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ARTICLE

Methods, Tools, and Technologies

Navigating the trade-offs between environmental DNA and conventional field surveys for improved amphibian monitoring

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Abstract

The need for efficient, accurate biodiversity monitoring is growing, especially for globally imperiled taxa, such as amphibians. Environmental DNA (eDNA) analysis holds enormous potential for enhancing monitoring programs, but as this tool is increasingly adopted, it is imperative for users to understand its potential benefits and shortcomings. We conducted a comparative study to evaluate the efficacy of two eDNA methodologies (quantitative (q)PCR and metabarcoding) and conventional field sampling approaches (seining, dip-netting, and visual encounter surveys) in a system of 20 ponds containing six different amphibian species. Using an occupancy modeling framework, we estimated differences in detection sensitivity across methods, with a focus on how eDNA survey design could be further optimized. Overall, both metabarcoding and qPCR were competitive with or improved upon conventional methods. Specifically, qPCR (species-specific approach) was the most effective technique for detecting two rare species, the California tiger salamander (*Ambystoma californiense*) and California red-legged frog (*Rana draytonii*), with a detection probability of >0.80 per survey. Metabarcoding (community approach) estimated amphibian diversity with comparable rates to field techniques on average, and detected an additional 41 vertebrate taxa. However, for two abundant species (western toads, *Anaxyrus boreas*, and Pacific chorus frogs, *Pseudacris regilla*), field techniques outperformed metabarcoding, especially as individuals metamorphosed. Our results indicate that eDNA approaches would be most effective when paired with visual encounter surveys to detect terrestrial life stages, and that more optimization, specifically primer choice and validation, is needed. By comparing methods across a diverse set of ponds and species, we provide guidance for future studies integrating eDNA approaches into amphibian monitoring.

KEYWORDS
Ambystoma californiense, amphibian, biodiversity, environmental DNA, metabarcoding, ponds, *Rana catesbeiana*, *Rana draytonii*

INTRODUCTION

Current rates of environmental change have increased the demand for efficient and cost-effective approaches to monitoring biodiversity (Schmeller et al., 2017). Conventional methods are frequently being augmented with modern technologies, such as acoustic monitoring, remote sensing, and crowd-sourced data collection (Gibb et al., 2019; Pimm et al., 2015). A particularly promising technique is the analysis of environmental DNA (eDNA), which has grown exponentially in recent years and is now being applied in a wide variety of conservation and management contexts (Beng & Corlett, 2020; Bohmann et al., 2014). By analyzing genetic material in environmental samples, rather than relying on direct observations or capture of species, eDNA analysis offers numerous advantages to conventional biological surveys. For example, the collection of eDNA samples (often from soil or water) is noninvasive, minimizing the disturbance of sensitive species and the risk of spreading invasive taxa or disease (Valentini et al., 2016). Surveys to collect eDNA may also be lower in cost or time commitment than established field surveys (Sigsgaard et al., 2015; Thomsen et al., 2012). This in turn can broaden the scale of studies or lower the barriers for implementing monitoring programs (Biggs et al., 2015; Ruppert et al., 2019). Moreover, eDNA approaches often detect species where other methods fail (Biggs et al., 2015; Dejean et al., 2012; Schmelzle & Kinziger, 2016; Thomsen et al., 2012), and for morphologically indistinct taxa, they offer standardized means for identification (Niemiller et al., 2018; Taberlet et al., 2012). For these reasons, eDNA methods can enhance existing monitoring programs and provide increased insight into patterns of biodiversity (Bohmann et al., 2014; Thomsen & Willerslev, 2015).

Studies using eDNA analysis for species monitoring generally employ one of two approaches: species-specific or community assays. Species-specific eDNA approaches commonly use quantitative PCR (qPCR), in which DNA from a single target species is amplified with primers and, increasingly, a probe specific to that species. Owing to their highly sensitive and specific nature, qPCR assays are a powerful tool for monitoring cryptic or low-density taxa, for example, endangered species (Biggs et al., 2015; Katz et al., 2021; Pope et al., 2020; Schmelzle & Kinziger, 2016) and non-native species in the early stages of invasions (Larson et al., 2020; Takahara et al., 2013; Tingley et al., 2019). qPCR is also relatively low-cost and rapid, in some cases, even allowing real-time species detection in the field (Thomas et al., 2020). In contrast, community eDNA monitoring uses metabarcoding, in which generic (and often degenerate) primers enable simultaneous amplification of DNA from a wide array of taxa. These primers bind conserved sites (i.e., shared across taxa) flanking a region of highly variable DNA sequence that differs between taxa, and subsequent PCR amplicons are sequenced on a high-throughput sequencing platform (Taberlet et al., 2012). When the focus is on describing entire communities, metabarcoding can quickly become more efficient and less expensive than field surveys or eDNA surveys using qPCR (Bálint et al., 2018; Harper et al., 2018). Consequently, metabarcoding has become a popular approach for standardized biodiversity monitoring and is likely to gain further traction as sequencing costs fall (Borrell et al., 2017; Deiner et al., 2017).

While eDNA approaches can be highly effective tools for species monitoring, their utility is dependent on a host of factors (Goldberg et al., 2016). For example, eDNA dispersion and stability is influenced by aspects of the physical and chemical environment (Curtis et al., 2021; Goldberg et al., 2018; Kessler et al., 2020). Certain taxa may also be difficult to detect with eDNA surveys owing to behavior, DNA deposition rates, or primer bias (Furlan et al., 2020; Halstead et al., 2017). Collection protocols and laboratory techniques, including sample collection, the number of replicates, DNA extraction and PCR procedures, and primers used, can strongly impact the sensitivity of both metabarcoding and qPCR (Curtis et al., 2021; Deiner et al., 2017; Goldberg et al., 2016). Moreover, even after field and laboratory components are complete, the bioinformatics pipeline used to process high-throughput sequencing data can introduce false negatives and positives (Coissac et al., 2012; Cristescu & Hebert, 2018). For these reasons, conventional survey techniques may still more accurately estimate species occurrence for some systems and taxa (Baker et al., 2018; Rose et al., 2019; Walsh et al., 2019). Further, many of the focal aims of field surveys, such as quantifying demographic structure, individual body condition, and species behaviors, cannot currently be addressed using eDNA survey (Beng & Corlett, 2020). Consequently, eDNA analysis is not a replacement for existing methods but a complement, and end users monitoring biodiversity require information on (1) how eDNA surveys can be used to complement existing approaches and (2) methods for maximizing the utility of eDNA techniques.

Studies that directly compare methods across multiple axes of variation (e.g., species, season, sampling protocol) are essential for optimizing eDNA surveys. For example, although still comparatively rare, direct comparisons of qPCR and metabarcoding, especially those incorporating multiple species or sampling protocols, can inform the choice of technique for specific applications (Blackman et al., 2020; Bylemans et al., 2019; Deiner et al., 2017; Dritsoulas et al., 2020; Harper et al., 2018; Lacoursière-Roussel et al., 2016; Peixoto et al., 2021; Schneider et al., 2016). In addition, explicitly quantifying the mechanisms shaping detection probability (rather than using a descriptive approach to compare detections across protocols, environments, or species) provides concrete information for further refining eDNA survey methods (Fediajevaite et al., 2021; Willoughby et al., 2016). For example, Goldberg et al. (2018) established that detection rates of Chiricahua leopard frogs (*Lithobates chiricahuensis*) from qPCR were strongly related to waterbody size. This mechanistic insight enabled them to refine their spatial survey design to increase detection and thus efficiency.

