

***Bonamia exitiosa* transmission among, and incidence in, Asian oyster *Crassostrea ariakensis* under warm euhaline conditions**

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ABSTRACT: Previously reported in Australia, New Zealand, and more recently in Europe, the protistan parasite *Bonamia exitiosa* was also reported in the mid-Atlantic region of the USA after causing serious mortalities there in the Asian oyster *Crassostrea ariakensis*. At the time, this oyster was being considered for introduction, and the potential consequences of introducing this species were being assessed using field and laboratory studies. *B. exitiosa* emerged as the most serious disease threat for this oyster species, especially under warm euhaline conditions and for oysters <50 mm in size. To better evaluate how quickly this parasite may be able to spread among *C. ariakensis*, we investigated *B. exitiosa* transmission and incidence in *C. ariakensis*. During a first trial, potential direct transmission of *B. exitiosa* was evaluated by cohabitating infected *C. ariakensis* with uninfected *C. ariakensis* under *in vivo* quarantine conditions. In a second experiment, *B. exitiosa* incidence was estimated *in situ* by determining its prevalence in *C. ariakensis* deployed in an enzootic area after 4, 7, 14, 21 and 28 d of exposure. Results suggest that under warm euhaline conditions *B. exitiosa* can be transmitted among *C. ariakensis* without requiring any other parasite source and that parasite incidence may be at least as high as 40% after only 4 d exposure to an enzootic area. These results underscored the severity of the bonamiasis disease threat to *C. ariakensis* and provided further evidence that efforts to build an aquaculture industry based on *C. ariakensis* in the eastern USA might have been thwarted by parasitic disease.

KEY WORDS: Haplosporidia · *Bonamia exitiosa* · *Crassostrea ariakensis* · Disease transmission · Incidence

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INTRODUCTION

As was revealed during field trials over the last decade in waters of the southeastern USA, the most significant disease threat known for the Asian oyster *Crassostrea ariakensis* is the microcell haplosporidian parasite *Bonamia exitiosa* (Hine et al. 2001, Burreson et al. 2004, Hill et al. 2010). Infection by *B. exitiosa* was first reported in 2003 after triploid *C. ariakensis* that had been deployed as small seed in Bogue Sound, North Carolina, experi-

enced catastrophic and rapid mortality in mid-summer (Burreson et al. 2004). Previously unnoticed in native *Ostrea stentina* (= *Ostreola equestris*; Shilts et al. 2007) present throughout the region (Carnegie et al. 2006), *B. exitiosa* is particularly pathogenic in young *C. ariakensis* <50 mm in shell height and caused mortality within weeks of deployment in Bogue Sound (Bishop et al. 2006). Additional field and laboratory studies demonstrated that *B. exitiosa* was most infective and pathogenic under a combination of warm (>20°C) and

polyhaline to euhaline (>20 psu) conditions (Bishop et al. 2006, Audemard et al. 2008a,b, Carnegie et al. 2008). *B. exitiosa* has been reported worldwide in various hosts and locations, for example: *Ostrea chilensis* in New Zealand (Hine et al. 2001, Berthe & Hine 2003), *O. angasi* in Australia (Corbeil et al. 2006), *O. edulis* in Europe (Abollo et al. 2008), and *O. stentina* in Tunisian waters and in North Carolina, USA (Hill et al. 2010), although it has never been observed infecting *C. ariakensis* in the Asian environments to which it is native.

To further assess the epizootiology of *Bonamia exitiosa* in *Crassostrea ariakensis*, we evaluated the transmissibility and incidence (i.e. rate of infection acquisition) of the parasite among *C. ariakensis*. While the native oyster species *Ostrea stentina* is known to harbor *B. exitiosa* in North Carolina (Carnegie et al. 2006), we hypothesized that the parasite may be able to complete its life cycle in *C. ariakensis* in the absence of *O. stentina* and that transmission among *C. ariakensis* may be direct. This hypothesis was tested in a laboratory experiment by placing infected *C. ariakensis* as cohabitants with naïve *C. ariakensis*. A previous similar experiment had resulted in an absence of parasite transmission (Audemard et al. 2008a); however, we suspected that sub-optimal water quality in the experimental aquaria may have influenced these results and felt that this experiment had to be repeated under better conditions. Our second objective was to investigate *B. exitiosa* incidence on a time scale ranging from 4 to 28 d exposure. Previous studies revealed that *B. exitiosa* could be detected within a few weeks after deployment of *C. ariakensis* in Bogue Sound during summer and early fall (Carnegie et al. 2008). We suspected, however, that infection may occur even more rapidly. We investigated this hypothesis during the present study.

MATERIALS AND METHODS

Trial 1: *Bonamia exitiosa* transmission

On 20 June 2007, a subset of several hundred tri-ploid *Crassostrea ariakensis* produced in quarantine (Spawn 3MXWCA07-1) at the Virginia Institute of Marine Science (VIMS) Aquaculture Genetics and Breeding Technology Center was transferred to the University of North Carolina Institute of Marine Sciences (IMS), located in Morehead City, North Carolina. The oysters were placed in upwellers receiving unfiltered euhaline seawater (>30 psu) from Bogue

Sound, where *B. exitiosa* is enzootic (Carnegie et al. 2008, Hill et al. 2010).

