

## RESEARCH ARTICLE

# Evidence of ectoparasite-induced endocrine disruption in an imperiled giant salamander, the eastern hellbender (*Cryptobranchus alleganiensis*)

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## ABSTRACT

Parasitic leeches and trypanosomes release chemical signals into their hosts to evade immuno-detection, but it is unknown whether these compounds manipulate host behavior or endocrine physiology. We determined whether parasitic infections with leeches and/or trypanosomes affected the immune and stress response of an imperiled giant species of amphibian, the eastern hellbender (*Cryptobranchus alleganiensis* Daudin). We monitored corticosterone and white blood cell counts in response to restraint and injection with adrenocorticotropin hormone (ACTH) or saline for up to 50 h. The presence of leeches dampened hellbender corticosterone responses to restraint and reduced diel patterns of plasma corticosterone. Injection with ACTH restored the normal inter-renal responses of hellbenders, suggesting that leeches, possibly through neurotransmitters in leech saliva, cause down-regulation of corticosterone release at the level of the pituitary or hypothalamus. Infection with leeches also increased the relative abundance of eosinophils, white blood cells often recruited into circulation in response to parasitic infection. Lastly, neutrophil to lymphocyte (N:L) ratios increased in all animals after 24 h of capture and remained elevated for up to 50 h, but these temporal dynamics did not differ with parasite infection. Trypanosome infection did not affect any aspect of hellbender physiology that we measured. Our findings reveal a previously undocumented host–parasite dynamic. While the functional significance to the parasite is unclear, the physiological and behavioral implications for the host are great, given the important role of glucocorticoids in regulating physiology and behavior.

**KEY WORDS:** N:L ratio, Endoparasite, Leech, Trypanosome, Amphibian, Corticosterone

## INTRODUCTION

Parasites can alter the behavior of their host to aid parasite transmission, growth and reproduction, and ultimately their persistence in the environment (Escobedo et al., 2005; Klein, 2003). Often, the proximate mechanisms behind host behavioral manipulations by parasites include parasite-induced changes in hormones and neurotransmitters important in regulating behaviors [e.g. dopamine, glucocorticoids, noradrenaline (norepinephrine), etc.; Klein, 2003]. Although more commonly noted in endoparasites, both endoparasites and ectoparasites are known to cause physiological and behavioral changes in a variety of hosts

including mice, lizards and many invertebrates (Klein, 2003). Physiological changes elicited by parasites often promote host behaviors that improve transmission of the parasite, but in some cases (e.g. highly virulent parasites) physiological and behavioral effects can ultimately impede survival and reproductive capabilities of the host (Klein, 2003). For some sanguivorous ectoparasites like leeches, the possibility for parasite-induced host behavioral or physiological changes exists because their saliva contains a broad array of molecular signals (Hildebrandt and Lemke, 2011; Salzet et al., 2000). However, to our knowledge, the presence of signaling molecules in leech saliva has only been considered in relation to thwarting host immune responses.

Endoparasites and ectoparasites like trypanosomes and leeches can cause a plethora of physiological and pathological effects. In some host species, leech infection can lead to open wounds, reduced body condition, reduced growth and sometimes death (Berven and Boltz, 2001; Santoro et al., 2007). In addition, there is considerable molecular crosstalk between leeches and their hosts (Escobedo et al., 2005; Hildebrandt and Lemke, 2011). Leeches emit a variety of hormones and enzymes [e.g. adrenocorticotropin hormone (ACTH), steroid hormones, histamines, opioids and endocannabinoids] via their salivary glands that serve to prevent blood coagulation, diminish firing of pain receptors and downregulate immunocyte activity of their hosts (Hildebrandt and Lemke, 2011; Salzet et al., 2000). Like leeches, trypanosomes can also cause pathology and engage in molecular crosstalk (Escobedo et al., 2005; Mazon et al., 2006; Oladiran and Belosevic, 2012). For instance, some hematozoans are known to influence receptor abundance for corticotropin releasing hormone (CRH) in the skin and gills of their host (Mazon et al., 2006), which may influence localized swelling. Others are known to directly modulate production of cytokines by their host, presumably to avoid destruction by the host's immune system (reviewed in Oladiran and Belosevic, 2012), but cytokines are known to also have other neurological effects that could influence host behavior (Goshen et al., 2007). Still other protozoans can increase corticosterone and decrease testosterone, glucose, aggression, social displays, dominance and territoriality in their hosts (Schall, 1982; Schall and Dearing, 1987; Dunlap and Schall, 1995). Furthermore, several trypanosomes and other flagellated protozoans are even known to manipulate the physiology and behavior of their sanguivorous vectors (Poulin and Levri, 2012).

