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Using environmental DNA methods to improve detectability in a hellbender (*Cryptobranchus alleganiensis*) monitoring program

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ABSTRACT

Isolation of environmental DNA (eDNA) is becoming a valuable tool for detecting presence of rare or secretive aquatic species. The recent use of quantitative PCR (qPCR) with eDNA sampling presents the possibility of using this method to infer population abundance and status. This approach would be especially useful for species such as the Eastern hellbender (*Cryptobranchus alleganiensis alleganiensis*), a declining, secretive, aquatic salamander that requires intense field survey effort to study. In 2012, we conducted eDNA sampling at sites across the range of the species in North Carolina. Our objectives were to assess presence across 61 sites, test for a correlation of abundance and biomass with eDNA estimates at a subset of 23 sites, and sample at multiple spatial and temporal scales in three river systems. Overall, we detected hellbender eDNA at 33 sites, including all sites with 2012 hellbender records, 71% of all recent or historic sites with hellbender presence, and at nine sites that lack species occurrence records. We did not find a correlation between eDNA estimates and field survey counts of individuals or biomass. We detected a strong temporal increase in eDNA during the September breeding period, but no consistent evidence of a spatial relationship with eDNA. Overall, our results demonstrate the efficacy of eDNA for detecting hellbender populations. Furthermore, the potential utility of qPCR to assess population status in hellbenders requires further study and testing, although it may be promising for determining population reproductive status.

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1. Introduction

Over the past several years, researchers have repeatedly demonstrated the ability to detect aquatic organisms based on isolation of DNA from water samples, a technique known as environmental DNA analysis (hereafter referred to as eDNA) (Thomsen and Willerslev, 2015). Example taxa and environments include amphibians in lentic systems (Ficetola et al., 2008; Dejean et al., 2012; Thomsen et al., 2012a), amphibians in lotic systems (Goldberg et al., 2011; Olson et al., 2012), invasive fish (Jerde et al., 2011; Takahara et al., 2012), invertebrates (Goldberg et al., 2013; Deiner and Altermatt, 2014) and marine species (Foote et al., 2012; Thomsen et al., 2012b). The major appeal of this technique is that it only requires collecting or filtering water, and then subsequent lab extraction and amplification of DNA. In most scenarios, the method requires much less effort and time than intensive surveys, and when personnel costs are incorporated, it may have a greater cost effectiveness than surveys that require individual observations, at least for detecting presence of the species (Biggs et al., 2015; Sigsgaard et al., 2015).

To be fully useful as a monitoring method, eDNA techniques ideally would be able to make inferences regarding population size or status, such as by testing a relationship between amount of eDNA detected as a function of abundance or biomass. This goal was initially addressed by running multiple samples and PCR replicates and using the proportion of eDNA positives as a proxy for abundance (Ficetola et al., 2008; Goldberg et al., 2011; Olson et al., 2012). For instance, with invasive American bullfrogs (*Lithobates catesbeianus*), Ficetola et al. (2008) found that high density sites had sample replicates that almost always amplified, whereas low density samples generally amplified in only a third of the samples. On the other hand, Goldberg et al. (2011) found no evidence

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for a relationship with summer density and spring eDNA sampling using Rocky Mountain tailed frog (*Ascaphus montanus*) populations. However, both studies had relatively low sample sizes, and the frequency of PCR detection is limited by autocorrelation of PCR replicates and its reliance on only categorical presence/absence.

A better approach to this problem is through the implementation of quantitative PCR (qPCR), in which PCR amplification is followed in real-time, which allows for an estimate of amount of original target DNA. Therefore, in addition to being more sensitive to lower copy numbers than traditional PCR (Wilcox et al., 2013), it has the potential for researchers to directly correlate their results with actual population estimates. For instance several studies have used qPCR to detect significant associations between DNA estimates and density or biomass in captive environments (Takahara et al., 2012; Thomsen et al., 2012a; Goldberg et al., 2013; Klymus et al., 2015), demonstrating the potential to infer relative population size from such methods. A strong relationship using qPCR was also demonstrated in the natural environment for density and biomass of Rocky Mountain tailed frogs and Idaho giant salamanders (*Dicamptodon aterrimus*) across streams that had intense survey data as comparisons (Pilliod et al., 2013). However, questions remain as to the application of qPCR for population estimation in the field, particularly regarding a number of confounding environmental factors. These include water volume, flow rate, aquatic type, and life history differences among species that may include differences in shedding rate among individuals (see Klymus et al., 2015) or differences in activity at different times of year (i.e., periods of dormancy or mating activities).

Eastern hellbenders (*Cryptobranchus alleganiensis alleganiensis*) are giant, secretive, aquatic salamanders that are declining across their range and are currently being evaluated as a candidate for listing under the U.S. Endangered Species Act (J. Applegate, personal communication). Hellbenders are the largest salamanders by mass in North America and are completely aquatic. They primarily use cutaneous respiration and rely on spaces under large rocks for microhabitats, so they have been strongly affected by water pollution and siltation (Phillips and Humphries, 2005). However, although declines from historic sites have been documented, the detailed distribution and status of hellbenders is still poorly known. Recently, researchers have documented the ability to detect hellbenders with eDNA methods (Olson et al., 2012; Santas et al., 2013). Although study results varied with respect to detectability of hellbenders, they all consistently determined the presence of hellbenders at known sites. Determining hellbender presence is important, but that alone might provide little information on population status. For instance, hellbenders are long-lived organisms (likely 30–50 years), and larger individuals may survive for many years even if no reproduction takes place (Unger et al., 2013). Thus, presence/absence of eDNA would not be able to distinguish functionally extinct populations represented by a single geriatric individual from reproducing populations. However, estimation of eDNA amounts through qPCR may allow researchers to infer factors such as population size and reproductive activities that would be quite important for assessing population status.

