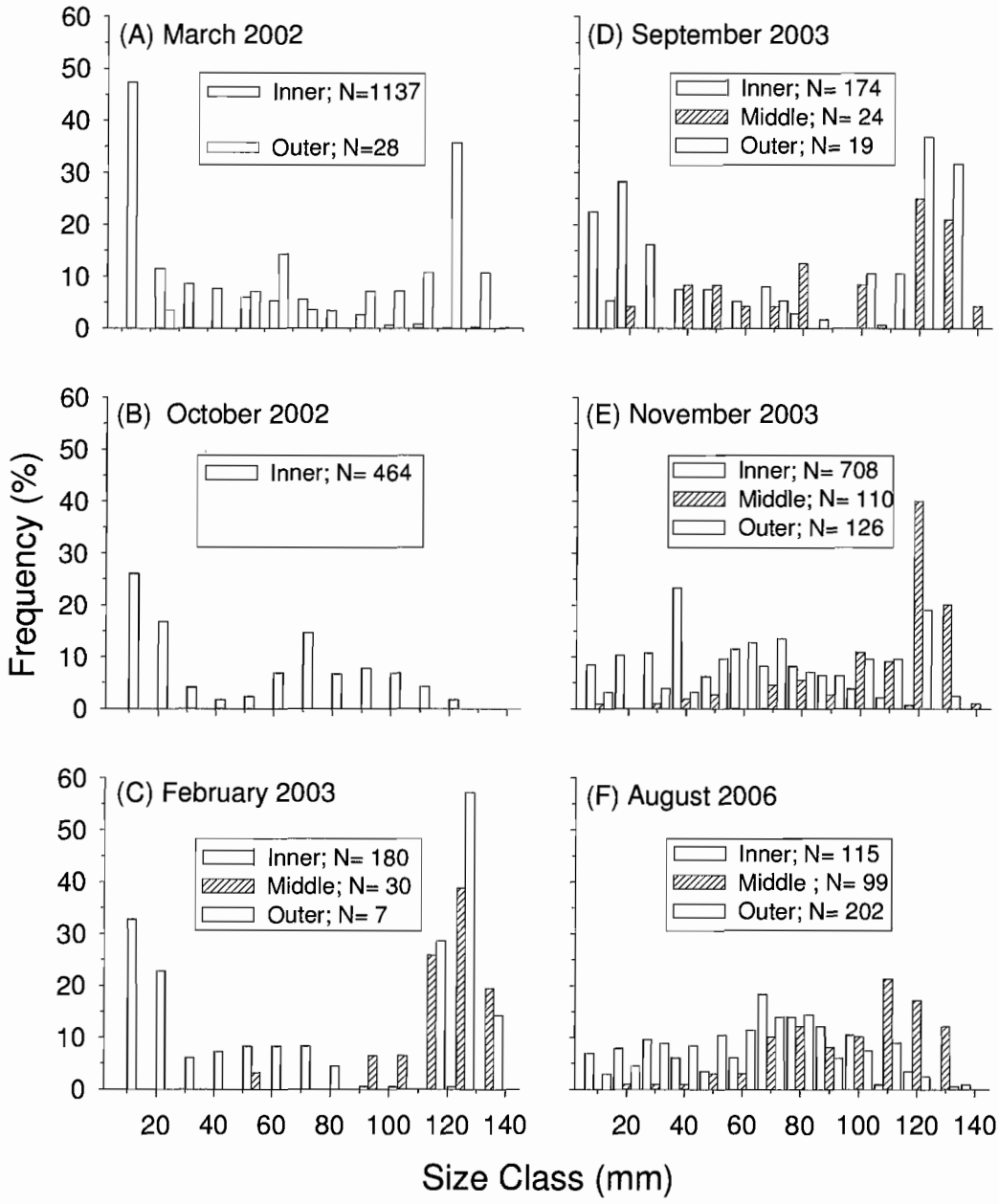

Figure 2. *"Bathymodiolus" childressi*. Length-percent frequency histograms from the inner, middle, and outer zones of the western side of the BP mussel bed in (a) March 2002, (b) October 2002, (c) February 2003, (d) September 2003, (e) November 2003, and (f) August 2006.

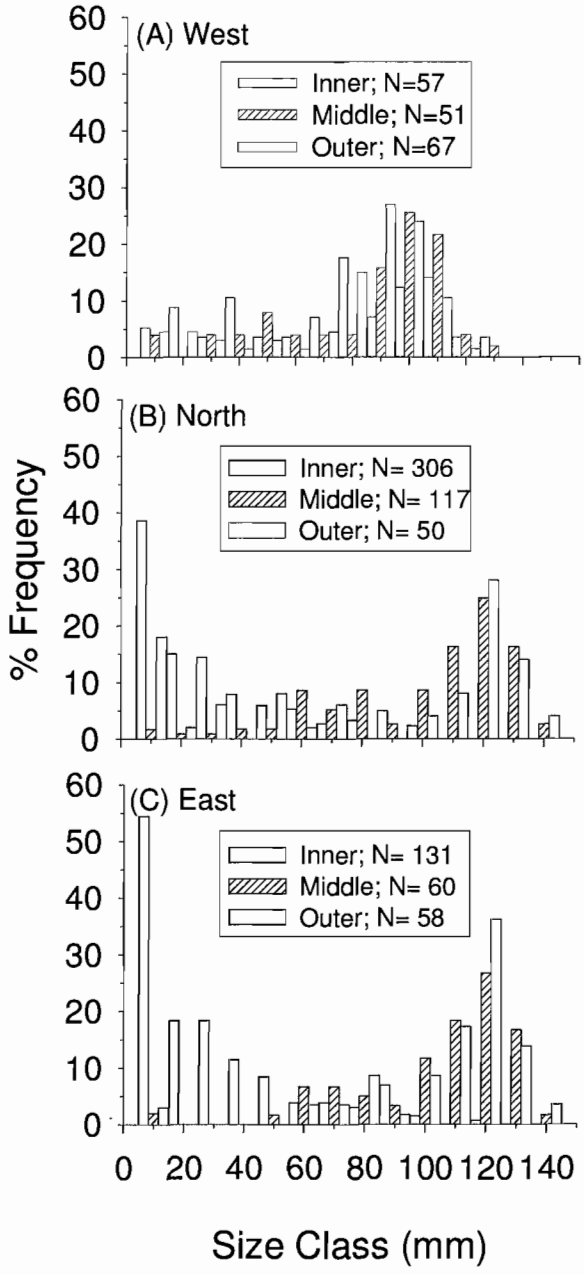

Figure 3. *Bathymodiolus" childressi.* Length-percent frequency histograms from the inner, middle, and outer zones of the BP mussel bed at the (a) western, (b) northern, and (c) eastern sides during July 2004.

