Diseases of oysters *Crassostrea ariakensis* and *C. virginica* reared in ambient waters from the Choptank River, Maryland and the Indian River Lagoon, Florida

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ABSTRACT: To assess potential benefits and liabilities from a proposed introduction of Asian Suminoe oysters, susceptibilities of exotic *Crassostrea ariakensis* and native *C. virginica* oysters were compared during exposures to pathogens endemic in temperate, mesohaline waters of Chesapeake Bay and sub-tropical, polyhaline Atlantic waters of southern Florida, USA. Cohorts of diploid, sibling oysters of both species were periodically tested for diseases while reared in meso-cosms receiving ambient waters from the Choptank River, Maryland (>3 yr) or the Indian River Lagoon, Florida (10 to 11 mo). *Haplosporidium* sp. infections (e.g. MSX disease) were not detected in oysters from either site. *Perkinsus* sp. infections (dermo disease) occurred among members of both oyster species at both sites, but infections were generally of low or moderate intensities. A *Bonamia* sp. was detected by PCR of DNAs from tissues of both oyster species following exposure to Florida waters, with maximum PCR prevalences of 44 and 15% among *C. ariakensis* and *C. virginica* oysters respectively during June 2007. Among *C. ariakensis* oysters sampled during April to July 2007, a *Bonamia* sp. was detected in 31% of oysters by PCR (range 11 to 35%) and confirmed histologically in 10% (range 0 to 15%). Among simultaneously sampled *C. virginica* oysters, a *Bonamia* sp. was detected in 7% by PCR (range 0 to 15%), but histological lesions were absent. Although this is the first report of a *Bonamia* sp. from Florida waters, sequences of small subunit (SSU) rDNA and *in situ* hybridization (ISH) assays both identified the Florida pathogen as *Bonamia exitiosa*, which also infects oysters in the proximate waters of North Carolina, USA.

KEY WORDS: Dermo · MSX · *Perkinsus marinus* · *Haplosporidium nelsoni* · *Bonamia exitiosa* · Chesapeake Bay · Atlantic–Florida · Suminoe oyster · Eastern oyster

INTRODUCTION

Among considerations for proposed introductions of exotic bivalve molluscs to new locations, avoiding the spread or exacerbation of marine diseases is critical. Conservative methods for avoiding inadvertent

pathogen introductions include the Code of Practice on Introductions and Transfers of Marine Organisms by the International Council for the Exploration of the Sea (ICES 1995). In some cases where exotic bivalve molluscs have been introduced to coastal ecosystems without such precautions, collateral in-

troductions of exotic pathogens have had severe impacts on native molluscs. Examples include the probable introduction of *Haplosporidium nelsoni* along North American Atlantic coasts with experimental introductions of *Crassostrea gigas* oysters, which decimated native *C. virginica* oysters (Burreson et al. 2000, Fincham 2006), and the probable introduction of the destructive clam pathogen *Perkinsus olseni* along European Atlantic coasts with introductions of Asian *Ruditapes philippinarum* clams (Hine 2002, Villalba et al. 2004).

Conversely, introduced molluscs have shown lethal susceptibilities to native pathogens present in recipient environments, even when such pathogens cause negligible or unrecognized pathologies among native molluscs. During experimental exposures to French–Atlantic waters endemic for *Bonamia ostreae*, Asian Suminoe oysters *Crassostrea ariakensis* suffered lethal infections by a *Bonamia* sp. pathogen (Cochennec et al. 1998). Unanticipated effects from an unrecognized local pathogen include the lethal susceptibility of *C. ariakensis* oysters to *B. exitiosa* infections when reared in Atlantic waters of North Carolina, USA (Burreson et al. 2004, Bishop et al. 2006, Carnegie et al. 2008). Although *B. exitiosa* was previously unrecognized from southern US Atlantic waters and is without known disease impacts among native *C. virginica* oysters there, it is now known to infect native *Ostrea stentina* oysters (= *Ostreola equestris*, Shilts et al. 2007) in North Carolina waters (Hill et al. 2010, R. B. Carnegie unpubl. data).

The Suminoe oyster *Crassostrea ariakensis* was proposed for possible introduction to Chesapeake Bay waters by the Maryland Department of Natural Resources in 2002, as a mechanism to replace or bolster depleted fishery production and ecological services from native *C. virginica* oyster populations that have been diminished by a century and a half of over-harvesting and habitat destruction (Newell 1988, Rothschild et al. 1994, Kirby 2004) and more recently by diseases (Andrews 1962, Burreson & Andrews 1988). To evaluate potential benefits and detriments to native oysters and ecosystems from such an introduction, results of diverse investigations were evaluated within the framework of a formal Programmatic Environmental Impact Statement (USACOE 2009).

This report describes infections by major pathogens that occurred among native *Crassostrea virginica* oysters and exotic *C. ariakensis* oysters when groups of diploid sibling oysters of both species were reared in waters from 2 locations: the temperate, mesohaline Choptank River, Maryland, tributary of Chesapeake Bay, and the sub-tropical, polyhaline Indian River Lagoon on the Atlantic coast of southern Florida. It includes the first detailed report on infections by *Bonamia exitiosa* among oysters reared in Atlantic waters of southern Florida, USA.

