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Neutrophil: Lymphocyte Ratios as a Measure of Chronic Stress in Populations of the Hellbender (*Cryptobranchus alleganiensis*) across a Habitat Quality Gradient

Allison R. Litmer[1], Michael Freake[2], and Christopher M. Murray[3,4]

Amphibians are currently facing widespread population declines, primarily due to the introduction of anthropogenic stressors, which have the potential to alter ecosystem dynamics and elicit long-term physiological responses resulting in overall population declines. Population assessments typically rely upon genomics, demography, and geographic isolation; however, when physiological parameters are included, mechanistic explanations for population declines can be determined. Rapid population assessments that can be related to specific microhabitat characteristics for management purposes can be achieved by implementing a chronic stress proxy, such as neutrophil: lymphocyte (N:L) ratios. As a long-lived habitat specialist, facing dramatic population declines with state and federally protected populations, the Hellbender (*Cryptobranchus alleganiensis*) is a good candidate species for applying N:L ratios to assess population vulnerability and habitat quality. This study used N:L ratios as a proxy of chronic stress among Hellbender populations to determine environmental variables potentially correlated with chronic stress. Additionally, comparisons of N:L ratios were made among Hellbender populations to examine applicability of this method for assessing among-population differences. Of the microhabitat variables assessed, high conductivity, low pH, and low dissolved oxygen correlated with elevated N:L ratios. In addition, N:L ratios differed significantly among Hellbender populations, which suggests the utility of N:L ratios as an indicator of population-level differences. Specifically, where traditional methods lack the ability to detect concerns, physiological assessment suggested certain populations may be of concern in regard to experiencing chronic stress. Including physiological parameters in viability and vulnerability assessments more frequently, such as the one described here, can provide evidence of population concerns earlier than traditional methods, and allow for better management strategy by elucidating specific environmental variables contributing to stress.

AMPHIBIANS face widespread population declines primarily due to anthropogenic stressors, a major concern for population sustainability. Physical habitat and water chemistry alteration, land-use and atmospheric change, and direct species addition or removal (Malmqvist and Rundle, 2002; Stuart et al., 2004; Gangloff et al., 2016) are all anthropogenic stressors with the potential to elicit physiological responses in organisms resulting in long-term dysfunction, decrease in population sustainability (Hopkins and DuRant, 2011), and drastic alterations in the biological composition and ecological function of aquatic systems (Helmuth, 2009). Therefore, understanding the physiological responses of organisms to environmental stressors in a particular location is important when attempting to manage populations and natural systems (Wikelski and Cooke, 2006).

Among vertebrates, when a stressor is introduced, a physiological response occurs that acts to minimize the impact and promote survival through the activation of the hypothalamic-pituitary-adrenal (HPA) axis (Moore and Jessop, 2003). The HPA axis activation promotes a cascade of physiological effects, including the production and release of adrenal-synthesized glucocorticoid hormones (GC; Goessling et al., 2015) and alteration of circulating white blood cells, resulting in an increase in detectability of neutrophils and a decrease in lymphocytes (Davis et al., 2008). While acute elevation in glucocorticoid production allows maintenance of homeostasis, chronic release causes long-term changes in energy allocation influencing various physiological processes, including the immune system, digestion, and reproductive output and behavior (Sapolsky et al., 2000; Romero, 2004; Wikelski and Cooke, 2006; Romero and Butler, 2007; Davis et al., 2008; Goessling et al., 2015). Therefore, assessing levels of chronic stress could act as a useful metric of habitat condition and overall population viability at a scale not always considered by current management practices (Hopkins et al., 1997; Homan et al., 2003; Moore and Jessop, 2003; Romero, 2004; Hayes et al., 2006; Müller et al., 2011).

Assessing the relationship between stress response and human disturbance is increasingly being used with a conservation initiative (Hopkins et al., 1997; Romero and Wikelski, 2002; Hopkins and DuRant, 2011; Burraco and Gomez-Mestre, 2016; Navas et al., 2016). Proxies of stress are used as an indicator of the physiological stress response of an organism to stimuli. Proxies of chronic stress can be used to assess population vulnerability, as long-term physiological changes have detrimental consequences (Navas et al., 2016). While conservation physiology is not a new concept, management strategies tend to focus on metrics relating to fluctuation in population size, intraspecific variation, genetics, and geographic location (Groom et al., 2006), which have proven to be useful indicators of population viability and for designating a species, or population, as "in need of

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conservation" (Foster et al., 2009; Olson et al., 2013; Benson et al., 2016; Kirchhoff et al., 2017; Freake et al., 2018). Incorporating physiological parameters more frequently in population assessments, such as targeted stress-response proxies, would provide a metric that is not always considered, elucidating influencers of population declines and early anticipation of where problems may occur (Wikelski and Cooke, 2006). Stress response as a conservation metric would offer a rapid assessment tool for determining population vulnerability and improving management strategies.

Among reptiles and amphibians, measurements of corticosterone are being used to predict individual and population viability in response to environmental changes (Hopkins et al., 1997; Homan et al., 2003; Romero, 2004; Hopkins and DuRant, 2011; DuRant et al., 2015). Many studies have implemented methods of measuring circulating corticosterone concentration to assess stress on individual and population levels (Gendron et al., 1997; Hopkins et al., 1997; Homan et al., 2003; Hopkins and DuRant, 2011). However, plasma corticosterone decreases in concentration over time, attenuating even when the stressor is still present. Therefore, corticosterone concentrations are useful for measuring acute stress response in conjunction with other methods, but potentially misleading as a stand-alone assessment of chronic stress (Goessling et al., 2015). An additional method for measuring chronic stress that does not rely upon hormone concentrations—but rather hematological parameters—is relative white blood cell counts (Davis et al., 2008), where the increase in detection of neutrophils and decrease in lymphocytes in response to a chronic stressor can be quantified using a ratio (neutrophil: lymphocyte, or 'N:L' ratio). The change in circulating leukocytes provides a more useful indication of chronic stress in comparison to plasma GC concentrations because leukocytes do not attenuate over chronic periods of time (Goessling et al., 2015). Noticeable changes in ecosystems due to anthropogenic stressors may lag (Malmqvist and Rundle, 2002) when using traditional metrics of population assessments, making the incorporation of N:L ratios useful to conservation for earlier detection for deteriorating population viability (Wikelski and Cooke, 2006), and when assessed in relation to microhabitat quality, conservation assessment can focus on the direct cause of population declines.

