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Parasitism as a potential contributor to massive clam mortality at the Blake Ridge Diapir methane-hydrate seep

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Vesicomyid clam species are abundant in many deep-sea chemosynthetic communities, including cold seeps. They rely primarily on thiotrophic (sulphide-oxidizing) gill symbionts for nutrition and thus require sulphide-rich environments. Submersible surveys of megafaunal distributions at the Blake Ridge Diapir, a deep-sea methane-hydrate seep located ~200 miles off the coast of Charleston, South Carolina, documented massive mortalities of vesicomyid clams. The cause of these mortalities is unknown, but sulphide deprivation, sulphide toxicity, and disease are possible agents of mortality in this system. Similar redox profiles in sediment cores from live and dead clam beds do not support the hypothesis that there has been a transient shift in the flux of sulphide. To address the potential for disease as a cause of mortality, we undertook a histological survey of microparasites and other indications of disease in clam tissues. Six morphological types of parasites were identified using light microscopy, including two viral-like inclusions, *Rickettsia*-like gill inclusions, possible bacterial gut inclusions, bacterial gill infections, and a protistan inclusion. Of these parasites, two were pathogenic: viral-like inclusions in mantle tissues caused tissue degradation; bacterial gill infections resulted in localized disruption and degradation of gill filaments. Infection prevalence and densities were low for all parasites observed. The majority of clams examined showed intense haemocytic responses in the absence of any obvious etiologic agent, suggesting the presence of parasites not detectable by our methods. Our findings indicate that the clam population at the Blake Ridge seep was in relatively good health at the time of sampling.

INTRODUCTION

The Blake Ridge Diapir is a methane seep located approximately 200 miles off the coast of Charleston, South Carolina, at a depth of 2155 m (Figure 1). An undescribed species of vesicomyid clam (previously referred to as *Vesicomya* cf. *venusta* in Van Dover et al. (2003), but now considered to belong to an undescribed genus and species [E. Kryolova, personal communication]), and *Bathymodiolus heckerae* mussels are the dominant megafauna at the Blake Ridge Diapir (Van Dover et al., 2003). The clams and the mussels derive their nutrition from endosymbiotic, chemoautotrophic, sulphide-oxidizing bacteria harboured within specialized gill epithelial cells known as bacteriocytes; the mussels also host methanotrophic endosymbionts in their bacteriocytes (Van Dover et al., 2003). The digestive tract and filter-feeding apparatus of vesicomyid clams are reduced (Boss & Turner, 1980), suggesting that organic carbon produced by the endosymbionts is the primary source of nutrition in these bivalves.

Large bands (~1 m width, 10 m to 20 m length) and smaller patches (<1 m diameter) of live clams blanketed the sea-floor at the Blake Ridge Diapir in 2001, as did patches of clam shells (Van Dover et al., 2003). Because of their reliance on sulphide, vesicomyid clams at seeps serve as flux indicators (e.g. Levin et al., 2003). It is unclear, however, whether clam shells indicate mortality due to a decrease in sulphide availability, or if mortality results from other causes. Predation was deemed an unlikely explanation, as clam shells did not display structural damage associated with predation by the likely predators in the system (crab, octopus; Van Dover et al., 2003). The clam shells at the Blake Ridge site were relatively uniform in size and extent of erosion, suggesting that the die-off was synchronous and pandemic (Van Dover et al., 2003). Although massive bivalve mortality has frequently been observed at seeps (e.g. Mayer et al., 1988; Jollivet et al., 1990; Olu et al., 1996), the cause of such mortality is a matter of conjecture. Seeps like the one at the Blake Ridge Diapir have greater longevity and stability compared to hydrothermal vents (Sibuet & Olu, 1998), but they are still subject to transient shifts in the loci and intensity of seepage (e.g. Sibuet & Olu, 1998; Levin et al.,

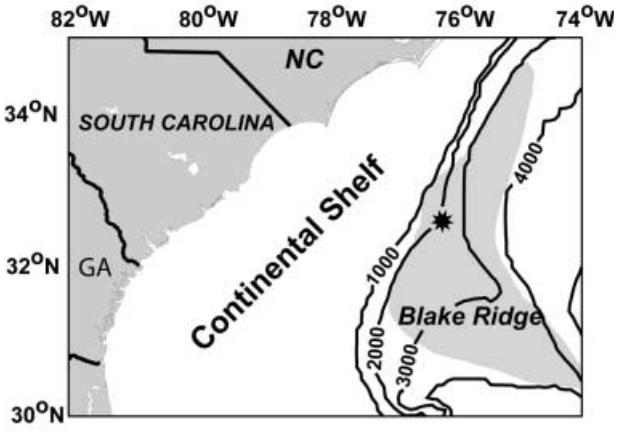

Figure 1. Location of the Blake Ridge Diapir methane-hydrate seep site (✱).

