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Research Article

Seasonal and Diel Signature of Eastern Hellbender Environmental DNA

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ABSTRACT Examination of environmental DNA (eDNA) is a non-invasive conservation tool that has been used for the detection of aquatic organisms. When coupled with quantitative polymerase chain reaction (qPCR), eDNA sampling may be used to infer seasonal or diel activities of target species. To survey the status of eastern hellbenders (*Cryptobranchus a. alleganiensis*), fully aquatic cryptic salamanders of conservation concern, through eDNA analyses, we collected water samples monthly from 13 sites across 8 tributaries of the West Branch Susquehanna River in Pennsylvania, USA, from June through October 2014. We also examined the effects of the breeding season, diel activity, and stream environmental variables (e.g., temp, pH) on eDNA concentration estimates. We repeatedly detected hellbender eDNA from all 4 tributaries known to contain hellbenders, and from downstream sites of 2 of the 4 tributaries without known records of hellbenders. In the tributaries known to contain hellbenders, we observed notable increases in eDNA concentrations during the September breeding season, suggesting possible reproductive events. However, such seasonal eDNA signature was lacking from the eDNA positive sites of the tributaries without known records of hellbenders. There was no difference in eDNA estimates between diurnal and nocturnal samples, indicating that diel activity was inconsequential to eDNA estimates. Our statistical analyses of the eDNA positive sites revealed no effects of the stream variables on eDNA estimates. Yet, the presence of hellbenders was positively associated with stream temperature and negatively with pH. The positive association with temperature was likely to be an artifact of the sampling design, whereas the negative association with pH may indicate negative effects of farming and livestock on hellbenders. Our findings concur with recent studies on the importance of temporal sampling in interpreting eDNA signature in relation to life histories of target species. Further studies are needed to characterize the core habitats of newly found populations for future management of the declining hellbender populations. © 2017 The Wildlife Society.

KEY WORDS *Cryptobranchus a. alleganiensis*, diel activities, eDNA, hellbender, monitoring, Pennsylvania, seasonal activities, stream environment variables.

Non-invasive sampling and detection of genetic materials of wildlife found in the environment (environmental DNA [eDNA]) is widely used to characterize populations of aquatic organisms that are traditionally difficult to sample because of their secretive nature or low densities. The method has been used to detect rare and secretive native insects, fish, amphibians, and mammals (Goldberg et al. 2011, Olson et al. 2012, Thomsen et al. 2012, Pilliod et al. 2014, Biggs et al. 2015), and non-native aquatic species (Ficetola et al. 2008, Jerde et al. 2011, Dejean et al. 2012, Goldberg et al. 2013, Fukumoto et al. 2015). The use of eDNA is increasing in popularity because it allows for the detection of animals without time- and cost-consuming manual labor of physically locating animals, which also risks disrupting their habitats and directly harming animals. Moreover, eDNA analysis coupled with quantitative polymerase chain reaction (qPCR) can offer more reliable detectability than traditional physical survey methods (Takahara et al. 2013, Wilcox et al. 2013, Biggs et al. 2015, Smart et al. 2015, Spear et al. 2015). These studies present the promising outlook of this novel conservation tool; yet, there are still some challenges left such as biotic and abiotic factors that influence the detectability and concentration estimates of eDNA (Rees et al. 2014).

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In addition to presence-absence data, eDNA estimates through qPCR can provide valuable information about biomass and population density of target species (Takahara et al. 2012, Goldberg et al. 2013, Pilliod et al. 2013, Klymus et al. 2015). However, the relationship between eDNA estimates and known density of animals is often obscured in the field (Goldberg et al. 2011, Spear et al. 2015). In addition to temporal changes in population sizes, one of the difficulties of inferring a population density is that eDNA concentration in the field may fluctuate daily and seasonally depending on diel and seasonal activities of target species. For example, Spear et al. (2015) reported that eDNA estimates of the eastern hellbender (*Cryptobranchus a. alleganiensis*), a secretive fully aquatic giant salamander, sharply increased during their breeding month of September across multiple sampling sites. This temporal signature of eDNA is likely caused by male–male combat, gamete release, or increased baseline metabolism of animals during the breeding season, which presents a challenge in estimating population density. In the meantime, an analysis of such temporal signature may provide an exciting opportunity of assessing reproductive status of populations through eDNA (Spear et al. 2015).

Another challenge associated with eDNA analysis is to control for influences of environmental conditions. One obvious factor is change in stream discharge, which affects eDNA concentration through diffusion and transport. A few recent studies also explored how other conditions such as water temperature, pH, and solar radiation affect eDNA concentration through degradation processes (Pilliod et al. 2014, Strickler et al. 2015). Testing eDNA from American bullfrog (*Lithobates catesbeianus*) in a mesocosm setting, Strickler et al. (2015) reported that degradation rates became faster under warmer, higher ultraviolet-B, and neutral to acidic conditions. These new findings demonstrate the importance of incorporating environmental variables into estimation of eDNA concentrations in the field-collected samples, which is not commonly practiced to date.

