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# Experimental cross-infections by *Perkinsus marinus* and *P. chesapeaki* in three sympatric species of Chesapeake Bay oysters and clams

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ABSTRACT: In controlled laboratory transmission experiments, uniform doses of axenic *in vitro* isolate cultures of *Perkinsus marinus* from a *Crassostrea virginica* oyster, and of independent *P. chesapeaki* isolates from Chesapeake Bay *Mya arenaria* and *Macoma balthica* clams, were used to reciprocally challenge *Perkinsus* sp.-free *C. virginica*, *M. arenaria*, and *M. balthica* experimental hosts. Following mantle cavity inoculations, all 3 experimental hosts acquired high incidences (30 to 100%) of infections by each of the 3 *Perkinsus* sp. isolates, based on PCR assays of DNAs from experimental host tissues that were collected through 60 d post-inoculation. Lesions containing proliferating pathogen cells were documented histologically in tissues of all experimental host species challenged with all isolates of both *Perkinsus* species. Experimental *Perkinsus* sp. challenge isolates were re-isolated and propagated *in vitro* from infected tissues of host molluscs from most (5 of 9) experimental treatment groups. Koch's postulates were generally satisfied to confirm experimental infections in all bivalve molluscs that were challenged with 3 isolates of 2 *Perkinsus* spp. These results suggest potential broad and overlapping host specificities for the 2 current Chesapeake Bay-endemic *Perkinsus* species: *P. marinus* and *P. chesapeaki*.

KEY WORDS: Dermo disease · *Crassostrea virginica* · Host-specificity · *Macoma balthica* · Mollusc · *Mya arenaria*



## INTRODUCTION

Based on results of then-novel Ray's fluid thioglycollate medium (RFTM) assays (Ray 1952), an early survey of Chesapeake Bay molluscs reported that diverse non-oyster hosts are infected by *Perkinsus marinus* (= *Dermocystidium marinum*) (Andrews 1953). At that time, *P. marinus* had recently been described as a lethal pathogen of Gulf of Mexico oysters (Mackin et al. 1950), and had also been detected in the earliest (1949) Chesapeake Bay oyster samples examined for that parasite (Ray & Chandler 1955, Andrews & Hewatt 1957). A distinctive *Perkinsus* sp. parasite was subsequently reported to infect Chesapeake Bay *Macoma balthica* clams (Valiulis & Mackin 1969), and a parasite from that clam host was recently described as *P. andrewsi* (Coss et al. 2001). *Perkinsus* sp. infections were also reported as prevalent among *Mya arenaria* (McLaughlin & Faisal 2000) and *Tagelus plebeius* (Dungan et al. 2002) commercial clams in mesohaline Chesapeake Bay waters, and a parasite of the former host was described as *P. chesapeaki* (McLaughlin et al. 2000). The *P. chesapeaki* description was subsequently emended to include parasites infecting both *T. plebeius* and *M. balthica* clams, and *P. andrewsi* was recognized as a junior synonym of *P. chesapeaki* (Burreson et al. 2005).

Histological and RFTM assays in current and historic use for diagnoses of *Perkinsus* spp. infections in mollusc hosts do not differentiate *P. marinus* and *P. chesapeaki*. Thus, species identities of parasites detected by such generic assays are uncertain in light of the several

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*Perkinsus* spp. now known to infect sympatric Chesapeake Bay bivalve molluscs. However, species-specific polymerase chain reaction (PCR) assays now allow sensitive detection and discrimination of *Perkinsus* species whose DNAs may be present in tissue samples (Coss et al. 2001, Burreson et al. 2005, Moss et al. 2006) or environmental samples (Audemard et al. 2004).

With the recent synonymy of *Perkinsus chesapeaki* and *P. andrewsi*, only *P. marinus* and *P. chesapeaki* are now recognized to co-occur in Chesapeake Bay waters (Burreson et al. 2005). Based on PCR results, both of these *Perkinsus* spp. are inferred to cross- or co-infect several sympatric Chesapeake Bay bivalve molluscs (Kotob et al. 1999, Coss et al. 2001). With a single apparent exception (McLaughlin & Faisal 1998), however, *in vitro Perkinsus* sp. isolates propagated from several Chesapeake Bay clam species have uniformly proven to be *P. chesapeaki*, while numerous *in vitro* isolates from Chesapeake Bay oysters have uniformly been identified only as *P. marinus* (La Peyre et al. 2006). Results from species-specific *in situ* assays to confirm PCR-inferred co-infections by multiple *Perkinsus* species are lacking, as are experimental results that confirm or refute the broad host specificities inferred for the 2 *Perkinsus* spp. that are currently endemic in Chesapeake Bay.

To empirically resolve the possible host specificities of *Perkinsus marinus* and *P. chesapeaki*, we report results of controlled laboratory experiments in which 3 *in vitro* isolate cultures of *P. marinus* and *P. chesapeaki* (= *P. andrewsi*) were used to reciprocally challenge *Perkinsus* sp.-free oysters and clams that are sympatric source-hosts for these parasite species in Chesapeake Bay waters.

