Pre- and post-settlement factors controlling spatial variation in recruitment across a cold-seep mussel bed

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ABSTRACT: The size structure of a population of the mussel *'Bathymodiolus' childressi* shifts dramatically across the mussel bed encircling the Brine Pool NR 1 cold seep in the Gulf of Mexico. While the fringes of the bed are inhabited by large mussels, small juveniles are abundant only at the edge of the pool, leading to the inference that larvae settle preferentially near the source of methane. We used *in situ* experiments to test whether this shift in size distribution is structured by differences in larval supply, settlement, substratum selection by settling larvae, juvenile mortality, growth, or predation across the mussel bed. We investigated the variation of these factors adjacent to the pool, at the fringes of the mussel bed, and also 2 m away from the bed. Neither the supply of larvae nor the density of settlers (<0.5 mm long) collected on settlement plates differed among the 3 zones. Juveniles (mean length: 9 mm) survived and grew equally well in all 3 zones. Survival was higher in caged than in uncaged juveniles (5 to 15 mm long), but mortality of uncaged juveniles was similar at all distances from the pool, suggesting that predation alone does not cause the observed differences in size structure. We reject the prevailing hypothesis that the distribution pattern can be attributed to settlement preferences of larvae alone. We hypothesize that very early post-settlement mortality, secondary settlement, and/or later migration of juveniles play a role in establishing and maintaining the persistent pattern of size structure across this mussel bed.

KEY WORDS: *Bathymodiolus childressi* · Chemosynthetic communities · Deep sea · Mussel · Predation · Larval supply · Recruitment

INTRODUCTION

In many ecosystems, recruitment establishes the initial ecological patterns upon which all later processes act — variations in recruitment influence later spatial distributions, population size and structure, and community interactions. For marine species with dispersive larvae, larval settlement patterns and preferences are sometimes inferred from recruitment or population size structures (e.g. Bergquist et al. 2002, Kelly & Metaxas 2008), but such inferences should generally be regarded as hypotheses unless they have been tested experimentally. Settlement, which is the initial establishment of larvae onto a substratum, is affected by pre-settlement factors such as larval dispersal, supply of larvae to a habitat, settlement cues, and habitat selection. Recruitment, which is the first record of the settled juvenile by an observer (*sensu* Keough & Downes 1982), is also influenced by post-settlement processes such as juvenile migration, mortality, predation, and competition. Thus, inferences about settlement from recruitment or population patterns may be incorrect for species with dramatic post-settlement mortality and are especially problematic for species that are capable of movement after settlement (reviewed by Hunt & Scheibling 1997). By testing mul-

tiple working hypotheses (Platt 1964, Chamberlin 1965), we can provide insights not only into the relative importance of pre- and post-settlement processes in establishing population structure, but also of structuring processes such as early post-settlement mortality or behaviors that are not easily tested with manipulative field experiments. This is especially useful in inaccessible deep-sea habitats, where frequent sampling or complementary laboratory experiments are generally not possible. Here, we use multiple working hypotheses to test the role of various pre- and post-settlement processes in establishing recruitment patterns and variations in the fine-scale distribution of a deep-sea mussel bed at a cold seep on the upper continental slope of Louisiana.

Hydrocarbon seepage is widespread in this region and most sites host dense assemblages of benthic invertebrates that are dependent upon chemosynthetic bacterial symbionts for nutrition. Like other chemosynthesis-based ecosystems, there is a high degree of endemism at cold seeps. In contrast to hydrothermal vents, cold seeps are quite long-lived; for example, some cold-seep tubeworms in the Gulf of Mexico have been estimated to be 170 to 250 yr old (Bergquist et al. 2000). Brine Pool NR1 cold seep (BP) is a collapsed brine-filled salt diapir that is supersaturated with methane (see Supplement 1, available at www.int-res.com/articles/suppl/ m414p131_supp/ for video). The pool is surrounded by a dense bed of '*Bathymodiolus*' *childressi* mussels, which are endemic to cold seeps and dependent on methanotrophic endosymbionts (Childress et al. 1986). The size structure of this mussel population shifts dramatically across a short environmental gradient. New recruits and older juveniles of '*B.*' *childressi* are abundant only at the edge of the brine and only larger mussels are found at the outer fringes of the mussel bed (Fig. 1), leading to the inference that larvae settle preferentially at the pool's edge (MacDonald et al. 1990a,b,c, MacDonald & Fisher 1996). Some researchers have suggested that methane at the pool's edge acts as a cue to induce high settlement there (MacDonald et al. 1990c, MacDonald & Fisher 1996). However, the underlying cause of the high density of juveniles near the pool's edge remains untested. We hypothesize that methane is unlikely the sole cause of the observed recruitment pattern because concentrations of methane are highly variable

and not significantly different among different areas of the BP mussel bed (Smith et al. 2000), meet the minimum requirements necessary for mussel growth (Cary et al. 1988, Kochevar et al. 1992), and are often higher than methane concentrations at nearby seep sites that also host juvenile '*B.*' *childressi* (MacDonald et al. 1989, MacDonald et al. 1990a, Nix et al. 1995).

Alternatively, the observed population structure of mussels at the BP could be explained by a number of pre-settlement, settlement, and post-settlement events. First, appropriate physical substrata or biogenic cues for settlement could be associated with the abundant living mussels at the pool's edge rather than the mostly dead mussels around the outer fringes of the bed. Second, although the conditions throughout the BP mussel bed are suitable for survival and growth of adult '*Bathymodiolus*' *childressi* (Smith et al. 2000),
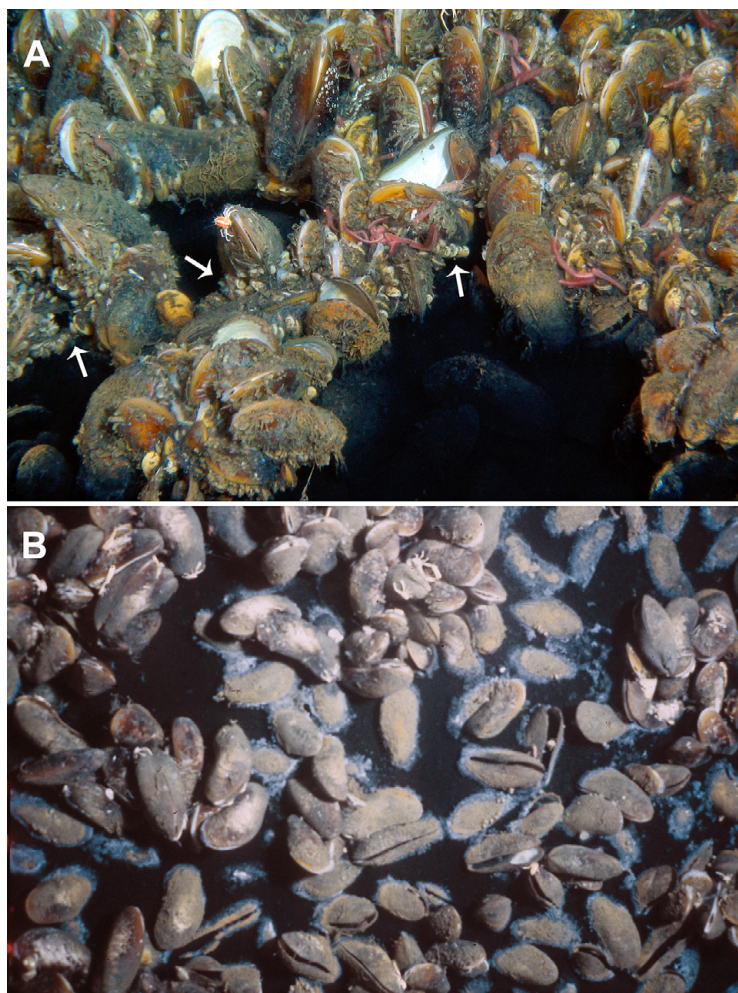