Eastern hellbenders are in the family Cryptobranchidae, which is one of the oldest salamander lineages consisting of 3 species (Pyron and Wiens 2011). Eastern hellbenders inhabit fast-flowing streams and are historically found throughout the eastern United States (Phillips and Humphries 2005). It is difficult to find these giant salamanders because they are nocturnal and often hide under large slab rocks (Noeske and Nickerson 1979, Phillips and Humphries 2005). Also, as seen in many amphibian populations across the globe (Houlahan et al. 2000, Collins and Storfer 2003, Collins et al. 2009), hellbenders are currently in a state of decline because of a combination of natural and anthropogenic factors including siltation and eutrophication, illegal harvesting, habitat destruction through pollution and construction, and possibly chytrid fungal infection (Mayasich et al. 2003, Wheeler et al. 2003, Nickerson and Briggler 2007, Foster et al. 2009, Souza et al. 2012). Once common in Pennsylvania (Mayasich et al. 2003, Phillips and Humphries 2005) where our research sites were located, eastern hellbenders are currently listed as a species of special concern.

Because of their secretive nature and confusion with another large fully aquatic salamander, mudpuppy (*Necturus maculosus*), the current distribution range of eastern hellbenders is not fully understood (Phillips and Humphries 2005). Given their declining status, it is an urgent conservation task to determine their distribution range at a stream level and establish an effective and sustainable monitoring program. The traditional survey techniques for hellbenders involve physically finding animals by snorkeling and turning rocks (Hillis and Bellis 1971, Nickerson and Mays 1973, Peterson 1987, Nickerson et al. 2002). This requires specialized skill, time, and effort because large rocks may need several people to lift and diving gear is often needed to reach them. It also risks disturbing the population and its habitat especially when traditional field surveys are conducted during breeding seasons when the detectability of animals is the greatest. Thus, eDNA analysis makes it a particularly useful tool for accessing population status of hellbenders. A few recent studies established hellbender eDNA protocols by using traditional PCR (Olson et al. 2012, Santas et al. 2013) and qPCR (Spear et al. 2015). Although both have successfully detected hellbender eDNA, the qPCR protocol developed by Spear et al. (2015) was more sensitive and provided more consistent results among PCR replicates.

We used the qPCR protocol developed by Spear et al. (2015) to achieve 4 goals. Our first was to survey hellbender population status (presence-absence) in streams with and without known hellbender records in the West Branch Susquehanna River Basin, central Pennsylvania, USA. Our second goal was to determine whether the seasonal pattern of eDNA fluctuation found from streams in North Carolina, USA (Spear et al. 2015), would be replicated in streams in central Pennsylvania. Third, we examined whether hellbender eDNA concentration changes in relation to their diel activity. We predicted that nocturnal samples would contain greater amount of eDNA than diurnal samples because hellbenders are nocturnal (Noeske and Nickerson 1979). It is also likely that eDNA degradation rate slows down during nighttime without solar radiation (Strickler et al. 2015). Finally, we examined whether stream conditions (i.e., conductivity, dissolved oxygen, pH, water temperature, stream current) affect eDNA concentration. Because DNA degradation rates become greater under environments favoring microbial growth (Strickler et al. 2015), we predicted that higher conductivity, which can be caused by greater input of phosphate and nitrate, higher dissolved oxygen, neutral pH, and higher water temperature would be negatively associated with eDNA concentrations. We also predicted that greater stream current would be negatively associated with eDNA concentrations because of faster replacement of stream water, preventing eDNA accumulation in a given section.

STUDY AREA

We analyzed water samples collected from 13 sites across 8 tributaries of the West Branch Susquehanna River in central Pennsylvania (Fig. 1), which has a drainage area of

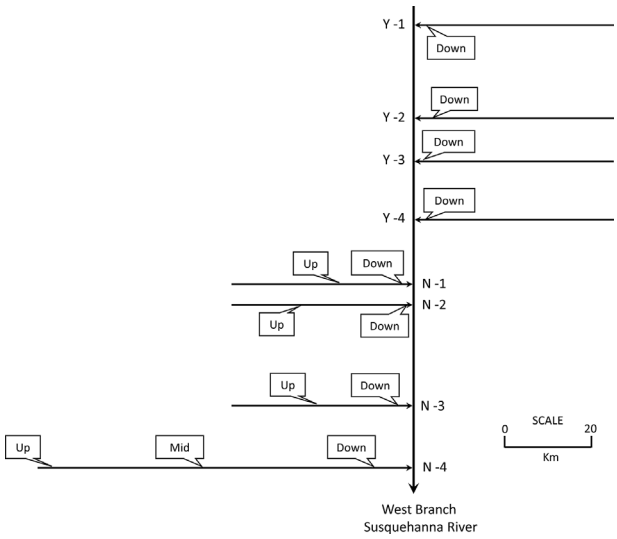

Figure 1. Sampling sites along 8 tributaries of the West Branch Susquehanna River, central Pennsylvania, USA, 2014. Y-1 through Y-4 are tributaries with known hellbender records. N-1 through N-4 are tributaries with no known hellbender records.