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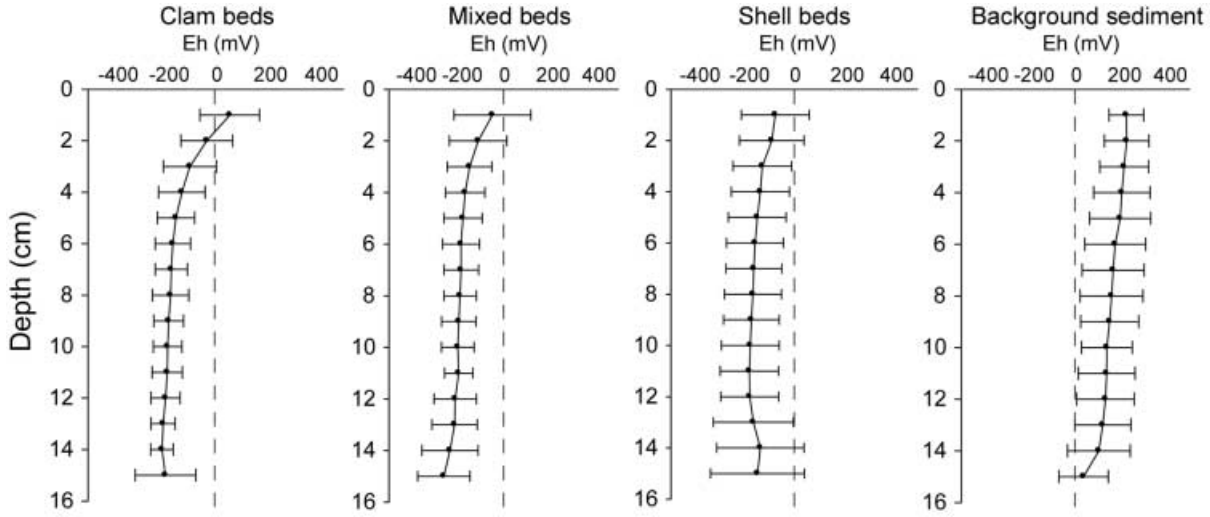

Figure 2. Redox profiles (mean ±SD) in push cores from the Blake Ridge seep site. (A) Live clam beds (N=13); (B) mixed live/dead clam beds (N=5); (C) dead clam beds (N=19); (D) background sediment (N=7).