On 24 July 2007, 35 d after deployment, these oysters (= NC *Crassostrea ariakensis*) were retrieved and shipped overnight back to VIMS. Our subsequent experiment was performed under quarantine conditions and was begun when the NC *C. ariakensis* were returned to VIMS, on 25 July 2007. *Bonamia exitiosa* prevalence and mean infection intensity were determined in a sample of 30 NC *C. ariakensis* using PCR and histology (see 'B[.] *exitiosa* prevalence and ... intensity'). On the same date, 30 *C. ariakensis* from the same cohort maintained in polyhaline conditions at the VIMS hatchery and not sent to North Carolina for parasite exposure (= VA *C. ariakensis*) were sampled for *B. exitiosa* diagnosis in order to confirm that these control oysters had indeed remained free of the parasite. No *Bonamia* sp. has ever been observed in samples from Chesapeake Bay, Virginia. Average sizes of these animals were 22 ± 3 and 22 ± 4 mm (mean \pm SD) for NC and VA *C. ariakensis*, respectively.

On the day the trial began, the NC and VA *Crassostrea ariakensis* were carefully rinsed and any dead animals (empty shells) were removed. As treatment aquaria, 3 replicate 40 l aquaria containing 50 NC *C. ariakensis* and 50 VA *C. ariakensis* were established. To distinguish these 2 oyster groups, the VA *C. ariakensis* were placed in a mesh bag, while the NC *C. ariakensis* were free in the aquaria. As controls, 3 replicate aquaria each contained 50 free VA *C. ariakensis* and 50 bagged VA *C. ariakensis*. Aquaria were filled with 1 μ m filtered water of ~20 psu from the York River adjusted to ~33 to 35 psu by adding artificial sea salts (Crystal Sea, Marinemix) and maintained at ~26 to 28°C to mimic environmental conditions under which *Bonamia exitiosa* transmission to *C. ariakensis* occurs in the field (Carnegie et al. 2008). Oysters were fed a commercial algal paste (Shellfish Diet, Reed Mariculture) containing *Isochrysis* sp., *Pavlova* sp., *Thalassiosira weissflogii* and *Tetraselmis* sp. daily, and two-thirds of the water in each aquarium was changed 3 times a week. The water removed from the tanks was bleached and discarded as previously described (Audemard et al. 2008b). Mortality was checked daily, and dead animals were removed.

To assess the potential transmission of *Bonamia exitiosa* from infected (NC *Crassostrea ariakensis*) to uninfected *C. ariakensis* (i.e. the VA *C. ariakensis*), 10 and 20 VA *C. ariakensis* per treatment aquarium were sampled after 28 and 49 d of cohabitation, respectively. On the same dates, 23 August and 11 September 2007, an equal number of oysters was sampled from the VA *C. ariakensis* held in mesh bags in the

control aquaria. The animals sampled were analyzed by PCR and histology to evaluate *B. exitiosa* infections (see '*B. exitiosa* prevalence and ... intensity').

Trial 2: *Bonamia exitiosa* incidence in *Crassostrea ariakensis*

During this trial, the incidence of *Bonamia exitiosa* in *Crassostrea ariakensis* deployed in Bogue Sound euhaline waters in which *B. exitiosa* is enzootic was investigated. Incidence was defined as the percentage of oysters that became infected by this parasite during a specific time period. Since initial infections can be light and undetectable by histology and PCR, after uninfected animals were exposed to Bogue Sound waters for specific lengths of time, a subset of these animals was returned to VIMS and maintained under warm euhaline conditions in quarantine aquaria for several weeks, so infections acquired in Bogue Sound might develop to detectable levels.

On 2 August 2007, several hundred *Crassostrea ariakensis* produced at VIMS were deployed in upwellers located at the IMS and receiving Bogue Sound water as described above. These oysters were 18 ± 2 mm (mean \pm SD) in shell height. After 4 d and subsequently after 7, 14, 21 and 28 d of exposure to Bogue Sound waters, subsets of the deployed *C. ariakensis* (NC *C. ariakensis*) were shipped back to VIMS (Fig. 1). In order to assess the general water temperature trend during the exposure of *C. ariakensis* to Bogue Sound waters, water temperature data collected at hourly intervals at the NOAA monitoring station at Beaufort, North Carolina (Station I.D. 8656483), <5 km from the Bogue Sound upweller system in which all the oysters were held, were used.

