THE INFLUENCE OF ENVIRONMENTAL FACTORS AND CO-OCCURRING PARASITES ON BLOOD PHYSIOLOGY IN EASTERN HELLBENDERS (*CRYPTOBRANCHUS ALLEGANIENSIS*)

Katherine Louise Slack

Thesis submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Master of Science

In

Fisheries and Wildlife Conservation

William A. Hopkins

Ignacio T. Moore

Luis E. Escobar

March 13, 2024

Blacksburg, Virginia

Keywords:

Amphibian, Hellbender, Ecophysiology, Parasites, Vector, Hematology, Acute Stress

The Influence of Environmental Factors and Co-occurring Parasites on Blood Physiology in Eastern Hellbenders (*Cryptobranchus alleganiensis*)

Katherine L. Slack

ACADEMIC ABSTRACT

Climate change, habitat degradation, and infectious disease are major drivers of global amphibian declines. Amphibians are particularly susceptible to these factors due to their unique physiology and habitat requirements. Thus, investigating components of amphibian physiology and evaluating the influence of environmental conditions, perceived threats, and encounters with infectious agents is essential to conserving imperiled amphibian species. Here, I measured hematocrit, hemoglobin, relative proportions of polychromatic red blood cells, and white blood cell differentials in a wild population of male Eastern Hellbender salamanders (*Cryptobranchus alleganiensis*) which remain with their nest for up to ~8 months and have frequent encounters with parasitic leeches (*Placobdella appalachiensis*) that transmit hematophagous endoparasites (*Trypanosoma sp.*), often resulting in coinfection. Results indicate that hematocrit and hemoglobin increase in response to acute stress and with temperature. Additionally, the magnitude of the stress-induced hemoconcentration response was greater at lower temperatures. Hellbenders exhibited an increase in the proportion of neutrophils and eosinophils in circulation as temperatures decreased while the proportions of lymphocytes and basophils had an inverse effect. Furthermore, the proportion of neutrophil precursors also increased as temperature decreased, which signifies recruitment of innate immune cells during seasonally cold periods. Coinfection of the leech and trypanosome parasites resulted in decreased hematocrit and hemoglobin and a marked increase in polychromatic red blood cells which is indicative of regenerative anemia in the hellbender host. However, these effects were not present in individuals only infected with trypanosomes, implicating the leech vector as the key contributor to anemia in hellbenders. Moreover, the proportion of neutrophils and eosinophils increased, while lymphocytes decreased, in response to leech attachment. However, as parasitemia of leeches and trypanosomes increased concurrently, the proportion of lymphocytes increase in circulation while neutrophils and eosinophils decrease, underscoring the complexities associated with coinfection and multi-parasite interactions. Together, this research provides novel insights into the blood physiology of an imperiled salamander by establishing reference values essential for population surveillance while also describing how these values fluctuate across season and in response to extrinsic factors, with an emphasis on co-occurring hematophagous parasites.

The Influence of Environmental Factors and Co-occurring Parasites on Blood Physiology in Eastern Hellbenders (*Cryptobranchus alleganiensis*)

Katherine L. Slack

GENERAL AUDIENCE ABSTRACT

While large losses of biodiversity are a global trend among vertebrate populations, amphibian species are most at risk of extinction due to the compounding effects of infectious disease, habitat degradation, and climate change. Understanding how these factors impact the health, condition, and survival of imperiled species is vital to monitoring populations and developing successful conservation strategies. Here, I used hematological metrics that are associated with respiratory, metabolic, and immune responses to monitor a wild population of imperiled eastern hellbender salamanders (*Cryptobranchus alleganiensis*). Eastern hellbenders are fully aquatic giant salamanders that engage in an ~8-month solitary paternal care period and have faced precipitous declines in recent decades. Furthermore, hellbender populations in southwest Virginia harbor parasitic leeches (*Placobdella appalachiensis*) which act as vectors for blood-feeding endoparasites (*Trypanosoma sp.*), frequently leading to coinfection. My aim was to gain mechanistic insight into how hellbender blood physiology varies with environmental conditions, how they respond to potential threats like acute stressors, and how parasites and coinfection impact their health. Through my research, I discovered fascinating insights into the physiology of hellbenders. When faced with environmental temperature changes and acute stress, hellbenders adapt by modulating the red blood cell concentration in their blood, enhancing blood-oxygen-carrying capacity, to meet increased metabolic demands. Furthermore, white blood cell parameters of hellbenders exhibit seasonal changes, which may indicate an increased

reliance on mechanisms of innate immune responses during colder temperatures, when cells associated with adaptive immune responses are less active. My study also revealed that coinfection of the leech vector and trypanosome parasites in hellbenders can trigger immune responses and lead to regenerative anemia in the host. However, the pathologies associated with anemia in hellbenders were attributed to the leech vector rather than the trypanosome parasite it transmits. These findings shed light on the physiology of amphibians and have important implications for population surveillance, particularly in identifying potential environmental factors that threaten imperiled species.

ACKNOWLEDGEMENTS

I am immensely grateful to have had the guidance of my advisor and coach, Dr. Bill Hopkins, during this challenging journey. Bill has been a constant source of encouragement, urging me to delve deeper into my scientific curiosity while also keeping me grounded and focused (albeit an extremely difficult task). I am deeply thankful for all the lessons I have learned from him and for the countless ways he has helped me become a better scientist and researcher. I also extend my sincere appreciation to my committee members, Dr. Ignacio Moore and Dr. Luis Escobar, for their patience, insights, and invaluable support throughout this process.