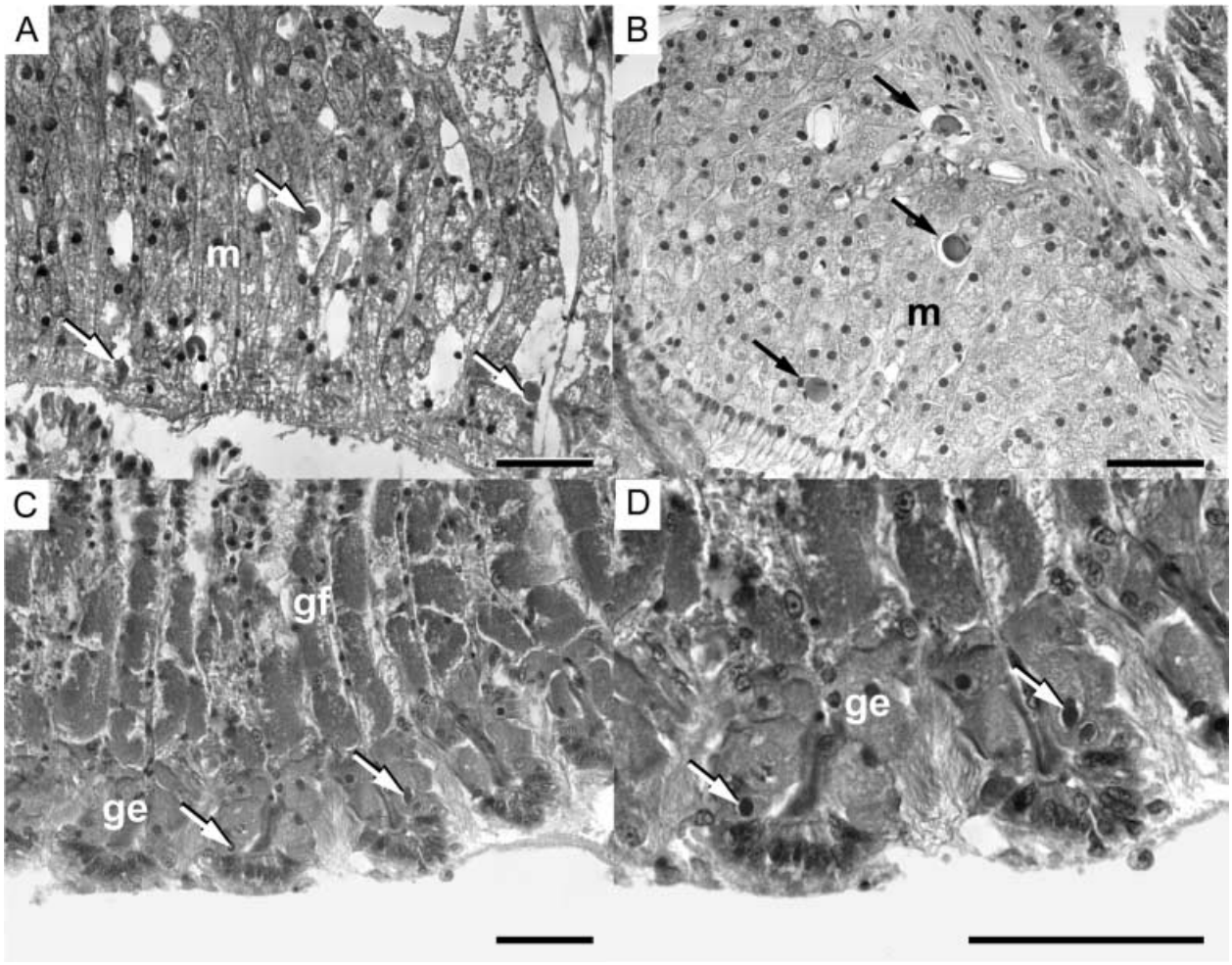

Figure 3. Putative viral-like parasites in Blake Ridge vesicomyid clams. (A) Viral-like Inclusion I (arrows) in mantle, with associated necrosis; (B) Viral-like Inclusion I (arrows) in mantle, not associated with tissue pathology; (C) Viral-like Inclusion II (arrows) in gill epithelium; (D) higher magnification of (C). g, gill filament; ge, gill epithelium; m, mantle. Scale bars: 50 μm.

2003). Cessation or reduction of fluid flux was considered a likely cause for bivalve death at the Blake Ridge Diapir (Van Dover et al., 2003), based on the nutritional reliance of vesicomyid clams on the delivery of sulphides to the sea-floor. Narrow sulphide requirements of at least some vesicomyid clam species (Goffredi & Barry, 2002) suggest that death could also be due to sulphide toxicity caused by an increase in fluid flux.

Shifting seepage is not the only potential cause for clam death at the Blake Ridge Diapir. Additional factors,

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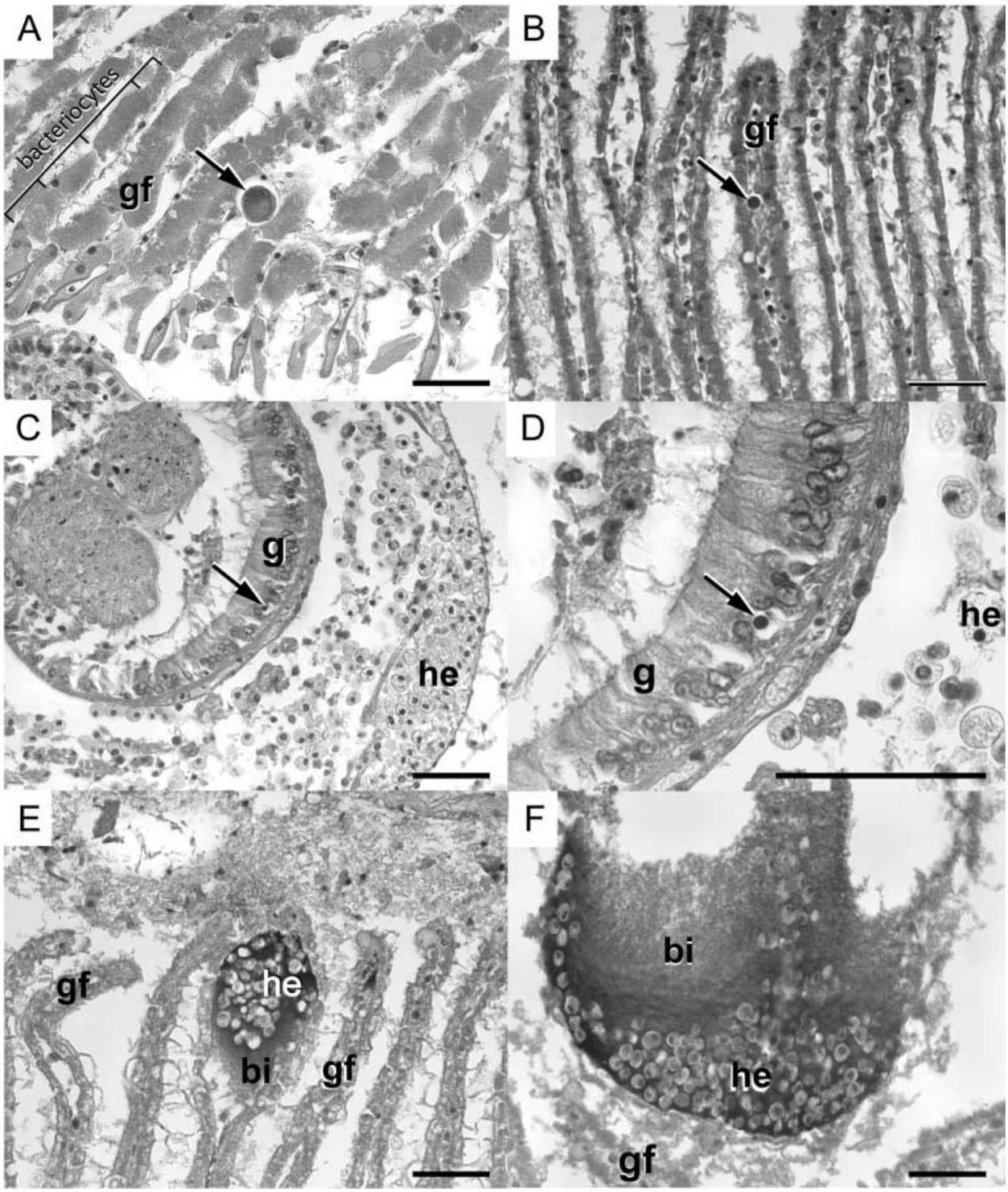