MATERIALS AND METHODS

Experimental mesocosms

Effluent-quarantined plastic or fiberglass oyster rearing tanks at each facility were segregated in biologically secure modules that received raw ambient waters pumped directly from their respective local sub-estuaries. At the Chesapeake Bay–Maryland site, 500-l oyster rearing tanks received mesohaline Choptank River waters at 20 l min^{-1}. At the Atlantic–Florida site, 700-l oyster rearing tanks received polyhaline Indian River Lagoon waters at 10 l min^{-1}. Local ambient waters were aerated from the rearing tank bottoms to maintain ambient particle suspensions during water transits through experimental oyster rearing tanks, and accumulated sediments were periodically flushed from tank bottoms. Experimental oysters were reared during experimental exposures to ambient waters at each site in mesh containers that were suspended off-bottom in mesocosm rearing tanks. Before discharge, effluent mesocosm waters were sanitized by continuous chlorination to a free chlorine concentration ≥2 ppm for ≥45 min (Kelly et al. 2011).

Experimental oysters

Experimental oysters of 3 contiguous year-classes of each species were set on adult oyster shells during July of 2004, 2005, and 2006 at the Maryland facility. Competent pediveliger larvae were obtained during different years from either a Taylor United Shellfish oyster hatchery (Shelton, WA, 2004) or a Virginia Institute of Marine Science (VIMS) oyster hatchery (Wachapreague, VA, 2004 to 2006). When scheduled genetic analyses (Cordes et al. 2008) revealed that both 2005 year-classes of VIMS hatchery larvae were *Crassostrea virginica* oysters, the planned 2005 *C. ariakensis* year-class was eliminated from all experimental deployments and analyses (Kelly et al. 2011). Other than the missing 2005 *C. ariakensis* year-class, new year-classes of each oyster species were exposed to Choptank River waters during July 2004 through November 2007. The maximum exposure

period to extant local disease pressures was 50 mo for 2004 year-class experimental oysters, while the minimum exposure time was 14 mo for 2006 year-class oysters.

During September 2006, groups that included all year-classes of experimental oysters of both species were transferred from the Maryland mesocosm facility to the southern Florida facility. Experimental oysters that had been exposed for variable durations to pathogens present in Choptank River waters were subsequently exposed to pathogens from Indian River Lagoon waters for 10 mo (*Crassostrea ariakensis*) or 11 mo (*C. virginica*). Siblings of experimental oysters that were transferred for exposure to pathogens in Florida waters were sampled at the Maryland facility for disease analyses within 30 d of those transfers.

Disease assays and schedules

Samples of 20 (Florida) or 24 (Maryland) experimental *Crassostrea ariakensis* and *C. virginica* oysters were taken haphazardly for pathological assays during periodic sampling iterations at each site (Table 1). To secure sufficient tissue volumes from small juvenile oysters for histological analyses, alternative Ray's fluid thioglycollate medium assays (ARFTM, La Peyre et al. 2003) and various PCR assays, duplicate samples from each group were required when cohorts of young oyster spat at the Maryland facility had modal shell heights <15 mm. At the Maryland facility, 24 small oysters from *C. ariakensis* and *C. virginica* mesocosm populations were processed whole for histological analyses, and an additional 24 oysters from each analyzed group were aseptically shucked, weighed, and inoculated into ARFTM for detection of *Perkinsus* sp. infections. When taken, tissue samples for PCR assays were excised ventral to adductor muscles of oysters sampled for histological assays. Once oysters in a year-class/species cohort attained a modal shell height >15 mm, adequate tissue sub-samples for histologi-

cal, ARFTM, and PCR assays were taken from the same individual oysters.

Analyses for dermo disease were conducted by genus-specific, tissue wet weight-normalized ARFTM assays (La Peyre et al. 2003, McCollough et al. 2007) to determine *Perkinsus* sp. infection prevalences and intensities. Samples from each experimental oyster cohort were collected annually during April, July, and November 2004 to 2007 at the Maryland facility. Samples containing multiple year-classes of oysters from each species cohort were collected for monthly ARFTM assays during March, June, July, and August 2007 from oyster groups reared at the Florida facility (Table 1).

Transverse histological tissue samples from oysters of each experimental oyster cohort were fixed, embedded, sectioned, and stained with Mayer's hematoxylin and eosin (MHE, Howard et al. 2004). Histological slides were exhaustively analyzed microscopically at high magnification for detection of *Bonamia* sp. and *Haplosporidium* sp. infections, as well as for detection of other infectious or pathological conditions.

When needed, template DNAs for PCR assays were preserved in sub-samples of gill and mantle tissues that were excised from oysters processed for histopathological assays and preserved in >5 volumes of 95 to 100% ethanol. Genomic DNA from each analyzed oyster tissue was extracted using a QIAamp DNA Kit (QIAGEN) and was subsequently analyzed for the presence of *Bonamia* spp. by a genus-specific PCR assay (Carnegie et al. 2000, Hill et al. 2010).