## MATERIALS AND METHODS

**Experimental molluscs**. *Perkinsus* sp. parasites are not reported in USA Pacific coast molluscs, which include conspecifics of several *Perkinsus* sp.-infected Chesapeake Bay mollusc hosts. Wild, *Perkinsus* sp.-free *Mya arenaria* (16 to 70 mm shell length) and *Macoma balthica* (9 to 25 mm shell length) clams were collected from the Yaquina Bay estuary near Newport, Oregon. *Perkinsus* sp.-free *Crassostrea virginica* oysters (31 to 52 mm shell height) that were propagated from descendents of broodstocks imported during the early 1900s to British Columbia, Canada, were acquired from Taylor Shellfish (Shelton) facilities in southern Puget Sound. Clams and oysters were shipped to Oxford, Maryland, under humidified refrigeration, and maintained upon arrival at 20 to 23°C in separate, covered aquaria containing 25 ppt artificial seawater (ASW).

**Pathogen isolate cultures**. Three *in vitro* isolates of 2 *Perkinsus* species from Chesapeake Bay bivalve molluscs were obtained from the American Type Culture Collection (ATCC, Manassas, VA) for use as challenge experiment inocula. These included *P. marinus* isolate ATCC 50439 from a *Crassostrea virginica* oyster (Dungan & Hamilton 1995), *P. chesapeaki* neohapantotype isolate ATCC PRA-65 from a *Mya arenaria* clam (Burreson et al. 2005), and *P. chesapeaki* (= *P. andrewsi* holotype) isolate ATCC 50807 from a *Macoma balthica* clam (Coss et al. 2001). Isolate suspension cultures were propagated at 27°C in 850 mOsm $kg^{-1}$ (29 ppt) Dulbecco's modified Eagle's (DME):Ham's F-12 *Perkinsus* sp. culture medium (Burreson et al. 2005) that was supplemented to the following final concentrations with fetal bovine serum (FBS, 3% v/v), HEPES buffer (25 mM), sodium bicarbonate (7 mM), yeast extract ultrafiltrate (Sigma Y4375, 0.2% v/v), lipid mixture (Sigma L5146, 0.1% v/v), glucose (0.05% w/v), trehalose (0.01% w/v), galactose (0.01% w/v), L-glutamine (2 mM), penicillin (100 U $ml^{-1}$), and streptomycin (100 µg $ml^{-1}$) (DME/F12-3).

**Experimental inocula**. *Perkinsus* spp. cultures were expanded *in vitro* to the desired inoculum cell numbers by continuous, exponential propagation in ventilated T75 tissue culture flasks. Experimental inoculum cells from isolate cultures were harvested separately on 3 successive days, when replicate cell suspensions were pooled, and cells were pelleted by centrifugation at 300 × *g*. Cell pellets were re-suspended in 10 ml of 25 ppt sterile artificial seawater (SASW) for counting. Viable pathogen cell concentrations were estimated by hemacytometer counts of neutral red-stained suspension aliquots, and $1 \times 10^8$ viable pathogen cells were transferred into 50 ml of SASW for overnight, stationary-phase holding ($2 \times 10^6$ cells $ml^{-1}$) at 20°C.

For inoculum suspension preparation on the following morning, cells from 10 ml of quantified pathogen cell suspensions in SASW were concentrated by centrifugation for 5 min at 300 × *g*, and cell pellets were resuspended in 1 ml of SASW as experimental inocula. Inoculum suspensions were loaded into 1 ml insulin syringes for delivery of standard 50 µl boluses containing $1 \times 10^6$ pathogen cells, into the mantle cavities of 16 to 20 potential hosts of each challenged mollusc species. For each challenged host species, 20 negative-control molluscs received 50 µl placebo inoculations of the SASW inoculum diluent without pathogen cells, and 20 uninoculated individuals were processed as additional negative controls. DNAs in 100 µl aliquots of each inoculum suspension were preserved in 1 ml of absolute ethanol in a sterile tube, for use as PCR templates.

**Experimental inoculations**. On 4 successive days, different groups of 16 to 20 individuals from each chal-

lenged mollusc species, received 50 µl pallial cavity injections of one of the following inocula: SASW diluent (negative control), *Perkinsus marinus* (ATCC 50439) suspension, *P. chesapeaki* (ATCC PRA-65) suspension, *P. chesapeaki* (ATCC 50807) suspension. Access of the inoculating needle to the mantle cavity gill areas of experimental oysters was through a small notch made the previous day with veterinary clippers at the postero-ventral oyster valve margins opposite the gills. Clam mantle cavities were inoculated by insertion of the inoculating needle into the gill area, through their continuously exposed proximal siphon musculatures. Following inoculations, challenged mollusc groups were separately held out of water for 6 h, humidified at 12 to 13°C, before re-immersion in separate, covered treatment group aquaria (20 or 40 l, containing recirculating 25 ppt ASW) that were maintained at 22 ± 1°C (mean ± SD) during the experiment. Uninoculated groups of each experimental host species were separately retained in the same covered holding aquaria that were established upon their arrivals, until sacrificed for tissue and DNA samples following the last inoculation sequence.

**Experiment maintenance**. Depending on mortalities among experimental molluscs, treatment groups were maintained for up to 60 d post-inoculation (p.i.). Mollusc hosts in experimental treatment aquaria were fed daily with 100 µl ind.$^{-1}$ of Shellfish Diet 1800™ phytoplankton concentrate (Reed Mariculture), and a sample of phytoplankton feed cells was preserved in 10 volumes of absolute ethanol for subsequent extraction and analysis by PCR assays for the presence of *Perkinsus* sp. template DNA. Experimental treatment aquaria were monitored once or twice daily for removal of dead or moribund molluscs. Where possible, tissue samples from experimental hosts were preserved for both histological analyses and for PCR assays. DNAs for PCR assays were preserved by aseptic excision of mantle and gill tissues with flamed instruments, and immersion in sterile tubes containing 10 volumes of absolute ethanol. Transverse histological tissue samples were preserved for 48 h in Davidson's alcohol, formalin, acetic acid (AFA) fixative, before subsequent processing by standard methods for paraffin histology. Necrotic tissues from dead hosts were only preserved in ethanol for PCR assays.