Figure 4. Bacterial inclusions in Blake Ridge vesicomyid clams. (A&B) *Rickettsia*-like gill inclusion (arrows); (C) possible bacterial gut inclusion (arrow) surrounded by haemocytes; (D) higher magnification of (C); (E&F) bacterial infections between gill filaments; note numerous haemocytes within bacterial clusters. bi, bacterial infections; g, gut; gf, gill filament; he, haemocytes. Scale bars: 50 μm.

including disease, could account for or contribute to massive mortality. Parasite epizootics and die-offs are well known in populations of commercially important, shallow-water bivalve species (e.g. *Perkinsus marinus* and *Haplosporidium nelsoni* in oysters [*Crassostrea virginica*; Andrews, 1996]; a protistan parasite [QPX, Quahog Parasite Unknown] in clams [*Mercenaria mercenaria*; Whyte et al., 1994] and in other bivalve populations [Lee et al., 2001; Park & Choi, 2001]). Parasitism and disease can influence the dynamics of bivalve populations by reducing reproductive effort, impairing growth, and altering population density. The interaction between parasites and their hosts may also be innocuous (e.g. the infection of seep mussel [*Bathymodiolus heckerae*] gill tissue by *Rickettsia*-like bacteria [Ward et al., 2004]).

Studies of disease and parasitism in deep-sea organisms are scarce, primarily due to the relative inaccessibility of the deep-sea environment, the low benthic biomass

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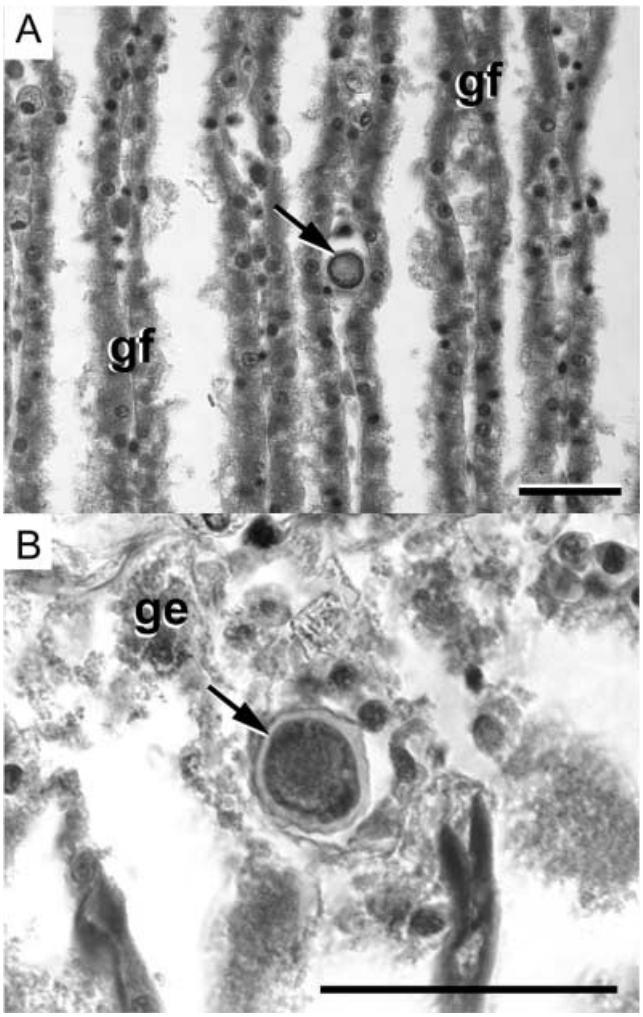

Figure 5. Protist I in Blake Ridge vesicomyid clams. (A) Protist I (arrow) within a gill filament; (B) Protist I (arrow) in gill epithelium. ge, gill epithelium; gf, gill filament. Scale bars: 50 μm.

throughout most of the deep sea, and the lack of any commercial interest in understanding disease processes in deep-sea populations. Deep-sea chemosynthetic communities (cold seeps, hydrothermal vents, and whale skeletons), however, are characterized by high biomass, and some communities are repeatedly visited with submersibles and other deep-submergence assets. Chemosynthetic ecosystems thus offer an opportunity for characterization of disease and parasitism at bathyal depths, although there continues to be a dearth of studies of parasites even in these environments. Recent studies suggest that microparasites may be major determinants of community structure in some (Powell et al., 1999; Ward et al., 2004), but not all (Terlizzi et al., 2004) molluscan species endemic to chemosynthetic habitats. In *Bathymodiolus* spp. mussels from hydrocarbon seeps off the Gulf of Mexico, for example, infections of *Bucephalus*-like trematodes caused sterilization and left large portions (40%) of the mussel population reproductively compromised (Powell et al., 1999). Parasitism and disease may also be important in *Bathymodiolus heckerae* mussels at the Blake Ridge Diapir, where a viral-like inclusion in the digestive tract was associated with tissue necrosis (Ward et al., 2004). This parasite may have contributed directly to mortality or exacerbated the failing condition of already stressed animals (Ward et al., 2004).

