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Characteristics of vesicomyid clams and their environment at the Blake Ridge cold seep, South Carolina, USA

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ABSTRACT: Spatial distributions and patchiness of dominant megafaunal invertebrates in deep-sea seep environments may indicate heterogeneities in the flux of reduced chemical compounds. At the Blake Ridge seep off South Carolina, USA, the invertebrate assemblage includes dense populations of live vesicomyid clams (an undescribed species) as well as extensive clam shell beds (i.e. dead clams). In the present study, we characterized clam parameters (density, size-frequency distribution, reproductive condition) in relation to sulfur chemistry (sulfide and sulfate concentrations and isotopic compositions, pyrite and elemental sulfur concentrations) and other sedimentary metrics (grain size, organic content). For clams >5 mm, clam density was highest where the total dissolved sulfide concentration at 10 cm depth (ΣH_2S_{10cm}) was 0.4 to 1.1 mmol l^{-1}; juvenile clams (<5 mm) were most dense where ΣH_2S_{10cm} was lowest. Clams were reproductively capable across a broad range of ΣH_2S_{10cm} (0.1 to 6.4 mmol l^{-1}), and females in the sampled populations displayed asynchronous gametogenesis. Sulfide concentrations in porewaters at the shell–sediment interface of cores from shell beds were high, 3.3 to 12.1 mmol l^{-1}, compared to <1 mmol l^{-1} sulfide concentrations at the clam–sediment interface in live clam beds. Concentration profiles for sulfide and sulfate in shell beds were typical of those expected where there is active microbial sulfate reduction. In clam beds, profiles of sulfide and sulfate concentrations were also consistent with rapid uptake of sulfide by the clams. Sulfate in shell beds was systematically enriched in ^{34}S relative to that in clam beds due to microbial fractionation during sulfate reduction, but in clam beds, sulfate $\delta^{34}S$ matched that of seawater (~20‰). Residual sulfide values in clam and shell beds were correspondingly depleted in ^{34}S. Based on porewater sulfide concentrations in shell beds at the time of sampling, we suggest that clam mortality may have been due to an abrupt increase in sulfide concentration and sulfide toxicity, but other alternatives cannot be eliminated.

KEY WORDS: Cold seep · Gas hydrate · Sulfur isotope · Fractionation · Flux indicators · Chemosynthesis · Bivalve reproduction · Gametogenesis

INTRODUCTION

Biological investigations in deep-sea chemosynthetic systems generally focus on how species manage to thrive and survive in these extreme environments. Vesicomyid clams are one of the conspicuous components of many chemosynthetic systems. They and other bivalves at vents and seeps host chemoautotrophic endosymbionts that constrain the bivalves to occupy environments with suitable fluxes of reduced compounds (e.g. sulfide, methane). Vent and seep bivalves typically occupy discrete habitats with sharp boundaries that are

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likely to be controlled more by physico-chemical conditions than by biological interactions (Van Dover 2000). Patchy distributions of vesicomyid clams within chemosynthetic environments have been linked to patchiness in the availability of reduced compounds for endosymbiotic microbial oxidation (e.g. Barry et al. 1997, Sahling et al. 2002, Levin et al. 2003) and to variations in requirements and tolerances of clam species to these compounds (Barry et al. 1997).

Little attention has been devoted to the causes of massive mortalities of clams evident at vents and seeps, although at vents common causes of mortality must be waning of hydrothermal activity or overrun by volcanic eruptions. As the flux of reduced compounds in a system becomes naturally diminished below the optimum concentration for growth, or is arrested altogether, the condition of the bivalves deteriorates, resulting in resorption of gonad, loss of symbionts (Raulfs et al. 2004) and, ultimately, if flow is not restored, death (Hessler et al. 1988). Extensive beds of mussel shells and clam shells have been mapped along mid-ocean ridges where hydrothermal venting has ceased (e.g. Haymon et al. 1991), and the condition of mussels transplanted from areas of high to low sulfide flux have a lower tissue dry-weight to shell-length ratio than the reciprocal transplants (Smith 1985).

Massive bivalve mortality at seeps has also been attributed to cessation of fluid flux (e.g. Jollivet et al. 1990, Van Dover et al. 2003). But at seep sites, where the causes and timing of changes in fluid flux are not well understood nor are as dramatic as the shutdown of hydrothermal activity, alternative explanations for mortality cannot be excluded without geochemical assessment. For example, bivalve mortality might occur in beds receiving excess flux of reduced chemicals. Sulfide in particular is a potent toxin that interferes with the cytochrome *c* oxidase enzyme system of cellular respiration (National Research Council Committee on Medical and Biological Effects of Environmental Pollutants 1979), and enhanced flux of sulfide has been implicated in the mortality of at least some vesicomyid clam species (Goffredi & Barry 2002). A further cautionary note regarding inferences about causes of mortality was highlighted in recent work by Ward et al. (2004), who described a pathogenic, viral-like inclusion in mussels *Bathymodiolus heckerae* from the methane-hydrate seep on the Blake Ridge Diapir, where extensive mussel shell beds were found adjacent to live mussel beds.

In addition to live mussel beds and dead mussel shell beds, the Blake Ridge Diapir site is characterized by live clam beds (<1 m diameter) and extensive (up to 10 m long, <1 m wide) beds of clam shells. The clam, referred to as *Vesicomya* cf. *venusta* by Van Dover et al. (2003), is now considered likely to belong to an undescribed genus and species (E. Krylova pers. comm.). As in other vesicomyid clams, the digestive tract and filter-feeding apparatus are reduced in the Blake Ridge seep species. Microscopic and isotopic evidence indicates that the gills of Blake Ridge clams contain sulfide-oxidizing bacteria, from which the clams are inferred to derive most of their nutrition (Van Dover et al. 2003).

Clam shells in Blake Ridge shell beds were relatively uniform in size (~1 to 2 cm length) and in the limited extent of erosion of the periostracum and shell, suggesting that die-off was synchronous and pandemic (Van Dover et al. 2003). Unlike in Blake Ridge mussels, parasite burdens in Blake Ridge clams were light, and there was no evidence of tissue pathology (Mills et al. 2005). Predation is a common cause of clam mortality in shallow water, but clam shells collected from Blake Ridge shell beds did not show evidence of mortality due to crab, octopus, or fish predation (Van Dover et al. 2003).

In this study, we explored the relationship between sediment characteristics (sulfur chemistry, grain size, organic carbon and total nitrogen content) and clam characteristics (density, size, reproductive condition) in clam beds, shell beds, and background habitats in the Blake Ridge area to determine the optimal environmental characteristics for the clams and to gather evidence for the possibility that enhanced or diminished sulfide flux might have contributed to clam mortality or compromised tissue quality.

MATERIALS AND METHODS

Study site. The Blake Ridge cold seep (32° 29.623′ N, 76° 11.467′ W; 2155 m) is a soft-sediment, chemosynthetically based ecosystem that lies at the summit of a salt diapir ~300 km off the coast of South Carolina (Fig. 1). The site lies within an area of the South Atlantic Bight that represents a major gas hydrate province within the US Exclusive Economic Zone (Paull & Dillon 1981). Methane is the predominant gas in the hydrates and is generated through bacterially mediated reactions (Paull et al. 2000). The seep site was drilled by the Ocean Drilling Program in 1996 (Leg 164; Shipboard Scientific Party 1996), and the first submersible observations of the site were made in 2001. A description of the site, including attributes of the associated community, is provided in Van Dover et al. (2003).

Sampling and initial core processing. Fifty-three push cores (6.35 cm inner diameter, 30 cm length; penetration depths: 11 to 28 cm) were collected from 4 microhabitat types (clam beds: n = 20; shell beds: n = 19; clam/shell beds: n = 6; background: n = 8) at the Blake Ridge Diapir in 2003 using the deep-sea sub-

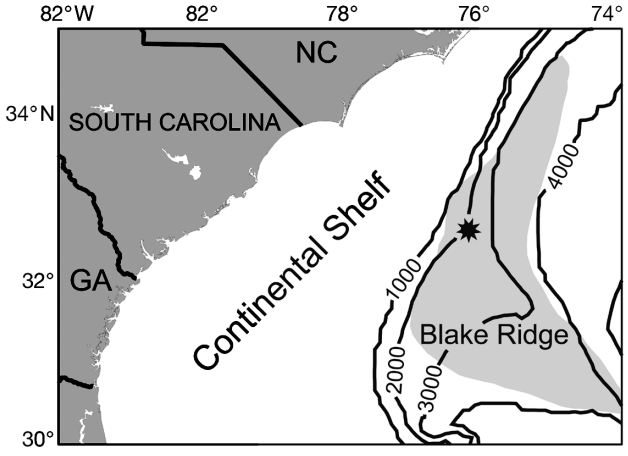

Fig. 1. Blake Ridge (light grey shaded area) and seep site (*). Isobaths (m)

mersible 'Alvin'. Microhabitat designations for push-cores were based on submersible characterization of the seabed and shipboard visual examination of intact push-core samples, without reference to the porewater chemistry. Clam beds contained large numbers of mature clams on the sediment surface and few or no clam shells (Fig. 2A); shell beds contained a thick layer of shells (Fig. 2B) and 0 to 1 live clams per push core; push cores from clam/shell beds contained 5 to 10 live clams plus shells. Five of the background samples were taken at the erosional face of the Blake Ridge depression, ~2 km NE of the active seep site; 3 background cores were taken within 200 m of active seeps at the Blake Ridge Diapir, in areas devoid of vesicomyid shells and megafaunal organisms. Clam densities (for clams visible without the aid of a dissecting microscope, i.e. ~5 mm length) were determined for all cores.

