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DEVELOPMENT AND APPLICATION OF AN eDNA METHOD TO DETECT AND QUANTIFY A PATHOGENIC PARASITE IN AQUATIC ECOSYSTEMS

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Abstract

Approaches based on organismal DNA found in the environment (eDNA) have become increasingly utilized for ecological studies and biodiversity inventories as an alternative to traditional field survey methods. Such DNA-based techniques have been largely used to establish the presence of free-living organisms, but have much potential for detecting and quantifying infectious agents in the environment, which are necessary to evaluate disease risk. We developed an eDNA method to examine the distribution and abundance of the trematode *Ribeiroia ondatrae*, a pathogenic parasite known to cause malformations in North American amphibians. In addition to comparing this eDNA approach to classical host necropsy, we examined the detectability of *R. ondatrae* in water samples subject to different degradation conditions (time and temperature). Our test exhibited high specificity and sensitivity to *R. ondatrae*, capable of detecting as little as 14 fg of this parasite's DNA (1/2500th of a single infectious stage) from field water samples. Compared to our results from amphibian host necropsy, quantitative PCR was ~ 90% concordant with respect to *R. ondatrae* detection from 15 field sites and was also a significant predictor of host infection abundance. DNA was still detectable in lab samples after 21 days at 25 °C, indicating that our method is robust to field conditions. By comparing the advantages and disadvantages of eDNA versus traditional survey methods for determining pathogen presence and abundance in the field, we found that the lower costs and effort associated with eDNA approaches provide many advantages. The development of alternative tools is critical for disease ecology as wildlife management and conservation efforts require reliable establishment and monitoring of pathogens.

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Ecological Archives material:

Appendix A (hand-held filter unit details)

Keywords

disease; survey; environment; DNA; parasite; amphibian

Introduction

Traditional survey methods to determine the presence of particular species in the environment are often labor- and time-intensive. They are also susceptible to "false negatives", whereby surveys fail to detect cryptic or rare species that are actually present (Dejean et al. 2011, Schmidt et al. 2013). This has pushed researchers to search for ways to quantify sampling uncertainty, such as occupancy modeling (Schmidt et al. 2013), and to develop detection tools that are less vulnerable to observer error. For the latter, methods that rely on the detection of environmental DNA (eDNA) have been advanced as a complementary approach to identify those species that are inconspicuous or difficult to find within a range of habitats (see Bohmann et al. 2014 for a review). Cellular DNA (e.g. sloughed living cells) and extracellular DNA (after cell death and destruction) are typical sources of total organismal DNA that are often very persistent in the environment (Taberlet et al, 2012). Detection of eDNA could be utilized for many types of ecological studies, including diet analysis, biodiversity inventories, and determining species distributions (Yaccoz 2012). Techniques employing eDNA are also useful for monitoring purposes, such as evaluating the status of endangered and invasive species (e.g. Goldberg et al. 2011, Piaggio et al. 2014). For example, the spread of the invasive Asian carp within North America has been effectively tracked through eDNA methods that are vital for efforts to prevent their colonization of the Great Lakes (Jerde et al. 2011).

Techniques involving eDNA also have enormous potential for monitoring disease risk, as they can facilitate our ability to establish the presence, diversity, and quantity of infectious agents. Given that infectious diseases have been recognized as a significant wildlife conservation issue (Daszak et al. 2000, Thompson et al. 2010), there is a need for effective tools that reliably evaluate the presence and abundance of pathogens in the field. Classical methods employed to detect pathogens are just as resource-intensive, if not more so, than those used for free-living organisms because they typically involve culturing (prokaryotes) or examination of host tissues via necropsy (Audemard et al. 2004, Reinitz et al. 2007). As a result, DNA-based methods have been developed to decrease the time required for in vivo detection (i.e. within hosts) while also increasing precision. For instance, the development of fluorescent probes employing real-time PCR to determine the presence of human schistosome parasites within snail hosts has led to a better understanding of local transmission dynamics and assessment of intervention efforts (Kane et al. 2013). However, this approach still requires host collection and the associated logistical issues identified above, as well as others unique to assessing infectious diseases in the field, such as extensive host sampling. Because macroparasites typically show a highly aggregated distribution in host populations, with most occurring in/on a few heavily infected hosts (Shaw et al. 1998), a large sample size is often required in order to establish their presence with confidence. Such collections become particularly challenging in remote or difficult to access locations or when rare/endangered species are involved (Bohmann et al. 2014). Host examination and

the identification of parasites requires specific expertise as well (Gordon et al. 2011), collectively emphasizing the importance of detecting parasites through eDNA. To date, such methodology has been almost exclusively developed for human parasites (e.g. Worrell et al. 2011, Kao et al. 2013) but holds much promise for wildlife diseases (e.g. Audemard et al. 2004, Bridle et al. 2010), particularly to elucidate parasite ranges and determine the level of host risk.

In the present study, we aimed to develop (and apply) an eDNA method for detecting the presence of the pathogenic trematode *Ribeiroia ondatrae* in wetland habitats. This flatworm parasite has been documented to cause high levels of amphibian mortality and various malformations in North America (Johnson et al. 1999, 2011, Goodman and Johnson 2011a, b), and there is evidence that it has become more common over the past few decades (Johnson et al. 2003). Thus far, most reports of *R. ondatrae* and amphibian malformations are limited to select regions in North America; reports of malformed frogs are noticeably absent or rare in the southern United States and most of Canada (Johnson et al. 2005, Roberts and Dickinson 2012). Importantly, however, it is not clear whether this signifies a genuine absence of *R. ondatrae*, a presence of *R. ondatrae* that does not cause obvious amphibian deformities, or simply insufficient investigation in some geographic areas. Given the highly pathogenic effects of this parasite and its possible role in amphibian population declines (Johnson et al. 1999, 2011), it is crucial to determine its distribution and how its abundance is affected by anthropogenic changes, such as water temperature, nutrient concentrations, and biodiversity (Johnson et al. 2007, Paull and Johnson 2011, Koprivnikar et al. 2012).

