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Determining Presence of Rare Amphibian Species: Testing and Combining Novel Survey Methods

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Abstract.—**Data deficiencies exist for ~20–25% of amphibian and reptile species worldwide, typically excluding them from conservation consideration and funding opportunities. Current species status is often unknown at historic locations or previously unsurveyed areas. We present an iterative study to assess and optimize novel survey methods for a declining amphibian species (Boreal Toad, *Anaxyrus boreas boreas*) using a combination of methods. We found that if toads are present in a drainage, searching riparian areas multiple times during the active season is an efficient way of detecting the occurrence of previously unknown breeding populations. Once a breeding population has been located, traditional visual encounter surveys yield high probabilities of species detection for monitoring efforts ($\hat{p}_{vis} \approx 0.80$). Supplementing streamside surveys when toads are not detected with other survey methods (e.g., environmental DNA [*e*DNA] samples) at suitable breeding locations can help confirm the species is absent, provided ≥10 *e*DNA samples are collected. Moreover, employing both visual surveys and *e*DNA samples can simultaneously yield distributional information on amphibian species and target pathogens, if pathogen presence is evaluated for all captured amphibian species and environmental samples are tested for both amphibian and pathogen DNA. Our iterative process of designing, testing, optimizing, and combining sampling methods to determine current species distribution should serve as a model for other rare amphibian and reptile species and provide managers better information with which to plan mitigation and conservation efforts.**

Determining distribution and status of a species is fundamental to ecology (Andrewartha and Birch, 1954) and species conservation and management (Leopold, 1933; Thompson, 2004; MacKenzie et al., 2018). Such information is lacking for many amphibian and reptile species, with data deficiencies reported by the International Union for Conservation of Nature (IUCN) Red List (https://www.iucnredlist.org/) for many species (25.4% amphibians, 21.8% reptiles; Bland et al., 2016). Monitoring changes in a species distribution is especially important for rare or declining taxa where efficient sampling is needed to definitively establish: 1) species presence, to protect previously unknown populations from potential threats, or 2) species absence, to identify locations for translocation, reintroduction, or natural recolonization.

The Boreal Toad (*Anaxyrus boreas*) in the southern Rocky Mountains (SRM), United States, is an example of a once-common species that has undergone rapid and extensive decline over the last four decades (Hammerson, 1999; Muths et al., 2003; Scherer et al., 2005; Mosher et al., 2018b). The amphibian fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*; Longcore et al., 1999) was first detected in the SRM in 1998 and has been implicated in Boreal Toad declines throughout the region (Muths et al., 2003; Scherer et al., 2005; Mosher et al., 2018b). The Boreal Toad Conservation Team, formed in 1994, monitors toad status at wetlands with known contemporary or historic breeding activity (Loeffler, 2001). In addition, the Boreal Toad Recovery Plan recommends conducting visual encounter surveys of potentially suitable habitat to detect unknown, extant breeding populations and identify unoccupied habitat for potential reintroductions within the species historic range (Loeffler, 2001). These surveys typically target lentic habitats above 2,440 m, utilize a single site visit, and yield limited success. For example, Hammerson (1992) surveyed 377 potentially suitable sites (wetlands, wet meadows, beaver ponds, and oxbows) and observed only a single population of Boreal Toads. Because sites were never resurveyed, it was impossible to separate the two possibilities for nondetection at a given site: 1) toads were truly absent, or 2) toads were present, but undetected.

Contemporary studies have suggested alternatives to traditional visual encounter surveys of lentic habitats. In Montana, Boreal Toads use riparian areas extensively during the summer, and hoop nets can capture large numbers of individuals (juveniles and adults of both sexes; Schmetterling and Young, 2008; Young and Schmetterling, 2009). A simultaneous radiotelemetry study revealed that ~80% of toad relocations were in the riparian zone or stream habitat (Schmetterling and Young, 2008), suggesting that searching these areas may be an efficient and complementary means of detecting the species within occupied drainages. Similarly, environmental DNA (*e*DNA) has been used to detect a variety of low-density or secretive aquatic species including amphibians (e.g., Goldberg et al., 2018), reptiles (e.g., Hunter et al., 2015), fish (e.g., Matter et al., 2018), invertebrates (e.g., Currier et al., 2018), and fungal pathogens (e.g., Mosher et al., 2018c).

In this study, we iteratively tested and incorporated different survey approaches to determine efficient, effective sampling methods to estimate the occurrence of Boreal Toads in drainages with little to no previous survey effort. Our primary objectives were to: 1) determine if hoop net sampling or stream corridor surveys could be used to detect and locate active breeding

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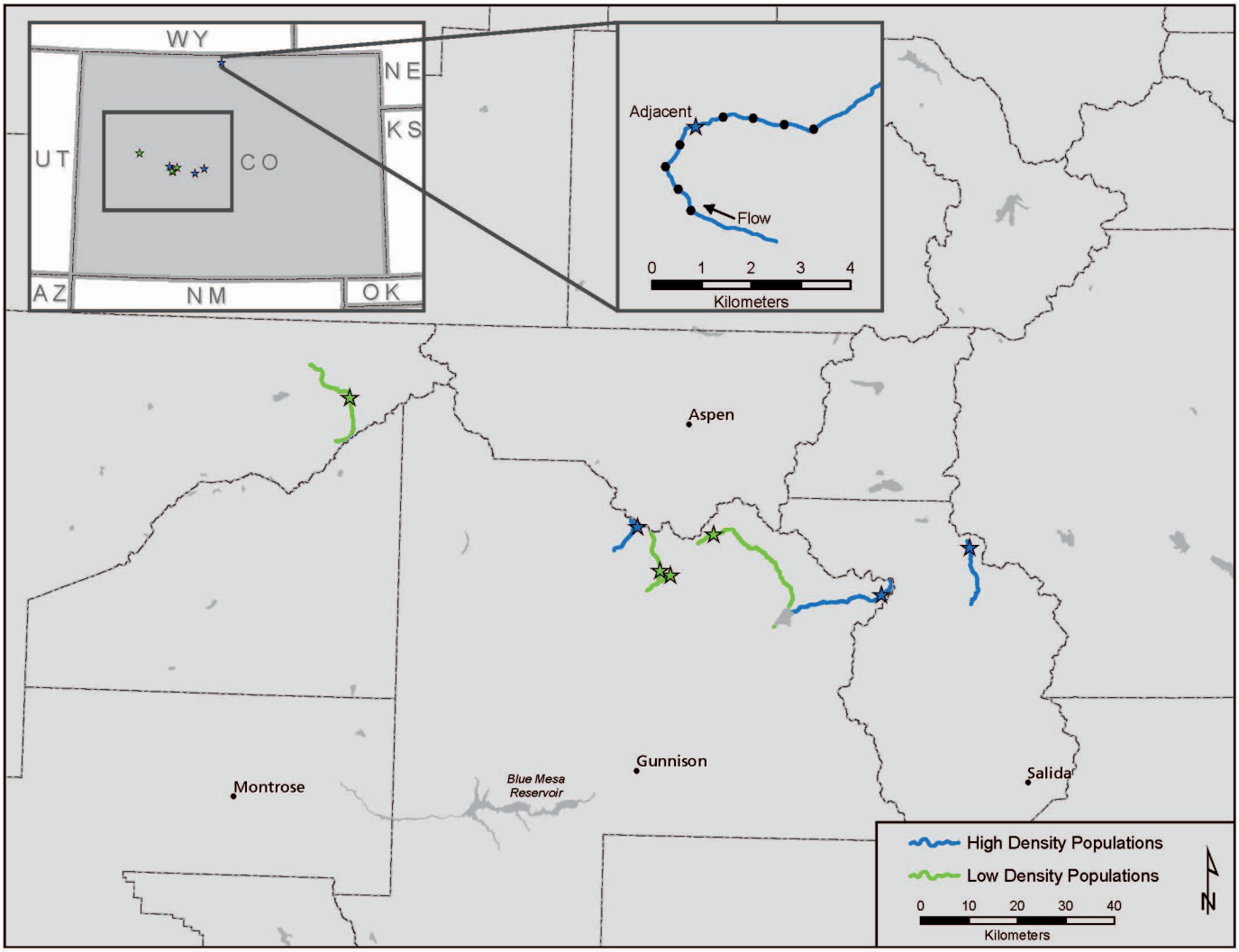

