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HEALTH AND HABITAT QUALITY ASSESSMENT FOR THE EASTERN HELLBENDER (*CRYPTOBRANCHUS ALLEGANIENSIS ALLEGANIENSIS*) IN INDIANA, USA

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ABSTRACT: The eastern hellbender (*Cryptobranchus alleganiensis alleganiensis*) has experienced precipitous population declines throughout its range. Numerous factors are speculated to be involved, but no empirical evidence has been presented for any. We implemented a population-wide health assessment in Indiana, USA, examining both the physical well-being of individuals and the quality of their habitat. Physicochemical parameters were analyzed directly in the field and later in the laboratory, when appropriate. Samples were collected June 2008–October 2008 and June 2009–September 2009 for reproductive analysis, blood screening, and disease prevalence. Of 27 chemicals screened in water samples, three were found in the study site, including atrazine. Atrazine was found at levels reported to cause reproductive problems in other amphibians. Vitellogenin was detected only in females and proved a reliable indicator of sex. Sperm parameters were generally of high quality and similar to other populations. Most plasma parameters were similar between sexes, although there were significant differences in calcium and potassium concentrations. Abnormalities were common, occurring in 68% of individuals. No hemoparasites were found, but amphibian chytrid fungus (*Batrachochytrium dendrobatidis*) was detected on one individual. Our findings establish a baseline for hematology and water-quality parameters that can be used as a model for evaluating population health throughout the hellbender range.
Key words: Chytrid, hematology, reproduction, salamanders, sperm, vitellogenin, water quality.

INTRODUCTION

Amphibian populations throughout the world have been declining for several decades (Stuart et al., 2004). Although habitat destruction and climate change remain primary concerns among biologists, there is mounting evidence that disease plays a role in amphibian population declines (Daszak et al., 2003). Water quality can have a profound effect on individual health and potentially contribute to the continued decline of aquatic amphibian populations, many of which spend part or all of their lives in aquatic environments. Recent research suggests that pesticides and other contaminants may result in male feminization (Hayes et al., 2002a), decreased survival (Storrs and Kiesecker, 2004), and increased susceptibility to disease (Forson and Storfer, 2006). An amphibian chytrid fungus (*Batrachochytrium dendrobatidis*) found in many amphibian populations around the world may be partially responsible for the decline and extinction of infected populations (Wake and Vredenburg, 2008). We examined the relationship between water quality and animal health using a long-lived, obligate paedomorphic salamander, the eastern hellbender (*Cryptobranchus alleganiensis alleganiensis*).

Given its life history, the eastern hellbender is especially sensitive to water quality. Hellbenders are restricted to cool, rocky, flowing rivers and streams with high levels of dissolved oxygen (Smith, 1907). Flowing water facilitates optimal respiration via cutaneous oxygen diffusion (Ultsch and Duke, 1990). The effects of chemical contamination on hellbenders are unknown, but continual exposure to endocrine disrupting chemicals could lead to feminization in males and disrupt reproduction (Hayes et al., 2002a). Vitellogenesis is the process whereby yolk is deposited in developing eggs and the associated vitellogenin (VTG) protein is usually only present in females. Vitellogenin assays are used as a biomarker in fish to indicate exposure to estrogenic chemicals in the environment (Heppell et al., 1995). However, VTG production has been induced in the laboratory for several species of amphibians and reptiles (Palmer and Palmer, 1995). Vitellogenin assays are especially important for permanently aquatic salamanders that could be exposed throughout their lifetime and experience continuous exposure during the pesticide application season.

Chemical contaminants not only act as estrogen mimics but also exposure to certain commonly used agricultural chemicals can have significant effects on semen quality of human males (Hauser et al., 2002). Given the hellbender's fully aquatic nature and mode of reproduction, pesticide exposure could greatly reduce the species reproductive output. Many hellbender populations comprise primarily adults in older age classes and exhibit low reproductive output (Wheeler et al., 2003). Assessing the reproductive output of males is one method of addressing this lack of recruitment at the prezygotic stage.

Lastly, animal health assessments that incorporate hematologic screens, parasite analysis, and estimates of disease prevalence at the population level are critical to evaluate amphibian declines. Another measure of population health is estimates of physical abnormalities. Amphibian abnormality rates have recently been reported to exceed the expected rates of 0–5% (Johnson et al., 2002; Williams and DeWoody, 2009). Although some hellbender abnormalities are thought to result from intraspecific confrontations, abnormalities are reported at rates much higher than expected (Miller and Miller, 2005).

