CSIRO PUBLISHING

Wildlife Research, 2012, **39**, 629–636
http://dx.doi.org/10.1071/WR12114

An eDNA approach to detect eastern hellbenders (*Cryptobranchus a. alleganiensis*) using samples of water

Zachary H. Olson[A,C], *Jeffrey T. Briggler*[B] *and Rod N. Williams*[A]

[A]Department of Forestry & Natural Resources, Purdue University, West Lafayette, IN 47907, USA.
[B]Missouri Department of Conservation, PO Box 180, Jefferson City, MO 65102, USA.
[C]Corresponding author. Email: olson.z.h@gmail.com

Abstract

Context. Environmental DNA, or eDNA, methods are a novel application of non-invasive genetic sampling in which DNA from organisms is detected via sampling of water or soil, typically for the purposes of determining the presence or absence of an organism. eDNA methods have the potential to revolutionise the study of rare or endangered taxa.

Aims. We evaluated the efficacy of eDNA sampling to detect populations of an amphibian of conservation concern, the eastern hellbender (*Cryptobranchus a. alleganiensis*), indirectly from their aquatic environments.

Methods. We developed species-specific primers, validated their specificity and sensitivity, and assessed the utility of our methods *in silico* and in laboratory trials. In the field, we collected water samples from three sites with known densities of hellbenders, and from one site where hellbenders do not occur. We filtered water samples, extracted DNA from filters, and assayed the extraction products for hellbender DNA by using polymerase chain reaction (PCR) and gel electrophoresis.

Key results. Our methods detected hellbenders at densities approaching the lowest of reported natural densities. The low-density site (0.16 hellbenders per 100 m^2) yielded two positive amplifications, the medium-density site (0.38 hellbenders per 100 m^2) yielded eight positive amplifications, and the high-density site (0.88 hellbenders per 100 m^2) yielded 10 positive amplifications. The apparent relationship between density and detection was obfuscated when river discharge was considered. There was no amplification in any negative control.

Conclusion. eDNA methods may represent a cost-effective means by which to establish broad-scale patterns of occupancy for hellbenders.

Implications. eDNA can be considered a valuable tool for detecting many species that are otherwise difficult to study.

Additional keywords: density, detection, DNA-based, monitoring, non-invasive, occupancy, presence.

Received 26 June 2012, accepted 15 August 2012, published online 17 September 2012

Introduction

Non-invasive genetic sampling is an appealing framework for the study of elusive, secretive and rare taxa because the individuals under study do not need to be located physically (Waits and Paetkau 2005), and a variety of information can be garnered that otherwise would be very difficult to obtain. For example, non-invasive genetic sampling has been used in the context of conservation and management to describe population sizes (Solberg *et al*. 2006; Sawaya *et al*. 2012), dispersal (Valière *et al*. 2003), survival (Marucco *et al*. 2009) and parentage (Constable *et al*. 2001) among other population-level metrics (see reviews by Taberlet and Luikart 1999; Waits and Paetkau 2005; Beja-Pereira *et al*. 2009). The utility of non-invasive genetic sampling hinges in part on the availability of a suitable substrate from which an animal's DNA can be isolated. Genetic material is often obtained indirectly from target species by collecting hair or faeces (Waits and Paetkau 2005). However, other substrates such as urine in snow (Hausknecht *et al*. 2007) and shed feathers (Rudnick *et al*. 2005) have also been used to obtain DNA. Many non-invasive genetic studies have focussed on charismatic mega-fauna such as wolves (*Canis lupus*; Sastre *et al*. 2009), grizzly bears (*Ursus arctos*; Kendall *et al*. 2008) and bighorn sheep (*Ovis canadensis*; Epps *et al*. 2005). Far fewer studies have focussed on non-invasive approaches to sample herpetofauna.

The lack of non-invasive genetic approaches to study amphibian species in particular is surprising, considering their importance in terrestrial and aquatic food webs (Blaustein *et al*. 1994), their potential role as indicator species (Welsh and Ollivier 1998) and the conservation status of many amphibian populations worldwide. Over 42% of amphibians (2030 of 4727 species) for which sufficient data exist are listed as threatened according to International Union for the Conservation of Nature (IUCN) Red List criteria (IUCN 2008). It is therefore of paramount importance that non-invasive techniques be developed and validated for these vulnerable taxa (Waits

2004). We have selected the eastern hellbender (*Cryptobranchus a. alleganiensis*) to evaluate non-invasive genetic methods because aspects of its natural history make it well suited to serve as an amphibian model.

The eastern hellbender is a large, aquatic salamander that historically occurred throughout much of the eastern United States (Phillips and Humphries 2005). Hellbenders occupy cool, clear and well oxygenated streams and rivers and feed primarily on crayfish (Decopoda). Adults are long-lived (over 20 years in the wild), yet hellbender populations throughout much of the mid-western United States suffer from extremely low reproduction, with declining populations being skewed towards adult age-classes (Wheeler *et al*. 2003; Burgmeier *et al*. 2011*b*). As a consequence of these declines, hellbenders are listed as threatened or endangered in nearly every state in which they occur. Although the ultimate cause of the declines is unknown, factors such as habitat degradation (Nickerson and Mays 1973; Williams *et al*. 1981), collection for scientific investigations or for the pet trade (Nickerson and Briggler 2007), and angling mortality (Kern 1984; Olson *et al*. 2013) have undoubtedly affected hellbenders. Because of their sensitivity to environmental degradation, hellbender populations are often seen as important indicators of water quality and the overall health of aquatic ecosystems.

