Causes, Diagnostics, and Distribution of an Ongoing Penaeid Shrimp Black Gill Epidemic in the U.S. South Atlantic Bight

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CAUSES, DIAGNOSTICS, AND DISTRIBUTION OF AN ONGOING PENAEID SHRIMP BLACK GILL EPIDEMIC IN THE U.S. SOUTH ATLANTIC BIGHT

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ABSTRACT Penaeid shrimp including *Litopenaeus setiferus* (white shrimp), *Farfantepenaeus aztecus* (brown shrimp), and *Farfantepenaeus duorarum* (pink shrimp) support one of the most valuable commercial fisheries in the U.S. Southeast Atlantic. Since the late 1990s, the fishery, especially in coastal Georgia and South Carolina, has experienced a significant decline. A contributing factor to this decline has been hypothesized to be a severe outbreak of shrimp black gill caused by a ciliate parasite. DNA sequence-based analysis of the gill parasite small subunit rRNA gene identifies the proposed causative agent as being closely related to the apostome ciliate *Hyalophysa chattoni*; however, morphological characteristics of the ciliate observed by electron microscopy are inconsistent with this identification. Although studies are ongoing to identify this ciliate, it was possible to develop a diagnostic polymerase chain reaction-based assay targeting the small subunit rRNA gene and use it to investigate the seasonal and geographic distribution of the parasite in wild shrimp populations. Next-generation sequencing of the gill microbiome confirmed that when black gill is at its peak, microeukaryotic communities were dominated by apostome-related ciliates, but over the year highly diverse communities of gill associates, including other potential ciliate, fungal, euglenozoid, and amoeboid parasites are commonly detected. Improved molecular diagnostics and the ability to explore the diversity of potential parasites in shrimp provide important new insights into the relationship between the shrimp fishery and black gill.

KEY WORDS: penaeid shrimp, *Apostome ciliate,* black gill, parasite, fishery, next generation sequencing

INTRODUCTION

The condition of darkened gill tissue in crustaceans known generically as black gill, black spot, and black death is reported widely among shrimp and other crustaceans in both the wild and aquaculture settings (Bower et al. 1994, Stentiford et al. 2012). Black gill in crustaceans is the result of a generalized immune response to a number of viral, bacterial, and protist pathogens and parasites that activate the crustacean innate immune response (Cerenius et al. 2010). Metal and organic contaminants have also been reported to illicit black gill symptoms in various crustacean species (Fontaine & Lightner 1975, Rao & Doughtie 1984). In aquaculture settings, ascorbic acid deficiency is also known to evoke black gill in penaeid shrimp (Magarelli et al. 1979). Since the early 2000s, there has been an ongoing epidemic of shrimp black gill (sBG) in wild penaeid shrimp populations along the southwestern Atlantic coastal zone with its epicenter off the coasts of Georgia and South Carolina (Gambill et al. 2015). The primary species that have been affected are *Litopenaeus setiferus* (white shrimp) and *Farfantepenaeus aztecus* (brown shrimp). The cause of the outbreak has been attributed to a ciliate parasite, although the identity of the ciliate remains unclear. As described earlier, the symptomatic melanized gills associated with sBG may be a result of an innate immune system response to the ciliate.

This response involves phagocytosis, hemocyte aggregation, nodulation/encapsulation, synthesis of antimicrobial peptides, and activation of proteolytic cascades leading to tissue melanization (Hall 1999, Burnett & Burnett 2015). The presence of tissue invasive ciliates is understood to trigger the activation of the prophenoloxidase system in the hemocytes that catalyzes the oxygenation of phenols, leading to the synthesis of the dark pigment melanin and thus resulting in the symptomatic darkened gills (Aspan et al. 1995, Cerenius & Soderhall 2004, Cerenius et al. 2010, Amparyup et al. 2013). The immune system is thought to cause the death of invading ciliates as a result of asphyxiation, production of cytotoxic quinones via the prophenoloxidase system, free radicals, and antimicrobial peptides (Nappi et al. 1995, Gillespie et al. 1997, Hall 1999). In penaeid shrimp, unidentified apostome ciliate cysts are associated with black gill disease in the Gulf of Mexico (Couch 1978, Overstreet 1978). Additionally, the apostome ciliate *Synophrya* has been associated with black gill in crabs from the Gulf of Mexico (Landers 2010), in prawns from Australia (Lester & Paynter 1989), and in various decapods found on the North Carolina coast (Johnson & Bradbury 1976).

As recently discussed by Burnett and Burnett (2015), it is quite likely that crustacean immune responses that result in the formation of hemocytic melanized nodules impair cardiovascular function and metabolism. The presence of nodules in gill tissues is thought to interfere with critical gill functions including respiration and ion regulation that rely on unimpeded flow of hemolymph through the gill vasculature (White et al.

1985, Martin et al. 2000, Burnett & Burnett 2015). Respiratory impairment in the white shrimp *Litopenaeus vannamei* (Scholnick et al. 2006) and in the blue crab *Callinectes sapidus* (Burnett et al. 2006, Thibodeaux et al. 2009) by the presence of hemocytic nodules induced by bacterial infection has been experimentally confirmed. Preliminary studies with *Litopenaeus setiferus* indicate physiological impairment, including reduced physical endurance, is associated with shrimp with visible black gill compared with animals that do not exhibit visible symptoms (A. Fowler, personal communication). Although in experimental systems, crustaceans can generally survive diminished respiratory and metabolic function associated with damaged gill tissue, in nature where predation pressure can be intense, secondary mortality may be considerably greater on black gill–impacted shrimp.

In this study, the cause and the geographic and seasonal distribution of the current black gill epidemic in the Southwestern Atlantic coastal fishery in Georgia and South Carolina and its impact on the fishery were investigated. An important component of this study was to identify the causative agent of the current black gill epidemic and to develop novel molecular diagnostic tools to detect its presence in symptomatic and asymptomatic shrimp and other possible reservoirs.

MATERIALS AND METHODS

Shrimp Collection and Tissue Preservation

Shrimp were collected routinely in cooperation with the Georgia Department of Natural Resources; Coastal Resources Division (GA DNR CRD) fishery-independent Ecological Monitoring Trawl Survey (EMTS) program (Page 2012) and with the educational trawl programs conducted by the Georgia Marine Extension Service (GA MAREX) (2016). The EMTS program has continuously operated since 1976 collecting shrimp from 42 fixed stations (stratified by sector: creeks, sounds, and ocean) each month from each of six major sound systems along the length of the Georgia coast. Shrimp were collected from stations in Wassaw, Ossabaw, Sapelo, St. Simons, St. Andrews, and Cumberland Sounds. Tow duration is standardized to 15-min bottom time with a 12.2-m (40 ft) flat shrimp net with 4.8-cm (1 7/8 inch) stretch-mesh webbing throughout the body and bag. The GA MAREX trawl program operates exclusively in Wassaw Sound using similar fishing protocols except that a 7.6-m (25 ft) trawl net is used. Shrimp were randomly sampled from each trawl and replicate gill tissue samples were preserved in 70% nondenatured ethanol for polymerase chain reaction (PCR)-based molecular analysis and Zinc Formalin Fixative (Sigma-Aldrich no. Z2902) for histological examination. Gill tissue was preserved immediately upon collection, shipboard. shrimp collected outside the EMTS and GA MAREX programs were collected and preserved in a similar fashion. White shrimp (9) with and without obvious black gill from South Carolina (Charleston Bay) were collected in October 2014. Brown shrimp with obvious black gill (6) were collected in the Gulf of Mexico (29° 0′14′′ N, 92° 17′6′′ W) in October 2015. White shrimp (8) with and without obvious black gill were collected in the Atlantic from Florida near the St. Johns River (30° 29′45′′ N, 81° 7′21′′ W) in September 2014, from several stations in Pamlico Sound, NC (60), in September 2015 and from the Chesapeake Bay (10) (37° 16′36′′ N, 76° 33′45′′ W) in October 2014. Specimens (10) of the northern shrimp

(*Pandalus borealis*) with and without black spot were collected from the Gulf of Maine in April 2016. As with the Georgia samples, replicate gill tissue samples were preserved in 70% molecular grade ethanol for DNA-based analyses and in Zn–formalin for histological analysis. Preservative in vials and instructions were provided to the collectors who collected and preserved gill tissues immediately upon collection and returned them to our laboratories. Information about each shrimp including length, sex, and disease state was also collected. In most cases, routine water quality parameters including water temperature, salinity, and dissolved oxygen concentration were also recorded.

Histology and Microscopy

Gross symptoms of black gill were noted at the time of collection. All shrimp with obvious gill tissue melanization were identified as shrimp with black gill and the severity of melanization was recorded. Severity was ranked by color development as clean (no melanization visible), light brown, dark brown, and black. Light microscopy (LM) of fresh tissue or tissue squashes was used to confirm the presence of encysted ciliates and melanized tissue in representative samples. Tissue fragments were observed using a Leica M205 FA stereomicroscope and tissue squashes were examined using a Zeiss Standard 25 compound microscope.

Routine Histology (Light Microscopy)

Zinc-formalin-fixed gill tissues from white and brown shrimp were processed for routine LM and embedded in paraffin. Five micrometer sections were cut, mounted on glass slides, stained with hematoxylin and eosin, coverslipped, and examined using a light microscope. The ciliates and nodules in 10 high power (×400) microscopic fields of each specimen were counted and reported as numbers/mm^2 of gill tissue.