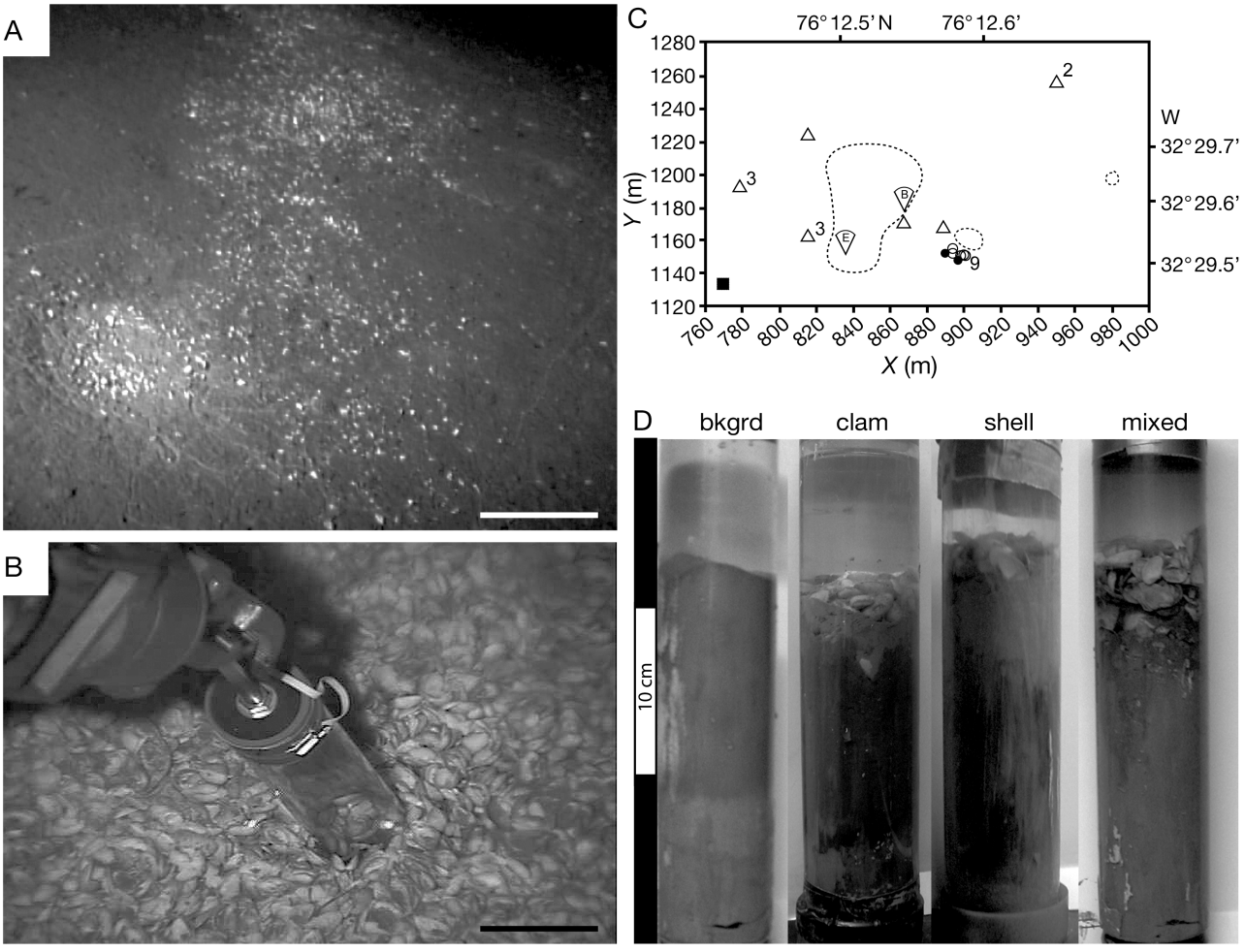

Fig. 2. Vesicomyid clams at the Blake Ridge seep site. (A) Clam and shell beds; scale bar ≈50 cm. (B) Push core sampling in a clam shell bed; scale bar = 10 cm. (C) Relative location of push cores analyzed for porewater chemistry, grain size, organic C and total N content, and reproductive condition of adult clams. ■: background; ○: clam beds; ●: clam/shell beds; △: shell beds. Additional background cores were collected ~2 km NE of the clam beds. Numbers indicate number of closely spaced cores; letters indicate markers at the site; areas enclosed by dashed lines indicate approximate distribution of mussel beds in 2001 (from Van Dover et al. 2003). (D) Representative push cores from 4 microhabitats at Blake Ridge

Cores were stored in a cold room (4°C) on recovery and vertical redox potential (Eh in mV) profiles (1 cm intervals) were obtained from undisturbed cores by inserting a UNISENSE platinum Eh electrode (0.5 mm diameter; 3 mm length) through the sediment surface. Redox potential was read on a portable pH-millivolt meter (Denver Instruments) connected to a saturated calomel electrode suspended in the overlying water. Values were corrected to the hydrogen reference electrode scale by adding +244 mV to each measurement (Bagander & Niemisto 1978). Calibration of the electrodes was verified by measuring the redox potential of quinhydrone dissolved in buffers (pH 4 and 7; Bohn 1971).

Geochemical and physical analyses. After the Eh profiles were taken, representative push cores from each microhabitat (9 from clam beds, 2 from clam/shell beds, 10 from shell beds, and 6 from background sediments: 1 from within 120 m of the sampled clam beds; 4 from ~2 km to the NE; Fig. 2C), were sub-sampled for chemical analyses (sulfide and sulfate concentration) of the porewater, grain-size analyses, and organic carbon and total nitrogen (i.e. ammonia and organic nitrogen) content. Each core was sectioned into 2 cm intervals under nitrogen. For push cores with layers of live clams and/or clam shells at the sediment–water interface, the '0' index was the surface of the sediment layer underlying the shells. Porewater was squeezed from core sections using Reeburgh-style squeezers (Reeburgh 1967) and collected in 7 ml vacutainers purged with nitrogen and pretreated with 0.3 ml of saturated zinc acetate solution.

Sediment samples (1 cm^3) from the 1 cm and 8 cm intervals of push cores after porewater extraction were analyzed for mineral sulfide: acid volatile sulfide (AVS: particulate FeS and residual dissolved H_2S) and chromium reduced sulfide (CRS: particulate FeS_2 and elemental sulfur). A 1 N HCl extraction was used for acid volatile sulfide; a boiling chromium concentrated acid extraction was used for chromium reduced sulfide (Fossing & Jørgensen 1989). Both extractions were followed by sulfide analysis (Cline 1969). To compare the relative abundance of dissolved sulfide (ΣH_2S) and mineral sulfide in push cores, ΣH_2S values were averaged for all intervals within each core and concentrations of mineral sulfides were averaged across the 2 sample intervals for each core.

Sediment sub-samples for grain-size analyses were taken from the 0 to 1 cm interval after extraction of porewater. Sediments were dried and homogenized and % sand, silt, and clay (± SD) were determined by sieving and gravimetric techniques. Dried, homogenized sediment from the 0 to 1 cm interval (~15 mg acidified with 10% HCl) was analyzed for total organic C and total N content using an EA 1108 Elemental Analyzer.

Porewater sulfide concentration was determined colorimetrically (Cline 1969) and sulfate concentration was determined turbidimetrically (Gieskes et al. 1991). Analytical precision was typically within ± 5% for both methods. Where sulfate concentrations were high (~30 mmol l^{-1}), analytical precision dropped to ±15%.

To determine the sulfur isotopic profiles of porewater H_2S and SO_4, sulfur isotope composition of dissolved H_2S and SO_4 was measured using a high-temperature combustion method consisting of an elemental analyzer (EA) coupled under continuous flow with an Optima stable isotope ratio mass spectrometer (GV Instruments, Manchester, UK). Sulfur isotope compositions are reported in the standard delta notation (δ) as per mil (‰) deviations from the international sulfur isotope standard Canyon Diablo Troilite (CDT), according to the equation:

$$\delta^{34}S = \left(\frac{^{34}S/^{32}S_{sample}}{^{34}S/^{32}S_{standard}} - 1 \right) \times 10^3 \quad (1)$$

A laboratory sulfur reference gas standard was calibrated against NBS-127. Analytical precision for internal sulfur combustion standards was better than ±0.5‰.

We also estimated the isotopic fractionation factor between sulfate and sulfide. Microbial sulfate reduction preferentially breaks the weaker bonds formed by ^{32}S over those formed by ^{34}S. Consumption of sulfate during the production of sulfide results in porewater that is isotopically enriched in the residual sulfate and depleted in the produced sulfide. If one assumes a nearly closed system with respect to available sulfate, the isotopic fractionation factor (ε) between the reactant sulfate and product sulfide for down-core variations in porewater sulfate can be modeled according to $\varepsilon = \delta^{34}SO_4^{2-}/\ln f$, where f is the fraction of sulfate remaining in reaction and ε is the slope of the regression between $\delta^{34}SO_4^{2-}$ and ln$f$ (Mariotti et al. 1981).

Sulfate reduction rates were calculated from sulfate porewater profiles using a porewater diffusion model (Jørgensen 1978). This model uses an exponential equation with the parameters of diffusion and sedimentation rate to evaluate changes in sulfate concentration with depth, according to the equation, $f(x) = ae^{-bx}$, where x is depth, and a and b are constants determined by the best fit line to the down-core porewater sulfate profile. In the absence of evidence to the contrary, we assume that fluid advection rates are negligible. The diffusion coefficient for sulfate given the porosity and temperature for Blake Ridge surficial sediments is estimated to be 93 $cm^2\ yr^{-1}$ (Dickens 2001) and the sedimentation rate is 0.48 mm yr^{-1} (Shipboard Scientific Party 1996).