Here our goal was to build upon a PCR-based test developed by Reinitz et al. (2007) for *R. ondatrae* detection within snails to create an eDNA method that would not require host collection, thus representing a relatively simple and cost-effective alternative for establishing the presence of this parasite in the field, particularly for a host group (amphibians) that is widely declining. In spite of the recent increase in ecological studies employing eDNA-related methods, standard field protocols have yet to be established and the environmental persistence of DNA is not well-understood (Barnes et al. 2014). eDNA methods may not represent a significant improvement over classic field sampling techniques if they are also prone to false negatives due to spatial or temporal issues, e.g. DNA degradation due to mistimed sampling or particular environmental conditions. We therefore compared the *R. ondatrae* status of 15 field sites using both amphibian host necropsy and eDNA analysis of water samples. Because trematode infectious stages have a relatively short lifespan, typically <24 hours (Combes et al. 1994), we also conducted lab experiments to determine the detectability of *R. ondatrae* DNA through time within water samples maintained at two different temperatures. In addition, we compared the cost and time required to establish parasite presence at field sites through our eDNA and classical methods. By considering the reliability, robustness, and resources associated with both approaches, we aimed to create a reliable field test for the presence of *R. ondatrae* that can be extended to other wildlife parasites.

Methods

Host and parasite collection

The life cycle of *R. ondatrae* is complex and involves multiple hosts. Adult worms are found within the digestive tract of avian definitive hosts and shed eggs that pass with the host's fecal material. In water, the eggs will embryonate and hatch into free-swimming infectious stages (miracidia) that infect suitable gastropod first intermediate hosts (*Planorbella/ Helisoma* spp. snails), undergoing multiple rounds of asexual reproduction to produce another free-swimming infectious stage (cercaria) which seeks out a second intermediate host (amphibians or fish). Within the second intermediate host, the cercaria forms a cyst and the life cycle is completed upon host consumption by an appropriate bird (see Johnson et al. 2004 for a review).

Planorbella spp. snails infected with *R. ondatrae* were collected from ponds near San Jose (California, USA) and St. Catherines (Ontario, Canada). The snails were maintained in the laboratory in separate 20 L aquaria containing dechlorinated tap water, under a 16:8 light-dark cycle, and were fed a diet of boiled spinach. Trematode cercariae were collected by placing individual snails in water-filled Petri dishes overnight (for *R. ondatrae*) or under a lamp for 1 h during the day (for all other trematode species). The cercariae were then identified using previously published descriptions (Schell 1985, Szurocki and Richardson 2009). Trematode cercariae of each species (~300–800 μm total body length) were pooled in groups of 25 in 1.5 ml microfuge tubes, chilled on ice to slow their movement, and we then removed most of the water using a micropipette. DNA was extracted from the pooled cercariae using the DNA Wizard Purification Kit (Promega) according the manufacturer's protocols. The concentration was determined using a Nanodrop spectrophotometer (General Electric).

To compare the *R. ondatrae* detection ability of our eDNA method versus host necropsy, we also collected larval or newly-metamorphosed amphibians at field sites using sweep nets, euthanized them in a 0.2% solution of buffered MS-222 (Sigma-Aldrich), and conducted necropsies. We examined all major organ systems of the hosts, with particular focus on the skin for *R. ondatrae* cysts (see Fig. 1), while identifying and quantifying all macroparasites present. This allowed us to determine *R. ondatrae* presence at each field site (infection present in at least one host), as well as individual infection abundance (number of cysts/host – see Bush et al. 1997 for standard terminology).

PCR sensitivity and specificity to R. ondatrae DNA

A pair of PCR primers, designed by Reinitz et al. (2007), and two pairs of PCR primers designed using Primer3 (Version 0.4.0) software, were tested for their ability to PCR-amplify a 164 to 290 bp fragment of the internal transcribed spacer 2 (ITS-2; GeneBank ID: AY761142.1) sequence of *R. ondatrae's* ribosomal DNA sequence (Table 1). The primers were compared to all sequences within GenBank using BLAST software to assess their specificity to the *R. ondatrae* sequence. End-point PCR reactions were performed using 10-fold serial dilutions of template DNA, ranging from 0.1 pg to 1 ug DNA, with primers at a concentration of 0.8 μM and EconoTaq Plus Green Master Mix (Lucigen). A gradient PCR

method that systemically varied temperature and time was first used to select the optimal annealing temperature, using the following cycling conditions: 94°C for 5 minutes, 40 cycles of 94°C for 30 seconds, a variable annealing temperature ranging from 40°C to 60°C (the optimal temperature was concluded to be 46°C) for 30 seconds, 72°C for 30 seconds, and then 72°C for 5 minutes. After PCR amplification, the PCR products were resolved on 1.5% agarose gels, either stained with ethidium bromide or SYBR Gold (Invitrogen), and visualized with a BioRad Universal Hood II UV transilluminator. To avoid cross-contamination, DNA extractions and handling of samples were performed in a laminar flow cabinet and all equipment and water was UV irradiated for 15– 30 minutes between steps and prior to use. Negative control samples lacked any template DNA. A total of 5 replicate experiments were performed for each DNA dilution.

The species-specificity of the primers was assessed by determining their ability to detect 0.01 to 10 individual *Echinoparyphium* spp. and fasciolid-type cercariae in a single PCR reaction. DNA from these two trematode species was extracted using the DNA Wizard Purification Kit (Promega) and we used the primers, thermal cycling conditions, and DNA visualization methods described above.

PCR detection of degraded R. ondatrae DNA

In addition to testing the ability of the PCR to amplify small amounts of fresh *R. ondatrae* DNA, the efficacy of the PCR following possible DNA degradation in water over time was evaluated. Five live *R. ondatrae* cercariae were placed into each of 16 different 1L glass jars containing 900 ml of dechlorinated tap water and 100ml of water from an aquarium containing only zebrafish (simulating microfauna from a fish-populated habitat). The 16 jars were divided among 4 treatment groups to examine the separate and combined effects of time (10 or 21 days) and temperature (20°C or 25°C). The water from each jar was then filtered and processed as described below for eDNA water samples to determine DNA detectability.

eDNA collection and extraction

An inexpensive (∼ $18.00 USD) hand-held eDNA collection prototype for use with water samples was developed (see Fig. 1 and Appendix for details). Ten units were constructed consisting of a filter body capable of holding 500 mL of water and a removable filter support unit to collect particulates. Water was collected from 21 different wetlands in California and southern Ontario in July 2012 and July 2013 for which *R. ondatrae* infection presence/absence was verified through the collection and necropsy of larval or newly-metamorphosed amphibians from each of the sites. Particulate matter (including any eDNA present) was collected by pushing 500 ml of water through the filtration support unit equipped with 3μm pore size Whatman cellulose nitrate membrane filters (Sigma-Aldrich). While smaller pore sizes capture more eDNA, clogging prevents the filtering of large water volumes (e.g. no more than 250 mL for 0.2 μm pore size) and thus we chose a larger pore size and adjusted the filtration volume to compensate (see Turner et al. 2014). A separate, unused filtration apparatus was used for each of the 10 sites in 2012 to avoid the possibility of cross-contamination and these were thoroughly washed and dried prior to re-use in 2013. Five water subsamples (5 m apart) per site near the shoreline (15–45 cm water depth) were

collected by lowering the filter body just below the surface of the water and filling it to the 500 ml mark. Following attachment of the filter and its support, a bicycle pump was used to push the water through the filter, using pressures less than 40 PSI to prevent filter membrane rupture. Filters were stored in 15 ml disposable plastic tubes containing 10 ml of 70% ethanol.