Thin Section Histology and Electron Microscopy

Gill tissue was fixed in cold 4% glutaraldehyde buffered with 0.05 M sodium cacodylate, pH 7.5. The samples were then rinsed in buffer and postfixed in buffered 2% OsO$_4$. After postfixation, the samples were dehydrated and stored for either plastic embedding [LM histology and transmission electron microscopy (TEM)] or for scanning electron microscopy (SEM). Plastic sections for LM (Spurr epoxy, EMS Cat no. 14300) were cut at 1-μm thickness with a diamond knife on a Sorvall MT2B ultramicrotome and stained with sodium borate-buffered toluidine blue for 10 sec on a hot plate. Tissue sections were observed using a Nikon E600 light microscope. Tissue for SEM was dehydrated to 100% ethanol, critical-point dried and sputter-coated with gold. Prepared tissue was examined using a Zeiss Evo 50 SEM at the Auburn University Research Instrumentation Facility, using both the backscatter and secondary electron detectors. For TEM, Spurr sections of gill tissue were cut at 80–90 nm using a Sorvall MT2B ultramicrotome and stained with uranyl acetate and lead citrate. Sections were photographed using a Zeiss EM10 TEM at Auburn University Research Instrumentation Facility.

Polymerase Chain Reaction Amplification, Cloning, and Sequencing of 18S rRNA Genes from the Georgia sBG Ciliate and *Synophrya* sp. from the Northern Shrimp *Pandalus borealis*

Nearly, the complete small subunit rRNA gene was sequenced from the Georgia sBG-associated ciliate and

Synophrya sp. Prior to this study, the 18S rRNA gene from *Synophrya* sp. had not been sequenced. Total genomic DNA was purified from ethanol (70%)-preserved gill tissue collected from shrimp exhibiting obvious gill tissue melanization using the DNeasy blood and tissue kit following the manufacturer instructions (QIAGEN Inc., Valencia, CA). DNA was initially purified from a collection of 10 brown shrimp (*Farfantepenaeus aztecus*) and white shrimp (*Litopenaeus setiferus*) collected from near Sapelo and Wassaw Sounds, GA, in August 2013 that all had black gill. The presence of the sBG ciliate was confirmed in these samples by microscopy (data not shown). Initially, the V4–5 region of the rRNA gene was amplified using a set of general ciliate-primers based on those originally published by Guo et al. (2012) with degenerate (ambiguous) bases added to capture a larger diversity of ciliates. The modified general ciliate primers were 18SF-ciliate-458 (5′-AGC AGG CGC GHA AAT TRC CCA ATC Y) and 18SR-ciliate-1260 (5′-CCG TGT TGA GTC AAA TTA AGC CG). All primers used in this study were synthesized by Integrated DNA Technologies. *In Silico* analysis using the Silva TestPrime utility (Quast et al. 2013) suggests that these primers would amplify a large diversity of ciliates but are far from universal for ciliates. The primers are predicted to amplify approximately 35% of known Intramacronucleata ciliates and 83% of Postciliodesmatophora ciliates available in the Silva SSU r126 database (data not shown). All primer sequences are provided in Table 1. All samples of shrimp gill tissue with black gill symptoms produced the expected sized fragment of approximately 800 bp. Polymerase chain reaction product from each of the 10 shrimp samples were cloned using the TOPO-TA cloning kit (ThermoFisher Scientific). Standard Sanger sequencing was completed by Functional Biosciences, Inc. (Madison, WI). Based on the sBG ciliate sequences that were recovered, two *Hyalophysa chattoni* and close relative-specific primers were designed; Hyalo-18SF-754 (5′-GGA CAG TTG GGG GCA TTA GT) and reverse Hyalo-18SR-952

(5′-GAC CAA GTT ATA AAA TGG CCA). These primers were paired with universal eukaryotic 18S rRNA primers Univ-18SF-15 and Univ-18SR-1765S (Gruebl et al. 2002) to amplify the full-length rRNA gene from the sBG ciliate. Because the ciliate targeted primers, Hyalo-18SF-754 and Hyalo-18SR-952 also were able to amplify the 18S rRNA gene from *Synophrya* sp. identified microscopically in the northern shrimp specimens from the Gulf of Maine, this same strategy was used to amplify and sequence nearly the complete 18S rRNA gene from *Synophrya* sp.

In addition to PCR amplification of ciliate sequences from shrimp gill tissues using the general ciliate primers, the identity of the ciliate was further explored by partially sequencing the 18S rRNA gene from sBG ciliates (8) that had been individually removed and isolated from gill tissue.

Phylogenetic analysis was facilitated using the MEGA6 evolutionary analysis software package (Tamura et al. 2013) after alignment of the recovered sBG ciliate sequence with all available ciliate 18S rRNA sequences available at the time of analysis (50).

Primer design strategy essentially followed previously described procedures (Frischer et al. 2014) using a reference alignment of ciliates and crustacean sequences and facilitated using the tools available in PrimerPlus 3 (Untergasser et al. 2012), BioEdit (Hall 1999), and MEGA6 (Tamura et al. 2013). All amplicons were cloned and sequenced as described earlier. Sequence fragments were assembled using the CAP Contig utility in BioEdit (Hall 1999).

Development of a sBG Ciliate-Specific Diagnostic PCR Assay

The sBG-specific primers Hyalo-18SF-754 and Hyalo-18SR-952 were evaluated as a specific diagnostic tool for the detection of the sBG ciliate. This primer set produces a 198-bp amplicon of the V5 rRNA region of the sBG 18S rRNA gene. Before evaluation of primer sensitivity and specificity, routine

TABLE 1.

PCR primer sets used in this study.

Forward primer	Reverse primer	Amplicon size (bp)	Annealing temperature (°C)	Specificity	Source
18SF-ciliate-458 5′- AGC AGG CGC GHA AAT TRC CCA ATC Y	18SR-ciliate-1260 5′ CCG TGT TGA GTC AAA TTA AGC CG	802	68	General ciliate	Modified from Guo et al. 2012
Hyalo-18SF-754 5′ GGA CAG TTG GGG GCA TTA GT	Hyalo-18SR-952 5′ GAC CAA GTT ATA AAA TGG CCA	198	56	sBG specific	This Study
Univ-18SF-15 CTG CCA GTA GTC ATA TGC	Hyalo-18SR-92 5′ GAC CAA GTT ATA AAA TGG CCA	937	52	sBG specific	This Study
Hyalo-18SF-754 5′ GGA CAG TTG GGG GCA TTA GT	Univ-18SR-1765S 5′ ACC TTG TTA CGA CTT	198	56	sBG specific	This Study
Univ-18S-577F 5′ CAG CAG CCG CGG TAA TTC C	18S-1180R 5′ CCC GTG TTG AGT CAA AAG C	630	56	General eukaryote	Hadziavdic et al. 2014

diagnostic PCR reaction conditions using Qiagen Master Mix PCR reagents (QIAGEN Inc., Valencia, CA) were determined. Optimized conditions included a 3-min initial denaturation step at 94°C followed by 30 cycles of denaturation at 94°C, annealing at 56°C, and extension at 72°C with a duration of 30 sec for each thermal cycling step. Following the amplification cycles, a final extension at 72°C for 5 min was included. Routine PCR amplification was facilitated using an Applied Biosystems GeneAmp PCR System 9700 (ThermoFisher Scientific). The specificity of the sBG diagnostic assay was examined empirically by attempting to amplify the target 18S rDNA fragment from *in vitro* cultures of several ciliate and algal species, and genomic DNA extracts from grass shrimp (*Palaemonetes* sp.), muscle tissue from a white shrimp (*Litopenaeus setiferus*) that did not exhibit black gill symptoms and gill tissue from a *L. setiferus* specimen with black gill. All shrimp specimens were collected from the Wassaw Sound estuary, GA. The ciliates *Uronema marinum*, *Cohnilembus reniformis*, and *Euplotes vannus* were purchased from the Culture Collection of Algae and Protozoan. Algal cultures, *Thalassiosira weissflogii*, *Rhodomonas* sp., and *Isochrysis* sp. were purchased from the National Center for Marine Algae and Microbiota. The sensitivity of the assay was determined by real time PCR (qPCR). Standard curves for qPCR assays were generated from a cloned fragment of the sBG 18S rRNA gene. qPCR was facilitated using the SsoFast EvaGreen Supermix regents and a CFX96 Real-Time PCR Detection System (BIO-RAD Life Sciences Research).