Occupancy modeling (MacKenzie et al., 2002) is a highly effective approach for estimating these kinds of associations and is increasingly employed in studies using eDNA analysis (McClenaghan et al., 2020; McColl-Gausden et al., 2021; Schmelzle & Kinziger, 2016). Critically, occupancy models estimate both the probability of a species' presence at a location (site occupancy, ψ) and the probability that a species is detected in a given survey (detection probability, p). This enables users to account for false negatives (i.e., cases where a species is present but undetected), and to evaluate the influence of covariates on detection probability. By extending occupancy modeling to include additional hierarchical levels, one can estimate detection at multiple levels of nested survey designs, a common feature of eDNA surveys (Dorazio & Erickson, 2018). This information can guide survey design, for instance, by quantifying whether effort is best spent collecting additional environmental samples or running additional qPCR replicates (Davis et al., 2018). Studies that simultaneously incorporate multiple methods and species while controlling for detection bias can therefore offer practical information on the relative efficacy and optimization of eDNA approaches (Ruppert et al., 2019). Yet, the vast majority of published eDNA studies do not quantify detection probabilities to rigorously compare methodologies (Fediajevaite et al., 2021).

In this study, we evaluated multiple methods for surveying amphibian communities, including a suite of conventional field techniques (dipnet sweeps, seines, and visual encounter surveys) and species-specific and community eDNA approaches (qPCR and metabarcoding). We compared these methods in a system of 20 ponds within the California Bay Area, which contain a community of six amphibian species varying in their phenology and abundance. Using occupancy modeling, we estimated species- and method-specific detection probabilities and their relationship with variables pertaining to sample collection and pond attributes. Our study builds upon a field survey protocol already in use for the past decade to evaluate whether and under what circumstances eDNA surveys can enhance species monitoring. We show that eDNA methods increase the detection of two protected species in particular, and that metabarcoding approaches provide additional biodiversity data. Finally, we present recommendations to assist end users in deciding whether and how to incorporate eDNA monitoring into studies of amphibian biodiversity and ecology.

METHODS

Study system and species

Our 31-km^2 study area was located in Santa Clara County, California, within Joseph D. Grant County Park (Santa Clara County Parks) and the adjacent Blue Oak Ranch Reserve (University of California). We chose 20 ponds within these properties, for which we had a decade's worth of prior data on amphibian occurrence (Joseph et al., 2016; Moss et al., 2021). Ponds ranged in surface area from 105 to 5884 m^2 and were primarily surrounded by grassland and oak woodlands.

Up to six species of amphibian utilize these waterbodies for breeding: Pacific chorus frogs (*Pseudacris regilla*), California newts (*Taricha torosa*), California red-legged frogs (*Rana draytonii*), western toads (*Anaxyrus boreas*), California tiger salamanders (*Ambystoma californiense*), and American bullfrogs (*Rana catesbeiana*). Detection of three of these species is of especially high relevance to managers. The American bullfrog is an exotic species known to threaten native amphibian populations and is the target of eradication efforts throughout the region (Lawler et al., 1999). Both the California tiger salamander and California red-legged frog are federally protected species (U.S. Fish and Wildlife Service, 2002, 2017) emphasizing the importance of further details regarding their distribution and temporal trends (Moss et al., 2021).

After eggs are laid during winter and spring, amphibian larvae develop within ponds and metamorphosis generally occurs between June and September (Lannoo, 2005). Western toads are generally the first to metamorphose, followed by Pacific chorus frogs, California tiger salamanders, California red-legged frogs, and California newts (Johnson et al., 2012; Lannoo, 2005). Bullfrog larvae are an exception; eggs are laid during summer and develop over 2 years, such

that both first-year larvae and metamorphosing second-year larvae are commonly observed at sites. During the late spring and summer (May–July), when our sampling occurred, larval and recently metamorphosed individuals are the most abundant life stages at ponds and comprise the majority of the amphibian biomass.

Field and eDNA sampling

We sampled ponds between May and July 2018, representing the peak period of larval development for most species. We conducted two separate field surveys for each pond (Appendix S1: Table S1), in which we assessed amphibian communities and water quality using conventional wetland survey techniques: net sweeps, seines, and visual encounter surveys (Figure 1; see detailed methods in Johnson et al., 2013; Joseph et al., 2016). At each survey visit, we conducted between 3 and 5 seines of approximately 10 m length each, as well as a visual encounter survey in which one observer walked the entire perimeter of the pond, recording the species and life stages of all amphibians encountered. Dipnet surveys were only conducted at the first visit and consisted of 10–15 habitat stratified dipnet sweeps, roughly one sweep every 10 m of shoreline. We also recorded pond surface area (in square meters), water salinity (in microsiemens), and turbidity (in nephelometric turbidity units [NTU]) (McDevitt-Galles & Johnson, 2018; Appendix S1) at each visit. Finally, we calculated an index of density for each species at each visit, which was the number of larval individuals per meter of seine (Appendix S1).

We also conducted two eDNA surveys per site, during which water samples were collected and filtered (Figure 1). We collected water samples once during early summer (between 25 May and 1 June) and once during mid-summer (between 14 July and 17 July), with the second eDNA survey generally occurring after the second field survey (Appendix S1: Table S1). During each eDNA survey, we collected approximately 170 ml of water from each of three separate locations along the perimeter of a pond, which were pooled to obtain 500 ml of water in total (Appendix S1). We also collected field negative controls, consisting of 500 ml tap water handled identically to field samples (Appendix S1).

We filtered each water sample using disposable filter funnels (Appendix S1), splitting the 500 ml sample evenly across two filters to obtain replicates (Figure 1). When filters clogged due to the abundance of suspended sediment in water, we either filtered a smaller volume of water, used a third filter, or used a coarser filter (Appendix S1). The volume of water filtered and type of filter were recorded and used as covariates in models. We followed a protocol developed by Goldberg and Strickler (2017) to minimize contamination potential during water sampling and filtering (Appendix S1).

From each filter, we performed one DNA extraction using methods previously established by Goldberg et al. (2011) (Appendix S1). We included an extraction negative control in each batch of extractions. Each DNA extract was split into two aliquots, which were analyzed with both qPCR and metabarcoding (Figure 1).

