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Perkinsus chesapeaki in stout razor clams *Tagelus plebeius* from Delaware Bay

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ABSTRACT: *Perkinsus chesapeaki* is reported from stout razor clams *Tagelus plebeius* in Delaware Bay, extending the known range of *P. chesapeaki* north of Chesapeake Bay. *P. marinus*, which causes dermo disease, is prevalent in cultured and wild oysters at this site, but was not detected in *T. plebeius.* Evidence for the presence of disseminated neoplasia, also reported from Chesapeake Bay, was equivocal. Although *P. chesapeaki* infections were associated with mortality events, light infection intensities and a general lack of histopathological evidence of disease limit inferences about a causal relationship. A comparison of Ray's fluid thioglycollate medium (RFTM)-based and PCR-based detection assays highlight differences in detection capabilities related to the quantity and type of tissue processed rather than assay sensitivity per se, a point that should be considered when surveying populations for disease prevalence. Investigators are further cautioned to use care when applying and interpreting diagnostic assays when used with novel species.

KEY WORDS: Perkinsosis · Range extension · *Tagelus plebeius* · *Perkinsus chesapeaki* · Stout razor clam

INTRODUCTION

The stout razor clam *Tagelus plebeius* is a deep burrowing bivalve common to the western Atlantic Ocean from Cape Cod to the Argentinean Patagonia, including the Gulf of Mexico (Gosner 1979, Vazquez et al. 2006). It is a filter-feeder that occupies sand-mud habitats in both intertidal and shallow subtidal areas (Holland & Dean 1977a). In some areas it is commercially harvested; for example, a small commercial fishery exists in upper Chesapeake Bay, USA, where it is used as bait for eel and crab pots (Dungan et al. 2002; see also www.marylandgis.net/metadataexplorer, keyword 'Tagelus'). A *T. plebeius* fishery does not exist in Delaware Bay, USA, but the species is abundant on the Cape Shore sand flats along the eastern shore of the lower bay. Cape Shore is used for oyster culture, and dermo disease (caused by *Perkinsus marinus*) is prevalent in both cultured and wild oysters at this site. Despite anecdotal reports of *T. plebeius* mortalities during spring and/or fall, little effort has been put forth to investigate the cause of the clam mortalities, due to the lack of commercial interest in the species. The reports of *T. plebeius* mortalities along with descriptions of perkinsosis in *T. plebeius* from neighboring Chesapeake Bay (Dungan et al. 2002) motivated us to determine whether perkinsosis was present in *T. plebeius* at this site. Dungan et al. (2007) found that *P. chesapeaki* and *P. marinus* readily cross-infect *Crassostrea virginica*, *Mya arenaria* and *Macoma balthica* in the laboratory, leading us to look for both species in *T. plebeius*. Here we report various efforts to determine the cause of these mortality events in Delaware Bay.

MATERIALS AND METHODS

Samples were collected from the intertidal zone during low tide at the Cape Shore flats (39° 04′ 25″ N, 74° 54′ 45″ W). This site consists of an extensive tidal flat that extends 500 to 1000 m from the shore depending upon the amplitude of the tidal excursion. A more complete site description is presented by Hidu (1978).

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Between August 2004 and November 2005, clams were collected opportunistically in response to reports of mortality and examined for evidence of potential pathogenic organisms (Table 1). The protocols and assays used varied among dates as described below. In fall 2006, a concerted effort was made to systematically collect a sample of live clams before and after a mortality event.

To assess the magnitude of the November 2006 mortality event, 1 m^2 quadrats were haphazardly sampled along a 100 m transect to determine the density of dead clams at the surface. Dead clams were easily enumerated as the empty articulated valves from adult clams protruded 1 to several cm above the sediment surface. Then, to determine the density of live clams remaining in burrows beneath the surface, two 100 m^2 plots (each approximately 5 × 20 m) were sampled hydraulically along the mortality zone. Sediments were liquefied with a water pump and live clams collected when they rose to the surface. Based on the mortality transect, one plot was centered in a zone with a high density of dead clams and the other in a zone with a relatively low density of dead clams. No dead or moribund clams were recovered from below the surface. All live clams were measured (shell length and wet meat weight), shucked and examined for any gross abnormalities; evidence of parasites was assessed as described later.

