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PATHOLOGY, EFFECTS, AND TRANSMISSION OF BLACK GILL IN COMMERCIAL PENAEID SHRIMP FROM THE SOUTH ATLANTIC BIGHT

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ABSTRACT Severe outbreaks of black gill (BG), heavily melanized gills of crustaceans, have been reported in white (*Litopenaeus setiferus*) and brown shrimp (*Farfantepenaeus aztecus*) from coastal Georgia and South Carolina during late summer and fall since the mid-1990s. The cause of this condition is an apostome ciliate that elicits the innate immune response of the shrimp, resulting in the formation of melanized nodules in shrimp gill tissue. In the absence of a definitive identification, the causative ciliate is referred to as the shrimp black gill (sBG) ciliate. During outbreaks, necrosis of gill tissue was often seen in microscopic sections; in some cases, there appeared to be penetration of the ciliate into gill tissue. Shrimp with BG exhibited reduced physical endurance and escape responses compared with shrimp without symptomatic BG. Physical impairment due to BG may contribute to higher predation rates and increased vulnerability to environmental conditions. The infection transmission rate of the sBG ciliate appears to be atypically low for apostome ciliates, raising the question of how epidemic levels of BG reoccur annually. Asymptomatic shrimp placed in direct contact with carcasses (heads) from BG symptomatic shrimp exhibited a significant increase in the development of BG symptoms after 7 days ($P = 0.028$), but waterborne transmission was not detected. A preliminary survey of sympatric crustacean species, including syntopic species of grass shrimp (*Palaemonetes* spp.), indicated the possible presence of the sBG ciliate, suggesting that other crustacean species may serve as infection reservoirs. These studies support the conclusion that BG is negatively impacting the penaeid shrimp fishery and highlight the challenges that remain in understanding and managing the ongoing sBG epidemic in the southeastern United States.

KEY WORDS: penaeid shrimp, apostome ciliate, black gill, pathology, South Atlantic Bight

INTRODUCTION

Penaeid shrimp, including *Litopenaeus setiferus* (white shrimp) and *Farfantepenaeus aztecus* (brown shrimp), support one of the most valuable commercial fisheries in the South Atlantic Bight (Gillet 2008), but since 2000, they have been in dramatic decline (Frischer et al. 2017). One contributing cause to this decline is hypothesized to be a severe outbreak of a gill infection caused by a parasitic ciliate. The infection results in tissue melanization and is, therefore, generally referred to as shrimp black gill (sBG). The name derives from the macroscopic appearance of darkened gills that results from the host immune response to the parasite (Sritunyalucksana & Söderhäll 2000, Jiravanichpaisal et al. 2006, Hauton 2012).

Over the past two decades, severe outbreaks of BG in penaeid shrimp have been reported off the coast of Georgia and South Carolina (Gambill et al. 2015, Fowler et al. 2018). The sBG ciliate is present in shrimp populations from approximately May through January, with peak infection rates and visibly melanized gills occurring in the late summer through the fall (August to October). Monitoring using molecular diagnostic assays and histological studies indicate that the sBG ciliate is absent from shrimp populations during the late winter and early spring (Frischer et al. 2017). Environmental reservoirs of the associated ciliate during the remainder of the year have been speculative (Price 2016), as has the epidemiology. In the case of current BG outbreaks impacting the South Atlantic Bight, the invading pathogen has been identified as an apostome ciliate. Based on its 18S gene sequence, the sBG ciliate is closely related to the nonpathogenic apostome ciliate *Hyalophysa chattoni*; however, morphological characteristics of the ciliate are inconsistent with this identification and suggest that the sBG ciliate is an undescribed species (Frischer et al. 2017). Because the identity of sBG ciliate has not been determined, its life history including potential vectors, transmission pathways, and pathogenicity is currently unknown. As recently discussed by Burnett and Burnett (2015), however, it is quite likely that crustacean immune responses that result in gill tissue melanization also result in impaired cardiovascular function and metabolism. The presence of melanized 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 Pacific white shrimp *Litopenaeus vannamei* (Scholnick et al. 2006) and in the Atlantic blue crab *Callinectes sapidus* (Burnett et al. 2006, Thibodeaux et al. 2009) by the

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presence of hemocytic nodules induced by bacterial infection has been experimentally confirmed. 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 BG-impacted shrimp.

The present work reports on the microscopic examination of this ciliate in symptomatic and asymptomatic shrimp, the physiological impact of BG and potential transmission pathways, and infection reservoirs.

MATERIALS AND METHODS

Collection and Experimental Facilities

In Georgia, 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). Specimens collected approximately monthly in 2014 were examined to investigate the dynamics of ciliate and nodule concentration. For experimental purposes, shrimp were exclusively collected from the Wassaw Sound estuary. In South Carolina, shrimp were collected by the South Carolina Department of Natural Resources' (SCDNR) Crustacean Research and Monitoring Section in James Island Creek using a 1.27 cm mesh cast net, or in Charleston Harbor using trawl gear as described in DeLancey et al. (2005, 2008). Physiological experiments were conducted at the SCDNR Marine Resources Research Institute (Charleston, SC) and transmission studies were conducted at the University of Georgia, Skidaway Institute of Oceanography (SkIO) (Savannah, GA).

In South Carolina, shrimp were held in 75 L tanks filled with water from Charleston Harbor (salinity: 30–32 ppt). Temperature (26°C–28°C), photoperiod (10 h light: 14 h dark), and oxygen concentrations (minimum oxygen concentration—5 mg/L) were maintained. Twenty percent of the seawater was changed every 3 days. Temperatures, salinities, and dissolved oxygen were recorded weekly (YSI model 556 MPS). Shrimp were fed commercial shrimp feed (Hyper-Intensive Shrimp 35; Zeigler Bros., Inc.). In Georgia, experiments were conducted at SkIO in 125 L tanks receiving flowing natural seawater, and maintained at ambient temperatures and salinity in dim light to simulate conditions where the shrimp were collected. Shrimp were fed fresh squid (~1 g per animal daily).

Routine Histology

Zinc formalin–fixed gill tissues from white and brown shrimp were processed for routine light microscopy 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. Hundreds of white, brown, and occasional tiger shrimp (*Penaeus monodon*) collected routinely from Georgia coastal waters were examined in this study. In addition, representative shrimp, including pink shrimp (*Farfantepenaeus duorarum*) from South Carolina, North Carolina, Virginia, Florida, and several locations in the Gulf of Mexico, were also examined. Freshly discarded carapaces collected within 2 h of molting ($n = 3$) from BG-infected white shrimp were also examined for the presence of ciliates.