In this study, we used redox profiles in push core samples as a preliminary assessment of habitat characteristics and sulphide availability (Boulegue, 1978) beneath live clam beds (>90% of individuals alive), clam shell beds (no live individuals), and mixed beds (<10% of individuals alive) at the Blake Ridge seep site. Parasite burdens (parasite morphotypes, prevalence, and intensity) were documented for clams collected from live clam beds and from mixed beds using histological methods. Tissue infiltration by haemocytes, the primary internal defence mechanism in molluscs (Lauckener, 1983; Pipe & Coles, 1995), was also recorded as an indicator of host response to disease and stress.

MATERIALS AND METHODS

Push cores (6.35-cm diameter, 30-cm length) were taken from live clam beds (N=13), clam shell beds (N=19), and mixed (N=5) beds at the Blake Ridge Diapir (32°29.623′N 76°11.467′W; 2155 m; July–August 2003) using the deep-sea submersible 'Alvin'. Push cores (N=7) were also collected from non-seep, background sediments devoid of vesicomyid shells or other flux indicators.

Cores were stored in a cold room (4°C) on recovery and vertical redox potential (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. Calibration of the electrodes was verified by measuring the redox potential of quinhydrone dissolved in buffers (pH 4, 7).

Zero to 48 live clams (maximum shell length: 28.3 mm) were collected per push core. Clams from each core were maintained at 4°C for up to 6 h before being processed. Shell lengths (±0.1 mm) were measured and the number of nautiliniellid polychaetes (*Vesicomyicola trifurcatus*; Dreyer et al., 2004) living within the clam mantle cavities was also recorded. Dissected clam tissues were fixed in Davidson's solution for 24 h and stored in 70% ethanol.

A subset of 60 clams (43 from live beds, 17 from mixed beds) representing all available sizes was dehydrated in an ethanol series and embedded in paraffin. Whole-animal cross sections (6-μm thick) were cut through the anterior, middle, and posterior regions of the clam and stained with haematoxylin and eosin (H&E). In the absence of any prior studies of parasite diversity in this species of clam, we relied on recognition of abnormal cell types or conditions in tissues followed by consultation with a shellfish pathologist (E. Burreson, Virginia Institute of Marine Science) for classification of parasite morphotypes to the level of viral- or bacterial-like inclusion. Size, shape, texture, colour, and infected tissue were used to distinguish different morphotypes. Gill, mantle, foot, gonad, and digestive tissue were examined in 50 cross sections per clam using light microscopy. The collection site for each clam (live or mixed bed) was not known at the time of evaluation of slides.

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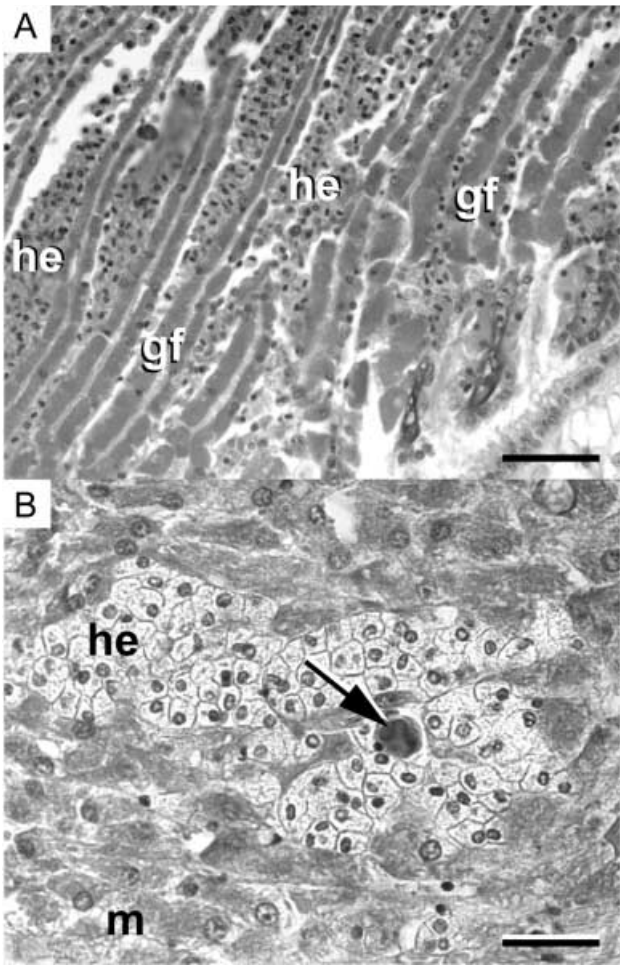

Figure 6. Haemocytic infiltrations in vesicomyid clams. (A) Haemocytes between and among gill filaments; (B) haemocytes surrounding an unidentified (possibly acellular) inclusion (arrow) in mantle tissue. gf, gill filaments; he, haemocytes; m, mantle. Scale bar: 50 μm.

