The Influence of Environmental Conditions and Coinfection by Blood-Feeding Parasites on Red Blood Cell Physiology of an Ectothermic Host

Katherine L. Slack*
Jordy Groffen
William A. Hopkins
Department of Fish and Wildlife Conservation, Virginia Tech, Blacksburg, Virginia 24061

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ABSTRACT

Vector-borne blood parasites cause myriad sublethal effects and can even be deadly to endotherms, but far less is known about their impacts on ectothermic hosts. Moreover, the pathologies documented in endotherms are generally linked to infection by blood parasites rather than by their vectors. Here, we measured hematocrit, hemoglobin, and relative proportions of immature red blood cells to evaluate the physiological effects of two blood-feeding parasites and coinfection on ectothermic hosts, differentiating among pathological responses, extrinsic factors, and natural variations. We investigated a population of wild eastern hellbender salamanders (*Cryptobranchus alleganiensis*), which harbor leeches (*Placobdella appalachiensis*) that transmit blood parasites (*Trypanosoma* spp.) to their hosts, often resulting in coinfection. We observed seasonal changes in host hematology corresponding to water temperature and demonstrated their ability to modulate hematological parameters in response to acute stress. We reveal seasonal relationships between parasite dynamics and host physiology, in which peak parasitemia occurred when hosts had seasonally high hematocrit and hemoglobin concentrations. We found that coinfected individuals expressed symptoms of anemia, including a regenerative response to depletion of their red blood cells. We also documented a more pronounced pathological response to leech vectors than to the trypanosomes they transmit. Our research underscores the complex interactions between host physiology, multiple parasites, and environmental factors and highlights the pathologies associated with the vector in coinfections. Given the contributions of climate change and disease in the rapid global decline of ectotherms such as amphibians, our study provides timely foundational insights into multiple factors that influence their red blood cell physiology.

Keywords: ectotherm, hematology, parasite, coinfection, anemia, stress, season, temperature.

Introduction

Blood-feeding ectoparasites are ubiquitous in their distribution and affect vertebrate health by transmitting endoparasites to the host, in some cases leading to epidemics of vector-borne disease in humans, livestock, and wildlife populations (Greenwood et al. 2008; Schmunis and Yadon 2010; Martínez-García et al. 2021). Well-studied examples include *Plasmodium*, *Babesia*, and *Trypanosoma* species that are vectored to their mammalian and avian hosts by biting arthropods (Tizard et al. 1977; Tuteja 2007; Brun et al. 2010; Pérez-Molina and Molina 2018; Martínez-García et al. 2021). In these cases, the virulent pathology is typically from the blood parasite. However, sanguinivorous vectors themselves can have a variety of effects on host physiology, including depletion of red blood cells (Kaufman 1989; Nuttall 1998) and effects on the endocrine system (DuRant et al. 2015). Moreover, the saliva of some vectors contains multiple compounds, such as anticoagulants and immune modulators, capable of altering host physiology to optimize conditions for parasites (Nuttall 1998; Salzet et al. 2000; Caljon et al. 2006). Although we know that blood-feeding vectors can influence host physiology (Caljon et al. 2006; Hildebrandt and Lemke 2011; DuRant et al. 2015; Hopkins et al. 2016), most research neglects the possible interactive effects of the coinfecting vector and blood parasite on the host.

The vast majority of research on vectors and vector-borne disease also centers on endothermic hosts, despite the fact that wild ectotherms are known to commonly carry multiple species of vector-borne parasites (e.g., Densmore and Green 2007; Hopkins et al. 2016; González et al. 2021). The literature on blood-feeding vectors transmitting parasites to ectotherms is relatively scarce and mostly relates to experimental infection (Desser et al. 1990; Lainson and Paperna 1995; Davies and Johnson 2000; Bricknell 2017) and aquaculture management rather than to wild populations (Bricknell 2017). Thus, to elucidate the pathology of blood-feeding parasites on wild ectothermic hosts, we need a better understanding of the

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*Corresponding author; email: kslack@vt.edu.

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seasonality of infection and how intrinsic characteristics of the host and environmental conditions influence host physiology. For example, red blood cell physiology of ectotherms can vary depending on circadian and circannual oscillations (Bridges et al. 1976; Frangioni and Borgioli 1984) as well as temperature (da Silva et al. 2013), which need to be understood to differentiate natural variation from pathological changes caused by parasites.

In this study, we used giant hellbender salamanders (*Cryptobranchus alleganiensis alleganiensis*) to investigate the modulation of hematological parameters in a wild ectotherm and to explore the interplay between blood-feeding endoparasites, their ectoparasitic vectors, and their collective impacts on host health. Eastern hellbenders are fully aquatic giant salamanders of the family Cryptobranchidae, which represents one of the oldest lineages of salamanders (Pyron and Wiens 2011). It is the heaviest amphibian in North America, with a mass of up to 2.2 kg, growing up to 74 cm in total length (Petranka 1998), and a lifespan of 25 yr or more (Nickerson and Mays 1973; Taber et al. 1975). Hellbenders are an imperiled species (USFWS 2018) that tend to thrive in cool, fast-flowing, oxygen-rich streams (Nickerson and Mays 1973) with low organic matter and fine sediments (Pugh et al. 2016), extensive boulder habitat, and high upstream forest cover (Pugh et al. 2016; Bodinof Jachowski and Hopkins 2018). Hellbenders in our study system are parasitized by freshwater leeches (*Placobdella appalachiensis*; Hopkins et al. 2014, 2016), which serve as the vector for a newly identified species of trypanosome (W. A. Hopkins, unpublished manuscript) that has yet to be described (*Trypanosoma* sp.; Davis and Hopkins 2013). In this study, we focused on male hellbenders as they engaged in extended solitary paternal care of their eggs and larvae under large boulders (Bishop 1941; Nickerson and Mays 1973; Hopkins et al. 2023). Although the timing of the breeding season can vary depending on environmental factors and geographic location (Nickerson and Mays 1973), in our study in Virginia the timing of oviposition ranged from August 30 to September 22. Following a ~60-d period of embryonic development, larvae hatched from eggs and remained in the nest with the guarding male until the following April–May (Hopkins et al. 2023).

We hypothesized that seasonal fluctuations in temperature, acute stress, and blood-feeding endo- and ectoparasites would influence red blood cell physiology in hellbenders. We predicted that hellbenders infected with one or both parasites would have lower baseline hematocrit and hemoglobin because of the lysing and consumption of circulating red blood cells. We also predicted that the proportion of newly recruited immature red blood cells would increase with the intensity of the infection, indicative of a regenerative response, and be inversely correlated with baseline hematocrit and hemoglobin. We predicted that hellbenders' physiological responses to acute stress would be consistent with those of other vertebrates (Romero and Butler 2007), with hematocrit and hemoglobin concentrations increasing in circulation in response to standardized acute handling stress. Because hellbenders' metabolic demands should decrease in winter and thus reduce their need for circulating red blood cells to meet their oxygen requirements, we also predicted that they would have lower baseline hematocrit and hemoglobin and greater hemoconcentration response to stress during colder months. Taken together, we provide one of the most comprehensive assessments of the red blood cell responses of an amphibian to parasite infection and coinfection within the context of environmental variation that is expected to intensify in the face of future climate change and deforestation.

Material and Methods

Site Description and Sample Collection

Our study was conducted in four stream reaches (~100–200 m long) along a ~13-km segment of a stream within the upper Tennessee River Basin in Virginia. We selected reaches with similar site quality, upstream catchment-wide riparian forest cover (64.3%–67.9%), and high population densities of healthy hellbenders that successfully reproduce annually (Bodinof Jachowski and Hopkins 2018; Hopkins et al. 2023). Underwater artificial shelters ($N = 30$–35) were deployed up to 10 yr before this study in each stream reach (Hopkins et al. 2023). These shelters mimic cavities under large boulders that hellbenders use for year-round shelter and nesting habitat, and they are used to monitor population trends, annual reproduction, physiology, and behavior of hellbenders (Bodinof Jachowski et al. 2020; Button et al. 2020*a*, 2020*b*; Galligan et al. 2021; Hopkins et al. 2023; Brooks et al. 2024; Case et al. 2024; O'Brien et al. 2024). We monitored the hourly temperature of each stream reach using U24-001 HOBO conductivity data loggers (Onset Computer, Bourne, MA; range: 0–10,000 μS/cm).

We captured reproductive male hellbenders nesting in our artificial shelters across the paternal care period in 2020 and 2021. Sex was determined based on external morphology, with males showing obvious cloacal swelling during the breeding season (Makowsky et al. 2010). Focusing on reproductive males enabled us to remove the confounding influences of sex and life history stages on hematology and is also important because of recent evidence that hellbender population declines may be influenced by male physiology and parental care decisions (Hopkins et al. 2023). We sampled each individual at oviposition (day 0 [August 30–September 22], $N = 58$) and at attempted recapture at midembryonic development (~day 30 [October 2–17], $N = 48$), at larval hatching (~day 60 [November 5–21], $N = 44$), and before juvenile emergence from the nest in the spring (~day 200 [March 25–April 20], $N = 25$) or until nest failure ($N = 33$). Our prior work demonstrated that adult male hellbenders are tolerant of repeated capture and blood collection and that repeated handling does not affect their nesting success (Hopkins et al. 2023) or parental care behaviors (O'Brien et al. 2024). However, to eliminate the possibility that seasonal changes in red blood cell physiology could be caused by repeated phlebotomy of nesting males, in 2021 we also sampled an out-group of nonnesting male hellbenders. Specifically, we sampled one group of unique nonnesting males at midembryonic development ($N = 8$) and another at hatching ($N = 7$). These males were not bled before their single sampling event, allowing us to compare their red blood profiles to those of breeding males that had been sampled repeatedly. This out-group also provided the opportunity for a hematological comparison between males without nests and males providing parental care to their eggs.

