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Comparative efficacy of eDNA and conventional methods for monitoring wetland anuran communities

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Documenting biodiversity, species occurrence, and species status require reliable monitoring techniques, but the complex life history and cryptic behavior of many anurans create challenges for conventional monitoring approaches. Environmental DNA (eDNA) surveys are a promising alternative (or complement) to conventional anuran monitoring, but their relative success has not been fully tested. We assessed the comparative efficacy of targeted eDNA detection via quantitative PCR (qPCR) and three conventional amphibian survey methods (visual encounter, breeding call, and larval dipnet surveys) for detecting nine anuran species in natural wetlands in southern Ontario, Canada. Our analyses revealed that all assessment methods yielded imperfect detection, with visual encounter and eDNA surveys detecting the greatest species richness and eDNA surveys requiring the fewest sampling events. Amphibian community composition results differed among survey methods and sampling events, and detection efficacy was markedly variable, with some species requiring two to three methods to maximize detection success. Notably, two relatively terrestrial species (*Anaxyrus americanus* and *Hyla versicolor*) had relatively low and seasonally variable eDNA detection rates, suggesting that species-specific ecology likely affects eDNA presence or detection. These findings suggest that optimized monitoring for complex anuran communities may require application of multiple monitoring methods, which may need to be tailored to individual target species or communities.

KEYWORDS

environmental DNA (eDNA), quantitative PCR (qPCR), environmental monitoring, species richness, community composition, amphibian communities

Introduction

Population monitoring is critical for identifying species declines and mitigating biodiversity loss, particularly for data-deficient and rapidly declining taxa. Amphibians are perhaps the most threatened vertebrate group, with >40% of species showing evidence of numerical decline, including multiple instances of local extinction and extirpation (Stuart et al., 2004; Johnson et al., 2017; Leung et al., 2017). This alarming decline highlights the need to develop effective amphibian monitoring programs so that conservation status of populations and communities can be assessed quickly and reliably. Amphibian species vary considerably in their habitat requirements (Mattfeldt et al., 2009), breeding periods (Bridges and Dorcas, 2000), and life cycles (Wells, 2007), creating challenges for selecting the best monitoring tools for tracking changes in their distribution and abundance. It follows that gaps in our understanding of amphibian population trends can be attributed, in part, to a lack of robust, reliable, and universally applicable monitoring techniques.

Amphibians are commonly surveyed using a variety of conventional monitoring methods that exhibit varying levels of success and efficacy. These approaches usually involve conducting visual or auditory (i.e., breeding call) surveys, which can be influenced by a range of biotic and abiotic factors affecting assessment reliability (De Solla et al., 2005; Schmidt, 2005; Petitot et al., 2014). Indeed, visual and auditory surveys typically require environmental conditions (i.e., rainfall, humidity, and temperature; see Mazerolle et al., 2005; Schmidt, 2005; Asad et al., 2020) and habitat characteristics (i.e., vegetation type and coverage; see Bailey et al., 2004) that are conducive to high probability of detection when animals are present. Likewise, target species must be reasonably abundant in the surveyed area for reliable detection (see Gu and Swihart, 2004; Tanadini and Schmidt, 2011). Observer error also can contribute to unreliable amphibian detections from conventional surveys because individuals have different levels of expertise in identifying amphibian species in the field (Lotz and Allen, 2007; McClintock et al., 2010; Barata et al., 2017). In addition, because amphibians undergo multiple life stages and are sensitive to environmental conditions, detectability via conventional surveys may vary through space and time. It follows that these complexities challenge the reliability of conventional amphibian surveys and should encourage consideration and evaluation of alternative methods.

Additional challenges arise when amphibian monitoring programs attempt to track changes in multiple species concurrently, using a one-size-fits-all approach. This common practice ignores differences in species ecology and corresponding detectability, leading to under-detection when surveys are ill-timed or poorly suited for a particular species (Bridges and Dorcas, 2000). Detection rates for individual species within amphibian communities can be improved via strategic use of multiple survey methods and sufficient effort (Ryan et al., 2002; Petitot et al., 2014) or accounting for detection probabilities during data collection and analysis (Schmidt, 2005; Mazerolle et al., 2007). Accordingly, even in the best circumstances, conventional amphibian monitoring methods remain imperfect.

Environmental DNA (eDNA) detection is growing in acceptance as an alternative method for monitoring wildlife (Taberlet et al., 2018; Pawlowski et al., 2020), including a variety of amphibian species, communities (e.g., Goldberg et al., 2011; Valentini et al., 2016; Malekian et al., 2018) and pathogens of primary concern for amphibians (e.g., ranavirus, chytridiomycosis, and *Saprolegnia* spp.; Vilaça et al., 2020; Congram et al., 2022; Pavić et al., 2022). Sloughed cells, feces, gametes, and other sources of eDNA can be detected by sampling water from aquatic habitats, without requiring physical capture or direct observation of the organism itself (Taberlet et al., 2018; Pawlowski et al., 2020). These advancements are especially promising for low-density and cryptic amphibian species that pose challenges to conventional monitoring (Pilliod et al., 2013; Spear et al., 2015). Despite these advantages, some aspects of eDNA technology are still being refined. Occupancy can be underestimated for a variety of reasons, and current challenges include improving sampling designs by identifying ecologically relevant sampling periods (Eiler et al., 2018), determining sufficient sampling replication (Goldberg et al., 2018), and counteracting inhibition that interferes with DNA amplification (Jane et al., 2015).

We conducted eDNA and conventional amphibian surveys (visual encounter, breeding call, and larval dipnet surveys) to assess their relative performance in detecting nine anuran species in southern Ontario, Canada (Figure 1). We predicted that eDNA methods would detect greater species richness than conventional surveys, and that the efficacy of different methods would vary by species. Specifically, we expected that explosive breeders would have high detectability across all survey methods during their respective breeding seasons, whereas common, primarily aquatic species would have consistent detectability throughout the sampling period, with higher detectability using eDNA. More broadly, this study contributes to efforts to improve amphibian population monitoring through assessment, refinement, and ultimate adoption of new survey methods.

Methods

Study area and target species

The study was conducted during April to August 2016 across 30 waterbodies near Peterborough, Ontario, Canada (Figure 2; Supplementary Table 1). The local anuran community is composed of nine species with diverse breeding and larval periods: American toad (*Anaxyrus americanus*), gray treefrog (*Hyla versicolor*), spring peeper (*Pseudacris crucifer*), boreal chorus frog (*P. maculata*), American bullfrog (*Lithobates catesbeianus*), green frog (*L. clamitans*), northern leopard frog (*L. pipiens*), mink frog (*L. septentrionalis*), and wood frog (*L. sylvaticus*; Tattersall and Ultsch, 2008; Mills, 2016). During the study, all field equipment was decontaminated with 10% bleach solution and rinsed to destroy residual DNA (Wilson et al., 2014), thereby avoiding cross-contamination from amphibian pathogens known to be present in the area (Vilaça et al., 2020; Congram et al., 2022).