Based on the seasonality and oyster size susceptibilities reported for *Bonamia* sp. infections among triploid *Crassostrea ariakensis* oysters reared in coastal North Carolina waters (Burreson et al. 2004, Bishop et al. 2006, Carnegie et al. 2008), tissue DNA was routinely extracted and analyzed for the presence of *Bonamia* spp. from 24-oyster samples of yearling cohorts with shell heights ≤40 mm during July of each year (2005 to 2007) at the Maryland site. Members of all year-class/species cohorts at the Maryland site were also tested by PCR for *Bonamia* spp. during

Table 1. Annual schedule for disease samples and analyses among oysters in Maryland mesocosms during 2004 to 2007 (MD) and Florida mesocosms during 2007 (FL). ARFTM: alternative Ray's fluid thioglycollate medium assays

| Disease assay | Month | | | | | | | | | | |
	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Histopathology analyses			FL	FL	FL	MD, FL	FL				MD	
ARFTM assays for *Perkinsus* spp.			FL	MD		FL	MD, FL	FL			MD	
PCR assays for *Bonamia* spp.		FL	FL	FL	FL	FL	MD, FL				MD	

November 2006, which was approximately 1 mo after their sibling oysters were transferred to the Florida site.

Experimental oysters exposed to Florida waters were analyzed more frequently, following PCR-detection of a *Bonamia* sp. among samples of *Crassostrea ariakensis* oysters experiencing high mortalities at the Florida site during March 2007. PCR assays were conducted on samples of approximately 20 *C. ariakensis* oysters that were taken during February to July 2007, and on similar *C. virginica* oyster samples taken during May to July 2007 (Table 1).

Small subunit (SSU) rDNA sequencing

Sequenced PCR products of a *Bonamia* sp.-positive *Crassostrea ariakensis* oyster from the Florida site were used to generate a *Bonamia* sp. small subunit (SSU) rDNA sequence for parasite identification. In order to sequence the entire SSU rDNA region, 3 PCR amplifications were performed: 16S-A (Medlin et al. 1988) + Bon-745R (Carnegie et al. 2006) for the 5′ end; Bon-1310F (Carnegie et al. 2006) + 16S-B (Medlin et al. 1988) for the 3′ end; and C_F + C_R (Carnegie et al. 2000) to complete the middle portion. For each primer pair, a 25-µl total volume reaction contained 1× PCR buffer (Applied Biosystems), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 µg µl^{-1} bovine serum albumin (BSA), 0.25 µM primer mix, 0.024 units µl^{-1} Ampli*Taq* DNA polymerase (Applied Biosystems), and 200 to 250 ng (= 0.5 to 1.6 µl) of template DNA. Purified PCR products were cloned into the plasmid vector pCR4-TOPO, using the TOPO TA Cloning kit (Invitrogen), and were then transformed into One Shot TOP10 competent *E. coli* cells (Invitrogen). Clones with inserts of desired size were propagated, and their plasmids were extracted using the QIAprep Spin Miniprep Kit and protocol (QIAGEN) for sequencing on a 16-capillary 3130*xl* Genetic Analyzer (Applied Biosystems). A consensus sequence was compared to sequences deposited in the National Center for Biotechnology Information (NCBI) GenBank database using a basic local alignment search tool (BLAST) search (Altschul et al. 1990).

In situ hybridization assays

Confirmatory chromogenic *in situ* hybridization (ISH) assays were performed on histological sections from 2 experimental oysters exposed to Florida waters, and that tested positive by *Bonamia* spp. PCR assays. A cocktail of 3 digoxigenin-labeled probes specific for *B. exitiosa* was employed, which included the SSU rRNA/rDNA-targeted probes CaBon166, CaBon461, and CaBon1704 (Hill et al. 2010). For each sample, experiments included the following treatments applied to serial histological sections: a no-probe control (25 µl hybridization buffer only), a positive control, and a standard experimental treatment using the CaBon166/461/1704 probe cocktail (Hill et al. 2010). Tissue sections for ISH assays were processed as previously described (Stokes & Burreson 1995), except that sections were treated with 50 µg ml^{-1} Proteinase K in PBS for 18 min at 37°C, or with 125 µg ml^{-1} pronase in P buffer (50mM Tris-HCl, pH 7.5, 5mM EDTA) for 30 min at 37°C, rather than 100 µg ml^{-1} Proteinase K for 15 min. The hybridization solution contained a digoxigenin-labeled oligonucleotide probe cocktail (50 µl total volume; 2 ng µl^{-1} each of CaBon166, CaBon461, and CaBon1704 probes for the *B. exitiosa*-specific ISH). After enzymatic color development, sections were counterstained with Bismarck brown Y (Stokes & Burreson 2001) and analyzed with an Olympus BX51 microscope.

RESULTS

MSX disease

Histopathological examinations revealed no *Haplosporidium* sp. infections among 631 oysters of both species and all year classes reared at either facility during 2004 to 2007. Depending on the number of year classes present on sampling dates (Table 1), 39 to 120 oysters were analyzed from each facility at each sampling interval.

Dermo disease

Oysters of both species contracted *Perkinsus* sp. infections during exposures to dermo disease-enzootic Choptank River waters. Infection prevalences among members of each oyster species/year-class cohort cycled seasonally, with annual maxima during late summer and autumn (August and November samples). Maximum annual prevalences for species/year-class cohorts frequently increased with cumulative years of exposure to ambient local infection pressures. Among November samples from 3 yr (2005 to 2007) at the Maryland facility, maximum

prevalences of *Perkinsus* sp. infections were greater among oysters of the 2004 *Crassostrea ariakensis* cohort (29%, 42%) during two of those years, than among members of the coincident 2004 *C. virginica* cohort (8%, 29%) (Fig. 1).