FIG. 1. Map of sampled stream drainages in Colorado. Inset gives an example of a subset of hoop nets including the adjacent net location (star location on each stream) and additional hoop net locations (inset) set at 500-m intervals up and downstream of the adjacent net ($n_{max} = 17$ nets on a given stream).

populations, and 2) explore the ability of *e*DNA sampling to detect Boreal Toads at potential breeding locations. Our study consisted of two phases; first, we tested and refined streamside surveys to optimize detection probabilities in drainages with relatively low or high abundances of Boreal Toads. In the second phase, we employed these optimal streamside surveys in drainages with unknown toad status and simultaneously tested *e*DNA sampling methods at both potential and known active breeding sites. We estimated detection probabilities for each method (streamside and *e*DNA surveys) and compared these estimates to those from conventional visual encounter surveys currently used in monitoring efforts for this rare amphibian. Our approach should serve as a model for other systems with widely distributed habitats occupied by relatively few extant populations.

MATERIALS AND METHODS

Phase One: Field Methods.—In 2010, a pilot study was conducted in four mountain stream drainages in Colorado with relatively high annual counts of Boreal Toad adults, egg masses, and/or tadpoles at known breeding locations (termed high-population drainages): Texas Creek and Copper Creek (Gunnison County), Panhandle Creek (Larimer County), and Fourmile Creek (Chaffee County) (Fig. 1). Breeding locations were lentic habitats, typically $\leq$50 m from the stream. A hoop net was set at the stream location closest to the known breeding location, hereafter referred to as the adjacent net (distance = 0 m; Fig. 1 inset). Additional nets were set at 500-m intervals up to 4 km upstream and downstream of the adjacent net (maximum $n = 17$ hoop nets per stream). All, or a proportion, of the stream bank between each hoop net was also searched for toads during each visit and the location of toad detections was recorded with a handheld global positioning system (GPS). Streams were visited 2–4 times from June to early September.

In 2011–2012, we focused on refining our streamside surveys and tested the methodology in both high-population drainages and low-population drainages (drainages with relatively low counts of adults and inconsistent annual breeding activity). We revisited three of the four high-population drainages in 2011, in late June, mid-July, and early August (three visits per drainage). The following year, we surveyed four low-population drainages (Fig. 1) three times during the same period (late June–early August). We employed a more standardized survey in both

TABLE 1. Model selection results for 16 models fit to 2010 Boreal Toad detection–nondetection data from 53 stream reaches (units) in four high-population drainages. K is the number of estimated parameters in the model, ΔAICc is the relative difference in AICc values, w is the model weight, and $-2l$ is twice the negative log-likelihood. Occupancy probability (ψ) was modeled as constant among all reaches (.) or as a function of distance (Dist) and/or the direction (upstream or downstream, Direction) from the adjacent net location nearest the known breeding location. ψ(Dist × Direction) represents an interactive function between these two factors. Detection probability (p) was modeled as a function of sampling effort (Effort), relative flow (Flow), different (t) or constant (.) among visits.

Model	K	ΔAICc	w	$-2l$
ψ(Dist × Direction) $p(t)$	6	0.00	0.33	70.90
ψ(Dist × Direction) p(Effort)	5	0.84	0.22	74.29
ψ(Dist × Direction) p(.)	4	1.34	0.17	77.23
ψ(Dist) $p(t)$	5	3.07	0.07	76.52
ψ(Dist × Direction) p(Flow)	5	3.20	0.07	76.64
ψ(Dist) p(.)	3	4.58	0.03	82.82
ψ(Direction) p(Effort)	4	5.01	0.03	80.90
ψ(Dist) p(Effort)	4	5.04	0.03	80.93
ψ(Dist) p(Flow)	4	6.26	0.01	82.16
ψ(Direction) $p(t)$	5	6.26	0.01	79.71
ψ(Direction) p(.)	3	7.01	0.01	85.24
ψ(.) $p(t)$	4	7.06	0.01	82.95
ψ(.) p(Effort)	3	7.54	0.01	85.77
ψ(.) p(.)	2	8.31	0.01	88.79
ψ(Direction) p(Flow)	4	8.59	0.00	84.48
ψ(.) p(Flow)	3	9.84	0.00	88.08

years wherein an observer searched a 10-m wide transect on each stream bank and the location of every encountered toad was determined by handheld GPS.

Phase One: Biological Hypotheses and Analyses.—We used single season occupancy models (MacKenzie et al., 2002, 2018) to analyze Boreal Toad detection–nondetection data in our study. These models estimated two types of parameters, both of which could be modeled as a function of covariates:

ψ_i = (occupancy probability) = probability that Boreal Toads occur in given unit i, and
p_{it} = (detection probability) = probability of detecting Boreal Toads at an occupied unit i during survey t.

Detection of toads via hoop nets proved ineffective in 2010 (see Results), but toads were detected in all drainages via streamside surveys. We analyzed the 2010 streamside detection–nondetection data by defining a sample unit as a 500-m stream reach centered on each net location; for example, the 'adjacent stream reach' extended 250 m upstream and 250 m downstream of the adjacent net location (Fig. 1). Detection–nondetection information was compiled for repeated visits to each 500-m stream reach (unit).

