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C Audemard

RB Carnegie

NA Stokes

MJ Bishop

CH Peterson

See next page for additional authors

Authors

C Audemard, RB Carnegie, NA Stokes, MJ Bishop, CH Peterson, and Eugene M. Burreson

EFFECTS OF SALINITY ON *BONAMIA* SP. SURVIVAL IN THE ASIAN OYSTER *CRASSOSTREA ARIAKENSIS*

CORINNE AUDEMARD,[1]* RYAN B. CARNEGIE,[1] NANCY A. STOKES,[1] MELANIE J. BISHOP,[2] CHARLES H. PETERSON[3] AND EUGENE M. BURRESON[1]

[1]*Virginia Institute of Marine Science, School of Marine Science, College of William and Mary, Gloucester Point, Virginia 23062;* [2]*University of North Carolina at Chapel Hill, Institute of Marine Sciences, 3431 Arendell St., Morehead City, North Carolina 28557;* [3]*University of Technology, Sydney, Department of Environmental Sciences, P.O. Box 123, Broadway, NSW 2007, Australia*

ABSTRACT   A novel *Bonamia* sp. discovered in Bogue Sound, NC, has recently emerged as a parasitic threat to the Asian oyster *Crassostrea ariakensis*. Because this oyster is being considered for introduction to the mid-Atlantic region, more data are needed to better evaluate the risks associated with this parasite. Field observations collected from North Carolina and information on other *Bonamia* spp. suggest an affinity for higher salinities, and direct transmissibility; in the absence of explicit experimental tests, however, this is largely hypothetical. Consequently, we used laboratory trials to test the direct transmissibility and the persistence of *Bonamia* sp. in infected triploid *C. ariakensis* transferred to and maintained at three salinities, 10, 20, and 30 psu for at least 15 wk. Under these experimental conditions, there was no evidence of direct *Bonamia* sp. transmission. Average parasite intensity in infected oysters transferred to and maintained at 10 and 20 psu decreased compared with oysters placed at 30 psu. At the same time, host mortality was significantly reduced at salinities below 30 psu. These experimental results suggest that the survival of *Bonamia* sp. in *C. ariakensis* may be limited in mesohaline areas. The persistence, pathogenicity, and transmission of *Bonamia* sp. under polyhaline conditions will need to be further evaluated to better describe the geographic areas at risk in the event of *C. ariakensis* introduction.

KEY WORDS: *Crassostrea ariakensis*, *Bonamia*, salinity, persistence, transmission, experimental, introduction, Suminoe oyster

INTRODUCTION

As the Asian oyster *Crassostrea ariakensis* (Fujita 1913) continues to receive consideration for possible introduction to mid-Atlantic waters of the United States, parasitism by a *Bonamia* sp. has emerged as the most significant disease-related concern. In the summer of 2003, triploid *C. ariakensis* maintained in Bogue Sound, NC (34°43′N, 76°45′W), experienced unexpected and catastrophic mortality caused at least in part by a novel microcell parasite, a *Bonamia* sp. (Burreson et al. 2004). Morphological characters were typical of microcells (Carnegie & Cochennec-Laureau 2004), because parasites were small (<5 μm), presented a "fried egg" appearance (Bower et al. 1994), and were observed in and around hemocytes (Burreson et al. 2004). DNA sequencing of the small subunit ribosomal DNA gene indicated clear similarity to southern hemispheric *Bonamia* spp. (Burreson et al. 2004), suggesting a possible recent introduction through the nearby North Carolina State Port in eastern Bogue Sound at Morehead City (Bishop et al. 2006). *Bonamia* sp. is now considered to be enzootic within Bogue Sound, as it has reappeared annually and has continued to cause heavy mortalities in each triploid seed *C. ariakensis* deployment to Bogue Sound within a few weeks of any warm-season transfer (water temperature >20°C).

