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ORIGINAL ARTICLE

Testing the effectiveness of environmental DNA (eDNA) to quantify larval amphibian abundance

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Abstract

Environmental DNA (eDNA) monitoring is rapidly becoming an established approach for detecting the presence of aquatic organisms and may also be useful for indexing or estimating species abundance. However, the link between eDNA concentration and abundance of individuals (i.e., density or biomass) remains tenuous and may vary widely across species and environmental conditions. We investigated the relationship between eDNA concentration and abundance in two common and closely related amphibians in eastern North America, the wood frog (*Rana sylvatica*), and northern leopard frog (*R. pipiens*). We manipulated tadpole density in 80-L mesocosms and documented the relationship between tadpole density and biomass and eDNA concentration through time. The two species differed in the amount of detectible genetic material produced, despite having comparable biomass. Concentration of eDNA increased with tadpole numbers and was primarily correlated with tadpole density in wood frogs and biomass in leopard frogs. eDNA degradation rates were rapid and comparable between species, with tadpoles becoming indetectable within 5 days post-removal from the mesocosm, irrespective of tadpole density. Overall, our findings support that eDNA concentration has potential for tracking amphibian abundance in wetlands, but that indices of abundance are likely to be coarse and species-specific calibration will be required. Future research should address how biotic and abiotic factors influence eDNA production, degradation, and recovery across species and through time before relying on eDNA for monitoring amphibian abundance in nature.

1 | INTRODUCTION

Detecting rare and elusive species in their natural environment is becoming an increasing priority in conservation and management, especially in the context of monitoring patterns of biodiversity loss and ecosystem structure in changing environments (Díaz et al., 2006; Johnson et al., 2017; Mace et al., 2012). Recent efforts in biodiversity monitoring highlight the need to develop reliable survey techniques for documenting patterns of species occurrence and abundance (e.g., Ceballos et al., 2015; Hoffmann et al., 2010; Livore et al., 2021). These efforts are especially relevant for rare or cryptic species, which typically have low detection probability in the field and thus present challenges for reliable assessment (Butchart et al., 2010; Evans et al., 2016; Pomezanski, 2021). For example, cryptic aquatic vertebrates are notably difficult to survey using traditional methods such as visual encounter or dipnet surveys (Gunzburger, 2007). Efforts to improve species detection and numerical estimation include improved monitoring systems for aquatic vertebrates that tend to be constrained by precision, accuracy, and the spatial and temporal extent of monitoring (Tillotson et al., 2018). Accordingly, there is a high priority to develop robust tools that accurately and effectively monitor different species to obtain estimates of spatial distribution and population size.

Environmental DNA (eDNA) refers to genetic material suspended in water or submerged in sediment that can be used to track biodiversity (Beng & Corlett, 2020; Ficetola et al., 2008). As such, eDNA may be relevant to biodiversity monitoring programs and in the last decade, this approach has moved beyond proof-of-concept and is now increasingly used for tracking rare or invasive species in real-world settings (Biggs et al., 2015; Jerde et al., 2013; Sigsgaard et al., 2015; Wang et al., 2021). However, despite these advances, eDNA technology remains largely focused on detecting species presence/absence (Goldberg et al., 2011; Laramie et al., 2015; Valentini et al., 2016; Vilaça et al., 2020). Studies often report high variation in eDNA concentration from collected samples, offering an incomplete understanding of how environmental factors affect eDNA and how these may relate to population density of the target species (Klymus et al., 2015; Pilliod et al., 2014; Wilcox et al., 2016; Yates et al., 2019). Despite these uncertainties, several studies report positive relationships between eDNA concentration and species abundance and/or biomass (e.g., Kelly et al., 2014; Klymus et al., 2015; Lacoursière-Roussel, Rosabal, & Bernatchez, 2016; Pilliod et al., 2013; Takahara et al., 2012; Tréguier et al., 2014), although there remains uncertainty as to which abundance estimator is the most appropriate and best-fit. In a recent meta-analysis, Yates et al. (2019) revealed a preponderance of studies yielding equivocal results in terms of linking eDNA concentration to species abundance metrics, highlighting the overall state of uncertainty surrounding this area of investigation. Thus, while it is not surprising that there is interest in extending eDNA-based monitoring to infer levels of abundance of aquatic organisms, it remains premature to conclude that the method is sufficiently robust to be widely used for such purposes.

Although high variability in eDNA concentration is observed among species with divergent life history traits (e.g., Lacoursière-Roussel, Côté, et al., 2016; Lacoursière-Roussel, Rosabal, & Bernatchez, 2016; Pilliod et al., 2013), it is less clear whether closely related species with similar life histories show low variability in eDNA concentration. Indeed, most eDNA validation studies focus on single species, though differences in amount of eDNA produced or its pattern of release or persistence in the environment may vary according to taxon. This uncertainty is important if eDNA will be used to monitor aquatic amphibians, given that many species worldwide are in decline and require closer attention (Green et al., 2020; Stuart et al., 2004). Accordingly, it is imperative that eDNA detection methods be fully investigated and validated to establish a reliable baseline for future amphibian monitoring.

We evaluated the reliability of eDNA for determining the abundance of tadpoles of two closely related and widely distributed amphibian species that served as models of the aquatic amphibian community in eastern North America. Wood frog (*Rana sylvatica*) and northern leopard frog (*R. pipiens*) distributions are widespread in permanent and ephemeral wetlands in central Ontario, where at a finer scale their abundance can vary considerably across space and time (MacCulloch, 2002). We conducted a mesocosm experiment to determine the relationship between eDNA and tadpole abundance and predicted that similar size and larval life history of *R. sylvatica* and *R. pipiens* would lead to eDNA production that is i) comparable for both species, and ii) related to tadpole density or biomass for both species. We also predicted that iii) species would exhibit similar relationships between eDNA concentration, eDNA degradation, and numerical abundance (i.e., density or biomass) metrics. Ultimately, this study serves as an important baseline for the broader adoption of eDNA as a tool to index amphibian populations in natural wetlands in eastern North America.