Fig. 1. '*Bathymodiolus*' *childressi*. Photographs of the Brine Pool NR1 cold seep (BP) mussel bed at the (A) inner- and (B) outer-seep zone. Note the clumps of small juvenile mussels evident in the inner-seep zone (arrows) and the large, mostly dead mussels that make up the outer zone bed. Also visible in (A) are dead mussels that have sunk beneath the brine.

we do not know if the conditions at the outer fringes of the BP mussel bed can support new recruits. Third, initial settlement patterns of *'B.' childressi* may not reflect recruitment patterns since, like intertidal mytilid mussels, the larvae may settle in an initially suitable location then drift later as plantigrade juveniles to another part of the bed where they may undergo secondary settlement (Bayne 1964). Finally, differential predation on recruits, which can influence the structure of hydrothermal vent communities (Micheli et al. 2002), could also influence the size distribution of mussels across the bed.

In this study, we used manipulative field experiments to simultaneously test multiple hypotheses about the roles of pre- and post-settlement processes in establishing the differential size distribution of *'Bathymodiolus' childressi* across the BP mussel bed. Specifically, we set out to test whether the observed size distribution is structured by differences in (1) larval supply, (2) settlement, (3) substratum selection, (4) juvenile mortality correlated with physico-chemical variations, (5) growth, and (6) predation on juveniles across the BP mussel bed. All 6 hypotheses are rejected by the experimental results. We discuss alternative hypotheses including differential early post-settlement mortality, secondary settlement, and juvenile migration that could establish the population structure of *'B.' childressi* across the BP mussel bed.

MATERIALS AND METHODS

Study site and terminology. The BP is a collapsed salt diapir filled with methane-saturated brine, located ~120 km south of Louisiana (27° 43′ 24″ N, 91° 16′ 30″ W) at a depth of ~650 m. A bed of *'Bathymodiolus' childressi* mussels encircles the brine pool. The morphology (Gustafson et al. 1998) and molecular phylogeny (Jones et al. 2006) of *'B.' childressi* reveal uncertainty about the placement of this species into the genus *Bathymodiolus*; thus, we follow the recommendations of Gustafson et al. (1998) to place the genus name of *'B.' childressi* in quotation marks. *'B.' childressi* is a mixotrophic mussel that harbors methanotrophic endosymbionts in its gills (Childress et al. 1986) and is known from seep sites in the northern and western Gulf of Mexico at depths ranging from 546 to 2222 m (Gustafson et al. 1998).

The mussel bed surrounding the BP can be divided into 3 zones: inner-, middle-, and outer-seep (Smith et al. 2000, Bergquist et al. 2005). The inner-seep zone is operationally defined as the one-third of the mussel bed that is directly adjacent to the brine pool, and the outer-seep zone is the one-third of the bed that is adjacent to the bare sediment. The inner-seep zone is char-

acterized by high methane and oxygen concentrations (>200 µM and ≤160 µM, respectively) and non-detectable hydrogen sulfide (Smith et al. 2000). The outer-seep zone has similar (but variable) methane concentrations, lower average oxygen levels (sometimes <50 µM), and high hydrogen sulfide (>1000 µM) (Smith et al. 2000). The middle-seep zone is transitional between the inner- and outer-seep zones and is patchy in mussel composition and water chemistry (Smith et al. 2000). We characterized mussel size distributions and community composition in the middle-seep zone, but all field experiments took place at the inner- and outer-seep zones and at a site ~2 m away from the outer periphery of the mussel bed in bare sediment. We refer to this latter location, which was presumably not influenced by methane seepage, as the 'non-seep' zone.

Definitions. Frequent sampling is generally necessary to describe patterns of settlement. However, for *'Bathymodiolus' childressi*, the conspicuous reddish color of the larval shell (Arellano & Young 2009) starkly contrasts with the yellow dissoconch (adult shell) that forms immediately following metamorphosis (Bayne 1976), making new 'settlers' easily identifiable. Thus, throughout this study, settlers were defined as individuals up to 0.5 mm in length, which is the size of the larval shell of *'B.' childressi* at settlement (Gustafson et al. 1998, Arellano & Young 2009). Unless otherwise noted, the term 'juveniles' is applied to individuals from 0.5 to 10 mm long. This size category was chosen because these are the smallest sized juveniles we could physically manipulate and that were available in sufficient numbers for experimentation. In 2 experiments (second trial of predation and growth), we had to use individuals up to 15 or 20 mm long, respectively, due to the low supply of smaller juveniles during those months.

Size distribution and juvenile density. To determine the persistence of the size distribution and monitor recruitment across the BP mussel bed throughout the year, samples of *'Bathymodiolus' childressi* were taken from the BP in March 2002 (inner- and outer-seep zones only), October 2002 (inner-seep zone only), February 2003, September 2003, November 2003, July 2004, and August 2006 (inner-, middle-, and outer-seep zones). All samples were taken from the western side of the BP, except in July 2004 when additional samples were collected at the northern and eastern sides to determine if the observed patterns were consistent in other regions of the mussel bed. Mussel collections were made with the hydraulic clam-shell scoop of the 'Johnson-Sea-Link I' and 'II' (J-S-L) submersibles (Harbor Branch Oceanographic Institution) and 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 recov-

ery, we counted the mussels collected from each zone and measured the lengths (mm) of all individuals using vernier calipers. Preliminary measurements (October 2002) showed that length is strongly correlated with both width and height; thus, only length was used as a measure of size throughout this study (Fig. S1 in Supplement 2, available at www.int-res.com/articles/suppl/m414p131_supp.pdf, see also Gustafson et al. 1998). Length data are presented as percent-frequency histograms. Since length data were not normally distributed, size distributions between zones within each month were compared using Kolmogorov-Smirnov tests (SPSS 14.0 software). When the size distributions in the 2 zones did not differ significantly at $\alpha = 0.05$, they were combined for comparison to the third zone.