The most effective method currently available to monitor hellbender populations involves teams of biologists flipping large shelter or nest rocks in streams and rivers (Browne *et al*. 2011). Not only is this work extremely labour-intensive, time-consuming and logistically challenging, but it is also invasive. For example, lifting large rocks is potentially dangerous for both the researchers and the hellbenders (e.g. hellbenders can be injured or killed during such sampling). Further, although great care is taken to preserve the original orientation of each shelter rock, the micro-environment under that rock is altered during sampling (Burgmeier *et al*. 2011*a*). Anecdotal evidence suggests that disturbing nest or shelter rocks can result in abandonment for several years (J. Briggler, unpubl. data). Thus, there is a critical need for a methodological advance to facilitate the non-invasive detection of hellbenders.

We evaluated a novel application of non-invasive genetic sampling methods that takes advantage of the hellbender's aquatic environment to facilitate their detection. The use of environmental DNA (or eDNA) to non-invasively survey for species is a recent development in non-invasive genetics. The adaptation of non-invasive genetic methods to detect DNA in water sampled directly from the environment originated in marine studies that used sloughed skin cells to genetically identify individual whales from ocean water (e.g. Valsecchi *et al*. 1998). In contrast to more traditional non-invasive genetics studies where epithelial cells are targeted from a known substrate such as hair or faeces, eDNA methods require blind sampling of an environmental medium such as soil or water to retrieve DNA. Ficetola *et al*. (2008) provided the first validation of eDNA methods, and used the new techniques to document the occurrence of bullfrogs in ponds across France. Importantly, Jerde *et al*. (2011) described a methodological advance by validating eDNA approaches for lotic (i.e. flowing water) systems to investigate the distribution of invasive fish species in a network of rivers and canals in Chicago, IL, USA.

The goal of our study was to assess the efficacy of eDNA sampling methods for use in the context of potential broad-scale occupancy studies of eastern hellbender populations. Our objectives were to (1) develop species-specific primers and test their sensitivity and specificity, and (2) determine the relationship between hellbender population density, flow rate of the river and measures of detection. Although our methods are specific to hellbenders, we hope that the present study will provide a useful framework for the development of eDNA methods across a wide variety of taxa.

Materials and methods

Primer selection and evaluation

We used Primer3 software (Rosen and Skaletsky 2000) to develop a suite of primer pairs in the hellbender mitochondrial Cytochrome-*b* region. We restricted the search to primer locations that would produce fragments <150 base pairs (bp) in length (i.e. smaller fragments are less susceptible to degradation; Deagle *et al*. 2006), and we eliminated any primers the program returned that targeted annealing sites known to contain polymorphisms in the hellbender genome. Known polymorphisms were identified from haplotypes resolved in previous genetic studies (Sabatino and Routman 2009). We used the search option primerBLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast, verified 28 August 2012) in the National Center for Biotechnology Information (NCBI) nucleotide database to further parse the suite of primers identified by Primer3 down to those without potential non-specific targets.

Primer pairs were then optimised in the laboratory by using high-quality, tissue-extracted hellbender DNA at concentrations of $20\,\mathrm{ng\,uL^{-1}}$ (quantitated using a Nanodrop 8000, Thermo Fischer Scientific, Wilmington, DE, USA). Further adjustments to reaction concentrations and thermocycler profiles were made on the basis of results from 10-fold serial dilutions of hellbender DNA, ranging from $20\,\mathrm{ng\,uL^{-1}}$ down to $2 \times 10^{-8}\,\mathrm{ng\,uL^{-1}}$. Finally, the best subset of primers was tested against DNA collected as part of a different study from eight non-target species at concentrations of $20\,\mathrm{ng\,\mu L^{-1}}$ (fish: mottled sculpin, *Cottus bairdii*; green sunfish, *Lepomis cyanellus*; rainbow darter, *Etheostoma caeruleum*; northern hogsucker, *Hypentelium nigricans*; central stoneroller, *Campostoma anomalum*; largemouth bass, *Micropterus salmoides*; smallmouth bass, *Micropterus dolomieu*; amphibian: mudpuppy, *Necturus maculosus*). These non-target species were selected because they are common sympatric species in hellbender habitats. The primer pair that most reliably amplified eastern hellbender mtDNA, was most sensitive in serial dilution trials and failed to amplify target fragment lengths from non-target species was advanced for use in the remainder of the study.

Laboratory trials

Mitochondrial DNA (mtDNA) is present in eukaryotic cells in copy numbers at least several orders of magnitude greater than is nuclear DNA (Birky *et al*. 1989). We expected to recover very low abundances of cells from environment samples, and we hypothesised that mitochondrial primers would be more

sensitive (and thus preferable for eDNA studies) because their target should exist at higher copy numbers when compared with primers targeting the nuclear genome (Waits and Paetkau 2005). We tested this hypothesis by using a multiplex of three hellbender microsatellite loci (Call127, Call171 and Call351; loci and conditions in Unger *et al*. 2010) against the 10-fold serial dilution above, and qualitatively compared their amplification success with that of the final mtDNA primers.

Prior to field sampling, we conducted a laboratory trial of our methods by sampling water from a 110-L fish tank containing a hellbender initially captured in Indiana. The hellbender was placed in the tank 2 weeks before sampling, and we changed 20% of the tank water daily to maintain husbandry standards. We then collected three separate samples (2, 4 and 8 L) of water from the tank, and used an additional negative control sample consisting of 2 L of autoclaved water from our laboratory. We otherwise followed field sampling protocols exactly (see below) to filter the samples and extract DNA. We assayed the four samples by scoring 12 polymerase chain reactions (PCRs) per filter: 10 reactions incorporating the sample, one negative reaction in which we added water in place of the template, and one positive reaction into which we added 20 ng of hellbender DNA as below. We used the tank samples to evaluate the utility of the following three polymerases: MyTaq (BioLine, Taunton, MA, USA), NEB Taq (New England BioLabs, Ipswich, MA, USA), and ApliTaq Gold (Applied Biosystems, Foster City, CA, USA) by quantifying the number of positive reactions per sample for each polymerase.