**Experiment termination**. After 60 d, or whenever the number of surviving experimental hosts in a treatment group fell to 10 (approximate 50% mortality), remaining treatment-group hosts were sacrificed and sampled to insure acquisition of at least 10 high-quality host tissue samples from each experimental treatment for both histological and PCR assays, and for parasite *in vitro* re-isolation attempts. Experimental groups that endured through 60 d were sacrificed and processed for tissue samples on 4 successive days, in the same order that the experimental inoculations occurred. For subsequent use of enlarged *Perkinsus* sp. hypnospores as *in vitro* re-isolate culture inocula, tissues from experimental hosts inoculated with *Perkinsus* spp. were incubated in RFTM that was supplemented with penicillin (200 U ml$^{-1}$), streptomycin (200 µg ml$^{-1}$), gentamicin (200 µg ml$^{-1}$), chloramphenicol (50 µg ml$^{-1}$), and nystatin (50 U ml$^{-1}$).

***In vitro* parasite recovery**. From each experimental host mollusc that survived to treatment-group terminations, duplicate gill (clams) or mantle (oysters) tissue biopsies were inoculated into 2 ml of RFTM in wells of duplicate 24-well culture plates. Plates were incubated for 48 h at 27°C before tissues in wells of one replicate plate were stained with 30% (v/v) Lugol's iodine for enumeration of enlarged *Perkinsus* sp. hypnospores. Based on relative *Perkinsus* sp. cell densities estimated by RFTM assays, enlarged *Perkinsus* sp. hypnospores from unstained duplicate tissues of the 6 most promising individuals from each experimental treatment group were used as inocula for *in vitro* propagation of *Perkinsus* sp.

For re-isolation of *Perkinsus* sp., RFTM was aspirated from selected experimental tissues, and enlarged parasite hypnospores were released into suspension by trituration of tissues in 2 ml of DME/F12-3 culture medium. Resulting inoculum suspensions were serially diluted into DME/F12-3 medium in 6 wells of a 24-well culture plate, and incubated at 27°C with daily microscopic observation for *Perkinsus* sp. proliferation. Proliferating re-isolate cultures were expanded in culture flasks, an aliquot of cells from each re-isolate was preserved in ethanol for extraction of PCR template DNA, and viable re-isolate cells were cryopreserved in a DME/F12 freezing medium that was supplemented to 8% (v/v) with cell culture dimethyl sulfoxide (DMSO) and 6% (v/v) FBS.

**PCR assays and amplicon sequencing**. Ethanol-preserved DNAs from control and challenged mollusc host tissues, *Perkinsus* spp. inoculum cells, phytoplankton feed cells, and *Perkinsus* sp. re-isolate cells were extracted using the DNAeasy Tissue Kit™ (Qiagen) following the manufacturer's protocol. DNAs isolated from *Perkinsus* sp. inoculum cells, the *Perkinsus* sp. re-isolate cultures, and experimental mollusc tissues, were each used as templates in 3 separate amplifications by each of the 4 diagnostic PCR assays described below.

Genus *Perkinsus*-specific PCR assays (85-750-ITS [internal transcribed spacer]) were performed with methods and primers targeting rDNA sequences that are conserved among all known *Perkinsus* species, except *P. qugwadi* (*incertae sedis*) (Casas et al. 2002). PCR to test for the presence of *P. marinus* DNA was

performed using *P. marinus*-specific primers (Pmar-ITS), as previously described (Audemard et al. 2004). *P. chesapeaki*-specific primers (Pches-ITS) (Burreson et al. 2005) were used to test for the presence of *P. chesapeaki* DNA (forward: 5′-AAACCAGCGGTCTCTTCT-TCGG-3′ and reverse: 5′-CGGAATCAACCACAACA-CAGTCG-3′). The Pches-ITS primers were designed to target sequences of the ITS region of the rRNA gene complex that are conserved within the species *P. chesapeaki*, based on alignments of these sequences from GenBank for *P. chesapeaki* G117 (AF091541), ATCC PRA-65 (AY876302–AY876318), ATCC 50864 (AF440464–AF440467), ATCC 50866 (AF440468–AF440471), ATCC 50807 (= *P. andrewsi*, AY305326), and *Perkinsus* sp. (AF252288). *P. chesapeaki* (= *P. andrewsi*)-specific rDNA non-transcribed spacer region primers (Pand-NTS) were used according to published protocols (Coss et al. 2001).

PCR amplifications were performed with 10 to 50 ng of genomic template DNAs in 25 µl reactions. Reagent concentrations were as follows: 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each of dATP, dGTP, dCTP, dTTP, 25 pmol of each primer, 0.625 units of *Taq* DNA polymerase (Invitrogen), and 0.2 mg $ml^{-1}$ of bovine serum albumin (BSA, Idaho Technology). Touchdown™ PCR amplification cycling parameters for the Pches-ITS reactions were as follows: initial DNA denaturing for 4 min at 94°C; followed by 10 cycles of 30 s at 94°C and 30 s at 64°C, with a –1°C change in annealing temperature for each cycle and 1.5 min extension at 72°C; followed by 30 cycles with 30 s at 94°C, 30 s at 54°C, 1.5 min at 72°C, and a final extension of 5 min at 72°C. Amplification products were stained with ethidium bromide, separated on gels of 2% (w/v) agarose in Tris-borate-EDTA buffer (1× TBE), and visualized under UV light.

