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RESEARCH ARTICLE

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Designing environmental DNA surveys in complex aquatic systems: Backpack sampling for rare amphibians in Sierra Nevada meadows

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Abstract

1. Surveys for environmental DNA (eDNA) can provide an efficient and effective means of detecting aquatic organisms in various types of aquatic systems.
2. In the summer of 2017, the efficacy of a new, integrated eDNA backpack sampler to detect two native amphibians (*Rana sierrae* and *R. cascadae*) at risk was tested in complex mountain meadows in California. Samples were collected at 65 locations in 15 meadows where the target species were known to be present or were historically present.
3. Collection and preservation of individual samples took less than 10 min on average. Environmental DNA analysis methods detected each species at all meadows with visual detections (N = 11) except one with one frog seen away from sampling sites. Bayesian multi-scale occupancy modelling indicated that conditional detection probabilities at the sample level ranged from 0.30 (CL 0.07–0.65) at meadow heads where no frogs were observed during visual surveys to 0.93 (CL 0.77–1.00) at the meadow foot with at least one frog observed in the vicinity.
4. Compared with visual surveys, eDNA methods more frequently detected amphibians at the sampling-location scale. The improvement in detection using eDNA methods was most pronounced for samples collected at the downstream ends of meadows where water converges, where eDNA methods detected target species at 10 of 11 occupied meadows.
5. These results suggest that the addition of eDNA sampling to visual surveys in mountain meadows will improve survey accuracy and increase the probability of detecting rare frogs.

KEYWORDS
anuran distribution, multi-scale occupancy modelling, self-desiccating filters, visual surveys

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1 | INTRODUCTION

Accurate knowledge of where imperilled or invasive organisms occur allows the strategic allocation of limited management resources, yet determining the occupancy and distribution of these species can be difficult, especially in complex habitats with few individuals (Tyre et al., 2003; Mackenzie & Royle, 2005; Chades et al., 2008). Researchers have developed a molecular tool to address this problem in aquatic environments by surveying for the presence of trace genetic evidence (such as shed skin, faeces, urine and mucus) of species of interest in the water (Belle, Stoeckle, & Geist, 2019; Dejean et al., 2012; Ficetola, Miaud, Pompanon, & Taberlet, 2008; Goldberg, Pilliod, Arkle, & Waits, 2011; Rees, Maddison, Middleditch, Patmore, & Gough, 2014). Instead of time-consuming survey techniques that often involve disturbing animals through netting or electroshocking, surveyors obtain water samples at strategic locations within the aquatic habitat, reducing field time, costs and risk to target organisms (Thomsen & Willerslev, 2015). This environmental DNA (eDNA) method has proved effective at detecting both rare native species (Brozio et al., 2017; Harper et al., 2019; Ikeda, Doi, Tanaka, Kawai, & Negishi, 2016; Jerde, Mahon, Chadderton, & Lodge, 2011; McKee et al., 2015; Strickland & Roberts, 2019) and potentially detrimental non-native species (Dejean et al., 2012; Goldberg, Sepulveda, Ray, Baumgardt, & Waits, 2013; Jones et al., 2018; Kamoroff & Goldberg, 2017; Wilcox et al., 2018; Wilson et al., 2014). Yet even though it has proven effective, eDNA methods are only beginning to be embraced by managers as a monitoring tool (Goldberg, Strickler, & Pilliod, 2015; Hering et al., 2018; Roy, Belliveau, Mandrak, & Gagne, 2018).

Hesitancy to apply eDNA sampling stems in part from uncertainties about how its effectiveness varies with environmental conditions and across taxa (Barnes et al., 2014; Goldberg, Strickler, & Fremier, 2018; Strickler, Fremier, & Goldberg, 2015). For example, DNA breaks down more quickly in warm or acidic water compared with cool or neutral water (Strickler et al., 2015), and does not travel far from its source in still water conditions (Dunker et al., 2016; Goldberg et al., 2018). In addition, the production of eDNA varies among individuals and within individuals over time (Stewart, 2019). However, a better understanding of the drivers of uncertainty in eDNA detectability allows adjustments in sampling and analysis to account for this variability. For example, Goldberg et al. (2018) investigated eDNA detection for a suite of amphibian species across a gradient of environmental conditions to determine the different physiological, ecological and hydrological processes limiting detection. The findings were then used to adjust sampling protocols for the different species and environments in a way that increased detection probability to 0.95.

Advances in sampling methods should also improve the effectiveness of eDNA sampling as a monitoring tool. Recently, a backpack sampler was developed with a negative pressure inline filtration system to collect eDNA samples efficiently (Thomas, Howard, Nguyen, Seimon, & Goldberg, 2018). Using this system, water samples are pumped through a filter mounted at the end of a pole extension. Flow rate, sample volume and filtration pressure can be programmed and recorded for each sample collected (Thomas et al., 2018). In addition, Thomas, Nguyen, and Goldberg (2019) developed self-desiccating filter packs that may further increase collection efficiency and decrease contamination risk for field samples because field crews would no longer have to remove, fold and place filters in ethanol-filled vials in the field. Although promising, the effectiveness of these new tools has yet to be demonstrated for many sampling applications.

Advances in eDNA sampling methodology could facilitate the management of complex montane wet-meadow areas that provide key habitat for native amphibians at risk. For example, amphibians have experienced severe declines in California's mountains over the last century owing to habitat alteration, disease and invasive species (Adams et al., 2017; Knapp & Matthews, 2000; Piovia-Scott et al., 2015; Pope, Brown, Hayes, Green, & Macfarlane, 2014; Rachowicz et al., 2006; Wake & Vredenburg, 2008). Declines in the Sierra Nevada yellow-legged frog (*Rana sierrae*) and Cascades frog (*Rana cascadae*) have been particularly pronounced. *Rana sierrae* is a federally listed endangered species that inhabits high-elevation lakes, streams and meadows in the Sierra Nevada. *Rana cascadae* is under review for listing on California's endangered species list and occurs in similar habitats in the southern Cascade and Klamath ranges of California. Severe declines and extinctions have been documented for both species in lake habitats at higher elevations resulting from a deadly fungal pathogen and the pervasive introduction of non-native sport fishes (De Leon, Vredenburg, & Piovia-Scott, 2017; Fellers, Pope, Stead, Koo, & Welsh, 2008; Knapp & Matthews, 2000; Rachowicz et al., 2006; Vredenburg, Knapp, Tunstall, & Briggs, 2010). Mountain meadows may play an increasing role in supporting these native amphibians because they provide refuge from fish and possibly disease (Pope et al., 2014). Owing to the complexity of meadows, native frogs can be difficult to detect using conventional survey techniques, especially when populations are small. If eDNA surveys can provide an efficient and effective alternative detection method, they have the potential to greatly facilitate the conservation of rare amphibians and management of their meadow habitats.