Responding to the need to better describe *Bonamia* sp. and the factors potentially influencing its distribution and pathogenicity, field studies have been conducted in North Carolina. In these trials, susceptibility of *C. ariakensis* to *Bonamia* sp. was demonstrated to be size dependent, with greater infection prevalence and associated mortality observed in small (<40 mm) oysters (Bishop et al. 2006). Affinity of *Bonamia* sp. for

higher salinity waters (31–35 psu) was also suggested based on results from the deployment of *C. ariakensis* along a salinity gradient (Bishop et al. 2006). In that trial, initially uninfected oysters were deployed at stations from Beaufort Inlet into the Newport River and through Core Creek to the Neuse River, covering a gradient in salinity ranging from 35 psu to 8–14 psu. *Bonamia* sp. infections were observed only at the higher salinity areas closest to Bogue Sound and Beaufort Inlet. Although these data may relate to *Bonamia* sp. salinity tolerance, other explanations are possible. First, *Bonamia* sp. distribution may still be spatially limited if it is a recent ballast-water introduction from a ship using the Morehead City port. Second, the source(s) or reservoir(s) of *Bonamia* sp. in Bogue Sound may be restricted to high-salinity waters. The only native Bogue Sound species found to date that hosts *Bonamia* sp., the native oyster *Ostreola equestris* Say 1834 (unpublished data), inhabits euhaline to polyhaline waters and consequently could limit the distribution of the parasite to high-salinity waters. However, the low *Bonamia* sp. abundance in *O. equestris* populations (never more than 3.3% prevalence, with most infections light) raises questions as to whether *O. equestris* could be the only parasite source, given the predictably rapid and intense infections of any *C. ariakensis* deployed during warm-water conditions.

In the context of a potential introduction of *C. ariakensis* to mesohaline-polyhaline waters of Chesapeake Bay (National Research Council 2003), more empirical data are needed to better evaluate the effects of salinity on survival and transmission of *Bonamia* sp. Chesapeake Bay is characterized by salinities lower than 25 psu except near its mouth, which contrasts with the high salinity environment (generally >30 psu) where *Bonamia* sp. has been observed in Bogue Sound. The key question, therefore, is whether *Bonamia* sp. would persist within, and be transmitted among, *C. ariakensis* at the

intermediate to low salinities of Chesapeake Bay. Laboratory trials to begin evaluating the low-salinity tolerance of *Bonamia* sp. are the focus of this study.

MATERIALS AND METHODS

Source of C. ariakensis *for Laboratory Trials*

Crassostrea ariakensis used in this study were triploid oysters spawned at the Virginia Institute of Marine Science (VIMS) hatchery on 2 June 2004 (spawn 3MXWCA04-1). Absence of *Bonamia* sp. infection in these oysters was confirmed in 30 *C. ariakensis* by polymerase chain reaction (PCR) prior to any transfer to North Carolina and deployment in the field (see details later). On September 20, 2004, a subset of these oysters, ranging in size from 10–30 mm, was transferred to upwellers on a NC Division of Marine Fisheries pier, adjacent to the University of North Carolina Chapel Hill Institute of Marine Sciences (UNC-IMS). The upwellers received raw seawater from Bogue Sound. The rest of the animals were maintained at VIMS under quarantine conditions. Thirty *C. ariakensis* were sampled from the upwellers on October 6 and 19 for PCR and histological diagnosis of *Bonamia* sp. (see details later). By the second sampling date, *Bonamia* sp. prevalence was estimated to be >50%, so on October 25, 400 deployed *C. ariakensis* were returned to VIMS for the first laboratory trial, designed to evaluate *Bonamia* sp. transmission as a function of salinity. On the same date, 30 of the *C. ariakensis* being maintained at the VIMS hatchery were sampled for *Bonamia* sp. PCR and histological diagnosis to confirm that these oysters had remained uninfected before being placed in cohabitation with the *C. ariakensis* returned from North Carolina. On November 9 an additional 600 *C. ariakensis* were returned to VIMS to begin the second laboratory trial, evaluating simple persistence of *Bonamia* sp. within *C. ariakensis*, again as a function of salinity. In both trials, the initial level of infection was determined by PCR on a sample of 30 oysters, and the remaining oysters were placed in tanks as described later.