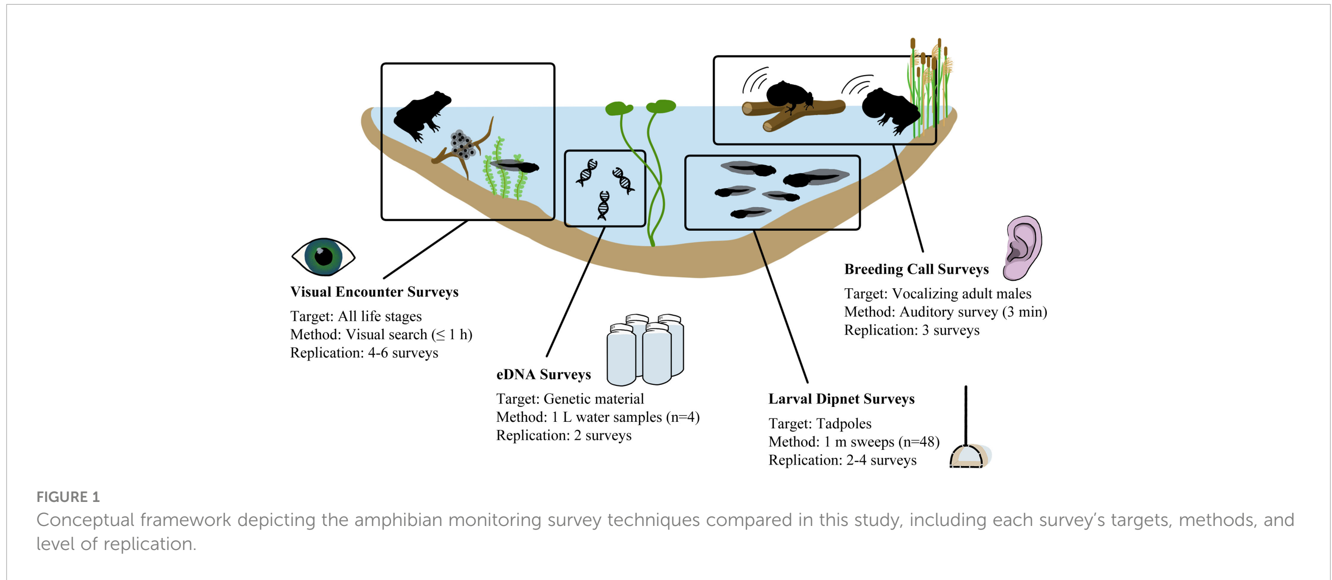

FIGURE 1
Conceptual framework depicting the amphibian monitoring survey techniques compared in this study, including each survey's targets, methods, and level of replication.

Conventional surveys

We surveyed anurans in waterbodies using visual encounter (visual), breeding call (call), and larval dipnet (dipnet) surveys; different versions of these approaches form the basis of many contemporary anuran monitoring programs (e.g., Muths et al., 2005; Badzinski et al., 2008; Mattfeldt et al., 2009). Visual surveys targeted all life stages during spring (20 April to 19 May), early summer (6 June to 8 July), and later summer (28 July to 19 August) survey periods. Representative samples were obtained by surveying twice within each survey period, at 2- to 10-day intervals (Crisafulli, 1997). Two-member crews searched littoral (water depth ≤ 1.25 m) and riparian zones (transitional habitat between the water's edge and a change in terrestrial vegetation/substrate, or up to 6 m from shore; Crisafulli, 1997) following parallel transects 2 m apart (Crump and Scott, 1994). We overturned all moveable cover and scanned water, leaf matter, and vegetation for organisms. When water was too deep to wade, we made observations along the waterbody's perimeter (Crisafulli, 1997). Surveys ended once all accessible habitats had been searched or 1 h had elapsed. For sites that could not be completely searched within 1 h, new transects were established during subsequent visits.

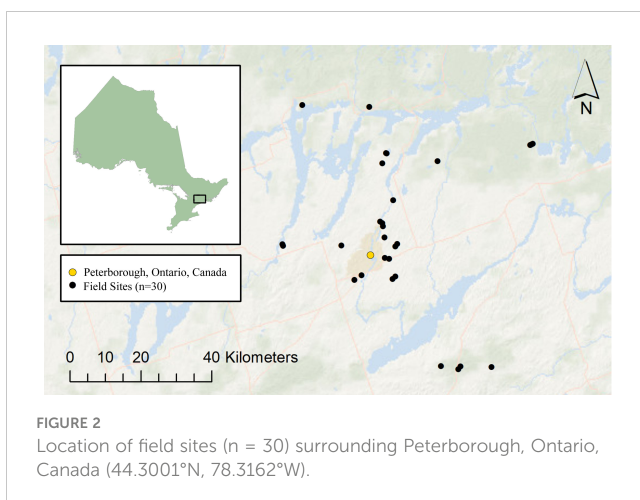

FIGURE 2
Location of field sites (n = 30) surrounding Peterborough, Ontario, Canada (44.3001°N, 78.3162°W).

Dipnet surveys targeted larvae and took place during the early summer and late summer sampling periods to ensure sufficient development of tadpoles for species identification. Two surveys were conducted within each sampling period, at 2- to 10-day intervals (Crisafulli, 1997). We established transects extending the length of the waterbody's perimeter or until 100 m was reached and divided each transect into 12 equal-length segments that were further divided into four equivalent depth zones (to a maximum possible depth of 1.4 m; Shaffer et al., 1994). An equal-volume 1-m sweep was taken haphazardly within each depth zone, and netted larvae were identified to species before being returned to their habitat at survey's end (Shaffer et al., 1994). This process was repeated for each of the 12 segments, totaling 48 sweeps per survey (Shaffer et al., 1994). Shallow water level precluded dipnetting at one site.