2 | METHODS

2.1 | Study site

In April 2016, we collected six egg masses of *R. pipiens* and *R. sylvatica* near Peterborough, ON (44°28′15.7″N, 78° 16′59.7″W). Clutches were pooled and eggs were hatched and reared in 10-L bins filled with aged tap water. Once hatched tadpoles reached Gosner stage 25 (approximately 7–10 days, Gosner, 1960), they were transferred to 80-L mesocosms housed outdoors and inoculated with de-tannined tree leaves (Hossie & Murray, 2010). Tadpoles were fed ground algae discs (Wardley Algae Discs™) ad libitum, and water levels were maintained at 80 L with aged tap water, in compliance with Trent University Animal Care Protocol #24250.

2.2 | Mesocosm experiment

We populated randomly assigned species-specific mesocosms at densities of 1, 4, 16, and 64 tadpoles per container, with six replicates per density. Four mesocosms remained unpopulated (mesocosm blanks), to

assess potential contamination from sampling. We collected 1-L water samples in Nalgene bottles 24 h after tadpole introduction, and twice weekly thereafter for a total of 14 water samples per mesocosm for *R. pipiens* (N_{total} = 336 water samples) and 10 water samples per mesocosm for *R. sylvatica* (N_{total} = 240 water samples). Visual assessment of mesocosms was conducted daily and dead individuals were promptly removed. We refrained from sampling tadpole biomass and density for 2 weeks after tadpole introduction to mesocosms, to allow for proper acclimation as well as degradation of any genetic material released from early deaths (Maruyama et al., 2014; Thomsen et al., 2012). The density and total biomass of tadpoles in a mesocosm (hereafter "biomass") were then measured weekly by capturing all tadpoles within each mesocosm. Density values were adjusted to reflect mortalities that were recorded prior to water sample collection so that there was one density observation for each collected water sample. While density was determined for all mesocosms during each capture period, biomass was measured on rotation for a single replicate per density per species to reduce handling stress and mortality. Species-specific biomass was then extrapolated to other density replicates. While we acknowledge that the lack of replicate biomass measurements per time period reduces variation in that metric, biomass measurement was seen as stressful to tadpoles and thus we sought to obtain a representative sample by sampling rotationally through time. Once ~10% of tadpoles reached metamorphosis (emergence of first forelimb), the experiment ended for all treatments. We continued collecting water samples for 3 weeks post-removal, collecting seven samples per mesocosm during that time, to assess degradation of genetic material. All water samples were refrigerated at 4°C until filtration.

2.3 | Sample filtration and DNA extraction

Sample filtration, DNA extraction, and quantitative PCR (qPCR) analysis were conducted in separate rooms to avoid contamination. All samples were filtered within 12 h of sample collection through a three-manifold filtering unit (EZ-Stream™ vacuum pump) using 1.5 µm pore size 691 glass microfiber filters (VWR). Filter funnels and their bases were immersed in a 10% bleach solution for 15 minutes, then rinsed with deionized water between each sample to minimize inter-sample contamination. Likewise, the manifold and counter were bleached, and forceps were flame-sterilized in 70% ethanol. Filters were placed in 2 ml internal-thread cryogenic vials (PROGENE) and stored at −80 °C until DNA extraction. Prior to and postsample filtration, 500 ml of deionized water was filtered to check for potential contamination (i.e., filter blanks).

Filters were extracted using Qiashredder/DNeasy Blood & Tissue kits (Qiagen), following Goldberg et al. (2011), with the following modifications: Full filter papers were extracted in halves, and ATL (lysis buffer) and proteinase K were doubled to accommodate the filter. After buffer AL and ethanol precipitation, sample halves were recombined. Samples were eluted twice with 100 µl of $T_{low}E$ to maximize yield (Xue et al., 2009), for a total elution volume of 200 µl. After extraction, samples were stored at 4°C until further analysis. Extraction negatives were extracted alongside filters to control for contamination.

2.4 | qPCRs

All samples were assayed within 6 h of extraction, with 3 test replicates run per sample. Each qPCR was run with 20 µl of the following cocktail: 10 µl of TaqMan® Environmental PCR Master Mix 2.0 (Applied Biosystems), 0.2 µM reverse primer, 0.2 µM forward primer, 0.2 µM species-specific probe (Beauclerc et al., 2019), 0.2 µM synthetic control probe, 3.4 µl ddH_2O, and 5 µl of undiluted DNA. Primer pairs and probes developed by Beauclerc et al. (2019) were shown to be species-specific, with reported efficiencies of 96.8% and 102.5% for *R. sylvatica* and *R. pipiens*, respectively. Two negative controls were included per 96-well plate. StepOnePlus thermocycling conditions for Environmental Master Mix were as follows: initial warm-up for 2 min at 50°C, followed by 10 minutes of denaturation at 94°C, and a 2-step process of 15 seconds of denaturation at 94 °C and 1 min of annealing at 60°C, repeated for 40 cycles.