Perkinsus sp. infection prevalences increased among oysters of both species cohorts following their transfer to the Florida mesocosms (Fig. 2). In contrast to the trend of higher *Perkinsus* sp. infection prevalences among some *Crassostrea ariakensis* year-class cohorts exposed to Maryland waters, members of the *C. virginica* species cohort showed prevalences greater than or equal to those among *C. ariakensis* oysters during their exposures to Florida waters (Fig. 2). Due to high mortalities among *C. ariakensis* oysters in the Florida mesocosms, July 2007 was the final month when samples of both species were available for testing. Species year-classes were pooled among samples from the Florida site, so results are shown for pooled cohorts of 2 and 3 year-classes for *C. ariakensis* and *C. virginica* oysters, respectively.

Despite relatively high prevalences, intensities of *Perkinsus* sp. infections remained generally low among oysters of both species and all year-classes that were exposed to ambient waters at both sites. Among oysters exposed to Maryland Chesapeake Bay waters, maximum infection intensities consistently occurred among oysters from November samples. Among all oysters from the Maryland mesocosms, the maximum infection intensity of *Perkinsus*

Fig. 2. *Perkinsus* sp. infecting *Crassostrea ariakensis* and *C. virginica*. Prevalence (%) of infections among oyster samples (n = 20) of experimental *C. ariakensis* and *C. virginica* oysters reared in Florida mesocosms during 2007. Oysters were transferred from the Maryland mesocosms during September 2006. Infection prevalences are also plotted for sibling oysters (n = 24 to 72) that remained in the Maryland mesocosms (Nov-2006 MD) at 1 mo after those transfers

sp. cells g^{-1} oyster tissue wet weight was much higher among *Crassostrea virginica* oysters (7.8 × 10^5) than among *C. ariakensis* oysters (2.0 × 10^2). However, only two 2004-cohort *C. virginica* oysters from the same November 2006 Maryland mesocosm sample showed infection intensities of that maximum magnitude (>1 × 10^5 g^{-1}). Among oysters from the Florida mesocosms, maximum infection intensities were consistently below 100 *Perkinsus* sp. cells g^{-1} oyster tissue wet weight for oysters of both species and were slightly higher among *C. ariakensis* (59) than *C. virginica* oysters (24).

Bonamia sp. infections

Bonamia sp. infections were not detected among oysters of any year-class of either species during their 1 to 3 yr exposures to Maryland, Chesapeake Bay waters, by quarterly histological assays or annual PCR assays (July, November). Among *Crassostrea ariakensis* oysters reared during 2007 in Atlantic–Florida waters, PCR prevalences for *Bonamia* sp. DNA increased from 6 to 42% during February to June and decreased to 11% in July (Fig. 3). Histological lesions of low to high intensities were confirmed among *C. ariakensis* oysters at prevalences of 11, 15, and 14% during April, June, and July, respectively. Native *C. virginica* oysters reared in Florida waters during 2007 were also PCR-positive for a *Bonamia* sp. at 15% prevalence in June and 5% prevalence in
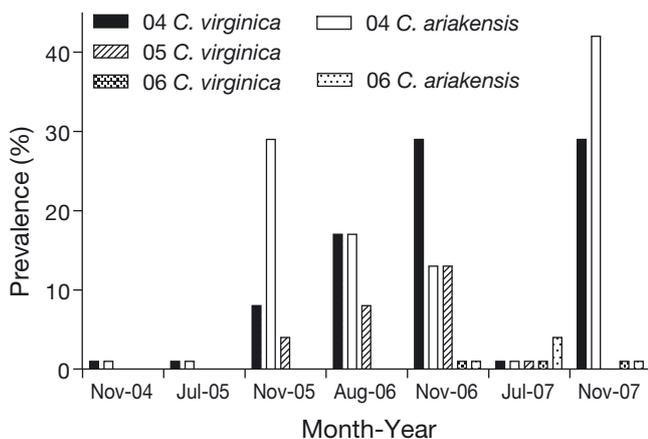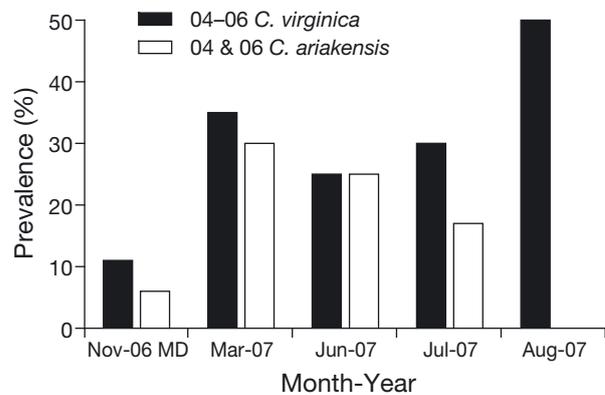
Fig. 1. *Perkinsus* sp. infecting *Crassostrea ariakensis* and *C. virginica*. Prevalence (%) of infections during selected months, among members of 2 *Crassostrea ariakensis* and 3 *C. virginica* oyster year-classes that were exposed to ambient Choptank River, Maryland, waters over more than 3 yr. Among oyster samples (n = 24) of each species/year-class cohort, infection prevalences were highest during August or November of each year. Bars showing prevalence <4% mark analyzed samples with actual prevalences of 0%

July, but histological lesions were never detected (Fig. 3).

