Long distance (> 20 km) downstream detection of endangered stream frogs suggests an important role for eDNA in surveying for remnant amphibian populations

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Abstract

Globally, amphibian species have suffered drastic population declines over the past 40 years. Hundreds of species are now listed as Critically Endangered, with many of these considered "possibly extinct". Most of these species are stream-dwelling frogs inhabiting remote, montane areas, where traditional surveys limit our ability to find remnant populations. Environmental DNA (eDNA) monitoring could revolutionize surveys for missing and endangered amphibian populations by enabling sampling of downstream sections to screen the vast upstream catchments. However, this is dependent on quantifying downstream detection probability and distances. Here we tested this in two endangered stream frogs (Litoria nannotis and L. lorica) that co-occur in a remote stream catchment, and for which we know precise downstream distributional limits from traditional surveys. Importantly, the last populations of L. lorica persist in this catchment; one small (˜1,000 frogs) and one very small (˜100 frogs). We conducted eDNA screening at set distances downstream from the populations (up to 22.8 km) using precipitation from two water volumes and via filtering, during moderately high and low flow conditions. During high flow, we reliably detected both species as far as 22.8 km downstream using the larger water volumes and filtering techniques. We could only detect the very small population of L. lorica immediately downstream. Detection success was higher at the sites further downstream for both species during high flow compared to low flow. Our downstream detection distances (> 20 km) suggest eDNA is a valuable tool for detecting rare stream amphibians. We provide recommendations on optimal survey techniques.

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Running title

Endangered frogs' eDNA downstream transport

Authors

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Abstract. Globally, amphibian species have suffered drastic population declines over the past 40 years. Hundreds of species are now listed as Critically Endangered, with many of these considered "possibly extinct". Most of these species are stream-dwelling frogs inhabiting remote, montane areas, where traditional surveys limit our ability to find remnant populations. Environmental DNA (eDNA) monitoring could revolutionize surveys for missing and endangered amphibian populations by enabling sampling of downstream sections to screen the vast upstream catchments. However, this is dependent on quantifying downstream detection probability and distances. Here we tested this in two endangered stream frogs (*Litoria nannotis* and *L. lorica*) that co-occur in a remote stream catchment, and for which we know precise downstream distributional limits from traditional surveys. Importantly, the last populations of *L. lorica* persist in this catchment; one small (~1,000 frogs) and one very small (~100 frogs). We conducted eDNA screening at set distances downstream from the populations (up to 22.8 km) using precipitation from two water volumes and via filtering, during moderately high and low flow conditions. During high flow, we reliably detected both species as far as 22.8 km downstream using the larger water volumes and filtering techniques. We could only detect the very small population of *L. lorica* immediately downstream. Detection success was higher at the sites further downstream for both species during high flow compared to low flow. Our downstream detection distances (> 20 km) suggest eDNA is a valuable tool for detecting rare stream amphibians. We provide recommendations on optimal survey techniques.

KEYWORDS

downstream detection, eDNA, endangered species, monitoring, rainforest frog, tropics

INTRODUCTION

Amphibians contain a greater proportion of Critically Endangered and Endangered species than any other Class of animal (IUCN, 2020). Of particular concern is that 587 amphibians are listed as Critically Endangered, which is double the number of similarly listed mammals, birds or reptiles (IUCN, 2020). These species are on the brink of extinction; indeed, the persistence of many is uncertain (Scheele et al., 2019; Stuart et al., 2004). Within the amphibian species listed as Critically Endangered, 143 are categorized as "possibly extinct" (CR[PE]) or "possibly extinct in the wild" (CR[PEW]) (IUCN, 2020). It is not possible to enact conservation measures for these species without knowing if, or where, they persist, and conservation actions for known Critically Endangered species are often limited by uncertainty regarding how many populations remain (Gillespie et al., in press).

The key threat to amphibians is chytridiomycosis disease, which has particularly impacted montane, stream-associated species (Scheele et al., 2019; Stuart et al., 2004). In the last three decades, globally, hundreds of such species have been reduced to small remnant populations or are 'missing' due to this disease. Montane stream environments are a challenge for traditional surveys (i.e., on foot, usually at night when frogs are active) due to the remote, rugged terrain and seemingly countless small tributaries to search. Further, the activity of many species is dependent on weather (e.g., rain), impacting the probability of detection on any one survey (Scheele & Gillespie, 2018). The chances of rediscovering a small population on one section of stream (e.g. Puschendorf et al., 2011) are akin to 'finding a needle in a haystack'. On the other hand, these environments offer a theoretically ideal scenario for using eDNA for threatened species detection and monitoring because the myriad tributaries all flow downstream into a few major drainages for each mountain.

