BioInvasions Records (2024) Volume 13, Issue 1: 121–140

Research Article

First detection of white spot syndrome virus (WSSV) and infectious hypodermal and hematopoietic necrosis virus (IHHNV) from wild-caught giant tiger prawn, *Penaeus monodon* Fabricius, 1798 (Penaeoidea: Penaeidae) from the Gulf of Mexico and Northwestern Atlantic Ocean

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Citation: Krol JD, Hill JM, Smith PRK, Kendrick MR, Gooding EL, Fuchs C, Whelan NV, Bullard SA (2024) First detection of white spot syndrome virus (WSSV) and infectious hypodermal and hematopoietic necrosis virus (IHHNV) from wild-caught giant tiger prawn, *Penaeus monodon* Fabricius, 1798 (Penaeoidea: Penaeidae) from the Gulf of Mexico and Northwestern Atlantic Ocean. *BioInvasions Records* 13(1): 121–140, https://doi.org/10.3391/bir.2024.13.1.11

Received: 21 June 2022

Accepted: 29 September 2023

Published: 26 December 2023

Handling editor: Jamie Bojko

Thematic editor: April Blakeslee

Abstract

We screened the gill and somatic muscle of 152 wild-caught invasive giant tiger prawns (GTPs), *Penaeus monodon* Fabricius, 1798 (Penaeoidea: Penaeidae) for infection by white spot syndrome virus (WSSV), infectious hypodermal and hematopoietic necrosis virus (IHHNV), and Taura syndrome virus (TSV) using molecular methods (PCR and qPCR) and transmission electron microscopy (TEM). The sampled GTPs comprised 1 freshly-collected specimen from Mississippi Sound (Northern Gulf of Mexico) during 2020; 54 frozen specimens originally captured from the Northern Gulf of Mexico off Mississippi, Alabama, and Florida during 2014–2016; 76 frozen specimens originally captured from the Northwestern Atlantic Ocean off North Carolina, South Carolina, Georgia, and Florida during 2014–2020; and 21 museum-accessioned specimens (19 initially ethanol-preserved; 2 initially formalin-fixed) captured from the Gulf of Mexico and Northwestern Atlantic Ocean off Florida during 1988, 2011–2013, and 2016. Molecular viral detection relied upon qPCR with TaqMan chemistry for WSSV, conventional PCR for IHHNV, and rt-PCR for Taura virus. TEM was performed on WSSV qPCR+ positive GTP gill to confirm viral infection. A total of 18 GTPs were positive for WSSV by qPCR, 1 was positive for IHHNV by conventional PCR, and none were positive for Taura virus. This is the first report of a WSSV or IHHNV infection in a wild-caught GTP from the Gulf of Mexico or Northwestern Atlantic Ocean and first detection of an IHHNV infection in a wild-caught host in the Northwestern Atlantic Ocean. The phylogenetic analyses indicated that, broadly, sympatric WSSV isolates (unless identical) do not share a recent common ancestor (they are paraphyletic), suggesting that the virus has been repeatedly translocated and introduced into the Gulf of Mexico and Northwestern Atlantic Ocean and that it originated from different localities.

Key words: *P. monodon*, WSSV, IHHNV, viral taxonomy, molecular marker, wild infection, invasive, virus

Introduction

The giant tiger prawn (GTP), *Penaeus monodon* Fabricius, 1798 (Penaeoidea: Penaeidae), is a large (270 mm maximum total length; 260 g total wet weight) and commercially-important penaeid. GTP capture fisheries historically existed off Indonesia, Malaysia, and the Philippines (Motoh 1981, 1985; Chan 1998). Lightner et al. (2012) reported that worldwide GTP production was only recently surpassed by domesticated whiteleg shrimp, *Litopenaeus vannamei* (Boone, 1931) (Penaeoidea: Penaeidae). The native geographic range of GTP comprises the Indo-West Pacific (between approximately 30°E to 155°E longitude and 35°N to 35°S latitude) and includes Australia, Bangladesh, Hong Kong, India, Japan, Kenya, Korea, Madagascar, Oman, Pakistan, Papua New Guinea, Saudi Arabia, Somalia, South Africa, Sri Lanka, Taiwan, and Tanzania (Motoh 1985; Fuller et al. 2014). Commercial GTP landings have steadily declined from overfishing (Alam et al. 2022) and the destruction of mangrove nursery habitat (Mohamed 1967; Motoh 1985; Chaudhari and Jalihal 1993). The capture of live GTP broodstock and post-larvae for spawning and grow-out have also likely contributed to population declines and decreased fisheries landings (Mohamed 1967; Chaudhari and Jalihal 1993; Kautsky et al. 2000; Shinji et al. 2019; Alam et al. 2022).

GTPs were introduced for aquaculture in the Atlantic Ocean Basin and are now established in the Gulf of Mexico (Wakida-Kusunoki et al. 2013, 2016), Northwestern Atlantic Ocean (Fuller et al. 2014; Zink et al. 2018; present study), Northeastern Atlantic Ocean off Africa, the Caribbean Sea (Altuve et al. 2008; Gómez-Lemos and Campos 2008; Giménez et al. 2014; Alfaro-Montoya et al. 2015), and the Southwestern Atlantic Ocean off South America from Venezuela to Brazil (Coelho et al. 2001; Silva et al. 2002; Fuller et al. 2014). Fuller et al. (2014) suggested three potential pathways of tiger shrimp introduction to the northwestern Atlantic Ocean, including larvae released from ballast water, migrations from established populations in the Caribbean Sea and South America, and escapement from aquaculture facilities. The first release of GTPs in North America was accidental and originated from a culture pond in South Carolina during 1988 (Fuller et al. 2014). Approximately 300 GTPs were soon thereafter trawled from adjacent locales off South Carolina, Georgia, and northeastern Florida. No GTP was reported subsequently off the United States until a specimen was collected in 2006 from the Northern Gulf of Mexico (Mississippi Sound) off Dauphin Island, Alabama. A string of subsequent sightings and collections during 2006–2013 initially off the coast of North Carolina, South Carolina, Florida, and Louisiana and later confirmed along the coast of Texas, Mississippi, and Georgia (Fuller et al. 2014) led to concern about the ecological impacts of an established GTP population as well as the concomitant introduction of viruses that could impact aquaculture production and wild fisheries. Especially concerning was that ~ 30 kg of GTPs were captured off northeastern Florida in 2013

and a putative juvenile GTP was captured in Biscayne Bay, Florida, in 2016 (Zink et al. 2018); both indicating that an established, breeding population of GTP existed there. Despite the concerns about established populations of GTP in the Northwestern Atlantic Ocean and Gulf of Mexico, to date, relatively few studies have surveyed the viruses of wild-caught GTPs (de la Peña et al. 2007; Lightner 2011; Knibb et al. 2018; Oakey et al. 2019; Arbon et al. 2022). In fact, no record of a virus infecting a wild-caught GTP exists from North America.