We compared the density of juvenile mussels (<10 mm long) across the BP mussel bed through quantitative sampling in November 2003 and August 2006. The samples taken from the inner- and outer-seep zones were from the most extreme edges of the zones still containing living mussels. The collections from the middle were approximately halfway between the 2 edges of the mussel bed. In the November 2003 samples, the scoop and suction tools on the 'J-S-L' submersible were used to collect all organisms within a 2500 cm² PVC quadrat. The scoop samples were placed in a hydraulically operated sealed acrylic box and were identified and counted upon recovery at the surface. Suction samples from the same quadrats were recovered in the acrylic buckets, capped with 1 mm nylon mesh. Because of space and dive constraints, only one 2500 cm² quadrat was sampled in each zone in November 2003 only. Smaller samples from each zone were collected on subsequent dives, and these were treated as replicates of the 2500 cm² quadrat collections in November 2003 (for a total of 3 replicate samples). These samples consisted of 2 adjacent scoops that were combined from each zone (total area = ~690 cm²), after first using suction to collect associated fauna and any small mussels within the area. In August 2006, 3 replicate samples were collected with the sub's scoop and suction alone (~690 cm²) from each of the 3 zones. Data were standardized to area for analysis and a 4th-root transformation was applied to normalize the data. The transformed density (no. m⁻²) of small mussels was compared across the 3 zones using a mixed-model 2-factor ANOVA (SPSS 14.0 software), with trial (collection date) as a random factor. Because the effect of trial was not significant at $\alpha = 0.25$ (Quinn & Keough 2002), trials were pooled and the data were reanalyzed using a 1-factor ANOVA, with zone as a fixed factor.

Larval supply. To determine the supply of *'Bathymodiolus' childressi* veligers to the inner-, outer-, and non-seep zones of the BP mussel bed, 3 replicate tube traps were placed in each zone alongside settlement experiments (see below). Tube traps were 30 cm tall

PVC pipes (5 cm diameter opening, aspect ratio = 6:1) that were mounted on 2 kg iron discs (dumbbell weights) and filled with 10% formalin buffered with seawater (Yund et al. 1991). We took 6 integrated current readings at the mouth of one tube trap in each zone with a flow meter (Marsh McBirney Model 2000) in February 2003 and detected no significant differences among zones ($F = 3.246$, p = 0.067), suggesting that capture rate should not vary among zones as a function of flow. Tube traps were deployed for 271 d, from February 12 to November 10, 2003 and again for 247 d from November 11, 2003 to July 15, 2004. After recovery, contents were transferred to 70% ethanol until the larvae of *'B.' childressi* were identified visually and counted; none of the larvae had begun forming the dissoconch, suggesting that none of the individuals that were counted had been resuspended from the benthos. Based on the density of juveniles across the mussel bed calculated here and the differential density of adult mussels across the bed (Smith et al. 2000), we expected the mean number of larvae captured per day to decrease from the inner- to the outer- to the non-seep zones ($M_{non} \leq M_{outer} \leq M_{inner}$). Thus, we analyzed each trial separately with a Jonckheere-Terpstra test for ordered alternatives (Daniel 1989).

Settlement. To evaluate spatial variation in settlement density across the 3 zones and recruitment on different substrata across the 3 zones, we placed 5 replicate settlement racks in the inner- and outer-seep zones and 3 in the non-seep zones of the BP mussel bed (13 racks total) on October 10, 2002 and recovered them on July 9 to 15, 2004. Settlement racks consisted of 4 settlement substrata that were placed in random order within caged (0.5 cm Vexar mesh) PVC racks ($90 \times 23 \times 4$ cm). Caging the racks was necessary to exclude potential predators and grazers and to hold settlement substrata, which included live mussels. Fluorescein injection around the cages at the time of deployment suggested that the cages did not alter flow on a gross scale. Settlement substrata within the racks were large (>90 mm length) mussel shells; large, living mussels; small (30 to 50 mm length) mussel shells; and small, living mussels. Mussels and shells that were used for the substratum treatments were collected from the BP and the size classes were chosen based on the range of sizes available. The numbers of mussels or shells that were used in each treatment were adjusted to maintain similar total surface areas between treatments. The surface areas of the various substratum treatments were calculated from mussel shell length using the following equation (D. C. Bergquist pers. comm., Bergquist et al. 2005): $SA = 0.5794 \times L^{2.06}$, where $SA =$ surface area (cm²) and $L =$ length (cm). The average total surface area of the settlement substrata in each treatment was 110 ± 14 cm² ($\pm SD$). The average surface area available for settlement on each settlement rack (=cage

plus plate substratum treatments) in the inner- and outer-seep zones was 0.39 m^2. The racks that were placed in the non-seep zone rested on bare sediment. Thus, the bottoms were not available for settlement, leaving an average potential settlement area of 0.30 m^2 on the non-seep treatments.

To determine whether there is an effect of zone (i.e. physico-chemical properties within the 3 zones) on total settlement, all settlers (<0.5 mm long) on the racks (cages plus substratum treatments) were summed to calculate the 'total settlement density' on each of the 3 replicate settlement racks in each zone. Settlement density (settlers m^{-2}) in each zone was analyzed with a 1-factor ANOVA, with zone as a fixed factor, followed by Tukey's HSD post-hoc test. In addition, to determine if the numbers of settlers reflect juvenile *'Bathymodiolus' childressi* density in each zone, we used a single-classification goodness-of-fit test and applied William's correction to reduce the chance of error resulting from comparing only 2 classes (Sokal & Rohlf 1981). Based on the mean juvenile density data presented in this paper (see 'Results: Size distribution and juvenile density'), we expected a settlement ratio of 6:1 in the inner:outer-seep zones if settlement alone could explain the observed size distribution pattern. We expected 0 settlement in the non-seep zone. Thus, we used the 6:1 ratio to generate expected values for the numbers of settlers in the inner- and outer-seep zones after scaling the total observed numbers of settlers to the available settlement surface area in the non-seep zone.

To determine whether substratum selection is an important determinant of settlement distribution, we intended to compare settler (<0.5 mm length) density on each substratum treatment (large mussel shells; large, living mussels; small mussel shells; and small, living mussels). However, due to low density of settlers on the substratum treatments, we could not analyze settlers, but instead counted recruits (up to 14 d old) in the analysis. We calculated age of recruits using our mean growth rates (see below and Table 2) and assumed initial lengths of 0.5 mm. Because there was no recruitment on some substratum treatments in the outer- and non-seep zones, we analyzed the data from only the inner-seep zone using a 2-factor randomized block ANOVA, with shell size (large or small) × state (live mussel or mussel shell) as fixed factors, and settlement rack as the block. The data were not normally distributed, but we chose to analyze these data using ANOVA anyway because this analysis is robust to violations of the normality assumption (Underwood 1981).

Growth. To assess variance in growth across the 3 zones, we measured and glued (with cyanoacrylate adhesive, 'super glue') 10 juveniles (<20 mm long) from the inner-seep zone of the BP mussel bed to small pieces of 1 mm mesh (plastic window screen) that were

fastened within cages of 0.5 cm Vexar mesh. In preliminary experiments, glued individuals began laying down byssal threads almost immediately and survived in the lab indefinitely. Cages were placed in the inner-, outer-, and non-seep zones on November 12, 2003 and recovered on July 9, 2004 (after 239 d). Due to limited availability of juvenile mussels, only one set of treatments was placed in each zone; hence, individuals were treated as replicates. Upon recovery, the surviving individuals were measured and the individual changes in length were noted. Changes in individual lengths were analyzed using a 1-way ANOVA, with zone (inner-, outer-, and non-seep) as a fixed factor (SPSS 14.0 software).