We address these questions within the context of declining hellbender populations in Indiana, which are now relegated to a single river within the Ohio River basin (Kern, 1984). The river passes through areas of intensive agriculture, including livestock operations and row cropping, which has raised concerns about how water quality may influence the health and persistence of the population. Our goals were to 1) monitor water-quality parameters, including physical, nutrient, and chemical properties on a weekly basis; 2) examine blood plasma for the presence of VTG and its use in gender determination; 3) establish baseline hematologic characteristics of captured individuals; 4) evaluate sperm quality; and 5) document the occurrence and frequency of physical abnormalities, parasites, and amphibian chytrid fungus.

METHODS

Study area and surveys

Surveys were conducted along a 112-km stretch of the Blue River in southern Indiana. The Blue River runs through a complex of agriculture and forest until connecting with the Ohio River. Adult hellbenders were captured across 40 sites selected as part of a separate mark-recapture study (Burgmeier et al., 2011). Samples were collected in September 2007, June 2008–October 2008 and June 2009–September 2009. For a detailed description of the study site and sampling regime, see Burgmeier et al. (2011).

Hellbenders were captured by hand or net after lifting all possible boulder-sized rocks within the stream. Individuals were given unique passive integrated transponder (PIT) tags for identification if recaptured. Stream flow conditions were evaluated before surveying to ensure conditions were within suitable ranges for effective surveys (depth, <1 m). Equipment was rinsed in a 1% bleach solution between daily surveys.

Water quality and analysis

Seven Indiana Department of Natural Resources (IDNR) survey sites were selected for continual water-quality sampling. These sites were spread throughout the study area and are representative of most land-use types surrounding the river. Physical and nutrient properties were sampled at all seven sites, and five sites were selected for a 27-chemical, multiresidue pesticide-screening of water samples (Table 1). Grab samples were taken once weekly using 500-ml Nalgene bottles for nutrient sampling and 1-l amber-glass bottles for pesticide samples. Water samples were collected from November 2008 through August 2009. All water samples were taken as close to the thalweg of the river as safely possible at a depth of >0.5 m. Water samples for pesticide analysis were shipped on ice within 24 hr of collection to A&L Great Lakes Laboratories, Inc. (Fort Wayne, Indiana, USA) and analyzed via EPA-525.2 Modified/NPD. A Quanta Hydrolab (Hach Company, Loveland, Colorado, USA) was used to determine the dissolved oxygen (mg/l, % saturation), specific conductivity (mS/cm), pH, temperature (C), and turbidity (nephelometric turbidity units [NTU]) at each site (Table 1). A Hach DR 2400 and a Hach DR 2800 (Hach Company) were used to analyze water samples for orthophosphate (mg/l PO_4^{3-}), ammonia-nitrogen (mg/l NH_3–N), and nitrate-nitrogen (mg/l NO_3^-–N).

Blood collection and analysis

From all hellbenders, including those previously captured, approximately 1.3 ml of blood was drawn from the caudal vein, 2.5 cm posterior to the cloaca, using a 25-gauge 1-inch (2.5 cm) needle (Solis et al., 2007a). Blood smears were made in the field and fixed in 100% methanol. Blood was placed in 3-ml heparinized vials and held on ice for analysis. A heparinized capillary tube was used to extract blood from each vial and then centrifuged at 12,000 × G for 2 min to assess packed red blood cell volume (hematocrit %). The remaining blood was centrifuged at 11,200 × G for 30 sec, and plasma and red blood cells were placed in separate tubes in liquid nitrogen for short-term storage. We used an Abaxis Vetscan Classic whole-blood analyzer and Avian/Reptile Profile Plus reagent rotor (Abaxis, Union City, California, USA) to conduct plasma-chemistry analyses for 12 parameters (Table 2).

Abnormalities, parasites, and amphibian chytrid fungus

Hellbenders were examined in the field for injuries, abnormalities, and ectoparasites. A Giemsa stain with azure blue was used to stain blood smears. Slides were scanned under a 10× lens for extracellular parasites and to determine smear quality, and 1,000 cells were examined under 100× oil immersion to rule out intraerythrocytic parasite infection. Duplicate slides were examined for half of the slides for quality control. Cotton swabs were used to sample the ventrum, feet, and mouth for amphibian chytrid zoospores following the protocol by Briggs and Vredenburg (2007). Swab samples were stored in 95% ETOH until submission to Pisces Molecular LLC (Boulder, Colorado, USA) for quantitative polymerase chain reaction (qPCR) analysis (Annis et al., 2004).