Trial 1: Transmission of Bonamia *sp.*

Our first trial was designed to test if *Bonamia* sp. could be transmitted directly between infected and uninfected *C. ariakensis*, and to determine the influence of salinity on transmission. Salinity treatments encompassed salinities characteristic of Chesapeake Bay (10 and 20 psu), and a salinity at which *Bonamia* sp. has been observed in Bogue Sound (30 psu). Animals were maintained in 40-L aquaria and each experimental parameter was tested in triplicate tanks. For the 10 psu treatment, oysters were first placed in experimental tanks at 20 psu for one day before decreasing the salinity to 10 psu. Filtered (1 μm) York River water was adjusted to the desired salinity by adding artificial sea salts or fresh water. Each tank was stocked with 30 *Bonamia* sp.-exposed *C. ariakensis* returned from North Carolina on October 25, 2004 ("NC *C. ariakensis*") and 30 of the unexposed *C. ariakensis* that had been retained in Virginia ("VA *C. ariakensis*"). The VA *C. ariakensis* were placed in a mesh bag (5 mm mesh size) to distinguish them from the NC *C. ariakensis*. Three control tanks, one at each salinity, were each stocked with 60 VA *C. ariakensis*. The trial began on October 26 and ran under quarantine conditions. Oysters were fed a

commercial algal paste (Shellfish Diet, Reed Mariculture) containing *Isochrysis* sp., *Pavlova* sp., *Thalassiosira weissflogii* (Grunow) G. Fryxell and Hasle 1977, and *Tetraselmis* sp. daily; two thirds of the water was changed twice weekly; and temperature was maintained at 24°C to 25°C. Mortality was checked daily. Sampling of 10 VA *C. ariakensis* from the experimental and control tanks and 5 NC *C. ariakensis* occurred after three weeks (November 16) and six weeks (December 7) cohabitation; after approximately 18 weeks (February 25) all remaining animals were sampled. The rationale for the number of NC oysters sampled was a compromise between describing the dynamics of *Bonamia* sp. in these oysters and providing a parasite source in the experimental tanks. Animals sampled were fixed for PCR and histology for disease analysis (see following sections for details).

Trial 2: Persistence of Bonamia *sp.*

The second laboratory study was designed to evaluate the ability of infected *C. ariakensis* to purge *Bonamia* sp. at salinities characteristic of Chesapeake Bay. Two 40-L aquaria were filled with water at each of three salinities—10, 20, and 30 psu—as earlier mentioned. Ninety *Bonamia* sp.-exposed NC *C. ariakensis* transferred from North Carolina on November 8, 2004 were placed in each of these tanks on November 9. In this trial, limited laboratory space prevented establishment of VA *C. ariakensis* controls. Oysters to be exposed to 10 psu were first conditioned at 20 psu for one day. Feeding, water changes, and monitoring of mortality were as above. Samples of 15 oysters/tank were collected after one week (November 17), three weeks (December 1), and 10 weeks (January 20), and all remaining animals were sampled after 15 weeks (February 25). The number of oysters sampled per tank (15) was greater than in the first trial (5) to better describe the effects of salinity on *Bonamia* sp. infection in *C. ariakensis*. Animals were fixed for subsequent molecular and histological analyses.

Assessment of Cumulative Oyster Mortality

At each sampling date, percent mortality was calculated for each replicate tank by dividing the number of oysters that died during the time interval encompassed between two sampling dates by the number of live oysters at the beginning of the interval and multiplying by 100. Successive interval mortalities were added to obtain cumulative mortality. Average cumulative mortalities and standard deviations were calculated for each treatment.

Bonamia *sp. Prevalence and Average Parasite Intensity*

Sampled oysters were processed for disease diagnosis using PCR. Histology was subsequently performed on PCR-positive oysters to confirm infection and document intensities. Oysters were shucked and small pieces of gill, mantle, and visceral tissues (~3–5 mm^3) were 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. DNA was extracted from the ethanol-preserved tissue using a DNeasy Tissue Kit (QIAGEN, Santa Clarita, CA) following the manufacturer's protocol. DNA was

quantified with a GeneQuant *pro* spectrophotometer (Amersham Biosciences, Cleveland, OH) and adjusted to 200 ng/μL. PCR was performed using *Bonamia* genus SSU rDNA-specific primers $C_F + C_R$ (Carnegie et al. 2000). Reaction and cycling conditions were as reported by Carnegie et al. (2000), except that primer amount was increased to 25 pmol. Amplification products (expected size of 760 bp) were electrophoresed in 2% agarose gels (in 1X TAE), stained with ethidium bromide, and visualized under UV light.