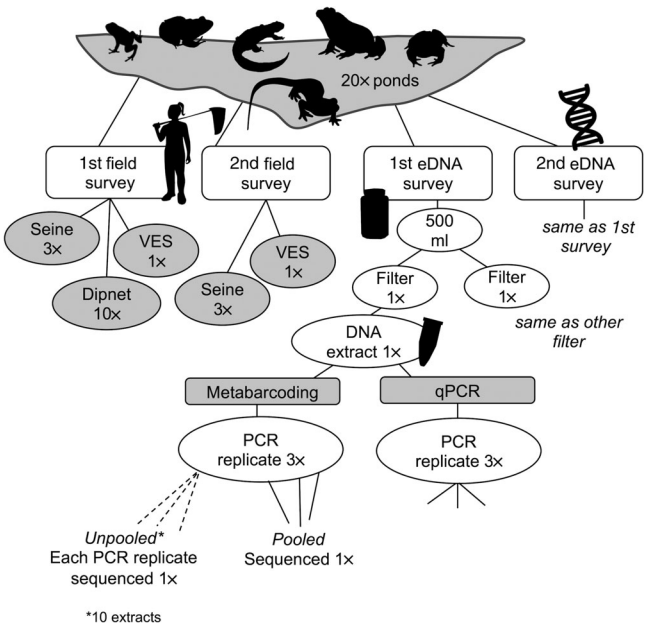

FIGURE 1 Diagram of hierarchical sampling design. We sampled 20 ponds using five techniques: metabarcoding (eDNA), qPCR (eDNA), seines (field), visual encounter surveys (VES; field), and dipnets (field). Field surveys and metabarcoding targeted all six amphibian species, whereas qPCR targeted only two protected species. For eDNA sampling, we collected one water sample (500 ml) per visit that was split across two filters, each of which underwent DNA extraction. DNA extracts were subjected to metabarcoding and qPCR analysis. Amphibian images: www.phylopic.org (*Ambystoma* credit: Matt Reinbold [modified by T. Michael Keesey])

qPCR assays

Quantitative PCR was used to assess the presence of the two protected species, California red-legged frogs and California tiger salamanders. We used a previously validated species-specific assay for California red-legged frogs (Halstead et al., 2018), which targeted a 98 bp region of the cytochrome b gene (Appendix S1: Table S2). For California tiger salamanders, we developed and used a novel

assay to target a 108 bp fragment of the mitochondrial control region (for details of assay development and validation, see Appendix S1). We ran each qPCR assay in triplicate for each DNA extract, including field, extraction, and qPCR (molecular grade water) negative controls (Figure 1). If inhibition was detected, we purified the sample using an inhibitor removal kit and repeated the PCRs, discarding data from the first round of PCRs that were inhibited (Appendix S1). We defined a positive detection as a sample where all three qPCR replicates were amplified. If at least one replicate did not amplify, we reran the sample, and defined the sample as positive if amplification was observed in any of the replicates again (Appendix S1). For details on reaction conditions, see Appendix S1.

Metabarcoding

We conducted metabarcoding on each DNA extract to estimate the presence of all amphibian species and additional vertebrates. Vertebrate-specific primers were used to target the mitochondrial 12S ribosomal RNA (rRNA) gene (Kelly et al., 2014; Riaz et al., 2011). We first verified primer suitability in silico using ecoPCR (Ficetola et al., 2010) by comparing primer sequences with custom reference databases for vertebrates that occur in our study system (Appendix S1). All DNA extracts were purified prior to PCR to remove inhibitors (Appendix S1). Next, we used a two-step PCR protocol with a nested tagging approach for library preparation (Harper, Lawson Handley, Carpenter, et al., 2019; Li et al., 2019). The PCR and library preparation protocols are fully described in Appendix S1.

In the initial PCR, we used 12S rRNA primers modified to include unique tags, heterogeneity spacers, sequencing primers, and pre-adapters. We performed three PCR replicates per extraction, after which replicates were purified, normalized, and pooled into sublibraries (Appendix S1). A subset of 10 extracts from five different ponds were used to evaluate the effect of pooling and quantify PCR replicate-level variation. For these extracts, we repeated PCR in triplicate and sequenced PCR replicates independently without pooling (Figure 1; Appendix S1). In addition to field and extraction negative controls, we included a PCR negative control and positive control (Eastern Massasauga *Sistrurus catenatus* DNA) in each sublibrary (Appendix S1).

Sublibraries were purified and used as template DNA for a second PCR, which attached pre-adapters, unique tags, and Illumina adapters. PCRs were conducted in duplicate, after which replicates were again purified, normalized, and pooled (Appendix S1). The library was run on an Illumina MiSeq (Illumina, Inc., CA, USA) at the Roy J. Carver Biotechnology Center Functional Genomic Unit, University of Illinois at Urbana-Champaign.

We demultiplexed raw sequence reads using a custom python script and used metaBEAT v0.97.11 (https://github.com/HullUni-bioinformatics/metaBEAT) for bioinformatic processing, which included quality trimming, merging, chimera removal, clustering, and taxonomic assignment (Hänfling et al., 2016; Harper et al., 2018). We used a BLAST identity of 95% to assign sequences (Appendix S1). Finally, we used the R package *microdecon* v.1.0.2 (McKnight et al., 2019) in the software program R v.4.0.2 (R Core Team, 2020) to identify and remove contaminant sequences.

Detection sensitivity of all amphibian survey methods

We fit single-season occupancy models (MacKenzie et al., 2002) to compare detection probabilities from field methods and eDNA methods. We estimated the probability of a site being occupied by a given species (ψ) and the probability of a species being detected at a visit with a specific method, given that the site is occupied (p). To obtain a detection matrix for each species, we pooled all replicates of a certain method taken on the same date; for instance, we took the overall detection from all replicate dipnets or seines conducted at a site visit (Appendix S1). We fit occupancy models using the R package *unmarked* v.1.0.1 (Fiske & Chandler, 2011), with a separate suite of models for each species. Our primary focus was on comparing detection probabilities across methods rather than estimating occupancy probability; we therefore fit an intercept-only model for the occupancy component ($\text{logit}(\psi) \sim 1$). As predictor variables for the probability of detection, we included survey method, pond area, survey date, and an interaction between survey method and date (Appendix S1: Table S3). The survey method was modeled as a categorical variable with five levels (seine, dipnet, visual encounter survey [VES], qPCR, or metabarcoding [MB]). To improve model convergence, we centered and scaled continuous predictor variables (Appendix S1: Table S3). We verified that included predictor variables were not collinear ($|r| < 0.5$).

We fit separate models for all possible combinations of predictor variables (10 candidate models per species) and used the corrected Akaike information criterion (AIC_c) to rank models (Burnham & Anderson, 2004). Models within two AIC_c values of the best-ranking model ($\Delta AIC_c < 2$) were considered informative. We report

coefficient estimates from the top-ranking model (lowest AIC_c), as well as model-averaged predictions and 95% confidence intervals produced with the *predict* function in the package *unmarked*.

Detection sensitivity of qPCR and metabarcoding approaches

To better compare the two eDNA methods (qPCR and metabarcoding) and inform survey design, we used a single-season Bayesian hierarchical occupancy model (fit with the R package *eDNAoccupancy* v.0.2.7; Dorazio & Erickson, 2018) to estimate the probability of detection at three hierarchical levels: (1) the probability of eDNA presence at a site (ψ), (2) the probability of the eDNA presence in a given DNA extract, given that eDNA was present at a site (θ), and (3) the probability of eDNA detection in a single qPCR or PCR/sequencing replicate, given its presence in the DNA extract (p).