Recently, a new species of leech (*Placobdella appalachiensis*) and an unknown species of trypanosome (*Trypanosoma* sp.), were detected in a population of giant salamanders (eastern hellbenders, *Cryptobranchus alleganiensis* Daudin) in Southwest Virginia (Hopkins et al., 2014; Hopkins and DuRant, 2011; Davis and Hopkins, 2013). It is possible that leeches serve as the vector for trypanosome transmission to hellbenders (Hamilton et al., 2007, and citations therein). Because leeches and trypanosomes interact

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with hormones and receptors directly involved in the stress response (e.g. ACTH, CRH and their receptors) and can cause pathological effects that could elicit a stress response, we hypothesized that these parasites could affect the stress physiology of their hellbender hosts. Understanding what factors influence how hellbenders respond to their environment is particularly important because these imperiled amphibians are of great conservation concern. They are the only giant salamander (i.e. Family Cryptobranchidae) in North America and are experiencing severe population declines across much of their range (Taber et al., 1975; Petranks, 1998). Recently, the Ozark subspecies (*Cryptobranchus alleganiensis bishop* Grobman) was offered protection as a federally endangered species (Federal Register, 2010). The eastern subspecies (*Cryptobranchus alleganiensis alleganiensis* Daudin) is currently being considered for this designation because they suffer from similar factors to Ozark hellbenders, including habitat degradation, parasites and disease, and degraded water quality (Mayasich et al., 2003; Hiler et al., 2005; Briggler et al., 2007a,b; Bodinof et al., 2011, 2012; Hopkins and DuRant, 2011; Davis and Hopkins, 2013). However, despite their dramatic population declines we know little about the basic physiology of hellbenders in the wild (but see Hopkins and DuRant, 2011; Solís et al., 2007), and nothing about how parasite infection influences their physiology.

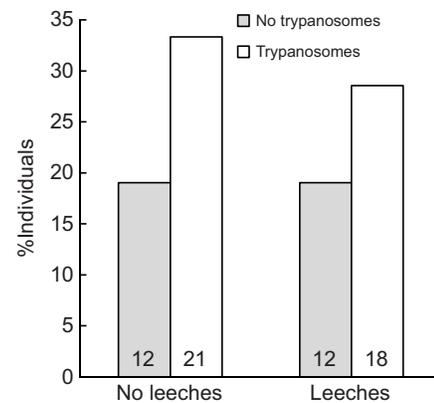
The objective of our study was to determine whether hellbender stress responsiveness could be influenced by parasitic infection, which could have behavioral and/or physiological consequences for the hosts. Two of the most common ways to monitor responses to stress in wildlife involve measures of glucocorticoid (e.g. corticosterone) concentration and white blood cell count, specifically neutrophil to lymphocyte (N:L) ratios. Glucocorticoids are important in regulating blood glucose, and become elevated in plasma when animals respond to stressors, like disease and habitat modification (Herman, 1992; Dallman and Bhatnagar, 2001; Homan et al., 2003). Assessing white blood cell count can provide indications of disease, inflammation or parasite infection (Davis et al., 2008). In addition, N:L ratios change in response to acute and chronic stressors and also can be used to assess stress responses of animals (Davis et al., 2008; Dhabhar, 2002). To achieve our objective, we captured hellbenders from a stream in Virginia, USA, then injected individuals with saline or ACTH, a pituitary hormone that stimulates the interrenal glands (or adrenals in birds and mammals) to secrete corticosterone, and monitored their plasma corticosterone and white blood cell count for 50 h. We assessed hellbenders for leech and trypanosome infection and compared hormone and cellular changes in relation to their infection status.

## RESULTS

### Leeches and trypanosomes

We captured a total of 69 individuals in this study ( $N=7$  juveniles,  $N=31$  females,  $N=31$  males). For six of these individuals we did not collect trypanosome data ( $N=5$  juveniles,  $N=1$  male). For the remaining 63 individuals we detected leeches and trypanosomes in 18 hellbenders, only leeches in 12 hellbenders, only trypanosomes in 21 hellbenders, and neither leeches nor trypanosomes in 12 hellbenders (Fig. 1). We did not detect a significant relationship between leech abundance and trypanosome abundance ( $r^2=0.014$ ;  $P=0.35$ ).

We did not detect an effect of leeches, trypanosomes or their interaction on body mass, snout–vent length (SVL) or body condition (in all cases  $P\geq 0.16$ ). Lastly, there was no relationship between the date hellbenders were captured and trypanosome abundance ( $r^2=0.01$ ,  $P=0.55$ ), but there was a significant positive

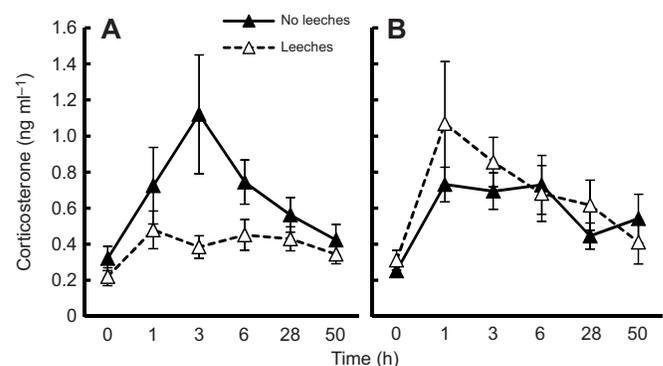


**Fig. 1.** The percentage of eastern hellbenders (*Cryptobranchus alleganiensis*) with and without leeches and/or trypanosomes. Numbers inside bars indicate the number of hellbenders represented in each group.