For each of these exposure times, on the date of return of the NC *Crassostrea ariakensis* to VIMS, 3 treatment aquaria were established, each containing 50 NC *C. ariakensis* and 50 VA *C. ariakensis* from the same cohort, which had been maintained at the VIMS hatchery (Fig. 1). These VA *C. ariakensis* were placed in mesh bags

to distinguish them from the oysters returning from Bogue Sound. By placing oysters returning from NC in cohabitation with unexposed and presumably healthy oysters, we intended to assess potential *Bonamia exitiosa* transmission under these laboratory conditions. Upon arrival in the laboratory, an additional 30 NC *C. ariakensis* and 30 VA *C. ariakensis* were fixed for further PCR and histological analyses to determine whether *B. exitiosa* could be detected prior to initiation of laboratory exposure. As a control, 3 aquaria each containing 50 free VA *C. ariakensis* and 50 VA *C. ariakensis* held in a mesh bag were established on the date of the deployment of the oysters in Bogue Sound, i.e. on 2 August 2007.

All the oysters (treatment and control aquaria) were maintained at 33 to 35 psu and ~26 to 28°C. Mortality was checked daily, and any dead animals were removed. After the last aquaria (28 d exposure

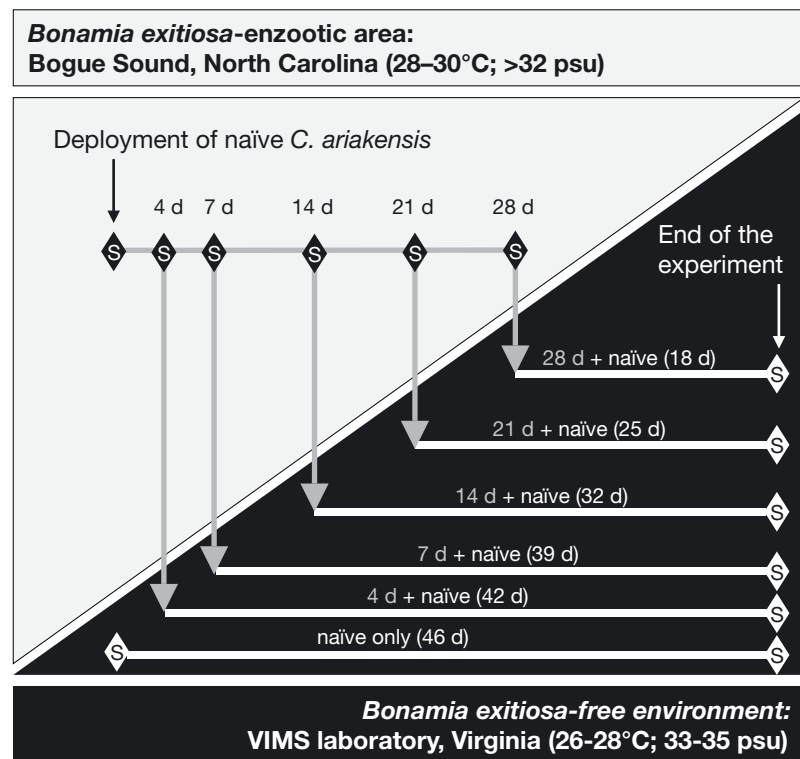


Fig. 1. Experimental design for investigation of *Bonamia exitiosa* incidence in *Crassostrea ariakensis*. Upper left corner with light grey background represents the enzootic area, Bogue Sound, NC; lower right with black background represents the Virginia Institute of Marine Science (VIMS) quarantine laboratory. After deployment of naïve *C. ariakensis* in Bogue Sound, samples were collected after 4, 7, 14, 21 and 28 d of exposure and returned to VIMS. Some of these sampled oysters were analyzed for *B. exitiosa* infections directly upon collection, while a subset was placed in aquaria in cohabitation with naïve *C. ariakensis*, free of *B. exitiosa* infections, for the number of days specified in parentheses. 'S' indicates when an oyster sample was collected for PCR and histology analyses

treatment) were established, all the aquaria were maintained for an additional 18 d under laboratory conditions (Fig. 1). At the end of the experiment, 10 NC *Crassostrea ariakensis* and 10 VA *C. ariakensis* were sampled from each aquarium to assess the timing of *Bonamia exitiosa* infection acquisition in the field and potential transmission of the parasite under laboratory conditions. Ten free VA *C. ariakensis* and 10 bagged VA *C. ariakensis* were sampled from each control aquarium to confirm that these oysters had remained free of *B. exitiosa*. These animals were analyzed by PCR and histology as described below.

Assessment of cumulative oyster mortality

Cumulative mortality was calculated for each aquarium and at each sampling date (28 and 49 d exposure) for the transmission trial. For the incidence trial, cumulative mortalities within the aquaria were assessed at the 4 and 7 d time points of the experiment and subsequently on a weekly basis until the end of laboratory exposure. At each time point, average cumulative mortalities and standard errors were calculated as described by Audemard et al. (2008b).

Bonamia exitiosa prevalence and average parasite intensity

After each sampling, all the oysters collected were shucked, and a tissue sample of ~3 to 5 mm³ comprising some gill, mantle and visceral mass tissue was fixed in 95% ethanol for DNA extraction. A ~5 mm thick standard section of each oyster (anterior to the adductor muscle, including digestive gland, stomach/intestine, gonad, mantle and gills) was fixed for 24 h in Davidson's fixative (Shaw & Battle 1957) for subsequent paraffin histology performed using standard methods.