To confirm *Perkinsus* spp. identities and assay specificities, amplification products from 2 to 5 experimental host tissue DNAs representing each treatment regime were sequenced by simultaneous bi-directional cycle-sequencing as previously described (Reece & Stokes 2003). DNAs were extracted from the *P. chesapeaki in vitro* cultures that were re-isolated from tissues of challenged hosts, and PCR amplification products were sequenced to confirm isolate identities.

**Histological assays**. Histological sections of paraffin-embedded experimental tissue samples were cut at 5 to 6 µm for collection and drying onto poly-L-lysine-coated microscope slides. Sections were de-waxed, rehydrated, and stained with Mayer's hematoxylin and eosin (H&E) for histopathological analyses.

## RESULTS

The PCR primers specific for the genus *Perkinsus* (85-750-ITS) yielded approximate 750 bp amplicons, and primers specific for *P. marinus* (Pmar-ITS) and *P. chesapeaki* (Pches-ITS) amplified products of approximately 509 and 554 bp, respectively, from DNA templates of axenic isolate inocula, and from tissue DNAs of oysters and clams experimentally challenged with the *P. marinus* inoculum or the 2 *P. chesapeaki* inocula. These PCR products were confidently differentiated and identified by size, following electrophoretic separation on standard agarose gels. Template DNA from the commercial phytoplankton concentrate used to feed experimental molluscs was negative by all PCR assays, as were tissue DNAs from experimental molluscs that received no inoculation, or that received the negative-control SASW diluent inoculum (Table 1).

Tissue DNAs from *Perkinsus* sp.-inoculated experimental host molluscs were positive at variable incidences, and only by PCR assays specific for inoculated pathogen species. Based on pathogen-species PCR assay results, all 3 experimental mollusc host species acquired infections by all 3 *Perkinsus* sp. challenge isolates at 33 to 100% incidences (Table 1). The highest experimental infection incidences (83 to 100%) consistently occurred in experimental hosts

Table 1. *Crassostrea virginica*, *Mya arenaria* and *Macoma balthica*. Proportions (%) of experimental mollusc host treatment groups in which species-specific rDNA internal transcribed spacer (ITS)-region PCR assays detected experimental parasite species' DNA at host death or treatment termination. SASW: sterile artificial seawater; Pmar-ITS: *Perkinsus marinus*-specific assay; Pches-ITS: *P. chesapeaki*-specific assay

| Treatment inocula | % infected (n) | | | | | |
|---|---|---|---|---|---|---|
| Host: | *C. virginica* | | *M. arenaria* | | *M. balthica* | |
| PCR assay: | Pmar-ITS | Pches-ITS | Pmar-ITS | Pches-ITS | Pmar-ITS | Pches-ITS |
| Uninoculated | 0 (0/20) | 0 (0/20) | 0 (0/20) | 0 (0/20) | 0 (0/20) | 0 (0/20) |
| SASW | 0 (0/20) | 0 (0/20) | 0 (0/20) | 0 (0/20) | 0 (0/20) | 0 (0/20) |
| *P. marinus* ATCC 50439 | 45 (9/20) | 0 (0/20) | 30 (6/20) | 0 (0/20) | 83 (15/20) | 0 (0/18) |
| *P. chesapeaki* ATCC PRA-65 | 0 (0/20) | 100 (20/20) | 0 (0/18) | 33 (6/18) | 0 (0/20) | 70 (14/20) |
| *P. chesapeaki* ATCC 50807 | 0 (0/20) | 90 (18/20) | 0 (0/16) | 38 (6/16) | 0 (0/20) | 45 (9/20) |

that were not the original, cognate source-hosts for experimental *Perkinsus* spp. challenge isolates. Thus, 90 and 100% of oyster host treatments were infected when challenged with the 2 *P. chesapeaki* isolates, ATCC 50807 and PRA-65, respectively, that were originally isolated from clams. Likewise, 83% of experimental *Macoma balthica* clams were infected when challenged with the *P. marinus* isolate (ATCC 50439) from an oyster.

Infection incidences estimated by results of PCR assays on DNAs from challenged molluscs were consistent between the genus-*Perkinsus* 85-750-ITS assay and both of the species-specific Pmar-ITS and Pches-ITS PCR assays. In contrast, the Pand-NTS assay (Coss et al. 2001) detected only 17 to 56% of the infections that were detected by either the 85-750-ITS or Pches-ITS assays among experimental hosts challenged with either of the 2 *P. chesapeaki* isolates (Table 2). Sequence analyses of amplification products from the species-specific Pmar-ITS and Pches-ITS PCR assays confirmed the identities of all detected *Perkinsus* spp. to be the same as those of the *in vitro* isolates used to challenge specific host groups.