The data used in this analysis were restricted to the five sites for which metabarcoding PCR replicates were sequenced independently. Within these five sites, metabarcoding and qPCR generated identical detection histories for California red-legged frogs, with no missed detections; consequently, there was no variation between methods and we did not fit hierarchical models for this species. However, the two methods generated different detection histories for California tiger salamanders. For this species, we fit a model using method (qPCR or metabarcoding, a categorical variable) as a covariate for both θ and p. Site-level occupancy (ψ) was modeled using an intercept-only model. For details on prior distributions and model-fitting procedures, see Appendix S1.

Detection sensitivity of qPCR

We ran a separate suite of hierarchical occupancy models using only data from qPCR to estimate how sample collection and pond characteristics affected detection at the extract (θ) and PCR replicate level (p). We fit separate models for California tiger salamanders and California red-legged frogs and used qPCR data from all 20 sites. We considered sampling date, pond area, filter type (0.45 or 5 μM), and water volume filtered as potential covariates for detection at the DNA extract level (θ; Appendix S1: Table S3). As covariates of detection at the PCR replicate level (p), we considered pond salinity, turbidity, water volume, and whether the sample was purified due to the presence of inhibitors (Appendix S1: Table S3). No covariates were included at the site level (ψ). Predictor variables for each component of the model were uncorrelated ($|r| < 0.5$). For California red-legged frogs, only two out of 77 DNA extracts had disagreement among qPCR replicates, with the remaining samples amplifying in 100% or 0% of replicates. Therefore, our power was limited to test covariates for replicate-level detection (p) and we only tested variables influencing DNA extract-level detection (θ), using an intercept-only model for p.

For each species, we fit models for all possible combinations of covariates at both levels (California tiger salamanders: $n = 256$; California red-legged frogs: $n = 16$) using code adapted from Harper, Griffiths, Lawson Handley, et al. (2019). Models were ranked using the widely applicable information criterion (WAIC) for which models with lower values are considered to have greater predictive power (Dorazio & Erickson, 2018; Watanabe, 2013). We evaluated model fit, convergence, and parameter estimates from the top-ranking model (e.g., lowest WAIC), as well as a null model (ψ, θ, and p modeled with intercept only). Prior distributions and model-fitting procedures for each model were the same as for the previously described hierarchical models (Appendix S1).

Detection sensitivity of metabarcoding

For metabarcoding data, we could not investigate covariates of detection probability at multiple hierarchical levels because PCR replicates of each DNA extract were typically pooled prior to sequencing. Instead, we used generalized linear mixed models to evaluate the effects of pond characteristics and sample collection on probability of detection. We fit a separate suite of models for each species but did not fit models for American bullfrogs because there were only four missed detections in total and thus insufficient statistical power.

The response variable was the detection (1 or 0) of the species from a given DNA extract, modeled with a binomial distribution. We filtered the metabarcoding detection data to sites with known occupancy for a species (e.g., sites where the species was detected at least once with any method) so that any non-detections were false negatives. Predictor variables included sampling date, pond area, filter type, water volume filtered, and turbidity (Appendix S1: Table S3). We included a random effect for site because there were multiple DNA extracts per site (two from each round of sampling). We assessed collinearity of variables within each species' filtered dataset and retained only those that were not correlated ($|r| < 0.5$). We fit models using the package *glmmTMB* in R (Brooks et al., 2017). We fit all

combinations of each predictor variable and, as described above, ranked models using AICc.

RESULTS

Environmental DNA collection and verification

We visited most of the 20 sites at least twice, obtaining two eDNA filter replicates on each of two site visits, with a few exceptions (Appendix S1: Table S1). We collected a total of 79 DNA extracts from 39 water samples, with the second round of eDNA sampling generally occurring after the second round of field visits (Appendix S1: Table S1).

Metabarcoding generated 19.5 million reads, with 7.3 million remaining after metaBEAT processing. The majority of reads (99%) were assigned to a taxonomic unit (Appendix S1). Amphibians were the most well-represented taxa with 2.8 million (38%) assigned reads, although we also detected 41 other vertebrate taxa (Appendix S1). All six focal amphibian species were detected using metabarcoding, with American bullfrogs generating the most reads (886,716) and Pacific chorus frogs the fewest (16,181).

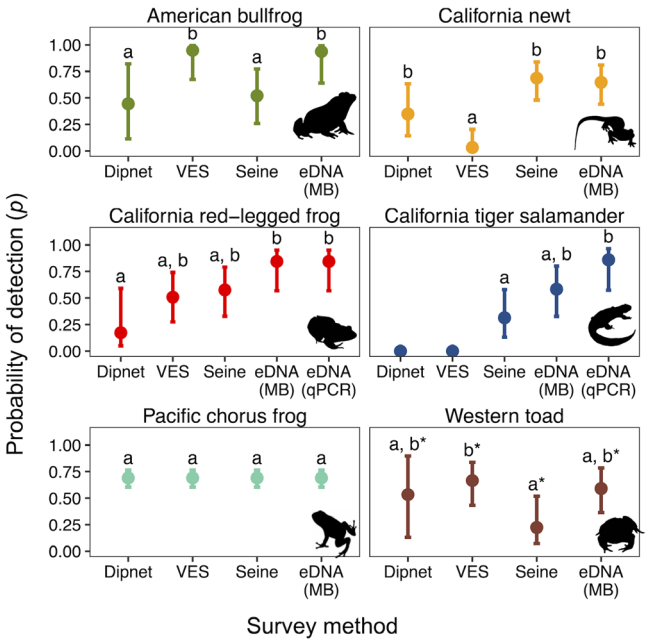

FIGURE 2 Detection probability (p) as a function of survey method and species. We plot the mean and 95% CI of model-averaged predictions, holding other covariates (pond area and sample date) at the average value. Letters designate methods that are significantly different. Field survey methods included dipnets, seines, and visual encounter surveys (VES). Environmental DNA (eDNA) methods included metabarcoding (MB) and qPCR. Sampling method interacted with date for western toads such that the best method depended on date (see Figure 5a)

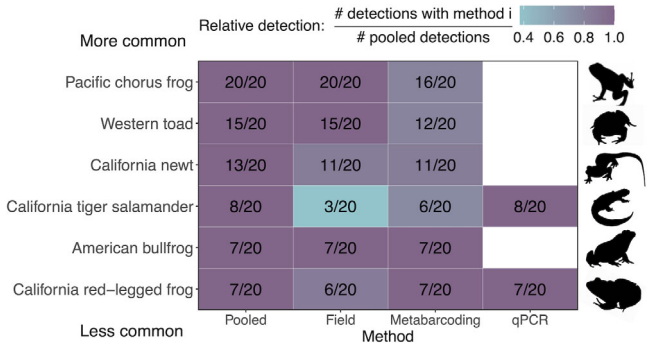

FIGURE 3 Number of ponds (out of 20) where each amphibian species was detected. Pooled detections give the number of ponds where a species was detected with any method and can be considered a naïve occupancy rate. We compared eDNA detections (metabarcoding and qPCR approaches) with those from established field surveys (dipnets, seines, and visual encounter surveys). We only used qPCR on two species (California tiger salamanders and California red-legged frogs). Color indicates the relative effectiveness of a single method compared with the pooled detections. Image credits: www.phylopic.org (*Ambystoma* credit: Matt Reinbold [modified by T. Michael Keesey])

We did not observe amplification in any of the negative controls (field, extraction, and qPCR negatives) with qPCR (Appendix S1). For metabarcoding, the only contaminants observed in the negative controls were domestic species (Appendix S1).