This article describes tests carried out in the summer of 2017 on whether eDNA monitoring with the backpack sampler could be used to detect *R. sierrae* and *R. cascadae* at 15 meadows in the Sierra Nevada and southern Cascade ranges. Meadows were selected with known or expected occupancy by either species, including occupied sites with a range of abundances, and visual surveys were conducted concurrently with eDNA sampling. In addition to evaluating occupancy at the whole-meadow scale, associations between the results from eDNA sampling and specific habitat categories within meadows were also evaluated. The study had two further objectives: a beta version of the Smith-Root eDNA Sampler Backpack (Thomas et al., 2018) was tested, and newly developed self-desiccating filter packs were evaluated that may increase collection efficiency and decrease contamination risk for field samples (Thomas et al., 2019).

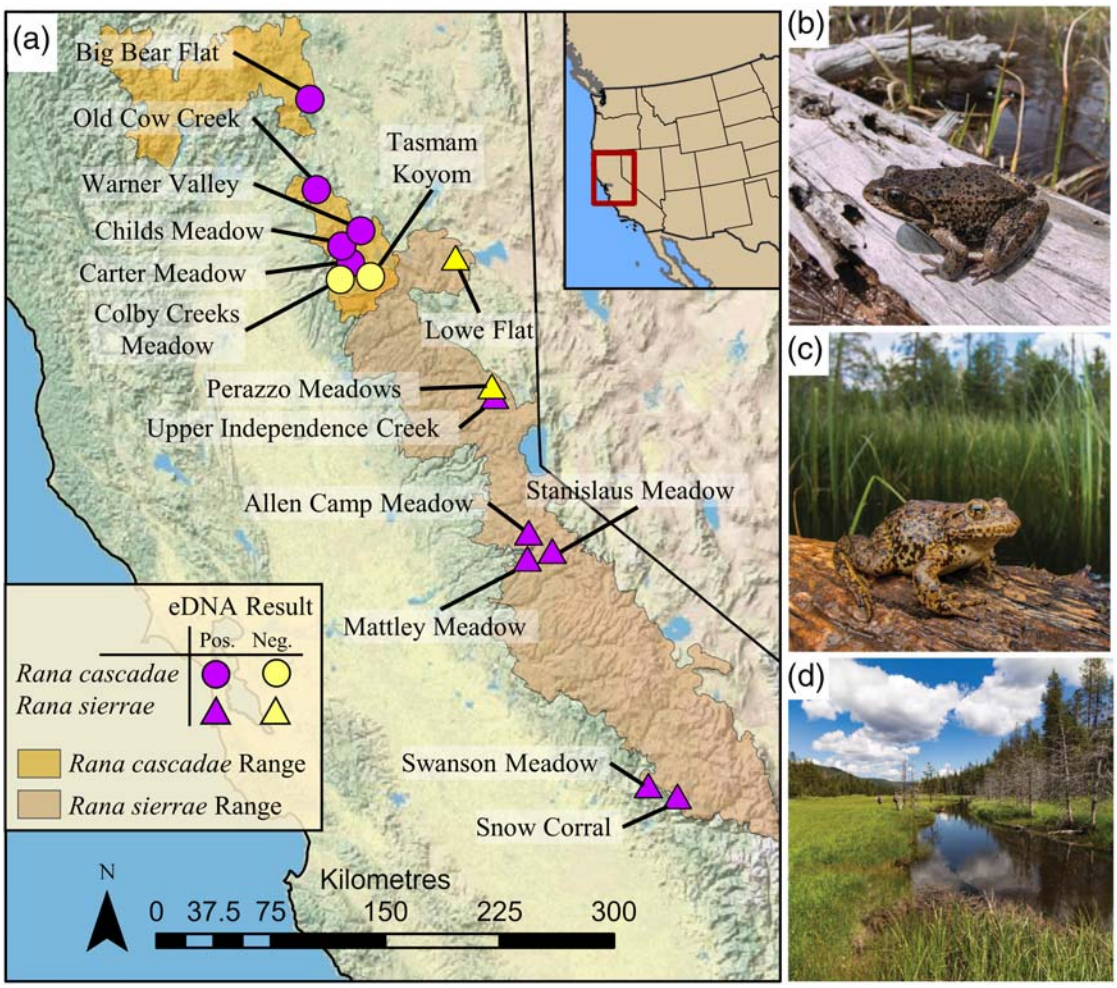

FIGURE 1 Meadows sampled for eDNA of Cascades frogs (*Rana cascadae*) and Sierra Nevada yellow-legged frogs (*R. sierrae*) in the Sierra Nevada and southern Cascades ranges of California (a). Images include target amphibians, *R. cascadae* (b) and *R. sierrae* (c), and an example of channel habitat in Childs Meadow. Photo credits: S. Riffle (b and c) and A. Bearer (d)
[Correction added on 11 September 2020, after first online publication: The photo credits on Figure 1 has been corrected in this version.]

2 | METHODS

2.1 | Site selection

Meadows were sampled in the Sierra Nevada Mountains from the Sierra National Forest north to the Plumas National Forest for *R. sierrae*, and meadows in the southern Cascades Range in California for *R. cascadae* (Figure 1, Table 1). Specific meadows were chosen based on determinations of frog presence or absence from previous visual surveys. Sites were included with high densities of animals, with few animals remaining, and where target species were historically present but presumably extirpated.