18,073 km^2. We collected water samples from June through October 2014. All 13 sampling sites were distributed within a 35-km radius from the central point in Union County (41° 1′53.86″N, 77° 1′4.94″W) and their elevations ranged from 134 m to 404 m ($\bar{x}$ SD =189.02 23.00 m). The sampling sites were located within the Ridge and Valley Physiographic Province where the ridges were dominated by the northeastern hardwood forest and the valleys were predominantly covered with farmland. Annual average temperature in this area was 10.8°C (range = − 28.9–41.1°C) and annual total precipitation was 103 cm (annual total range = 70–178 cm; National Weather Service). Climate in these regions is characterized by 4 distinct seasons with relatively long, cold winter. Among the 8 tributaries, 4 had known records of hellbenders (stream ID: Y-1, Y-2, Y-3, Y-4), whereas the other 4 did not (stream ID: N-1, N-2, N-3, N-4; confidential information obtained upon request through Pennsylvania Natural Heritage Program). These tributaries were comparable in size (10–20 m wide). All of these tributaries were easily accessible by the general public, and all but 1 sampling site (the upstream site of N-2) were located within or adjacent to privately owned residential or farming areas (i.e., the valley section of the Ridge and Valley Province). The upstream site of N-2 was located in a state forest in the ridge section of the Province where temperate broadleaf deciduous trees were dominant. There was no implementation of conservation management of eastern hellbenders in our study area. Dominant predatory aquatic species that may compete with hellbenders for resources in these tributaries include brook trout (*Salvelinus fontinalis*), brown trout (*Salmo trutta*), rainbow trout (*Oncorhynchus mykiss*), and smallmouth bass (*Micropterus dolomieu*). In addition, American eels (*Anguilla rostrata*) were recently reintroduced to Y-1 and N-3. The Pennsylvania Fish and Boat Commission lists eastern hellbenders as a species of special concern and is protective of their locality data. Accordingly, the details of our sampling sites were not disclosed. Instead, we provided a schematic diagram of the sampling sites (Fig. 1).

METHODS

Field Sampling

We collected diurnal and nocturnal water samples each month between June and October 2014 from the 13 sites, resulting in the collection of 150 samples including 20 negative controls over the study period (see the description of negative controls in Laboratory Procedures section). We collected water samples from surfaces of the streams (i.e., 10 cm depth) in 1L plastic jars that we placed in re-sealable zipper storage bags individually and kept samples on ice in a cooler until we returned to the lab. We wore nitrile gloves and changed them between samples. At each sampling, we also collected stream environmental data (i.e., water temperature, stream current, pH, dissolved oxygen, conductivity). We used a portable flow meter (Marsh McBirney Flomate model 2000, Loveland, CO, USA) for the collection of stream current data and a water quality meter (YSI 6920 multi probe, Yellow Springs, OH, USA) for the rest of the variables. We recorded stream current at a similar depth to water sampling (10 cm depth) as an average value (m/sec) of 10 readings with a 10-second interval between readings at each sampling. We designated 1 sampling site (downstream) to each known tributary (Y-1, Y-2, Y-3, Y-4), 2 sampling sites (upstream and downstream) to 3 of the 4 tributaries without records of eastern hellbenders (N-1, N-2, N-3), and 3 sampling sites (upstream, midstream, downstream) to the last tributary without records (N-4). We avoided collecting water samples during and after heavy rains to avoid extreme cases of eDNA diffusion. In each month, we collected a set of diurnal and nocturnal samples from the same sites within 24 hours; day sampling occurred between 1000 and 1600 and night sampling between 2200 and 0400.

Laboratory Procedures

Using vacuum pumps (Welch, Mount Prospect, IL, USA) and 0.45-μm cellulose filters (Whatman International GE Healthcare, Maidstone, United Kingdom), we filtered all samples within 24 hours of collection. Because filtration of a 1-L water sample would take hours to complete with only 1 filter, we used 3 filters per sample to expedite the filtration process. This process is unlikely to have affected the concentration of extracted DNA (Olson et al. 2012). We cut each filter in half to save one as a backup. We stored filtered DNA at 20°C until extraction. After finishing the filtration of each sample, we rinsed all the equipment used in distilled water, 50% ethanol-water solution, 50% bleach-water solution, and then DNA Away^{TM} (Molecular Bioproducts Thermo Scientific, Waltham, MA, USA) to prevent cross-contamination. At the end of each filtering cycle, we added a negative control by analyzing distilled water filled in one of the cleaned sample bottles after we used them to collect water samples in the sampling cycle.

We performed DNA extraction and subsequently ran qPCR in accordance with the standard protocol of the DNeasy Blood and Tissue Kit (Qiagen, Germantown, MD,

USA) and the qPCR protocol developed by Spear et al. (2015). We used Applied Biosystems StepOne™ Real-Time PCR system (Thermo Scientific) to run all samples in triplicate, along with PCR negative controls and hellbender DNA standards that we created through serial dilutions of DNA extracted from a tail clip and measured using a NanoDrop™ fluorospectrometer (Thermo Scientific). We used StepOne™ Software version 2.3 (Life Technologies Thermo Scientific) with the manual threshold option to establish the standard curve and estimate the amount of DNA for each sample. When there was only 1 amplification success out of triplicates, we considered those samples suspicious. When there were 2 amplification successes out of triplicates, we conducted a fourth qPCR (i.e., quadruplicate) and considered samples positive when replication success was 3 out of 4 and suspicious when replication success was 2 out of 4.