Biological analyses. The top sections of cores containing clams and/or shells were washed through a 65 µm sieve. All large clams (>5 mm length; includ-

ing shells) and shells were removed, measured (shell length, ±0.1 mm), and live clams were counted. Material retained on the sieve was preserved in 10% buffered formalin, stored in 70% ethanol, and subsequently sorted under a dissecting microscope to collect and count the smallest clams within a subset of push cores (clam beds: n = 8; shell beds: n = 7; clam/shell beds: n = 2; background: n = 8).

Subsets of 37 female and 24 male adult individuals were selected for histology from among the largest clams (11 to 25 mm shell length) within push cores analyzed for chemical and physical parameters. Clam tissues were fixed in Davidson's solution for 24 h, and then stored in 70% ethanol. Serial sections through several entire clams of both sexes were examined to determine an optimal standard section for analysis. Tissue slices taken from the mid-body region, where the gonad was found to be concentrated, were dehydrated through a graded ethanol series, and embedded in paraffin. Transverse sections (6 µm) were stained with Gill's hematoxylin and eosin (H&E) for assessment of reproductive condition.

Gonadal tissues were analyzed under light microscopy with a compound microscope (females) or a dissecting microscope (males). Oocyte feret diameter (the theoretical diameter of an object if it were spherical in shape) in females and areas of somatic and reproductive tissues in males were determined from digital photographs taken with a Diagnostic Instruments Spot camera, using Sigma Scan Pro 2.0 software. Oocyte characteristics were analyzed in 3 histological sections from each female; within an individual, sections were systematically selected from the mid-dorsal region (i.e. the region of greatest gonadal development, based on study of serial sections of this species; T. P. Heyl unpubl.) and were separated by 200 µm. All previtellogenic (Stage 1), early vitellogenic (Stage 2), and late vitellogenic (Stage 3) oocytes with the nucleus and nucleolus evident on each section (i.e. 50 to 150 oocytes ind.$^{-1}$) were staged, counted, and measured. From these data, mean oocyte diameter and volume, oocyte stage, and oocyte size-frequency distributions were determined. A gonadal index (modified from Kennedy 1977, Eversole 1980) was used to rank different developmental stages of gametogenesis within an individual according to whether they were inactive, early developing, developing, ripe, or spent. Somatic and reproductive areas in males were measured from single histological sections from the mid-dorsal body region and were used to calculate the relative proportion of reproductive to somatic tissue (referred to herein as % gonad).

Statistical analyses. Data collected from individual push cores for percentage composition of sand, silt and clay, and organic carbon and total nitrogen were square-root transformed and analyzed using one-way ANOVAs and Tukey's post-hoc analysis (MINITAB) to investigate differences among the 4 microhabitats sampled.

Differences in mean and maximum oocyte diameter, oocyte volume, density of oocytes, oocyte stage, and gonad size in males were analyzed statistically using a stratified ANOVA (2 error terms were tested — 'within' clam and 'among' clam variance) as part of the R-software system (Ihaka & Gentleman 1996). The 'among' clam strata tested for correlations between reproductive characteristics and sulfide concentration in all push cores.

RESULTS

Sediment characterization by habitat

Eh, sulfide and sulfate concentration, and isotopic profiles for all push cores are provided in on-line appendices at www.int-res.com/articles/suppl/m339p169_app.pdf.

Sediments in push cores from the 4 different microhabitats (background, clam beds, clam/shell beds and shell beds) were visually (Fig. 2D) and chemically different. Layers of light brown or tan sediments in push cores from background sites suggested oxidizing conditions, which was confirmed by Eh values between 0 and +200 mV throughout the cores (Fig. 3A). Sulfide concentration in porewater of background sediments was low, ranging from 0.7 to 19.3 µmol l^{-1} (mean ± SD: 3.5 ± 4.28 µmol l^{-1}); sulfide concentration >10 µmol l^{-1} was restricted to sediments below 7 cm. Porewater sulfate profiles showed little variability with depth but varied among cores. The 4 background cores collected from the Blake Ridge Depression 125 km southeast of the Blake Ridge Diapir had sulfate concentrations of 23 to 33 mmol l^{-1}, near that of seawater (29 mmol l^{-1}). Two background cores were collected at the seep but distal to any evidence of methane seepage (Alvin 3909-22 and 3909-24). These cores had sulfate concentrations on the order of 14 mmol l^{-1}, approximately half that of seawater.

Push cores from live vesicomyid clam beds had 2 to 4 cm thick layers of clams (Fig. 2C). Clams were stacked on one another, with no consistent orientation. Posterior margins were not necessarily oriented vertically into the water column and exposed clam surfaces were free of any covering of sediment. Clam density (clams >5 mm shell length) per push core ranged from 11 to 48 ind. Clam shells were present but rare in the clam-bed push cores. Sediment immediately beneath the clams (0 to ~2 cm interval) was light-brown, followed by a steep color gradation to dark brown-black.

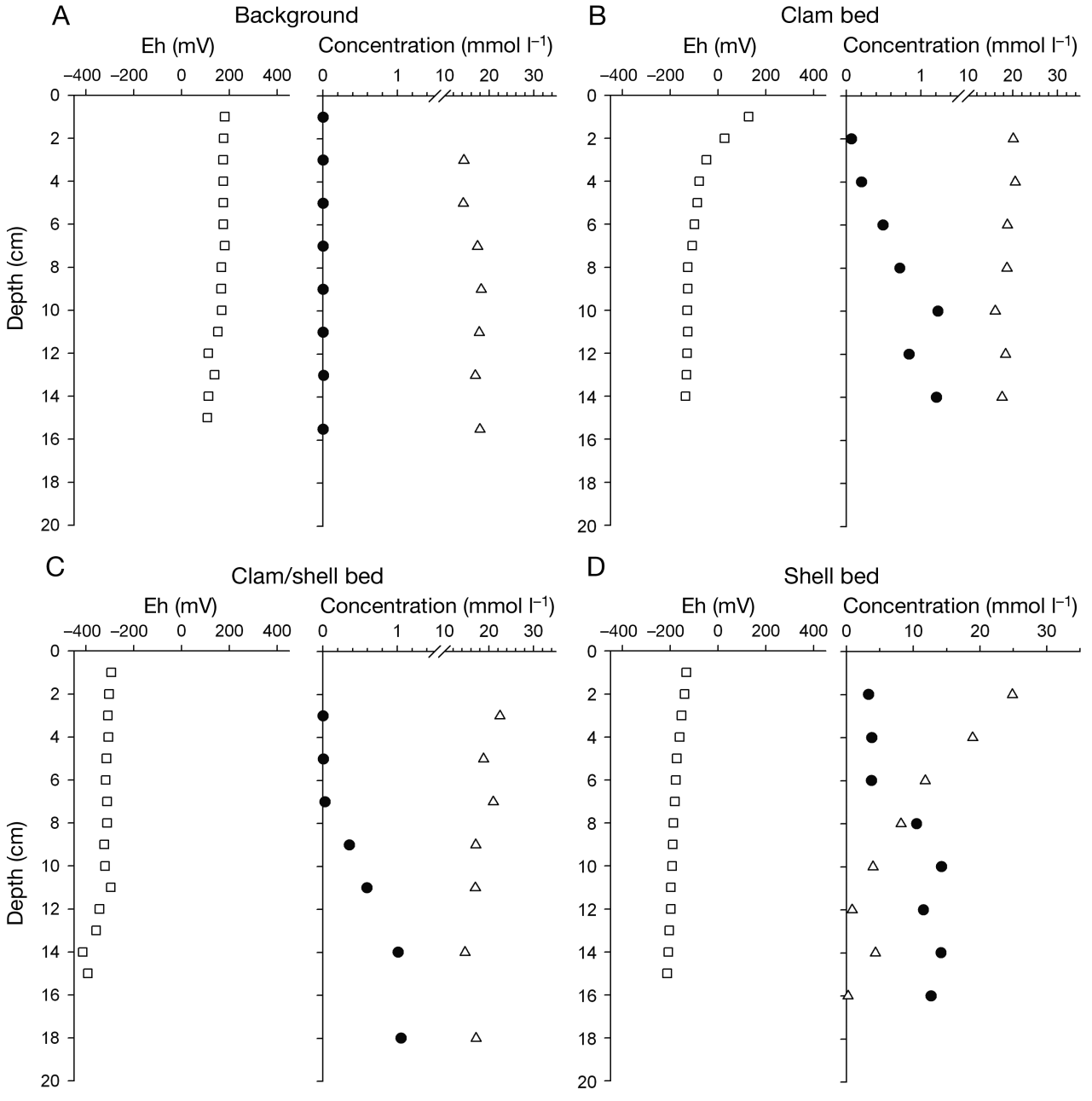

Fig. 3. Redox potential profiles (□) (Eh [mV]) and sulfide (●) and sulfate (△) concentrations (mmol l^{-1}) in representative push cores from 4 microhabitats at the Blake Ridge seep. (A) Background, Alvin 3909-22. (B) Clam bed, Alvin 3910-7. (C) Clam/shell bed, Alvin 3912-19. (D) Shell bed, Alvin 3909-5

A typical Eh profile for push cores from clam beds included a 0 to 2 cm oxidizing layer, with reducing conditions below 2 cm (Fig. 3B). Three of the 20 push cores from clam beds were completely reducing throughout the sediment profile. Sulfide concentrations in porewater below 3 cm ranged from 0.01 to 3.6 mmol l^{-1} (1.4 ± 1.95 mmol l^{-1}), except in core 3910-1, where porewater sulfide concentration was as high as 8.8 mmol l^{-1} at 18 cm and 1.3 mmol l^{-1} at the sediment–seawater interface. The depth at which sulfide was maximum in clam-bed push cores was always at or below 10 cm. Sulfate concentration ranged from 10 to 33 mmol l^{-1} (16.0 ± 5.70 mmol l^{-1}) and typically varied by only a few mmol l^{-1} with depth within a given core. Sulfide and sulfate concentrations in core 3910-1 did not match this pattern; instead, sulfate concentration decreased as sulfide concentration increased with depth into the core.