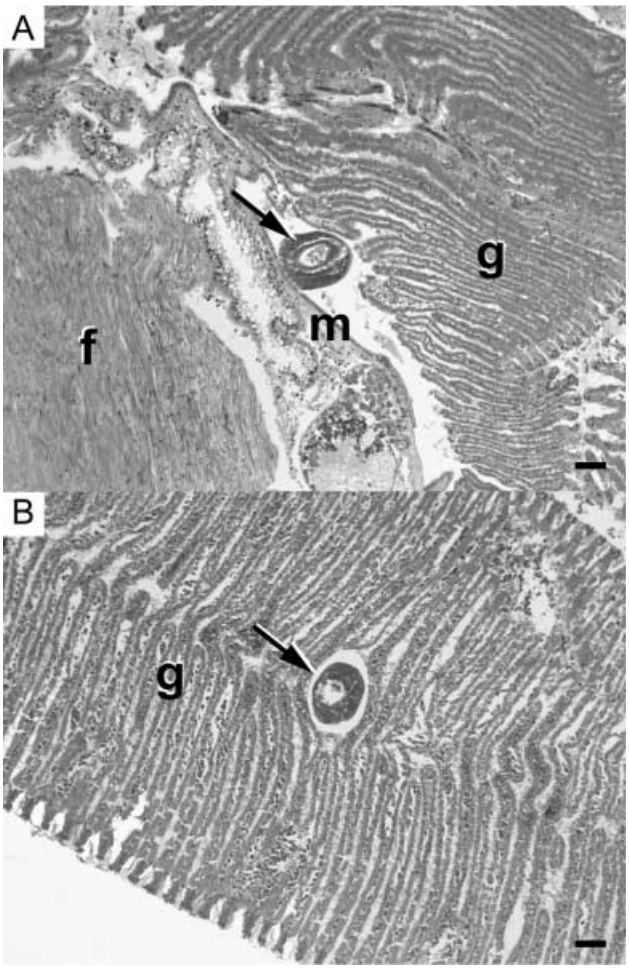

Figure 7. *Vesicomyicola trifurcatus* (Polychaeta: Nautiliniellidae) in Blake Ridge vesicomyid clams. (A) Nautiliniellid cross section (arrow) between mantle and gill; (B) nautiliniellid cross section (arrow) between gill filaments; note displacement of surrounding filaments. f, foot; g, gill; m, mantle. Scale bar: 50 μm.

Parasite dimensions are reported as averages of measurements taken from 5 to 25 individuals. Parasite density (average number per cross-section, based on examination of 50 cross-sections per individual) and presence/absence of haemocyte infiltration were determined for each individual, and parasite prevalence (% of individuals infected) was calculated from these data.

A Spot camera (Diagnostic Instruments) was used to document tissue quality, parasite morphotypes, and immune responses in clam sections. Adobe Photoshop was used for contrast adjustment.

RESULTS

Sediment chemistry

The upper 2 cm of sediment in push cores from clam beds (Figure 2A) and mixed (Figure 2B) beds were oxidizing ($\sim +200$ mV); below 2 cm, the sediment was reducing (~ -200 mV). Sediments in push cores from shell beds (Figure 2C) were reducing (~ -200 mV) throughout the cores. Sediments in push cores collected from background sediments (Figure 2D) were oxidizing ($\sim +200$ mV) throughout the cores.

Microparasite types

Six morphological types of microparasites were observed in histological sections of Blake Ridge Diapir vesicomyid clams: viral-like mantle and gill epithelium inclusions, bacterial gut inclusions, *Rickettsia*-like colonies in gills, bacterial infections of the gill tissue, and a protistan parasite. More than half (60%) of all individuals examined were host to at least one of these parasitic agents.

Viral-like Inclusion I. Viral Inclusion I (10 μm diameter) was located in mantle tissue (Figure 3A,B). Spherical or oval inclusions were eosinophilic, lacked internal structures discernable with light microscopy, and had a waxy, cellophane-like character. Mantle tissue surrounding the inclusions appeared degraded, and light haemocytic infiltration was occasionally associated with these inclusions. Viral-like Inclusion I was present in 8% of the individuals examined; infection density ranged from 0 to 0.24 inclusions per cross-section.

Viral-like Inclusion II. A smaller (5 μm diameter), viral-like inclusion was found in gill epithelial cells (Figure 3C,D). As with Viral-like Inclusion I, no internal structure was apparent at the level of light microscopy, and

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Table 1. *Comparison of microparasite types described in seep molluscs.*

Host species Location	Parasite type	Site of infection	Size	Reference
Bathymodiolus sp. Gulf of Mexico hydrocarbon seeps 550–560 m	*Rickettsia*-like colonies	gill epithelium digestive tissue	10–15 μm 25 μm	Powell et al., 1999
	extracellular gill ciliates	among gill filaments	20 μm	
	gill rosettes	gill epithelium	2–5 μm cells, 12 to 28 cells per vacuole	
	Bucephalus-like inclusions	gonad (entire body in heavy infections)	na	
Bathymodiolus heckerae Blake Ridge methane seep 2155 m	*Rickettsia*-like colonies	gill and mantle epithelium	20 μm	Ward et al., 2004
	extracellular gill ciliates	among gill filaments	30 μm	
	Chlamydia-like inclusions	digestive tissue	50 μm	
	viral-like inclusions	digestive tissue	5 μm	
Paralepetopsis floridensis Florida Escarpment brine seep 3288 m	*Rickettsia*-like colonies	digestive tissue	25–30 μm	Terlizzi et al., 2004
	bacterial inclusions	digestive tissue	15 μm	
Vesicomyid clam Blake Ridge methane seep 2155 m	*Rickettsia*-like colonies	gill epithelium	20 μm 8 μm	this study
	possible bacterial inclusions	digestive tissues	2–3 μm	
	bacterial infections	gill filaments	200 μm	
	viral-like inclusions	gill epithelium mantle epithelium	5 μm 10 μm	
	protists	gill epithelium	15 μm	

na, data not available.

inclusions were characterized by ovate or spherical shape and cellophane-like, eosinophilic appearance, with a narrow ring (~1 μm) of cleared tissue surrounding the inclusions. Tissue damage was not apparent and a haemocytic response was never observed in association with Viral-like Inclusion II. Viral-like Inclusion II was recorded in 3% of clams and infection density ranged from 0 to 0.06 inclusions per cross-section.

