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Evaluation of potential health risks to Eastern Elliptio (*Elliptio complanata*) (Mollusca: Bivalvia: Unionida: Unionidae) and implications for sympatric endangered freshwater mussel species

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Abstract

Conservation efforts for freshwater mussel species require identification and evaluation of potential health risks to populations. Sampling large numbers of individual mussels, however, could damage the small, fragile, extant populations of imperiled mussel species. In this study, a small sample size was designed to reduce lethal sampling, costs, and environmental disruption, while still allowing 95% confidence in detecting health risks of 25% or greater prevalence in the population. Health assessments were conducted on twenty specimens of the Eastern Elliptio, *Elliptio complanata*, collected from two North Carolina sites as part of a survey to evaluate potential disease threats to mussels in the region. Bacteriological sampling of the gastrointestinal tracts yielded 18 aerobic bacterial species, of which *Aeromonas hydrophila* (55.0%), *Enterobacter* spp. (40.0%), and *Bacillus* spp. (30.0%) were predominant. Histological lesions of internal organs included mild to moderate digestive gland atrophy and inflammation in one mussel, and mild to moderate parasitism in several individuals. A distinct difference in parasite prevalence was evident between infections in *E. complanata* from the two collection sites. The trematode metacercaria of *Homalometron armatum* and what appears to be three gill ciliate species, the most abundant being tentatively identified as the scyphidiid peritrich *Mantoscyphidia* sp., were found in mussels from one site only. This study demonstrates a comprehensive diagnostic approach incorporating multiple modalities to assess the health status of mussel populations, while minimizing the sample size required to obtain valuable information. Furthermore, this study provides baseline health data of *E. complanata* at two sites in south-central North Carolina and suggests the potential usefulness of *E. complanata* as an environmental bioindicator of health risks to sympatric threatened freshwater mussel populations.

Introduction

North America supports the greatest diversity of freshwater mussel fauna in the world (Williams et al., 1993; National Native Mussel Conservation Committee, 1998). Unfortunately, anthropogenic factors including habitat loss and alteration, commercial exploitation and exotic species introduction, along with non-anthropogenic factors such as predation and disease imperil this diversity (Williams et al., 1993). The

national strategy for freshwater mussel conservation includes efforts to research threats, such as disease risks, for wild populations and reintroduction programs (U.S. Fish and Wildlife Service, 1996; National Native Mussel Conservation Committee, 1998). Currently, relatively little is known about diseases that affect freshwater mussels. Methods are needed to assess health risks that could imperil fragile extant native mussel populations or impair successful augmentation or reintroduction efforts.

Developing and testing diagnostic methods require sampling which could potentially damage small populations. While certain statistical benefits exist in sampling large numbers of animals for health assessments, smaller sample sizes pose less risk to populations with limited distribution and can still offer statistical power in identifying potential health risks. A sample size of ten animals allows detection of disease or potential pathogens occurring at prevalences of greater than 25% with 95% confidence (Ossiander & Wedenmeyer, 1973). Small sample sizes minimize lethal sampling, costs, and environmental disruption needed to obtain health information, while still permitting the detection of highly prevalent potential health risks with reasonable confidence, particularly important in managing threatened and endangered species. Furthermore, an approach using multiple diagnostic techniques instead of a single modality on each sampled mussel would not only provide more comprehensive health assessments of animals, but also minimize the numbers of mussels required to acquire valuable health information.

The Eastern Elliptio, *Elliptio complanata* (Lightfoot, 1786), in the family Unionidae is commonly found in Atlantic slope rivers along the east coast of North America and has been used as a biomonitor for bacterial, organic pollutant, and trace metal water contamination (Tessier et al., 1984; Turick et al., 1988; Muncaster et al., 1989). This species is sympatric with several endangered mussel species including the Carolina heelsplitter, *Lasmigona decorata* (Lea, 1853). This sympatry and availability make *E. complanata* a potentially useful surrogate for evaluation of health risks to some endangered mussel species. This study incorporates a multiple-modality diagnostic approach to examine the health status of *E. complanata* from the two sites where the Carolina heelsplitter persists in the wild.