We modeled occupancy probability, or the probability that at least one toad occurred at a given stream reach, as a function of the distance (m, Dist) and direction (upstream or downstream, Direction) from the adjacent net location. If toads were using streams as movement corridors, we expected toad occupancy to be highest at the adjacent stream reach near the known breeding location and to decline for stream reaches that were farther away. We expected an interaction between distance and direction, as toad occurrence may decline more sharply for upstream stream reaches.

We modeled detection probability as a function of survey effort, visit, or flow conditions. Survey effort (Effort) values varied among reaches and visits (t): a value of 1 denoted that the entire 500-m stream reach was searched, a value of 0.5 was assigned when approximately half of the stream reach was searched, and 0.1 was given when only the area around the hoop net location was searched. There were large differences in flow levels across streams and visits that could influence toad detection probabilities. Using the nearest gauging station to each study reach, we averaged flows for 3 days surrounding each visit and scaled the averages relative to the highest flows experienced during the June 6–8 visit to Texas Creek (relative Flow = 1). We expected lower detection probability during visits with high flow, lower survey effort, or as a result of seasonal variation associated with toad activity.

Our candidate model set consisted of models where occupancy probability was constant over stream reaches ψ(.) or varied by distance, direction, or an interaction between distance and direction. We considered detection probability structures that were constant over visits (.) or varied as a function of effort, flow, or among visits. We fit 16 models representing all combinations of these occupancy and detection structures (Table 1).

We used similar covariates to model occupancy and detection probability for streamside surveys conducted in 2011–2012 with the following exceptions. Most reaches and visits were surveyed using the protocol detailed above (Effort = 1). Exceptions include a visit when three observers searched a subset of reaches (Effort = 1.5) and a reach whose spatial extent was not searched completely (Effort = 0.95). We replaced the largely ineffective flow covariate (see Results) with stream gradient measured as percent slope (StrGrad), hypothesizing that toad detection probability would be higher at reaches with lower stream gradients. Finally, the focus of the 2011–2012 surveys was to test the streamside survey methodology in both high- and low-population drainages and determine the effect of relative toad abundance and breeding activity on occupancy and detection probabilities; hence, we included a categorical covariate differentiating reaches in the high- and low-population drainages (Pop). Similar to our 2010 analysis, we fit all possible combinations of occupancy and detection probability structures, with and without an additive population effect (34 total models, Appendix 1).

Using resulting occupancy and detection probability estimates, we determined the optimal number of visits and length of streamside surveys needed to insure a high probability of detecting toads if they existed within a drainage (Appendix 2). Briefly, we calculated the probability of detecting toads in an occupied drainage by surveying different lengths of stream (l = 2.5, 3.5, or 4.5 km) one to three times (t = 1, 2, or 3) during the postbreeding season. This analysis yielded the optimal streamside survey (OSS) protocol used in phase two of the project (detailed below).

Phase Two: Field Methods.—We employed OSS in drainages with unknown Boreal Toad status in three regions of Colorado from 2014–2016. We sampled a different region each year. Surveys consisted of sampling 4.5 km of riparian habitat centered on wetland complexes that historically supported toad breeding or contained suitable breeding habitat located via aerial photos. Surveys were conducted by two observers, with each observer searching a 10-m wide transect on each stream bank. We conducted 1–3 OSS in each drainage and *e*DNA samples were collected at the historic or suitable breeding location(s).

We collected *e*DNA samples with modified Nalgene water bottles (1 L) with a replaceable Swinex Luer-lok filter (5–10 μm)

FIG. 2. Photo of modified Nalgene bottles used to collect *e*DNA samples.

attached to the bottom and an air valve inserted into the lid (Fig. 2). A bicycle pump attached to the air valve produced enough pressure to filter up to 10 L of water per sample. We collected one to six *e*DNA samples from each historic or potential breeding location. Surveyors forced as much water as possible through the filter, recorded the amount of water filtered, then removed the filter and sealed it in a Ziplock bag. Each Nalgene bottle was used only once during a visit and thoroughly cleaned with 10% bleach solution between visits to prevent cross-contamination. Surveyors placed individually bagged filters in coolers as soon as possible, stored them in a freezer, and shipped to Pisces Molecular (Boulder, Colorado, USA) at the end of the field season.

We also collected *e*DNA samples at known breeding (occupied) locations during annual monitoring visits to ensure *e*DNA detection probability could be estimated. We sampled these locations using an established monitoring protocol consisting of visual encounter surveys (VES); observers walk along the perimeter of the breeding site scanning for adult toads, eggs, or larvae (see Appendix B in Loeffler, 2001). Observers usually collected *e*DNA samples during visual encounter surveys, but occasionally *e*DNA samples and VES were conducted on different days. Collectively, visual surveys (both OSS and VES) and *e*DNA samples were collected throughout the breeding and active season (late May–September).

Phase Two: eDNA Lab Methods.—Filter samples were analyzed by Pisces Molecular using standard extraction procedures and real-time polymerase chain reaction protocols (qPCR; Kirshtein et al., 2007) and used an inhibitor-removal reagent (GeneReleaser™, BioVentures, Murfreesboro, Tennessee, USA) to remove compounds that might inhibit the qPCR reaction (McKee et al. 2015). In 2014, a subset of *e*DNA samples was extracted by researchers at Colorado State University (Mosher et al. 2017) using a commercially available inhibitor removal kit (OneStep™ PCR Inhibitor Removal Kit, Zymo Research, Irvine, California, USA) before qPCR analysis was performed by Pisces Molecular. We tested for potential detection probability differences associated with the two extraction procedures in our analysis.

Phase Two: Biological Hypotheses and Analysis.—We compiled detection–nondetection information for each survey of each drainage (unit) with known or potential breeding locations. Surveys consisted of OSS, VES, and *e*DNA samples. Occupancy probability was fixed to 1 for drainages with known occupied breeding locations and estimated for those with unknown occupancy status (historic and potential breeding locations). We allowed occupancy probabilities to vary among years (Yr), as different regions were sampled each year.

Toad detection probabilities, and the factors that influence these probabilities, may be similar or differ for visual surveys (OSS and VES) and *e*DNA samples. For example, we expected toad detection probabilities to vary seasonally and among years regardless of the type of survey; however, different extraction methods and the volume of water filtered would only influence *e*DNA detection probabilities. Accordingly, we modeled detection probabilities for visual surveys separately from *e*DNA detection probabilities. We expected visual detection probabilities might vary: 1) seasonally, and we considered linear and quadratic functions of day of the year (Date); 2) among years (Yr); 3) among survey type (OSS or VES, Survtype); or 4) among units with known breeding activity and those with unknown toad status (Unittype). Because survey type and unit type are highly correlated, we never included both of these factors in the same model structure.

