Innate immunity and stress physiology of eastern hellbenders (*Cryptobranchus alleganiensis*) from two stream reaches with differing habitat quality

William A. Hopkins , Sarah E. DuRant

Dept. of Fish and Wildlife Conservation, Virginia Tech, VA, USA

a b s t r a c t

In addition to depriving amphibians of physical habitat requirements (e.g., shelter, moisture, and food), habitat modification may also have subtle effects on the health of amphibians and potentially precipitate interactions with other deleterious factors such as pathogens, contaminants, and invasive species. The current study was designed to evaluate the physiological state of imperiled giant salamanders, the eastern hellbender (*Cryptobranchus alleganiensis*), experiencing different surrounding land use that influences in-stream habitat quality. When we compared hellbenders from a stream reach with greater anthropogenic disturbance to a more forested site, we found that baseline and stress-induced plasma levels of corticosterone were similar in the two areas, but were very low compared to other amphibians. Males consistently had higher plasma corticosterone levels than females, a finding congruent with the known territorial activities of males early in the breeding season. Innate immune responsiveness (measured as bactericidal ability of blood; BKA) was also similar at the two sites, but juveniles had less robust BKA than adults. We found a positive relationship between restraint time and BKA, suggesting that the bactericidal ability of hellbenders may improve following acute stress. Finally, there was a tendency for hellbenders with skin abnormalities to have higher BKA compared to individuals with normal integument, an observation consistent with patterns observed in other animals actively responding to pathogens. Our study provides foundational physiological information on an imperiled amphibian species and reveals important knowledge gaps that will be important for understanding the ecology, evolution, and conservation of hellbenders.

2011 Elsevier Inc. All rights reserved.

1. Introduction

Amphibian populations have experienced dramatic declines in recent decades [17,18], and in many cases the factors that underlie these declines are likely multifactorial [46]. Similar to other vertebrates, habitat loss and degradation is perhaps the greatest direct threat to many amphibians [15,46]. Habitat modification not only reduces or eliminates important resources (e.g., breeding sites, moisture, food) for amphibians, but may also have subtle effects on their health and precipitate deleterious interactions with other factors such as pathogens, contaminants, and invasive species. For example, changes in habitat structure can influence the production of glucocorticoids [19], hormones that are critical for blood glucose regulation and the ability of amphibians to respond to stressful or energetically demanding situations [9,16]. Chronic shifts in glucocorticoid levels also influence a myriad of other physiological processes including digestion, reproduction, and the immune system

[39]. Thus, it is plausible that degradation of amphibian habitats could lead to long-term physiological disruptions that may influence individual fitness and ultimately, if many individuals are adversely affected, population viability. Because of the complex ways in which anthropogenic disturbances can interact within different ecological contexts [26,31], basic information about physiological performance of amphibians in different environmental settings is needed to resolve key uncertainties underlying amphibian declines.

The decline of the hellbender (*Cryptobranchus alleganiensis*) in North America embodies many of the characteristics surrounding the plight of amphibians around the world. This long-lived (exceeding 30 years, [47]) and extremely large (up to 74 cm total length; [37]) salamander is fully aquatic and spends its entire lifecycle within cool, well oxygenated streams. Occurrence of juvenile and adult hellbenders is strongly associated with large, stable boulders or bedrock used for cover, feeding, and reproduction [2,32]. Although larval ecology of hellbenders is poorly understood, surveys suggest that hellbender larvae have additional habitat requirements that include the interstitial spaces of gravel and cobble bottom for early development (Personal observation, [33]). Across the species' range, which includes the Ozark (*Cryptobranchus*

Corresponding author. Address: Wildlife Ecotoxicology and Physiological Ecology, Dept. of Fish and Wildlife Conservation, Virginia Tech, 106 Cheatham Hall, Blacksburg, VA 24061, USA. Fax: +1 540 231 7580.

E-mail address: hopkinsw@vt.edu (W.A. Hopkins).

allegaiensis bishopi) and the eastern (*Cryptobranchus alleganiensis alleganiensis*) subspecies, degraded water quality and sedimentation that disrupts microhabitat are factors associated with population declines [3,12,28,51]. Widespread declines in the Ozark hellbender have generally been more severe than in the eastern hellbender [4,13,53], leading to recent proposed listing of the Ozark hellbender as federally endangered [Federal Register: 8 Sep 2010 (Vol. 75 No. 173)]. Recent evidence suggests that in addition to habitat degradation, other factors such as disease may play a key role in the decline of the Ozark hellbender [2–4,17,50]. However, whether habitat modification, physiological performance, and the manifestation of disease are related to one another remains unknown.

The current study was designed to provide basic background information about the physiological state of eastern hellbenders from sites experiencing different surrounding land use that influences in-stream habitat quality. Specifically, we sought to determine whether two different reaches of a single stream characterized by varying levels of land disturbance (development and agriculture) harbored hellbenders with different endocrinological or immunological profiles. We sampled hellbenders from these two reaches and quantified baseline and stress-induced plasma levels of glucocorticoids because of the known responsiveness of the hypothalamo–pituitary–interrenal axis to a wide range of disturbances [20,54,55]. To characterize innate immune function, we adopted a widely used assay to determine the ability of hellbender blood to kill a foreign pathogen [23,27,48]. Our work provides novel information about the physiology of hellbenders, which has rarely been studied, especially in the wild [45]. In addition, we provide one of the first assessments of the microbicidal capacity of an amphibian and how this innate immune response relates to acute stress.

2. Materials and methods

2.1. Site description

The stream selected for this study is one of several in the Tennessee River Basin currently being surveyed by our research team to understand the health and abundance of hellbenders in Virginia, USA. Because of the sensitive status of this species and threats of illegal collection, we are not permitted to reveal the name of our study stream. We selected this stream because it drains a predominantly forested watershed and still harbors a relatively large population of hellbenders, but is increasingly subjected to a wide range of surrounding land use that threatens in-stream water and microhabitat quality. The uppermost portion of the stream is mountainous, predominantly surrounded by forest, and has limited access by major roads. Progressing downstream 15 km, there is increasing runoff from agricultural fields, numerous pig and cattle farms that permit animals to enter the stream, increased rural development, and major roads that parallel and cross the stream frequently (Table 1). During significant rain events (>2 cm) small tributaries feeding these mid-reaches of the stream frequently exceed their banks and provide sediment to the stream in such high quantities that the water often transitions from very clear to nearly 0% visibility in <2–3 h. Progressing 20 km further into the lowermost reaches of this stream (not sampled in this study), the cumulative impacts of upstream anthropogenic activities result in heavy sedimentation and very little habitat for hellbenders. Our recent surveys demonstrated that hellbenders are still very abundant in the upper and mid reaches of the stream, but are sparse in the lowermost reaches [21]. Thus, for this study we delineated a 500 m reach in the uppermost (hereafter "forested") and middle (hereafter "impacted") sections of the stream for comparison (Fig. 1). Because it is possible that habitat in the impacted reach will continue

Table 1
Land use, microhabitat, and water quality of two stream reaches from which hellbenders were collected for this study. Land use and stream habitat were characterized using a 100 m reach centered within each study site. Embeddedness is presented as the mean (±1 SE) of all rocks measured in each reach. Water quality parameters are presented as the mean (±1 SE) of the 6–7 days we surveyed each site. Percent differences are presented for the impacted site relative to the forested site.