Juvenile survival and predation. This experiment was designed to determine juvenile survival across the 3 zones in the presence and in the absence of predators (the latter reflecting the variation in survival due to physiological intolerances associated with physico-chemical variations). We attached 15 young juveniles (<10 mm long) that were collected from the inner-seep zone of the BP mussel bed to sheets of 1 mm plastic mesh using cyanoacrylate adhesive and placed them in treatment cages (0.5 cm Vexar mesh, 13 × 13 × 8 cm^3), control cages (cages with parts of sides and tops open), and no cages. The glue was necessary to keep small mussels from escaping the cages and as a means for transplanting small, uncaged mussels. On October 10, 2002, three replicates of each treatment were placed in the inner-, outer-, and non-seep zones. Experiments were recovered on February 11, 2003 (after 124 d). The experiment was rerun from February 11 to September 15, 2003 (after 216 d). However, due to limited collections of small juvenile mussels for the second deployment, individuals that were <15 mm were used and only 2 replicates were placed in each zone.

If variation in juvenile survival due to physiological intolerances explained the size distribution pattern, we expected survival of transplanted juveniles to decrease from the inner- to the outer-seep zone and we expected no survival in the non-seep zone. Thus, we calculated the percent survival of individuals that were placed within the caged treatments only and analyzed each trial separately with 2 Jonckheere-Terpstra tests for ordered alternatives to test the hypothesis that $M_{non} \leq M_{outer} \leq M_{inner}$ (Daniel 1989).

To determine if predation differed among zones, the percentages of juvenile mussels surviving within the caged, uncaged, and cage control treatments were calculated and arcsine transformed for initial analysis with a 3-factor, mixed model ANOVA (zone × treatment × trial), using trial as a random factor (SPSS 14.0 software). Since the effect of trial was not significant at $\alpha = 0.25$ (Quinn & Keough 2002), trials were pooled and the data were reanalyzed with a 2-factor ANOVA.

Distributions of potential predators. To determine the distribution of potential predators at the BP, 3 replicate quantitative samples were collected in November 2003 and August 2006 from the inner-, middle-, and outer-seep zones of the BP mussel bed (see 'Materials and methods: Size distribution and juvenile density'). The mean densities (no. m^{-2}) for each of 3 potential predators, including unidentified polyclad flatworms, a buccinid snail (*Eosipho canetae*), and small galatheid crabs (*Munidopsis* sp.), and one potential 'bulldozer' (*Bathynerita naticoidea*, 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. naticoidea* among the zones were analyzed in separate 2-factor, mixed model ANOVAs, with zone as a fixed fac-

tor and collection month as a random factor (SPSS 14.0 software). If the effect of month was not significant at $\alpha = 0.25$, months were pooled (Quinn & Keough 2002) and the data were reanalyzed using a 1-factor ANOVA. Because we did not collect any *E. canetae* in August 2006, only the November 2003 data were analyzed using a 1-factor ANOVA.

RESULTS

Size distribution and juvenile density

A distinct difference in size distributions among the 3 zones of the BP mussel bed was evident throughout the sampling period (Fig. 2). Although the size distrib-
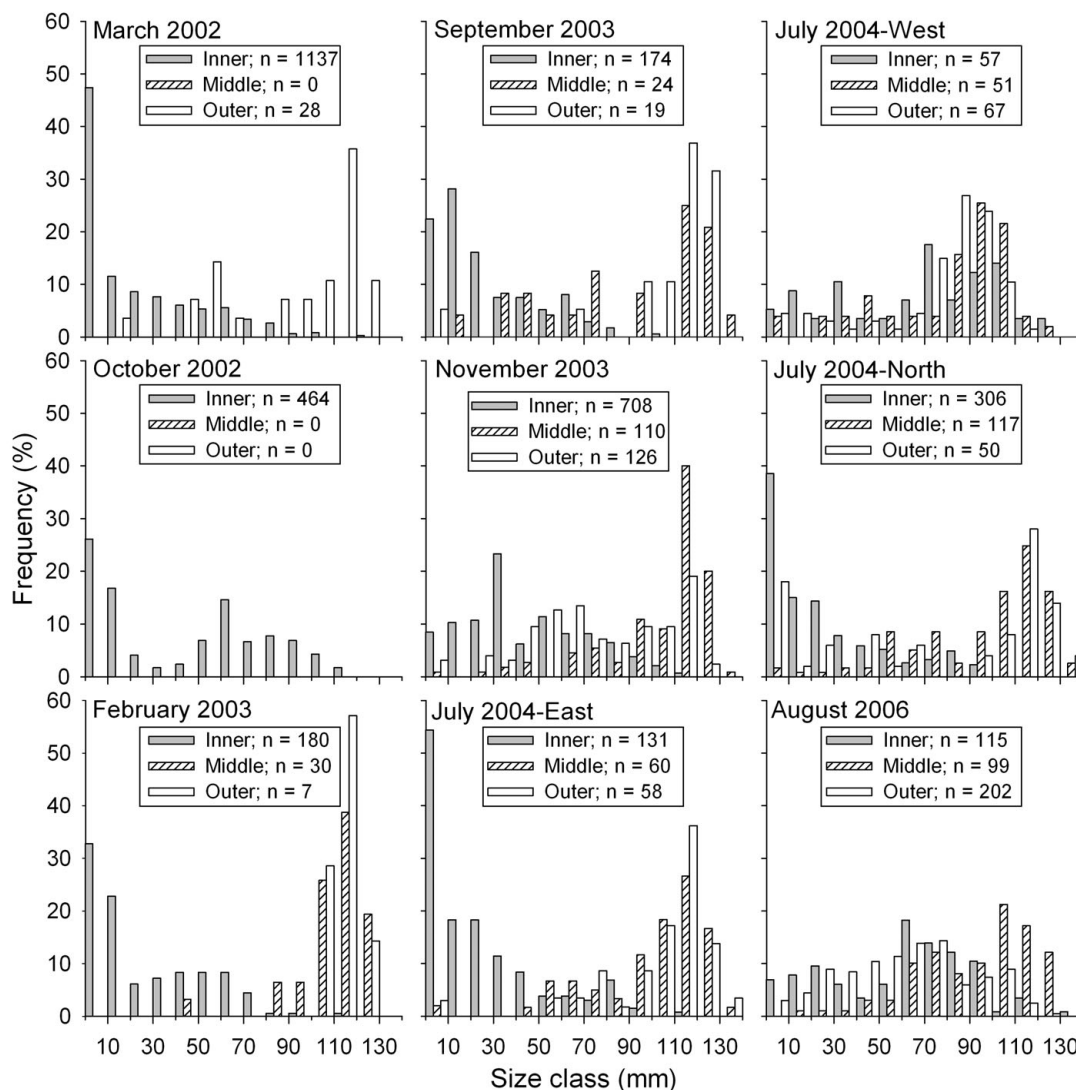

Fig. 2. *'Bathymodiolus' childressi*. Length frequencies (%) from different zones of the Brine Pool NR1 cold seep (BP) mussel bed in different months between 2002 and 2006. All samples were taken from the western side of the pool except those indicated in July 2004

utions of mussels in the middle- and outer-seep zones were not always significantly different from each other, the size distributions of mussels in the inner-seep zone samples were significantly different from the distributions in the other 2 zones in all samples except in August 2006 (Table 1).