The Hellbender (*Cryptobranchus alleganiensis*) is a species currently facing population declines, with federally endangered populations in Arkansas and Missouri, and state protected populations in Ohio, Illinois, Maryland, North Carolina, Alabama, and Indiana. Hellbenders are long-lived, fully aquatic salamanders (Nickerson and Krysko, 2003) inhabiting well-oxygenated, swift-flowing, cool streams, with low siltation, large rocks for adults, and gravel for juveniles (Wheeler et al., 2003; Humphries and Pauley, 2005; Miller and Miller, 2005; Pugh et al., 2015; Spear et al., 2015; Jachowski, 2016). Conservation assessment for relatively long-lived and widely distributed amphibians, like Hellbenders, using common population viability metrics require long-term studies, as juvenile recruitment and population responses to stochastic events are variable (Wheeler et al., 2003; Freake and DePerno, 2017). Early detection of chronic stress among Hellbender populations could allow identification of underlying threats and an improved conservation assessment. A sensitive physiology and increased longevity make Hellbenders ideal indicators of in-stream conditions and good candidates to use N:L ratios to assess contributing environmental factors to chronic stress. Additionally, the relationship between increased stress and elevated N:L ratio for salamanders has been quantified, where salamanders injected with corticosterone exhibit elevated N:L ratios within 24 hours (Davis and Maerz, 2009). More specifically for Hellbenders, DuRant et al. (2015) found that after approximately six hours of inflicted capture stress or ACTH injection on Hellbenders N:L ratios dramatically increased from initial capture values (∼0.3–0.4) to N:L ratios up to 1.5, providing evidence that N:L ratios are indicative of chronic stress in Hellbenders.

This study aimed to assess the relationship between N:L ratio, a proxy of chronic stress, and microhabitat quality among wild Hellbender populations as an indicator of population viability. Low quality microhabitat for Hellbenders has been associated with high turbidity, high water temperature, high embeddedness, and high conductivity (Keitzer et al., 2013), small rock size, low dissolved oxygen, low flow velocity, and low substrate roughness, as well as a pH outside of an optimal range between 7 and 9 (Nickerson and Mays, 1973; Solís et al., 2007). Diagnosing a correlation between specific microhabitat characteristics and an indicator of chronic stress among populations could improve vulnerability and management assessments among populations. We tested the hypotheses that Hellbender populations in areas with poor microhabitat and surrounding developed land cover types exhibit increased N:L ratios relative to populations in areas with high quality streams and that N:L ratio differs among populations. The specific objectives of this study were to: 1) locate Hellbenders and diagnose microhabitat characteristics; 2) assess the applicability of N:L ratio as an indicator of chronic stress in relation to specific microhabitat characteristics; 3) determine if Hellbender N:L ratio correlates with land cover type and proximity to roads; and 4) utilize N:L ratios to identify populations of Hellbenders that may be of conservation concern. Two previous studies assessed white blood cell counts in Hellbenders (DuRant et al., 2015; Hopkins et al., 2016) with a focus on the impact of parasites on Hellbender physiology. Therefore, this study elaborates on the use of N:L ratios in Hellbenders with a chronic stress perspective by reporting ratios for multiple populations that have not been previously assessed with this method, with respect to varying habitat quality.

MATERIALS AND METHODS

Study sites.—To assess the ability of N:L ratios to indicate poor habitat quality conditions, and to assess N:L ratio across a gradient of environmental parameters, sites predicted to be of low habitat quality, intermediate quality, and high quality for Hellbenders were intentionally selected and surveyed in Tennessee, North Carolina, and Kentucky (Fig. 1). Five streams were surveyed within federally protected habitat and three streams were surveyed outside of federally protected habitat (Table 1). Within streams surveyed across years, such as the Little River and Little Buffalo River, location of survey sites varied by as much as ∼300 m; however, the streams within the same physiographic regions were separated by water drainages.

Survey techniques.—We assessed all microhabitat variables at the specific point and time of capture for each Hellbender. In-

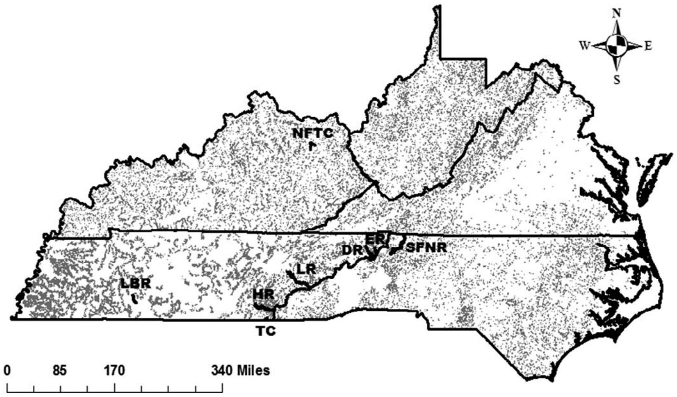

Fig. 1. Map of eight sites surveyed between 2017 and 2018 for Hellbenders, including North Fork Triplett Creek (NFTC) in Kentucky, Little Buffalo River (LBR), Hiwassee River (HR), Little River (LR), Doe River (DR), Tumbling Creek (TC), and Elk River (ER) in Tennessee, and South Fork New River (SFNR) in North Carolina.

stream microhabitat was assessed by measuring water depth, pH, conductivity, dissolved oxygen, temperature, flow velocity, turbidity, substrate type, embeddedness, canopy cover, cover object presence and size, and substrate roughness. A Yellow Springs Instrument (YSI) ProDSS was used to measure all in-stream characteristics (pH, conductivity, dissolved oxygen, temperature, and turbidity) and flow velocity was assessed using the float method (Bain and Stevenson, 1999). The YSI was periodically calibrated between sites. Canopy cover was assessed using the Brown Canopy Scope method (Brown et al., 2000). A 1 m chain was placed along the substrate contour of the stream parallel to flow to measure substrate roughness (k), which was calculated as $k = 100/d$, where d is the distance between the two ends of the chain after placement (Hardison and Layzer, 2001; Black et al., 2015). Embeddedness and dominant substrate type were visually determined based on methods and categories described by Bain and Stevenson (1999). Cover object size was determined by measuring the greatest distance across the object.

Diurnal and nocturnal surveys conducted for Hellbenders occurred between April and early August in 2017 and 2018 to avoid negatively influencing reproductively active Hellbenders in late August, and the potential influence of reproductively active animals exhibiting different N:L ratios (Davis and Maerz, 2008). Diurnal surveys consisted of skin diving between 0900–2000 hours (Nickerson and Krysko, 2003) and nocturnal surveys consisted of wading through streams with headlamps, searching for active Hellbenders just after sunset. During all surveys, individuals were captured by hand or net, and morphometric measurements, including mass, snout–vent length (SVL), total length (TL), and mass (Table 1), abnormalities, and presence of external parasites were recorded for analyses. Measurements, reference photos, and GPS points were referenced to reduce replicate samples from the same animal.