Parameter	Forested	Impacted	(%) Difference
Land use			
Watershed area (ha)	12,930	21,416	+65.6
Forest (%)	77.3	70.8	8.4
Developed (%)	3.3	3.8	+15.2
Agriculture (%)	19.1	25.1	+31.4
Microhabitat			
% Boulder (P256 mm)	49.0	12.0	75.5
% Embeddedness	25.7 (5.0)	35.3 (5.0)	+37.4
Water quality			
Water temperature	16.1 (0.11)	16.9 (0.17)	+5.0
Dissolved oxygen (%)	90.1 (0.31)	89.0 (0.99)	1.2
pH	8.7 (0.04)	8.6 (0.02)	1.1
Salinity	0.09 (<0.01)	0.10 (<0.01)	+11.1
Total dissolved solids	0.12 (<0.01)	0.13 (<0.01)	+8.3
Conductivity	153.83 (2.20)	172.71 (2.78)	+12.3

to be degraded in the future unless changes to land management are implemented, we postulated that this site also provided an opportunity to examine the health of hellbenders before further habitat degradation and localized hellbender population declines occur.

We characterized the land surrounding each stream reach and in-stream microhabitat of our two stream reaches to provide evidence of gradual degradation along a downstream gradient. Briefly, we characterized surrounding land use, relative to the midsection of each reach, as % land cover for the entire watershed using ESRI's ArcGIS v. 9.1 and National Land Use Cover Data from 2001 (http://www.mrlc.gov/index.asp). Land cover categories included open water, developed (rural/urban), barren land, forest, shrub/scrub, agriculture, and woody wetlands. Forest, agriculture, and developed lands accounted for >99% of all land cover at both study sites; thus, we only report these three categories to compare human disturbance at the two sites.

We selected a 100 m segment in the middle of both reaches to characterize in-stream microhabitat quality. To quantify the percentage of the substrate occupied by boulders large enough to conceal adult hellbenders (>25 cm diameter), we used a modified Wolman Pebble Count Method at 5 linear transects (0, 25, 50, 75, and 100 m) established perpendicular to stream flow. Because siltation causes rocks to become embedded and thus unfavorable habitat for hellbenders [12], we characterized the embeddedness of rocks along each transect by calculating the % of vertical rock height embedded in the underlying substrate. Finally, prior to our surveys each morning we measured standard water quality parameters using a YSI 556 MPS portable water probe (Yellow Springs, OH).

2.2. Hellbender surveys and blood collection

We collected hellbenders during diurnal surveys by turning cover objects while skin-diving, which is the best method for obtaining all age classes of hellbenders [22,34]. We collected all hellbenders between 8-July-2010 and 4-August-2010, between 0941 and 1812 h using an approved IACUC protocol (08-085-FIW). We conducted our study during mid/late summer because it represents the beginning of the breeding season in this stream, when male and female adult hellbenders can be distinguished by the swollen cloaca of males [25]. However, we completed our
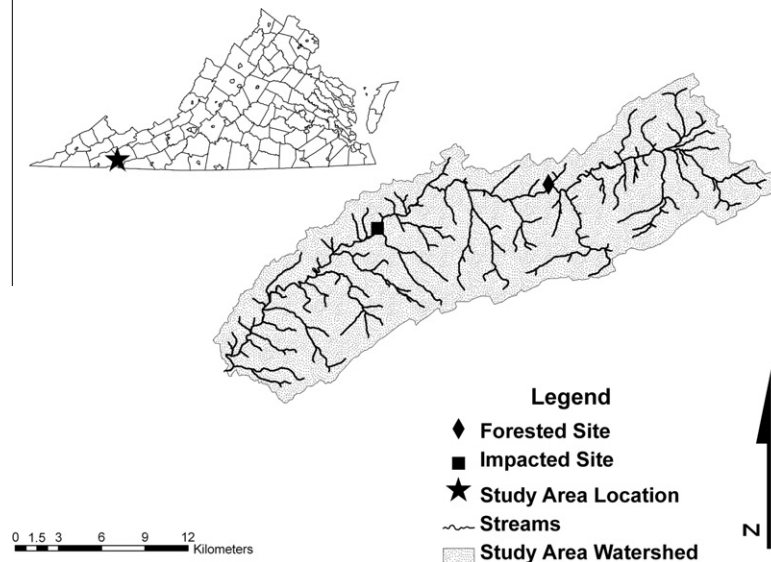
Fig. 1. Map showing locations of the two stream reaches in Southwest Virginia, USA sampled for hellbenders in this study. The downstream site is impacted by higher levels of surrounding landuse by humans that influences habitat quality within the stream.

study several weeks before oviposition typically starts in this stream (September) to avoid disturbing active nests.

Because blood samples were to be used for determination of baseline corticosterone concentrations, we had to capture, restrain, and bleed hellbenders within a few minutes [40]. This was achieved by bleeding hellbenders at their point of capture within the stream. We lifted each rock by hand or peavey, while a skin-diver captured the hellbender. We then transferred the hellbender to a team member responsible for restraining the individual with neoprene gloves on a flat plastic board with wet towels. The skin diver then retrieved a 1 cc blood sample from the caudal vein. A fourth team member used a stopwatch to keep track of handling time, recorded all pertinent data, and provided gear (e.g., syringes, sterilized towels, etc.) and logistical assistance. Using these techniques we were able to obtain an initial blood sample from >90% of individuals within 3 min (mean time for all individuals = 2.17 ± 0.11 min) of capture.

Once we collected the initial blood sample, we transported each hellbender to the stream bank. We measured total and snout-vent length (TL and SVL), weighed, and sexed (based on cloacal morphology of adults) each hellbender and subjected them to a physical exam. We classified hellbenders <30 cm TL (<19.2 cm SVL) as juveniles based on the average size at maturity reported in the literature [35,36]. Although the pivotal size we designated for maturity was likely accurate for most individuals, particularly females which mature later and at larger sizes than males [35,36], one small male (SVL = 18.1 cm) had a swollen cloaca suggesting that some males in this stream may reach sexual maturity at slightly smaller sizes. We counted and removed ectoparasites (leeches) from each hellbender and stored them in fixative. Distinguishing marks such as recent or healing injuries, scars, missing toes or limbs, or other aberrations were noted. In addition, we occasionally noted hellbenders with severely discolored skin blotches, typically gray in color, localized primarily around the junction of the limbs and torso, but also occasionally on the dorsum, head, and tail. We then injected a passive integrative transponder tag (PIT tag) into the tail of each hellbender for future identification. We bled hellbenders a second time (0.3–0.5 cc) at 30 min post-capture and then restrained them in a 3-l plastic container filled with fresh stream water where they remained until a third blood sample (0.3–0.5 cc) was collected at 60 min post-capture. We then

released each hellbender under the rock where it was initially collected.