Materials and methods

Twenty adult specimens of *E. complanata* were collected in early November 1998, ten from each of the two sites in Union County (Goose Creek [site 1] and Waxhaw Creek [site 2]), south-central North Carolina, where the endangered *L. decorata* is known to exist. The sample size minimized lethal sampling while accommodating detection of disease or potential pathogens occurring at prevalences of greater than 25% with 95% confidence (Ossiander & Wedenmeyer, 1973). Mussels were placed in damp mesh bags in coolers at ambient temperature for no longer than 5 h prior to processing. Mussels were evaluated externally and rinsed in sterile water to remove debris. Lengths of mussel shells were measured by ruler (Hinch et al., 1989). Shells were opened at the hinge with a blunt metal spatula, and the digestive gland was biopsied aseptically with a scalpel after serosal disinfection with 70% isopropyl alcohol. These samples were placed in BBL Port-a-Cul tubes (Becton Dickinson and Company, Cockeysville, MD 21030) for transport to the clinical microbiology laboratory of North Carolina State University College of Veterinary Medicine (NCSU-CVM) for bacterial culture. Within 24 hr of collection, digestive gland samples were transferred to three thioglycollate broth-enriched media, Columbia blood agar, phenyl ethyl alcohol agar, and desoxycholate agar, and incubated at both 20 °C and 35 °C. After 48 h incubation, colonies were subcultured by plating isolates on the three media at both 20 °C and 35 °C. Pure cultures were isolated after 24 h incubation, and bacterial species were identified using a bioMerieux Vitek Jr. biochemical identification system (bioMerieux Vitek, Inc., Hazelwood, MO 63042).

The dorsal valve was removed from each mussel, and the remainder of the body placed in 10% neutral buffered formalin after transverse incisions were made in foot and gonadal tissue to allow for adequate formalin penetration. A diagonal cross-section was trimmed from each formalin-fixed mussel and processed for routine paraffin embedding (Howard & Smith, 1983). Four-micron sections were stained with hematoxylin and eosin (H&E) and evaluated by light microscopy. Mussel gender was determined histologically. Internal organs evaluated histologically included mantle, gill, gastrointestinal tract, digestive gland, kidney, cardiovascular tissue, and foot. Special stains, including Fite's acid fast, Gomori's methanamine silver (GMS), Macchiavello, and periodic acid Schiff (PAS) stains,

were used when appropriate to evaluate histological lesions (Luna, 1968). Parasites were identified on the basis of both sectioned material and whole mount preparations of small representative pieces of gill tissue stained with Van Cleave's hematoxylin.

The 2-tail Fisher's exact test (Glantz, 1997) was used to evaluate potential associations between site of collection and clinical, microbiological, and histological findings. A P value of less than 0.05 was considered significant.

Results

Length of mussels ranged from 6.8 to 9.6 cm. The median length of mussels collected from site 1 was 7.6 cm with quartiles of 7.1 and 7.9 cm. The median length of mussels collected from site 2 was 8.6 cm with quartiles of 8.1 and 8.8 cm. The heights of the collected mussels varied by site, appropriate to the length measurements (site 1: median 4.0 cm, quartiles 3.9 and 4.2 cm; site 2: median 4.9 cm, quartiles 4.6 and 5.5 cm). All mussels from both sites had some degree of bilateral periostracal loss associated with the hinge. No gross internal lesion was noted in any mussel sampled.

Mixed bacterial cultures were obtained from eight of ten mussels from each site sampled. One to four bacterial species were isolated from each mussel's digestive gland, with the exception of one mussel from site 2, which produced no bacterial colony at either 20 or 35 °C. Of the 46 total bacterial isolates recovered, 18 grew at 20 °C only, 12 grew at 35 °C only, and 16 grew at both temperatures. Some bacterial species had colonies with varying temperature dependence for growth (Table 1). For example, one isolate of *Vibrio alginolyticus* grew at 35 °C only, another grew at 20 °C only, and a third grew at both temperatures.

