

Novel quantitative PCR assay specific for the emerging *Perkinsea* amphibian pathogen reveals seasonal infection dynamics

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ABSTRACT: Amphibians are suffering from large-scale population declines worldwide, and infectious diseases are a central driving force. Most pathogen-mediated declines are attributed to 2 pathogens, the fungus *Batrachochytrium dendrobatidis* and iridoviruses in the genus *Ranavirus*. However, another emerging pathogen within *Perkinsea* is associated with mass mortality events in anurans throughout the southeastern USA. Molecular resources for detecting amphibian *Perkinsea* have been limited to general protistan primers that amplify a range of organisms, not all of which are disease agents. Moreover, the only quantitative method available involves histopathology, which is labor intensive, requires destructive sampling, and lacks sensitivity. Here, we developed a novel quantitative (q)PCR assay that is sensitive and specific for amphibian *Perkinsea*, providing a resource for rapid and reliable pathogen diagnosis. We used histopathology to confirm that qPCR burdens track the severity of *Perkinsea* infections across multiple anuran tissues. We also sampled 3 natural amphibian communities in Florida, USA, to assess the prevalence and intensity of amphibian *Perkinsea* infections across species, seasons, tissues, and life stages. Anurans from 2 of 3 sampling locations were infected, totaling 25.1 % of all individuals. Infection prevalence varied significantly among locations, seasons, species, and life stages. Infection intensity was significantly higher in larval tissues than adult tissues, and was significantly different across locations, seasons, and species. Understanding relationships between amphibian *Perkinsea* infection, other pathogens, and biotic and abiotic cofactors will allow us to assess what drives population declines, improving our ability to develop conservation strategies for susceptible species to reduce global amphibian biodiversity loss.

KEY WORDS: Emerging disease · Amphibian decline · Protozoan · Parasite · Frog · *Rana* · *Hyla* · *Acris* · Anuran · Mortality · Florida

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INTRODUCTION

Amphibians are experiencing elevated rates of population declines and species extinctions across the globe (Collins & Storfer 2003, Alroy 2015). A variety of factors contributes to these declines, but infectious disease has played a central role since at least the 1970s (Berger et al. 1998, Daszak et al. 1999,

Wake & Vredenburg 2008, Grant et al. 2016). Two infectious pathogens, the fungus *Batrachochytrium dendrobatidis* (*Bd*) and iridoviruses in the genus *Ranavirus*, are thought to be leading drivers of disease-related mortalities in amphibian species (Berger et al. 1998, Daszak et al. 2000, Grant et al. 2016). Furthermore, novel infectious agents with potentially large host impacts continue to be identi-

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fied, such as the salamander fungus *B. salamandrivorans* (Spitzen-van der Sluijs et al. 2016). Ongoing identification, characterization, and assessment of novel amphibian pathogens is therefore necessary to understand the role infectious pathogens play in global patterns of amphibian declines.

A novel, as-yet undescribed, protistan pathogen within the Perkinsea clade has been linked to several mass mortality events of larval ranid frogs within the last 2 decades (Green et al. 2003, Davis et al. 2007, Landsberg et al. 2013, Chambouvet et al. 2015, Isidoro-Ayza et al. 2017). It is genetically similar to the *Perkinsus* pathogen that infects bivalves, which leads to its being referred to as a *Perkinsus*-like organism (Rothermel et al. 2008, Chambouvet et al. 2015, Isidoro-Ayza et al. 2017). Amphibian Perkinsea has spore and zoospore life stages, and the spores can survive desiccation and can tolerate a wide variation in temperature, pH, and salinity, to hatch into zoospores when the rainy season refills ponds (Cook 2008). After polyhedral zoospores hatch, they can penetrate anuran embryos, hatchlings, and tadpoles (Davis et al. 2007, Cook 2008). The zoospore then embeds in the liver of the anuran, proliferates throughout the host's internal organs, and degrades the tissue, ultimately killing the host (Green et al. 2003, Davis et al. 2007, Cook 2008). Amphibian Perkinsea has also been associated with subcutaneous muscle inflammation, suggesting that infections may be detectable using non-destructive swab, toe, or tail clip methods (Jones et al. 2012).

Amphibian Perkinsea infection is the likely cause of mass mortality events throughout the USA, from as far south as Florida to as far north as Alaska (Green et al. 2002, 2003, Landsberg et al. 2013, Isidoro-Ayza et al. 2017). Perkinsea infections are also linked to anuran population declines outside of the USA, including French Guiana, Cameroon, Tanzania, the island of Sao Tome, and the UK (Green et al. 2002, Chambouvet et al. 2015). Several declining species of special concern have been impacted by Perkinsea, including the federally endangered dusky gopher frog *Rana sevosa* and the Florida gopher frog *R. capito* (Green et al. 2002, 2003, Davis et al. 2007, Cook 2008, Gahl & Calhoun 2010, Jones et al. 2012, Landsberg et al. 2013). *R. sevosa* has experienced high levels of mortality attributed to Perkinsea, reaching over 95% mortality in some cases and decimating this federally protected species (Cook 2008, Atkinson 2016). Perkinsea-associated mortalities typically progress at a steady rate over the course of a breeding season, causing almost complete larval extirpation without a

noticeably large mortality event (Atkinson 2016). Mass mortality events attributed to amphibian Perkinsea (identified through histopathology) occur infrequently and can vary drastically depending on the water chemistry of the pond where the outbreak is occurring (Davis et al. 2007, Cook 2008, Atkinson 2016). Given the apparent lethality and potential to cause mass mortality events, but a lack of definitive causal relationships, further research into amphibian Perkinsea is urgently needed.

To better characterize the links between amphibian Perkinsea exposure, infection, and disease in anuran populations, pathogen-specific polymerase chain reaction (PCR) and quantitative PCR (qPCR) assays are necessary. PCR allows for the detection of a pathogen by amplifying a specific segment of pathogen DNA present in a DNA sample (Kessing et al. 1989, Innis et al. 1990). qPCR is a much more sensitive method that uses an oligonucleotide probe labeled with a fluorescent reporter to detect even minute amounts of nucleic acid present in a sample, and to quantify the amount of target DNA present in a sample by comparison with standards of known concentration (Bustin et al. 2009). Because qPCR can detect extremely small quantities of pathogen DNA, it can also be used to confirm infection from tissue biopsies and even skin swabs, potentially eliminating the need for destructive sampling to diagnose infections such as Perkinsea that largely reside in internal organs, but which can be present on the skin. This technique has become a powerful tool for the routine surveillance and detection of *Bd* (e.g. Retallick et al. 2006), but has yet to be validated for amphibian Perkinsea.