Physical Endurance and Behavior Studies

Physical endurance trials were conducted at the SCDNR Marine Resources Research Institute with shrimp collected during July and August 2014. Studies were conducted using a specially designed aquatic treadmill (described in Scholnick et al. 2006, Thibodeaux et al. 2009) to quantify time to exhaustion and behavior for both shrimp with visible BG ($n = 13$) and those that did not exhibit macroscopic melanization of the gills (asymptomatic) ($n = 16$). Before each experiment, the length (80–100 mm) and weight of each shrimp were recorded, and it was noted whether shrimp exhibited macroscopic evidence of BG. Temperature, salinity, and dissolved oxygen were recorded at the beginning and end of each trial (YSI model 556 MPS). The treadmill was filled with water from the shrimp holding tank and dissolved oxygen concentrations were maintained with an air stone and bubbler. Each shrimp was fed before the start of the experiment and allowed to acclimate for 10 min before beginning the trial. The treadmill was set to a speed at which shrimp would walk forward steadily and quickly without swimming (5.45 rpm), and behavioral observations were recorded every 30 min for 7 h or until the shrimp was exhausted. At the end of each trial, shrimp were removed from the treadmill and returned to the holding tank. To minimize response to external stimuli, a box was placed over the treadmill with a small opening to allow for viewing. Behaviors recorded were placed into one of the following three categories: (1) forward motion on the treadmill (i.e., walking steadily forward and swimming); (2) exhaustion behaviors (i.e., walking only on front appendages with pleopods off the treadmill and riding the treadmill); or (3) escape behaviors (i.e., jumping vertically off the treadmill, sprinting forward, and tail flipping). Occurrence of these behaviors was divided into two portions (0–3 h and 3.5–7 h) of the trial for analyses. The time to exhaustion (i.e., shrimp lying on their sides against the back of the treadmill and not moving) was noted for all individuals as appropriate. Trials were terminated if shrimp became exhausted before the end of the 7 h period. Student's *t*-tests were used to compare the percent of time individual shrimp spent performing each of the behaviors in the two time categories.

Transmission Studies

The infectivity and transmission pathways of the sBG ciliate were investigated in fully aerated flow-through 125-L seawater tanks at SkIO in Georgia during the fall of 2013 and 2014. In each treatment, 15 shrimp that did not exhibit macroscopic melanization of the gills (asymptomatic) were placed in seawater tanks. Experimental treatments were as follows: (I) asymptomatic shrimp only (control); (II) asymptomatic shrimp with heads from five shrimp with visible gill melanization (referred to as direct). In this treatment, shrimp heads were completely consumed within 24 h; (III) asymptomatic shrimp with heads from five shrimp with visible black gill separated by a porous Plexiglas partition (referred to as waterborne-dead); and (IV) asymptomatic shrimp with living shrimp with visible gill

melanization separated by a porous Plexiglas partition (referred to as waterborne-live). After 3 and 7 days, five randomly collected shrimp were sampled and examined for evidence of BG. Studies were conducted on October 7–14, 16–23, 2013, and October 9–17 and 17–24, 2014. These dates were during the peak of black gill prevalence in each respective year. In the 2013 studies, the waterborne-live (treatment IV) was not included.

Potential Reservoirs of the sBG Ciliate

To identify other potential crustacean hosts (reservoirs) of the sBG ciliate, a broad range of crustacean species were collected during August to October in 2015, when BG prevalence is typically highest, and tested for the presence of the sBG ciliate using a recently developed sBG ciliate-specific polymerase chain reaction (PCR) diagnostic assay (Frischer et al. 2017). This assay uniquely targets the sBG ciliate 18S rRNA gene but in practice likely hybridizes to closely related apostome ciliates. With the exception of grass shrimp (*Palaemonetes* spp.) and zooplankton collected using a 64-μm mesh towed plankton net, all specimens were captured in trawl nets in association with trawl surveys conducted by in the GA DNR EMTS program and GA MAREX Trawl Program. Grass shrimp were collected weekly from the Skidaway River from the main dock at the SkIO from March to November 2015 in association with the Skidaway River Monitoring Program (SRiMP 2017). Grass shrimp were captured with a dip net that was dragged along the side of the dock. Approximately, 12 grass shrimp (*Palaemonetes* spp.) were collected each week. Replicate gill tissue samples were immediately excised from each sample using dissection forceps and preserved in both 70% non-denatured ethanol for molecular analysis and 10% zinc formalin (Sigma Aldrich Z2902) for routine histological analysis as previously described (Frischer et al. 2017). Zooplankton samples were collected in October and November 2015 using a 30-cm ring diameter, nylon mesh plankton net with a 64-μM filtering cod end assembly. Samples were preserved in 70% non-denatured ethanol for molecular analysis. All samples were stored at 4°C until processing. Sediment and water samples were also collected and examined to determine if these environments might serve as a reservoir of sBG infection. Samples were collected from the Wassaw Sound estuarine system and offshore of the Georgia coast in October 2014 ($n = 3$), and August ($n = 3$) and October 2015 ($n = 3$) when BG prevalence in penaeid shrimp was high. Water was collected ~1 m above the bottom using 10 L Niskin bottles and dispensed into 25 L carboys. Triplicate water samples (5 L) from each carboy were filtered first through 200-μm mesh netting and collected onto 3.0 μm, 47 mm diameter, polycarbonate membrane filters (Millipore cat #TSTP04700). The filters were cut in half, placed in 15 mL tubes, and stored at –80°C for subsequent DNA extraction and PCR analysis. Sediment samples were collected using a Ponar grab sampler. Five aliquots of each sediment sample were preserved in polypropylene vials containing 70% non-denatured ethanol for molecular analysis. DNA was purified from crustacean samples as previously described (Frischer et al. 2017). DNA was purified from sediment and filters using the MO BIO Laboratories PowerSoil DNA Isolation Kit. Analysis for the presence of sBG ciliate DNA from all sample types was performed using the PCR and DNA extraction techniques described in Frischer et al. (2017).

RESULTS

Microscopic Examination of South Atlantic Bight Shrimp

Light microscopy studies of gill tissues from shrimp with macroscopic evidence of BG revealed the presence of a large ciliate (32–38 μm in diameter), melanized nodules, and variable amounts of host tissue necrosis (Fig. 1A, B). Only shrimp with large numbers of melanized nodules appeared macroscopically to have blackened gills (Frischer et al. 2017). Many macroscopically normal animals, however, were still found to have microscopic evidence of low-level infections that induced focal underlying tissue changes (Fig. 2). Often, multinucleate ciliates were observed, suggesting that these ciliates were actively feeding and dividing. In macroscopically affected animals, large ciliates were frequently observed adjacent to gill lamellae and in close proximity to melanized nodules (Fig. 3A). Some ciliates appeared to be enclosed by a delicate membrane or cyst wall (Fig. 3B), and occasional ciliates were observed to be encapsulated or encompassed by a melanized nodule (Fig. 3C, D). In