Blood Processing and Hematology

Each time we removed a hellbender from its artificial shelter, a team member wearing neoprene gloves would immediately restrain the animal on a flat sterilized plastic board with clean wet towels. Then we used a heparinized syringe to obtain a baseline (within 3 min of capture [average time: 126 ± 6 s]; hereafter, time 0 [T0]) sample of whole blood (collecting an average of ~69 μL/100 g body mass) from the caudal vein (Hopkins and DuRant 2011; DuRant et al. 2015; Hopkins et al. 2016, 2020; Galligan et al. 2021; Case et al. 2024). We then transported the individual in a plastic bin containing fresh stream water to the stream bank for processing. We assessed the hellbender's body condition; noted any abnormalities in physical appearance, such as injury; and obtained standard body morphometrics, including snout-to-vent length (cm), total length (cm), and mass (g). We recorded the number of leeches attached to the individual and the number of leech bite wounds, which are distinct from any other markings on hellbenders. After 60 min of being held in the container of stream water, we bled the hellbender again (hereafter, time 60 [T60]) to assess stress-induced changes in hematology. This method of capture, handling, and confinement has been shown to provoke the glucocorticoid stress response in hellbenders (DuRant et al. 2015; Hopkins et al. 2020). Because our capture-and-restraint protocol should also provoke the release of catecholamines, which increases heart rate and blood pressure, we expected hemoconcentration to occur because of plasma efflux and/or splenic release of red blood cells (Romero and Butler 2007). We used uniquely coded passive integrated transponder (PIT) tags that had been implanted in prior years to identify each hellbender. If individuals were newly captured in this study, we inserted a PIT tag subcutaneously along the tail's dorsolateral region, approximately 5 cm posterior to the tail base, before releasing it back to its artificial shelter.

At each interval where a blood sample was collected, we injected ~75 μL of whole blood into two heparinized capillary tubes and transported them on ice to the lab for later determination of hematocrit (also referred to as packed cell volume). We determined hemoglobin in the field using a handheld hemoglobinometer (HemoCue Hb 201+; HemoCue, Brea, CA) by placing one or two drops of fresh blood onto duplicate microcuvettes before inserting them into the device (Velguth et al. 2010). The performance of the instrument was periodically checked during the study using R&D Glu/Hgb trilevel liquid controls (R&D Systems, Minneapolis, MN). Although the HemoCue device has not yet been clinically validated for use in salamanders, it has been successfully used in studies examining hellbender hematology (Hopkins et al. 2016; Bodinof Jachowski et al. 2024), as well as in other salamander research (Bazar et al. 2009). Alternative methods, such as Sahli's hemoglobinometer, have also been used for studying hellbenders (Jerrett and Mays 1973) and have produced results consistent with our findings. The average of the duplicate hemoglobin values (high reproducibility; intraclass correlation coefficient [ICC] = 0.99; Sokal and Rohlf 1995) was used to represent the individual's blood hemoglobin concentration at each time point. We also made duplicate blood smears with fresh whole blood using a standard two-slide wedge technique. The remaining volume of whole blood from the T0 and T60 samples was placed on ice for transport to the lab.

Back at the lab and within 8 h of sample collection, we centrifuged duplicate capillary tubes for each individual at 5 *g* for 5 min. We quantified hematocrit levels using a standard hematocrit capillary tube reader (McCormick Scientific, St. Louis, MO) to calculate the proportion of packed red blood cells in each sample. The average of the duplicate values (ICC = 0.99) was used to represent each individual's hematocrit at each time point. We used a Hamilton syringe to remove plasma from the upper layer of the sample, which was then archived at −80°C for companion endocrinology studies. We then extracted the buffy coat, the thin white layer of white blood cells on top of the packed red blood cells, which is also where trypanosome parasites partition during centrifugation (Hopkins et al. 2016). We made duplicate buffy coat smears using the standard two-slide wedge technique to quantify trypanosome infection status, a technique that greatly increases the detectability of trypanosomes compared to standard whole-blood smears (as described in Hopkins et al. 2016). Buffy coat and whole-blood smears were stained using Camco Quik Stain II and air-dried in preparation for microscopy.

Microscopy

For microscopy, a single observer (K. L. Slack) was blinded to the identity of each slide and examined 50 random fields of view in both whole-blood and buffy coat smears. We used a light microscope at ×400 magnification to quantify the proportion of polychromatic cells in whole-blood smears and the presence of trypanosomes in buffy coat smears. Trypanosomes were identified using morphological descriptions (Davis and Hopkins 2013) that have been used consistently in other studies (DuRant et al. 2015; Hopkins et al. 2016). Before the study started, we confirmed the reliability and reproducibility of the observer by blindly quantifying 20 whole-blood smears in triplicate. We then calculated the ICC score for polychromasia (ICC = 0.97) and trypanosomes (ICC = 0.98) to demonstrate a high degree of reproducibility.

Quantifying Polychromasia

Polychromatic cells are immature red blood cells that can be distinguished from mature red blood cells by the former's basophilic cytoplasm, rounder form, nucleus occupying a greater portion of the cellular body, and more dispersed chromatin (supplemental fig. 1; supplemental figs. 1–5 are available online). Because there is no established protocol to estimate the relative abundance of polychromatic blood cells compared to mature cells in amphibians, we modified a method described by González et al. (2021). Their method calculated the average number of red blood cells within a single field of view and multiplied it by 50, which was used as the standard denominator to quantify the proportion of polychromatic cells within 50 fields of view. However, we adjusted their protocol for our study because the number of red blood cells within a monolayer decreased and was more dispersed as hematocrit decreased. Since we evaluated individuals with varying levels of hematocrit, we could not estimate the proportion of polychromatic red blood cells using a single average number as the standard denominator. Thus, our modification of their protocol accounted for the variation in circulating red blood cells associated with differences in hematocrit among individuals. We counted the total number of red blood cells within 50 fields of view for 15 slides with variable corresponding hematocrit (15%–50%) and performed a linear regression ($y = 3{,}100.2 + 26.647 \times \text{hematocrit}$; $R^2 = 0.70$) between these two parameters. Based on this relationship, we were able to estimate the total number of red blood cells per 50 fields of view from each individual's hematocrit, which was then used as the denominator for each individual to calculate the proportion of their red blood cells that were polychromatic.

We quantified polychromasia from whole-blood smears using T0 and T60 blood samples from the 2021 season. However, in the 2020 season, we lacked T60 whole-blood smears and were unable to quantify polychromasia for that particular subset. To resolve this, we performed a Pearson's correlation between the proportion of polychromatic red blood cells at T0 and T60 during the 2021 paternal care period to determine whether metrics differed following exposure to 60 min of acute stress. Polychromasia at T0 and polychromasia at T60 were highly correlated ($r = 0.92$) and did not change appreciably because of restraint. Therefore, we used the linear model describing this relationship ($y = 0.015 + 1.064 \times \text{T0}$; supplemental fig. 2) to estimate the proportion of polychromatic red blood cells at T60 for the 2020 season, so that it could be included in our statistical models.

Statistical Analysis

We used R (R Core Team 2022) to conduct all statistical analyses. We created linear mixed effects models and generalized linear mixed effects models to analyze the relationship between hematological parameters and acute stress, body size, seasonal changes in temperature, and parasitic infection. For the red blood cell response variables, we chose a multivariate approach to reduce the dimensionality of the dataset and to account for their interdependence. Since hematological parameters were measured on different scales, we standardized each variable to have a mean of 0 and a standard deviation of 1. We performed a principal component analysis (PCA) based on a correlation matrix to provide a response variable representing hematocrit, hemoglobin, and proportion of polychromatic red blood cells at T0 and T60. The resulting PC scores were used as response variables in our models.

Sampling day (oviposition, midembryonic development, hatching, emergence) and 24-h median temperature (°C) before capture were strongly correlated predictors and produced comparable model outputs. Thus, both variables could not be included within the same model. We ultimately chose to use the 24-h median temperature to represent seasonal effects in the model because temperature is a dominant physiological driver of ectothermic metabolism (da Silva et al. 2013) and is inversely related to dissolved oxygen concentration (Li and Liu 2019), a key influence on vertebrate red blood cell physiology. We confirmed this choice statistically by subsetting the data by sampling day and running models with those subsets to see whether temperature still had an effect. The results showed that temperature significantly affected the PC1 within each sampling day, indicating that temperature is the overarching driver of red blood cell parameters across seasons (supplemental fig. 3).

We also assessed seasonality among the predictors for parasites. We used a Poisson distribution in our models to evaluate how parasite abundance varied across the parental care period. The number of leeches, leech bites, and trypanosomes greatly varied among individuals, so we scaled each variable representing the intensity of parasitic infection to have a mean of 0 and a standard deviation of 1 (Schielzeth 2010) before using them as predictors in the models for PC1 and PC2. For our categorical model predictors, we used the timing of blood samples (T0 or T60). We also chose to include total length (cm) as a covariate after preliminary analysis confirmed the influence of body size on red blood cell parameters. A previous study found positive correlations between body size and leech infection in hellbenders (Hopkins et al. 2014). However, in our exploratory analysis, we found no correlation between body size and parasite abundance. Thus, the potential interaction was not considered in our models. Additionally, we examined the interactions between capture restraint and temperature, between capture restraint and body size, and between parasite infection intensity and temperature.

We used the lme4 package to analyze PC1 and PC2, which were included as response variables with a normal distribution and an identity link function. The individual PIT tag ID was included as a random effect in all models to account for repeated measures on the same individuals. Following model composition, we used likelihood ratio tests and Akaike's information criterion corrected for small sample sizes (AICc; Burnham and Anderson 2002) to determine the best-fitting model.

Last, we compared male hellbenders with nests to the out-group of nonnesting males to assess whether seasonal changes in red blood cell physiology could be caused by repeated phlebotomy of the nesting males. We subset the data for captures that occurred only at midembryonic development and hatching and then performed a separate PCA that included both nesting males and nonnesting males from the out-group. We used the resulting PC scores as the response variable. For the predictors, we included an interaction between reproductive status (nesting, nonnesting) and temperature, in addition to the same set of predictors from the top-ranking AICc model, which best represented the variation in red blood cell parameters in nesting male hellbenders across the full parental care period.

Following the results from our statistical analysis, we also performed a secondary post hoc analysis on the relationship between the proportion of polychromatic red blood cells and the mean corpuscular hemoglobin concentration (MCHC) to explore the potential influence of immature red blood cells on oxygen-carrying capacity of the blood. We calculated MCHC by dividing hemoglobin (g/dL) by hematocrit (%) to obtain the average hemoglobin content per cell volume (Witeska 2015; Hopkins et al. 2016; Johnstone et al. 2017). We then scaled and centered the values and performed a linear mixed effects model with the transformed MCHC as a response variable and the proportion of polychromatic red blood cells as the predictor variable.