The objectives of our study were to investigate the utility of quantitative eDNA for a hellbender monitoring program currently occurring in the state of North Carolina, USA. Specifically, we had three objectives:

(1) Determine status (presence/absence) and relative abundance of hellbenders at sites across their range in North Carolina.

(2) Collect multiple samples along known hellbender rivers to test for the influence of within-stream distance on quantification of hellbender eDNA.

(3) Collect samples over multiple time periods to examine whether eDNA can be used to assess reproductive status in hellbenders.

To address the first objective, we collected water samples from 61 sites spread across the range of the species in the state. At 23 of these sites, visual and rock-lifting surveys were conducted in conjunction with eDNA collections, which allowed us to correlate eDNA estimates with actual survey numbers. We addressed the second objective by selecting three stream systems with known hellbender presence and collecting samples from three points along each stream system separated by various distances. We investigated the third objective by collecting samples at multiple points in time from May to November at the same sites as used in objective 2. This allowed us to test whether there were increased levels of eDNA leading up to and during the hellbender breeding season.

2. Methods

2.1. General field collection protocol

At each site, we collected water samples using vacuum filtration with a hand pump. We filtered one liter of water at each site. We collected water either from the shore or upstream of collecting personnel at each site in a disposable cup and poured the water into a filter cup inserted into a one liter vacuum flask. All water collections were conducted before survey personnel entered the water. We used a 0.45 μm cellulose nitrate filter (Whatman International, Ltd.). After filtering, the filter paper was removed using forceps treated with DNA Away™ (Molecular Bioproducts) to ensure no contamination between samples. Filters were placed in a centrifuge tube filled with 95% ethanol. Personnel wore disposable gloves during sample collection. We also filtered deionized water from a laboratory or household well water at every fifth site to test for the presence of sample contamination. To evaluate an additional potential source of contamination, we tested the possibility of transmitting eDNA through sampling equipment by sampling a wetsuit used in hellbender surveys. After immersion in river water at a known high-density hellbender site, we squeezed the wetsuit and associated gear (gloves, boots, etc.) to extract one liter of water.

2.2. Laboratory methods

We extracted DNA from filters using a modified protocol described in Goldberg et al. (2011) of the DNeasy® Blood and Tissue Kit (Qiagen). Filters were cut in half (with the other half being stored in 95% ethanol as a backup) and ripped into a few pieces and allowed to dry overnight. The standard protocol of the extraction kit was then followed with the exception of the use of a Qiashredder® (Qiagen) spin column after the lysis buffer step.

We amplified eDNA using a qPCR protocol. We designed a diagnostic primer set for hellbenders using a consensus mitochondrial cytochrome b sequence representing populations across the range of hellbenders (Sabatino and Routman, 2009). Primers and probes were designed using Primer Express v3.0 (Applied Biosystem, Inc.) and were checked for potential cross-amplification with syntopic taxa using Primer-BLAST software (Ye et al., 2012). The selected

primer/probe combination amplifies a 104 bp region. The sequence of the forward primer, reverse primer, and probe are as follows:

Forward primer   5′ GTTTGCATGAGTATTRCGGATT 3′
Reverse primer   5′ TCGCTATRCATTATACAGCAGATACA 3′
Probe   5′ 6FAM – CATCTCGGCAGATATG – MGB-NFQ 3′

We included degenerate bases in the primer sequence to allow for amplification across all hellbender populations. Hellbenders are not closely related to any North American salamanders, so it is less likely that we would have non-target amplification with other amphibians, and there was no evidence for potential cross-amplification *in silico*. However, we tested for non-specific amplification with the mudpuppy, *Necturus maculosus*, a sympatric species in North Carolina which uses similar microhabitats as hellbenders. Furthermore, we tested the primers on the two other members of the family Cryptobranchidae (*Andrias davidianus* and *Andrias japonicus*). Although the other cryptobranchids are found in Asia, the exclusion of the two closest related species would increase confidence in the specificity of the test. We also sequenced results from five initial positive water samples during development (collected in Tennessee; Spear et al., unpublished) to confirm that we were amplifying the target hellbender sequence.

We ran 15-μL qPCR reactions with 7.5-μL QuantiTect Multiplex PCR Mix (Qiagen, Inc.), 0.4 μM of each primer, and 0.2 μM of probe, 0.6-μL of TaqMan® Exogenous Internal Positive Control 10X Exo IPC Mix (Applied Biosystems), 0.3-μL of TaqMan® Exogenous Internal Positive Control 50X Exo IPC DNA (Applied Biosystems), and 3-μL of sample extract on an Applied Biosystems® 7500 Fast Real-Time PCR system (Life Technologies™). The qPCR cycling protocol began with 15 min at 95 °C followed by 50 cycles of 94 °C for 60 s and 60 °C for 60 s. All samples were run in triplicate with both extraction and PCR negative controls. All extractions and PCR setup were conducted in a "clean room" dedicated exclusively to low-copy DNA extractions and PCR setup at the University of Idaho Laboratory for Ecological, Evolutionary and Conservation Genetics. No species tissue or PCR product is allowed in the clean room and all personnel must shower and change clothes before going into the clean room from another lab. We used DNA extractions from tail tip clips from hellbenders to serve as standards for the qPCR analysis. We used a NanoDrop™ fluoro-spectrometer (Thermo Scientific) to estimate the DNA quantities for the tissue extract. We then diluted a 117 ng/μl DNA extract at four levels to include in the qPCR runs: 10^{-3} ng/μl, 10^{-4} ng/μl, 10^{-5} ng/μl, and 10^{-6} ng/μl. These dilutions cover the range of DNA concentrations that are typically seen with eDNA extractions. Amplification of the IPC was used as a test of whether PCR inhibition was occurring within each reaction.