Here, we optimized an existing PCR assay and developed a novel qPCR assay that is sensitive and specific for amphibian Perkinsea. We compared the reliability of both assays to detect the presence of amphibian Perkinsea across a range of host species and tissues, including some that can be sampled without euthanizing the host. We also conducted histopathological examinations on a subset of anurans and directly compared qPCR loads to visual metrics of infection severity and tissue damage to assess whether qPCR loads are a reliable indicator of disease severity. Finally, we used our qPCR assay to measure the prevalence and intensity of amphibian Perkinsea infections across anuran species, sampling locations, tissues, and seasons in Florida, USA, to better understand pathogen and disease dynamics in regions with and without previously documented Perkinsea-associated mortalities.

MATERIALS AND METHODS

Sample collection

Samples were collected monthly from 3 amphibian communities from Florida in August 2015 through January 2017, when water was present (27 surveys total). Sampling sites included Pebble Lake at Mike Roess Gold Head Branch State Park (GH; 29° 50' 52" N, 81° 57' 43" W; N = 13 surveys), Archbold Biological Station (ABS; 27° 10' 58" N, 81° 21' 8" W; N = 4 surveys), and Alaina's Pond within the University of Central Florida's Arboretum (AP; 28° 36' 1" N, 81° 11' 26" W; N = 10 surveys). ABS and AP were selected because they are wetlands in central Florida containing high anuran species diversity throughout most of the year; GH was included because of previously documented *Perkinsea*-associated die-offs at this site (Davis et al. 2007, Landsberg et al. 2013). Samples for histological examination were collected at GH in September–November 2017, and February 2018 (N = 13 individuals). We sampled 11 species across 4 anuran families, including southern cricket frogs *Acris gryllus* (N = 123 tissue samples), southern toads *Anaxyrus terrestris* (N = 5), eastern narrow-mouthed toads *Gastrophryne carolinensis* (N = 1), green treefrogs *Hyla cinerea* (N = 2), pine woods treefrogs *H. femoralis* (N = 1), barking treefrogs *H. gratiosa* (N = 23), American bullfrogs *Rana catesbeiana* (N = 41), pig frogs *R. grylio* (N = 2), southern leopard frogs *R. sphenoccephala* (N = 29), Cuban treefrogs *Osteopilus septentrionalis* (N = 44), and Florida gopher frogs *R. capito* (N = 6), along with individuals which could not be identified to species (N = 21 tissue samples).

Upon capture, animals were euthanized by an overdose of tricaine methanesulfonate (MS-222) into the coelomic cavity. A fresh pair of gloves was worn for each captured individual, and all tissue samples were stored separately to ensure pathogens were not spread among individuals or tissues. Whole adult specimens were dissected, and we collected a lobe of the liver and a piece of the intestine. Whole tadpole specimens were also dissected, and we collected a tail clip, a lobe of the liver, and the mouthparts. DNA was extracted from all tissues following the manufacturer's protocol using DNeasy Blood and Tissue kits (Qiagen). Once extracted, DNA elutions were stored in a -20°C freezer until molecular analysis.

PCR optimization

Because amphibian liver tissue was previously found via histology and PCR to be most commonly

infected with *Perkinsea* (Davis et al. 2007, Cook 2008), liver DNA was used to optimize the PCR assay. The primer pair 300F-B and 600R (see Table 2), developed by Chambouvet et al. (2015), was used to amplify an 800 base pair (bp) central region of the 18S ribosomal RNA gene specific for the NAG-01 clade of *Perkinsea* (Chambouvet et al. 2015). The optimal reaction mixture included 1× concentration OneTaq standard buffer with MgCl₂ (New England BioLabs), 800 μM dNTPs, 10 mM DMSO, 0.2 μM of forward and reverse primers (300F-B and 1294R), 0.625 units of OneTaq polymerase (Applied Biosystems), 1 μl of DNA template, and molecular-grade water to reach a total reaction volume of 10 μl. Optimal cycling conditions included an initial denaturation stage of 2 min at 95°C followed by 36 cycles of 30 s at 95°C, 30 s at 55°C, 2 min at 72°C, and an additional extension stage of 10 min at 72°C. Controls were included with each run and consisted of molecular-grade water (negative controls) and DNA extractions from livers of 2 *R. sevosa* individuals confirmed via liver smear to be positive for *Perkinsea* (positive controls). PCR products were visualized on 2% agarose gels. To confirm the presence of *Perkinsea*, PCR products from all apparent positives (samples with bands of the appropriate size) were Sanger sequenced at the Eurofins Genomics sequencing facility (Louisville, Kentucky, USA).

qPCR assay development

Initial use of primers 300F-B and 600R (Chambouvet et al. 2015) produced low amplification rates but generated enough sequence data to develop qPCR primers and probes. All recovered *Perkinsea* sequences were compared to existing sequences in GenBank using BLAST searches implemented in Geneious (BioMatters). We aligned our *Perkinsea* sequences with GenBank sequences showing significant homology, then used Primer3 (Untergasser et al. 2012) implemented in Geneious to develop qPCR primers and a probe targeting regions that were conserved across our *Perkinsea* sequences but not in other distinct, but alignable, protistan sequences.

All qPCR samples were run on the CFX96 Real-Time System (Bio-Rad). Because *Perkinsea* is genetically similar to bivalve pathogens in the genus *Perkinsus*, and is sometimes referred to as a '*Perkinsus*-like' pathogen (Chambouvet et al. 2015), DNA from several *Perkinsus* species was tested against positive and negative controls to determine whether our probe was specific for amphibian *Perkinsea* only.

All samples were analyzed using Bio-Rad CFX Manager software, and threshold amplification (C_q) values through cycle 35 were considered true positives, whereas samples amplifying above cycle 36 were conservatively attributed to non-specific late-stage amplification. All samples with C_q values before cycle 36 were run a second time to confirm the presence and quantity of *Perkinsea*; if 2 runs differed in intensity by more than an order of magnitude, a third run was conducted and the 2 most consistent results were retained. Infection intensity was determined using C_q values generated from qPCR amplification curves converted into starting quantities based on comparison to the standard curve run on every qPCR plate (Fig. S1 in the Supplement at www.int-res.com/articles/suppl/d129p085_supp.pdf). The average starting quantity across replicate qPCR runs was used as the final intensity value. Infection intensity values were then log transformed to account for exponential pathogen growth dynamics.