Field sampling

Although population densities of eastern hellbenders are not well known, several states have surveyed their eastern hellbender populations extensively. Among these, Indiana and Missouri have recent survey data that were used in the present study. Hellbender habitat is not ubiquitous in streams (Nickerson and Mays 1973; Humphries and Pauley 2005), and we refer to the discrete sections of river with known hellbender habitat as 'sites'. We sampled one site in Indiana (site dimensions: 600 m × 20 m) and two sites in different rivers in Missouri (Missouri 1–200 m × 32 m; Missouri 2–200 m × 12 m; see Table 1). We do not report the location of our sites because illegal collection has been indicted in the decline of some hellbender populations (Nickerson and Briggler 2007).

Abundance of hellbenders was determined at each site using a combination of snorkeling, rock lifting, and visual searches (Browne *et al*. 2011) during annual sampling from 2008 to 2011 in Indiana, from 2006 to 2011 at Missouri 1, and from 2004 to 2010 at Missouri 2 (Table 1). The data from these searches should be considered the best-available estimates of the number of hellbenders at each site. We calculated the density of hellbenders at each site (as hellbenders per 100 m^2) by dividing the number of hellbenders in the site by the area of the site (i.e. site length × wetted width/100). We also recorded stream flow (in $m^3 s^{-1}$) at the date of sampling, using the nearest United States Geological Survey gauging station to determine the potential effects of water volume on our methods. Sites in Missouri represented higher-density populations, and the site within Indiana represented low population densities for hellbenders (Burgmeier *et al*. 2011*b*; Table 1). We sampled sites during October–December to capitalise on relatively low water levels, which we assumed would increase our probability of detecting hellbenders.

At each site, we filled 30–40 autoclaved 2-L polypropylene screw-top bottles (Dynalab Corporation, Rochester, NY, USA) with water by submerging open bottles 1–2 inches below the surface by using gloved hands. Sampling was conducted 50 m downstream from the end of each site and was centred at the

Table 1. Results of eDNA field sampling to detect hellbenders from water samples

Site characteristics and positive amplifications from filters that received four 2-L bottles of sample water each. Positive amplifications are recorded for each filter, as the number of positive PCR reactions of the 10 replicate PCR reactions conducted per filter. Indiana was sampled 17 October 2011, the negative control was sampled 18 November 2011, and Missouri 1 and 2 were sampled 8 December 2011. For the negative control, discharge was unknown, but lower than that for the experimental sites

Parameter	Site			
	Indiana	Missouri 1	Missouri 2	Negative control
Hellbender density (individuals per 100 m^2)	0.16	0.38	0.88	0
Discharge ($m^3 s^{-1}$)	0.74	54.37	13.20	–
Filter				
1	1/10	0/10	2/10	0/10
2	0/10	1/10	1/10	0/10
3	0/10	0/10	1/10	0/10
4	0/10	0/10	0/10	0/10
5	0/10	1/10	1/10	0/10
6	0/10	0/10	1/10	0/10
7	1/10	0/10	1/10	0/10
8	0/10	0/10	0/10	0/10
9	0/10	0/10	0/10	0/10
10	–	6/10	3/10	0/10
Total positive reactions	2	8	10	0
Average positives per filter (s.e.)	0.22 (0.15)	0.80 (0.59)	1.0 (0.30)	–
No. of positive filters	2	3	7	0

deepest point in the river. Filled bottles were placed on ice in large plastic containers until their contents could be filtered (i.e. approximately 1–6 h) so as to slow any DNA degradation. We applied 8 L (i.e. four bottles) of the sampled water to a sterile 1.5-µm pore-size fibreglass filter (Whatman, Piscataway, NJ, USA) by using sterile filter cups (Nalgene, Rochester, NY, USA) on a 4-L glass filter flask (i.e. four bottles through each filter). We determined in preliminary filtration attempts that 8 L was near the maximum amount of water that a filter could support before failure (data not shown). Filters were removed from filter cups and placed into a 5-mL screw-cap tube by using flame-sterilised forceps, were transported on ice, and were then stored at −20°C for <1 week until DNA could be extracted.

Filtration occurred in areas where no hellbender DNA had ever been amplified, and we changed gloves between each filter. DNA was extracted from each filter within a sterile laminar-flow hood by using the PowerWater DNA isolation kit (MO BIO Laboratories, Carlsbad, CA, USA) following manufacturer instructions, except that we performed final elutions using 50 µL of nanopure water (c.f. Jerde *et al*. 2011). Extracted samples were then sealed and moved to a laboratory in which no hellbender DNA had ever been amplified, first for storage at −20°C and then to take aliquots for PCR reaction mixtures. For each sample, we prepared 12 reactions across one row of a 96-well PCR plate, including 10 replicate PCR reactions incorporating the sample being tested, one negative control reaction (i.e. water added) and one positive control reaction (i.e. 20 ng of tissue-extracted hellbender DNA added later; see below). After the sample and PCR reaction mixtures were dispensed into each well, the wells were sealed with a bleach-sterilised PCR mat, and the reactions were moved to a laboratory dedicated to DNA amplification. In this laboratory, only the positive control well was unsealed to add 20 ng of hellbender DNA before the reactions were placed in a thermocycler. After amplification, we visualised 3 µL of each PCR product by using electrophoresis (100 V for 40–60 min) in a 2% agarose gel stained with ethidium bromide. Size standards (50-bp ladder) were included in each gel to verify fragment sizes.

We used amplification data from field samples to assess the power of our methods to detect hellbenders on the basis of a sample of filters. To determine the number of filters needed to achieve a certain probability of receiving a false-negative result (i.e. the probability of failing to produce ≥1 amplification from 10 reactions per filter when hellbenders are present), we solved the following equation:

$$y_{i,j} = \prod_{i=1}^{j}(1 - \hat{d}),$$

where y is the probability of a false negative (in this case, we solved for $y = 0.05$, or a 5% chance of receiving a false negative), j is the number of filters to be assayed, and $\hat{d}$ was the proportion of filters with ≥1 positive amplification from our data at each site (see Smyser *et al*. 2010).