Shrimp viruses, as severe pathogens of cultured shrimps (Lightner 1993, 1996a, b, 1999; Lightner and Redman 1998 a, b; Flegel 1997, 2006; Flegel and Alday-Sanz 1998), are of particular concern in the context of this biological invasion. As summarized by Lightner et al. (2012), the shrimp aquaculture industry grew rapidly world-wide and long before rapid and cost-effective nucleotide-based diagnostic tests were available to detect shrimp viruses. As live shrimp stocks and commodity shrimp (Durand et al. 2000) were translocated among countries and introduced outside of their native range, shrimp viruses were also likely unknowingly co-introduced and caused epizootics among naive (endemic) shrimps with scant or no innate resistance to exotic viruses (Lightner 2011). Lightner (2011) considered white spot syndrome virus (WSSV) and Taura syndrome virus (TSV) as the most virulent in this context, and, to a lesser extent, infectious hypodermal and hematopoietic necrosis virus (IHHNV), infectious myonecrosis virus (IMNV), and yellowhead virus (YHV). The collective economic impact of these viruses to the shrimp aquaculture industry in Asia and the Americas could exceed $12B in losses due to captive epizootics as well as lost jobs and export revenue (see Table 2 of Lightner [2011]). Stentiford et al. (2012) estimated that shrimp viruses cost the global industry ~ $3 B/yr (40% of tropical shrimp production). As a result, the industry began breeding specific-pathogen free (SPF) or specific-pathogen resistant (SPR) shrimp stocks (OIE 2020). Arbon et al. (2022) asserted that these technologies have resulted in a shift from culturing native shrimps to culturing domesticated lines of putatively disease resistant Pacific white shrimp. Likewise, domesticated stocks of GTP exist in Hawaii, Madagascar, and Thailand but the efficacy of these domesticated stocks regarding disease resistance is unknown and needs further investigation. Arbon et al. (2022) further noted that developing disease resistant GTP stocks and successful culture and biosecurity of broodstock benefits from knowledge of the pathogens that infect them in the wild. We concur and recognize this as further justification for screening wild decapod populations for these viruses.

The viruses studied herein (WSSV, IHHNV, TSV) are known in the Americas and listed as reportable pathogens by the World Organization for Animal Health (WOAH) (formerly World Organization for Animal Health; OIE) (OIE 2022). The GTP is considered a natural host for WSSV and IHHNV (Lightner 1999) and has been experimentally infected with

TSV (Srisuvan et al. 2005). WSSV, IHHNV, and TSV emerged as problematic in shrimp aquaculture in 1992, 1981, and 1992, respectively (see Table 2 of Walker and Winton [2010]).

WSSV (monotypic *Whispovirus*; monotypic *Nimaviridae*) is the etiological agent of white spot disease (WSD) (Wang et al. 1995). It is a large, enveloped, double stranded DNA virus with a rod-shaped particle having an apical envelope extension (Durand 1997). It infects numerous penaeid and non-penaeid decapod hosts and has been isolated from shrimps in every country supporting shrimp farming (Pradeep et al. 2012; Diggles 2017). It has been reported in wild-caught decapods (none from GTP) in the Gulf of Mexico and northwest Atlantic Ocean (Chang et al. 2001; Shields and Overstreet 2007; Blaylock et al. 2019; Muhammad et al. 2020; Vazquez-Sauceda et al. 2016). WSSV was originally discovered in 1992 from an epizootic of kuruma shrimp, *Penaeus japonicus* Spence Bate, 1888 in northern Taiwan (Chou et al. 1995; Lightner et al. 1998). The first documented case of disease caused by WSSV in the Western Hemisphere was reported from pond-reared northern white shrimp, *Penaeus setiferus* (Linnaeus, 1776) in south Texas in 1995 (Lightner 1996b).

IHHNV, also known as *Decapod penstylhamaparvovirus 1* [monotypic *Penstylhamaparvovirus*; Parvoviridae], is the etiological agent of infectious hypodermal and hematopoietic necrosis disease as well as runt deformity syndrome (RSD) (Bonami et al. 1990; Kalagayan et al. 1991; Lightner 1996a, 1999, 2011; Walker and Winton 2010; Pénzes et al. 2020). Three genotypes are known: Type 1 from Australia; Type 2 from eastern Asia and the Americas; and Type 3 from Southeast Asia. Non-infectious homologous insertions into the genome of GTPs are also described as Type A and Type B (Shen et al. 2015; Tang et al. 2002, 2006, 2007). It is the smallest of the known shrimp viruses (22 nm in diameter) and has a non-enveloped icosahedron particle with a single strand of DNA genome. Approximately 30 decapods, including GTPs, and non-decapod host have been reported to be infected with or carriers of IHHNV (Yu et al. 2021). The first reports of IHHNV are from late 1980 through 1981 from the University of Arizona's experimental shrimp culture facility in Hawaii, with acute mortalities in cultured blue shrimp, *Penaeus stylirostris* Stimpson, 1871 (see Lightner et al. 1983). IHHNV is known from each country where shrimp farming occurs and has been reported from wild stocks in the Indo-Pacific and along the Pacific coast of the Americas (Gulf of California to Peru) (Lightner 1996a; Aguilera et al. 2010). In the last 15 yrs, IHHNV has been reported from wild, native decapods from the Atlantic coast of Argentina, Brazil, and Mexico (Martorelli et al. 2010; Cavalli et al. 2013; Guzmán-Sáenz et al. 2009; Hernández-Pérez et al. 2017). Guzmán-Sáenz et al. (2009) reported IHHNV in wild decapods collected during 2005–2006 from Tamaulipas (Mexico), which also comprises the northern-most locality record for a wild IHHNV infection within the Gulf of Mexico.