Synthetic oligonucleotide positive controls for both species were developed as per Wilson et al. (2016) to serve as quantitative standards and enable identification of potential false positives resulting from laboratory contamination. The oligonucleotide consists of a sequence of the COI region of each species (as per Beauclerc et al., 2019) with an 18-bp insert designed by Wilson et al. (2016) placed between the species-specific probe and the reverse primer regions. The sequence of the insert is not found within amplified region of the species and has a corresponding TaqMan probe allowing for its detection to only be associated with the control. Synthetic qPCR standards were developed by resuspending oligonucleotides in sterile water as per manufacturer instructions and diluted in $T_{low}E$ to create a serial dilution of 10^6, 10^5, 10^4, 10^3, 10^2, 10^1, and 1 target copy per 5 µl volume. Two serial dilution series were included per 96-well plate. If the synthetic probe was detected in the negatives, the plate was re-run. Inhibition was evaluated by running a TaqMan exogenous internal positive control (IPC) qPCR assay (Life Technologies, Carlsbad, California) following the manufacturer's protocol adjusted to a 20 µl reaction volume. No evidence of inhibition was detected in the samples.

2.5 | Statistical analyses

To minimize type I and type II error (i.e., false-positive and false-negative detections), we established qPCR thresholds that included a limit of detection (LOD) and limit of quantification (LOQ). LOD was determined following Hunter et al. (2017), which uses the cycle threshold (Ct) values of the qPCR standard dilution series of all sample runs (n = 27 and 31 for *R. sylvatica* and *R. pipiens*, respectively) to determine the lowest amount of target species DNA that is detectable and distinguishable from the concentration plateau. For LOQ, we used a receiver operator characteristic (ROC) approach to determine

TABLE 1 Threshold diagnostics for *Rana sylvatica* and *R. pipiens* samples, respectively, at 1, 3, 6.5, and 10 target DNA copies/reaction

Threshold (copies/reaction)	True negative	True positive	False positive	False negative	Sensitivity	Specificity
R. sylvatica						
1	36.6% (144)	75.3% (236)	2.3% (9)	1% (4)	98.3%	94.1%
3	38.4% (151)	59.0% (232)	0.5% (2)	2% (8)	96.7%	98.7%
6.5	38.7% (152)	57.8% (227)	0.2% (1)	3.3% (13)	94.6%	99.3%
10	38.9% (153)	56.5% (222)	0% (0)	4.6% (18)	92.5%	100%
R. pipiens						
1	18.6% (107)	80.9% (334)	0% (0)	0.5% (2)	99.40%	100%
3	18.6% (107)	80.9% (334)	0% (0)	0.5% (2)	99.40%	100%
6.5	18.6% (107)	80.1% (331)	0% (0)	1.2% (5)	98.50%	100%
10	18.6% (107)	79.4% (328)	0% (0)	1.9% (8)	97.60%	100%

Note: Numbers in brackets represent the number of samples classified as true negative, true positive, false positive, and false negative, respectively.

optimal copy/reaction thresholds for both larval amphibian species. This method, unlike others (e.g., Bustin et al., 2009; Hunter et al., 2017), assesses sensitivity (i.e., true-positive target detections in proportion to false-negative target detections) and specificity (i.e., true-negative target detections in proportion to false-positive target detection in control samples) of eDNA detections at each candidate data threshold by quantifying tradeoffs between acceptance or rejection of false positives and false negatives at any given threshold (Serrao et al., 2017). Sensitivity and specificity of mesocosm samples and negative controls (i.e., unpopulated mesocosm samples, cooler, filtration, and extraction controls) were assessed at LOQ thresholds 1, 3, 6.5, and 10 copies/reaction following Serrao et al. (2017). Samples that fell below determined thresholds were classified as non-detections.

Analysis of covariance (ANCOVA) was used to determine the effects of species (*R. sylvatica*, *R. pipiens*), time (five and 7 weeks for *R. sylvatica* and *R. pipiens*, respectively), and their interaction, on biomass and eDNA concentration; the latter was log-transformed prior to analysis to meet assumptions of normality. The ANCOVA was run using the "stats" package in R version 4.1.2 (R Core Team, 2021). Linear mixed-effects models (LMM) were constructed using the "lme4" package in R version 4.1.2 (Bates et al., 2015; R Core Team, 2021) to examine the relationship between biomass, density, allometrically scaled mass (ASM), day, and eDNA copy number. For the ASM models, individual mass estimates were obtained by dividing the mesocosm biomass by the observed density. We then determined the optimal scaling coefficient for the ASM models by iteratively fitting linear mixed-effects models with scaling coefficients that ranged from 0 to 1 at intervals of 0.01 and comparing the models using AIC. Following the observed results of Yates, Glaser, et al. (2021), Yates, Wilcox, et al. (2021), the AIC values observed for the range of scaling coefficients should have an upward parabolic shape, with the lowest AIC value indicating the scaling coefficient with the best fit (i.e., the "optimal scaling coefficient"; Figure S1). To meet the assumption of linearity, we log-transformed biomass, ASM, and density prior to analysis (Kennedy et al., 1992). Mesocosm was treated as a random effect. We used Akaike's information criterion (AIC) to determine the best model for each species, where well-supported models were defined as those having ΔAIC <2.0 (Johnson & Omland, 2004). We used AIC weight (w_i) to compare models, and conditional R^2 determined goodness-of-fit.

We calculated *R. sylvatica* and *R. pipiens* eDNA degradation using an exponential decay model fit to copy number using nonlinear least squares regression in the "stats" package in R version 4.1.2 (R Core Team, 2021). The exponential decay model was

$$N_t = N_0 e^{-rt}$$

where N_t = concentration of eDNA in copies/reaction, N_0 = initial eDNA concentration at time 0, t = time (days) since tadpole removal and r = decay rate. Effects of species (*R. sylvatica*, *R. pipiens*), density (1, 4, 16, or 64 tadpoles/mesocosm), time (day), and the interaction between density and day were estimated using an ANCOVA.

