FINAL REPORT

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Developing a Coordinated Research Approach for Hellbender Conservation in the Northeast Region

Photo credit: **Steven Johnson**

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Executive Summary

Although the hellbender has been identified as a Species of Greatest Conservation Need by the Northeast Association of Fish and Wildlife Agencies (NEAFWA), there remain substantial data gaps in its distribution. Hellbender conservation efforts in the northeast are largely state-specific and could benefit from a more coordinated approach. Through this collaborative, multi-state project, our objectives were to 1) better document hellbender distribution in the northeast region through environmental DNA (eDNA) surveys, and 2) develop a communication framework and standardized methodologies to facilitate hellbender conservation efforts. In Year 1, our team established a communication framework and worked collaboratively to develop standard, optimized protocols for eDNA sample collection and analysis. Over the following two years, we collected eDNA samples from a total of 200 sites in New York (59), Pennsylvania (42), Maryland (22), West Virginia (15), and Virginia (62). Through an extraordinary amount of volunteer help, we used our NEAFWA funding more efficiently and exceeded our original target number of 130 sites by almost 50%.

We confirmed the presence of hellbender eDNA at 9 of 10 sites (90%) with recent hellbender observations and at 25 of 51 sites (49%) with historic records of the species. Additionally, we detected hellbender eDNA at 5 sites with anecdotal records and 34 sites with no previous records of hellbenders. Rock-turning surveys were conducted for 43 of the eDNA-negative sites. Hellbenders were found only at a single site, suggesting a low incidence (2.3%) of false negatives. Rock-turning surveys also were conducted at 31 of the eDNA-positive sites, and the species was detected at 11 of these locations. Based on the lack of contamination in our negative controls, we consider it unlikely that the remaining 23 sites represent false positives. More likely, the eDNA signal represents a low-density or upstream population. Across all sampling sites in NY, PA, and VA, eDNA concentrations were correlated positively with both detectability (i.e., number of positive eDNA sample replicates per site) and hellbender abundance, as estimated by rock-turning surveys.

Through this project, we engaged a total of 54 students and 53 citizen scientists in hellbender conservation, with 2,469 hours of volunteer effort contributed to sample collection and analysis. Importantly, our archive of frozen eDNA samples represents a ‘snapshot’ of entire biological communities at the time of sample collection. This archive is a valuable resource for future inventory and monitoring of native fauna, introduced species, and aquatic pathogens. Through the support of the Northeast Association of Fish and Wildlife Agencies, we have established an effective framework for region-wide hellbender conservation that has resulted in a better understanding of the species’ distribution, public engagement in conservation efforts, and tangible resources to benefit wildlife conservation in the northeastern U.S.

Introduction

The eastern hellbender (*Cryptobranchus alleganiensis alleganiensis*) is declining in many parts of its range [1] and has been identified as a Species of Greatest Conservation Need by the Northeast Association of Fish and Wildlife Agencies (NAFWA). The species' historic range in the northeast includes New York, Pennsylvania, Maryland, West Virginia and Virginia. Despite a significant amount of research effort, substantial gaps remain in our knowledge of the hellbender's current distribution, particularly in NY, PA and VA. Given the broad distribution and cryptic nature of this species, generating a comprehensive distribution map is challenging using traditional approaches. Conventional hellbender surveys rely on rock-turning, which is time-intensive, physically demanding, and potentially destructive to the species' microhabitat. In contrast, environmental DNA (eDNA) surveys can provide information about species occurrence (and potentially abundance) without disturbing sensitive habitat [2]. Such information is urgently needed to guide ongoing efforts to protect and restore wild hellbender populations.

Environmental DNA is a relatively new approach to determining the presence/absence of aquatic vertebrates in targeted locations [2]. In the past decade, eDNA has been used in aquatic systems to estimate species richness [3, 4], detect rare or threatened vertebrates [4-10], track species migrations [11], identify parasites [12], document potential routes of species invasions [13], and detect a diverse group of invasive species [14-25]. This approach has been successfully applied to detect stream-dwelling amphibians [26] and was recently used to discover previously unknown hellbender populations in North Carolina [27]. The hellbender is an ideal candidate for eDNA monitoring because it is rare, secretive, and presumed to be sensitive to disturbance. Furthermore, hellbenders exude multiple sources of DNA into the environment, including skin, feces, blood, spermatozoa, and eggs. Recent research indicates that concentrations of hellbender eDNA are elevated during the breeding season, likely due to a combination of external fertilization events, increased activity, and male-male aggression [27]. Thus, eDNA surveys conducted during the breeding season (Aug – Oct) may be more likely to detect small or remnant populations.

In contrast to presence/absence studies, relatively little research has focused on correlating eDNA quantity with estimates of animal abundance. Concentrations of eDNA have been correlated

with fish abundance in large lakes and closed mesocosms [28, 29], fish biomass in laboratory tanks and closed mesocosms [29-32], and the abundance and biomass of amphibians in streams [33]. However, numerous studies have failed to detect a consistent relationship between eDNA quantity and animal abundance or biomass, including in bullfrog tadpoles [30], great crested newts [34], and hellbenders [27]. This lack of correlation is unsurprising, given that eDNA concentration can be influenced by many variables, including stream volume, water temperature, pH, and UV exposure [35], as well as sample collection protocol [36] and extraction/processing methods [37]. Still, ongoing advancements in PCR technology, survey protocols, and data analysis may result in more reliable eDNA-based estimates of abundance.

In addition to knowledge of the species' distribution, hellbender conservation efforts would benefit from better coordination among researchers and population managers. In the northeast, hellbender conservation efforts vary by state and include population monitoring surveys, occupancy modeling, habitat augmentation, disease surveys, and 'head-starting' (i.e., captive rearing and release) programs. These efforts involve a diverse group of stakeholders, including state and federal wildlife agencies, universities, non-profit organizations, and zoos. Better coordination among states would facilitate knowledge transfer and increase the success and efficiency of these programs. Furthermore, data or biological samples often can be collected or used more efficiently through state partnerships. For example, researchers in VA are collecting blood from hellbenders for physiological research, and these samples could provide a DNA source for NY researchers studying population genetics.

The overall goal of this project was to create a better understanding of hellbender distribution in the northeast and increase coordination among researchers and managers working with this species. Our specific objectives were to 1) fill major data gaps in hellbender distribution throughout the northeast, 2) develop efficient, standardized protocols for hellbender monitoring, and 3) generate an archive of eDNA samples that can be used for future research. Through these collective efforts, the project directly addressed Topic 3 of the NAFWA's Regional Conservation Needs framework: Identify NE Species of Greatest Conservation Need Data Gaps, Design Data Collection Protocols, and Collect Data.