Blood draw and N:L ratio determination.—Upon capture, each Hellbender was restrained using a "bender board," where the salamander was placed dorsal side up and held in place by foam on a board (Burgmeier et al., 2010). This method of restraint is applicable for Hellbenders of varying sizes, although proved best for large Hellbenders, and allows for safe restraint. Less than 1 cc of blood was drawn from the caudal vein of each Hellbender (Hopkins and DuRant, 2011) immediately upon capture (less than 10 minutes) to reduce the influence of capture stress on the sample. At least two slides were prepared directly after blood draws, using 5 μL of whole blood, and stained with Giemsa-Wright stain for leukocyte types (Murray et al., 2013). The N:L ratios were obtained by identifying and counting the first 100 leukocytes using 40X magnification on a Leica. All permitting and animal welfare regulations were abided in this study (2017 scientific collection permit numbers: TN, 1873; GSMNP, GRSM-2017-SCI-2010; 2018 scientific collection permit numbers: TN, 1370; GSMNP, GRSM-2018-SCI-2010; KY, SCI1811143; NC, 18-ES00542; and IACUC number: TTU-IACUC-16-17-005).

Statistical analyses.—All statistical analyses were conducted with $\alpha = 0.05$. Spearman Rho rank-order correlations were performed to determine if Julian date (sampling time), mass, or SVL were associated with N:L ratios and potentially confounded results. To determine if N:L ratio differed between Hellbenders with abnormalities (including abrasion, scarring, and external parasites) and without abnormalities, we ran a Kruskal-Wallis rank-sum test.

Linear mixed models were performed to determine the influence of microhabitat variables (water quality parameters and physical habitat parameters) on N:L ratio while taking into account the influence of site and year, with year as a fixed factor and site as a random factor, using the package 'car' (Fox and Weisberg, 2011) and 'lme4' (Bates et al., 2015)

Table 1. Total number of captures (n), average masses, average snout–vent lengths (SVL), average total lengths (TL), standard deviations (±), and percentage of abnormalities (abrasions, scarring, external parasites, or missing limbs) for Hellbenders captured in 2017 and 2018 at Little River (LR), Tumbling Creek (TC), Hiwassee River (HR), Elk River (ER), North Fork Triplett Creek (NFTC), Little Buffalo River (LBR), Doe River (DR), and South Fork New River (SFNR). Asterisks are noted for sites that are within federally protected habitat.

Site	n	Mass (g)	SVL (mm)	TL (mm)	% Abnormalities
*LR	8	376.9±119.2	251.3±34.7	386.3±46.0	37.5
*TC	12	262.7±193.7	215.0±73.5	320.4±104.8	50.0
*HR	11	307.3±62.0	273.7±24.2	364.4±36.6	72.7
*ER	3	380.0±52.9	260.0±17.3	420.0±20.0	33.3
*NFTC	4	722.5±302.1	310.0±50.2	491.3±62.0	75.0
LBR	4	690.0±240.0	315.0±44.3	475.0±51.8	25.0
DR	1	400.0	245.0	420	0
SFNR	5	569.0±143.1	305±31.6	490.0±38.2	60.0
Average		408.0±221.4	255.3±57.2	394.5±87.9	59.6

Table 2. Descriptive statistics (±SD) of water quality data from Little River (LR), Tumbling Creek (TC), Hiwassee River (HR), Elk River (ER), North Fork Triplett Creek (NFTC), Little Buffalo River (LBR), Doe River (DR), and South Fork New River (SFNR) Hellbender locations in 2017 and 2018.

Site	Average N:L ratio	Depth (cm)	pH	Conductivity (μs/cm)	DO (%)	Temperature (°C)	Velocity (m/s)	Turbidity (ntu)
2017								
LR	0.569	44.25±4.79	8.26±0.17	17.63±2.65	99.48±1.86	21.15±0.24	0.37±0.07	2.68±0.33
TC	0.254	48.9±61.83	7.53±0.16	16.13±0.26	96.16±1.38	17.6±0.79	0.22±0.23	1.9±0.18
HR	0.654	28.8±15.58	8.22±0.33	33.53±1.34	103.97±7.43	21.77±1.45	0.29±0.22	2.29±0.48
ER	—	—	—	—	—	—	—	—
NFTC	—	—	—	—	—	—	—	—
LBR	0.315	51.0±1.41	8.10±0.01	78.15±0.07	105.7±1.56	23.2±0.56	0.11±0.05	3.2
DR	1.410	62.00	8.18	95.30	90.80	15.50	0.279	4.2
SFNR	—	—	—	—	—	—	—	—
2018								
LR	0.643	33.75±15.97	7.55±0.32	14.3±0.08	97.7±0.50	15.4±0.76	0.16±0.06	1.75±0.06
TC	0.405	33.6±10.53	7.2±0.12	15.7±0.09	96.5±0.84	15.0±1.09	0.27±0.27	2.08±0.21
HR	0.559	21.18±4.14	7.62±0.52	16.58±0.93	109.2±2.99	16.58±0.93	0.19±0.02	0.50±0.08
ER	0.723	73.33±7.64	7.69±0.09	75.07±0.06	95.53±0.81	15.87±0.23	0.17±0.04	3.27±0.12
NFTC	1.668	63.75±8.30	7.63±0.52	80.7±0.44	97.3±2.40	22.58±0.59	0.10±0.02	7.10±0.48
LBR	1.092	61±4.24	7.27±0.89	65.65±7.14	101.20±3.68	16.75±7.14	0.24±0.25	4.8±4.38
DR	—	—	—	—	—	—	—	—
SFNR	0.554	55.20±18.34	8.32±0.17	82.14±0.54	101.1±1.59	23.44±0.35	0.30±0.09	7.64±1.9

in R i386 3.4.1 (R Core Team, 2017). To facilitate comparison of parameter estimates in our models, we standardized covariates by subtracting the mean and dividing by the standard deviation (Wenger et al., 2017). However, as mentioned above, we intentionally selected various sites to incorporate a wide range of habitat parameters assessed (i.e., sites with high versus low flow velocity, for example). Therefore, we expected site to be influential on the dataset.

To determine how habitat parameters influenced N:L ratios from a physiological perspective, we conducted additional separate multiple regression analyses for water characteristics and physical microhabitat for 2017 and 2018. Water characteristics included pH, dissolved oxygen, conductivity, velocity, water temperature, water depth, and turbidity in the exact location where Hellbenders were located. Physical habitat characteristics included percent canopy cover, rock size, substrate roughness, and rock embeddedness. Spearman Rho rank-order correlations were performed prior to running multiple linear regressions to assess collinearity among environmental variables to determine if specific variables should be removed from analyses.