3 | RESULTS

3.1 | Limit of detection/limit of quantification

The limit of detection (LOD) for *R. sylvatica* and *R. pipiens* was estimated at 0.16 (0.10–0.25, 95% CI) and 0.33 (0.23–0.47, 95% CI) copies/reaction, respectively. In determining the limit of quantification (LOQ), we found that *R. sylvatica* sample sensitivity was highest (98.3%) at one copy/reaction, while specificity was lowest (94.1%, Table 1). By contrast, at 10 copies/reaction sensitivity was lowest (92.5%), and specificity was highest (100%). Accordingly, we chose the intermediate threshold of 3 copies/reaction to limit both false-positive (0.5%) and false-negative (2.0%) rates, while optimizing the ratio between sensitivity (96.7%) and specificity (98.7%, Table 1). Sensitivity for *R. pipiens* samples was highest (99.4%) at one copy/reaction, while the lowest (97.6%) was at 10 copies/reaction (Table 1). As no false positives were detected at any threshold for *R. pipiens*, 100% specificity was maintained at all thresholds. The false-negative rate for *R. pipiens* at 10 copies/reaction was highest (1.9%)

of all thresholds, which was similar to the false negative rate of *R. sylvatica* samples (2.0%) at 3 copies/reaction. Therefore, although LOD and ROC calculations both justified a lower data acceptance threshold, we chose a conservative threshold of 10 copies/reaction for LOQ in *R. pipiens* to facilitate comparison between species. We note that the optimal *R. pipiens* threshold identified by ROC was 1 copy/reaction; however, lower thresholds yielded qualitatively similar results to those presented here.

3.2 | Mesocosm experiment

Tadpole mortality was low throughout the experiment but differed between species and density treatments. On average, 1.46 [0.66, 2.38] (skewness = 1.55, kurtosis = 4.00; mean [bootstrapped 95% CI]) *R. pipiens* tadpoles died per mesocosm over the course of the experiment, compared to 0.75 [0.33, 1.17] (skewness = 1.42, kurtosis = 3.53) tadpoles for *R. sylvatica* mesocosms. For both species, more tadpoles died in the 64 tadpoles/mesocosm treatments (*R. pipiens* – 5.33 [4.17, 6.5] (skewness = 0.19, kurtosis = 1.51) tadpoles/mesocosm, *R. sylvatica* – 2.33 [1.5, 3.17] (skewness = 0.05, kurtosis = 1.61) tadpoles/mesocosm) than in the lower density treatments (all <1 tadpole/mesocosm on average). Given that the measured density was different for some mesocosms than the starting density treatments, we have specified which density value was used in the analyses below by using "starting density" to refer to the initial treatments established in each mesocosm and "observed density" to refer to the number of tadpoles recorded in a mesocosm, accounting for mortality.

We observed an overall increase in *R. sylvatica* and *R. pipiens* eDNA concentrations in all densities over the course of the experiment (Figures S2 and S3). We found no difference in the overall biomass of *R. sylvatica* and *R. pipiens* in mesocosms over the course of the experiment ($F_{1,572}$ = 0.04, p = 0.85), but observed different rates of eDNA production between the two species ($F_{1,572}$ = 25.48, p < 0.001). *R. pipiens* produced almost twice the detectible genetic material after controlling for study duration (estimated marginal mean [95% CI] copies/reaction, *R. sylvatica*: 246 [194, 312]; *R. pipiens*: 571 [467, 696]). Biomass and eDNA concentrations both increased over the course of the experiment (*R. sylvatica*: $F_{1,572}$ = 199.55, p < 0.001; *R. pipiens*: $F_{1,572}$ = 72.97, p < 0.001). Interestingly, *R. sylvatica* and *R. pipiens* were comparable in their rate of change of eDNA concentration through time, across density treatments ($F_{1,572}$ = 1.27, p = 0.27, Figures S2 and S3).

Overall, 98.3% (n = 236) of *R. sylvatica* eDNA samples successfully amplified in all three test replicates. Of all extracted qPCR negative controls (n = 136, includes mesocosm, cooler, and filter blanks, and extraction and qPCR negatives), 92.9% contained no target DNA. Detection levels in the negative control samples for this species ranged from 0 to 6.45 copies/reaction, with a median of 0 copies/reaction (mean copies/reaction [bootstrapped 95% CI]; 0.22 [0.11, 0.35], skewness = 5.52, kurtosis = 36.52). We saw a similar positive detection rate with *R. pipiens* eDNA (99.4%, n = 336), and 100% of extracted qPCR negative controls (n = 81) contained no target DNA.

Of samples taken from populated mesocosms, 2.9% (n = 7) of *R. sylvatica* samples fell below the determined threshold of 3 copies/reaction, while 2.4% of *R. pipiens* mesocosm samples (n = 8) fell below the determined threshold of 10 copies/reaction.