Methods

Site Identification

Sites for eDNA sampling were identified in coordination with state wildlife agencies using a combination of species records, anecdotal reports, and ground surveys to identify potentially suitable habitat. Sites with state-confirmed records of hellbenders within the last 5 years (at the time of eDNA sample collection) were considered "known", while those with older records were considered "historic". "Anecdotal" sites were those with questionable records of hellbender occurrence, regardless of when the information was recorded. These included citizen sightings or 'expert' reports where the original information was deemed unreliable (e.g., GPS coordinates located far from the waterbody). Finally, "potential" sites were those of apparently suitable habitat with no previous information regarding the presence or absence of the species.

Sample Collection and Filtration

Stream samples were collected between Jul 15 – Oct 15 in 2014 and 2015. Prior to sample collection at each site, all equipment was soaked in 10% bleach for 30 minutes, rinsed with tap water, and allowed to dry completely. Because they came into direct contact with eDNA filters, tweezers were sterilized with 50% bleach for 30 min and rinsed with distilled water. Bottles were rinsed an additional three times in stream water immediately before sample collection.

Surface water samples were collected 50 m downstream of suitable habitat, from areas of low stream flow whenever possible. A total of three samples (two liters each) were collected from each site, one from each bank, and a third from the middle. In a few cases, river conditions restricted the collection

Fig. 1. Filtration system for eDNA capture. *Top:* Stream water is collected in 1-L bottles and poured into filter cups attached to a vacuum flask. *Bottom:* The ultra-fine filter paper captures DNA, sediment, and other microscopic debris in the water.

of all three samples to a single bank. In all cases, the surveyor entered downstream of the collection site and faced the bottle upstream to avoid contamination. Bottles were immediately capped and placed in a cooler within 1 h of collection. Samples were transported to a hellbender-free facility, maintained at approximately 4°C, and filtered within 48 h of collection. Gloves were worn throughout the filtration process and were changed between sites. Each 2-liter sample was passed through a separate 0.45µm (pore diameter) filter paper (MoBio Laboratories Inc., Carlsbad, CA) using a vacuum filtration system (Fig. 1). Filter papers were removed from the filtration cup and folded using two pairs of tweezers. Folded filter papers were placed in a microcentrifuge tube with ~1 ml of 95% ethanol and frozen (≤-20°C) within 5 days. Samples were transferred to -80°C for long-term storage. Samples that took longer than 12 h to filter (due to high sediment loads) were discarded. One negative control (i.e., distilled water) was included in each filtering session, corresponding to 1-8 sites. All filtration equipment was bleach sterilized (as described above) in between sites.

Rock-Turning Surveys

Data from rock-turning surveys conducted within the last five years were compared to results of eDNA sampling. Because many of these surveys were conducted prior to this project, methodology varied among states. Given the variation in habitat type (e.g., round boulders versus thin rock slabs) and manpower among states, it is not feasible to standardize all aspects of rock-turning surveys. However, the RCN participants agreed to the following methodology for current and future rock-turning surveys of eDNA sites: surveys will begin 200 m downstream of the eDNA collection site and continue until either 1) a hellbender is found, 2) all suitable habitat has been surveyed, or 3) the collection site is reached. Because hellbenders often inhabit inaccessible habitats (e.g., large boulders), data are normalized to survey effort rather than area. We report hellbender abundance as the number of individuals caught per hour per survey team. We use survey-team-hours rather than person-hours

eDNA Extraction

Gloves were worn when handling all samples and were changed between sample sites. Extractions were performed on a laboratory bench sterilized with 10% bleach. Tweezers (used to handle filter papers) were sterilized by autoclaving or immersion in 50% bleach for 30 min and rinsed with

deionized water. Each filter paper was torn in two equal halves; one half was extracted and the other was transferred to a new tube of 95% ethanol for long-term storage (-80°C). This provided a 'back up' for each sample in case the extraction failed or the sample was subsequently compromised. Filter tips were used whenever directly pipetting the DNA sample. Samples were extracted using the DNeasy Blood and Tissue Kit (Qiagen Inc, Valencia, CA) as previously described [27]. Extracted DNA was stored at -80°C until analysis by qPCR.

PCR Analysis

Amplification of a species-specific region of the mitochondrial cytochrome b gene was performed using a quantitative PCR (qPCR) protocol, with primers and probe designed by Spear et al. [27]. A 15-µl qPCR reaction containing 7.5 µl Qiagen QuantiTecht Multiplex PCR NoRox Master Mix, 0.75 µl primer probe mix (8 µM each forward and reverse primer, 4 µM probe), 3.75 µl RNase-free water, and 3 µl sample was run on a Bio-Rad CFX96 real time PCR system coupled with a Bio-Rad C1000 Touch Thermal Cycler. The thermal regime was modified from Spear et al. [27], with an initial denaturing step of 95 °C for 15 minutes and 50 cycles of a 60 second denaturing step at 94°C followed by a 60 second annealing step at 53°C. Bio-Rad CFX Manager Software with an automatically calculated baseline was used to estimate starting DNA quantities for each sample. Samples (including negative controls) were randomly arranged on each 96-well plate, along with PCR negatives (RNase-free water) in 10% of wells. To ensure repeatability of results, 10% of samples were randomly selected for replicate runs. These samples were considered positive only if at least 2 replicate runs were positive.

Concentrations of eDNA are reported in nanograms (ng) per microliter (µl). In contrast, some studies report the total amount of eDNA captured by the filter paper. Because we extracted ½ of the filter paper and eluted into a final volume of 100 µl, the total amount of eDNA captured by the filter paper can be calculated by multiplying the concentration value by 200. For example, a concentration of 1×10^{-4} ng/µl would correspond to a total amount of 2×10^{-2} ng of eDNA captured by the filter paper.

Results

Public Engagement

Across all states, a total of 2,469 hours of volunteer effort was contributed to this project by 54 students and 53 citizen scientists (Table 1). These hours included 24 months of part-time (40%) volunteer effort by Co-PI Foster, who conducted all PCR analysis. About half (52%) of the samples in Virginia were collected by volunteers through a Citizen Science program coordinated by Co-PI Terrell (Fig. 2). This program included individuals from the Virginia Master Naturalists, as well as students from University of Virginia (Wise, VA), Southwest Virginia Community College (Richlands, VA), and Cornerstone High School (Abingdon, VA). All of the samples from eastern Maryland were collected by citizen scientists from the Susquehannock Wildlife Society. Finally, biologists from Western Pennsylvania Conservancy engaged a large number of volunteers in sample collection and rock-turning surveys.