The Shrimp Gill Microbiome

To detect and identify potential shrimp parasites and other symbionts that were not targeted by the ciliate-directed PCR-based approaches, the presence of eukaryotic associates was detected using generic eukaryotic 18S rRNA-targeted primers followed by high-throughput next-generation sequencing of resultant amplicon mixtures. The collection of all eukaryotic organisms detected in shrimp gill tissue is referred to here as the "shrimp gill microbiome." The shrimp gill microbiome from 10 white shrimp collected monthly from March to December 2014 in Wassaw Sound, GA was determined. An approximate 600-bp fragment spanning the V4 and V5 regions of the18S rRNA gene was amplified using the universal eukaryotic primers Univ-18S-557F and Univ-18S-1180R (Hadziavdic et al. 2014). To inhibit amplification of the shrimp (*Litopenaeus setiferus*) 18S gene, amplification reactions were performed in the presence of a specific penaeid shrimp peptide nucleic acid (PNA) oligonucleotide blocker essentially as previously described except that a PNA blocker specific for *L. setiferus* was used (Troedsson et al. 2008). The penaeid shrimp PNA–blocking oligonucleotide Ls-PNA-18S-735 (5′-Lys-GCC GGC GCA CAT GGG) was designed, developed, and validated in this study essentially as previously described by Troedsson et al. (2008). The PNA was synthesized by PNA Bio Inc (Thousand Oaks, CA). The blocking efficiency of shrimp PNA was empirically determined to prevent the amplification of 99.99% of all shrimp 18S rRNA gene copies in a PNA–PCR reaction (Fig. 1). PNA–PCR reactions (50 μl) were facilitated using 0.5 U/25μl Phusion High Fidelity Taq DNA polymerase (New England Biolabs) 200 μM (final concentration) dNTPs (Promega) 250 nM each (final concentration) of primers and 1.5 μM of the PNA. Template DNA concentration ranged from 5 to 50 ng of template DNA (shrimp gill genomic extracts) per reaction. PNA blocking was
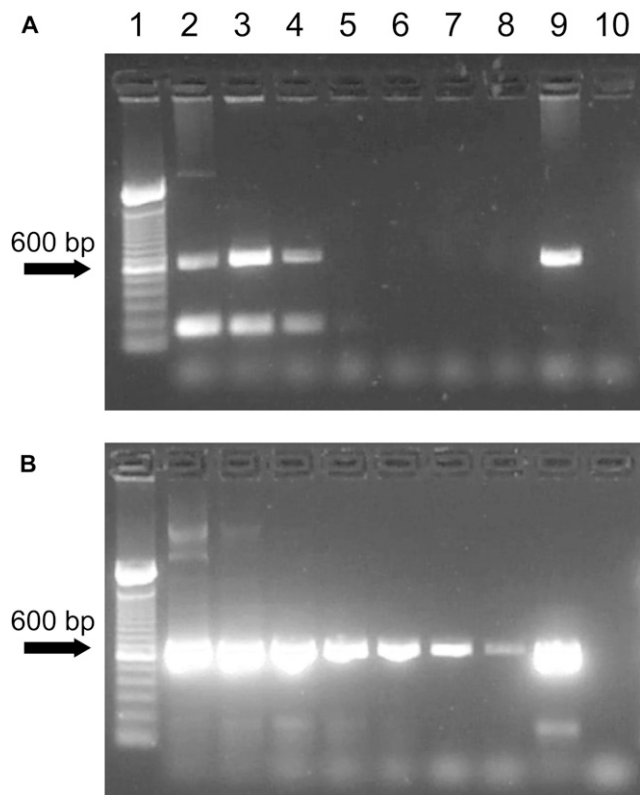

Figure 1. Shrimp PNA blocking PCR (PNA–PCR) assay. PCR amplification of the 18S rRNA gene (V4 and V5 region, ~600 bp) with the universal eukaryote targeted primer set Univ-18S-557F and Univ-18S-1180R in the (A) presence and (B) absence of the PNA blocking oligonucleotide Ls-PNA-18S-735. Approximately four orders of magnitude of blocking were achieved. Lane 1, molecular weight ladder (100 bp); lanes 2–7, serial dilution from 10^8 to 10^2 copies of the 18S rRNA gene from *Litopenaeus setiferus*. The *L. setiferus* 18S gene was cloned into a plasmid vector and cloned plasmid DNA used as the quantitative reference standard. Lane 9, positive control. Lane 10, negative control (water).

incorporated into a standard PCR cycle by including a PNA annealing step (66°C) following denaturation at 98°C (30 sec). The specific PNA–PCR conditions were 25 cycles of 98°C (10 sec), 66°C (30 sec), 60°C (30 sec), and 72°C (30 sec). Following the 30 amplification cycles, an additional 5-min final extension step at 72°C was completed. Amplification reactions were facilitated using an Applied Biosystems GeneAmp PCR System 9700 (ThermoFisher Scientific).

Sequencing of barcoded amplicon libraries (e.g., Mäki et al. 2016) was carried out using Ion Torrent procedures on a Personal Genome Machine (PGM) similar to those described by Clare et al. (2014) except that barcoded libraries were prepared from randomly sheared (~400 bp) preparations of the 630-bp 18S rRNA amplicon and were sequenced on a 316v2 chip with 400 bp chemistry. Standard protocols for library preparation (Ion Xpress Plus Fragment Library Kit MAN00077044 Revision A.0), library templating (Ion PGM Template OT2 400 Kit PGM template preparation MAN0007218 Revision 3.0), and sequencing (Ion PGM Sequencing 400 kit, MAN0007242 Revision 2.0) were followed. Raw sequence reads were filtered using the Ion Torrent Suite software (version 4.2.1) to trim adapter sequences and to remove low-quality and polyclonal

sequences. Sequences were further curated for quality using the Mothur pipeline (Schloss et al. 2009). Quality controlled sequences were uploaded to the SILVAngs pipeline (Quast et al. 2013) where libraries were aligned, dereplicated, and classified.

RESULTS

Gross and Microscopic Black Gill Pathology

The visible symptoms of black gill in both *Litopenaeus setiferus* and *Farfantepenaeus aztecus* include darkened gills ranging in color from light brown to black (Fig. 2A, B). Gill discoloration from black gill is distinct and easily distinguished from general gill fouling. It is not uncommon to observe shrimp that exhibit asymmetric coloration between the right and left laterals. Microscopic and histological observations indicate the presence of melanized hemocytic nodules most commonly occurring at the distal tips of the gill lamellae (Fig. 2C, D). Histological examination often, but not always, reveals the presence of large (30–38 µm) round ciliates in the vicinity of nodules (Fig. 2D). Thin section histology most frequently reveals the invading ciliate in the encysted form attached to the gill exoskeleton rather than internal to the gill tissue (Fig. 3A, B). In rare instances, direct evidence of the ciliate's invasive nature was observed. In one occasion, a change in the host exoskeleton was observed at the sBG ciliate attachment location (loss of the epicuticle at places), and in another occasion tissue infiltration was observed (Fig. 3C, D).

Relationship Between Black Gill and Ciliate/Nodule Abundance

The relationship between ciliate and nodule abundance and gill coloration was investigated in wild shrimp collections. A total of 354 wild brown and white shrimp collected from August 2013 to November 2014 were examined histologically. White and brown shrimp were pooled as the presentation of black gill
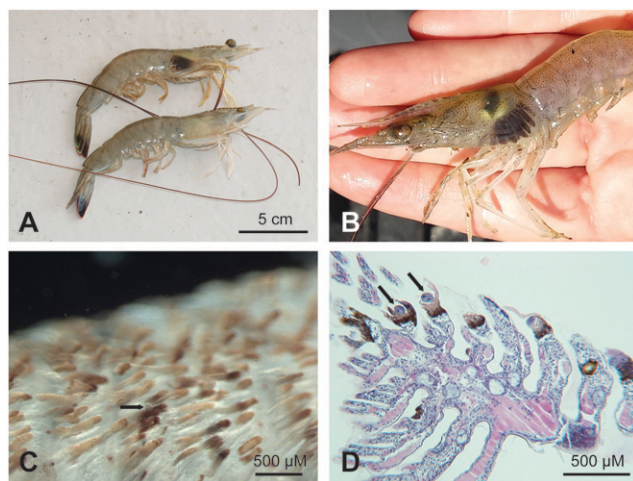

Figure 3. The sBG ciliate. Noninvasive (A) divided and (B) undivided encysted sBG ciliate attached to the gill exoskeleton of a white shrimp. Thin plastic tissue sections stained with toluidine blue. (C) Transmission electron micrograph of the sBG ciliate invading the gill tissue of a white shrimp. Arrows indicate edges of the entrance wound on the gill exoskeleton. (D) Enlargement from Figure 2C revealing that the invading ciliate is surrounded by a thin barrier or cyst wall which separates the cell from the host tissue, and appresses the cilia against the cell surface (arrows).

Figure 2. Shrimp black gill. (A) White shrimp (*Litopenaeus setiferus*) with and without black gill. (B) Gill of white shrimp with severe black gill. (C) Gill tissue from white shrimp with black gill observed under a stereomicroscope. Arrow highlights melanized distal tips of gill lamellae. (D) Hematoxylin and eosin–stained paraffin-embedded histological section (5 µm) of gill tissue from a brown shrimp (*Farfantepenaeus aztecus*) with black gill. Arrows identify invading ciliates.
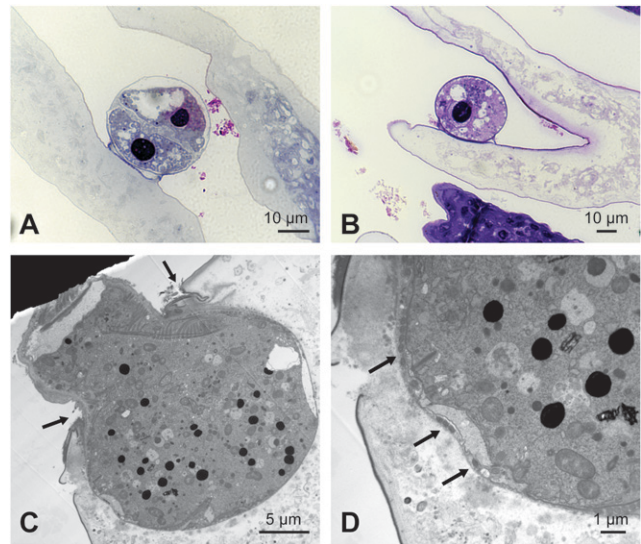
symptoms was indistinguishable in these two species (data not shown). Shrimp were collected from sites distributed across the entire length of the Georgia coast. There was a significant linear relationship between gill color and both ciliate ($r^2 = 0.90$) and nodule ($r^2 = 0.95$) abundance (Fig. 4). Although variability was high, visible symptoms of black gill were observed in shrimp that had on average greater than 3 ciliates or 9 nodules/mm^2 of gill tissue. The significant correlation between the symptoms of black gill and the abundance of ciliates and nodules and a lack of correlation with other parasites supports the conclusion that this ciliate is the likely causative agent of black gill in the South Atlantic Bight region. In the absence of a definitive taxonomic identification, this ciliate is referred to as the Georgia sBG ciliate.