We used Applied Biosystems 7500 software v2.0.6 (Life Technologies™) with the manual threshold option to calculate the standard curve and estimate the DNA amount for each sample. We transformed the raw DNA concentration estimate based on the
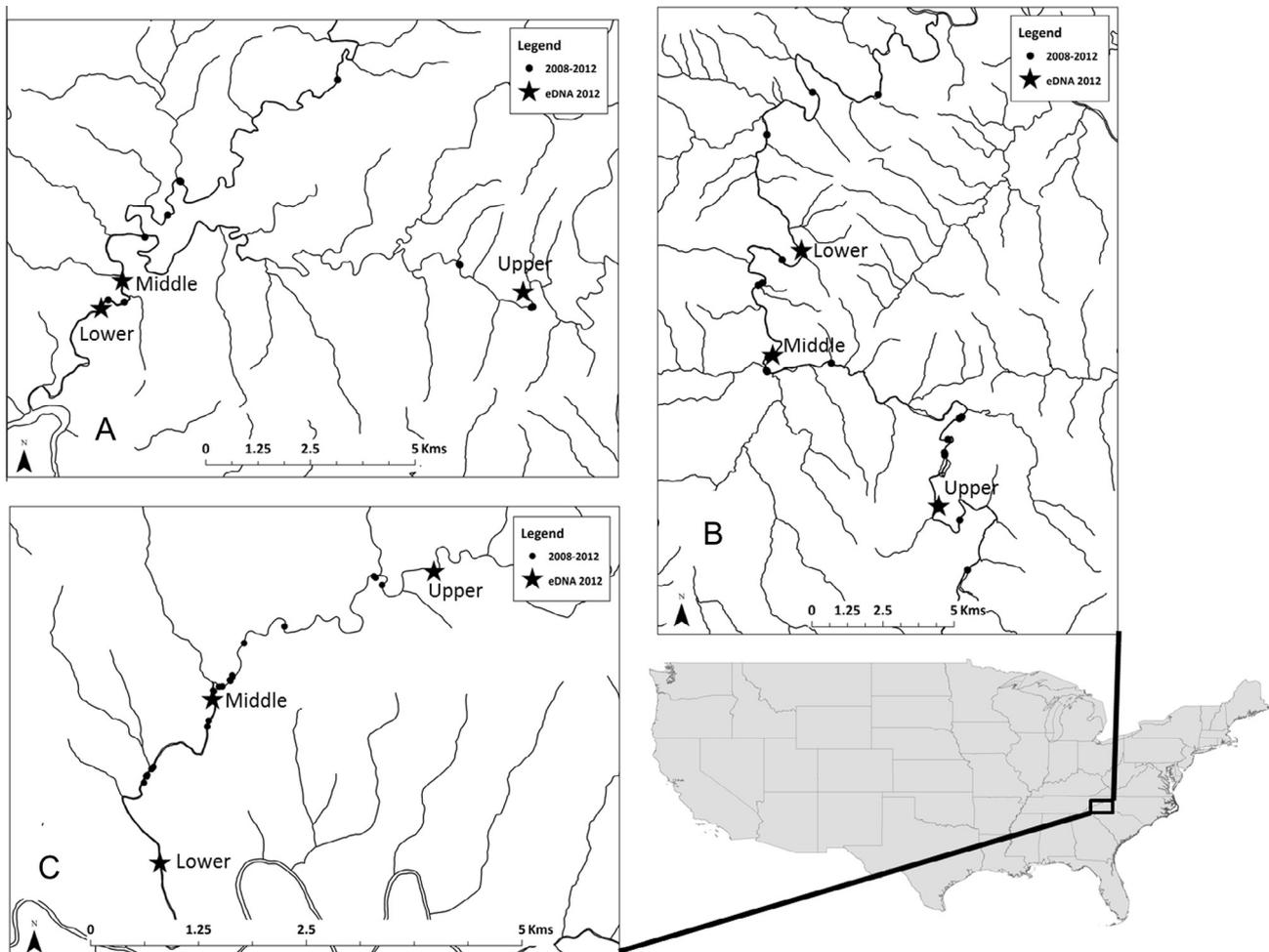

Fig. 1. Collection sites for hellbender eDNA and recent survey positives for the three drainages used in temporal and spatial analyses. Map demonstrates 2008–2012 hellbender encounters and eDNA samples. Labels indicate position of sample points in the study system. Inset represents approximate location of study sites in the United States. (A) French Broad A sites, (B) French Broad B sites, and (C) Hiwassee A sites.

qPCR run to an estimate of actual DNA amount in nanograms across the entire filter by multiplying the qPCR estimate by the concentration of the DNA extract used for the standards. We then extrapolated this amount to represent the total contained within the filter, assuming a constant concentration throughout the extract and filter. Although this adjustment to estimate total filter DNA should not affect any analytical results, we decided to extrapolate to provide a general estimate of how much eDNA might be captured through filtration of one liter. However, we recognize that our standard DNA (extracted from tail tips) could represent different cell types than those captured through the eDNA process and thus somewhat skew our absolute values, but should not affect our conclusions as all eDNA samples were run with the same tissue standard.