Results

We obtained blood metrics from 73 adult male hellbenders (nesting, $N = 58$; nonnesting out-group, $N = 15$) in 2020 and 2021. Over the 2 yr of the study, which included multiple recaptures across the paternal care period, we obtained data from 190 capture events (nesting, $N = 175$; nonnesting out-group, $N = 15$). We collected 189 sets of paired blood samples from T0 and T60, which were used to determine hematocrit (T0, $N = 189$; T60, $N = 189$) and hemoglobin (T0, $N = 190$; T60, $N = 189$) concentrations as well as to create blood smears for microscopy and to estimate polychromasia (T0, $N = 189$; T60, $N = 102$). We obtained morphometrics and metrics to evaluate parasitic infection (leeches, leech bites, and trypanosomes) for all 73 individuals.

We identified seasonal differences in environmental conditions across the paternal care period. The 24-h median temperature before capture varied across seasons, ranging from 6.91°C to 19.24°C. Temperatures were highest at oviposition, followed by a decline during midembryonic development, reaching their lowest levels at hatching and overwintering and rising again during larval emergence in the spring (fig. 1).

We observed seasonal differences in the prevalence and intensity of parasite infections. All individuals with leeches attached were also infected with trypanosomes. Total prevalence of trypanosome infection remained high throughout the year, ranging between 84.5% and 89.6%, while coinfection decreased following midembryonic development (fig. 2). Among infected individuals, the intensity of trypanosome infection varied significantly seasonally; it was highest at oviposition and similar at midembryonic development, but it then decreased at hatching and decreased further at emergence in the spring (fig. 3*A*; table 1; supplemental table 1; supplemental tables 1–3 are available online). The intensity of leech infection was significantly greater at oviposition than during the rest of the parental care period (fig. 3*B*; table 1; supplemental table 1). The number of leech bites within bitten individuals was significantly lower during spring emergence than during the rest of the parental care period (fig. 3*C*; table 1; supplemental table 1). Parasite prevalence, intensity of infection, and average red blood cell parameters within each sampling period are reported in table 1.

The PCA performed on red blood cell parameters of reproductive males produced two PC scores that together explained 98% of the variance (table 2). Red blood cell PC1 explained 71.6% of the variance and was positively loaded with hematocrit and hemoglobin but negatively loaded with polychromasia. Red blood cell PC2 explained 25.9% of the variance, and all parameters were positively loaded. However, polychromasia strongly influenced PC2, whereas hematocrit had a moderate influence and hemoglobin had a weak influence on the PC.

Among the candidate models for PC1, the two top-ranking models representing the effects of stress, temperature, total length, and parasitic infection accumulated all the AICc weight (table 3). The top-ranked model had an AICc weight of 0.93, while the second-ranked model had an AICc weight of 0.07. The difference in AICc values (ΔAICc) between the two competing models was greater than 2, indicating that the top-ranked model was more likely to explain the variation in PC1 than the second-ranked model. Predictions and fit for the top-ranking model representing PC1 are displayed in figure 4. PC1 increased in response to stress ($\beta = 1.733$, $\mathrm{SE} = 0.355$, $t = 4.880$, $P < 0.001$) and with temperature ($\beta = 0.266$, $\mathrm{SE} = 0.019$, $t = 13.688$, $P < 0.001$; fig. 5), but the interaction between stress and temperature was not statistically conclusive ($\beta = -0.04$, $\mathrm{SE} = 0.025$, $t = -1.682$, $P = 0.094$). PC1 decreased as total body length ($\beta = -0.094$, $\mathrm{SE} = 0.029$, $t = -3.254$, $P = 0.002$; fig. 6) and leech bites ($\beta = -0.507$, $\mathrm{SE} = 0.308$, $t = -1.650$, $P = 0.099$) increased, but the latter was not statistically significant regardless of the temperature (leech bites × temperature: $\beta = -0.001$, $\mathrm{SE} = 0.020$, $t = -0.040$, $P = 0.968$). PC1 was negatively influenced by the number of leeches attached ($\beta = -3.249$, $\mathrm{SE} = 0.569$, $t = -5.708$, $P < 0.001$), but the effect of leech infection was dependent on temperature (leeches × temperature: $\beta = 0.189$, $\mathrm{SE} = 0.033$, $t = 5.746$, $P < 0.001$). Specifically, the negative effect of leeches on PC1 was more pronounced in warmer temperatures. To visualize the effects of leeches, leech bites, and temperature on PC1 scores, we added the number of leeches and bites together as a single predictor variable (supplemental fig. 4) and incorporated it into a three-dimensional scatterplot (fig. 7). Both figures (fig. 7; supplemental fig. 4) demonstrate how PC1 values decrease with the temperature and the combined number of leeches and leech bites.

The top-ranked model for PC2, which included the predictors of stress, temperature, and infection status, had an AICc weight of 0.99 and a ΔAIC greater than 2, confirming that it outcompeted the lower-ranking candidate models (table 4). PC2 increased in response to stress ($\beta = 1.576$, $\mathrm{SE} = 0.271$, $t = 5.807$, $P < 0.001$), and the magnitude of the stress response decreased as temperature increased (stress × temperature: $\beta = -0.049$, $\mathrm{SE} = 0.019$, $t = -2.597$, $P = 0.009$; supplemental fig. 5). Infection status influenced PC2; specifically, individuals with coinfection had much higher PC2 scores ($\beta = 2.159$, $\mathrm{SE} = 0.486$, $t = 4.439$, $P < 0.001$) than those with only trypanosomes present ($\beta = 0.260$, $\mathrm{SE} = 0.486$, $t = 0.555$, $P = 0.580$). As temperature decreased, PC2 increased in coinfected individuals (coinfection × temperature: $\beta = -0.140$, $\mathrm{SE} = 0.031$, $t = -4.546$, $P < 0.001$). However, there was no statistical relationship between PC2 and individuals infected with only trypanosomes, even after accounting for temperature (only trypanosomes present × temperature: $\beta = -0.012$, $\mathrm{SE} = 0.030$, $t = -0.410$, $P = 0.682$). Moreover, PC2 had strong positive loadings for the proportion of polychromasia (table 2; fig. 8*A*). The trends in figure 8*A* reflect increased polychromasia as the parental care period progressed and depended on infection status. Finally, the results from our secondary analysis on the relationship between MCHC and polychromasia indicate that as the proportion of polychromatic

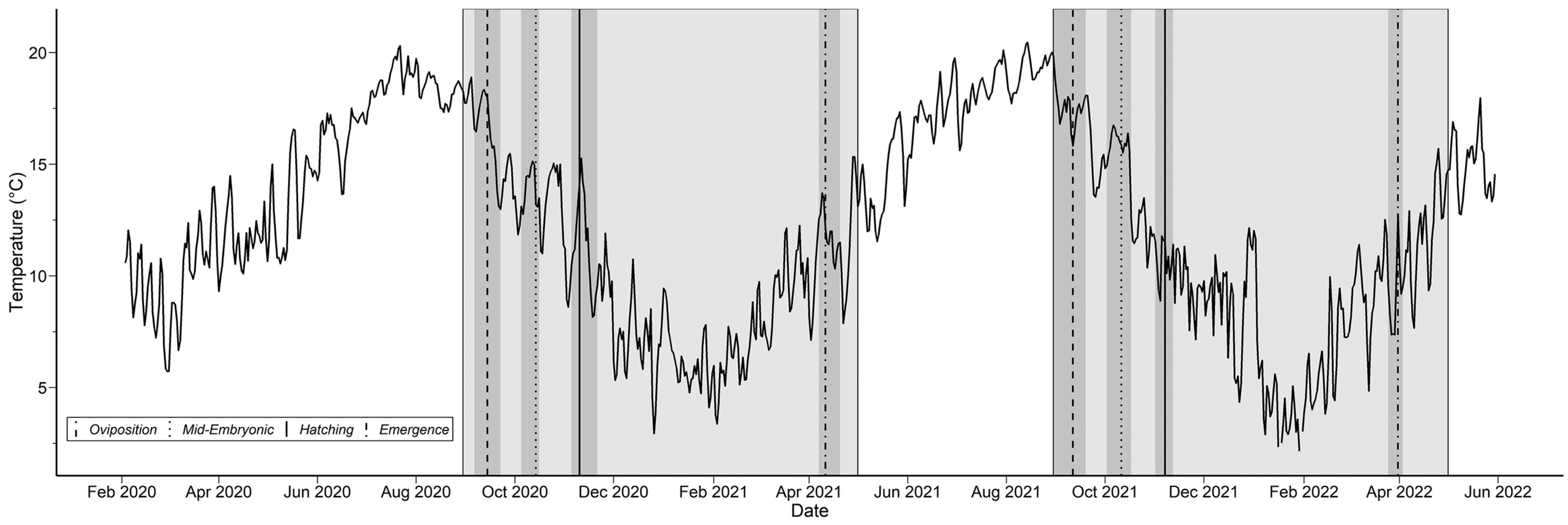

Figure 1. Seasonal variation in stream temperature across the paternal care period of eastern hellbenders (*Cryptobranchus alleganiensis*) in 2020 and 2021. Stream temperature (°C) is presented as the 24-h daily median stream temperature averaged across four stream reaches. The two light gray shaded areas indicate the range of the 8-mo-long paternal care periods in 2020 and 2021, respectively. Vertical lines within these areas indicate the average date of oviposition (dashed lines) and sampling at midembryonic development (dotted lines), larval hatching (solid lines), and spring emergence (dot-dashed lines).