In histological views, *Bonamia* sp. microcells oc-curred and apparently proliferated within circulating *Crassostrea ariakensis* hemocytes, at 1 to 10 micro-cells or microcell plasmodia hemocyte^{-1}. Most infec-tions were of low intensities, but 2 oysters showed systemic infections of moderate or high intensities, with 1% or more of hemocytes among visceral con-nective and vascular tissues infected. The largest intra-hemocytic *Bonamia* sp. microcells had diam-eters of 3 µm and showed central acidophilic nuclei with diameters of 0.5 µm. Plasmodium-like bodies containing numerous, small *Bonamia* sp. nuclei or cells also infected circulating hemocytes (Fig. 4).

Sequencing of SSU rDNA from a *Bonamia* sp.-infected Florida *Crassostrea ariakensis* oyster pro-duced a single 1749-bp sequence (GenBank acces-sion number JF831807) that was identical to the *Bonamia* sp. sequence characterized earlier from *C. ariakensis* from North Carolina, which is indistin-guishable from the SSU rDNA sequence of *B. exitiosa* (Hill et al. 2010) and which should therefore be iden-tified as such. The cocktail of DNA probes CaBon166, CaBon461, and CaBon1704 that are specific for *B. exitiosa* (Hill et al. 2010) hybridized to *Bonamia* sp. cells in sections from 2 infected *C. ariakensis* that were evaluated in this study, demonstrating the sometimes abundant presence of *B. exitiosa* in oys-ters exposed to Indian River Lagoon waters (Fig. 5). All positive and negative controls performed as expected, with *B. exitiosa* positive control sections showing strong hybridization by the probe cocktail, and the no-probe negative control sections from the infected Florida oysters showing no hybridization (Fig. 5).

DISCUSSION

MSX disease

Haplosporidium nelsoni infections decimated *Cras-sostrea virginica* populations of the Delaware and Chesapeake bays when they first occurred there dur-ing 1957 to 1960 (Ford & Haskin 1987, Burreson &
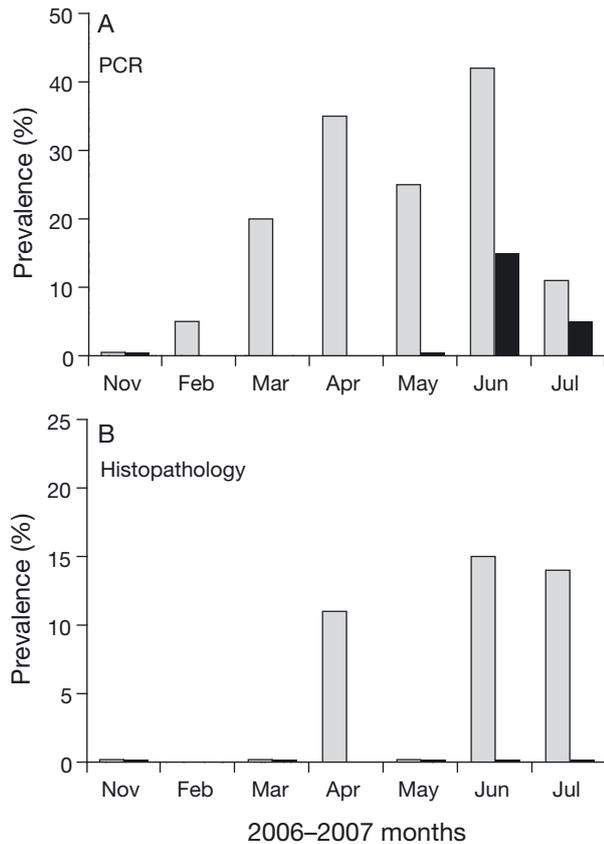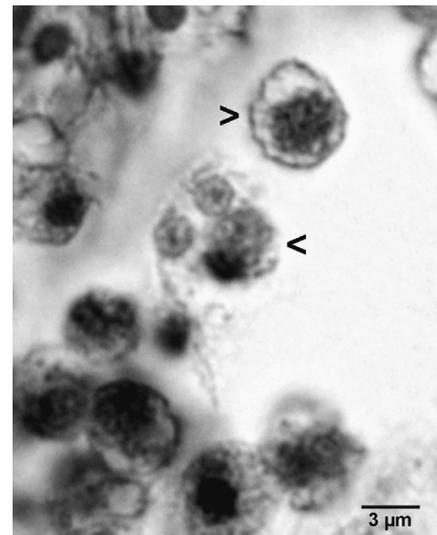

Fig. 3. *Bonamia* sp. infecting *Crassostrea ariakensis* and *C. virginica*. Prevalence (%) from (A) PCR assay (n = 9 to 48 oysters) and (B) histological detection (n = 14 to 48 oysters) of *Bonamia* sp. DNA and lesions among (gray) *C. ariakensis* and (black) *C. virginica* oyster cohorts after exposure for 2 to 26 mo in Maryland waters (Nov) and for 10 mo in Florida waters (Feb–Jul). Bars showing prevalences <5% mark ana-lyzed samples with actual prevalences of 0%. *Y*-axis scales differ between plots