Environmental DNA has been used extensively in stream environments to detect vertebrate and invertebrate

species (e.g. Deiner & Altermatt, 2014; Goldberg, Pilliod, Arkle, & Waits, 2011; Jane et al., 2015; Jerde et al., 2016; Thomsen et al., 2012). In flowing streams, eDNA production, decay, transport, retention and resuspension are the most important factors accounting for target species detectability (Barnes, Turner, & Turner, 2016). The production and decay of eDNA depend on the eDNA source and the biotic and abiotic factors surrounding it (Nielsen, Johnsen, Bensasson, & Daffonchio, 2017). However, eDNA transport is a more complex phenomenon (Shogren, Tank, Egan, Bolster, & Riis, 2019). Studies assessing eDNA transport in freshwater systems, and its relationship with detectability, reported contrasting trends. While some have found the intuitive pattern of reduced detectability with stream distance from the source (Deiner & Altermatt, 2014), others have found the opposite. Wood, Erdman, York, Trial, & Kinnison (2020) found that eDNA is almost undetectable just below a small number of caged Atlantic salmon (*Salmo salar*) but detectability increases downstream after eDNA has been mixed in the water, and then it declines steadily with distance (Wood et al., 2020). An increasing number of recent studies are showing that mean eDNA transport distance and retention in a system are highly influenced by water discharge and physico-chemical characteristics of a stream (Shogren et al., 2017, 2019). Among the physical characteristics of a stream, substrate accounts for a significant amount of eDNA retained, with finer substrates, such as pea gravel, being more efficient at eDNA retention, and hence higher downstream detection probability, than coarser substrates, such as cobble stone (Jerde et al., 2016).

Remarkably few studies have been published on eDNA detection of stream amphibians (Pilliod, Goldberg, Arkle, & Waits, 2014; Santas, Persaud, Wolfe, & Bauman, 2013; Sasso et al., 2017; Spear, Groves, Williams, & Waits, 2015). Most of these studies did not determine eDNA detectability at different downstream distances from a source population, primarily because the downstream distributional limits of species were not known. Santas et al. (2013) and Spear et al. (2015) used targeted eDNA surveys to detect Hellbender salamanders (*Cryptobranchus alleganiensis*) at known, and previously unknown, sites. Sasso et al. (2017) used a metabarcoding approach to detect amphibian communities across four streams from separate drainages in the Brazilian Amazon. They showed that a four-day eDNA sampling event captured all stream species found during a five-year long survey in the area using traditional techniques. These studies showed that eDNA surveys are efficient for amphibian species detection, but did not determine downstream detection distances. In a study measuring eDNA downstream transport from introduced caged salamanders, Pilliod et al. (2014) found that eDNA could be detected 5 m downstream, but their study was limited to 50 m. It is currently unknown whether eDNA of stream-dwelling amphibians could travel a long distance downstream from a small population, allowing for their detection via eDNA analysis.

Environmental DNA could revolutionize the way we survey for missing and critically endangered amphibians (and, potentially, other stream taxa) (Ficetola, Manenti, & Taberlet, 2019), especially if we can capture it in streams carrying waters from hydrographical basins located in mountain areas. This is because hundreds of small streams flow off mountains into a small number of large streams. Frequently there are roads crossing these large streams at the base of mountains, allowing easy sampling points for collecting water. Detections in large streams would then focus further eDNA or traditional field survey efforts upstream to more efficiently locate populations. However, this is dependent on demonstrating significant downstream eDNA detection distances for small amphibian populations. Here we assess this in a thoroughly-surveyed endangered stream frog system in north Australia.

Rainforest stream frogs inhabiting the mountains of eastern Australia have been heavily impacted by chytridiomycosis disease (Scheele et al., 2017). Declines started in the late 1970s near Brisbane (south-east Queensland) and progressed north to impact frogs of the Wet Tropics region, in north-east Queensland, in the late 1980s and early 1990s (Laurance, Mcdonald, & Speare, 1996; Scheele et al., 2017). Six species were believed to have gone extinct, while population numbers of others declined substantially, and now persist in a fraction of their former range (Scheele et al., 2017). The persistence of several species remains uncertain because although they have not been seen for two or three decades, and surveys have not been conducted in remote, rugged parts of their former ranges (Gillespie et al., in press). The number of populations of several known extant Critically Endangered species is not known for the same reason, and locating these has been identified as a priority research action (Gillespie et al., in press).

The Armoured Mistfrog (*Litoria lorica*) was one species of the Wet Tropics considered to be extinct after the severe declines in the early 1990s. However, it was rediscovered as a single, small population in 2008 during research on populations of a co-occurring Endangered species, the Waterfall Frog (*Litoria nannotis*) (Puschendorf et al., 2011). These two 'torrent frogs' are semi-aquatic species restricted to cascades and waterfalls. They differ in size, *L. nannotis* being bigger than *L. lorica* (55 mm and 37 mm adult size, respectively), but have near-identical ecologies, foraging side-by-side at night in the splash zone of waterfalls and hiding by day in the flowing water or associated rock cracks (Puschendorf et al., 2011). The tadpoles of both species have suctorial mouth discs and are also restricted to the fast flowing rocky sections (Puschendorf et al., 2011). The rediscovery of *L. lorica* triggered on-ground surveys of most potential habitat in the region, including detailed surveys of streams throughout the large catchment where *L. lorica* was rediscovered (Hoskin & Puschendorf, 2014). These surveys found populations of *L. nannotis* in all sections of suitable habitat but did not find any additional populations of *L. lorica* . Permission was obtained for a trial reintroduction of *L. lorica* , which involved translocating adults during three consecutive years (2013–2015) to establish a small population in a discrete area of habitat approximately 4 km upstream of the rediscovered population (Hoskin & Puschendorf, 2014) (Figure 1). Based on regular monitoring of these two populations over the last five years, the rediscovered population is estimated to consist of approximately 1,000 frogs (plus an unknown number of aquatic tadpoles at any one time) along a 4 km stretch of stream, and the reintroduced population is estimated at about 100 frogs (and a small number of tadpoles at any one time) along an approximately 1 km section of stream (Hoskin & Puschendorf, 2014; Hoskin & Puschendorf, in prep) (Figure 1).