Table 1. Quantitative measures of public engagement through this project*.

State	Students (#)	Citizen Scientists (#)	Volunteer Effort (h)
MD	-	3	98
NY	9	3	2,063
PA	-	35	100
VA	45	12	208
TOTAL	**54**	**53**	**2,469**

*Includes students and citizen scientists involved in eDNA sample collection, PCR analysis, and/or rock-turning surveys.

eDNA Concentration versus Detection Probability

Concentrations of eDNA were correlated (r = 0.329, P = 0.005) with detectability (i.e., # of positive replicates) across all sites in NY, PA, and VA (Fig. 2A). This relationship was not observed among WV or western MD sites, and eDNA concentrations of positive samples from these states were high compared to NY, PA, and VA (Fig. 2B). Given these inconsistencies, WV and western MD data were omitted from subsequent analyses.

eDNA Concentration versus Hellbender Abundance

Across all eDNA-positive sites in NY, PA, and VA, eDNA concentrations were correlated (r = 0.931, P < 0.001) with hellbender abundance, as estimated by the number of individuals caught per hour of rock-turning survey (Fig. 3A). This relationship also was observed when non-detections (i.e., sites with no hellbender observations) were omitted (r = 0.973, P < 0.001; Fig. 3B).

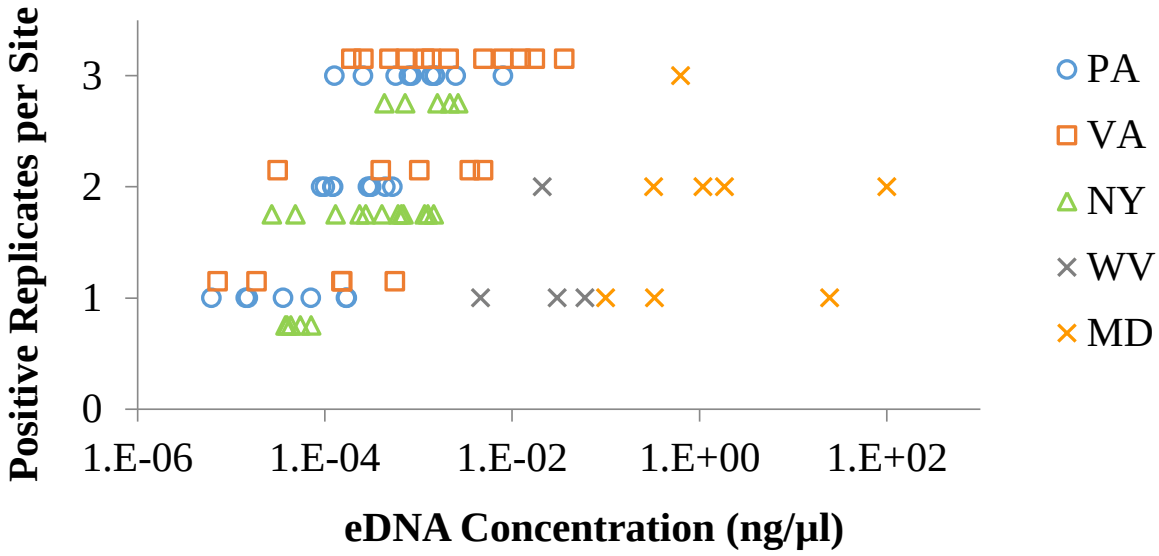
eDNA Results by State

Eastern Maryland

Volunteers from Susquehannock Wildlife Society (Darlington, MD) collected eDNA samples from a total of 12 sites in the Susquehanna River drainage of northeastern Maryland. Samples were collected at historic sites in Conowingo Creek (n = 3) and Octoraro Creek (n = 4), one anecdotal site in Peddler's Run, and potential sites in Deer Creek (n = 2) and broad Creek (n = 2). Historic records were from newspapers/books published >100 years ago. All 12 sites (100%) tested negative for hellbender eDNA.

Above: A citizen science volunteer collects eDNA samples in Tazewell County, VA.

A.



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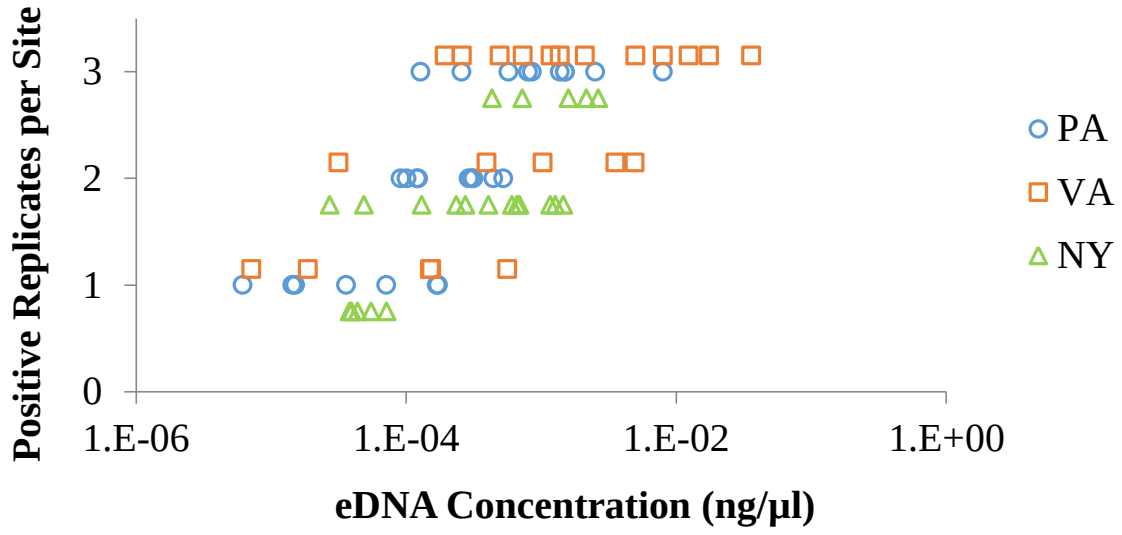


Figure 2. Detectability versus eDNA concentration (A) across all sites and (B) with suspect data from WV and MD omitted (r = 0.329, P = 0.005). Detectability is measured as the number of eDNA-positive replicates (1-3) from a given site. Data are jittered by state along the vertical axis.