Detection sensitivity of all amphibian survey methods

Environmental DNA methods were outperformed or were not significantly different from field methods for every species (Figure 2). When averaged across species, the best detection method was qPCR ($p = 0.85$), followed by metabarcoding ($p = 0.72$), seines ($p = 0.50$), VES ($p = 0.47$), and dipnets ($p = 0.37$). Full results of occupancy modeling for each species are given in Appendix S1 (coefficient estimates) and Tables S4–S9 (model selection tables).

For California tiger salamanders, eDNA techniques yielded more detections than conventional field techniques. Quantitative PCR detected California tiger salamanders at every site where they were known to occur (eight of the eight sites where they were found with at least one method), whereas MB missed them at two sites (6/8). The probability of detection was not significantly different between eDNA methods (qPCR: $p = 0.86$, 95% CI = 0.57–0.97; MB: $p = 0.58$, 95% CI = 0.33–0.80; Figure 2; Appendix S1). Field-based methods only detected California tiger salamanders at three sites in total, and only using seines (Figure 3; Appendix S1).

The eDNA survey was similarly effective at detecting the California red-legged frog. Both qPCR and metabarcoding detected red-legged frogs at seven sites, with identical survey-level detection ($p = 0.84$, 95% CI = 0.57–0.95), and did not miss them at any site where they were detected with other methods (Figure 3). Detection was significantly lower with dipnetting, which only detected red-legged frogs at one site ($p = 0.17$, 95% CI = 0.05–0.59), while other methods were intermediate (Figure 2; Appendix S1).

For American bullfrogs (qPCR not performed), metabarcoding ($p = 0.94$, 95% CI = 0.64–0.99) and VES ($p = 0.95$, 95% CI = 0.67–0.99) were the most effective methods, and both detected bullfrogs at the same seven sites. Dipnets and seines were less effective for bullfrogs (Figure 2; Appendix S1). For California newts (qPCR not performed), metabarcoding ($p = 0.65$, 95% CI = 0.44–0.81) and seining ($p = 0.69$, 95% CI = 0.48–0.84) were the most effective methods, detecting them at 11 sites (Figure 2), but both methods missed newts at two sites where they observed with other methods (Figure 4; Appendix S1).

For Pacific chorus frogs (qPCR not performed), which were known to be present at all 20 sites, method was not a strong predictor of detection probability (Appendix S1), and the best method (seines: 18/20 sites) was not significantly better than any others (metabarcoding: 16/20; dipnets: 15/20; and VES: 15/20; Figure 2). The estimated probability of detection for a given survey was 0.69 (95% CI = 0.61–0.76) across all methods. This was highly dependent upon date ($\beta_{\text{date}} = -0.44$, 95% CI = −0.83 to −0.06) with improved detections earlier in the season for all methods (Figure 5a).

Similarly, no single method detected western toads (qPCR not performed) at all 15 sites where they were known to be present (MB: 12/15; VES: 12/15; dipnets: 11/15; and seines: 9/15; Figure 3). Method interacted with date to influence probability of detection (Appendix S1), such that the effectiveness of VES significantly increased over the summer ($\beta_{\text{VES}\times\text{date}} = 1.58$, 95% CI = 0.40–2.73), whereas metabarcoding and seining declined ($\beta_{\text{MB}\times\text{date}} = -1.25$, 95% CI = −2.08 to −0.41; $\beta_{\text{seine}\times\text{date}} = -2.40$, 95% CI = −4.10 to −0.74). Therefore, by mid-July, VES were the most effective survey technique (Figure 5a) with a 0.96 probability of detecting western toads (95% CI = 0.70–0.99), whereas in mid-May, metabarcoding and seines were most effective (seines: $p = 0.93$, 95% CI = 0.61–0.99; MB: $p = 0.91$, 95% CI = 0.64–0.98).

Detection sensitivity of qPCR and metabarcoding approaches

In the five ponds where we had PCR replicate-level data for metabarcoding and qPCR, we did not detect a

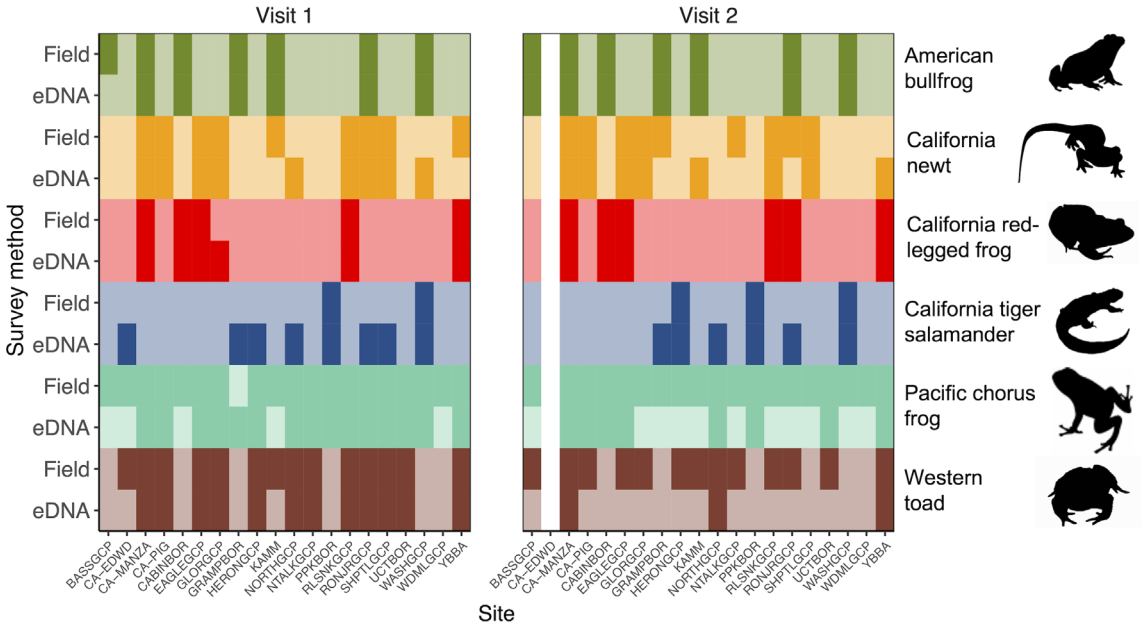

FIGURE 4 Detections of six amphibian species at 20 ponds. Each row gives the detection of a given species using a particular method across ponds (columns). We pooled detections from field methods (dipnets, seines, and visual encounter surveys) and eDNA methods (qPCR: California red-legged frog, California tiger salamander; metabarcoding: all species). Each site received two field visits and two eDNA visits, with the second eDNA visit occurring later in the summer than the second field visit (Appendix S1: Table S1). Color hue indicates the detection (1; dark) or non-detection (0; light) of a given species. For certain species (e.g., American bullfrogs), detection was consistent across methods and time, while for other species (e.g., Pacific chorus frog, western toad), detection declined across time for eDNA. One site (CA-EDWD) was dry on the second visit