Histopathologic examination

Tadpole and adult frogs were collected from GH and euthanized using an overdose of MS-222. Specimen 91 in September, specimens 109 and 110 in October, and specimens 189 and 190 were collected in November of 2017; specimens 217–224 were collected in February 2018 (see Table 3). Specimens were swabbed in the field before euthanizing; adults were swabbed a total of 25 times (5 strokes on the ventral surface, 5 strokes on each thigh, and 5 strokes on each hind foot), and tadpoles were swabbed a total of 20 times (5 strokes on the mouthparts, 5 strokes on the ventral surface, and 5 strokes on each side of the tail). Necropsies of the animals were then performed in the field, and we removed liver, intestines, tail (tadpoles only), mouth parts (tadpoles only), and toe (adults only) tissue. Portions of the tail, liver, and intestine were placed in molecular-grade ethanol, and were used for qPCR analysis. The remainder of the liver, intestinal tissue, and the remainder of the specimens were submerged in 10% buffered formalin for 2 d, and then transferred to ethanol for long-term storage. Histopathological examinations were performed by the Aquatic, Amphibian, and Reptile Pathology program at the University of Florida's College of Veterinary Medicine. All tadpoles were serially sectioned. For adult frogs, samples from all viscera, brain, skin, and skeletal muscle were collected. All samples were processed routinely, and histologic sections cut at

3 μ m were stained with hematoxylin and eosin (HE). A subset of sections was also stained with Gömöri methenamine silver (GMS), periodic acid-Schiff (PAS), and Giemsa.

Statistical analyses

We analyzed PCR-based *Perkinsea* prevalence using ANOVA comparing infection prevalence against site, species, and anuran family. We also assessed qPCR-based *Perkinsea* prevalence using ANOVA comparing infection prevalence against month, season, site, species, and anuran family, followed by Tukey's HSD post hoc tests to determine the statistical significance of each factor in explaining *Perkinsea* prevalence. To compare *Perkinsea* prevalence across life stages, we used chi-squared tests on 2×2 contingency tables. Additionally, to evaluate how these variables may be interacting to affect the prevalence of *Perkinsea*, we combined individually significant factors into various generalized linear models (GLMs; family = binomial) and then conducted model selection using AICc using the Package bblme (Bolker & R Development Core Team 2016). Additionally, we compared the detection rates of PCR and qPCR using a chi-squared test to determine if the methods differed significantly in their ability to detect *Perkinsea*.

To assess significant differences in infection intensity, we conducted an ANOVA comparing intensity against month, season, location, species, and family, followed by Tukey's HSD post hoc tests to determine the statistical significance of each factor in explaining *Perkinsea* intensity. We then combined individually significant factors into various linear mixed effect models (using sample ID as a random effect) and conducted model selection using AICc. This was done to determine how these variables may be influencing *Perkinsea* infection intensity. All analyses were conducted in R using the packages binom, bblme, lme5, and ggplot2 (Wickham 2009, R Core Team 2012, Dorai-Raj 2014, Bates et al. 2015, Bolker & R Development Core Team 2016).

RESULTS

Amphibian *Perkinsea* detection via PCR

Among the 230 tissue samples (from 173 individuals) tested for amphibian *Perkinsea* using conventional PCR (Table 1; 17 larvae and 156 adults), significantly

Table 1. Amphibian *Perkinsea* infections detected using conventional PCR, grouped by species, sampling location in Florida (USA), and tissue sample. Values in parentheses are percentages; dashes (–) indicate no tissue from that species and location. GH: Gold Head (Clay County); AP: Alaina's Pond (Orange County); ABS: Archbold Biological Station (Highlands County)

Species	Location	Liver	Tail	Mouthparts	Intestine
<i>Acris gryllus</i>	GH	0/55 (0)	0/1 (0)	–	0/4 (0)
	AP	0/23 (0)	–	–	–
	ABS	0/6 (0)	–	–	–
<i>Anaxyrus terrestris</i>	GH	1/3 (33.3)	–	–	0/2 (0)
<i>Gastrophryne carolinensis</i>	GH	0/1 (0)	–	–	–
<i>Hyla cinerea</i>	GH	0/1 (0)	–	–	0/1 (0)
<i>Hyla femoralis</i>	GH	0/1 (0)	–	–	–
<i>Hyla gratiosa</i>	GH	0/8 (0)	0/1 (0)	–	0/3 (0)
<i>Rana capito</i>	GH	2/2 (100)	2/2 (100)	2/2 (100)	–
<i>Rana catesbeiana</i>	GH	0/20 (0)	0/10 (0)	–	–
<i>Rana grylio</i>	AP	0/2 (0)	0/1 (0)	–	–
<i>Rana sphenoccephala</i>	GH	0/5 (0)	–	–	0/4 (0)
	AP	0/3 (0)	–	–	–
<i>Osteopilus septentrionalis</i>	GH	4/6 (66.7)	4/6 (66.7)	6/6 (100)	–
	AP	0/8 (0)	–	–	0/6 (0)

more larvae were infected (64.7%) than adults (a single *Anaxyrus terrestris*, 0.64%; $\chi^2 = 97.5$, $df = 1$, $p < 0.0001$). All positive individuals were sampled from our northernmost location, GH (Table 1). Infection prevalence was significantly different among species (Tukey's HSD = 9.72, $df = 9$, $p < 0.0001$). Specifically, *Rana capito* and *Osteopilus septentrionalis* had significantly higher *Perkinsea* prevalence detected via PCR compared to all other species (Tukey's HSD = 9.72, $df = 9$, $p < 0.01$; Table 1). However, PCR positives differed significantly by tissue type and were inconsistent across tissues within individuals (Table 1).

Two unique haplotypes were recovered from sequencing 12 *Perkinsea* PCR positives (GenBank accession numbers MF103770 and MF103771; Table S1 in the Supplement). All 12 sequences were fixed for 1 bp substitution relative to the previously characterized *R. sphenoccephala* pathogen isolate (EF675616) and uncultured *Perkinsea* isolates (KP122567-9), and 1 sequence also had a second nucleotide substitution (Fig. S2 in the Supplement).