2.2 | eDNA sample collection and visual surveys

Two replicate eDNA samples were collected from each of two to six localities within each meadow (Table 1, Appendix A). These included the head and foot of most meadows to determine if these were effective sampling locations to determine presence and extent of occupancy (e.g. present in meadow, but not above meadow). Additional sampling localities were selected within meadows based on the known or suspected habitat use of the focal species (Figure 2). Sampling localities were assigned to one of four categories: (i) head of the meadow; (ii) foot of the meadow; (iii) stream channel; or (iv) off-channel pool. In conjunction with each eDNA sample collected, a visual count of amphibians within the expected area of influence of the sample locality was also conducted. The area of influence was defined as the pool where the sample was collected or, for stream samples, the reach extending 30 m upstream of the sample locality. When field crew members did not know the current or historical location of target species within a meadow, surveys were guided by local biologists with knowledge of frog distribution in that meadow. To minimize the risk of sample contamination, eDNA samples were collected by crew members who had not handled target species.

At each sampling locality, the Smith-Root eDNA Sampler Backpack (Thomas et al., 2018) was used with a split in the sampling line for replicate sampling. An extension pole was used to collect

TABLE 1 Meadows sampled for Sierra Nevada yellow-legged frogs (*Rana sierrae*) and Cascades frogs (*Rana cascadae*), including the number of sampling locations per meadow (*n*), whether or not the species was detected by eDNA or visual surveys, mean sample volume filtered (±SE) and mean time to collect a sample (±SE)

Meadow	Target species	Meadow size (ha)	Elevation (m)	Date surveyed	*n*	eDNA?	Visual?	Sample volume (L)	Sample time (min)
ALC	*R. sierrae*	3.7	2,468	20 August 2017	4	Yes	Yes	1.1 (0.4)	6.0 (0.7)
IND	*R. sierrae*	12.9	2,149	17 August 2017	5	Yes	Yes	1.5 (0.3)	6.4 (1.0)
LF	*R. sierrae*	5.7	1,832	21 June 2017	2	No	No	1.2 (0.4)	13.5 (3.5)
MAT	*R. sierrae*	18.4	2,180	19 August 2017	6	Yes	Yes	1.4 (0.4)	8.3 (1.8)
PRZ	*R. sierrae*	152.6	1,986	18 August 2017	6	No	Yes	0.9 (0.3)	6.7 (1.1)
SNC	*R. sierrae*	7.8	2,144	22 June 2017	3	Yes	Yes	1.2 (0.5)	13.0 (2.3)
STA	*R. sierrae*	10.4	2,355	22 August 2017	4	Yes	Yes	0.9 (0.4)	7.2 (2.3)
SWM	*R. sierrae*	17.5	1,720	23 June 2017	6	Yes	Yes	0.5 (0.1)	8.7 (2.1)
BBF	*R. cascadae*	40.3	1,232	19 June 2017	4	Yes	Yes	1.8 (0.1)	6.5 (1.3)
CAR	*R. cascadae*	25.1	1,868	9 July 2017	5	Yes	Yes	1.7 (0.3)	6.4 (0.5)
CHI	*R. cascadae*	19.2	1,468	8 July 2017	4	Yes	Yes	2.0 (0.0)	6.0 (1.2)
COL	*R. cascadae*	9.4	1,518	12 July 2017	3	No	No	2.0 (0.0)	4.1 (0.6)
TAS	*R. cascadae*	14.4	1,318	20 June 2017	4	No	No	1.7 (0.2)	8.0 (3.0)
OCC	*R. cascadae*	12.5	1,786	7 July 2017	6	Yes	Yes	1.8 (0.2)	5.2 (0.4)
WAR	*R. cascadae*	105.3	1,577	11 July 2017	3	Yes	Yes	1.0 (0.5)	6.7 (1.5)

Abbreviations: ALC, Alan Camp Meadow; BBF, Big Bear Flat; CAR, Carter Meadow; CHI, Childs Meadow; COL, Colby Creek; IND, Independence Creek; LF, Lowe Flat; MAT, Mattley Meadow; PRZ, Perazzo Meadow; SNC, Snow Corral Meadow; STA, Stanislaus Meadow; SWM, Swanson Meadow; TAS, Tasmam Koyom; WAR, Warner Valley.

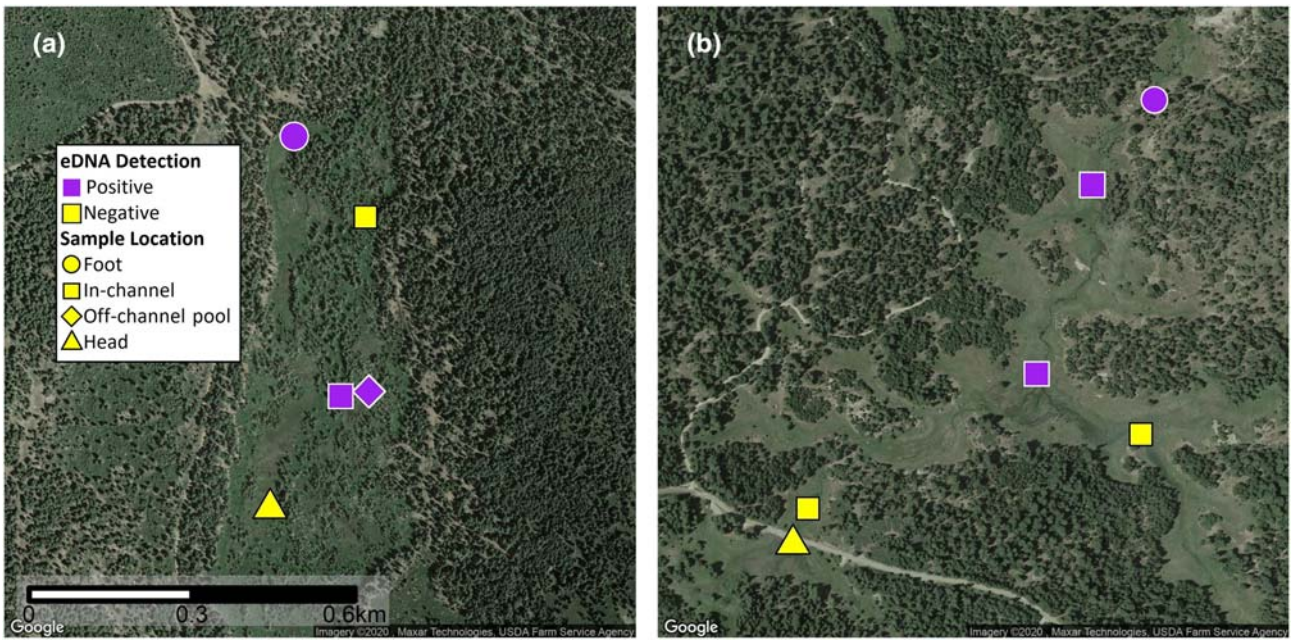

FIGURE 2 Examples of eDNA sampling designs at Carter Meadow for Cascades frogs (a) and at Swanson Meadow for Sierra Nevada yellow-legged frogs (b). Both meadows flow to the north