For the multiple regression comparing water quality parameters and N:L ratio in 2017, water temperature and turbidity were removed due to high collinearity between water temperature and dissolved oxygen (Spearman Rho $= 0.811$, $P < 0.001$), pH (Spearman Rho $= 0.605$, $P = 0.003$) and conductivity (Spearman Rho $= 0.547$, $P = 0.008$), and high collinearity between turbidity and pH (Spearman Rho $= 0.552$, $P = 0.008$), conductivity (Spearman Rho $= 0.582$, $P = 0.005$), and depth (Spearman Rho $= 0.440$, $P = 0.040$). In the 2018 multiple regression comparing water quality parameters, water temperature and turbidity were also removed due to high collinearity between water temperature and pH (Spearman Rho $= 0.663$, $P < 0.001$), turbidity (Spearman Rho $= 0.678$, $P < 0.001$), and conductivity (Spearman Rho $= 0.795$, $P < 0.001$), and high collinearity between turbidity and pH (Spearman Rho $= 0.496$, $P = 0.010$) and depth (Spearman Rho $= 0.606$, $P = 0.001$). No physical habitat parameters were removed from any analysis. Only two dominant substrate types, boulder and cobble, were observed at Hellbender locations. Therefore, to determine if dominant substrate type influenced N:L ratio, a Mann-Whitney U test was conducted.

To compare land cover data and N:L ratios, we obtained land cover data from the National Land Cover Database (NLCD) using the most recent dataset (2011 dataset; Homer et al., 2015), with a system modified from the Anderson Land Cover Classification System (Anderson et al., 1976). Using ArcMap 10.5, Hellbender coordinates were converted to a shape file, and six coordinates were removed from the analyses due to inaccurate GPS coordinates. Using the spatial analyst tool in ArcMap, land cover data were extracted for the specific Hellbender localities from surveys. To determine if there was a difference in N:L ratio based on land cover type, a Kruskal-Wallis rank-sum test was conducted. To determine if there was a correlation among N:L ratio and proximity to roads, distance of each Hellbender location to nearest road was measured using ArcMap. A linear regression was conducted to assess the correlation between N:L ratio and distance from road.

To determine if there were differences in N:L ratio across sample sites, Kruskal-Wallis rank-sum tests with a Dunn's test for pairwise comparisons were conducted for 2017, 2018, and a combined dataset. The Spearman Rho rank-order correlation, Kruskal-Wallis rank-sum, and Mann-Whitney U tests were conducted in IBM SPSS Version 22.0 (Statistical Package for the Social Sciences; IBM Corp., 2013), and the mixed linear models, linear regression, and multiple linear regression analyses were conducted using R i386 3.4.1 (R Core Team, 2017).

RESULTS

Hellbenders were found in reaches of streams with a water depth ranging from 10.00–200.00 cm and flow velocity ranging from no flow to 0.76 m/s (Table 2). In-stream characteristics in locations where Hellbenders were located

Table 3. Physical habitat data with standard deviations from Little River (LR), Tumbling Creek (TC), Hiwassee River (HR), Elk River (ER), North Fork Triplett Creek (NFTC), Little Buffalo River (LBR), Doe River (DR), and South Fork New River (SFNR) Hellbender locations in 2017 and 2018.

Site	Average N:L ratio	Embeddedness (%)	Canopy cover (%)	Rock size (mm)	Roughness (k)
2017					
LR	0.569	25±24	68±30	101.13±22.60	1.12±0.07
TC	0.254	21±13	54±31	105.85±67.63	1.16±0.23
HR	0.654	15±10	18±30	85.79±13.23	1.09±0.05
ER	—	—	—	—	—
NFTC	—	—	—	—	—
LBR	0.315	93±4	45±16	189.00±41.01	1.08±0.06
DR	1.410	60	0	119	1.07
SFNR	—	—	—	—	—
2018					
LR	0.643	6±3	31±37	85.50±11.24	1.15±0.08
TC	0.405	65±37	88±13	75.75±10.75	1.08±0.03
HR	0.559	5	8±9	92.50±17.94	1.06±0.04
ER	0.713	5	0	132.00±64.13	1.11
NFTC	1.668	86±11	91±1	158.75±41.11	1.10±0.01
LBR	1.092	65±50	81±7	154.00±41.01	1.07±0.05
DR	—	—	—	—	—
SFNR	0.554	11±9	0	118.00±14.83	1.24±0.15

had a range in pH of 6.64–8.64, conductivity of 14.2–95.3 μs/cm, dissolved oxygen from 90.80–111.6%, temperatures from 11.7–23.8°C, and turbidity from 0.4–9.8 ntu (Table 2). Hellbenders were found under rocks with a range of zero to complete embeddedness and from 60–240 m in size, with a range of no canopy cover to complete coverage, and roughness ranging from 1.02–1.67 (Table 3).

A positive association between N:L ratio and survey date for 2017 was detected (Spearman Rho $= 0.460$, $P = 0.031$), but no significant correlation was detected for N:L ratio and survey date for 2018 or both years combined. We found no significant correlation between mass, or SVL, and N:L ratio. We also found no significant difference in N:L ratio among Hellbenders with the presence of external abnormalities for 2017 or 2018.

No significant trends in N:L ratio were detected for water quality or physical habitat parameters using a linear mixed model. In 2017, we found overall significance between tested water quality parameters and N:L ratio ($F = 7.385$, df $= 5, 16$, $P < 0.001$; Fig. 2). High conductivity ($t = 2.371$, $P = 0.031$, $R^2 = 0.427$; Fig. 3A), alkaline pH ($t = 3.269$, $P = 0.005$, $R^2 = 0.410$; Fig. 3B), and low dissolved oxygen ($t = -4.096$, $P < 0.001$, $R^2 = 0.189$; Fig. 3C) were significantly correlated with elevated N:L ratios. In 2018, multiple linear regression analysis did not reveal overall significance between tested water quality parameters and N:L ratio ($F = 1.614$, df $= 5, 20$, $P = 0.148$; Fig. 4); however, acidic pH ($t = -2.243$, $P = 0.034$, $R^2 = 0.249$; Fig. 5) was significantly correlated with elevated N:L ratios in 2018. The multiple regression analyses did not reveal any significance among physical habitat characteristics and N:L ratio for 2017 or 2018. We did not detect any significant influence of dominant substrate type on N:L ratio.

We found four land cover categories overlaying the Hellbender occurrence data: deciduous forest, evergreen forest, developed–open space, and open water. No significant differences in N:L ratio based on land cover data for 2017, 2018, or both years combined were detected. However, the most common land cover type was deciduous forest ($n = 28$, $\bar{x} = 0.688$ N:L ratio), followed by developed–open space ($n = 9$, $\bar{x} = 0.743$), open water ($n = 4$, $\bar{x} = 0.655$), and evergreen forest ($n = 1$, $\bar{x} = 0.127$). No significant correlations were detected between Hellbender N:L ratio and distance to road.

In 2017, a significant difference was detected in N:L ratio among sites (H $= 11.156$, df $= 4$, $P = 0.025$; Table 2, Fig. 6). *Post hoc* pairwise comparisons using a Dunn's test revealed that Tumbling Creek had significantly lower N:L ratios than Hiwassee River ($P = 0.006$) and Doe River ($P = 0.023$). No significant among-site differences in 2018 (Table 3) were detected. However, when 2017 and 2018 data were combined, significant differences among sites were detected (df $= 4$, $P = 0.009$, H $= 18.849$; Fig. 7). *Post hoc* pairwise comparisons found that Tumbling Creek exhibited a significantly lower N:L ratio than Hiwassee River ($P = 0.013$), North Fork Triplett Creek ($P < 0.001$), Elk River ($P = 0.044$), and Doe River ($P = 0.029$), and that Little River was significantly lower than North Fork Triplett Creek ($P = 0.032$).