TSV (*Apavirus*; *Discoviridae*) is the etiological agent of Taura syndrome. The disease was originally blamed on a fungicide but later the true etiological viral agent was discovered (Brock 1997; Lightner 2011). TSV is a non-enveloped, 31–32 nm in diameter, icosahedron with a positive sense single stranded RNA genome (Hasson et al. 1995; Bonami et al. 1997). Susceptible hosts are primarily penaeid shrimps; including greasyback shrimp (*Metapenaeus ensis*), northern brown shrimp (*Penaeus aztecus*), GTP, northern white shrimp, blue shrimp, Indian white shrimp (*Penaeus indicus*), kuruma shrimp, and whiteleg shrimp (= the most susceptible to Taura syndrome) (OIE 2019; Lightner 1996b, 2011; Lightner and Redman 1998b; Tang et al. 2012; Brock 1997). Taura syndrome was first reported in 1992 from shrimp farms near the Taura River, Gulf of Guayaquil, Ecuador. Shortly after being recognized as a viral disease, it spread to other shrimp farming regions of Latin America and parts of the United States (Jimenez 1992; Brock 1997; Hasson et al. 1999; Lightner 1999). However, it has not been reported in wild shrimps along the Atlantic, Caribbean, or Gulf of Mexico coast of the Americas (Lightner 1996a; OIE 2019). In 1998, TSV was reported from whiteleg shrimp from Taiwan and is now recognized in most shrimp farming countries throughout Asia, the Middle East, and the Americas (Lightner 2011; Lightner et al. 2012).

Materials and methods

Shrimp samples and collections

Gill or, if gill was not available, somatic muscle from 152 wild-caught GTP were screened for WSSV, IHHNV, and TSV. The sampled GTPs comprised 1 freshly-collected specimen trawled by a commercial shrimper during 2020 from Mississippi Sound (Northern Gulf of Mexico) (JDK and SAB collection; gill preserved in 95% ethanol); 54 specimens trawled by commercial shrimpers, recreational fishers, and researchers during 2014–2016 from the Northern Gulf of Mexico off Mississippi, Alabama, and Florida and initially frozen (JMH collection; whole frozen GTPs were gill biopsied by JDK at JMH's laboratory and preserved in 95% ethanol before being shipped to the Southeastern Cooperative Fish Parasite and Disease Laboratory [SCFPDL]); 76 specimens trawled by commercial shrimpers during 2014–2020 from the Northwestern Atlantic Ocean off North Carolina, South Carolina, Georgia, and Florida and initially frozen (PRKS, MRK, and ELG collection; whole frozen GTPs were gill biopsied and preserved in 95% ethanol before being shipped to the SCFPDL); and 21 specimens (19 initially ethanol-preserved; 2 initially formalin-fixed) in the collection of the Florida Fish and Wildlife Conservation Commission's Fish and Wildlife Research Institute (FWRI) that were trawled by commercial shrimpers and researchers during 1988, 2011–2013, and 2016 from the Atlantic and Gulf of Mexico coast of Florida and initially fixed in formalin or preserved in 95% ethanol (gill was

biopsied and preserved in 95% ethanol before being shipped to the SCFPDL) (FSBC Nos. I–33739, I–078598, I–078599, I–078600, I–095572, I–102510, I–106260, I–112186, I–133251, I–138202) (Supplementary material Table S1). The code FSBC follows the annotated codes for natural history collections detailed by Sabaj (2020). Common names for penaeids and the use of "shrimp" and "prawn" (neither of which have any taxonomic standing nor define monophyletic groups) follows Chan (1998).

Nucleic acid extraction

DNA and RNA were extracted from the gill and somatic muscle samples using the Qiagen DNeasy Blood and Tissue Kit and the Qiagen RNeasy plus mini kit, respectively, according to the manufacturer's instructions. DNA and RNA were each eluted in 30 µL of RNase-free water, diluted to a 50 ng/ µL concentration if needed, and stored at −20 °C until further testing. WSSV screening was performed using a Taqman qPCR assay with primers WSS1011F/WSS1079R and probe designed by Durand and Lightner (2002). We used a positive control to generate a standard curve (Muhammad et al. 2020). The assay was performed using Taqman Universal Mastermix II, with no UNG. A 5 µL of template DNA was added to the PCR master mix containing 0.3 µm of each primer plus 0.15 µm of Taqman probe for a 25 µL final reaction volume. Amplification was carried out using the following PCR cycle in a QuantStudio 5 Real Time PCR System: 50 °C for 2 min, 95 °C for 10 min followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. A cut off C_T value of < 38 was used to determine positive DNA amplification. Copy number of positive samples were calculated based on the oligonucleotide length and the assumption of average weight of a nucleotide base pair is 650 Da along with conversions from weight to volume as described by Blaylock et al. (2019). IHHNV detection first used recommended WHOA conventional PCR primer sets 309F/R (Tang et al. 2007) followed by primer set 389F/R (OIE 2009). The PCR reactions contained: 5 µL of 5x goTaq reaction buffer, 0.2 µm of each primer, 0.8 µm of dNTP mix, 2.5 mM $MgCL_2$, and 0.15 µL Taq polymerase (5U/µL) for a 25 µL reaction volume. Amplification for primer set 309F/R was carried out using PCR cycle: 95 °C for 5 min, followed by 45 cycles of 30 s at 95 °C, 30 sec at 53 °C, and 1 min at 72 °C. Amplification using 389F/R primer set follows protocol detailed by OIE (2009). TSV screening used RT-PCR primers 9992F/9195R (Nunan et al. 1998; OIE 2009) and QIAGEN OneStep Ahead RT-PCR Kit according to the manufacturer's instructions with the extracted RNA.

Sequencing

WSSV qPCR positives with a Ct value < 25 were used for conventional PCR and sequencing using primer sets (146, USC4, USC5, VP28) as per Knibb et al. (2018). Three samples (Ocean Springs, Mississippi, 8 August

2014; Charleston Harbor, South Carolina, October 17 2017; Ashley River, South Carolina 28 July 2015) had C_T values < 25, indicating higher viral concentrations than those with greater C_T values, and were therefore specifically chosen to have a higher chance of containing enough intact DNA for amplification by all primer sets. PCR products for WSSV and IHHNV were analyzed on 1% agarose gels with ethidium bromide before being purified with QIAquick PCR purification kit and sequenced by Azenta life sciences (South Plainfield, New Jersey) using the same primer sets for amplification. All 18 WSSV+ gill tissue was processed for whole genome sequencing. Additional tissues were resampled and purified with Polyethylene glycol 6,000 at 30% in 1.5 mole $NaCl_2$ at a 2:1 volume for 2 hrs; 3 µL of RNase was subsequently added to the mixture and incubated for an additional 30 min at 37 °C to remove host RNA. The viral capsule was disrupted to release viral DNA using 3 µL proteinase K for 1 hr at 37 °C. Purification to further remove host DNA and amplification of genomic DNA used AMPure XP beads (Beckman Coulter) and GenomiPhi V2 DNA Amplification Kit (Cytiva) as per the manufacturer's instructions. Amplified DNA was sent to the Genomics and Bioinformatics Resources Core at the University of Idaho for whole genome sequencing on an Illumina MiSeq using 2 × 300 bp chemistry. Given failures associated with genome sequencing (see Results), we amplified and sequenced four fragments of the genome for phylogenetic and network analyses; these fragments were chosen because proven-effective primers exist for them.