The eDNA was extracted from the filters using a modified TRIZOL reagent (Invitrogen) protocol. Filters from different sites were processed separately to ensure no cross contamination. The filters from each site were air-dried in Petri dishes until all of the ethanol evaporated. The dried filters were chopped into small pieces with flame-sterilized scissors, and packed into 15 ml tubes, to which 1.5 mL of TRIZOL was added. This mixture was vortexed for 15 sec, followed by incubation for 5 minutes at room temperature to dissolve the DNA from the filters. The TRIZOL solution was then transferred to another 2 ml tube. Chloroform (300 μl) was added to the TRIZOL mixture, vortexed for 15 seconds, and incubated at room temperature for 15 minutes. The tube was then centrifuged at 15,000 xg for 15 minutes at 4°C. The aqueous phase (containing the RNA) was removed using a pipette and discarded. The DNA in the interphase and phenol phase was precipitated by adding 450 μl of 100% ethanol, followed by a gentle vortex and incubation at room temperature for 3 minutes. The DNA was pelleted by centrifuging at 15,000 xg for 5 minutes at room temperature. The DNA pellet was washed twice in 1 ml of 0.1M sodium citrate for 30 minutes on a rocking table. The DNA was then washed briefly with 1 ml of 70% ethanol and pelleted by centrifugation. The pellet was dried using a vacuum centrifuge and re-suspended in 100 μl of nuclease-free water. To enhance re-suspension, the DNA was warmed at 55°C for 10 minutes. The DNA concentrations were determined by spectrophotometry and then stored at −20°C until analyzed by PCR.

Qualitative end-point PCR eDNA detection

R. ondatrae eDNA (1 ul of extracted DNA) derived from filter collections was detected by PCR using the Ro-ITS 3 primer pair, as described above. Each site sub-sample was replicated 3 times for a total of 15 PCR reactions per site (5 replicate sub-samples from each site). PCR products were resolved on 1.5% agarose gels in TBE stained with ethidium bromide. A replicate was considered positive if a band of 164 bp was observed. Field sites were scored as negative for end-point PCR if <3 of 15 replicate water samples produced faintly visible PCR products after 40 cycles of PCR amplification. Sites were considered positive if 4 or more faint PCR products were observed, or one or more intensely-staining PCR products were observed in the electrophoresis gels.

Quantitative PCR eDNA detection

Quantitative PCR (q-PCR) was used to obtain estimates of the number of cercariae collected from each 500 ml sample of pond water. DNA was extracted from five cerariae as described above, and the DNA concentration was determined, to enable the amount of DNA/ceraria to be calculated. Based on three replicates, 2.5 ng of DNA could be extracted from a single cercaria. Serial dilutions of the DNA in water were then used as template for q-PCR, using BioRad's iQ SYBR Green Supermix on a BioRad iQ5 Multicolor Real-Time Detection System as per the manufacturer's protocols. Samples were analyzed in 10μl reactions (5μl

iQ SYBR Green Supermix, 0.5µl of each Ro-ITS 3 primer, 3.5µl sterile water, and 0.5µl site DNA template) using the manufacturer's protocol. Real time data was compiled using iQ (BioRad) software. A melt curve analysis was performed on all samples to confirm that only one amplicon was produced in each reaction. The cycle threshold (C_t) values for each DNA dilution were plotted to generate a standard curve, and linear regression was used estimate the concentrations of *R. ondatrae* DNA, and therefore the number of cercariae in each 500 ml pond sample. Note that relatively high C_t values correspond to a greater number of PCR cycles needed to amplify the DNA, i.e. less was initially present in a sample.

Because the q-PCR method showed greater sensitivity and accuracy compared to standard end-point PCR for our 2012 samples (detection of 5.54×10^{-6} versus 4.0×10^{-4} of a cercaria, and 90% versus 70% accuracy compared to host necropsy, respectively), only q-PCR was used to analyze the 2013 samples. Each DNA sub-sample was analyzed by q-PCR in duplicate to calculate the *R. ondatrae* DNA concentration for each sampling site. The q-PCR assay could consistently detect 14 fg of *R. ondatrae* DNA, whereas amounts below this value produced more variable C_t values. Accordingly, a sample was considered "negative" (i.e. no *R. ondatrae* present) below this DNA threshold, while samples with values above this threshold considered the site to be "positive." A pond was considered positive if any one of the five sub-samples were positive.

Statistical analysis

We used a Linear Mixed Model (LMM) to determine if C_t (q-PCR Cycle Threshold) values differed between sites with *R. ondatrae* present or absent (categorical fixed factor based on examination of amphibians from that sampling year), with site identity and sampling year for each sub-sample as categorical random factors. To meet the assumptions of a normal distribution, the C_t values for each sub-sample were log10-transformed before analysis. We excluded sites for which we were unable to obtain any PCR reactions, or those where we could not examine at least 9 larval/newly metamorphosed amphibians from the collection year, leaving 15 sites for the LMM (Table 2). To assess whether site C_t values predicted individual infection abundance for the amphibians examined, we used a generalized linear mixed model (GLMM). As infection abundance represents count data, we used a Poisson distribution with log link function. In addition to site C_t value as a fixed effect, site identity, sampling year, and host species were included as categorical random effects. All analyses were done with SPSS 21.0.