Call surveys targeted vocalizing adult males during their breeding period. To encompass breeding periods of all target species, 3-min point surveys (Shirose et al., 1997) were conducted three times at intervals of >15 days (Bird Studies Canada, 2009). Sampling periods spanned early breeding (29 April to 5 May), mid breeding (20 May to 24 May), and late breeding (18 June to 29 June). As per the Marsh Monitoring Program's Amphibian Survey Protocol, surveys ran between 30 min after sunset and midnight, with minimum air temperatures of 5°C, 10°C, and 17°C, respectively (Bird Studies Canada, 2009). We used two observers/survey to account for inter-observer variation (Pierce and Gutzwiller, 2007). Surveys were conducted at least 5 min after observer arrival at the site and composed of identifying anuran calls <100 m from their point location and spanning 180° in front of them (Bird Studies Canada, 2009). Calls were indexed as follows: no calls (0); calls were not overlapping and individuals could be counted (1); some calls overlapped and individuals could be estimated (2); and calls were continuous/overlapping (full chorus) and individuals could not be reliably estimated (3) (Bishop et al., 1997; Bird Studies Canada, 2009).

eDNA sample collection, filtration, and extraction

eDNA surveys were conducted twice per site, during the spring and early summer sampling periods. For each survey, we collected four 1-L surface water samples from each of the four cardinal directions or ≥ 20 m apart. Samples were transported in a cooler with ice, and each cooler contained a negative water sample (cooler blank).

Water samples were refrigerated at 4°C for ≤ 12 h (Hinlo et al., 2017) and filtered through 4.7-cm-diameter grade 691 1.5-μm Glass Microfibre Filters (VWR, Radnor, PA, USA) on a Filter Funnel Manifold (Pall Corporation, Port Washington, NY, USA) by an EZ-stream vacuum pump (Millipore Sigma, Billerica, MA, USA). Filtration equipment was sanitized using 10% bleach solution and thorough rinsing with deionized water, and 500 mL of deionized water was filtered pre- and post-sample filtration (filter blanks) to monitor for contamination (Wilson et al., 2014). Clogged filters were replaced ≤ 4 times, and detritus was removed prior to storage at −80°C (Wilson et al., 2014).

We extracted filters in halves using the DNeasy Blood & Tissue DNA Kit (Qiagen Inc., Valencia, California, USA; Hinlo et al., 2017). Following Goldberg et al. (2011), samples digested overnight in 360 μL of lysis buffer and 40 μL of Proteinase K (20 mg/mL), and undigested material was removed using QIAshredders (Qiagen Inc., Valencia, CA, USA). Extracted DNA was eluted twice with 100 μL of 70°C $T_{low}E$ (10 mM Tris, pH 7.5, 0.1 mM EDTA) to increase yields (Xue et al., 2009) and purified using the OneStep™ PCR Inhibitor Removal Kit (Zymo Research, Irvine, CA, USA). We stored recombined sample halves at −20°C.

qPCR eDNA assays

Standard curves were generated using two standard dilution series from 1 to 10^6 copies of synthetic oligonucleotides per 5-μL volume, designed as per Wilson et al. (2016). Species-specific amplicons (71–130 bp) within the mitochondrial cytochrome *c* subunit 1 (CO1) barcoding region were amplified using primers and probes developed by Beauclerc et al. (2019). Each 20 μL of qPCR reaction contained 10 μL of Taqman® Environmental Master Mix 2.0 (Applied Biosystems, Waltham, MA, USA), 0.4 μM forward primer, 0.4 μM reverse primer, 0.4 μM species-specific probe, 0.4 μM synthetic probe, 3.4 μL of ddH_2O, and 5 μL of extracted DNA. Samples were amplified in triplicate (three qPCR replicates) using the StepOnePlus™ Real-time PCR System (Applied Biosystems, Waltham, MA, USA) under the following parameters: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and a species-specific temperature for 1 min (Beauclerc et al., 2019).

eDNA validation

During preliminary tests, inconsistencies between eDNA and visual survey results from the previous year (2015) led us to suspect inhibition at one site. While internal positive controls (IPCs) did not indicate inhibition, assays returned unexpectedly low DNA yields when this site's samples were combined with uninhibited ones. Thus, we attempted to minimize inhibition by using the Zymo's OneStep™ PCR Inhibitor Removal Kit (Williams et al., 2017) and the Environmental Master Mix (Applied Biosystems, Waltham, MA, USA; Jane et al., 2015).

To minimize the likelihood of false-positive detections, a limit of detection (LOD) was calculated using threshold cycle (Ct) values from qPCR standard dilution series (n = 24–29) for each species (Hunter et al., 2017). The upper 95% confidence interval was selected as a conservative LOD, ranging from 0.13 to 0.47 copies per reaction depending on species (Supplementary Table 2). A limit of quantification such as that suggested by Serrao et al. (2018) was not calculated, as samples were not collected from wetlands where species were known to be absent *a priori*. To monitor for contamination, we also analyzed nine extraction negatives and 19–24 qPCR negatives for all species. Green frog was used as a proxy for additional contamination monitoring as it was detected at 100% of field sites, and we analyzed all cooler blanks (n = 28) and 40 randomly selected filter blanks (27% of total) for green frog DNA.

To assess variability among qPCR assays, a coefficient of variation (CV) was calculated for qPCR triplicates of positive eDNA samples. An eDNA detection required that at least one of the three qPCR replicates produced an amplification curve with a copy number above the species-specific LOD (see Dejean et al., 2012; Sigsgaard et al., 2015; Smart et al., 2015). Replicates that fell below the species-specific LOD were converted to zero-values, and triplicates were averaged to determine a final quantity for each sample. A site-level detection required that a species be detected in at least one sample.

Statistical analysis

We assessed whether sampling period affected prevalence of detections by scoring presence–absence species detections for conventional surveys (visual, n = 160; dipnet, n = 98; call, n = 90) and above species-specific LOD thresholds for all eDNA samples (n = 240). Generalized linear models used sampling period as the predictor and presence-absence as the response (package *stats*, glm function; R Core Team, 2019), using a type III likelihood ratio chi-square test (package *car*, Anova function; Fox and Weisberg, 2019). Statistical significance is a function of sample size and should not be conflated with biological significance (Gibbons and Pratt, 1975; Yoccoz, 1991); we report p-values to help contextualize results, but we also interpret trends in the data rather than exclusively relying on an arbitrary threshold p-value.

We assessed community similarity across time by calculating Jaccard similarity coefficients (*J*) between each sampling event (pooling data from all sites) using

$$J = \frac{C}{A + B - C}$$

where A is the number of species detected during sampling event a, B is the number of species detected during sampling event b, and C is the number of species detect in both (Jaccard, 1908; Real and

Vargas, 1996). For ephemeral waterbodies with seasonal drying, analyses were limited to periods when water was present.

We tested whether survey method influenced species prevalence assessment via generalized logistic mixed-effects regressions (GLMER) for each species with site-level detection (presence-absence) as the response, survey type as the predictor, and site as a random effect to account for repeated sampling (package lme4, GLMER function; Bates et al., 2019). We estimated marginal means (package *emmeans*, emmeans function with Tukey adjustment; Lenth, 2019) to determine p-values of each survey type. Boreal chorus frog, American bullfrog, and mink frog were excluded due to low sample size. In addition, low variance in green frog's prevalence among survey methods resulted in the GLMER failing to converge as expected. Thus, we used a chi-square test and *post-hoc* pairwise analysis (package *stats*, chisq.test function; R Core Team, 2019) as a substitute for a GLMER in this case; visual and eDNA surveys were analyzed as a single category because they yielded identical site-level detections for green frog.