Identity of the Black Gill Ciliate

Nearly, the complete small subunit ribosomal RNA (18S rRNA) gene (1634 bp) from the black gill–associated ciliate was recovered and sequenced. The sBG sequence has been deposited in the NCBI Genbank database (KX906567). Phylogenetic analysis places the sBG sequence with high confidence within the Apostomatia and most closely related to *Hyalophysa chattoni* strains HpC-1 (EU503536) and HpC-2 (EU503537) (Fig. 5). Nucleotide similarity between *H. chattoni* and the sBG ciliate is 99.6% and 99.5%, respectively. This level of similarity is consistent with a species-level identity (Clamp et al. 2008). Another representative of the genus *Hyalophysa*, *H. lwoffi* strain HpL-1 (EU503538), shares a 97.5% nucleotide identity with the sBG ciliate though *H. lwoffi* is strictly a freshwater species. To confirm that the sequence derived from gill tissue samples belongs to the rounded sBG ciliate, the *Hyalophysa*-specific
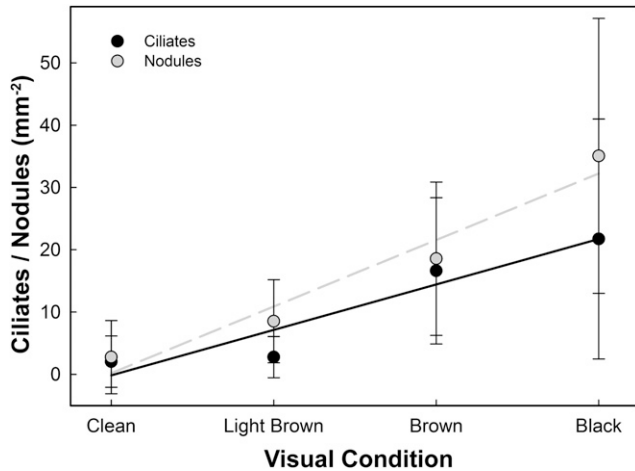
Figure 4. Relationship between the visual symptoms of sBG (gill color) and the concentrations of ciliates (●) and melanized hemocytic nodules (○) in gill tissues from white and brown shrimp. There was a significant relationship between gill color and ciliate ($r^2 = 0.90$, $n = 354$) and nodule ($r^2 = 0.95$, $n = 169$) abundance.

PCR assay was used to amplify the 198-bp rRNA gene fragment from individual sBG ciliates isolated from gill tissue. Sequences obtained from eight individual ciliates were all identical to the sequence derived from the gill tissue samples confirming that the commonly observed rounded encysted ciliate is the sBG ciliate. Because the apostome ciliate *Synophrya* is known to cause black gill in decapod crustaceans (Johnson & Bradbury 1976) and the 18S rRNA from this organism had not previously been sequenced, it was sequenced in this study. *Synophrya* sp. associated with *Pandalus borealis* was sequenced and included in this

phylogenetic analysis. The *Synophrya* sp. sequence was also deposited in Genbank (KX906568).

Despite the species-level similarity between the sBG ciliate and *Hyalophysa chattoni* inferred from small subunit rRNA gene sequence comparisons, morphological investigation by SEM and TEM reveals several inconsistences with this conclusion. Scanning electron microscopy of melanized gills confirms the presence of the encysted, rounded sBG ciliate that is observed in LM paraffin and plastic sections. This gross morphology of the sBG ciliate is not consistent with apostomes such as *Hyalophysa* because *Hyalophysa* is not rounded and it does not divide within the cyst while on the host. Transmission electron microscopy of the rounded sBG ciliate, both divided and undivided forms, reveals a cell with an abundance of lipid droplets and endoplasmic reticulum within the cytoplasm and has been observed on the shrimp exoskeleton and invading gill tissue (Figs. 3C, D and 6C, D). Additionally, the sBG ciliate has disks or plates of membrane within the cytoplasm, which appear to align inside the outer pellicle of the cell, possibly to provide new membrane for growth or food vacuole formation (Fig. 6D). Similar "membrane organelles" are known from apostome ciliates. The invasive ciliate interestingly has a cyst wall surrounding the cell, even as it expands within the host tissue (Fig. 3C, D). The presence of the cyst wall surrounding the internalized ciliate and the numerous lipid droplets is also known for the invasive apostome *Synophrya*. This rounded ciliate corresponds to the sBG organism identified via 18S sequencing, providing strong evidence that the invasive ciliate is an apostome. Additional TEM data are being sought to confirm whether the sBG ciliate is related to the apostome *Synophrya*, which is known to cause gill melanization. In addition to the abundant sBG ciliate, other ciliates are occasionally observed including one with an oblong cyst and an internal morphology
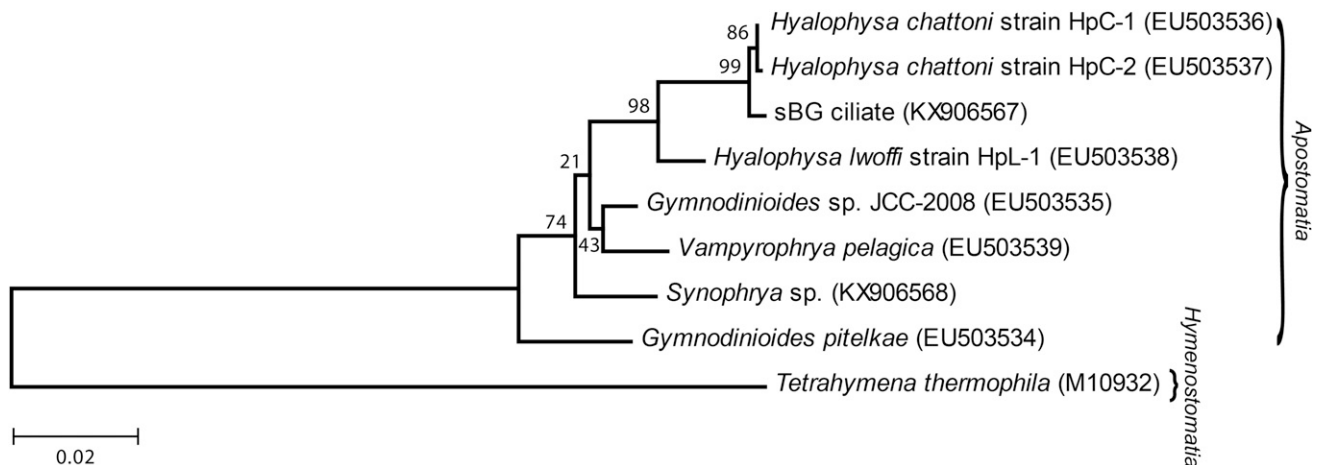

Figure 5. Inferred phylogenetic identity of the sBG ciliate. Phylogenetic analysis was based on the nearly complete 18S gene sequence from the Georgia sBG ciliate determined in this study (KX906567). Based on this analysis, the sBG ciliate is identified as an apostome ciliate closely related to *Hyalophysa chattoni*. Evolutionary history was inferred by using the maximum likelihood method using the Tamura–Nei model (Tamura & Nei 1993). The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained by applying the neighbor-joining method to a matrix of pairwise distances estimated using the maximum composite likelihood approach. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The tree shown includes all available rRNA sequences from Apostome ciliates available at the time of analysis. The Hymenostomatia ciliate *Tetrahymena thermophila* (M10932) was also included as an outgroup. All positions containing gaps and missing data were eliminated. There were a total of 1,588 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al. 2013).
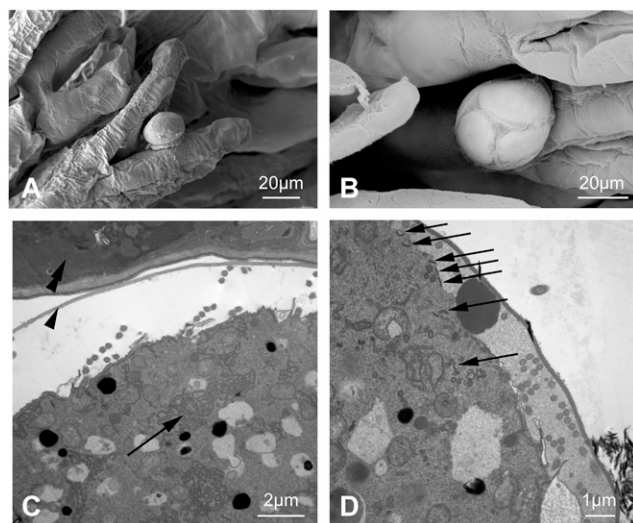
Figure 6. Electron microscopic identification of the Georgia sBG ciliate. Scanning electron microscopy revealed most commonly (A) a rounded encysted ciliate attached to the shrimp gill exoskeleton of specimens with visible black gill. (B) Dividing encysted forms were also observed. Transmission electron microscopy of (C) undivided, noninvasive ciliates reveal large tracts of endoplasmic reticulum (arrows) and lipid droplets (black). This type of ciliate matches the invasive stage shown in Figure 2C, D. Arrowhead, ciliate cyst wall; double arrowhead, shrimp gill tissue. (D) Section through a sBG ciliate showing membrane plates within the cytoplasm and aligning under the outer cell pellicle (arrows).

consistent with a noninvasive apostome such as *Gymnodinioides* or *Hyalophysa*.