Here we aimed to test the utility of eDNA as a method for surveying endangered stream frogs. We used several sampling and laboratory techniques to determine downstream detection probabilities at a range of distances for populations of *L. lorica* and *L. nannotis* . This system is ideal for testing downstream detection distances for the following reasons. First, adults of the two species have extensive (daily) contact with water, so eDNA can enter the stream at all times regardless of the presence of tadpoles. Second, the catchment containing*L. lorica* has been thoroughly surveyed (i.e., on foot, using head torches at night), including all areas of potentially suitable habitat for these two species (Hoskin & Puschendorf, 2014; Hoskin, unpub. data). These surveys have resolved downstream limits for the two species on all sections of streams. Third, *L. nannotis* is present and common in discrete upstream sections of all streams, whereas *L. lorica* is restricted to two sections—a small population (approx. 1,000 frogs) and a very small population (approx. 100). We were therefore able to select downstream localities, at known distances from source frog populations, of both species to test eDNA detection. We use these results to outline the feasibility of eDNA for detecting small populations of stream frogs kilometers upstream.

MATERIALS AND METHODS

Study site

The present study was conducted in a perennially-flowing, rainforest-fed stream catchment in the Wet Tropics of north-east Australia. Tropical Australia exhibits high seasonality (Feng, Porporato, & Rodriguez-Iturbe, 2013) and is characterised by periods of no rain (dry season, generally May to September) and periods of increased rainfall (wet season, generally October to April). Environmental DNA detection of*Litoria* species was determined by sampling during two stream flow conditions— moderately high flow (herein termed 'high flow') and low flow. The most comprehensive sampling was conducted during high flow, 8–10 April 2019, and consisted of: three sites downstream from the translocated *L. lorica* population (sites 2, 4, 5); three sites downstream from the main *L. lorica* population (sites 7, 9, 10); a site about 10% into the main *L. lorica* population (site 6); and three sites in tributaries where only *L. nannotis* has been found upstream during on-foot surveys (sites 1, 3, 8) (Figure 1). Of note is that sites 7, 9 and 10 were 10.0, 17.1 and 22.8km downstream, respectively, from a known *L. lorica* frog population. Site 2 (immediately downstream from the translocated population) and site 6 (at the upper end of the main *L. lorica* population) are comparable because there is an estimated 100 adult *L. lorica* immediately upstream from both sites (C. Hoskin, unpub. data). Flow was only calculated at site 1 (0.12 m^3/sec) during this sampling trip. Environmental DNA

was captured across these sites via two methods: (1) direct water collection and preservation, and (2) water filtration.

Sampling during low flow was conducted on 24–25 October 2019, and only included a subset of the key sites (sites 5-10) most informative for comparing long distance eDNA transport. Flow rate was not calculated on that trip because site 1 was not accessed and measuring flow at any other site would have not allowed for direct comparisons to the previous sampling event. Environmental DNA sampling during low flow was by direct water collection and preservation only.

Direct water collection and preservation

For the high flow sampling trip, 15 mL and 375 mL water samples were taken. At each site, five replicate 15 mL samples were collected using a new, clean falcon tube of 50 mL capacity and decanting into another falcon tube containing 10 mL of Longmire's preservative solution (Renshaw, Olds, Jerde, Mcveigh, & Lodge, 2015) (Figure 2). At each site, a field blank was also taken to ensure that the process of sample collection did not introduce contamination. The field blank consisted of decanting 15 mL of MilliQ water into a falcon tube containing 10 mL of preservative solution. Environmental DNA was extracted from the entire water volume collected.

Additionally, three replicate 350 mL samples were collected at sites 1-4 (hard-to-access sites where larger water volumes could not be collected and carried) and five replicated 350 mL samples were collected at sites 5-10. However, one replicate of site 1-4 and 6-10 as well as two replicates from site 5 were destined to different analyses (data not included here) (see Table S1 for info on final number of replicates per site).

Sampling was performed using a new, clean jar of 500 mL capacity to take each 375 mL sample from the stream and decanting it into a bottle containing 125 mL of Longmire's preservative solution (Table S1). A field blank was also included after collecting these samples. It consisted of decanting 375 mL of MilliQ water into a falcon tube containing 125 mL of preservative solution. Environmental DNA was extracted from a 100 mL aliquot of each replicate (Figure 2).