DISCUSSION

Neutrophil: lymphocyte ratios in Hellbenders.—The results of this study identify environmental attributes that may result in elevated N:L ratios, a proxy of chronic stress, among Hellbenders. The proposed hypotheses that Hellbenders in streams with poor microhabitat characteristics exhibit elevated N:L ratios, and that N:L ratio as a proxy of chronic stress could provide indication of differences among populations were supported by the results of this study. Using N:L ratios as a proxy of chronic stress, we found that high conductivity, acidic or basic pH, and low dissolved oxygen correlate with elevated N:L ratios among Hellbenders in the surveyed populations. Conductivity, pH, and dissolved oxygen also exhibited collinearity with high turbidity and warm temperatures, suggesting that these variables may also be influential on Hellbender chronic stress. When taking into account site and year in the analyses for this study, we detected no significant differences using a linear mixed model. However, a variety of sites were intentionally surveyed to include a wide range of values for the habitat parameters and various sites were surveyed across 2017 and

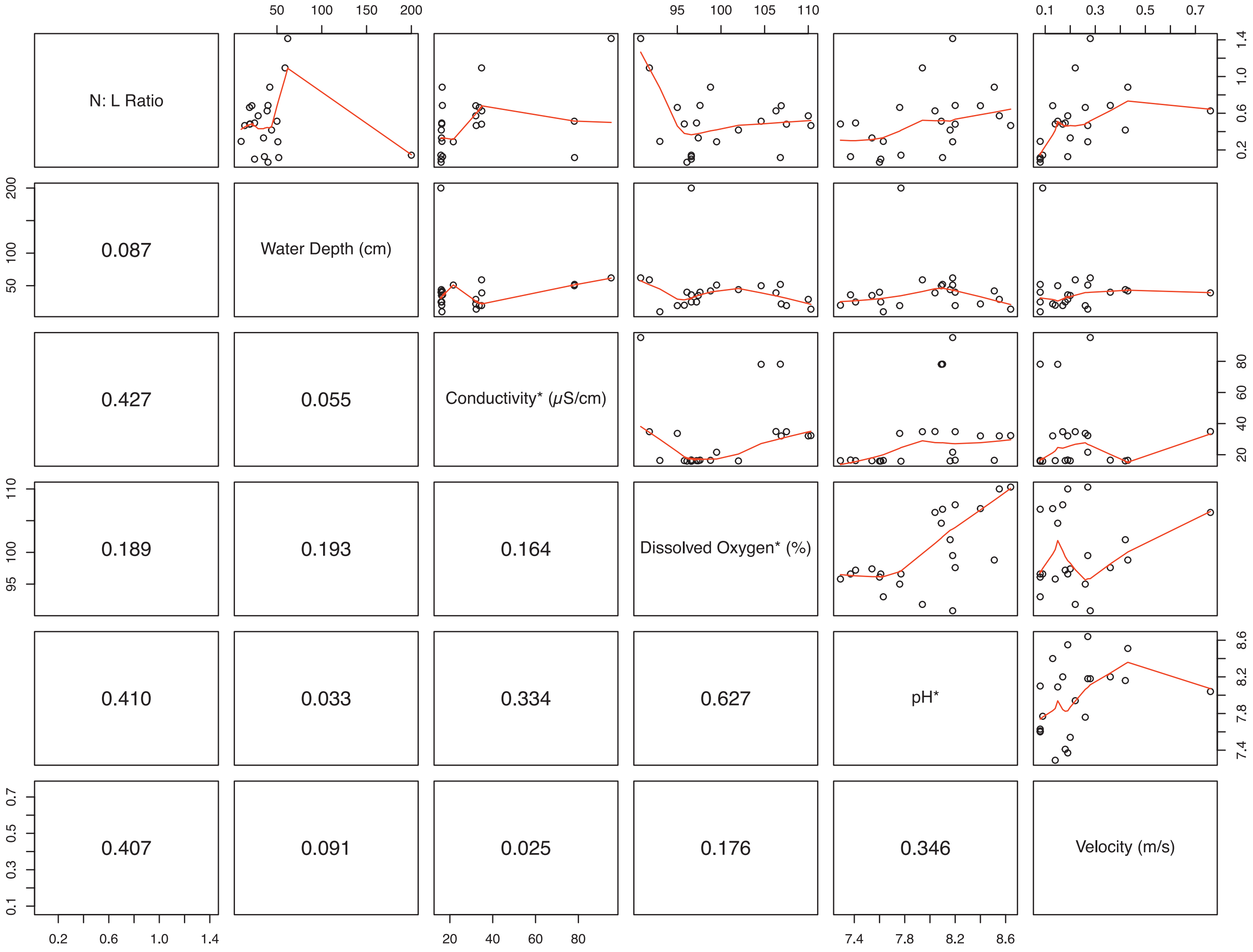

Fig. 2. Scatterplots showing multiple linear regression comparing Hellbender neutrophil: lymphocyte ratio and water characteristics for 2017. The first row of graphs depicts neutrophil: lymphocyte ratio on the y-axis and corresponding environmental variables on the x-axis; all other rows depict the relationship among water quality parameters assessed. R^2 values are provided for each graph, the red line represents the overall trend line, and significant variables are indicated with an asterisk.

2018. Therefore, we expected site and year to contribute to variation in N:L ratio and habitat parameters. In order to determine biologically meaningful results regarding which environmental variables may correlate with N:L ratio, we decided to run multiple regressions without site as a factor.

The results of this study did not support the hypotheses that N:L ratio would correlate with specific land cover types, and that N:L ratio would become elevated with increasing proximity to roads. However, the majority of land cover types overlaying Hellbender localities was "deciduous forest." Additionally, the population of Hellbenders from Tumbling Creek had consistently low N:L ratios and a thick riparian zone aside from one road that ran alongside the river to a campground. Therefore, the results suggesting that proximity to roads is not correlated with elevated N:L ratios among Hellbenders may have been skewed by this population. A previous study found current land cover type to be poor predictor of Hellbender presence (Bodinof Jachowski et al., 2016) and suggested that the long life span of Hellbenders may result in a lag in population level responses to land use changes, which has also been suggested to occur in certain fishes (Utz et al., 2010). However, a study focusing on historic land-use change was able to document changes in Hellbender populations and habitat within Hellbender streams with corresponding land-use change resulting in declines (Nickerson et al., 2017), corroborating the suggestion that current land-use data do not accurately predict the influence land-use has on Hellbenders, but historic land-use change might be a better predictor.