We deemed 4 of the 60 eDNA measurements made available for analysis as suspicious because of <3 out of 4 replication success. To ensure these 4 measurements were not associated with all covariates of interest and could thus be removed, we fit logistic regression models for suspicious versus non-suspicious measurements using a single covariate at a time. We determined that no covariates were associated with the suspicious values. Thus we removed these 4 observations from the data. The 4 suspicious measurements were the diurnal sampling of Y-1 in June, the nocturnal sampling of Y-4 in June, the diurnal sampling of N-1 in October, and the diurnal sampling of N-2 in July (Table 1).

Statistical Methods for Temporal Analysis

There were 7 sampling sites where we never detected hellbender eDNA; thus, we excluded these sampling sites from the following analyses. To account for the repeated, temporal sampling of streams, we used linear mixed-effects models with stream included as a random intercept. Because of the small sample size, we could not incorporate random slopes. These models assume normality in the response; thus, we transformed eDNA using a square-root transformation to reduce skewness. We selected the square-root transformation because of the presence of zeros. We fit 9 models; each contained at least the main effect of season and a random intercept for stream. We modeled season using 1 parameter for each month of measurement to account for possible month-to-month fluctuations in eDNA concentrations. A preliminary analysis revealed that eDNA concentrations were overall higher in the streams with known hellbender records than those in the streams without records. Thus, we adjusted all models for the known presence of hellbenders via the inclusion of an indicator variable denoting known presence. We also investigated the effect of season adjusted for pH, conductivity, stream current, dissolved oxygen, and sampling timing (i.e., diurnal vs. nocturnal). To adjust for each, we added the corresponding covariate to the model one at a time for investigation. We also examined a model containing all environmental variables. We did not include the temperature of the stream in the analysis because of its high correlation with season. To determine the best models, we used Akaike's Information Criterion (AIC) and corrected Akaike's Information Criterion (AIC_c) for correction of small sample sizes suggested by Burnham and Anderson (2002). We also performed tests of significance using confidence intervals, Wald tests, and likelihood ratio tests to characterize potential associations between eDNA concentrations and primary covariates of interest including season, known presence of hellbenders, and timing of the sampling.

Table 1. Seasonal and diel detectability and replicate means (1 SE) of estimated concentrations (μg/L) of hellbender environmental DNA (eDNA) across all the surveyed stream sections in central Pennsylvania, USA throughout the sampling season June through October in 2014. Y-1 through Y-4 are streams with known hellbender records. N-1 through N-4 are streams with no known hellbender records. D denotes diurnal samples and N denotes nocturnal samples. Question marks (?) indicate suspicious samples, which we did not include in the analyses because of their low replication successes. Dashed line (—) indicates no eDNA detected.

		Jun		Jul		Aug		Sep		Oct	
Stream	Stream section	D	N	D	N	D	N	D	N	D	N
Y-1	Down	?	—	0.01869	0.01524	0.00310	0.01291	0.07134	0.10620	0.00362	0.03133
				0.01062	0.00662	0.00109	0.00439	0.00762	0.03251	0.00049	0.01635
Y-2	Down	—	—	0.13180	0.12997	0.00158	0.00004	0.14208	0.25365	—	—
				0.00563	0.03392	0.00079	0.00001	0.03841	0.03798		
Y-3	Down	0.10854	0.08978	0.01742	0.02135	0.02355	0.00065	0.87783	0.80678	0.00350	0.00395
		0.08337	0.08453	0.00817	0.00579	0.00546	0.00007	0.08861	0.07913	0.00130	0.00016
Y-4	Down	—	?	0.01676	0.01071	0.00611	0.01444	0.02798	0.12431	0.05328	—
				0.00629	0.00504	0.00112	0.01094	0.00356	0.00817	0.05294	
N-1	Up	—	—	—	—	—	—	—	—	—	—
	Down	—	—	—	0.00615	0.01748	0.01065	—	0.00738	—	?
					0.00501	0.01222	0.01064		0.00481		
N-2	Up	—	—	—	—	—	—	—	—	—	—
	Down	—	—	?	0.00120	0.00844	0.05804	—	—	—	—
					0.00083	0.00488	0.05803				
N-3	Up	—	—	—	—	—	—	—	—	—	—
	Down	—	—	—	—	—	—	—	—	—	—
N-4	Up	—	—	—	—	—	—	—	—	—	—
	Mid	—	—	—	—	—	—	—	—	—	—
	Down	—	—	—	—	—	—	—	—	—	—

To assess the associations between eDNA and stream environmental data, we conducted inference using confidence intervals on the effects of stream conductivity, pH, stream current, and dissolved oxygen.