Fig. 4. *Bonamia exitiosa*. Cells proliferating within circulat-ing hemocytes among visceral connective tissues of a *Cras-sostrea ariakensis* oyster exposed to Florida waters. Three *B. exitiosa* cells are focused above the dark nucleus of one hemocyte (left arrow), and a multinucleate, proliferating body of smaller *B. exitiosa* cells occupies an adjacent hemo-cyte (right arrow). Scale bar = 3 µm

Ford 2004). That pathogen is now redundantly confirmed to infect *C. gigas* oysters in Japan (Friedman et al. 1991, Kamaishi & Yoshinaga 2002), Korea (Kern 1976, Burreson et al. 2000), and China (Wang et al. 2010) with negligible pathological consequences. Such evidence supports the hypothesis that *H. nelsoni* was coincidentally introduced to mid-Atlantic USA estuaries along with Asian *C. gigas* oysters that were unsuccessfully introduced there prior to 1950 to assess their growth and reproductive potential as aquaculture species (Burreson & Ford 2004, Fincham 2006). One report of low-intensity *H. nelsoni* infections among several triploid *C. ariakensis* oysters exposed to Chesapeake Bay waters confirms some susceptibility of *C. ariakensis* oysters to *H. nelsoni* infections (Kingsley-Smith et al. 2009).
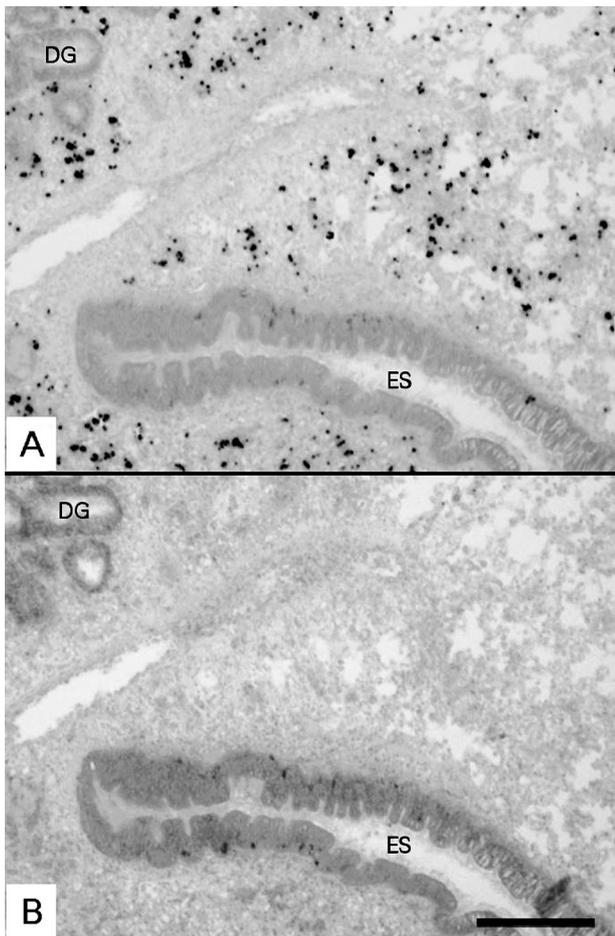

Fig. 5. *Bonamia* sp. infecting *Crassostrea ariakensis*. *In situ* hybridization of the parasite infecting oysters in Florida mesocosms. (A) Positive hybridization with *B. exitiosa*-specific probes. Dark staining indicates numerous *B. exitiosa* cells distributed in and among hemocytes in oyster connective tissues. (B) No-probe negative control. DG: digestive gland; ES: esophagus. Scale bar = 100 µm

Reports of MSX disease among oysters from US southern Atlantic and Gulf of Mexico coasts are limited. Haskin & Andrews (1988) cite several personal communications reporting MSX disease among *Crassostrea virginica* oysters from US Atlantic locations south of Chesapeake Bay. Systemic *Haplosporidium nelsoni* infections are reported among 2 to 28% of *C. virginica* oysters in samples collected during 1989 from coastal bays of northern North Carolina (Morrison et al. 1992). Among South Carolina coastal oysters surveyed during 1994 and 1995, geographically widespread *H. nelsoni* infections of variable intensities are reported at local prevalences up to 48% (Bobo et al. 1997). For *C. virginica* oyster samples collected during 1966, 1968, 1986, and 1987 from numerous sites along the Georgia coast, low prevalences of *H. nelsoni* infections, including some of lethal intensities, are reported among 2 to 6% of samples from 1986 and 1987 (Lewis et al. 1992). In oysters from the St. Johns River estuary on the Atlantic coast of northern Florida, *H. nelsoni* infections are reported without data on infection prevalence or intensities (Hillman et al. 1988).