2.3. Presence/absence and relative abundance methods

A single one liter sample was taken from 61 sites across 9 watersheds in the known and potential range of hellbenders in North Carolina. The species was considered present if amplification occurred in at least two out of three qPCR replicates; we re-ran samples that did not amplify at all three replicates to ensure we could repeat amplification. Sites that only amplified once in six replicates (two sets of three replicates) were considered ambiguous. Twenty-three of the sites were surveyed for hellbenders from June–August 2012 using a combination of snorkeling upstream and rock-flipping with a log peavey (a long pole with a moveable hook that can be used as a lever to lift heavy objects) for at least 150 m to a maximum of 300 m upstream of eDNA sampling sites. We attempted to flip all rocks that were possible to lift with the peavey. Although exact personnel varied somewhat between surveys, they were all supervised by experienced hellbender surveyors (JG and LAW). Surveys did not take place if river levels were too high or clarity too low, based on surveyor judgement. We recorded numbers of individuals detected, total mass of captured individuals, and number of individuals per linear meter. We were unable to incorporate a measure of hellbenders/ m^2 because stream width was not measured when the physical surveys were conducted. To provide an initial test of whether eDNA concentration can serve as a proxy for abundance, we tested the correlation of the average of eDNA estimates across three replicates with both number of individuals encountered and total mass. This analysis was done using a Spearman rank correlation coefficient test, implemented using the pspearman package (Savicky, 2009) in R v 2.15.1 (R Core Team, 2012).

2.4. Spatial and temporal sampling methods

We collected multiple spatial and temporal samples along three rivers across two drainages (Fig. 1; sites labelled French Broad A, French Broad B, and Hiwassee A). In each river system, we sampled at three different points separated by distances from 1.25 km to 18.5 km. Furthermore, each site was sampled at five points in time: May/June, July, September, October, and November. Based on our field observations in the region (unpublished), the months from May–July represent the time before mating, September is the month where mating activities take place in the study streams, and October and November represent the time after mating when eggs have been laid and larvae hatch. To examine whether the eDNA estimates were influenced by either season or stream position, we used mixed effect modeling with season, stream position, and the interaction of these two variables as fixed effects. The sampling site and river were included as random effects and modeled as random slopes based on the fixed effects. Mixed effect modeling was done using the lme4 package (Bates et al., 2013) in R v2.15.1 (R Core Team, 2012) and models were ranked based on AIC weight (Burnham and Anderson, 2002). In addition, to address temporal

change in eDNA in a controlled setting, we collected samples from the same time period from a captive adult male hellbender in a 284 liter indoor tank with a full spectrum daylight fluorescent bulb, recirculating water, and agitation with a wave maker on the bottom. Despite being in captivity, the hellbender showed signs of breeding condition (i.e., cloacal swelling) during each year at the same time as the general hellbender breeding season, and this progression was documented in 2012.

3. Results

3.1. Presence/absence and relative abundance

The qPCR protocol consistently amplified hellbender DNA, but did not amplify *N. maculosus* or either *Andrias* species. We also successfully sequenced the target region from our test water samples. We detected no evidence of PCR inhibition in any sample and had no hellbender amplification in any of our field or laboratory negative controls, suggesting that we had no contamination between water filtering events or during laboratory extractions or assays. However, we amplified hellbender DNA from river water extracted from the wetsuit, thus sampling equipment is a potential source of contamination, although in our study we collected all water samples before surveyors entered the water. We detected hellbender DNA at 33 sites, had two ambiguous sites, and had 26 sites for which no evidence of hellbender DNA was detected (Tables 1 and A1). The lowest concentration detected at any site was 0.00002 ng/μl. Hellbender eDNA was detected at all nine sites in which 2012 snorkel/rock-flipping surveys documented hellbenders and at 12 of 14 sites (86%) in which 2012 surveys failed to detect hellbenders. Hellbender eDNA was detected at 24 of 34 sites (71%) with past records of hellbender occurrence (combining sites both with and without field surveys). We detected hellbender eDNA at three of eight sites (38%) with anecdotal reports of hellbenders. Finally, at 18 sites in which hellbenders had never been reported, we detected hellbender eDNA at six sites. Therefore, we confirmed current presence at 24 known sites and added nine sites to the list of hellbender sites in the state. We detected hellbenders with snorkeling surveys at nine of the 23 surveyed sites; eDNA positives were found at 21 of these same sites (Table 2). However, we were unable to confidently quantify the amount of eDNA at three of these sites due to a baseline and curve that shifted up during the entire course of the qPCR run. The shift leads to a very low C_t value that greatly inflated any estimate of DNA quantification, and is clearly not representative of actual DNA quantity. It is

Table 1
Summary of hellbender survey (both eDNA and field snorkel/rock-flipping) detections organized by category of the past site detection history for hellbenders. In this case, historical record implies any verified record before 2012. 2012 survey represents whether there was a snorkel survey conducted in 2012, and 2012 field detection represents whether hellbenders were captured during the 2012 surveys. Ambiguous results were tallied as a non-detection for the table.