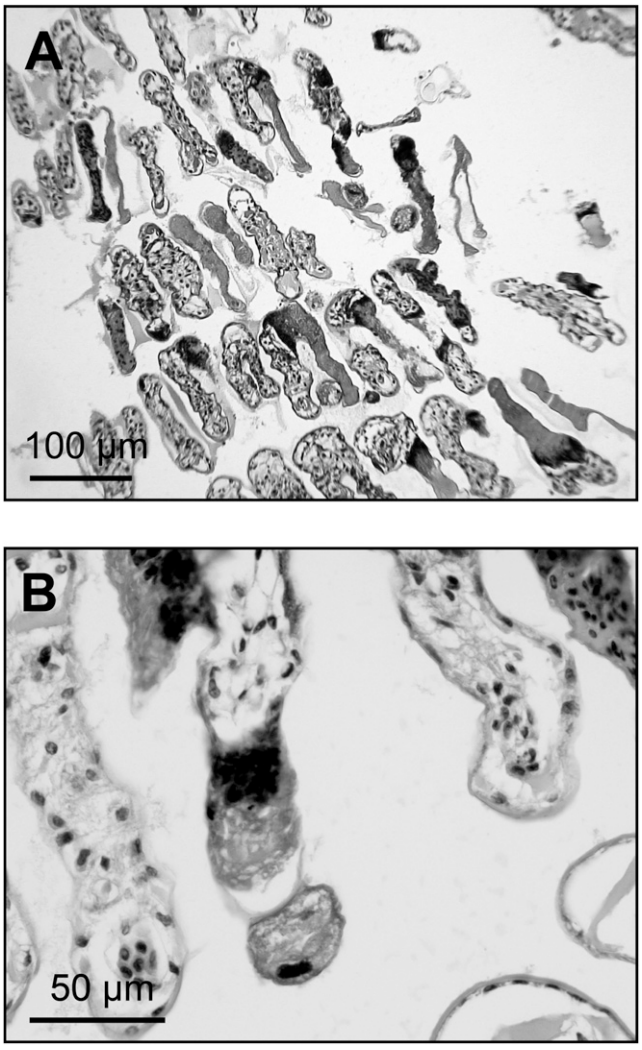


Figure 1. Histological section of shrimp gill tissue with macroscopic evidence of BG. Low (A) and high (B) power micrographs are shown. Note the large apostome ciliate, melanized nodules, and host tissue necrosis. [Hematoxylin and eosin (H & E); A bar = 100 μm; B bar = 50 μm].

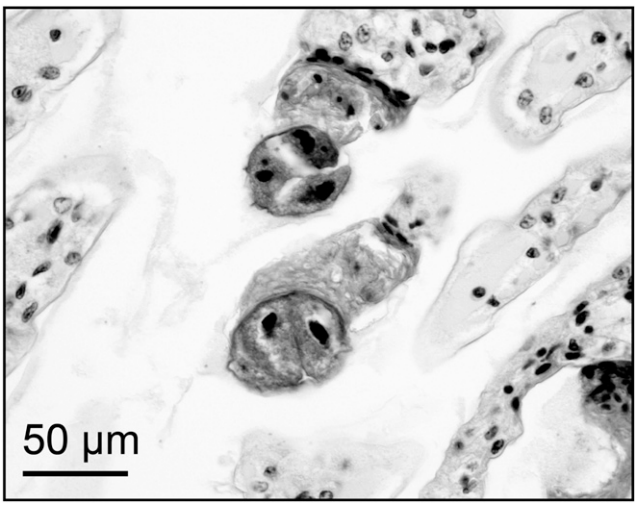


Figure 2. Histological section of shrimp gill tissue that was macroscopically normal. The average concentration of hemocytic nodules and ciliates was 7 mm^{-2} and 3 mm^{-2}, respectively. Both ciliates in the shown micrograph appear to be dividing and are adjacent to host tissue that has undergone melanization and focal necrosis. (H &E, bar = 50 µm).

some cases, only remnants of the encapsulated ciliate could be seen (Fig. 3C), but in others, the encapsulated ciliate appeared morphologically intact with a visible nucleus (Fig. 3D). In gills with high ciliate numbers, necrotic host tissues were often observed adjacent to the ciliates, and in some cases, ciliates penetrated the underlying tissue (Figs. 3A and 4A). In specimens with low ciliate numbers and high nodule counts, it appeared that most of the ciliates had been destroyed by encapsulation (Fig. 4B). For example, in one such specimen, there were on average 83 nodules/mm^2 but only 1 ciliate/mm^2. Many ciliates and remnants of melanized nodules were observed in recently molted exoskeletons from macroscopically infected shrimp (Fig. 4C), indicating that ciliates and nodules were removed by the molting process.

Changes in the Ciliate and Nodule Concentration from July to November

The concentration of sBG ciliates and associated hemocytic nodules in shrimp gill tissue was investigated in monthly shrimp collections in 2014. Shrimp black gill ciliates and nodules were low or absent from December through June and remerged in July (Fig. 5). This pattern is consistent with previous reports of seasonal prevalence of BG symptomatic shrimp (Frischer et al. 2017). From July to August, sBG ciliate concentrations increased 80-fold from 0.1 ± 0.3 to 8.0 ± 2.4 ciliates/mm^2. Similarly, the concentration of hemocytic nodules increased approximately 15-fold from 1.5 ± 1.8 to 23.3 ± 3.0 during this same period. Following the peak concentrations in August, sBG ciliate concentration decreased exponentially from August through November. By contrast, hemocytic nodule concentrations remained high through October decreasing in November.

Physiological Effects Associated with BG

None of the tested shrimp died on the treadmill. BG symptomatic shrimp had a mean shorter time to exhaustion (2.75 h) compared with asymptomatic shrimp (4.3 h), but these were not significantly different from one another (t-test, $P = 0.218$) (Fig. 6A). Most (62%, 8 of 13) of the control shrimp walked on the treadmill for seven continuous hours, whereas only 50% (8 of 16) of the BG shrimp could walk that long.

There were different patterns in shrimp behavior depending on whether it was the first 3 h (Fig. 6B) or the later 3.5 h (Fig. 6C) of the trial. In the first portion of the trials, asymptomatic shrimp spent significantly more time in forward motion ($P = 0.01$), whereas the BG shrimp spent significantly more time performing exhaustion behaviors ($P = 0.01$). There was no difference in escape behavior ($P = 0.125$). There were, however, two BG outlier shrimp that spent 20% and 60% of the time performing escape behaviors. In the second portion of the trial, there was no difference in the amount of time the two groups of shrimp spent performing either forward ($P = 0.61$) or exhaustion ($P = 0.123$) behaviors, but asymptomatic shrimp spent a significantly higher portion of their time performing escape behaviors than BG shrimp ($P = 0.012$).

Shrimp Black Gill Potential Reservoirs

Fifteen crustacean species were analyzed for the presence of sBG ciliate DNA (Table 1). All samples were asymptomatic for BG. The sBG ciliate was detected in five species including the lesser blue crab (*Callinectes similis*), the common spider crab (*Libinia emarginata*), the mantis shrimp (*Squilla empusa*), the seabob shrimp (*Xiphopenaeus kroyeri*), and the grass shrimp (*Palaemonetes* spp.), suggesting the potential for these species to serve as reservoirs of sBG infection. The sBG ciliate was not detected in the other crustacean species examined or in the mixed zooplankton samples. The copepod *Acartia tonsa* dominated the zooplankton community at the time of sampling (data not shown).

sBG ciliate DNA was commonly detected in grass shrimp (25% prevalence), with highest prevalence in March. Prevalence decreased between April and August and was at its lowest in September when the prevalence of BG in penaeid shrimp is maximal (Frischer et al. 2017). The prevalence of the sBG ciliate in grass shrimp was inversely proportional to the prevalence of BG in commercial penaeid shrimp ($P = 0.05$, $r = –0.70$) (Fig. 7).

The sBG ciliate was not detected in any of the water or sediment samples (Table 1). Water and sediment samples spiked with gill tissue from sBG infected shrimp gill tissue were positive, indicating that the DNA extraction and PCR procedures can detect the sBG ciliate in these sample matrices (data not shown).