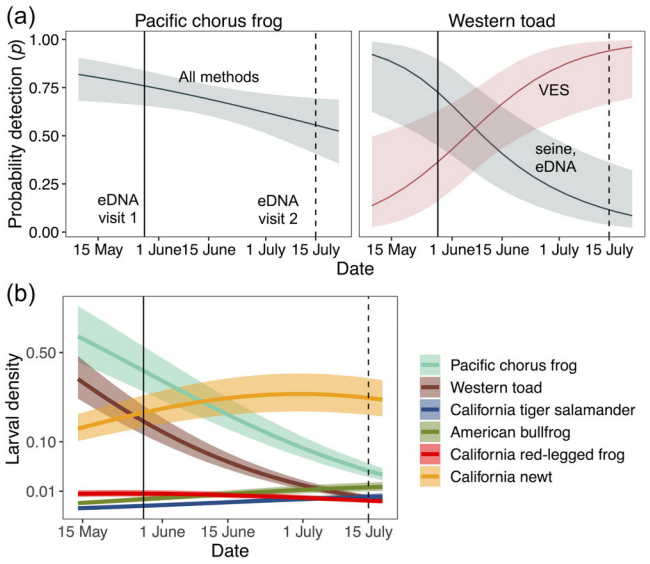

FIGURE 5 (a) Effect of sampling date on probability of detection. We show the two amphibian species for which date was an informative predictor. Vertical lines show the mean dates of eDNA surveys (first visit, solid; second visit, dashed). Best-fit lines show the predicted probability of detection from occupancy models, averaged across methods that did not significantly differ. For western toads, there was an interaction between survey method and date, with visual encounter surveys (VES) outperforming other methods later in the season. Seines and eDNA approaches relied upon detection of amphibians (primarily larvae) within the waterbody, whereas VES detected terrestrial life stages, primarily post-metamorphic juveniles. (b) Amphibian larval density as a function of survey date. Density (number of individuals per meter) was estimated using seine hauls (Appendix S1). Vertical lines show the mean dates of eDNA surveys (first visit, solid; second visit, dashed). We show best-fit lines from generalized linear models (Appendix S1). Western toads and Pacific chorus frogs show the strongest declines through time

difference between the two methods. The probability of detecting California tiger salamanders in a single PCR replicate (p) with metabarcoding was not significantly different than with qPCR (metabarcoding: $p = 0.43$, 95% CI = 0.12–0.81; qPCR: $p = 0.72$, 95% CI = 0.56–0.85). At the DNA extract level, methods also did not strongly differ ($\beta_{\text{method},\theta} = -0.29$, 95% CI = −1.57 to 1.23). The probability of detecting California tiger salamanders in a DNA extract, given that their eDNA was present at a site (θ), was 0.65 (95% CI = 0.37–0.87) for qPCR and 0.50 (95% CI = 0.14–0.97) for metabarcoding. For California red-legged frogs, the two methods produced identical results at each of the five ponds.

Detection sensitivity of qPCR

Detection of California red-legged frogs by qPCR analysis was relatively robust to variation in sampling protocol and water quality, and overall detection was high at both hierarchical levels. The probability of detecting California red-legged frogs in a single qPCR replicate, given that DNA was present in an extract (p), was 0.93 (95% CI = 0.93–0.97). The probability of detecting California red-legged frogs in a DNA extract (θ), given that the species was present at the site, was 0.77 (95% CI = 0.59–0.90). The best-fitting model for California red-legged frogs included the volume of water filtered as a covariate for the probability of detection at the DNA extract level (θ). However, water volume was uninformative, with the coefficient estimate overlapping 0 ($\beta_{\text{Volume},\theta} = -0.13$, 95% CI = −0.60 to 0.34), and the next best model was a null model (see Table A2 in Zenodo archive at: DOI: 10.5281/zenodo.5668507).

As with California red-legged frogs, detection of California tiger salamanders via qPCR was robust to both pond attributes and variation in sampling protocol. That is, we did not identify covariates that strongly impacted detection at either the DNA extract or qPCR replicate level (see Table A3 in Zenodo archive at: DOI: 10.5281/zenodo.5668507). The best-supported model included water volume filtered as a predictor of p and day as a predictor of θ, but the effects were uninformative and overlapping 0 ($\beta_{\text{volume},p} = -0.33$, 95% CI = −0.71 to 0.04; $\beta_{\text{day},\theta} = 0.24$, 95% CI = −0.31 to 0.78).

Detection sensitivity of metabarcoding

For most amphibian species, the volume of water filtered, pond area, turbidity, and date had no effect on the probability of detection with metabarcoding (Appendix S1: Table S11). For two species (Pacific chorus frog and western toad), DNA extractions from later collection dates had significantly reduced detections (Appendix S1: Table S11) as was consistent with occupancy models pooled at the survey level. Turbidity also reduced detection for one species, the Pacific chorus frog ($\beta_{\text{turbidity}} = -0.82$, 95% CI = −1.64 to 0.00).

DISCUSSION

Here, we compared eDNA metabarcoding, qPCR, and conventional field survey methods across 20 natural ponds to understand how multiple techniques could be combined to best survey amphibian communities. eDNA approaches yielded higher detection than conventional survey techniques for two protected species; however, other species showed mixed results. As a result, a combination of eDNA and conventional field surveys provided a more complete picture of amphibian communities than either method alone. Despite the rapid growth of eDNA

TABLE 1 Potential considerations for the integration of eDNA approaches into amphibian monitoring programs

Question	Yes	No
Is the amphibian community of interest highly diverse and/or is there uncertainty about which species may be present?	Metabarcoding may provide more standardized and efficient information on community composition than field or qPCR approaches	Multiple single-species qPCR assays may be suitable for describing amphibian community
Are rare/endangered amphibians or invasive species a specific target of the monitoring program?	qPCR assays for target species (especially assays that have already been validated) may enhance or replace field surveys	Increased sensitivity may not be necessary and metabarcoding could suffice
Does the study take place across a large spatial scale or are sites logistically difficult to sample?	eDNA methods may improve sampling efficiency. Consider either spatial or temporal replication in sampling	Field surveys, if already effective, may suffice. Users should consider whether eDNA surveys will provide additional information
Are other traits of amphibians (e.g., morphology, relative abundance, reproductive status, age-sex class) under study?	eDNA methods cannot provide this information, and field surveys will be required	eDNA surveys can complement field surveys to increase detection probabilities
Are there other components of biodiversity of interest (e.g., macroinvertebrates, pathogens, predators)?	Consider pairing qPCR and metabarcoding to target additional components of interest	Choice of survey method will depend on other study aims and questions
Have previous monitoring efforts been undertaken in this system?	Employ a pilot study alongside existing methods to evaluate where eDNA survey can enhance monitoring efforts	Use metabarcoding as an initial survey to identify overall biodiversity and follow up with more targeted surveys

analysis as a survey tool, few studies to date have compared the relative efficacy of different eDNA approaches alongside conventional sampling (Fediajevaite et al., 2021). Our study provides valuable insight into choosing an optimal survey design and methods for particular monitoring applications. To facilitate this decision-making, we pose several questions that should be addressed before integrating eDNA approaches into studies of amphibian ecology and conservation (Table 1) and highlight alterations to survey design, which could improve detections from eDNA analysis.