This pattern held true in July 2004 on all sides (west, north, and east) of the pool, but the bimodal size distribution of the mussels on the west side did not appear as prominent as in other months or on the other two sides (Figs. 2,3). The mean lengths of mussels in the inner zone were significantly larger ($F = 47.35$, $p < 0.001$) on the west side than on the east or north sides.

Juveniles were always more abundant in the inner zone than in the middle or outer zones (Figs. 4, 5). Analysis of quantitative samples showed a significant effect of zone on fourth-root-transformed density of juveniles ($F = 10.603$, $p = 0.001$; Fig. 5). Juvenile density in the inner zone was greater than the density at both the middle zone (Tukey HSD: $p = 0.001$) and the outer zone (Tukey HSD: $p = 0.049$), but we detected no significant difference between the middle and outer zones (Tukey HSD: $p = 0.151$). The average densities of juveniles (number m^2) at the inner, middle, and outer zones were 117.0 ± 159.2, 2.4 ± 5.9, and 18.9 ± 27.1 ($\bar{x} \pm$ S.D.), respectively.

Larval supply and settlement

The average current velocities in February 2003 at the mouths of the tube traps were 0.012 ± 0.002, 0.017 ± 0.005, and 0.024 ± 0.002 m s^{-1} ($\bar{x} \pm$ S.D.) in the inner, outer, and away zones, respectively. Significant differences among these current velocities were not detected ($F = 3.246$, $p = 0.067$). Likewise, we could not reject the null hypothesis that the numbers of veligers captured per day were not ordered across the three zones (H_o: $M_{away} = M_{outer} = M_{inner}$) for either trial ($J_{3,3,3} = 14$, $p = 0.50$; Fig. 6).

Figure 4. Photographs of the Brine Pool mussel bed at the (a) inner and (b) outer zone. Note the small mussel recruits in the inner zone (indicated by arrows) and the large, mostly dead mussels that make up the outer zone bed. Also shown in (a) are *Methanoaricia dendrobrachiata* worms and galatheid crabs. Some *Bathynerita naticoidea* snails are visible in (b).

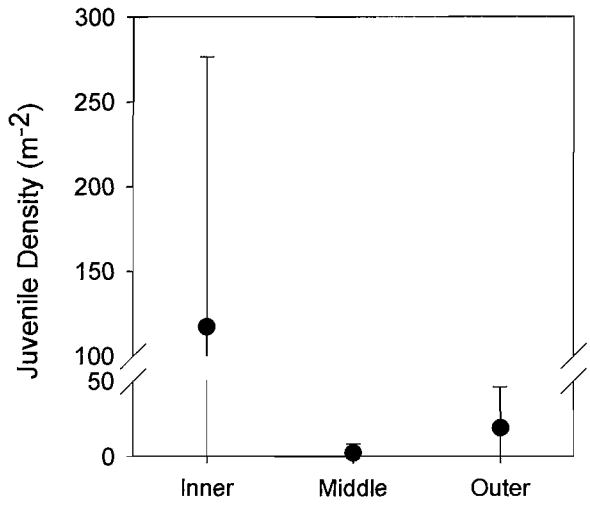

Figure 5. *"Bathymodiolus" childressi.* Mean density of juvenile mussels (m^{-2}) in the inner, middle, and outer zones. Note the break on the y-axis between 50 and 100. Error bars are +/- 1 S.D.

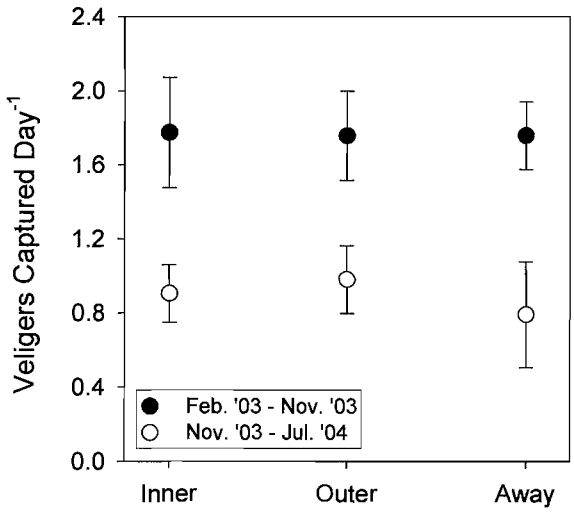

Figure6. *"Bathymodiolus" childressi.* Mean number of veligers captured day^{-1} in tube traps placed at the inner, outer, and away zones of the BP mussel bed from February 12 to November 10, 2003 (filled circles) and November 11, 2003 to July 15, 2004 (open circles). Error bars are +/- 1 S.D.

Similarly, although we detected significant differences in settlement density across all three zones ($F = 5.71$, $p = 0.022$; Fig. 7), we did not detect any difference in settlement density between the inner and outer zones (Tukey HSD: $p = 0.097$). A difference in settlement density was detected between only the inner and away zones (Tukey HSD: $p = 0.023$; Fig. 7). Although we expected zero settlement in the away zone, average settlement density there was 38.0 ± 9.7 settlers m^{-2} ($\bar{x} \pm$ S.D.). We reject the hypothesis that total settlement in the inner zone should be six times greater than in the outer zone to explain the adult mussel distribution and juvenile mussel density ($G_{adj} = 88.44$, $p < 0.001$; Table 3). Instead, there were only twice as many settlers in the inner than there were in the outer zone (Table 3).

Table 3. Total number of settlers on settlement racks at the inner and outer zones. Expected frequencies are based on a hypothesized 6:1 ratio for the inner to outer zones. G_{adj} is the Williams' adjusted log likelihood statistic G.