Last, we used Jaccard coefficients to assess the community similarity inferred from the different detection methods. All analyses were conducted in R version 3.6.1 (R Core Team, 2019).

Results

Conventional surveys

Conventional survey methods differed in their species detections: Visual surveys resulted in detections for all nine target species, but call surveys failed to detect American bullfrog or mink frog at all, and dipnet surveys never detected American bullfrog, mink frog, or boreal chorus frog. Notably, visual surveys rarely detected boreal chorus frogs but successfully detected green frogs in all 30 waterbodies. In contrast, call and dipnet surveys each detected green frogs at only 73.3% of sites (Table 1).

Species were detected inconsistently through sampling periods. For visual surveys, gray treefrogs were detected most frequently in early summer, boreal chorus frogs only in spring, and green frogs least frequently in spring (Table 1). Of the six species detected via dipnet surveys, four were mostly or entirely captured in early summer (Table 1). Dipnet surveys yielded comparable detections across sampling periods for green frogs and a trend of more frequent American toad detections in early spring (Table 1). For call surveys, boreal chorus frogs were detected at only one site during early breeding. For the remaining six species detected via call surveys, each had significantly different detection rates between sampling periods (Table 1).

Expectedly, breeding call surveys exhibited high variability in community composition among temporally distinct sampling events (Figure 3). To a lesser extent, community composition from dipnet and visual surveys also shifted through time. In addition, for call and dipnet surveys, sampling events separated by shorter time intervals yielded more similar communities compared to more temporally distant events (Figure 3).

eDNA—qPCR assays and contamination monitoring

Amplification efficiencies for qPCR assays were within the generally accepted bounds of 90%–110% (Raymaekers et al., 2009), where the ideal amplification efficiency is 100% (Rogers-Broadway and Karteris, 2015). Primer-probe sets also performed as expected, detecting DNA at all standard concentrations. Standards containing one copy per reaction had the lowest amplification frequency for gray treefrog (8.7%), boreal chorus frog (12.0%), and American toad (16.7%), with all others amplifying in 32.0%–60.9% of replicates (Supplementary Table 2). Standards containing 10 copies per reaction amplified in between 79.2% and 100% of assays, and all other standards (10^2–10^6 copies per reaction) amplified in 100% of assays.

eDNA procedures yielded low cross-contamination, with green frog DNA detected in one of the 28 cooler blanks (2.47 copies per reaction) and two of the 40 filtration blanks (0.82 and 0.29 copies per reaction). These assays ran separately from field samples and may indicate contamination between filtration manifolds. Of the qPCR and extraction negatives, 0.27 copies per reaction of mink frog DNA was detected in a single qPCR negative—a concentration below the species' LOD (0.30 copies per reaction). This assay also detected mink frog DNA in three field samples: two detections were verified via another assay or conventional survey, and the third (2.2 copies per reaction) was both well above the LOD and taken from appropriate mink frog habitat. Thus, we included all samples in our analysis.

eDNA—field samples

Effectiveness of eDNA detection varied considerably among species and sites. Mean CV for qPCR triplicates ranged from 50.1% (American toad) to 173.2% (boreal chorus frog and American bullfrog; Supplementary Table 2). Across all species and sampling periods, intra-site variation ranged from 26.6% (n = 53) of detections occurring in one of the four samples compared to 34.7% (n = 69) in four of the four samples (Table 2). Species with notably low spatial repeatability included mink frog, which was never detected in more than two of the four spatial replicates at a given site. American bullfrog and boreal chorus frog were detected only in early summer and never in more than a single eDNA sample.

When using eDNA, most species were detected at between 30% and 80% of study sites (n = 30), with the exceptions of American toad (one site), boreal chorus frog (two sites), and green frog (all 30 sites; Table 3). Two species, American toad and gray treefrog, had greater detections in early summer than spring (both $p < 0.001$). All other species had comparable detections across sampling periods ($p > 0.05$). Notably, the inclusion of a second sampling period increased the number of occupied sites for all species (Table 3), with an evident shift in community composition between spring and early summer ($J = 0.52$).

TABLE 1 Outcome of conventional amphibian monitoring surveys across 30 waterbodies in southern Ontario, Canada.

Species	Total Sites	Survey Period 1		Survey Period 2		Survey Period 3		χ^2	p-value
Visual Encounter Surveys		(n = 30)	(n = 30)	(n = 30)	(n = 29)	(n = 21)	(n = 20)		
American toad	15	4	9	8	9	4	5	0.99	0.611
Gray treefrog	18	0	2	11	10	6	3	22.61	<0.001
Spring peeper	15	8	2	5	7	4	2	0.59	0.745
Boreal chorus frog	4	4	0	0	0	0	0	8.02	0.018
American bullfrog	1	0	0	1	0	0	0	-	-
Green frog	30	21	27	30	29	21	20	25.20	<0.001
Northern leopard frog	26	17	21	18	17	15	11	0.26	0.879
Mink frog	3	0	1	1	2	2	1	2.14	0.342
Wood frog	13	7	9	9	6	3	2	3.72	0.156
Larval Dipnet Surveys		(n = 29)	(n = 29)	(n = 20)	(n = 20)	N/A			
American toad	7	5	6	2	1	-		2.73	0.099
Gray treefrog	16	12	14	5	4	-		5.31	0.021
Spring peeper	13	11	8	3	2	-		5.60	0.018
Boreal chorus frog	0	0	0	0	0	-		-	-
American bullfrog	0	0	0	0	0	-		-	-
Green frog	22	15	13	12	11	-		0.81	0.368
Northern leopard frog	6	3	4	0	0	-		7.71	0.005
Mink frog	0	0	0	0	0	-		-	-
Wood frog	7	6	6	0	0	-		13.73	<0.001
Breeding Call Surveys		(n = 30)		(n = 30)		(n = 30)			
American toad	15	6		12		0		19.67	<0.001
Gray treefrog	20	0		13		13		26.10	<0.001
Spring peeper	27	27		23		0		71.55	<0.001
Boreal chorus frog	1	1		0		0		-	-
American bullfrog	0	0		0		0		-	-
Green frog	22	0		8		20		38.61	<0.001
Northern leopard frog	15	13		4		0		22.62	<0.001
Mink frog	0	0		0		0		-	-
Wood frog	6	6		0		0		14.06	<0.001

The number of positive sites is reported in total and classified according to survey period. Visual survey periods included spring (20 April to 19 May), early summer (6 June to 8 July), and late summer (28 July to 19 August); dipnet survey periods also included early summer and late summer. Call survey periods included early breeding (29 April to 5 May), mid breeding (20 May to 24 May), and late breeding (18 June to 29 June). The number of sites surveyed is in parentheses, and p-values denote whether prevalence differed among survey periods.