We allocated a portion of all blood samples to a corticosterone assay, and smaller subset of samples at all three time points was used for a bacterial killing ability (BKA) assay. Whole blood to be used for BKA was immediately flash frozen in the field on liquid nitrogen. We placed blood for corticosterone analysis on ice and transported it back to the field house within 8 h of collection where it was centrifuged at 3.5g. We then removed plasma from the samples and froze it on liquid nitrogen for transport to the lab where we stored it at 80 C.

2.3. Enzyme immunoassay

We determined plasma corticosterone levels using enzyme immunoassay kits (Cat: No. 900-097, Assay Designs). These kits have low cross-reactivity with other major steroids such as cortisol (0.046%). To validate our assay, we tested for parallelism of serially diluted hellbender plasma and corticosterone standards. Hellbender plasma was pooled from several individuals with high plasma corticosterone and assayed at 1:2, 1:4, 1:5, 1:10, and 1:20 dilutions. Our test for parallelism confirmed that serial dilution of hellbender plasma was parallel with the standard curve.

We optimized the assay for hellbender plasma using a technique described in Wada et al. [52]. Based on our optimization results we determined that the hellbender samples should be analyzed at a 1:5 plasma dilution with 2% steroid displacement buffer. Each 96-well plate included a standard curve ranging from 15.63 to 2000 pg/ml with standards (non-specific binding and total binding) assayed in triplicate. Each plate also contained a 500 pg/ml standard assayed in quadruplet to allow us to estimate inter-plate variability. We ran samples in duplicate. Assay detection limit ranged from 0.033 to 0.083 ng/ml. When plasma corticosterone concentrations fell below the detection limit, which occurred in 32 out of 183 samples ($n = 25$ adults; $n = 7$ juveniles; 24 of which were BDL at the initial sample time), we used the plate's detection limit as the plasma corticosterone concentration for that individual. We calculated intra-assay variation as the average coefficient of variation across all samples within each plate and inter-assay variation across plates as the coefficient of variation of the 500 pg/ml standards. Intra- and inter-assay variation was 18.8%

and 9.9%, respectively. To further verify the validity of our technique, we also reran a subset of samples ($n = 15$) using radioimmunoassay (RIA) following the methods of Wingfield et al. [54]. However, this method was not sensitive enough to detect the low levels of corticosterone in hellbender plasma (Ignacio Moore, unpublished); only one of these samples fell above the RIA detection limit (1.34 ng/ml).

2.4. Bactericidal assay

To determine bacteria killing ability of hellbender blood we followed methods similar to those outlined in Liebl and Martin [23]. First, we optimized the assay for hellbenders to identify optimal incubation conditions as well as appropriate blood and bacterial dilutions. For the optimization we pooled four hellbender blood samples and ran them at three blood dilutions (1:5, 1:10, and 1:20). For each blood dilution, samples were exposed to one of 3 dilutions of *Escherichia coli* (10^4, 10^5, and 10^6). We reconstituted *E. coli* pellets in 10 μl PBS, incubated the solution for 30 min at 37 C, then vortexed the solution until the pellet was fully dissolved. We then achieved our target bacteria concentrations by further diluting with PBS. Next we incubated samples in triplicate for 1 h at one of three temperatures (16, 20 and 24 C). These temperatures were chosen because they are similar to the range of temperatures that hellbenders experience at our sampling sites during the months we collected blood samples (July–August). After the incubation period we added 250 μl tryptic soy broth (TSB) to each sample and prepared triplicate positive control samples. Positive controls contained 48.5 μl of either 10^4, 10^5, or 10^6 *E. coli* solution and 250 μl TSB. Next, we incubated all samples and positive controls at 37 C and measured the absorbance of samples using a nanodrop after 6, 8, 12, and 24 h incubation. We measured absorbance at a wavelength of 300 nm and killing was calculated as 1 − (sample absorbance/absorbance of control). We chose the blood dilution, bacteria concentration, and sample measurement time in which 50% of the bacteria were killed. Sample measurement time also corresponded with the inflection point for bacteria growth.

Based on the results of our optimization assay we ran hellbender samples in triplicate using the following procedure. To assess bacterial killing ability, we added 10^5 bacteria concentration to a 1:10 blood dilution and incubated the samples for 1 h at 20 C. We chose this temperature because our optimization demonstrated similar bacteria killing at all of the temperatures that we tested. We then added the TSB and allowed bacterial growth to occur for 8 h at 37 C.

2.5. Statistical analyses

We ran all statistical analyses in SAS 9.1 (SAS Institute, Inc., Cary, NC, USA) or Microsoft Excel and recognized statistical significance at $a < 0.05$. Landuse and habitat variables were not compared statistically between reaches because these measures were unreplicated. Likewise, water chemistry variables were not compared with statistical models because a single location within each reach was monitored repeatedly, leading to no spatial replication and low variance within each stream reach. Where appropriate, we tested for normality and homoscedasticity using Ryan-Joiners and Bartlett's tests, respectively. Unless otherwise noted, we used raw data in statistical analyses. Two individuals (a severely injured male and a female found free roaming in open water; see results and discussion for details) were not included in corticosterone and BKA statistical analyses because these individuals had unusually high (3–10-fold higher than other individuals) plasma corticosterone concentrations. Some of the juveniles were difficult to

obtain blood from and as a result, sample sizes for juveniles and their inclusion in statistical models varied (details below).

We calculated body condition by regressing $\log_{10}$-body mass against $\log_{10}$-SVL. We used the residuals from this regression as our estimate of body condition in statistical comparisons.

To determine whether baseline corticosterone or baseline BKA was influenced by the time it took to obtain an initial blood sample, we regressed each physiological variable against time (the time between capture and initial blood collection). We $\log_{10}$-transformed corticosterone values and time to better meet assumptions of normality and equal variance. Because circadian rhythms and seasonal variance influence a myriad of physiological processes, we also used repeated measures ANCOVA to determine whether time of day or sample date influenced baseline or stress-induced corticosterone levels or BKA.

To determine the effect of sampling site, restraint time (initial, 30 and 60 min), and sex on plasma corticosterone we conducted a repeated-measures ANOVA (SAS Proc Mixed). We $\log_{10}$-transformed corticosterone values to better meet assumptions of normality and equal variance. In the model, we included site, time (<3 min, 30 min, and 60 min), sex, and their two-way interactions as independent variables. We initially included $\log_{10}$-body mass and body condition as covariates in the statistical model, but they were dropped due to insignificance (in both cases $p \geq 0.18$).

We investigated the effects of sampling site and restraint time on bacteria-killing ability of hellbender blood using a repeated-measures ANOVA (SAS Proc GLM). Due to small sample sizes we were unable to test for the effects of sex (impacted: males $n = 5$; females $n = 7$; forested: males $n = 8$; females $n = 5$) on BKA, therefore males and females were pooled within sites for statistical comparisons. Site, restraint time, and their interaction were included as independent variables in the model. Initial models included $\log_{10}$-body mass and body condition as covariates, but we dropped these variables from final models because they had little influence on BKA (in both cases $p \geq 0.52$).