Quality assurance and quality control

Because contamination is a concern in non-invasive genetic studies in general (Taberlet *et al*. 1996), and in eDNA studies specifically (Darling and Mahon 2011), we incorporated ‘equipment blank’ bottles and ‘cooler blank’ bottles as negative controls for each sampled field site in the study (c.f. Jerde *et al*. 2011). Before filtering sampled water from the field site, we first filtered 4 2-L equipment blank bottles that contained autoclaved ultrapure water from our laboratory. Second, we filtered 4 2-L cooler blank bottles (autoclaved ultrapure water from our laboratory) that were treated identically to field-sampling bottles, with the exception that they were not opened at the field sites. These negative control filters were extracted and tested via PCR (identical conditions) along with the sample filters to allow us to identify any equipment or background contamination, respectively. We also sampled an additional field site (40°25′52.9″N, 87°2′15.6″W) in Indiana in a stream several hundred kilometres outside of the historic or current range of hellbenders (Minton 2001) as a study-wide negative control. This site was sampled using methods identical to the positive field sites. Finally, to directly combat contamination, all equipment potentially contacting site water was sterilised using a 10-min exposure to 10% bleach solution before and after sampling.

To verify that any positive bands from field sites were in fact hellbender DNA, we sequenced a subset of PCR products from the field samples. PCR fragments were found to be too short to provide reliable sequences via Sanger sequencing during preliminary attempts (i.e. reads were not scoreable until near the end of the fragment). Additionally, cleaning the 72-bp fragments for sequencing often resulted in sample loss, because the fragments could pass through spin-column media or into suspension in ethanol due to their size and be discarded. Thus, we used a kit (pGem-T Vector, Promega Corporation, Madison, WI, USA) to clone fragments into competent cells (JM109 cells from Promega), following manufacturer instructions. We then used universal primers T7 and SP6 to amplify our target fragment from within the cloned plasmids by using colony PCR.

We picked five well isolated colonies from each of two replicate plates prepared for each fragment. We touched a sterile toothpick to a colony, and then submerged and agitated the end of the toothpick directly in a PCR reaction mixture. We used 20-µL reaction volumes consisting of 0.25 mM of each primer (T7 and SP6), 0.2 mM of each dNTP, 1 mM $MgCl_2$, 0.5 units *Taq*, and 1× reaction buffer. The thermocycler profile included an initial denature step of 95°C for 4 min, 35 cycles of 95°C for 30 s, 52°C for 30 s, and 72°C for 30 s, and a final extension step of 72°C for 10 min.

Colony PCR products were cleaned by precipitating the DNA with a cold ethanol solution (0.12 mM NaOAc in 100% ethanol), centrifuging to form a pellet, washing the pellet with 70% ethanol and re-suspending the cleaned product in water. We then cycle-sequenced ~10 ng of cleaned PCR product in 10-µL reaction volumes containing 5 pmol of SP6 primer, 1 µL Big Dye Terminator version 3.1 (Applied Biosystems) and 3 µL of 5× buffer (Applied Biosystems). The sequencing-reaction profile was as follows: 98°C for 5 min, followed by 35 cycles of 98°C for 30 s, 50°C for 15 s, and 60°C for 2 min. Sequences were cleaned using the same sodium-acetate protocol as above or by using a kit (DyeEx 2.0, Qiagen, Germantown, MD, USA) before being rehydrated in 30 µL of sterile water. We submitted 15 µL of

each sequence to the Purdue Genomics Core Facility where they were run on an ABI 3730xl automated DNA sequencer (Applied Biosystems). We trimmed and edited the resulting sequences using Sequencher version 4.1 (GeneCodes Corporation, Ann Arbor, MI, USA). Finally, we determined the likely origin of the sequences visually by assessing their alignment with expected eastern hellbender sequences, and by conducting BLAST searches for similar sequences in the NCBI nucleotide database. We note that researchers could also verify the sequences of positive bands using next-generation sequencing technologies (see Ficetola *et al*. 2008) rather than the protocols we report here.

Results

Primer selection and evaluation

We evaluated 10 primer pairs in pre-sampling trials. The primer pair that performed best, and that we used in all subsequent trials, was CRAL1-F: 5′-TCAATTGCCCATATCTGCCGA-3′, CRAL1-R: 5′-AGAGGCTCCGTTTGCATGAGT-3′. PCRs were conducted in 10-μL volumes consisting of 0.25 mM of each primer, 0.2 mM of each dNTP, 1 mM $MgCl_2$, 0.5 units *Taq* and 1 μL of extraction product in 1× reaction buffer (BioLine). The final thermocycler profile included an initial denature step of 95°C for 5 min, then eight touchdown cycles of 95°C for 30 s, 62°C for 30 s (annealing step decreasing by one degree each cycle) and 72°C for 30 s. The touchdown cycles were followed by 32 additional cycles of denaturing at 95°C for 30 s, annealing at 58°C for 30 s and extension at 72°C for 30 s, with a final extension step of 72°C for 10 min. Using these reaction specifications, we amplified hellbender DNA during serial-dilution trials from DNA concentrations as low as $\sim 2.0 \times 10^{-6}$ ng μL^{-1}, and did not amplify non-target DNA.

Laboratory trials

Using the multiplex of nuclear microsatellite markers, we successfully amplified hellbender DNA from concentrations as low as 2 ng μL^{-1}. This represented a sensitivity of six magnitudes of order lower than what was achieved with the mitochondrial primer set, and indicated that primers targeting sequences in the mitochondrial genome will find more utility than those targeting the nuclear genome for future eDNA studies focusing on detecting organisms. Thus, for the remainder of the study, we report results from attempts to amplify mtDNA. The average number of positive reactions per filter (±s.e.) from the three tank-test filters was 9.33 ± 0.67 of 10 possible for My*Taq*, 7.00 ± 0.00 for Ampli*Taq* Gold and 8.67 ± 0.67 for NEB *Taq*. No amplification was evident for any polymerase when assayed against the negative control filter. From these preliminary data, we concluded that our methods would allow the detection of hellbenders if they were to occur at very high densities (one hellbender per 110-L aquarium), and we proceeded to use My*Taq* in the field trials to determine whether our methods would also find utility under natural conditions.