Finally, using our eDNA results, we examined whether the stream environmental parameters were associated with presence-absence of hellbenders by using binary logistic regression. For this analysis, we included all 13 sites and used the stream environmental data collected in July and August nocturnally, and diurnally in August, during which the detectability (presence-absence) of hellbender eDNA was greatest (Table 1). Our limited sample size ($n = 13$) precluded multiple logistic regression; thus, we tested an effect of each variable individually on the probability of detecting the presence of hellbenders. We did not design our study to examine environmental parameters associated with hellbender distribution. Therefore, our limited sample size prevents drawing broad inferences from these analyses. Nonetheless, such information can be useful for future studies and conservation management. We conducted statistical analyses using the software R, version 3.3.1 (R Foundation for Statistical Computing, Vienna, Austria). We fit mixed models using the R package lme4 (Bates et al. 2015).

RESULTS

We detected hellbender eDNA from all known streams (Y-1, Y-2, Y-3, Y-4) and from the downstream sites of 2 unknown streams (N-1, N-2; Table 1). Hellbender eDNA was never detected from the upstream sites of N-1 and N-2 and all sites of N-3 and N-4 throughout the sampling period (10 water samples analyzed at each site; Table 1). The exogenous internal positive control (Applied Biosystems® TaqMan®) included in each qPCR reaction indicated no sign of PCR inhibition. The concentration estimates of hellbender eDNA in the known streams were relatively stable at a low level in June through August with a slight decline in August, and then notably increased in September followed by the quick decline to the pre-September level in October (Fig. 2). In the unknown streams, on the other hand, eDNA estimates were consistently low and there was no increase during their breeding month of September (Fig. 2).

The results of the model selection suggested that the model consisting of an interaction between season and known (presence-absence of known hellbender records) was the best model supported by the lowest AIC and AIC_c values (Table 2). This interaction model accounted for most of the AIC_c and AIC weight (0.998 and 0.988, respectively). Further, the likelihood ratio test of all interaction terms simultaneously was highly significant ($\chi^2_4 = 23.117$, $P < 0.001$); thus, the inclusion of the interaction is warranted. This also suggests, in general, a difference between streams with prior records of hellbender presence and those without. Contrary to our prediction, there was no notable difference in the eDNA estimates between diurnal and nocturnal samples (Fig. 2). The addition of diel sampling timing did not improve the model (Table 2), and the

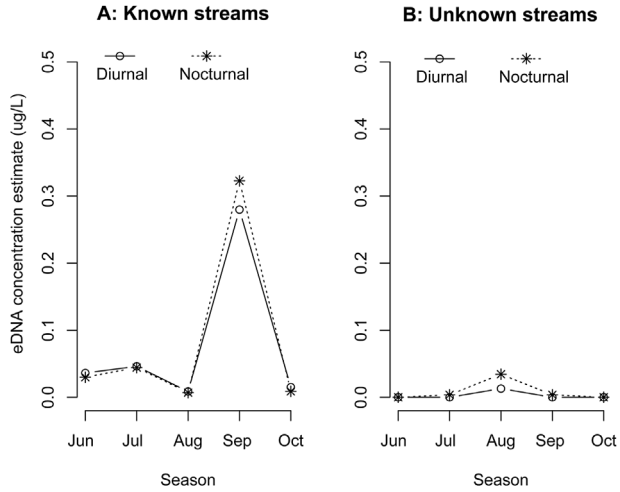

Figure 2. Seasonal changes in mean environmental DNA (eDNA) concentration estimates (μg/L) of eastern hellbenders in central Pennsylvania, USA, separated by diel sampling timing (diurnal and nocturnal) for the 4 streams that had known record of hellbenders (A) and for the 2 streams that did not have known records (B). We collected water samples monthly, June through October in 2014. In each month, we collected diurnal and nocturnal samples from each site within 24 hours.

association between diurnal and nocturnal samplings and the square-root of eDNA was nonsignificant ($\beta = 0.015$, 95% CI $= -0.066$, 0.097). The addition of the other environmental variables (i.e., pH, conductivity, stream current, dissolved oxygen) increased AIC_c and AIC values. The all covariate model, which contained all variables of interest, had the largest AIC and AIC_c values. There were no significant associations between these variables and the square-root of eDNA (pH: $\beta = -0.001$, 95% CI $= -0.007$, 0.004; conductivity: $\beta = -0.001$, 95% CI $= -0.002$, 0.0004; stream current: $\beta = -0.006$, 95% CI $= -0.086$, 0.074; dissolved oxygen: $\beta = 0.011$, 95% CI $= -0.021$, 0.043). Thus, these environmental variables could, in conjunction with the AIC and AIC_c results (Table 2), be excluded from the model. Of the interaction effects, only that of September was significant (Table 3). The remaining interaction effects were nonsignificant, suggesting no difference when compared to the measurements from known streams taken in June (Table 3). None of the seasonal effects were statistically significant in streams with no known presence (Table 3). The estimated eDNA concentration in streams with a known presence was higher in each month, with the exception of August, than the concentration in the streams with no known presence of hellbenders. However, the only month where the difference between types of streams was significant was September (Fig. 3).