water from a depth of about 5–10 cm through the two inline filters attached 25 cm apart on the end of the pole. Samples were taken from either a single point or along a transect up to 15 m long, depending on the size of the area being sampled and whether constant submersion could be maintained while moving through the water. The total volume pumped, start time and end time were recorded for each sample. Attempts were made to filter 2.0 L per filter (i.e. 4.0 L total) at each sampling locality, but in some instances filters clogged before this volume was obtained. Water depth, water temperature, transect length and coordinates (using a handheld GPS) were also recorded for each sample. In the standard sampling protocol used, filters were removed from the filter packs upon completion of the sample and stored in 95% ethanol before analysis. At four localities, samples were collected using both the ethanol preservation method and newly developed self-desiccating filters (Thomas et al., 2019) to determine (via paired *t*-tests) whether they provided comparable results. Instead of being removed from the filter housing, filters with desiccating filter housings were left in their housing and stored in their individual packaging.

A field negative sample (distilled water) was collected at every site. In addition, recommended best practices for preventing cross-contamination between samples were followed (Goldberg et al., 2016). New gloves were used whenever filters were handled, filter housings and forceps were single-use, and all other field gear was decontaminated with bleach between meadows to prevent the transfer of DNA and pathogens.

2.3 | eDNA assay development

An existing assay was available for *R. sierrae* (Bedwell & Goldberg, 2020), but not for *R. cascadae*, so a range-wide assay was developed for *R. cascadae*. Sequence data were provided by K. Monsen (Monsen & Blouin, 2003) and an inclusive consensus sequence for the D-loop sequence was created using Sequencher version 5.2.4 (GeneCodes Corp., Ann Arbor, MI, USA). Primer Express 3.0.1 (Applied Biosystems, Foster City, CA, USA) was used for assay design and assays were hand-checked against a sequence of American bullfrog (*Lithobates catesbeiana*) from Genbank (D12694.1). Primer-BLAST (Ye, Coulouris, & Zaretskaya, 2012) was used to test for specificity *in silico* against all species in the database with the criteria of at least 2 bp differences on each primer, with one within 5 bp of the 3′ end, and at least 1 bp difference in the middle of the probe. For ease of multiplexing, the probe was extended for *R. cascadae* using OligoAnalyzer (Integrated DNA Technologies) so that non-Taqman probes could be ordered. The final assay was: forward primer (RACAF) TAATCGAATACCCCGTGCATT; reverse primer (RACAR) ATACCAGGTGTTCTCGCTGTCAC;probe (RACAProbe) Cy5-TGCGTTAAA/TAO/TACCTCGATACAAGCTCAGG-3IaBrqSP.

For validation, each qPCR plate included 3 μl of DNA extract in a total volume of 15 μl, with 10 samples of the target species and five samples of each other closely related species in the region (*R. sierrae*, *Anaxyrus boreas*, *Rana boylii*, *L. catesbeiana* and *Pseudacris sierra*). Reactions were run using 1X NoROX QuantiTect Multiplex PCR Mix (Qiagen Inc.), with recommended multiplexing concentrations (0.2 μM of each primer and probe) on a Bio-Rad CFX96 Touch Real-Time PCR Detection System. To test for inhibition of the qPCR, each well included an exogenous internal positive control (IC; Qiagen). Reactions were activated for 15 min at 95°C then ran for 50 cycles of 94°C for 60 s followed by 62°C for 60 s. After single-species validation, the *R. cascadae* assay was validated in a multiplex with the *R. sierrae* assay and IC, confirming no cross-amplification or reduction in signal of each assay when run together for samples testing positive for either or both target species.

2.4 | eDNA filter processing

Environmental DNA was extracted from the field-collected filters using the Qiashredder/DNeasy method (Goldberg et al., 2011) in a laboratory dedicated to low-quantity DNA samples, using best practices for preventing and detecting contamination (Goldberg et al., 2016). A negative extraction control was included with each set of extractions and an additional negative qPCR control was run with each plate of field samples. Reactions were as described above. A well was considered as testing positive if exponential growth was produced. Each sample was analysed in triplicate and any sample that produced inconsistent results (one or two positive) was rerun in triplicate. An inconsistent sample was considered positive if it tested positive in one or more well in both triplicate runs. A sample was considered inhibited if the IC curve was ≥3 Cq less than those of the standards. Samples testing as inhibited were cleaned using a OneStep™PCR Inhibitor Removal Kit (Zymo, Inc., Irvine, CA, USA). Quantitative standards for *R. cascadae* consisted of DNA samples derived from tissue from external skin, diluted 10^{-3}–10^{-6} in dilution buffer (Qiagen) and run in duplicate on each plate. Quantitative standards for *R. sierrae* consisted of gblock standard (Integrated DNA Technologies), in a 10-fold series dilution 10,000 to 10 in dilution buffer (Qiagen) and run in duplicate. To convert the units from the *R. cascadae* dataset to copy number for comparison with *R. sierrae*, a gblock standard curve was created and used to create a conversion to copy number. Efficiency of all reactions was 92–104% with $r^2 > 0.99$. Samples from three of the 65 meadow localities were found to be strongly inhibited on both filters and were excluded from further analyses.