We expected detection probabilities for *e*DNA samples might also vary seasonally and among years, between different extraction methods (Lab), and with the amount of water filtered (Vol). Finally, *e*DNA samples were often taken from areas within a wetland where toads had been recently observed via visual surveys. To account for the likely higher detection probability of these *e*DNA samples (i.e., nonindependence), we included a 'trap-effect' differentiating when an *e*DNA sample was collected in the same 2-wk period that a toad was observed via visual surveys (Prev_det).

Rather than fitting an extremely large model set incorporating all plausible combinations of covariates for detection probability parameters, we adopted a sequential approach to identify supported hypotheses (models). First, using a global model structure, ψ_{unk}(Yr) p_{vis}(Unit_or_Survtype + Date + Date^2 + Yr) p_{eDNA}(Lab + Vol + Prev_det + Date + Date^2 + Yr), we identified the most parsimonious structure for visual detection probability (18 model structures, Appendix 3). Retaining the best supported structure for p_{vis}, we explored seasonal variation in *e*DNA detection probability (six structures) and then tested the influence of extraction method, volume of filtered water, and trap-effect (eight structures). This stepwise approach to model selection is a reasonable and practical alternative to fitting hundreds of models ($18 \times 6 \times 8 = 864$ possible model structures) and is effective at identifying factors influencing model parameters (Doherty et al., 2012). We used Program MARK (White and Burnham, 1999) to fit all models and obtain parameter estimates, and we used an information-theoretic approach for model selection (Burnham and Anderson, 2002).

RESULTS

Phase One.—During the 2010 pilot study, heavy flows or small stream size prohibited setting hoop nets at all upstream locations; only 2–7 nets were set upstream of the adjacent net location in each of the four high-population drainages. Toads were captured in hoop nets in only one stream but detected in all drainages via streamside surveys. Fifty-three, 500-m stream reaches (units) were searched up to four times and toads were detected in 10 reaches (naïve occupancy = 0.19). Model selection results strongly suggested that toad occupancy varied by both distance

TABLE 2. Model selection results for the top eight models fit to Boreal Toad detection–nondetection data from 73 stream reaches (units) surveyed in 2011–2012. Results from models with AIC weight, w, >0.03 are given; K is the number of estimated parameters, ΔAICc is the relative difference in AICc values, and $-2l$ is twice the negative log-likelihood. Occupancy probability (ψ) was modeled as different among high- and low-population drainages (Pop) or as a function of distance (Dist) and/or the direction (upstream or downstream, Direction) from the adjacent net location nearest the known breeding location. Detection probability, p, was modeled as a function of sampling effort (Effort), stream gradient (StrGrad), and different among high- and low-population drainages (Pop). Additive and interactive functions were represented by + and × symbols, respectively.

Model	K	ΔAICc	w	$-2l$
ψ(Dist × Direction) p(Pop + StrGrad)	6	0.00	0.34	104.89
ψ(Dist) p(Pop + StrGrad)	5	1.33	0.17	108.60
ψ(Pop + Dist × Direction) p(Pop + StrGrad)	7	1.87	0.13	104.32
ψ(Dist × Direction) p(Pop)	5	2.98	0.08	110.25
ψ(Pop + Dist) p(Pop + StrGrad)	6	3.55	0.06	108.44
ψ(Dist × Direction) p(Pop + Effort)	6	3.85	0.05	108.74
ψ(Pop + Dist × Direction) p(Pop)	6	4.60	0.03	109.49
ψ(Dist) p(Pop)	4	5.03	0.03	114.61

and direction from the known breeding location (Table 1, cumulative weight $w_+ = 0.79$). The model-averaged occupancy estimate for an adjacent stream reach near the known breeding location was high (0.68 [$\widehat{SE} = 0.20$]), but declined quickly with distance. Estimated distance effects varied with direction with more-pronounced declines for upstream reaches ($\hat{\beta}_{UpStrDist} = -1.98$ [$\widehat{SE} = 1.08$]) relative to downstream reaches ($\hat{\beta}_{DnStrDist} = -0.46$ [$\widehat{SE} = 0.20$]; estimates given on the logit scale using the best-supported model, Table 1). Detection probability appeared to vary seasonally among visits ($w_+ = 0.42$) or with survey effort ($w_+ = 0.29$), but there was little evidence that flow accounted for temporal variation in detection ($w_+ = 0.08$). Model-averaged detection probability was 0.40 during the first two visits ($\widehat{SE} = 0.16$), but declined later in the season, $\hat{p}_{t=3} = 0.28$ ($\widehat{SE} = 0.14$) and $\hat{p}_{t=4} = 0.20$ ($\widehat{SE} = 0.19$). For the models that included survey effort, the direction of the relationship was positive, consistent with our a priori expectations.

We surveyed 73 stream reaches (units) in seven drainages in 2011–2012. Seventy-two toads were detected at 11 of 38 reaches

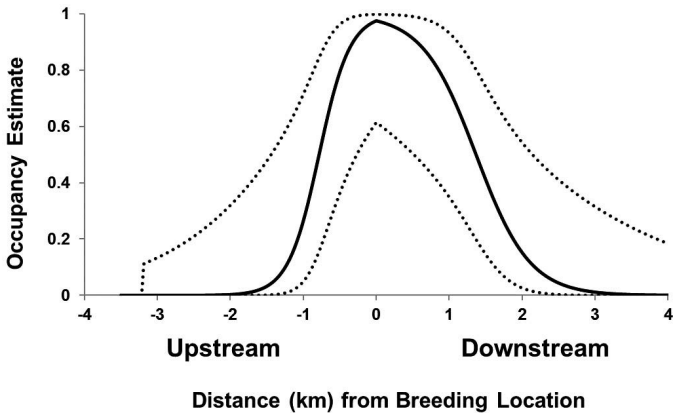

FIG. 3. Boreal Toad occupancy probability estimates ($\hat{\psi}$) for stream reaches (units) sampled in 2011–2012 in high- and low-population drainages. Estimates (solid line) and 95% confidence intervals (dotted lines) are obtained from the best supported model, ψ(Dist × Direction) p(Pop + StrGrad) ($w = 0.34$).