Site detection history	2012 survey?	2012 field detection	eDNA detection	Number of streams
Historical record	Y	+	+	9
Historical record	Y	–	+	7
Historical record	Y	–	–	1
Historical record	N	NA	+	8
Historical record	N	NA	–	9
Anecdotal	Y	–	+	2
Anecdotal	N	NA	+	1
Anecdotal	N	NA	–	5
None	Y	–	+	3
None	Y	–	–	1
None	N	NA	+	3
None	N	NA	–	12
Total		9	33	61

unclear what led to this pattern, as there was no evidence of inhibition based on the internal control. Therefore, our sample size was limited to 20 sites total, and only six sites that had both successful hellbender captures and eDNA estimation. We did not find a relationship between eDNA estimate and survey numbers, regardless of whether we tested the relationship with number of individuals, cumulative mass, or individuals/m. The large number of survey negatives likely influenced this result, but we detected no significant associations when zeroes were omitted, although this was based on only six data points.

3.2. Spatial and temporal sampling results

The best supported model included season as a fixed effect and a random slope effect based on both site and season (Table 3). This model had an AIC weight of 0.84, and therefore was the best supported model based on this criterion. Therefore, there is very little evidence that estimates of eDNA were dependent on the position of the site in the stream system, as this model only had an AIC weight of 0.11. The major difference based on season was clearly during the breeding season (Fig. 2), as each site had elevated levels in September that returned to reduced levels in October and November. This pattern was also strongly evident in the captive male hellbender, in which the September value was two–three orders of magnitude higher than all other samples (despite the presence of visible skin sheds in the tank in early summer that was not there in September) (Fig. 2d). Interestingly, while there was no general pattern of eDNA estimates by stream position, the two French Broad samples have a pattern of increasing eDNA with increasing distance downstream during September. This correlation was quite strong, although it was based on just three data points (Fig. 3). This pattern did not hold at the Hiwassee site, with the upper portion having a greater amount of eDNA than the middle (Fig. 3C).

4. Discussion

4.1. Utility of eDNA for assessing occurrence

Our results suggest that eDNA is likely to be an excellent tool for assessing presence of hellbenders and has much greater success

Table 2
Average hellbender eDNA estimates (adjusted for filter estimate) and field survey numbers for 20 sites in which field surveys were conducted and eDNA quantity was estimated. Average (with standard error in parentheses) is across three qPCR replicates, individuals is the total number of individuals captured during a survey, mass is cumulative mass of the individuals, and individuals/m is the number of individuals per linear stream meter. Site names are as in Table 1.

Site	Average (ng)	Individuals	Mass (g)	Individual/m
French Broad 3	0.0702 (0.027)	0	0	0
French Broad 4	0	0	0	0
French Broad 5	0.3588 (0.078)	0	0	0
French Broad 6	0.1404 (0.027)	0	0	0
French Broad 7	0	0	0	0
French Broad 9	0.1716 (0.078)	0	0	0
French Broad 11	0.1092 (0.0156)	1	388	0.01
French Broad 17	0.1404 (0)	7	1848	0.04
French Broad 21	0.1482 (0.1365)	2	1170	0.02
French Broad 22	0.6708 (0.1651)	0	0	0
Hiwassee 1	0.0468 (0.0156)	0	0	0
Hiwassee 2	0.5772 (0.0312)	3	312.9	0.015
Hiwassee 3	0.1404 (0.0936)	4	1550	0.023
Hiwassee 4	0.2808 (0.027)	0	0	0
Hiwassee 5	0.1716 (0.0156)	0	0	0
Little TN 1	0.0702 (0.027)	2	740	0.013
Little TN 6	0.1248 (0.0156)	0	0	0
Little TN 7	0.0936 (0.027)	0	0	0
Little TN 8	0.1404 (0.0715)	0	0	0

Table 3
AIC model selection results for the three hellbender eDNA models with non-zero AIC weight. Season is divided into three categories (pre-breeding, breeding, post-breeding) and Position is divided into the three categories of upper, middle, and lower. Random slope effects are represented by (Season|Site) and (Position|Site), with site representing the sampling locality.

Model	AIC value	AIC weight
Season + (Season|Site)	603	0.84
Season + Position + (Season|Site) + (Position|Site)	607	0.11
1 + (Season|Site)	609	0.04

than current methods involving snorkeling surveys. We detected hellbenders with eDNA at all sites with 2012 captures, and we also had 12 detections at sites where rock-flipping surveys yielded no hellbenders. Twenty-five of the 33 eDNA detections were positive for all three replicates, demonstrating very consistent amplification within a sample. This is in contrast to the findings of Olson et al. (2012), which found low detectability of hellbenders at sites in Indiana and Missouri. They took ten filter samples of eight liters each (compared to one liter in our study) and ran ten PCR replicates for each filter. Of the three sites they sampled, the number of filters with any positives ranged from three to seven, and the average number of successes per filter ranged only from 0.2 to 1. There were three main differences between our study and Olson et al. (2012). First, Olson et al. (2012) used conventional PCR instead of qPCR, and qPCR appears to be more sensitive than conventional PCR (Wilcox et al., 2013). Second, they used a different filter type (glass filter), although it is unclear how much the filter type affected results. Finally, they sampled in October and December, which our results suggest is a time at which hellbenders may be less detectable, although we note that there can be some variation in breeding season across the species range and it is possible individuals could still be breeding in October in some areas. Santas et al. (2013) had higher success in amplifying hellbender eDNA using the traditional PCR approach but required filtering at least two liters for consistent amplification, whereas our study only required filtering one liter. Although we did not take multiple samples at each site, the high frequency of positive results at sites with known presence strongly suggest that a qPCR protocol has the highest detection rates, and it includes the benefit of providing DNA quantification. There were ten sites with past records of hellbenders where we did not detect hellbenders with eDNA. Given the success of the eDNA method to detect populations that have been recently confirmed, we believe it is likely that at least some of the ten sites no longer support hellbenders (at least at the sampling locations). Finally, in addition to confirming presence at many known sites, we identified several locations through eDNA that were not previously known to have hellbenders. These sites are now candidates for monitoring to see if viable populations occur there. Finally, our ability to obtain and amplify eDNA off wetsuits demonstrates the care that must be taken to avoid contamination between sites, and that eDNA samples should always be taken before surveyors enter streams.