DNA was extracted from ethanol-preserved tissue using a DNeasy Tissue Kit (QIAGEN™) following the manufacturer's protocol. DNA was quantified with a GeneQuant *pro* spectrophotometer (Amersham Biosciences).

A PCR utilizing *Bonamia* genus small subunit rDNA-specific primers C_F and C_R was used to analyze all the oysters collected during the transmission and incidence trials (Carnegie et al. 2000). The only other *Bonamia* species present in North Carolina, *B. perspora* (Carnegie et al. 2006), has never been observed to infect *Crassostrea ariakensis* and has never been detected by PCR in association with this

oyster. Thus, all *C. ariakensis* tested to be *Bonamia*-positive using this generic PCR assay were assumed to be infected with *B. exitiosa*. PCR-positive animals were analyzed by histology to confirm infection and to document its intensity. The initial screening by PCR saved time and the costs that would be associated with the histological analysis of all the samples. A 25 µl total PCR reaction volume included 1× PCR buffer, 1.5 mM MgCl₂, 0.2 mM dNTPs, bovine serum albumin (BSA) at 0.4 µg µl⁻¹, 0.25 µM primers, AmpliTaq polymerase at 0.024 U µl⁻¹ and 0.5 to 1.0 µl (= 200 to 250 ng) template DNA. Each PCR experiment included a known *B. exitiosa*-positive sample as a positive control and a no-template sample (DNA replaced with water) as a negative control. Temperature cycling included a 4 min initial denaturation at 94°C; followed by 35 cycles of denaturation at 94°C, annealing at 59°C, and extension at 72°C for 1 min each; and then a final extension at 72°C for 10 min. Amplification products were electrophoresed in 2% agarose gels (in 1× TAE), stained with ethidium bromide and visualized under UV light. PCR prevalence was calculated as the percentage of oysters in which *B. exitiosa* DNA was amplified, and standard errors were calculated.

After fixation in Davidson's fixative, tissue from PCR-positive oysters was processed for standard paraffin histology. Slides were evaluated under oil immersion at 400 to 1000× magnification. Intensity of *Bonamia exitiosa* infection was rated at 0.5 (rare infection: 1 to 10 *B. exitiosa* cells were observed in the entire section), 1 (light infection: 11 to 40 *B. exitiosa* cells were observed), 3 (moderate infection: >40 cells were observed, but at densities of <5 cells in some fields), or 5 (heavy infection: *B. exitiosa* was present in all fields at densities of ≥5 cells field⁻¹). Prevalence was calculated as the percentage of oysters with *B. exitiosa* infections, as confirmed by histology. Average *B. exitiosa* infection intensity was calculated as the sum of all infection intensity scores divided by the total number of infected animals. Standard errors were calculated for both prevalence and average parasite intensity.

RESULTS

Trial 1: *Bonamia exitiosa* transmission

After an exposure of 35 d to Bogue Sound waters, 96.5% of the NC *Crassostrea ariakensis* were found to be infected with generally light to moderate *Bonamia exitiosa* infections (average intensity score:

2.3). These oysters were placed in cohabitation with initially uninfected VA *C. ariakensis* to assess potential transmission of the parasite. Cumulative mortality observed for the bagged and free VA *C. ariakensis* from the control aquaria, as well as for the VA *C. ariakensis* from the treatment aquaria, remained negligible (<4%) during the duration of the experiment (Fig. 2A). NC *C. ariakensis*, on the contrary, showed cumulative mortality of $\geq 85\%$ within 28 d.

After 28 d of cohabitation, *Bonamia exitiosa* DNA was detected by PCR in an average of 13% of the initially uninfected VA *Crassostrea ariakensis* (Fig. 2B). Rare *B. exitiosa* infections (average score: 0.17) were confirmed by histology at this time point for an average histological prevalence of 3% (Fig. 2B). After 49 d of cohabitation, PCR and histological prevalence of *B. exitiosa* in the treatment VA *C. ariakensis* had