Vitellogenin

Plasma proteins (1–4 µg) were separated using denaturing sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) on 4% stacking, 8% resolving gels under discontinuous conditions. Rainbow trout (*Oncorhynchus mykiss*) VTG was used as a positive control (250 ng) for the phosphoprotein staining assay. Following electrophoresis, gels were stained with Pro-Q Diamond Phosphoprotein gel stain (Molecular Probes, Eugene, Oregon, USA) according to the manufacturer's instructions, and bands were visualized under ultraviolet light. The putative VTG protein band was excised from the gel, trypsin digested, and sequenced using matrix-assisted laser desorption ionization (MALDI)–time of flight mass spectrometry. Peptides were identified using Mascot (Matrix Science Ltd., Boston, Massachusetts, USA) with the National Center for Biotechnology Information (NCBI) protein database. According to this analysis, Mascot ion scores greater than 53 indicate significant homology ($P<0.05$). All samples were screened at least three times.

Sperm screening

Sperm samples were collected from eight adult males (five from 22 September 2008 to 24 September 2008; and three from 16 September 2009 to 19 September 2009). Sperm was collected from males actively producing milt and placed in 1.5-ml microcentrifuge tubes. All estimates of sperm quality (motility, viability, and concentration) were made in the field. Sperm motility was determined by placing 10 µl of milt on a slide and counting all motile sperm cells. Two hundred sperm cells were counted and expressed as the percentage of total motile cells. Sperm viability was quantified by staining with a 5% eosin solution, which preferentially stains dead cells (live cells are left unstained; Bearden and Fuquay 1999). A blow dryer was used to quickly heat-fix the stain to prevent sperm cells from slowly dying and absorbing the stain. Sperm concentration was calculated using a hemocytometer by mixing 1 µl of undiluted sperm with 9 µl of water. Sperm cells were then counted using a

TABLE 1. Water quality parameters and their estimated levels from the Blue River in Indiana, USA, November 2008–August 2009.[a]

Parameter	Detection limit	Mean±SD	Median	Range
DO (mg/l)	—	10.15	9.88	6.83–14.98
DO % saturation	—	94.2±11.51	95%	56–124
pH	—	8.11±0.21	8.16	7.53–8.66
SpC (mS/cm)	—	0.38±0.07	0.37	0.15–0.66
Temperature (C)	—	13.51±7.35	13.58	0.02–25.94
Turbidity (NTU)	—	9.31±15.48	4.40	0–82.5
Ammonia (NH_3–N) (mg/l)	0.01–0.5	0.02±0.03	0.02	0.00–0.33
Nitrate ($NO_3^- - N$) (mg/l)	0.1–10.0	1.59±0.76	1.60	0.00–3.50
Phosphate (PO_4^{3-}) (mg/l)	0.02–2.5	0.21±0.23	0.14	0.00–1.70
Atrazine (μg/l)	0.5	2.92±2.85	2.00	0.50–11.10
Metolachlor (μg/l)	0.5	1.46±0.65	1.40	0.60–2.70
Simazine (μg/l)	0.5	0.74±0.22	0.70	0.50–1.10
Alachlor (μg/l)	0.5	ND		
Acetochlor (μg/l)	0.5	ND		
Butylate (μg/l)	0.5	ND		
Clomazone (μg/l)	0.5	ND		
Cyanazine (μg/l)	0.5	ND		
EPTC (μg/l)	0.5	ND		
Ethalfluralin (μg/l)	0.5	ND		
Fluchloralin (μg/l)	0.5	ND		
Metribuzin (μg/l)	0.5	ND		
Pebulate (μg/l)	0.5	ND		
Pendimethalin (μg/l)	0.5	ND		
Prometon (μg/l)	0.5	ND		
Propachlor (μg/l)	0.5	ND		
Propazine (μg/l)	0.5	ND		
Trifluralin (μg/l)	0.5	ND		
Vernolate (μg/l)	0.5	ND		
Carbofuran (μg/l)	0.5	ND		
Ethyl chlorpyrifos (μg/l)	0.1	ND		
Diazinon (μg/l)	0.1	ND		
Ethyl parathion (μg/l)	0.1	ND		
Fonofos (μg/l)	0.1	ND		
Isofenphos (μg/l)	0.1	ND		
Malathion (μg/l)	0.1	ND		
Methyl parathion (μg/l)	0.1	ND		