After fixation in Davidson's fixative, tissue from PCR-positive oysters was processed for standard paraffin histology. Intensity of *Bonamia* sp. infection determined by histology was scored as 0 (negative), 0.5 (rare infection: less than 10 parasite cells on the entire section), 1 (light infection: more than 10 cells total but only focal to multifocal infections), 3 (moderate infection: 2–3 cells in almost every field observed using a $\times 100$ objective lens) and 5 (heavy infection: 3–5 cells per field). Prevalence was calculated as the percentage of oysters with *Bonamia* sp. infections, as confirmed by histopathology. Average *Bonamia* sp. intensity was calculated as the sum of all infection intensity scores determined by histology divided by the total number of infected animals. Standard deviations were calculated for both prevalence and average parasite intensity.

Statistical Analysis

Prior to analysis, *Bonamia* sp. prevalence and cumulative mortality were arcsine-transformed, and parasite infection intensity was ln(x + 1)-transformed (Underwood 1997). Analyses were performed on each of the sampling dates separately. This approach was necessary because of the nonindependence of times and because independency of the observations is one of the assumptions of the ANOVA. For the transmission trial, differences in mean cumulative mortality between salinity treatments (10, 20, or 30 psu) and between oyster groups (NC *C. ariakensis* or VA *C. ariakensis*) were determined using a two-way ANOVA (α = 0.05). For mean prevalence and average intensity of infection in both trials, and for mean cumulative mortality in the persistence trial, differences between salinity treatments were determined using one-way ANOVAs (α = 0.05). When appropriate, Scheffé tests were performed for multiple comparisons. Analysis of cumulative mortality was performed on each of the sampling dates. For prevalence and average parasite intensity, however, the analysis was performed on each sampling date except the final sampling because of the loss of oysters at 30 psu and the absence of disease data for this treatment. Analyses were performed using StatView software.

RESULTS

Bonamia sp. Infection Acquisition in Bogue Sound

No infection by *Bonamia* sp. was observed in a sample of 30 *C. ariakensis* from the VIMS hatchery prior to deployment in Bogue Sound upwellers. Sixteen days after deployment (October 6), 3.3% of the oysters were PCR-positive for *Bonamia* sp. During this exposure period, water temperatures in Bogue Sound ranged from 22°C to 26°C. One month after deployment (October 19) the prevalence had reached 76.5% and temperatures remained higher than 20°C. Oyster sizes on this date ranged from 18–37 mm.

Trial 1: Transmission of Bonamia sp.

At the beginning of the trial (October 26), 80% of NC *C. ariakensis* were infected with *Bonamia* sp. The average intensity score was 3.3, an indication that the average infection was moderate to heavy in intensity. The VA *C. ariakensis* (sample of 30 animals) maintained at VIMS hatchery were confirmed to be *Bonamia* sp.-free. Experimental VA *C. ariakensis* placed in cohabitation with NC *C. ariakensis* (total of 270 animals sampled), as well as those in control aquaria, remained free of *Bonamia* sp. during the entire trial.

Salinity influenced *Bonamia* sp. prevalence and parasite intensity in NC *C. ariakensis* (Fig. 1). After three weeks of exposure, prevalence of *Bonamia* sp. in NC *C. ariakensis* was zero at 10 psu, and had decreased to $13 \pm 11\%$ at 20 psu, but was still $60 \pm 20\%$ at 30 psu (Fig. 1A). After six weeks of exposure, *Bonamia* sp. was observed only at 30 psu, with $58 \pm 33\%$ of the NC *C. ariakensis* infected with generally moderate *Bonamia* sp. infections (average intensity: 2.8 ± 1.9). At each of these time points, *Bonamia* sp. prevalence was significantly lower at 10 and 20 psu compared with 30 psu ($P = 0.0040$ after three weeks, $P = 0.0027$ after six weeks exposure). After three weeks of exposure, parasite infection intensity was significantly lower ($P < 0.0001$) at 20 psu, where generally light infection intensities were observed (average score: 1.3 ± 1.5), than at 30 psu, where infections remained moderate to heavy (average score: 3.8 ± 1.4) (Fig. 1B). Observed high standard deviations associated with prevalence were likely related to the small number of NC *C. ariakensis* individuals (5) sampled per tank in this trial. On the final sampling date, no oysters remained alive at 30 psu and no *Bonamia* sp. was detected in the surviving NC *C. ariakensis* held at 20 and 10 psu. All *Bonamia* sp. cells observed during this trial were uni- or binucleate, and were observed in hemocytes primarily in the digestive gland but also in the gills, palps, and mantle.

