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Detection and quantification of a keystone pathogen in a coastal marine ecosystem

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ABSTRACT: The pathogenic amoeba *Paramoeba invadens* causes recurrent mass mortalities of green sea urchins *Strongylocentrotus droebachiensis* in coastal Nova Scotia, Canada, driving regime shifts from urchin barrens to kelp beds. Outbreaks of the disease (paramoebiasis) are sporadic, and the source population(s) and epizootiology of the amoeba are poorly understood. We developed PCR-based detection of *P. invadens* in urchin tissue, sediment, and seawater. Primers specific to the *P. invadens* nuclear SSU rRNA gene were designed and used in PCR and qPCR analyses to better detect and quantify *P. invadens* during, following, and in the absence of a natural disease outbreak. A comparison of pathogen load in asymptomatic and symptomatic sea urchins indicated a lower threshold of ~1 cell mg^{-1} tissue for observing overt signs of paramoebiasis in urchins. *P. invadens* was detected for the first time in sediment during and following an outbreak of disease in 2014. It also was detected in low abundance (<10 cells l^{-1}) in seawater in fall 2015 in the absence of sea urchin mass mortality or a strong storm event, but not under similar conditions in summer/fall 2016 and 2017. The ability to detect and quantify this pathogen in sea urchins and environmental samples sheds new light on mechanisms of introduction, spread, and persistence of *P. invadens* along the Nova Scotian coast and the role of large-scale meteorological events and ocean warming in these processes.

KEY WORDS: Epizootic · Ecosystem dynamics · *Paramoeba invadens* · *Strongylocentrotus droebachiensis* · Quantitative real-time PCR · Pathogenic amoebae · Sea urchins

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INTRODUCTION

Epizootics play a major role in structuring marine communities (Lafferty et al. 2004, Ward & Lafferty 2004, Feehan & Scheibling 2014), yet despite an increasing frequency of these events in recent decades (Ward & Lafferty 2004, Wilcox & Gubler 2005), the aetiology of disease, host–pathogen interactions, and transmission dynamics in marine ecosystems remain poorly understood (Harvell et al. 2004, Burge et al. 2014). The frequency of marine epizootics is predicted to increase with climate change (Burge et al. 2014), making it increasingly important to understand the dynamics of disease outbreaks in order to manage and conserve ecologically and economically important marine species and ecosystems.

The green sea urchin *Strongylocentrotus droebachiensis* plays a key role in determining the structure of the shallow subtidal ecosystem along the Atlantic coast of Nova Scotia, Canada, where increases in sea urchin density can drive regime shifts from a kelp-bed to an urchin-barrens state (Mann 1977, Scheibling et al. 1999). Since the early 1980s, recurrent outbreaks of the pathogenic amoeba *Paramoeba invadens* have significantly reduced sea urchin populations in Nova Scotia, resulting in reestablishment of the kelp-bed state and collapse of the sea urchin fishery along this coast (Scheibling et al. 2013). *P. invadens* (also known as *Neoparamoeba invadens*; see Nowak & Archibald 2018) was initially reported as a new species (Jones 1985), and the distinction from other *Paramoeba*/*Neoparamoeba* spe-

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cies was recently confirmed by molecular data (Feehan et al. 2013). The pathology of paramoebiasis in *S. droebachiensis* has been well characterized (Jones et al. 1985) and Koch's postulates fulfilled (Jones & Scheibling 1985). A direct relationship between seawater temperature and the growth and progression of *P. invadens* outbreaks in coastal waters is well documented in field and laboratory studies. These outbreaks typically occur between August and October, when surface seawater temperatures are above 10–12°C (the proposed lower thermal threshold for propagation of paramoebiasis) and attain an annual peak around 16–18°C (Scheibling et al. 2013, Feehan et al. 2016). Below 5°C, *P. invadens* ceases to grow in culture, and 2.5°C has been proposed as the lower threshold for survival of the pathogen in shallow coastal waters (Jellett & Scheibling 1988, Buchwald et al. 2015).

These studies suggest that *P. invadens* is typically eliminated from coastal waters during winter, when minimum temperatures usually range from –1 to 2°C (Buchwald et al. 2015, Feehan et al. 2016). The sporadic nature of disease events further supports this hypothesis, as seawater temperatures in Nova Scotia extend above the lower thermal threshold for propagation of paramoebiasis every year, but are not always accompanied by a disease outbreak (Scheibling & Hennigar 1997, Scheibling & Lauzon-Guay 2010). Recurrent outbreaks of the disease have been statistically linked to strong storms and hurricanes, suggesting that *P. invadens* is repeatedly re-introduced to Nova Scotia from distant sources (Scheibling & Lauzon-Guay 2010). The mechanism of introduction remains elusive, although there is indirect evidence that *P. invadens* may be transported by horizontal advection of surface seawater from an offshore source population(s) where temperatures are conducive to year-round survival (Feehan et al. 2016).

To date, the natural occurrence of *P. invadens* has been documented only in its sea urchin host and only during outbreaks of disease, either through histological observation (Jones et al. 1985) or by raising cultures from infected individuals (Jellett & Scheibling 1988, Jellett et al. 1989, Feehan et al. 2013, 2016). Histological observations indicate that *P. invadens* is present in low abundances in tissues of infected sea urchins (Jones et al. 1985), and waterborne transmission experiments (using symptomatic individuals as an upstream source) indicate that the infection rate of healthy downstream individuals depends on the number of infected sea urchins at the source (Scheibling & Stephenson 1984). *P. invadens* has been isolated only from sea urchins showing clinical signs of paramoebiasis (Jellett & Scheibling 1988, Jellett et al. 1989), suggesting that culturing methods may not be sufficiently sensitive to detect amoebae during initial (pre-symptomatic) stages of infection or during recovery of individuals at low temperatures, when the growth rate of *P. invadens* in culture is slow (Jellett & Scheibling 1988). Also, since *P. invadens* is likely transported to Nova Scotia via seawater (Feehan et al. 2016), tracking the presence of *P. invadens* in the environment as well as in sea urchins is critical to understanding the transmission and progression of this disease.