There are no reports of MSX disease among native *Crassostrea virginica* oysters from the Indian River Lagoon, where anecdotal observations also suggest that *Haplosporidium nelsoni* may be absent or generally negligible as a pathogen of native *C. virginica* oysters. During the current investigation, salinity conditions in mesohaline Choptank River waters were relatively low (5 to 14 psu), and MSX disease was not detected in annual surveys of wild Choptank River oysters during 2004 to 2007 (Tarnowski 2011). Thus, the absence of *H. nelsoni* infections among experimental oysters of both species that were exposed to either Choptank River waters or Indian River Lagoon waters was consistent with low known or assumed MSX disease pressures at those locations during our investigation. Since a large body of work suggests that acquisition of MSX disease among oysters reared in mechanically distributed waters of enclosed oyster culture facilities is extremely rare for unknown reasons (Burreson & Ford 2004), interference of experimental systems in natural host–parasite interactions must also be considered when results from such studies are used to predict possible impacts from introductions of non-native species.

Dermo disease

Experimental oysters of both species contracted *Perkinsus* sp. infections during their exposures to

dermo disease-endemic Choptank River waters (Tarnowski 2011), confirming the introduction of infectious *Perkinsus* sp. cells to the Maryland meso-cosms through their Choptank River water supplies. *Perkinsus* sp. infections were detected as early as 90 d after metamorphosis among juvenile *Crass-ostrea virginica* oysters exposed in the Maryland mesocosms, which is consistent with rapid infections (10 d) reported among juvenile oysters exposed dur-ing field deployments in other mesohaline Chesa-peake Bay waters (McCollough et al. 2007).

Like caged *Crassostrea virginica* oyster spat de-ployed in mesohaline Chesapeake Bay waters (Abbe et al. 2010), infection prevalence among oyster species/year–class cohorts reared in our Maryland mesocosms showed cyclic seasonal fluctuations and generally increased with cumulative years of expo-sure (Fig. 1). Although dermo disease prevalence increased notably following transfers of both species cohorts to our Florida mesocosms (Fig. 2), existing infections among transferred members of both species-cohorts precludes specific differentiation of infections from Choptank River sources and those that were acquired during exposures to Indian River Lagoon waters. Despite uncertainty about the source of such infections, *Perkinsus* sp. infection prevalence increased dramatically among oysters of both species during rearing in Florida waters.

The frequent higher prevalence of dermo disease among some of our *Crassostrea ariakensis* oyster year classes exposed to Choptank River waters apparently belies the relatively high resistance to dermo disease reported for *C. ariakensis* oysters reared in Virginia waters (Calvo et al. 2001), but is consistent with prevalent *Perkinsus* sp. infections among some laboratory-reared *C. ariakensis* oysters (Moss et al. 2006, Schott et al. 2008). Trends for prevalence of *Perkinsus* sp. infections among oyster species-cohorts exposed to Atlantic–Florida waters were marginally reversed. Following a dramatic ini-tial increase, prevalence of *Perkinsus* sp. infections declined over time among *C. ariakensis* oysters rea-red in the Florida mesocosms, while prevalences among oysters of the *C. virginica* cohort increased with time to reach 50% (Fig. 2).

Only two 2004-*Crassostrea virginica* oysters from the Maryland mesoscosm showed high *Perkinsus* sp. infection intensities on the order of 10^5 pathogen cells g^{-1} oyster tissue wet weight, which approached the nominal lethal threshold of 10^6 pathogen cells g^{-1} oyster tissue wet weight that has been broadly inferred (Choi et al. 1989, Bushek et al. 1994, Al-bright et al. 2007). However, the maximum infection

intensity recorded for any *C. ariakensis* oyster from either site was dramatically lower than that thresh-old, at 200 *Perkinsus* sp. cells g^{-1} oyster tissue wet weight in a single oyster from the Maryland meso-cosms. These results suggest that infectious *Perkin-sus* sp. cells were consistently present in the waters of both Maryland and Florida mesocosms, but that conditions in neither location fostered widespread intensification of prevalent infections. Despite the report of prevalent high-intensity *P. marinus* infec-tions among laboratory-reared *C. ariakensis* oysters (Moss et al. 2006), our results are consistent with other results suggesting that enhanced functional resistance of *C. ariakensis* oysters to dermo disease may partly reflect that oyster's general ability to min-imize pathological consequences from *Perkinsus* sp. infections (Calvo et al. 2001, Kingsley-Smith et al. 2009).

The ARFTM assay that we used to detect *Perkinsus* sp. infections is genus-specific. Although results of species-specific assays confirm that *Perkinsus* sp. infections among *Crassostrea virginica* oysters in Chesapeake Bay are almost exclusively caused by *P. marinus* (Reece et al. 2008), the lack of similar information for *C. ariakensis* oysters and Florida waters compels our generic reference here to *Perkin-sus* sp. infections detected by our ARFTM assays.