Genome assembly and phylogenetic analysis of WSSV

Raw Illumina data were trimmed for low-quality reads and sequencing adapters using fastp and default settings (Chen et al. 2018). Genome assembly for the sequenced individual was done by mapping raw reads to the NCBI Reference Sequence for WSSV (NC_003225) with Bowtie2 (Langmead and Salzberg 2012) and default parameters. For phylogenetic analyses, we used Sanger-sequenced fragments (Ocean Springs, Mississippi, 8 August 2014; Charleston Harbor, South Carolina, October 17 2017; Ashley River, South Carolina, 28 July 2015) of WSSV and orthologous fragments of publicly available whole WSSV genomes. Orthologous genomic regions were retrieved by aligning Sanger sequenced fragments to whole genome sequences in Geneious Primer (Biomatters) with the Mauve Plugin for genome alignment (Darling et al. 2004) and then extracting the aligned regions. Aligned gene regions were concatenated with FASconCAT-G (Kück and Longo 2014). We inferred a maximum likelihood phylogeny with gaps (indels) coded as a fifth state because most of the sequence variation was in gappy regions of the alignment. The maximum likelihood tree, which was midpoint rooted, was inferred in RAxML-ng using the 5-character multistate model GTR + F0 + Γ. Branch support was estimated with non-parametric

bootstrapping; the appropriate number of bootstrap replicates was determined with bootstrapping method in RAxML and a maximum of 1000 replicates. Given the close relationship among sequenced WSSV isolates, we also inferred a median-joining network (Bandelt et al. 1999) in NETWORK (https://www.fluxus-engineering.com/) using the concatenated alignment.

Transmission electron microscopy (TEM)

TEM was performed using a Zeiss EM10 TEM using EtOH-preserved, WSSV qPCR+ gill. A 1.5 mm^3 cube of gill tissue was placed in 6% glutaraldehyde for 24 hrs before post-fixing in 2% osmium tetraoxide for 1 hr in darkness and at room temperature. Samples were dehydrated in an ethanol series with 2 final 1-hr washes with absolute EtOH. Samples were transferred to 100% propylene oxide for 8 hrs before transitioning to Spurr's resin. Transition steps initially comprised a 3:1 ratio of propylene oxide to Spurr's resin, then 1:2, then 1:3, and finally 2 steps at 100% Spurr's resin (each step for 8 hrs). Samples were finally placed in a BEEM embedding capsule with resin and hardened in a 65 °C oven for 24 hrs. A total of 60–70 1 µm sections were placed on a size 200 mesh copper grid. Grids were stained with uranyl acetate for 1 hr in the dark, washed 3 times with carbon dioxide free water, and stained for 15 min with lead citrate in a carbon dioxide reduced environment.

Results

qPCR and PCR

A total of 152 GTPs (Table S1) were screened for WSSV, IHHNV, and TSV: 18 (12%) were qPCR+ for WSSV (3 from the northern Gulf of Mexico off Mississippi, 1 from the eastern Gulf of Mexico off Florida, and 14 from the Northwestern Atlantic Ocean off South Carolina and Georgia), 1 was PCR+ for IHHNV (Northwestern Atlantic Ocean off South Carolina), and none was positive by rtPCR for Taura Virus. The RNA was stored at −20 °C, which can lead to sample degradation, possibly affecting testing and leading to false negatives. Thus, this result should be viewed with skepticism and repeated upon collection of fresh GTPs for RNA viral screening. WSSV+ samples, C_T value, and estimated copy number are detailed in Table 1. Gill from 2 of the WSSV qPCR+ samples (1 from the Gulf of Mexico; 1 from off South Carolina) were screened by TEM (Figure 1) for confirmation of viral infection (the gill of the IHHNV+ shrimp was inadequate for TEM). One sample from the Gulf of Mexico (Ocean Springs, Mississippi, 8 August 2014) and 2 from South Carolina (Charleston Harbor, South Carolina, 17 October 2017; Ashley River, South Carolina, 28 July 2015) had relatively lower C_T values of 22.36, 17.9, and 24.4, respectively, and were used for the phylogenetic analysis. One GTP (Cooper River, South Carolina, 7 July 2020) of 152 GTPs sampled was PCR+ for IHHNV using both primer sets 309F/R and 389F/R.

Table 1. Giant tiger prawn, *Penaeus monodon*, that were qPCR+ for WSSV infection in the present study. Copy number estimated from reported cycle threshold value.

WSSV Positives	Location	Collection Date	Ct value	Copy Number
Northwest Atlantic Ocean	Coosaw Creek, SC	7/23/2014	37.017	11
	Folly River, SC	7/24/2014	32.78	241
	Murrells Inlet, SC	8/18/2014	37.66	7
	Cooper River, SC	10/30/2014	37.3	9
	Folly River, SC	7/13/2015	33.56	136
	Ashley River, SC	7/28/2015	24.14	140, 672
	Ashley River, SC	7/28/2015	35.32	37
	Rock Springs Creek, SC	7/29/2015	36.8	12
	Cowen Creek, SC	9/19/2015	35.11	43
	Atlantic Ocean (off GA)	10/8/2015	37.79	6
	Winyah Bay, SC	7/20/2017	36.7	13
	Charleston Harbor, SC	10/17/2017	17.904	13, 984, 489
	Ashley River, SC	8/13/2019	33.56	136
	Bull Creek, SC	10/15/2020	35.7	28
Gulf of Mexico	Ocean Springs, MS	8/15/2014	22.36	522, 383
	Gulf of Mexico	10/12/2015	37.838	6
	Peace River, FL	5/26/2016	35.64	29
	D'Iberville, MS	10/1/2020	37.9	6