Rickettsia-like *gill inclusions.* Bacterial gill inclusions were observed in 14% of the clams. In one case, a larger, granular, *Rickettsia*-like colony with a diameter of 20 μm was recorded in the gill filaments (Figure 4A). Other *Rickettsia*-like gill inclusions were smaller, ~8 μm in diameter (Figure 4B). No pathology was observed with the *Rickettsia*-like inclusions, although there was minor distortion of gills caused by accommodation of the inclusions. *Rickettsia*-like gill inclusions infection density ranged from 0 to 0.06 inclusions per cross-section.

Bacterial gut inclusions. One or more small (2–3 μm diameter), spherical inclusions in digestive epithelial cells were observed in 10% of the clams (Figure 4C,D). These inclusions were associated with localized (within 1–2 μm) degradation of digestive tissue and are interpreted as bacteria, although we cannot rule out other possibilities (e.g. necrotic haemocytes). Infection density ranged from 0 to 0.04 inclusions per cross-section.

Bacterial Infection I. The most pathogenic illustration of bacterial parasitism was present in 28% of individuals. This infection, termed Bacterial Infection I, was characterized by large, diffuse aggregations of rod-shaped bacteria between and among the gill filaments (Figure 4E,F). Because these infections were not always entirely encircled in host tissue, the term 'inclusion' has not been employed. Bacterial Infection I was always associated with haemocytic infiltration (aggregates [200 μm diameter] of several to hundreds of haemocytes per cross section). Haemocytes surrounded and infiltrated the bacterial mass and were sometimes surrounded themselves by a clear border, suggesting vacuolization by the bacteria. Gill filaments adjacent to bacterial infections showed evidence of bacteriocyte degradation, breakage, and spatial disruption (Figure 4F).

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Protist I. Protist I was the most prevalent parasite, present in gills of 35% of clams examined. Infection density for Protist I ranged from 0 to 0.24 inclusions per cross-section. Protist I was spherical (15 μm diameter; range=8 μm to 22 μm), enclosed in a cell wall, and often surrounded by a diaphanous, irregular halo 1 μm to 2 μm thick (Figure 5A,B). The halo of larger specimens was most pronounced and the interior of the parasite assumed a distinctly granular appearance, whereas the interior of smaller Protist I parasites tended to be less opaque and lacked granularity. Smaller specimens of Protist I were often not associated with a host response, but larger individuals (>14 μm) sometimes provoked encapsulation, a characteristic defence reaction involving the recognition and sequestration of foreign bodies (Smolowitz et al., 1997). Other than localized disruption of gill structure and the lucent halo, there was no pathology associated with this parasite type.

Haemocyte responses

Intense haemocytic infiltration was recorded in 42% of clams analysed. Haemocyte aggregations appeared primarily within and among gill filaments (Figure 6A) and in mantle tissues (Figure 6B), although haemocytic infiltration was also observed in the visceral mass. Dense aggregations of haemocytes in the gills often resulted in patent disruption of gill structure. Haemocytic responses were occasionally observed in conjunction with obvious inclusions (Figure 7B), but more frequently no etiologic agent was identified.

Comparison of parasite burdens in clams from live and mixed clam beds

Uneven sample sizes and low parasite densities preclude a statistically robust comparison of the parasite burdens in clams from clam beds and mixed beds. Of the two most prevalent parasite types, Protist I was nearly twice as prevalent in clam beds (39%) than in mixed beds (23%). Bacterial Infections were more prevalent in mixed beds (35%) than in clam beds (25%), as was haemocytic infiltration of tissues (mixed beds: 59%; clam beds: 35%).

Macroorganisms

More than 60% of the clams examined contained one or more nautiliniellid polychaetes. In most cases, the polychaetes were not closely associated with clam tissues, but in three instances, juvenile nautiliniellids were intimately associated with gill tissue, causing displacement of gill filaments (Figure 7).

DISCUSSION

Complicating any explanation of massive bivalve mortalities at seeps is our inability to know when a die-off took place and what environmental conditions were like at that time. Lack of substantial sediment cover and the unbroken and unweathered condition of the shells suggest that clam mortality at the Blake Ridge site was relatively recent (months to years, rather than decades).