Push cores from mixed beds of shells and clams contained mostly shells but included from 5 to 10 clams (>5 mm length). The shells formed layers up to 4 cm deep on the sediment surface. The sediment was light brown to ~8 cm beneath the shells before turning dark brown to black. Eh profiles indicated that the reducing zone began within 1 cm below the shell layer (Fig. 3C). Porewater sulfide concentration ranged from 0 to 1.1 mmol l^{-1} (0.3 ± 0.39 mmol l^{-1}). Sulfate concentration ranged from 14 to 23 mmol l^{-1} (18.7 ± 2.71 mmol l^{-1}).

Of 19 push cores from shell beds, 16 contained only shells, 3 contained 1 live clam (>5 mm) each; of 7 shell-bed push cores examined for juvenile clams, 1 push core (3909-20) contained 13 ind. and 2 push cores contained 1 juvenile. Shell layers in push cores from shell-beds were dusted with sediment at the surface and were up to 8 cm thick, with broken shells mixed with sediment at depth. The periostracal layer of the shells were abraded in patches extending from the umbo region to the edge of the shell, but the shells on the surface of the sediment were not markedly dissolved, nor did they show signs of predation. Shell-bed sediments were characteristically dark, with the exception of one anomalous core, 3909-10, discussed separately below. Eh profiles indicated the sediments were reducing throughout cores from shell beds (Fig. 3D). Sulfide concentration was high (3.3 to 12.1 mmol l^{-1}) in the surface sediment layers (≤4 cm) in 7 of 10 cores from shell beds. For these 7 cores, sulfide concentrations in porewater just below the shell layer were about 4× greater than concentrations observed at the same level in clam beds. Sulfate concentrations in cores from shell beds decreased from the surface to ~5 cm and sulfide concentrations increased from the surface to ~5 cm. Porewater sulfide concentration below the sulfate reduction zone in shell-bed sediments was an order of magnitude higher than in any other push core sediments, up to 17.5 mmol l^{-1}. Sulfate concentration below the sulfate reduction zone in push-core sediments from shell beds was typically <1 mmol l^{-1}, i.e. an order of magnitude lower than those observed from other sample sites. Push core 3909-10 did not contain live clams; this anomalous core had sediment coloration and porewater chemistry profiles characteristic of cores from background sediments. Push core 3909-20, with 13 juveniles, had Eh, sulfide, and sulfate profiles similar to those in cores from clam beds. Push core 3910-12 had an anomalously low sulfide concentration at the top of the sediment.

Mineral sulfides (FeS and FeS_2) were detected in samples from all microhabitats where dissolved sulfide was detectable (i.e. clam beds, clam/shell beds, shell beds). There was no significant difference in mineral sulfide concentration among these microhabitat types (ANOVA, $p > 0.05$; Fig. 4A).

With 1 mole of sulfide produced for every mole of sulfate consumed during the process of dissimilatory sulfate reduction, the stoichiometric relationship between sulfide and sulfate concentration for each push-core interval provides an index of the degree to which sediments retain dissolved sulfide. The slope of the regression line between sulfate and sulfide concentration data below the clam beds was −0.061 with an R^2 value of 0.019 (Fig. 4B). One core from a clam bed (3910-1, containing 20 adult clams) falls along the regression line for shell beds. A deficit of 94% of the sulfide produced from sulfate reduction existed in the dissolved sulfur species in sediments below clam beds. Sulfide retention below shell beds is considerably greater, with a slope of −0.45 and R^2 of 0.45 ($p < 0.0001$). As a result, approximately 55% of dissolved sulfide is thought to have been retained in the sediment porewater.

Sulfate and sulfide isotopic profiles for sedimentary porewater reveal different patterns of isotopic fractionation below clam beds and shell beds (Fig. 5). At the surface of clam-bed sediments, $\delta^{34}S_{SO4}$ was ~20.2‰, similar to that of seawater sulfate values ($\delta^{34}S_{SO4}$ = 20.5 ± 0.1‰, n = 4), and became slightly but systematically enriched in ^{34}S with depth, to a maximum of 21.3‰. Sulfide isotopic values ($\delta^{34}S_{H2S}$) in cores from clam beds ranged from 4.3 to 8.0‰ and were thus depleted in ^{34}S relative to porewater sulfate in clam beds. In shell-bed sediments, $\delta^{34}S_{SO4}$ ranged from 4.3 to 8.0‰ and were depleted ^{34}S relative to porewater sulfate in clam beds. Sulfide isotopic values in shell-bed cores ($\delta^{34}S_{SO4}$ = −5.5 to −1.1) were markedly depleted in ^{34}S (Fig. 5A).

Throughout all of the pushcores analyzed, the isotopic composition of sulfide in clam beds ranged from −18.8 to 16.2‰ and was distributed around a mean $\delta^{34}S_{sulfide}$ value of −1.3 ± 1.4‰ (Fig. 5B) . $\delta^{34}S_{sulfide}$ values from shell-bed cores ranged from −12.1 to 21.0‰ and was characterized by 2 modes: −6.6 ± 1.0‰ and 17.8 ± 0.6‰ (Fig. 5B).

Isotopic enrichment factors observed in clam beds were fairly uniform (average $\varepsilon_{clambed}$ = 10.3 ± 0.9‰), with the exception of one outlier (ε_{clam} = 6‰). Enrichment factors below shell beds varied from 0.9 to 30.4‰, with an average $\varepsilon_{shellbed}$ = 16.1 ± 4.6‰. Shell-bed fractionation factors grouped according to the 2 modes of ^{34}S-enriched sulfides (where $\varepsilon_{shellbed\text{-}enriched}$ = 11.3 ± 5.0‰) and ^{34}S-depleted sulfides ($\varepsilon_{shellbed\text{-}depleted}$ = 25.8‰).

Sulfate reduction rates in clam beds ranged from 0.016 to 0.24 µmol SO_4^{2-} cm^{-2} d^{-1} and averaged 0.091 ± 0.038 µmol SO_4^{2-} cm^{-2} d^{-1} (n = 6). Calculated sulfate reduction rates for shell beds varied from 0.0029 to 0.92 µmol SO_4^{2-} cm^{-2} d^{-1}, with an average consumption rate of 0.27 ± 0.14 µmol SO_4^{2-} cm^{-2} d^{-1} (n = 6).

Percent sand differed significantly among the 4 microhabitats ($p < 0.05$). Clam beds had a lower percentage of sand (12.9% ± 11.0) than shell beds (30.4% ± 10.7) but no other significant differences were found between other microhabitats. Percent silt did not differ significantly among habitats (48.6% ± 12.6; $p = 0.461$), but there were significant differences among sites in percent clay ($p < 0.001$), with a greater percentage of clay in clam beds (33.0% ± 2.0) than in shell beds (23.9% ± 3.3) or background sediments (23.8% ± 5.5).

Sediments from the 4 microhabitats at the Blake Ridge seep had low organic carbon (0.9% ± 0.22) and total nitrogen (0.1% ± 0.03) content. There was a greater percentage of C and N in clam beds and shell beds (clam beds: 1.04% organic C, 0.15% total N; shell beds: 0.95% organic C, 0.13% total N) than in background sediments (0.6% organic C, 0.09% total N; $p \leq 0.003$).

General clam characteristics

The size-frequency distribution for clams from all push cores (n = 818 ind.) was characterized by 2 modes: one centered on juveniles (1 to 2 mm shell length) and one centered on adults (15 to 20 mm) (Fig. 6A). Maximum shell length was 28.3 mm. There was no correlation between juvenile (clams <5 mm shell length) and adult (>5 mm shell length) clam density within push cores (ANOVA, $p > 0.05$; Fig. 6B).

As reported above, sulfide concentrations where the clams lived (the upper 3 cm of sediment through which the clam foot extends) were typically too low (0.0 to 1.3 mmol l^{-1}) and unvarying (average = 0.3 ± 0.51 mmol l^{-1}) to be a useful index of the underlying sulfide environment. We chose to use the sulfide concentration at 10 cm depth within each core (herein referred to as ΣH_2S_{10cm}) as the reference sulfide concentration for all subsequent analyses.