Methods comparison

eDNA surveys detected the greatest mean species richness across sites (4.37 ± 0.28 species, mean ± SE), outperforming both call and dipnet surveys (Figure 4). While estimated species richness was comparable between eDNA and visual surveys (4.17 ± 0.26 species), eDNA sampling achieved these results more efficiently, with fewer site visits. Call surveys (3.53 ± 0.25 species) and visual surveys (3.63 ± 0.27 species) detected comparable mean species richness by the third survey, after which the field effort for call surveys ended. Dipnet surveys yielded in the lowest species richness (2.45 ± 0.23 species).

Community composition was most similar between visual and eDNA surveys (J = 0.68), and least similar between dipnet and call surveys (J = 0.47). Visual and call surveys (J = 0.53), visual and dipnet surveys (J = 0.52), eDNA and dipnet surveys (J = 0.50), and

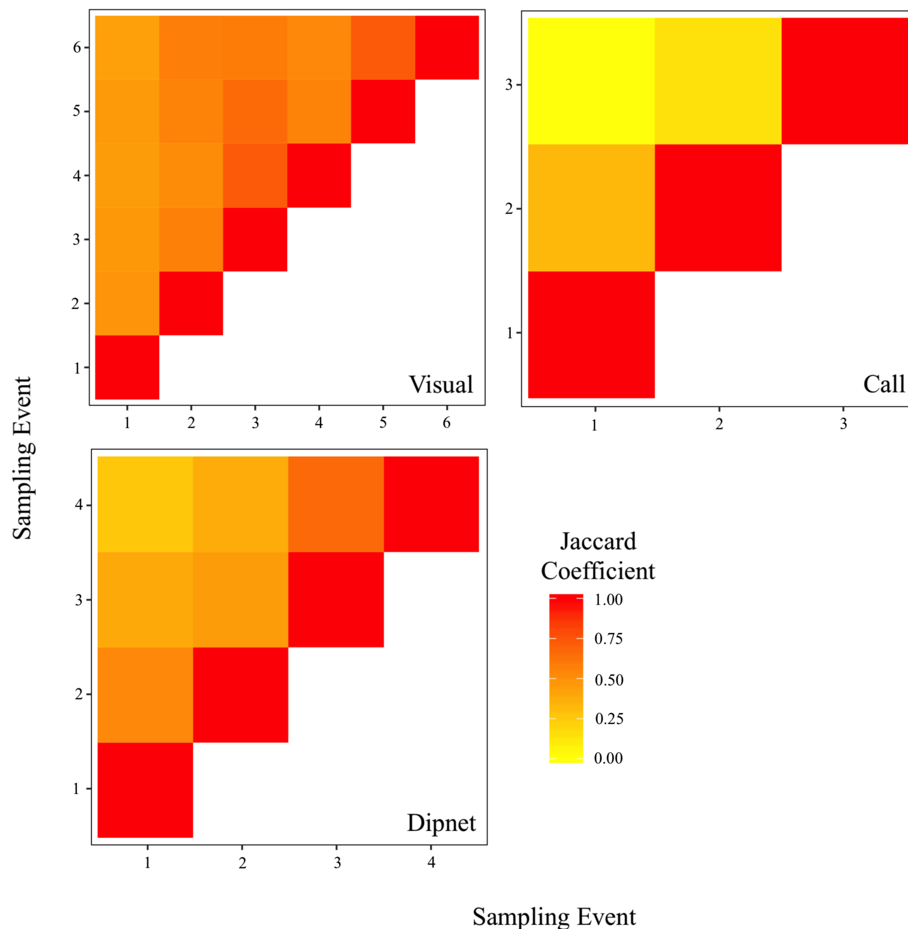

FIGURE 3
Community similarity between sampling events for three conventional amphibian monitoring methods. Surveys targeted nine amphibian species across 30 waterbodies in southern Ontario, Canada. Jaccard coefficients (*J*) are expressed on a gradient from 0.00 (completely dissimilar; yellow) to 1.00 (identical; red). Sampling events occurred during distinct survey periods: visual surveys 1 and 2 occurred in spring (20 April to 19 May); visual surveys 3 and 4, and dipnet surveys 1 and 2 occurred in early summer (6 June to 8 July); visual surveys 5 and 6, and dipnet surveys 3 and 4 occurred in late summer (28 July to 19 August); call surveys occurred during early breeding (29 April to 5 May), mid breeding (20 May to 24 May), and late breeding (18 June to 29 June).

TABLE 2 **Intra-site variation in eDNA amphibian monitoring surveys across 30 waterbodies in southern Ontario, Canada.**

	Spring Detections				Early Summer Detections			
Species	1 Sample	2 Samples	3 Samples	4 Samples	1 Sample	2 Samples	3 Samples	4 Samples
American toad	3	0	1	1	2	0	1	6
Gray treefrog	1	0	0	0	3	5	3	4
Spring peeper	5	9	2	5	1	7	2	6
Boreal chorus frog	0	0	0	0	2	0	0	0
American bullfrog	0	0	0	0	1	0	0	0
Green frog	4	3	6	14	4	5	5	16
Northern leopard frog	4	2	8	5	4	6	4	3
Mink frog	5	1	0	0	4	0	0	0
Wood frog	5	1	3	4	5	2	1	5

The number of positive sites from spring (20 April to 11 May 11) and early summer (6 June to 8 July) survey periods is reported by the number of water samples in which eDNA was detected (one of the four, two of the four, three of the four, or four of the four samples).

TABLE 3 Outcome of eDNA amphibian monitoring surveys across 30 waterbodies in southern Ontario, Canada.

Species	Spring		Early Summer		Cumulative		χ^2	p-value
	Positive Sites	Positive Samples	Positive Sites	Positive Samples	Positive Sites	Positive Samples		
American toad	5	10	9	29	10	39	11.46	<0.001
Gray treefrog	1	1	15	38	15	39	51.61	<0.001
Spring peeper	21	49	16	45	23	94	0.28	0.597
Boreal chorus frog	0	0	2	2	2	2	-	-
American bullfrog	0	0	1	1	1	1	-	-
Green frog	27	84	30	93	30	177	1.75	0.186
Northern leopard frog	19	52	17	40	24	92	2.54	0.111
Mink frog	6	7	4	4	9	11	0.87	0.352
Wood frog	13	32	13	32	17	64	−5.68E-43	1.000

The number of positive sites and positive samples is reported by survey period, including spring (20 April to 11 May), early summer (6 June to 8 July), and the cumulative total of both. P-values denote whether prevalence differed between the two survey periods.

eDNA and call surveys (J = 0.54) all exhibited differences in community composition.