Field sampling

We amplified fragments of the predicted size (72 bp) from each of the three field sites with known densities of hellbenders (Table 1). The number of positive reactions, the average positive reactions per filter, and the number of filters with ≥1 positive reaction appeared to increase with increasing density of hellbenders at each site (Table 1). This pattern was no longer evident when the volume of water flowing over each site was considered (Table 1). We estimated that 12 filters (i.e. 48 2-L bottles) would need to be assayed using the methods presented in the current paper to reduce the probability of obtaining false-negative results to <5%, given hellbender densities similar to our low-density site in Indiana (0.16 hellbenders per 100 m^2; Fig. 1). The number of filters needed to achieve the same power decreased when sites with higher hellbender densities were considered (Fig. 1).

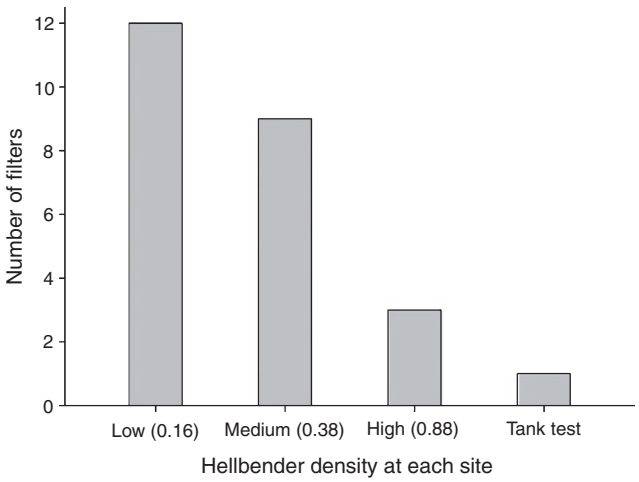

Fig. 1. Results of a power analysis, indicating the number of filters to be assayed given a certain density of hellbenders at a site, so as to reduce the probability of obtaining false-negative results (no filters with ≥1 positive amplification) to <5%. Densities of hellbenders at sampled sites are indicated in parentheses, and refer to the number of hellbenders per 100 m^2. The tank test refers to results obtained by collecting multiple samples from a 110-L aquarium containing a hellbender, this being a much higher 'density' than would occur in nature.

Quality assurance and quality control

No amplification was evident from equipment-blank or cooler-blank filters from any site. The negative-control site yielded no positive amplifications (i.e. 0 of 10 filters had positive amplifications). We sequenced fragments from five positive reactions from the field sites, including two from Indiana, one from Missouri 1 and two from Missouri 2 (Table 1). BLAST results for fragments from each site returned 100% positive matches (E-value 7.0×10^{-29}) to other archived hellbender sequences, whereas the next-nearest match was a salamander species endemic to Italy (*Salamandrina perspicilatta*; 86% match; E-value 6×10^{-20}). We truncated one of the two sequences from Missouri 2 because of poor read quality; however, it still aligned best with hellbender sequences (E-value 0.014), whereas the next-closest species was a lizard from Papua New Guinea (*Lygisaurus curtus*; E-value 0.056). Thus, we are confident that the positive amplifications in our study were in fact hellbender DNA.

Discussion

The eDNA sampling protocol we adopted involved four main steps after an initial effort of selecting primers. First, water was sampled from a pre-determined location at the thalweg of a stream, and then vacuum-pumped through micro-pore filters to capture any cells (or particle-bound DNA) suspended in the water column. Second, the filters were subjected to a DNA-extraction protocol to release DNA from the filters and from inside any captured cells. Third, products of the extraction step were subjected to PCR, using species-specific primers that amplified only pre-determined sequences of mtDNA. Finally, PCR products were visualised using traditional gel electrophoresis, and a small subset of positive amplifications were sequenced to verify their origin. We expect that as these methods become more refined, eDNA will have the potential to revolutionise non-invasive sampling for rare, elusive or endangered species.

Using eDNA methods optimised specifically for an amphibian species of conservation concern, we were able to detect the species from samples of river water even where the organism occurred at very low densities (Burgmeier *et al*. 2011*b*). Interestingly, measures of amplification success at each site seemed to increase along with the density of hellbenders at that site, although we surveyed too few sites to evaluate this relationship in more detail. The population density of organisms under investigation using eDNA methods has previously been shown to affect amplification success in mesocosms and wetland systems (Ficetola *et al*. 2008). However, Goldberg *et al*. (2011) reported no relationship between their measure of amplification success and the density of Rocky Mountain tailed frogs (*Ascaphus montanus*), in a stream in Idaho, USA. These authors also achieved 100% amplification success among trials involving a second amphibian species that varied in density across sites within one stream (Goldberg *et al*. 2011). Thus, the relationship between amplification success and the density of the population under study may not always be clear, particularly in lotic systems.