During low flow sampling, only 375 mL water sampling was conducted. Five replicate 375 mL samples were collected from sites 5–10 (Table S1), using the procedures outlined above.

Environmental DNA was extracted from 100 mL water sample plus preservative solution out of the 375 mL samples, given that any volume larger than 100 mL becomes impractical in standard laboratory centrifuges. These are now termed "100 mL samples" from here on.

Water filtration

Water was filtered through a string filter of 1 µm nominal pore size using a large volume sampler designed to collect suspended sediment in flood plumes (Lewis et al., 2018) at five sites that have relatively easy access (Table S1), given that this filtering device was large and heavy ($>$ 5 kg after water filtration). Water filtration was performed for one hour and a single replicate was collected at each site. After filtration was completed, the filter was preserved in 700 mL of Longmire's solution diluted to 25% in MilliQ water. Three field blanks consisting of preserving a new, clean filter into 700 mL of Longmire's buffer diluted to 25% were included. The diluted Longmire's solution in which the filter had been preserved was stored at room temperature without exposure to ambient light for one week after collection, in order to allow the eDNA captured in the filter to come into solution. After this period, a 20 mL aliquot from the diluted Longmire's solution in which the filter had been preserved was taken and eDNA was extracted (Figure 2). Each water sample was mixed by inversion ten times in order to ensure that all eDNA was resuspended in the water column before taking the aliquot for extraction.

Environmental DNA extractions

Upon arrival to the laboratory, eDNA from preserved collected water was extracted via a glycogen-aided isopropanol based precipitation protocol (Edmunds, Villacorta-Rath, Huerlimann, & Burrows, 2019) in a dedicated eDNA laboratory at James Cook University (JCU), Australia. The only exception was the 100

mL samples from sites 1-5 and 9-10 that were screened for *L. nannotis* . These sites were extracted on the return from the field but the screening was unsuccessful due to a failure of the real-time PCR instrument. They were then extracted again five months after sample collection to repeat the screening for *L. nannotis* . In the intervening five months, the water samples mixed with Longmire's solution were stored at room temperature and in the dark, which has been demonstrated to protect eDNA from degradation (Renshaw et al., 2015; Williams, Huyvaert, & Piaggio, 2016).

For all extractions, 20 mL sample aliquots were mixed with 5 µL glycogen (200 mg/mL), 20 mL isopropanol and 5 mL NaCL (5M). Samples were then incubated overnight at 4°C and subsequently centrifuged at 6,750 g for 10 min to form a pellet. The supernatant was then discarded and pellets were dissolved in 600 µL of lysis buffer (guanidinium hydrochloride and TritonX; pH 10), transferred into a 2 mL tube, and frozen overnight. Environmental DNA present in the samples was lysed at 50°C for five hours and a subsequent precipitation step was carried out by adding 1 µL glycogen and 1,800 µL polyethylene glycol (PEG) buffer to the samples. Samples were centrifuged at 20,000 g for 30 min to form a pellet that was then washed twice using 70% ethanol. After the ethanol washes, the pellet was dried and eDNA was resuspended in 100 µL MilliQ water. Finally, eDNA was purified using the Qiagen DNeasy® PowerClean® Pro Cleanup kit and eluted in 100 µL elution buffer.

Given that we sampled very large volumes of water with our filtration system, samples were highly pigmented (Figure S1). After eDNA extraction, samples still contained a high level of coloration and therefore we applied a 1:10 dilution and a subsequent 1:2 dilution, which did not exhibit any coloration. We applied inhibition tests (Jane et al., 2015) on the final 1:20 dilution.

Real-time PCR (qPCR)

Target species detection was performed using two different species-specific primer pairs, developed at TropWATER (JCU), targeting the cytochrome *c* oxidase subunit I (COI) mitochondrial gene of*L. lorica* and *L. nannotis* (Table S2; Edmunds, Villacorta-Rath, Huerlimann, & Burrows, 2019). The limit of detection (LOD) was estimated using a 10-fold serial dilution of artificial DNA (aDNA) ranging from 2.82 x 10^7 to 0.70 copies/µL for*L. lorica* and 2.17 x 10^7 to 0.55 copies/µL for*L. nannotis* . Additionally, the LOD was determined using a 7-fold serial dilution of genomic DNA (gDNA) from each of the target species. For *L. lorica* gDNA dilutions ranged between 1.24 and 3.1 x 10^{-5} ng/µL, whereas *L. nannotis* ranged between 6.45 x 10^{-4} and 1.6125 x 10^{-5} ng/µL. Four technical replicates per dilution were used and the LOD was determined through a discrete threshold and defined as the lowest concentration showing amplification of at least one technical replicate. Based on the serial dilutions, the LOD was determined to be 1.64 x 10^{-5} ng/µL or 0.6 copies/µL for *L. lorica* , and 1.61 x 10^{-5} ng/µL or 0.6 copies/µL for *L. nannotis* , respectively.