Variation in eDNA efficacy across species and methods

Detection rates varied strongly across species and methods, with no single method consistently generating the highest detection across all species. However, for the two species where both eDNA techniques (metabarcoding and qPCR) were used, they resulted in higher detection rates than field techniques, with qPCR showing an advantage over metabarcoding. This was particularly true for conservation priority California tiger salamanders in which qPCR outperformed both metabarcoding and field surveys (Figure 2). We were limited in statistical power to identify why qPCR had enhanced detection relative to metabarcoding; however, nonsignificant trends pointed to qPCR having increased detection probability at the PCR replicate level ($p = 0.72$ for qPCR compared with 0.43 for metabarcoding). Other studies comparing single-species approaches with metabarcoding also observed lower detection sensitivity using metabarcoding, with species masking, primer and amplification bias, technical replication, and sequencing depth given as potential reasons for decreased sensitivity (Bylemans et al., 2019; Harper et al., 2018; Schneider et al., 2016; Wood et al., 2019). While slightly less effective than qPCR, metabarcoding was still superior to field surveys, detecting California tiger salamanders at three more sites than even pooled field methods (Figure 3).

The qPCR assay we used for California tiger salamanders has not been previously published; herein, we have demonstrated its high sensitivity in situ. The probability of detecting California tiger salamanders at a given survey was 0.86 for qPCR compared with 0.31 for seines, and dipnets did not detect them at all (Figure 2). Given their precipitous declines across central California (U.S. Fish and Wildlife Service, 2017), California tiger salamanders are a target of numerous monitoring programs.

Existing methods, which often combine seining, dipnetting, and minnow trapping (U.S. Fish and Wildlife Service and California Department of Fish and Wildlife, 2003), are time-intensive and can disturb or injure larvae (Gray et al., 2013). Despite these efforts, California tiger salamanders are still notoriously difficult to detect (Kieran et al., 2020). Moreover, owing to California tiger salamanders' protected status, researchers must receive extensive training and obtain federal permits to conduct surveys. Beyond improving detection and reducing disturbance, an added benefit of eDNA analysis is the possibility of monitoring hybridization—a pervasive threat for this species (Ryan et al., 2009; U.S. Fish and Wildlife Service, 2017)—although nuclear markers would be necessary to do so. Thus, for California tiger salamanders, eDNA methods have strong potential to complement existing approaches, facilitating more efficient, widespread monitoring.

For California red-legged frogs, another protected species, qPCR and metabarcoding yielded similar detection probabilities, which were higher than even combined field methods (Figure 3). For both eDNA techniques, the detection probability at the DNA extract level was 0.77 compared with 0.93 at the PCR replicate level. As a result, effort and cost would be better spent on collecting additional samples (e.g., DNA extracts) than running additional PCRs, as detection of DNA within the sample with a small number of PCR replicates is already close to 1. While half of our DNA extracts were pseudoreplicates taken from the same water sample, we suggest that a better approach would be to take two separate water samples at each visit to produce two extracts. This level of sampling would achieve a 95% probability of detection and would generate true biological replicates.

The invasive American bullfrog showed the highest probability of detection overall (>0.90 for both VES and metabarcoding), and agreement was also high among VES and metabarcoding, detecting bullfrogs at the same sites and consistently across time (Figure 4). Thus, while past studies have demonstrated the potential of eDNA analysis to improve bullfrog detection during the early stages of invasions (Dejean et al., 2012), our results indicate that eDNA monitoring may provide no additional benefits where bullfrog populations are already established. Similarly, for California newts, eDNA metabarcoding is likely to be a complement, rather than a replacement to field surveys. Although metabarcoding and seining had similar rates of detection, metabarcoding detected newts at certain sites and time points where field surveys did not and vice versa.

For the two most widespread and abundant species in our system, metabarcoding produced mixed results. Pacific chorus frogs were ubiquitous, occurring at all 20 sites, yet metabarcoding failed to detect them at four sites where they were observed with other methods. Occupancy models estimated that detection probabilities for metabarcoding were equivalent to each individual field method (Figure 2). However, when field detections were combined, they detected Pacific chorus frogs at all 20 sites and missed them at only a single survey visit, whereas by the second eDNA survey, metabarcoding only detected Pacific chorus frogs at eight sites (Figure 4). Species life history and survey timing appear to partially explain this phenomenon. Densities of larval Pacific chorus frogs in waterbodies dropped consistently through summer as individuals metamorphosed (Figure 5b), and biomass was likely below detectable levels by the second eDNA sampling visit, which occurred later in the summer than the second field survey (Appendix S1: Table S1). Moreover, VES, which target terrestrial, postmetamorphic stages, continued to identify metamorphic Pacific chorus frogs into late summer. We observed a similar pattern for western toads, which also underwent metamorphosis by mid-summer and were rarely present as larvae by the second eDNA survey (Figure 5b). Similar temporal declines in detection were noted for western toads in a previous study using qPCR, suggesting that a more sensitive analysis method may not ameliorate this problem (Franklin et al., 2018). In short, metabarcoding failed to find a signal in ponds shortly after metamorphosis, emphasizing the low persistence of eDNA in aquatic environments in the absence of the source organism. Other studies have similarly described the rapid loss of eDNA signals, which can become undetectable within just 1 week after organisms are no longer present (Brys et al., 2021). In systems like ours, pairing an early-season eDNA survey with a late-season visual survey may be a more efficient way to monitor both species. The rapid loss of eDNA following metamorphosis further underscores the importance of designing surveys based on a priori knowledge of species natural history and phenology (De Souza et al., 2016).

While metamorphosis and survey timing appear to partially explain low rates of detection of Pacific chorus frogs and western toads with metabarcoding, false negatives for Pacific chorus frogs could additionally be driven by PCR bias and taxonomic resolution of primers. This could explain why read counts for Pacific chorus frogs were comparatively low (Appendix S1) and detection was imperfect even in early summer when biomass was high (Figure 4). Pacific chorus frogs had one mismatch at the reverse primer binding site (see Table A1 in Zenodo archive at: DOI: 10.5281/zenodo.5668507), but amplification was predicted to occur with this relatively small degree of mismatch using ecoPCR (Ficetola et al., 2010). Other species (California newt, western toad) also had

one mismatch at the reverse primer binding site but produced higher read counts (Appendix S1). We used broad-spectrum primers designed to amplify DNA from all vertebrates (Riaz et al., 2011), and it is possible that other vertebrate DNA was preferentially amplified and sequenced due to "species masking" (Harper, Lawson Handley, Carpenter, et al., 2019; Kelly et al., 2014; Klymus et al., 2017). Indeed, several of the ponds located in areas open to grazing contained a high proportion of reads from cows.