[a] DO = dissolved oxygen; SpC = specific conductance; NTU = nephelometric turbidity units; ND = not detected at specified detection limit; EPTC = *S*-ethyl dipropyl carbamothioate.

phase-contrast microscope over five diagonal squares on the hemocytometer. The total amount of sperm was averaged, and a formula (*dilution factor* × *hemocytometer factor* × *number of sperm cells counted* × *conversion factor*) was used to calculate sperm cells per milliliter (Unger, 2003).

Statistical analysis

A Student's *t*-test was used to test for differences between genders for each hematologic parameter. Data were log or square-root transformed when normality assumptions were not met. If assumptions were not satisfied, a nonparametric Mann-Whitney *U*-test or Satterthwaite *t*-test was used. Student's *t*-tests and Satterthwaite *t*-tests were conducted using SAS statistical software (SAS Institute Inc., Cary, North Carolina, USA). Mann-Whitney *U*-tests were conducted in JMP (SAS Institute). Descriptive statistics (mean, variance, standard deviation, etc.) were developed using Microsoft Excel (Redmond, Washington, USA). Data are reported as mean±SD. For average pH, values were converted to their associated hydrogen ion concentrations; an average of those values was taken and was then converted back to pH (EIFAC, 1986).

TABLE 2. Comparison between genders for mean blood parameters of eastern hellbenders (*Cryptobranchus alleganiensis alleganiensis*) from the Blue River in Indiana, USA, September 2007–September 2009.[a]

Parameter	Males	Females	P-value[b]
Hematocrit (%)	34.37±7.59	31.65±6.18	0.0821
Aspartate aminotransferase (U/l)	74.52±38.1	76.48±47.12	0.8909
Bile acids (μmol/l)	<35*	<35*	—
Creatine kinase (U/l)	661.7±788.7	488.52±300.1	0.2732
Uric acid (mg/dl)	<0.3*	<0.3*	—
Glucose (mg/dl)	23.27±6.41	21.61±5.49	0.2297
Calcium^{++} (mg/dl)	7.89±0.58	10.93±2.1	<0.0001
Phosphorus (mg/dl)	5.14±1.33	5.11±1.36	0.8232
Total protein (g/dl)	3.15±0.62	3.19±0.56	0.7428
Albumin (g/dl)[c]	1.22 (<1)±0.08	1.19 (<1)±0.12	—
Globulin (g/dl)[d]	2.6 (>1.99)±0.36	2.34 (>2.03)±0.59	—
Potassium^{+} (mmol/l)	5.07±1.42	4.38±1.18	0.0239
Sodium^{+} (mmol/l)[e]	110.83 (<110)±0.75	111 (<110)±1.20	—

[a] Asterisk (*) indicates the value was out of the range of the whole-blood analyzer.

[b] Dash (—) indicates insufficient data for statistical comparisons.

[c] No. in parentheses is the detection limit. Of 60 male samples and 29 female samples, 54 (90%) and 22 (76%), respectively, were below this limit.

[d] No. in parentheses is the mean estimate based on the formula TP−ALB=GLOB (total protein−albumin=globulin), which was used for individuals with ALB_BCG (albumin measured by bromocresol green) levels below detectable limits that also had readings for total protein (46 males, 22 females). This would represent the minimum estimate for these samples.

[e] No. in parentheses is the detection limit. Of 54 male and 31 female samples, 48 (89%) and 23 (74%) samples, respectively, were below this limit.

RESULTS

Water quality, VTG, and sperm screening

Levels of physical parameters collected are listed in Table 1. Orthophosphate was present throughout the year, but the highest levels were present during summer at a mean of 0.38±0.24 mg/l. Dissolved oxygen was high throughout the year. Ammonia was present in the system at low levels (Table 1). The 27-chemical, multiresidue, pesticide screening of water samples detected three pesticides. Atrazine was detected from 1 April 2009 to 8 July 2009, metolachlor from 26 May 2009 to 30 June 2009, and simazine from 2 June 2009 to 12 June 2009, with June being the month of highest concentration (Fig. 1). These chemicals, and all others tested, were below detectable limits (<0.5 μg/l) the remainder of the year.