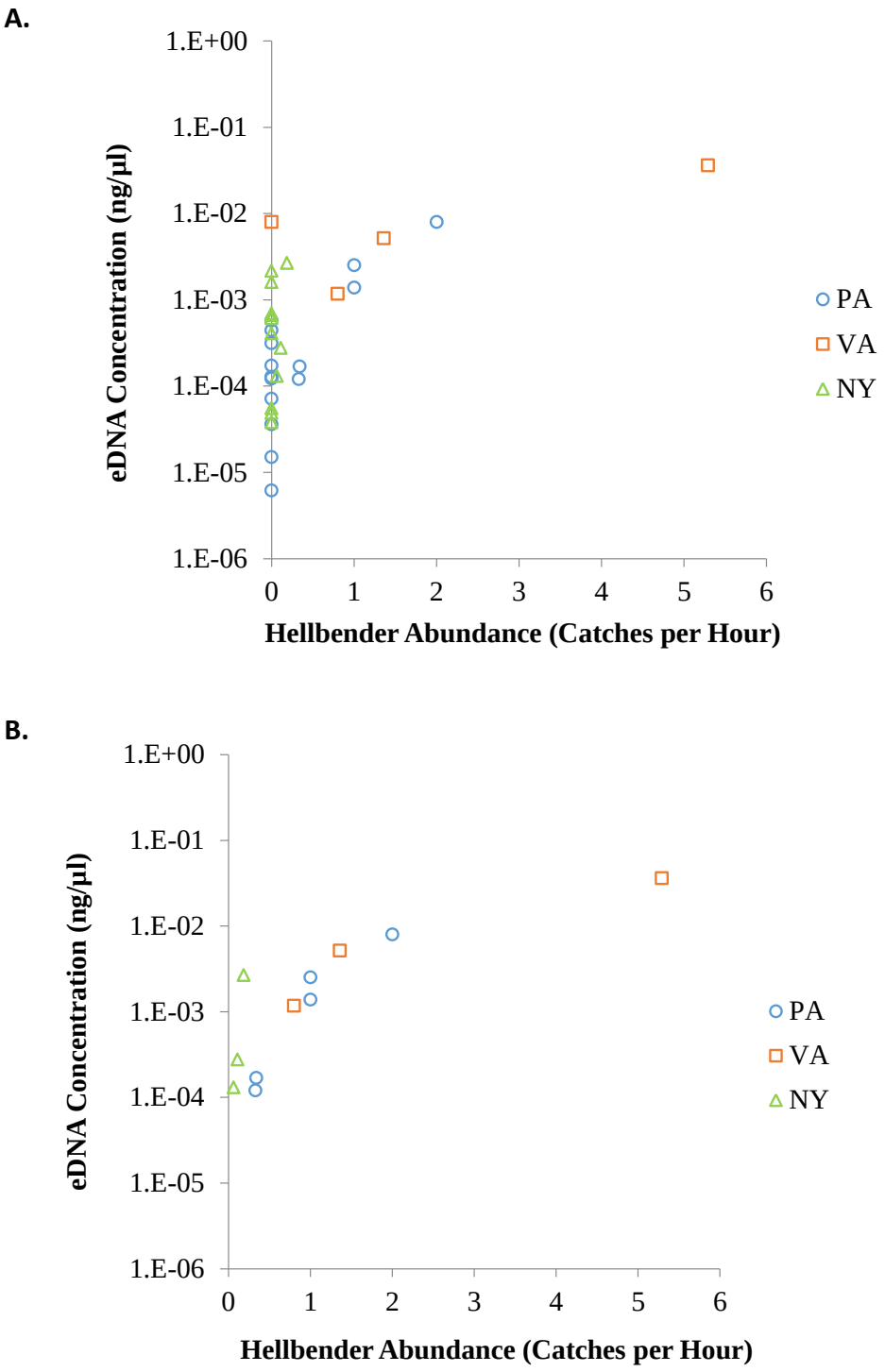


Figure 3. Concentration of eDNA versus hellbender abundance among (A) all eDNA-positive sites and (B) only sites where hellbenders were detected by both eDNA and rock-turning surveys ($r = 0.931$, $P < 0.001$ and $r = 0.973$, $P < 0.001$, respectively.

New York

Researchers from Buffalo State College and University at Buffalo (Buffalo, NY) collected eDNA samples from a total of 59 sites in New York's Allegheny and Susquehanna watersheds, representing 3 known, 22 historic, and 34 potential localities (Fig. 4). Of these sites, 22 (37%) tested positive for hellbender eDNA, including all known sites, as well as 10 historic and 9 potential localities. Among positive sites, eDNA concentrations (average of 3 replicates) ranged from 2.73×10^{-5} to 2.66×10^{-3} ng/µl. In most cases (77.3%), at least two of the three replicates tested positive.

Rock-turning surveys were conducted during the summer in 2014 and 2015 at 20 eDNA-negative sites and 9 eDNA-positive sites. Hellbenders were found only at three eDNA-positive sites, where they occurred at relatively low abundance (<0.2 catches per hour).