Adult clam density was low (10 ind. $core^{-1}$) in one push core where the ΣH_2S_{10cm} was lowest (0.1 mmol l^{-1}), and clams were absent where ΣH_2S_{10cm} was >13 mmol l^{-1} (Fig. 6C). Adult clam density was maximal (48 ind. $core^{-1}$) at ~1 mmol l^{-1} ΣH_2S_{10cm}; density was variably low (8 ind. $core^{-1}$) to high (to 48 ind. $core^{-1}$) between 0.6 and 1.1 mmol l^{-1} ΣH_2S_{10cm} (Fig. 6C). Juvenile densities were maximal (47 ind. $core^{-1}$) in the core where ΣH_2S_{10cm}

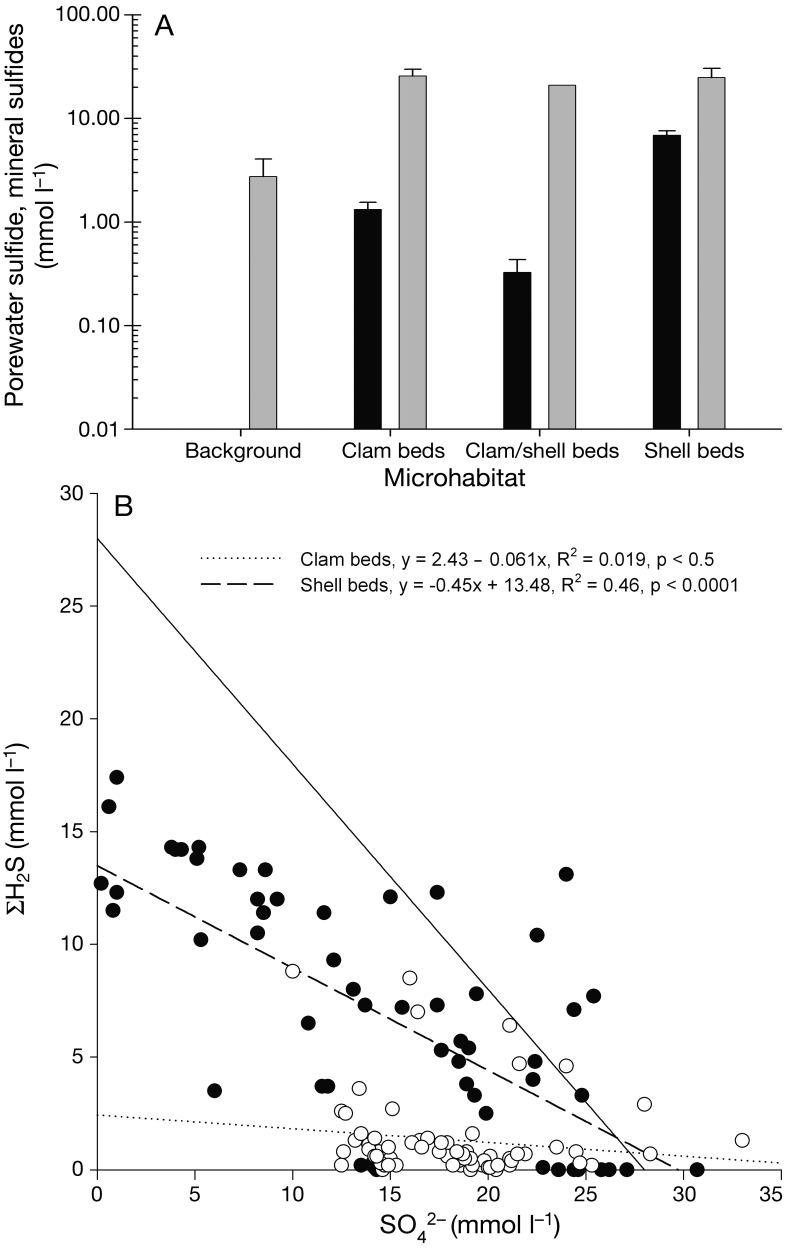

Fig. 4. (A) Average concentrations (± SE) of porewater sulfide and mineral sulfides (AVS + CRS) within push cores from 4 microhabitats at the Blake Ridge seep. Black bars = dissolved sulfide from: background cores (n = 37 intervals, 5 push cores); clam beds (n = 68 intervals, 9 push cores); shell beds (n = 59 intervals, 2 push cores); clam/shell beds (n = 14 intervals, 10 push cores). Grey bars = mineral sulfide concentrations averaged from 1 and 8 cm intervals in 2 push cores from each microhabitat. (B) Sulfide retention (ΣH_2S vs. SO_4^{2-}) in clam bed (○) and shell bed (●) push cores from Blake Ridge. Solid regression line represents quantitative reduction of sulfate to sulfide (1:1). Shell bed (– – – – –) and clam bed (·····) regressions suggest approximately 55% of the sulfide produced during reduction is lost from the sediment below shell beds and a sulfide loss of up to 94% from clam bed sediments

was lowest and were less than 13 ind. core^{-1} at other ΣH_2S_{10cm} concentrations (Fig. 6C). The greatest number of juveniles collected (47) was from one of the push cores designated a clam/shell bed. One push core from shell beds had a large number of juveniles (13), but the ΣH_2S_{10cm} concentration from this core was low (2.5 mmol l^{-1}) relative to the other shell beds. There was no significant correlation between shell length and ΣH_2S_{10cm} in the sub-sample of clams collected from Blake Ridge ($t_{[35]} = -0.802$, p = 0.428; Fig. 6D).

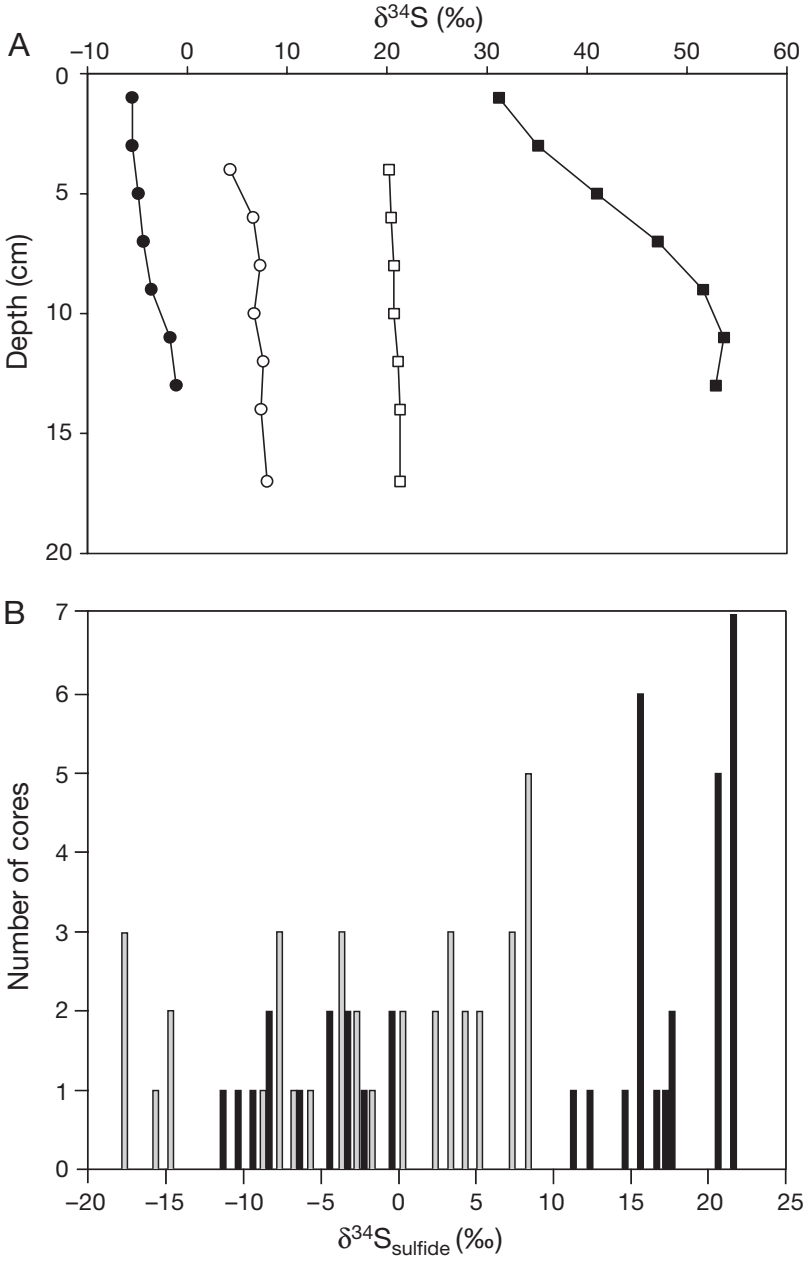

Fig. 5. (A) Down-core $\delta^{34}S$ profiles of sulfate (squares) and sulfide (circles) dissolved in representative cores of clam-bed (3912-4; open symbols) and shell-bed (3909-19; filled symbols). (B) Distribution of sulfide isotopic compositions for porewater collected below clam beds (grey bars) and shell beds (black bars)

Clam reproductive characteristics

General reproductive anatomy and condition

The reproductive anatomy of Blake Ridge clams matches that described for other species of vesicomyid clams, namely there was no evidence for hermaphroditism and gonads occupied the bulk of the visceral mass (Fig. 7A). The gonads extend from the dorsal epithelium to the foot muscle and surround the remnants of the digestive system, which is reduced to a single tubule. Developing gametes were arranged peripherally around a central lumen within reproductive acini (Fig. 7 inset; Lisin et al. 1996). There was no evidence for follicle cells associated with oocytes in females at the level of light microscopy. Female gametes were categorized into 3 stages: pre-vitellogenic (~15 to 30 µm; dark-pink-staining with H&E), and 2 vitellogenic stages (early vitellogenic: ~30 to 60 µm, pink-staining, fine granules inferred to be yolk; late vitellogenic: ~60 to 200 µm with large, light-pink-staining granules). Mature oocytes were large (~200 µm) and presumably develop into non-planktotrophic larvae, as inferred in other vesicomyid species (Lisin et al. 1996). The earliest gametogenic stages, the oogonia (<15 µm) were too small to be distinguished reliably from other cell types. Pre-vitellogenic and vitellogenic gametogenic stages were found within each female acinus (Fig. 7B), although the relative proportions of these stages varied within individuals. Only mature sperm were observed in acini of male specimens examined (Fig. 7C).