Cloning and sequencing of R. ondatrae ITS-2 gene

DNA of *R. ondatrae* cercariae was collected from snails originating from our California and southern Ontario sites, extracted from gel bands of approximately 164bp, and then purified using QIAquick Gel Extraction Kit (Qiagen) as per the manufacturer's instructions. The PCR products were ligated into the pJET PCR cloning vector (Thermo Fisher Scientific), were transformed into Sub-cloning Efficiency™ DH5α Chemically Competent *E. coli* cells (Invitrogen) following the manufacturer's protocol. Plasmid DNA was subsequently purified from three bacterial clones derived from both the Californian and Ontario *R. ondatrae* isolates using a Qiagen QIAprep Miniprep kit as per the manufacturer's instructions. The

PCR fragments within the plasmids were then sent to the Robarts Sequencing Facility (London, Canada) for DNA sequencing.

Comparison of eDNA and traditional methods

We determined the time and cost needed to assess the *R. ondatrae* status of a site (presence and abundance) using eDNA and traditional necropsy-based approaches (Table 3). For the eDNA method, we considered the time required to collect water samples, extract the DNA, and perform the q-PCR steps detailed above in order to generate a site *R. ondatrae* DNA concentration via the C_t value. The costs associated with both approaches were determined based on the specified materials, transport from field sites to the lab, and an estimate of labor using the time per site and a standard wage. For the traditional field-sampling approach, we estimated the time associated with collecting 18 larval/newly metamorphosed amphibians (mean sample size of our 15 retained sites), and the subsequent necropsy of each to determine *R. ondatrae* infection (presence and cyst abundance). As the collection and use of vertebrates in research requires both institutional and government permits, we also considered the time associated with this. We ignored time required for driving to and from field sites, as well as that to obtain access permission, as this would presumably be equivalent for both methods.

Results

PCR detection sensitivity and specificity

All three pairs of PCR primers amplified the expected (164–290 bp) sized PCR products from *R. ondatrae*, and DNA sequencing confirmed their identities. No differences in the sequences were observed between the *R. ondatrae* samples isolated from California and southern Ontario. Using the Ro-ITS 3 primer set, we could detect as little as $1/2500^{th}$ of a single cercaria under optimal conditions using molecular grade water and directly extracting DNA without a membrane filter (Figure 2a). The Ro-ITS 3 primer set was also the most stringent as it did not amplify genes from *Echinoparyphium* sp. or a fasciolid-type of cercaria (Figure 2b and 2c). In contrast, primer pairs Ro-IRS 1 and Ro-ITS 2 showed reduced specificity under the PCR conditions used, both detecting 2.4×10^{-2} of a cercaria from these other trematode species. Neither time (10 or 21 days) nor temperature (20°C or 25°C) caused enough degeneration to affect the ability of the PCR to amplify the target sequence, suggesting our method was robust for detecting parasite eDNA at field sites with different conditions. Our BLAST pairwise alignment of the Ro-ITS 3 primer set amplicon (see Appendix 2) resulted in a 100% identity to the *R. ondatrae* ITS-2 gene in Genbank (AY761142.1) and not to any other North American species.

eDNA presence and abundance

The end-point PCR method was determined to be 70% accurate for our 2012 field samples when compared to *R. ondatrae* infections detected via amphibian necropsy. Thus, three of the 2012 sites appeared as “false negatives” (i.e. *R. ondatrae* infection was found in amphibians but not via PCR); however, the end-point PCR method did not show any false positives.

The calibration curve generated for our q-PCR tests indicated the effective range of this method to be from 10 to 5.54×10^{-6} cercariae; below this range, the C_t values were too high and quite variable, making the test inaccurate. To reduce the chances of a false positive, we consequently considered sites as negative if the mean C_t value was 35 or higher (5.54×10^{-6} cercariae or less). We considered C_t scores of <35 to indicate *R. ondatrae* presence and our line of best fit had a R^2 value of 0.81, indicating that C_t values lower than 35 provided a reasonably strong estimation of cercariae abundance in field samples. The q-PCR technique showed 86.6% accuracy (correct for 13/15 field sites in 2012 and 2013) when compared to the host necropsy data (Table 2).

The results of the LMM indicated a significant difference in the mean q-PCR C_t value between field sites characterized as *R. ondatrae* positive or negative based on amphibian necropsy ($F_{1,8}3 = 7.101$, $P = 0.009$). Sites with *R. ondatrae*-infected amphibians had a mean C_t value of 32.51 (± 0.34 S.E.) compared to a mean of 35.04 (± 0.44 S.E.) for those where infected frogs were not found (Figure 3). In addition, the GLMM results showed that mean site C_t value was a significant predictor of individual *R. ondatrae* infection abundance within examined amphibians ($F_{1,266} = 4.721$, $P = 0.031$, coefficient = −0.518; Figure 4). Our calculation of the average cost and time needed to assess the *R. ondatrae* status of each field site in the present study indicates that the necropsy-based approach took almost 5 times as long and cost double compared to using eDNA (Table 3).

Discussion

Our eDNA method for field-detection of the pathogenic amphibian parasite *R. ondatrae* proved to be both sensitive and specific, closely matching results derived from traditional host necropsy, and illustrating the broad potential of this approach for large-scale spatial investigations related to disease ecology. We were able to detect as little as 14 fg of *R. ondatrae* DNA from environmental water samples compared to the detection limit of 100 fg for PCR of snail host tissues described by Reinitz et al. (2007). This eDNA approach also allowed us to confirm the presence of *R ondatrae* in southern Ontario, considerably expanding the known range of this parasite and illustrating how such methodology can be used to investigate pathogen distribution. These results further demonstrate the complexity of evaluating pathogen presence based on restricted criteria. The presence of malformed frogs has often prompted a subsequent examination to determine whether *R. ondatrae* is present; however, amphibian species vary in their propensity to develop malformations following exposure (Johnson et al. 2012). In southern Ontario, only grey tree frogs (*Hyla versicolor*), green frogs (*Lithobates clamitans*), and American bullfrogs (*Lithobates catesbeianus*) have been detected at our field sites, all of which are highly resistant to *R. ondatrae* -induced malformations (Johnson et al. 2012, LaFonte and Johnson 2013). While amphibian deformities have been reported in eastern Canada (Ouellet et al. 1997), the role of *R. ondatrae* has not been specifically examined in relation to these observations, and has only been confirmed as a causative agent in a host species (*Pseudacris regilla*) restricted to western Canada (Roberts and Dickinson 2012).