Amphibian *Perkinsea* qPCR development

We named the qPCR primer pair producing the highest rate of *Perkinsea* amplification PerF and PerR (Table 2), which together amplified a 126 bp fragment of the amphibian *Perkinsea* 18S rRNA gene. The custom Taqman probe (*Perkinsea*P: 6-FAM-5'-ACC GTG TTG ATC GAG GCA TT-3'-Tamra) was designed from the most conserved region internal to this primer pair. The optimal reaction mixture included 1× concentration Supermix (Bio-Rad), 10 µM forward primer (PerF) and reverse primer (PerR), 1 µM probe (PerP), 5 µl of sample DNA, and molecular-grade water to reach a total reaction volume of 25 µl. Cycling conditions were optimized at 95°C for 5 min, followed by 40 cycles of 95°C for 15 s and 58°C for 1 min. Standard curves were generated from serial dilutions of a gBlock gene fragment consisting of 250 bp of synthesized DNA identical to the consensus target region of the amphibian *Perkinsea* genome (Integrated DNA Technology) serially diluted from

Table 2. PCR and qPCR primers used to amplify fragments of the amphibian *Perkinsea* 18S rRNA gene from DNA extracted from amphibian tissues

Primer	Sequence (5'–3')	Specificity	Reference
300F-B	GGG CTT CAY AGT CTT GCA AT	NAG-01 cluster	Chambouvet et al. (2015)
600R	GAA TTT CAC CTC TGA CSG TTT G	Eukaryotes	Chambouvet et al. (2015)
1294R	CCA GRA CAT CTA AGG GCA TCA	Eukaryotes	Chambouvet et al. (2015)
PerF	GAA CGA CCG TCC TAC CTT GG	Amphibian <i>Perkinsea</i>	This study
PerR	AGG CCT GCT TGA AAC ACT CT	Amphibian <i>Perkinsea</i>	This study

concentrations of 2×10^8 to 2×10^9 copies reaction⁻¹ which were run in duplicate (Fig. S1). DNA extracted from *Perkinsus olsenii*, *P. beihaiensis*, *P. chesapeakei*, and *P. marinus* were all qPCR negative, confirming that our assay is specific for amphibian *Perkinsea* and does not broadly amplify species within the *Perkinsea* clade.

Histopathologic examination

Tissues from adult and larval *R. sphenoccephala* and a single liver of an *R. catesbeiana* individual revealed varying degrees of *Perkinsea*-associated tissue damage that were positively correlated with infection intensity measured from tissue samples and skin swabs via our *Perkinsea* qPCR assay (Table 3). In the most severely affected individuals, *Perkinsea* organisms replaced 25–90% of the parenchyma of various viscera, including the liver, kidney (mesonephros), gill, spleen, heart, stomach, intestine, and skin/dermis and were associated with variable amounts of necrotic cellular debris but minimal inflammation (Figs. 1 & 2). Fewer organisms were noted throughout other tissues, including the central nervous system, eye/retina, oral cavity, pancreas, coelomic fat bodies, and gonads, but were not associated with significant tissue damage and/or inflammation (Fig. 2). Small to moderate numbers of organisms were routinely associated with degeneration and necrosis of skeletal muscle, primarily in the tail (Fig. 1). *Perkinsea* organisms ranged in size from 2–6 μ m in diameter, with 2 apparent subpopulations of organisms. A larger, round 4–6 μ m population was characterized by a pale basophilic to amphophilic core surrounded

by a 1–2 μ m thick wall with positive histochemical reactivity to GMS, PAS, and Giemsa (Fig. S3 in the Supplement). A second, smaller population of organisms were 2–3.5 μ m in diameter, stained pale eosinophilic with HE, and lacked a prominent wall. Organisms were readily identifiable on light microscopy in individuals with high qPCR loads (Table 3), but in animals with low loads, organisms were rare or not apparent with routine HE or special histochemical (GMS, PAS, or Giemsa) stains.

Perkinsea infection dynamics in central Florida

In 298 tissue samples collected from 211 individuals and tested using our qPCR assay, a total of 53 individuals (25.1%) were *Perkinsea* positive in at least 1 tissue (Table 4). Excluding 1 *R. capito* larva that was collected dead at GH (cause of death unknown), all individuals included in this study appeared healthy at the time of sampling. A significantly higher proportion of larval tissues (73.4%) was infected with *Perkinsea* compared to adult tissues (18.2%; $\chi^2 = 85.6$, $df = 1$, $p < 0.0001$) (Tukey's HSD = 24.66, $df = 3$, $p < 0.0001$; Fig. 3). *Perkinsea* infection prevalence was significantly higher at GH compared to the other 2 sampled amphibian communities (Tukey's HSD = 7.35, $df = 2$, $p = 0.018$; Fig. 3). *Perkinsea* prevalence was also significantly different across months (Tukey's HSD = 10.96, $df = 10$, $p < 0.001$), seasons (Tukey's HSD = 23.89, $df = 3$, $p < 0.001$), and species (Tukey's HSD = 5.563, $df = 11$, $p < 0.001$; Fig. 3). The most informative GLMs explaining *Perkinsea* prevalence included life stage and/or sampling location as explanatory variables (Table S2 in the Supplement).

Table 3. Absent, mild, moderate, and severe amphibian *Perkinsea* infections in *Rana sphenoccephala* and *R. catesbeiana* detected with histopathology compared to the intensity measured via quantitative PCR (qPCR; in gene copies)

Individual	Perkinsea severity			qPCR intensity		
	Liver	Mesonephros (kidney)	Skeletal muscle	Liver	Skeletal muscle	Swab
91 (<i>R. sphenoccephala</i>)	None	None	None	0	0	0
109 (<i>R. catesbeiana</i>)	None	None	None	0	0	0
110 (<i>R. catesbeiana</i>)	None	None	None	0	0	0
189 (<i>R. catesbeiana</i>)	None	None	None	0	0	0
190 (<i>R. sphenoccephala</i>)	None	None	None	0	0	0
217 (<i>R. catesbeiana</i>)	Severe	–	–	–	4898596	13312280
218 (<i>R. sphenoccephala</i>)	None	None	None	6141	31789	5028
219 (<i>R. sphenoccephala</i>)	Mild	None	None	4239	5878	1080
220 (<i>R. sphenoccephala</i>)	Moderate	Moderate	Mild	520371	9294945	7627
221 (<i>R. sphenoccephala</i>)	Mild	None	None	200910	1338066	449
222 (<i>R. sphenoccephala</i>)	Severe	Severe	Moderate	106449025	19588508	43987
223 (<i>R. sphenoccephala</i>)	None	None	None	87914	3515	4641
224 (<i>R. sphenoccephala</i>)	Severe	Severe	Severe	26930664	0	47464133