The MALDI sequencing of the putative (VTG) band (Fig. 2) resulted in a peptide that demonstrated significant homology with two known peptide sequences from the NCBI database. Peptide mass measured in the mass spectrometer correlated to a theoretical peptide, I/LQTDAVMALR, which showed strong homology to *Xenopus laevis* VTG A2 precursor (gi|139636) with a Mascot ion score of 62 and an NCBI protein Basic Local Alignment Search Tool (BLAST) E value 0.82. The second peptide sequence match was to that of *Xenopus laevis* VTG B1 (gi|155369239) with Mascot ion score of 62 and an NCBI protein BLAST E value 1.1.

Vitellogenin was used as a sex-specific biomarker on 73 hellbenders. Each individual was screened at least three times, and products were consistent among assays. Of the 62 individuals whose sex had been determined in the field, all 43 males were negative for the VTG band, and 18 of the19 females were positive. The VTG screening accurately matched 61 of 62 (i.e., >98% accuracy) to the field-identified gender. Of the 11 unknown individuals, seven males and four females were identified using the VTG band.

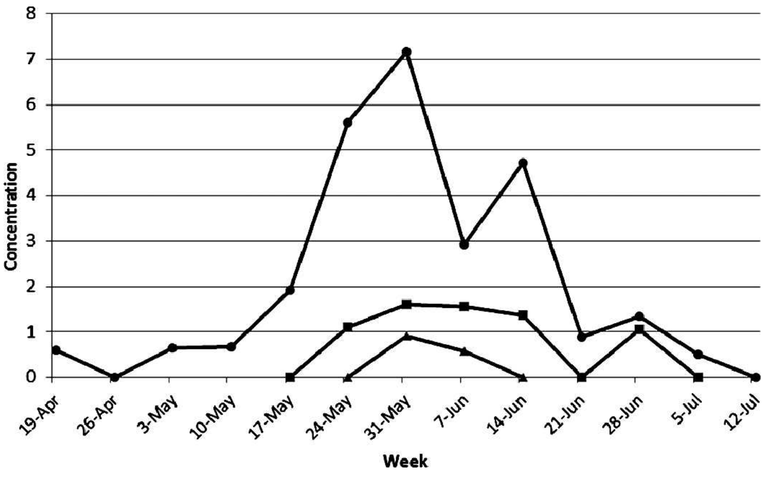

FIGURE 1. Mean pesticide concentration (μ/l) per week for three chemicals detected during weekly sampling of water from the Blue River, Indiana, USA, April 2008–July 2009. Circles = atrazine, squares = metolachlor, triangles = simazine.

Average sperm motility was 85.25± 21.30%. Sperm viability was high with 95.0±5.4% of sperm categorized as live with intact acrosomes. Sperm concentration averaged $31.4\times10^6\pm7.6\times10^5$ sperm/ml for both years. Collectively, sperm quality was high across all sampled individuals.

Hematology and blood chemistry

We collected 122 samples from 86 hellbenders over three seasons (53 male, 22 female, 11 unknown). Levels of most parameters were similar between genders (Table 2), except that calcium (t=5.26, df=33.9, P<.0001) and potassium (t=−2.3, df=80, P=0.0239) levels differed significantly between males and females. Three apparently sick hellbenders (one bloated, two emaciated) had notable differences from baseline population values. Two hellbenders, one emaciated and one bloated, had differences in hematocrit (%; a measure of packed red blood cell volume), aspartate aminotransferase (AST; elevated for all three), creatine kinase (CK; elevated, one bloated, one emaciated), glucose (GLU; depressed, both emaciated), and phosphorus (PHOS; elevated, one emaciated and depressed, one bloated). There was no apparent pattern in the respective ailment.

Abnormalities, parasites, and amphibian chytrid fungus

Of 88 hellbenders examined for abnormalities, 68% (n=60) had ≥1 abnormality, 47% (n=42) had ≥2, and 22% (n=19) had ≥3. Abnormalities included missing