Surveys for this study were conducted during summer months in an attempt to remove the influence of sampling date and reproduction on N:L ratios. In 2017, a correlation between survey date and N:L ratio was detected; however, this was a result of surveying a population that had consistently low N:L ratios, Tumbling Creek, at the beginning of the year in 2017 and populations that had consistently higher N:L ratios later in the season. In 2018, Tumbling Creek was surveyed later in the season which removed the correlation between survey date and N:L ratio. Overall, the data from this study suggest that in order to reduce the likelihood of elevated N:L ratios, an indicator of chronic stress that could potentially lead to a decline in

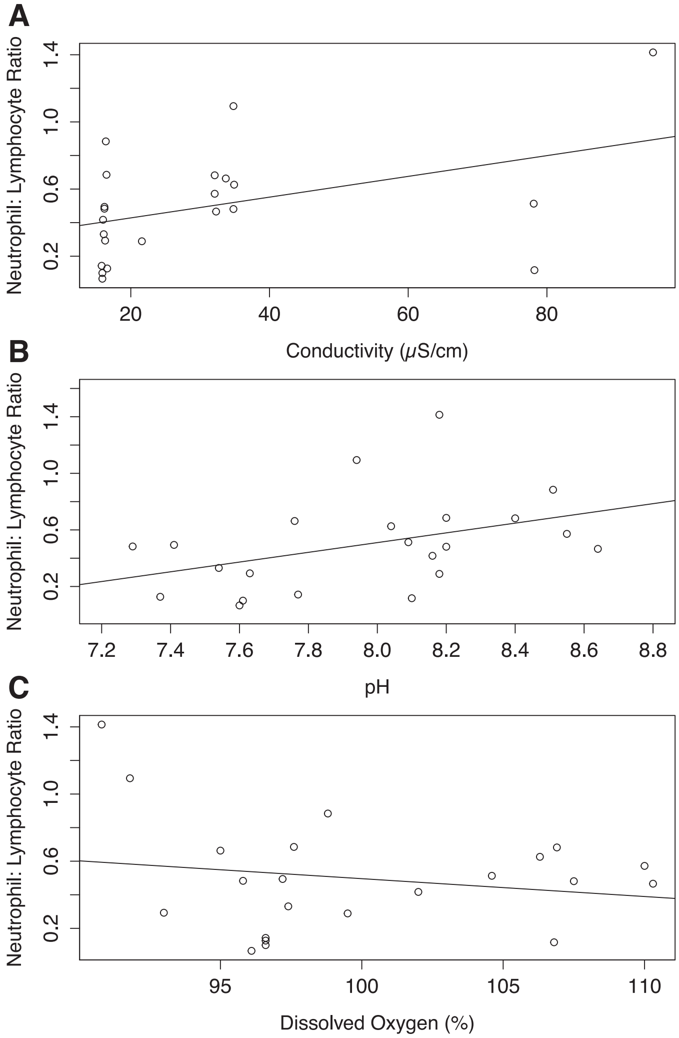

Fig. 3. Scatterplots showing regressions between Hellbender neutrophil: lymphocyte ratios and conductivity (A), pH (B), and dissolved oxygen (C), in 2017.

Hellbender survivorship, actions must be taken to mitigate influencers that result in streams with high conductivity, acidic or alkaline water pH, low dissolved oxygen, high turbidity, and warm water temperature. However, stream characteristics are variable across Hellbender range, and historic conditions should be considered when making assessments. Environmental values deemed as "high" or "low" will vary depending on underlying geology and geographic location. Therefore, the specific values found in this study are only representative of the regions surveyed, and habitat parameters in other specific regions of interest should be assessed for specific management implementations.

In addition to determining environmental variables associated with potential chronic stress, N:L ratios were significantly different among populations, which may be indicative of certain populations of conservation concern. Specifically, Hellbender populations in Hiwassee River, North Fork Triplett Creek, Doe River, and Elk River experienced significantly elevated N:L ratios compared to other populations. The N:L ratios found among Hellbenders within the significantly high N:L ratio populations residing on federal lands were similar to that found in a Hellbender at Doe River, a site with low Hellbender abundance in an urban environment. However, only one Hellbender was found at Doe River and therefore the results from this population should be taken in consideration with the low sample size. While anthropogenic impacts may be greater at sites without federal protection, federal lands are still susceptible to human impacts which could influence Hellbenders. However, it should be noted that aspects of microhabitat within sites may be suitable for Hellbenders, and other environmental factors could be contributing to the N:L ratios that were not considered here.

Comparisons of N:L ratios among populations could provide a useful way to discern populations exposed to stressors that may be of concern. Habitat quality assessments can be time consuming when looking at multiple sites across a distribution, and therefore N:L ratios could provide a starting point to indicate which populations may be of concern. N:L ratio and the analogous heterophil: lymphocyte (H:L) ratio has been implemented with the conservation initiative of making population comparisons for a variety of taxa, including birds, amphibians, marsupials, and mammals (Owen et al., 2005; Hinam and St. Clair, 2008; Johnstone et al., 2012; Banbura et al., 2013; Dananay, 2013). Studies relating H:L and N:L ratio to habitat have found elevated ratios among populations in habitats considered to be of poor quality compared those that were less disturbed (Banbura et al., 2013), and elevated ratios among birds in areas with lower vegetation and producing lower quality offspring (Ortego, 2007). Therefore, the differences in N:L ratio found among sites in the current study could be indicative of habitat quality and populations experiencing chronic stress.

For amphibians, N:L ratios are being used increasingly as a proxy to indicate chronic stress and have been implemented to elucidate the influence of intra- and inter-specific interactions (Davis and Maerz, 2009; Davis and Milanovich, 2010), elucidate the influence of parasites (Shutler et al., 2009; Shutler and Marcogliese, 2011; Hopkins et al., 2016), compare populations (Shutler and Marcogliese, 2011), and assess the influence of habitat (Homan et al., 2003). The results of this study elaborate on two previously conducted studies assessing white blood cell counts in Hellbenders, with previous studies detecting ranges of N:L ratios from 0.3–1.5 (DuRant et al., 2015) and 0.36–0.77 (Hopkins et al., 2016). While thresholds indicative of chronic stress have not been clearly diagnosed for amphibians to our knowledge, for reptiles an analogous heterophil: lymphocyte ratio above ~1:1 has been considered indicative of chronic stress (Lance et al., 2010). However, at what specific point allostatic overload occurs, or when the energy required to maintain a homeostatic balance exceeds the organism's capacity to do so occurs, in Hellbenders is still unclear for determining a specific N:L ratio threshold.