Development, Sensitivity, and Reliability of an 18S rRNA-Targeted Diagnostic Assay

Despite the ambiguity associated with the identity of the sBG ciliate, it was possible to design a unique PCR-based diagnostic assay that was specific to the genus *Hyalophysa*. *In silico* exploration of the theoretical specificity of the primer set using the Silva TestPrime utility (Quast et al. 2013) loaded with the ssu-126 database indicated that the primer pair *Hyalophysa*-754F and *Hyalophysa*-952R uniquely matched *Hyalophysa chattoni*. When three nucleotide mismatches were allowed, other apostome ciliates were matched including *Gymnodinoides pitelkae* (EU503534), *Gymnodinoides* sp. JCC-2008 (EU503535), *Hyalophysa lwoffi* (EU503538), and *Vampyrophyra pelagica* (EU503539). Comparison with the sequence obtained from the apostome ciliate *Synophrya* sp. associated with *Pandalus borealis* (KX906568) also indicated that the *Hyalophysa*-targeted primer pair would match the *Synophrya* sp. 18S rRNA gene target with three mismatches. Because it was possible to amplify the *Synophrya* sp. 18S rRNA gene using the *Hyalophysa*-specific primer set, it can be concluded that in practicality the primer set is not species specific but specific to apostome ciliates closely related to *Hyalophysa* utilizing routine PCR reaction conditions. Empirical specificity testing was consistent with *in silico* analysis (Fig. 7). In empirical studies, the primer pair did not amplify gene products from a variety of common ciliates, algae, or uninfected shrimp. Amplicon was produced from the grass shrimp (*Palaemonetes pugio*). The grass shrimp *P. pugio* is known to commonly harbor *H. chattoni*

(Grimes 1976). Quantitative real-time PCR (qPCR) investigations indicated a routine detection sensitivity of the *Hyalophysa*-specific PCR assay of 10 gene copies (Fig. 8A). However, due to extreme variability in rRNA gene copy number in ciliates (Gong et al. 2013), it was not possible to quantify the abundance of the sBG ciliate using a qPCR approach (Fig. 8B). For example, especially for shrimp with a relatively low abundance of ciliates (0–10 ciliates/mm^2 gill tissue), gene copy number estimates ranged by 4–5 orders of magnitude. In extreme cases, animals with 1–5 ciliates/mm^2 tissue estimated by direct microscopic observation contained the same number of sBG 18S rRNA gene copies as animals that had 22–23 ciliates/mm^2 of gill tissue.

The reliability of the PCR diagnostic assay as a means to detect the presence and absence of the sBG ciliate compared reasonably well with visual and histological approaches (Fig. 9). There was a 70.7% (395 of 559) agreement (shaded quadrats) between visual presence of black gill and PCR detection. Discrepancies included 70 of 559 shrimp (12.5%) that visibly did not exhibit black gill symptoms but that were positive by PCR and 16.8% (94 of 559) that exhibited black gill symptoms but were PCR negative. Compared with histological observations, there was a 67.2% agreement (shaded quadrats) with PCR detection (125 of 186). Discrepancies included 24 of 186 shrimp (12.9%) in which ciliates were not observed in histological slides but were PCR positive and 19.9% (37 of 186) samples in which nodules or ciliates were observed in histological preparations but that were PCR negative.

The Shrimp Gill Microbiome

To further investigate the association of the sBG ciliate with black gill symptoms, the total community of gill associates was identified using next-generation sequencing approaches in individual shrimp collected from March to December 2014. The total community of gill associates is referred to here as the gill microbiome. After sequence culling to remove low-quality sequences, sequences that did not have at least 10 replicates in the dataset, sequences identified as shrimp, human, or derived from terrestrial plants, the dataset included 668,725 sequences with an average length of 306 bp. Consistent with histological and PCR-based analysis, excluding metazoan sequences, the gill microbiome of shrimp with symptomatic black gill was dominated by sequences identified as the sBG ciliate (Table 2). Shrimp black gill ciliates dominated the shrimp gill microbiome from July to October, further supporting the correlation between the sBG ciliate and black gill (Fig. 10). The exception to this pattern was the sample collected in September, which was dominated by trematode sequences (data not shown). Other potential parasites included other types of ciliates, protists, fungi, and a variety of trematodes, cestodes, and nematodes. However, none of these groups exhibited a seasonal pattern consistent with the prevalence of black gill. Interestingly, a highly diverse array of metazoan-associated sequences was also recovered from the shrimp gill samples (Table 3). It seems likely that these sequences represent larval forms or are derived from ubiquitous detritus (environmental DNA) trapped on shrimp gills as shrimp forage and occupy the estuarine benthos.

Prevalence and Seasonality of sBG in Georgia

Shrimp with black gill were first officially reported in Georgia in the fall of 1996 (Page 2012). With the exception of
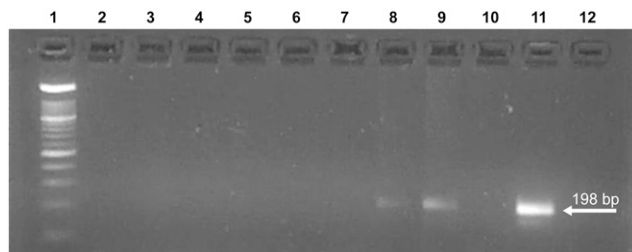
Figure 7. Validation of the specificity of the sBG-specific 18S rDNA-targeted PCR-based diagnostic assay. PCR amplification of genomic DNA extracts from several ciliates, algae, grass shrimp, and black gill symptomatic and asymptomatic white shrimp using the PCR primer set Hyalo-18SF-754 and Hyalo-18SR-952. The diagnostic 198-bp amplicon was only produced from symptomatic white shrimp and grass shrimp and visualized by gel electrophoresis (2% agarose). Lane 1, molecular weight ladder (100 bp); lanes 2–4, ciliates (lane 2—*Uronema marinum*, lane 3—*Cohnilembus reniformis*, lane 4—*Euplotes vannus*); lanes 5–7, algae (lane 5—*Thalassiosira weissflogii*, lane 6—*Rhizosolenia* sp., lane 7—*Isochrysis* sp.); lanes 8–9, grass shrimp (*P. pugio*); lane 10, healthy white shrimp (muscle tissue); lane 11, white shrimp with black gill (gill tissue); lane 12, negative control (water). PCR reactions (30 cycles) were performed in 25-μl volumes using Qiagen PCR Master Mix reagents (Qiagen Inc). Cycling conditions included an initial 10-min denaturation step (94°C) followed by 30 cycles of 94°C (30 sec), 56°C (30 sec), and 72°C (60 sec). The final cycling step was followed by an additional extension period of 10 min at 72°C.
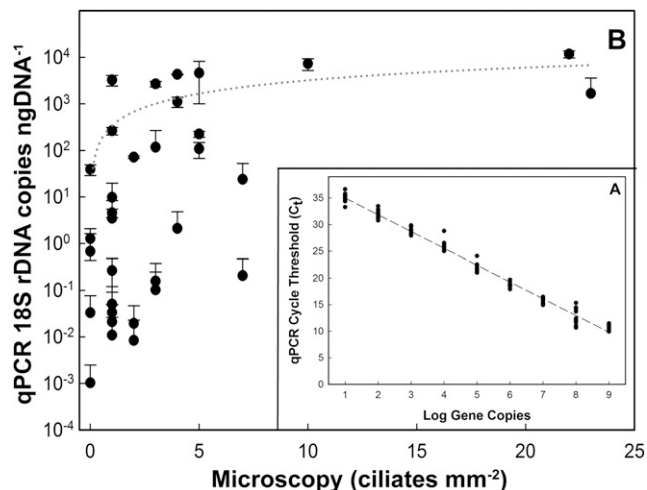

Figure 8. Real-time PCR (qPCR) quantification of the sBG ciliate targeting the 18S rRNA gene with the primer set Hyalo-18SF-754 and Hyalo-18SR-952. (A) Detection sensitivity was ~10 gene copies with a dynamic range of nine orders of magnitude. (B) Particularly at typical levels of ciliate infestation (0–10 ciliates/mm^2 gill tissue), there was not a significant correlation between 18S rRNA gene copy number and the abundance of ciliates observed in histological gill preparations. qPCR assays were facilitated using a CFX96 Real-Time PCR detection system (BIO-RAD Life Sciences Research) with SsoFast EvaGreen Supermix reagents.

1997, 1998, and 2001, black gill has been observed every year since 1996 (Fig. 11A). Shrimp with visible black gill generally first appear in August, peak in September and October, and decline through November and December (Fig. 11B). An exception was 2016 when symptomatic black gill appeared in late May (M. Frischer unpublished observations). Examination of shrimp using the more sensitive PCR diagnostic assay suggests that the ciliate is present from as early in the year as May and can persist through the following March. At least in 2014 and 2015, the prevalence of the sBG ciliate as detected using the PCR diagnostic assay, reached considerably higher prevalence levels than suggested by visual symptoms, especially preceding and following peak prevalence in September and October (Fig. 11B). During the months when shrimp with black gill symptoms are common (August–December), average black gill prevalence has ranged from 6.5% to 45.7% since 1996. Although the long-term annual average prevalence of black gill is ~20%, in the late summer and fall, it is not uncommon for the prevalence of shrimp with black gill in an individual trawl to be nearly 100%.