Zone	Obs.	Exp.	G_{adj}	P
Inner	252	325	88.44	< 0.001
Outer	127	54		
Total	379	379		

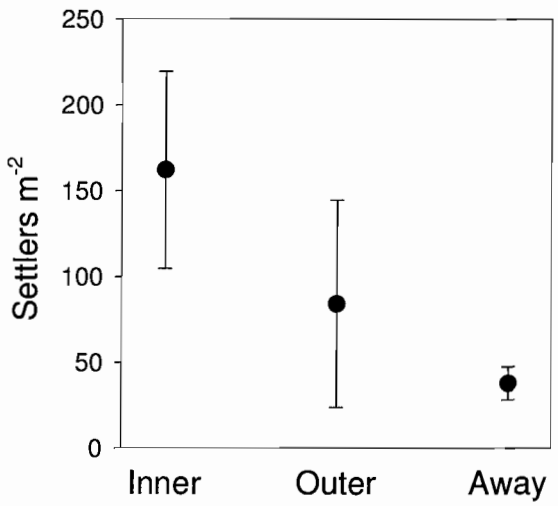

Figure 7. *"Bathymodiolus" childressi.* Mean number of settlers m^{-2} on settlement racks placed in the inner, outer, and away zones of the BP mussel bed. Error bars are +/- 1 S.D.

We were unable to test for zone effect with substratum size (large or small) or state (living mussel or empty shell) on recruitment because we only found recruits on all four substratum treatments within the inner zone (Fig. 8). We were unable to detect a significant effect of substratum size ($F = 0.003$, $p = 0.955$), state ($F = 0.249$, $p = 0.625$), or their interaction ($F = 0.313$, $p = 0.583$) on recruitment of 14-day-old mussels (Table 4, Fig. 8). At the outer zone, we found 53.0 ± 81.2 recruits m^2 ($\bar{x} \pm$S.D.) on the large empty mussel shell treatments and in the away zone we found of 47.4 ± 82.1 recruits m^2 ($\bar{x} \pm$ S.D.) on small empty shells, but none on living mussels in these zones (Fig. 8).

Table 4. Two-factor ANOVA table showing the effect of size (large or small) and state (living mussel or mussel shell) on density (recruits m^{-2}) of 14-day-old recruits in the inner zone only.

Source	df	Type III SS (10^2)	MS (10^2)	F	Sig.
Size	1	0.103	0.103	0.003	0.955
State	1	7.694	7.694	0.249	0.625
Size * State	1	9.701	9.701	0.313	0.583
Error	16	495.1	30.95		

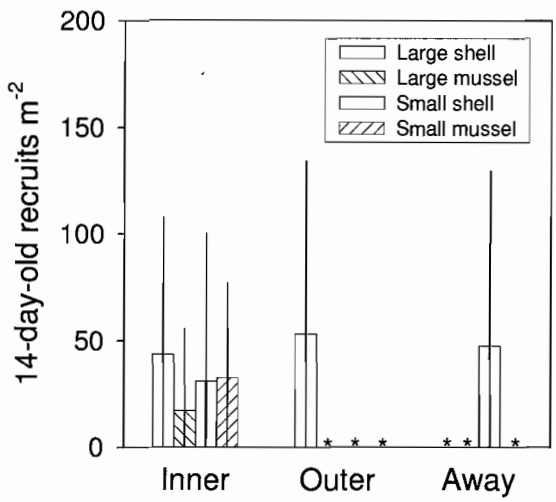

Figure 8. *"Bathymodiolus" childressi.* Mean number of 14-day-old recruits m^{-2} on four substratum treatments placed within settlement racks in the inner, outer, and away zones of the BP mussel bed. Asterisks indicate treatments on which zero settlement occurred. Error bars are +/- 1 S.D.

Survival and growth

Survival of juvenile *"Bathymodiolus" childressi* transplanted from the inner zone to the inner, outer, and away zones was high in all experiments—more than 88.9 % survived in all inner and outer zone treatments, and more than 77.8 % survived in all away zone treatments (Table 5). We could not reject the null hypothesis that the percent survival of transplanted juveniles did not differ among zones (H_o: $M_{away} = M_{outer} = M_{inner}$) for either trial (Trial 1: $J_{3,3,3} = 12$, $p > 0.50$; Trial 1: $J_{2,2,2} = 5$, $p > 0.50$). Similarly, in the bag transplants, the difference between the mean percent survival of juveniles transplanted to the inner zone and those transplanted to the outer zone was not outside our equivalence tolerance limit of 50%, leading us to reject the null hypothesis that the difference in means is not trivial (Table 5, Fig. 9). As a matter of fact, we also reject the null hypothesis that the means are equal because a difference of 0% is within the 95% confidence interval (Fig. 9).

Table 5. *"Bathymodiolus" childressi.* Average percent survival of juveniles transplanted from the inner zone to the outer and away zones in two different experiments. Cage experiments in Trial 1 were in position from October 10, 2002 to February 11, 2003, and those in Trial 2 were in position from February 11, 2003 to September 15, 2003. Bag transplants were in position from March 12, 2002 to October 9-11, 2002.

Experiment	Zone	n	% Survival	S.D.
Cage Exp. 1, Trial 1	Inner	3	88.89	7.70
	Outer	3	93.33	0.00
	Away	3	77.78	16.76
Cage Exp. 2, Trial 2	Inner	2	96.67	4.72
	Outer	2	96.67	4.72
	Away	2	86.67	18.86
Bag Transplant	Inner	3	96.67	2.89
	Outer	2	95.00	0.00

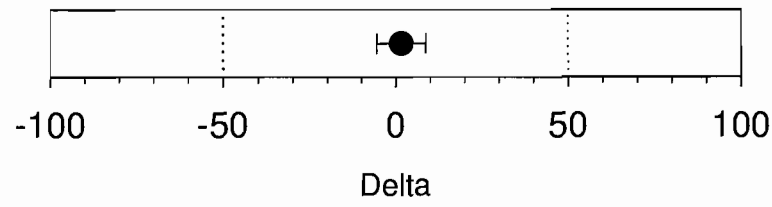

Figure 9. Equivalence test for mean percent survival of juveniles transplanted to the inner zone and those transplanted to the outer zone. Dotted lines indicate the equivalence tolerance of 50% around zero. Error bar is 95% confidence intervals of the delta between the two means.

Likewise, survival of juveniles placed within cages on the bottom and floating 30 cm above the bottom at the three zones were similar. We cannot reject the null hypothesis that survival of juveniles placed within cages across the three zones was independent of whether they were benthic or floating ($G_{adj.} = 0.5614$, $X^2_{0.05(2)} = 5.991$; Table 6).