To address these challenges, we developed polymerase chain reaction (PCR) and quantitative realtime PCR (qPCR) protocols to detect and quantify *P. invadens* in sea urchin tissue and environmental samples. Molecular tools such as PCR-based techniques can be used to rapidly and effectively track the distribution and abundance of pathogens in hosts and in the environment. Various PCR-based tools have been employed for other *Paramoeba*/*Neoparamoeba* species, especially *P.*/*N. perurans*, the causative

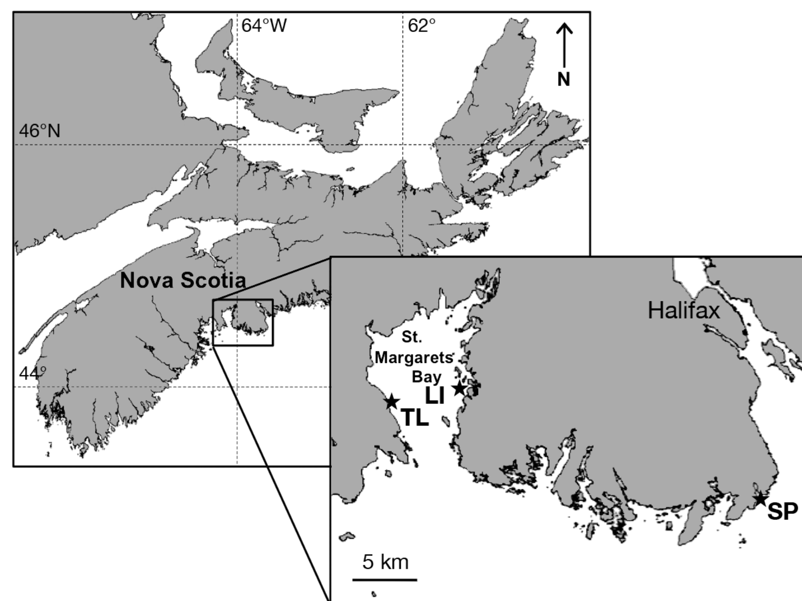

Fig. 1. Study sites along the Atlantic coast of Nova Scotia, Canada, showing The Lodge (TL, with sampling locations at 8 and 18 m depth) and Luke Island (LI) in St. Margarets Bay, and Splitnose Point (SP) along the headland southwest of Halifax Harbour

agent of amoebic gill disease (Young et al. 2007, Bridle et al. 2010, Downes et al. 2017, Nowak & Archibald 2018). For example, qPCR techniques have been used to detect and monitor *P./N. perurans* in gill tissue of farmed Atlantic salmon in Ireland (Downes et al. 2015) and in the water column at salmon farms in Tasmania, Australia (Wright et al. 2015), where they have informed management strategies to prevent the spread of the pathogen.

Our *P. invadens*-specific methods were employed in monitoring the presence or abundance of this species at 3 sites near Halifax, Nova Scotia, during and after an outbreak of paramoebiasis following a strong storm in September 2014, with monitoring continuing up to November 2017 at 1 site. Our study is the first to document the presence of *P. invadens* in the environment (i.e. outside of the sea urchin host) and provides new insights into the distribution and population dynamics of the pathogen on this coast. By facilitating rapid detection of *P. invadens* in *S. droebachiensis*, including tissues from asymptomatic individuals, these methods present new possibilities for effective monitoring and early detection of disease in this ecologically and economically important species. They also provide the capability for broadscale environmental sampling to locate source populations and track the spread of paramoebiasis in coastal waters.

MATERIALS AND METHODS

Field sampling and sample preparation

In 2014, as part of a broader field experiment to examine mechanisms of introduction of *Paramoeba invadens* to the Atlantic coast of Nova Scotia, we monitored the presence of the amoeba in sea urchins (*Strongylocentrotus droebachiensis*) transplanted from a source population (Splitnose Point), where sea urchins had persisted in barrens for more than a decade, to cages in kelp beds at 2 sites at 8 m depth (Luke Island and The Lodge) in St. Margarets Bay, where sea urchins had experienced recurrent mass mortalities due to paramoebiasis (Fig. 1). We also monitored urchins in cages at 18 m depth at 1 of the bay sites (The Lodge) and those in a back-transplant control group at Splitnose Point. Transplantation took place in August 2014, just prior to an increase in seawater temperature above the lower thermal threshold for propagation of paramoebiasis (~10°C). At each location, 20 adult sea urchins (>30 mm test diameter) were added to each of 2 or 4 cages placed 2–5 m apart on the rocky seabed, and fed kelp from the surrounding area (for details, see Feehan et al. 2016). Divers monitored the cages every 2 wk to count surviving sea urchins, remove dead individuals, and collect those showing signs of paramoebiasis (drooping spines, gaping peristome, loss of attachment to the substratum). Water temperature was recorded using a thermograph (StowAway TidbiT Temp Logger, Onset Computer) at 8 m depth at The Lodge.

Sampling of caged sea urchins for *P. invadens* began on 21 October 2014, amid an outbreak of paramoebiasis after the passing of a strong storm on 22 September 2014 (Feehan et al. 2016). At each sampling interval, 4 symptomatic urchins (1 cage^{-1}) were collected. If 4 symptomatic urchins were not available at the time of sampling, asymptomatic urchins were haphazardly selected to make up the difference. Sea urchins were transferred in a cooler to the laboratory where they were dissected within 6 h. Two to 4 radial nerves (10–20 mg total weight) were excised and stored at −80°C until DNA extraction. For confirmation purposes, we also attempted to cultivate *P. invadens* from 1 of the remaining radial nerves of 19 urchins, following the methods described by Jones & Scheibling (1985) and Feehan et al. (2013) (see Supplement 1 at www.int-res.com/articles/suppl/m606p079_supp.pdf). In addition to sea urchins, 2 samples of surface sediment were collected by divers into 50 ml centrifuge tubes at each sampling interval, beginning 7 October 2014. One sediment sample was collected from within a randomly selected cage, the other ~5 m from the cage site. Samples were transferred to the shore where they were processed within 1 h of collection. Approximately 5 g of sediment were placed into 3.5 ml phenol:chloroform:isoamyl alcohol (25:24:1) mixed with reagents from the MoBIO PowerSoil RNA Extraction Kit: 0.2 ml buffer SR1, 0.8 ml buffer SR2, and 2.5 ml bead solution. Sediment samples were stored overnight in buffers at 4°C, and DNA was extracted the next day.