3.3 | Relationship between biomass, ASM, density, and eDNA

For *R. pipiens*, the optimal scaling coefficient was 0.72 (range of scaling coefficients with ΔAIC <2: 0.66–0.79) and for *R. sylvatica* the optimal scaling coefficient of 0.79 (range of scaling coefficients with ΔAIC <2: 0.64–0.93). In these models, biomass, density, and ASM were included independently and in models with additive and interactive effects of day. For *R. sylvatica*, models containing ASM and density were supported (Table 2; Figure 1). The most supported model for *R. sylvatica* contained observed density and day (log(observed density) β (95% CI) = 0.89 (0.75, 1.03), day β = 0.06 (0.04, 0.08), ΔAIC = 0, w_i = 0.48) (Table 2). Although the model containing the interaction term for observed density and day also received support, the interaction term was small and 95% CI included zero (log(observed density) = 0.88 (0.50, 1.3), day = 0.06 (0.03, 0.09), log(observed density)*day = 0.0002 (−0.01, 0.01), ΔAIC = 2.0, w_i = 0.18). The model containing ASM interacting with day was also supported, although the 95% CI of the coefficient for day also included zero (log($ASM^{0.79}$) = 0.68 (0.24, 1.12), day = −0.01 (−0.04, 0.02), log($ASM^{0.79}$)*day = 0.02 (0.002, 0.03), ΔAIC = 1.4, w_i = 0.24). The model containing ASM and day received moderate support (ΔAIC = 4.7, w_i <0.05). Models containing biomass were also moderately supported for *R. sylvatica* (all ΔAIC >4.3, w_i <0.06). By contrast, for *R. pipiens* the best-fit model was the ASM mass rather than observed density or biomass, with the model including the interaction term between ASM and day being the most supported (log($ASM^{0.72}$) = 1.55 (1.37, 1.72), day = 0.03 (0.01, 0.04), log(biomass)* day = −0.02 (−0.02, −0.01), ΔAIC = 0, w_i = 1.0, Table 2, Figure 2). Notably, there was not support that *R. pipiens* observed density had either direct or interactive effects on eDNA concentration (all ΔAIC >62.1, w_i <0.001). For both species, null models had the least support of all candidates (all ΔAICc >88.5).

3.4 | eDNA degradation

eDNA degraded rapidly after tadpoles had been removed from the mesocosms, with tadpoles mostly being undetectable within 5 days from removal (Figure 3). eDNA degradation rates did not differ between species ($F_{1,332}$ = 0.18, p = 0.67). Tadpole starting density affected rate of *R. sylvatica* eDNA degradation ($F_{3,160}$ = 21.23, p < 0.001), with starting density treatments of 1, 4, 16 and 64 tadpoles/mesocosm providing decay rate constants ranging from 0.52 ± 0.19 to 0.66 ± 0.66 day^{-1}. However, for *R. pipiens* decay rate

TABLE 2 Relationship between eDNA copy number relative to predictors including biomass, density, allometrically scaled mass (ASM), and day for *Rana sylvatica* and *R. pipiens*

Model	Df	Delta AIC	AIC weight	R^2_{cond}
R. sylvatica				
Log(Density) + Day	5	0	0.482	0.53
Log($ASM^{0.79}$) + Day + (Log($ASM^{0.79}$) * Day)	6	1.4	0.238	0.53
Log(Density) + Day + (Log(Density) * Day)	6	2.0	0.177	0.53
Log(Biomass) + Day + (Log(Biomass) * Day)	6	4.3	0.056	-
Log($ASM^{0.79}$) + Day	5	4.7	0.046	-
Log(Biomass) + Day	5	12.5	<0.001	-
Day	4	48.1	<0.001	-
Null Model	3	88.5	<0.001	-
R. pipiens				
Log($ASM^{0.72}$) + Day + (Log($ASM^{0.72}$) * Day)	6	0	0.9975	0.71
Log(Biomass) + Day + (Log(Biomass) * Day)	6	12.0	0.0025	0.70
Log(Biomass) + Day	5	30.3	<0.001	-
Log($ASM^{0.72}$) + Day	5	35.5	<0.001	-
Log(Density) + Day + (Log(Density) * Day)	6	62.1	<0.001	-
Log(Density) + Day	5	100.2	<0.001	-
Day	4	170.8	<0.001	-
Null Model	3	261.8	<0.001	-

Note: eDNA concentration was the response variable, and mesocosm was a random effect. R^2 shown is the conditional R^2.

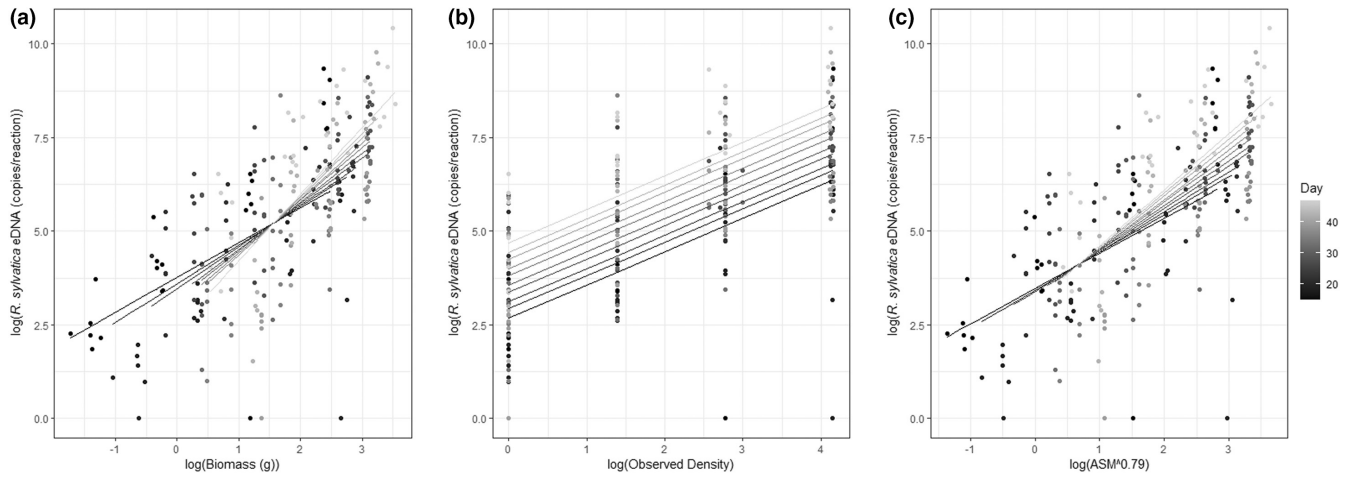

FIGURE 1 The relationship between eDNA (copies/reaction) and (a) total tadpole biomass per mesocosm, b) the density of tadpoles recorded in each mesocosm, and (c) allometrically scaled mass (individual $mass^{0.79}$) for *Rana sylvatica*. X-axes are log-transformed for normality. Lines on each figure are the predicted values from models containing the variable on the x-axis of each figure interacting with day

was consistent regardless of starting density ($F_{3,160} = 1.20$, $p = 0.31$), with decay rate constants ranged from 0.43 ± 0.26 to 0.79 ± 0.51 per day across density treatments.