qPCR assays were performed on a QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific Australia Pty Ltd) in white 96 or 384-well plates sealed with optical films (Thermo Fisher Scientific Australia Pty Ltd). Twelve technical replicates of each sample were screened in all qPCR analyses, representing 36% of the total available DNA elution volume. Additionally, three no template controls (NTC) were used. The NTC samples did not contain the target species DNA and their lack of amplification indicated that no contamination was introduced during plate handling. Each qPCR assay consisted of 3 µL of template DNA and 7 µL of master mix (5 µL PowerUp SYBR Green Master Mix; 0.5 µL forward primer at 10 µM; 0.5 µL reverse primer at 5 µM; 1 µL MilliQ® water). Thermal cycling conditions were as follows: initial denaturation and activation at 95°C for 2 min then 55 cycles of 95°C for 15 secs and 60°C for 1 min. A subsequent melt curve analysis was performed to generate dissociation curves by transitioning from 60°C to 95°C at 0.15°C sec^{-1}.

All plates were analysed with a common fluorescence threshold (0.2) using QuantStudio Design and Analysis Software (version 1.4.2; Thermo Fisher Scientific Australia Pty Ltd) before export and subsequent analyses in Microsoft Excel. Samples were considered putative positive detections if: (1) the amplification curve crossed the common fluorescence threshold within 50 cycles; (2) the amount of eDNA was above the LOD; and (3) the melt curve analysis showed a dissociation temperature peak at 79.66degC (+-0.75 – 99% confidence interval) for*L. nannotis* , 78.52degC (+-0.62 – 99% confidence interval) and for*L. lorica* . Amplicons

from putative positive detections were sequenced at the Australian Genome Research Facility (AGRF) to confirm that they were true detections. A nucleotide BLAST was performed and amplicon sequences from the samples considered putative positive detections were considered as true detections if there was [?] 97% pairwise identity with the *COI* gene of *L. nannotis*(GenBank accession number JC130908; matching between positions 219–286 bp) or *L. lorica* (no sequences on GenBank) matched at 99% pairwise identity to in-house sequences, with the targeted *COI*section being: (CGACACTTATTATGTTGTAGCCCATTTCCATTATG-TATTGTCTATAGGAGCTGTATTCGCCATTATAGC) (Edmunds et al., 2019). This section of *L. lorica* sequence has at least three mismatches to other frog species.

Detection analysis and presentation

Detection rate for each species was calculated as the proportion of qPCR replicates of each sampling technique at each site that yielded a positive detection in relation to the total number of qPCR replicates at that site. This was calculated separately for the high and low flow sampling trips. Standard errors of these proportions were calculated by dividing the standard deviation of the number of positive qPCR replicates detections by the square root of the total number of qPCR replicates. The downstream limit of each species on each tributary was known accurately (i.e., +/- 20 m) from field surveys (C. Hoskin, unpub. data) and the distance from these limits to downstream sites was estimated by measuring stream distances in Google Earth. Of particular relevance were the downstream distances from the two populations of*L. lorica* (see stars and inset on Figure 1).

Inhibition test

We tested inhibition in water samples by spiking 80 copies of artificial DNA (aDNA) into triplicated samples from the sites without presence of*L. lorica* (sites 1, 3 and 8). Additionally, we spiked the same number of DNA copies into three technical replicates containing only MilliQ water. We compared the Ct values of each negative control site to those of the spiked MilliQ water samples to detect any shifts that would suggest qPCR inhibition.

RESULTS

The qPCR of all field control samples and extraction control samples were devoid of *L. lorica* and *L. nannotis* DNA. The three sampling methods gave positive detections of *L. nannotis* and*L. lorica* at most of the sampling sites where each of the species is known to occur at the site or upstream. Importantly, we did not detect *L. lorica* at the three upstream sites where this species is not known to occur (sites 1, 3, 8) (Table 1; Figure 1). The assessment criteria for positive detections listed were satisfied in the following ways: (1) amplification occurred within 50 cycles; (2) amount of eDNA was above the LOD; (3) the melt curve peak corresponded to that of each species; and (4) amplicon sequences from *L. nannotis* and*L. lorica* samples considered putative positive detections matched the COI regions targeted in each species with [?] 97% match for *L. nannotis* and [?] 99% for *L. lorica* detections.

Detection rates and distances from direct water collection and preservation

Detection of both species using the two water collection methods was variable. Extraction from the 100 mL samples (obtained from the 375 mL water collections) was more successful for detecting *L. lorica*than the 15 mL samples (Table 1). *Litoria lorica* was detected at only three sites (sites 4, 7, 9) using the 15 mL samples in very low frequency (< 4%; Table 1). No detections were made with the 15 mL samples at either site where there are approximately 100 *L. lorica* adults upstream (sites 2 and 6). In comparison, *L. lorica*was detected at more sites using the 100 mL samples (sites 2, 6, 7, 9, 10) and at considerably higher frequencies (> 12.5%, up to 37.5% at site 7; Table 1). The 100 mL samples gave detections at both sites where there are small populations of this species immediately upstream (site 2 and 6).