Juveniles were always more abundant in the inner- than in the middle- or outer-seep zones (Fig. 1). The average densities of juveniles (no. m^{-2}) at the inner-, middle-, and outer-seep zones were 117.0 ± 159.2, 2.4 ± 5.9, and 18.9 ± 27.1 (±SD), respectively. Zone significantly affected the 4th-root transformed density of juveniles (F = 10.603, p = 0.001). Juvenile density was greater in the inner-seep zone than in both the middle- and outer-seep zones (Tukey's HSD: p = 0.001 and 0.049, respectively), but no significant difference was detected between the middle- and outer-seep zones (Tukey's HSD: p = 0.151).

Larval supply and settlement

We could not reject the null hypothesis that larval supply (nos. of veligers captured d^{-1}) was not ordered across the 3 zones for either trial (Fig. 3A; $J_{3,3,3}$ = 14, p = 0.50). Zone significantly affected total settlement density (F = 5.71, p = 0.022; Fig. 3B), but there was no significant difference in total settlement density between the inner- and outer-seep zones (Tukey's HSD: p = 0.097). Moreover, we rejected the hypothesis that total settlement in the inner-seep zone should be 6× greater than in the outer-seep zone to reflect juvenile mussel density (G_{adj} = 88.44, p < 0.001; Table S1 in Supplement 2). Instead, there were only about twice as many settlers in the inner- than in the outer-seep zone: mean settlement density (±S.D) was 162.2 ± 57.4 in the inner-seep and 84.2 ± 60.6 settlers m^{-2} in the outer-
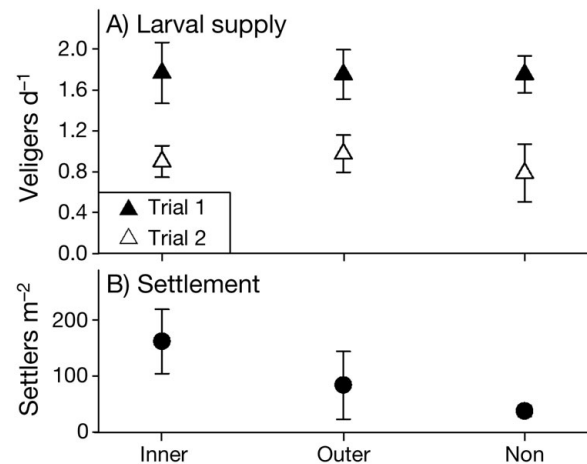

Fig. 3. *'Bathymodiolus' childressi*. Variations in larval supply and total settlement in the inner-, outer- and non-seep zones. (A) Mean number of veligers captured per day in tube traps (n = 3); Trials 1 and 2 were in place for 271 and 247 d, respectively. (B) Mean densities of settlers (m^{-2}) on the settlement racks (inner- and outer-seep, n = 5; non-seep, n = 3). Error bars: ±1 SD

seep zone. Although we expected 0 settlement in the non-seep zone, mean settlement density there was 38.0 ± 9.7 settlers m^{-2} (Fig. 3B).

Recruitment variation on different substrata

Although recruitment occurred in all 3 zones, we could not test for the interaction between zone and substratum size (large or small) or state (living mussel or empty shell) because we found recruits on all 4 substratum treatments only within the inner-seep zone (Fig. 4). Within the inner-seep zone, we were unable to detect a significant effect of substratum size (F = 0.100, p = 0.754), state (F = 2.390, p = 0.131), their interaction (F = 0.582, p = 0.450) or of blocking (F = 0.807, p = 0.642) on recruitment of 14 d old mussels (Fig. 4; Table S2 in Supplement 2).

Juvenile survival and growth

Survival of juvenile *'Bathymodiolus' childressi* that were transplanted from the inner-seep to the inner-, outer-, and non-seep zones was high in all experiments (>88.9% survived in all inner- and outer-seep zone treatments, and >77.8% survived in all non-seep zone treatments; Fig. 5). Percent survival of transplanted juveniles did not differ among zones for either trial (Trial 1: $J_{3,3,3}$ = 12, p > 0.50; Trial 2: $J_{2,2,2}$ = 5, p > 0.50).

Table 1. *'Bathymodiolus' childressi*. Results of pairwise Kolmogorov-Smirnov tests comparing the size distributions in the inner-, middle-, and outer-seep zones during each sampling period. When size distributions were not significantly different at α = 0.05, the middle- and outer-seep zones were pooled (mid/out) for comparison with the inner-seep zone. In March 2002, mussels were not collected from the middle-seep zone

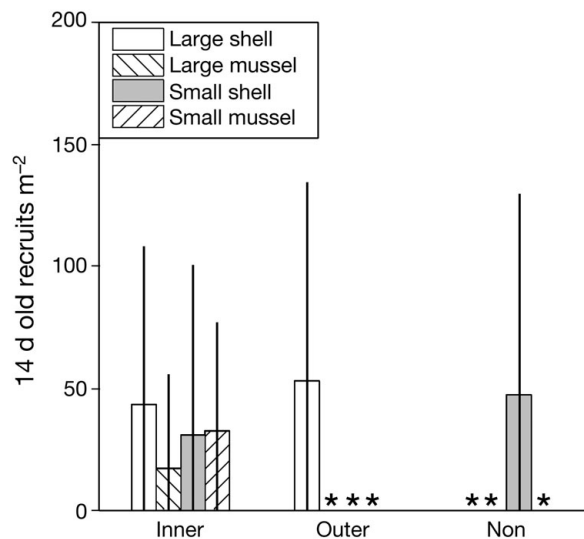| Sampling period	Zone 1	Zone 2	Z	p
March 2002	Inner	Outer	3.993	< 0.001
September 2003	Inner	Mid/Out	4.508	< 0.001
February 2003	Inner	Mid/Out	5.345	< 0.001
November 2003	Inner	Mid/Out	7.103	< 0.001
July 2004-West	Inner	Mid/Out	1.555	0.016
July 2004-North	Inner	Mid/Out	7.140	< 0.001
July 2004-East	Inner	Mid/Out	6.102	< 0.001
August 2006	Inner	Middle	3.471	< 0.001
	Inner	Outer	1.024	0.245
	Middle	Outer	3.714	< 0.001

Fig. 4. *'Bathymodiolus' childressi*. Up to 14 d old recruits m^{-2} on 4 substratum treatments placed within settlement racks in the inner-, outer-, and non-seep zones of the Brine Pool NR1 cold seep (BP) mussel bed. (∗) Treatments with 0 recruitment. Inner- and outer-seep zone, n = 5; non-seep zone, n = 3. Error bars: ±1 SD
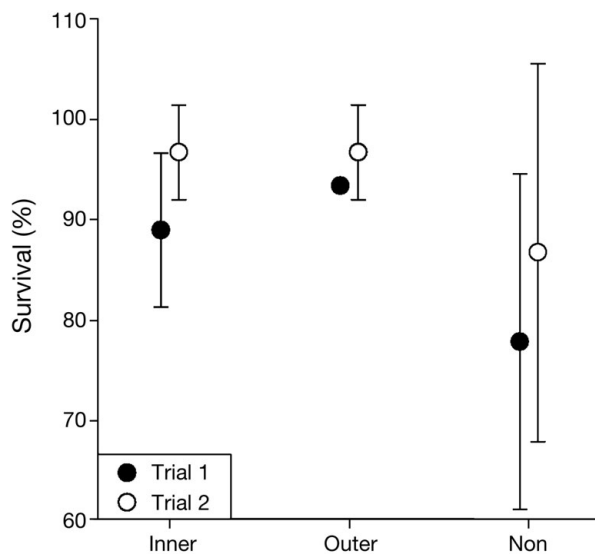