In November 2014, once the seawater temperature dropped below 10°C, the main experiment was terminated. Surviving sea urchins were left to overwinter in cages at Splitnose Point. All sea urchins in cages at Luke Island had died or were sampled prior to this date. At The Lodge, there was little or no morbidity or mortality in cages at either 8 or 18 m depth (Feehan et al. 2016). The cages at Splitnose Point were swept away at some point in the winter of 2014/2015, and only sediment was sampled in the experimental area when sampling was resumed in April 2015.

In June 2015, 4 cages at Luke Island and Splitnose Point were replaced and restocked with wild sea urchins from Splitnose Point (20 cage^{-1}). Monitoring of caged sea urchins and sampling of sea urchins and sediments to test for the presence of *P. invadens* was resumed at 2 wk intervals (as above) until October 2015. At each interval, 4 haphazardly selected sea urchins were collected (1 cage^{-1}) along with 4 sediment samples (1 from within each of 2 randomly selected cages and 2 at ~5 m from the cage site in opposing directions), and processed as described above. In addition, duplicate 4 l seawater samples were collected in clean Ziploc bags from ~2 m above the cage site, and prior to any sediment disturbance, to test for *P. invadens* in the water column. Seawater samples were transported on ice to the laboratory and processed upon arrival: first pre-filtered through 100 µm mesh, then vacuum-filtered onto 2.0 µm Isopore membranes (Merck Millipore) with a filtration time <30 min and/or volume <1.5 l per membrane. All filtering equipment was thoroughly cleaned using a 10% bleach solution and rinsed with autoclaved distilled water prior to filtration. Immediately following filtration, membranes were placed into cryogenic tubes, flash-frozen in liquid nitrogen, and stored at −80°C until DNA extraction.

After October 2015, once seawater temperature had again dropped below 10°C, caged sea urchins were monitored and sampled along with sediment and seawater every 2 to 3 mo until May 2016. The cages at Splitnose Point had once again been swept away by 17 December 2015, concluding sampling at this site. From July to December in 2016 and 2017, caged sea urchins were monitored and seawater samples were collected monthly at Luke Island; 4 sea urchins were collected in October 2016, and September, October, and November 2017, and processed as described above.

DNA extraction

DNA was extracted from radial nerve tissue of *S. droebachiensis* using the DNeasy blood and tissue kit (Qiagen) following the manufacturer's instructions with the following modifications: tissue was thawed (5 min at room temperature), then suspended in 180 µl buffer ATL (Qiagen) and 20 µl Proteinase K (Qiagen), then incubated at 56°C for 1 h on a rocking platform with vortexing every 15 min. Subsequent steps were as per the manufacturer's protocol. DNA was eluted in 100 µl AE buffer (Qiagen) and stored at −20°C.

Sediment DNA was extracted using the MoBIO PowerSoil Total RNA Isolation Kit and DNA Elution Kit (MoBIO) following the manufacturer's protocol. DNA was eluted in 50 µl buffer SR7 and stored at −20°C.

Seawater DNA was extracted from the frozen filter membranes using the DNeasy Blood and Tissue Kit (Qiagen) following the manufacturer's protocol for 'Purification of Total DNA from Animal Tissues', with sample handling modified as follows: membranes were thawed for 5 min, then suspended in 400 µl sterile distilled water (dH_2O), 360 µl buffer ATL (Qiagen), and 40 µl Proteinase K (Qiagen). Reactions were vortexed, then incubated at 56°C for 1 h. Reactions were then re-vortexed, and 400 µl buffer AL (Qiagen) and 400 µl ethanol were added. Subsequent steps were as per the manufacturer's protocol. Seawater DNA was eluted in 200 µl AE buffer and stored at −20°C.

Detection of *P. invadens* by PCR

A primer set 'Nuc1' consisting of forward primer ParNssu905F (5'-TCA AGG CAA GCA TAA TTT TAA TG-3') and reverse primer ParNssu1094R (5'-CAA GAA TTT CAC CTC TGA CAT C-3') was designed to specifically amplify 190 bp of the nuclear small subunit ribosomal DNA (SSU rDNA) of *P. invadens*. Primer design and testing, and PCR optimization are described in Supplement 1. PCR reactions using DNA extracted from radial nerves, sediment, and seawater as template were conducted in 20 µl volumes containing 2 µl of 10× Reaction Buffer (Invitrogen); 0.2 mM each dNTP; 1.5 mM (radial nerve) or 2.0 mM (sediment and seawater) $MgCl_2$; 0.5 µM of each primer, 1 µl template DNA at 1/2 (radial nerve, sediment) or 1/1 (most seawater samples) dilution of the stored DNA solution, and 1 U *Taq* DNA polymerase (Invitrogen). Additionally, 1.2 µg of bovine serum albumin (BSA) was added for seawater DNA only. Appropriate positive and negative PCR controls were applied throughout. The thermocycling protocol was as follows: 94°C for 2 min, 30 cycles of 94°C for 15 s, 58°C for 30 s, 72°C for 15 s, and then a final step at 72°C for 1 min. PCR products were visualized using gel electrophoresis (1.5% TAE agarose gel; 6 µl PCR product per well). Some seawater samples from 2017 were inhibitory for PCR at standard dilution (assessed from universal SSU rRNA primer controls), and were instead examined at 1/2 dilution (with equivalent down-dilution of these qPCR experiments; see below).