The same *Perkinsus* sp. isolates that were used to challenge experimental hosts were re-isolated and propagated *in vitro* from tissues of all 3 host species following termination of some, but not all, inoculated treatments (Table 3). Viable, histozoic *Perkinsus* sp. hypnospores enlarged, and were universally detected at 10 to 89% incidences, following RFTM incubations of tissues from *Perkinsus* sp.-inoculated experimental host treatments. *Perkinsus* spp. challenge isolates were re-isolated *in vitro* from tissues of challenged hosts in 5 of 9 inoculated treatment groups (Table 3),

Table 2. *Crassostrea virginica*, *Mya arenaria* and *Macoma balthica*. *Perkinsus* spp. infections in experimental host tissues detected by each of 4 different PCR rDNA assays. ITS: internal transcribed spacer; Pmar-ITS: *Perkinsus marinus*-specific assay; Pches-ITS: *P. chesapeaki*-specific assay; Pand-NTS; *P. chesapeaki* (= *P. andrewsi*)-specific non-transcribed spacer assay; SASW: sterile artificial seawater

| Host species | Inoculum | % infected by PCR assays | | | |
|---|---|---|---|---|---|
| | | *Perkinsus* spp. 85-750-ITS | *P. marinus* Pmar-ITS | *P. chesapeaki* Pches-ITS | *P. chesapeaki* Pand-NTS |
| All tested | Uninoculated | 0 | 0 | 0 | 0 |
| All tested | SASW | 0 | 0 | 0 | 0 |
| *C. virginica* | *P. marinus* ATCC 50439 | 45 | 45 | 0 | 0 |
| *M. arenaria* | | 40 | 30 | 0 | 0 |
| *M. balthica* | | 83 | 83 | 0 | 0 |
| *C. virginic*a | *P. chesapeaki* ATCC PRA-65 | 95 | 0 | 100 | 40 |
| *M. arenaria* | | 22 | 0 | 33 | 6 |
| *M. balthica* | | 65 | 0 | 70 | 15 |
| *C. virginica* | *P. chesapeaki* ATCC 50807 | 90 | 0 | 90 | 50 |
| *M. arenaria* | | 31 | 0 | 38 | 6 |
| *M. balthica* | | 45 | 0 | 45 | 10 |

Table 3. *Crassostrea virginica*, *Mya arenaria* and *Macoma balthica*. *In vitro* re-isolation and propagation of experimental *Perkinsus* sp. challenge isolates from infected experimental mollusc host tissues. RFTM: Ray's fluid thioglycollate medium; ITS: internal transcribed spacer; SASW: sterile artificial seawater; nd: not done; na: not applicable

| Host species | Inoculum | Infected by RFTM assay % (n) | Re-isolation frequency % (n) | Re-isolate rDNA-ITS identities | |
|---|---|---|---|---|---|
| | | | | *P. marinus* % | *P. chesapeaki* % |
| All tested | SASW | 0 (0/10) | nd | na | na |
| *C. virginica* | *P. marinus* ATCC 50439 | 50 (5/10) | 0 (0/6) | na | na |
| *M. arenaria* | | 70 (7/10) | 0 (0/6) | na | na |
| *M. balthica* | | 89 (8/9) | 0 (0/6) | na | na |
| *C. virginic*a | *P. chesapeaki* ATCC PRA-65 | 80 (8/10) | 33 (2/6) | 0 | 100 |
| *M. arenaria* | | 60 (6/10) | 33 (2/6) | 0 | 100 |
| *M. balthica* | | 50 (5/10) | 17 (1/6) | 0 | 100 |
| *C. virginica* | *P. chesapeaki* ATCC 50807 | 40 (4/10) | 83 (5/6) | 0 | 100 |
| *M. arenaria* | | 10 (1/10) | 17 (1/6) | 0 | 100 |
| *M. balthica* | | 40 (4/10) | 0 (0/6) | na | na |

confirming the presence of viable cells of *Perkinsus* sp. challenge isolates in experimental host tissues sampled at 9 to 44 d p.i. Sequence analyses of PCR products amplified from DNAs of all 11 re-isolates confirmed their identities with respective *P. chesapeaki* treatment inocula.

The *Perkinsus marinus* experimental isolate (ATCC 50439) was not re-isolated *in vitro* from any of the challenged host species, due in part to rapid overgrowth of all *P. marinus* re-isolation cultures by vigorous thraustochytrid contaminants. The *P. chesapeaki* neohapantotype isolate from a *Mya arenaria* clam (ATCC PRA-65) was re-isolated from members of each experimentally challenged mollusc species. The *P. chesapeaki* experimental isolate that originated from a *Macoma balthica* clam (ATCC 50807) was frequently re-isolated from tissues of challenged oysters (5 of 6), and from tissues of 1 of 6 *M. arenaria* clams, but was not re-isolated from tissues of any *M. balthica* experimental clams.