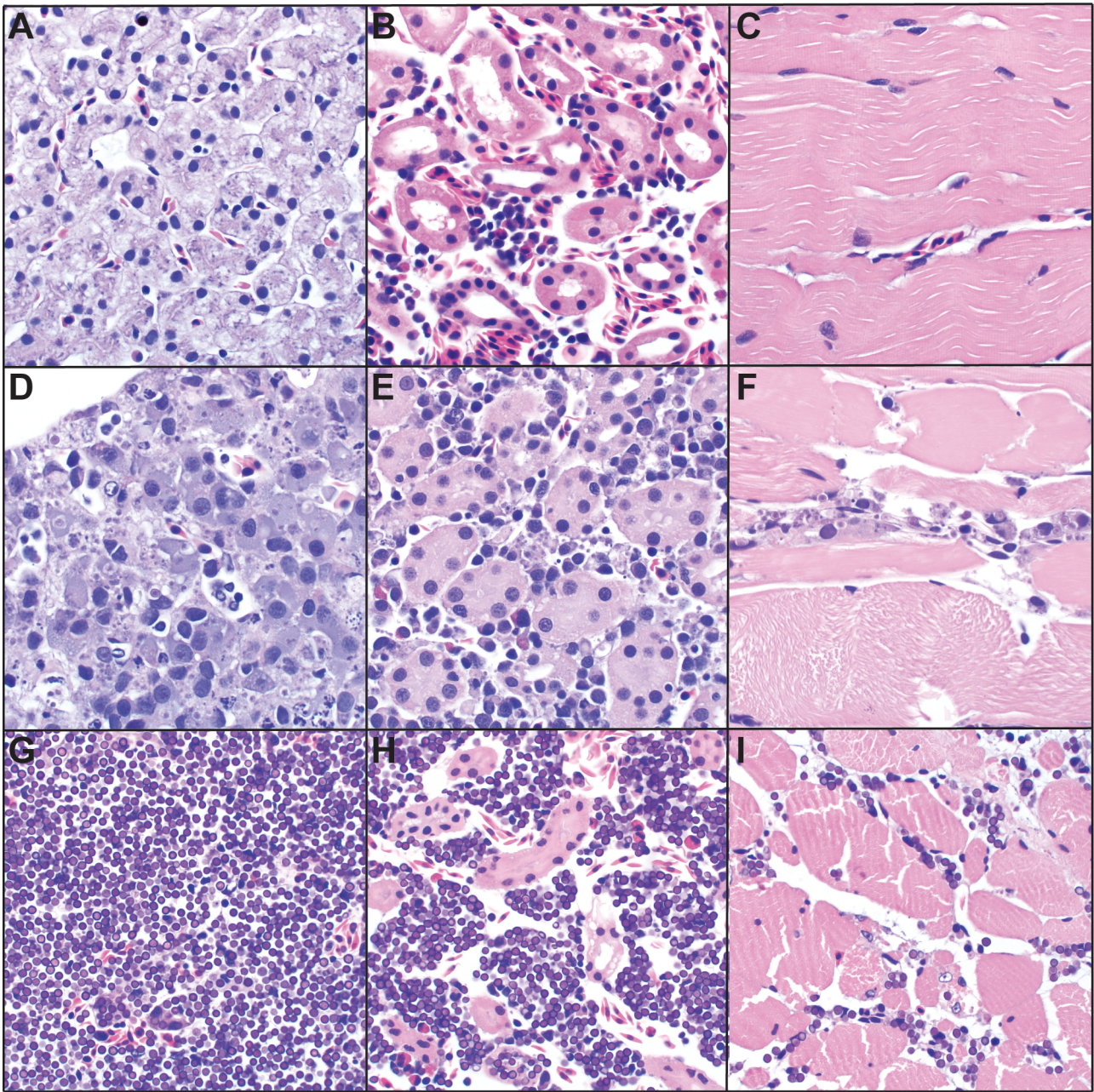


Fig. 1. Comparison of (A–C) liver, (D–F) kidney (mesonephros), and (G–I) skeletal muscle histopathology in larval southern leopard frogs *Rana sphenoccephala*. Uninfected control tissues (A,D,G) are compared to individuals with mild to moderate (B,E,H) and severe (C,F,I) histologic evidence of perkinsiosis. Panels A and D are from specimen PL 91, panel G is from specimen PL 190, panels B, E, and H are from specimen PL 220, and panels C, F, and I are from specimen 224 (see Table 4). 500× magnification

Infection intensity was not significantly different across tissue types, but this was confounded by life stage (Fig. 4). *Perkinsea*-positive liver and intestine sampled from adult frogs had significantly lower infection intensities compared to *Perkinsea*-positive liver, mouthparts, and tail muscle sampled from larvae (Tukey's HSD = 10.53, df = 4, $p = 0.0011$; Fig. 4).

Samples collected in January, February, and March showed significantly higher intensity than all other months (Tukey's HSD = 6.626, df = 8, $p < 0.01$; Fig. 4). Infection intensity also differed significantly across host species (Tukey's HSD = 15.46, df = 9, $p < 0.001$), and members of the Ranidae had significantly higher infection intensity compared to other anuran families