We had one “false negative” whereby *R. ondatrae* infection was found in hosts from a site but not in the collected from among the 5 site subsamples. This probably reflects the highly

heterogeneous distribution of trematode-infected snails (and therefore of infectious stages) within water bodies (Fernandez and Esch 1991) but can also result from low sample size. The results of a recent eDNA study to detect an amphibian fungal pathogen (*Batrachochytrium dendrobatidis*) indicated that 6 subsamples were sufficient to ensure high accuracy (Schmidt et al. 2013), but the optimal number is likely pathogen-dependent and highly influenced by target organism density (Moyer et al. 2014). Consequently, large volumes of water are required to achieve a high probability of detecting the eDNA of species with low abundance, thereby minimizing false negatives (Moyer et al. 2014). DNA degradation is a less likely explanation for host versus water sample differences given our ability to detect *R. ondatrae* after 21 days in lab water samples kept at 25°C, conditions in which breakdown should be rapid (Dejean et al. 2011, Barnes et al. 2014). Our choice of target DNA probably facilitated detection under such circumstances given its relative abundance compared to other sequences within the genomic DNA. Notably, the ITS-2 sequence is tandemly repeated within the ribosomal DNA (Prokopowich et al. 2003), often in thousands of copies, resulting in a high probability that some of this relatively short target sequence will be intact after prolonged exposure in an aquatic environment (Deagle et al. 2006). Because our eDNA field collections took place in mid-summer when we have previously collected snails with active *R. ondatrae* infections from some sites included in the present study, it is doubtful that no cercariae had emerged for over 21 days, or site water temperatures were so high that DNA breakdown occurred much more rapidly than what we observed at 25°C. Environmental inhibitors of the PCR reaction, such as humic acids and heavy metals, may instead have played a role (Wilson 1997, Matheson et al. 2010). While commercially available kits can deal with such inhibitors to a certain extent, this increases the cost of sample processing and our aim was to develop a test that was as economical as possible in order to allow future large-scale field testing.

We also had a possible “false positive” for one southern Ontario site in which *R. ondatrae* DNA was detected but infected amphibians were not found. If the prevalence of infection was low at that site, the number of frogs examined may have been inadequate to establish parasite presence. There is wide variation in the proportion of amphibians infected with *R. ondatrae* across field sites in North America, ranging from 0–100% (Johnson and McKenzie 2009). Given that our chosen primer set had a high specificity, failing to amplify the DNA of two other trematode species commonly found in our field sites (Koprivnikar and Redfern 2012, Johnson et al. 2013), our results likely indicate true *R. ondatrae* presence rather than that of other parasites; however, further testing with other parasites will allow us to assess this possibility. Rather, host species identity is likely the primary explanation. As discussed above, grey tree frogs were primarily sampled in the southern Ontario wetlands, and are highly resistant to *R. ondatrae* infection (Johnson et al. 2012). Because tadpoles of this species are capable of actively clearing cysts within 72 hours (LaFonte and Johnson 2013), collected hosts would have to represent recent infection events in order to detect *R. ondatrae* through necropsy. The eDNA results may therefore provide a more accurate assessment of parasite presence in this case.

However, the detection of *R. ondatrae* DNA from a site is not necessarily indicative of amphibian infection given the complex life cycle of most trematodes, particularly the

occurrence of multiple life history stages. Avian definitive hosts may deposit trematode eggs at sites lacking suitable gastropod first intermediate hosts. Consequently, the DNA of eggs or hatched miracidia could be collected in water samples and amplified through the same PCR protocol as for the stage infectious to amphibians (cercariae). Environmental RNA (eRNA) represents another approach to determine which stages of the trematode life cycle are present. Due to the relatively rapid degradation of RNA compared to DNA, there is a smaller temporal window of collection, possibly permitting the detection of recently-emerged cercariae rather than eggs originating from transient birds. In addition, the identification of genes up-regulated during specific points of the life cycle may allow detection of relevant parasite stages (Juthikumar et al. 2010).

More importantly, eDNA tests for pathogens requiring multiple hosts can be used not only to assess whether a location is currently functionally colonized (i.e. the entire life cycle is maintained on site), but also the potential risk of host disease under the right circumstances. For instance, a site may be considered low-risk for *R. ondatrae-induced* malformations if infected amphibians are not found, or appropriate intermediate host species not present. However, avian definitive hosts may continually introduce parasite eggs that are detectable via eDNA, indicating future risk if the site is colonized by competent first or second intermediate hosts. In this case, the development of a test with combined primers to simultaneously detect the presence of both *R. ondatrae* and the required first intermediate host (*Planorbella* spp. snails) would be ideal to best evaluate amphibian infection risk. Such multi-faceted tests will be important in order to extend from the current focus on single-species detection (free-living or symbiotic) and allow assessment of community composition (Goldberg et al. 2011, Lodge et al. 2012, Thomsen et al. 2012). Collected eDNA can also be useful for other purposes, such as assessing pathogen diversity through metagenomics approaches (Smith et al. 2012).

Our eDNA-based detection of *R. ondatrae* constitutes an effective replacement for traditional host collection and examination for macroparasite infection. In terms of accuracy, it would appear that the two methods are relatively comparable. We had an almost 90% match between these approaches but classical sampling methods are also prone to methodological issues, especially for rare or cryptic species, and are thus not completely reliable either (Schmidt et al. 2013). If our “false positive” indicates true *R. ondatrae* presence, then this balances the false negative, making the two methods equal. This is comparable to previous studies contrasting eDNA and classic sampling approaches for free-living organisms (e.g., Jerde et al. 2011, Pilliod et al. 2011). Consequently, a site-occupancy approach with repeated sampling should generally be considered for eDNA studies (Schmidt et al. 2013), although it may not be appropriate for detecting the presence of certain wildlife pathogens such as *R. ondatrae* given the brief window for collecting infected hosts (larval and newly-metamorphosed amphibians). We note that while we found our q-PCR approach more sensitive than end-point PCR, these platforms often provide similar non-quantitative results and the latter approach is less expensive and typically more accessible (Nathan et al. 2014).