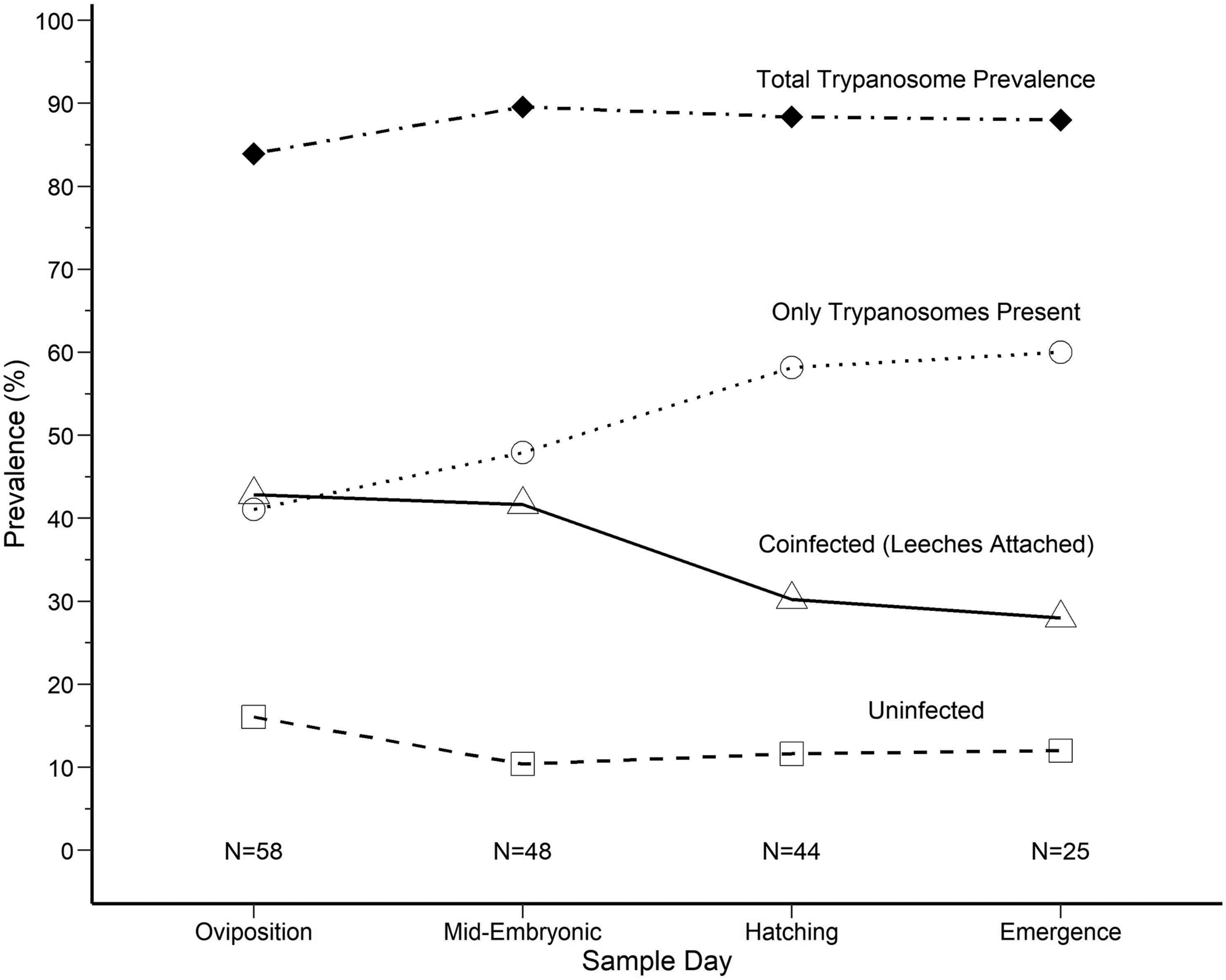

Figure 2. Prevalence of parasite infection in male eastern hellbenders (*Cryptobranchus alleganiensis*) with nests across the paternal care period. Prevalence is the proportion of individuals in the sample size that are within a given infection status category at each capture period (labeled on the graph above the corresponding sample day). Individuals with no leeches attached and no trypanosomes detected in blood were classified as "uninfected." Individuals with no leeches attached at capture but with trypanosomes detected in blood were classified as "only trypanosomes present." Individuals with leeches attached at capture and trypanosomes detected in blood were classified as "coinfected (leeches attached)." Note that all individuals with leeches attached were also infected with trypanosomes and that there were no instances in which an individual was infected with only leeches. Also present on the graph is the total proportion of hellbenders infected with trypanosomes regardless of leech attachment, which is classified as "total trypanosome prevalence."

red blood cells increased, MCHC decreased ($\beta = -7.708$, SE = 0.803, $t = -9.601$, $P < 0.001$; fig. 9).

After performing a separate PCA that included both the nesting males and the nonnesting out-group at midembryonic development and hatching, the resulting loadings and proportion of variance for each PC barely changed compared to the initial PCA that included only nesting males (supplemental table 2). In our subsequent models, we included the same predictor variables from the top-ranked model explaining PC1 and PC2 in only nesting males but added the additional categorical predictor representing nesting status interacting with temperature. Statistical outcomes from the top-ranked models are included in supplemental table 3. The effects of stress, temperature, and total length are similar to the effects previously stated regarding PC1. In contrast, the PC1 with the out-group included was not influenced by leeches or leech bites, likely due to the exclusion of data from oviposition when leech abundance was seasonally highest (see "Discussion"). Stress, temperature, and infection status influenced PC2, with the out-group similar to PC2 excluding the out-group. Most importantly, individuals in the nonnesting out-group had comparable polychromasia to the nesting males at midembryonic development and hatching (fig. 8). Because the nonnesting males were not previously bled that season, this observation provides evidence that red blood cell regeneration is not a result of repeated phlebotomy across the parental care period. In

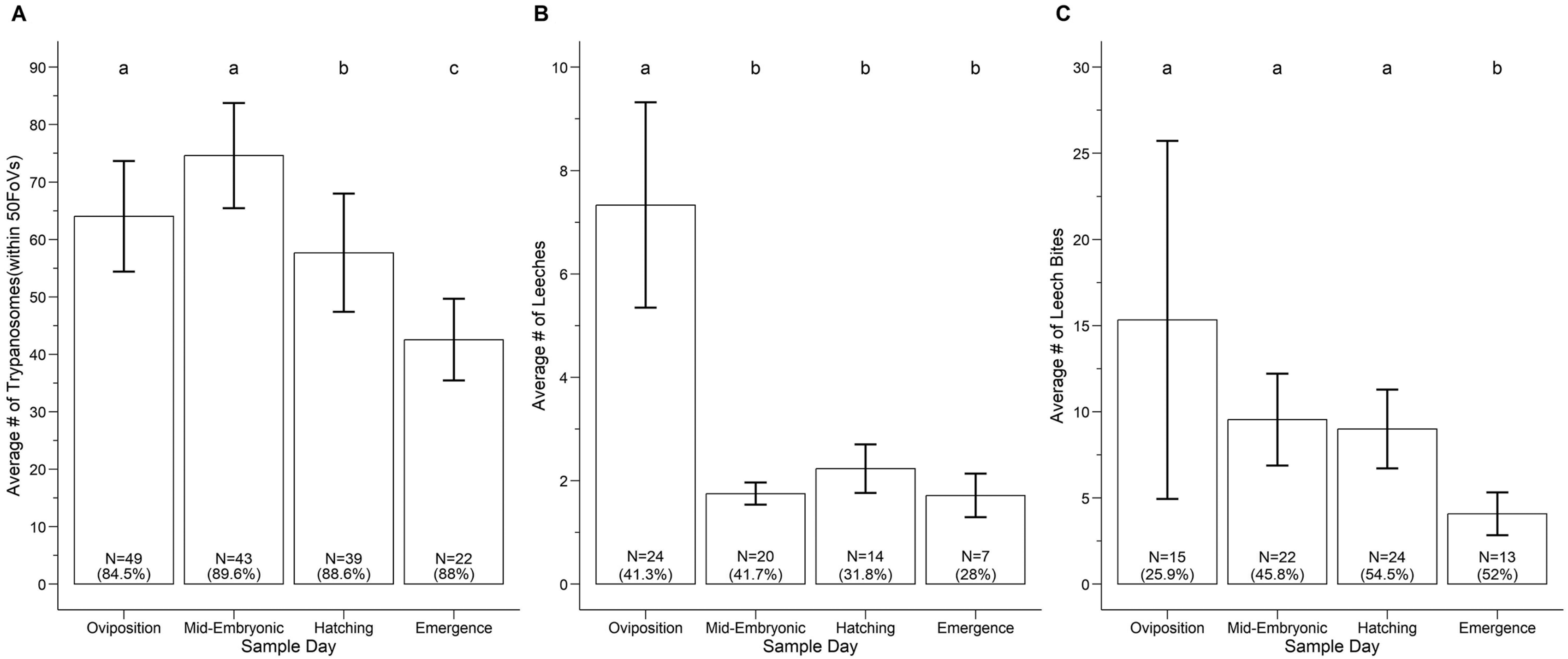

Figure 3. Intensity of parasitic infection among infected eastern hellbenders (*Cryptobranchus alleganiensis*) with nests across the paternal care period. Columns represent arithmetic means, and error bars represent ±1 SEM. The sample size and parasite prevalence for each sampling day are noted at the bottom of each column. *A*, Average number of trypanosomes within infected individuals (at least one trypanosome present) across the paternal care period. *B*, Average number of leeches attached at capture among infected individuals (at least one leech attached) across the paternal care period. *C*, Average number of leech bites within individuals with bites (at least one leech bite present) across the paternal care period. Different lowercase letters indicate the results from the mixed effects model used to assess how the intensity of infection differed across the parental care period. The statistical differences in parasite intensity across sampling day were assessed at $\alpha = 0.05$. FoVs = fields of view.

Table 1: Characteristics of hematological parameters and parasitic infection of adult male eastern hellbenders (*Cryptobranchus alleganiensis*) across the paternal care period from a population in southwest Virginia

		Midembryonic development		Hatching		
Parameter	Oviposition ($N = 58$)	Nesting ($N = 48$)	Out-group ($N = 8$)	Nesting ($N = 44$)	Out-group ($N = 7$)	Emergence ($N = 25$)
T0 Hct (%)	38.1 ± .94	36.1 ± .99	35.6 ± 2.02	27.0 ± 1.10	22.8 ± 1.88	33.6 ± 1.70
T60 Hct (%)	48.3 ± .69	44.3 ± .88	47.9 ± 1.07	41.1 ± .97	33.6 ± 1.40	44.0 ± 1.59
T0 Hb (g/dL)	9.9 ± .25	8.7 ± .26	9.0 ± .41	6.41 ± .27	5.1 ± .47	8.0 ± .42
T60 Hb (g/dL)	12.0 ± .21	10.3 ± .26	10.5 ± .30	9.1 ± .21	7.6 ± .36	9.6 ± .45
T0 MCHC (g/dL)	26.0 ± .27	24.1 ± .28	25.4 ± .72	23.9 ± .44	22.2 ± .67	23.9 ± .61
T60 MCHC (g/dL)	24.8 ± .28	23.3 ± .33	21.9 ± .43	22.3 ± .36	22.6 ± .90	21.8 ± .45
Polychromasia (%)	1.6 ± .24	5.4 ± .93	1.0 ± .35	6.7 ± .91	9.7 ± 1.27	7.3 ± 1.27
Leech bites	15.3 ± 10.39	9.6 ± 2.66	3.0 ± 1.52	8.9 ± 2.19	20.5 ± 13.09	4.1 ± 1.24
Leech prevalence (%)	41.3	41.7	25.0	31.8	71.4	28.0
Leech intensity	7.3 ± 1.98	1.8 ± .22	5.5 ± 4.50	2.2 ± .44	2.2 ± .490	1.7 ± .42
Trypanosome prevalence (%)	84.5	89.6	75.0	88.6	100	88.0
Trypanosome intensity	65.8 ± 9.40	74.6 ± 9.15	52.7 ± 9.2	58.2 ± 10.04	64.7 ± 18.88	42.5 ± 7.1

Note. Arithmetic means (±1 SEM) are presented for blood parameters and parasite presence. Parasite prevalence represents the proportion of the population infected, and intensity is determined by the number of parasites present in an infected individual. Also displayed are parameter values from nonnesting males (out-group) who were sampled only once during the study (one group at midembryonic development and another at hatching). Hb = blood hemoglobin concentration; Hct = hematocrit (also referred to as packed cell volume); MCHC = mean corpuscular hemoglobin concentration; T0 = time zero (baseline sample); T60 = time 60 (sample following 60 min of acute stress).

addition, nesting status did not influence red blood cell metrics for either PC1 or PC2, even when accounting for temperature (see supplemental table 3).