Transmission Studies

The results from four transmission experiments after 7 days of exposure are provided in Table 2. There was no evidence of transmission in any of the experiments or treatments after 3 days of exposure (data not shown). Under these experimental conditions, infection transmission was minimal. Combining normalized transmission rates for each of the 4 independent trials, statistically significant ($P = 0.03$) evidence of transmission was observed when shrimp were allowed to consume heads of shrimp with visible symptoms of black gill. When analyzing each trial separately, significant transmission was only observed during one of the four experiments (Table 2, Experiment IV).

DISCUSSION

Shrimp BG has been frequently reported in wild penaeid shrimp populations in the southeastern Atlantic and Gulf of

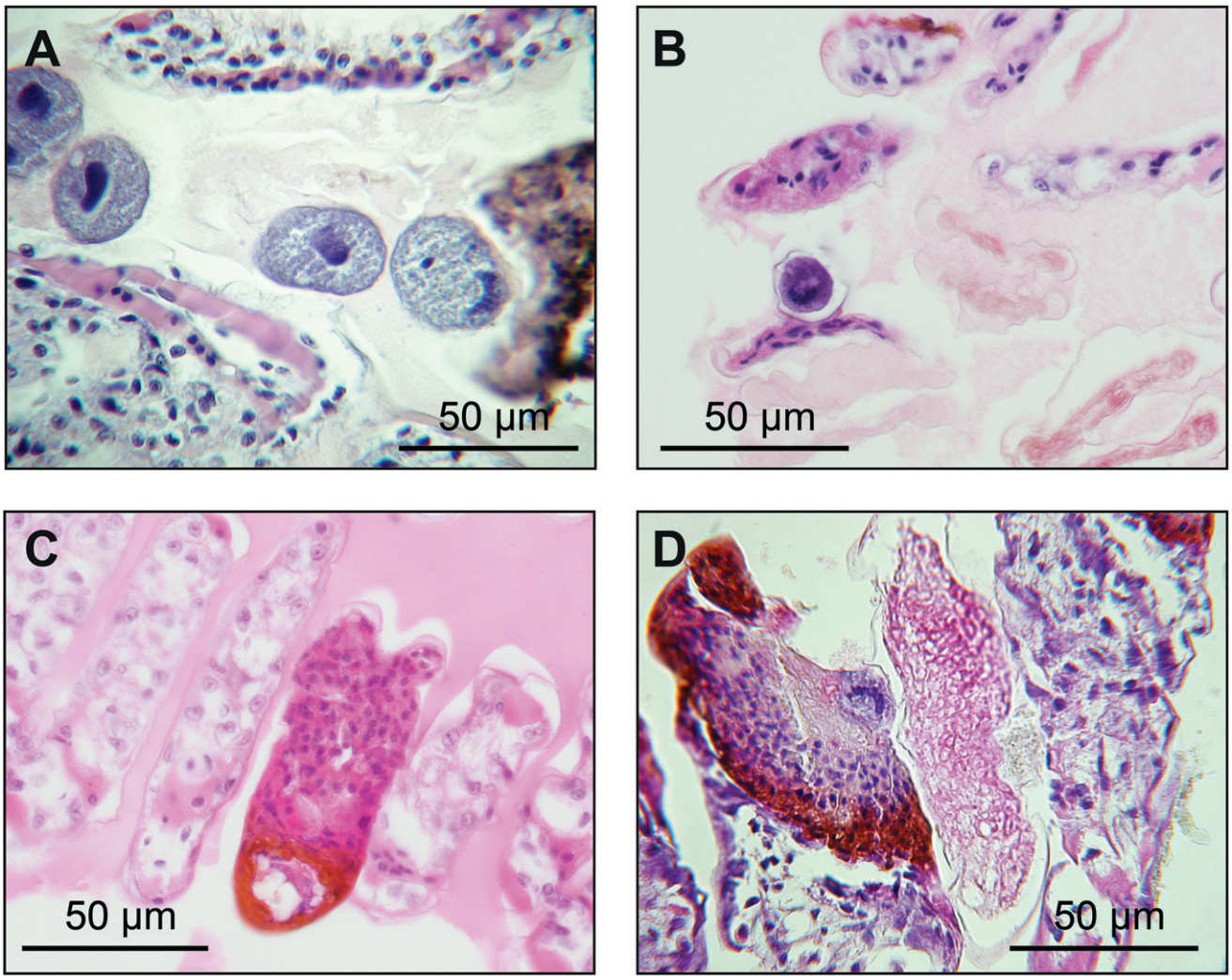
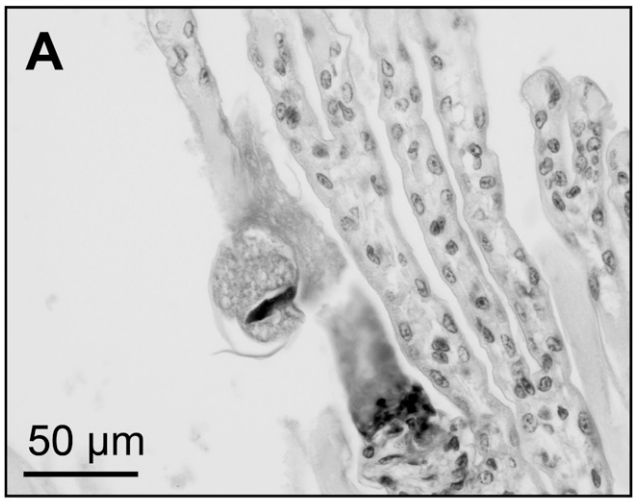


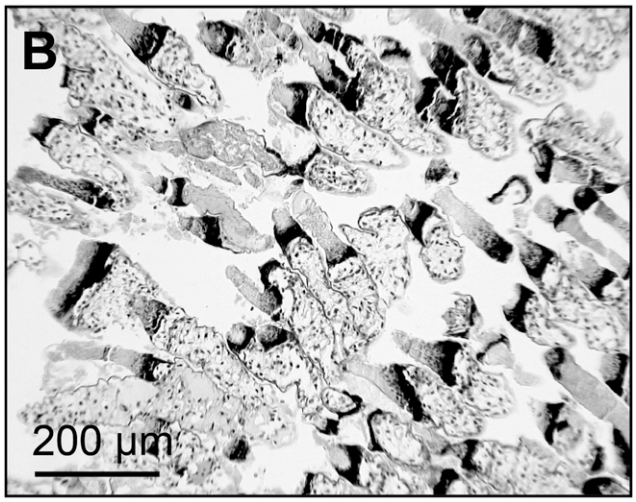
Figure 3. Histological sections of shrimp gill tissue exhibiting various processes involved in the sBG infection and response processes. (A) The large ciliate at the right appears to be in close proximity to a forming nodule. (B) A ciliate contained within a cyst wall or membrane. (C) Remnants of a ciliate within a nodule. (D) A ciliate entrapped within a melanized nodule that appears to be undergoing necrosis. (H & E; each bar = 50 µm).

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 & Roberson 2014, Gambill et al. 2015, Frischer et al. 2017). The evidence presented here supports that gill melanization is the result of the immune response by shrimp to the presence of a large apostome ciliate that has previously been identified as the likely causal agent of sBG in the South Atlantic Bight (Frischer et al. 2017).