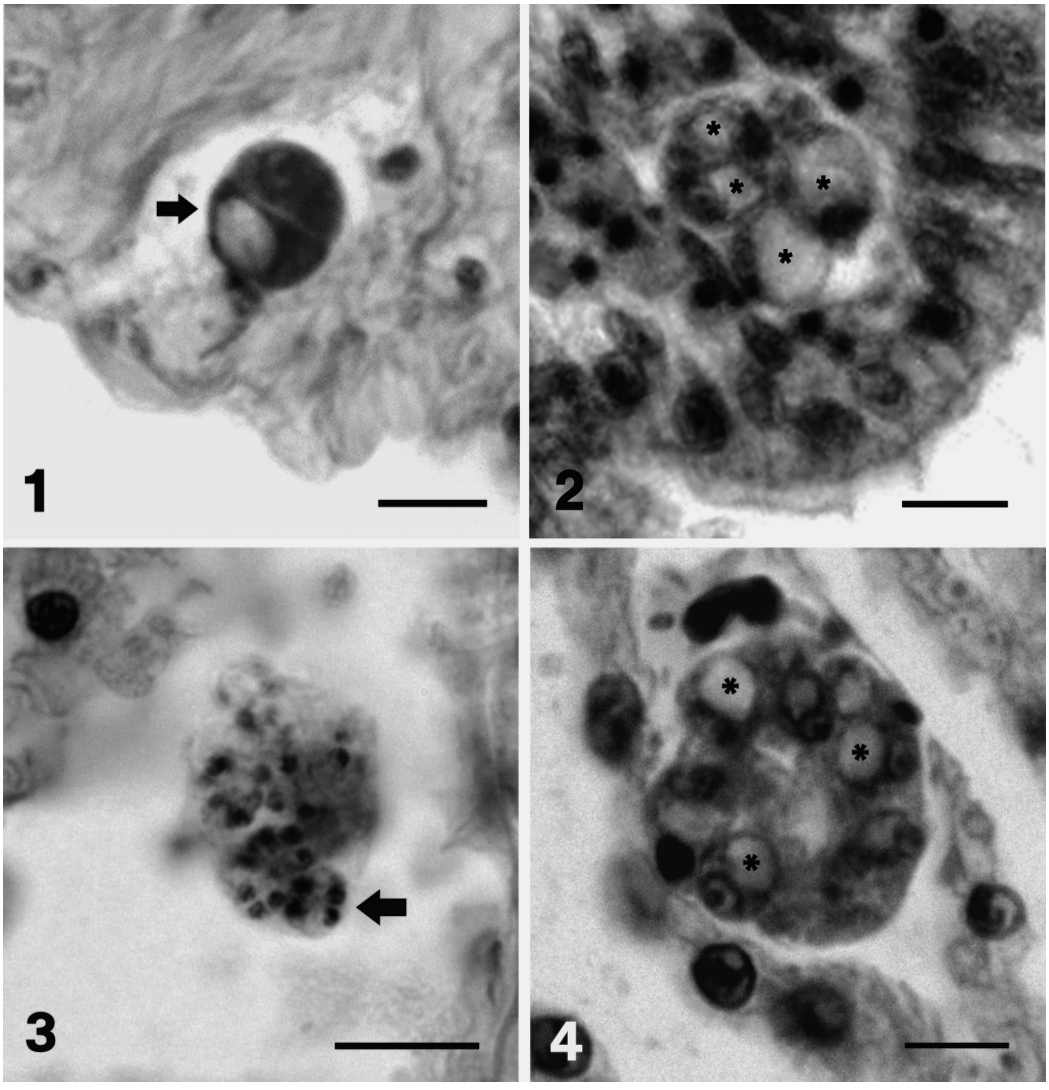
Lesions containing proliferating *Perkinsus* sp. cells were detected at variable frequencies (7 to 55%) in histological sections of available tissues from all challenged, experimental mollusc host groups, providing *in situ* confirmation of active infections in all groups of experimentally challenged hosts (Table 4). Detection frequencies of histological assays fell consistently and often dramatically below those of both RFTM (Table 4) and PCR assays (Table 2) for all experimental treatments. Among experimental host groups challenged with each of the 3 experimental *Perkinsus* sp. isolates, histological lesions were consistently detected at highest frequencies among challenged *Macoma balthica* clams, at lowest frequencies among challenged *Mya arenaria* clams, and at intermediate frequencies among challenged *Crassostrea virginica* oysters. Among hosts challenged with different experimental *Perkinsus* sp. isolates, *P. marinus* lesions were found in both epithelial and connective tissues, while experimental lesions from either of the *P. chesapeaki* challenge isolates were found almost exclusively in host connective tissues (Table 4).

Experimental *Perkinsus marinus* (ATCC 50439) lesions were found in host mantle and intestinal epithelia among challenged *Crassostrea virginica* oysters, and lesions from both *P. chesapeaki* challenge isolates (ATCC PRA-65, ATCC 50807) were found in challenged *C. virginica* mantle (Figs. 1 & 2), gill, and visceral connective tissues, but rarely in oyster digestive epithelia (Table 4). In experimental *Mya arenaria* clam hosts, *P. marinus* lesions were found in gill epithelia, and in connective tissues of mantle (Fig. 3), gill, kidney, and viscera. In experimental *Macoma balthica* clam hosts, *P. marinus* lesions were found in gill epithelial and connective tissues (Fig. 4), mantle epithelial and connective tissues, and in visceral, kidney, and nervous connective tissues. Among both challenged experimental clam hosts, *P. chesapeaki* lesions were found in connective tissues of gills, mantle, viscera, gonad, kidney, and adductor muscle, as previously documented (Burreson et al. 2005).