Bonamia exitiosa infections

Both the morphologies and the intra-hemocyte tis-sue tropism of the microcells infecting diploid *Crass-ostrea ariakensis* oysters exposed to Indian River Lagoon waters of southeastern Florida closely resem-ble those of *Bonamia exitiosa* infecting triploid *C. ari-akensis* oysters along the Atlantic coast of southeast-ern USA (Burreson et al. 2004). The central position of the nucleus in the Florida parasite is also consis-tent with *B. exitiosa*, and not with the eccentric nucleus of *B. ostreae* (Abollo et al. 2008, Hill et al. 2010), another *Bonamia* sp. that occurs in Atlantic waters of northeastern USA (Friedman & Perkins 1994, Carnegie et al. 2000). *Bonamia perspora* is also present along the US southeast coast (Carnegie et al. 2006), but it was described from *Ostrea stentina* (= *Ostreola equestris*, Shilts et al. 2007) and has never been observed in a *Crassostrea* sp. oyster. Haplo-sporidian characteristics of *B. perspora* include abun-dant plasmodia and sporogonic forms in addition to uninucleate cells and an extracellular tissue tropism which excludes intra-hemocytic infections (Carnegie et al. 2006).

Histological evidence and host specificity both suggest that the Florida parasite is the same as the *Bonamia* sp. that infects *Crassostrea ariakensis* oysters in North Carolina waters, and is thus *B. exitiosa*. The genetic finding that the *Bonamia* sp. parasite from Florida waters is identical to *B. exitiosa* from North Carolina, over the 1749-bp sequence of SSU rDNA that we obtained, confirms that identification. Therefore, the range of *B. exitiosa* in eastern North America extends at least as far south as the Indian River Lagoon, Florida.

Although diploid *Crassostrea ariakensis* oysters exposed to polyhaline Atlantic waters of southern Florida for as few as 8 mo showed compelling evidence of *Bonamia* sp. infections by both PCR and histological assays, their siblings that were exposed for 3 yr to mesohaline waters in Chesapeake Bay never showed such infections. Therefore, while our results extend the US Atlantic range for *B. exitiosa* to include southern Florida, they also support results of extended investigations that consistently report the absence of *Bonamia* sp. infections among *C. ariakensis* or *C. virginica* oysters from mesohaline or polyhaline waters of Chesapeake Bay and Virginia coastal bays (Calvo et al. 2001, Kingsley-Smith et al. 2009) and of experimental work suggesting that the parasite would be intolerant of intermediate salinities that occur there (Bishop et al. 2006, Audemard et al. 2008a,b).

Bonamia sp. PCR amplifications were reported from low numbers of triploid *Crassostrea ariakensis* oysters that were variously exposed to polyhaline and mesohaline waters in Chesapeake Bay before subsequent rearing in re-circulating laboratory waters prior to testing. However, the lack of histological confirmation and the apparent opportunities for acquisition of *Bonamia* sp. cells or DNA during experimental rearing in facilities housing *B. ostreae*-infected *Ostrea edulis* oysters collectively weaken the conclusion that those rare PCR and sequencing results confirm the presence of several *Bonamia* species in Maryland or Virginia coastal waters (Schott et al. 2008).

We obtained *Bonamia* sp. PCR amplifications from both *Crassostrea ariakensis* and *C. virginica* oysters reared in our Florida mesocosms, but we confirmed *Bonamia exitiosa* infections histologically only among PCR-positive *C. ariakensis* oysters. These results suggest that *B. exitiosa* cells are enzootically suspended in waters of the Indian River Lagoon, that such cells are infectious for *C. ariakensis* oysters exposed to those waters, and that native *C. virginica* oysters exposed to the same waters are physically

associated with *B. exitiosa* without showing patent infections. Positive *Bonamia* sp. PCR results have been similarly reported from *C. virginica* oysters exposed to enzootic waters of North Carolina, but neither patent infections nor pathological consequences were associated with positive PCR results among native *C. virginica* oysters there (Burreson et al. 2004).

Since no evidence for patent infections of *Crassostrea virginica* by *Bonamia exitiosa* has been found, that oyster cannot be credibly viewed as a likely local source of the parasite in coastal waters of the southeastern USA. Neither can our experimental *C. ariakensis* be the source of *B. exitiosa* cells and infections, since those exotic oysters harbored no infections before exposure to Indian River Lagoon waters. To understand the source and epizootiology of *B. exitiosa* in southern Florida, we must consider a possible central role for *Ostrea stentina* (= *Ostreola equestris*, Shilts et al. 2007), which is now known to be a host for that pathogen (Hill et al. 2010). We must also consider potential host roles for numerous other oyster species that occur in the American tropics and subtropics, but for which parasite fauna remain unknown, including *Cryptostrea permollis*, *Teskeyostrea weberi*, and *Dendostrea frons*.

Results of the current investigation were inadequate to evaluate the relative susceptibility of *Crassostrea ariakensis* oysters to *Haplosporidium* spp. pathogens endemic in US Atlantic waters. However, they confirm the reported susceptibility of *C. ariakensis* oysters to *Perkinsus* sp. pathogens that infect native oysters in those waters (Moss et al. 2006, Schott et al. 2008, Kingsley-Smith et al. 2009). *C. ariakensis* reared in Florida waters during the current investigation were infected by the same *Bonamia exitiosa* pathogen that causes lethal infections among such oysters in North Carolina waters, so the known geographic range for that pathogen is now extended southward. Potential ecosystem ramifications of introducing a new susceptible host to USA waters endemic for *B. exitiosa* can only be speculated, but survival of such introduced oysters is threatened by their documented lethal susceptibilities to *Bonamia* sp. infections in Atlantic waters of both France (Cochennec et al. 1998) and the USA (Burreson et al. 2004).

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