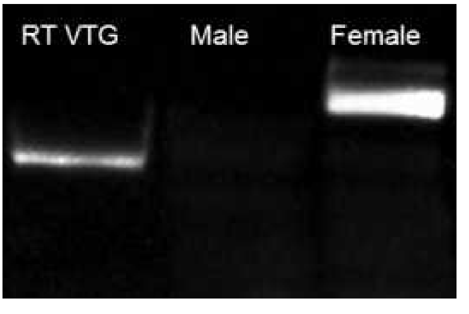

FIGURE 2. Sex determination in eastern hellbenders (*Cryptobranchus alleganiensis alleganiensis*) from the Blue River, Indiana, USA, using phosphoprotein staining of vitellogenin (VTG). Lane 1, positive control containing purified rainbow trout VTG (250 ng); lane 2, male hellbender plasma sample (1 μg of total protein), negative for VTG; lane 3, female plasma sample (1 μg of total protein), positive for VTG. The discrepancy in the position of the VTG band between the control and the female hellbender is due to the expected difference in band size between species.

digits and limbs, scars, open wounds, and abnormal or missing eyes. Missing or deformed digits were the most common, occurring in 52% (n=46) of individuals (Table 3). No significant difference in the number of abnormalities was detected between genders ($z=-0.997$, $P=0.32$). One hellbender appeared grossly distended nearly to the point of floating. This individual was observed in this condition in 2007 and 2008.

We analyzed 168 blood smears from 80 hellbenders and found blood to be free of hemoparasites. Of 80 samples from 77 individuals analyzed for amphibian chytrid fungus; one sample from an adult male was positive. Extracted blood was nearly clear, and that hellbender exhibited severe anemia and hypoproteinemia with a hematocrit value of 4% and undetectable protein levels. When compared with other individuals from the same population (Table 2), calcium (5.4 mg/dl) and creatine kinase (114 μg/l) levels were also greatly reduced.

DISCUSSION

Water quality

Habitat destruction and degradation are cited as the leading causes of decline in many wildlife populations, including amphibians (Stuart et al., 2004). An important component of degradation is decrease in the quality of aquatic systems due to anthropogenic chemical and nutrient input (Karr et al, 1985). Considering the hellbender's aquatic life history, it is a prime candidate for population decline due to poor water quality. All physical parameters we measured were well suited for aquatic life. Standard levels for orthophosphate in streams do not exist; however, the US Geological Survey (USGS) recommends levels of total phosphates be below 0.1 mg/l (Mueller and Helsel, 1999). Orthophosphate levels in our study were slightly higher than the recommended 0.1 mg/l of total phosphate by the USGS. Moreover, it has been suggested that the reference level for total phosphate in streams be decreased, and signs of eutrophication have been seen at levels far lower than we detected (Dodds and Welch, 2000). Levels of both orthophosphate and nitrate exceeded the US Environmental Protection Agency (USEPA)–recommended criteria for total phosphate and total nitrogen for ecoregion IX, subecoregion 71 of 30 μg/l and 0.8 mg/l, respectively (USEPA, 2002). Despite these levels, there were no noticeable signs of eutrophication (e.g., high densities of submerged macrophytes) even during low water, suggesting that eutrophication is not a major concern in the Blue River.

Atrazine, metolachlor, and simazine were present in low levels in late April to early July 2009. Exposure to ecologically relevant levels of atrazine may produce effects ranging from multiple gonads and demasculinization in male frogs to increased disease and parasite sensitivity in amphibians (Hayes et al., 2002a; Forsen and Storfer, 2006). Williams and Semlitsch (2009) reported that low-dose exposures to atrazine and metolachlor did not result in a significant increase in larval mortality in three species of anurans but noted that combinations of commonly used pesticides have elicited negative effects in some studies (Hayes et al., 2006; Relyea, 2009). Metolachlor and several other industrial chemicals have been detected in two Missouri rivers containing Ozark hellbenders (*Cryptobranchus alleganiensis bishopi*), but the effects of these exposures have not been studied (Solis et al., 2007b). The three pesticides we detected were present concomitantly for 2 wk, whereas atrazine and metolachlor were found together at detectable levels for up to 1 mo. Whether this combination of chemicals has manifested noticeable effects in the population will be difficult to study given the species protected status, but we found no detectable effects concerning the endpoints of VTG production and sperm quality. Despite the hellbender's life history and the

TABLE 3. Abnormalities in eastern hellbenders (*Cryptobranchus alleganiensis alleganiensis*) from the Blue River in Indiana, USA, the total number of each type, and the frequency of occurrence in individual animals captured between September 2007 and September 2009.

Type	Total per type	Frequency[a]
Missing/abnormal digits	64	0.52
Bite marks/scarring	32	0.28
Open wounds	21	0.18
Missing appendages	9	0.091
Other[b]	7	0.08
Eye injury	5	0.057

[a] Frequency of individual hellbenders with specified type of abnormality ($n=88$).