Geographic Range

To determine the range of the sBG ciliate, black gill symptomatic shrimp were collected from as wide a geographic range as possible and examined for the presence of the Georgia sBG ciliate. Shrimp with visible black gill symptoms were collected in the Gulf of Mexico (29° 0′14′′ N, 92° 17′6′′ W) and in the Atlantic from Florida near the St. Johns River (30° 29′45′′ N, 81° 7′21′′ W) up to Virginia in the Chesapeake Bay (37° 16′36′′ N, 76° 33′45′′ W). Specimens of the northern shrimp (*Pandalus borealis*) were collected from the Gulf of Maine. Although it is not possible to confirm a continuous

distribution from the sampling effort, all the black gill symptomatic penaeid shrimp (Gulf of Mexico to Chesapeake Bay) harbored the same ciliate that occurs in the South Atlantic Bight. Identity was confirmed by both histological and genetic examination.

Establishing the Relationship Between Black Gill and the Fishery

A causal link between black gill prevalence and fishery performance has been difficult to establish or to refute (Fowler et al. 2016). In Georgia, commercial shrimp landing records and black gill prevalence are significantly and negatively correlated although the correlations are weak (Fig. 12). The prevalence of black gill is slightly better correlated with fall shrimp landings ($r^2 = 0.415$, $P = 0.001$) than with total landings ($r^2 = 0.304$, $P = 0.008$). Fall white shrimp comprise the majority of Georgia's shrimp commercial shrimp landings accounting for an average of 55.7% of total landings during the period before black gill became established in Georgia waters (1957–1999) (Fig. 13A); however, since 2000, fall harvests have declined 7.5% from pre-black gill rates of 55.7% of total shrimp landings to an average of 48.2%. The decline in the fall shrimp harvest, in excess of the general decline in the fishery that has occurred, indicates a disproportional effect on fall shrimp populations (Fig. 13B) and is consistent with the hypothesis that black gill has negatively impacted the Georgia wild shrimp fishery. However, fall shrimp landings as a proportion of total shrimp landings appear to have been at the lowest during the mid-1980s and 1990s prior to the emergence of black gill and therefore may not be attributable to black gill during this period. Cold winters may be responsible for these poor harvests. Significant mortality of overwintering white shrimp occurs if temperatures drop below 6°C (DeLancey et al. 2008). Examination of the fishery-independent
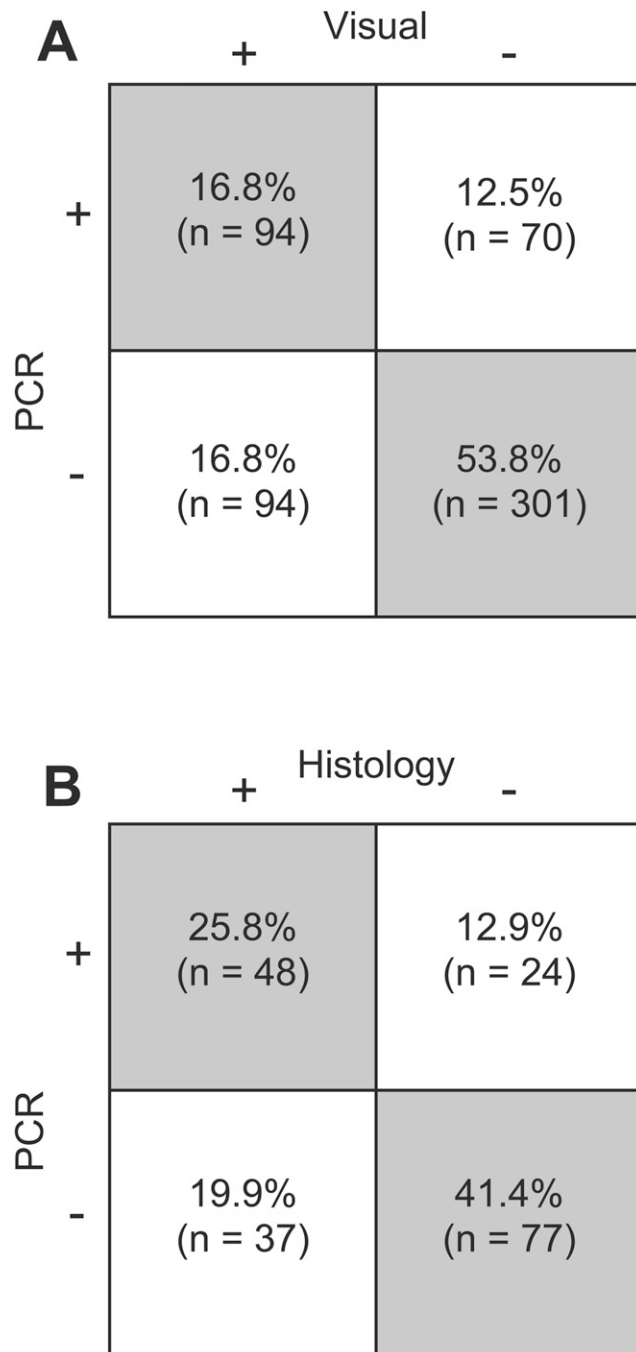
Figure 9. Comparison of (A) visual and (B) histological detection of sBG to PCR-based presence/absence detection in shrimp gill samples. The proportion of samples where both diagnostics were in agreement is shaded.

TABLE 2.

Taxon-level classification of sequences recovered from the *Litopenaeus setiferus* gill parasitome, March–December 2014.

Taxonomic group	Sequences (*n*)	Taxa (*n*)
Metazoa	344743	73
Ciliophora (all)	201052	35
Ciliophora (*Hyalophysa* sp.)	*153736*	*1*
Diatomea	4304	*27*
Syndiniales	1332	5
Discicristata	1224	4
Fungi	1020	10
Chlorophyta	843	5
Bicosoecida	738	1
Cercozoa	625	11
Dinoflagellata	501	10
Retaria	243	4
Cryptomonas	85	1
Protostelium	79	1
Leucocryptos	76	1
Rhodellophyceae	73	1
Schizoplasmodiopsis	66	1
FV18–2D11 (Alveolata)	65	2
Isochrysidales	62	1
Bolidomonas	35	1
Apicomplexa	30	1
NAMAKO-2	29	1
Labyrinthulomycetes	22	2
Jakobida	19	1
Gracilipodida (LEMD267)	17	1
NIF-4C10	14	1
Pelagophyceae	13	1

Only sequences that occurred 10 or more times in the data are shown.

DISCUSSION

Shrimp black gill is well established in wild penaeid shrimp populations in the southeastern Atlantic and Gulf of Mexico although many different causative agents have been implicated (Couch 1978). In coastal Georgia and South Carolina, sBG regularly reaches high levels in the late summer and fall (Geer 2013, Gambill et al. 2015). The cause and consequences of this condition, however, are poorly understood. This study reports on fishery and fishery-independent monitoring of sBG and suggests that the cause of the epidemic is an apostome ciliate closely related to *Hyalophysa chattoni*. The correlative evidence is convincing, but in the absence of further morphological, genetic, and experimental data, there remains some level of uncertainty that the sBG ciliate is the causative agent of black gill. This study also describes the development of a PCR-based diagnostic assay for the sBG ciliate and determined its geographic and seasonal distribution.

The origin of current sBG outbreaks in the southeastern Atlantic is still not known. Based on the earliest reports in Georgia, the emergence of sBG is consistent with the hypothesis that the sBG ciliate is the result of an exotic invasive species introduction with multiple occurrences of introduction (Wilson et al. 2009). Although there may have been some inconsistences in reporting black gill prior to 2003 when standardized protocols were adopted by the GA DNR CRD EMTS program, during the mid- to late-1990s, black gill was

catch data normalized for fishing effort did not reveal a significant relationship between shrimp catch and the prevalence of black gill ($P = 0.311$) or year ($P = 0.172$) (data not shown). The lack of a relationship between effort-normalized catch and the prevalence of black gill suggests that black gill has not contributed significantly to the decline of the fishery and thus is at odds with the commercial landings record. Because of these contradicting datasets, it is difficult to definitely conclude that recent declines in the fishery are directly related to the prevalence of shrimp with visually apparent black gill symptoms.
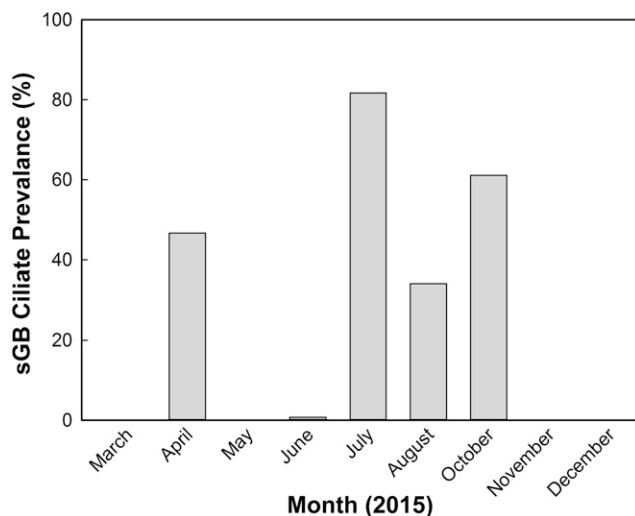
Figure 10. Fraction of shrimp gill microbiome protist community accounted for by sBG 18S rRNA gene sequences in next-generation sequencing libraries. Libraries were prepared from white shrimp gill samples collected monthly (March–December, 2014). All shrimp samples were collected from the Wassaw Sound estuary, GA. The sample from September 2014 was omitted because it was dominated (99.9%) by a single trematode sequence. *Hyalophysa*-like sequences were not recovered in March, May, November, and December.