4.2. Relationship between eDNA and abundance

We did not see a relationship between eDNA estimates and field survey numbers. This result is in contrast to previous studies in which estimates of eDNA correlated with abundance or biomass (Thomsen et al., 2012a; Takahara et al., 2012; Goldberg et al., 2013; Pilliod et al., 2013; Klymus et al., 2015), but is more consistent with Biggs et al. (2015) who also did not find a clear correlation with abundance. However, there are a number of confounding factors that could have obscured the relationship in our study. First, our lack of stream width data precluded us from calculating
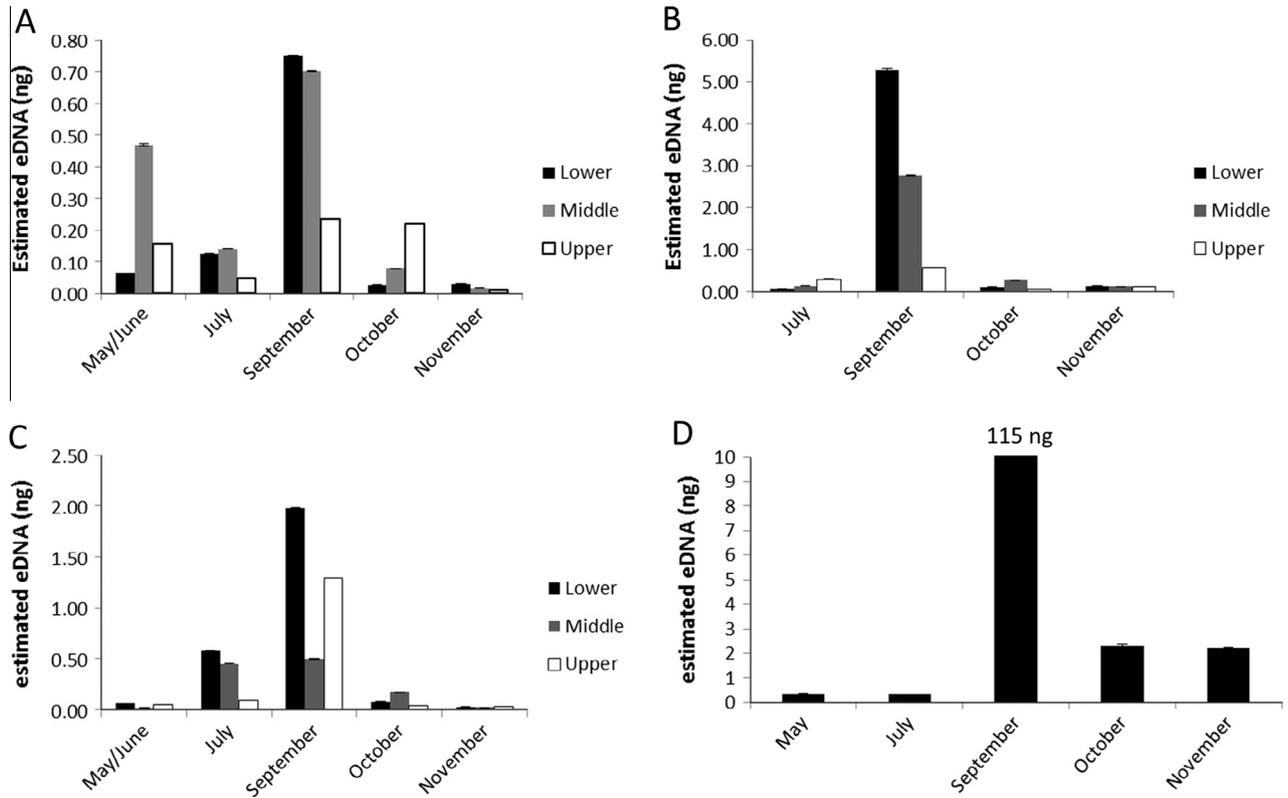
Fig. 2. Estimates of hellbender eDNA quantity (in nanograms) for three river systems that were sampled across five time periods, as well as for a captive male hellbender housed in a 284 liter tank. (A) French Broad A, (B) French Broad B, (C) Hiwassee A, and (D) captive hellbender (in order to better depict low values, we constrained the y-axis to ten ng; the text box above the September bar represents the actual estimate). French Broad B is missing data for the May/June time point because eDNA quantity could not be reliably estimated due to a shifted baseline leading to inflated estimates of eDNA during analysis for this time point. Error bars represent the standard error of three qPCR replicates. Distances between sampling sites in the same river are presented in Fig. 3.
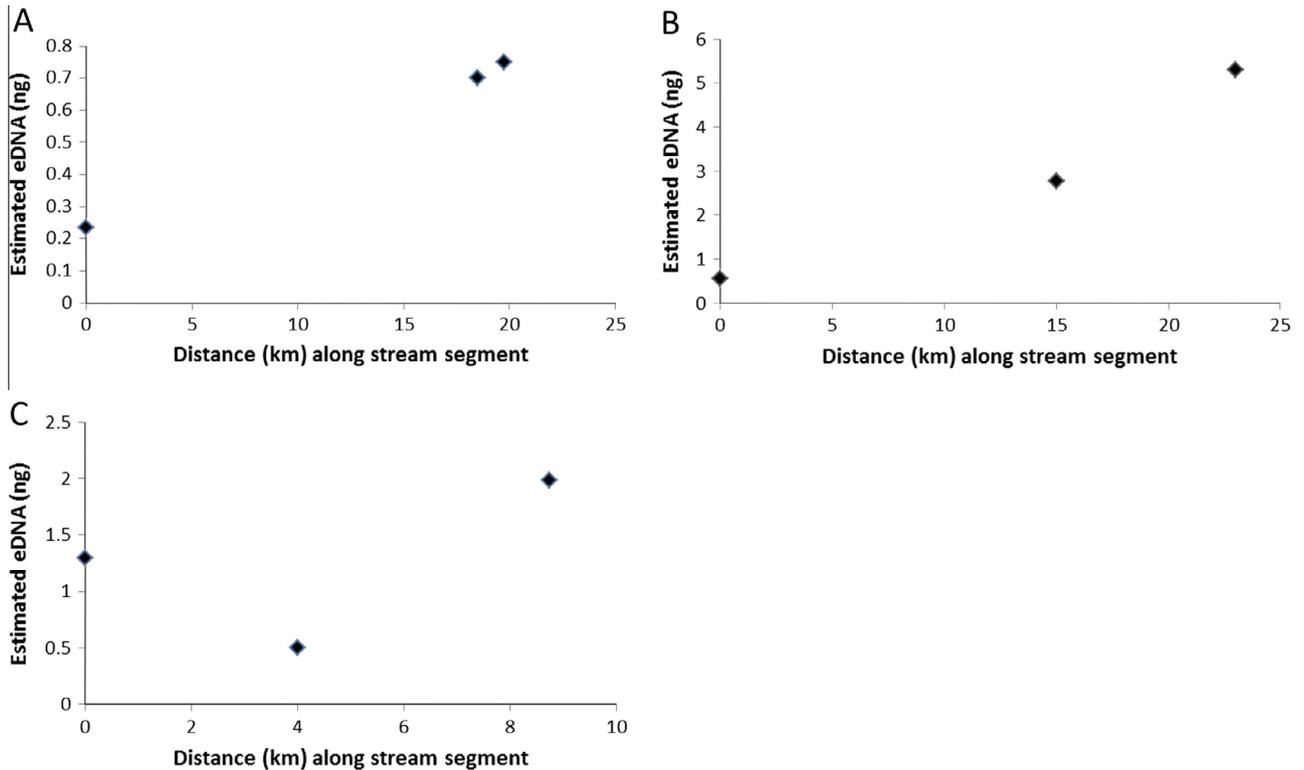