Table 6. *"Bathymodiolus" childressi.* Two-factor contingency table showing numbers of surviving juveniles (<20mm long) placed at the Brine Pool in cages from November 12, 2003 to July 9, 2004. A two factor *G*-test of independence does not reveal an interaction ($G_{adj} = 0.5614$, $X^2_{0.05\,(2)} = 5.991$).

	Zone (b = 3)			
Height (a = 2)	Inner	Outer	Away	Totals
Floating	10	9	9	28
Benthic	10	6	10	26
Totals	20	15	19	54

Growth of juvenile *"Bathymodiolus" childressi* varied significantly with zone and height of the cage ($F = 41.101$, $p < 0.001$; Table 7, Fig. 10). Figure 10 shows more growth in juvenile mussels in the benthic cages in the inner zone than in the outer or away zones. Mean growth rates ($\bar{x} \pm$ S.D.) for benthic individuals < 20 mm long were 1.44 ± 0.30 mm $30d^{-1}$ (n = 6) in the inner zone, 0.52 ± 0.16 mm $30d^{-1}$ (n = 6) in the outer zone, and 0.22 ± 0.20 mm $30d^{-1}$ (n = 10) in the away zone. Conversely, in the floating cages differences in the growth of individuals in these two zones is not evident (Fig. 10). Here, mean growth rates ($\bar{x} \pm$ S.D.) for individuals < 20 mm long were 0.19 ± 0.24 mm $30d^{-1}$ (n = 10) in the inner zone, 0.52 ± 0.16 mm $30d^{-1}$ (n = 8) in the outer zone, and 0.10 ± 0.04 mm $30d^{-1}$ (n = 9) in the away zone.

Table 7. *"Bathymodiolus" childressi.* Two-factor ANOVA table comparing mean growth rates (mm 30 d^{-1}) of juveniles within cages across three zones and two heights.

Source	df	Type III SS	MS	F	Sig.
Zone	2	3.821	127.809	56.801	< 0.001
Height	1	4.299	4.299	127.809	< 0.001
Zone * Height	2	2.765	1.382	41.101	< 0.001
Error	43	1.446	0.034		

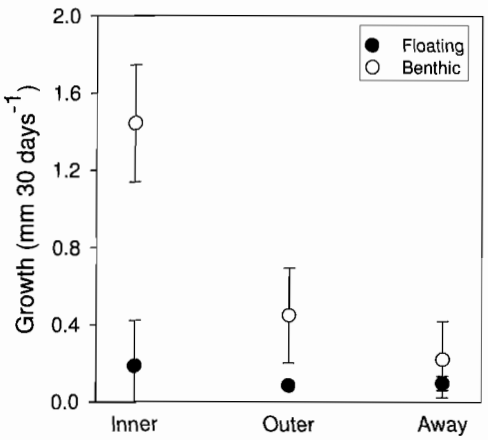

Figure 10. *"Bathymodiolus" childressi.* Mean growth (mm 30 $days^{-1}$) of caged juveniles placed on the benthos (open circles) or floating 30 cm above the benthos (filled circles) at the inner, outer, and away zones of the BP mussel bed. Error bars are +/- 1 S.D.

Predation

Caging experiments

In caging experiment 1, caging treatment (caged, uncaged, and partially caged) significantly affected survival of juvenile mussels ($F = 10.374$, $p < 0.001$) (Fig. 11; Table 8). Post hoc pairwise contrasts showed significantly higher survival in caged treatments than in uncaged treatments (Tukey HSD: $p < 0.001$), but no difference partially caged and uncaged or caged treatments (Tukey HSD: $p = 0.068$ for uncaged vs. partially caged, and $p = 0.097$ for caged vs. partially caged). We did not detect a significant effect of zone on survival of juveniles in cages, partial cages, or no cages ($F = 0.028$, $p = 0.972$) (Fig. 11; Table 8). Although we expect a large effect if predation alone causes the observed differential distribution of juveniles across the BP mussel, the effect size of zone on percent survival was low. The partial eta (η^2) was just 0.002, indicating that zone by itself accounted for only 0.2% of the overall (effect + error) variance.

Table 8. *"Bathymodiolus" childressi.* Two-factor ANOVA table showing the effect of transplant zone (inner, outer, and away zones) and treatment (caged, partially caged, and uncaged) on the arcsine transformed survival ratio of juvenile *"Bathymodiolus" childressi* (<15 mm long).

Source	df	Type III SS	MS	F	Sig.
Zone	2	0.005	0.002	0.028	0.972
Treatment	2	1.798	0.899	10.374	< 0.001
Zone * Treatment	4	0.748	0.187	2.158	0.094
Error	35	3.032	0.087		

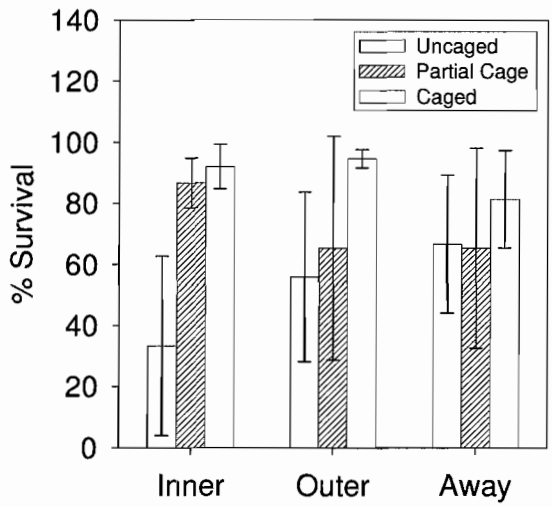

Figure 11. *"Bathymodiolus" childressi.* Percent survival of juveniles (<15 mm long) that were uncaged, partially caged, and caged and placed at the inner, outer, and away zones of the BP mussel bed. Error bars are +/- 1 S.D.

Results from caging experiment 2, the predation experiment testing the effect of height, caging, and zone on the survival of juvenile *"Bathymodiolus" childressi*, were not as clear. Although survival of the benthic uncaged individuals was lower in all three zones (Table 9), a *G*-test of goodness of fit to test for independence fits a completely independent log-linear model ($\ln \hat{f}_{ijk} = \mu + \alpha_i + \beta_j + \gamma_k$), indicating no interactions between any of the three factors ($G = 9.214$, $X^2_{0.05\,(7)} = 14.067$).