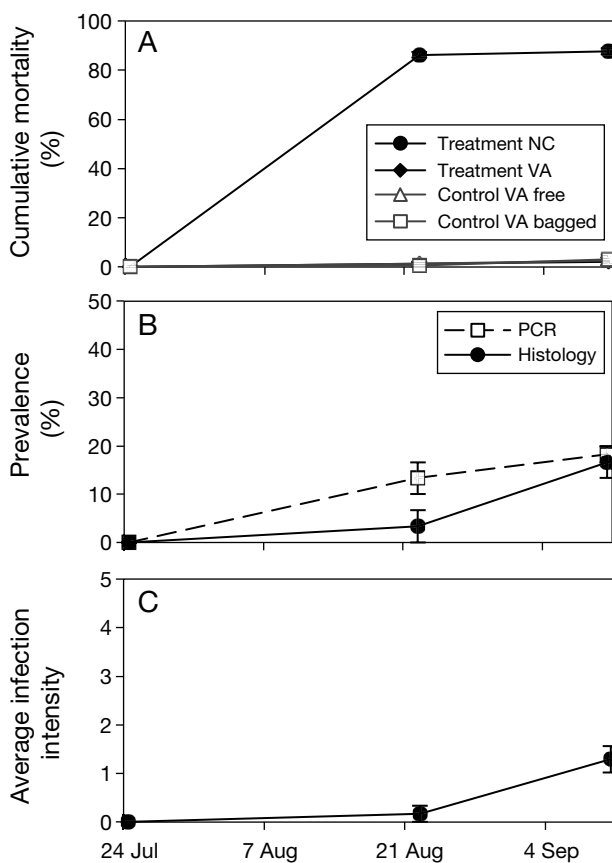


Fig. 2. *Bonamia exitiosa* infecting *Crassostrea ariakensis*. Results of the transmission trial. (A) Cumulative mortality measured in the oysters from the treatment and control aquaria after 28 d (23 August) and 49 d (11 September) of cohabitation. (B) *B. exitiosa* PCR and histology prevalence in the VA *C. ariakensis* placed in cohabitation with NC *C. ariakensis*. (C) Average parasite infection intensity in VA *C. ariakensis* determined by histology. Error bars = 1 SE

increased to an average of 18 and 16%, respectively. Observed infection intensities (Fig. 2C) were higher than at the 28 d time point and ranged from rare to moderate (average score: 1.29). None of the VA *C. ariakensis* samples from the control aquaria were found to harbor *B. exitiosa*.

Trial 2: *Bonamia exitiosa* incidence

Samples collected directly after exposure to Bogue Sound waters

During the exposure to Bogue Sound waters from 2 to 30 August 2007, average daily temperatures measured at the nearby Beaufort NOAA station ranged from 28 to 30°C and salinities were >32 psu.

Using PCR, *Bonamia exitiosa* DNA was first detected after 14 d exposure in 3.3% of the oysters collected (Fig. 3A). When analyzed by histology, all the oysters that were positive by PCR were confirmed to harbor *B. exitiosa* infections that were rare in intensity (Fig. 3B). After 21 d of exposure, 30% of the oysters were positive using PCR, but infection by *B. exitiosa* was confirmed in only a minority of these oysters; histological prevalence remained as low as 3.3% and *B. exitiosa* infections were rare in intensity

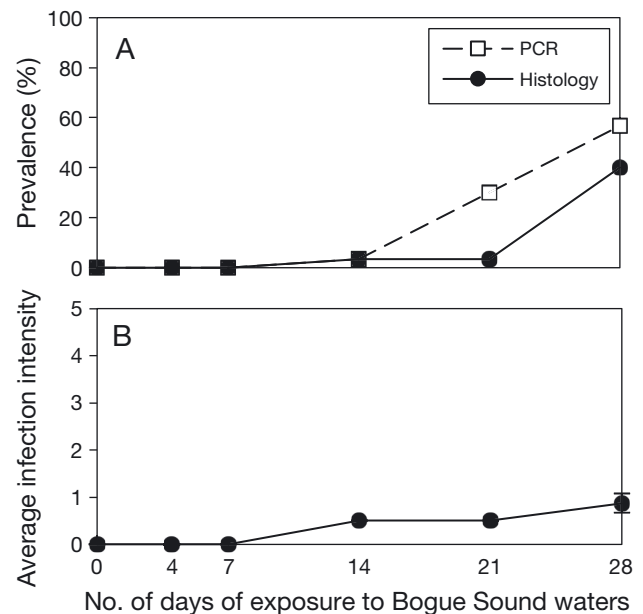


Fig. 3. *Bonamia exitiosa* infecting *Crassostrea ariakensis*. *B. exitiosa* in NC *C. ariakensis* from the incidence trial directly upon return from their exposure to Bogue Sound, NC, waters from 2 to 30 August. (A) PCR and histology prevalence and (B) average infection intensity determined by histology. Error bars = 1 SE

(average score: 0.5). Finally, after 28 d of exposure, 56.7% of the oysters were positive by PCR and 40% were confirmed to harbor *B. exitiosa* infections ranging from rare to moderate in intensity (average score: 0.8).

Samples collected after additional laboratory exposure

Independent of the treatment (control or treatment aquaria) and of oyster origin (VA or NC), average cumulative mortalities remained <21% (Fig. 4) at the final sampling date (20 September 2007). By the end of the laboratory exposure, the highest cumulative mortality values were measured in NC *Crassostrea ariakensis* that had been exposed to Bogue Sound waters for 21 and 28 d (Fig. 4A). The bagged VA *C. ariakensis* exposed to the NC *C. ariakensis* 4 d treat-