2.5 | Statistical analyses

To evaluate the effectiveness and consistency of the eDNA sampling method, eDNA occupancy and detection probabilities were estimated for *R. sierrae* and *R. cascadae* at the meadow, sampling locality and replicate filter scales with Bayesian, multi-scale occupancy models using the R package eDNAoccupancy v0.2.0 (Dorazio &

Erickson, 2018). These models accommodate three levels of sampling so it was possible to model the probability of species occurrence among meadows (Ψ), the conditional probability of species occurrence at a sampling locality within a meadow given that the species is present in the meadow (θ) and the conditional probability of species detection on replicate filters collected at a sampling locality given that the species is present at that sampling locality (p). Only meadows where at least one of the two focal frog species were observed were included. Owing to their similar biologies, both species were included in the same models to maximize sample size. For estimating θ, habitat category (head, foot, in-channel, off-channel pool) and the presence or absence of frogs in the visual survey of the area of influence were included as covariates. For estimating p, the volume of water filtered was included as a covariate. The inclusion of additional sample locality-level covariates (e.g. water temperature) led to models that would not converge. Estimates of the parameters were computed using the Markov chain Monte Carlo algorithm in the eDNAoccupancy package, set at 20,000 iterations with the first 2,000 discarded as burn-in and the posterior sample thinned to every tenth sample to reduce autocorrelation. The posterior mean and 95% credible limits for each parameter of the multi-scale model were estimated using the function posteriorSummary and convergence was assessed by plotting traces and estimating levels of autocorrelation (Dorazio & Erickson, 2018). Where duplicate ethanol-preserved and self-desiccating filter samples were collected, only the ethanol-preserved filters were used for this analysis.

Because the occupancy models could only be fitted with a limited number of covariates and only used presence/absence data as a response variable, linear mixed models (LMMs) were also fitted using the R package glmmTMB (Brooks et al., 2017). As with the occupancy models, only the meadows where *R. sierrae* or *R. cascadae* were found were included. The LMMs used eDNA quantity (as determined by qPCR) as a response variable, natural log-transformed to meet model assumptions. Each model included random intercepts for Meadow (12 levels) and eDNA sample locality (53 levels) to account for the nested data structure. Additional predictor variables included target species (*R. cascadae* or *R. sierrae*), the number of frogs observed within the expected area of influence for the sample, sample volume and sample habitat category as fixed model covariates. In addition, two interaction terms were included: visual count × habitat category and visual count × species, because different meadow habitats and interspecific variation in behaviour may influence the visual detection of frogs (e.g. larvae can easily hide in silty off-channel pools). Model selection was then conducted using an information theoretic approach (AICc; Burnham & Anderson, 2002). Differences between levels of categorical covariates in all LMMs were evaluated with estimated marginal means using the R package emmeans (Lenth, 2018).

Based on results from both modelling exercises, additional *post-hoc* analyses were run to explore further the influence of certain predictors. Specifically, an assessment was made of whether the volume of water filtered differed among habitat categories within meadows using an LMM and estimated marginal means.

3 | RESULTS

3.1 | Assay validation

The *R. cascadae* assay was successfully validated *in silico*, with no matches in the search. However, many species do not have sequences of the D-loop region in the database. The assay was also successfully validated against tissue samples from all co-occurring anurans.

3.2 | eDNA sample collection

Sample collection usually took <10 min (mean for *R. cascadae* samples = 6.1 min, mean for *R. sierrae* samples = 8.7 min). Filtered water volumes ranged from 0.2 to 2.2 L per filter and averaged 1.4 L. The two-sample preservation methods yielded no differences in the quantity of eDNA (mean copy number for ethanol-preserved, 1.5 per ml; for self-desiccating filters, 1.6 per ml; t = 0.7, d.f. = 11, P = 0.5).

3.3 | Detection of *R. sierrae* and *R. cascadae* using eDNA sampling

Rana sierrae was detected from at least one eDNA sample at six of eight meadows sampled for the species, and occurrence was

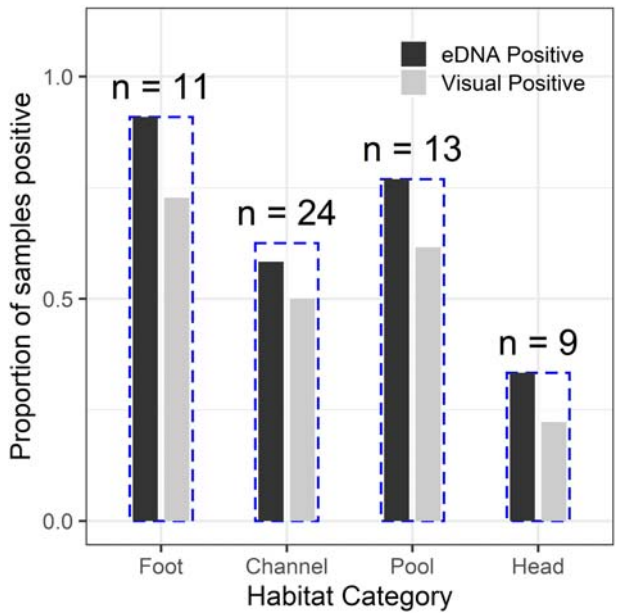

FIGURE 3 Proportion of samples with positive detections of Cascades frogs (*Rana cascadae*) or Sierra Nevada yellow-legged frogs (*R. sierrae*) at each location sampled within meadows where occupancy was confirmed. Black bars are eDNA-positive detections and grey bars are visual positive detections in the area of influence for each sample location (see text for details). The blue dashed lines indicate the cumulative proportion of positive samples using either method so that a gap between the height of a bar and the dashed line represents failed detections using that method