Although the prevalence of shrimp exhibiting the symptoms of BG generally peaks in September and October (Gambill et al. 2015), the highest prevalence and intensity of sBG ciliates detected using microscopy-based approaches were observed in August (Table 1). Similar seasonal patterns have been reported with other symbiotic crustacean ciliates including the blue crab–associated peritrich ciliate *Lagenophrys callinectes* in Maryland Bay and the Gulf of Mexico (Couch 1983, Arias 2013). It may be that the high-water temperatures and lower oxygen concentrations found in August are conditions that allow for the proliferation of the sBG ciliate to such an extent that the shrimp's immune system is overwhelmed. Alternatively, it may be that during summer conditions shrimp experience higher levels of stress because of increased temperature and lower available dissolved oxygen and are, therefore, more susceptible to BG infections. The presence of high ciliate concentrations in shrimp gills during August suggests that this month would be the period of highest mortality associated with BG.

In some cases, but not universally, necrosis was apparent in tissues adjacent to sBG ciliates and in a small fraction of samples, tissue penetration of the ciliate into the necrotic tissues was observed (see Figs. 3A and 4A). Often dividing ciliates (multinucleate) were observed in association with tissue necrosis and melanization, suggesting attached ciliates are actively growing and, therefore, likely feeding. Tissue necrosis has been observed in several crustacean species infected with protozoan parasites (Armstrong et al. 1981, Sheppard et al. 2003, Athanassopoulou et al. 2004, Shields & Overstreet 2007, Small et al. 2005a, 2005b, Sparks et al. 1982, Walker et al. 2009). The most commonly studied apostomes are exuviotrophic symbionts of crustaceans that feed on the exuvial fluid in cast-off





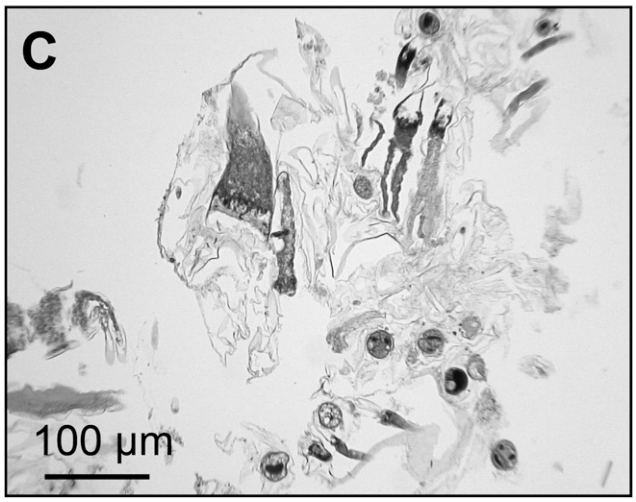


Figure 4. (A) In this specimen, there appears to be necrosis of the gill tissue subjacent to the ciliate and possible penetration into the necrotic area. Note the absence of melanization. (B) This specimen had macroscopic changes of BG. Microscopically, there were high numbers of nodules (average 83/mm^2) but few ciliates (average 1/mm^2). (C) Exoskeleton of a recently molted shrimp showing the presence of ciliates and nodules. (H & E, A bar = 50 µm; B, C bar = 100 µm).

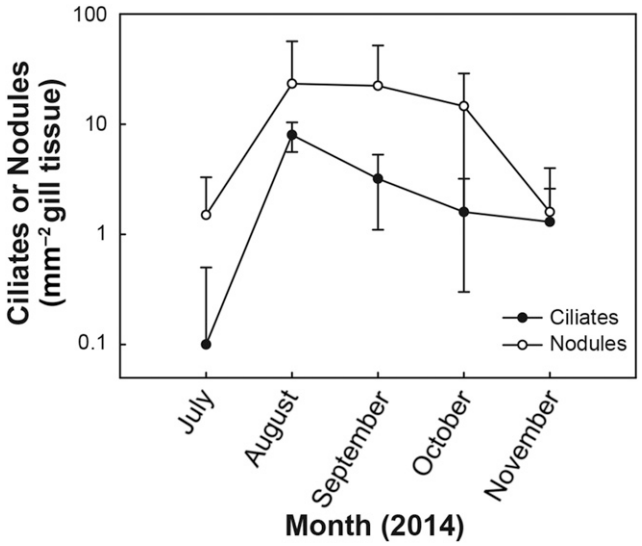


Figure 5. Concentration of sBG ciliates (black filled circle) and hemocytic nodules (white filled circle) in shrimp gill tissue. Shrimp (*Litopenaeus setiferus* and *Farfantepenaeus aztecus*) were collected monthly from July through November 2014 when symptomatic black gill was present and examined histologically. Shrimp were collected from each of Georgia's major sound systems in association with the GA DNR CRD EMTS trawl survey program. A total of 107 shrimp were collected and examined with monthly samples ranging from 15 to 33 individuals.

molted exoskeletons (Landers 2010). Other apostome species, however, including *Synophrya* sp., *Collinia* sp., and *Vampyrophrya* sp., are known to penetrate crustacean gill tissues during their feeding stages and produce extensive tissue necrosis (Chatton & Lwoff 1935, Johnson & Bradbury 1976, Haefner & Spacher 1985, Capriulo & Small 1986, Ohtsuka et al. 2004, Landers 2010). Based on the observations in this study, we speculate that the sBG ciliate is capable of penetrating and feeding on shrimp gill tissues. Apostome ciliates exhibit a complicated multiphasic life history with feeding often taking place during a single stage in the life history (Bradbury et al. 1987). In some apostome species, however, feeding can occur at multiple life history stages. For example, *Synophrya hypertrophica* has two feeding stages, including the parasitic histotrophic hypertrophont that feeds on the host tissue before molting and the benign exuviotrophic trophont that feeds on exuvial fluids after molting (Chatton & Lwoff 1935, d'Avila-Levy et al. 2016, Landers 2010). The life history of the sBG ciliate has not yet been fully described, but we speculate that it may be similar to *S. hypertrophica* in that the sBG may have both exuviotrophic and histotrophic forms during its life cycle. During its histotrophic phase, it likely triggers the shrimp innate immune response that results in the characteristic symptoms of BG. Small et al. (2005a) present evidence that extracellular proteases secreted by a ciliate parasite of the Norway lobster, *Nephrops norvegicus*, cause tissue necrosis. We speculate that the sBG ciliate associated with BG could be producing similar proteases and resulting in gill necrosis.

In this study, we observed that the physical endurance and predator escape behavior was reduced in BG symptomatic shrimp compared with shrimp that did not have melanized gills. On average, BG shrimp became tired almost 1.5 times as quickly as asymptomatic shrimp; although variability was high,