Table 9. *"Bathymodiolus" childressi.* Three-factor contingency table showing numbers of surviving juveniles (<20mm long) of *"Bathymodiolus" childressi* placed at the Brine Pool from November 2003 to July 2004. A *G*-test of goodness of fit does not reject a completely independent log-linear model ($G = 9.214$, $X^2_{0.05\,(7)} = 14.067$).

		Zones (c = 3)			
Height (a = 2)	Treatment (b = 2)	Inner	Outer	Away	Totals
Floating	Caged	10	9	9	28
	Uncaged	10	9	9	28
	Subtotals	*20*	*18*	*18*	*56*
Benthic	Caged	10	6	10	26
	Uncaged	1	7	5	13
	Subtotals	*11*	*13*	*15*	*39*
Totals		31	31	33	95

Potential predator distributions

We pooled collection dates in analyses of all potential predators because collection date was not significant at the $\alpha = 0.25$ level (Quinn & Keough 2002). We could not detect a significant difference in the square- root-transformed density (number m^{-2}) of polyclad flatworms ($F = 0.161$, $p = 0.853$) or the buccinid gastropod *Eosipho canetae* ($F = 1.337$, $p = 0.292$) (Fig. 12a, b). Tests for differential densities of these were not powerful because mean densities were low with large variances; potential predators

were absent from many samples. The square root transformed densities of galatheid crabs varied significantly across the BP mussels bed ($F = 15.73$, $p < 0.001$) (Fig. 12c). There were significantly fewer galatheids in the inner than middle or outer zones (Tukey HSD: $p = 0.001$), but the density of galatheids in the middle and outer zones did not differ from each other (Tukey HSD: $p = 0.812$). There were significantly fewer *Bathynerita naticoides* (a common snail that could be a bulldozer) in the inner zone than in the middle or outer zones ($F = 22.049$, $p < 0.001$; Tukey HSD: $p < 0.001$) of the BP mussel bed (Fig. 12d). No significant difference in the square root density of *B. naticoides* between the middle and outer zones was detected (Tukey HSD: $p = 0.578$).

Because scoop collections cannot sample large, mobile fauna we were able to make only observations on the distribution of some larger organisms that might bepredators on *"Bathymodilus" childressi*. The large spider crab *Rochina crassa* was observed frequently around the fringes of the mussel bed, but never near the pool itself. The asteroid *Sclerasterias tanneri* was observed often near the outer zone and in the middle zone of the bed, but not in the inner zone.

Shipboard predation experiments

There was no evidence of predation on small mussels by polyclad flatworms, *Eosipho canetae*, *Rochina crassa*, or *Sclerasterias tanneri* in shipboard experiments conducted in November 2003 or July 2004. Some mortality of juvenile mussels placed with galatheid crabs (average 16.6%) was evident in November 2003. However, we did not directly observe predation on the mussels by the galatheids. In July 2004, galatheid crabs was observed scavenging on the two mussels that died in one replicate.

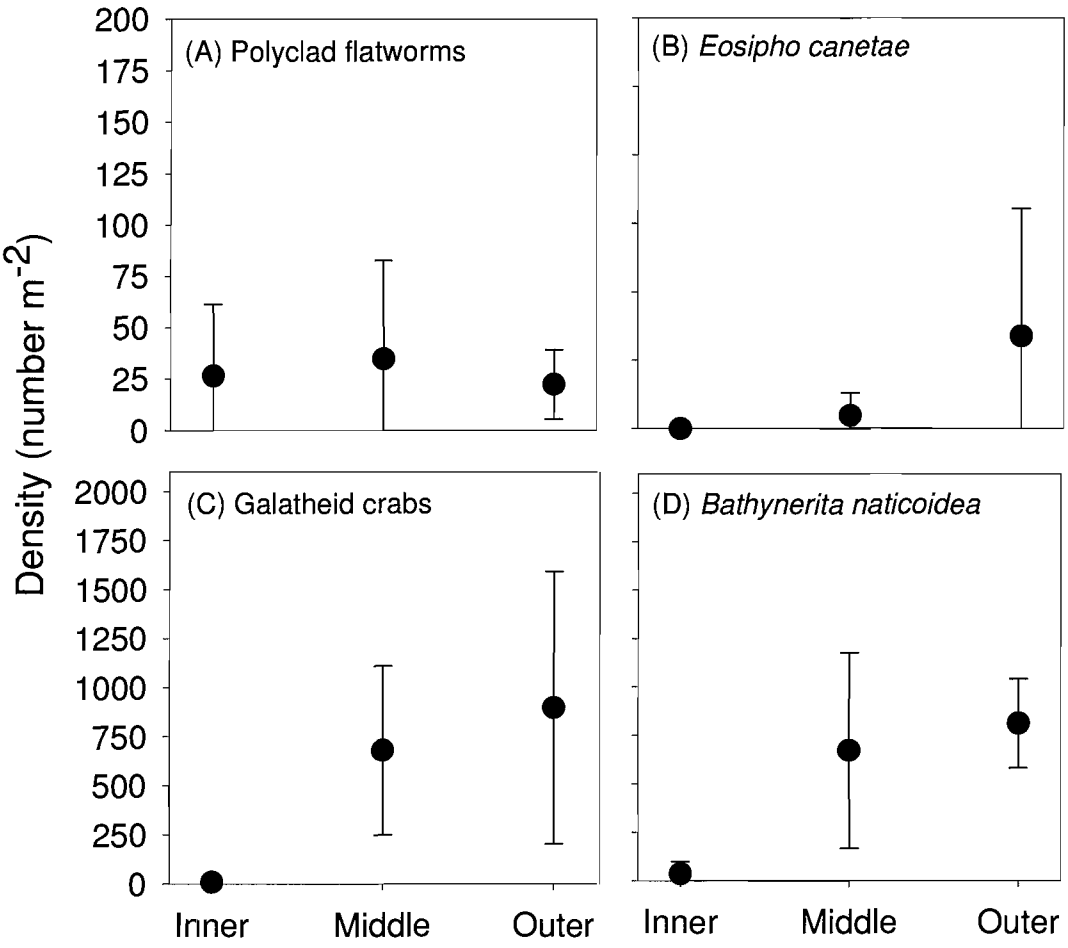

Figure 12. Mean densities (number m^{-2}) of (a) polyclad flatworms, (b) *Eosipho canetae*, (c) galatheid crabs, and (d) *Bathynerita naticoides* in the inner, middle, and outer zones of the BP mussel bed. Means are pooled over November and August trials. Note the scales for (a) and (b) are 10 fold less than those for (c) and (d). Error bars are +/- 1 S.D.