Discussion

Blood-feeding parasites have significant impacts on host physiology, but their effects on ectothermic hosts remain understudied compared to their impacts on endotherms. Likewise, compared to the deep knowledge on the blood parasites that blood-feeding vectors transmit, much less is known about the effects of vectors on their hosts. However, to effectively monitor the impact of blood-feeding parasites on ectotherm hosts in wild systems, it is imperative to distinguish physiological variation from pathological influences. Thus, fundamental knowledge of how hematological parameters are influenced by stress, body size, and environmental conditions is essential. In this study, we evaluated how hellbender salamanders modulate blood parameters in response to these factors and also investigated seasonality in vector attachment and parasite abundance, as well as the cumulative effect of coinfection on the hellbender host.

Table 2: Results from principal component analysis of red blood cell metrics in nesting male eastern hellbenders (*Cryptobranchus alleganiensis*)

Parameters	PC1	PC2	PC3
Hemoglobin	.659	.185	.729
Hematocrit	.626	.402	−.669
Polychromasia	−.417	.897	.149
Eigenvalue	2.148	.777	.074
Proportion of variance	.716	.259	.025
Cumulative variance	.716	.975	1.000

We found evidence of anemia, as well as a regenerative response to anemia, caused by coinfection by two species of blood-feeding parasites. We demonstrated that hematocrit and hemoglobin were closely correlated and were inversely related to polychromasia (PC1; table 2). Moreover, the proportion of polychromatic young red blood cells increased, while hematocrit and hemoglobin decreased, in coinfected hellbenders compared to in uninfected individuals and individuals infected only with trypanosomes. This increase in polychromatic cells is indicative of increased erythropoietic activity and a regenerative response to anemia (Fallon et al. 2017, 2020). Importantly, the distinctive blue-gray coloration of young red blood cells (see supplemental fig. 1) is due to residual RNA and a deficiency of hemoglobin (Latimer et al. 2003). Among other taxa, immature red blood cells have a lower affinity for oxygen than their mature counterparts (Edwards et al. 1971). Indeed, the inverse relationship we observed between MCHC and the proportion of immature red blood cells in hellbenders indicates that average hemoglobin content in red blood cells decreases as polychromasia increases. Thus, our findings collectively suggest that coinfected individuals with a high proportion of polychromatic red blood cells and reduced hematocrit and hemoglobin may have lower oxygen-carrying capacity than uninfected individuals and those infected singly with trypanosomes.

Contrary to our predictions, we found no evidence that trypanosomes alone elicit a pathological response in hellbenders, but coinfection with the leech vectors did. Increased proportions of polychromasia have been linked to the presence of trypanosomes in other amphibians (González et al. 2021) and freshwater fish

Table 3: Results from linear mixed effects model selection examining the effects of seasonal variation in temperature, acute stress, body size, and parasites on red blood cell parameters in nesting male eastern hellbenders

Linear mixed effects model	K	AICc	ΔAICc	AICc weight	Cumulative weight	R^2 marginal	R^2 conditional
PC1 ~ time × temp + TL + leeches × temp + bites × temp	11	879.83	0	.93	.93	.54	.75
PC1 ~ time × temp + TL + leeches × temp + bites × temp + tryps	12	884.89	5.06	.07	1.00	.55	.75
PC1 ~ time × temp + TL + infection × temp	11	91.71	30.88	0	1.00	.51	.74
PC1 ~ time × temp + TL	7	917.57	37.74	0	1.00	.45	.74
PC1 ~ time × temp + TL + leeches × temp	9	917.97	38.14	0	1.00	.49	.71
PC1 ~ time × temp + leeches × temp	8	918.63	38.80	0	1.00	.46	.69
PC1 ~ time × temp	6	918.82	38.99	0	1.00	.41	.71
PC1 ~ time × temp + TL + tryps	8	921.80	41.97	0	1.00	.45	.73
PC1 ~ time × temp + leeches × temp + tryps	9	923.58	43.75	0	1.00	.47	.69
PC1 ~ time × TL	6	1,084.09	204.26	0	1.00	.27	.59
PC1 ~ time	4	1,090.33	210.50	0	1.00	.15	.49
PC1 ~ 1	3	1,173.50	293.67	0	1.00	.0	.31

Note. Body size is shown as total length (TL; cm). AICc = Akaike's information criterion corrected for small sample sizes; ΔAICc = difference in AICc values; tryps = trypanosomes.

(Lapirova and Zabotkina 2018), but we do not know of any studies that have linked polychromasia to a leech vector. Typically, in endotherms, hemolytic anemia elicits a more intense regenerative response than hemorrhagic anemia because the iron from lysed cells is more readily available for erythropoiesis than the iron stored as hemosiderin (Latimer et al. 2003). Thus, we expected to see a greater hematological response to trypanosomes, which lyse red blood cells and consume hemoglobin for iron and protein (Pishchany and Skaar 2012; Siqueira-Neto et al. 2018), than to biting vectors like leeches. In contrast to this expectation, the proportion of polychromasia was much higher in coinfected individuals, while uninfected individuals and those infected only with trypanosomes did not statistically differ (fig. 8*A*). Trypanosome infection can cause a diversity of pathologies in ectothermic hosts, including anemia (Densmore and Green 2007), but most aquatic trypanosomes are thought to be nonpathogenic to their fish (Woo 1994, 2006) and amphibian (Omonona and Ekpenko 2011) hosts. Our results support other work on infected hellbenders, which found slightly lower hematocrit in stream reaches with parasites present, but the confounding influence of variable habitat quality among stream reaches prevented the prior study from attributing lower hematocrit to parasitic infection (Bodinof Jachowski et al. 2024). Although our current study overcame this confound by focusing on a narrower segment of a single stream with comparable habitat quality, a limitation of both the current study and the prior work by Bodinof Jachowski et al. (2024) is the lack of individuals in the sample population harboring leeches without simultaneous infection with trypanosomes. This precludes us from disentangling whether the effects of coinfection are driven by the presence of leeches alone or from a multiparasite interaction within the host, as can occur when multiple parasites compete for resources (Budischak et al. 2018). Nevertheless, our research demonstrates that the attachment of the leech vector contributes to symptoms of anemia in hellbender hosts, even after eliminating the possibility that repeated phlebotomy could contribute to symptoms (fig. 8*B*). The literature on anemia caused by leeches is scarce and mostly encompasses rare instances of leech infestation in the orifices of humans and livestock (Kose et al. 2008). Leech saliva contains a suite of chemical compounds, including immunomodulators, vasodilators, and anticoagulants (Salzet et al. 2000; Hildebrandt and Lemke 2011). Whether compounds in leech saliva are capable of affecting red blood physiology is unknown, but prior work has shown that they can affect hellbenders' endocrine system (DuRant et al. 2015). Additional research is needed to determine the mechanisms by which leeches influence red blood cell physiology in hellbenders and whether leeches can induce anemia in other host-parasite systems.

Our study design enabled us to demonstrate seasonal interactions between parasite infection dynamics and red blood cell physiology. Leech infection intensity peaked at oviposition (fig. 3*B*), supporting our previous work on leech feeding ecology (Bodinof Jachowski et al. 2024). The intensity of trypanosome infection peaked at oviposition and midembryonic development, but it then decreased at hatching and decreased further at emergence (fig. 3*A*). Leeches exhibit brooding and parental care of their offspring before leading them to their first blood meal (A. Blumenthal and W. A. Hopkins, unpublished manuscript) during the time when the hosts have their seasonally highest amount of circulating red blood cells and when multiple hosts are interacting for breeding (O'Brien et al. 2024). Leech offspring of similar species require a rapid growth period, feeding three to five times, to survive over winter (Wilkialis 1984; Moser et al. 2009), which means that they not only repeatedly consume blood meals from the same host but also potentially reinfect themselves and the host with trypanosomes. This finding is important because it logically indicates that peak transmission of trypanosomes corresponds to seasonal attachment of the leech vectors. Importantly, the negative effect of leeches on