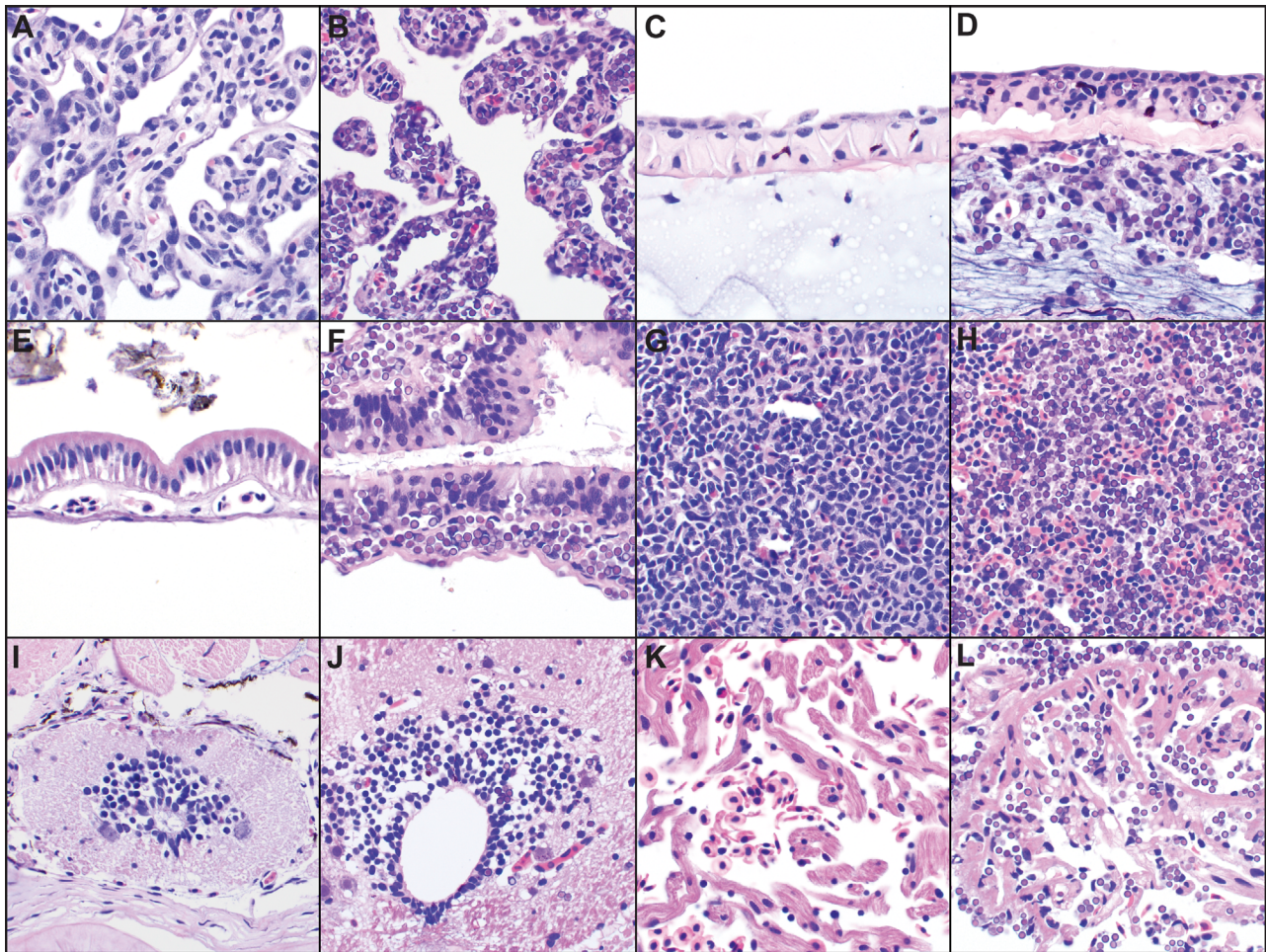


Fig. 2. Histopathologic evidence of perkinsiosis in other tissues of larval southern leopard frogs *Rana sphenoccephala*. The normal histologic appearance of (A) gill, (C) skin, (E) intestine, (G) spleen, (I) spinal cord, and (K) heart is compared to infected (B) gill, (D) skin, (F) intestine, (H) spleen, (J) caudal brain stem, and (L) heart with various loads of *Perkinsia* organisms. All unaffected tissues are from PL91 or PL190, while all affected tissues are from PL222 and PL224 (see Table 4). 500× magnification

(Tukey's HSD = 7.281, $df = 4$, $p < 0.001$; Fig. 4). The one exception was *O. septentrionalis*, a member of the family Hylidae, which had significantly higher average infection intensity compared to all non-Ranidae species sampled (Tukey's HSD = 15.46 $df = 9$, $p < 0.001$; Fig. 4). Finally, infection intensity varied significantly based on sampling location (Tukey's HSD = 5.463, $df = 2$, $p < 0.001$; Fig. 4). The most informative mixed effects LM explaining *Perkinsia* intensity was the global model, which had a marginal R^2 value of 0.728 and a conditional R^2 value of 0.892 (Table S2).

Overall, across the 230 tissue samples tested using both PCR and qPCR, PCR detected *Perkinsia* infection in 12.2% of sampled tissues (28/230) while qPCR detected infection in a significantly higher proportion (75/230) of sampled tissues (32.6%; $\chi^2 = 16.739$, $df = 1$, $p < 0.0001$).

DISCUSSION

Pathogen molecular diagnostics can be a powerful tool for monitoring wildlife populations, understanding host–pathogen dynamics, and preventing disease outbreaks (Kriger et al. 2006, Dong et al. 2008, Vredenburg et al. 2010). Here, we optimized an existing PCR protocol and developed a novel qPCR assay that is specific for the anuran pathogen *Perkinsia* and more effective at detecting amphibian *Perkinsia* infections than traditional PCR or histopathology. Using qPCR to quantify *Perkinsia* across a range of species and tissue types revealed high prevalence and intensity in multiple species and at 2 locations within Florida, USA. In contrast to previous studies that only detected *Perkinsia* in larval anurans and primarily in the liver (Davis et al. 2007, Landsberg et al. 2013, Chambouvet et al. 2015), our study con-

Table 4. Amphibian *Perkinsea* infections detected using quantitative PCR (qPCR) grouped by species, sampling location, and tissue type. Values in parentheses are percentages; dashes (–) indicate no tissue from that species and location. GH: Gold Head (Clay County, FL); AP: Alaina's Pond (Orange County, FL); ABS: Archbold Biological Station (Highlands County, FL). Full species names are given in Table 1

Species	Location	Larvae			Adults	
		Tail	Mouthparts	Liver	Liver	Intestine
<i>A. gryllus</i>	GH	0/1 (0)	1/1 (100)	1/1 (100)	18/64 (28.1)	0/4 (0)
	AP	–	–	–	8/41 (19.5)	1/1 (100)
	ABS	–	–	–	0/10 (0)	–
<i>A. terrestris</i>	GH	–	–	–	1/3 (33.3)	1/2 (50)
<i>G. carolinensis</i>	GH	–	–	–	1/1 (100)	–
<i>H. cinerea</i>	GH	–	–	–	0/1 (0)	0/1 (0)
<i>H. femoralis</i>	GH	–	–	–	1/1 (100)	–
<i>H. gratiosa</i>	GH	0/3 (0)	0/3 (0)	3/3 (100)	0/7 (0)	0/7 (0)
<i>R. capito</i>	GH	2/2 (100)	2/2 (100)	2/2 (100)	–	–
<i>R. catesbeiana</i>	GH	0/1 (0)	–	–	0/22 (0)	1/18 (5.6)
<i>R. grylio</i>	AP	–	–	–	0/2 (0)	–
<i>R. sphenoccephala</i>	GH	5/5 (100)	5/5 (100)	3/5 (60)	1/6 (16.7)	1/5 (20)
	AP	–	–	–	1/3 (33.3)	–
<i>O. septentrionalis</i>	GH	4/6 (66.7)	6/6 (100)	6/6 (100)	–	–
	AP	–	–	–	0/20 (0)	0/6 (0)