DISCUSSION

The abundance of juvenile *"Bathymodiolus" childressi* directly adjacent to the brine pool has been noted repeatedly since the site was first described (MacDonald et al. 1990a, b, c MacDonald & Fisher 1996, Smith et al. 2000). Here, we document the persistence of the bimodal size distribution of mussels across the BP mussels bed, with the highest density of the smallest mussels in the inner zone and the largest mussels in the outer zone. This pattern is consistent around the pool and throughout the sampling period (Figs. 2, 3).

We did not expect to find the smallest size classes of mussels at the Brine Pool throughout the year because Tyler et al. (2006) indicate that gametogenesis and spawning in *"Bathymodiolus" childressi* is seasonal. Gametogenesis begins in November and continues through February, followed by a prolonged spawning period beginning as early as October and continuing through February (Tyler et al. 2006, Chapter 2). The highest periods of recruitment corresponded with the known spawning season of *"B." childressi* in October 2002, September 2003 (but not November 2003), February 2003, and March 2002. Conversely, recruitment was not evident in high abundance on the west side of the pool in July 2004 and August 2006, nor were there recruitment events evident in samples taken by Smith et al. (2000) in August 1992, September 1994, and August 1995. However, an abundance of mussels <10 mm long was evident at the northern and eastern sides of the pool in July 2004 (Fig. 3). Nevertheless, while individuals less than 5 mm long make up 15-16% of the population in February 2003 and March 2002, only 1.75%, 8.5%, and 3% of the samples at the western, northern, and eastern sides of the pool,

respectively, were less than 5 mm long in July 2004 (Fig. 3). Considering our calculated mean growth rate of 1.44 ± 0.30 mm 30 days^{-1} ($\bar{x}$ ± S.D.), if mussels settled in March 2004 they would still be 5-10 mm long, providing an explanation for the bimodal distribution in even the summer months. In addition, individual and interannual variability in gametogenesis combined with the extended spawning season may account for the persistence of individuals <10 mm in length within the inner zone throughout the sampling period.

Smith et al. (2000) quantitatively sampled the mussel bed in the inner, middle, and outer zones and showed a relative density of about six times more mussels residing in the inner than in the outer zone. On average we found about 10 times more juveniles <10 mm long in the inner than the outer zone (Fig. 5). Although individuals in the middle size classes are found in the middle and outer zones, peaks of the smallest size class mussels are conspicuously absent in these zones (Fig 2, 3; Smith et al. 2000). If juvenile mussels are so much less abundant at outer zone, then how are median-sized mussels showing up in the population in the middle and outer zones? Below, we discuss several pre-settlement, settlement, and post-settlement factors that may contribute to structuring the observed bimodal size distribution of mussels across the BP mussel bed.

Pre-settlement and settlement

Larval supply can often be sufficient to explain variability in settlement and recruitment (Underwood & Keough 2001). At larger spatial scales there should be a tighter correlation between settlement and larval supply due to passive transport than at smaller spatial scales where settlement behavior and cues may play a more important role

(Underwood & Keough 2001). Larval supply has been shown to correlate with settlement at coastal scales (Jonsson et al. 2004) and between zones of the intertidal (Minchinton & Scheibling 1991). In July 2004, differential larval supply due to prevailing currents may explain the observed recruitment along the northern and eastern sides of the brine pool when a recruitment pulse was absent at the western side of the pool.

At the smaller scale of the mussel bed width (which ranges from 3 to 7 m, but is ~3 m wide at the western side of the pool), we expect that settlement cues and substrate selection would be more likely than larval supply to influence settlement distribution. At the BP cold seep, high densities of small mussels are found primarily in the inner zone, while large mussels are found in both zones. Conversely, empty mussel shells are available in the outer zone, but in the inner zone empty mussel shells are found only at the very edge of the pool where they are often under the surface of the brine pool or covered in black hydrocarbons (pers. obs.) and are probably not suitable settlement substrata. Since small and large living mussels are available substrata for settlement at the inner zone and if increased settlement caused the higher density of juveniles at the inner than the outer zone, we expected more settlement on the living mussels than dead mussel shells. However, we were unable to identify a substratum preference for settlement of larvae of *"Bathymodiolus" childressi* when we tested large verses small, living mussels and empty mussel shells. Also, shallow-water mytilid mussels tend to settle most readily onto filamentous surfaces, and settlement is induced in some mytilids by conspecific byssal threads (Bayne 1976, Lutz and Kennish 1992). The mussels

residing at the inner zone usually are covered with residual byssal threads that should be ideal for settlement. Despite the availability of apparently appropriate substrata, there was no evidence that settlement was increased in the inner zone.

Both larval supply and settlement were equally high at the inner and outer zones of the mussel bed, suggesting that neither of these pre-settlement nor settlement factors explain the variation in juvenile density across the mussel bed. Ever since the first descriptions of the BP mussel bed, methane has been implicated as a settlement cue (MacDonald et al. 1990c, MacDonald & Fisher 1996) and larvae have been assumed to preferentially settle at the inner zone (MacDonald et al. 1990a, b, c, MacDonald & Fisher 1996, Smith et al. 2000). Additionally, environmental variability has been associated with physiological condition of adult mussels and community structure at the brine pool (Nix et al. 1995, Smith et al. 2000, Bergquist et al. 2004, 2005). Environmental cues such as methane or brine seepage may indeed cue settlement, but there is no evidence from our observations that larvae settle preferentially in the inner zone.

Perhaps the most striking result of this series of experiments was the high supply of larvae, number of settlers, and percent survival of juveniles in the away zone—2 meters outside of the BP mussel bed and over bare sediment. Although, we assumed the away zone was not influenced by methane seepage, we have no evidence that it is not. It has been hypothesized that substratum may be a limiting factor for colonization of a seep site by mussels (MacDonald et al. 1990c, Smith et al. 2000) and tubeworms (Bergquist et al. 2002). In this study, mussels settled in a location that was not colonized by other mussels when we supplied them with a settlement surface (pvc racks and mesh cages),

providing evidence that an appropriate settlement surface may be a necessity for *"Bathymodiolus" childressi* to colonize a seep site. Conversely, Levin et al. (2006) showed that outside a seep on the northern California margin (525 m depth) appropriate chemical cues alone were sufficient to induce colonization by some seep infauna. The fact that a high percentage of juvenile mussels survived at the away zone may be due to either sufficient seepage of methane or their ability to filter feed (Page et al.1991, Pile & Young 1999).