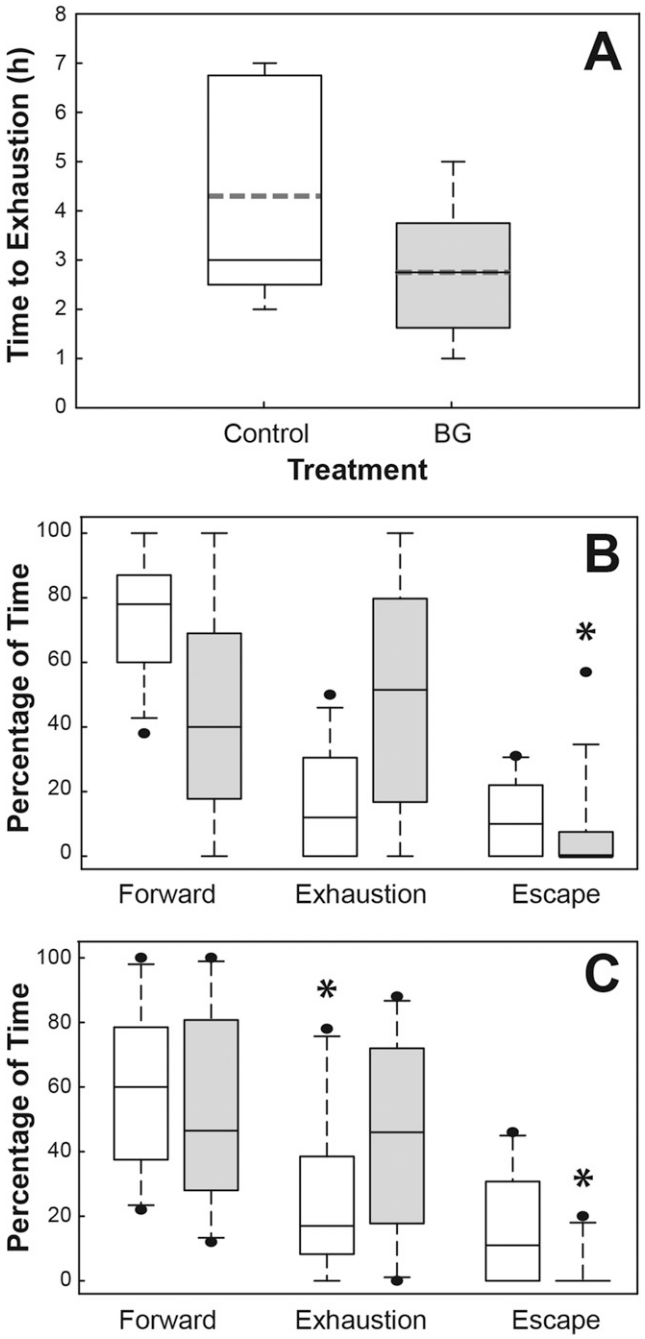


Figure 6. (A) Cumulative number of asymptomatic ($n = 13$) and sBG shrimp ($n = 16$) exhausted during the 7-h treadmill trials in August and September 2014. (B) The percentage of time asymptomatic ($n = 13$) and sBG shrimp ($n = 16$) spent performing forward, exhaustion, or escape behaviors during the 7-h treadmill trials divided into the first half of the experiment (0–3 h) and (C) during the second half (3.5–7 h). Asterisks denote a significant ($P < 0.05$) difference between pairs.

these results were not statistically significant ($P = 0.218$). Interestingly, half (8 of 16) of the BG shrimp were able to walk on the treadmill for the entire 7-h trial. These animals were, however, noted to have less obvious macroscopic gill tissue discoloration (data not shown). It is possible that these individuals had been recently infected and thus did not have the same amount of gill tissue melanization as other individuals. In the behavior trials, there was a notable difference between asymptomatic shrimp and BG shrimp during both the first and second half of the trial period, with asymptomatic shrimp performing more forward behaviors and BG shrimp exhibiting more behaviors associated with exhaustion. After 3 h, none of the BG shrimp performed escape behaviors. The BG shrimp were already more lethargic than asymptomatic shrimp during the first half of the trial and became increasingly impacted during the second half and did not perform energy-taxing escape behaviors. The necrosis associated with BG seems likely to be responsible for reduced respiratory capacity resulting in a reduction in physical endurance. We hypothesize that mortality in the wild may be induced by the sBG ciliate by lowering the shrimp's respiratory capacity and compromising its behavioral abilities to avoid predation, leading to a differential in mortality rates of infected and uninfected shrimp. High temperatures, low oxygen, and limited food are stressors that may also accentuate this differential.

TABLE 1.

Detection (PCR) of the Georgia sBG ciliate in common estuarine and coastal crustacean species in the South Atlantic Bight.

Species or environmental matrix	*N*	% sBG ciliate positive
Callinectes similis (lesser blue crab)	19	21.1
Libinia emarginata (common spider crab)	10	10.0
Squilla empusa (mantis shrimp)	7	28.6
Xiphopenaeus kroyeri (seabob shrimp)	5	40.0
Palaemonetes spp. (grass shrimp)	153	25.0
Menippe mercenaria (stone crab)	1	0.0
Callinectes sapidus (blue crab)	1	0.0
Ovalipes ocellatus (lady crab)	1	0.0
Portunus gibbesii (iridescent swimming crab)	5	0.0
Portunus spinimanus (blotched swimming crab)	1	0.0
Panopeus herbstii (mud crab)	1	0.0
Acartia tonsa (copepod)	9	0.0
Unidentified isopod	1	0.0
Unidentified crab	2	0.0
Mixed zooplankton sample	9	0.0
Sediment	9	0.0
Water	9	0.0

Because the sBG ciliate and symptomatic shrimp are generally not present during the late winter and spring (Frischer et al. 2017), the question of where it goes and how it reinfects shrimp arises. Apostome ciliates are frequently associated with a broad range of crustaceans (Landers 2004), and, therefore, we explored the possibility that the sBG ciliates can reside in crustacean species other than the commercially important penaeid shrimp species. We also investigated possible transmission pathways including via the water and consumption of infected shrimp. Although the survey of potential host crustacean species was quite small and, therefore, likely underestimates possible sources, the sBG ciliate was detected in five other crustacean species, indicating that other crustaceans may be a source of infection. Given that the 18S rRNA–targeted diagnostic assay has the potential to hybridize to closely related apostome ciliates, however, these observations should be confirmed independently. Interestingly, the prevalence of the sBG ciliate in grass shrimp was highest during periods when

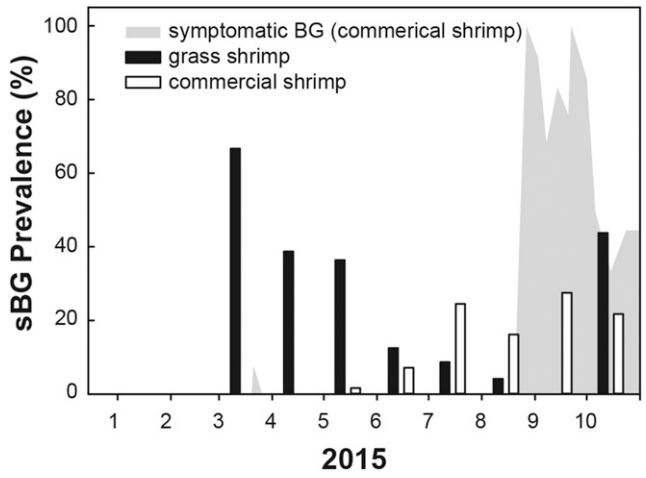


Figure 7. Seasonal prevalence of the sBG ciliate in grass shrimp *Palaemonetes* spp. collected from Skidaway River, GA (black filled rectangle) and commercial penaeid shrimp collected from Wassaw Sound estuary system during the Georgia Department of Natural Resources Ecological Monitoring Trawl Survey from March to November in 2015 (white filled rectangle). Commercial shrimp samples were not collected in November. A significant negative correlation ($r = -0.70$) was observed ($P < 0.05$). The prevalence of commercial shrimp with visible symptoms of BG is indicated by the shaded regions.

sBG ciliate prevalence is lowest in commercially important penaeid shrimp, including in the early spring when it is generally absent in penaeid shrimp. Perhaps, the sBG ciliate is capable of overwintering in grass shrimp that are present throughout the year in the estuary? The sBG ciliate was not detected in either water or sediment samples that were examined, suggesting that it is unlikely that these environments serve as overwintering reservoirs of the sBG ciliate.