Fig. 5. *'Bathymodiolus' childressi*. Average percent survival of juveniles transplanted from the inner-seep to the other zones. Trials 1 (n = 3) and 2 (n = 2) were in place for 124 and 216 d, respectively. Error bars: ±1 SD

Table 2. *'Bathymodiolus' childressi*. Mean growth rate of juveniles placed within cages at the inner-, outer-, and non-seep zones of the Brine Pool NR1 cold seep (BP) mussel bed

Zone	n	Mean growth rate (mm 30 d^{-1} ± 1SD)
Inner-seep	6	1.44 ± 0.30
Outer-seep	6	0.52 ± 0.16
Non-seep	10	0.22 ± 0.20

Juvenile growth rate varied significantly with zone (F = 58.201, p < 0.001). Growth was greatest within the inner zone (Table 2). Smith et al. (2000) estimated that mussels within the 10 to 20 mm size class in the inner-seep zone grew at a rate of 1.4166 mm 30 d^{-1} (n = 1), which is within the range of the growth rates we determined for benthic juveniles in the inner-seep zone. At the outer-seep zone, however, Smith et al. (2000) predicted much higher mean growth rates of mussels under 10 mm long (0.833 ± 0.33 mm 30 d^{-1}; n = 2) than we show.

Predation on recruits

The caging treatment significantly affected the survival of juvenile mussels (F = 10.374, p < 0.001) (Fig. 6; Table S3 in Supplement 2). There was a significantly higher survival in caged than in uncaged treatments (Tukey's HSD: p < 0.001), but no difference between cage controls and uncaged or caged treatments (Tukey's HSD: p = 0.068 for uncaged vs. cage control, and p = 0.097 for caged vs. cage control). We did not detect a significant effect of zone on survival of juveniles in cages, cage controls, or without cages (F = 0.028, p = 0.972) (Fig. 6; Table S3). Although we expected a large zone effect if predation alone caused 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 alone accounted for only 0.2% of the overall (effect + error) variance (Table S3).
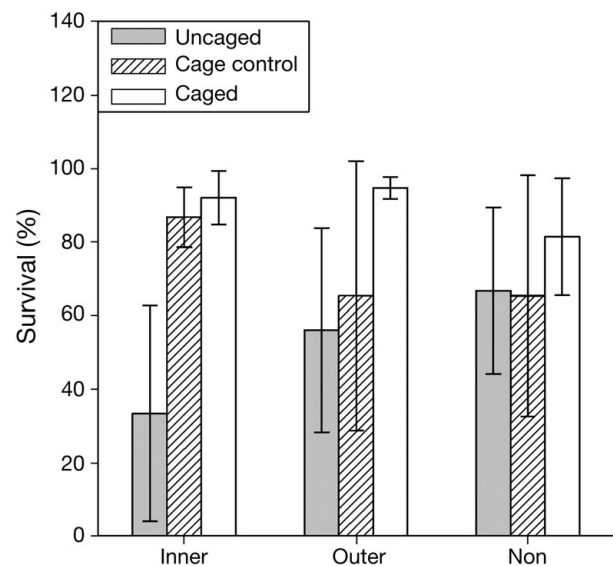

Fig. 6. *'Bathymodiolus' childressi*. Average percent survival of juveniles (<15 mm long) that were in uncaged, cage control, or caged treatments and placed at the inner-, outer-, and non-seep zones of the Brine Pool NR1 cold seep (BP) mussel bed (n = 5). Error bars: ±1 SD

We could not detect a significant difference in the square-root transformed densities across the zones for either the polyclad flatworms ($F = 0.161$, p = 0.853) or the buccinid gastropod *Eosipho canetae* ($F = 1.337$, p = 0.292) (Fig. 7A,B). Tests for differential densities of these 2 species were not powerful because mean densities were low, with large variances due to their absence in many samples. The square-root transformed densities of the galatheid crabs varied significantly across the BP mussel bed ($F = 15.73$, p < 0.001) (Fig. 7C). There were significantly fewer galatheids in the inner- than in the middle- or outer-seep zones (Tukey's HSD: p = 0.001), but the density of galatheids in the middle- and outer-seep zones did not differ from each other (Tukey's HSD: p = 0.812). There were also significantly fewer *Bathynerita naticoidea* in the inner- than in the middle- or outer-seep zones ($F = 22.049$, p < 0.001; Tukey's HSD: p < 0.001) of the BP mussel bed (Fig. 7D). We did not detect a significant difference in the square-root transformed densities of *B. naticoidea* between the middle- and outer-seep zones (Tukey's HSD: p = 0.578).

DISCUSSION

The high density of *'Bathymodiolus' childressi* juveniles adjacent to the brine pool has been noted repeatedly since the BP was first described (MacDonald et al. 1990a,b,c MacDonald & Fisher 1996, Smith et al. 2000). Smith et al. (2000) reports a density of ~3× more

Fig. 7. Mean densities (no. m^{-2}) of (A) polyclad flatworms, (B) *Eosipho canetae*, (C) galatheid crabs, and (D) *Bathynerita naticoidea* in the inner-, middle-, and outer-seep zones of the Brine Pool NR1 cold seep (BP) mussel bed. Error bars: ±1 SD, n = 3 for each

mussels in the inner- than in the outer-seep zone and we found ~6× more juveniles (<10 mm long) in the inner- than in the outer-seep zone. We also document the persistence of this distributional pattern year-round, throughout the sampling period, and around the pool (Fig. 2). We did not expect this pattern to persist throughout the year; instead, we expected to find seasonal recruitment pulses following the spawning period of *'B.' childressi* from October through February (Tyler et al. 2006, Arellano & Young 2009). Although we found large, mostly uni-modal peaks of the smallest juveniles in sampling months at the end of and just after the spawning season (February 2003, March 2002, and July 2004), the pattern of distribution of mussel sizes across the bed remained throughout the year (Fig. 2). Individual and interannual variability in gametogenesis combined with the extended spawning season, long larval durations (Arellano & Young 2009), and relatively slow growth rates probably account for the persistence of individuals <10 mm long within the inner-seep zone throughout the sampling period. Using our calculated mean growth rate of 1.44 ± 0.30 mm 30 d^{-1} (±SD), settlers would need >5 mo to grow out of the 0 to 10 mm size bin. Moreover, our inability to effectively sample mussels <5 mm long via the submersible should deflate the relative height of the 0 to 10 mm size bin, making peaks in recruitment following spawning less evident.

Nevertheless, the percentages of the smallest mussels were always conspicuously low in middle- and outer-seep zones (Fig. 2; Smith et al. 2000). If recruitment is so much lower in the middle and outer-seep zones, then how do median-sized mussels persist in the population in these zones? Below, we discuss the pre-settlement, settlement, and post-settlement factors that may contribute to the structuring of the spatial variation in mussel sizes across the BP mussel bed.