To determine whether individuals with discolored skin or ectoparasites had different baseline plasma corticosterone concentrations or BKA compared to individuals without these anomalies, we conducted a series of one-way ANOVAs. Due to small sample size of individuals with skin discoloration (e.g., for CORT; forested: $n = 3$ males; impacted: $n = 2$ males and 4 females) or ectoparasites (e.g., for CORT; forested: $n = 0$; impacted: $n = 3$ males, 3 females, and 3 juveniles), we combined individuals from both sites and sexes for statistical comparison. Only a single individual had both ectoparasites and skin discoloration.

We also tested for correlations between baseline corticosterone and baseline BKA using linear regression. We $\log_{10}$-transformed corticosterone values to better meet assumptions of normality and equal variance.

3. Results

3.1. Habitat and survey results

The watershed surrounding the impacted site was considerably larger than that surrounding the more forested upstream reach (Fig. 1; Table 1). Landcover in both areas was dominated by forest, but the percentage of land converted to agriculture and rural development increased downstream. Taken together, the larger percentage of disturbed habitat contained within a larger watershed results in much greater drainage from degraded lands. Whereas the watershed surrounding the more forested reach is surrounded by 2896 ha of agriculture/developed land, 6189 ha of modified land drains into the more impacted reach.

Siltation is a common side effect of converting forest to other land cover types for human use. Our downstream reach had considerably fewer boulders than the more forested site, and siltation in the downstream reach has caused available boulders to become increasingly embedded (Table 1). Qualitatively, siltation in the more impacted reach was also commonly evidenced by decreased water clarity after storm events and high sediment loading from small tributaries entering from surrounding agricultural fields. Despite these increased anthropogenic inputs into the stream, both of our sites had similar water quality parameters, with the exception of conductivity (Table 1).

In total, we captured 74 hellbenders at our two sites. At the forested site, we captured 11 females, 14 males, and 2 juveniles (total = 27). From the impacted site we captured 15 females, 12 males and 20 juveniles (total = 47). Our sampling effort was not equivalent at the two sites, so these sample sizes do not reflect relative abundance of hellbenders at the sites. Body mass of individuals ranged from 54 g to 1.03 kg, TL ranged from 19.3 to 56.5 cm, and SVL ranged from 12.5 to 35.5 cm. With all individuals included in a regression model, there was a strong significant positive relationship between hellbender mass and SVL ($r^2 = 0.98$, $p < 0.001$; Fig. 2).

3.2. Physiological measures

In most cases baseline blood samples were taken in <3 min [40], however it took longer to collect blood from 7 individuals (up to nearly 6 min). To determine whether or not we could include these individuals in our study, we relied on visual inspection of the data and linear regression (Fig. 3). There was no relationship between the amount of time it took to obtain an initial blood sample and baseline corticosterone (Fig. 3) or baseline BKA (Corticosterone: $r^2 = 0.002$, $p = 0.52$; BKA: $r^2 = 0.08$, $p = 0.58$). Based on these observations, these 7 individuals were retained in subsequent statistical comparisons of corticosterone and BKA. Neither the time of day when an individual was captured nor the date an individual was sampled influenced corticosterone concentrations (time of day: $p = 0.93$; date: $p = 0.39$) or BKA (time of day: $p = 0.18$; date: $p = 0.89$).

Hellbenders of both sexes and from both sites exhibited a pronounced increase in plasma corticosterone concentrations with restraint time (time: $F_{2,92} = 43.35$, $p < 0.001$; Fig. 4A). Post hoc comparisons revealed that plasma corticosterone concentrations

Fig. 3. The relationship between baseline plasma corticosterone concentrations of hellbenders and initial handling time (time between capture and blood sample). Males: $n = 23$; females: $n = 25$; juveniles $n = 18$. Regression was run on $\log_{10}$-transformed plasma corticosterone and time.

differed significantly from one another at all three time points ($p < 0.005$ in all cases). Individuals exhibited 30–59% and 97–228% higher plasma corticosterone concentrations at 30 min and 60 min post capture, (respectively) than at initial capture. There was no difference in plasma corticosterone concentrations of hellbenders between the two sites (Site: $p = 0.95$; site time: $p = 0.77$; sex site: $p = 0.34$;). However, we detected a strong effect of sex on plasma corticosterone (sex: $F_{1,46} = 12.62$, $p < 0.001$; Fig. 4A). At all time points, males had 41–85% higher plasma corticosterone concentrations than females (pooled site means ± 1 SE of plasma corticosterone for males vs. females, respectively: baseline: 0.30 ± 0.03 vs. 0.21 ± 0.02 ng/ml; 30 min: 0.42 ± 0.05 vs. 0.30 ± 0.04 ng/ml; 60 min: 0.86 ± 0.10 vs. 0.47 ± 0.06 ng/ml). Sexes did not differ in their responsiveness to capture (sex time: $p = 0.16$). In addition, we analyzed plasma corticosterone of several juvenile hellbenders from the impacted site (Fig. 4B). Because we only had blood samples from juveniles from one site and our sample size was relatively small, we simply report values for qualitative comparisons to adults. Mean ± 1 SE plasma corticosterone of juvenile hellbenders and sample size for each time point were as follows: baseline ($n = 15$): 0.35 ± 0.08 ng/ml; 30 min ($n = 7$): 0.88 ± 0.31 ng/ml; 60 min ($n = 7$): 1.10 ± 0.49 ng/ml. However, sample sizes were adequate to detect a significant effect of restraint time on juvenile corticosterone using repeated measures ($F_{2,12} = 11.14$; $p = 0.002$), with plasma corticosterone levels reaching an apparent plateau after 30 min.