Surprisingly, given the epidemic proportions that sBG reaches and the apparent disappearance of the ciliate in the winter, transmission rates observed in the experimental studies were low or nonexistent. Transmission was observed only when shrimp were allowed to consume infected shrimp, and, even in this treatment, variability was high and transmission was not always observed. A contributing problem in these experimental studies is the lack of ciliate-free control shrimp. By necessity, these experiments were conducted during the fall when BG was prevalent in the wild population. The shrimp designated as "asymptomatic" likely included shrimp with low levels of sBG infection. Thus, it is not clear if infection was transmitted from macroscopically infected shrimp to asymptomatic or if asymptomatic shrimp developed the macroscopic condition from prior infection without acquiring a new infection during the experimental period. The lack of a relevant ciliate-free control group limits the sensitivity of the experimental design. Efforts are underway to address this problem but, to date, a method to obtain sBG-free shrimp has not been developed. Although molting did occur in each of the transmission studies, a second potential experimental limitation may have been that the infectious life history stage was not produced during the 7-day experimental period. Nonetheless, the studies conducted suggest feeding behavior as an important mechanism of transmission and highlights the need to describe the full life history of the sBG ciliate.

Although the life history of the sBG ciliate remains unresolved, the observation that the ciliate can elicit an immune response and that there is tissue invasion, on occasion, suggests that the life history of the sBG ciliate is similar to *Synophrya hypertrophica* in that it may involve both exuviotrophic and histotrophic feeding stages. Although genetic analyses indicate that the sBG ciliate is most closely related to the exuviotrophic nonpathogenic ciliate *Hylophysa chattoni*, recent electron microscopy studies suggest that the sBG ciliate shares many morphological characteristics with *S. hypertrophica* (Frischer et al. 2017). Additional studies are required to resolve the life history and to identify the sBG ciliate. To date, investigations of transmission rates and pathways suggest that the sBG is not highly infectious. Transmission was observed only via direct contact when asymptomatic shrimp were allowed to consume symptomatic shrimp. These studies are, however, likely incomplete as they lacked appropriate controls and may not have involved all life stages of the ciliate. The conclusion that the sBG is not highly infectious is inconsistent with the annual cycle of disappearance in the late winter and reemergence at epidemic levels in the summer. Initial surveys of common crustaceans for the presence of the sBG ciliate indicates that the ciliate has a broad host range and suggests that other crustaceans may serve as a reservoir of infectious agents. Sediments and water do not appear to be long-term reservoirs of infection but they may be involved in transient transmission processes. Interestingly, the seasonal prevalence of the sBG ciliate in grass shrimp (*Palaemonetes* spp.) was inversely correlated to its prevalence in commercial food shrimp, suggesting that grass shrimp may be an important overwintering reservoir for the sBG ciliate.

The relationship between the state of the fishery and the current epidemic of sBG remains unclear. Based on the

TABLE 2.

Experimental transmission of the sBG ciliate.

Experiment	Date	Treatment (average ciliates/mm^2 ± SD)			
		Control	Direct	Waterborne (dead)	Waterborne (live)
I	October 7–14, 2013	4.75 (5.6)	11.0 (9.1)	12.25 (11.6)	ND
II	October 16–23, 2013	2.7 (2.5)	3.0 (2.0)	1.7 (2.1)	ND
III	October 9–17, 2014	1.3 (1.5)	3.0 (1.9)	1.4 (0.9)	1.4 (0.9)
IV	October 17–24, 2014	0.0 (0)	1.0 (0.1)*	0.6 (0.9)	0.8 (0.8)

ND, not done.
* Significant treatment effect ($P < 0.05$).

studies reported here, it can be concluded that sBG, given its high prevalence and apparent pathogenicity, is likely to be having a negative impact on the overall physiological condition of shrimp in this commercially important fishery. Especially because the causative ciliate is widespread and appears to be present in many other species of common crustaceans, it is unlikely that it will be possible to eradicate it. Improved understanding of the life history, mechanisms of transmission, and pathology may provide important insights for the development of future management and mitigation strategies.

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LITERATURE CITED

Arias, M. 2013. Prevalence of the parasite *Lagenophrys callinectes* in blue crabs *Callinectes sapidus*. Report of Undergraduate Research Opportunity, Louisiana State University. Accessed July 23. Available at: sapidus http://nsgl.gso.uri.edu/lsu/lsut13003.pdf.

Armstrong, D. A., E. M. Burreson & A. K. Sparks. 1981. A ciliate infection (*Paranophrys* sp.) in laboratory-held dungeness crabs, *Cancer magister*. *J. Invertebr. Pathol.* 37:201–209.

Athanassopoulou, F., D. Speare, R. J. Cawthorn, R. MacMillan & B. Despres. 2004. Pathology of *Anophryoides haemophila* (Scuticociliatida: Orchitophryidae), parasite of American lobster *Homarus americanus* kept under experimental conditions. *Aquaculture* 236:103–117.

Bradbury, P. G. D. & A. Campillo. 1987. The feeding apparatus of a chitinivorous ciliate. *Tissue Cell* 19:351–363.

Burnett, K. G. & L. E. Burnett. 2015. Respiratory and metabolic impacts of crustacean immunity: are there implications for the insects? *Integr. Comp. Biol.* 55:856–868.

Burnett, L. E., J. D. Holman, D. D. Jorgensen, J. L. Ikerd & K. G. Burnett. 2006. Immune defense reduces respiratory fitness in *Callinectes sapidus*, the Atlantic blue crab. *Biol. Bull.* 211:50–57.

Capriulo, G. M. & E. B. Small. 1986. Discovery of an apostome ciliate (*Collinia beringensis* n. Sp.) endoparasitic in the Bering Sea euphausiid *Thysanoessa inermis*. *Dis. Aquat. Organ.* 1:141–146.

Chatton, É. & A. Lwoff. 1935. Les Ciliés Apostomes. 1. Aperçu historique et general; étude mongraphique des genres et des espées. *Arch. Zool. Exp. Gén.* 77:1–453.

Couch, J. A. 1978. Diseases, parasites and toxic responses of commercial penaeid shrimps of the Gulf of Mexico and south Atlantic coasts of North America. *Fish Bull.* 76:1–44.

Couch, J. A. 1983. Diseases caused by protozoa. In: Provenzano, A. J., editor. The biology of the crustacea, vol. 6. New York, NY: Academic Press. pp. 79–111.

d'Avila-Levy, C. M., V. Yurchenko, J. Votypka & P. Grellier. 2016. Protist collections; essential for future research. *Trends Parasitol.* 32:840–842.

DeLancey, L. B., J. E. Jenkins, M. B. Maddox, J. D. Whitaker & E. L. Wenner. 2005. Field observations on white shrimp, *Litopenaeus setiferus*, during spring spawning season in South Carolina, U.S.A., 1980–2003. *J. Crustac. Biol.* 25:212–218.