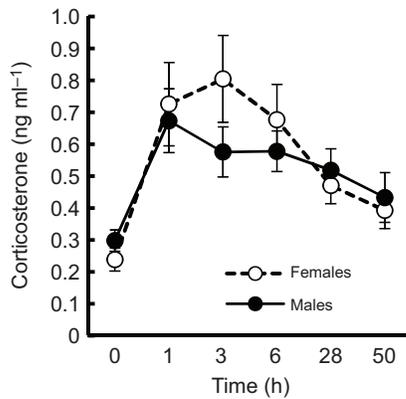
relationship between capture date and leech abundance ( $r^2=0.22$ ,  $P<0.001$ ). Hellbenders tended to harbor more leeches later in the season than earlier in the season.

### Corticosterone

We detected a significant effect of restraint time on hellbender plasma corticosterone concentrations (Figs 2, 3; restraint time:  $F_{5,221}=18.82$ ,  $P<0.001$ ) that was similar across treatments and parasite infections (treatment $\times$ restraint time:  $P=0.28$ ; leech $\times$ restraint time:  $P=0.49$ ; trypanosome $\times$ restraint time:  $P=0.89$ ). Corticosterone increased within the first hour of restraint and remained elevated for up to 6 h post-capture, but began to decrease by 28 h post-capture. However, hellbenders' response to saline or ACTH injection differed depending on leech infection (Fig. 2; treatment $\times$ leech:  $F_{1,42}=10.91$ ,  $P=0.002$ ). Hellbenders with leeches that were injected with saline had reduced corticosterone secretion compared with individuals injected with saline that did not have leeches (Fig. 2A), yet the presence of leeches did not alter corticosterone responses of hellbenders injected with ACTH (Fig. 2B). In contrast, the presence of trypanosomes had no effect on hellbender corticosterone levels (trypanosome:  $P=0.69$ ). There was evidence for sex-based differences in plasma corticosterone levels; males had 6% higher baseline corticosterone level than females at capture, but females achieved greater corticosterone



**Fig. 2.** Plasma corticosterone concentrations of eastern hellbenders (*C. alleganiensis*) with or without leeches following injection with saline or adrenocorticotropic hormone. (A) Saline; (B) adrenocorticotropic hormone (ACTH). Hellbenders were sampled upon capture (0 h), and at 1, 3, 6, 28 and 50 h post-capture.

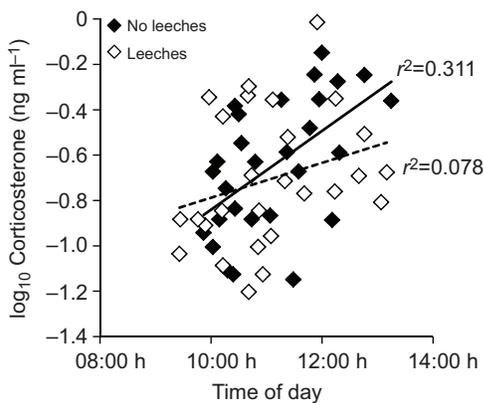


**Fig. 3.** Plasma corticosterone concentrations of male and female eastern hellbenders (*C. alleganiensis*) up to 50 h post-capture following ACTH or saline injection (treatments are combined). Hellbenders were sampled upon capture, and at 1, 3, 6, 28 and 50 h post-capture.

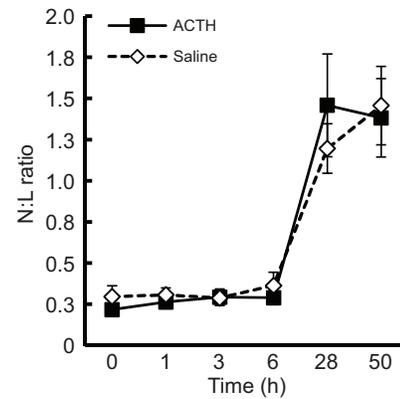
concentrations in response to restraint (Fig. 3; sex×time:  $F_{5,221}=2.16$ ,  $P=0.059$ ). However, there was no difference in how the sexes responded to saline or ACTH injection or to infection with either parasite (sex×treatment:  $P=0.52$ ; sex×leech:  $P=0.72$ ; sex×trypanosome:  $P=0.22$ ).

Regression revealed a significant interactive influence between infection with leeches and the time of day that hellbenders were captured on plasma corticosterone levels (Fig. 4; capture time×leech:  $P=0.028$ ). Baseline corticosterone was higher later in the day in hellbenders that did not have leeches, but there was no significant diel pattern in hellbenders with leeches. Regardless of the presence of leeches (leech:  $P=0.18$ ; leech×date:  $P=0.11$ ), hellbenders captured earlier in the season had higher baseline corticosterone concentrations than hellbenders captured later in the season ( $y=-0.0049x+198.17$ ;  $r^2=0.086$ ,  $P=0.030$ ).