Detection rates differed according to survey type for some species. Specifically, eDNA and call surveys both performed well for spring peepers, detecting them at 76.7% and 90.0% of sites, respectively, outperforming visual and dipnet surveys (Figure 5). eDNA and visual surveys detected green frogs and northern leopard frogs at high rates, outperforming dipnet and call surveys. In addition, eDNA performed the best for wood frogs, detecting them at 56.7% of sites, with visual surveys following at 43.3% (Table 4).

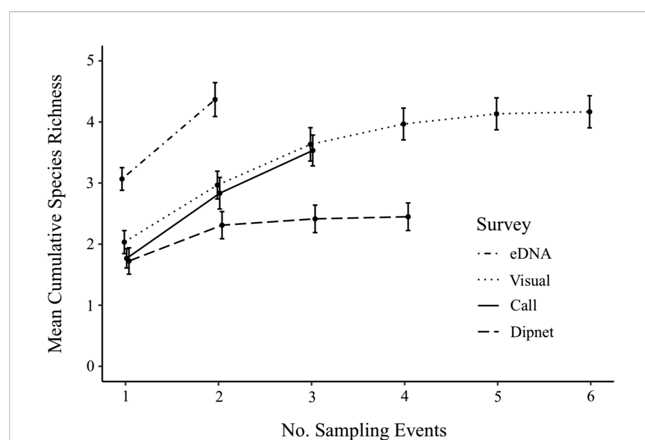

FIGURE 4
Cumulative species richness (± SE) from eDNA and conventional amphibian monitoring surveys across 30 waterbodies in southern Ontario, Canada. Surveys targeted nine amphibian species during distinct sampling periods: eDNA survey 1, and visual surveys 1 and 2 occurred in Spring (20 April to 19 May); eDNA survey 2, visual surveys 3 and 4, and dipnet surveys 1 and 2 occurred in early summer (6 June to 8 July); visual surveys 5 and 6, and dipnet surveys 3 and 4 occurred in late summer (28 July to 19 August); call surveys occurred during early breeding (29 April to 5 May), mid breeding (20 May to 24 May), and late breeding (18 June to 29 June).

Each method yielded unique detections, with eDNA leading with 14 unique detections across four species. Most notably, both mink frog and wood frog were detected solely by eDNA at six of the 30 sites each (Figure 6). Call surveys made 12 unique detections, compared to nine and one unique detection for visual and dipnet surveys, respectively. Notably, eDNA methods performed poorly when targeting American toad and gray treefrog, with eDNA detected at 47.6% (10 of 21) and 60.0% (15 of 25) of known occupied sites, respectively. Call and, to a lesser extent, visual surveys were most successful at detecting American toads and gray treefrogs (Figure 6).

Discussion

We found that the four amphibian survey methods differed in their ability to detect the presence of nine anuran species in southern Ontario, Canada. In general, eDNA and visual surveys performed comparably, with both detecting a greater species richness than call or dipnet surveys. Notably, eDNA methods were more efficient than visual surveys, detecting most species after fewer sampling events. Species detections via all methods varied through time, and each method produced false-negative detections that reflected survey limitations, timing of field sampling, or variation in species life history. These findings suggest that eDNA-based methods can serve as a valuable complement to conventional methods, improving the success and efficacy of amphibian monitoring programs (see Lacoursière-Roussel et al., 2016; Moss et al., 2022). However, we emphasize that eDNA-based methods require species- and system-specific testing and refinement before they can be fully integrated into amphibian monitoring programs.

Our finding that eDNA-based surveys detected more anuran species compared to dipnet and call surveys is consistent with previous research (see Smart et al., 2015; Lacoursière-Roussel et al.,

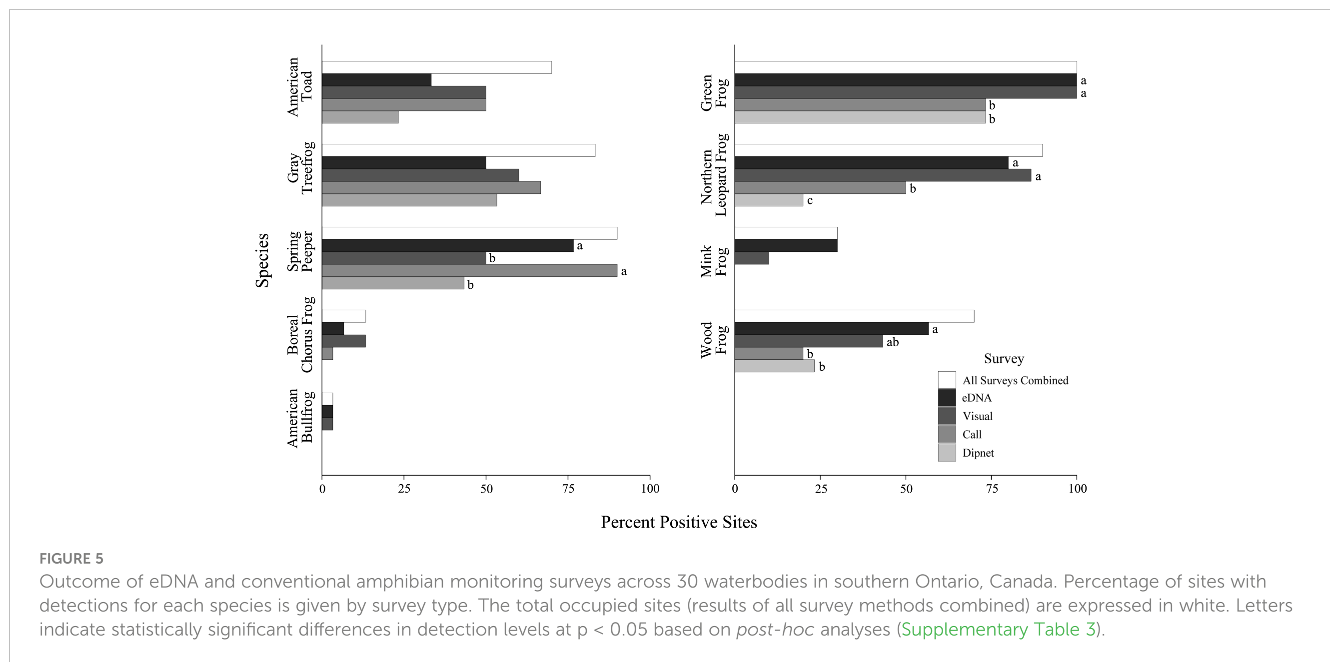

FIGURE 5
Outcome of eDNA and conventional amphibian monitoring surveys across 30 waterbodies in southern Ontario, Canada. Percentage of sites with detections for each species is given by survey type. The total occupied sites (results of all survey methods combined) are expressed in white. Letters indicate statistically significant differences in detection levels at $p < 0.05$ based on *post-hoc* analyses (Supplementary Table 3).