Cumulative mortalities among inoculated experimental hosts varied with general consistency between challenged host species, and varied with less consistency between host groups challenged with different *Perkinsus* sp. isolates (Fig. 5). Among challenged host species, *Macoma balthica* clams generally showed the highest mortality rates, and *Perkinsus* sp.-inoculated *M. balthica* treatments were all terminated upon reaching their 50% mortality threshold at 15 to 17 d p.i. In contrast, the SASW placebo-inoculated *M. balthica* treatment endured to termination at 26 d p.i. *Cras-*

Table 4. *Crassostrea virginica*, *Mya arenaria* and *Macoma balthica*. Frequency and tissue locations of *Perkinsus* sp. lesions detected by histological assays of experimental mollusc hosts. RFTM: Ray's fluid thioglycollate medium

| Host species | Inoculum | Positive RFTM assay frequencies % (n) | Histological lesion frequencies % (n) | Histological lesion sites |
|---|---|---|---|---|
| *C. virginica* | *P. marinus* ATCC 50439 | 50 (5/10) | 15 (3/20) | Mantle and intestine epithelia |
| *M. arenaria* | | 70 (7/10) | 7 (1/15) | Gill epithelium |
| *M. balthica* | | 89 (8/9) | 55 (6/11) | Mantle, gill, visceral, kidney, nerve connective tissues; gill epithelium |
| *C. virginic*a | *P. chesapeaki* ATCC PRA-65 | 80 (8/10) | 11 (2/18) | Mantle, visceral, vascular connective tissues |
| *M. arenaria* | | 60 (6/10) | 6 (1/16) | Gill connective tissue |
| *M. balthica* | | 50 (5/10) | 50 (6/12) | Mantle, gill, visceral, kidney connective tissues |
| *C. virginica* | *P. chesapeaki* ATCC 50807 | 40 (4/10) | 18 (3/17) | Mantle connective tissue; intestine epithelium |
| *M. arenaria* | | 10 (1/10) | 8 (1/12) | Gill connective tissue |
| *M. balthica* | | 40 (4/10) | 40 (6/15) | Mantle, gonad, adductor muscle connective tissues |




Figs. 1 to 4. *Crassostrea virginica*, *Mya arenaria* and *Macoma balthica*. Histological lesions in experimental oysters and clams challenged with 3 *Perkinsus* sp. *in vitro* isolates of 2 species. All scale bars = 10 µm. Fig. 1. *C. virginica* pallial mantle epithelium lesion containing a pair of dividing *P. chesapeaki* (ATCC PRA-65) trophozoites (arrow) that show an eccentric vacuole (lower) and a nucleus with prominent nucleolus (upper). Fig. 2. Pallial mantle epithelium lesion in *C. virginica* containing a group of proliferating *P. chesapeaki* (ATCC 50807) cells (*). Fig. 3. *M. arenaria* mantle vasculature containing a large aggregate of small, vacuolated *P. marinus* cells, including a dividing 4-cell schizont (arrow). Fig. 4. *M. balthica* gill epithelium lesion containinga colony of *P. marinus* trophozoites (*)

*sostrea virginica* oysters generally experienced the lowest mortality rates among pathogen-challenged experimental hosts, with *Perkinsus* sp.-inoculated treatments terminated at 11 to 59 d p.i. *Mya arenaria* clams showed generally intermediate mortality rates, with *Perkinsus* sp.-inoculated treatments terminated at 9 to 44 d p.i. The *C. virginica* treatment group challenge with its cognate *P. marinus* pathogen isolate (ATCC 50439) showed the lowest overall mortality rate among oyster treatment groups, while among both clam species, the lowest mortality rates were found among SASW placebo-inoculated treatments.

## DISCUSSION

The results reported here demonstrate that each of the 3 species of experimental mollusc hosts that we challenged with mantle cavity inoculations of cultured pathogen cells were infected by both *Perkinsus marinus* and *P. chesapeaki*. Conspecifics of each Chesapeake Bay mollusc host from which the tested *Perkinsus* spp. isolates originated became infected, as did 2 other mollusc species that are sympatric with the origin-host in many Chesapeake Bay habitats. These experimental results are consistent with the broad host

specificities inferred from results of PCR assays for *P. marinus* and *P. chesapeaki* that have previously been reported from some of the same species of wild Chesapeake Bay clams and oyster (Kotob et al. 1999, Coss et al. 2001). These previous PCR results and our current experimental results both remain inconsistent, however, with the narrow host specificities inferred by the extensive and exclusive historical propagation only of *P. marinus in vitro* isolates from Chesapeake Bay oysters, and of the nearly exclusive propagation of *P. chesapeaki* isolates from diverse Chesapeake Bay clam species (La Peyre et al. 2006).