Fig. 3. Correlation of hellbender eDNA estimates (in nanograms) in September with distance along sampled stream segments. The upstream site was represented as distance zero for the purposes of this analysis. (A) French Broad A, (B) French Broad B, and (C) Hiwassee A.

true density of hellbenders at sites. However, the lack of even a correlational trend with the other metrics leads us to assume that incorporation of stream width would be unlikely to significantly change conclusions. Most importantly, there were a high proportion of field survey negatives at sites with eDNA positives. This is not only problematic from a pure statistical perspective, but it also indicates that the field survey methods underestimate the true numbers of hellbenders in the area. Such error is likely to be highly site dependent, which would also obscure a relationship. Such a scenario would not be unexpected as hellbenders are secretive and occur in microhabitats that are difficult to access (under large slab rocks and bedrock ledges), and streams vary in their ease of accessing microhabitats. Second, working in a lotic system increases the chances that eDNA estimates are influenced by individuals that occur upstream of a sampling site. For instance, Pilliod et al. (2013) used survey estimates of *A. montanus* and *D. aterrimus* along 30 random transects across a one km stream length and detected a significant relationship with eDNA estimates immediately downstream of this segment. Our surveys also typically took place upstream of eDNA sampling sites and surveys generally continued for up to 250–300 m. Thus, the characteristics of a lotic system alone likely do not explain our lack of pattern. Instead, the significant relationship observed by Pilliod et al. (2013) is probably more related to the ability to more completely census stream amphibian populations, as well as a greater number of individuals, in the Idaho stream system (Cossel et al., 2012). Third, the samples for this portion of the study were collected from June to August, which is confounded by potential temporal variation, and this is supported by the fact that the three highest eDNA values were collected in August, close to the breeding season (Table A1). Another issue we encountered in a few samples was a shifted baseline in the eDNA amplification that allowed for characterization of presence, but not accurate estimation of eDNA quantity. This pattern was repeatable, although we are unsure of the mechanism behind the pattern. Finally, we only took one sample per site and therefore were unable to assess the variability between samples at the same site. If there is a large amount of variability between samples, then we would likely need to take multiple samples per site to detect a relationship. We are currently collaborating with partners across Georgia, North Carolina, and Tennessee to collect a much wider sample of eDNA and field survey estimates to further investigate this issue, as the sample size in our current study is limited. However, our work so far suggests relatively low variability among samples (Spear et al., unpublished).