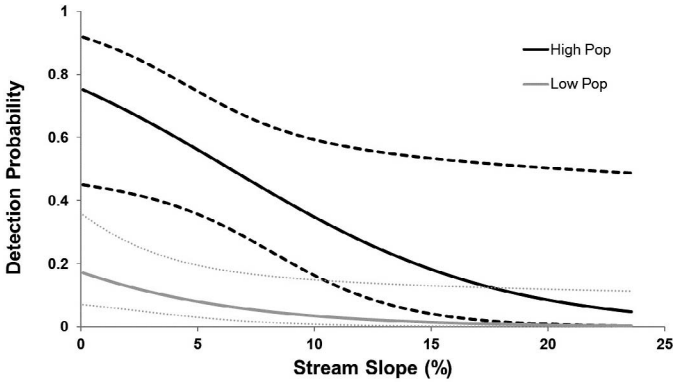

FIG. 4. Boreal Toad detection probability estimates, $\hat{p}$ for stream reaches (units) sampled in 2011–2012 in high- and low-population drainages as a function of stream slope. Estimates are obtained from the best-supported model, ψ(Dist × Direction) p(Pop + StrGrad) ($w = 0.34$); dotted and dashed lines represent 95% confidence intervals.

surveyed in the three high-population drainages (2011 naïve occupancy = 0.29) and 12 toads were detected at 6 of 35 reaches surveyed in the four low-population drainages (2012 naïve occupancy = 0.17). Consistent with our 2010 analysis, model selection results suggested that occupancy probabilities varied with distance and direction from the known breeding locations ($w_+ = 0.68$; Table 2, Appendix 1). Estimates obtained from the best-supported model suggested that occupancy probabilities for stream reaches near a breeding site are high ($\hat{\psi} = 0.98$ [$\widehat{SE} = 0.04$], but declined quickly for both upstream and downstream reaches (Fig. 3). With our more-standardized surveys conducted during a narrow sampling window (~6 wk: late June–early August), there was little temporal variation in detection probability (Table 2). Detection probability did vary among reaches, with higher detection probability at low gradient reaches in high-population drainages (Fig. 4).

Using these occupancy and detection probability estimates, we determined that OSS should search at least 4.5 km of stream habitat three times during the postbreeding season. In relatively high-population drainages, this would ensure that toads would be detected at least once (Appendix 2). In drainages with relatively low toad abundance and inconsistent breeding activity, the probability of detecting toads with this level of sampling ranged between 0.40–0.65, depending on the actual breeding location relative to the sampled stream length (Appendix 2). Our results emphasized the importance of surveying the reach adjacent to breeding locations within the surveyed stream section; thus, OSS employed in drainages of unknown toad status (2014–2016) were centered on historic breeding locations or suitable breeding habitat determined via aerial photos.

Phase Two.—Multiple visual surveys (OSS or VES) and *e*DNA samples were collected at 75 drainages (units) from 2014–2016. Units consisted of approximately twice as many drainages with unknown toad status (55 units) as drainages with known breeding locations (20 units). Toads were detected in 3 of 23 unknown-status drainages in 2014 (naïve occupancy = 0.13), 4 of 21 unknown-status drainages in 2015 (naïve occupancy = 0.19), and 6 of 11 unknown-status drainages in 2016 (naïve occupancy = 0.55).

Not surprisingly, toad detection probability via visual surveys was higher for units with known breeding locations ($\hat{p}_{vis}$(Kn) ≈ 0.80) compared to drainages with previously unknown toad status ($\hat{p}_{vis}$(Unkn) ≈ 0.25–0.30); however, there was little

TABLE 3. Model selection results for detection structures for *e*DNA samples fit to Boreal Toad detection–nondetection data from 75 units (drainages with known and unknown breeding locations) from 2014–2016. First, we tested six detection structures representing seasonal variation in *e*DNA detection probability, including linear and quadratic functions of day of the year (Date) and variation among years (Yr). Retaining the best-supported seasonal detection structure, we fit models where *e*DNA detection probability was influenced by extraction method (Lab), volume of filtered water (Vol), and previous detection or 'trap' effect (Prev_det). *K* is the number of estimated parameters in the model, ΔAICc is the relative difference in AICc values, *w* is the model weight, and $-2l$ is twice the negative log-likelihood. All models were fit using the best-supported structures for occupancy and visual detection probabilities: ψ_{unk}(Yr) p_{vis}(Unittype).

Model	*K*	ΔAICc	*w*	$-2l$
Seasonal Structures: p_{eDNA}(Lab + Vol + Prev_det + Seasonal structure)				
p_{eDNA}(... + Date + Yr)	12	0.00	0.43	368.75
p_{eDNA}(... + Yr)	11	0.08	0.42	371.67
p_{eDNA}(... + Date + Date^2 + Yr)	13	2.91	0.10	368.72
p_{eDNA}(... + Date)	10	5.03	0.04	379.37
p_{eDNA}(... + Date + Date^2)	11	7.47	0.01	379.07
p_{eDNA}(... + .)	9	10.44	0.00	387.46
Additional Effects				
p_{eDNA}(Prev_det + Date + Yr)	10	0.00	0.62	369.10
p_{eDNA}(Vol + Prev_det + Date + Yr)	11	2.42	0.18	368.76
p_{eDNA}(Lab + Prev_det + Date + Yr)	11	2.74	0.16	369.09
p_{eDNA}(Lab + Vol + Prev_det + Date + Yr)	12	5.24	0.04	368.75
p_{eDNA}(Date + Yr)	9	32.30	0.00	404.07
p_{eDNA}(Vol + Date + Yr)	10	34.85	0.00	403.95
p_{eDNA}(Lab + Date + Yr)	10	34.95	0.00	404.05
p_{eDNA}(Lab + Vol + Date + Yr)	11	37.52	0.00	403.87

temporal variation among years, or among dates within years, after accounting for the spatial variation among units (Appendix 3). Most visual surveys in drainages with unknown toad status were OSS, and the estimated detection probabilities were nearly identical to our expected values based on our previous work (expected probability of detection = 0.30 for a single OSS centered on an occupied breeding location, Appendix 2). The consistency in detection probability among years/regions suggested that conducting three OSS centered on potential breeding habitat can be used to verify Boreal Toad presence and identify additional breeding locations in drainages of unknown toad status.

In contrast to visual surveys, *e*DNA detection probability varied within and among years (Table 3, Fig. 5); it was similar in 2014 and 2016 and higher earlier in the season. In 2015, the majority of *e*DNA samples were collected late in the season (August and September) when *e*DNA detection probability was extremely low (Fig. 5). Neither extraction method nor the volume of water filtered (range: 0.05–10 L) influenced *e*DNA detection probability (Table 3). Including these variables in the detection structure did not improve model fit (−2LogL) and thus they are uninformative or 'pretending' variables (Burnham and Anderson, 2002; Arnold, 2010). If toads were detected via visual surveys during the same 2-wk period, often on the same day that *e*DNA samples were collected, the probability of *e*DNA detection was much higher, likely because filter samples were collected near the detected toads (Table 3, Fig. 5). This lack of independence, if unaccounted for, would result in an overestimate of *e*DNA detection probability. Using a combination of visual and *e*DNA surveys, we estimated that toads occurred in 20–75% of drainages of unknown status sampled in 2014–2016, representing a 35–140% increase relative to naïve occupancy values (model-averaged estimates: $\hat{\psi}_{2014} = 0.21$ [$\widehat{SE} = 0.12$], $\hat{\psi}_{2015} = 0.46$ [$\widehat{SE} = 0.25$], and $\hat{\psi}_{2016} = 0.74$ [$\widehat{SE} = 0.22$]).