Among the 18 recognized bacterial species cultured from the 20 specimens of *E. complanata*, 4 were found in mussels from both sites (Table 1). *Aeromonas hydrophila* (55%), *Enterobacter* spp. (40%), and *Bacillus* spp. (30%) were predominant at these two sites. There was an apparent difference in composition of bacterial species detected in mussels at the different sites. *Enterobacter intermedius*, *Enterobacter amnigenus* biogroup 2, *V. alginolyticus*, *Escherichia coli*, *Serratia marcescens*, *Proteus mirabilis*, and *Klebsiella pneumoniae* were found in mussels from site 1. *Enterobacter cancerogenus*, *Pantoa agglomerans*, *Vibrio fluvialis*, *Escherichia hermanii*, *Morganella morganii*, and Group D *Streptococcus* were isolated from mussels at site 2.

Table 1. Incubation temperatures for bacterial species isolated from gastrointestinal tracts of *Elliptio complanata* at the two collection sites

Site	Bacterial species	Number of isolates obtained		
		35 °C	20 °C	20 °C and 35 °C
Site 1 (Goose Creek)	*Aeromonas hydrophila*	0	3	4
	Bacillus spp.	1	1	0
	Enterobacter amnigenus biogroup 2	0	1	0
	Enterobacter cloacae	0	1	1
	Enterobacter intermedius	0	1	0
	Escherichia coli	2	0	0
	Hafnia alvei	1	0	0
	Klebsiella pneumoniae	0	2	0
	Proteus mirabilis	1	0	1
	Serratia marcescens	0	0	2
	Vibrio alginolyticus	1	1	1
Site 2 (Waxhaw Creek)	*Aeromonas hydrophila*	0	2	2
	Asaccharolytic gram negative rod	0	0	1
	Bacillus spp.	1	0	3
	Enterobacter cancerogenus	1	0	1
	Enterobacter cloacae	0	2	0
	Escherichia hermanii	1	0	0
	Group D *Streptococcus*	1	0	0
	Hafnia alvei	0	2	0
	Morganella morganii	1	0	0
	Pantoea agglomerans	0	2	0
	Vibrio fluvialis	1	0	0
	No growth	0	0	1
	Totals	12	18	16

Histological evaluation of *E. complanata* indicated that three females and seven males were collected from site 1 and five females and five males from site 2. Nine of ten mussels from site 1 had moderate infections of the encysted trematode *Homalometron armatum* in the foot muscle (4–20 per histological section). No trematode cysts were observed in animals from site 2. Those mussels with heavier infections of *H. armatum* also had cysts in the mantle, gonad, digestive gland, and kidney. Cysts of this trematode were characterized by an oval to circular eosinophilic homogenous cyst wall up to 12 μm thick and 104 to 233 μm in diameter (Figures 1 and 2). The cyst wall consisted primarily of parasite material with a thin outer basophilic layer of host origin surrounded by fibroblasts. Little or no local tissue inflammation was associated with the trematode cysts. The difference in prevalence of infection was statistically significant between collection sites ($P < 0.01$). No other trematodes were identified in mussel tissues in this study.

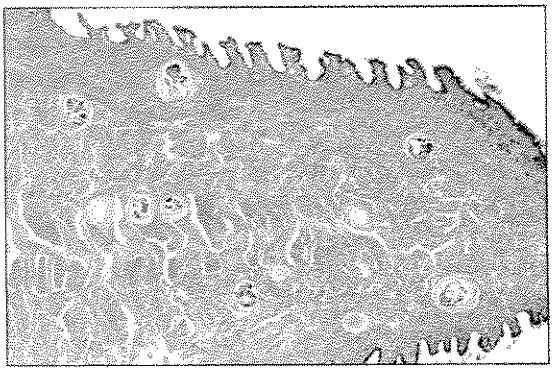

Figure 1. Size variation of metacercariae of *H. armatum* in foot tissue of *E. complanata* from site 1 (H&E, actual magnification 34×).