Five stages of reproductive condition were observed in female clams from Blake Ridge seeps (Fig. 8): 'Inactive': dominated by small (<25 µm) oogonia; 'Early-developing' dominated by pre-vitellogenic oocytes; 'Developing': dominated by pre-vitellogenic and early vitellogenic oocytes; 'Ripe': dominated by late vitellogenic oocytes; 'Spent': central lumens of acini are expansive, interacinal tissue (epithelium surrounding gonad) structure is disrupted, and vitellogenic oocytes are rare. In some Ripe individuals (3 of 37), there was evidence for partial release of gametes (i.e. a

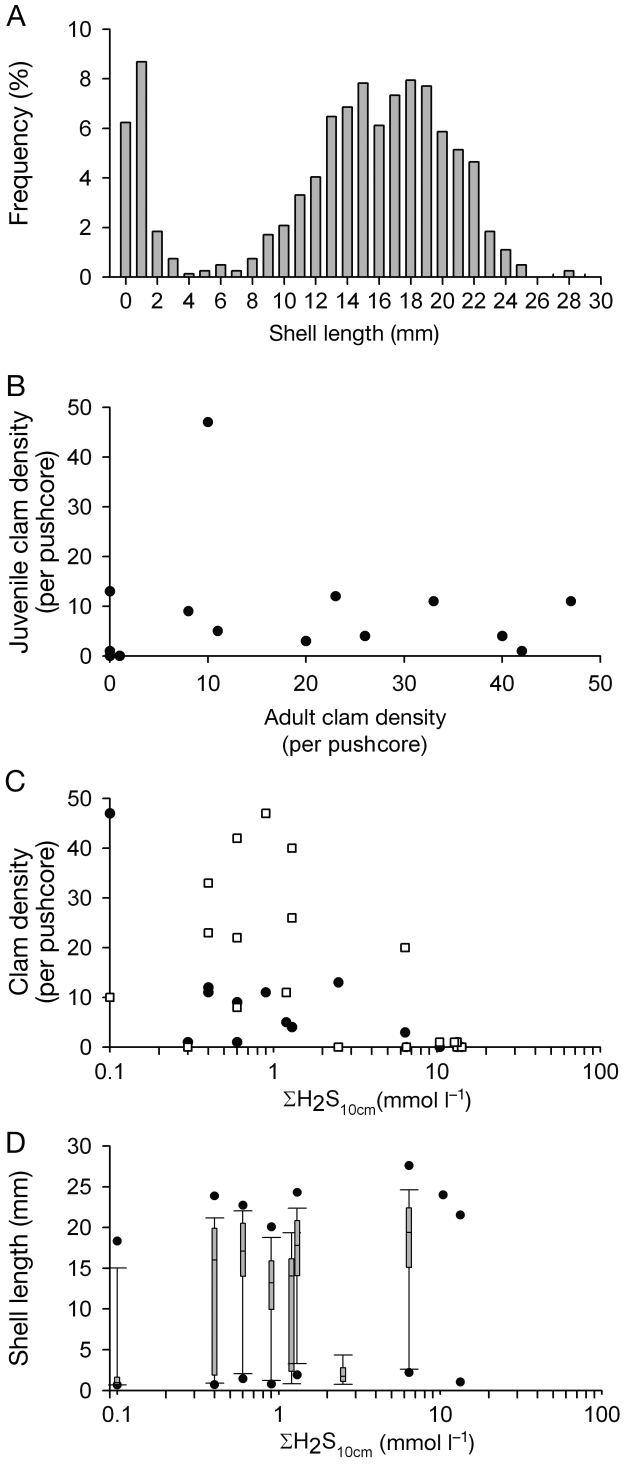

Fig. 6. Vesicomyid clams from Blake Ridge. (A) Size-frequency distribution of clam shell lengths (total n = 818) from clam beds. (B) Relationship between juvenile (<5 mm) clam density and adult (>5 mm) clam density per push core. (C) Relationship between clam density per pushcore and ΣH_2S_{10cm}; □: adult clams; ●: juvenile clams. (D) Relationship between clam shell length (mm) and ΣH_2S_{10cm}. Box-plots: median, 5th, 10th, 25th, 75th, 90th, and 95th percentiles; for $\Sigma H_2S_{10cm} > 10$ mmol l^{-1}, values are plotted as scatter plots

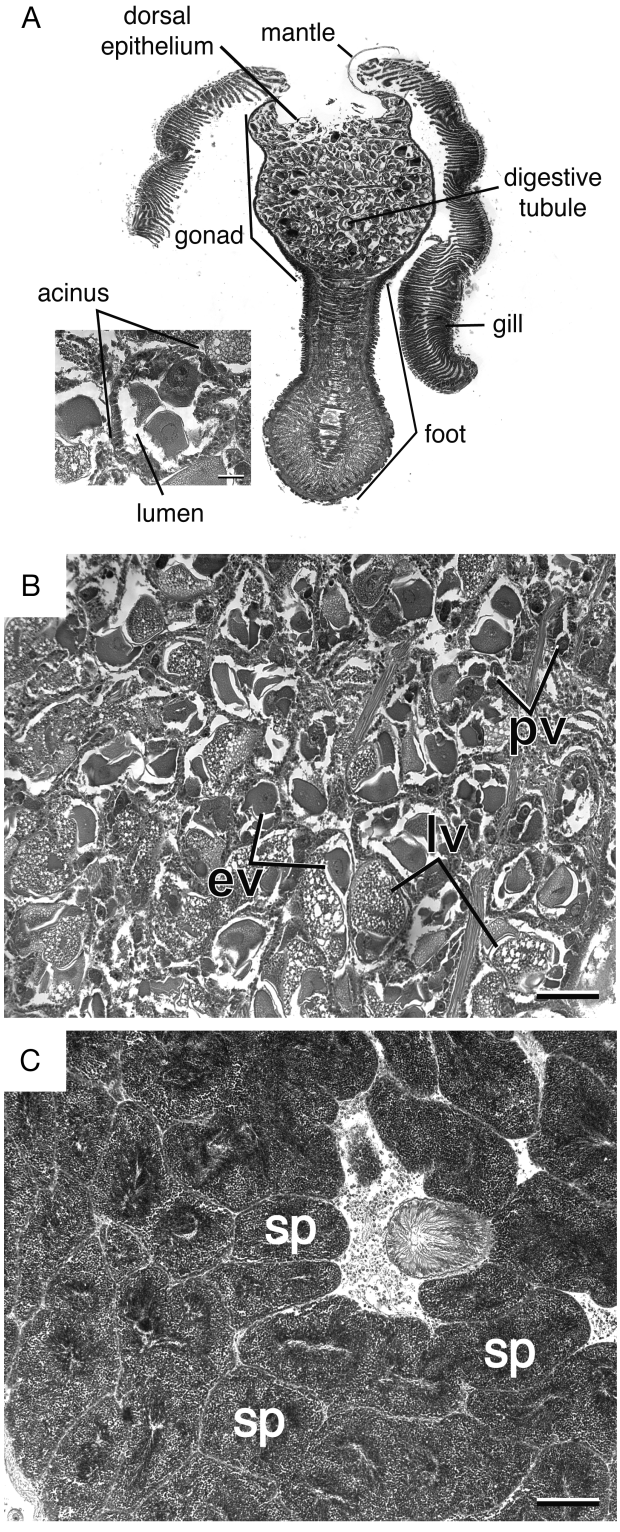

Fig. 7. Internal anatomy of vesicomyid clams from Blake Ridge. (A) Cross-section through the middle region of a female clam. Inset: Acinus of female clam. Scale bar = 20 µm. (B) Female gonad. pv: pre-vitellogenic oocytes; ev: early vitellogenic oocytes; lv: late vitellogenic oocytes. Scale bar = 150 µm. (C) Male gonad. sp: mature sperm. Scale bar = 150 µm