For Ray's fluid thioglycollate medium (RFTM) assays, clam tissues (mantle, gill or whole animal) were placed into culture tubes with RFTM and antibiotics, incubated in the dark, then processed using either tissue squash or body burden methods as described by Bushek et al. (1994). The Mackin Scale (Ray 1954, Mackin 1962) was used to rank infection intensities in tissue squashes from 0 (= no infection detected) to 5 (= heavily infected). Body burdens were enumerated as described by Fisher & Oliver (1996). For histology, a transverse section containing visceral mass, gonad, mantle and gill tissues was dissected as described by Dungan et al. (2002), then processed by means of standard molluscan protocols described by Howard et al. (2004). Histology sections (n = 1 per individual) were examined with standard light microscopy for evidence of parasites or pathological conditions, including disseminated neoplasia (DN disease) as detected in *Tagelus plebeius* from Chesapeake Bay (Dungan et al. 2005). A genus-specific PCR assay (Casas et al. 2002) was performed to identify individuals infected with *Perkinsus* spp. using 50 ng of template DNA extracted from 20 to 25 mg of labial palp tissue (Dungan et al. 2002). DNA extractions were performed using a DNeasy Blood and Tissue Kit (Qiagen) following manufacturer's protocols. DNA quantity (range 146 to 642 ng μl^{-1}) and quality (260/280 ratio > 1.862) was verified using a GeneQuant Pro Classic RNA/DNA Calculator (GE Healthcare Bio-Sciences). Species-specific PCR assays for *P. marinus* (Audemard et al. 2004) and *P. chesapeaki* (Burreson et al. 2005) were subsequently applied regardless of the genus-specific assay results. *P. marinus* and *P. chesapeaki* were selected over other *Perkinsus* species because *P. marinus* is known to infect oysters at the study site, *P. chesapeaki* is known to infect *T. plebeius* in neighboring Chesapeake Bay, and both parasites have been shown to cross-infect oyster and clam hosts in the laboratory (Dungan et al. 2007). All PCR products were discriminated on 2% agarose gels stained with SYBR green (Invitrogen) at 1:10 000 dilution in Tris-Borate-EDTA. All negative PCR assays were repeated to confirm the absence of any *Perkinsus* spp. parasites.

Table 1. *Tagelus plebeius.* Collection dates, sample size and tissues used for assays completed. RFTM: Ray's fluid thioglycollate medium. BB: body burden. Tissue type analyzed: m = mantle, lp = labial palps, w = whole body, cs = transverse cross-section; X = mortality assessment conducted

Date	n	RFTM	BB	Histology	PCR	Mortality assess.
Aug 2004	18		m			
Mar 2005	2		w			
Nov 2005	20	m		cs	lp	
Oct 2006	20		w	cs	lp	
Nov 2006	24		w	cs	lp	X

RESULTS

Cape Shore mortality events were reported in August 2004, March 2005, November 2005 and November 2006. Evidence of mortality consisted of large numbers of empty articulated *Tagelus plebeius* valves protruding through the surface sediments of the intertidal zone. The November 2006 event provides an example of the scale of mortality at this site. Surface counts along a 100 m transect on the second sand bar running parallel to shore revealed up to 26 dead clams m^{-2} with a mean density of 5.5 m^{-2} (± 6.9 SD). The greatest density of dead clams occurred on the south end of the transect. Hydraulic subsurface sampling recovered 4 live clams from a 100 m^2 plot (0.04 live clams m^{-2}) at the southern end of the transect and 20 live clams from a similar 100 m^2 plot (0.2 clams m^{-2}) at the northern end. These data indicate that mortality exceeded 90% at the study site.

Following the August 2004 mortality report, 18 clams, ranging in size from 55 to 83 mm in shell length (mean = 69 mm, SD = 8 mm), were collected for stan-

Table 2. *Tagelus plebeius.* Size data and results for *Perkinsus* assays. RFTM: Ray's fluid thioglycollate medium. nd = no data

Collection date	Shell height (mm) Mean (SD)	Range	*Perkinsus* prevalence (%) RFTM	PCR	Mean body burden (cells g^{-1} wet wt)	*Perkinsus* cells per 20 mg PCR sample[a]
11 Aug 2004 (n = 18)	69 (8)	55–83	83	nd	1430	28.61
9 Mar 2005 (n = 2)	75	74–76	100	nd	1280	25.62
18 Nov 2005 (n = 20)	nd	nd	100	90	5344[b]	106.00
2 Oct 2006 (n = 20)	72 (10)	44–91	95	0	13	0.26
2 Nov 2006 (n = 24)	70 (7)	56–86	33	0	1	0.03

[a]Calculated from mean body burden
[b]Estimated from Choi et al. (1989)

dard RFTM tissue assays. Samples were inadvertently stored at 8°C until February 2005, resulting in extensive tissue degradation. As a result, tissue squashes could not be performed; therefore, the samples were examined using body burden protocols. Fifteen clams (83%) were positive for *Perkinsus* parasites via RFTM methods (Table 2). Mean parasite load was 1430 cells g^{-1} wet tissue weight (SE = 395), assuming tissue samples originally weighed approximately 0.2 g (estimated from subsequent samples). There was no relationship between infection level and shell length (r^2 = 0.0033, p = 0.845). These results indicated the presence of *Perkinsus* spp. parasites. At this time, no samples were collected for PCR analysis.