Transient shifts in the flux of sulphide from the seabed have been implicated in massive bivalve mortalities in deep-sea seep environments (e.g. Jollivet, 1990; Van Dover et al., 2003). Had sediment redox profiles beneath the clam shells at the Blake Ridge Diapir matched the oxidizing redox profiles of nearby background sediments, we would have concluded that chemoautotrophic symbionts in the clams were likely unable to obtain sufficient sulphide for survival. But reducing conditions in sediments immediately beneath the clam shell beds suggest that sulphate reduction and sulphide generation remained robust and that starvation was an unlikely cause of clam mortality in this instance. Without quantitative measures of sulphide concentrations and flux, however, we cannot be sure that habitat quality was uncompromised by toxic levels of sulphide. Sulphide requirements for vesicomyid clams can be narrowly constrained within species and variable among species (Goffredi & Barry, 2002). For example, two co-existing vesicomyid species (*Calyptogena pacifica* and *Calyptogena kilmeri*) at cold seeps in Monterey Bay have different modes of sulphide uptake and assimilation (Goffredi & Barry, 2002). *Calyptogena pacifica* shows greater efficiency in sulphide uptake, transport, and oxidation than *C. kilmeri*, and is thus poised to inhabit environments with lower sulphide availability (Goffredi & Barry, 2002). The less efficient sulphide metabolism of *C. kilmeri* and its symbionts necessitates higher environmental sulphide levels (Goffredi & Barry, 2002). Even small changes in sulphide availability could have dramatic effects on clam health and survivorship (Goffredi & Barry, 2002) and may account for clam mortalities at the Blake Ridge site. Despite uncertainty about the specific sulphide requirements of Blake Ridge vesicomyids, the similar redox profiles beneath clam beds, mixed clam and shell beds, and shell beds suggests that factors other than shifting fluid flux, including parasitism and disease, might contribute to vesicomyid mortality.

The potential impact of disease in Blake Ridge bivalves has been demonstrated in a histopathological analysis of the mussel, *Bathymodiolus heckerae* (Ward et al., 2004). A pathogenic viral-like inclusion was found in the digestive tract of these mussels and was associated with degraded tissue quality and decreased reproductive capacity (Ward et al., 2004). While disease seems to play a role in mussel population structure at the Blake Ridge Diapir (Ward et al., 2004), the impact of parasitism on the Blake Ridge clams was not obvious. Although the number of parasite types observed in mussels (5) and clams (6) from the Blake Ridge was similar, infection densities and number of individuals infected with any parasite were higher in the mussels. Digestive tissue is often a portal for infection in bivalves (Lauckner, 1983; Pipe & Coles, 1995), and its reduced condition in vesicomyids (Boss & Turner, 1980; Goffredi & Barry, 2002) may limit points of entry for pathogenic agents. None of the clam parasites had a large impact on overall tissue quality. The most common parasite, Protist I, appeared to be innocuous. Viral-like Inclusion I (sometimes associated with tissue necrosis) and Bacterial Infection I (associated with localized disruption and degradation of gill filaments), appear to be potential agents of disease that could, if present in higher densities, contribute to mortality among these populations. Viral infections have been linked with severe mortalities in

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shallow-water molluscs (Lauckner, 1983). Bacterial Infection I may be secondary colonizers, taking advantage of tissue that had been compromised by wounding or other factors. The possible gut bacterium was linked to degradation of digestive epithelial tissue, but extremely low infection prevalence and density, combined with the relative unimportance of the digestive tract in vesicomyid nutrition (Goffredi & Barry, 2002), suggest that this parasite was not likely to play a structuring role in clam communities at Blake Ridge seeps.

To date, microparasites of four seep molluscs (two mussels [Powell et al., 1999; Ward et al., 2004], one limpet [Terlizzi et al., 2004], and one clam [this study]) have been described using light microscopy (Table 1). *Rickettsia*-like inclusions in gill and/or digestive tissues and protists (including ciliates) associated with gill filaments were observed in all four species. Viral-like inclusions are so far only reported for Blake Ridge clams and mussels.

No severe necrosis was correlated with the nautiliniellid polychaete *Vesicomyicola trifurcatus*, although the gross displacement of gill structure by juvenile polychaetes may interfere with gill function. These histological observations are consistent with a commensal relationship between nautiliniellid polychaetes and their vesicomyid hosts (Van Dover et al., 2003).

Phagocytosis by haemocytes is the primary internal immune defence in molluscs (Pipe & Coles, 1995), and haemocytic infiltration may be a sign of parasitic infection. The presence of haemocytic responses in the majority of animals analysed suggests the presence of etiologic agents not detected with light microscopy. Many infections can only be inferred by the presence and intensity of host response (Lauckner, 1983), although haemocytic invasion is not always necessarily indicators of disease; haemocytic aggregations also occur in response to wounding or other noninfectious stresses (Pipe & Coles, 1995).

Although no intensely pathogenic parasites were detected in the clams examined, survivorship often reveals little about disease, and it is premature to eliminate parasitism as a potential contributor to mortality in the Blake Ridge clam populations, especially since the prevalence of Bacterial Infection I and of haemocytic responses was 10 to 20% greater in clams collected from the mixed beds than in clams from the clam beds. Infection density was light for all parasites, so the potential impact of heavy infestation by these parasites cannot be gauged; parasites that appear innocuous in low numbers can become pathogenic at higher densities (Smolowitz, 1997). The QPX-like protistan parasites in shallow-water bivalves, for example, have been recorded in low numbers in healthy individuals but have been linked to severe mortalities when present in high densities (Whyte et al., 1994; Smolowitz, 1997).

Further study of parasitism in vesicomyids at the Blake Ridge Diapir is merited and should include electron microscopic investigation of parasites, particularly the viral-like mantle and gill epithelial inclusions, to confirm the nature of the infective agents. Use of specific stains to target putative parasites is also warranted, as is use of molecular methods to characterize parasitic infections.

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