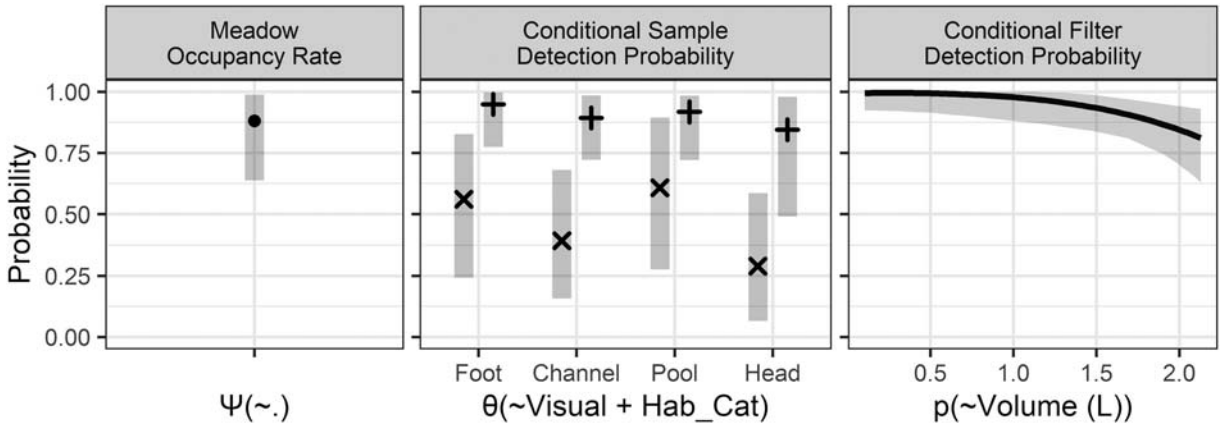

FIGURE 4 Bayesian occupancy model results. Light grey bars and ribbon indicate 95% credible intervals and black shapes and lines indicate estimated median values. The first panel shows the modelled occupancy rate for meadows in the analysis. The second panel shows the sample-level detection probability, conditional on the meadow being occupied. Samples collected from locations with positive visual detections of the target species (+) had higher detection probabilities than those collected with no adjacent visual detections (×). The third panel shows the relationship between volume of water filtered and the detection probability per filter in each habitat category (conditional on the sample location having eDNA to detect)

visually confirmed at seven of those eight meadows (one frog was encountered at Perazzo Meadow while walking between sample localities). *Rana cascadae* was detected from at least one eDNA sample at five of seven meadows, and occurrence was also confirmed using visual surveys at the same five meadows (Table 1). Results also indicated the capability of eDNA surveys to determine occupancy at the sample scale: eDNA sample results matched local visual surveys 84% of the time and, of the nine differences between eDNA sample results and visual survey detections, eight involved detections of eDNA where no animals were observed (Figure 3). At 10 of the 11 meadows where eDNA analysis detected frogs, eDNA samples from the foot of the meadow tested positive (Figure 3).

The multi-scale occupancy model estimated a meadow occupancy rate of 0.88 (95% credible limit, CL: 0.64–0.99, Figure 4) for *R. sierrae* and *R. cascadae* at meadows known to support them (n = 12). Conditional detection probabilities at the sample level ranged from 0.30 (CL 0.07–0.65) at meadow heads where no frogs were observed during visual surveys to 0.93 (CL 0.77–1.00) at the meadow foot with at least one frog observed in the vicinity (Figure 4). The conditional detection probability for individual filters ranged from 0.83 to 1.0 depending on the volume of water filtered. Occupancy models found a negative relationship between volume of water filtered and detection probability (Figure 4). However, *post-hoc* analyses found that habitat category and volume were confounded – samples collected at the head of the meadow filtered greater volumes of water than samples collected at the other localities in the meadow (estimated marginal mean, EMM, for the head = 1.8 L per filter; EMM for other sampling localities = 1.2 L per filter; t = 4.1, P = 0.0001; Figure 5). Removing the volume covariate from the model did not alter the other model estimates substantially. All negative controls tested negative for both frogs.

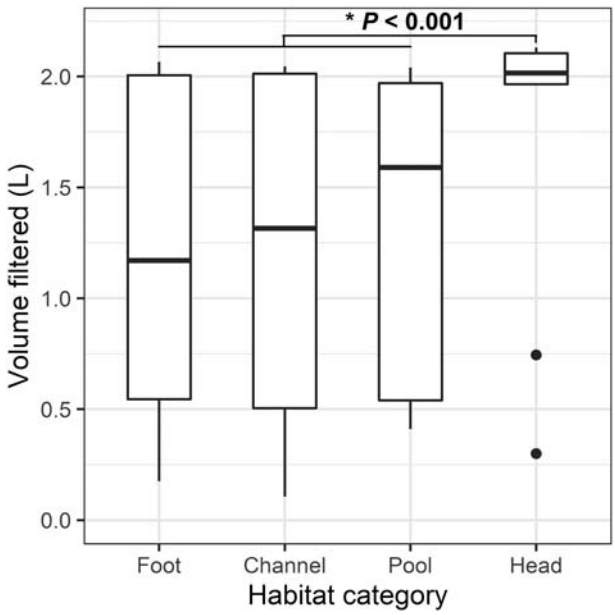

FIGURE 5 Volume of water filtered by habitat category. Thick horizontal lines represent the median; the bottom and top of the bars represent the 25th and 75th percentiles, respectively; whiskers below and above bars represent 10th and 90th percentiles, respectively; and dots represent points outside the 10th and 90th percentiles

3.4 | Factors affecting eDNA quantity for *R. sierrae* and *R. cascadae*

Analyses yielded two similarly well-supported models of eDNA quantity (Appendix B). Both models included the visual count of the target species in the vicinity of the sample and sample habitat category, but only one contained species (*R. sierrae* vs. *R. cascadae*)

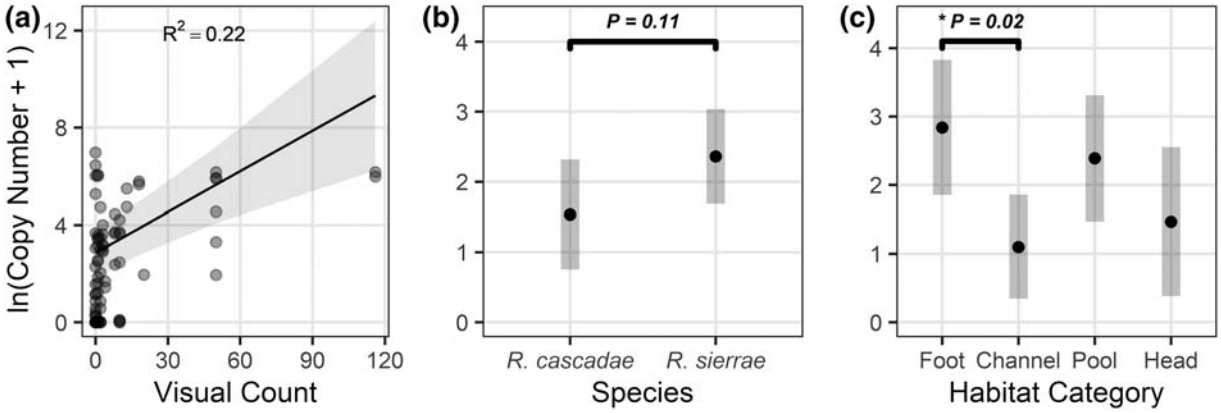

FIGURE 6 Estimated influence of the linear mixed model covariates (a) visual count of frogs within the expected area of influence of the sample (see text for details), (b) species, and (c) habitat category on eDNA quantity. Estimates for each variable account for the influence of the other covariates. Grey shading signifies 95% confidence intervals; points in (a) represent raw data, while points in (b) and (c) represent estimated marginal means