Environmental attributes influencing Hellbenders.—High conductivity has previously been documented to negatively affect aquatic organisms through low egg survivorship and hatchling success, decreased tadpole body condition, decreased sperm mobility (Ettling et al., 2013), and low abundance (de Solla et al., 2002; Kirchberg et al., 2016; Soto-Rojas et al., 2017). Previous studies looking at Hellbender habitat selection found similar results as presented here; human-disturbed stretches with low Hellbender abundance

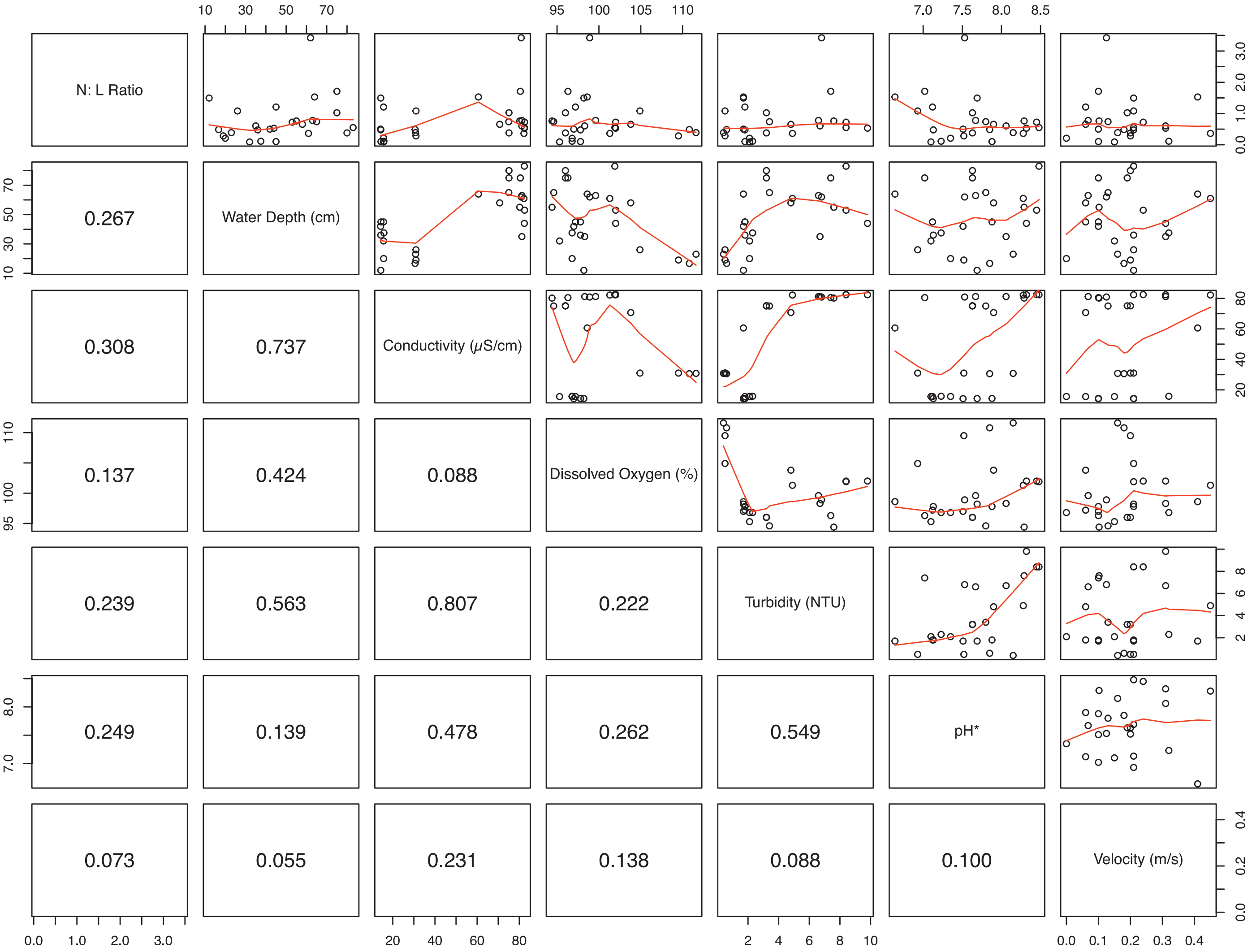

Fig. 4. Scatterplots showing multiple linear regression comparing Hellbender neutrophil: lymphocyte ratio and water characteristics for 2018. The first row of graphs depicts neutrophil: lymphocyte ratio on the y-axis and corresponding environmental variables on the x-axis; all other rows depict the relationship among water quality parameters assessed. R^2 values are provided for each graph, the red line represents the overall trend line, and significant variables are indicated with an asterisk.

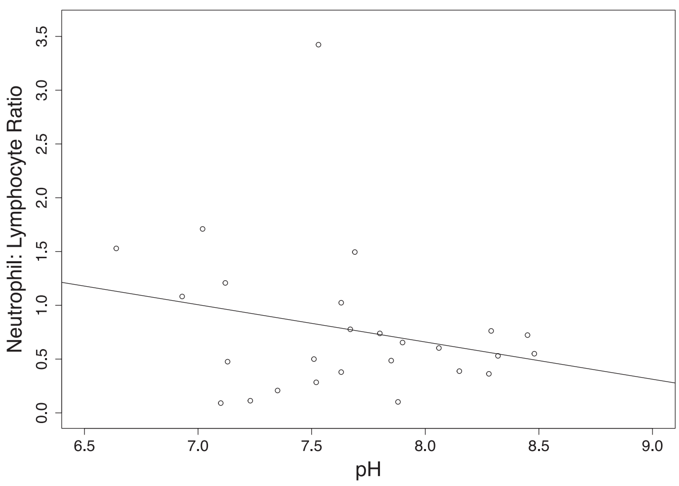

Fig. 5. Scatterplot showing regression between Hellbender neutrophil: lymphocyte ratio and water pH observed in 2018.

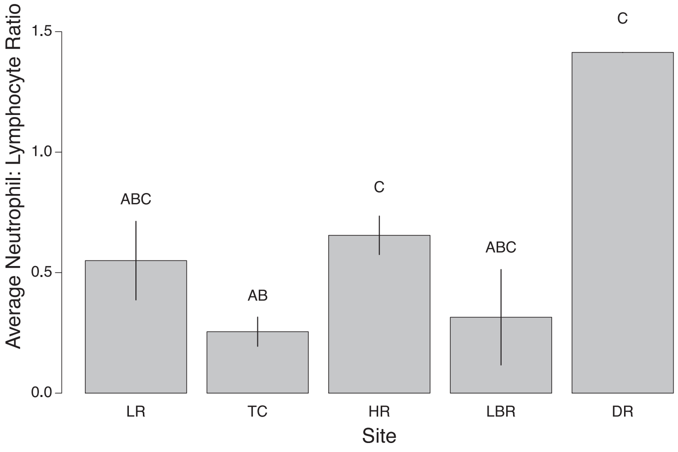

Fig. 6. Hellbender neutrophil: lymphocyte ratio in 2017 at Little River (LR; $n = 4$), Tumbling Creek (TC; $n = 9$), Hiwassee River (HR; $n = 6$), Little Buffalo River (LBR; $n = 2$), and Doe River (DR; $n = 1$). Letters indicate among-site differences, where sites that are significantly different have different letters, and error bars represent two standard deviations from the mean.