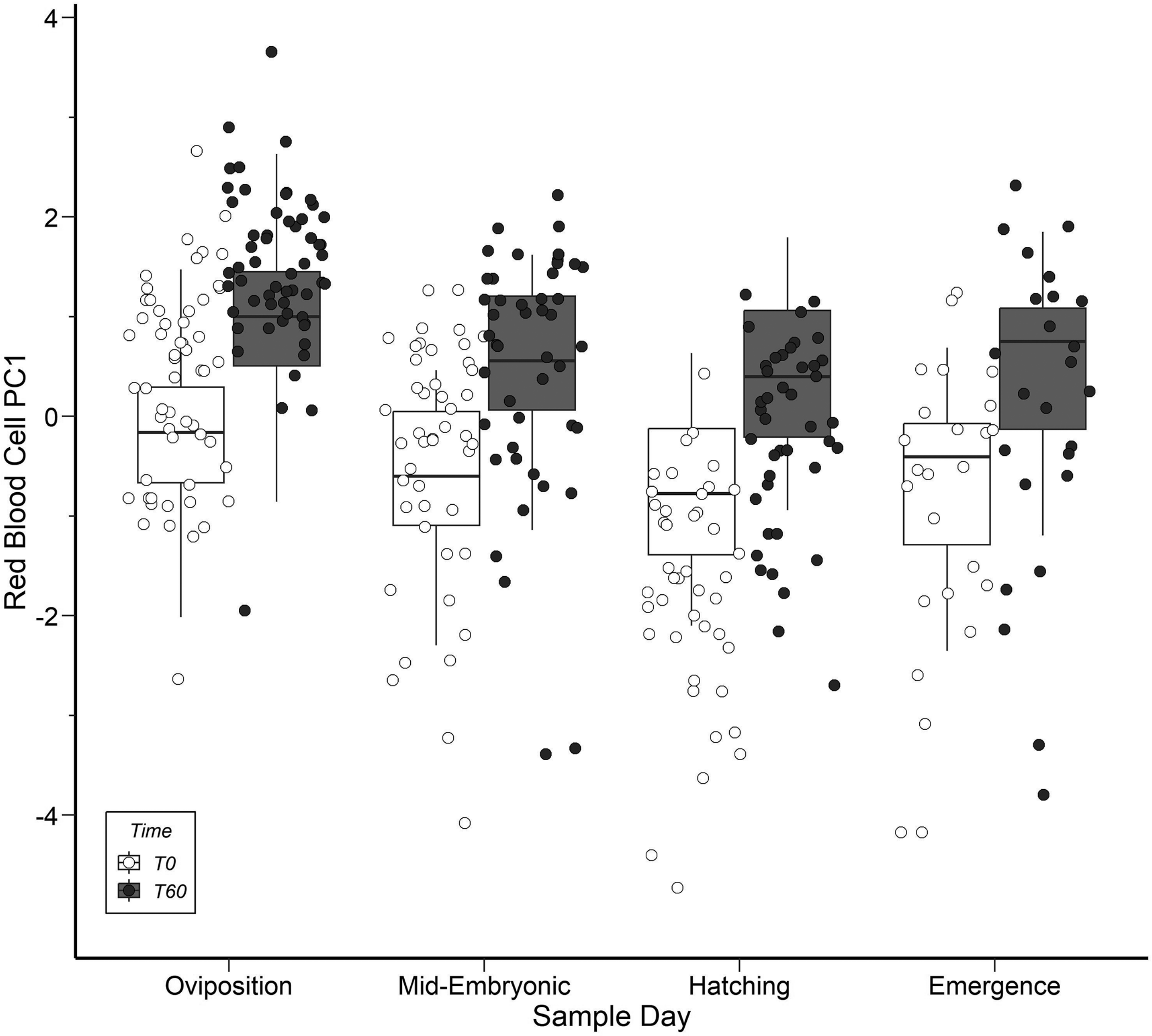

Figure 4. Model predictions from top-ranking Akaike's information criterion corrected for small sample sizes model (boxplots) overlaid with raw data (points) predicting PC1 scores across the paternal care period of hellbenders (*Cryptobranchus alleganiensis*) at capture (time zero [T0]) and after 1 h of restraint (time 60 [T60]). Rankings of Akaike's information criterion corrected for small sample sizes suggest that stress, total length, temperature, leeches, and leech bites influence red blood cell metrics from nesting male hellbenders. The center horizontal line of each boxplot reflects the median value. The lower and upper hinges of each boxplot correspond to the first and third quartiles (the 25th and 75th percentiles), respectively.

hematocrit and hemoglobin was also most pronounced with peak leech attachment during oviposition (fig. 3*B*). However, as temperature decreased in the fall, polychromasia increased in coinfected individuals. We postulate that this time lag in the regenerative response is attributable to the time it takes for the host to generate new red blood cells in response to the gradual, cumulative effect of leeches feeding on their hosts in late summer and early fall, which is supported by the accumulation of leech bites from oviposition to hatching (figs. 3*C*, 8*A*). The timing of peak infection, as well as the lag time between the peak leech infection and the observed regenerative red blood cell response, should be considered when evaluating the pathology of blood-feeding vectors. For example, previous work evaluating hematological parameters in hellbenders did not identify a relationship between parasites and symptoms of anemia (Hopkins et al. 2016), likely due to the fact that the sampling occurred a month before the breeding season, before peak leech infection occurs.

Contrary to expectations based on the literature, hematocrit and hemoglobin decreased as total length increased (fig. 6).

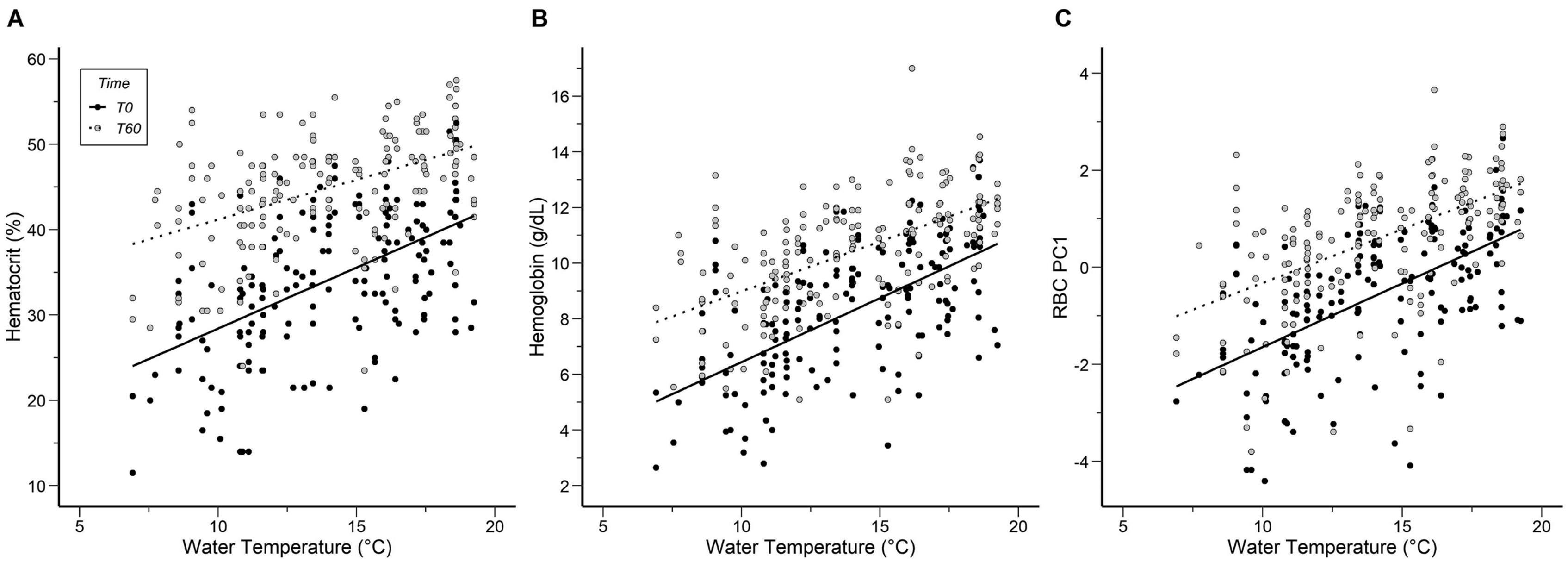

Figure 5. Relationship between stream temperature (°C) and red blood cell (RBC) metrics in nesting male eastern hellbenders (*Cryptobranchus alleganiensis*). *A*, Relationship between stream temperature and hematocrit (%) in blood collected at capture (time zero [T0]) and after 1 h of restraint (time 60 [T60]). *B*, Relationship between stream temperature and hemoglobin concentrations (g/dL) at T0 and T60. *C*, Relationship between stream temperature and PC1 scores from the principal component analysis conducted on RBC metrics: hematocrit, hemoglobin, and polychromasia. Lines represent model predictions (T0, solid lines; T60, dotted lines), while points represent raw data. While hematocrit and hemoglobin are presented here for visual purposes, the final statistical analysis used only principal component scores.

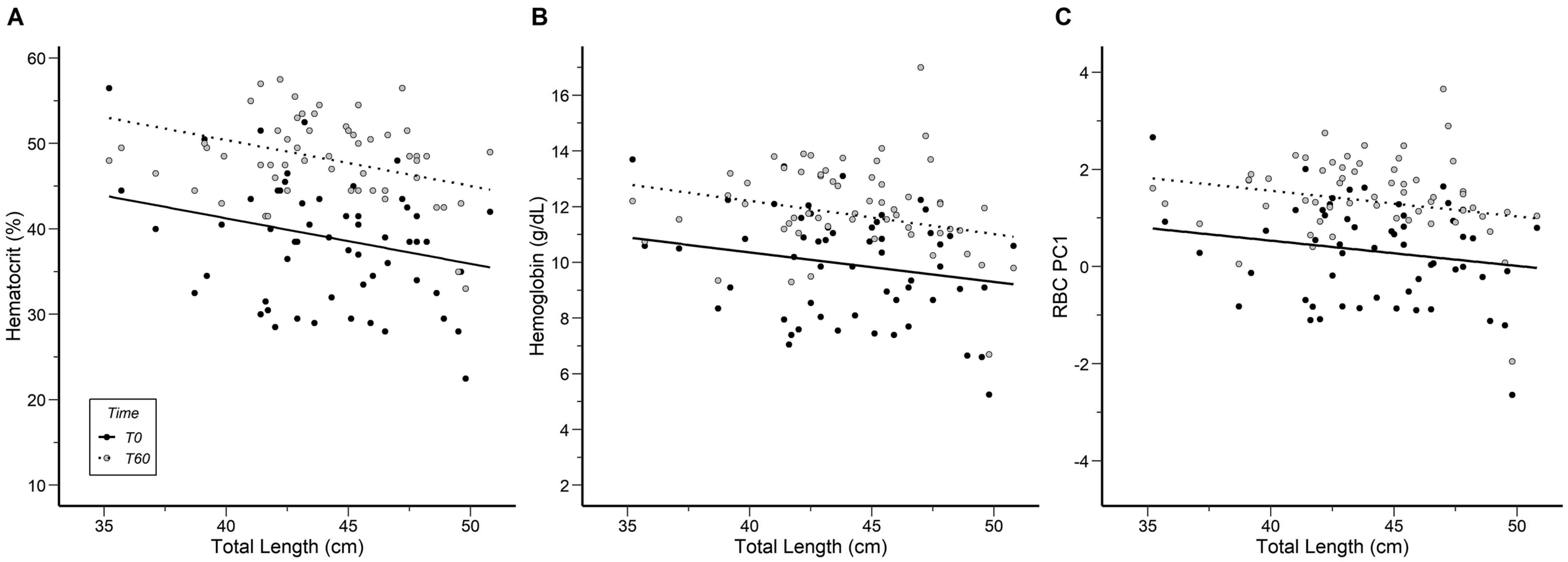

Figure 6. Relationship between total length (cm) and red blood cell (RBC) metrics at oviposition in nesting male eastern hellbenders (*Cryptobranchus alleganiensis*). *A*, Relationship between total body length and hematocrit (%) in blood collected at capture (time zero [T0]) and after 1 h of restraint (time 60 [T60]). *B*, Relationship between total body length and hemoglobin concentrations (g/dL) at T0 and T60. *C*, Relationship between total body length and PC1 scores from the principal component analysis conducted on RBC metrics: hematocrit, hemoglobin, and polychromasia. Lines represent model predictions (T0, solid lines; T60, dotted lines), while points represent raw data. While hematocrit and hemoglobin are presented here for visual purposes, the final statistical analysis used only principal component scores. For ease of visualization, data show only the relationship between total length and RBC metrics during the oviposition capture period, but this pattern is maintained across all sampling periods.