DeLancey, L. G., J. E. Jenkins & E. L. Wenner. 2008. Long-term monitoring of white shrimp, *Litopenaeus setiferus* (Linnaeus), stocks within the ACE Basin National Estuarine Research Reserve, South Carolina. *J. Coast. Res.* 55:193–199.

Fowler, A. E., J. W. Leffler, S. P. Johnson, L. B. DeLancey & D. M. Sanger. 2018. Relationships between meterological and water quality variables and fisheries-independent white shrimp (*Litopenaeus setiferus*) catch in the ACE Basin NERR, South Carolina. *Estuaries Coasts* 41:79–88.

Frischer, M. E., R. F. Lee, A. R. Price, T. L. Walters, M. A. Bassette, R. Verdiyev, M. C. Torris, K. Bulaski, P. J. Geer, S. A. Powell, A. N. Walker & S. C. Landers. 2017. Causes, diagnostics, and distribution of an ongoing penaeid shrimp black gill epidemic in the U.S. south Atlantic bight. *J. Shellfish Res.* 36:487–500.

Gambill, J. M., A. E. Doyle, R. F. Lee, P. J. Geer, A. N. Walker, L. G. Parker & M. E. Frischer. 2015. The mystery of black gill: shrimpers in the south Atlantic face off with a cryptic parasite. Current. *J. Mark. Educ.* 29:2–7.

Geer, P. J. & D. E. Roberson. 2014. Assessment of Georgia marine fishery resources. SEDAR-PW6-RD29. Accessed May 29. Available at: http://sedarweb.org/docs/supp/SEDAR_PW6_RD29_Geer%26Roberson2003.pdf.

Georgia Marine Extension Service. 2016. Educational experiences in marine science and coastal ecology; scientific sampling cruise. p. 7. Accessed December 12. Available at: http://marex.uga.edu/uploads/documents/MAREX_edu_booklet_web.pdf.

Gillet, R. 2008. FAO fisheries technical paper 475. Global study of shrimp fisheries. Part II shrimp fisheries in selected countries. Shrimp fishing in the United States of America. pp. 289–312. FAO Fiji. Accessed July 23. Available at: http://www.fao.org/docrep/011/i0300e/i0300e00.htm.

Haefner, P. A. & P. J. Spacher. 1985. Gill meristics and branchial infestation of *Ovallipes stephensoni* (Crustacea, Brachyura) by *Synophrya hypertrophica* (Ciliata, Apostomida). *J. Crustac. Biol.* 5:273–280.

Hauton, C. 2012. The scope of the crustacean immune system for disease control. *J. Invertebr. Pathol.* 110:251–260.

Jiravanichpaisal, P., B. L. Lee & K. Söderhall. 2006. Cell-mediated immunity in arthropods: hematopoiesis, coagulation, melanization and opsonization. *Immunobiology* 211:213–236.

Johnson, C. A. & P. Bradbury. 1976. Observations on the occurrence of the parasitic ciliate *Synophrya* in decapods in coastal waters off the southeastern United States. *J. Protozool.* 23:273–280.

Landers, S. C. 2004. Exuviotrophic apostome ciliates from crustaceans of St. Andrew Bay, Florida, and a description of *Gymnodinioides kozloffi* n. sp. *J. Eukaryot. Microbiol.* 51:644–650.

Landers, S. C. 2010. The fine structure of the hypertrophont of the parasitic apostome *Synophrya* (Ciliophora, Apstomatida). *Eur. J. Protistol.* 46:171–179.

Martin, G. G., M. Quintero, M. Quigley & H. Khosrovian. 2000. Elimination of sequestered material from the gills of decapod crustaceans. *J. Crustac. Biol.* 20:209–217.

Ohtsuka, S., M. Hora, T. Suzaki, M. Arikawa, G. Omura & K. Yamada. 2004. Morphology and host-specificity of the apostome ciliate *Vampyrophrya pelagica* infecting pelagic copepods in the Seto Inand Sea, Japan. *Mar. Ecol. Prog. Ser.* 282:120–142.

Page, J. 2012. Ecological monitoring surveys. Attachment 3 in Geer, P. J. and C. Belcher. 2012. Georgia Marine Research, Surveys, and Development. Annual Report to USFWS Sportfish Restoration Project GA F-79-R-7. July 2011 to June 2012. GA DNR Coastal Resources Division, Brunswick, GA. 17 pp.

Price, A. R. 2016. Environmental reservoirs and mortality associated with shrimp black gill. MS Thesis: Marine Sciences, Savannah State University, Savannah, GA. 140 pp.

Scholnick, D. A., K. G. Burnett & L. E. Burnett. 2006. Impact of exposure to bacteria on metabolism in the penaeid shrimp *Litopenaeus vannamei*. *Biol. Bull.* 211:44–49.

Sheppard, M., A. Walker, M. E. Frischer & R. F. Lee. 2003. Histopathology and prevalence of the parasitic dinoflagellate, *Hematodinium* sp. In crabs (*Callinectes sapidus*, *Callinectes similis*, *Neopanope sayi*, *Libinia emarginata*, *Menippe mercenaria*) from a Georgia estuary. *J. Shellfish Res.* 22:873–880.

Shields, J. D. & R. M. Overstreet. 2007. Diseases, parasite, and other symbionts. In: Kennedy, V. S. & L. E. Cronin, editors. The blue crab *Callinectes sapidus*, College Park, MD: Maryland Sea Grant College. pp. 299–417.

Small, H. J., D. M. Neil, A. C. Taylor & G. H. Coombs. 2005a. Identification and partial chracterisation of metalloproteases secreted by a *Mesanophrys*-like ciliate parasite of the Norway lobster *Nephrops norvegicus*. *Dis. Aquat. Organ.* 67:225–231.

Small, H. J., D. M. Neil, A. C. Taylor, K. Bateman & G. H. Coombs. 2005b. A parasitic scuticociliate infection in the Norway lobster (*Nephrops norvegicus*). *J. Invertebr. Pathol.* 90:108–117.

Sparks, A. K., J. Hibbits & J. Fegley. 1982. Observations on the histopathology of a systemic ciliate (*Paranophrys* sp.?) disease in the dungeness crab, *Cancer magister*. *J. Invertebr. Pathol.* 39:219–228.

SRiMP (Skidaway River Monitoring Program). 2017. Skidaway river monitoring program. Accessed July 23. Available at: http://www.skio.uga.edu/research/biological-oceanography/skidaway-river-monitoring-program/.

Sritunyalucksana, K. & K. Sönderhäll. 2000. The proPO and clotting system in crustaceans. *Aquaculture* 191:53–69.

Thibodeaux, L. K., K. G. Burnett & L. E. Burnett. 2009. Energy metabolism and metabolic depression during exercise in *Callinectes sapidus*, the Atlantic blue crab: effects of the bacterial pathogen *Vibrio campbellii*. *J. Exp. Biol.* 212:3428–3439.

Walker, A. N., R. F. Lee & M. E. Frischer. 2000. Transmission of the parasitic dinoflagellate *Hematodinium* sp. infection in blue crabs *Callinectes sapidus* by cannibalism. *Dis. Aquat. Organ.* 85:193–197.

White, K. N., N. A. Ratcliffe & M. Rossa. 1985. The antibacterial activity of hemocyte clumps in the gills of the shore crab, *Carcinus maenas*. *J. Mar. Biol. Ass. U.K.* 65:857–870.