[b] Other injuries include, but are not limited to, bloating of the body cavity, unusual skin protrusions, and skeletal deformation.

high level of agricultural production around our study area, our results indicate that the water quality of the Blue River is not likely the reason for the apparent lack of reproduction and the continued decline of the population.

Vitellogenin and sperm screening

The potential for endocrine disruption in amphibians has been a growing concern in recent decades. Atrazine has been found in laboratory studies to induce effects including the castration and demasculinization of developing amphibian larvae (Hayes et al., 2002a, 2002b). However, Kloas et al. (2009) reported that atrazine had no significant effect on sexual differentiation. Unfortunately, the biomarkers used in those laboratory studies required sacrificing the individual for testing. For less-invasive field studies that require the survival of the animal, VTG has been proposed as a viable alternative, and its production has been induced in male herpetofauna at ecologically relevant doses of some pesticides (Palmer and Palmer, 1995; Brande-Lavridsen et al., 2008). However, on examining five previous studies, Rohr and McCoy (2010) found no effects on VTG production by atrazine. Vitellogenin production can end in males once exposure to a particular estrogenic substance has ceased (Hayes, 2005). We found no evidence of VTG production in known hellbender males. The absence of detectable VTG in males does not entirely eliminate endocrine disruption as a potential problem in the population, but it does suggest that it is likely not the cause behind the continued decline and apparent lack of reproduction.

Hellbenders in Indiana, USA, attain breeding condition in late August to early October. Outside of that 8–9 wk period, gender determination in the field is impossible. In the absence of poor water quality, however, VTG assay may be a valuable tool for sex determination because of the VTG female specificity. Our findings illustrate the utility of VTG as a means of hellbender sex determination up to 2 mo before the breeding season. Through intensive surveys and radiotelemetry, we positively identified the sex of 62 individuals captured throughout the study by traditional methods and matched those results with those found through our VTG assays. The only nonmatching individual was recorded as a female in the field but was negative for VTG. The most likely explanation is a gender misidentification in the field because the date of capture was near the beginning of the breeding season when males and females would typically be morphologically distinct. Another possible explanation is that the lack of VTG production was due to a lack of egg production. Little is known regarding the production of VTG in amphibians. Vitellogenin has been detected throughout the year in female Italian crested newts (*Triturus carnifex*; Zerani et al., 1991); however, the VTG cycle of female hellbenders is unknown outside of the sampling period of this study and requires further investigation.

Sperm quality may decrease with senescence in humans (Kidd et al, 2001). This is especially concerning because the Indiana, USA, hellbender population consists solely of adults 15 yr or older, and no signs of successful reproduction have been reported for 25 yr (Kern, 1984; Burgmeier et al., 2011). However, estimates of sperm health indicated reproductively active and "healthy" males. Overall motility and viability estimates were high, indicating that most hellbender milt sampled contained live, motile sperm cells. Sperm concentration estimates were on average lower, but similar to, previous estimates for eastern hellbenders from Missouri, USA, and North Carolina, USA (Unger, 2003). Variation in local, seasonal, male breeding conditions could explain these numbers. However, it is likely that these sperm concentrations, along with relatively high rates of motility and viability, are enough to fertilize a clutch of eggs.

Hematology and blood chemistry

We found few differences in blood chemistry between sexes in the Blue River hellbender population. Calcium levels in females were significantly higher than in males. Calcium levels have been associated with folliculogenesis in females, which likely explains the discrepancy (Campbell, 1996). Males, however, had significantly higher potassium, which, although not explicitly stated, was a pattern in Solis et al. (2007a). In general, our numbers were similar to those reported from populations of eastern hellbenders with a few noticeable exceptions (Solis et al., 2007a; Huang et al., 2010). In our study, AST, CK, and uric acid (UA) values were considerably lower than those found in Solis et al. (2007a), although only the AST and CK differed from that found by Huang et al. (2010). Aspartate aminotransferase originates from the liver, heart, and skeletal muscle and can indicate liver disease. Creatine kinase is found in muscles and can increase during stress, capture, and handling. Uric acid is a waste product in amphibians, reptiles, and birds, and increased levels can indicate dehydration and renal compromise associated with disease. Solis et al. (2007a) anesthetized their individuals and performed routine processing before blood draw, which could explain increased levels of CK and UA. Handling in Rock Pigeons (*Columba livia domestica*) was associated with substantial increases in CK and decreases in UA (Scope et al., 2002). However, Halliday et al. (1977) showed increased levels of UA in domestic chickens (*Gallus domesticus*). Baseline differences between populations might exist that explain all or part of the differences, but further research is needed to rule out handling-stress. Our numbers, in conjunction with those of Solis et al. (2007a), provide important baselines for assessing the individual and population health of hellbenders for future monitoring and management.