For each sampling date, average cumulative mortalities and standard deviations (for the experimental tanks) or raw data in the absence of replication (for the control tanks) are presented (Fig. 1C, 1D and 1E). In cases where all the oysters from one or two of the replicate tanks were removed (either because of mortality or to sampling), the cumulative mortality values were considered to be 100% for mean and standard deviation calculations.

Average cumulative mortality of the NC *C. ariakensis* placed at 30 psu was 58% after three weeks and 70% after six weeks exposure, whereas it remained below 8% at 10 and 20 psu (Fig. 1C). The second sampling date (6 weeks exposure) removed all the remaining NC animals in two of the three replicate tanks at 30 psu. After about nine weeks exposure, all the remaining NC *C. ariakensis* at 30 psu were dead. Mortality of NC *C. ariakensis* placed at 10 and 20 psu remained below 27% and 40%, respectively, through the end of the trial. Mortality of VA *C. ariakensis* in cohabitation with the NC *C. ariakensis* oysters remained <18% through six weeks exposure for all treatments (Fig. 1D). By the end of the trial, however, all VA *C. ariakensis* in cohabitation with NC *C. ariakensis* at 30 psu were dead, whereas cumulative mortality was 12% at 20 psu and 4% at 10 psu. After three and six weeks exposure, the interaction between salinity and oyster origin was not significant ($P = 0.4157$ at 3 weeks, $P = 0.1483$ at 6 weeks). For each of these sampling dates, cumulative mortality was
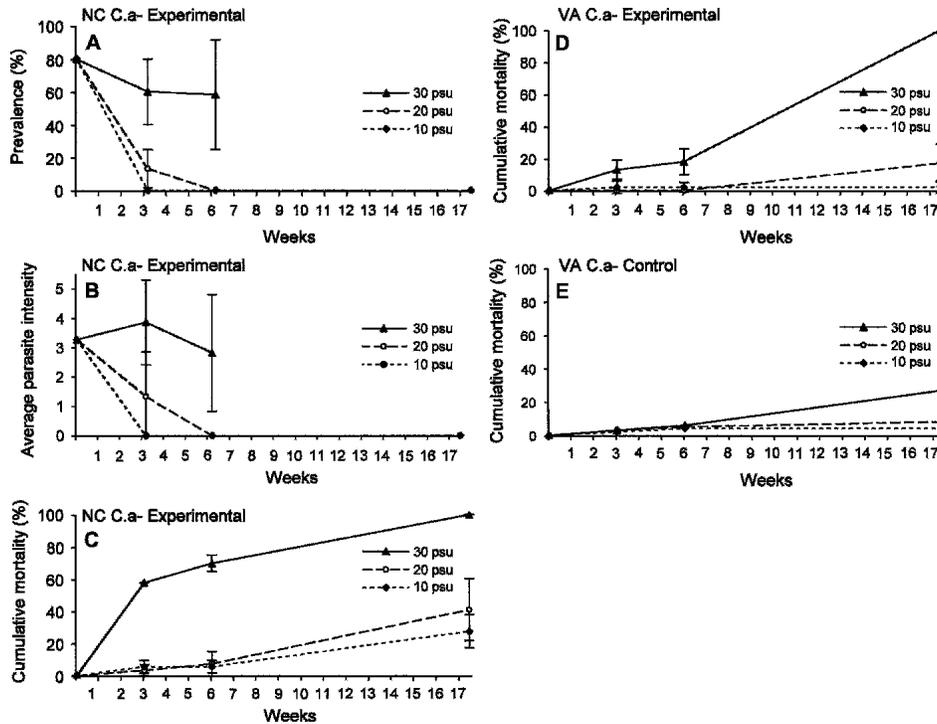
Figure 1. Transmission trial. NC *C. ariakensis*: (A) prevalence of *Bonamia* sp. detected by PCR and confirmed by histology, (B) average parasite intensity, and (C) cumulative mortality. VA *C. ariakensis*: (D) cumulative mortality of the experimental oysters and (E) of the control oysters. *Bonamia* sp. was never detected in VA *C. ariakensis*, thus VA *C. ariakensis* was not included in graphs related to parasite prevalence and average infection intensity. Error bars correspond to one standard deviation.