Only 2 clams were collected in March 2005. They measured 76 and 74 mm with wet tissue weights of 14.6 and 13.6 g, respectively. Body burden assays revealed that both contained *Perkinsus* spp. infections: 1804 parasites g^{-1} wet weight in the larger clam and 758 parasites g^{-1} wet weight in the smaller clam (Table 2).

All subsequent samples were processed for routine histopathology, PCR assays and either RFTM tissue or body burden assays. *Perkinsus* spp. were detected by PCR, RFTM and body burden assays, though not by histology. While no lesions or other indications of disease were observed histologically, some apparent hemocyte abnormalities were present (e.g. generally larger hemocytes with higher nucleus to cytoplasm ratios) that were suggestive of disseminated neoplasia; however, no mitotic structures indicative of cell division were observed.

Following a mortality event in November 2005, empty articulated clam shells were observed protruding from the sediments at densities indicating several dead clams m^{-1}. Twenty live clams were collected with a water pump. Standard RFTM tissue assays of mantle and gill tissues revealed 100% prevalence of *Perkinsus* parasites (Table 2) with mostly very light to light infections on the Mackin Scale (Mackin 1962), although some clams were scored as having light to moderate intensity infections. On the Mackin Scale, the weighted prevalence was 0.9, which is equivalent to a mean parasite load of 5344 cells g^{-1} wet weight of sampled tissues (based on Choi et al. 1989). Genus-specific PCR assays of gill tissues detected *Perkinsus* infections in 18 of 20 samples indicating 90% prevalence. Using species-specific PCR assays, none of these clams were positive for *P. marinus* whereas 15 (75%) were positive for *P. chesapeaki*, including 1 individual that was negative with the genus-specific PCR assay.

In fall 2006, 20 clams were sampled in October prior to any reported mortality, and an additional 24 clams were collected in November following the mortality event assessed above. Clams were similar in size to previous samplings with a range in shell length of 44 to 91 mm (mean = 72 mm, SD = 10 mm) in October and 54 to 86 mm (mean = 70 mm, SD = 7 mm) in November. *Perkinsus* prevalence detected by the RFTM body burden assays was 95% for October 2006 and 33% for November 2006. Body burdens were light during both months (Table 2) with October burdens ranging from 2 to 45 cells g^{-1} wet tissue weight and November burdens ranging from 1 to 18 cells g^{-1} wet tissue weight. By comparison, genus-specific and species-specific PCR assays of labial palp tissues did not detect any *Perkinsus* DNA (Table 2). However, PCR template was extracted from <25 mg of tissue; therefore, mean body burden cell densities indicate that PCR tissue samples in October and November 2006 may not have contained any parasites (Table 2).

DISCUSSION

This study reveals that the known range of *Perkinsus chesapeaki* extends north to Delaware Bay and represents the first report of *P. chesapeaki* in *Tagelus plebeius* outside of Chesapeake Bay. Like Reece et al. (2007), we did not find *P. marinus* infecting *T. plebeius* despite the high prevalence of dermo disease in adja-

cent oysters. While *P. chesapeaki* infections were weakly associated with mortality events, other factors may be the cause of reported mortality patterns at this site. Finally, our data highlight subtle but significant differences in detection capabilities between RFTM and PCR assays as well as some precautions that should be taken or at least acknowledged when transferring assay protocols among species.

Despite the broad distribution of *Tagelus plebeius* along the western Atlantic Coast, few studies have examined the species for parasites and disease. In the only pathology study we are aware of from southern latitudes, Vazquez et al. (2006) found no serious pathogens in *T. plebeius* from Argentinean populations. In northern latitudes, Holland & Dean (1977b) reported mortalities from South Carolina and attributed them to predation and old age. Dungan et al. (2002, 2005) documented perkinsosis and DN disease in *T. plebeius* from upper Chesapeake Bay populations following declines in fishery landings, putatively resulting from extensive mortality events. *Tagelus plebeius* mortalities have been anecdotally reported from lower Delaware Bay for many years. Following a mortality episode in April 1992, *Perkinsus*-like parasites were noted in fresh hemolymph smears (S. Ford unpubl. data), but the work was not pursued further. The present study represents the first documented efforts to identify the cause of *T. plebeius* mortalities in Delaware Bay.