Litoria nannotis was detected at all but one site (site 9) using the 15 mL water samples. Detection rates were low at many sites using this method (< 10 % at six out of the nine sites with detections) but high at three sites (> 25%; Table 1). Interpretation of *L. nannotis* detection from the 100 mL samples (from the 375 mL water samples) is complicated by the lag between sample collection and eDNA extraction, which could have

reduced our ability to detect the species presence. The extraction and screening conducted immediately on return from the field was successful for sites 6, 7 and 8, yielding high detection frequencies (27–46%; Table 1), but was unsuccessful for the other seven sites due to failure of the real-time PCR instrument. Re-extraction from those water samples was conducted 5 months later and detection results appears to have been obviously impacted compared to results from sites 6–8 in the initial screening (Table 1). No detections were made at four of these seven sites, and the remaining three sites had low detection frequency ($< 10\%$; Table 1).

Detection results of particular note from the water sampling include the following. Accounting for the difference in time to DNA extraction in*L. nannotis* , detection from the 100 mL water samples was higher than the 15 mL water samples, particularly for the rarer species,*L. lorica* . Very small upstream populations of *L. lorica*(i.e., approx. 100 adults) were detected using the 100 mL water samples but not the 15 mL water samples (i.e., results for sites 2 and 6). The 100 mL samples gave detections of both species at most far downstream sites (i.e., 10–22.8 km), including detection of both species at the furthest downstream site (22.8 km) (Figure 1).

Comparing detection during high and low water flow

Downstream detection of *L. lorica* was considerably higher at all sites downstream of the main population during high flow (Figure 3; Table S3). Detection among the frogs at the main population (site 6) was similar in both flow conditions but differences are noticeable further downstream, with high flow detection being high at 10 km (37.5 % at site 7) and declining to 22.8 km (12.5 % at site 10). In comparison, there were no (site 7) or negligible (sites 9, 10) far downstream detections during low flow (Figure 3). Results for *L. nannotis*are complicated by the difference in time to eDNA extraction outlined above, which likely greatly reduced detection success at the furthest downstream sites (9 and 10) during high flow. Detection during high flow was high in the main population (44.4%, site 6) and 10 km downstream (45.8%, site 7) but then decreased to 0% at site 9 and near-zero at site 10 (Figure 3). Detection during low flow was very high at site 6 in main the population (78.3%) but declines to very low frequency detection ($< 3.3\%$) at the downstream sites (Figure 3; Table S3). Given the difference in time to eDNA extraction for *L. nannotis* high flow samples from sites 9 and 10, this plot also suggests higher detection during high flow conditions.

Detection rates and distances from water filtration

Filtration was only performed during high flow at the four downstream sites (7–10). *Litoria lorica* was detected at all three sites that it occurs upstream (7, 9, 10), with a clear decrease in detection rate with distance (Table 1). *Litoria nannotis* was detected at all four sites, once again with a general pattern of decreased detection with distance (Table 1). Detection rate for both species was considerably higher at these sites compared to the water collection methods, and included 100% detection for *L. lorica* at 10 km downstream (site 7), 91.6% detection for *L. nannotis* at site 8 (which is 10.5 km downstream from the lowest distribution of *L. nannotis* in that catchment) $> 50\%$ detection of both species at 17.1 km (site 9) and 17% and 25% detection of *L. lorica* and *L. nannotis* , respectively, at 22.8 km (site 10). The exception to these high detection rates was site 5, where neither*L. lorica* and *L. nannotis* were detected via filtration. This site is only 2.7 km downstream from the lower limit of both species in the upstream tributaries (Figure 1). No detections for *L. lorica* can be attributed to the small upstream population of only about 100 frogs but the lack of *L. nannotis* detections is unexpected because there are large upstream populations (C. Hoskin, unpub. data).

Inhibition tests

Mean Ct values for the two spiked field samples collected during high flow were not significantly different from the spiked MilliQ water (L1:$P = 0.32$; L3: $P = 0.49$; L8: $P = 0.1$) (Table S4), indicating that no inhibition was present in the qPCR assays. The field sample collected through water filtration during high flow showed significant differences in Ct values when compared to the spike MilliQ water (L8: $P = 0.039$); however, the difference in mean Ct values was only 0.6 cycles. The field sample collected during low flow also showed a significant difference in Ct value compared to MilliQ water (site 8: $P < 0.001$) but this difference was only 0.4 cycles (Table S4). A shift in Ct values of less than 1 cycle is not suggested to indicate sample

inhibition (Cao, Griffith, Dorevitch, & Weisberg, 2012; Hartman, Coyne, & Norwood, 2005), so we conclude no inhibition in the extracted eDNA from any sampling events.