Several factors are likely to confound the relationship between density and amplification success in eDNA studies. First, persistence of DNA in the water column, a function of the rates of production and degradation of DNA either as cell- or particle-bound molecules (Pietramellara *et al*. 2009; Dejean *et al*. 2011; Taberlet *et al*. 2012), may lead to the detection of organisms from some distance upstream of the intended sampling location in lotic systems. Conversely, the persistence of DNA in lentic systems is expected to translate to a length of time (rather than a distance) a species may be detected after it is no longer present (Thomsen *et al*. 2012). Dejean *et al*. (2011) found overall persistence times of 25 and 14 days, respectively, for eDNA from two species in separate removal experiments in lentic systems. In addition to some degree of variability associated with the persistence of DNA in water, we also expect that different stream morphologies will affect the distance suspended molecules will travel in lotic systems via different rates of suspension and deposition (Howard 1994). These uncertainties contribute complexity to any expectation regarding our ability to detect organisms at distances above sampling sites using eDNA sampling methods. However, specifically for hellbenders, our methods should provide an efficient means by which to establish broad-scale patterns (i.e. watershed level) of site occupancy for a species that is otherwise very labour-intensive to study.

Because our sites were located in different rivers, we were able to collect a limited amount of data with which to assess the effects of flow rate on the relationship between density and detection probability. Flow rate has been cited as a factor affecting detection probabilities in eDNA studies (Goldberg *et al*. 2011; Jerde *et al*. 2011); however, no data exist regarding the magnitude of its effects. Taking into account river discharge (in $m^3 s^{-1}$) on the day we sampled, the relationship between density and amplification success was less clear. The Missouri 1 site occupied the middle position among our three sites, in terms of density and amplification success, but was located in a substantially larger river with a discharge four times higher than that of the river containing the higher-density site, Missouri 2 (Table 1). Notably, variation (s.e.) around the average positive reactions per filter for Missouri 1 was nearly twice that for Missouri 2, and nearly four times that of our low-density, low-discharge site in Indiana. More data points will be necessary to determine whether greater variation in amplification success (and detection probability) among samples can be expected in rivers with greater discharge. We also intentionally sampled at a time of year when we expected low water levels to increase our detection rates. Although our study was not designed to test this assumption, establishing any seasonal patterns in detection related to flow or animal-movement behaviour would be an interesting area for future research.

The benefits of eDNA sampling over more traditional methods will depend, in large part, on the species and questions of interest. Because the risk of contamination is high in eDNA studies (Darling and Mahon 2011), two separate laboratories are needed to separate extraction from amplification in the workflow. Although our methods required very little in the way of specialised equipment or training, eDNA methods such as ours probably require more space than do traditional sampling methods. The greatest logistical benefit of eDNA sampling in our study system was savings associated with person-hours. A single researcher can collect and filter water, whereas teams of biologists must be assembled to safely sample hellbender habitats via rock-lifting (e.g. Burgmeier *et al*. 2011*b*). In addition, eDNA sampling eliminates contact with the study organism altogether, which limits stress to the animals associated with capture (Putman 1995) and mitigates the growing potential of transporting emerging diseases (Berger *et al*. 1998; Lips *et al*. 2006) among populations. Therefore, although not without difficulties, we expect that eDNA sampling will find utility for researchers interested in a variety of species. In particular, the eastern hellbender served as a good model for those species that occur at low densities or are of particular conservation concern.

Acknowledgements

This study was funded by a grant (the Christine Stevens Wildlife Award) from the Animal Welfare Institute, and was supported by the Department of Forestry and Natural Resources at Purdue University. Animal husbandry considerations were approved by the Purdue Animal Care and Use Committee under protocol #08-025-11. We thank the Williams Laboratory for helpful

comments on an earlier draft of this manuscript, E. Routman for providing sequences of hellbender haplotypes, and the Goforth Laboratory for providing the non-target samples.

References

Beja-Pereira, A., Oliveira, R., Alves, P. C., Schwartz, M. K., and Luikart, G. (2009). Advancing ecological understandings through technological transformations in noninvasive genetics. *Molecular Ecology Resources* **9**, 1279–1301. doi:10.1111/j.1755-0998.2009.02699.x

Berger, L., Speare, R., Daszak, P., Green, D. E., Cunningham, A. A., Goggin, C. L., Slocombe, R., Ragan, M. A., Hyati, A. D., McDonald, K. R., Hines, H. B., Lips, K. R., Marantelli, G., and Parkes, H. (1998). Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and central America. *Proceedings of the National Academy of Sciences, USA* **95**, 9031–9036. doi:10.1073/pnas.95.15.9031

Birky, C. W. Jr, Fuerst, P., and Maruyama, T. (1989). Organelle gene diversity under migration, mutation, and drift: equilibrium expectations, approach to equilibrium, effects of heteroplasmic cells, and comparison to nuclear genes. *Genetics* **121**, 613–627.

Blaustein, A. R., Wake, D. B., and Sousa, W. P. (1994). Amphibian declines: judging stability, persistence, and susceptibility of populations to local and global extinctions. *Conservation Biology* **8**, 60–71. doi:10.1046/j.1523-1739.1994.08010060.x

Browne, R. K., Li, H., Mcginnity, D., Okada, S., Zhenghuan, W., Bodinof, C. M., Irwin, K. J., McMillan, A., and Briggler, J. T. (2011). Survey techniques for giant salamanders and other aquatic Caudata. *Amphibian & Reptile Conservation* **5**, 1–16.

Burgmeier, N. G., Sutton, T. M., and Williams, R. N. (2011*a*). Spatial ecology of the eastern hellbender (*Cryptobranchus alleganiensis alleganiensis*) in Indiana. *Herpetologica* **67**, 135–145. doi:10.1655/HERPETOLOGICA-D-10-00027.1

Burgmeier, N. G., Unger, S. D., Sutton, T. M., and Williams, R. N. (2011*b*). Population status of the eastern hellbender (*Cryptobranchus alleganiensis alleganiensis*) in Indiana. *Journal of Herpetology* **45**, 195–201. doi:10.1670/10-094.1

Constable, J. L., Ashley, M. V., Goodall, J., and Pusey, A. E. (2001). Noninvasive paternity assignment in Gombe chimpanzees. *Molecular Ecology* **10**, 1279–1300. doi:10.1046/j.1365-294X.2001.01262.x