In view of the comparable accuracy of our eDNA and necropsy approaches, the advantages of the former primarily relate to the ease of obtaining permits and conducting field

collection, particularly time and sample transport, and expense. Eliminating the need to collect and euthanize also obviously spares wild hosts, which can be critical for populations of at-risk and endangered species. Unlike the detection of most free-living organisms, assessing the presence and intensity of pathogens typically requires hosts to be collected in the field and then later examined in a lab setting. Given the high among-host aggregation observed for many parasites (Shaw et al. 1998), an adequate number of individuals must also be gathered. This number will vary for different parasites but will likely exceed the number of environmental subsamples for each site. We gathered 5 water subsamples at each of our field sites and this generally took less than 30 person-minutes with our pump-action collection apparatus. However, the time needed to collect 9–32 amphibians/site was often considerably longer (sometimes double or more). Once collected, there is also the issue of properly preserving/storing host tissues for later necropsy, necessitating the use of chemicals or access to freezers, as well as shipping costs, adequate storage space, and expertise for identification. For remote sites, or when dealing with host species that are endangered, this could prove to be extremely challenging, if not impossible. In comparison, our cellulose nitrate membrane filters were preserved in small amounts of ethanol and appeared to remain stable until processing.

With respect to cost, eDNA-based tests are generally considered more affordable than traditional sampling methods; our materials costs were approximately $12 (USD) per site, which is comparable to costs reported by other studies (Goldberg et al. 2011, Worrell et al. 2011). By considering the total cost and effort to assess the *R. ondatrae* status of a site, we still found an overall advantage to using an eDNA approach. This is primarily driven by the greater labor associated with obtaining permits, sample collection, and processing time for the classical necropsy-based approach (almost five times more), particularly since the q-PCR method allows multiple sites to be run at the same time. As a result, the cost per site using the traditional method was double. While there are obvious advantages to eDNA methods for detecting wildlife pathogens, we caution that necropsies should not be replaced. For instance, our site C_t values were strongly related to host infection abundance but not a perfect predictor (coefficient of −0.518), illustrating the value of retaining classical methods in order to collect certain data. Because macroparasite pathology is often intensity-dependent, as is the case for *R. ondatrae* (Johnson et al. 2012), such information can be critical to understanding the effects of parasites on individual hosts and populations.

Here we present a reliable and resource-minimizing approach to detect the trematode *R. ondatrae* via eDNA that will enable us to better understand the distribution of this highly pathogenic macroparasite and the risk it poses to amphibians, as well as aiding in the investigation of other wildlife diseases. This will facilitate a reduction in the number of hosts collected, an increase in the number of inspected sites, an expansion into areas that are difficult to access, and the inclusion of multiple temporal scales. Given the documented and projected effects of environmental perturbations on wildlife diseases (Daszak et al. 2001, Altizer et al. 2013), it is essential that we develop tools to determine current pathogen distributions and monitor these for changes as such considerations are critical to conservation and management efforts.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Equipment and methods used for eDNA and necropsy-based assessment of *Ribeiroia ondatrae* status of field sites: (a) sweep net collection of larval/metamorphic amphibians, (b) *R. ondatrae* cysts within infected host (indicated by arrows), (c) *R. ondatrae* cercaria - likely the largest eDNA contributor, and (d) hand-held water filtration unit for *R. ondatrae* eDNA collection.

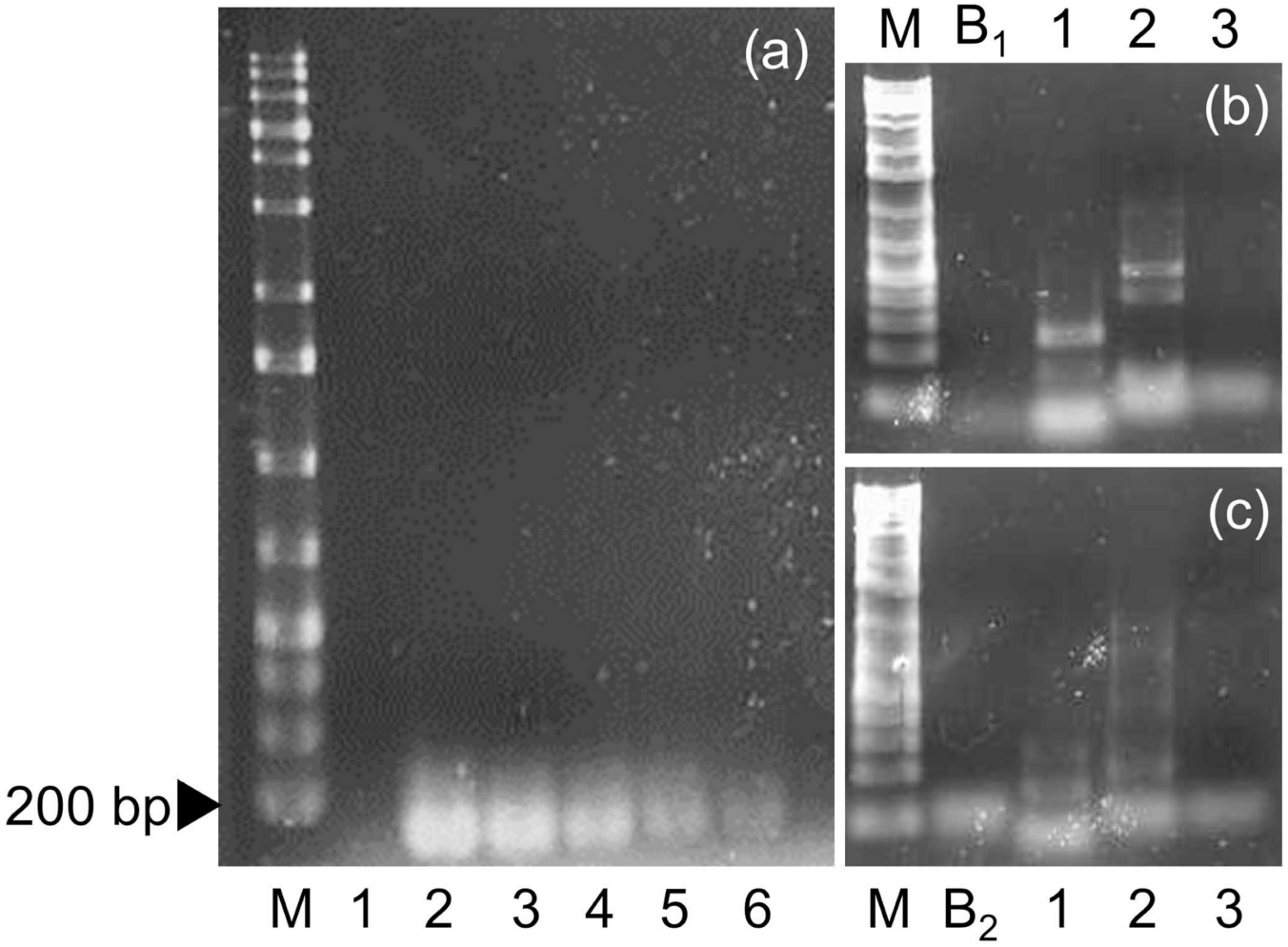

Figure 2.