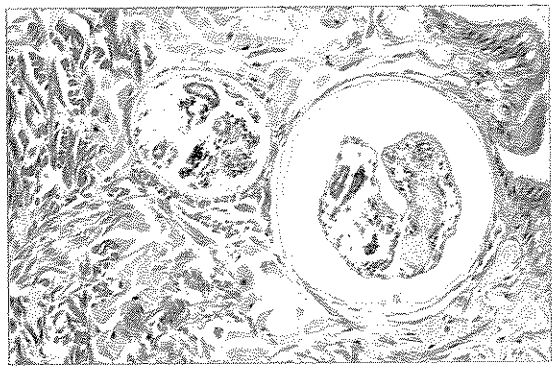

Figure 2. Higher magnification of metacercaria of *H. armatum* in foot tissue of *E. complanata* from site 1 (H&E, actual magnification 178×). Note the lack of an associated inflammatory response.

Six mussels from site 1 hosted mild to moderate infections of at least one and possibly three gill ciliates (Figures 3 and 4). Scattered along the internal gill arch surface, the most abundant ciliate, a species tentatively identified as the scyphidiid peritrich *Mantoscyphidia* sp., ranged from 11 to 29 μm in diameter and had both a deeply basophilic, C-shaped macronucleus and a pale eosinophilic, adoral ciliary zone. No host inflammatory response was associated with any gill parasite. No gill protozoans were observed in gill sections from *E. complanata* collected at site 2 and no ciliates were found in any other tissues from either site.

Only one mussel from site 2 had any histological evidence of infection. A rare microorganism, 11 to 28 μm in diameter, deeply basophilic, acid fast positive, PAS negative, and GMS negative, was evident in the digestive gland epithelium (Figure 5). A similar microorganism was also noted in the digestive gland epithelium of one mussel from site 1. No inflammatory infiltrate was associated with this unidentified agent.

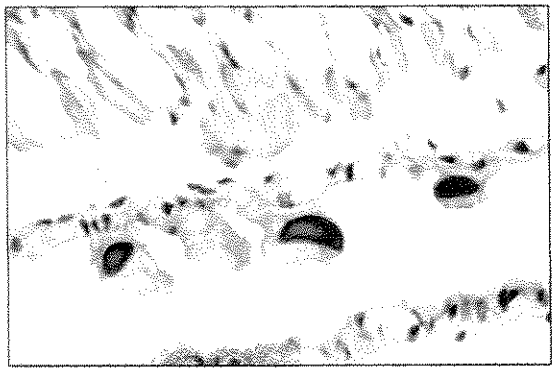

Figure 3. Gill tissue of *E. complanata* from site 1 exhibiting specimens of the most abundant ciliate, a species tentatively identified as the scyphidiid peritrich *Mantoscyphidia* sp. (H&E, actual magnification 476×).

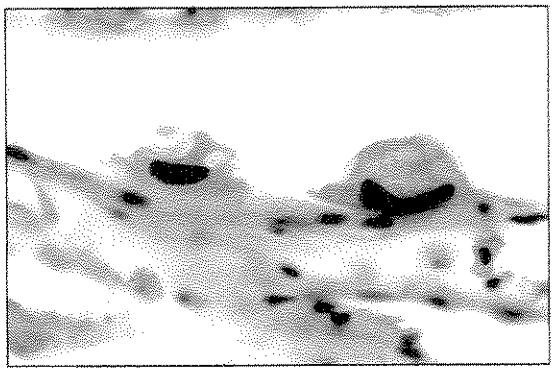

Figure 4. Two specimens of what may be a trichodinid peritrich ciliate on gill of *E. complanata* from site 1 (H&E, actual magnification 611×).