Darling, J. A., and Mahon, A. R. (2011). From molecules to management: adopting DNA-based methods for monitoring biological invasions in aquatic environments. *Environmental Research* **111**, 978–988. doi:10.1016/j.envres.2011.02.001

Deagle, B. E., Eveson, J. P., and Jarman, S. N. (2006). Quantification of damage in DNA recovered from highly degraded samples – a case study on DNA in faeces. *Frontiers in Zoology* **3**, 11. doi:10.1186/1742-9994-3-11

Dejean, T., Valentini, A., Duparc, A., Pellier-Cuit, S., Pompanon, F., Taberlet, P., and Miaud, C. (2011). Persistence of environmental DNA in freshwater ecosystems. *PLoS ONE* **6**, e23398. doi:10.1371/journal.pone.0023398

Epps, C. W., Palsbøll, P. J., Weyhausen, J. D., Roderick, G. K., Ramey, R. R. II, and McCullough, D. R. (2005). Highways block gene flow and cause a rapid decline in genetic diversity of desert bighorn sheep. *Ecology Letters* **8**, 1029–1038. doi:10.1111/j.1461-0248.2005.00804.x

Ficetola, G. F., Miaud, C., Pompanon, F., and Taberlet, P. (2008). Species detection using environmental DNA from water samples. *Biology Letters* **4**, 423–425. doi:10.1098/rsbl.2008.0118

Goldberg, C. S., Pilliod, D. S., Arkle, R. S., and Waits, L. P. (2011). Molecular detection of vertebrates in stream water: a demonstration using Rocky Mountain tailed frogs and Idaho giant salamanders. *PLoS ONE* **6**, e22746. doi:10.1371/journal.pone.0022746

Hausknecht, R., Gula, R., Pirga, B., and Kuehn, R. (2007). Urine – a source for noninvasive genetic monitoring in wildlife. *Molecular Ecology Notes* **7**, 208–212. doi:10.1111/j.1471-8286.2006.01622.x

Howard, A. (1994). A detachment-limited model of drainage basin evolution. *Water Resources Research* **30**, 2261–2285. doi:10.1029/94WR00757

Humphries, W. J., and Pauley, T. K. (2005). Life history of the hellbender, *Cryptobranchus alleganiensis*, in a West Virginia stream. *American Midland Naturalist* **154**, 135–142. doi:10.1674/0003-0031(2005)154[0135:LHOTHC]2.0.CO;2

IUCN, Conservation International, and NatureServe (2008). 'An Analysis of Amphibians on the 2008 IUCN Red List.' Available at http://www.iucnredlist.org/amphibians [verified 30 May 2012].

Jerde, C. L., Mahon, A. R., Chadderton, W. L., and Lodge, D. M. (2011). 'Sight-unseen' detection of rare aquatic species using environmental DNA. *Conservation Letters* **4**, 150–157. doi:10.1111/j.1755-263X.2010.00158.x

Kendall, K. C., Stetz, J. B., Roon, D. A., Waits, L. P., Boulanger, J. B., and Paetkau, D. (2008). Grizzly bear density in Glacier National Park, Montana. *The Journal of Wildlife Management* **72**, 1693–1705. doi:10.2193/2008-007

Kern, W. H. (1984). The hellbender, *C. alleganiensis* in Indiana. M.Sc. Thesis, Indiana State University, Terre Haute, IN.

Lips, K. R., Brem, F., Brenes, R., Reeve, J. D., Alford, R. A., Voyles, J., Carey, C., Livo, L., Pessier, A. P., and Collins, J. P. (2006). Emerging infectious disease and the loss of biodiversity in a Neotropical amphibian community. *Proceedings of the National Academy of Sciences, USA* **103**, 3165–3170. doi:10.1073/pnas.0506889103

Marucco, F., Pletscher, D. H., Boitani, L., Schwartz, M. K., Pilgrim, K. L., and Lebreton, J.-D. (2009). Wolf survival and population trend using non-invasive capture-recapture techniques in the western Alps. *Journal of Applied Ecology* **46**, 1003–1010. doi:10.1111/j.1365-2664.2009.01696.x

Minton, S. A., Jr (2001). 'Amphibians and Reptiles of Indiana.' (Indiana Academy of Science: Indianapolis, IN.)

Nickerson, M. A., and Briggler, J. T. (2007). Harvesting as a factor in population decline of a long-lived salamander; the Ozark hellbender, *Cryptobranchus alleganiensis bishopi* Grobman. *Applied Herpetology* **4**, 207–216. doi:10.1163/157075407781268354

Nickerson, M. A., and Mays, C. E. (1973). A study of the Ozark hellbender *Cryptobranchus alleganiensis bishopi. Ecology* **54**, 1164–1165. doi:10.2307/1935586

Olson, Z. H., Burgmeier, N. G., Zollner, P. A., and Williams, R. N. (2013). Survival estimates for adult eastern hellbenders and their utility for conservation. *Journal of Herpetology* **47**, in press.

Phillips, C. A., and Humphries, W. J. (2005). *Cryptobranchus alleganiensis* Daudin, 1803. In 'Amphibian Declines: the Conservation Status of United States Species'. (Ed. M. Lannoo.) pp. 648–651. (University of California Press: Los Angeles, CA.)

Pietramellara, G., Ascher, J., Borgogni, F., Ceccherini, M. T., Guerri, G., and Nannipieri, P. (2009). Extracellular DNA in soil and sediment: fate and ecological relevance. *Biology and Fertility of Soils* **45**, 219–235. doi:10.1007/s00374-008-0345-8

Putman, R. J. (1995). Ethical considerations and animal welfare in ecological field studies. *Biodiversity and Conservation* **4**, 903–915. doi:10.1007/BF00056197

Rosen, S., and Skaletsky, H. (2000). Primer3 on the WWW for general users and for biologist programmers. In 'Bioinformatics Methods and Protocols: Methods in Molecular Biology'. (Eds S. Misener and S. A. Krawetz.) pp. 365–386. (Humana Press Inc.: Totowa, NJ.)