significantly lower for the VA *C. ariakensis* compared with the NC *C. ariakensis* ($P = 0.0060$ at 3 weeks, $P = 0.0006$ at 6 weeks). Salinity did not significantly influence cumulative mortality ($P = 0.4157$ at three weeks, $P = 0.1483$ at six weeks). At the final sampling date, however, the interaction between salinity and oyster origin was significant ($P < 0.0001$). Cumulative mortality was significantly lower for the VA *C. ariakensis* compared with the NC *C. ariakensis* ($P < 0.0001$) and was significantly lower at 10 and 20 psu compared with 30 psu ($P < 0.0001$). Cumulative mortality of VA *C. ariakensis* in the control tanks was higher at 30 psu than at 20 and 10 psu, but by the end of the trial mortality was only 27% at 30 psu, 8% at 20 psu, and 4% at 10 psu (Fig. 1E).

Trial 2: Persistence of *Bonamia sp.*

At the beginning of the trial, 80% of NC *C. ariakensis* were infected with *Bonamia* sp., with infections generally moderate to heavy in intensity (average score: 3.5). *Bonamia* sp. prevalence and infection intensity observed in this trial were not as strongly influenced by salinity as in the transmission trial (Fig. 2A and B, respectively). After one week exposure, decreases in prevalence and in parasite infection intensity were observed but differences between salinity treatments were marginally significant ($P = 0.0714$ and $P = 0.0540$, respectively). After three weeks exposure, differences in mean *Bonamia* sp. prevalence between salinity treatments were significant ($P = 0.0218$), with prevalence at 10 and 20 psu being significantly lower than at 30 psu ($P = 0.0461$ and $P = 0.0256$, respectively). The influence of salinity on average parasite intensity was nonsignificant after 3 and 10 weeks exposure ($P = 0.4073$ and $P = 0.4648$). During

this trial, pycnotic cells were observed within hemocytes in some individuals regardless of the salinity treatment, whereas pale *Bonamia* sp. cells and debris were observed in oysters maintained at 20 and 10 psu. After 10 weeks exposure, *Bonamia* sp. was observed only at 30 psu, with $5 \pm 7\%$ of the oysters infected and with infections generally rare (0.3 ± 0.3). On the final sampling date (15-wk exposure), no oysters remained at 30 psu and no *Bonamia* sp. was detected in surviving NC *C. ariakensis* at 20 and 10 psu.

In this trial, the absence of controls (VA *C. ariakensis* placed under the same conditions as in the first trial) limits the ability to interpret cumulative mortality data. Nonetheless, mortality of NC *C. ariakensis* was significantly higher at 30 psu than at 20 and 10 psu at each sampling time point ($P = 0.0017$ at 1 week, $P = 0.0013$ at 3 weeks, $P = 0.0059$ at 6 weeks and $P = 0.0007$ at the final sampling). After the third sampling (10-wk exposure), when cumulative mortality had reached an average of $54 \pm 2\%$ at 30 psu, all the remaining oysters left in the tanks at 30 psu were sampled. By the end of the trial, mortality at 20 psu and 10 psu was $28 \pm 8\%$ and $16 \pm 4\%$, respectively.