DISCUSSION

Conservation of Critically Endangered amphibians is dependent on finding remnant populations (Gillespie et al., in press), which is a serious challenge in remote, rugged environments using traditional survey techniques. Conventional frog surveys consist of night-time spotlighting, which is time consuming and challenging in remote, montane areas. In any one day it is only possible to cover a small area in the catchment. In contrast, given long detection distances of fish and invertebrates, eDNA surveys can cover many downstream sites quickly, effectively surveying vast upstream areas. Environmental DNA has been used to detect stream-associated amphibians (Santas et al., 2013; Sasso et al., 2017; Spear et al., 2015) but the key question remains: how far downstream can small amphibian populations be detected?

In the present study, far downstream detection was reasonably consistent for large water samples and filtering, including both species being detected at the furthest site (22.8 km downstream) using both techniques. While detection of the small population of *L. lorica*(approximately 1,000 adults) was reasonable, downstream detection of very small populations of *L. lorica* (approximately 100 adults) was poor. The small populations could be detected immediately downstream of the 100 individuals (sites 2 and 6) but there was essentially no detection at the two sites 1.2 km and the 4 km, respectively, downstream of site 2. Our other major finding was that frog detection was generally better during high flow, and this is discussed below. The fact that we could detect both endangered frog species at sites $>$ 20 km downstream from the closest populations suggests eDNA surveys could revolutionize the way we conduct surveys for the hundreds of missing and critically endangered amphibian species.

Influence of eDNA capture method on species detectability

Water filtration is the most widely used method of eDNA sampling, given that it maximises the probability of capturing trace amounts of DNA that are not evenly distributed in the water (Eichmiller, Bajer, & Sorensen, 2014; Pilliod, Goldberg, Arkle, & Waits, 2013; Sepulveda et al., 2019). The amount of water processed by our large volume sampler was up to two orders of magnitude larger than the commonly used sample volumes for eDNA studies (Rees, Baker, Gardner, Maddison, & Gough, 2017), with the exception of Sepulveda et al. (2019). However, water filtration of such large volumes is a time-consuming activity and requires expensive gear that is not readily available. Additionally, carrying large amounts of gear is not practical for sites without vehicle access.

Filtering was highly successful for far downstream detection in our study. However, we only used this technique at the five sites with nearby road access; the other sites involve lengthy, challenging access on foot. Our filtering volumes were large, averaging about 1,500 L (Table S1). Although we had good detection downstream from the main*L. lorica* population, filtering 2.7 km downstream from the very small translocated population (at site 5) gave no detections (Table 1). Sepulveda et al. (2019) found that downstream detection of low density trout populations was dependent on filtering large volumes ($>$ 1,500 L). Similarly, Stoeckle, Kuehn, & Geist (2016) did not detect small oyster populations ($<$ 800 individuals) 0.5 km and 1 km from the population, and Pilliod, Goldberg, Arkle, & Waits (2014) could detect five caged giant salamanders 5 m downstream but not 50 m. In contrast, other filtering studies have detected five caged trout 200 m downstream (Jane et al., 2015) and one caged salmon 1 km downstream (Wood et al., 2020).

Rather than a real lack of detections, it is possible that our inability to detect the very small *L. lorica* population downstream could reflect inhibition. The fact that *L. nannotis* was also not detected in the filtered sample at site 5 supports this because there are large upstream populations of that species (Table 1; Figure 1). False negatives can arise from sample inhibition (Jane et al., 2015; Williams, Huyvaert, & Piaggio, 2017), which is a particular concern when filtering a very large volume of water because filters can concentrate a large amount of qPCR inhibitors. These inhibitors can be environmental compounds such as sediment, humic acids and phenolic compounds act as qPCR inhibitors (Wilson, 1997) and therefore vary greatly in each study system (Jane et al., 2015). The solution from our filtered samples were clearly high in

dissolved organic matter (Figure S1) and we had complete inhibition of undiluted samples, but no inhibition only after applying a 1:20 dilution.

Direct water collection and preservation was generally more successful for detection using the larger water samples (100 mL) than the smaller water samples (15 mL), particularly for the rarer species, *L. lorica* . Far downstream detection of *L. lorica* was incomparably better with the larger water samples (Table 1). For *L. nannotis* , this was complicated by differences in eDNA extraction time since sample collection for many of the 100 mL samples, but for samples screened immediately after field collection, detection was much higher using the larger water samples (sites 6–8; Table 1). This simply reflects the fact that eDNA was precipitated from more water in the larger samples. We conclude that screening for rare species is unreliable using 15 mL samples, and 100 mL samples (water sample plus preservative solution) are recommended. This volume of water is practical to carry in the field and precipitating eDNA from any volume larger than 100 mL becomes impractical in standard laboratory centrifuges.

Influence of water flow on eDNA downstream detection

We generally had higher frequencies of downstream detection during high flow conditions (Figure 3). We believe the difference relates directly to eDNA transport rather than temporal variation in target species' ecology or the amount of eDNA coming from the source. Our study species inhabit cascades/waterfalls all year and they do not move away from these discrete sections of suitable habitat (Puschendorf et al. 2011). Spear et al. (2015) found higher eDNA concentrations during the Hellbender salamander breeding season, possibly due to gamete release into the study streams. This is unlikely the cause of differences in our system because *L. lorica* and *L. nannotis* breeding primarily occurs between September and January (C. Hoskin, unpub. data), so there were probably many tadpoles in the stream at the time of the low flow sampling (late October) and few at the time of the high flow sampling (early April). Yet, detection was higher during high flow.