DISCUSSION

Data deficiencies exist for ~20–25% of amphibian and reptile species worldwide, typically excluding them from conservation consideration and funding opportunities (Bland et al., 2016). Deficiencies often include uncertain population status at historic locations with few or old records and at previously unsurveyed areas with potentially suitable habitat (Bland et al., 2016; Currier et al., 2017; Matter et al., 2018). Such deficiencies are common for declining or rare species; the same species with potentially high extinction-risk (Bland et al. 2016).

We present an iterative study to test and optimize novel survey methods for a high-elevation, declining amphibian species. In our region, hoop nets were ineffective at sampling Boreal Toads, likely because of lower population densities and high pathogen prevalence relative to systems in the northern Rocky Mountains (Young et al., 2007; Mosher et al., 2018b). Using our occupancy and detection probability estimates from occupied drainages with consistent, relatively high breeding populations, we determined that three streamside surveys at least 4.5 km in length and centered on historic breeding locations or suitable breeding habitat (termed optimal streamside survey) would ensure a high probability that toads would be detected if they were present (Appendix 2). In Phase 2, we combined these optimal streamside surveys with *e*DNA sampling to determine the species status in 55 drainages with little to no previous survey effort. We documented Boreal Toads in 13 drainages and located three new breeding locations by searching lentic habitats near stream detection locations. In drainages where toads were not detected on multiple (≥3) streamside surveys (length = 4.5 km), we feel confident that these drainages do not contain robust breeding populations of toads (Appendix 2). Our results suggest detection probability differs among occupied stream reaches in drainages with relative low and high populations of breeding toads. Accordingly, more (≥7) and/or longer stream surveys would be necessary to confirm (with 90% confidence) that no toads occurred within a drainage using streamside surveys alone (Appendix 2).

Toad detection probabilities from *e*DNA were lower than those for visual surveys, unless samples were collected during the same period that a toad was seen. The *e*DNA detection probability also declined during the postbreeding season (Fig. 5;

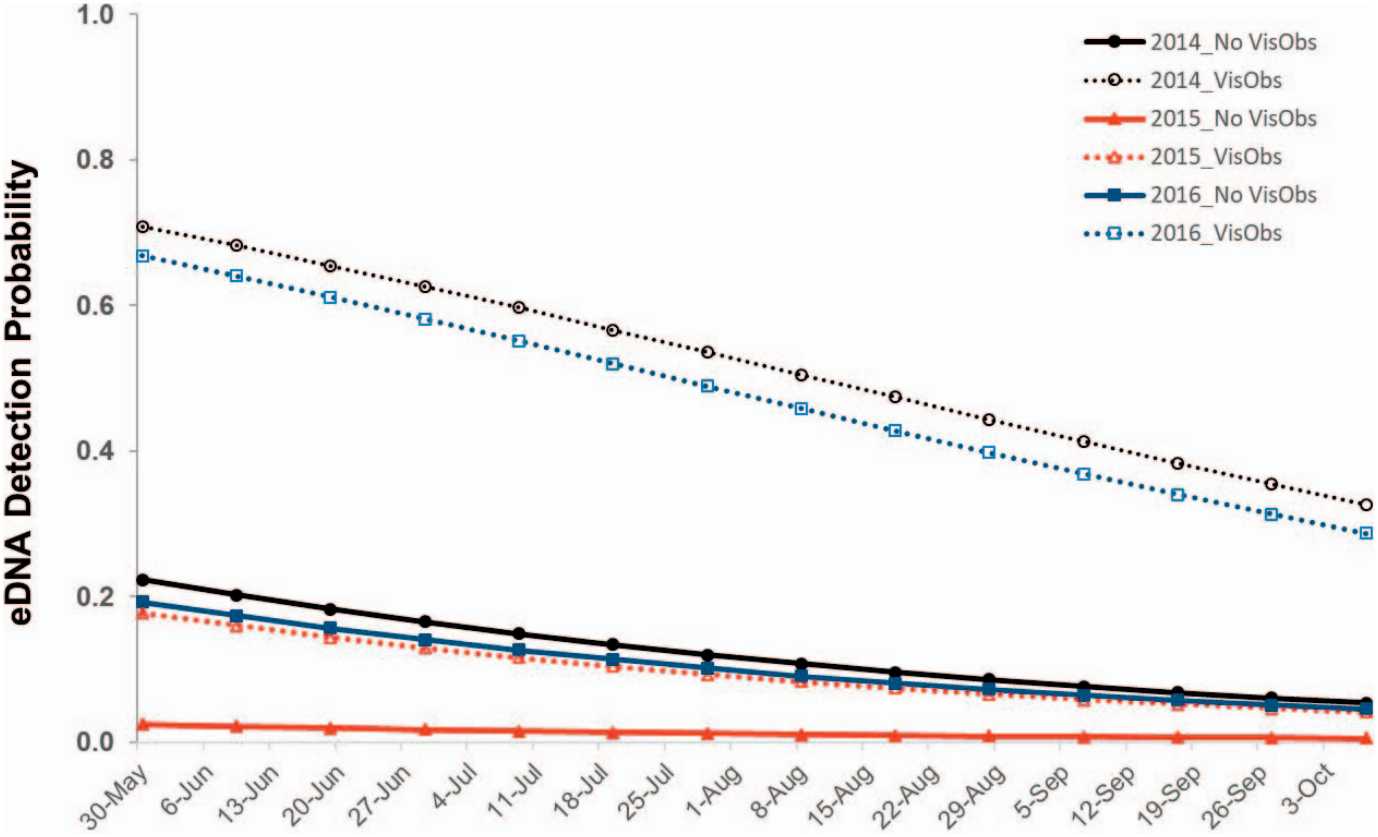

FIG. 5. Boreal Toad *e*DNA detection probability estimates across dates for samples collected in 2014–2016. Estimates are obtained from the best supported model, ψ_{unk} (Yr) p_{eDNA} (Prev_det + Date + Yr) p_{vis} (Sitetype). Detection probabilities are given for *e*DNA samples taken when toads were and were not observed via visual surveys in the same 2-wk period (dotted and solid lines, respectively).