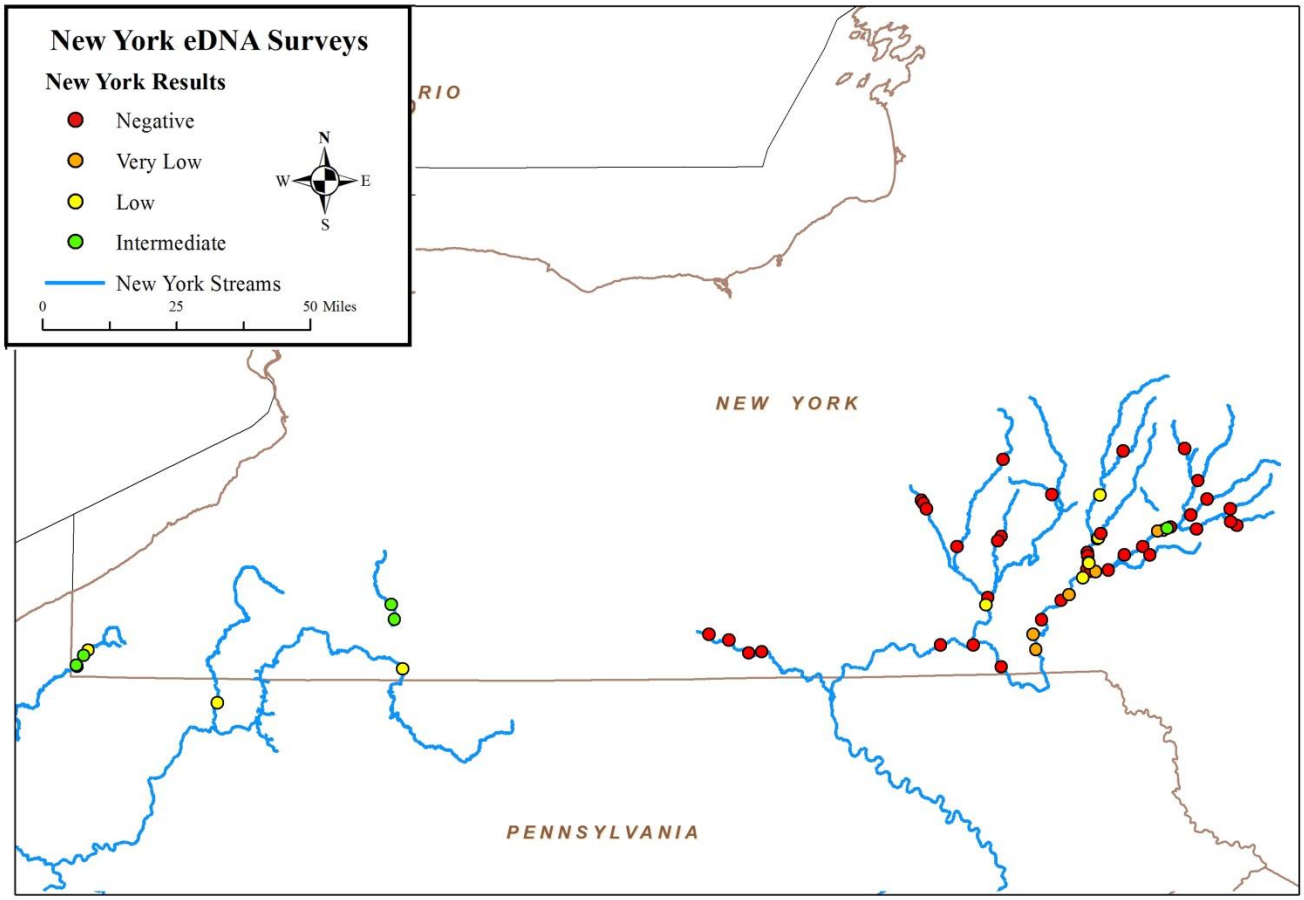


Figure 4. Results of eDNA sampling in New York. Sites testing positive had very low ($\leq 10^{-5}$), low (10^{-4}), or intermediate (10^{-3}) concentrations of eDNA.

Pennsylvania

Biologists from Western Pennsylvania Conservancy (Indiana, PA) collected eDNA samples from a total of 42 sites in the Allegheny and Susquehanna River drainages of north-central Pennsylvania (Fig. 5). These samples represented 4 known, 18 historic, and 20 potential sites. Of these sites, 26 (61.9%) tested positive for hellbender eDNA, including 3 known, 12 historic, and 11 potential localities. Among positive sites, eDNA concentrations ranged from 6.15×10^{-6} to 7.99×10^{-3} ng/µl. In most cases (73.1%), at least two of the three replicates tested positive.

Rock-turning surveys were conducted during the summer in 2014 and 2015 at 8 eDNA-negative and 15 eDNA-positive sites. Hellbenders were detected at 5 of the eDNA-positive sites, all of which had historic occurrence records of the species. Unexpectedly, hellbenders also were found at one eDNA-negative site, where they occurred at a moderate abundance (0.75 catches per survey hour).

Above: Eric Chapman and Alysha Trexler from Western Pennsylvania Conservancy conduct rock-turning surveys to validate eDNA results.