Other studies have also found higher percentage of downstream detection during high flow (e.g. Jane et al., 2015; Jerde et al., 2016; Shogren et al., 2017, 2018). Downstream detection probability is dependant on many factors associated with stream flow, including eDNA retention in the system, speed of eDNA transport, resuspension of eDNA previously retained in the system and bacterial degradation of eDNA (Barnes et al., 2016).

Environmental DNA can settle into the stream substrate and bind to sediment and biofilms on substrate surfaces (Fremier, Strickler, Parzych, Powers, & Goldberg, 2019; Jerde et al., 2016; Pedersen et al., 2015), limiting downstream transport from the source (Fremier et al., 2019; Jerde et al., 2016; Shogren et al., 2017). Therefore, slower flow can result in more passive retention of eDNA in the system (Fremier et al., 2019; Webster et al., 1987). Conversely, high flow results in fast particle transport and resuspension (Wipfli, Richardson, & Naiman, 2007), potentially transporting eDNA further downstream (Fremier et al., 2019; Webster et al., 1987). Additionally, bacterial biomass (and hence eDNA degradation) is likely higher during low flow (Ann, Freixa, Butturini, & Romanı, 2019; Proia, Schiller, Gutierrez, & Marcé, 2016) and low flow may increase exposure time to the damaging effects of UV and temperature (Pilliod et al., 2014; Strickler, Fremier, & Goldberg, 2015). Our better detection during high flow therefore likely reflects increased eDNA transport speed and more continuous suspension, and less degradation from bacteria, UV and higher temperatures.

Species-specific detection rates

We generally had considerably higher detection frequency for *L. nannotis* than *L. lorica* . The difference is obvious for the 15 mL samples and for the 100 mL samples screened directly after field collection (i.e., sites 6–8) (Table 1). Detection frequency was also higher for *L. nannotis* in the filtered samples from the three furthest downstream sites (Table 1). This reflects the much larger populations of *L. nannotis* in all tributaries (Figure 1). The two species are at similar abundance where they co-occur (C. Hoskin, unpub data) but *L. nannotis* is larger in size and it occurs in much more extensive areas of the catchment (totalling thousands of individuals). The review by Yates, Fraser, & Derry (2019) found a positive correlation between eDNA

concentration and population abundance. Although we did not measure eDNA concentration our detection frequency data supports this trend.

Implications for monitoring threatened species

Litoria lorica in our study system allowed us to test how small a target population size can be and still be detected at distances of kilometers. We could detect the small population of about 1,000 frogs (plus unknown number of tadpoles) at most of the downstream sites using the larger water samples and the filtering technique (including our furthest site at 22.8 km). In contrast, we had low success detecting the very small population 100 frogs (plus unknown number of tadpoles) at downstream sites. This suggests there may be a population size threshold for long distance eDNA detection using the techniques and replicate numbers we used.

From our results, we suggest the following methodology for detection of threatened stream frogs. Firstly, the method that best balances reliability and practicality is collecting moderate water volumes (e.g., 500 mL, including buffer) and precipitating eDNA from 100 mL subsamples. The smaller water volumes (15 mL) had limited detection and the filtering requires heavy ($>$ 5 kg) and specialist gear. Collecting replicates of 100 mL of water does not require training or special equipment and many bottles can be carried in a backpack. Secondly, we would suggest water collection during moderately high flow conditions. We did not test very high flow conditions in this system (which occur briefly following heavy rain) but we predict lower detection during these due to the dilution of eDNA in massive water volumes. Thirdly, we suggest screening in readily accessible downstream areas and then working upstream from there if: (i) a positive detection is made, or (ii) there is strong reason to believe a very small population remains undetected upstream (e.g., the last known historic site or an unconfirmed recent sighting). It is important to note that water filtration is recommended if a stream site is accessible (e.g. where there is a road crossing). However, based on our results, we can only recommend filtering $>$ 1,000 L of water. In our system, the next step will be screening downstream sites in all catchments where*L. lorica* was known to occur before disease-induced declines or likely occurred undetected pre-decline. Our results suggest this strategy would be valuable for detecting remnant amphibian populations, globally.

DATA ACCESSIBILITY

DNA sequences of *L. lorica* and *L. nannotis* forward and reverse primers are found under Table S2, Supplemental information. All other data available on request from the authors.

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AUTHORS CONTRIBUTIONS

C.J.H and D.B. conceived the project; C.V.R, C.J.H and D.B. designed the research; C.V.R and C.J.H performed the field work; C.V.R. led the laboratory work; C.V.R, C.J.H, D.B. and J.M.S. analyzed and interpreted the data; C.V.R and C.J.H wrote the draft, and all authors edited the manuscript.

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