(a) Sensitivity of the Ro-ITS 3 primer under lab conditions to dilutions of *Ribeiroia ondatrae*. M = ladder and 1 = primer control, with 2, 3, 4, 5, and 6 representing 4.0, 0.4, 0.04, 0.004, and 0.0004 of a cercaria, respectively. Also shown are stringency tests of primer pairs to *Echinoparyphium* sp. (b) and a fasciolid-type cercariae (c). M = ladder, B1 and B_2 = DNA template blanks containing Ro-ITS 1 and Ro-ITS 2, respectively; 1 = Ro-ITS 1, 2 = Ro-ITS 2, and 3 = Ro-ITS 3.

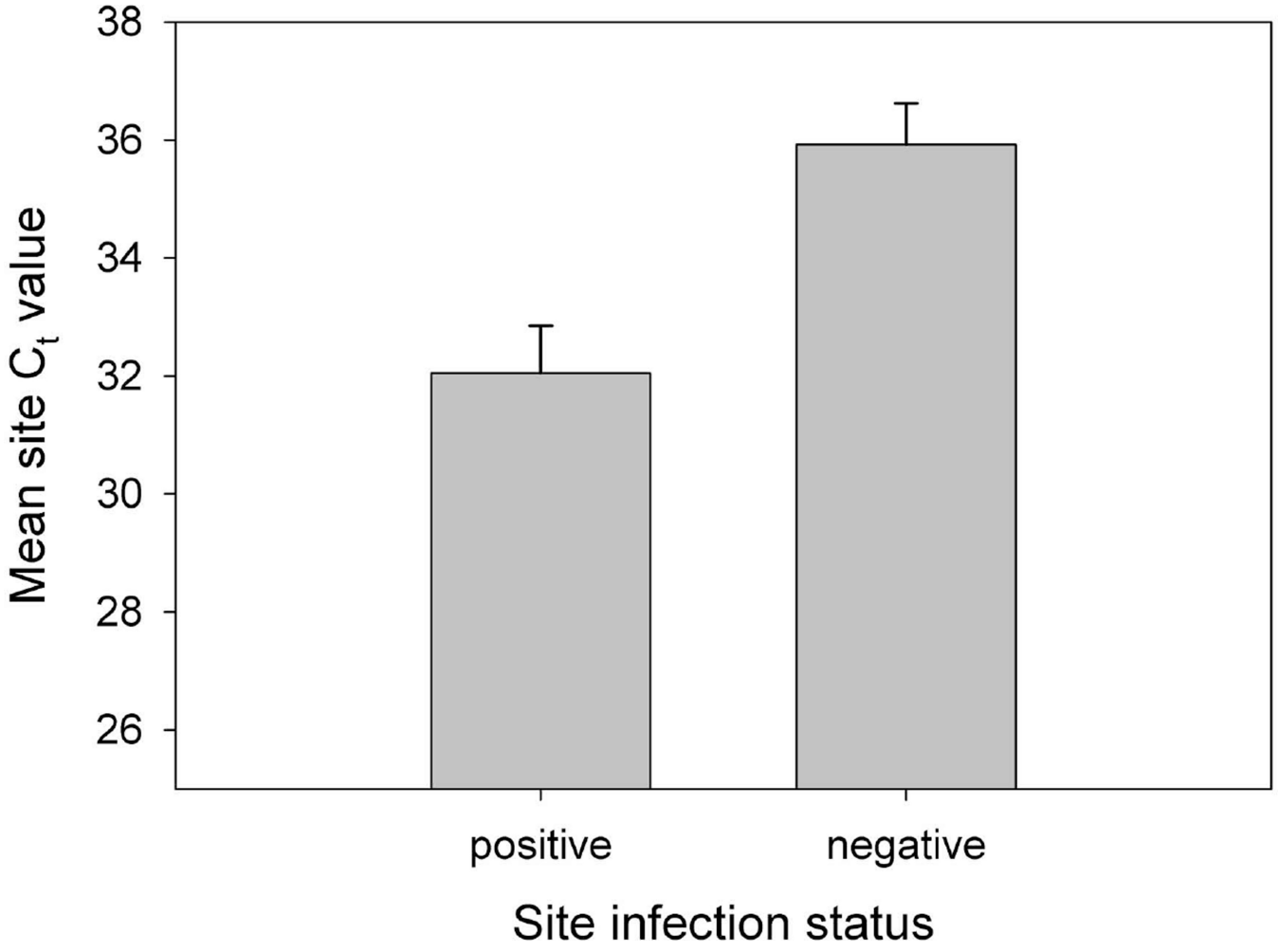

Figure 3.

Mean Cycle Threshold (C_t) value (± S.E.) resulting from quantitative PCR of field-collected *Ribeiroia ondatrae* eDNA in water samples. Note that higher C_t values correspond to a greater number of PCR cycles required to amplify the DNA, i.e. less was initially present in a sample. Site status (negative or positive) reflects the absence or presence of *R. ondatrae*-infected amphibians as determined by necropsy.