4 | DISCUSSION

Our findings confirmed that tadpole eDNA concentrations from two common frog species in eastern North America were correlated to a variety of abundance metrics. Species differed in the amount of genetic material produced as well as in the relationship between eDNA and indices of abundance, but had comparable rates of eDNA degradation when tadpoles were removed from mesocosms. The observed broad similarities between species responses suggests that eDNA may have the potential to be developed as an index of tadpole abundance, under suitable conditions. These results add to a growing body of literature, showing that eDNA might be extended beyond merely detecting species presence/absence in aquatic systems (e.g., Eichmiller, Best, & Sorensen, 2016; Ficetola et al., 2008; Wilcox et al., 2013), but we caution that additional work is needed

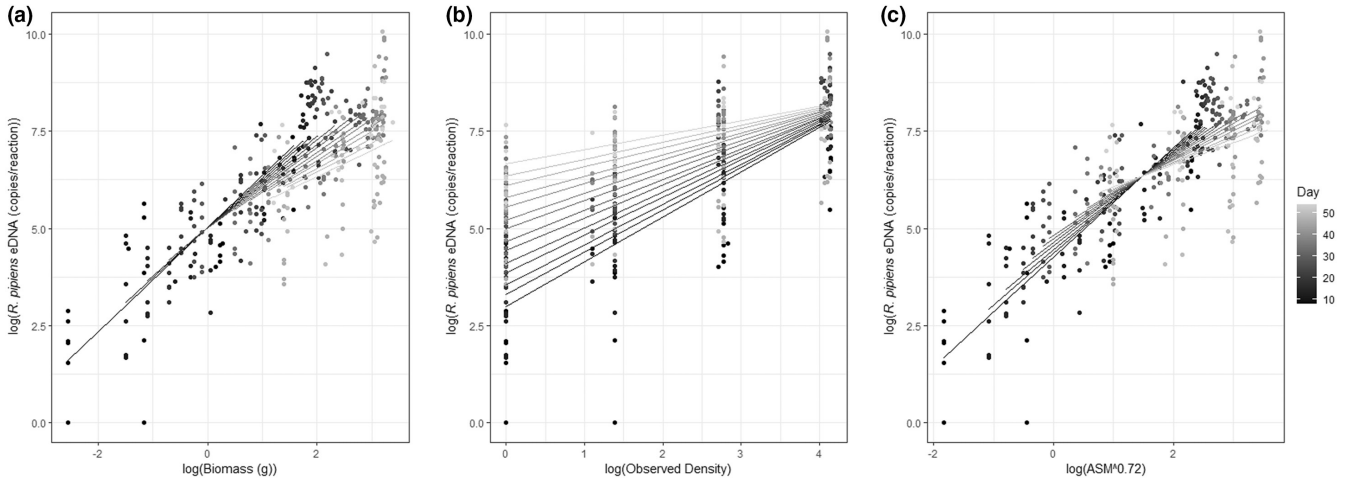

FIGURE 2 The relationship between eDNA (copies/reaction) and (a) total tadpole biomass per mesocosm, (b) the density of tadpoles recorded in each mesocosm, and (c) allometrically scaled mass (individual mass$^{0.72}$) for *Rana pipiens*. X-axes are log-transformed for normality. Lines on each figure are the predicted values from models containing the variable on the x-axis of each figure interacting with day

to more fully understand the determinants of eDNA concentration across species and environments, and how these relate to indices of abundance (Beng & Corlett, 2020; Yates et al., 2019).

Some previous studies successfully correlated eDNA concentration to different abundance metrics, including density, biomass, and allometrically scaled biomass, but in a recent meta-analysis Yates et al. (2019) summarized the existing literature and surmised that the relationship between eDNA concentration and a most appropriate and best-fit abundance metrics remained largely equivocal to accurately predict abundance. One reason for this lack of consistency across studies may be that the most appropriate abundance metric for fitting to eDNA concentration is not consistent and may vary across species or environmental conditions (e.g., Yates, Glaser, et al., 2021). Past studies assessing the performance of abundance metrics included fish (e.g., Doi et al., 2017; Lacoursière-Roussel, Côté, et al., 2016; Lacoursière-Roussel, Rosabal, & Bernatchez, 2016; Takahara et al., 2012) and amphibians (e.g., Iwai et al., 2019; Pilliod et al., 2013; Thomsen et al., 2012), which illustrates that efforts to validate these tools are being appropriately directed across taxa. Further, studies revealing correlation between eDNA and organism density (e.g., Doi et al., 2015), biomass (e.g., Evans et al., 2016; Maruyama et al., 2014), or neither metric (e.g., Doi et al., 2017) should be considered with caution given the unresolved uncertainty about the most appropriate abundance metric for correlating with eDNA concentration. Indeed, the best metric should be that which most strongly associates with eDNA production, release, and recovery, but these mechanisms remain poorly understood for many species and systems. Regardless, the persistent challenge in identifying the most appropriate abundance correlate highlights that variability in the biological relationship between organism abundance and eDNA remains difficult to resolve. Further, different studies addressing this question can have markedly different experimental designs and laboratory procedures, leading to variation in quality control, assay sensitivity and specificity, and other factors affecting the performance of abundance metrics (see Beng & Corlett, 2020). For example, in our study system the relationship between eDNA concentration and tadpole abundance metrics was intermediately strong (model R^2 ranging from 0.53 to 0.71), with differences in the best-fit metrics also differing between species (Table 2). It follows that many variables can account for these differences, including that eDNA production and release can vary according to age and size of organisms (Maruyama et al., 2014; Mizumoto et al., 2018), which could be different even between closely related species reared in identical conditions, as was the case in our experiment. Thus, it is safe to surmise that persistent uncertainty in liking eDNA concentration with the "best" abundance metric may continue to challenge efforts to develop robust eDNA-based abundance metrics, especially for organisms that undergo rapid growth or that have age-related changes in cell production and release (Doi et al., 2017; Tillotson et al., 2018; Werner, 1986). Ultimately, it is important to remind that any eDNA-based abundance metrics that may be considered for species monitoring programs probably will need to remain coarse and categorical to reflect unavoidable uncertainties in their precision. Accordingly, our study provides a preliminary proof-of-concept for further exploring eDNA-based amphibian population indices and highlights the need to not only conduct species-specific controlled experiments to gain an understanding of the relationship between eDNA concentration and abundance metrics, but also to develop rigorous experiments that test links between organism density, biomass, and eDNA production and recovery in natural systems.