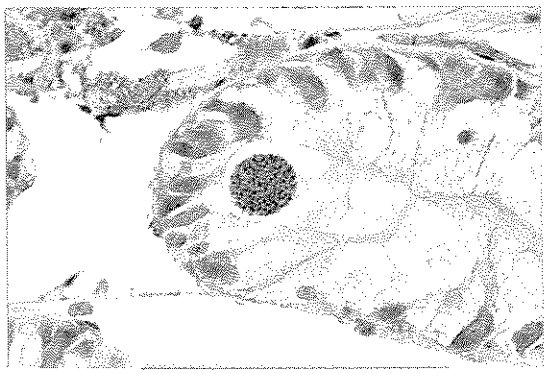

Figure 5. Unidentified microorganism in digestive gland epithelium of *E. complanata* from site 2 (PAS, actual magnification 468×).

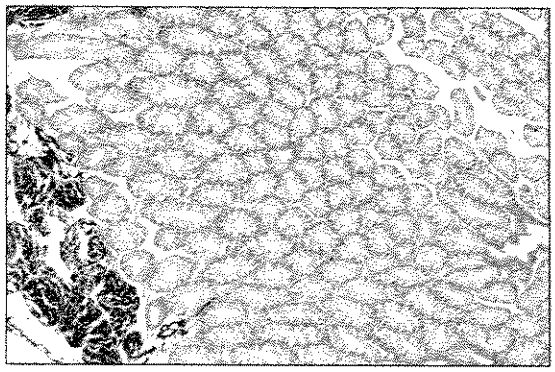

Figure 6. Histologically normal digestive gland of *E. complanata* from site 2 (H&E, actual magnification 32×).

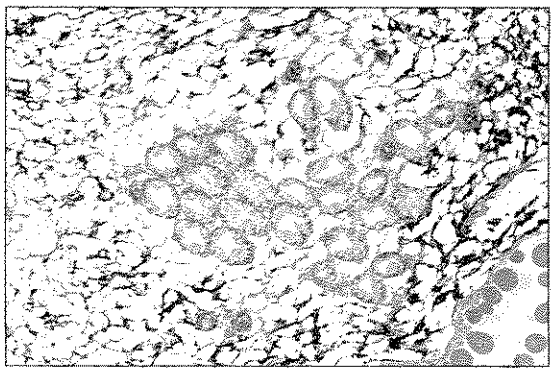

Figure 7. Moderate digestive gland atrophy and inflammation of *E. complanata* from site 2 (H&E, actual magnification 32×).

Mild to moderate, focal digestive gland atrophy associated with moderate granular hemocytic inflammation was found in one mussel from site 2 (compare Figure 7 with Figure 6). No associated pathogen was seen, and the underlying cause for this lesion was not determined.

Discussion

This study provided a health assessment of a freshwater mussel species using a combination of morphometric, microbiological, and histological techniques. The sampling of only ten animals at each site minimized the amount of lethal sampling and environmental disruption required to perform this survey, which is particularly important should similar assessments be applied to threatened and endangered mussel populations. This sample size still allowed us to detect highly prevalent potential health risks with reasonable confidence, a necessary step in evaluating ecosystem health and managing threatened and endangered species. The strategy of using multiple diagnostic modalities further reduced sampling mortality, time, and costs.

Lengths of the mussels in this project were consistent with those previously reported for *E. complanata* by Johnson (1970) and Clarke (1981). The loss of the periostracum seen in specimens from both sites could have resulted from mechanical or biochemical dissolution of the shell, or a disease process damaging the outer shell layer as has been described in marine bivalves (Carriker, 1996). No concurrent disease condition was identified histologically in soft tissues of affected mussels that would have accounted for the high prevalence of periostracal changes. We suspect that mechanical wear primarily contributed to development of these lesions, though chemical dissolution of the shell cannot be ruled out because concurrent water quality or sediment assessments were not conducted.