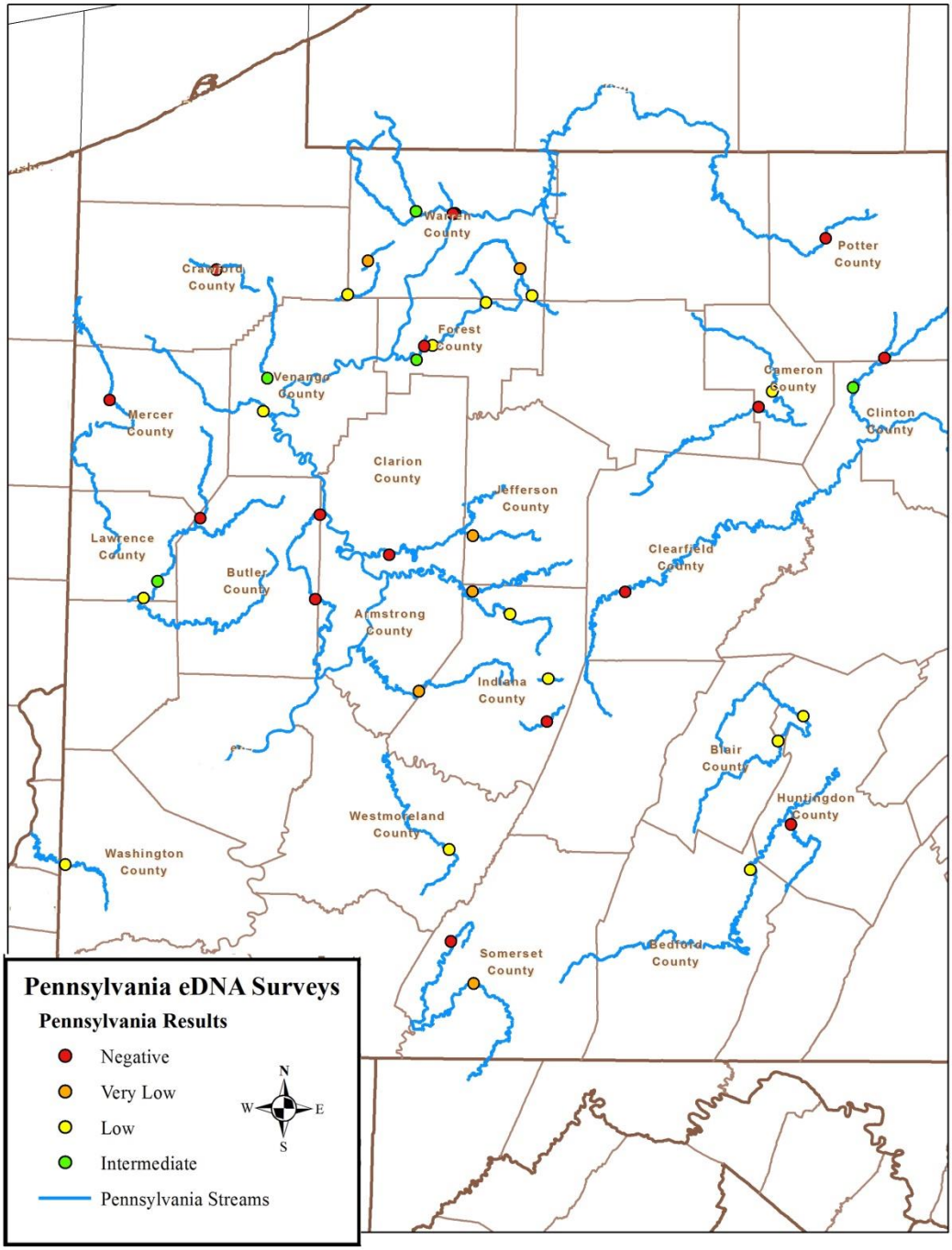


Figure 5. Results of eDNA sampling in Pennsylvania. Sites testing positive had very low ($\leq 10^{-5}$), low (10^{-4}), or intermediate (10^{-3}) concentrations of eDNA.

Virginia

A total of 62 sites were sampled for eDNA in the Big Sandy, Tennessee, and New River drainages of southwestern Virginia (Fig. 6). These samples were collected by Dr. Terrell and a team of citizen scientists from the Virginia Master Naturalists, University of Virginia-Wise, and Southwest Virginia Community College. Collectively, the samples represented 1 known, 4 historic, 7 anecdotal, and 49 potential sites. Of these sites, 21 (34%) tested positive, including the known site, as well as 3 historic, 5 anecdotal, and 14 potential localities. Among sites testing positive, eDNA concentrations (average of 3 replicates) ranged from 7.18×10^{-6} to 3.61×10^{-2} ng/µl. In most cases (81.0%), at least two of the three replicates tested positive. Rock-turning surveys conducted during the summer in 2013 – 2015 at 4 eDNA-negative sites and 2 eDNA-positive sites. Hellbenders were found only at the eDNA-positive sites, where they occurred at moderate to high abundance (0.8 – 5.3 catches per hour).

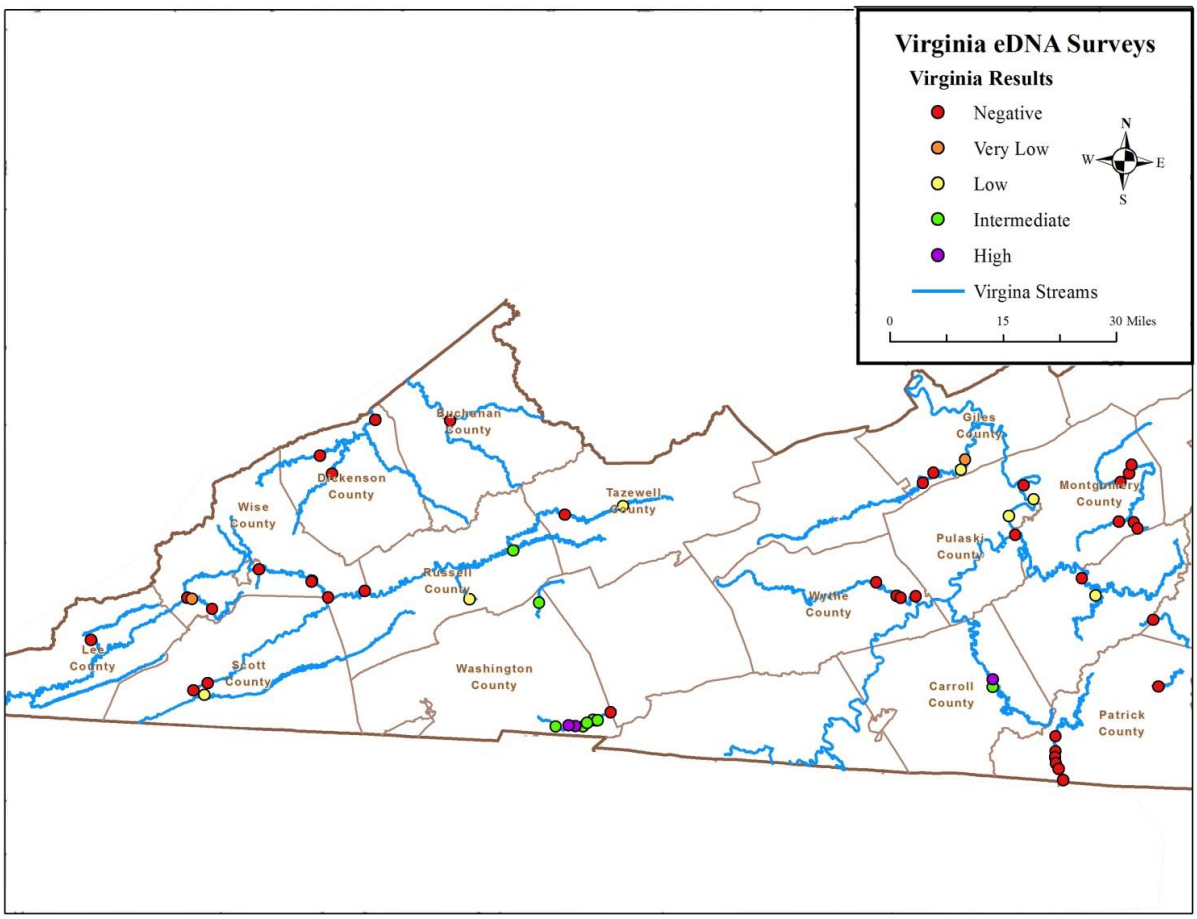


Figure 6. Results of eDNA sampling in Virginia. Sites testing positive had very low ($\leq 10^{-5}$), low (10^{-4}), intermediate (10^{-3}), or high ($\geq 10^{-2}$) concentrations of eDNA.