Baseline corticosterone did not correlate with any measure of body size (in all cases  $r^2<0.001$ ,  $P\geq 0.94$ ). We also did not detect any relationships between leech or trypanosome abundance and  $\log_{10}$  baseline corticosterone (in both cases  $r^2<0.001$ ,  $P\geq 0.61$ ).



**Fig. 4.** The relationship between time of day and baseline corticosterone in eastern hellbenders (*C. alleganiensis*) with or without leech infection. Hellbenders were sampled between 09:26 h and 13:15 h. The solid line corresponds to hellbenders that were not infected with leeches ( $y=0.174x-2.575$ ), whereas the dashed line corresponds to hellbenders infected with leeches ( $y=0.077x-1.557$ ). There was no relationship between time of day and corticosterone in hellbenders with leeches; the dashed line is included for visual purposes only.



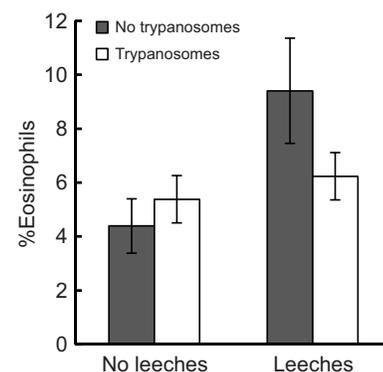
**Fig. 5.** Effect of ACTH or saline injection over time on neutrophil:lymphocyte (N:L) ratios of eastern hellbenders (*C. alleganiensis*). Hellbenders were sampled upon capture, and at 1, 3, 6, 28 and 50 h post-capture. Animals were pooled for this figure regardless of sex and infection status.

### White blood cells

N:L ratios remained similar to baseline ratios (at capture, 0 h) during the first 6 h after capture but increased dramatically by 28 h after capture and remained high at 50 h post-capture (Fig. 5; time:  $F_{5,230}=9.50$ ,  $P<0.001$ ). However, there was no influence on N:L ratio of sex, presence/absence of leeches, presence/absence of trypanosomes, treatment or any of the two-way interactions (in all cases,  $P>0.14$ ). However, at capture (0 h), hellbenders with leeches had 25% higher N:L ratios than uninfected hellbenders, but no such trend was evident in relation to trypanosome infection status. We did not detect a significant correlation between  $\log_{10}$  baseline N:L ratios and  $\log_{10}$  baseline corticosterone ( $r^2=0.016$ ,  $P=0.40$ ).

At the time of capture, the percentage of hellbender white blood cells that were eosinophils was related to leech and trypanosome infection (Fig. 6). In general, individuals infected with leeches had a greater percentage of eosinophils than individuals without leeches (Fig. 6; leech:  $F_{1,52}=4.67$ ,  $P=0.014$ ; trypanosome:  $F_{1,52}=0.90$ ,  $P=0.35$ ; leech×trypanosome:  $F_{1,52}=3.28$ ,  $P=0.076$ ). There was no statistically significant influence of trypanosome or leech infection on any of the other white blood cell types we quantified (in all cases,  $F\leq 3.23$ ,  $P\geq 0.08$ ).

The percentage of white blood cells that were neutrophils increased with increasing baseline corticosterone ( $r^2=0.10$ ,  $y=5.52x+14.42$ ,  $P=0.017$ ), whereas the percentage of white blood



**Fig. 6.** Effect of leech and trypanosome infection on the relative percentage abundance of eosinophils in eastern hellbenders (*C. alleganiensis*). Hellbenders were sampled immediately upon capture.

cells that were lymphocytes decreased with increasing baseline corticosterone ( $r^2=0.09$ ,  $y=-7.05x+79.51$ ,  $P=0.023$ ). However, neither basophils nor eosinophils were correlated with baseline corticosterone (in all cases,  $r^2\leq 0.05$ ,  $P\geq 0.10$ ).

## DISCUSSION

In this study, we found that infection with leeches dampened the normal corticosterone responses of hellbenders to physical restraint, but injection with ACTH restored their normal circulating levels of corticosterone. Hellbenders infected with leeches also tended to have a higher percentage of eosinophils relative to other white blood cells than uninfected hellbenders. Our findings provide important basic insights into hellbender physiology, and suggest that infection with leeches alters fundamental physiological processes that may be important to the health of this imperiled amphibian species.

### Leeches and trypanosomes

The majority ( $N=51$ ; 81%) of the 63 hellbenders captured in the 1.4 km stretch of stream that we sampled were infected with leeches and/or trypanosomes (Fig. 1). Previous studies have documented leeches on Ozark hellbenders (Dundee and Dundee, 1965; Nickerson and Mays, 1973; Moser et al., 2008; Huang et al., 2010; Bodinof et al., 2012); however, only recently have leeches been detected in populations of the eastern hellbender (Hopkins and DuRant, 2011; Davis and Hopkins, 2013; Hopkins et al., 2014). Genetic analyses revealed that this leech is a new species in the genus *Placobdella*, and is distinct from the species of leech that parasitizes Ozark hellbenders (Hopkins et al., 2014). Leech infection frequency (48%) and mean ( $\pm$ s.d.) intensity of infection ( $5.1\pm 6.1$ ) of the hellbenders that we sampled were lower than the infection rates (71%) and mean intensity ( $8.7\pm 22.1$ ) of Ozark hellbenders sampled in three streams in Arkansas and Missouri (Moser et al., 2008). Interestingly, we found that the probability of infection with leeches increased as the summer progressed, an observation that suggests annual cyclicality in attachment dynamics of this leech to its hellbender host. Seasonal host attachment has been observed in other leeches; *Oligobdella biannulata* (Moore), a leech that infects some *Desmognathus* salamanders, leaves its host from May to July to lay eggs, then reattaches between late August and early October and overwinters on the host (Moser et al., 2005). Although the natural history of the newly discovered species of leech infecting eastern hellbenders is currently unknown, a similar phenomenon could account for the increase in leech detection later in the summer.