A need exists to standardize methods for health assessment of mussels and other freshwater bivalves. Bacteriological sampling in this study was designed to help identify components of a complex flora in a mussel species. Microbial analysis of the bacterially rich gastronintestinal tract as compared to other internal tissues enhanced detection of mussel flora and potential pathogens (Al-Jebouri & Trollope, 1981). The finding that bacteria isolated from the mussels had different thermal growth characteristics emphasized the importance of culturing mussel tissue at multiple temperatures to maximize isolation of organisms. Bacteriological findings were consistent with those previously reported in freshwater and saltwater bivalves. Marine- and brackish-water mussels have been shown to have resident gastrointestinal flora predominately of the genera *Vibrio*, *Pseudomonas*, *Bacillus*, and *Staphylococcus* (Hariharan et al., 1995). Bacterial cultures of soft tissues from freshwater mussels in the Ohio River have grown predominately motile *Aeromonas* and *Pseudomonas* spp. (Starliper et al., 1998; Starliper & Morrison, 2000). Isolation of enteric bacteria such as *Lactobacillus* spp., *Aeromonas hydrophila*, *Enterobacteriaceae*, and fecal coliforms has been associated with environmental fecal contamination in previous studies involving freshwater and estuarine mussels (Al-Jebouri & Trollope, 1984; Turick et al., 1988; Hariharan et al., 1995). The digestive gland flora identified in this study likely reflect local bacterial microhabitats. The predominance of *Aeromonas hydrophila* and *Enterobacter* spp.

isolated from our samples offers potential evidence of environmental contamination in these two south-central North Carolina streams. Further study would be required to establish this relationship, but the use of our standardized method for establishing baseline digestive gland bacterial flora facilitates future studies including evaluation of morbidity or mortality events.

Several trematodes have been known to infect freshwater unionid clams (e.g., Genter & Hopkins, 1966; Fuller, 1974; Bower et al., 1994). Trematodes in this study were identified only by characteristics apparent on histological sections of the organisms. Most encysted metacercariae resembled *Homalometron armatum*, a species already known to North Carolina and previously described by Hopkins (1937) and Miller (1959). The spinous tegument of trematodes in this study differentiated these trematodes from *Polylekithum ictaluri*, which also encysts in unionids (Seitner, 1951). Additionally, diagnostic dark excretory refractile concretions are more pronounced in *P. ictaluri* metacercariae than those of *H. armatum*. We noted an obviously longer prepharynx than pharnyx in a few sectioned metacercariae from *E. complanata*. This characteristic is consistent with *H. armatum* in contrast to a related smaller digenean, *Microcreadium parvum*, known to encyst in unionid clams and bear a typically shorter prepharynx than pharnyx (Simer, 1929; Hopkins, 1937). The encysted specimens of *H. armatum*, unlike those of *M. parvum*, continue to grow after the first week of infection in the clam intermediate host. We noted a wide range in cyst size as would be expected with *H. armatum*, but because few sections revealed features that could differentiate species, some of the individuals could have been *M. parvum*. A mixed infection of trematodes could not be ruled out given the limitations of histological evaluation of sections as the only method for parasite identification. No other trematodes, such as *Aspidogastor* or *Cotylogastor* spp., were definitely identified in this study. Confirmation of more than one trematode species might be achieved by teasing whole live specimens from their cysts for examination and feeding cysts to potential definitive hosts to assess infectivity. These techniques should be considered in future studies of mussel health and parasitism in the region.

The complex life cycles of trematodes give some insight into the ecology of the hosts. *Homalometrum armatum* infects a variety of unionid clams and matures in several species of freshwater fish. Known definitive hosts for both *H. armatum* and *M. parvum* include the freshwater drum, *Aplodinotus grunniens*, and a wide range of centrarchids for at least *H. armatum* (Simer, 1929; McGraw & Allison, 1967; Underwood & Dronen, 1984). In Texas, McGraw and Allison (1967) found the mature adult parasite in 1 of 25 specimens of the centrarchid *Lepomis megalotis*, while Genter and Hopkins (1966) identified *H. armatum* infections in seven of nine unionid species in the same river system. Infections relate at least partially to the specific feeding behavior of these fishes on the mussel second intermediate host, as well as to appropriate conditions allowing mussels to become infected with cercariae shed from the appropriate amnicolid snail first intermediate host. A moderate infection usually indicates an intact ecosystem with necessary hosts for completion of the trematode life cycle. In contrast, the absence of an infection may be due to lack of or damage to local biodiversity, disrupting the worm's life cycle (Overstreet, 1997). Disparity in the degree of trematode infections in *E. complanata* between the two sampling sites could indicate differences in host prevalence between these sites.