DISCUSSION

The two laboratory trials performed in this study indicated that *Bonamia* sp. infections were rapidly purged when infected oysters were placed at 10 and 20 psu. Under these salinities, decreases in infection intensity (from moderate to light infections) were noticeable after only one week in the persistence trial. This decrease was observed after three weeks exposure to the reduced salinities in both trials, and parasites were completely purged from their hosts after a maximum of 6–10 wk for
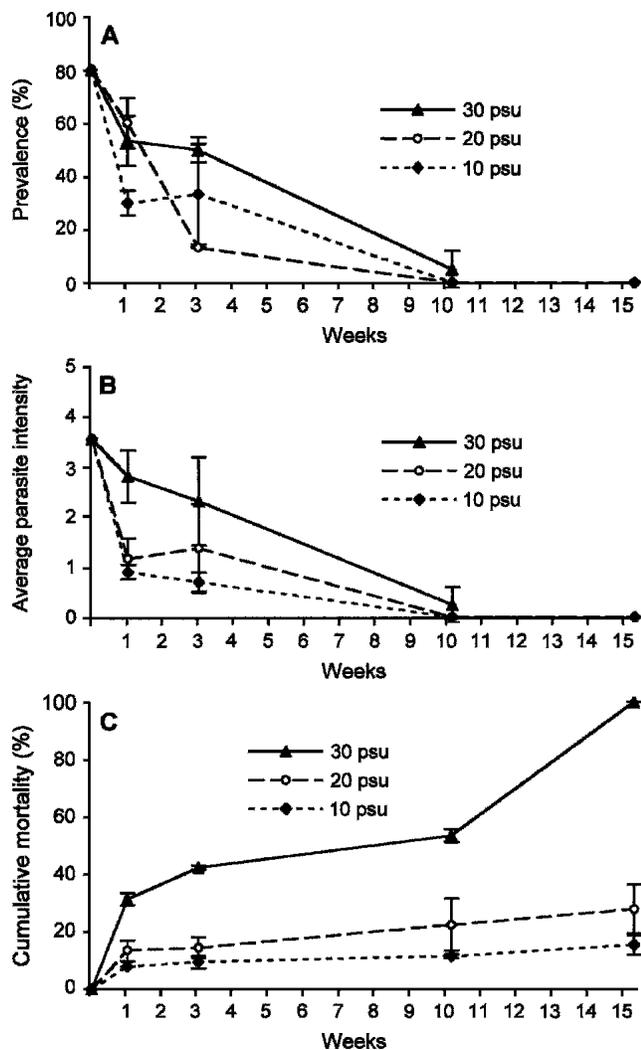
Figure 2. Persistence trial. (A) Prevalence of *Bonamia* sp. in NC *C. ariakensis* detected by PCR and confirmed by histology, (B) average parasite intensity, and (C) cumulative mortality. Error bars correspond to one standard deviation.

the first and second trials, respectively. Histological examinations of oysters maintained at 10 or 20 psu revealed irregularly stained *Bonamia* sp. cells as well as debris within hemocytes that appeared to be degraded parasites. These data suggest that low to moderate salinities detrimentally affected the survival of the parasite within *C. ariakensis*, presumably through some combination of factors relating to parasite physical tolerances and host defenses, subsequently leading to a decrease in parasite infection intensities.

Mortality of infected oysters appeared to depend on the level of parasite infection. Decrease in infection intensity observed at 10 and 20 psu was accompanied by a decrease in host mortality relative to oysters maintained at 30 psu. Similarly, persistence of *Bonamia* sp. at 30 psu induced significant host mortalities, which reached 58.0% and 42.5% for the transmission and persistence trials, respectively, after three weeks exposure. Mortality of infected animals likely contributed to the decrease in prevalence; however, even at 30 psu, as at lower salinities, decreasing prevalence seemed to be related to the

purging of the parasite. Between the 3 and 10 week sampling points in the persistence trial, significant decreases in prevalence (from 50% to 5%) and infection intensity (from moderate-light to rare infections) were observed at 30 psu, whereas mortality showed no significant increase (+11%). The oysters that died at the beginning of the trials were likely already highly infected when placed in the tanks, but it is probable that the development of light infections was limited and never led to the death of the hosts. Purging of the parasite at 30 psu may have been related to experimental conditions that hindered parasite survival and multiplication. The highest salinity tested in these trials (30 psu) may actually be lower than optimal for parasite multiplication; in Bogue Sound salinities can be somewhat higher (to 35 psu). However, other experimental conditions may be involved, as described later.