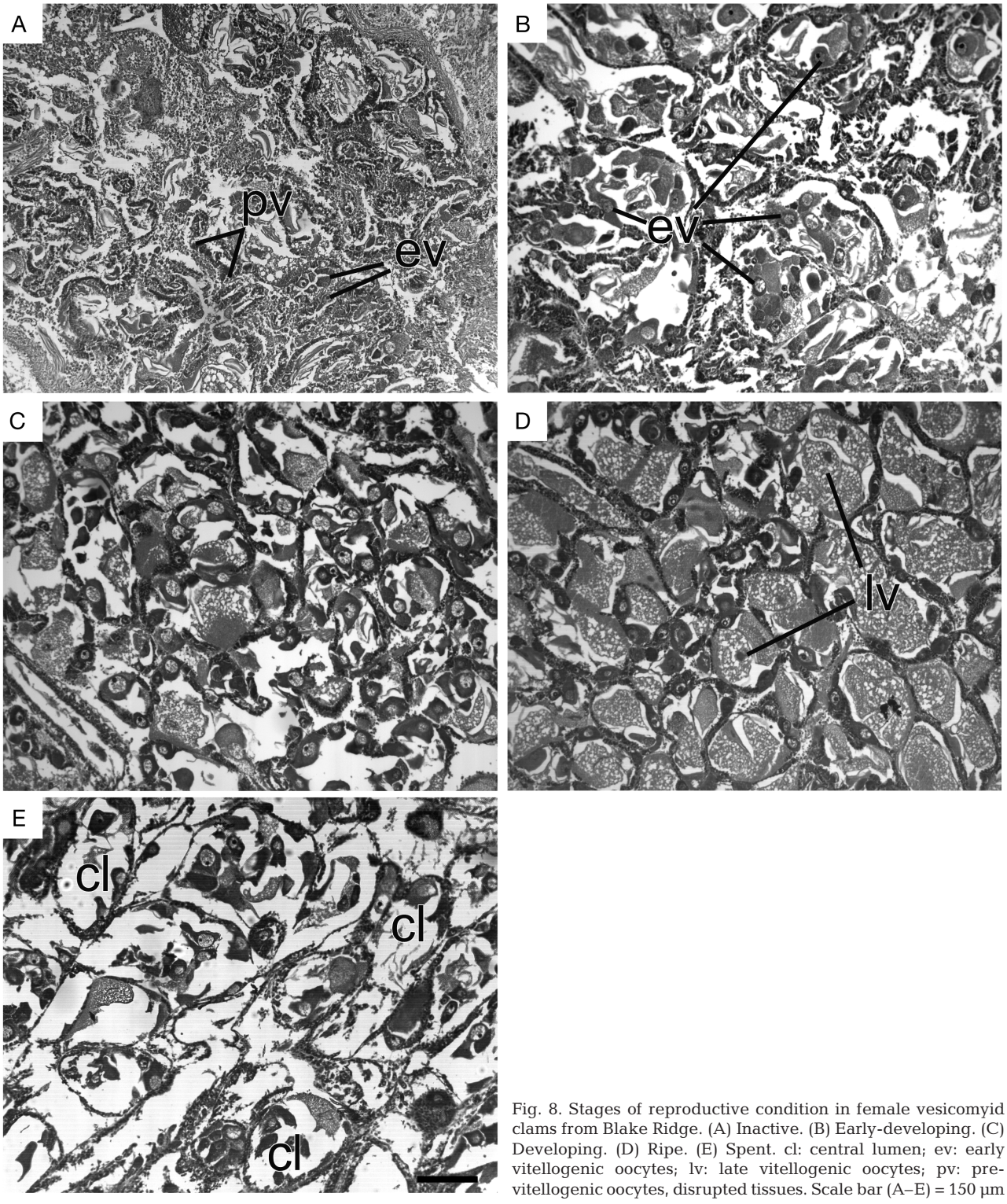

Fig. 8. Stages of reproductive condition in female vesicomyid clams from Blake Ridge. (A) Inactive. (B) Early-developing. (C) Developing. (D) Ripe. (E) Spent. cl: central lumen; ev: early vitellogenic oocytes; lv: late vitellogenic oocytes; pv: previtellogenic oocytes, disrupted tissues. Scale bar (A–E) = 150 µm

portion of the gonad appeared to be spent). Most females examined were in the 'Developing' to 'Ripe' reproductive stages (see Fig. 9). There was no detectable variation in reproductive condition in male clams from Blake Ridge; all males were in a Ripe condition, i.e. with only mature sperm (Fig. 7C). The ratio of gonadal to somatic tissue (% gonad) ranged from 9 to 38% in males. There was a weak correlation between % gonad and shell length for males ($t_{[19]}$ = 1.844, p = 0.08).

Relationship between reproductive characteristics and the environment

There was no significant correlation between mean oocyte diameter and ΣH_2S_{10cm} (stratified ANOVA; $F_{[1, 30]} = 0.169$, p = 0.684; Fig. 9A) or mean oocyte volume and ΣH_2S_{10cm} (stratified ANOVA; $F_{[1, 30]} = 0.289$, p = 0.622). There was no correlation between reproductive stage of females and ΣH_2S_{10cm} within sediments ($t_{[35]} = 0.101$, p = 0.92; Fig. 9A) or between % gonad in males and ΣH_2S_{10cm} ($t_{[19]} = 1.087$, p = 0.290).

DISCUSSION

The availability of sulfide to chemosynthetic megafauna at methane-hydrate seeps is spatially discrete (Sahling et al. 2002) and may be variable over spatial and temporal scales (Levin 2005). Records of fluid flow at seeps document transience on time scales of hours to months, with variation coinciding with tidal, lunar, or much longer cycles (Carson & Screaton 1998). Patchy distributions of megafaunal species at seeps reflect the patchy character of fluid expulsion and the availability of reduced compounds that support chemoautotrophic symbiont-invertebrate relationships, giving rise to the designation of megafaunal species as 'flux indicators'. Exactly what kind of flux they indicate is not always well known, and for a given species, an occupied habitat may reflect sediment characteristics and biological interactions (e.g. competition, predation). Our study provides the first documentation of sediment characteristics, including porewater sulfur chemistry, beneath clam and shell beds at the Blake Ridge seep.

Vertical profiles of sulfide and sulfate chemistry in push cores from Blake Ridge environments reflect the activities of clams and microbes. Sulfate concentrations were lower below clams and shells relative to background sediments, and the base of the sulfate reduction zone was much deeper below the clam beds than below the shell-bed habitats. Although sulfate gradients were steeper below clam beds than below shell beds (Fig. 3), a Student's *t*-test comparison of calculated sulfate reduction rates indicates no statistical difference between rates of sulfate consumption. Upper sediment layers in push cores from clam beds and mixed beds were characterized by steep gradients in sulfate concentrations, from ~29 mmol l^{-1} in the overlying seawater to half that value within 2 cm. These gradients suggest high rates of sulfate reduction, but sulfide concentrations in the upper sediment layers of cores from clam beds were generally low (0.1 to 1.0 mmol l^{-1}). Porewater chemistry profiles document lower sulfide retention (ΣH_2S vs. SO_4^{2-}) in clam-bed porewaters compared to porewaters of shell-bed sediments. We estimate that >90% of the sulfide produced from sulfate reduction is missing from the sediment below clam beds. Dissolved sulfide can be removed from a sediment system through diffusion and chemical oxidation at the sediment–water interface, uptake by chemosynthetic organisms (including endosymbiotic bacteria in the gills of the clams), or precipitation as mineral sulfides. Mass balance estimates of sulfide production and retention in anoxic water columns (Mandernack et al. 2003) and cold-seep sediments (Aharon & Fu 2000, 2003) suggest that physical, chemical, and free-living microbial activity in these systems only removes up to 35% of dissolved sulfides. The extensile foot of the clam, where sulfide uptake presumably takes place (Scott & Fisher 1995), reaches to the interface between oxidized and reduced sediments, while ciliated gills pump overlying oxygenated water into the mantle cavity though the

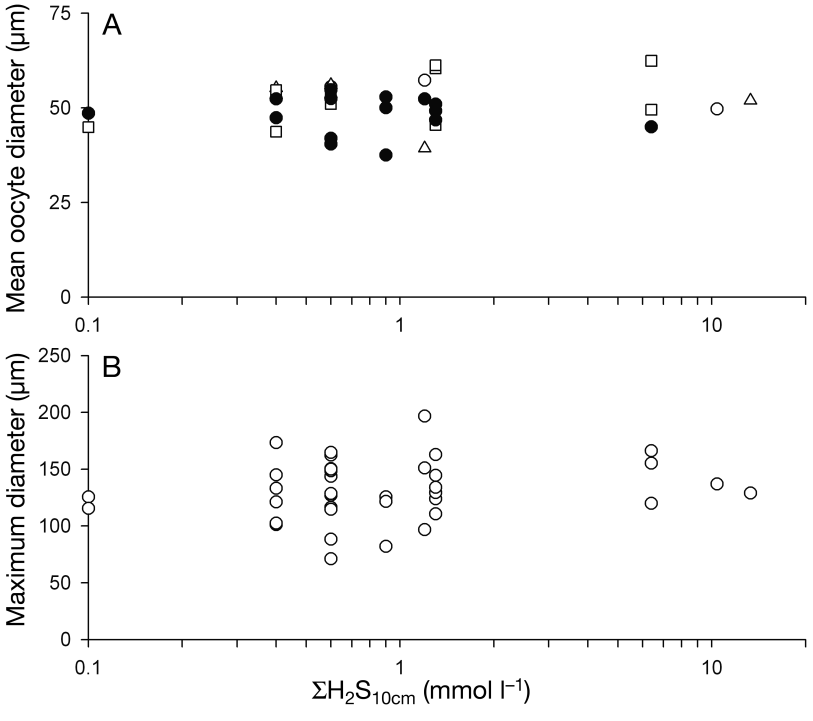

Fig. 9. Oocyte characteristics of vesicomyid clams from all push cores. (A) Mean oocyte diameter vs. ΣH_2S_{10cm}. (B) Maximum oocyte diameter vs. ΣH_2S_{10cm}. Symbols represent different stages of reproductive condition: ○: inactive and spent; △: early-developing; □: late developing; ●: ripe

siphons. We infer that the clams are extracting most of the missing sulfide and that they are remarkable in their capacity for sulfide uptake.

We are intrigued by the negligible sulfide concentrations in 2 cores from 'background' sites (Alvin 3909-22 and 3909-24), where sulfate concentrations in the top 2 cm of the cores dropped to half that of seawater values. Low sulfate concentrations in these cores, taken adjacent to sites where seepage was evident, suggest that sulfate reduction is taking place in these sediments, but the negligible sulfide concentrations and oxidizing conditions of the cores are not consistent with this hypothesis. The oxidizing condition of the cores and lack of sulfide could be explained if the sulfide was 'fixed' by iron oxides into pyrite, but we should have seen higher pyrite levels in these cores if this was true. At other seep sites, transformations between dissolved sulfate and solid phases such as barite ($BaSO_4$) have been implicated as controlling factors in observed sulfate profiles (Orcutt et al. 2005).