4.3. Temporal and spatial changes in eDNA concentration

The only supported pattern seen in our samples collected at the three river systems was the marked increase in eDNA in September compared to months before and after. Although the magnitude of increase differed among sites, every site showed elevated eDNA during this time. We had hypothesized this relationship because of the anticipated increase in DNA during the breeding season as hellbender mating season is characterized by combat between males for control of egg-laying sites as well as the release of gametes into the stream due to external fertilization in this species. The consistent increase indicates that September may be the optimal time to collect eDNA samples when attempting to identify new populations, although detection rate is likely high across seasons given the 100% success in detecting eDNA at known positives from field surveys. The rapid increase in eDNA in the captive male hellbender suggests that individuals in reproductive condition are sufficient to cause this peak without actual reproductive activities; it remains to be investigated if we will be able to use eDNA to determine whether successful reproduction has occurred. For instance, while mating occurs in September, the presence of eggs in nests

guarded by male hellbenders and subsequent hatching would occur in October and November. In our samples, at most sites, there was a large drop-off in eDNA concentration from September to October. We are currently investigating temporal signatures of eDNA estimates in both captive and wild populations to assess whether eDNA will be a promising technique for identifying reproducing populations.

The other interesting pattern associated with the September spike in eDNA is that two of the three river systems correlated strongly with river distance and position. Therefore, it is likely that the higher values of eDNA at downstream sites is due to a cumulative increase of DNA across the stream system as the concentrations increase at this time period. The exception was Hiwassee A in which the upstream site was higher than the middle site, despite several documented captures in the middle area (Fig. 3C). A relationship of eDNA with stream distances at a scale of kilometers might suggest a relatively constant presence or density of salamanders along the stream course such that eDNA is constantly being generated and spread downstream, whereas a situation in which there are river segments without hellbenders might lead to the pattern we see in Hiwassee A in which eDNA does not accumulate linearly. Further testing is needed since we do not know the actual distribution along the full length of any of the three study rivers. However, if hellbenders are not distributed continuously throughout the river segment, eDNA approaches could be used to delineate larger segments with and without hellbender populations at a broader scale. Note that this pattern was only seen in September, and eDNA estimates in the other months did not have any obvious correlation with stream position, and thus may be largely independent of other sites. The lack of a consistent spatial pattern in the eDNA concentrations of multiple samples along the same river has also been observed in Chinook salmon (Laramie et al., 2015).

One important assumption we have made is that streamflow and stream depth do not significantly influence our results. We cannot directly test this assumption due to the lack of streamflow data for our study sites. While flow and depth tend to be lower in September than earlier in the summer across the area (which would be expected to increase eDNA estimates), the difference is small compared to the increase in eDNA during this time period. Furthermore, there can be more variation on a day to day basis (i.e., due to high rainfall events) than across seasons. Laramie et al. (2015) collected Chinook salmon eDNA samples at both high and low flow times and found that eDNA was only elevated in one of the four study areas during the low-flow period; in the other three, there was no difference. Therefore, while stream depth and flow likely have some influence on eDNA concentrations, we would not expect the consistent result we observed if depth and flow were the main determining factors.

A second concern is that we took only a single sample per time point and location and therefore could not assess variability. For instance, eDNA might move in pulses within the stream system (i.e., one might incidentally filter a raft or clump of cells). Our typical filtering protocol involved collecting water at several intervals because we used an approximately 250 ml cup to collect water from the stream, and so we would avoid being influenced by one raft of cells. We also chose this design because previous hellbender eDNA work demonstrated a general pattern of consistent results among replicates (Spear et al., unpublished data) and so we chose to allocate resources toward a wider sampling effort. Finally, our temporal results were quite consistent, in that every site had its greatest value in September and the captive hellbender had two separate time points that were virtually identical (May/July and September/October). We would expect much less consistency if between-sample variability was a major concern. However, it is true that more replicates would increase confidence in our findings.

4.4. Conclusions

Our study builds upon previous work (Olson et al., 2012; Santas et al., 2013) demonstrating that eDNA is a highly effective tool for documenting presence of hellbenders and should be a component of any monitoring plan given its apparent higher efficacy for detecting presence. Our results using qPCR have begun to reveal the potential advantages and limitations of using eDNA to elucidate population processes beyond presence/absence. We demonstrate a strong temporal trend associated with timing of reproductive activities, a relationship that requires more exploration, but one that could be invaluable for researchers who study longer-lived aquatic organisms for which successful reproduction is a major concern. Furthermore, such temporal differences should encourage researchers to conduct pilot eDNA studies over several months to determine the best sampling times, especially for low density populations; trials during species' breeding periods might be particularly productive. Ultimately, we might expect a similar pattern in any aquatic species with external fertilization and could provide a useful template for researchers studying threatened fish or mussels in similar streams. Hellbenders appear to have high eDNA detection rates regardless of season, but this may not be the case for smaller species that likely have a lower baseline rate of eDNA production.

Our study shows the difficulties in attempting to correlate abundance with eDNA in a system in which field surveys have low success rates. We need to generate a greater sample size before we rule out meaningful correlations between eDNA and abundance with hellbenders and other similar systems, but given the difficulties outlined previously, we expect that a strong correlation with survey numbers is unlikely. Instead, a more realistic goal may be to develop bins of eDNA quantity (i.e., the eDNA score of Biggs et al. (2015)) that would match up with broad characterizations of population status – for instance, populations characterized by all age classes compared to a stream with only a few surviving adults remaining. As the ability to detect eDNA in multiple systems is becoming firmly established (Ficetola et al., 2008; Goldberg et al., 2011; Thomsen et al., 2012b), we see tests of the ability of eDNA to answer demographic questions as the next avenue in this rapidly growing field.

Acknowledgments

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biocon.2014.11.016.

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