Under the described experimental conditions, there was no evidence of direct *Bonamia* sp. transmission, given that the parasite was not detected in VA *C. ariakensis* placed in cohabitation with NC *C. ariakensis*. This result could be a consequence of (1) absence of direct transmissibility of *Bonamia* sp. from *C. ariakensis* to *C. ariakensis,* or (2) non-optimal experimental conditions for the transmission of *Bonamia* sp. Although direct transmission has been documented for *B. ostreae* and possibly for *B. exitiosa* (Bachère et al. 1986, Elston et al. 1986, Elston et al. 1987, Hine 1996, Hine et al. 2002), the life cycle of *Bonamia* sp. observed in North Carolina may be more complex. Based on the discovery of the spore-forming species *Bonamia perspora* (Carnegie et al. 2006), we now recognize *Bonamia* as a haplosporidian genus with at least some spore-forming, presumably indirectly transmissible members. Our failure to detect direct parasite transmission over many weeks in the experimental conditions contrasts strongly with observations in Bogue Sound, where *C. ariakensis* deployed even to areas free of *C. ariakensis* will display >70% *Bonamia* sp. prevalence within a few weeks in warm summer months. This suggests that the true source of *Bonamia* sp. in the North Carolina environment is not *C. ariakensis*. It is also unlikely to be *O. equestris* alone, as crested oysters are sometimes distant from *Bonamia* sp.-affected *C. ariakensis*. This issue highlights the great uncertainty concerning *Bonamia* sp. in waters north or south of enzootic areas in North Carolina: we will not be able to gauge the true risk of *Bonamia* sp. activity in these waters without knowing its source(s) in the environment.

Non-optimal experimental conditions may also have compromised the transmission of *Bonamia* sp. Degraded experimental conditions were revealed by the mortality of VA *C. ariakensis* placed in cohabitation with NC *C. ariakensis* at 30 psu. In these tanks, death of the infected NC *C. ariakensis* may have lead to bacterial proliferation and low dissolved oxygen, although the actual cause of death was not identified in this study. Maintenance in mesh bags of the VA *C. ariakensis* placed in cohabitation with NC *C. ariakensis* may also have affected the survival of these oysters. As the control oysters that underwent low mortality were not held in bags, the negative influence of this factor cannot be excluded. The potential detrimental effects of *P. marinus* on *C. ariakensis* survival observed in previous laboratory trials (Moss et al. 2006) was not identified as the cause of VA *C. ariakensis* mortality in this trial as histological analysis of experimental and control VA *C. ariakensis* sampled after six weeks exposure and during the final sampling revealed *P. marinus* infection (moderate) in only one

of 139 oysters. The period of exposure of uninfected *C. ariakensis* to infected *C. ariakensis* was limited to three weeks, and after this period all the remaining NC *C. ariakensis* were removed (through sampling or death), suggesting that after this period the presence of potential *Bonamia* sp. infective stages was limited. Field data suggest, however, that this exposure period to potential infective *Bonamia* sp. stage was long enough to allow transmission, because uninfected oysters deployed in the Bogue Sound environment showed intense infections after only 3–5 wk exposure (Burreson et al. 2004). Finally, as postulated earlier, 30 psu may still not be optimal for the parasite persistence, multiplication, and transmission. In the future, direct transmission of *Bonamia* sp. will need to be studied at higher salinities. To minimize deterioration of water quality, more frequent water changes should be performed, and to better identify causes of mortality, water quality should be analyzed on a regular basis.

Based on the earlier mentioned experimental results, we find that salinity could play a major role in *Bonamia* sp. distribution. *Bonamia* sp. has an affinity for estuarine and coastal waters of relatively higher salinity (>20 psu). The risks associated with *Bonamia* sp. in the event of an introduction of *C. ariakensis* to Chesapeake Bay may initially be restricted to areas where salinities are greater than 20 psu, primarily the waters of Virginia. However, under changing environmental conditions such as climate warming, a potential range expansion of the parasite may occur as has been observed recently for Eastern oyster parasite *Perkinsus marinus* (Ford 1996, Ford & Chintala 2006). The actual threshold salinity under which *Bonamia* sp. is rapidly purged, if such exists, as well as the influence of pulsed freshets of varying duration and intensity will be investigated in future trials and should improve our appreciation of the areas at risk.

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