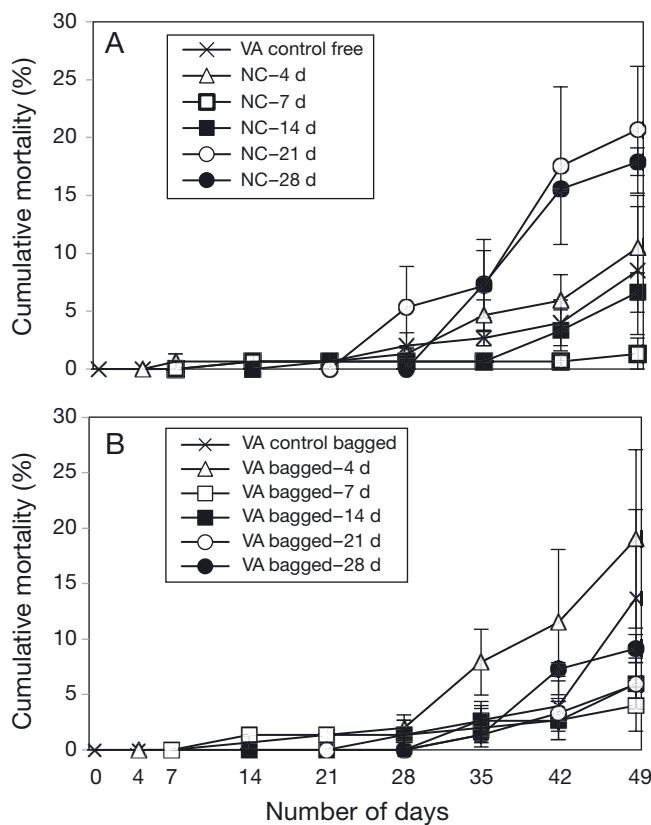


Fig. 4. *Bonamia exitiosa* infecting *Crassostrea ariakensis*. Cumulative mortality measured in oysters from the control and treatment aquaria from the incidence trial. (A) Cumulative mortality in the VA *C. ariakensis*-free control and in the NC *C. ariakensis* exposed to Bogue Sound waters for the specified length of time and (B) Cumulative mortality in the bagged VA *C. ariakensis* placed in cohabitation with NC *C. ariakensis* exposed for the specified length of time to Bogue Sound waters. Error bars = 1 SE

ment also showed some of the highest values recorded during this trial (Fig. 4B).

By the end of laboratory exposure (Fig. 5A), the PCR and histological analyses of the NC *Crassostrea ariakensis* from the different treatments showed higher prevalence of *Bonamia exitiosa* than was measured directly upon return of oysters from the field (Fig. 3A). *B. exitiosa* was not detected by PCR in any of the sampled VA *C. ariakensis* placed in cohabitation with these NC *C. ariakensis*. Average PCR prevalence in the NC *C. ariakensis* was 73 and 70% for 4 and 7 d treatments, respectively, and histological prevalence was 43.3% for both treatments. The 14 d treatment showed an increase in both PCR (90%) and histological prevalence (77%) compared to the 4 and 7 d treatments. The 21 d treatment was characterized by 100% *B. exitiosa* prevalence as measured by both PCR and histology. Finally, the 28 d treatment showed a decrease in both PCR (90%) and histological (77%) prevalence. For all these different treatments average parasite infection intensities were light to moderate (Fig. 5B).

DISCUSSION

In the present study, transmission of *Bonamia exitiosa* from infected *Crassostrea ariakensis* to uninfected *C. ariakensis* held together under warm euha-

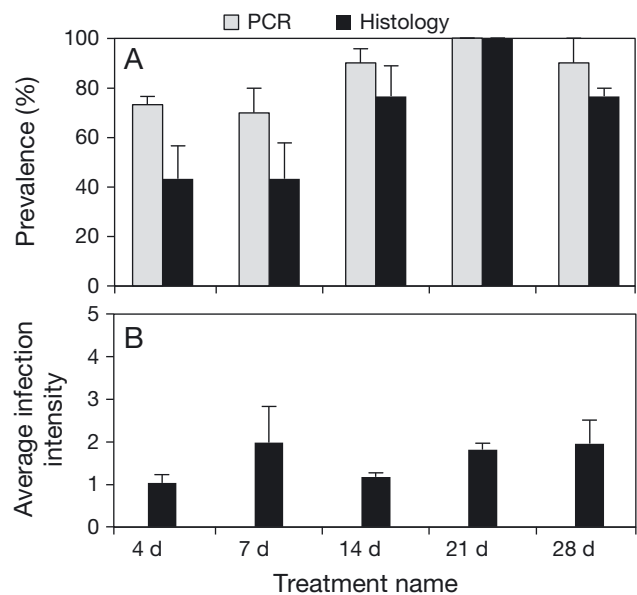


Fig. 5. *Bonamia exitiosa* infecting *Crassostrea ariakensis*. *B. exitiosa* in NC *C. ariakensis* from the incidence trial after additional laboratory exposure. (A) PCR and histology prevalence and (B) average infection intensity determined by histology. Error bars = 1 SE

line laboratory conditions was observed after only 28 d of cohabitation. The experimental transmission of *Bonamia* spp. parasites during cohabitation of infected and non-infected hosts has previously been documented for *B. ostreae* in the flat oyster *Ostrea edulis* (Bachère et al. 1986, Elston et al. 1986, 1987, Cullopy et al. 1999, Lallias et al. 2008). Results from a previous experiment also suggested that *B. exitiosa* may be directly transmitted among *O. chilensis* (Hine 1996). Similarly, the results of the present experiment suggest that *B. exitiosa* may be directly transmitted among *C. ariakensis*. It may be pointed out, however, that in spite of the filtration (1 µm) of the water used during this experiment, the presence of small invertebrates such as harpacticoid copepods and annelids was noted in the experimental tanks by the end of the experiment. It remains possible that these invertebrates may have been involved in *B. exitiosa* transmission; however, these were not screened for *B. exitiosa* in this study, leaving their potential involvement in the *B. exitiosa* life-cycle unresolved. This trial, nevertheless, demonstrated that *B. exitiosa* does not require the native oyster *O. stentina* besides *C. ariakensis* to complete its life cycle.