Pre-settlement and settlement

There was no significant variation in larval supply between the inner- and outer-seep zones, suggesting that supply of larvae alone is not sufficient to explain the variation in juvenile density across the mussel bed. Larval supply can often explain variability in settlement and recruitment patterns (Underwood & Keough 2001), and has been shown to correlate with recruitment 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 might explain the observed recruitment peaks along the northern and eastern sides of the brine pool, even though a recruitment pulse was absent at the western side of the pool (Fig. 2); however, little is known of the current patterns
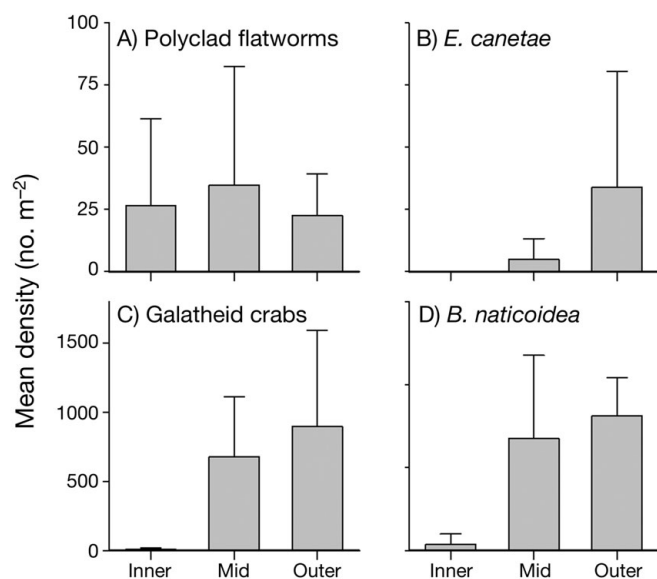
at the BP cold seep. A correlation between settlement and larval supply due to passive transport should be more evident at larger than at smaller spatial scales where behavior and cues play a more important role in determining settlement distribution (Underwood & Keough 2001). Indeed, the supply of larvae was similar at the scale of the width of the mussel bed, which ranges from 3 to 7 m but is ~3 m wide where sampling took place on the western side of the pool.

Instead, we expected substrate selection and variation in settlement cues to influence settlement distribution across the small scale of the mussel bed. Different settlement substrata, and presumably biogenic cues, are available in the various zones of the mussel bed. Small mussels are found primarily in the inner-seep zone, while large mussels are found in both zones (Fig. 1). In the outer-seep zone, empty mussel shell hashes are prevalent, but empty mussel shells are found only at the very edge of the pool in the inner-seep zone, where they are usually under the surface of the brine or covered in toxic hydrocarbons (see Fig. 1A). Thus, if the persistent size distribution of mussels at the BP is driven primarily by settlement substratum preferences, then we would expect highest settlement on living mussels regardless of size. However, we were unable to identify a substratum preference for settlement of larvae of *'Bathymodiolus' childressi* by analyzing recruitment of 14 d old recruits on the various substrata. Recruitment of individuals in this size class obviously did not directly reflect settlement, since we found an insufficient number of settlers on the substratum treatments for analysis. The caging probably altered flow on a small scale and may have led to the observed low settlement densities on the substratum treatments themselves. However, given the small spatial scales between treatments (~3 to 6 m) and low average benthic flow rates (on average, 2 cm s^{-1}; Pile & Young 1999), it is unlikely (albeit possible) that the cages would alter settlement on the substratum treatments differentially. Nevertheless, even when we presented an artificial substratum (settlement racks), there was no evidence of a zone effect on total settlement within the mussel bed. In other words, total settlement was not significantly higher in the inner-seep than in the outer-seep zone (Fig. 3B), which does not support the hypothesis that larvae preferentially settle in this zone due to chemical settlement cues such as methane or biogenic cues (MacDonald et al. 1990a,b,c, MacDonald & Fisher 1996). However, although the observed distribution of settlers did not fit the expected 6:1 distribution (Table S1), there was a trend of decreasing total settlement from the inner- to the outer-seep zone (Fig. 3B). Even with high spatial variation in settlement, it is possible that the

high density of juveniles in the inner-seep zone could result from the slightly higher settlement density accumulating over time or compounding with other factors that also slightly, albeit insignificantly, affect the size structure of the mussels across the bed.

Post-settlement factors

Neither differential survival associated with variations in the physico-chemical environment and growth of juvenile mussels (<10 mm long), nor predation appear to have caused the observed differences in size distributions of mussels across the BP mussel bed. Environmental variability has been correlated with variations in physiological condition of adult mussels at the brine pool; in general, mussels in the outer-seep zone are in poorer physiological condition than those in the inner-seep zone (Nix et al. 1995, Smith et al. 2000). However, juvenile mussels survived and grew in all 3 zones in the absence of predators. If growth causes the observed size distributions, we would expect the growth rate of small individuals in the inner-seep zone to be slow, leading to the persistence of small individuals, and the growth rate at the outer-seep zone to be fast, requiring frequent sampling to observe an abundance of small individuals. On the contrary, growth rate decreased away from the inner-seep zone. Similarly, Smith et al. (2000) found that mussels in the outer-seep zone grew slower, even though the concentration of methane available for assimilation by bacterial symbionts remains high in this zone. Low oxygen levels at the outer-seep zone probably limit methane oxidation rates, inhibiting growth (Smith et al. 2000). On the other hand, low diffusion of methane beyond the mussel bed probably accounts for the slowed growth of mussels that were placed in the non-seep zone. Although data on methane concentrations are not available in this zone, Smith et al. (2000) detected no methane but high oxygen in water samples that were taken just 1 m above the mussel bed.

Similarly, there was no differential predation on juveniles across the mussel bed. However, caging increased the survival of juveniles, suggesting potential predation, although we were unable to positively identify a predator of juvenile *'Bathymodiolus' childressi* mussels. There are 3 species at the BP cold seep that we considered to be likely predators on adult and juvenile mussels. Through isotopic analysis, MacAvoy et al. (2002) showed that the spider crab *Rochina crassa* collected on seep sites obtained from 8 to 48% of its nutrition within the cold seep community, while cold-seep colonists like the seastar *Sclerasterias tanneri* and the buccinid gastropod

Eosipho canetae each contained from 50 to 100 % chemosynthesis-based material. We attempted to obtain direct evidence of predation by these colonists through several shipboard predation experiments, but experiment durations were short and no predation was observed (Text S1 in Supplement 2). Because survival rates in cage controls were not significantly different from those in the inner-seep zone cages, we suspect that the potential predator was large enough to be excluded from the cage controls, suggesting that the spider crab and the seastar are the more likely predators than the gastropod. Because scoop collections cannot sample large mobile fauna, we made anecdotal observations on their distributions during dives and while viewing video footage. *R. crassa* was frequently observed around the fringes of the mussel bed, but never near the pool itself, while *S. tanneri* was observed in the outer- and middle-seep zones, but not in the inner-seep zone.

Alternative hypotheses

Because of the limitations of doing field manipulations using a submersible, not all post-settlement factors have been tested. For example, we suggest that secondary settlement, migration, or early post-settlement mortality could all potentially explain the observed size distribution of mussels at the BP cold seep.