The mean and standard errors of the stream environmental data were within typical ranges of stream conditions (Table 4; Behar et al. 1996). Binary logistic regression analyses showed that the models with temperature or pH were significantly better than the null model in predicting presence or absence of hellbender eDNA for all of the sampling periods analyzed (i.e., Jul nocturnal, Aug diurnal and Aug nocturnal; Table 5). The logistic coefficients of the significant models suggested that temperature was positively associated with the

Table 2. Akaike's Information Criterion (AIC) and corrected Akaike's Information Criterion (AIC_c) values for the 9 hellbender environmental DNA (eDNA) models along with AIC weight and AIC_c weights. We collected water samples for eDNA analyses from 13 sites across 8 tributaries of the West Branch Susquehanna River, central Pennsylvania, USA, from June through October, 2014. The notation (1 | stream) denotes that we included a random intercept for stream identification. The variable time denotes sampling timing (diurnal or nocturnal). The variable DO denotes dissolved oxygen of stream water. The interactive model between season and known (presence-absence of known hellbender records) was the best supported model by both AIC and AIC_c. The AIC and AIC_c weights represent the probabilities of the given model being the best model.

Model	AIC	AIC weight	AIC_c	AIC_c weight
Season known + (1 | stream)	52.437	0.998	45.181	0.988
Season + known + (1 | stream)	37.320	0.001	34.256	0.004
Season + known + conductivity + (1 | stream)	36.649	0.000	32.736	0.002
Season + (1 | stream)	34.439	0.000	32.106	0.001
Season + known + DO + (1 | stream)	35.859	0.000	31.946	0.001
Season + known + pH + (1 | stream)	35.637	0.000	31.724	0.001
Season + known + time + (1 | stream)	35.475	0.000	31.562	0.001
Season + known + current + (1 | stream)	35.397	0.000	31.484	0.001
All covariates + (1 | stream)	30.759	0.000	22.092	0.000

detectability of hellbenders, whereas pH had negative associations (Table 5). We also converted pH values to hydrogen ion concentrations and re-ran all relevant analyses, which showed essentially identical results to those with pH. Thus, we presented the results for pH because interpreting coefficients of hydrogen ion concentrations from the logistic regression and the mixed models is not intuitive (i.e., higher pH = lower hydrogen ion concentration).

DISCUSSION

Detection of Hellbender eDNA in Streams With and Without Known Records

Our results corroborated the reliability and efficiency of the qPCR-based hellbender eDNA protocol developed by Spear et al. (2015) and further suggest that eDNA sampling can be used to characterize distribution ranges within the streams. We repeatedly detected hellbender eDNA from all the tributaries with known records. In addition, we also identified new localities from 2 of the 4 tributaries without known records (N-1 and N-2). We detected eDNA only from the downstream sites of N-1 and N-2, whereas we never detected eDNA from the upstream sites of the same tributaries. These findings suggest that the hellbender distribution in N-1 and N-2 is potentially limited to an approximately 10.6-km stretch in N-1 and a 27-km stretch in N-2 between the up and downstream sampling sites. Tributary N-1 and N-2 are frequently accessed by humans. Tributary N-1 runs through heavily farmed areas and its water quality is visibly worse than that of N-2. Tributary N-2, on the other hand, runs through some dispersed residential areas, but the upstream section is more forested and pristine. However, N-2 has been well-stocked with non-native trout and heavily fished. To develop conservation strategies at a stream level, future studies should conduct finer-scale eDNA sampling to better characterize their distribution range in each stream and ultimately to identify the core habitats for remaining hellbenders. Finally, we never detected eDNA from tributary N-3 and N-4, which runs through the heavily farmed area and also has been stocked for trout fishing.

Table 3. Estimated effects of month, known (presence-absence of known record), and their interactions from the best-supported hellbender environmental DNA (eDNA) model. We collected water samples for eDNA analyses from 13 sites across 8 tributaries of the West Branch Susquehanna River, central Pennsylvania, USA, from June through October, 2014. For each estimated effect, we calculate a 95% confidence interval and *P*-value based on a *t*-distribution with 46 degrees of freedom. Estimates and intervals are based on square-root transformation of eDNA concentration estimates. The main effects of June, July, August, September, and October are the estimated square-root eDNA levels for streams without a known hellbender record. June known denotes the coefficient for the interaction between June and known presence and thus is not included in the table because June is the reference variable.

Effect	Estimate (95% CI)	*P*
Jun	0.000 (0.155, 0.155)	1.000
Jul	0.038 (0.135, 0.210)	0.662
Aug	0.142 (0.013, 0.297)	0.072
Sep	0.021 (0.134, 0.177)	0.782
Oct	0.000 (0.173, 0.172)	0.999
Known	0.092 (0.105, 0.290)	0.351
Jul known	0.057 (0.183, 0.297)	0.636
Aug known	0.160 (0.388, 0.067)	0.163
Sep known[a]	0.365 (0.137, 0.592)	0.002
Oct known	0.018 (0.258, 0.221)	0.878

[a] Indicates interactions that are significant ($P < 0.05$).