Although Ozark hellbenders have been known to harbor leeches for several decades (Dundee and Dundee, 1965; Nickerson and Mays, 1973; Moser et al., 2008, 2013), nothing is known about their impact on hellbender health or physiology. Previously, a few hellbenders with leeches were captured in our study population ( $N=9$  of 74), but small sample sizes precluded our ability to rigorously evaluate whether their stress responses were compromised (Hopkins and DuRant, 2011). In the current study, we found evidence that hellbenders infected with leeches had disrupted circadian patterns of corticosterone secretion. Glucocorticoids like corticosterone often peak just before an active period, then later decrease (Dallman, 1993; Breuner et al., 1999). The increase in corticosterone is thought to influence metabolism and support the energetic demands of activity (Dallman et al., 1993; Santana et al., 1995; Breuner et al., 1999). Hellbender corticosterone was highest in individuals captured later in the day, a time when corticosterone would be predicted to be elevated as hellbenders are typically most active in the evenings (Noeske and

Nickerson, 1979; Humphries and Pauley, 2005). Disruption of circadian cyclicality of hormones could have ramifications for metabolic processes, foraging behavior and social interactions, particularly during the time of our study (late summer) when hellbender activity increases as a result of the onset of breeding (Dallman and Bhatnagar, 2001; Richardson and Martin, 1988).

In addition to altered circadian patterns of corticosterone, hellbenders with leeches also exhibited reduced adrenocortical activity in response to capture. Although the mechanism for suppressed adrenocortical responsiveness is not presently known, the ability of ACTH injection to restore corticosterone release indicates that inhibition of corticosterone secretion is not occurring at the inter-renal glands themselves. Instead, this suggests that the dampened stress response is caused by endocrine disruption at the level of the pituitary (e.g. reduced responsiveness to CRH, or reduced capacity for ACTH release) or hypothalamus (e.g. reduced CRH release). We propose that the most likely explanation for reduced corticosterone responses to both stress and time of day in hellbenders with leeches relates to chemical signals transmitted from leeches to their hosts.

Leech saliva is known to contain many different compounds like hormones (e.g. ACTH) and neurosignaling molecules (e.g. opiates and endocannabinoids) that are believed to directly affect host responses to infection (Hildebrandt and Lemke, 2011; Salzet et al., 2000). Some of these compounds could also influence the hypothalamo-pituitary-adrenal (HPA) axis in hosts. Because the physical stimuli associated with capture and restraint failed to elicit a normal adrenocortical response in leech-infected individuals, candidate compounds should be capable of disrupting perception and/or neuroendocrine signaling at the level of the hypothalamus or the pituitary. Exogenous ACTH, opiates and endocannabinoids from leech saliva could all interact with the HPA axis of hellbenders at this level (Patel et al., 2004; Romero and Wikelski, 2006).

It is also unclear whether there is any functional significance of leech-induced suppression of corticosterone release in the hellbenders. Corticosterone inhibition in hellbenders could be beneficial for the leeches. For instance, fish with lower plasma cortisol concentrations had higher copepod infections, likely due to suppression of cortisol-stimulated changes in the skin that can help to prevent parasite infection (Haond et al., 2003). Additionally, there is some evidence that parasites can manipulate host hormones to increase their success by reducing host immune responses, or by directly utilizing host hormones for growth and reproduction (Escobedo et al., 2005). In our system, leeches may have evolved mechanisms to reduce glucocorticoid production in their host if it helps to prevent host immune responses. Alternatively, the effect on hellbender corticosterone may have no bearing on leech success, and may instead be a by-product of the molecular signaling that serves other purposes such as evading detection by the parasitized host. Regardless of why leeches induce changes in adrenocortical activity or how these changes occur, a reduced corticosterone response to stress could impede an individual's ability to respond appropriately to and recover from natural stressors as corticosterone helps restore homeostasis (Sapolsky et al., 2000). Moreover, altered circadian patterns of corticosterone release could impact normal cyclical processes (e.g. appetite, activity and foraging). It remains unclear whether leeches influence other hellbender hormones, but because leeches interfere with communication at the level of the hypothalamus and/or pituitary, disruption of other hormonal pathways (e.g. hypothalamo-pituitary-gonadal axis) remains possible. Moreover, in light of the very low concentrations of corticosterone used by hellbenders, determining whether other

glucocorticoids (e.g. cortisol) are disrupted by leeches should be a priority.