Post-settlement factors

Differential survival, predation, or growth of juvenile mussels (up to 10 mm long) alone do not appear to cause the bimodal size distribution of mussels evident across the BP mussel bed. Juvenile mussels were capable of surviving in all zones. Similarly, there was no differential predation on juveniles across the mussel bed that would explain the bimodal size distribution of mussels. However, caging did increase survival of juveniles indicating potential predation, but we were unable to positively identify a predator of juvenile *"Bathymodiolus" childressi* mussels. There are three colonists (*sensu* Carney 1994) at the Brine Pool cold seep that we consider possible predators on adult and juvenile mussels. Through isotopic analysis, MacAvoy et al. (2002) showed that the atelecylid crab *Rochina crassa*, the seastar *Sclerasterias tanneri*, and the buccinid gastropod *Eosipho canetae* each obtain 50-100% of their nutrition within the cold seep community. Because survival rates in our partial cages were not significantly different from survival rates in our cages in the inner zones, we suspect the potential predator was large enough to be excluded from the partial cages, suggesting that the acetylid crab and

the seastar are more likely predators than the gastropod. Nevertheless, there is no evidence that these predators selectively prey upon juvenile mussels, nor did we see evidence that predation was differential across the bed.

Juvenile mussels grew in all three zones. If growth were to cause the observed bimodal size distribution, we would expect the growth rate of small individuals in the inner zone to be slow leading to the persistence of small individuals and the growth rate at the outer zone to be fast—requiring frequent sampling to observe an abundance of small individuals. On the contrary, growth rate decreased away from the inner zone and above the benthos. Diffusion of methane with height may account for the slowed growth of mussel above the benthos. Likewise, lack of methane in the away zone probably explains the similarity in growth rates between individuals placed in the "away" zone on the sediment and the growth rates of the individuals in any of the floating cages. Similar to our results for benthic individuals, Smith et al. (2000) found that, although methane was not significantly different across the mussel bed, mussels in the outer zone were in poorer physiological condition and grew slower. Smith et al. (2000) determined *in situ* growth rates for "*Bathymodiolus" childressi* in the three different zones through a mark-recapture growth study and showed that growth rates decline with mussel size. The growth rate Smith et al. (2000) determined for mussels within the 10-20 mm size class in the inner zone ($\bar{x} \pm$ S.D.: 1.4166 $\pm$ 0 mm 30 d^{-1}; n = 1) is within the range of our growth rates for benthic juveniles in the inner zone. At the outer zone, however, Smith et al. (2000) predict much higher growth rates of mussels under 10 mm long ($\bar{x} \pm$ S.D.: 0.833 $\pm$ 0.33 mm 30 d^{-1}; n = 2) than we show.

Alternative hypotheses

We have rejected the long-standing hypothesis that preferential settlement structures the size distribution across the BP mussel bed. In addition, we have shown that juveniles survive and grow equally well across the zones, and there is no evidence of differential predation across the bed. Not all post-settlement factors have been tested, however. Migration through secondary settlement (Lutz and Kennish 1992; Seed and Suchanek 1992) or migration of small juveniles, or early post-settlement mortality could all potentially explain the absence of individuals of the smallest size class in the outer zone.

Migration

In many shallow-water mytilid species, primary settlement of larvae onto a filamentous substratum (often algae or byssal threads) is followed by secondary settlement of small juveniles onto an established mussel bed (Lutz and Kennish 1992; Seed and Suchanek 1992). Between primary and secondary settlement, mussels up to 2 mm long can pass through a byssus drifting phase (Lutz and Kennish 1992). Byssal drifting by juvenile mussels that have settled in the outer zone of the BP mussel bed, which is physiologically less favorable for adult mussels (Smith et al. 2000), is a mechanism that would allow these settlers to relocate to a more favorable part of the mussel bed (such as the inner or middle zones) or disperse to other seep sites. Larger mussels can also move by depositing and releasing byssal threads. Mussels residing in the inner zone are often cantilevered over the brine, held at the surface of the pool of brine by byssal threads securing them to adjacent mussels lying on the sediment. We

show that the density of mussels in the inner zone is nearly 10 times greater than in the outer zone. Perhaps competition for space in the inner zone and the risk of sinking under the toxic brine induces juveniles to move outward toward the middle and outer zones as they grow, thus explaining the persistence of peaks of mid-sized mussels in the middle and outer zones, but not of small mussels.

Early post-settlement mortality

Early post-settlement mortality may significantly alter observed recruitment and adult distribution patterns and can be caused by delay of metamorphosis, physical disturbance and hydrodynamics, competition, physiological stress, predation, and biological disturbance such as bulldozing by grazers (reviewed by Gosselin and Qian 1997; Hunt and Scheibling 1997). We show that mortality of juveniles due to physiological tolerance and predation does not differ between the inner and outer zones. However, although we counted high numbers of settlers (<0.5 mm long) in all three zones, due to manipulation constraints our survival and predation experiments were conducted using individuals between 5 and 25 mm long. It is possible that there is high post-settlement mortality before recruits reach the size range possible for use in experimental manipulations.

Physiological stress tends to be higher in newly settled invertebrates and developmental abnormalities associated with extreme conditions are documented well (reviewed in Gosselin and Qian 1997; Hunt and Scheibling 1997). The outer zone of the mussel bed is low in oxygen and can have extremely high concentrations of hydrogen sulfide (Smith et al. 2000). Whether early settlers of *"Bathymodiolus" childressi* can

withstand the conditions in the outer zone is unknown. Likewise, *"B." childressi* settlers may avoid the unfavorable conditions by migrating out of the outer zone.

Potential predators are not evenly distributed across the BP mussel bed. Although we did not observe differential predation on juveniles (5-25 mm long) across the bed, some predators may selectively prey on small (< 5mm) *"Bathymodiolus" childressi* settlers. For example, the buccinid gastropod *Eosipho canetae*, which derives 71-100% of its nutrition from chemosynthetic material at the BP (MacAvoy et al. 2002), was found in only the middle and outer zones (this study, Bergquist et al. 2005). Predation by whelks is an important source of mortality of invertebrate recruits in intertidal habitats and there is evidence from the intertidal that some thaid whelk species prefer mytilid prey and preferentially consume mussels under 2 mm long (reviewed in Hunt and Scheibling 1997). Predation by *E. canetae* upon the new *"B." childressi* settlers is certainly a potential source of early post-settlement mortality in the middle and outer zones.