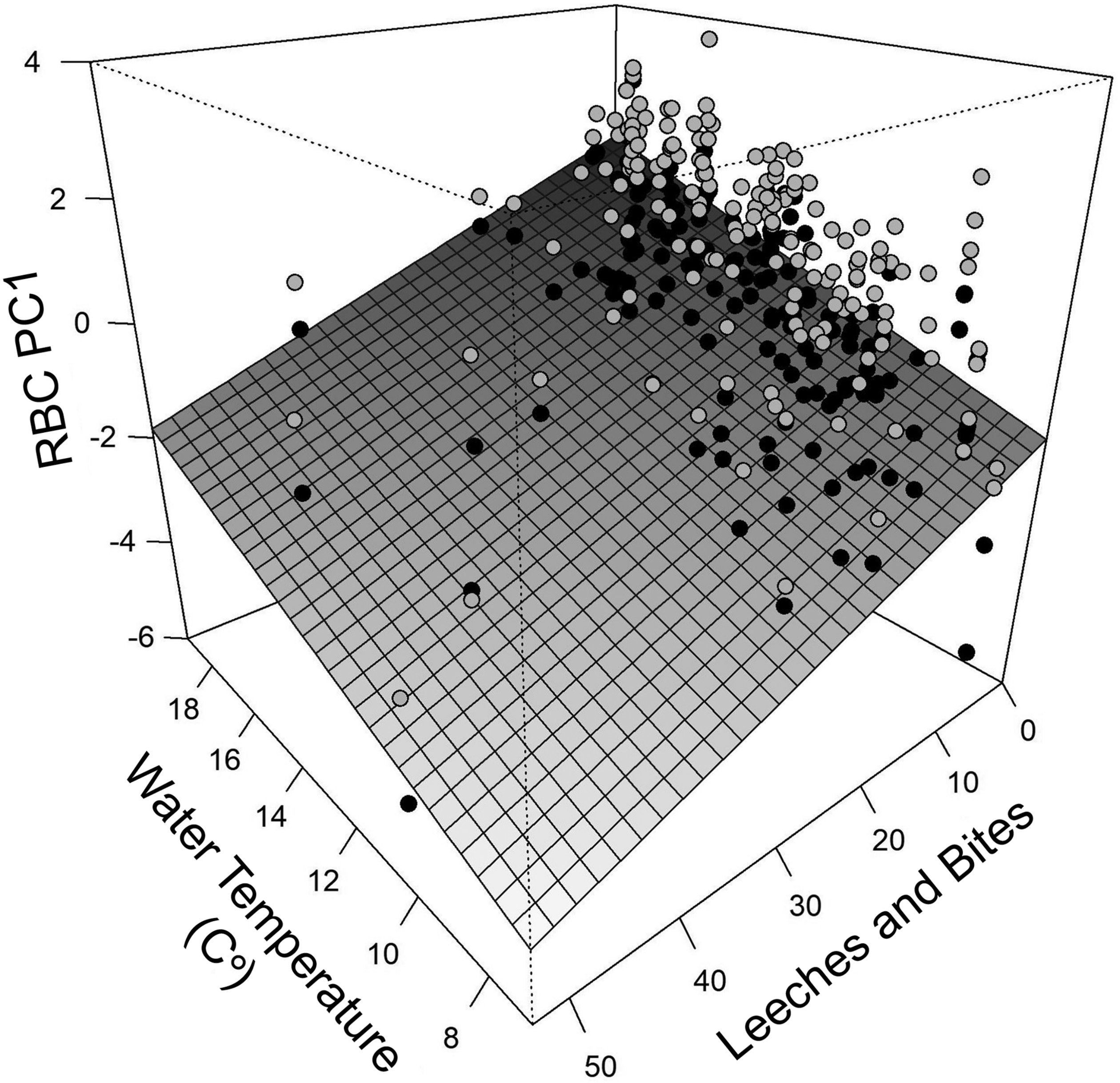

Figure 7. Relationship between stream temperature (°C), combined total of leeches and bites, and red blood cell (RBC) PC1 in nesting male eastern hellbenders (*Cryptobranchus alleganiensis*). Note that RBC PC1 had positive loadings for hematocrit (0.624) and hemoglobin (0.651) but negative loadings for polychromasia (−0.432). The raw values are plotted for clarity, and the plane was generated from simple linear regressions. Black points indicate baseline (at capture [time zero]) values, while gray points indicate stress-induced (after 1 h of restraint [time 60]) values. The single plane represents the effects of temperature, leeches, and bites on all PC1 values. See supplementary figure 5 to visualize the trends for both time zero and time 60. For visualization, we excluded one outlier from an individual during oviposition with 204 combined leeches and bites. This figure is only for visualization purposes. Leeches and bites were evaluated as separate predictor variables for statistical analysis.

Our findings contrast with previous research on hellbenders, which found a positive relationship between body size (snout-to-vent length) and red blood cell parameters (Hopkins et al. 2016), but that study included a wider range of age/size classes and both sexes, which likely contributed to these differences. Indeed, Bodinof Jachowski et al. (2024) demonstrated that seasonal variation in hematocrit differs between sexes. Our study controlled for sex and life history stage by focusing solely on adult males engaging in paternal care, which likely enhanced our ability to detect body size effects without these confounding variables. More broadly speaking, our findings also contrast with the bulk of existing literature on aquatic vertebrates including fish and amphibians (Murachi 1959; Joshi and Tandon 1977; Clark et al. 1979; Davis 2008; Maerz et al. 2009; Anthony et al. 2010; Baghizadeh and Khara 2015; Fazio et al. 2017; Liu et al. 2023; but see Zhang et al. 2018). However, research in male-brooding

Table 4: Results from linear mixed effects model selection examining the effects of seasonal variation in temperature, acute stress, and parasites on red blood cell parameters in nesting male eastern hellbenders

Linear mixed effects model	K	AICc	ΔAICc	AICc weight	Cumulative weight	R^2 marginal	R^2 conditional
PC2 ~ time × temp + infection × temp	10	674.51	.0	.99	.99	.36	.59
PC2 ~ time × temp + leeches × temp + bites × temp	10	684.64	10.13	.01	1.00	.35	.59
PC2 ~ time × temp	6	687.83	13.32	.00	1.00	.30	.55
PC2 ~ time × temp + leeches × temp	8	689.69	15.18	.00	1.00	.33	.56
PC2 ~ time × temp + leeches × temp + bites × temp + tryps	11	690.92	16.41	.00	1.00	.35	.59
PC2 ~ time × temp + tryps	7	693.93	19.42	.00	1.00	.30	.55
PC2 ~ time	4	694.16	19.65	.00	1.00	.27	.51
PC2 ~ time × temp + leeches × temp + tryps	9	695.71	21.20	.00	1.00	.32	.57
PC2 ~ time × temp + bites × temp	8	702.40	27.89	.00	1.00	.31	.54
PC2 ~ 1	3	833.60	159.09	.00	1.00	.0	.22

Note. AICc = Akaike's information criterion corrected for small sample sizes; ΔAICc = difference in AICc values; tryps = trypanosomes.

teleost fish suggests that smaller males are more susceptible to size-associated energetic constraints (Laroche et al. 2023) affecting nest success and reproductive phenology (Suski and Ridgway 2007), which is especially prominent in species with high reproductive costs, such as energetically costly gametes or parental care behavior (Ridgway et al. 1991; Tejedo 1992; Danylchuk and Fox 1994; Descamps et al. 2011). We postulate that similar constraints occur in male hellbenders, making paternal care more costly for smaller males than for larger males. Across the 8-mo paternal care period, hellbenders rarely leave their nests and engage in active behaviors (Settle et al. 2018; Unger et al. 2020; O'Brien et al. 2024) to promote the survival of their extremely large clutches (up to 5,300 cm^3; Hopkins et al. 2023), which may require proportionally more work for small males to care for. Thus, we speculate that