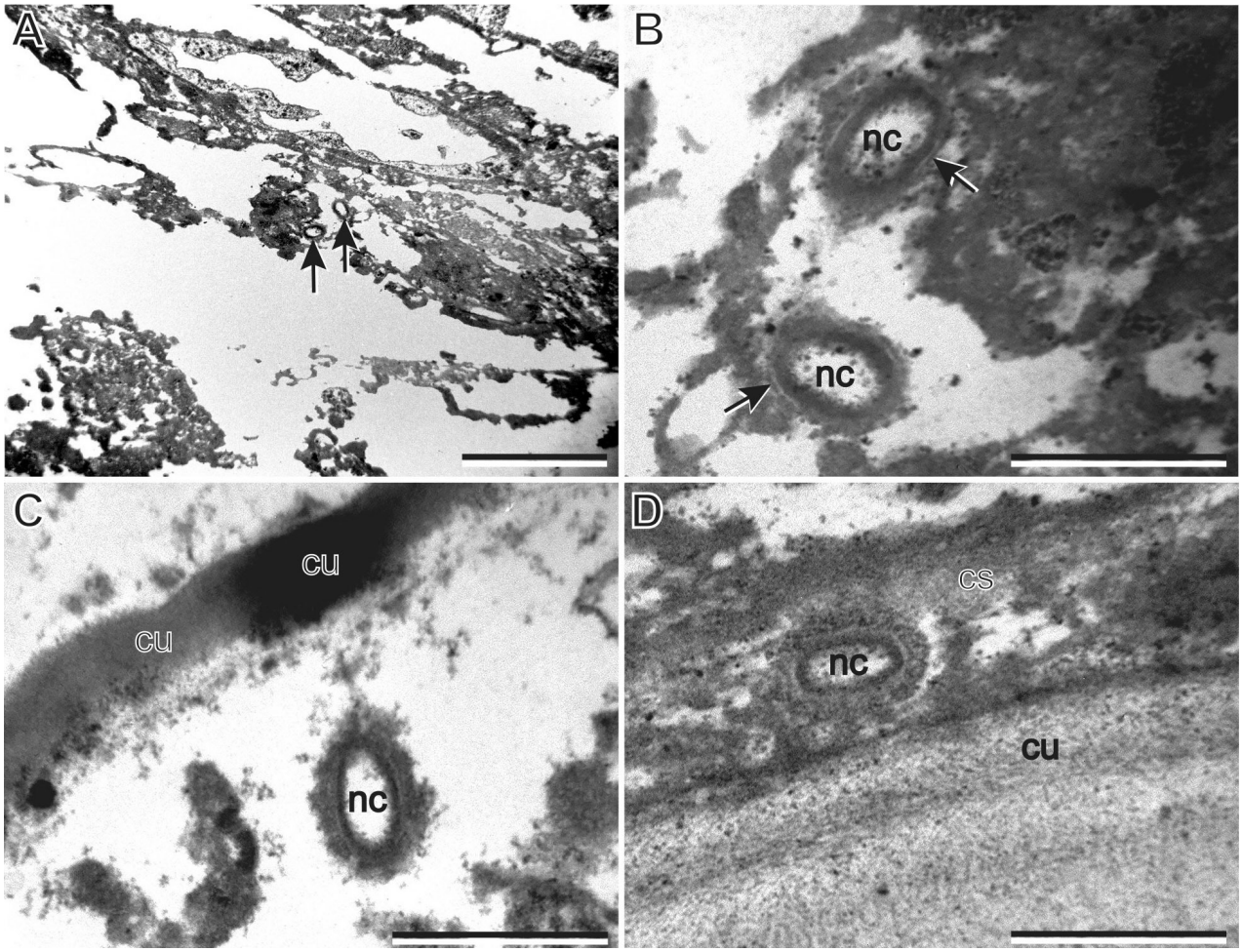

Figure 1. Gill tissue of giant tiger prawn (GTP), *Penaeus monodon*, that were qPCR+ for WSSV infection and imaged using transmission electron microscopy. (a) Virions (arrows) from GTP from the NW Atlantic Ocean off South Carolina. Scale bar = 2 µm. (b) Virions showing nucleocapsid lumen (nc) and segments of partly intact trilamilar envelope (arrows) from GTP from the NW Atlantic Ocean off South Carolina. Scale bar = 500 nm. (c) A virion showing the nucleocapsid lumen (nc) and the cuticle (cu) of GTP from the Gulf of Mexico off Mississippi. Scale bar = 500 nm. (d) A virion showing the nucleocapsid lumen (nc) within the subcuticular cytoplasmic sheet (cs) adjacent to the thick cuticle (cu) from a GTP from the NW Atlantic Ocean off South Carolina. Scale bar = 500 nm.

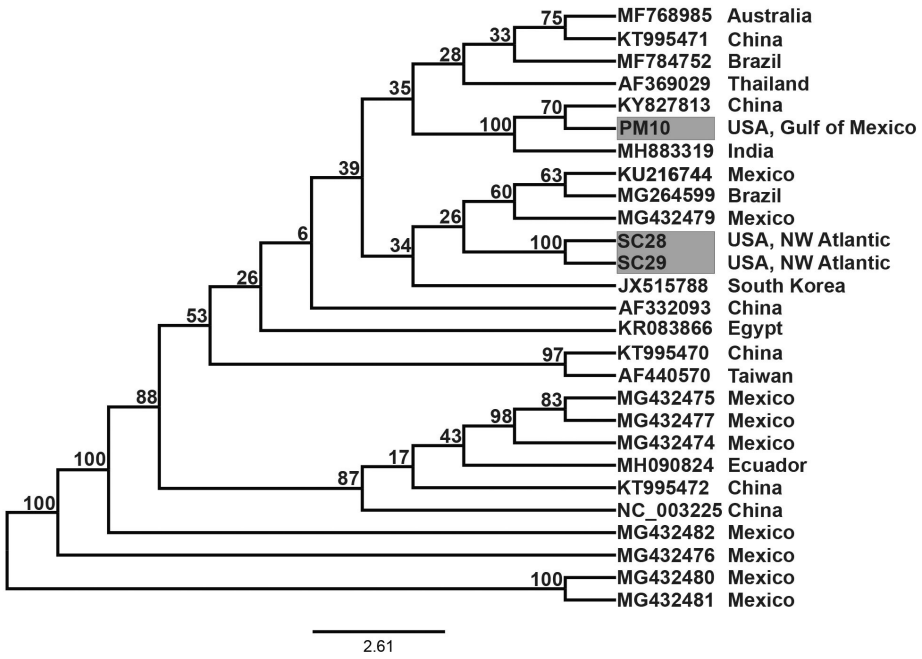

Figure 2. RAxML-g inferred maximum likelihood cladogram (midpoint rooted). Branches are labelled with non-parametric bootstrap support. GenBank accession numbers comprise the endnodes; gray boxes are sequences generated from the present study.