In addition to having altered adrenocortical activity, hellbenders harboring leeches also had higher levels of eosinophils than individuals not infected with leeches. Eosinophils are important in immune responses against parasitic infection, which is consistent with their increased abundance in parasitized hellbenders (Eales, 1997; Davis and Hopkins, 2013, and citations therein). Our findings are consistent with a previous study which found a higher occurrence of eosinophils in parasitized Ozark hellbenders versus non-parasitized eastern hellbenders (Huang et al., 2010). Though we did not detect a significant effect of leeches on N:L ratios in our repeated measures model, N:L ratios at the time of capture (0 h) were 25% higher in individuals harboring leeches, which is consistent with known shifts in N:L ratios that occur in response to infections in other animals (Davis et al., 2008).

Many hellbenders (62%) in the stream we sampled were also infected with trypanosomes. Leeches are most likely responsible for spreading the trypanosome, as trypanosomes are usually vector-borne parasites and are commonly transmitted by leeches (e.g. Hemmingsen et al., 2005). However, the co-association of leeches and trypanosomes was imperfect; not all individuals carrying leeches were infected with trypanosomes, and not all individuals with trypanosomes were actively carrying leeches. This result suggests that not all leeches carry trypanosomes, there is variation in the ability of individual leeches to transmit trypanosomes and/or that hellbenders are capable of clearing trypanosome and/or leech infections. It is also important to note that our method for detecting trypanosomes was imperfect; standard blood smears often produce false negatives (Murray et al., 1977) suggesting that trypanosome infection frequency may have been higher than we estimated. Future studies should evaluate the efficacy of other techniques, such as buffy coat slide preparation or molecular techniques, for detecting trypanosome infections in hellbenders.

Contrary to our predictions, neither trypanosome infection nor trypanosome abundance had any effect on hellbender corticosterone levels, N:L ratios, or other white blood cells, nor did trypanosome infection act additively or synergistically with leech infection. However, trypanosomes have been shown to cause anemia, weight loss, immunomodulation and mortality in other vertebrates (Silva et al., 1999; Oladiran and Belosevic, 2012). In addition, even in individuals not expressing obvious health effects of trypanosome infections, infection can still lead to higher mortality, reduced reproduction and reduced immune responses (Goossens et al., 1997; Smith et al., 2008). While hellbenders may be resilient to trypanosome infection, it is also possible that trypanosomes are affecting some other aspect of health or performance that we did not measure in our study.

#### Relationship between N:L and corticosterone responses

While it was not the goal of this study to compare stress metrics, we found both corticosterone and N:L ratios increased in response to handling stress. However, the timing of these two responses was very different and plasma corticosterone and N:L ratios were not correlated with each other. Corticosterone concentrations increased within 1 h of capture, peaked between 1 and 3 h of capture, and were returning to baseline concentrations by 6 h post-capture. In contrast, N:L ratios remained low until 28 h post-capture, at which time they increased by 400–660% and remained elevated at 50 h post-capture. Changes in N:L or heterophil (H):L ratios are known to lag behind corticosterone responses to stress, but this lag time is typically in the realm of 30 min to 2 h for endotherms (Davis et al., 2008). Other

ectotherms, like hellbenders, can take much longer for N:L ratios to respond to stress (Davis et al., 2008). For instance, newts injected with hydrocortisone did not exhibit altered N:L ratios until 3 days post-injection, whereas some frogs responded within 12–144 h of injection (Bennett and Harbottle, 1968). In addition, a study in poultry demonstrated prolonged elevation of H:L ratios even after corticosterone concentrations had returned to baseline levels (McFarlane et al., 1989), similar to our observations in hellbenders. Although N:L ratios are often used as an indicator of acute stress in individuals when it is difficult to obtain enough blood to analyze for corticosterone (Davis et al., 2008), N:L ratios may not be a good surrogate for detecting stress responses in hellbenders over short time scales (i.e. hours). However, for longer term monitoring of hellbender health (e.g. rehabilitation or relocation), N:L ratios may be very informative, as the effects of stress on N:L ratios may be long lasting after capture. Furthermore, the use of N:L ratios would not require that blood samples be collected within 3 min of capture. It is worth noting that individual counts of neutrophils and lymphocytes (but no other white blood cells nor N:L ratio) correlated with baseline corticosterone concentrations in the direction expected with increasing corticosterone levels (i.e. neutrophils increased and lymphocytes decreased; Davis et al., 2008).

#### Concluding remarks

This study provides evidence that a sanguinivorous parasite can influence fundamental aspects of host physiology that are critical to daily metabolic requirements and activity patterns, as well as responsiveness to environmental stimuli. Although more work is required to understand this parasite–host interaction, it is possible that the responses of hellbenders are related to molecular signals emitted by leeches that evolved to prevent detection and destruction by the host's immune system. Regardless, understanding whether such physiological changes in hellbender hosts can influence fitness-related traits is paramount to hellbender conservation efforts. Future studies that reveal environmental factors that influence rates of ectoparasitism in hellbenders across their declining range will clarify the conditions favoring leech populations and elucidate whether mitigation is possible for this imperiled species. Finally, from a much broader perspective, our study raises fundamental questions about whether the adrenocortical responses of other host species might be influenced by leeches and whether this is an adaptive form of host manipulation by parasites.