Abnormalities, parasites, and amphibian chytrid fungus

Abnormalities are common within amphibians (Blaustein and Johnson, 2003). Any population can be expected to exhibit a background abnormality rate of 0–5% (Meyer-Rochow and Asahima, 1988). However, hellbenders in Indiana, USA, exhibit a rate of 68.2%. Although high for most amphibian populations, it is similar to other studies of hellbenders that report rates as high as 90% (Hiler et al., 2005; Miller and Miller, 2005). Most injuries in the Indiana, USA, population seem to result from physical trauma rather than ontogenetic malformations. Hellbenders are long-lived, territorial, and regularly come into conflict for suitable shelter rock habitats. This, combined with contact with predators and human recreationalists, likely explains most abnormalities. It will be important to monitor the frequency and types of abnormalities in the future because the extreme types may indicate a serious underlying problem.

Parasite infections are routinely reported among many amphibian species (Wright and Whitaker, 2001). Although Ozark hellbenders have shown some signs of ectoparasites (leeches) and blood parasites, eastern hellbenders have shown few parasitic infections (Johnson and Klemm, 1977; Solis et al., 2007a). We found no ectoparasites or hemoparasites for hellbenders in our study. It does not appear that hemoparasites are a potential cause for the steady decline of hellbenders in Indiana, USA. None of the parasites we screened are those typically thought responsible for producing abnormalities in other amphibians (Johnson et al., 2002).

Amphibian chytrid fungus in naturally occurring wild populations is challenging, given its ability to spread quickly and eradicate entire populations (Lips et al. 2006). Amphibian chytrid fungus has been reported from hellbender populations in Arkansas, USA, and Missouri, USA, but no cases of symptomatic infections have been confirmed (Briggler et al., 2008). We found amphibian chytrid fungus on one hellbender. The individual showed no clinical signs of infection and was not one of the previously mentioned emaciated or bloated individuals; however, blood results revealed that the hellbender was severely anemic and hypoproteinemic. Voyles et al. (2009) reported a significant negative correlation between amphibian chytrid infection intensity and plasma potassium, sodium, and calcium levels in green tree frogs (*Litoria caerulea*). The infected individual from our study followed the same general pattern of reduced calcium and creatine kinase levels, but potassium levels could not be determined because of hemolysis, and the sodium was below detectable limits ($<$110 mmol/l). Based on the findings of hypoproteinemia and reduced calcium and CK levels, that individual was likely suffering from an underlying disorder, but a diagnosis could not be made, and the signs could not be linked to amphibian chytrid infection without further examination. Despite amphibian chytrid fungus being present at low levels, hellbenders have been regularly studied in Indiana, USA, for the past several decades with no reported symptomatic cases. It is unlikely that amphibian chytrid fungus is the major cause of the decline in Indiana, USA, but it will be important to monitor its presence to prevent or mitigate a future outbreak.

Management implications

Health issues are of growing concern to researchers because hellbender populations continue to decline. We provide a summary of several important factors often associated with amphibian health and effectively preclude them as the cause of the continued hellbender decline in Indiana. We found the Blue River maintains good water quality throughout the year. Hellbenders within the population seemed healthy with no signs of endocrine disruption, low levels of parasitemia, and high sperm quality comparable to known breeding populations from other watersheds. Comprehensive health assessments could provide a means of determining which factors potentially contribute to either the decline or the suppression of other hellbender populations and might serve to detect problems in otherwise healthy populations, so management actions can be implemented before decline. It is important to rule out all complicating variables when designing management plans to increase the chance of successfully fulfilling its goals. On a fine scale, our study provides important information for state agencies and management organizations in Indiana, USA, to evaluate the next step in hellbender conservation in the state. More broadly, we provide a framework for others looking to assess overall population health and eliminate potential causes of decline so future managers focus on the necessary components for successful conservation activities.

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