Western Maryland and West Virginia

In partnership with the Maryland Department of Natural Resources (DNR), co-PI Greathouse coordinated eDNA sample collection for 10 sites in the Casselman and Youghiogheny River drainages of western Maryland. Hellbenders are known to have occurred in both rivers historically. Eight of these sites tested positive for hellbender eDNA. However, eDNA concentrations ranged from 9.98×10^{-2} to 1.01×10^{2} ng/µl, equating to 3-4 orders of magnitude greater than those reported for NY, PA, and VA. In contrast to the other states, there was no relationship between eDNA concentration and detectability (i.e., # of positive replicates from a site). Rock-turning surveys were previously conducted at four of the eDNA sites (all of which tested positive) in the Casselman River by MD DNR in 2012 (1 – 4 survey hours per site, 9 total hours). A single hellbender was found at the Lower Big Bend site after four hours of surveying. No other hellbenders were found at any site.

Co-PI Greathouse also collected samples from a total of 15 sites in West Virginia, including 3 anecdotal and 12 potential localities. Four sites tested positive, and eDNA concentrations were relatively high, ranging from 4.61×10^{-3} to 5.99×10^{-2} ng/µl. In contrast to the NY, PA, and VA samples, only a single replicate tested positive at most (75%) of these sites. Rock turning surveys were conducted at all 15 sites, but failed to detect any hellbenders.

Given the lack of validation from rock-turning surveys and the exceptionally high eDNA concentrations in the western MD and WV samples, these data were considered unreliable and were omitted from correlations with eDNA detectability and hellbender abundance..

Above: Citizen Scientists from southwest Virginia learn how to collect eDNA samples during a training workshop in Tazewell County, VA.

Discussion

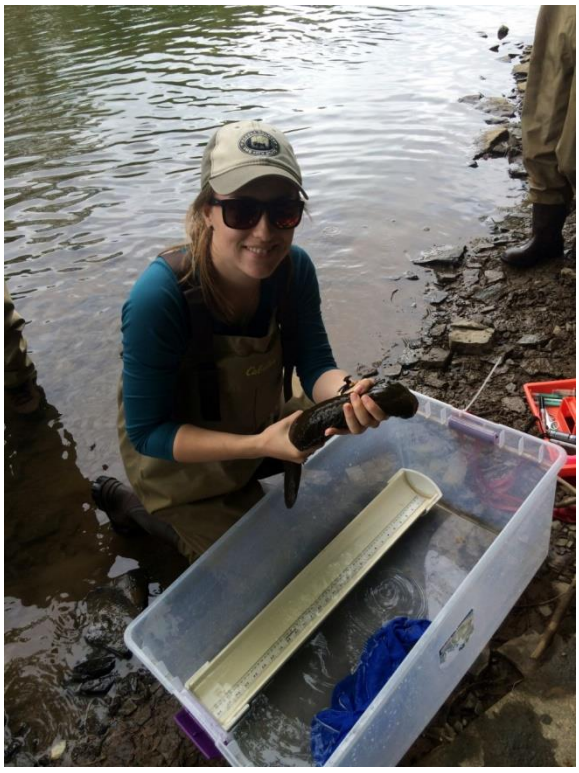
This project represents the broadest-scale eDNA survey of any *Cryptobranchid* to date, with 200 sampling sites distributed along more than 500 miles of northern Appalachia. We confirmed the presence of hellbender eDNA at 9 of 10 sites (90%) with recent hellbender observations and at 25 of 51 sites (49%) with historic records of the species. Additionally, we detected hellbender eDNA at 5 sites with anecdotal records and 34 sites with no previous records of hellbenders. Hellbenders were found only at one of 43 sites where eDNA was absent, suggesting a low incidence (3.2%) of false negatives. Rock-turning surveys also were conducted at 34 of the eDNA-positive sites, and the species was detected at 11 of these locations. Based on the lack of contamination in our negative controls, we consider it unlikely that the remaining 23 sites represent false positives. More likely, the eDNA signal represents a low-density or upstream population.

The strong correlation we observed between eDNA concentration and hellbender abundance (i.e., # caught per hour) was surprising, given the variation in physical and chemical characteristics among the sample sites. Furthermore, samples were collected and filtered by different groups of people in each state. In some states, as many as 57 different volunteers were involved in these sampling efforts over the course of the 2-year project. This finding contrasts with a recent study by Spear et al. that found no relationship between hellbender DNA quantity and abundance in North Carolina streams [27]. There are several possible explanations for these differences between the two studies. First, our sample size was larger than that of Spear et al. (n = 31 versus n = 23 sites), providing more statistical power to detect a correlation. Spear et al. had six sites with both hellbender capture and eDNA detection, compared to our 11 sites. Second, compared to the previous study, we collected 2× the volume of water per filter paper and 6× as much water per sampling site. Finally, all of our samples were collected during a 3-month period (July 15 – Oct 15) corresponding approximately with the fall breeding season, whereas Spear et al. collected samples during each calendar season [27]. Consistent with Spear et al., we observed a large number of field survey negatives at eDNA-positive sites [27].

Concentrations of eDNA also were strongly correlated with the number of eDNA-positive sample replicates per site. This relationship was remarkably consistent across NY, PA, and VA, given that samples were collected by many different people and extracted by different lab groups. In contrast,

samples from West Virginia and western Maryland that were collected by one group of researchers from The Wilds yielded findings that were inconsistent with the overall dataset. Specifically, eDNA concentrations of WV and western MD samples were increased by several orders of magnitude compared to other states. These concentrations did not correlate with eDNA detectability (i.e., # positive sample replicates per site) or hellbender catch per unit effort. Co-PI Adams is planning to conduct follow-up sampling this fall in western MD to attempt to independently validate these data.

In addition to generating broad-scale knowledge about the hellbender's distribution, this project generated direct, measurable benefits for public education and wildlife conservation. We engaged a large, varied group of citizen scientists in eDNA sample collection, with participants ranging from high-school students to retirees. Hellbender eDNA monitoring has proven to be a particularly effective and popular citizen science program because it 1) connects people with natural areas in their region, 2) does not require technical skills for sample collection, 3) results in a specific "yes or no" outcome, 4) has an obvious and direct benefit to species conservation, and 5) focuses on a charismatic species that is difficult to observe in nature. Conversely, the efforts of these volunteers substantially increased the impact of our project, by allowing an additional 70 sites to be collected. Importantly, the resulting archive of frozen eDNA samples represents a 'snapshot' of entire biological communities at the time of sample collection. These samples are a valuable resource for future inventory and monitoring of native fauna, introduced species, and aquatic pathogens. Through these collective efforts and the support of the Northeast Association of Fish and Wildlife Agencies, we have established an effective framework for region-wide hellbender conservation that has resulted in a better understanding of the species' distribution, public engagement in conservation efforts, and tangible resources to benefit wildlife conservation in the northeastern U.S.