Quantification of *P. invadens* in radial nerve tissue and seawater

To allow more sensitive detection of *P. invadens*, as well as quantification estimates when present, quantitative real-time PCR (qPCR) using the Nuc1 primer set (i.e. primers ParNssu905F and ParNssu 1094R) was performed and analyzed using the StepOnePlus Real-Time PCR System (Applied Biosystems) and SYBR Green chemistry. Reaction optimization for radial nerve and seawater samples is described in Supplement 1; attempts to establish a protocol for sediment DNA samples were unsuccessful. For each experimental plate, separate calibration curves for radial nerve and seawater DNA were generated by adding DNA from cultivated *P. invadens* cells (500, 50, 5, 0.5, 0.05, and 0.005 cells per reaction) to radial nerve and seawater DNA (at 1/2 the original DNA concentration) that had tested negative for *P. invadens* (to act as a sample-relevant 'background'). Calibration curves were conducted using triplicate reactions, and experimental samples were run in duplicate. Appropriate negative controls were also run. Quantitative PCR reactions were carried out in 20 µl volumes containing 10 µl Power SYBR Green PCR Master Mix (Applied Biosystems), 900 nM of each primer, 1.2 µg BSA for radial nerve samples or 2.5 µg BSA for seawater samples, and 1 µl template DNA, with the following thermocycling conditions: 95.0°C for 10 min, followed by 45 cycles of 95.0°C for 15 s and 60.0°C for 1 min. Template DNA was added at 1/2 the original DNA concentration, except for seawater samples from 2017, where 1/4 the original DNA concentration was used (see above). A melt-curve analysis was included to confirm specific amplification; samples or replicates with double peaks or aberrant melting temperatures were excluded from the analysis. The slope, R^2, and efficiency values of calibration curves were −3.41 to −3.31, >0.98, and 96.4 to 100.4%, respectively.

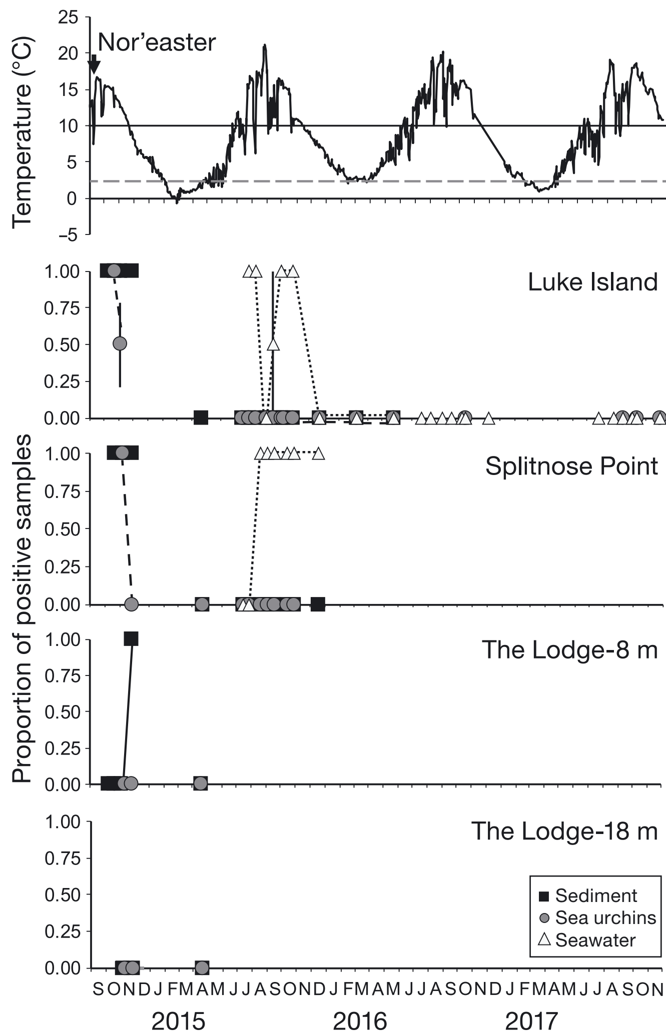

Fig. 2. Top panel: daily average seawater temperature at The Lodge-8 m. Arrow indicates the passing of a strong storm (nor'easter) on 22 September 2014. Solid horizontal line indicates the lower thermal threshold for propagation of paramoebiasis (10°C); dashed horizontal line indicates the lower thermal threshold for survival of *P. invadens* (2.5°C). Lower panels: mean (±SE) proportion of samples in which *Paramoeba invadens* was detected in sea urchins (n = 4), sediment (n = 2), and seawater (n = 2) DNA using PCR at 4 sampling locations (Luke Island, Splitnose Point, The Lodge-8 m, The Lodge-18 m) between October 2014 and November 2017 (except for a single sample on 26 November 2014 at The Lodge-8 m, in which *P. invadens* was detected using cultivation only)

Given the low precision of measurement for radial nerve weight (±10 mg), we used a conservative estimate of 20 mg tissue (the highest measured weight) to calculate pathogen load (i.e. number of *P. invadens* cells mg^{-1} of radial nerve tissue) for qPCR-positive radial nerve samples. A Mann-Whitney-Wilcoxon test was used to compare pathogen load between symptomatic and asymptomatic sea urchins.

RESULTS

Detection of *Paramoeba invadens* in sea urchins, sediments, and seawater using PCR

Following a strong storm (a nor'easter) on 22 September 2014, *P. invadens* was detected in all sea urchin tissue (n = 4) and sediment (n = 2) samples collected from Luke Island and Splitnose Point during a disease outbreak that was observed 2–6 wk post-storm (Fig. 2). As temperatures declined in November to below the lower thermal threshold for propa-

gation of paramoebiasis (10–12°C), a decrease in the proportion of sea urchins infected with *P. invadens* was observed at both sites, although *P. invadens* was still detected in sediment samples. *Paramoeba invadens* was not detected at The Lodge-8 m until the final sampling date at this site (26 November 2014), suggesting a lag in introduction of the pathogen to the western shore of St. Margarets Bay. At this time, *P. invadens* was detected in both sediment samples, but in only 1 (via cultivation only) out of 4 sea urchins sampled (Fig. 2). *P. invadens* was not detected in the sediment and sea urchins sampled from The Lodge-18 m in 2014.