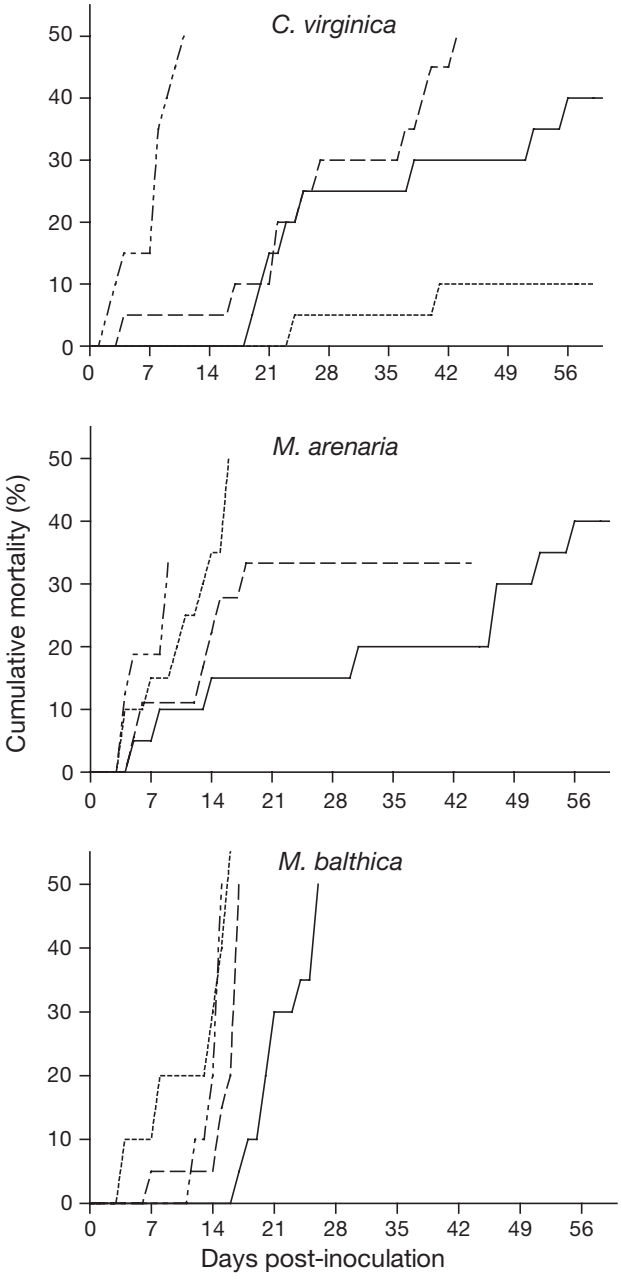



Fig. 5. *Crassostrea virginica*, *Mya arenaria* and *Macoma balthica*. Cumulative mortalities among 3 experimental mollusc host species challenged with different *Perkinsus* sp. isolates, or with sterile artificial seawater (SASW) diluent. Inoculum treatment series include SASW placebo (control) (——), *P. marinus* ATCC 50439 (·····), *P. chesapeaki* ATCC PRA-65 (– – –), and *P. chesapeaki* ATCC 50807 (– · – · –)

Our principal methods for detecting *Perkinsus* sp. infections in experimental mollusc tissues were species-specific PCR assays. However, such assays can also detect non-infecting parasite cells, and DNAs that may persist in experimental systems. Potential confounding PCR template sources include residual cell-free DNAs from experimental inocula, or DNAs from both live and dead, but non-infecting, parasite cells that may passively associate with experimental host tissues. The persistence of only inoculum-species-specific PCR results from assays on DNAs extracted from experimental host tissues collected throughout the duration of our experiment confirm effective prevention of experimental treatment cross-contaminations and suggest that these results reflect active *Perkinsus* sp. infections in experimental host tissues.

Despite a typically low probability for detection of *in situ* lesions in random histological sections from recently infected experimental hosts harboring potential light infections, we consistently documented lesions containing proliferating *Perkinsus* sp. cells in at least some histological sections of experimental host tissues from every treatment group that we challenged with *Perkinsus* sp. isolate cells. Those histological results confirm active infections by all of our experimental *Perkinsus* sp. isolates, and provide compelling evidence of subsequent pathogen proliferation in infected host tissues. Both PCR and histological results were further substantiated by positive RFTM assays of experimental host tissues, and by our consistent, although not universal, re-isolation and propagation of *Perkinsus* sp. inoculum cultures from such tissues.

Due to uncertainty on the number of *Perkinsus* spp. extant in Chesapeake Bay at the time of our experiment, parasite isolates originally described as *P. chesapeaki* and *P. andrewsi* were both used in our challenge experiments. These 2 isolates are now known to be synonymous representatives of *P. chesapeaki* (Burreson et al. 2005), and our similar results following experimental challenges of *Mya arenaria* and *Macoma balthica* clams with both *P. chesapeaki* isolates are consistent with the strong genetic and morphological evidence that those isolates are members of the same parasite species.

The generally high mortality rates among our experimental clam host species may reflect negative consequences of our sediment-free experimental system for host clams that normally live buried, supported, and compressed in benthic or intertidal substrates. Such

negative effects were probably compounded for deposit-feeding *Macoma balthica* host clams, by our deposit-free experimental design. However, *Perkinsus* sp.-inoculated treatments for both clam hosts consistently showed higher mortality rates than placebo-inoculated controls, reflecting probable differential pathogenic effects from experimental infections. Since hosts of different sizes were inoculated with the same total dose of *Perkinsus* sp. isolate cells, smaller *M. balthica* clams may have received higher effective challenge doses, if infectivity is modulated by the density of infectious pathogen cells impinging on smaller tissue surface areas available for colonization in smaller hosts.

Similar differential mortality trends among most *Perkinsus* sp.-challenged oyster treatments were apparently contradicted by the lowest overall mortality rate that occurred among oysters challenged with the *P. marinus* isolate of oyster origin. Whether that low mortality rate reflects relative specific resistance to *P. marinus* pathology by oysters in general, or by the specific oysters used in our experiment, is speculative. Arguing for random coincidence as the basis for that low experimental mortality is the fact that our experimental *Crassostrea virginica* hosts were propagated from a brood line that has not been exposed to *P. marinus* for at least 90 yr and has not been actively selected for dermo disease resistance during that time. In all cases, however, the highest mortality rates in our experimental treatments consistently occurred in naïve mollusc host groups challenged with a *Perkinsus* sp. isolate from a different host species.

Under the controlled conditions of our experimental design, all 3 challenged hosts were susceptible to infections by all 3 *Perkinsus* sp. isolates used to challenge them. Whether, and to what extent, *P. marinus* and *P. chesapeaki* naturally cross- or co-infect the same sympatric, wild mollusc hosts in Chesapeake Bay waters is the subject of a separate current investigation to specifically evaluate those possibilities.

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