The two individuals excluded from our statistical comparisons had plasma corticosterone concentrations notably higher than the rest of our study population. The first was an adult male from the forested site that had recently sustained severe injuries that included a large area of excised tissue from the dorsal surface of the tail that resembled a bite-mark and a 2.5 cm incision across the lower jaw that revealed underlying bone and muscle tissue. In addition, this individual had sustained serious injuries that had previously healed, which included a missing right eye, missing right limb, and partially excised hind right foot. This male's corticosterone levels were 4.46 (initial), 9.22 (30 min), and 12.48 (60 min) ng/ml. The second individual was an adult female from the impacted site that was captured while free roaming in open water, a behavior that we have not observed in 3 years of surveying this stream (Hopkins, pers obs). This individual had no external signs of trauma. It is possible that we accidentally flushed her while searching boulders downstream, which could have resulted in her roaming the stream for >5 h. Alternatively, she may have
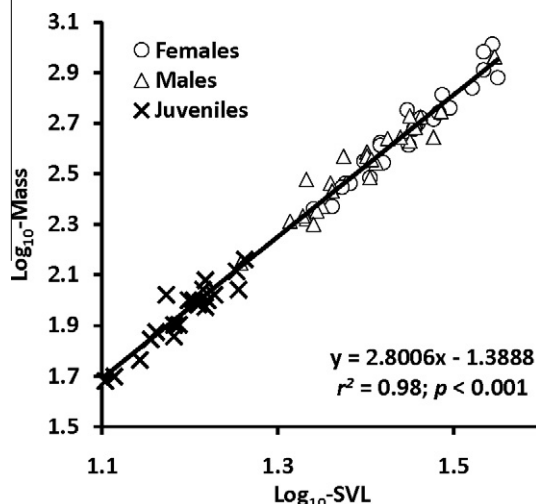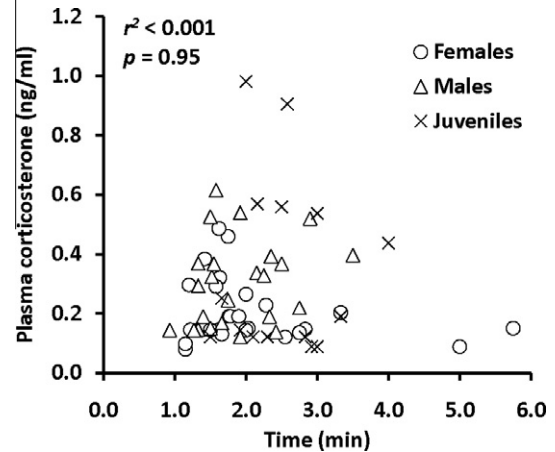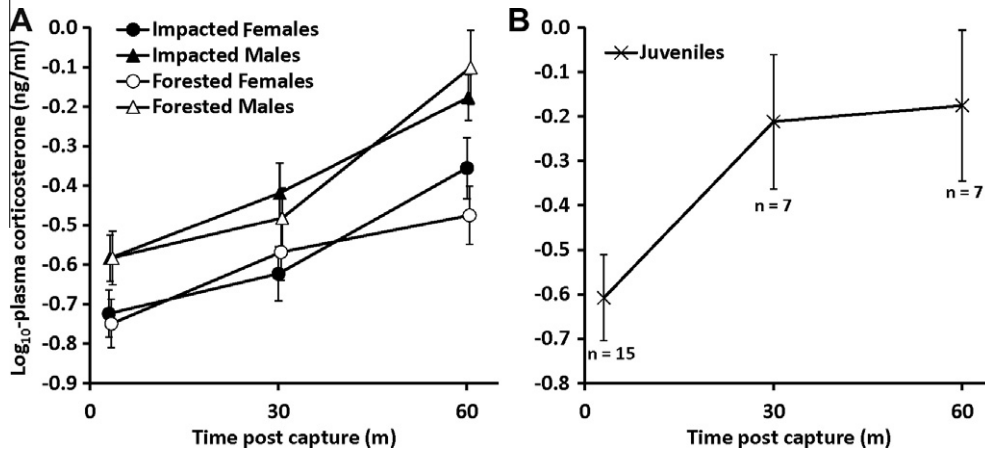

Fig. 2. The relationship between hellbender body mass and snout-to-vent length. Hellbenders were captured from two reaches of the same stream with differing habitat quality. Males: $n = 26$; females: $n = 26$; juveniles $n = 22$.

Fig. 4. Log$_{10}$-plasma corticosterone concentrations of adult (A) and juvenile (B) hellbenders at capture and at 30 and 60 min post-capture. Hellbenders were captured from two stream reaches of differing habitat quality (impacted and forested) within the same stream. Sample size for adults at all time points are as follows: impacted females $n = 14$ and males $n = 12$; forest females $n = 11$ and males $n = 13$. All juveniles were captured from the impacted reach of the stream. $n = 15$ at initial capture; $n = 7$ at 30 min post-capture; $n = 7$ at 60 min post-capture.

recently engaged in aggressive interactions with conspecifics, a common occurrence early in the breeding season as males are staking claim to breeding rocks [32]. This female's corticosterone levels were 0.99 (initial), 1.92 (30 min), and 2.85 (60 min) ng/ml.

All individuals exhibited a pronounced increase in their ability to kill bacteria with increased restraint time (Time: $F_{2,46} = 7.42$, $p = 0.002$; Fig. 5). The proportion of bacteria killed was 93–97% and 134–160% higher at 30 min and 60 min post-capture than at initial capture ($p = 0.05$ and $p = 0.003$, respectively). However, post hoc comparisons revealed that BKA at 30 and 60 min post capture did not differ from one another ($p = 0.25$). There was no difference in BKA among sites (site: $p = 0.50$; site time: $p = 0.72$). In addition, there was no relationship between baseline plasma corticosterone and baseline BKA ($R^2 = 0.038$, $p = 0.19$). We also determined BKA for several juvenile hellbenders from the impacted site, and report these data for qualitative comparisons. Mean ± SE proportion of *E. coli* killed by juvenile hellbenders and sample size for each time point were as follows: baseline ($n = 6$):

0.01 ± 0.08; 30 min ($n = 3$): 0.29 ± 0.11; 60 min ($n = 3$): 0.27 ± 0.05.

Several individuals at both sampling sites exhibited blotches of discolored skin (impacted: $n = 6$; forested: $n = 3$). In addition, 9 individuals from the impacted site harbored 1–24 leeches. No leeches were found on hellbenders at the forested site. There was no relationship between baseline plasma corticosterone and the presence of ectoparasites or skin discoloration (in all cases $p ⩾ 0.26$). Although statistically insignificant ($p = 0.11$), individuals with skin discoloration ($n = 9$) had baseline BKA twice as high as individuals without ($n = 38$) skin discoloration (mean proportion killed ± 1 SE discolored 0.36 ± 0.10 vs. normal 0.18 ± 0.05). There was no relationship between baseline BKA and the presence of ectoparasites ($p = 0.75$).

4. Discussion

Our study was designed to examine physiological characteristics of hellbenders in two physically distinct reaches of a stream that still harbors a robust population of these declining amphibians. Although the watersheds surrounding both reaches are predominantly (>70%) forested, the impacted reach is subjected to >15% and 30% more development and agriculture, respectively, compared to the upstream site. As a result of these anthropogenic activities, siltation has caused boulders to become 37% more embedded in the more impacted reach, decreasing the quality of microhabitat available for hellbenders. Although conductivity was the only notable difference in water quality during our surveys in this study (Table 1), we have previously documented significantly warmer water temperatures, lower dissolved oxygen, higher dissolved solids, and elevated conductivity in the more impacted site relative to the more forested area in late-summer surveys [21]. It is important to note that conversion of lands to agriculture and development could have a broad array of other effects on water quality including increased nitrogen, organic pollutants (e.g., pesticides and herbicides), and heavy metals, but quantification of such impacts was beyond the scope of our investigation. Despite the observed differences in land use and in-stream habitat quality, hellbenders are still abundant in the impacted reach. Thus, our impacted study site represents a moderately-impacted reach, falling within the large gradient of impacts present in this system (see site description).