Following an unusually cold winter, with seawater temperatures at 8 m depth dropping below 0°C in February 2015, *P. invadens* was not detected in sediment or sea urchins at any of the 4 sites sampled in April 2015 (Fig. 2). The amoeba also was not detected in sediment or sea urchins at either of the 2 sites (Luke Island and Splitnose Point) that we continued to sample during the summer/fall period of peak seawater temperatures in 2015, or throughout the following year at our single remaining site (Luke Island) when minimum winter temperatures (~2.5°C) and summer/fall maximum temperatures (18–20°C) were relatively warm.

There were no signs of paramoebiasis and no mortality of sea urchins in cages between June 2015 (when cages were restocked) and 28 October 2015 at Splitnose Point (prior to the loss of these cages) and 28 November 2017 at Luke Island (when the experiment was terminated), with the exception of 4 of 15 remaining urchins (1 and 3 individuals from 2 of the 4 cages) that were in an advanced state of putrification on 3 October 2017. This mortality event was associated with unusually warm summer seawater temperatures during the 10 d interval between sampling cages on 24 September and 3 October 2017 (mean ± SD: 18.0 ± 0.5°C; Fig. 2). Otherwise, sea urchins generally appeared healthy and strongly attached to the sides or tops of cages over this 2 yr period at Luke Island.

P. invadens was detected in seawater samples collected from Luke Island and Splitnose Point during the temperature peak between July and October in 2015, when temperatures at 8 m depth generally were above 10°C. The amoeba was also detected in seawater samples collected in December 2015 at Splitnose Point but not at Luke Island, when temperature had dropped to 7.4°C (Fig. 2, Table 1). However, *P. invadens* was not detected in subsequent seawater samples collected at Luke Island between March 2016 and November 2017.

Table 1. PCR (presence/absence) and qPCR (cells l^{-1}) analysis of *Paramoeba invadens* in seawater from 2 sites: Luke Island (LI) and Splitnose Point (SP), during Fall 2015; na: data were not available due to multiple melt curve peaks; nd: no data. Ongoing sampling at LI between March 2016 and November 2017 yielded no detection of *P. invadens* by PCR or qPCR (see Fig. 2)

Date	Location	Sample	PCR	qPCR (cells l^{-1})
13-Jul-15	LI	1	+	na
		2	–	0
	SP	1	–	0
		2	–	0
27-Jul-15	LI	1	+	1.25
		2	+	1.48
	SP	1	–	0
		2	–	0
11-Aug-15	LI	1	+	na
		2	+	1.94
19-Aug-15	SP	1	+	1.67
		2	+	3.12
02-Sep-15	LI	1	–	1.00
		2	–	nd
	SP	1	+	9.74
		2	+	na
16-Sep-15	SP	1	+	na
		2	+	1.77
17-Sep-15	LI	1	+	2.44
		2	–	0
04-Oct-15	LI	1	+	5.77
		2	+	3.07
08-Oct-15	LI	1	+	na
		2	+	na
13-Oct-15	SP	1	+	1.05
		2	+	na
28-Oct-15	LI	1	+	0
		2	+	3.94
	SP	1	+	4.09
		2	+	2.73
17-Dec-15	SP	1	+	0.76
		2	+	0.34
21-Dec-15	LI	1	–	0
		2	–	0

Quantification of *P. invadens* in sea urchins and seawater using qPCR

Pathogen load was low in radial nerve samples from 7 infected sea urchins (i.e. detected as positive for *P. invadens* DNA using PCR and qPCR), collected at Luke Island or Splitnose Point during an outbreak of paramoebiasis in 2014. The mean load was 2.25 cells mg^{-1} tissue (range: 0.20–6.72 cells mg^{-1}), excluding an outlier (94.55 cells mg^{-1} tissue) from Luke Island (Table 2). Once temperatures dropped below the lower thermal threshold for propagation of

Table 2. PCR (presence/absence) and qPCR (cells mg^{-1} tissue) analysis of *Paramoeba invadens* in radial nerve tissue of sea urchins from 3 sites: Luke Island (LI), Splitnose Point (SP), and The Lodge-8 m (TL-8 m), during and after a paramoebiasis outbreak in 2014. 'Outbreak status' indicates whether the sample was taken during or after the outbreak; there was no evidence of disease at TL-8 m, or later dates at LI and SP (July 2015 to November 2017), and all sea urchins sampled during that time were PCR-negative for *P. invadens* (see Fig. 2). Symp.: symptomatic; indicates whether sea urchins exhibited overt symptoms of paramoebiasis upon collection (+) or not (–)

Date	Outbreak status	Location	Sample	Symp.	PCR	qPCR (cells mg^{-1})
21-Oct-14	During	LI	1	–	+	1.66
			2	+	+	1.29
04-Nov-14	During	LI	1	–	–	0.25
			2	–	–	0
			3	–	+	0.2
			4	+	+	94.55
06-Nov-14	During	SP	1	+	+	3.4
			2	+	+	6.72
			3	+	+	1.08
26-Nov-14	After	SP	1	–	–	0.08
			2	–	–	0.05
26-Nov-14	None	TL-8 m	1	–	–	0
			2	–	–	0

the disease (~10°C), the pathogen load measured in radial nerve tissue from 2 infected sea urchins (from Splitnose Point) was 0.05 and 0.08 cells mg^{-1} tissue. This represents a decrease of 2 orders of magnitude (3 orders of magnitude if the outlier is included), although the data are too sparse to conduct a meaningful statistical analysis.