Phylogenetic analysis

The 2 WSSV isolates from the Northwestern Atlantic Ocean off South Carolina were identical and comprised a lineage distinct from the WSSV isolate from the Gulf of Mexico (Figures 2, 3). The phylogenetic and network analyses (RAxML-g inferred maximum likelihood cladogram (Figure 2) and the median joining network (Figure 3)) indicated that at least 2 lineages of WSSV infect GTPs in United States coastal waters off South Carolina, Georgia, Florida, and Mississippi (Figures 1–4). The WSSV isolates from the Northwestern Atlantic Ocean off South Carolina were sister to WSSV isolates from Mexico and Brazil and hence collectively comprise a clade of WSSV isolates from the Americas (Figure 2). This clade was sister to an isolate from South Korea (JX515788). The other WSSV isolate analyzed herein (from the Gulf of Mexico) was recovered within a clade of isolates from China and India (Figure 2). Genome sequencing for WSSV failed as a result of too few reads per sample mapping to the reference genome. Raw sequencing data will not be made publicly available.

Transmission electron microscopy

TEM of the sectioned gill tissue was challenged by the extremely poor condition of the frozen or EtOH-preserved shrimp tissues: the gill was expectedly extremely degraded and generally poor for TEM. Recognizable tissues primarily constituted the cuticle and residual subcuticular cytoplasmic sheet only. Within the tissue sections for 2 shrimp (Charleston Harbor, South Carolina, 17 October 2017; Ashley River, South Carolina, 28 July 2015) from off South Carolina and 1 shrimp (Ocean Springs, Mississippi, 8 August

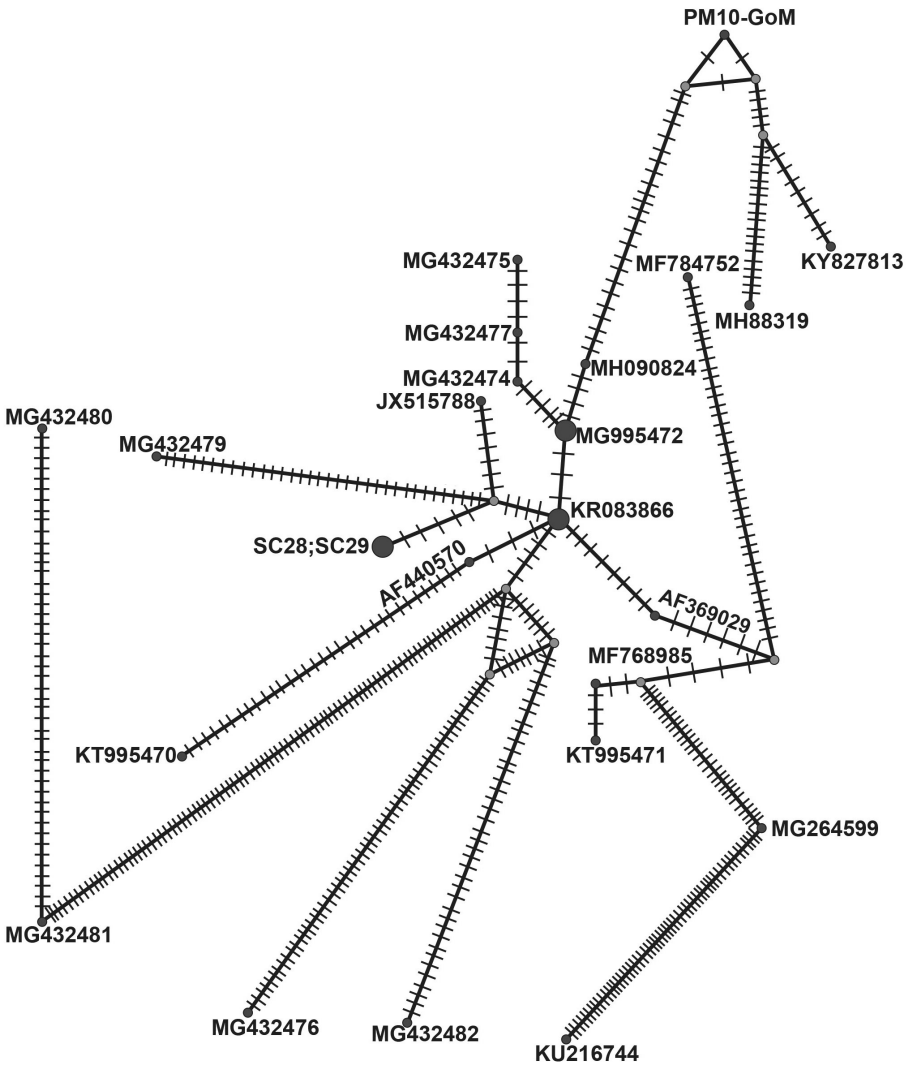

Figure 3. Median joining network of WSSV DNA sequences. Node size is proportional to the number of isolates with the same haplotype; gray nodes are inferred haplotypes; line ticks are mutations between isolates.

2014) from off Mississippi, we observed several viral particles that resembled published descriptions of WSSV (Figure 1; Durand et al. 1997; Lightner 2011). The gill tissue we studied was never initially fixed (*sensu stricto*; rather they were initially preserved in EtOH or frozen), and the resulting poor TEM images and poor differentiation at the cellular level was likely related to poor tissue stabilization. Our observations of virions in the studied gill tissue seemingly confirmed previous assertions that ethanol-based preservatives/solutions induce demonstrable artifactitious structural changes in virion particles and the virion envelop itself (Martín-González et al. 2020; Watts et al. 2021). Hence, the virions we observed with TEM in the WSSV+ gill samples cannot be definitively identified as WSSV based on morphology alone: they were ovoid or rod-shaped, with some specimens having a trilaminar envelope (Figure 1A–D). No tail like appendage could be discerned in a virion studied.