Rudnick, J. A., Katzner, T. E., Bragin, E. A., Rhodes, O. E. Jr, and DeWoody, J. A. (2005). Using naturally shed feathers for individual identification, genetic parentage analyses, and population monitoring in an endangered Eastern imperial eagle (*Aquila heliaca*) population from Kazakhstan. *Molecular Ecology* **14**, 2959–2967. doi:10.1111/j.1365-294X.2005.02641.x

Sabatino, S. J., and Routman, E. J. (2009). Phylogeography and conservation genetics of the hellbender salamander (*Cryptobranchus alleganiensis*). *Conservation Genetics* **10**, 1235–1246. doi:10.1007/s10592-008-9655-5

Sastre, N., Francino, O., Lampreave, G., Bologov, V. V., López-Martín, J. M., Sánchez, A., and Ramírez, O. (2009). Sex identification of wolf (*Canis lupus*) using non-invasive samples. *Conservation Genetics* **10**, 555–558. doi:10.1007/s10592-008-9565-6

Sawaya, M. A., Stetz, J. B., Clevenger, A. P., Gibeau, M. L., and Kalinowski, S. T. (2012). Estimating grizzly and black bear population abundance and trend in Banff National Park using noninvasive genetic sampling. *PLoS ONE* **7**, e34777. doi:10.1371/journal.pone.0034777

Smyser, T. J., Beasley, J. C., Olson, Z. H., and Rhodes, O. E. Jr (2010). Use of rhodamine b to reveal patterns of interspecific competition and bait acceptance in raccoons. *The Journal of Wildlife Management* **74**, 1405–1416.

Solberg, K. H., Bellemain, E., Drageset, O.-M., Taberlet, P., and Swenson, J. E. (2006). An evaluation of field and non-invasive genetic methods to estimate brown bear (*Ursus arctos*) population size. *Biological Conservation* **128**, 158–168. doi:10.1016/j.biocon.2005.09.025

Taberlet, P., and Luikart, G. (1999). Non-invasive genetic sampling and individual identification. *Biological Journal of the Linnean Society. Linnean Society of London* **68**, 41–55. doi:10.1111/j.1095-8312.1999.tb01157.x

Taberlet, P., Griffin, S., Goossens, B., Questiau, S., Manceau, V., Escaravage, N., Waits, L. P., and Bouvet, J. (1996). Reliable genotyping of samples with very low DNA quantities using PCR. *Nucleic Acids Research* **24**, 3189–3194. doi:10.1093/nar/24.16.3189

Taberlet, P., Prud'homme, S. M., Campione, E., Roy, J., Miquel, C., Shehzad, W., Gielly, L., Rioux, D., Choler, P., Clément, J.-C., Melodelima, C., Pompanon, F., and Coissac, E. (2012). Soil sampling and isolation of extracellular DNA from large amount of starting material suitable for metabarcoding studies. *Molecular Ecology* **21**, 1816–1820. doi:10.1111/j.1365-294X.2011.05317.x

Thomsen, P. F., Kielgast, J., Iversen, L. L., Wiuf, C., Rasmussen, M., Gilbert, M. T. P., Orlando, L., and Willerslev, E. (2012). Monitoring endangered freshwater biodiversity using environmental DNA. *Molecular Ecology* **21**, 2565–2573. doi:10.1111/j.1365-294X.2011.05418.x

Unger, S. D., Fike, J. A., Sutton, T., Rhodes, O. E. Jr, and Williams, R. N. (2010). Isolation and development of 12 polymorphic tetranucleotide microsatellite markers for the eastern hellbender (*Cryptobranchus alleganiensis alleganiensis*). *Conservation Genetics Resources* **2**, 89–91. doi:10.1007/s12686-009-9170-0

Valière, N., Fumagalli, L., Gielly, L., Miquel, C., Lequette, B., Poulle, M.-L., Weber, J.-M., Arlettaz, R., and Taberlet, P. (2003). Long-distance wolf recolonization of France and Switzerland inferred from non-invasive genetic sampling over a period of 10 years. *Animal Conservation* **6**, 83–92. doi:10.1017/S1367943003003111

Valsecchi, E., Glockner-Ferrari, D., Ferrari, M., and Amos, W. (1998). Molecular analysis of the efficiency of sloughed skin sampling in whale population genetics. *Molecular Ecology* **7**, 1419–1422. doi:10.1046/j.1365-294x.1998.00446.x

Waits, L. P. (2004). Using noninvasive genetic sampling to detect and estimate abundance of rare wildlife species. In 'Sampling Rare or Elusive Species: Concepts, Designs, and Techniques for Estimating Population Parameters'. (Ed. W. L. Thompson.) pp. 211–228. (Island Press: Washington, DC.)

Waits, L. P., and Paetkau, D. (2005). Noninvasive genetic sampling tools for wildlife biologists: a review of applications and recommendations for accurate data collection. *The Journal of Wildlife Management* **69**, 1419–1433. doi:10.2193/0022-541X(2005)69[1419:NGSTFW]2.0.CO;2

Welsh, H. H. Jr, and Ollivier, L. M. (1998). Stream amphibians as indicators of ecosystem stress: a case study from California's redwoods. *Ecological Applications* **8**, 1118–1132.

Wheeler, B. A., Prosen, E., Mathis, A., and Wilkinson, R. F. (2003). Population declines of a long-lived salamander : a 20+-year study of hellbenders, *Cryptobranchus alleganiensis. Biological Conservation* **109**, 151–156. doi:10.1016/S0006-3207(02)00136-2

Williams, R. D., Gates, J. E., Hocutt, C. H., and Taylor, G. J. (1981). The hellbender: a nongame species in need of management. *Wildlife Society Bulletin* **9**, 94–100.