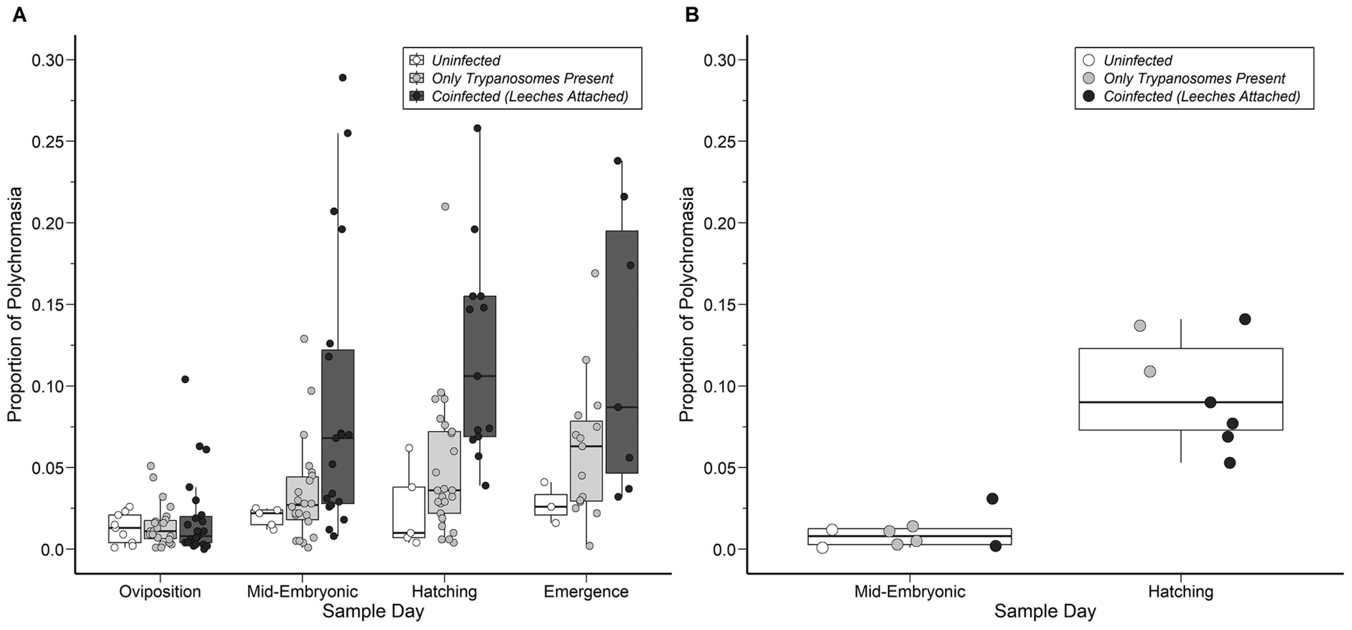

Figure 8. Proportion of polychromatic red blood cells and parasite prevalence across the parental care period. *A*, Variation in the proportion of polychromatic red blood cells at capture (time zero) grouped by infection status in nesting male eastern hellbenders (*Cryptobranchus alleganiensis*) across the paternal care period. *B*, Variation in the proportion of polychromatic red blood cells at time zero in the nonnesting out-group of male eastern hellbenders. The males in the out-group were captured and bled for only one capture period during midincubation or hatching in 2021. The out-group was used to assess whether repeated bleeds influenced red blood cell metrics. Since individuals in the nonnesting out-group (*B*) had comparable polychromasia to individuals with nests (*A*), we conclude that red blood cell regeneration is not a response to repeated bleeds across the parental care period. The center horizontal line of each boxplot reflects the median value. The lower and upper hinges of each boxplot correspond to the first and third quartiles (the 25th and 75th percentiles), respectively.

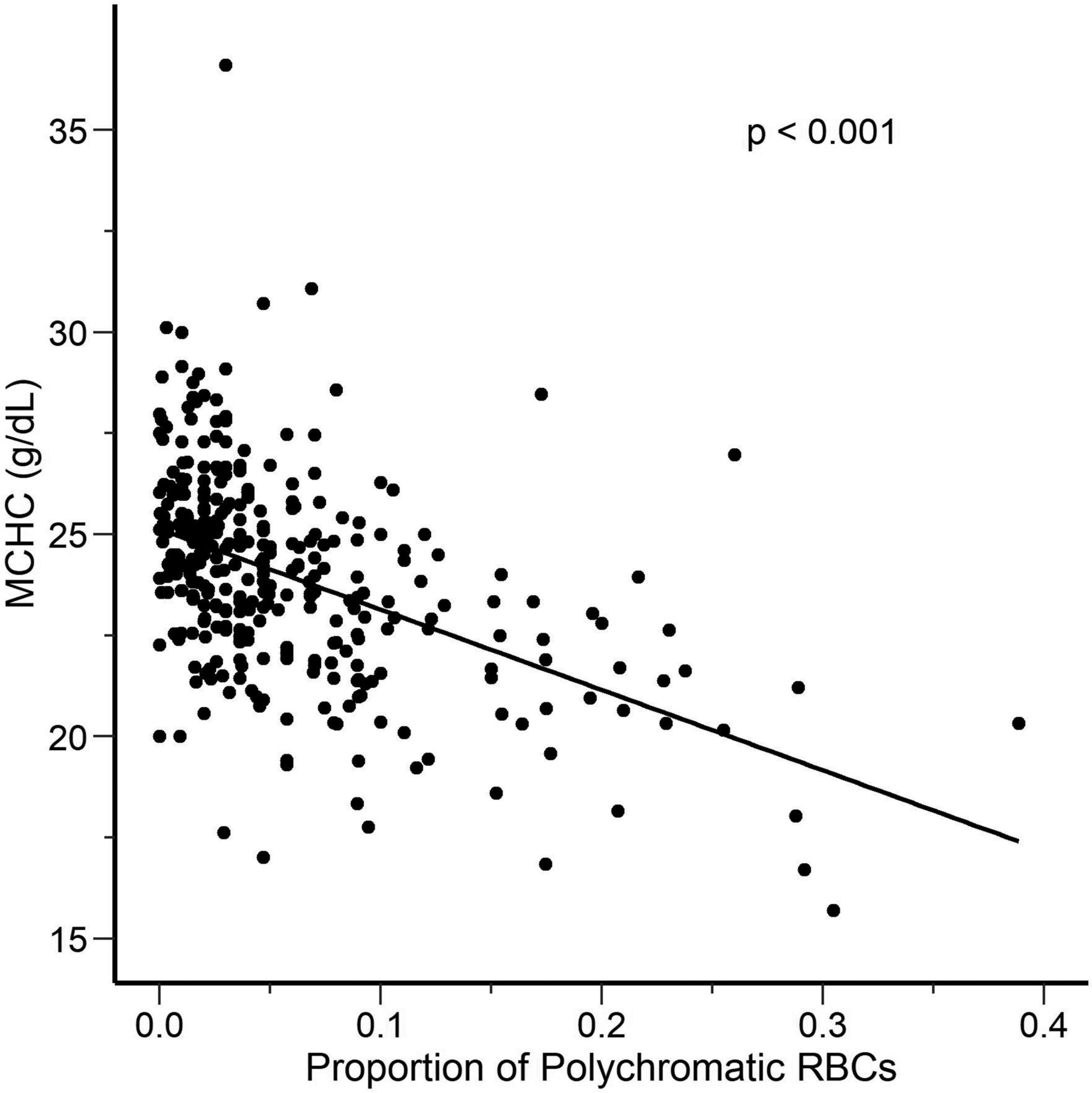

Figure 9. Relationship between proportion of polychromatic red blood cells (RBCs) and mean corpuscular hemoglobin concentration (MCHC; g/dL). Note that the figure reflects the results from the linear mixed effects model from a secondary analysis and that the MCHC values were not included in the principal component analysis with other RBC metrics. Line represents model predictions, while points represent raw data.

smaller nesting males may have elevated hematocrit and hemoglobin compared to their larger counterparts to increase oxygen-carrying capacity and delivery to tissue to meet those demands. We recommend that future research explore the influence of body size on paternal care behaviors and red blood cell metrics in male hellbenders to determine whether energetic constraints on smaller individuals affect their nest success.

As predicted, hellbenders exhibited hemoconcentration in response to acute stress from restraint, similar to other vertebrates (Romero and Butler 2007). Hematocrit and hemoglobin are essential in oxygen transport, and by modulating red blood cells in circulation, vertebrates can increase the oxygen-carrying capacity of their blood during times of increased respiratory or energetic demands, such as when evading a potential threat (Benfey and Biron 1994; Brill et al. 2008). As such, the catecholamine-mediated fight-or-flight response is highly conserved across vertebrates, increasing heart rate and blood pressure and resulting in plasma efflux out of the vascular space. Catecholamines can also provoke the release of red blood cells from spleen storage into circulation, maximizing gas exchange and delivery to tissues. For example, some cutaneously respiring urodeles utilize a compensatory and reversible process of splenic storage and release of red blood cells, allowing them to adapt to variable respiratory conditions, such as insufficient oxygen levels (Frangioni and Borgioli 1989, 1993*a*, 1993*b*). Because hellbenders rely primarily on cutaneous respiration (Guimond and Hutchison 1973), their physiology limits their ability to mechanically increase the exchange of respiratory gases to meet oxygen demands. Thus, it is possible that they utilize similar splenic storage of red blood cells and control mechanisms to optimize blood oxygen levels in response to environmental oxygen concentrations and metabolic demand.

We observed seasonal changes in red blood cell physiology corresponding to water temperature; baseline hematocrit and hemoglobin decreased with temperature (fig. 5). Ectotherm metabolic function is highly dependent on environmental temperatures (da Silva et al. 2013), and available dissolved oxygen in aquatic habitats is inversely related to temperature (Li and Liu 2019; Rajesh and Rehana 2022). Thus, hematocrit and hemoglobin were highest at oviposition, when temperatures were the highest in our study (fig. 1), hellbenders are known to be most active (Humphries and Pauley 2000), and available dissolved oxygen is at its lowest (Li and Liu 2019). Temperatures declined over midembryonic development and reached their lowest levels at hatching (fig. 1), which corresponded to the lowest levels of hematocrit and hemoglobin across the parental care period. This period of low temperature is when dissolved oxygen is at its highest and hellbender activity and metabolic demands have declined substantially, requiring hellbenders to have fewer red blood cells in circulation and thus optimizing the viscosity of their blood (Birchard 1997). Likewise, other aquatic vertebrates exhibit seasonal variation in hematocrit and hemoglobin levels, including higher levels corresponding to the onset of breeding behavior in cutaneously respiring urodeles (Frangioni and Borgioli 1984) and in fish (Bridges et al. 1976). Additionally, the magnitude of the stress response was greater in cooler temperatures (fig. 5), indicating that hellbenders had fewer red blood cells in circulation but more in storage, which were mobilized in response to stress. Variation in the magnitude of the hemoconcentration response has been observed in a salamander species relative to hypoxia (Frangioni and Borgioli 1993*a*), but our study is the first to establish the role of temperature in stress-induced hemoconcentration in an amphibian species. Our research confirms that hellbenders have the capacity to decrease the concentration of red blood cells in circulation, corresponding to lower water temperature, increased dissolved oxygen concentrations, and decreased metabolic activity, but that these cells can be readily released from storage during times of respiratory need, such as acute stress.

Elucidating the seasonal dynamics of attachment and transmission of blood-feeding parasites and their impact on red blood cell physiology in ectotherms is needed to understand the dynamics of vector-borne diseases in this group of vertebrates that has received comparatively less study than endothermic hosts. We provide fundamental insight into the red blood cell physiology of an imperiled amphibian species, which enhances our understanding of host–vector–blood parasite interactions and highlights a number of influential factors that should be considered for the diagnostic evaluation of wild ectotherms in the future. Additionally, we identified several novel findings that warrant future research, including the importance of leech vectors and parasitic coinfection in eliciting physiological responses in the host. Given the implications of disease and climate change in the global decline of amphibian populations, we recommend that future studies consider the seasonality of amphibian host physiology and parasite interactions to include coinfections.

Acknowledgments

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