as a covariate. Neither top model included sample volume or interactions between visual count and species or sample habitat category. The most inclusive of the highest ranked models was used for further analysis. The model-estimated eDNA quantity increased by 1 unit for each 18 target individuals counted within the expected area of influence of the sample (Figure 6). The quantity of eDNA was also higher at the foot of the meadow than in channel habitats within the meadow (Foot EMM, 2.8; in-channel EMM, 1.1 copies per ml; P = 0.02). Marginally more *R. sierrae* eDNA was detected than *R. cascadae* (P = 0.11; Figure 6).

4 | DISCUSSION

The analysis of eDNA samples collected with the Smith–Root eDNA Sampler Backpack efficiently and accurately detected the presence of rare native amphibians in complex mountain meadow habitats. The one meadow where we incidentally observed one individual *R. sierrae* but did not detect it in eDNA samples (Perazzo Meadow) is a large meadow with extensive off-channel aquatic habitats, suggesting that large, complex meadows require thorough visual and eDNA surveys to yield accurate assessments of occupancy for rare species. As expected, the Sampler Backpack allowed highly efficient sampling by filtering larger volumes of water more rapidly than standard hand-pumping methods (CSG, personal experience). The results also indicated similar effectiveness of self-desiccating filters vs. the standard method of sample preservation in ethanol.

Both visual surveys and eDNA sampling are effective at determining occupancy at the meadow scale; however, improved local detections using analysis of eDNA samples suggests that the addition of eDNA sampling to visual surveys in meadows will improve survey accuracy and increase the probability of detecting rare frogs. Environmental DNA sampling found eight localities with at-risk native amphibians where they were not seen using visual surveys. In contrast, we detected a frog visually and not in an eDNA sample only once. The difference between eDNA surveys and visual surveys was most pronounced when comparing samples collected at the foot of a meadow and in off-channel pools. The foot of a meadow tends to accumulate flowing water from the meadow, probably receiving transported eDNA from multiple habitats and increasing the probability of eDNA detection. Transport distances of eDNA are variable and dependent on abiotic retention in the benthos and degradation rates (Fremier, Strickler, Parzych, Powers, & Goldberg, 2019; Wilcox et al., 2016). Regardless, even with some loss of material, sampling at the foot of the meadow provides a more comprehensive sample than habitats further upstream in the meadow. It is also important to sample off-channel pools using eDNA methods because these habitats can be difficult to survey visually owing to the silty substrates where frogs and tadpoles can easily hide. In addition, sampling in pools is important for a thorough survey because transport of DNA out of the pools to downstream channel habitats is unlikely.

Our occupancy model found an inverse relationship between the probability of detecting eDNA and the volume of water sampled, contrary to the findings of others (Goldberg et al., 2018; Schultz & Lance, 2015). We suspect that this pattern resulted primarily from the fact that sample volume was confounded with sampling habitat type, as suggested by our LMM analysis of eDNA quantity. Samples collected at the head of the meadow tended to filter the maximum water volume (2 L per filter; Figure 5), but also tended to test positive for eDNA less often than samples collected at the foot of the meadow, in channels, or in off-channel pools (Figure 4). Samples collected at the foot of meadows and in off-channel pools often clogged early owing to fine silt in the water column, but these samples more frequently collected eDNA (Figure 4) and tended to contain larger quantities (Figure 6). Clearly, when sampling for rare species, it is more important to select the habitats where the eDNA of the species is likely to be found than sites where sample volume is maximized.

[Correction added on 1 September 2020, after first online publication: The second sentence in the second paragraph of the Discussion section has been updated in this version.]

4.1 | Sampling implications

Collecting replicate eDNA samples simultaneously at each locality in a meadow provided more accurate and robust results. In addition to allowing a calculation of detection probabilities for samples, it also enabled data to be used from sample localities even if one of the samples failed because of field sampling problems (e.g. prematurely clogged filter) or sample quality (e.g. inhibited PCR). For these reasons, we recommend the split line replicate sampling approach for the Sampler Backpack.

Based on these findings, eDNA sampling in mountain meadows is likely to improve survey accuracy and increase the probability of detecting rare frogs when combined with visual surveys. eDNA detection methods helped to identify specific habitats used by frogs, and the strength of the eDNA signal increased with the abundance of the target species. This adds to a growing literature linking the abundance of organisms to eDNA quantity (e.g. Bista et al., 2016; Chambert, Pilliod, Goldberg, Doi, & Takahara, 2018). For detection, we recommend always collecting a sample at the foot of the meadow, but additional sampling is likely to be necessary to determine occupancy accurately and robustly. The fact that target species were not detected at all localities sampled in occupied meadows underscores the importance of knowing the life history and habitat relationships of the target taxa so that samples can be collected from the most suitable localities. Assuming this knowledge, the high detection rates observed support the applicability of eDNA analysis to *R. sierrae* and *R. cascadae* surveying in mountain meadows where visual encounter surveys can be labour-intensive and inefficient.