Dissolved sulfur isotopic compositions revealed different extents of fractionation of sulfur below clam and shell beds. In both microhabitats, sulfate pools were enriched in ^{34}S and sulfide pools were depleted in ^{34}S, but the fractionation effect was much greater (and more variable) in the shell-bed sulfur system than in the clam-bed sulfur system. The process of clam-bed bioirrigation apparently influenced the isotopic composition of dissolved sulfate and sulfide. In clam beds, $\delta^{34}S_{sulfate}$ matched those of seawater (~20‰, Böttcher et al. 2000, this study) or increased only slightly with depth (to a maximum of 30 to 35‰ in one instance). Corresponding sulfide in clam beds was depleted in ^{34}S by 15 to 30‰ relative to sulfate, which is consistent with isotopic discrimination during dissimilatory sulfate reduction (Habicht & Canfield 2001). The modest down-core isotopic enrichment observed in the $\delta^{34}S$ of porewater sulfate relative to seawater sulfate (20.5‰) and the near parallel offset in the paired sulfide isotope signal suggests sediments inhabited by clams are open to solute exchange. Sediment mixing below clams would deepen the aerobic zone and replenish the sulfate supply, thus preventing the maximal sulfate isotope enrichments expected of Rayleigh-type closed system fractionations.

Sulfur fractionation observed within shell beds may be explained by termination of sediment bioturbation after clam mortality. In cores from shell beds, the downward decrease in sulfate concentrations and eventual disappearance of sulfate at depth below the shell beds suggest a nearly complete microbial reduction of sulfate within the upper 15 cm. As bioturbation ceased, the supply of ambient seawater into the sediments would be delivered primarily by diffusion. This condition, together with the potential for upward flux of fluids devoid of sulfate from the seabed (on the order of 2 to 40 cm yr^{-1}; Hornbach et al. 2005), could drive the sediments toward anoxia and high sulfide concentrations. Under closed system conditions, maximal fractionations were observed within shell bed cores 3909-8 (ε = 23.6‰), 3909-19 (ε = 30.4‰), and 3909-20 (ε = 21.1‰). The significantly lower fractionation factors (ε < 15‰) observed within shell bed cores 3909-4, 3909-5, and 3909-10 suggests that these sediments were in transition from a partially open system to one completely closed.

The proposed model of sulfur isotopic composition determined by the presence or absence of bioturbation may be influenced by other factors, such as rates of sulfate reduction and/or the composition of the microfaunal population within clam- and shell-bed sediments. Determination of whether the degree of openness of the sediment system is the principal mechanism for establishing the isotopic separation between sulfate and sulfide remains an important objective for future studies.

Sulfide concentrations (ΣH_2S_{10cm} = ~1 mmol l^{-1}) at which Blake Ridge clams occur in highest density were similar to sulfide concentrations (0.2 to 0.6 mmol l^{-1}) in fluids colonized by other vesicomyid species, including *Calyptogena magnifica* at hydrothermal vents on the Galapagos Spreading Center (Fisher et al. 1988) and *C. kilmeri* and *Vesicomya pacifica* from Monterey Canyon seeps (Barry et al. 1997). *V. pacifica* at northern California methane seeps tend to avoid sulfide concentrations >1 mmol l^{-1}, although they can be found in sediments with sub-surface sulfide concentrations up to 2 mmol l^{-1} (Levin et al. 2003). Large (~12 to 15 cm length) vesicomyid clams (*Calyptogena phaseoliformis*) that live in dense aggregations at the Kodiak seep (Gulf of Alaska) are reported to migrate meters, presumably to adjust their position to favorable sulfide conditions (Levin 2005). Blake Ridge clams are likely to adjust their positions to optimal sulfide conditions as well, although clam movement was not noted during the brief intervals of observation available. The broad sulfide tolerance of adult Blake Ridge clams (ΣH_2S_{10cm} = 0.1 to 6.4 mmol l^{-1}) may reflect the need and the capacity of the clams to adjust to transient sulfide fluxes. Although adult clams survive in sediments with $\Sigma H_2S_{10\,cm}$ > 6.4 mmol l^{-1}, juvenile density was low (0 to 3 clams per push core) at and above this concentration, suggesting that high sulfide concentration inhibits settlement and/or post-settlement survival.

A striking feature of the Blake ridge seep site is the abundance of shell beds adjacent to clam beds. Predation and disease were deemed unlikely to be responsible for this mortality (Van Dover et al. 2003, Mills et al. 2005), leaving porewater chemistry (i.e. either too little or too much sulfide) as a suspected factor. Methane

is also present in Blake Ridge Diapir sediments, but methane is not considered toxic to animals even at high concentrations (70 to 90% methane in oxygen; National Research Council Committee on Toxicology 2000). Too little sulfide seems an unlikely explanation, since shell beds sampled in this study were typically in areas where porewater sulfide concentrations at 10 cm depth were >6.5 mmol l^{-1} (up to 14.2 mmol l^{-1}). This is higher than sulfide concentrations at 10 cm in clam beds (this study), and similar to sulfide concentrations in microbial mat environments on the Cascadia Convergent Margin (Sahling et al. 2002) and in barren zones in the center of vesicomyid clam beds in Monterey Bay (Barry et al. 1997); i.e. they were high enough to exceed the tolerance of vesicoymid clams. In the absence of bioturbation and sulfide uptake by the clams, sulfide concentrations in sediment with active microbial sulfate reduction, especially under a cap of shells, would likely be elevated compared to sulfide concentrations in sediment supporting live clam beds, if the delivery of sulfide was identical between the 2 habitat types.

Without measurement of sulfide flux in shell beds at the time of clam mortality, it is impossible for us to point to sulfide toxicity as the definitive cause of mortality at the Blake Ridge Diapir, but we consider it plausible. *Calyptogena* species are known to survive periods of reduced or halted fluid flow or variable sulfide concentrations, however, by the cessation of pumping or feeding (Sibuet & Olu 1998), and clam trails in photographs (e.g. Fig. 2A) lead us to expect that Blake Ridge clams could move away from or avoid high concentrations of sulfide. Furthermore, vesicomyid clams have sulfide-binding proteins in their blood that prevent sulfide from poisoning the cellular cytochrome *c* oxidase system (Zal et al. 2000). These observations all imply that mortality due to sulfide toxicity (and concomitant hypoxia) might only occur if an increase in sulfide flux was substantial, abrupt, and extended in duration. Sulfide at high concentrations can have a narcotizing effect (Dubilier et al. 1993), which might prevent clams from relocating in response to a sudden increase in the flux of sulfide. While it is not known when the Blake Ridge clams died, the excellent condition of the shells in the Blake Ridge shell beds (no dissolution, intact periostraca, minimal coverage by sediment) suggests that the mortality was relatively recent. One recent disturbance at the seep site was the drilling activity of the Ocean Drilling Program: 5 boreholes, including 2 to depths of up to 10 m at the center of the diapir; i.e. within meters of clam beds (Shipboard Scientific Party 1996, Paull et al. 2000). There is no substantive evidence for burial of some clam beds by fine sediments generated during the drilling process followed by mortality. We consider it possible that clam mortality was coincident with disturbance and alteration of the subsurface hydraulic regime associated with the drilling activity, but our data and observations do not allow us to test this hypothesis. High-resolution, 3D imaging of the fluid migration pathways beneath the Blake Ridge Diapir seep reveal significant complexity and, in some cases, transport of fluids more than 1 km laterally away from the main, deep fluid conduit centered on the diapir (M. Hornbach, C. Ruppel pers. comm.). Even slight displacements on some of the fine-scale faults on top of the diapir could radically alter hydraulic regimes, leading to the activation or abandonment of some fluid pathways in favor of others. Activation of a fluid pathway would enhance the flux of reduced chemicals and might lead to the kind of massive mortality documented at the Blake Ridge Diapir.

We anticipated that reproductive condition would be a more sensitive indicator of optimal sulfide conditions for clams than clam density, so we characterized the reproductive attributes of adult clams from different push cores (i.e. different $\Sigma H_2S_{10\ cm}$). All male individuals examined were ripe, with mature sperm densely packed in the gonadal acini, regardless of the sulfide concentrations in the porewater. Females were more variable in condition, ranging from spent to ripe, but there was no apparent relationship between female reproductive status and sulfide concentration, nor did general tissue quality appear to be poor in any specimen. Thus we view the clams as reproductively capable over a broad range of sulfide availability (i.e. $\Sigma H_2S_{10\ cm}$ = 0.1 to 6.4 mmol l^{-1}), underscoring their ability to exploit spatially and temporally variable sulfide delivery.

Vesicomyid clams form large aggregations that are interpreted as being reproductively advantageous for broadcast spawners with external fertilization (Momma et al. 1995). The timing of reproduction in clams and other invertebrate populations living in deep sea chemosynthetic ecosystems is of perennial interest, given that environmental cues controlling gametogenesis and spawning (e.g. light, temperature) are relatively weak or absent in the deep sea. One vesicomyid clam species, *Calyptogena kilmeri*, is reported to undergo synchronous seasonal reproduction in Monterey Bay (Lisin et al. 1996). In contrast, observations of spawning events in populations of vesicomyid clams in Sagami Bay indicate that egg release is asynchronous and that spawning by females is induced by release of sperm from males (Fujiwara et al. 1998). We were surprised by the record of uniformly ripe vesicomyid males and females that were in different stages of reproductive condition. A similar discrepancy in reproductive condition between males and females has been reported for several deep-sea echinoderm species (Gage & Tyler 1991) that form

pairs, and seems better suited for males that might have chance encounters with females than for bivalves that engage in spawning events involving large numbers of individuals. The bimodal size distribution of clams at Blake Ridge features a large number of very small, juvenile clams and suggests that some combination of factors may favor synchronous delivery of clam larvae to the site. We find it difficult to reconcile the condition of uniformly ripe males, the combination of ripe and spent females, and the bimodal size distribution in clams from Blake Ridge. More details of gametogenic processes, spawning events, recruitment, and post-recruitment processes need to be elucidated if we are to understand the population dynamics of this species.

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