#### MATERIALS AND METHODS

##### 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 southwestern Virginia, USA, under VA permit no. 03-5981. 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, with individuals successfully reproducing. The area we chose to sample was located just upstream of the stream reach where we previously documented the presence of leeches and trypanosomes (Hopkins et al., 2014; Hopkins and DuRant, 2011; Davis and Hopkins, 2013).

##### 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 (Humphries and Pauley, 2005; Nickerson and Krysko, 2003). We collected hellbenders during summer 2011, between 09:26 h and

13:15 h using approved IACUC methods. 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 (Makowsky et al., 2010). However, we completed our study just before oviposition, which typically starts in this stream in September, to avoid disturbing active nests.

To obtain baseline blood samples from hellbenders, we captured, restrained and bled adults within 3 min (mean time for all individuals,  $2.53 \pm 0.13$  min) using heparinized syringes following previously used methods (Hopkins and DuRant, 2011). Immediately following the baseline bleed we injected all adult hellbenders with either 50 IU  $\text{kg}^{-1}$  (volumes were dependent on body mass to standardize dosage) of porcine ACTH (Sigma Chemical A6303) or an equivalent volume of saline (ACTH males:  $N=12$ ; ACTH females  $N=14$ ; saline males  $N=15$ ; saline females  $N=17$ ). Similar doses of porcine ACTH have been successfully used to stimulate adrenal release of corticosterone in other amphibians and reptiles (e.g. Glennemeier and Denver, 2002; Romero and Wikelski, 2006). To monitor the response of hellbenders to ACTH or saline injection, we then collected blood samples from hellbenders at 1, 3, 6 and 28 h post-capture. For a subset of individuals (ACTH males:  $N=3$ ; ACTH females:  $N=8$ ; saline males:  $N=9$ ; saline females:  $N=11$ ), we also collected a 50 h post-capture blood sample. The total volume of blood collected per animal was  $<1\%$  of the animal's body mass over the course of the experiment. We restrained hellbenders in mesh bags submerged in the stream until they were bled at 1, 3 and 6 h. For longer term restraint (28 and 50 h bleeds), we transported hellbenders in 18.9 liter buckets containing fresh stream water to a nearby state-operated fish hatchery where we held individual hellbenders in outdoor  $\sim 145$  liter individual flow-through tanks containing  $\sim 85$  liters water, which was continually refreshed with water from the same stream in which the hellbenders were captured. Each tank was also outfitted with an aerator to allow additional oxygenation of the water, and structure for hellbenders to use as cover.

Immediately after blood collection we made blood smears using a standard two-slide technique for subsequent screening for trypanosomes and white blood cell counts. Slides were air dried and stored dry until fixing and staining (see below). We placed the remaining blood on ice and transported it back to the field house within 8 h of collection, where it was centrifuged at 3.5 g. We then removed plasma from the samples and froze it on liquid nitrogen for transport to the lab to be stored at  $-80^\circ\text{C}$ .

We measured total length (TL) and SVL, weighed and sexed (based on cloacal morphology of adults) each hellbender, and subjected them to a physical examination. We classified hellbenders  $<30$  cm TL ( $<19.2$  cm SVL) as juveniles based on the average size at maturity reported in the literature (Peterson et al., 1983, 1988; Hadji-Azimi et al., 1987). Juveniles were not included in the time course because their small body size precluded repeated bleeding. We counted and removed ectoparasites to determine the leech abundance (number of leeches) from each hellbender and stored them in fixative. Distinguishing marks such as recent or healing injuries, scars or missing toes or limbs were noted. We then injected a passive integrative transponder tag (PIT tag) into the tail of each hellbender for future identification.

### 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%). This assay was previously validated and optimized for hellbenders (Hopkins and DuRant, 2011). Hellbenders have very low circulating concentrations of corticosterone compared with other amphibians (Hopkins and DuRant, 2011), so we further verified our assay results with two radioimmunoassays (Ignacio T. Moore, unpublished; S.E.D. and L.M.R., unpublished) and by liquid chromatography-mass spectrometry (LC-MS) using an assay that utilized a deuterated corticosterone standard and selective reaction monitoring (Richard Helm and W.A.H., unpublished data).

Based on our optimization (Hopkins and DuRant, 2011), we analyzed samples at a 1:3 plasma dilution with 2% steroid displacement buffer. Each 96-well plate included standards ranging from 15.63 to 2000  $\text{pg ml}^{-1}$  for standard curve construction, with standards (non-specific binding and

total binding) assayed in triplicate. Each plate also contained a 500  $\text{pg ml}^{-1}$  standard assayed in quadruplicate to allow us to estimate interplate variability. We ran samples in duplicate. Assay detection limit ranged from 0.03 to 0.15  $\text{ng ml}^{-1}$ . When plasma corticosterone concentrations fell below the detection limit, which occurred in 24 out of 322 samples, we used the plate's detection limit as the plasma corticosterone concentration for that individual sample. 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  $\text{pg ml}^{-1}$  standards. Intra- and inter-assay variation was 2.91% and 21.6%, respectively.