Post-settlement migrations can lead to drastic differences between initial recruitment patterns and adult distributions (reviewed by Hunt & Scheibling 1997). For example, migrations of juvenile gooseneck barnacles (*Pollicipes polymerus*) down the adult peduncle can be tracked by following a gradient of juvenile sizes from their settlement site near the top of the adult peduncle to large juveniles around the base of the adult peduncle (Hoffman 1984). Similar migrations may explain the spatial pattern of the sizes of *'Bathymodiolus' childressi* across the BP mussel bed. For many shallow-water mytilid species, primary settlement of larvae is often followed by a period of byssus drifting of juveniles up to 2 mm long before secondary settlement of small juveniles onto an established mussel bed (Bayne 1964). Byssus drifting by juvenile mussels that have settled in the outer-seep zone of the BP mussel bed, which is physiologically less favorable for adult mussels (Smith et al. 2000), is a mechanism that may allow these settlers to relocate to a different part of the mussel bed or cold seep. In addition, larger mussels can move by depositing and releasing byssal threads. Mussels in the inner-seep zone are cantilevered over the brine, and are held in place by byssal

threads securing them to adjacent mussels lying on the sediment (Fig. S2 in Supplement 2). The density of mussels in the inner zone is ~3× higher than in the outer zone (Smith et al. 2000). The risk of sinking under the toxic brine may induce juveniles to move toward the middle- and outer-seep zones as they grow, thus explaining the persistence of peaks of mid-sized mussels in these zones.

Early post-settlement mortality may significantly alter distribution patterns and can be due to delayed metamorphosis, hydrodynamics, competition, physiological stress, predation, and biological disturbances (reviewed by Gosselin & Qian 1997, Hunt & Scheibling 1997). We have shown that juvenile mortality due to physiological tolerances or predation does not differ between the inner- and outer-seep zones. However, our survival and predation experiments were conducted using individuals that were 5 to 25 mm long for practical reasons. Thus, we cannot discount the possibility that the observed ecological patterns may be influenced by differential 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 well documented (reviewed by Gosselin & Qian 1997, Hunt & Scheibling 1997). The outer-seep zone is low in oxygen and can have extremely high hydrogen sulfide (Smith et al. 2000); whether very early *'Bathymodiolus' childressi* settlers can survive the extreme conditions in the outer-seep zone is still unknown.

Moreover, although we could not detect differential predation on juveniles across the bed, some predators may selectively prey on *'Bathymodiolus' childressi* settlers. For example, the buccinid gastropod *Eosipho canetae*, which derives 71 to 100 % of its nutrition from chemosynthetically based material at the BP (MacAvoy et al. 2002), was found only in the middle- and outer-seep zones (this study, Bergquist et al. 2005). Predation by gastropods is an important source of mortality of invertebrate recruits in intertidal habitats and there is evidence that some gastropod juveniles preferentially consume mussels as small as 1 to 2 mm long (reviewed by Hunt & Scheibling 1997). Likewise, predation by *E. canetae* may be a potential source of early post-settlement mortality of *'B.' childressi* in the middle- and outer-seep zones.

Another likely source of early post-settlement mortality is 'bulldozing' by grazers (reviewed by Hunt & Scheibling 1997), which has been suggested as a possible structuring factor in hydrothermal vent communities (Micheli et al. 2002, Mullineaux et al. 2003, Kelly et al. 2007). The neritid gastropod *Bathynerita naticoidea* is endemic to cold seeps and grazes

the bacterial film from the shells of *'Bathymodiolus'* *childressi*. We suggest that *B. naticoidea* may bulldoze recently settled *'B.' childressi* off the shells of adults while grazing. This study and others have shown that *B. naticoidea* resides primarily in the middle- and outer-seep zones of the BP mussel bed (this study, Bergquist et al. 2005, Van Gaest et al. 2007); thus, bull-dozing of early settlers by *B. naticoidea* would explain the lack of small mussels in the outer-seep zone, despite high settlement there. Certainly, impacts of this grazer and other biological interactions on the population structure of *'B.' childressi* warrant further investigation.

Colonization of and recruitment on cold-seep mussel beds

Observations on the population structure and growth rates of cold-seep mussels and tubeworms have led to the formation of hypotheses about seep col-onization (e.g. Nix et al. 1995, Bergquist et al. 2002, 2004). In particular, Nix et al. (1995) showed that adult *'Bathymodiolus' childressi* that were transplanted to bare sediment outside of their mussel bed grew more and were in better physiological condition than those remaining in the original bed, suggesting that site chemistry alone is insufficient for colonization. Perhaps the most striking result of our experiments was the high number of settlers in the non-seep zone when we supplied them with a settlement surface alone, provid-ing evidence that a hard substratum may be a neces-sity for *'B.' childressi* to initially colonize a seep site. However, we cannot discount seepage as a settlement cue in the 'non-seep' zone; our only evidence of the lack of methane seepage was the lack of seep fauna in this zone, as our attempts to obtain water chemistry data were unsuccessful. In fact, like Nix et al. (1995), a high percentage of juvenile mussels survived when we transplanted them to the non-seep zone, suggesting that either seepage or advection of methane in the area is adequate to sustain juvenile mussels, or that *'B.' chil-dressi* juveniles can sufficiently supplement their nutri-tion by filter feeding (Pile & Young 1999). However, water chemistry cues may not be the only important settlement inducers for these mussels. Biological cues such as microbial biofilms are known to induce settle-ment in mytilid mussels (Satuito et al. 1995). Whether biofilms are important settlement cues for cold-seep endemics is still unknown, but a microbial community would certainly have been present on the settlement racks even in the non-seep zone. Moreover, it is unknown whether *'B.' childressi* acquires its methan-otrophic symbionts before or after settlement, although most studies suggest that bathymodiolins acquire their

symbionts from the environment (Won et al. 2003, Salerno et al. 2005). Indeed, it is possible that a hard substratum even in the absence of a 'seep chemical cue' is sufficient for initial colonization by *'B.'childressi* larvae, with subsequent survival and establishment of a mussel bed being dependent on whether seepage is sufficient to sustain the free-living form of the sym-biont, which could then infect the *'B.' childressi* juve-niles.

CONCLUSION

This study exemplifies both the value of multiple hypothesis testing for teasing apart a complex ecologi-cal process in a system that is inherently difficult to work in, as well as the value of using manipulative field experiments to test specific hypotheses in deep-sea systems. This approach is particularly useful for testing hypotheses about settlement and recruitment when complimentary lab experiments on settlement preferences are impossible. Based on empirical field experiments, we reject the long-standing hypothesis that the larvae of *'Bathymodiolus' childressi* preferen-tially settle near the source of methane at the edge of the BP. Instead, we suspect that other biological inter-actions or habitat selection processes are playing a role in structuring the juvenile distribution of *'B.' childressi* at the BP. We have also eliminated differential sur-vival, growth, and predation of juveniles as structuring factors. Recognizing that recruitment dynamics are inherently complex and that interactive effects of slight variations in the factors tested cannot be dis-missed, we suggest that both migration (via secondary settlement or migration of later juveniles) and early post-settlement mortality may play strong roles in structuring the spatial distribution of juvenile *'B.' chil-dressi* mussels at the BP cold seep. Furthermore, as has been suggested for hydrothermal vent populations (Micheli et al. 2002, Mullineaux et al. 2003, Kelly et al. 2007), we hypothesize that post-settlement mortality may be a result of biotic interactions such as bulldozing of new settlers. Testing these hypotheses via manipu-lative field experiments should be feasible in the future.

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