Pathogen load was significantly greater in symptomatic than asymptomatic urchins (Mann-Whitney-Wilcoxon test; $U = 28$, $p = 0.011$) during the disease outbreak. qPCR data are consistent with a threshold of ~1 cell mg^{-1} tissue, above which sea urchins display overt signs of paramoebiasis (Table 2). With the exception of 1 sample, sea urchins with pathogen loads of <1 cell mg^{-1} tissue were scored negative for *P. invadens* using conventional PCR, suggesting that ~1 cell mg^{-1} tissue is also the true detection limit for PCR. (Experiments conducted using radial nerve material spiked with cultured cells yielded a conservative estimate of the detection limit at ~10 cells mg^{-1} tissue; see Supplement 1). Quantitative real-time PCR is the more sensitive method of detection, yielding positive results from as little as an estimated 0.05 cells mg^{-1} radial nerve tissue. This approaches 2 plausible hard lower limits on detection: (1) it implies detection when there are only 1–2 *P. invadens* cells in the entire sample taken from a given sea urchin (assuming the DNA is from intact cells), and (2) it implies amplification when there are only a few copies (<5 on average) of the template DNA in the qPCR reaction. (Our qPCR-derived estimate of the copy number for nuclear SSU rDNA in *P. invadens* is ~475 $cell^{-1}$; see Supplement 1. Our qPCR protocol uses 0.5% of the radial nerve DNA sample per reaction.)

Mean estimated abundance of *P. invadens* in seawater samples pooled from Luke Island and Splitnose Point in 2015 increased from 0.7 cells l^{-1} in July to as high as 3.3–5.3 cells l^{-1} in mid-August and early October, coinciding with peaks in seawater temperature during this period, although there was considerable variability within and between sites and across dates (range: 0–9.74 cells l^{-1}; Fig. 3, Table 1). Mean cell abundance was below ~0.6 cells l^{-1} in December 2015 when seawater temperature was 7.4°C and decreasing. For reference, the abundance of *P. invadens* in seawater collected from

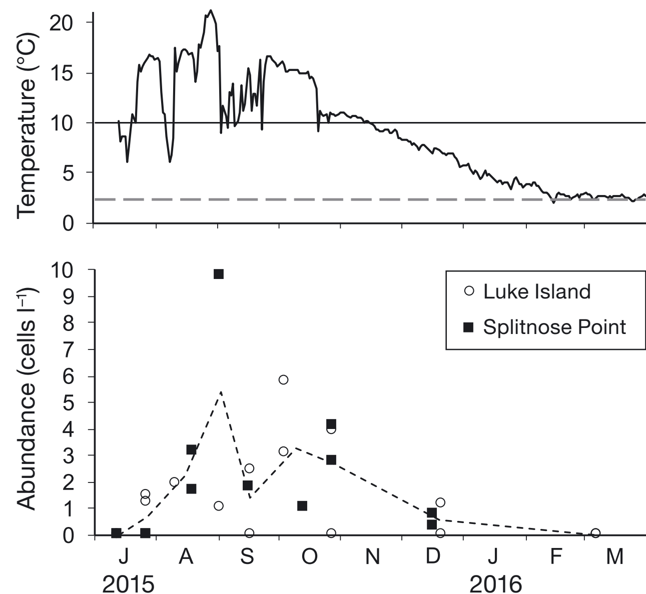

Fig. 3. Top panel: daily average seawater temperature at The Lodge-8 m. Solid horizontal line indicates lower thermal threshold for propagation of paramoebiasis (10°C); dashed horizontal line indicates lower thermal threshold for survival of *P. invadens* (2.5°C). Lower panel: estimated abundance (cells l^{-1}) of *Paramoeba invadens* in seawater from July 2015 to May 2016 at Splitnose Point and to December 2016 at Luke Island. Data points are duplicate seawater samples at a given site and date, except single samples (see Table 1) due to exclusion of a replicate because of methodological problems (multiple melting temperature peaks). Dashed line represents the mean abundance of *P. invadens* (data pooled between sites)

an aquarium containing infected sea urchins and sediment (estimated at 7860.5 cells l^{-1} by qPCR; Buchwald 2016) was 3 orders of magnitude greater than abundances observed in seawater samples collected during the annual peak in seawater temperature (although these may be underestimated by a factor of ~10, based on qPCR estimates of *P. invadens* abundance in seawater samples spiked with a known number of cells prior to DNA extraction; see Supplement 1). *P. invadens* was not detected using qPCR in subsequent seawater samples from Luke Island between March 2016 and November 2017, consistent with the PCR results (Table 2).

DISCUSSION

Detection of *Paramoeba invadens* in sea urchins and sediment

The PCR/qPCR-based methods introduced here enabled rapid and reliable detection of *Paramoeba invadens* in radial nerve tissue of *Strongylocentrotus droebachiensis*, providing the most direct insight yet into temperature-mediated host–pathogen dynamics during and after a disease outbreak following the passage of a strong storm. Our findings are consistent with predictions based on 3 decades of correlative field studies, laboratory experiments, and statistical modelling, which link overt signs of morbidity and mortality in sea urchins to post-storm seawater temperature (Scheibling et al. 2013, Feehan et al. 2016). Following a strong storm in late September 2014, we saw a decline in the proportion of infected sea urchins (and in the pathogen load of infected individuals) at Luke Island and Splitnose Point by late November, when seawater temperature was ~9°C, and had been below 10°C for 12 d. This pattern is consistent with the putative lower thermal threshold of 10°C for propagation of paramoebiasis (Scheibling & Stephenson 1984). The mechanism whereby the abundance of cells of *P. invadens* in sea urchin tissue declines at temperatures below 10°C is unknown. It may involve the temperature-dependent reduction in specific growth rate of the amoeba (Jellett & Scheibling 1988, Buchwald et al. 2015) or the ability of the sea urchin immune system to combat low levels of infection (Jellett et al. 1988).