### White blood cell count and N:L ratio

We air dried blood smears, then stained them with Giemsa (Camco Quik Stain II). We examined slides under  $400\times$  magnification and identified leukocytes as neutrophils, lymphocytes, eosinophils, basophils and monocytes (Hadji-Azimi et al., 1987; Turner, 1988; Thrall, 2004). At least 100 leukocytes were counted and only fields of view with even distributions of red blood cells were used. The relative number (i.e. proportion) of each cell type was calculated based on the number of cells of that type counted divided by the total number of leukocytes counted. The N:L ratio was calculated from the proportions of these cell types.

### Trypanosomes

We stained slides with a buffered Giemsa stain (Camco Quik Stain II) and a single observer (A.K.D.) examined them with a light microscope at  $400\times$  magnification. For each slide, 50 random fields of view were examined for the presence of blood parasites (Davis and Hopkins, 2013). If no parasites were found, the animal was assumed to be uninfected. The number of trypanosomes counted for each individual (within 50 fields) is hereafter referred to as trypanosome abundance.

### Statistical analyses

All statistical analyses were run in SAS 9.1 (SAS Institute Inc., Cary, NC, USA) or Microsoft Excel and statistical significance was recognized at  $\alpha < 0.05$ . Where appropriate, we tested for normal distribution of the data and homoscedasticity using Ryan-Joiners and Bartlett's tests, respectively. Corticosterone and N:L ratios were  $\log_{10}$  transformed to better meet assumptions of normality. Unless otherwise noted, raw data were used in statistical models.

### Leeches and trypanosomes

We tested for differences in body mass (g), SVL (cm) and body condition (calculated as the residuals of body mass versus SVL) among hellbenders with and without leeches and/or with and without trypanosomes using a MANOVA. The model contained leech, trypanosome and their interaction. In addition, we used linear regression to determine whether leech and trypanosome abundance co-varied, and to determine whether either leech or trypanosome abundance varied with date.

### Corticosterone and N:L ratios

We used two repeated-measures ANCOVAs to determine whether treatment (ACTH or saline injection), sex or presence of parasites affected hellbender corticosterone concentrations or N:L ratios. Models included treatment (ACTH or saline), sex, trypanosome (absence/presence), leech (absence/presence), and time as main effects and all two-way interactions. We tested the following specific hypotheses: (1) plasma corticosterone concentrations and N:L ratios over time would be dependent on treatment (ACTH or saline injection), with males having higher corticosterone concentration and N:L ratios than females (Hopkins and DuRant, 2011), (2) regardless of sex, infection with leeches would affect plasma corticosterone and N:L responses to treatment over time, and (3) regardless of sex, infection with trypanosomes would affect plasma corticosterone concentration and N:L responses to treatment over time. We initially included three-way interactions relevant to our hypotheses. However, all three-way interactions were highly insignificant for both models and were subsequently dropped from final models (in all cases,

$F \leq 1.22$ ,  $P \geq 0.25$ ). We retained all two-way interactions between main effects in models. Three hellbenders (two saline injected and one ACTH injected) had corticosterone concentrations that were 4–25 times higher than those of all other hellbenders at every time point; because they were outliers, we did not include them in analyses of corticosterone. We included the time of day and date hellbenders were captured as possible covariates in the corticosterone model, but dropped date because it was not significant in the repeated measures ANCOVA ( $P=0.81$ ). Neither time of day nor date of capture correlated with N:L ratios (in both cases,  $r^2 \leq 0.03$ ,  $P \geq 0.23$ ), so these variables were not included as covariates in the N:L ratio model. Based on the effects of leeches on adrenocortical responses to stress (see Results), we used robust regression to test for interactions between infection with leeches and time of day on baseline corticosterone concentrations. We also tested for interactions between leech abundance and the date that hellbenders were captured on baseline corticosterone concentrations.

We used linear regression to test for relationships between baseline corticosterone and N:L ratios, and for relationships between baseline corticosterone and measures of body size (mass, SVL and body condition).

### White blood cells

Monocytes were not detected in any hellbenders so this white blood cell was not included in analyses. We determined whether the relative abundance of the major white blood cells (lymphocytes, neutrophils, basophils and eosinophils) varied at the time of capture (0 h) among leech and trypanosome infected and uninfected hellbenders using four separate two-way ANOVAs, because white blood cells can respond independently of one another depending on the immune challenge (Eales, 1997). For instance, eosinophils tend to increase in response to ectoparasites, whereas neutrophils are important in phagocytosing non-viral infections (Eales, 1997; Davis et al., 2008). Finally, we also used linear regression to determine whether any of the individual white blood cells correlated with baseline corticosterone.

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### Competing interests

The authors declare no competing or financial interests.

### Author contributions

All authors contributed to the design of the study, sample analyses, and writing of the manuscript. S.E.D. and W.A.H. collected all data and conceived of the study.

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