Above: A student takes measurements of a hellbender collected during rock-turning surveys in New York.

References

1. Mayasich, J., D. Grandmaison, and C. Phillips, *Eastern hellbender status assessment report*2003, Duluth, MN: Natural Resources Research Institute.
2. Ficetola, G.F., et al., *Species detection using environmental DNA from water samples.* Biology Letters, 2008. **4**(4): p. 423-425.
3. Menotti-Raymond, M. and S.J. O'Brien, *Dating the genetic bottleneck of the African cheetah.* Proc Natl Acad Sci, 1993. **90**.
4. Carr, D. and A. Newell, *The role of A-kinase anchoring proteins (AKaps) in regulating sperm function.* Soc Reprod Fertil Suppl, 2006. **63**.
5. Lodge, D.M., et al., *Conservation in a cup of water: estimating biodiversity and population abundance from environmental DNA.* Molecular Ecology, 2012. **21**(11): p. 2555-2558.
6. Luo, R., et al., *SOAPdenovo2: an empirically improved memory-efficient short-read de novo assembler.* GigaScience, 2012. **1**.
7. *Liu B, Shi Y, Yuan J, Hu X, Zhang H, Li N, et al. Estimation of genomic characteristics by analyzing k-mer frequency in de novo genome projects. arXiv preprint. arXiv:1308.2012*.
8. *Smit AF, Hubley R, Green P. RepeatMasker Open-4.0. http://www.repeatmasker.org.. 2013-2015*.
9. Birney, E., M. Clamp, and R. Durbin, *GeneWise and Genomewise.* Genome Res, 2004. **14**.
10. Nawrocki, E.P., D.L. Kolbe, and S.R. Eddy, *Infernal 1.0: inference of RNA alignments.* Bioinformatics, 2009. **25**.
11. Altschul, S.F., et al., *Gapped BLAST and PSI-BLAST: a new generation of protein database search programs.* Nucleic Acids Res, 1997. **25**.
12. Griffiths-Jones, S., et al., *Rfam: annotating non-coding RNAs in complete genomes.* Nucleic Acids Res, 2005. **33**.
13. Lowe, T.M. and S.R. Eddy, *tRNAscan-SE: a program for improved detection of transfer RNA genes in genomic sequence.* Nucleic Acids Res, 1997. **25**.
14. Jerde, C.L., et al., *"Sight-unseen" detection of rare aquatic species using environmental DNA.* Conservation Letters, 2011. **4**(2): p. 150-157.
15. Cingolani, P., et al., *A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of Drosophila melanogaster strain w1118; iso-2; iso-3.* Fly, 2012. **6**.
16. Brown, P.R., et al., *A-kinase anchoring protein 4 binding proteins in the fibrous sheath of the sperm flagellum.* Biol Reprod, 2003. **68**.
17. Pontius, J.U., et al., *Initial sequence and comparative analysis of the cat genome.* Genome Res, 2007. **17**.
18. Dejean, T., et al., *Improved detection of an alien invasive species through environmental DNA barcoding: the example of the American bullfrog Lithobates catesbeianus.* Journal of Applied Ecology, 2012. **49**(4): p. 953-959.
19. Jurka, J., et al., *Repbase Update, a database of eukaryotic repetitive elements.* Cytogenet Genome Res, 2005. **110**.
20. Edgar, R.C., *MUSCLE: multiple sequence alignment with high accuracy and high throughput.* Nucleic Acids Res, 2004. **32**.
21. Benson, G., *Tandem repeats finder: a program to analyze DNA sequences.* Nucleic Acids Res, 1999. **27**.
22. Cunningham, F., et al., *Ensembl 2015.* Nucleic Acids Res, 2015. **43**.
23. Miki, K. and E.M. Eddy, *Identification of tethering domains for protein kinase A type I α regulatory subunits on sperm fibrous sheath protein FSC1.* J Biol Chem, 1998. **273**.
24. Santos, S., R. Chaves, and H. Guedes-Pinto, *Chromosomal localization of the major satellite DNA family (FA-SAT) in the domestic cat.* Cytogenet Genome Res, 2003. **107**.

25. Tesler, G., *Efficient algorithms for multichromosomal genome rearrangements.* J Comput Syst Sci, 2002. **65**.
26. Goldberg, C.S., et al., *Molecular Detection of Vertebrates in Stream Water: A Demonstration Using Rocky Mountain Tailed Frogs and Idaho Giant Salamanders.* PLoS ONE, 2011. **6**(7): p. 5.
27. Nielsen, R., et al., *SNP calling, genotype calling, and sample allele frequency estimation from new-generation sequencing data.* PLoS ONE, 2012. **7**.
28. Jones, P., et al., *InterProScan 5: genome-scale protein function classification.* Bioinformatics, 2014. **30**.
29. Griffiths-Jones, S., et al., *miRBase: microRNA sequences, targets and gene nomenclature.* Nucleic Acids Res, 2006. **34**.
30. Fanning, T.G., *Origin and evolution of a major feline satellite DNA.* J Mol Biol, 1987. **197**.
31. Stanke, M. and S. Waack, *Gene prediction with a hidden Markov model and a new intron submodel.* Bioinformatics, 2003. **19**.
32. Johnson, W.E., et al., *The late Miocene radiation of modern Felidae: a genetic assessment.* Science, 2006. **311**.
33. Lestrade, L. and M.J. Weber, *snoRNA-LBME-db a comprehensive database of human H/ACA and C/D box snoRNAs.* Nucleic Acids Res, 2006. **34**.
34. Li, H. and R. Durbin, *Fast and accurate short read alignment with Burrows–Wheeler transform.* Bioinformatics, 2009. **25**.
35. Paten, B., et al., *Cactus graphs for genome comparisons.* J Comput Biol, 2011. **18**.
36. Browning, S.R. and B.L. Browning, *Rapid and accurate haplotype phasing and missing-data inference for whole-genome association studies by use of localized haplotype clustering.* Am J Hum Genet, 2007. **81**.
37. Murphy, W.J., et al., *Dynamics of mammalian chromosome evolution inferred from multispecies comparative maps.* Science, 2005. **309**.