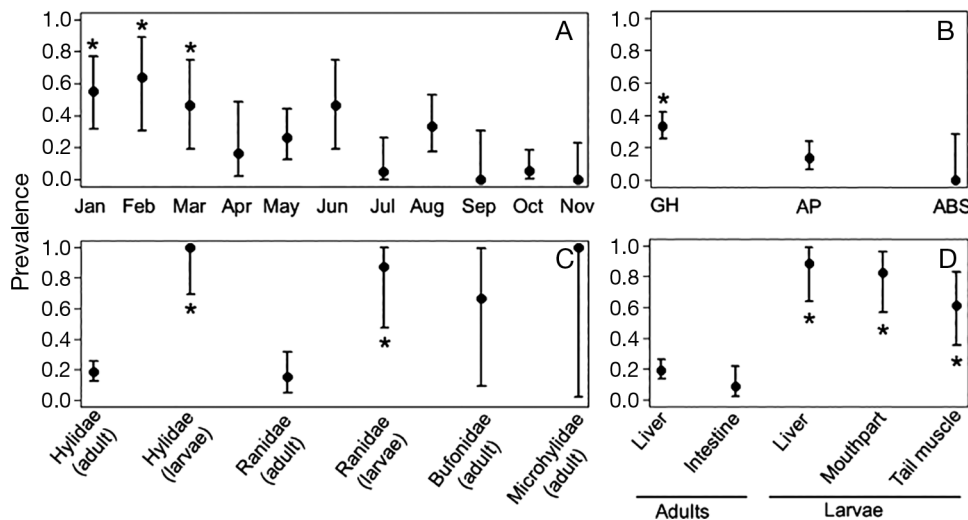


Fig. 3. Amphibian *Perkinsea* prevalence ($\pm 95\%$ binomial confidence interval) among (A) month, (B) location (GH: Gold Head, Clay County, FL; AP: Alaina's Pond, Orange County, FL; ABS: Archbold Biological Station, Highlands County, FL), (C) family, and (D) tissue type and life stage. Asterisks denote categories with significant differences compared to all other categories as inferred using an ANOVA with Tukey's HSD post hoc tests

firms, via molecular and histologic means, that both larval and adult life stages can be infected, and a variety of anuran tissues, as well as non-destructive swabs, have the capacity to harbor *Perkinsea*, including skin, tail muscle, mouthparts, and intestine. Of note, one examined specimen (224) had histologic evidence of muscle degeneration and necrosis with intralesional amphibian *Perkinsea* organisms in the tail while the qPCR results for that tissue were negative (Table 3). The cause of this single inconsistency between qPCR and histopathology results remains unclear, but suggests that sporadic distribution of infection in muscle tissue may cause infections to be missed from tail clips and that swabbing across a

broader region of the organism may be a preferred sampling technique for qPCR detection.

High infection intensities measured via qPCR were detected in both tail clips (skeletal muscle) and mouthparts of larvae. The high intensity detected in the tail tissue is consistent with previous reports of a skeletal muscle tropism in adult anurans (Jones et al. 2012), but high intensities in the soft tissues associated with the mouthparts suggests a more complex *Perkinsea* infection landscape than previously recognized. However, it is possible that our qPCR results for the mouthparts are due to infection of the surrounding skin, as mouthparts were never observed to harbor *Perkinsea* via histopathology.

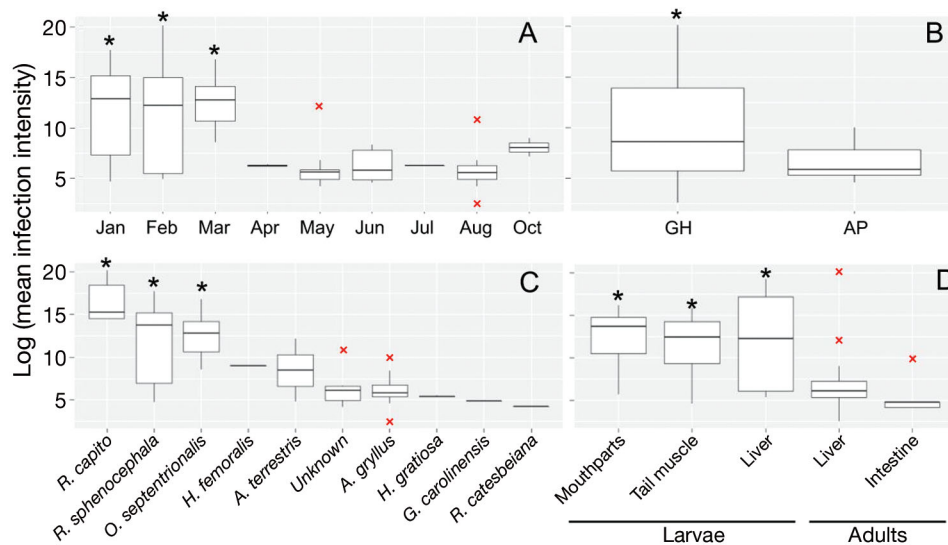


Fig. 4. Amphibian Perkinsia log-transformed mean infection intensity ($\pm$ SE) among (A) sampling month, (B) sampling locations (GH: Gold Head, Clay County, FL; AP: Alaina's Pond, Orange County, FL), (C) species sampled, and (D) tissue type and life stage sampled. When larval individuals could not be specifically identified, they were categorized as unknown. Outliers are depicted with red symbols. Asterisks denote categories with significant differences compared to all other categories as inferred using an ANOVA with Tukey's HSD post hoc tests

Swabbing has previously been shown to be a viable method for non-destructive sampling for *Bd* detection (Retallick et al. 2006, Hyatt et al. 2007). Although swab-based Perkinsia quantification may not provide an accurate representation of infection intensity at the level of the whole organism (Clare et al. 2016), it can provide a simple and minimally invasive tool to assess presence/absence of the pathogen for use in conservation monitoring. In this study, the specimens we analyzed via histopathology were also swabbed, and swab qPCR Perkinsia quantification was consistent with histological findings as well as qPCR results from other tissues (Table 3), demonstrating that skin swabs can be a viable method for amphibian Perkinsia detection.

Amphibians sampled from GH had the highest prevalence and intensity of Perkinsia (Table 4, Figs. 3 & 4), consistent with previous documentation of a mass mortality event due to amphibian Perkinsia at this location (Landsberg et al. 2013). Samples from GH also tended to have the most intense infections during the winter months, suggesting a seasonal pattern (Fig. 4). Species in the family Ranidae, which we found to be the predominant host for this pathogen in this study, tend to breed from January to May, although this can be extended later into the summer depending on the amount of rainfall (Palis 1998). The seasonality detected in this system may indicate a correlation between breeding phenology of the populations in these ponds and disease prevalence and intensity. Further monitoring of additional

amphibian communities, as well as tracking amphibian Perkinsia across multiple annual cycles, would increase confidence in our preliminary observation of seasonality, and may identify additional correlates of infection severity.