Identification of the peritrich ciliate symbionts in histological observations was difficult because the sections portrayed mostly lateral views of the organisms. Pieces of gill tissue were stained as whole mounts to better assess the ciliates. The most common species was a sessile scyphidiid peritrich, characterized by a relatively broad scopula and a compact C-shaped macronucleus. Fixed material contained individuals of varying sizes, but morphologically similar enough to be considered a single scyphidiid species. Until 1980, such ciliates would have been classified in the genus *Scyphidia*. Jankowski (1980, 1985) split the genus into five genera based solely on differences in host type or substratum. Members infesting marine and freshwater gastropods were placed into the genus *Mantoscyphidia*, which has 14 species reported in gastropods to date (Basson et al., 1999). There is no current generic taxon including such ciliates from bivalves, but the species found in *E. complanata* could tentatively be identified as *Mantoscyphidia* sp. To further characterize these parasites, evaluation of live specimens, Protargol impregnation of fixed tissues, and scanning electron microscopy would have been useful and is suggested for any future evaluation of such ciliates.

There also appeared to be a few specimens of a possible trichodinid with a C-shaped macronucleus and a conspicuous circular adoral ciliary zone, but

no cross-section demonstrated the diagnostic aboral adhesive disc. Members of the genus *Trichodina* are commonly associated with freshwater and marine molluscs (e.g., Haider, 1964), and at least a few of the suspect ciliate individuals in *E. complanata* might be *Trichodina* sp. Based on size and shape, this parasite does not appear to be *Trichodina unionis*, which infests several unionid species in Europe (Raabe & Raabe, 1961), reaches well over 100 μm wide by 20 μm high, and occurs primarily on the labial palps (Hampl, 1955). Very low numbers of a third concurrent species, a scuticociliatid ciliate, also occurred on the gills. As with trematode metacercaria, the prevalence and intensity of the trichodinid infestations may provide some insight into the environmental conditions and health between the two creeks in North Carolina. The infestation by ciliates, especially when in high intensities, is often associated with a correspondingly high level of organic material (Overstreet, 1993).

No predisposing factor or definitive cause was determined for the digestive gland atrophy noted in one mussel. Whereas digestive tubule atrophy could result from contamination or some other pathogenic cause, it could also result from normal environmental or biological conditions (Weinstein, 1997; Winstead, 1998). No conclusions can be drawn from a single clam in this study. The inflammatory response, however, does suggest a pathological condition. If acute pathogenesis was associated with mortality, the detrimental impact on mussels could be larger than statistics would indicate. This lesion should be considered in future health assessments of mussels.

Site differences in parasite load among *E. complanata* specimens were statistically significant, even with the small sample size in this study. Small sample sizes can identify potential disease risks to mussel populations while minimizing the negative impact of lethal sampling on a population. With expected future development and construction in areas of North Carolina where endangered mussels persist, it is important to establish current status and future indicators of aquatic animal health. This study provides a health assessment of *E. complanata* that can serve not only as a monitor of present environmental health status in North Carolina creeks but also as an initial baseline for future comparison and evaluation of overall mussel health in the region. While non-lethal methods would have been preferable in conducting these health assessments, non-lethal techniques have not been developed for comprehensive health determination of mussels. Development of standardized non-lethal protocols is needed to allow comparisons of disease risks over time within and among mussel populations. In the interim, use of a surrogate provides a valuable alternative to manipulation of endangered bivalve subpopulations in evaluating health risks (Milam & Farris, 1998). *Elliptio complanata* is a common freshwater mussel species sympatric with the endangered Carolina heelsplitter. As such, it could potentially serve as an important bioindicator of environmental and mussel health in the few remaining regions in North and South Carolina where specimens of *L. decorata* exist. As a surrogate, the Eastern Elliptio could provide critical information for management, augmentation, and reintroduction programs involving an endangered bivalve species.

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