reported sporadically. Black gill was first observed in 1996, absent in 1997 and 1998, reported again in 1999 and 2000, absent in 2001, and present from 2002 onward with considerable interannual variability in prevalence. This pattern is suggestive of several cycles of introduction and establishment failures until 2001when it became permanently established in the population. Alternatively, this pattern may suggest that the sBG ciliate is not an introduced species but rather

environmental conditions, possibly climate related, have changed sufficiently allowing it to proliferate to levels not previously observed. Parasitic microeukaryotes, including ciliates, are often associated with primary environmental drivers including temperature and salinity (Stentiford et al. 2012, Burge et al. 2014). In South Carolina, the prevalence of shrimp visibly affected by black gill is correlated with water temperature and salinity (Fowler et al. 2016) and in both Georgia and South Carolina black gill outbreaks exhibit a consistent seasonal pattern with symptoms coinciding with maximum water temperatures and minimum dissolved oxygen concentrations. Therefore, it is reasonable to suspect that temperature and other climate variable influence the process but additional studies, both observational and experimental, are required to explore these hypotheses further.

Based on its 18S rRNA gene sequence, the ciliate associated with sBG is closely related to the apostome ciliate *Hyalophysa chattoni*. Conventional interpretation of relatedness based on rRNA gene sequence similarity suggests that the sBG is a substrain of *H. chattoni*. This conclusion is, however, contradicted by morphology-based investigations and additional taxonomic and phylogenetic studies are required before the identity of the sBG ciliate is confirmed. If indeed it is an apostome ciliate it may redefine the features of this group. Sequence comparison with *Synophrya*, an apostome ciliate reported to cause black gill in decapods, distinguishes the sBG ciliate from this genus. Ongoing studies to amplify and sequence other commonly used phylogenetic markers including the mitochondrial COI gene and the ribosomal ITS region using a variety of general PCR primers have so far failed to produce target amplicons (data not shown). Efforts to capture the swimming tomite form from exuvial fluid associated with recent molts (in some cases collected within an hour of ecdysis) have also failed. Additionally, a vegetative trophont in shrimp gill tissue has not been observed though the invasive stage within

TABLE 3.

Metazoan sequences recovered from *Litopenaeus setiferus* gill parasitome, March–December, 2014.

|		Month (% of total sequences)									
Group	Total sequences	March	April	May	June	July	August	September	October	November	December
Trematoda	150581	32.42	0.12	0.01	0.00	0.00	0.00	33.76	1.28	0.06	32.36
Teleostei	115433	0.01	0.39	35.30	4.33	6.90	0.00	0.00	1.02	51.79	0.25
Anthozoa	24317	0.05	73.52	2.82	0.00	4.00	0.00	0.00	8.76	10.28	0.57
Chelicerata	23985	0.54	88.21	0.00	0.00	0.00	0.00	0.00	1.50	2.87	6.88
Hydrozoa	13205	0.00	0.41	71.20	1.31	21.41	0.00	0.00	0.27	5.40	0.00
Ascidiacea	7336	0.00	0.00	0.00	4.49	0.31	0.00	0.00	0.00	0.07	0.00
Scyphozoa	4699	0.00	0.00	86.68	0.00	0.00	0.00	0.00	0.00	13.32	0.00
Crustacea	2406	17.36	53.05	2.36	0.81	1.18	0.00	0.00	0.00	23.05	0.00
Chondrichthyes	2160	0.00	1.02	48.52	0.00	6.62	0.00	0.00	2.50	41.34	0.00
Bryozoa	1076	0.00	0.00	14.78	0.00	70.72	0.00	0.00	0.93	13.57	0.00
Appendicularia	417	0.00	0.00	0.00	100.00	0.00	0.00	0.00	0.00	0.00	0.00
Cephalopoda	402	0.00	2.49	86.57	0.00	10.95	0.00	0.00	0.00	0.00	0.00
Mammalia	252	0.00	0.00	63.49	17.46	3.97	0.00	0.00	0.00	15.08	0.00
Annelida	211	0.00	0.00	0.00	52.13	0.00	0.00	0.00	47.87	0.00	0.00
Demospongiae	115	0.00	0.00	0.00	0.00	100.00	0.00	0.00	0.00	0.00	0.00
Bivalvia	89	0.00	0.00	0.00	0.00	0.00	100.00	0.00	0.00	0.00	0.00
Turbellaria	88	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	100.00	0.00
Myriapoda	12	0.00	100.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Hexapoda	10	0.00	0.00	100.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
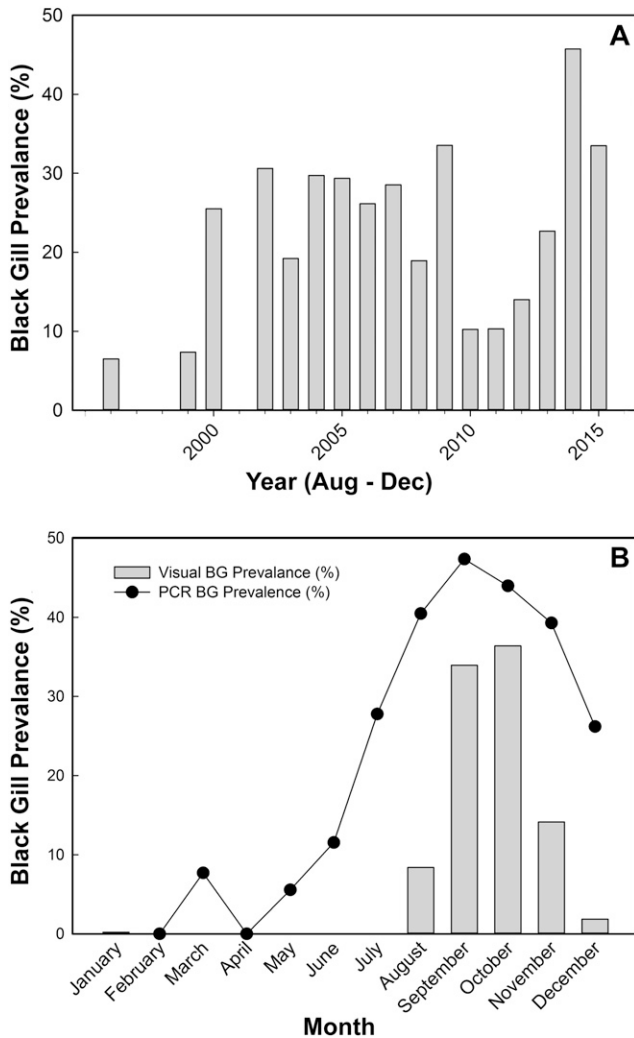
Figure 11. Prevalence of sBG in coastal Georgia. (A) Average annual (August–December) of visible black gill prevalence since its first appearance in the Georgia fishery. (B) Average monthly prevalence (2014–2015) of shrimp black based on visual observation of melanized gills (⬜) and PCR detection (—●—). Estimates of visible sBG prevalence based on fishery-independent coast wide surveys and collections of all commercial penaeid shrimp species conducted by the Ecological Monitoring Trawl Survey of the GA DNR's Coastal Resources Division.
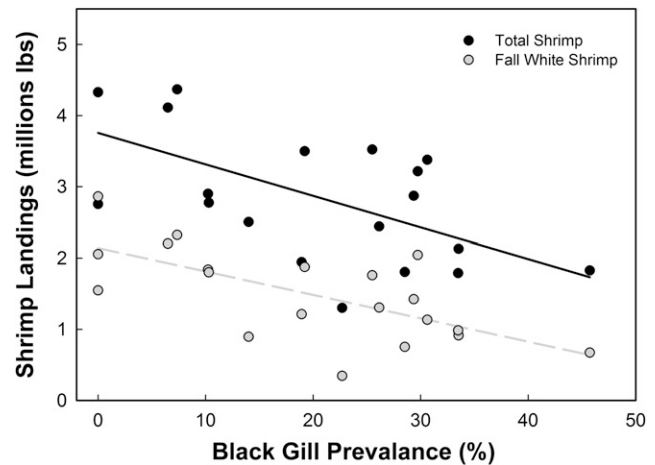

Figure 12. Relationship between black gill prevalence and (●) total and (○) fall season commercial shrimp landings in Georgia from 1957 to 2015. Both total ($r^2 = 0.30$, $P = 0.008$) and fall ($r^2 = 0.415$, $P = 0.001$) landings are significantly correlated with the prevalence of visible black gill. Data from the GA DNR Coastal Resources Division Ecological Monitoring Trawl Survey program.

the gill has been. The ciliary patterns on the surface of the trophont are the basis for species identification among apostome ciliates (Chatton and Lwoff 1935), though other characteristics can be used to identify whether a ciliate is an apostome or not, including internal structures; especially trichocysts, secretory dense bodies, a rosette, and membrane organelles (Bradbury 1966, 1973, Landers 1991a, 1991b, Landers et al. 2007). The current morphological data are consistent with the sBG ciliate being an apostome, but are not all consistent with the genus *Hyalophysa*. The sBG ciliate has membrane plates within the cytoplasm, similar to the membrane organelles of apostomes preparing to feed including *Hyalophysa* (Bradbury 1973, Landers et al. 2007). However, it also contains numerous lipid droplets and expands within its host's tissue while surrounded by a cyst wall, which is similar to the invasive apostome *Synophrya* (Chatton & Lwoff 1935, Landers 2010).