$\hat{p}_{eDNA}$ ranged from ~0.20 to <0.05). These results are not surprising, as *e*DNA detection is always imperfect and depends upon the species density and the ratio between DNA released by the organisms and the DNA degraded by environmental factors (Dejean et al., 2011). Boreal Toad abundance and activity is likely highest in lentic habitats during the breeding season, but released DNA likely degrades quickly in shallow, high-elevation habitats that experience high temperatures and ultraviolet (UV) exposure (Pilliod et al., 2014; Goldberg et al., 2018). Still, our *e*DNA detection estimates were comparable to probabilities reported for other low-density aquatic organisms (Ficetola et al., 2008; Mosher et al., 2017). Similar to other studies, we found no evidence that the volume of water filtered influences detection probability (Chestnut et al., 2014; Mosher et al., 2018c), emphasizing that collecting additional samples is more beneficial than increasing the filtered volume. Our estimates suggest that investigators should collect ≥10 filter samples during the breeding season to have a high degree of certainty (90% confidence) that toads do not exist at a potential breeding location. This increase in *e*DNA sampling effort does not necessarily imply increased processing cost, as there are various strategies for pooling samples to reduce cost (Boyle et al., 2004; Goldberg et al., 2018).

Our study highlights the importance of testing and combining a diversity of survey methods to maximize benefit for monitoring and recovery efforts. We found that if toads are present in a drainage, searching riparian areas multiple times during the active season is an efficient way of detecting the occurrence of previously unknown breeding populations. Once a breeding population has been located, traditional visual encounter surveys yield high probabilities of species detection for monitoring efforts ($\hat{p}_{vis}$(Kn) ≈ 0.80. Supplementing streamside surveys when toads are not detected with other survey methods (e.g., VES or *e*DNA samples) at suitable breeding locations can help confirm the species is absent. Employing both visual surveys and *e*DNA samples can simultaneously yield distributional information on other amphibian species and *Bd*, if captured amphibian species are swabbed and water filters are tested for both amphibian and *Bd* DNA (Young et al., 2007). Such information would increase our understanding of the mechanisms influencing the distribution and persistence of *Bd* in the southern Rocky Mountains independent of its presumed primary host (Boreal Toads; Mosher et al., 2018a,b) and thus provide managers better information to plan mitigation and conservation efforts (e.g., reintroductions; Garner et al., 2016; Gerber et al., 2018).

Finally, we encourage other investigators to employ a similar iterative process in determining optimal survey methods for their own study systems, as both occupancy and detection probabilities are likely to vary in different areas and among species. Our process is not limited to amphibian species; rather, it could apply to any rare species with limited distributional or detection information. Important steps in the process include the need to: 1) determine an appropriate spatial unit for which the species occurrence and detection probability is meaningful and potentially scalable (e.g., habitat of a certain size; in our case, this was a 500-m length of stream); 2) test potential survey methods within an area where the species is known to occur, perhaps across different levels of relative abundance or activity; 3) use estimates of occurrence and method-specific detection

probabilities to determine an optimal survey effort (number of visits, survey methods, number of units, etc.) to achieve a desired level of confidence in detecting the species if present or a desired precision of occupancy estimates (MacKenzie et al., 2018); 4) employ optimal survey methods in areas of unknown status and compare detection results to expected values; and 5) repeat the process if new survey methods become available.

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APPENDIX 1. Thirty-four models fit to Boreal Toad detection–nondetection data from 73 stream reaches (units) surveyed in 2011–2012. K is the number of estimated parameters in the model, ΔAICc is the relative difference in corrected Akaike information criterion (AICc) values, w is the model weight, and $-2l$ is twice the negative log-likelihood. Occupancy probability (ψ) was modeled as constant among all reaches (.), different among high- and low-population drainages (Pop), or as a function of distance (Dist) and/or the direction (upstream or downstream, Direction) from the adjacent net location nearest the known breeding location. Additive and interactive relationships were represented by + and × symbols, respectively. Detection probability (p) was modeled as a function of sampling effort (Effort), stream gradient (StrGrad), different (t) or constant (.) among visits. Detection probability differences were also explored among high- and low-population drainages (Pop).

Model	K	ΔAICc	w	$-2l$
ψ(Dist × Direction) p(Pop + StrGrad)	6	0.00	0.34	104.89
ψ(Dist) p(Pop + StrGrad)	5	1.33	0.17	108.60
ψ(Pop + Dist × Direction) p(Pop + StrGrad)	7	1.87	0.13	104.32
ψ(Dist × Direction) p(Pop)	5	2.98	0.08	110.25
ψ(Pop + Dist) p(Pop + StrGrad)	6	3.55	0.06	108.44
ψ(Dist × Direction) p(Pop + Effort)	6	3.85	0.05	108.74
ψ(Pop + Dist × Direction) p(Pop)	6	4.60	0.03	109.49
ψ(Dist) p(Pop)	4	5.03	0.03	114.61
ψ(Dist) p(Pop + Effort)	5	5.37	0.02	112.64
ψ(Pop + Dist × Direction) p(Pop + Effort)	7	5.40	0.02	107.84
ψ(Dist × Direction) p(Pop + t)	7	5.52	0.02	107.96
ψ(Pop + Dist) p(Pop)	5	7.03	0.01	114.30
ψ(Pop + Dist × Direction) p(Pop + t)	8	7.29	0.01	107.20
ψ(Dist) p(Pop + t)	6	7.37	0.01	112.26
ψ(Pop + Dist) p(Pop + Effort)	6	7.39	0.01	112.28
ψ(Pop + Dist) p(Pop + t)	7	9.50	0.00	111.95
ψ(.) p(Pop + StrGrad)	4	21.88	0.00	131.46
ψ(.) p(.)	2	29.49	0.00	143.48
ψ(.) p(Pop)	3	23.68	0.00	135.50
ψ(.) p(Pop + Effort)	4	23.11	0.00	132.69
ψ(.) p(Pop + t)	5	25.97	0.00	133.23
ψ(.) p(t)	4	31.84	0.00	141.42
ψ(Direction) p(Pop)	4	23.27	0.00	132.85
ψ(Direction) p(Pop + Effort)	5	23.23	0.00	130.50
ψ(Direction) p(Pop + StrGrad)	5	22.52	0.00	129.79
ψ(Direction) p(Pop + t)	6	25.68	0.00	130.57
ψ(Pop) p(Pop)	4	24.06	0.00	133.64
ψ(Pop) p(Pop + Effort)	5	23.45	0.00	130.71
ψ(Pop) p(Pop + StrGrad)	5	22.27	0.00	129.54
ψ(Pop) p(Pop + t)	6	26.51	0.00	131.40
ψ(Pop + Direction) p(Pop + t)	7	25.94	0.00	128.38
ψ(Pop + Direction) p(Pop)	5	23.37	0.00	130.64
ψ(Pop + Direction) p(Pop + Effort)	6	23.34	0.00	128.24
ψ(Pop + Direction) p(Pop + StrGrad)	6	22.41	0.00	127.30