Our study is the first to examine the stress physiology of wild hellbenders. Hellbenders from both stream reaches showed strong
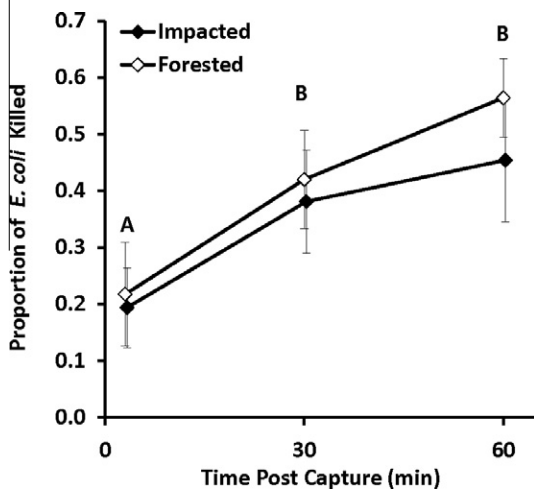

Fig. 5. The proportion of *E. coli* killed by adult hellbender blood collected at capture, 30 min, and 60 min post capture. Hellbenders were captured from two stream reaches of differing habitat quality (impacted and forested) within the same stream. Due to small sample sizes, sexes were combined for comparisons. Sample size at all time points are as follows: impacted $n = 12$; forested $n = 13$. Superscripts indicate which time points differed significantly from one another.

interrenal responsiveness to our restraint protocol, but overall plasma levels of corticosterone were quite low, even after 60 min of restraint. Average baseline corticosterone concentrations in hellbender plasma were below 0.3 ng/ml and average concentrations from adults at both sites did not exceed 1 ng/ml after restraint. These low levels were further confirmed by another lab using RIA on the same samples (see Results). The plasma levels of corticosterone are among the lowest concentrations reported for amphibians, even among salamanders which sometimes have very low baseline plasma levels of corticosterone [8,20,38,42,56]. Interestingly, we captured two hellbenders (see results) that were either severely injured or free-roaming in the stream, which provided fortuitous insight into the interrenal physiology of this species. Both individuals had corticosterone values 3–10 higher than the rest of our study population, clearly indicating that hellbenders have the physiological ability to achieve higher circulating corticosterone concentrations than what we observed in the other individuals sampled. The plasma concentrations in these two individuals were actually more similar to plasma concentrations in other salamanders (see above citations) than the rest of our sample population. It remains unclear whether hellbenders were relatively unresponsive or simply slow to respond to capture and restraint, but our observations beg for further studies using ACTH challenges and/or dexamethasone to better understand our unusual observations and how they relate to the interrenal physiology of hellbenders.

At both of our study sites, males had significantly higher baseline and stress-induced levels of corticosterone than females. This finding is most likely explained by the reproductive ecology of hellbenders. Most mating in our study system likely does not begin until September. However, when we sampled in July and August, male hellbenders were beginning to attain reproductive condition, as evidenced by their swollen cloacal morphology. At this time, males become more active and begin to aggressively stake claim to nest sites [32]. Males frequently exclude other hellbenders from optimal nest locations which are typically excavated under large flat rocks. Elevated corticosterone in males relative to females may be a byproduct of increased aggressive interactions instigated by males [29,44]. Corticosterone may even play an important permissive [41] role in the reproductive ecology of hellbenders. If males spend more time searching for, commandeering, and then defending nest sites at the onset of the reproductive season, corticosterone may be functionally important for maintenance of blood glucose levels that can sustain these energetically costly activities (e.g., see Hopkins et al. [20], Sapolsky et al. [41]. This would be particularly important if increased vigilance and territoriality results in decreased food consumption and the need to mobilize glucose from stores, but the relationship between hellbender feeding and reproductive ecology has not been studied.

We detected a marked increase in the BKA of hellbender blood as the stress associated with restraint increased. Similar work has demonstrated that innate immunity may be enhanced by acute physical stress or adverse social stimuli such as defeat [6,7,10]; but see [27,30], but our study is among the first to examine the relationship between stress and innate immunity in an ectotherm [14]. Our work was conducted early in the breeding season when hellbenders experience more frequent agonistic interactions with conspecifics, which can inflict injury [29] and be acutely stressful situations. Enhanced immunity after an acute stressor could be beneficial as it may aid in healing wounds and fighting bacteria at the wound site. Indeed, studies have demonstrated that exposure to an acute stressor results in redistribution of immune cells to areas likely to encounter pathogens, and several possible mechanisms may explain how acute stressors could act to enhance both innate and acquired immunity (reviewed in Dhabhar [10]). However, it is interesting to note that the two individuals (an injured

male and a wandering female, described above) that were clear outliers in their corticosterone profiles had BKA profiles that were similar to the rest of our study population. This anecdotal observation might be explained by differences in how acute and chronic stress influence immunity, and/or the manner in which different types of stressful stimuli are perceived by hellbenders [1,7,10].

There were notable similarities and differences in physiological profiles between age classes of hellbenders. Juvenile and adult hellbenders had similar interrenal and immune responses to capture and restraint; plasma corticosterone and the proportion of *E. coli* killed increased with time post-capture. Although juveniles had similar baseline and stress-induced plasma corticosterone concentrations as adults, they had relatively low blood BKA compared to adults at all time points. Similar effects of age on microbicidal capacity have been documented in stonechats (*Saxicola torquata*), where older birds have higher BKA than younger birds [49]. Because BKA was relatively low in all of the juveniles that we sampled, our results suggest that the microbicidal ability of hellbender blood improves as they mature. However, Tieleman et al. [49] also proposed the alternative hypothesis that differential juvenile survival based on juvenile microbicidal capacity could produce an adult population comprised of survivors with higher immune capabilities. Regardless of how ontogenetic shifts in immune function are interpreted, our findings are particularly interesting because juvenile recruitment is low and diseases are prevalent in declining Ozark hellbender populations (see below). Thus, future work on immune responsiveness of different aged hellbenders to pathogens may ultimately prove beneficial for their conservation.

Finally, we found no statistical relationships between skin abnormalities, ectoparasitism, and the two physiological parameters that we measured. However, we did observe noteworthy evidence of a relationship between skin discoloration and baseline BKA; individuals with patches of discolored skin had BKA twice as high as individuals without skin discoloration. This observation is consistent with other studies that have shown higher bactericidal activity in individuals testing positive for bacterial infection versus uninfected individuals (e.g., Desar et al. [11]). At this point it remains unclear what caused these skin abnormalities, but we highlight it here because integumental diseases have recently been documented in wild and captive Ozark hellbenders. For example, the fungus *Batrachochytrium dendrobatidis*, which has been implicated in amphibian declines around the world [24,43], is now known to be relatively widespread among Ozark hellbenders [2–5], and may contribute to their population declines. In addition, mysterious skin lesions, potentially caused by flesh-eating bacteria, are also under investigation in Ozark hellbenders (Jeff Briggler, Missouri Dept. Conservation, pers comm.). Clearly, additional research is needed to identify what causes skin abnormalities in our study population and whether it has more serious health implications for hellbenders.