The second trial demonstrated the high incidence of *Bonamia exitiosa* in *Crassostrea ariakensis* in natural waters under warm euhaline conditions. The absence of detectable *B. exitiosa* in cohabitating VA *C. ariakensis* during the incidence study suggested that the increased prevalence observed in NC *C. ariakensis* during their laboratory exposure was not a consequence of parasite transmission in aquaria. Rather, the prevalence increase represented increased detection of *B. exitiosa* infections as they became patent in the laboratory. The exposure of the NC *C. ariakensis* to favorable warm euhaline conditions in the laboratory for several weeks allowed the parasites acquired in Bogue Sound to proliferate within their hosts so that detectable levels of infection could be reached. As a consequence, the prevalence levels observed at the end of the laboratory exposure period reflected more accurately the incidence of *B. exitiosa* than the prevalence observed directly after the animals were sampled from the field. Although *B. exitiosa* was not detected by PCR or histology directly upon return from the field after a 4 d exposure, likely reflecting very small numbers of parasite cells focally distributed within host tissues and either missed during sampling or below amplifiable levels, the additional laboratory exposure revealed that >40% of these oysters were infected by *B. exitiosa*, as determined by histology. The PCR prevalence was 73% for the

4 d exposure treatment. PCR only indicates the detection of DNA, which may not always be associated with the presence of viable parasite cells responsible for an infection (Burreson 2008). In this study, however, the fact that the *B. exitiosa* DNA was not detected in the cohabiting VA *C. ariakensis* suggests that PCR detection in the NC *C. ariakensis* may actually be indicative of parasite infections. It has been documented in other studies that PCR is generally more sensitive than histology (Balseiro et al. 2006), which would support the lower prevalence obtained by histology compared to PCR.

Transmission was not observed in the present incidence trial during which NC *Crassostrea ariakensis* exposed to Bogue Sound waters for various lengths of time were placed in aquaria with VA *C. ariakensis*. In this experiment, however, the NC *C. ariakensis* from the incidence trial were only lightly infected at the onset of their cohabitation with non-infected *C. ariakensis*. The combination of potentially very light *Bonamia exitiosa* infections and the dilution of waterborne infective stages of *B. exitiosa* through water changes may explain the absence of transmission in this trial. In contrast, at the onset of the transmission trial, *B. exitiosa* prevalence in the NC *C. ariakensis* was 96.5%, with infection intensities ranging from light to moderate, which was likely associated with the release of sufficient *B. exitiosa* infective cells over time in the aquaria to infect the cohabitating non-infected oysters in spite of the water changes. As a final comment on transmission, we note again that transmission also failed in an earlier study (Audemard et al. 2008a). We must conclude that our small research aquaria are not conducive to the full *B. exitiosa* activity that would be displayed in natural waters. Whether this relates to biological factors or to physical factors such as water chemistry is not known.

In summary, this study emphasized 2 factors central to the rapid development of *Bonamia exitiosa* epizootics in experimental *Crassostrea ariakensis* populations in Bogue Sound under warm euhaline conditions: a high incidence of infection and, however imperfectly captured in our experimental systems, the high likelihood of direct transmissibility. Although Asian *C. ariakensis* were not introduced to open waters of the eastern USA as had been proposed, the threat of *B. exitiosa* will remain for hatcheries located in *B. exitiosa*-enzootic areas that produce *C. ariakensis* for aquaculture elsewhere (Grabowski et al. 2007). The high susceptibility of *C. ariakensis* to *B. exitiosa* should also not escape the notice of shellfish health managers in Asia.