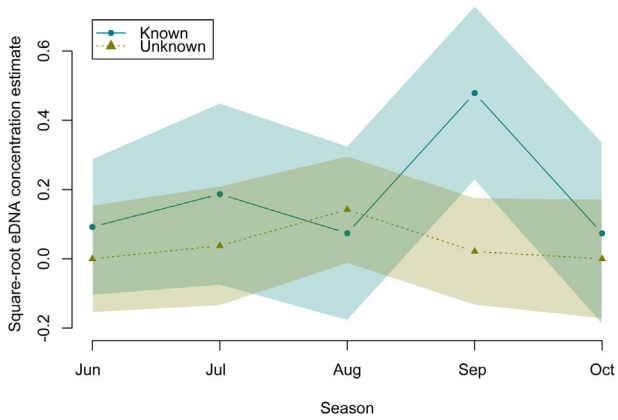

Figure 3. Seasonal changes in mean square-root concentration estimates of eastern hellbender environmental DNA (eDNA) in central Pennsylvania, USA, collected monthly from June through October 2014, with 95% confidence intervals by streams with or without known records of hellbenders.

Table 4. Means and standard errors of stream temperature, stream current, dissolved oxygen (DO), conductivity, and pH of all 13 sampling sites in central Pennsylvania, USA over the sampling period between June and October 2014 ($n = 10$, diurnal and nocturnal samples from each of 5 months). Y-1 through Y-4 are streams with known hellbender records. N-1 through N-4 are streams with no known hellbender records. The column eDNA indicates the stream sections in which we detected hellbender environmental DNA.

			Temp (°C)		Current (cm/sec)		DO (mg/L)		Conductivity (μS/cm)		pH	
Stream	Section	eDNA	$\bar{x}$	SE	$\bar{x}$	SE	$\bar{x}$	SE	$\bar{x}$	SE	$\bar{x}$	SE
Y-1	Down	X	19.89	1.77	8.97	0.77	9.36	0.32	108.4	4.1	7.35	0.15
Y-2	Down	X	17.67	1.51	28.08	2.84	10.01	0.46	93.1	3.8	7.35	0.12
Y-3	Down	X	19.13	1.50	63.77	6.62	9.28	0.46	87.2	1.4	7.44	0.12
Y-4	Down	X	18.00	1.35	54.01	3.23	10.31	0.66	125.1	5.4	7.33	0.11
N-1	Up		16.58	1.03	4.26	1.77	8.80	0.44	163.1	19.5	7.79	0.09
	Down	X	17.16	1.30	2.13	0.40	9.08	0.50	211.3	23.0	7.79	0.06
N-2	Up		14.10	0.76	8.09	1.37	9.81	0.25	26.7	0.5	7.96	0.10
	Down	X	16.25	1.13	33.27	2.36	9.81	0.19	55.3	2.5	7.88	0.07
N-3	Up		16.38	0.82	17.91	3.25	9.72	0.42	196.2	18.5	8.02	0.06
	Down		17.17	1.11	21.20	3.67	9.61	0.61	268.7	17.8	7.98	0.12
N-4	Up		13.89	0.68	35.29	2.68	9.68	0.33	396.9	12.9	7.77	0.07
	Mid		15.84	0.96	32.70	3.56	9.85	0.28	234.1	6.2	8.14	0.06
	Down		19.33	1.30	37.01	5.88	9.68	0.53	222.5	7.0	8.38	0.10

Seasonal Fluctuations of eDNA

Studies on eDNA had not considered the importance of sampling season in evaluating eDNA signature of aquatic organisms until recently. In addition to Spear et al. (2015), which detected seasonal change in hellbender eDNA, de Souza et al. (2016) reported significant associations between seasonal activities and detection probabilities of Black Warrior waterdog (*Necturus alabamensis*) and the flattened musk turtle (*Sternotherus depressus*). Hinlo et al. (2017) studied 3 invasive fish species in Australia, common carp (*Cyprinus carpio*), redfin perch (*Perca fluviatilis*), and Oriental weatherloach (*Misgurnus anguillicaudatus*), and also reported that eDNA concentrations were significantly affected by sampling season for common carp and redfin perch but not for Oriental weatherloach. Our results concur with these recent studies and emphasize the importance of evaluating seasonal eDNA fluctuations, in particular for our case, in relation to breeding season of target species.

In Pennsylvania, hellbenders typically breed in late August to early September (Smith 1907). As detected in streams in North Carolina (Spear et al. 2015), we also detected sharp increases in eDNA estimates during September in all of the tributaries with known hellbender records (Y-1 through Y-4). Such increases in eDNA estimates during breeding season suggest the possibility of successful reproduction, or at least suggest that animals are in reproductive conditions (Spear et al. 2015). A unique finding from the present study is the contrast in eDNA seasonal signature between the known and unknown tributaries; there were no eDNA increases during September in the downstream sites of N-1 and N-2, the tributaries without known records. There are 2 possible interpretations of this result: hellbenders migrated away from these sampling sites of N-1 and N-2 for breeding or animals stayed there but they were not in reproductive conditions. Our data rather support the former possibility because there were no increases in eDNA estimates and the detectability of eDNA notably declined in September in these 2 sites (Table 1). There is no consensus about breeding migration of hellbenders (Phillips and Humphries 2005). Breeding migration may be site- or population-specific, which may also be related to population size. Further studies are needed to examine the reproductive status and possible breeding migration of the newly found populations.