Although *Perkinsus chesapeaki* was associated with mortality events, we cannot attribute the mortality to this parasite. *Perkinsus* spp. infection intensities were generally light, especially in 2006, and no lesions were observed histologically. In other molluscan hosts, light *Perkinsus* spp. infection intensities were not associated with mortality (e.g. Figueras et al. 1992, Ragone & Burreson 1993, Casas et al. 2002, Dungan et al. 2007), and this may also be true of *Tagelus plebeius*. Other factors that may be responsible for the routine mortality events observed over the years at this site include freezing during winter when snow and ice accumulate on the flats, shifting sediments, age-dependent mortality (Holland & Dean 1977b) or DN disease (Dungan et al. 2005). While some of the attributes of DN were observed in the present study, others, like mitotic structures, were not. With regard to *T. plebeius* hemocytes, our assumption of normal cytological characteristics is based largely on experience with oysters and other commercial clam species, which may not be relevant. A standard reference for healthy *T. plebeius* histology is needed to resolve this issue.

The effect of prolonged storage on *Perkinsus* parasites detected in the first set of samples is unclear. Parasites could have proliferated during this period, although we are unaware of any reports suggesting this would occur; more probably, parasites died during this period, leading to a smaller number than were initially observed. Regardless, the assay indicated the presence of a *Perkinsus* species, which are known to cause mortalities in other molluscs. This observation led us to continue to pursue *Perkinsus* spp. as a potential pathogen of *T. plebieus* at this site.

The earliest report of *Perkinsus* spp. in *Tagelus plebeius* based on the RFTM assay is from Andrews (1954) in Chesapeake Bay. About 40 yr later, also using RFTM assays, F. O. Perkins (pers. comm.) reported *Perkinsus* spp. in *T. plebeius* from Chesapeake Bay with Mackin rank infection intensities of 0.5 to 1.0. Neither of these reports was associated with mortality events. When Dungan et al. (2002) began investigating *Perkinsus* spp. infections as a potential cause of mortality in Chesapeake Bay, they found 100% prevalence of *Perkinsus* spp. (0.8 and 2.3 weighted prevalence) at 2 sites using RFTM methods, while no more than 46% prevalence was detected by histology. Diagnostic assays using PCR were not performed; however, sequence analysis of *in vitro* isolates derived from several heavily infected individuals identified the *Perkinsus* species as *P. chesapeaki*. In the present study, PCR diagnostic assays can be compared with RFTM diagnostic assays. This comparison indicates that PCR was less likely than RFTM to detect infections when intensities were low (Table 2).

One reason for differences among assay results is the quantity of tissue examined by the different assays (Bushek et al. 1994). The body burden assay examines the entire individual, the tissue squash assay examines about 200 mg of tissue, and the PCR assays typically begin with the extraction of DNA from about 20 mg of tissue, from which only 50 ng is used for the template in the PCR reaction. Therefore, the PCR samples were the least likely to contain any parasite cells when infection intensities were low. To demonstrate this principle, we estimated the mean number of parasite cells per 20 mg PCR sample from the body burden counts (Table 2). During October and November 2006, PCR samples were not likely to contain a single *Perkinsus* parasite. Thus, sampling error is sufficient to explain the differences in detection (i.e. prevalence) between RFTM and PCR assays (Table 2).

Sampling error may also explain differences between genus- and species-specific PCR assays. In the November 2005 PCR assays, 1 sample that was negative with the genus-specific assay was positive with the species-specific assay. In addition, 4 samples that were positive with the genus-specific assay were negative with the species-specific assay; sampling error may explain these differences, although it is also possible that a *Perkinsus* species other than *P. chesapeaki* or *P. marinus* was present.

Another reason for differences among assay results is the type of tissue sampled. The RFTM assay was explicitly developed for the eastern oyster *Crassostrea virginica*, and studies have vetted which oyster tissues should be tested (e.g. Ray 1954, Oliver et al. 1998). Without knowing the pathology of perkinsosis in *Tagelus plebeius*, it is difficult to know which tissue to examine. We used labial palps for PCR assays based on the methods of Dungan et al. (2002), and used the mantle and gill or the whole body for the RFTM assays following methods typically employed for eastern oysters. Labial palps may not contain the heaviest infections in *T. plebeius*. Had PCR been performed on more heavily infected tissues, results may have been more congruent.

In conclusion, we advise investigators to use caution when interpreting results from disease diagnostic assays in novel applications to new species. First, no single assay may be adequate depending on the question being investigated. Second, which tissues should be targeted may change with species. Third, the mere presence of a parasite presumed or known to be pathogenic in one species may or may not be related to any pathological condition in another species. Finally, the established assignment of infection intensities and their interpretation may not translate across different species. In the present study we have identified *Perkinsus chesapeaki* as a parasite present in *Tagelus plebeius* in Delaware Bay. Further research is needed to determine its possible role in mortality of *T. plebeius*, its pathology and affinity for different tissues in *T. plebeius*, its occurrence in other host organisms and its co-occurrence with other *Perkinsus* species.

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