Importantly, the PCR protocol also provides the first evidence of *P. invadens* in sediment samples from the natural environment during and after a disease outbreak. The presence of *P. invadens* in sediment samples at Luke Island and Splitnose Point, following a decline in the proportion of infected sea urchins and decrease in pathogen load at Splitnose Point as temperature dropped below 10°C in late November 2014 (experimental sea urchins had been eliminated at Luke Island by that time), suggest that *P. invadens* can survive longer in the environment than in sea urchins immediately following a disease outbreak. A lag in the decline of *P. invadens* in sediment relative to sea urchin tissue may reflect active reduction of the amoeba by the urchin's immune system at low temperatures. Alternatively, it is possible that we are detecting residual DNA from dead cells in the sediment, although DNA is rapidly degraded upon cell death (Austin et al. 1997).

The generally low abundance of *P. invadens* in tissues of infected sea urchins inferred by qPCR is consistent with previous histological observations (Jones et al. 1985). A comparison of the abundance of *P. invadens* in radial nerve tissue between asymptomatic and symptomatic sea urchins indicates a threshold of ~1 cell mg^{-1}, above which sea urchins exhibit visible signs of paramoebiasis. This is likely similar to the detection limit using our conventional PCR protocol, constraining its value as a tool for identifying *P. invadens* presence in asymptomatic sea urchins (although it remains more convenient and potentially more sensitive than culturing for positive diagnosis of paramoebiasis). The qPCR protocol was much more sensitive, however, and could detect *P. invadens* at abundances as low as 1 cell in 20 mg of radial nerve tissue. These results indicate the utility of the qPCR-based protocol in particular as a means of detecting low levels of infection in an urchin population before the onset of overt signs of disease, which could have important implications for population monitoring for scientific or management objectives. The maximum abundance of *P. invadens* recorded in *S. droebachiensis*, 95 cells mg^{-1} tissue (observed in a single individual from Luke Island in November 2014), may reflect a rapid increase in pathogen load immediately before a sea urchin succumbs to the disease. Interestingly, abundances of amoebae in 3 other specimens collected simultaneously ranged from 0 to 2.5 cells mg^{-1}, indicating high individual variability in the experimental population at this time.

An apparent lag in the introduction of *P. invadens* to The Lodge-8 m, on the western shore of St. Margarets Bay, is consistent with previous observations that outbreaks of *P. invadens* can be patchy on scales of 10–100s km of coastline (Feehan et al. 2016). Feehan et al. (2016) provided evidence that *P. invadens* may be introduced to this coast via horizontal advection of

offshore surface waters associated with strong storms. Given that circulation in St. Margarets Bay is dominantly anti-clockwise (Heath 1973), we would expect a lag in the introduction of *P. invadens* to The Lodge on the western shore of St. Margarets Bay, compared to Luke Island on the eastern shore of the bay.

P. invadens was not detected in *S. droebachiensis* or sediment sampled between April 2015 and November 2017. The absence of the pathogen in sea urchins following an unusually cold winter in 2015–2016 (when temperatures were <2.5°C for 8.5 wk), and the absence of a strong storm thereafter, conforms to observations in previous years and is consistent with a putative lower thermal threshold around 2.5°C for over-winter survival of the amoeba along the coast of Nova Scotia (Buchwald et al. 2015). It has been proposed that outbreaks of disease after such cool winters require reintroduction of the pathogen by advection associated with strong storms (Scheibling & Hennigar 1997, Scheibling & Lauzon-Guay 2010, Feehan et al. 2016). By contrast, if an outbreak of paramoebiasis is followed by a relatively warm winter, a recurrence of disease may ensue once summer temperatures exceed the 10°C threshold for propagation of the disease (Buchwald et al. 2015, Feehan et al. 2016). Our findings from the PCR-based assay of *P. invadens* in sea urchins and sediments are consistent with this hypothesis.

The death of 4 caged urchins at Luke Island in October 2017 is attributed to a protracted period of unusually warm sea conditions, averaging 18°C over the 10 d interval between samples. Although the upper thermal tolerance limit of *S. droebachiensis* is ~22°C (Scheibling & Hatcher 2013), signs of stress (lowered activity, spine loss, weak attachment) and mortality have been observed in sea urchins when temperatures of 17°C are sustained for periods of days to weeks in the field or laboratory (Scheibling & Anthony 2001, Lauzon-Guay & Scheibling 2007). Reduced feeding and loss of attachment may have increased hypoxic stress as the cages became fouled, a condition that likely was exacerbated by calm sea conditions with little wind-driven mixing during the period of warm sea temperatures.

Interestingly, *P. invadens* was detected in seawater collected from Luke Island and Splitnose Point between mid-July and mid-December 2015, during the summer/fall peak in seawater temperature, at concentrations generally ranging between 1 and 5 cells l^{-1}. The pathogen was not detected in subsequent seawater samples from March 2016 to November 2017, even though temperatures >10°C extended from June to November in each year. The temporal pattern of occurrence of *P. invadens* in the water column in 2015 is consistent with previous studies that have measured temperature-dependent growth of the amoeba in culture (Buchwald et al. 2015), suggesting that DNA of *P. invadens* detected in seawater samples was from intact live cells experiencing growth. It is unlikely that *P. invadens* was locally resuspended by water movement since amoebae were not detected in sediment samples, and sea state was relatively calm during this period (see Conclusions below). It is possible that amoebae were resuspended further offshore and advected to the coast, although Feehan et al. (2016) found that orbital velocities at 60 m depth, even during the passage of strong storms, did not exceed the threshold (1–2 m s^{-1}) for resuspension of fine and medium grain-size sediments (particle sizes of 0.1 and 0.4 mm, respectively).