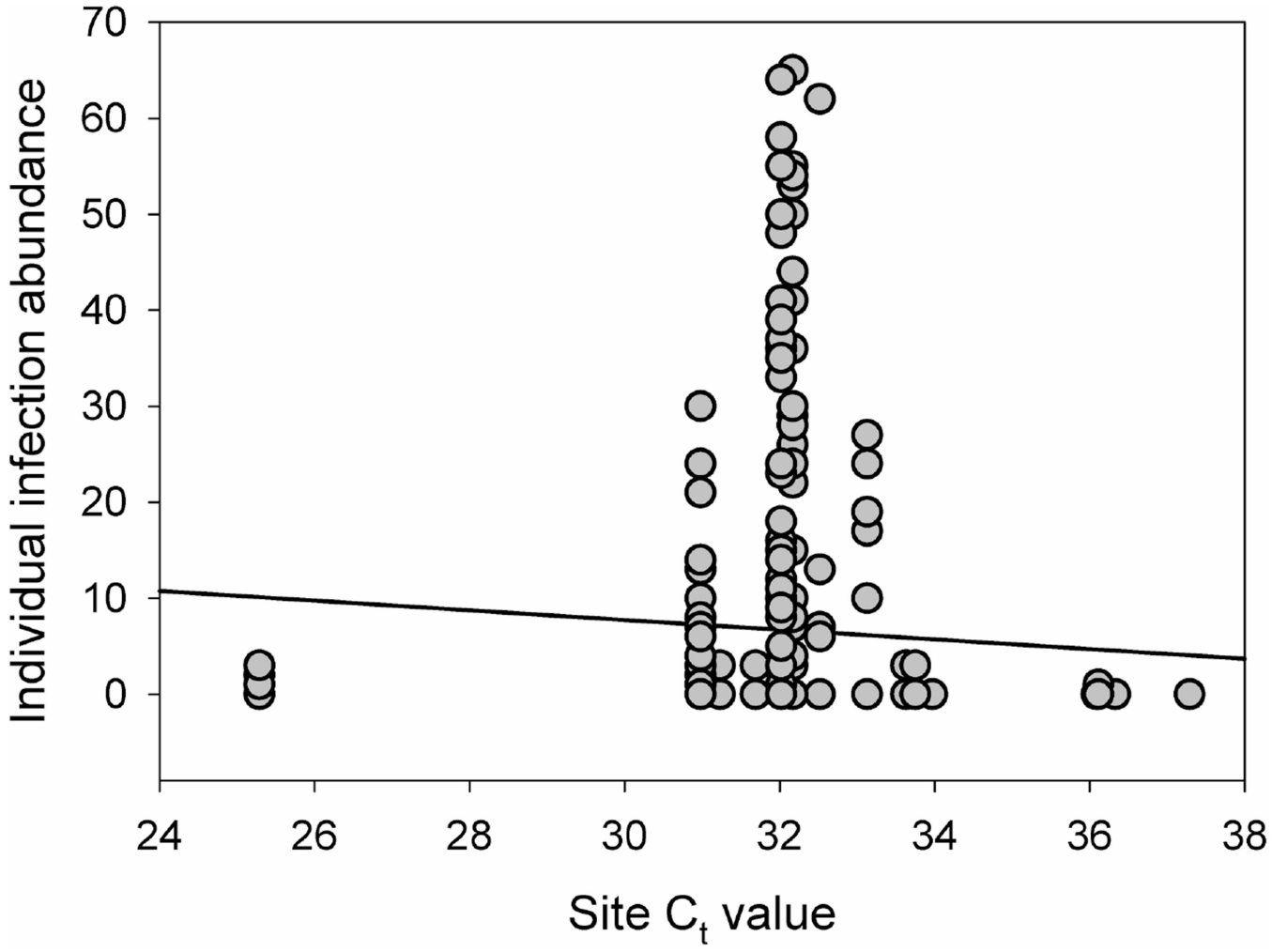

Figure 4.

Relationship between *R ondatrae* infection abundance within individual amphibians and mean site concentration of *R. ondatrae* cercariae as determined through quantitative PCR (C_t = Cycle Threshold value). Note that line of best fit is for illustrative purposes only.

Table 1

eDNA primer information

Primer Pair	Sequence	Product size (bp)
Ro-ITS 1	For: TCACGACGCTCAAATAGTCG Rev: GAGCATAGCTCCACCCGTAG	240
Ro-ITS 2	For: AGTCATGGTGAGGTGCAGTGA Rev: AGACCGCTTAGATAGCAG	290
* Ro-ITS 3	For: CGTGTTTGGCGATTTAGT Rev: TCAAAAATGAAGCAACAGT	164

* Derived using Primer3 software but identical to primers used by Reinitz et al. (2007).

Table 2

Ribeiroia ondatrae presence or absence at field sites (CA = California, ON = Ontario) based on eDNA results from quantitative real-time PCR (q-PCR with Cycle threshold – C_t values) and amphibian necropsies.

Site	# frogs examined	Mean frog infection intensity	Frog necropsy *Ribeiroia* status	Mean C_t value	q-PCR *Ribeiroia* status
1 (CA)	10	22	Present	32.52	Present
2 (CA)	9	0	Absent	37.29	Absent
3 (CA)	24	1.5	Present	25.29	Present
4 (CA)	21	61.3	Present	32.17	Present
5 (CA)	20	0.3	Present	33.63	Present
6 (CA)	32	64.8	Present	32.02	Present
7 (CA)	20	4.1	Present	30.98	Present
8 (ON)	15	**3**	Present	31.69	Present
9 (ON)	15	2.5	Present	31.23	Present
10 (ON)	15	0	Absent	33.97	*Present*
11 (ON)	19	0	Absent	36.09	Absent
12 (ON)	24	0	Absent	36.33	Absent
13 (ON)	20	1	Present	36.11	*Absent*
14 (ON)	10	3	Present	33.75	Present
15 (ON)	10	19.3	Present	33.13	Present

Table 3

Time and materials associated with eDNA (via quantitative PCR) and classical necropsy approaches to determine *Ribeiroia ondatrae* status (presence and quantity) for a single field site. All costs are in U.S. dollars.

	eDNA	Traditional field sampling
Time (h)		
Permits (vertebrate use)	n/a	2
Field sample collection	0.8 (5 water subsamples @ 10 min each)	1 (18 amphibians)
Sample processing[1]	0.5 (DNA extraction, q-PCR, and results interpretation)	3 (18 hosts @ 10 min each necropsy)
TOTAL TIME	**1.3**	**6**
Labor subtotal	$26 (1.33 h @ $20/h)	$120 (6 h @ $20/h)
Transportation[2]		
FedEx economy (by weight)	$37 (0.5 kg from S ON to MB) $76 (0.5 kg from CA to MB)	$37 (1 kg from S ON to MB) $87 (1 kg from CA to MB)
Transportation subtotal	$57 (mean)	$62 (mean)
Consumable materials[3]		
Preservatives	$4 (50 mL ethanol for 5 subsample containers)	$8 (100 mL ethanol for 15 specimens)
DNA extraction & PCR[4]	$12	n/a
TOTAL COST	**$95**	**$190**

[1] Ten sites (5 subsamples each) with standards can be run at once as thermal cycler can hold 96 samples. Per site time calculated by dividing total processing time for 96 samples (5 h) by 10.

[2] Based on field sites and sample processing locations in current study (S ON = southern Ontario, MB = Manitoba, CA = California)

[3] Re-usable equipment excluded (e.g. sweep nets, hand-held filtration units, thermal cycler)

[4] See text for specific quantities and products used