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LITERATURE CITED

- Abollo E, Ramilo A, Casas SM, Comesaña P, Cao A, Carballal MJ, Villalba A (2008) First detection of the protozoan parasite *Bonamia exitiosa* (Haplosporidia) infecting flat oyster *Ostrea edulis* grown in European waters. *Aquaculture* 274:201–207
- Audemard C, Carnegie RB, Stokes NA, Bishop MJ, Peterson CH, Burreson EM (2008a) Effects of salinity on *Bonamia* sp. survival in the Asian oyster *Crassostrea ariakensis*. *J Shellfish Res* 27:535–540
- Audemard C, Carnegie RB, Bishop MJ, Peterson CH, Burreson EM (2008b) Interacting effects of temperature and salinity on *Bonamia* sp. parasitism in the Asian oyster *Crassostrea ariakensis*. *J Invertebr Pathol* 98:344–350
- Bachère E, Comps M, Grizel H (1986) Infections expérimentales de l'huître plate *Ostrea edulis* L. par le protozoaire *Bonamia ostreae*. In: Vivares CP, Bonami JR, Jaspers E (eds) Pathology in marine aquaculture. Special Publication No. 9, European Aquaculture Society, Bredene, p 127–132
- Balseiro P, Fernandez Conchas R, Montes J, Gómez-León J, Novoa B, Figueras A (2006) Comparison of diagnosis techniques for the protozoan parasite *Bonamia ostreae* in flat oyster *Ostrea edulis*. *Aquaculture* 261:1135–1143
- Berthe F, Hine P (2003) *Bonamia exitiosa* Hine et al., 2001 is proposed instead of *B. exitiosus* as the valid name of *Bonamia* sp. infecting flat oysters *Ostrea chilensis* in New Zealand. *Dis Aquatic Org* 57:181
- Bishop MJ, Carnegie RB, Stokes NA, Peterson CH, Burreson EM (2006) Complications of a non-native oyster introduction: facilitation of a local parasite. *Mar Ecol Prog Ser* 325:145–152
- Burreson EM (2008) Misuse of PCR assay for diagnosis of mollusc protistan infections. *Dis Aquat Org* 80:81–83
- Burreson EM, Stokes NA, Carnegie RB, Bishop MJ (2004) *Bonamia* sp. (Haplosporidia) found in non-native oysters *Crassostrea ariakensis* in Bogue Sound, North Carolina. *J Aquat Anim Health* 16:1–9
- Carnegie RB, Barber BJ, Culloty SC, Figueras AJ, Distel DL (2000) Development of a PCR assay for detection of the oyster pathogen *Bonamia ostreae* and support for its inclusion in the Haplosporidia. *Dis Aquat Org* 42:199–206
- Carnegie RB, Burreson EM, Hine PM, Stokes NA, Audemard C, Bishop MJ, Peterson CH (2006) *Bonamia perspora* n. sp. (Haplosporidia), a parasite of the oyster *Ostreaolea equestris*, is the first *Bonamia* species known to produce spores. *J Eukaryot Microbiol* 53:232–245
- Carnegie RB, Stokes NA, Audemard C, Bishop MJ and others (2008) Strong seasonality of *Bonamia* sp. infection and induced *Crassostrea ariakensis* mortality in Bogue and Masonboro Sounds, North Carolina, USA. *J Invertebr Pathol* 98:335–343
- Corbeil S, Arzul I, Robert M, Berthe F, Besnard-Cochennec N, Crane M (2006) Molecular characterisation of an Australian isolate of *Bonamia exitiosa*. *Dis Aquat Org* 71:81–85
- Culloty SC, Novoa B, Pernas M, Longshaw M, Mulcahy MF, Feist SW, Figueras A (1999) Susceptibility of a number of bivalve species to the protozoan parasite *Bonamia ostreae* and their ability to act as vectors for this parasite. *Dis Aquat Org* 37:73–80
- Elston RA, Farley CA, Kent ML (1986) Occurrence and significance of bonamiasis in European flat oysters *Ostrea edulis* in North America. *Dis Aquat Org* 2:49–54
- Elston RA, Kent ML, Wilkinson MT (1987) Resistance of *Ostrea edulis* to *Bonamia ostreae* infection. *Aquaculture* 64:237–242
- Grabowski JH, Peterson CH, Bishop MJ, Conrad R (2007) The bioeconomic feasibility of culturing triploid *Crassostrea ariakensis* in North Carolina. *J Shellfish Res* 26:529–542
- Hill KM, Carnegie RB, Aloui-Bejaoui N, El Gharsalli R, White DM, Stokes NA, Burreson EM (2010) Observation of a *Bonamia* sp. infecting the oyster *Ostrea stentina* in Tunisia, and a consideration of its phylogenetic affinities. *J Invertebr Pathol* 103:179–185
- Hine PM (1996) The ecology of *Bonamia* and decline of bivalve molluscs. *NZ J Ecol* 20:109–116
- Hine PM, Cochennec-Laureau N, Berthe F (2001) *Bonamia exitiosus* n. sp. (Haplosporidia) infecting flat oysters *Ostrea chilensis* in New Zealand. *Dis Aquat Org* 47:63–72
- Lallias D, Arzul I, Heurtebise S, Ferrand S and others (2008) *Bonamia ostreae*-induced mortalities in one-year old European flat oysters *Ostrea edulis*: experimental infection by cohabitation challenge. *Aquat Living Resour* 21:423–439
- Shaw BL, Battle HI (1957) The gross and microscopic anatomy of the digestive tract of the oyster *Crassostrea virginica* (Gmelin). *Can J Zool* 35:325–346
- Shilts MH, Pascual MS, Ó Foighil D (2007) Systematic, taxonomic and biogeographic relationships of Argentine flat oysters. *Mol Phylogeny Evol* 44:467–473

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