2016; Valentini et al., 2016). For example, Moss et al. (2022) found that eDNA-based methods were generally comparable or superior to conventional approaches when surveying amphibian presence-absence. We attribute the comparable species richness results of our eDNA and visual survey methods largely to the rigor and intensity of conventional surveys that we performed (including repeated sampling, and per site average cumulative survey durations of 4.26 h for visual surveys, 4.48 h for dipnet surveys, and 0.15 h for call surveys). Our finding that eDNA methods detected relatively high species richness with minimal field investment (two survey events) emphasizes that eDNA methods may be more effective than conventional survey methods particularly when time and personnel are limited. However, robust amphibian community monitoring may require application of multiple monitoring methods.

Conventional amphibian survey methods are broadly understood to have limitations, as they can underestimate population abundance due to false-negative detections and generally contribute bias and imprecision to species monitoring (Tanadini and Schmidt, 2011). Common sources of false-negative error from conventional surveys include low population densities, variability in environmental and habitat conditions, species-specific behavioral traits, and observer error due to lack of experience or training (see Lotz and Allen, 2007; Tanadini and Schmidt, 2011; Asad et al., 2020; Hammond et al., 2021). In contrast, eDNA-based sampling is known to detect amphibians at low densities (Pilliod et al., 2013; Sepulveda et al., 2019), is less biased by cryptic and elusive behavior (see Kaganer et al., 2022), and can be performed with minimal expertise or training (e.g., Biggs et al., 2015). However, eDNA's unique utility necessitates novel considerations. Despite relatively high species richness results, our eDNA detections were highly variable among the four water samples collected per sampling event. This finding underscores eDNA's

TABLE 4 Outcome of conventional and eDNA amphibian monitoring surveys across 30 waterbodies in southern Ontario, Canada.

Species		Survey Method					
	Total Sites	eDNA	Visual	Call	Dipnet	χ^2	p-value
American toad	21	10	15	15	7	7.58	0.056
Gray treefrog	25	15	18	20	16	3.17	0.366
Spring peeper	27	23	15	27	13	13.09	0.004
Boreal chorus frog	4	2	4	1	0	–	–
American bullfrog	1	1	1	0	0	–	–
Green frog	30	30	30	22	22	9.24	0.010
Northern leopard frog	27	24	26	15	6	18.44	<0.001
Mink frog	9	9	3	0	0	–	–
Wood frog	21	17	13	6	7	12.67	0.005

The number of positive sites is reported by survey method. P-values denote whether prevalence differed among survey methods.

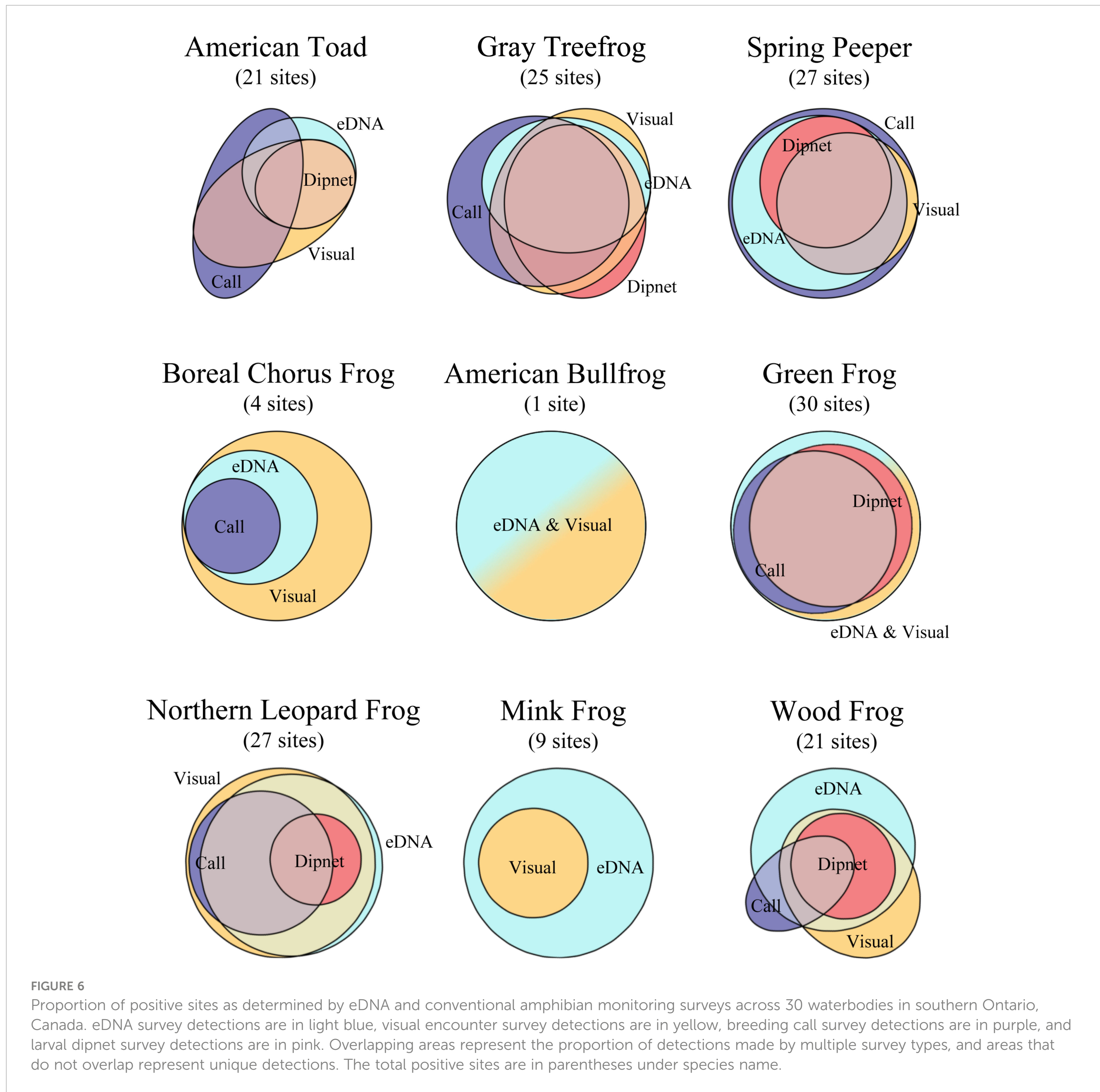

FIGURE 6
Proportion of positive sites as determined by eDNA and conventional amphibian monitoring surveys across 30 waterbodies in southern Ontario, Canada. eDNA survey detections are in light blue, visual encounter survey detections are in yellow, breeding call survey detections are in purple, and larval dipnet survey detections are in pink. Overlapping areas represent the proportion of detections made by multiple survey types, and areas that do not overlap represent unique detections. The total positive sites are in parentheses under species name.

sensitivity to complex natural processes and environmental factors (see Strickler et al., 2015; Harrison et al., 2019). Specifically, sampling heterogeneity is influenced by factors affecting eDNA dispersion, including wetland size, amphibian population and distribution, and water flow conditions (Strickler et al., 2015; Goldberg et al., 2018; Wood et al., 2021). High environmental variation is known to exist within localized amphibian breeding sites (Beentjes et al., 2019; Congram et al., 2022), necessitating thorough consideration of eDNA sampling heterogeneity during study design.