At the same time, false negatives could have arisen from low taxonomic resolution of primers. A large number of reads (>500,000 or 18% of amphibian reads) were assigned to a higher-level taxonomy (order Anura) despite the availability of reference sequences for all species of frogs in our study system (Appendix S1). The proportional read counts assigned to Anura (at the site visit level) were significantly and positively correlated with the densities of larval Pacific chorus frogs in seine hauls (Spearman rank-order correlation: $r_s = 0.37$, $p = 0.001$) but not positively correlated with the density of any other Anuran ($p > 0.05$). This finding supports, but does not prove, that many of the Anuran reads could have originated from Pacific chorus frogs. Our findings illustrate that in silico PCR provides a valuable but incomplete picture of primer performance. Testing primers in vitro would further elucidate the mechanisms underlying false negatives. In practice, more taxon-specific primers (e.g., Bálint et al., 2018; Valentini et al., 2016), blocking primers (Rojahn et al., 2021), or additional primer sets targeting different genetic markers (Collins et al., 2019) could be used to enhance the detection of focal taxa relative to other vertebrates.

Sensitivity of eDNA detections to water quality and sampling protocol

We found little evidence that pond water quality impacted rates of detection in our study, although one species (Pacific chorus frog) showed declining metabarcoding detection rates with higher turbidity (Appendix S1: Table S11). While the high organic matter and sedimentation commonly found in lentic waterbodies can impede eDNA capture (Bedwell & Goldberg, 2020; Franklin et al., 2018; Goldberg et al., 2016; Harper, Buxton, Rees, et al., 2019) and inhibit PCR (Franklin et al., 2018), this did not appear to occur in our samples, perhaps because we took measures to counter sample inhibition. We also found no evidence that modifying the filtration protocol to mitigate clogging (via larger filter sizes or smaller water volume) influenced detection probability, albeit with limited statistical power. Thus, our detections were relatively robust to modifications in collection protocol and natural variation in pond water properties.

Sampling date had a significant effect on detection for two species, suggesting that effort is better spent on expanding the number of visits rather than filtering more water or increasing the number of biological or technical replicates. Although we replicated our sampling temporally, collecting two water samples roughly seven weeks apart for each site, we did not replicate our sampling spatially within each site. Therefore, the samples and subsequent DNA extracts from the same visit were pseudoreplicates. Pooling water subsamples taken at multiple spatial locations allows one to better sample unevenly distributed eDNA, such as that present in stagnant waterbodies (Biggs et al., 2015; Goldberg et al., 2016), but there is some evidence that it can reduce the detection of rare species (Davis et al., 2018). Thus, a better approach might be to include at least two spatial biological replicates at each survey (Bálint et al., 2018) or use a "removal design," processing each additional sample only if the previous were negative (Davis et al., 2018). The optimal sampling protocol is likely to vary within each system making pilot studies like our own, which combine occupancy modeling and sampling across a gradient of natural sites, an important step for optimizing survey design.

Enhancing amphibian surveys with eDNA approaches

There is widespread interest in improving and expanding monitoring programs for amphibians due to their global declines (Stuart et al., 2004). Our study illustrates the potential advantages of incorporating eDNA surveys into existing monitoring programs. First, we found that both metabarcoding and qPCR improved the detection of two imperiled amphibians, beyond the best available field methods. For monitoring programs where rare species are the primary focus (Table 1), eDNA methods alone might provide more information and entail less disturbance than conventional surveys (Pope et al., 2020). In addition to improving amphibian detection, eDNA analysis of a single sample using qPCR and metabarcoding can provide data on some of the most pressing threats for amphibians: disease (Huver et al., 2015; Kamoroff & Goldberg, 2017), hybridization (Stewart & Taylor, 2020), and non-native species (Dejean et al., 2012). Moreover, the vertebrate primers we used identified 41 other vertebrate taxa in addition to our six focal amphibians, including birds, mammals, reptiles, and fish. Some of these species (e.g., fish) are important predators of amphibians (Joseph et al., 2016), while others (e.g., waterfowl) are involved in dispersing parasites of amphibians to ponds

(Wood et al., 2019). These additional detections therefore provide ecological information relevant to amphibian management and conservation (Kačergytė et al., 2021). For managers tasked with developing multi-species monitoring programs, metabarcoding offers the opportunity to estimate broader biodiversity alongside the presence of focal species (Table 1).

Detection rates from eDNA approaches varied across species, illustrating that eDNA analysis is not a one-size-fits-all solution to amphibian monitoring. Yet, the same was true of field approaches, with certain methods providing enhanced detection at specific time points or for particular species. Field researchers are well accustomed to employing a diverse suite of survey methods to target different species or questions, and rarely is one method expected to provide a complete ecological picture. Therefore, we suggest that the same expectation be applied to eDNA monitoring. To best help end users choose how to incorporate eDNA methods, we provide a general set of questions to guide decision-making (Table 1). We do not intend this to be an exhaustive list, but a general guide for identifying the types of questions that eDNA analysis is especially suited to target.

CONCLUSIONS

Environmental DNA surveys detected all six amphibian species in our study system, with varying success, and provided additional information that field surveys did not. When detecting rare species is a primary focus of a monitoring program, single-species approaches (qPCR) still appear to be the "gold standard" (Table 1). In small amphibian communities such as ours, a panel of species-specific qPCR assays could be employed to survey the whole community (Table 1). Yet, qPCR tends to become cost- and time-inefficient for speciose communities and at increasing sample sizes, whereas metabarcoding has high start-up costs but scales with the number of samples being processed (Hänfling et al., 2017; Harper et al., 2018; Wilcox et al., 2020). Metabarcoding was less sensitive than qPCR, but was still, on average, as effective as conventional survey approaches for detecting amphibians, with the added benefit of providing additional information on broader vertebrate biodiversity. Thus, for a number of applications, such as broad biodiversity monitoring or pilot studies, metabarcoding can generate efficient and accurate compositional data (Table 1). Metabarcoding was most limited by survey timing and potentially by primer bias, amplification bias, and taxonomic resolution; thus, these aspects require further investigation and refinement. However, as primer design, reference databases, bioinformatics pipelines, and survey designs are optimized, metabarcoding is likely to become part of the standard toolkit for amphibian monitoring. As users consider adopting eDNA techniques, we emphasize the need to employ methodological and statistical approaches that account for false negatives and false positives, and to employ eDNA surveys alongside conventional field surveys in studies such as our own. Together, these precautions will help design surveys where methods complement one another, producing more efficient and accurate biodiversity data.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data and code are available from Zenodo: https://doi.org/10.5281/zenodo.5668507. Raw read data from metabarcoding have been uploaded to the NCBI Sequence Read Archive (BioProject: PRJNA718336l; BioSamples: SAMN18528944–SAMN18529079l; SRA accessions: SRR14240072–SRR14240166).

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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