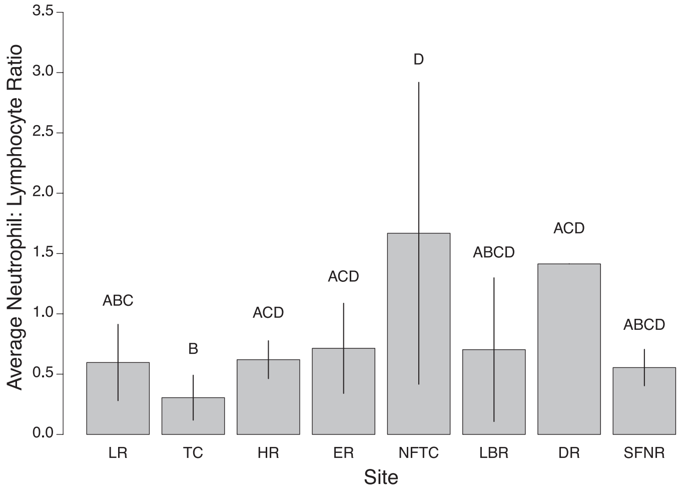

Fig. 7. Hellbender neutrophil: lymphocyte ratio across 2017 and 2018 at Little River (LR; $n = 8$), Tumbling Creek (TC; $n = 12$), Hiwassee River (HR; $n = 11$), Elk River (ER; $n = 3$), North Fork Triplett Creek (NFTC; $n = 4$), Little Buffalo River (LBR; $n = 4$), Doe River (DR; $n = 1$), and South Fork New River (SFNR; $n = 5$). Letters indicate among-site differences, where sites that are significantly different have different letters, and error bars represent two standard deviations from the mean.

had notably higher conductivity and turbidity, low pH and dissolved oxygen, and increased rock embeddedness, with conductivity being most notably different in occupied and unoccupied sites (Hopkins and DuRant, 2011; Keitzer et al., 2013; Quinn et al., 2013; Pugh et al., 2015; Pitt et al., 2017; Bodinof Jachowski and Hopkins, 2018). We found high collinearity among conductivity, turbidity, and water temperature in this study, suggesting that all of these characteristics could be interacting synergistically as contributors to chronic stress in Hellbenders.

In addition to high conductivity, acidic water has been found to negatively affect aquatic organisms through reduced larval growth and delayed metamorphosis (Burmeister, 2015; Farquharson et al., 2016), physiological changes (Neff et al., 2008), and increased mortality (Vatnick et al., 1999; Skei and Dolmen, 2006). Acidic deposition in rivers has long been of concern, especially in the Appalachian Mountains where acidification has caused documented salamander declines (Kucken et al., 1994), and many remaining Hellbender populations reside. Additionally, stream alkalization is of concern for Hellbenders, depending on the underlying geology in the specific location. Not only could stream acidification and alkalization influence Hellbender physiology and increase long-term stress, but also may be a cause of declining population recruitment, which has been documented throughout Hellbender distribution (Wheeler et al., 2003).

Hellbender skin permeability, dependence on cutaneous respiration, and sensitive physiology makes them susceptible to low dissolved oxygen and warm water temperatures. In 2017, we found that Hellbenders in areas with low dissolved oxygen and increased water temperature had N:L ratios indicative of chronic stress. Dissolved oxygen can decrease in areas with low flow, especially when paired with a nutrient increase, and cause physiological and behavioral changes in aquatic organisms (Kramer et al., 1983; Rutledge and Beitinger, 1989; Moore and Townsend, 1998). As river temperatures are continuing to increase in the United States (Kaushal et al., 2010), water temperature may become more important to Hellbender chronic stress and conservation in the future.

Other factors may influence N:L ratios and act as chronic stressors for Hellbenders that are outside of the scope of this study. These factors may include the prevalence of disease, heavy metals, nutrient loading, dams, nonnative aquatic organisms, and alteration of native prey and predator populations, among others (Mayasich et al., 2003; Gall and Mathis, 2009; Burgmeier et al., 2011). One hypothesis for elevated N:L ratios among Hellbenders that was not included in this study initially is the prevalence of human recreation, which was obseved at many sites in this study (Trauth et al., 1992; Mayasich et al., 2003). Hiwassee River is specifically of interest because we found elevated N:L ratios, despite recently documented high abundance of Hellbenders and age class distribution (Freake and DePerno, 2017), and areas with ideal Hellbender habitat. We hypothesize that contributing factors to the observed elevated N:L ratios among Hellbenders in Hiwassee River are impoundments that regulate water flow seasonally. Additionally, environmental variables, such as the water quality parameters and habitat assessed in this study, are influenced by physiographic region, which should be considered when assessing the influence of in-stream microhabitat on Hellbenders. Hellbenders may be locally adapted to the specific physiographic region they occur in, which may be reflected in their physiological tolerances to stream characteristics and fluctuation over time.

Management implications.—Using neutrophil: lymphocyte ratios as indicators of chronic stress in Hellbender populations, we were able to elucidate environmental characteristics that may be most valuable for Hellbender population sustainability using an approach with a mechanistic foundation. Specifically, reducing agricultural runoff near Hellbender populations and increasing riparian buffer could be beneficial to mitigate changes in conductivity, dissolved oxygen, and sedimentation and create shade to lower water temperatures (Cooper et al., 1986; Entrekin et al., 2018). A recent multi-scale study on Hellbenders diagnosed catchment-wide riparian (CWR) forest cover as the main predictor of Hellbender demography (Bodinof Jachowski and Hopkins, 2018). When looking at water quality in relation to CWR forest cover, Bodinof Jachowski and Hopkins (2018) found that conductivity, pH, and water temperature were highly correlated with increased CWR, and are the most important water quality parameters for maintaining viable Hellbender populations, corroborating the findings of this study and the implication of increasing riparian buffers along Hellbender streams. The majority of Hellbender populations with high abundance are within federal boundaries, indicating that reduced land-use change and protection are playing key roles in Hellbender survivorship. However, subtle changes in water chemistry and habitat can have drastic effects on Hellbenders (Pugh et al., 2015), and continuous monitoring of water quality is a key factor for conserving the Hellbender.

Neutrophil: lymphocyte ratios as indicators of chronic stress are applicable to all vertebrate taxa (Davis et al., 2008), and could prove useful for conservation of many species. Incorporating physiological parameters to conservation practices can provide an additional scale not typically considered. The results of this study demonstrate that stress proxies may provide a rapid tool for assessing population vulnerability and specific environmental attributes of concern, which may be important for conservation of long-lived, cryptic species, such as the Hellbender.

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