Our experiments revealed a higher production and/or release of genetic material by *R. pipiens* compared to *R. sylvatica* when reared under identical mesocosm conditions. Variation in eDNA release has been observed across other taxa (Goldberg et al., 2011; Maruyama et al., 2018, 2019; Sassoubre et al., 2016; Thomsen et al., 2012), but our comparison was conservative by involving congeneric and sympatric species with similar body size and larval life history. *R. pipiens* and *R. sylvatica* are common in wetlands

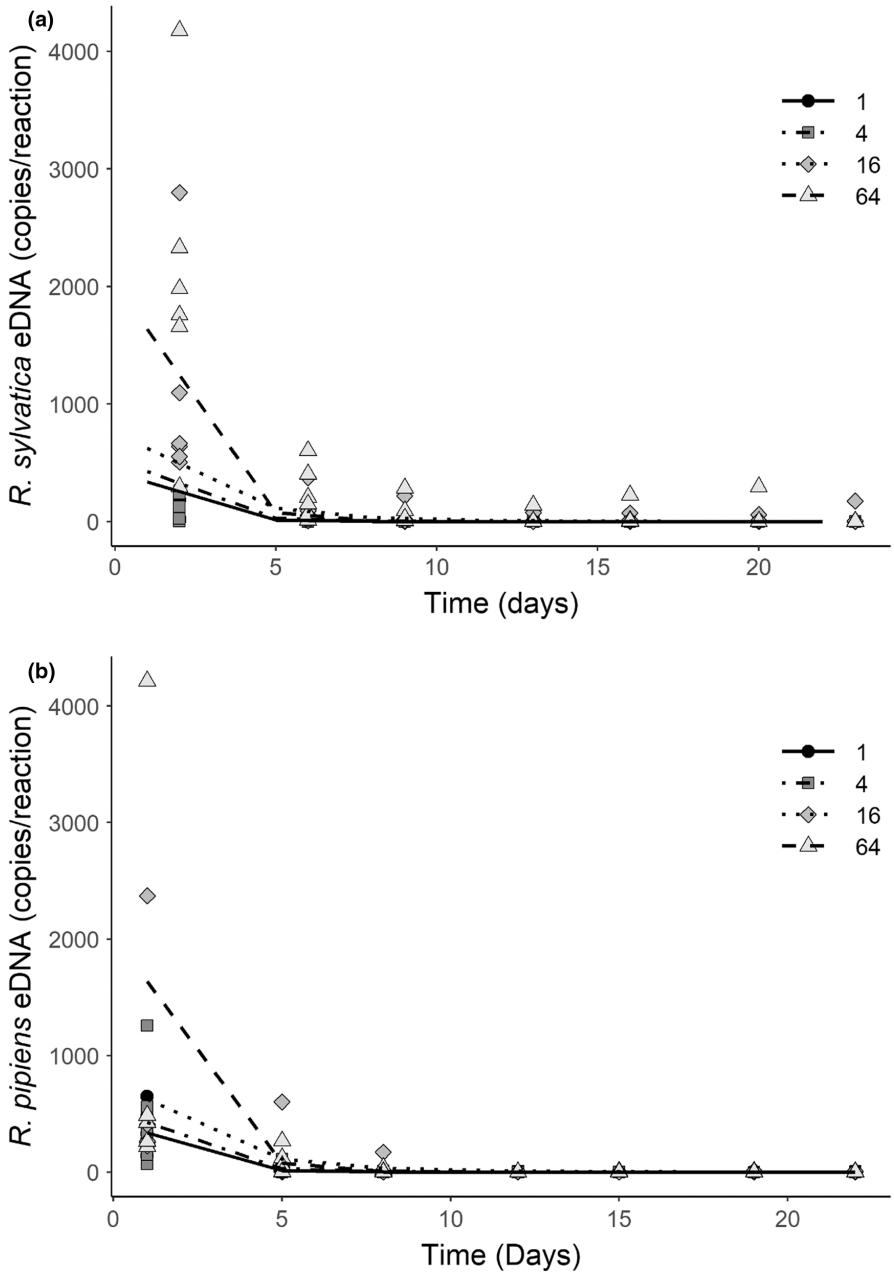

FIGURE 3 Degradation of *Rana sylvatica* (a) and *R. pipiens* (b) eDNA over time (days). Different line types represent the respective density treatments of 1, 4, 16, and 64 tadpoles per mesocosm and their exponential decay after tadpoles were removed. Points represent the mean copies/reaction for each mesocosm based on three qPCR replicates. For *R. sylvatica*, decay rate constants (r) were $0.58 \pm 0.48\,day^{-1}$ (1 tadpole), $0.66 \pm 0.66\,day^{-1}$ (4 tadpoles), $0.57 \pm 0.29\,day^{-1}$ (16 tadpoles), and $0.52 \pm 0.19\,day^{-1}$ (64 tadpoles), respectively. For *R. pipiens*, decay rate constants (r) were $0.78 \pm 0.51\,day^{-1}$ (1 tadpole), $0.68 \pm 0.58\,day^{-1}$ (4 tadpoles), $0.42 \pm 0.26\,day^{-1}$ (16 tadpoles), and $0.77 \pm 0.94\,day^{-1}$ (64 tadpoles), respectively