Our study provides valuable baseline physiological information on eastern hellbenders from a stream where they are still abundant. We found no evidence that corticosterone or BKA profiles were influenced by modest increases in anthropogenic activities, suggesting that these two parameters may not be affected by the specific suite of land uses surrounding our study area. Given that plasma glucocorticoid levels and measures of immune function (along with energetics) are among the most commonly used physiological responses in conservation physiology, our data form a foundation for future work that should include further monitoring of the physiological and population status of hellbenders in this stream if conditions are degraded further. Additional comparisons to other, more disturbed habitats (e.g., further downstream in our study system) should also be made, but such studies will be difficult because hellbenders have declined or disappeared in many of

these locations. Our study also highlights some of the basic knowledge gaps that, if filled, might prove helpful for hellbender conservation. For example, it remains unclear why hellbenders subjected to our capture and restraint protocol had such low circulating levels of corticosterone, but were capable of attaining much higher levels when subjected to different environmental stimuli. Likewise, sexual differences in physiology and how they relate to the ecology and behavior of hellbenders require further evaluation. While some of these questions might be addressed in captive populations currently established for their conservation (e.g., Ron Goellner Center for Hellbender Conservation at the St. Louis Zoo), additional field studies describing the ecophysiology of these long-lived stream salamanders is of utmost importance.

Acknowledgments

References

[1] E. Alleva, D. Santucci, Psychosocial vs. "physical" stress situations in rodents and Humans: Role of neurotrophins, Physiol. Behav. 73 (2001) 313–320.

[2] C.M. Bodinof, Translocation and conservation of hellbenders (*Cryptobranchus alleganiensis*) in Missouri, M.S. Thesis, University of Missouri, Columbia, 2010.

[3] J.T. Briggler, J. Ettling, M. Wanner, C. Schuette, M. Duncan, K. Goellner, *Cryptobranchus alleganiensis* (hellbender) Chytrid fungus, Herpetol. Rev. 38 (2007) 174.

[4] J.T. Briggler, J. Utrup, C. Davidson, J. Humphries, J. Groves, T. Johnson, J. Ettling, M. Wanner, K. Traylor-Holzer, D. Reed, V. Lindgren, O. Byers, Hellbender Population and Habitat Viability Assessment, IUCN/SSC Conservation Breeding Specialist Group, Apple Valley, MN, USA, 2007b.

[5] J.T. Briggler, K.A. Larson, K.J. Irwin, Presence of the amphibian chytrid fungus (*Batrachochytrium dendrobatidis*) on hellbenders (*Cryptobranchus alleganiensis*) in the Ozark highlands, Herpetol. Rev. 39 (2008) 443–444.

[6] D.M. Buehler, T. Piersma, K. Matson, B.I. Tieleman, Seasonal redistribution of immune function in a migrant shorebird: annual-cycle effects override adjustments to thermal regime, Am. Nat. 172 (2008) 784–796.

[7] E.M. Chester, T. Bonu, G.E. Demas, Social defeat differentially affects immune responses in Siberian hamsters (*Phodopus sungorus*), Physiol. Behav. 101 (2010) 53–58.

[8] M.D. Cooperman, J.M. Reed, L.M. Romero, The effects of terrestrial and breeding densities on corticosterone and testosterone levels in spotted salamanders, *Ambystoma maculatum*, Can. J. Zool. 82 (2004) 1795–1803.

[9] M.F. Dallman, S. Bhatnagar, Chronic stress and energy balance: role of the hypo-thalamo-pituitary axis, in: B.S. McEwen, H.M. Goodman (Eds.), Handbook of Physiology Section 7: The Endocrine System, Coping with the Environment: Neural and Endocrine Mechanisms, 4, Oxford University Press, New York, 2001, pp. 179–210.

[10] F.S. Dhabhar, A hassle a day may keep the pathogens away: the fight-or-flight stress response and the augmentation of immune function, Integr. Comp. Biol. 49 (2009) 215–236.

[11] I.M.E. Desar, M. van Deuren, T. Sprong, J. Jansen, F. Namavar, C.M. Vandenbroucke-Grauls, J.W.M. van der Meer, Serum bactericidal activity against *Helicobacter pylori* in patients with hypogammaglobulinaemia, Clin. Exp. Immunol. 156 (2009) 434–439.

[12] T.M. Fobes, Habitat analysis of the Ozark hellbender, *Cryptobranchus alleganiensis bishopi*, in Missouri, MS Thesis,, Southwest Missouri State University, Springfield, MO, USA, 1995.

[13] R.L. Foster, A.M. McMillan, K. Roblee, Population status of hellbender salamanders (*Cryptobranchus alleganiensis*) in the Allegheny River Drainage of New York State, J. Herpetol. 43 (2009) 579–588.

[14] S.S. French, D.F. DeNardo, T.J. Greives, C.R. Strand, G.E. Demas, Human disturbance alters endocrine and immune responses in the Galapagos marine iguana (*Amblyrhynchus cristatus*), Horm. Behav. 58 (2010) 792–799.

[15] M. Groom, G.K. Meffe, C.R. Carroll, Principles of Conservation Biology, third ed., Sinauer Associates, Sunderland, MA, 2006.

[16] C.A. Herman, Endocrinology, in: M.E. Feder, W.W. Burggren (Eds.), Environmental Physiology of the Amphibians, University of Chicago Press, Chicago, 1992, pp. 40–54.

[17] W.R. Hiler, B.A. Wheeler, S.E. Trauth, Abnormalities in the Ozark hellbender (*Cryptobranchus alleganiensis bishopi*) in Arkansas: a comparison between two rivers with a historical perspective, J. Arkansas Acad. Sci. 59 (2005) 88–94.

[18] M. Hoffman, et al. (>50 co-authors), The impact of conservation on the status of the world's vertebrates. Science, 2010.

[19] R.N. Homan, J.V. Regosin, D.M. Rodrigues, J.M. Reed, B.S. Windmiller, L.M. Romero, Impacts of varying habitat quality on the physiological stress of spotted salamanders (*Ambystoma maculatum*), Anim. Conserv. 6 (2003) 11–18.

[20] W.A. Hopkins, M.T. Mendonça, J.D. Congdon, Increased circulating levels of testosterone and corticosterone in southern toads, *Bufo terrestris*, exposed to coal combustion wastes, Gen. Comp. Endocr. 108 (1997) 237–246.

[21] W.A. Hopkins, S.E. DuRant, D.W. Garst, W.E. Moser. Population staus, habitat use, and physiological condition of eastern hellbenders (*Cryptobranchus alleganiensis*) in two stream reaches in southwestern Virginia, USA. Final Report to the Virginia Dept. of Game and Inland Fisheries, 2011.

[22] W.J. Humphries, T.K. Pauley, Life history of the hellbender, *Cryptobranchus alleganiensis*, in a West Virginia stream, Am. Midl. Nat. 154 (2005) 135–142.

[23] A.L. Liebl, L.B. Martin, Simple quantification of antimicrobial capacity of blood using spectrophotometry, Funct. Ecol. 23 (2009) 1091–1096.

[24] K.R. Lips, F. Brem, R. Brenes, J.D. Reeve, R.A. Alford, J. Voyles, C. Carey, L. Livo, A.P. Pessier, J.P. Collins, Emerging infectious disease and the loss of biodiversity in a neotropical amphibian community, Proc. Natl. Acad. Sci. USA 102 (2006) 3165–3170.

[25] R. Makowsky, L.A. Wilson, T.K. Pauley, Sexual dimorphism in the eastern hellbender, Herpetol. Conserv. Biol. 5 (2010) 44–48.