Another likely source of early post-settlement mortality is "bulldozing" by grazers. Bulldozing of early settlers is documented for many invertebrates (reviewed in Hunt and Scheibling 1997) and has been cited as possible a structuring factor in hydrothermal vent communities (Micheli et al. 2002). The nertid gastropod *Bathynerita naticoidea* is endemic to cold seeps and grazes the bacterial film from the shells of *"Bathymodiolus" childressi* (Van Gaest et al. 2007) and may bulldoze recently settled *"B." childressi* off the shells of adults while grazing. Moreover, this study and others have shown that *B. naticoidea* resides primarily in the middle and outer zones of the BP mussel bed (this study, Bergquist et al. 2005, Van Gaest et al. 2007). Thus, bulldozing of

early settlers by *B. naticoidea* could explain the lack of small mussels in the outer zones, despite high settlement to the outer zone. Finally, Micheli et al. (2002) showed that predation can structure the communities at hydrothermal vents indirectly, by reducing gastropods that bulldoze recruits of sessile invertebrates. Certainly, impacts of this grazer and impacts of other biological interactions on the population structure of *"B." childressi* warrant further investigation.

Supply-side ecology at cold seeps and hydrothermal vents

The recruitment of new individuals into an adult population depends on six basic processes: (1) larval production, (2) larval dispersal, (3) larval mortality, (4) settlement, (5) survival, and (6) growth of new recruits (Underwood and Keough 2001). Of these, the hypotheses about the causes and effects of settlement, survival, and growth are most readily tested by laboratory and *in situ* experiments. Experimental ecology has provided a wealth of information about hydrothermal vent communities (reviewed in Van Dover and Lutz 2004), where manipulative experiments have been used to test hypotheses about recruitment and have demonstrated biological facilitation and inhibition (Mullineaux et al. 2000, 2003), the effects of predation on recruitment (Micheli et al. 2002), and evidence of biological cues for colonization (Hunt et al. 2004). Taxa represented in hydrothermal vent communities are similar to those in cold-seep communities. Because seep sites are often shallower, seep organisms such as *"Bathymodiolus" childressi* are more easily maintained in the laboratory and cultured than vent organisms (Chapter 2), allowing for laboratory studies on supply-side ecology to correspond with *in situ* experiments. Yet, at cold seeps we know of only one published study that experimentally

tested hypotheses about recruitment (Levin et al. 2006). In the Gulf of Mexico, studies on demography and growth rates of cold-seep mussels and tubeworms have led to the formulation of hypotheses about recruitment dynamics (e.g. Nix et al. 1995, Smith et al. 2000, Bergquist et al. 2002, 2003, 2004), but until our study, those hypotheses have remained untested. We encourage further use of *in situ* manipulative experiments at cold seeps to provide answers to questions about recruitment dynamics, which may translate to explanations of dynamics of both cold-seep and hydrothermal-vent communities.

CHAPTER VI

GENERAL CONCLUSION

Like a few other cold-seep invertebrates, we were able to culture the larvae of *"Bathymodiolus" childressi* through the trochophore stage consistently and sometimes to D-shell veligers. Cultures of *"B." childressi* to the D-shell veliger stage show a definitive larval life of at least 8 days, although we kept trochophore larvae in the laboratory for more than 12 days without shell development or metamorphosis. We obtained indirect estimates of larval life spans by examining the size distribution of new recruits. We back-calculated the settlement dates from the length of new recruits and compared these settlement dates to the preceeding spawning period in order to determine larval durations from 3 months to perhaps as long as 13 months. Similarly, these estimates are within the range of larval durations we estimated based on spawning times and the presense of larvae in plankton.

Egg and prodissoconch sizes indicate planktotrophy in bathymodiolin mussels including *"Bathymodiolus" childressi.* We were unable to positively identify a food source for the larvae of *"B." childressi.* We investigated whether these larvae are capable of migrating from the Brine Pool cold seep (650 m depth) to the euphotic zone in order to feed. Early hypoetheses suggested that the larvae of hydrothermal-vent bathymodiolin mussels probably do not migrate to the surface waters, since migration

increases advection of larvae away from suitable habitats (Lutz et al., 1980; Lutz et al., 1984; Turner et al., 1985). However, we showed larvae of *"B." childressi* are energetically capable of migrating to the surface from 650 m depth (assuming typical veliger swimming speeds) without feeding. In addition, the larvae are tolerant of the temperature and salinities present in the upper water column of the Gulf of Mexico. Moreover, we found veligers of *"B." childressi* in the plankton up to at least 100 m depth. The large size of these veligers suggested they were up to 4 months old. We calculated a non-feeding metabolic lifespan for larvae of *"B." childressi* of up to 74 days, indicating that larvae could live at depth for extended periods without feeding.

A population genetics study suggests widespread dispersal of the larvae throughout the Gulf of Mexico (Carney *et al.*, 2006). Furthermore, Olu Leroy *et al.* (2007) show minimal genetic differentiation between the *"Bathymodiolus" childressi* and congeners from western and eastern Atlantic seeps, suggesting a historic connection between Gulf of Mexico and Atlantic seep mussels. We suggested larvae of *"B." childressi* remain at depth for a period of time before slowly swimming upward, with some reaching the surface. This strategy would allow larvae to take advantage of currents throughout the water column to disperse throughout the Gulf of Mexico and possibly across the Atlantic.

Finally, we examined whether pre- and post-settlment factors result in the distinct bi-modal size structure of the *"Bathymodiolus" childressi* population at the Brine Pool cold seep in the Gulf of Mexico. Using a series of *in situ* experiments and observations, we tested whether differential larval supply across the mussel bed, substratum selection

by settling larvae, juvenile mortality due to environmental conditions, differential growth, or juvenile mortality due to differential predation lead to the observed size structure. Larval supply and settlement were equal across the bed and juveniles survived and grew at all locations. Predation did not appear to cause the bimodal distribution either. We concluded that size structure at this mussel bed could not be attributed to either settlement preferences or mortality of juveniles. Instead, the pattern may result from migration or early post-settlement mortality.

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