in eastern North America, making them good test candidates for eDNA-based monitoring. However, our findings highlight that physiological and behavioral variation across species can underlie the relationship between abundance and eDNA concentration (see Sassoubre et al., 2016), which could contribute to observed interspecific variation. Previous investigations revealed an increase in eDNA detection immediately after animals were introduced to aquaria, with eDNA release rate decreasing as animals became acclimated to experimental conditions (Pilliod et al., 2014; Takahara et al., 2012; Thomsen et al., 2012). This variability highlights how physiological and behavioral variation that is well-characterized for fish (Barton, 2002; Brett & Groves, 1979) and larval amphibians (Hossie & Murray, 2012; Relyea, 2001) may influence detection of organisms via eDNA. Accordingly, the variability observed in our mesocosm study highlights that future experiments should aim to replicate field conditions to the fullest extent possible in terms of biotic and abiotic factors likely to influence eDNA production across species. Also, decay of genetic material in our study was rapid (<5 days) and largely comparable to that observed elsewhere (e.g., Barnes et al., 2014; Thomsen et al., 2012), but it is not clear how environmental factors including ultraviolet radiation, temperature, and water chemistry can contribute to eDNA decay (e.g., Eichmiller, Miller, & Sorensen, 2016; Mächler et al., 2018; Seymour et al., 2018; Strickler et al., 2015; Wang et al., 2021). Additional experiments will help refine our understanding of the role of abiotic factors on eDNA release, persistence, detection, and degradation in natural systems, as well as how these responses may vary through time or across species (Troth et al., 2021). However, it is worth highlighting that, ultimately, the rapid eDNA decay rates observed in our study provide a sobering assessment of the potential utility of eDNA-based

methods for tracking amphibian site occupancy after larvae have transformed and emerged from the aquatic environment.

In general, eDNA validation studies rarely report the outcome of sensitivity analyses for determining species detection thresholds. We suggest that such reporting is a crucial aspect of eDNA validation, with thresholds needing to be set using LOD (Hunter et al., 2017) and LOQ (Klymus et al., 2020; Nutz et al., 2011; Serrao et al., 2017). In our study, we confirmed that our lowest experimental concentration of tadpoles (1 tadpole per 80-L mesocosm) could be reliably detected at the established LOD and limited the number of false positives and false negatives with our LOQ. We deliberately set conservative detection thresholds to challenge the sensitivity of our assay, as well as to maximize detection efficiency and reliability. Therefore, our findings reinforce the potential utility of eDNA to monitor a variety of target species at low population density (e.g., Boothroyd et al., 2016; Lodge et al., 2012; Pilliod et al., 2013), albeit with some limitations. Despite the high sensitivity and specificity of our assays, imperfect detection at low eDNA concentration indicates that species detection and abundance estimation will be increasingly imprecise at low density, potentially resulting in false negative or false positive returns (Hunter et al., 2017). Imprecision in eDNA concentrations may be exaggerated in field samples due to environmental factors and inhibitors that were not present in our mesocosms (Goldberg et al., 2016; Harper et al., 2019), despite our best efforts to replicate field conditions. Because sampling methods and laboratory practices can contribute to detection error and are to some extent under the control of the individual research program, robust development of detection thresholds should be prioritized in the development of eDNA-based population monitoring programs (Ficetola et al., 2015; Serrao et al., 2017). For example, arbitrarily setting detection thresholds too high or too low will alter detection rates, which could have substantial impacts in terms of conservation or management efforts directly resulting from monitoring outcomes. Despite the recognized importance of thorough development and reporting of detection thresholds in eDNA validation studies, establishing and reporting detection thresholds continues to be rare in published studies (but see Currier et al., 2018; Roussel et al., 2015; Serrao et al., 2017). Accordingly, before eDNA-based monitoring protocols can be firmly established for field application, new standards of reporting are needed to increase transparency and repeatability of assay development and validation.

Our research supports the growing body of literature, indicating that eDNA has the potential for use beyond merely detecting species presence/absence, and that it may be possible to index species abundance using this method. However, variation in our system in terms of the best abundance metric and species-specific differences in eDNA production highlights the need to better understand how biological and environmental factors influence eDNA production, degradation, and recovery. Until such factors are better understood through robust mesocosm studies and their extension to field-based validation, we suggest that it will be premature to use eDNA for monitoring amphibian population trends beyond assessing presence vs. absence. This conservative approach will ensure that inference derived from monitoring programs is not extended beyond the confidence in the data until these promising tools are sufficiently validated.

AUTHOR CONTRIBUTIONS

DL, CJK, CCW, and DLM conceived the initial study and obtained funding; BAB, MJHW, and AMB performed and supported field sampling and laboratory experiments; LEB assisted with data analysis and interpretation; STV assisted with data interpretation. BAB and DLM drafted the manuscript, and all authors contributed to writing and revisions.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data are available on Dryad https://doi.org/10.5061/dryad.02v6wwq5v.

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SUPPORTING INFORMATION

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