Diel eDNA Fluctuation

To our knowledge, no studies to date tested associations between diel activity of aquatic organisms and eDNA signature. We predicted that eDNA estimates would be higher at night because hellbenders are typically nocturnal

Table 5. Results of binary logistic regression analyses testing the effect of each of the stream environmental variables on the detectability (i.e., presence-absence) of hellbender environmental DNA (eDNA). We collected the stream environmental data from 13 sites across 8 tributaries of the West Branch Susquehanna River in central Pennsylvania, USA, twice a month (diurnal [D] and nocturnal [N]) from June through October 2014. Because stream environmental variables fluctuate daily and seasonally, we did not test the relationship between the average values of the environmental variables and the overall detectability of hellbenders. Instead, we selected the data from 3 sampling trips (Jul nocturnal, Aug diurnal, and Aug nocturnal) for the logistic regression analyses because the detectability of hellbenders was greatest during these sampling trips. β denotes a logistic coefficient of a predictive variable in a predictive equation. Negative β values indicate negative associations between predictive variables and the detectability of hellbenders.

		Temperature (°C)		DO (mg/L)		Current (cm/sec)		Conductivity (μS/cm)		pH	
Month	Time	χ^2	β	χ^2	β	χ^2	β	χ^2	β	χ^2	β
Jul	N	5.91	0.83	0.64	0.66	1.22	0.03	2.91	0.01	5.67	7.68
Aug	D	5.87	0.68	1.19	0.85	1.17	0.31	3.63	0.13	4.93	4.26
Aug	N	4.23	0.76	0.56	0.69	0.41	0.02	3.65	0.01	8.38	11.73

Indicates predictive models that are significantly better than the null model ($P < 0.05$, $P < 0.01$).

(Noeske and Nickerson 1979), and also degradation rate of eDNA tends to increase under greater ultraviolet-B radiation (Strickler et al. 2015). However, we found no significant effect of diel sampling timing on eDNA estimates (Fig. 2; Table 2). It is still possible that diel eDNA fluctuation can be detected in a captive population where environmental variables are regulated. However, in the field setting, such diel eDNA patterns, if they exist, are most likely too subtle to be detected against the noises caused by numerous other factors including water discharge, water flow, subtle difference in sampling locations, and movement of animals. Our results suggest that unlike seasonal sampling, diel sampling timing is not a critical factor for hellbender eDNA sampling; thus, future research effort should be allocated toward covering more sampling sites.

eDNA and Stream Environmental Properties

Using American bullfrog as a model species in a controlled greenhouse mesocosm experiment, Strickler et al. (2015) reported that eDNA degradation rate was slowest under the combination of lower temperature, lower ultraviolet-B radiation, and higher pH conditions, emphasizing the importance of evaluating environmental factors in interpreting eDNA profiles. However, our statistical analyses on the eDNA positive sites showed negligible effects of the stream environmental variables collected (pH, conductivity, water current, and dissolved oxygen; Table 2). Natural systems may be too complex for the subset of environmental variables to show their effects without controlling for others. However, we found that stream temperature was positively and pH was negatively associated with the presence of hellbender eDNA (Table 5). Hellbenders in general prefer cool environments (Williams et al. 1981). The positive association with stream temperature is likely to be an artifact of the sampling design. We sampled water from only downstream sites of the known tributaries, whereas we collected water samples from upstream and downstream sites of the unknown tributaries. Water temperatures of the upstream sites, where we did not detect any eDNA positives, were noticeably colder than the downstream sites (Table 4), which likely resulted in the positive association.

The negative association between pH and the presence of hellbender eDNA may be worth being further examined. There was little seasonal fluctuation in pH, and some of the sampling sites in N-3 and N-4 had pH >8.0, which is the upper limit of its normal range (Behar et al. 1996). These 2 tributaries run through heavily farmed areas where fertilizers and livestock likely feed to the streams the excess amount of nitrogen-based compounds like ammonia. As pH rises, the amount of un-ionized ammonia that is toxic to aquatic life increases. Environmental factors determining the hellbender distribution are likely to be multifaceted and complex, among which ammonia toxicity may be one of the important factors.

MANAGEMENT IMPLICATIONS

Hellbender populations are in a state of decline throughout their range. Despite the declining trend, the current population status is unknown or not continuously monitored in the majority of the streams, mainly because of the insufficient number of trained field herpetologists available to conduct traditional field surveys over this wide range. Our study, together with several recent studies, showed that analyses of stream water samples for eDNA is a reliable and time-efficient method to survey hellbenders. Based on our results, we recommend that eDNA analysis should be more widely used to survey and monitor hellbender populations. Stream water should be sampled monthly from July to October and should be analyzed with qPCR to assess reproductive status of the populations. Sampling timing within a day and stream environmental variables likely have negligible effects on eDNA concentration estimates; thus, sampling effort should be maximized by prioritizing covering a wider range over sampling timing and collecting environmental data. Further studies are needed to better characterize the newly found localities for the future management of the declining hellbender populations in Pennsylvania.

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