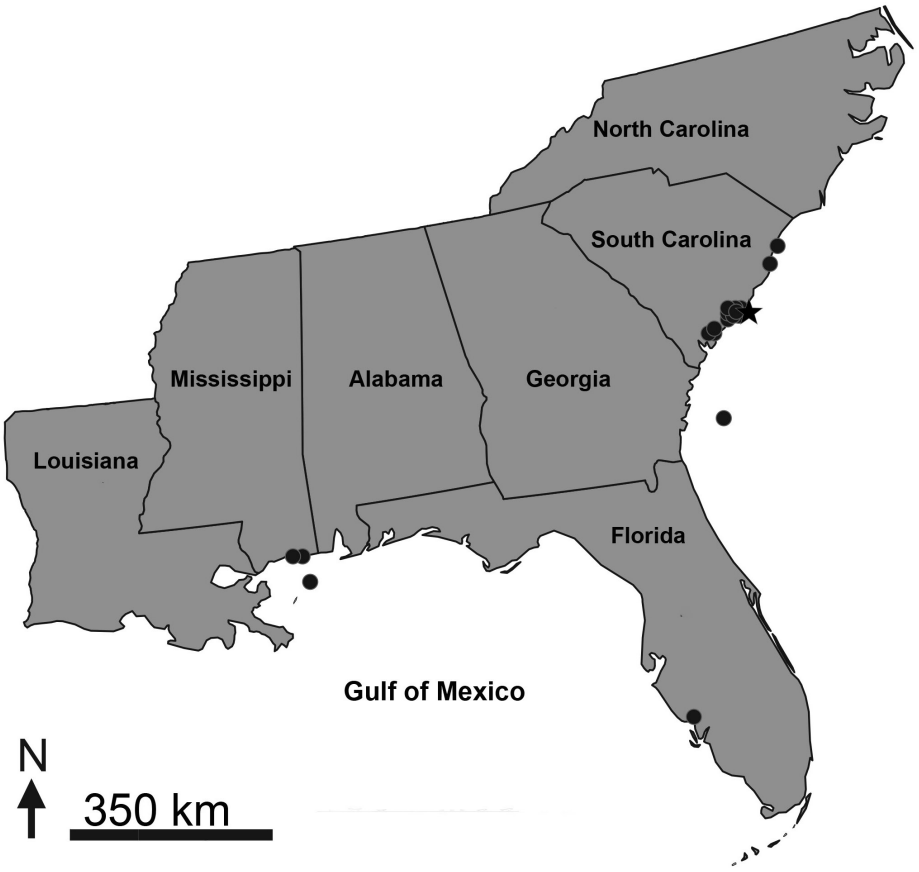

Figure 4. Geographic localities for the giant tiger prawns, *Penaeus monodon*, sampled herein that were qPCR+ for WSSV infection. The star represents the locality of the IHHNV+ detection.

The morphology of virions from TEM sections from South Carolina GTP (Figure 1A, B, D) follows: virion 220.55–311.45 nm (274.13 nm) long, 128.31–207.56 nm (173.35 nm) in maximum width at midsection, approximately 2.5× longer than wide; virion envelope 5.3–7.31 nm (6.42 nm) thick; gap between envelope and nucleocapsid 4.69–8.74 nm (6.33 nm) in breadth; nucleocapsid 191.08–286.37 nm (247.58 nm) long, 90.73–181.03 nm (146.48 nm) in maximum width at midsection. Morphology of virus from Gulf of Mexico GTP (Figure 1C): virion 281.69–310.81 nm (293.49 nm) long, 188.96–215.15 nm (211.51 nm) in maximum width at midsection; virion envelope 14.9–25.76 nm (22.17 nm) thick; gap between envelope and nucleocapsid 5.39–7.54 nm (6.02 nm) in breadth; nucleocapsid 237.49–265.35 nm (249.09 nm) long, 129.4–164.29 nm (148.35 nm) in maximum width at midsection. The envelope of the virions from the Gulf of Mexico (collected 3 yrs before the South Carolina samples) was degraded compared to that of the specimens from South Carolina.

Discussion

The present study documents the presence of WSSV and IHHNV infections among wild-caught GTP off North America (Northwestern Atlantic Ocean and Gulf of Mexico) and comprises the first published detection of a virus in a wild-caught GTP in the Atlantic Ocean Basin

(inclusive of the Gulf of Mexico). Whereas WSSV had been previously reported from decapods in the northern Gulf of Mexico and Northwest Atlantic Ocean (Chang et al. 2001; Vazquez-Sauceda et al. 2016; Blaylock et al. 2019; Muhammad et al. 2020), IHHNV has only been reported as far north as off Tamaulipas (Mexico) in the Atlantic Ocean Basin (Guzmán-Sáenz et al. 2009). The geographic gaps between previously reported infections and the infections reported herein are likely due to lack of sampling. Funding for conducting pathogen surveillance among apparently "healthy" wild aquatic animal populations can be difficult to attain. Hence, very little sampling effort has been focused on invasive GTP and their pathogens.

We herein detected 2 lineages of WSSV from the Gulf of Mexico and Northwestern Atlantic Ocean, respectively, based on a relatively small sample size. These lineages were not genetically related (they were paraphyletic) (Figures 2, 3). In specific and considering all available/comparable nucleotide sequences for WSSV, the phylogenetic analyses indicated that (i) WSSV isolates do not clade by locality (they are paraphyletic; an exception comprises the identical isolates of WSSV from South Carolina), (ii) the virus has been repeatedly translocated and introduced, and (iii) the Gulf of Mexico isolate and Northwestern Atlantic Ocean isolates do not share a recent common ancestor and were likely introduced independently and from different geographic localities. Analyzing additional viral isolates could reveal additional lineages that could further indicate independent WSSV introductions to the Atlantic Ocean Basin. However, too few WSSV isolates were studied herein to assess genetic variability as a tool for theorizing the time of this introduction. As an invasive species susceptible to both WSSV and IHHNV, GTP likely is a natural vector for both viruses, including different WSSV and IHHNV genotypes, to new geographic areas and naive hosts. Differential pathology attributable to viral strain/genotype is a concern for the conservation of native decapod populations (including commercially-important crabs and shrimps). In this way, describing these various strains is important in forecasting and/or understanding potential future disease outbreaks in wild and cultured populations.