Additional work is needed to determine if this sBG ciliate is an apostome and if it is a new species.

Although this study was not able to definitively identify the sBG ciliate, a PCR-based assay targeting its 18S rRNA gene was developed. The sensitivity of the assay was determined to be on the order of 10 gene copies suggesting that this assay is almost certainly sufficiently sensitive to detect a single ciliate. Real-time quantitative PCR studies indicate that the copy number of the target gene can be highly variable and therefore it is unlikely that quantitative estimates of ciliate abundance can be derived from PCR-based approaches. *In silico* and empirical specificity evaluation indicated that although the assay developed is theoretically specific to the genus *Hyalophysa*, in practice, the assay will amplify the 18S rRNA gene from *Hyalophysa* and closely related apostome ciliates. Unbiased next generation sequencing of the gill microbiome from white shrimp collected over nearly a year suggest that apostome ciliates other than the sBG ciliate are, if present, rare in penaeid shrimp from the South Atlantic Bight region. Thus, in practice, although the sBG PCR assay is capable of amplifying other apostome ciliates, detection in shrimp from the region is almost certainly confirmatory of the sBG ciliate. This interpretation is supported by the observation that isolated sBG ciliates yielded identical PCR amplicons to those obtained from shrimp tissue infected with ciliates.

In the majority of cases where visual, microscopic, and PCR diagnostics were compared, the results were self-consistent with samples exhibiting black gill being PCR positive and ciliates detected microscopically and those without being negative by PCR and microscopy but in a fraction of samples differential diagnoses were suggested by the different assays. Consideration of these discrepancies is likely useful for identifying the stage and condition of the infection. For example, 12.5% of the shrimp examined were PCR positive but did not exhibit visible signs of black gill and a similar fraction (12.9%) was PCR positive but ciliates were not detected by histological examination (Fig. 13). If indeed the sBG ciliate is the cause of black gill, a possible interpretation of these observations is that they result
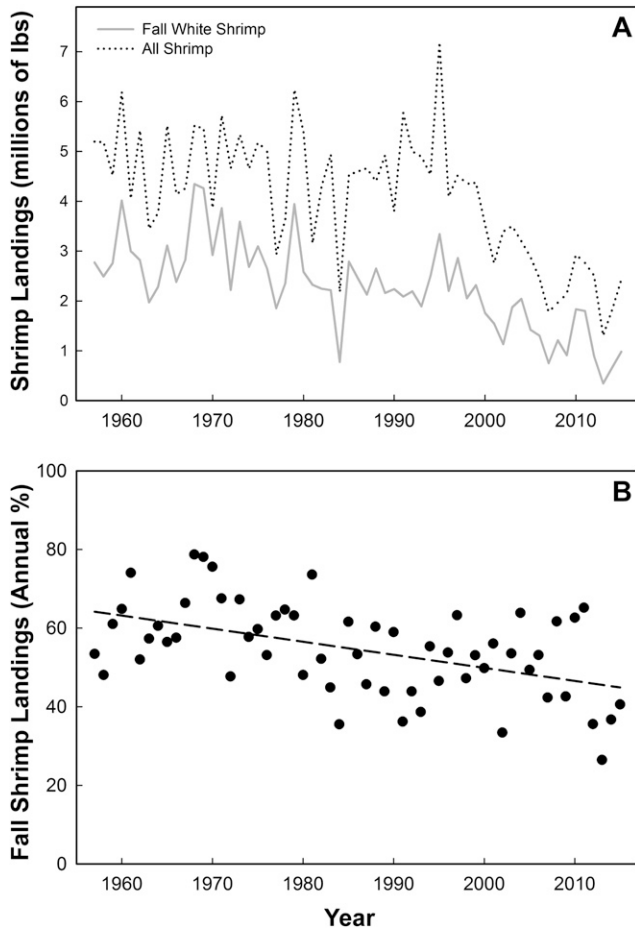
Figure 13. Total and fall season commercial shrimp landings in Georgia. (A) Total (······) and fall (——) shrimp landings. (B) The fall harvest relative to the total annual harvest has accounted for the largest fraction of the decline in shrimp landings.

The presence of the Georgia sBG ciliate has been confirmed by PCR and histological examination in shrimp collected from the Gulf of Mexico and along the eastern U.S. seaboard from Florida to Virginia (Chesapeake Bay). Although sBG does not currently result in epidemic-scale outbreaks outside the South Atlantic Bight region, that the sBG ciliate is so broadly distributed suggests that there is a real risk of epidemics occurring outside the South Atlantic Bight region and should be closely monitored in the future.

In addition to providing confirmatory evidence that the sBG ciliate is related to *Hyalophysa*, preliminary observation of the shrimp gill microbiome suggested the existence of diverse communities of previously uncharacterized potential parasites and pathogens associated with shrimp gills. Nearly 200 unique groups of organisms closely related to known parasites and pathogens were identified in a preliminary dataset of 10 shrimp collected from a single location over a 10-mo period (March–December 2014). The identity of these organisms is poorly resolved but provides an interesting new perspective and possible new approach to detect and identify emerging new disease threats. Interestingly, in addition to the detection of possible parasites, a large fraction of the sequences recovered from shrimp gill tissues was classified as metazoan sequences. Presumably, these sequences are derived either from larvae or detrital material containing environmental DNA (Rees et al. 2014) trapped on the gills. After trematode sequences clearly associated with trematode infections, the most commonly detected metazoan sequences were classified as fish (telostei), Anthozoa (corals), Chelicerata (horseshoe crabs), Hydrozoa, and Schyphozoa (jellyfish). At least 15 major classes of metazoan animals representative of the huge diversity of life in the productive estuaries of the South Atlantic Bight region were represented (Fautin et al. 2010). The presence of these sequences suggests that it might be possible using next-generation sequencing approaches to characterize gill associates of water filtering organisms as a means to monitor a huge range of diversity in aquatic environments. For example, it may be possible to identify reproduction events of cryptically spawning organisms such as corals or to detect the presence of invasive species.

The relationship between black gill and the fishery remains correlative. Commercial landings in Georgia have been declining since the early 2000s and are significantly correlated with the prevalence of black gill. Fishery independent assessments that normalize for fishing effort are, however, not significantly correlated with black gill prevalence. The poor correlation between black gill and shrimp harvests is likely in part due to the fact that visible black gill is a symptom rather than the cause. Shrimp with black gill are animals whose immune system is effectively managing infection. Shrimp incapable of defending themselves or recently molted shrimp that do not exhibit visible symptoms may be the individuals most impacted by the parasite but in the fishery data sets are identified as "healthy" animals. Routine inclusion of more definitive diagnostic data including direct microscopic observation and PCR detection that provide the means to detect the cause of black gill provide a more direct and likely relevant indicator of black gill. Regular use of these diagnostics may reveal stronger relationships between the fishery and the prevalence of the black gill causative agent. A second factor that likely limits the usefulness of visible black gill prevalence as a correlative factor is the frequency at which data are collected as part of the routine fishery independent EMTS

from animals in the early period of infection where either the abundance of invading ciliates is low such they are not detected in routine pathology examination or that the shrimp's immune system has not yet mounted a response resulting in the formation of melanized hemocytic nodules. Another explanation may be that the ciliates were present but in a nonpathogenic life stage and not recognized as the sBG ciliate. Alternatively, in cases where visibly melanized nodules were present and ciliates (or ciliate remains) were observed on microscopic examination but PCR results were negative may be indicative of a late stage infection. Presumably, after the immune system has mounted a successful response resulting in the digestion of the ciliate DNA and prior to molting, a shrimp would exhibit the visible symptoms of black gill but ciliates would be undetectable. Currently, an insufficient number of samples or controlled experimental studies have be conducted to rigorously test these hypotheses but, if this is indeed the case, the continued routine application of multiple diagnostic approaches may reveal important insights into the dynamics and development of sBG outbreaks.

The availability of the sBG diagnostic PCR assay used in combination with routine histological diagnosis indicates that the Georgia sBG ciliate is widely distributed geographically.

trawl survey program. The Georgia EMTS sampling program provides broad spatial coverage but individual sites are only sampled once per month, which is likely insufficient to identify direct relationships between black gill and mortality. Higher frequency data collection will also help to improve the resolution of the relationship between the black gill ciliate and the population. In addition to improved monitoring, experimental studies will be required to determine whether black gill is detrimentally impacting shrimp populations. Consistent with literature reports (Scholnick et al. 2006), shrimp with black gill appear to have reduced endurance, possibly making them more susceptible to predators (A. Fowler, personal communication). Preliminary experimental studies also suggest that there can be significant mortality associated with black gill (Price 2016).

Shrimp black gill, given its high prevalence and over 20-y history in the South Atlantic Bight shrimp fishery, is unlikely to dissipate. Likewise, it does not seem reasonable that a means to eradicate it exists. Thus, as with many other fishery issues, management and industry adaptation are the only reasonable options. Understanding the causes and processes that influence outbreaks and the effects on the fishery are key to managing the fishery and predicting the economic impacts necessary to maintain a viable fishery. It is unlikely that shrimp in the region will disappear but it is possible for the industry to experience irreparable economic harm.

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