P. invadens in laboratory cultures can form spherical cells with a well-defined cell margin, although it is unclear whether they represent a moribund form or some type of resistant stage or cyst (Buchwald et al. 2015). Encystment is common among freshwater or soil amoebae (Aguilar-Díaz et al. 2011), but may be less so in marine species (Sawyer 1990). True cysts have not been seen to our knowledge in any paramoebid, although a rounded 'pseudocyst' stage that resisted freshwater treatment has been defined in *Paramoeba/Neoparamoeba perurans* (Lima et al. 2017); the spherical cells of *P. invadens* might represent a similar phenomenon. (Pseudo-)cyst formation in *P. invadens* likely would enhance the potential for dispersal and survival in shallow waters or sediments, and may have contributed to the DNA of *P. invadens* detected in our seawater samples in 2015. The alternative hypothesis, that the DNA may have persisted from the disease outbreak in 2014, is unlikely. Without protection from enzymes, DNA is heavily degraded upon death of an organism (Austin et al. 1997). Studies on DNA in seawater suggest residence times ranging from 10 d to 2 mo (Nielsen et al. 2007).

It is important to note that our measures of the abundance of *P. invadens* in seawater may markedly underestimate actual abundance. Results from quantifying seawater samples spiked with a known number of cells of *P. invadens* using qPCR show ~10-fold lower estimates of cell number when compared to the number of cells added to the sample prior to filtration and DNA extraction (Supplement 1). Correcting for this discrepancy would rely on the assumption that *P. invadens* is present in the natural environment in the same form as the added cells in the spiking experiment (i.e. free-living amoebae).

Given that lobose amoebae are predominantly surface-associated (Rogerson & Gwaltney 2000, Rogerson et al. 2003), DNA from *P. invadens* detected in this study may be from cells attached to suspended particulate matter in the water column. Alternatively, *P. invadens* may be present within the body of another planktonic organism, and/or in an alternate life cycle stage such as a cyst. Any of these could plausibly reduce cell damage and loss of DNA during filtration, which in turn would reduce the magnitude of the underestimation.

Conclusions and prospects for future research

During our 3 yr study, *P. invadens* was only detected in sea urchins following a strong nor'easter on 22 September 2014 (Feehan et al. 2016). This storm caused a spike in significant wave height of 5.3 m off Halifax, within the range of wave heights recorded during previous hurricanes along this coast (Feehan et al. 2013). In contrast, daily significant wave height throughout summer/fall (May–September) 2015–2017 rarely exceeded 2 m (maximum 2.7 m, 30 June 2015; www.wunderground.com/marine-weather, Halifax Harbour Buoy ID C44258). Of 12 North Atlantic tropical storms or hurricanes from 2014 to 2017 that entered the area off Nova Scotia used in the logistic regression model linking hurricane activity and occurrence of sea urchin mass mortality (Scheibling & Lauzon-Guay 2010), only 1 (Gonzalo on 19 October 2014) had a high probability of mass mortality (P_m = 82%; Table S4 in Supplement 2 at www.int-res.com/articles/suppl/m606p079_supp.pdf), consistent with that recorded for 12 'candidate storms' (to which mass mortality events are attributed) between 1980 and 2009 (Scheibling & Lauzon-Guay 2010). Because this hurricane passed by the coast after a mass mortality was underway, it is not considered a candidate storm, although it may have played a role in furthering the spread of disease at that time (Feehan et al. 2016). The other 11 storms (between 1 and 4 yr^{-1}) were relatively far offshore or had diminished in intensity (wind speed) when they passed by the coast, or were followed by a 2 wk period of sea temperatures below the model threshold for mass mortality (12.2°C), resulting in estimates of P_m that ranged from 0.001 to 5.1% (Table S4), far below the average (57%) recorded for previous candidate storms (Scheibling & Lauzon-Guay 2010). Our study extends empirical support for this statistical link (the 'killer-storm hypothesis') across 38 consecutive years.

Our study presents the first evidence that *P. invadens* may be naturally present in the environment during the annual temperature peak (at least in seawater) in the absence of a hurricane or strong storm, without infecting sea urchins. This does not refute the hypothesis that a hurricane or strong storm is needed to introduce sufficient quantities of the pathogen from a distant source population(s) to trigger a disease outbreak and mass mortality (Scheibling & Lauzon-Guay 2010, Feehan et al. 2013). The concentrations we measured in the water column in 2015 may have been too low to effectively encounter and infect localized populations of sea urchins (as in our experimental cages). Our inability to detect *P. invadens* in seawater during the summer/fall in both 2016 and 2017 might suggest that, in the absence of strong storms, the presence of the pathogen in shallow coastal waters is ephemeral (or at least it is rarer than 0.5 cell l^{-1}). This sheds new light on the dynamics of this pathogen in nature, and elucidating the form in which *P. invadens* is occasionally present in seawater could be important to our understanding of the occurrence of paramoebiasis in sea urchins. For example, *P. invadens* may inhabit an unknown planktonic host where it does not interact directly with urchin populations. Interestingly, the presence of the amoeba in coastal waters in summer/fall 2015 was followed by a relatively warm winter in 2016, with minimum sea temperatures in February and March remaining at or above 2.5°C (Fig. 2). Under these conditions, *P. invadens* is predicted to survive and grow the following summer, potentially resulting in an outbreak of disease and sea urchin mortality. To date, this 'overwintering hypothesis' has been supported only in years following an outbreak of disease associated with a hurricane in the previous fall (Buchwald et al. 2015, Feehan et al. 2016).

This initial application of PCR and qPCR techniques demonstrates the promise of these techniques as a tool to expand our understanding of the population dynamics of *P. invadens* and transmission of paramoebiasis *in situ*. Importantly, the ability to detect *P. invadens* in seawater and sediments provides an opportunity to expand our sampling range to explore offshore waters and sediments at greater depths in our search for potential source populations that might seed shallow coastal areas. It also may allow examinations of the potential of *P. invadens* to maintain local populations under environmental conditions (e.g. warm winters) predicted by climate change (Buchwald et al. 2015, Feehan et al. 2016). These kinds of expanded studies are needed for a better mechanistic understanding of the outbreak

dynamics of this pathogen and the role of storms and sea temperature in mediating disease events of profound ecological and economical significance.

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