In addition, natural environments contain inhibitors (tannins, proteins, humic compounds, etc.) that can further confound eDNA survey results by reducing the efficacy of qPCR assays (Lance and Guan, 2020; Sidstedt et al., 2020). Constraints related to inhibition are common to eDNA studies (e.g., Sigsgaard et al., 2015; Adams et al., 2019), and we urge consideration of inhibition-reducing techniques during eDNA procedural optimization and validation. We used preliminary IPCs, an inhibitor removal kit (Williams et al., 2017), a qPCR master mix with low sensitivity to PCR inhibitors (Jane et al., 2015), and a pre-established/optimized extraction procedure (Goldberg et al., 2011) to minimize inhibition in our samples. Despite this concerted effort, our species detections via eDNA methods were still imperfect (see Supplementary Table 1). Our finding that all monitoring methods produced false-negative detections reflects the natural limitations of each method. It follows that a robust approach to monitoring amphibian communities should limit false negatives by using multiple survey methods, inclusive of eDNA and conventional surveys when time and personnel are available.

We found prominent temporal variation in our eDNA survey results, which likely reflects the substantive ecological differences

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across the anuran community that we targeted (see also Takahara et al., 2020). Seasonal changes in species life history, including variation in breeding, habitat use, and larval characteristics, are known to affect eDNA detection (Goldberg et al., 2011; Takahara et al., 2020). Furthermore, movement and behavior are known to be variable even within species, resulting in different breeding onsets and larval period durations among even neighboring wetlands (Benard and Greenwald, 2023). We attempted to target as many species as possible by incorporating two temporally distinct sampling periods into our study design. Much like previous studies of temporal eDNA replication (see Beentjes et al., 2019; Troth et al., 2021), we found that the addition of a second sampling period improved detection outcomes for most species and increased species richness results by an average of approximately one species per site. Our findings highlight that amphibian eDNA monitoring programs, especially at the community level, will benefit from repeated sampling events.

In addition, species-specific differences in physiology and behavior can lead to variable eDNA detections even among closely related species; Breton et al. (2022) found that wood frog (*L. sylvatica*) and northern leopard frog (*L. pipiens*) tadpoles produced detectable DNA at different rates under identical controlled conditions. While the biological processes behind this finding are lesser known, Breton et al. (2022) suggest the potential for a physiological origin. Building on this work, we found that temporal variation in eDNA detections *in situ* largely reflected species-specific seasonal activity patterns. In particular, eDNA surveys detected mid-season breeders (American toad and gray treefrog) less often in spring than in early summer (near their peak larval period), with eDNA surveys failing to outperform conventional methods for these species. In contrast, eDNA detection rates were consistent across time for early breeders, as well as late breeders with overwintering tadpoles; this is likely explained by their larval periods encompassing both of our eDNA survey periods. Finally, eDNA surveys also detected wood frogs (explosive breeders that can be challenging to monitor via conventional methods; De Solla et al., 2006) outside of their restrictive breeding window, as tadpole genetic material could be captured long after the adults had resumed their terrestrial lifestyle. While eDNA sampling has been shown to detect amphibians outside of active breeding and circadian periods (Rees et al., 2014; Eiler et al., 2018), a rapid loss of detectable eDNA has also been observed after tadpoles have been removed from a system (Breton et al., 2022). Therefore, our results suggest that ideal eDNA sampling windows for semi-terrestrial species are still restrictive, and survey optimization should involve identifying sampling periods that coincide with peak larval periods.

Finally, we recommend some additional methodological considerations that will improve the reliability and broaden the utility of eDNA monitoring. Many eDNA protocols now verify positive samples with low repeatability via reamplification (e.g., Goldberg et al., 2018; Kaganer et al., 2022); such efforts to improve reliability can be bolstered by additional means including our use of species-specific LODs (Hunter et al., 2017), synthetic oligonucleotides to eliminate contamination from qPCR controls (Wilson et al., 2016), and monitoring for contamination. In addition, recent advancements such as high-volume water sampling (Schabacker et al., 2020) and eDNA metabarcoding (e.g., Sasso et al., 2017; Taberlet et al., 2018) can greatly enhance the efficacy of comprehensive community surveys. Although eDNA metabarcoding is sensitive and particularly efficient for monitoring numerous and diverse targets (Deiner et al., 2017), targeted single-species qPCR testing can be more sensitive for detecting rare amphibians (Moss et al., 2022). While their utilities differ, both eDNA-based methods provide valuable ecological information. In particular, the suitability of eDNA-based presence–absence data in occupancy modeling (e.g., Smith and Goldberg, 2022) offers a useful tool for managers when developing conservation strategies.

To conclude, we highlight that best success in amphibian community monitoring can be achieved by adopting multiple survey techniques, including both conventional and eDNA-based methods. Similarly to other studies (Takahara et al., 2020; Svenningsen et al., 2022), we have shown that no single survey method reliably detected all amphibian species under a wide range of sampling conditions. Before eDNA-based methods can reliably replace conventional survey methods in amphibian monitoring programs, additional research into the sources of variation in species detectability and optimization of survey protocols must be a priority. Field and laboratory protocols should be optimized at the scale of individual studies or systems to account for species-specific, site-specific, and lab-specific sources of detection error. Adoption of these recommendations will help ensure that amphibian monitoring programs are supported by the best-available survey methods and thereby improve our ability to reliably assess changes in the prevalence and distribution of this imperilled group of organisms.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: Borealis, https://doi.org/10.5683/SP3/DCUGTW.

Ethics statement

The animal study was reviewed and approved by Trent University's Animal Care Committee.

Author contributions

All authors: conception and design. MW, BB, and AB: field data collection. MW, BB, and STV: laboratory analyses. MW: statistical analyses. MW, CW, and DM: drafted the manuscript. All authors: manuscript revision. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2023.1179158/full#supplementary-material

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