Rana catesbeiana, *R. sphenoccephala*, and *R. capito* are the species most affected by previous Perkinsia-associated disease events at GH (Landsberg et al. 2013). Consistent with this pattern, we detected high prevalence and intensity in *R. sphenoccephala* and *R. capito*. In contrast, we only found a single infected *R. catesbeiana*, and the high levels of infection detected in *R. sphenoccephala* were found in individuals that appeared healthy (Figs. 3 & 4).

Also surprising was the detection of the third highest average infection intensities in a non-ranid species, *Osteopilus septentrionalis* found at GH and AP, which is invasive in Florida and negatively impacts native anurans (Wyatt & Forsy 2004). High intensities in *O. septentrionalis* could indicate that the species was a vector that introduced Perkinsia to Florida, although more testing for Perkinsia within its native range is needed to confirm this. Alternately, *O. septentrionalis* could have been exposed to Perkinsia upon expansion to central Florida, which is the furthest south we detected the pathogen (AP), and high susceptibility to Perkinsia could be hampering the expansion of this species northward, as has been documented for native pathogens and non-native hosts in other systems (Torchin et al. 2003, Lee & Klasing 2004). More data are necessary to elucidate

how *O. septentrionalis* and *Perkinsea* are interacting and what the effects might be on native species' disease prevalence (Ortega et al. 2015).

Climate and other environmental factors impact disease dynamics of the widely studied amphibian pathogens *Bd* and ranavirus (Kiesecker et al. 2001, Carey & Alexander 2003, Daszak et al. 2005, Lips et al. 2008). We found that *Perkinsea* infections varied seasonally and became more severe during cooler months of the year, matching *Bd* infection dynamics for a variety of species and global regions (Berger et al. 2004, Kriger & Hero 2007, Todd-Thompson 2010, Savage et al. 2011, Nowakowski et al. 2016), and suggesting that similar environmental factors may be driving disease outbreaks for both pathogens. The colder months include January, February, and March, for which the average temperatures across our sites ranged from 11 to 29°C (Table S3 in the Supplement).

Non-invasive sampling, such as skin swabbing, removes barriers to more extensive monitoring and enables rapid pathogen surveys of large areas and many individuals (Piggott & Taylor 2003, Soto-Azat et al. 2009). Pre-screening sites for *Perkinsea* could be a powerful tool for management programs that actively translocate or head start and release amphibians into new habitats once seasonality and the most susceptible species can be confirmed. For example, *Perkinsea* has negatively contributed to the translocation efforts for the endangered species *R. sevosia* (Atkinson 2016), and qPCR-based *Perkinsea* testing, especially of non-destructive swabs, can enable managers to select pathogen-free release sites and captive populations, reducing the accidental spread of *Perkinsea* into new areas. Pre-release screening is already being employed for *Bd* and ranavirus, and will become increasingly important as more programs seek to reintroduce individuals from captivity into the wild (Seigel & Dodd 2002, Griffith et al. 1993). However, while non-destructive sampling provides a powerful tool for monitoring threatened populations, destructive sampling is still an important tool for understanding the health impacts of *Perkinsea* (Wasko et al. 2003) given the variation in infection intensity across tissues (Fig. 4).

The 4 *Perkinsea*-infected frog families we identified can also exhibit susceptibility to *Bd* and ranavirus (La Marca et al. 2005, Hoverman et al. 2011, Savage & Zamudio 2011, Van Rooij et al. 2015, Love et al. 2016). Of particular importance, *R. sevosia* demonstrates susceptibility to both pathogens when exposed in lab and field settings (Rothermel et al. 2008, Sutton et al. 2014). In contrast, *R. catesbiana*

demonstrates widespread tolerance to both pathogens (Daszak et al. 2004, Schloegel et al. 2009). Given that *Bd* and ranavirus co-occur at both of our *Perkinsea*-positive study sites (Horner et al. 2017), interactions with these other pathogens may impact *Perkinsea* infection dynamics, either through direct pathogen interactions or via pathogen-stimulated changes in host immunity (Gray et al. 2009, Blaustein et al. 2011, Rollins-Smith et al. 2011). The impact of co-infection has been a topic of interest in amphibians, with heavy emphasis on *Bd* and ranavirus interactions, although this relationship is not fully understood (Souza et al. 2012, Warne et al. 2016, Horner et al. 2017). Previous work confirms that *Bd* and ranavirus can co-infect *Perkinsea*-infected frogs (Cook 2008, Miller et al. 2011, Landsberg et al. 2013, Borteiro et al. 2014, Isidoro-Ayza et al. 2017), and ranavirus and *Perkinsea* both primarily infect larvae, while *Bd* and *Perkinsea* show similar seasonal fluctuations. This is especially important because mortality is often positively correlated with the number of pathogen infections of macroparasites within an individual (Johnson & Hoverman 2012, Whitfield et al. 2013). Therefore, future studies should focus on the relationship between *Perkinsea* and these other amphibian pathogens.

CONCLUSIONS

We have developed and validated a qPCR assay that can detect the emerging amphibian *Perkinsea* pathogen from a range of anuran tissue types and swabs. We also document that the highest *Perkinsea* prevalence and intensity in Florida occur in the winter months, at our northernmost sampling location (GH), and in members of the families Hylidae and Ranidae. Compared to previous studies that only detected *Perkinsea* in association with die-offs and within the family Ranidae, here we found high prevalence at a location in central Florida with no history or overt evidence of disease (AP), and we detected substantial infection in the invasive treefrog *Osteopilus septentrionalis*. Comparison of qPCR to histopathology confirms that high qPCR intensities correspond to severe perkinsiosis, whereas moderate to low qPCR intensities correspond to mild or undetectable histologic evidence of *Perkinsea* infection, demonstrating that qPCR is more sensitive than histopathology for detecting amphibian *Perkinsea* infections. Our qPCR protocol will thus facilitate a better understanding of co-infection dynamics between *Perkinsea* and other amphibian pathogens,

and the ability to detect *Perkinsea* using qPCR on non-destructive swab and tissue samples will allow wildlife managers to routinely monitor for *Perkinsea* at locations where amphibian disease is a concern. The variable presence and severity of *Perkinsea* infections across species, seasons, tissues, and locations, and evidence of tissue-damaging perkinsiosis in animals with high qPCR intensities, highlight the importance of understanding *Perkinsea* epidemiology if we are to mitigate further loss of global amphibian biodiversity.

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