The presence of viral sequences integrated into the genome of a host i.e., endogenous viral elements (EVE), has obvious relevance to PCR-based diagnostic tests because it could result in a false positive for a replicating virus (Alday-Sanz et al. 2020). An EVE comprises a viral genome that is integrated into the host germline leading to fixation and is most common among retroviruses as integration with the host genome is an obligatory component of the retroviral life cycle (Holmes 2011). DNA and RNA viruses can integrate with the host genome by interacting with cellular retroviral elements or via non-homologous recombination (Holmes 2011). WSSV and WSSV-like EVEs have been found in GTPs, kuruma shrimp, whiteleg shrimp, brush-clawed shore crab (*Hemigrapsus takanoi*), greasyback shrimp (*Metapenaeus ensis*), and flower crab (*Sesarma intermedium*)

(Hossain et al. 2021; Bao et al. 2020). The known variety and number of EVE present in crustacean genomes is continually growing with representatives from more than just the retroviruses (Thézé et al. 2014). The presence of EVEs of IHHNV were first reported by Tang and Lightner (2006, and these authors demonstrated the potential for false positives using the recommended WHOA primer sets. Saksmerprome et al. (2011) studied IHHNV EVEs in GTP genomes showing that the primer set 309F/R developed by Tang et al. (2007) to differentiate between exogenous IHHNV and endogenous elements had false positives in some instances because it detected unexpected endogenous elements. For this purpose, if there is an unexpected positive in otherwise healthy shrimp it has been recommended to use two confirmatory tests or primer sets coding for different regions of the genome (OIE 2019). We doubt that the PCR diagnosis of WSSV and IHHNV in our sample set was due to EVEs because our chemistry amplified multiple distinct gene fragments for both viruses. EVEs have the potential to benefit the host either by modulating the host response to exogenous viruses or, in the case of functional EVEs, by directly coding for proteins that act as immunogens (Holmes 2011). This has led to the study of EVEs for developing specific pathogen free/ specific pathogen resistant (SPF/SPR) shrimp stocks against some of the major viruses in the industry (Taengchaiyaphum et al. 2019).

The epidemiology of WSSV and IHHNV in wild decapod populations is understudied. We are aware of only 1 study that has documented the prevalence and intensity of viral infections among non-commercial decapods sympatric with invasive GTPs in the Gulf of Mexico (Muhammad et al. 2020). Muhammad et al. (2020) studied 11 non-penaeid decapod species native to the Gulf of Mexico and concluded that nearly all (10 of 11; 91%) were qPCR+ for WSSV. Given that WSSV is widespread throughout the Gulf of Mexico and northwest Atlantic Ocean (Chang et al. 2001; Chapman et al. 2004; Baumgartner et al. 2009; Muhammad 2016; Blaylock et al. 2019), Muhammad et al. (2020) theorized that the processing of imported seafood could comprise a chronic and long-term source of WSSV introductions on a regional scale. Given that these processing plants receive shrimp from across the world, this could lead to a genetically heterogenous WSSV population.

Surveying wild crustaceans for viral infections could improve the collective understanding of the natural geographic distribution, host specificity, and natural history of these viruses. Increased screening of wild decapods for shrimp viruses is needed from both applied and basic research perspectives. As parasites, viruses can show specificity to specific host lineages or to hosts that occupy a specific niche or habitat (Bandín and Dopazo 2011; Rothenburg and Brennan 2020). However, we lack host specificity information for most shrimp viruses. Each of these shrimp viruses could have evolved as a parasite of other decapods and crossed over to commercially-

valued shrimps, which are intensively screened for diseases by diagnostics laboratories and are thereby relatively less well documented from wild decapod populations. Regarding the applied value of viral diagnostics of wild decapod communities, given that shrimp farms are sited within aquatic ecosystems that also harbor a diverse decapod community, knowledge of crustacean vectors, pathways, and host life cycle specificity have obvious biosecurity relevance and best management practices implications.

Our data show that WSSV and IHHNV have been infecting GTP in their invasive range off North America since at least 2014 and 2020, respectively. All but 20 specimens were collected during 2014 through 2020. The lack of WSSV positives prior to 2014 could, in part, be a result of the small number of specimens available. Viral DNA could have also degraded beyond the detection threshold; as we suspect with the RNA virus TSV. Although the majority of GTPs with WSSV+ gill tissue were collected during June through October, this timeframe largely coincides with the commercial shrimping season. Thus, fewer prawns were collected outside of that period, which is bias. Without further and more consistent sampling, seasonality of WSSV in North America is indeterminate. There is also potential for false negatives or higher C_T values due to degradation of viral DNA from the specimens being initially frozen at –20 °C (for years) and then post-preserved in EtOH.

We doubt that IHHNV has moved from the western Gulf of Mexico to the South Carolina coast (or vice versa) without infecting decapods between these collection points. The IHHNV positive sample (Cooper River, South Carolina, 7 July 2020) was collected in 2020 from the coast of South Carolina and Guzmán-Sáenz et al. (2009) reported IHHNV in wild decapods from the coast of Tamaulipas, Mexico. As a single stranded DNA virus, IHHNV probably degraded more quickly than WSSV, perhaps preventing us from detecting it and therefore resulting in a false negative. Taura virus, as an RNA virus, would have fared the worst; with rapid degradation leading to decreased diagnostic sensitivity and a greater likelihood of false negatives.

Authors' contribution

Justin Krol and Stephen Bullard provided research conceptualization, investigation and data collection, data analysis and interpretation, and original draft writing. Dr. Jennifer Hill, Dr. Peter Kingsley-Smith, Dr. Michael Kendrick, Elizabeth Gooding, Corrine Fuchs provided preserved sampled giant tiger prawns captured from the Gulf of Mexico and Northwestern Atlantic Ocean as well as review and editing of the manuscript. Dr. Whelan provided data analysis and interpretation and review/editing.

Acknowledgements

We thank Capt. Frank Parker (Southern Shrimp Alliance, Ocean Springs, MS), Steve Curran (Aquatic Parasitology Laboratory, Auburn University), Michael Miller (Harrison School of Pharmacy, Auburn University), and Timothy Bruce (School of Fisheries, Aquaculture, and Aquatic Sciences, Auburn University) for methodological help. We thank the reviewers for the time and effort necessary to review this manuscript. The findings and conclusions in this article are those of the authors and do not necessarily represent the views of the U.S. Fish and Wildlife Service.

Funding

This work was funded by the Southeastern Cooperative Fish Parasite and Disease Project as well as by National Sea Grant (National Oceanic and Atmospheric Administration), United States Fish and Wildlife Service, United States Department of Agriculture (National Institute of Food and Agriculture), Federal Aid in Sport Fish Restoration (Alabama Department of Conservation and Natural Resources, Inland and Marine Resources Divisions), Alabama Agricultural Experiment Station, Auburn University College of Agriculture, and the South Carolina Saltwater Recreational License Fund.

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Supplementary material

The following supplementary material is available for this article:

Table S1. Records of giant tiger prawns, *Penaeus monodon*, screened for viruses in the present study.

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