APPENDIX 2

Optimal Streamside Surveys.—Using occupancy and detection probability estimates from the 2011–2012 analysis, we determined the optimal number and length of streamside surveys needed to ensure a high probability of detecting toads if they existed within a drainage. If toads used a 500-m stream reach, the probability of detecting toads in a given visit is p, and the probability of failing to detect toads is $(1 - p)$. If the occupied reach is surveyed t times, then the probability of detecting toads at least once is $p^* = 1 - (1 - p)^t$, or the complement of failing to detect toads on each of the t visits. This cumulative detection probability, p^*, is conditional on the reach being occupied, but an unconditional cumulative detection probability could be calculated for a given reach i as $(\psi_i \times p_i^*)$, where ψ_i is the probability of occupancy for stream reach i. Notice this unconditional detection probability is 0 if reach i is unoccupied. The probability of detecting toads in at least one reach of an occupied drainage is $p^*_{uncond} = 1 - \Pi_{i=1}^{I}(\psi_i \times p_i^*)$, where I was the number of stream reaches included in the calculation. The length of stream sampled (l) influenced the number of stream reaches included in the calculations while the number of visits (t) influenced the conditional probability of detection at occupied reaches (p_i^*).

We calculated the probability of detecting toads in an occupied drainage (p^*_{uncond}) by sampling $l = 2.5$, 3.5 or 4.5 km of stream, one to three times (t = 1, 2, or 3), during the postbreeding season using occupancy and detection estimates from the 2011–2012. Toad occupancy probability at a reach was strongly influenced by proximity to the breeding location and detection varied with stream gradient among high- and low-population drainages. Accordingly, we calculated p^*_{uncond} assuming that the sampled stream length l was: 1) centered on the breeding location, similar to our field study, 2) upstream of the breeding location, or 3) downstream of the breeding location. For example, when calculating p^*_{uncond} for a 4.5-km stream section "downstream" of an unknown breeding location, we used occupancy estimates from the adjacent stream reach (distance = 0 km) through the furthermost downstream reach (i = 9 stream reaches). When the stream length is reduced to $l = 3.5$ km, however, then the adjacent reach and the most downstream reach are removed in our calculation of p^*_{uncond}. We used detection probability estimates for a reach with average stream slope (4.84%) in drainages with relatively high ($\hat{\bar{p}}_{high} = 0.57$; Table A1) or low breeding toad populations ($\hat{\bar{p}}_{low} = 0.08$; Table A2).

TABLE A1. Expected probabilities of detecting toads in occupied drainages with relatively high breeding populations of Boreal Toads. Expected values vary as a function of the length of stream sampled (l), the location of the stream section relative to the unknown breeding location, and the number of visits. Italicized combinations indicate sampled stream lengths that do not include the adjacent stream reach (i.e., the stream reach nearest the breeding location).

Location of stream section	Stream length (l)	Number of visits (streamside surveys) $t = 1$	$t = 2$	$t = 3$
Centered	4.5 km	0.96	1.00	1.00
	3.5 km	0.96	1.00	1.00
	2.5 km	0.94	0.99	1.00
Upstream of breeding location	4.5 km	0.80	0.95	0.98
	3.5 km	*0.55*	*0.73*	*0.80*
	2.5 km	*0.17*	*0.24*	*0.27*
Downstream of breeding location	4.5 km	0.92	0.99	1.00
	3.5 km	*0.81*	*0.94*	*0.97*
	2.5 km	*0.60*	*0.77*	*0.83*

TABLE A2. Expected probabilities of detecting toads in occupied drainages with relatively low breeding populations of Boreal Toads. Expected values vary as a function of the length of stream sampled (l), the location of the stream section relative to the unknown breeding location, and the number of visits. Italicized combinations indicate sampled stream lengths that do not include the adjacent stream reach (i.e., the stream reach nearest the breeding location).

Location of stream section	Stream length (l)	Number of visits (streamside surveys) $t = 1$	$t = 2$	$t = 3$
Centered	4.5 km	0.30	0.50	0.65
	3.5 km	0.29	0.49	0.63
	2.5 km	0.26	0.45	0.59
Upstream of breeding location	4.5 km	0.16	0.29	0.40
	3.5 km	*0.09*	*0.16*	*0.23*
	2.5 km	*0.02*	*0.05*	*0.07*
Downstream of breeding location	4.5 km	0.23	0.41	0.55
	3.5 km	*0.17*	*0.31*	*0.42*
	2.5 km	*0.10*	*0.10*	*0.27*

APPENDIX 3. Model selection results for 18 detection structures for visual surveys fit to Boreal Toad detection–nondetection data from 75 units (drainages with known breeding locations or unknown Boreal Toad status) from 2014–2016. Visual detection probability ($\dot{p}_{vis}$) could vary temporally as a function of day of the year (Date) or years (Yr), among visual survey types (OSS or VES, Survtype), or among units with known or unknown breeding activity (Unittype). Because survey and unit type are highly correlated, we never included both factors in the same model structure. K is the number of estimated parameters in the model, ΔAICc is the relative difference in AICc values, w is the model weight, and $-2l$ is twice the negative log-likelihood. All models included general structures for occupancy and *e*DNA detection probability: ψ_{unk}(Yr) p_{eDNA}(Lab + Vol + Prev_det + Date + Date^2 + Yr).

Model	K	ΔAICc	w	$-2l$
p_{vis}(Unittype)	13	0.00	0.53	368.72
p_{vis}(Unittype + Yr)	15	2.14	0.18	364.69
p_{vis}(Unittype + Date)	14	2.34	0.16	368.03
p_{vis}(Unittype + Date + Yr)	16	4.22	0.06	363.53
p_{vis}(Unittype + Date + Date^2)	15	4.85	0.05	367.40
p_{vis}(Unittype + Date + Date^2 + Yr)	17	6.48	0.02	362.43
p_{vis}(Survtype)	13	22.20	0.00	390.92
p_{vis}(.)	12	24.32	0.00	395.97
p_{vis}(Survtype + Date)	14	25.22	0.00	390.91
p_{vis}(Survtype + Yr)	15	25.89	0.00	388.44
p_{vis}(Yr)	14	26.18	0.00	391.87
p_{vis}(Date)	13	27.16	0.00	395.88
p_{vis}(Survtype + Date + Date^2)	15	28.29	0.00	390.85
p_{vis}(Survtype + Date + Yr)	16	28.74	0.00	388.05
p_{vis}(Date + Yr)	15	28.84	0.00	391.40
p_{vis}(Date + Date^2)	14	30.13	0.00	395.82
p_{vis}(Date + Date^2 + Yr)	16	31.77	0.00	391.08
p_{vis}(Survtype + Date + Date^2 + Yr)	17	31.84	0.00	387.79