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APPENDIX A: RAW eDNA OCCUPANCY AND qPCR DATA

A result of 1 indicates the visual presence of the target amphibian species, a result of 0 indicates non-detection

Meadow	Sample	Species	Habitat category	Visual presence	Volume (L)	qPCR filter A	qPCR filter B
ALC	1	RASI	Pool	0	1.15	2.29	1.17
ALC	2	RASI	Foot	1	1.65	5.78	5.66
ALC	3	RASI	Channel	1	1.40	0.86	0.57
ALC	4	RASI	Head	1	4.26	4.00	2.97
BBF	1	RACA	Channel	1	3.91	1.96	I
BBF	2	RACA	Channel	1	3.91	6.15	4.54
BBF	3	RACA	Pool	0	4.04	0.00	0.00
BBF	4	RACA	Pool	1	3.81	3.71	0.00
CAR	1	RACA	Foot	1	4.05	I	0.00
CAR	2	RACA	Channel	0	4.05	0.00	0.00
CAR	3	RACA	Pool	1	1.67	5.91	5.91
CAR	4	RACA	Channel	1	4.03	0.07	0.09
CAR	5	RACA	Head	0	4.06	0.00	0.00
CHI	1	RACA	Channel	0	4.02	0.40	0.24
CHI	2	RACA	Pool	1	4.02	3.64	4.21
CHI	3	RACA	Channel	1	4.04	1.60	1.21
CHI	4	RACA	Foot	1	4.01	3.47	3.58
IND	1	RASI	Bottom	1	4.04	I	I
IND	2	RASI	Channel	1	2.10	3.66	3.71
IND	3	RASI	Channel	1	0.21	3.29	1.94
IND	4	RASI	Pool	1	4.08	5.48	4.73
MAT	1	RASI	Channel	0	3.94	0.00	0.00
MAT	2	RASI	Foot	1	4.13	0.00	1.85
MAT	3	RASI	Pool	0	0.82	3.06	3.67
MAT	4	RASI	Head	0	3.93	0.00	0.00
MAT	5	RASI	Channel	0	2.76	0.00	0.00
MAT	6	RASI	Channel	0	4.09	0.00	0.00
OLC	1	RACA	Head	0	4.03	0.00	0.00
OLC	2	RACA	Channel	0	4.01	0.00	0.00
OLC	3	RACA	Pool	1	1.08	3.01	4.72
OLC	4	RACA	Pool	1	3.94	0.05	0.09
OLC	5	RACA	Channel	0	4.05	0.86	0.00
OLC	6	RACA	Foot	0	4.01	1.14	1.57
PRZ	1	RASI	Head	0	4.21	0.00	0.00
PRZ	2	RASI	Channel	1	2.95	I	I
PRZ	3	RASI	Channel	0	0.64	0.00	0.00
PRZ	4	RASI	Channel	0	0.92	0.00	0.00
PRZ	5	RASI	Channel	0	1.10	0.00	0.00
PRZ	6	RASI	Foot	0	1.09	0.00	0.00
SNC	1	RASI	Foot	1	2.34	6.00	6.05
SNC	2	RASI	Pool	0	0.82	0.00	0.00
SNC	3	RASI	Head	1	4.22	1.44	1.68

Meadow	Sample	Species	Habitat category	Visual presence	Volume (L)	qPCR filter A	qPCR filter B
STA	1	RASI	Foot	0	1.20	6.02	6.96
STA	2	RASI	Channel	1	1.24	6.16	5.98
STA	3	RASI	Pool	1	1.00	3.41	3.15
STA	4	RASI	Head	0	4.02	6.45	5.27
SWM	1	RASI	Head	0	1.49	0.00	0.00
SWM	2	RASI	Channel	1	2.27	0.00	0.00
SWM	3	RASI	Channel	1	0.76	2.38	4.43
SWM	4	RASI	Channel	0	0.82	0.00	0.00
SWM	5	RASI	Channel	1	2.63	2.90	3.19
SWM	6	RASI	Foot	1	4.00	0.00	2.03
WAR	1	RACA	Head	0	0.60	0.00	0.00
WAR	2	RACA	Channel	0	4.03	0.57	0.27
WAR	3	RACA	Foot	1	1.05	2.51	2.55

Abbreviations: ALC, Alan Camp Meadow; IND, Independence Creek; LF, Lowe Flat; MAT, Mattley Meadow; PRZ, Perazzo Meadow; SNC, Snow Corral Meadow; STA, Stanislaus Meadow; SWM, Swanson Meadow; BBF, Big Bear Flat; CAR, Carter Meadow; CHI, Childs Meadow; COL, Colby Creek; HUM, Humbug Meadow; OCC, Old Cow Creek; WAR, Warner Valley.
RASI, *Rana sierrae*; RACA, *R. cascadae*. Quantitative PCR results are natural log-transformed copy numbers with 'I' indicating samples in which inhibition was detected during quality control checks in the laboratory. Inhibited samples were not included in analyses.

APPENDIX B: MODEL SELECTION TABLE

Candidate model set ordered by AICc for linear mixed models testing the relationship between ln qPCR copy number and several relevant predictors including habitat category (HabCat), species (Sp), nearby visual counts (VC), volume filtered (Vol), and the interaction between habitat category and visual counts. R^2m indicates the marginal R-squared value (proportion of the variance explained by the fixed effects within the model) for each model

Model	Intercept	HabCat	Sp	VC	Vol	HabCat × VC	d.f.	AICc	delta	Weight	R^2m
1	2.03	+	+	0.06			9	395.51	0.00	0.22	0.35
2	2.55	+		0.05			8	395.52	0.01	0.21	0.30
3	2.32	+		0.16		+	11	396.8	1.29	0.11	0.31
4	1.79	+	+	0.15		+	12	397.19	1.68	0.09	0.36
5	2.85	+		0.05	−0.11		9	397.43	1.92	0.08	0.31
6	2.06	+	+	0.06	−0.01		10	397.92	2.41	0.06	0.35
7	1.55			0.05			5	398.19	2.68	0.06	0.18
8	2.7	+		0.16	−0.14	+	12	398.6	3.10	0.05	0.32
9	1.11		+	0.05			6	398.92	3.42	0.04	0.21
10	2.01			0.05	−0.16		6	399.52	4.01	0.03	0.19
11	1.99	+	+	0.15	−0.06	+	13	399.65	4.14	0.03	0.36
12	1.47		+	0.05	−0.1		7	400.9	5.39	0.01	0.22
13	1.94						4	407.77	12.26	0.00	0.00
14	2.68				−0.28		5	407.77	12.26	0.00	0.03
15	1.44		+				5	408.79	13.29	0.00	0.04
16	2.78	+					7	409.33	13.82	0.00	0.06
17	2.25		+		−0.24		6	409.54	14.03	0.00	0.06
18	2.21	+	+				8	410.15	14.65	0.00	0.10
19	3.39	+			−0.23		8	410.27	14.77	0.00	0.08
20	2.79	+	+		−0.16		9	411.87	16.36	0.00	0.11