[26] L.B. Martin, W.A. Hopkins, L.D. Mydlarz, J.R. Rohr, The effects of anthropogenic global changes on immune functions and disease resistance, Ann. NY. Acad. Sci. 1195 (2010) 129–148.

[27] K.D. Matson, B.I. Tieleman, K.C. Klasing, Capture stress and the bactericidal competence of blood and plasma in five species of tropical birds, Physiol. Biochem. Zool. 79 (2006) 556–564.

[28] J. Mayasich, D. Grandmaison, C. Phillips, Eastern hellbender status assessment report, U.S. Fish and Wildlife Service, Ft. Snelling, MN, USA, 2003. p. 41.

[29] B.T. Miller, J.L. Miller, Prevalence of physical abnormalities in eastern hellbender (*Cryptobranchus alleganiensis alleganiensis*) populations of middle Tennessee, Southeast. Nat. 4 (2005) 513–520.

[30] S. Millet, J. Bennett, K.A. Lee, M. Hau, K.C. Klasing, Quantifying and comparing constitutive immunity across avian species, Dev. Comp. Immunol. 31 (2007) 188–201.

[31] I.T. Moore, W.A. Hopkins, Interactions and trade-offs among physiological determinants of performance and reproductive success, Integr. Comp. Biol. 49 (2009) 441–451.

[32] M.A. Nickerson, C.E. Mays, The Hellbenders: North American Giant Salamanders, Milwaukee Public Museum Press, Milwaukee, WI, USA, 1973.

[33] M.A. Nickerson, K.L. Kryssko, R.D. Owen, Habitat differences affecting age class distributions of the hellbender salamander, *Cryptobranchus alleganiensis*, Southeast. Nat. 2 (2003) 619–629.

[34] M.A. Nickerson, K.L. Krysko, Surveying for hellbender salamanders, *Cryptobranchus alleganiesis* (Dadin): a review and critique, Appl. Herpetol. 1 (2003) 37–44.

[35] C.L. Peterson, R.F. Wilkinson, M.S. Topping, D.E. Metter, Age and growth of the Ozark hellbender *Cryptobranchus alleganiesis bishop*, Copeia (1983) 225–231.

[36] C.L. Peterson, D.E. Metter, B.T. Miller, R.F. Wilkinson, M.S. Topping, Demography of the hellbender *Cryptobranchus alleganiesis* in the Ozarks, Am. Midl. Nat. 119 (1988) 291–303.

[37] J.W. Petranka, Salamanders of the United States and Canada, Smithsonian Institution Press, Washington DC, 1998.

[38] L.F. Ricciardella, J.M. Bliley, C.C. Feth, S.K. Woodley, Acite stressors increase plasma corticosterone and decrease locomotor activity in a terrestrial salamander (*Desmognathus ochrophaeus*), Physiol. Behav. 101 (2010) 81–86.

[39] L.M. Romero, L.K. Butler, Endocrinology of stress, Int. J. Comp. Psychol. 20 (2007) 89–95.

[40] L.M. Romero, J.M. Reed, Collecting baseline corticosterone samples in the field: is under three minutes good enough?, Comp Biochem. Physiol. A Mol. Integ. Physiol. 140 (2005) 73–79.

[41] R.M. Sapolsky, L.M. Romero, A.U. Munck, How do glucocorticoids influence stress-responses? Integrating permissive, suppressive, stimulatory, and adaptive actions, Endocr. Rev. 21 (2000) 55–89.

[42] S.N. Schubert, C.L. Wack, L.D. Houck, P.W. Feldhoff, R.C. Feldhoff, S.K. Woodley, Exposure to pheromones increases plasma corticosterone concentrations in a terrestrial salamander, Gen. Comp. Endocrinol. 161 (2009) 271–275.

[43] L.F. Skerratt, R. Speare, S. Cashins, K.R. Mcdonald, A.D. Phillott, H.B. Hynes, N. Kenyon, Spread of chytridiomycosis has caused the rapid global decline and extinction of frogs, Eco. Health 4 (2007) 125–134.

[44] B.G. Smith, The life history and habits of *Cryptobranchus allegheniensis*, Biol. Bull. 13 (1907) 5–39.

[45] M.E. Solís, J.M. Bandeff, Y.W. Huang, Hematology and serum chemistry of Ozark and Eastern hellbenders (*Cryptobranchus alleganiensis*), Herpetologica 63 (2007) 285–292.

[46] S.N. Stuart, J.S. Chanson, N.A. Cox, B.E. Young, A.S.L. Rodrigues, D.L. Fischman, R.W. Waller, Status and trends of amphibian declines and extinctions worldwide, Science 306 (2004) 1783–1786.

[47] C.A. Taber, R.F. Wilkinson Jr., M.S. Topping, Age and growth of hellbenders in the Niangua river, Missouri, Copeia 4 (1975) 633–639.

[48] B.I. Tieleman, J.B. Williams, R. Ricklefs, K.C. Klasing, Constitutive innate immunity is a component of the pace-of-life syndrome in tropical birds, Proc. R. Soc. B 272 (2005) 1715–1720.

[49] B.I. Tieleman, E. Croese, B. Helm, M.A. Versteegh, Repeatability and individual correlates of microbicidal capacity of bird blood, Comp. Biochem. Phys. A 156 (2010) 537–540.

[50] S.E. Trauth, J.D. Wilhide, P. Daniel, Status of the Ozark Hellbender, *Cryptobranchus bishopi* (Urodela: Cryptobranchidae), in the spring river, Fulton County, Arkansas, Proc. Arkansas Acad. Sci. 46 (1992) 83–86.

[51] S.E. Trauth, J.D. Wilhide, P. Daniel, The Ozark hellbender, *Cryptobranchus alleganiensis*, in Arkansas: distributional survey for Chicago Herpetol, Soc. Bull. 28 (1993) (1992) 81–85.

[52] H. Wada, T.P. Hahn, C.W. Breuner, Development of stress reactivity in white-crowned sparrow nestlings: total corticosterone response increases with age, while free corticosterone response remains low, Gen. Comp. Endrocrinol. 150 (2007) 405–413.

[53] B.A. Wheeler, E. Prosen, A. Mathis, R.F. Wilkinson, Population declines of a long-lived salamander: a 20+ year study of hellbenders, *Cryptobranchus alleganiensis*, Biol. Conserv. 109 (2003) 151–156.

[54] J.C. Wingfield, V.M. Vleck, M.C. Moore, Seasonal-changes of the adrenocortical-response to stress in birds of the Sonoran desert, J. Exp. Zool. 264 (1992) 419–428.

[55] J.C. Wingfield, L.M. Romero, Adrenocortical responses to stress and their modulation in free-living vertebrates, in: B.S. McEwen, H.M. Goodman (Eds.), Handbook of Physiology; Section 7: The Endocrine System; Volume IV: Coping with the Environment: Neural and Endocrine Mechanisms, Oxford Univ. Press, New York, 2001, pp. 211–234.

[56] S.K. Woodley, E.L. Lacy, An acute stressor alters hormone levels and activity but not sexual behavior in male and female Ocoee salamanders (*Desmognathus ocoee*), Horm. Behav. 58 (2010) 427–432.