I would like to express my gratitude to the individuals and institutions that were crucial to the success of my research. I am incredibly thankful to Jordy Groffen, who provided an indescribable amount of assistance in both the field and the lab. When I started my program in the summer of 2020 (peak COVID pandemic), Jordy was my first friend in Blacksburg. Our friendship helped me so much during a transitional time of isolation and loneliness and I feel lucky to have had his constant support and enthusiasm throughout this process. Thank you to my fellow Hopkins lab members Rebecca O'Brien, Brian Case, Holly Funkhouser, Austin Holloway, Justus Hargett, and the multitude of undergraduate researchers for their encouragement and assistance. This work would not have been possible without the dedicated efforts of hard-working field technicians including Emma Fralin, Katie Hahler, and Ajay Patel. Additionally, I would like to thank the Virginia Department of Wildlife Resources (DWR) and the National Science Foundation for funding this research. I especially thank J.D. Kleopfer from DWR for his unwavering support our research and hellbender conservation. I extend my appreciation to the Global Change Center at Virginia Tech, the Fralin Life Science Institute, and the academic and administrative staff within the Department of Fish and Wildlife Conservation for their assistance in various aspects of my research, including access to resources, facilities, and administrative support.

I greatly appreciate my family and friends for their insurmountable love and support. To my father and fellow wildlife biologist, thank you for teaching me to recognize the natural wonders around me, for instilling compassion for the voiceless, and for nurturing my curiosity. I would like to express my gratitude to my mother for teaching me the importance of persistence in the face of adversity and for always being my biggest cheerleader. I am also grateful to my sister and my niece for reminding me of my worth and helping me to overcome my self-doubt. I am grateful for the devoted patience and consistent presence of my long-time partner, Christian, who frequently made the seven-hour journey to share a weekend with me. I am excited that the long-distance portion of our relationship is finally coming to an end.

Finally, I would like to extend my appreciation to the support system that I have built throughout my grad school journey. To my dear friends Sharon Dorsey, Justice Madden, and Amber Wendler, thank you for the frequent check-ins, for our co-working sessions, and for constantly reminding me to be kind to myself. I am grateful for my dear friend Dr. Leonie Jacobs, whose continuous motivation and advocacy have been a constant source of strength and inspiration for me. Last but not least, I would like to give a special thank you to Brogan Holcombe, whom I met on my very first day of class and who has helped me overcome numerous challenges and obstacles throughout my time at Virginia Tech.

TABLE OF CONTENTS

INTRODUCTION

Amphibian Declines

The loss of biodiversity continues on a global scale, with nearly one-fifth of all living vertebrate species classified as Threatened on the IUCN Red List (Hoffman et al., 2010). Amphibians, in particular, are considered to be among the most endangered vertebrates, with 41% of amphibian species threatened with extinction (Hoffman et al., 2010, Luedtke et al., 2023). Within amphibians, Urodeles, such as salamanders and newts, face the highest risk of extinction (Luedtke et al., 2023). While amphibian population declines are attributable to global changes like habitat loss, disease, pollution, climate change, and introduced species (Collins and Storfer, 2003; Hof et al., 2011; Green et al., 2020), responses to these threats vary significantly among species and sub-populations (Campbell Grant et al., 2020). Some amphibian declines have been linked to various parasites and pathogens, including Ranavirus and chytrid fungus (Daszak et al., 1999; Yap et al., 2015; Fisher and Garner, 2020), resulting in mass mortalities and even extinction of species (Lips et al., 2008; Price et al., 2014). Additionally, climate change is expected to exacerbate these challenges, increasing the risk of diseases (Harvell et al., 2002; Greenspan et al., 2017; Cohen et al., 2019) and intensifying habitat loss for amphibians (Blaustein et al., 2010).

Amphibians are vital indicators of ecological health due to their physiology, which makes them sensitive to environmental stressors (Hopkins, 2007). Their permeable skin and distinct life cycle stages make them particularly responsive to even subtle changes in their habitat (Vitt et al., 1990; Wake, 1991; Blaustein et al., 1994, Hopkins, 2007), such as variations in water quality and UV radiation (Gerlanc and Kaufman, 2005; Taylor et al., 2005). Furthermore, their metabolic and respiratory demands fluctuate with environmental conditions, with temperature playing a crucial role in various physiological processes, including rates of oxygen uptake, heart rate, locomotion, water balance, digestion, developmental rate, sex determination, and immune function (Rome et al., 1992; da Silva et al., 2013). Thus, understanding the fundamental aspects of amphibian physiology can inform us about how they respond to changes in environmental conditions and disease, which can aid in monitoring populations and recognizing threats.

Study Species

The eastern hellbender (*Cryptobranchus alleganiensis*) is a species of fully aquatic giant salamander (Figure 1) of the family Cryptobranchidae and represents one of the oldest lineages of salamanders (Pyron and Wiens, 2011). It is the heaviest amphibian in North American with a weight up to 2.2 kg, growing up to 74 cm in total length (Petranka, 1998), and a lifespan of 25+ years (Taber et al., 1975; Nickerson and Mays, 1973). The historical distribution of eastern hellbenders spans from southern New York to northern Alabama (Nickerson and Mays, 1973), inhabiting the streams and rivers of the Ohio and Tennessee River Basins (Browne et al., 2014). Hellbenders inhabit cool, fast-flowing, oxygen-rich streams (Nickerson and Mays, 1973) with low organic matter and fine sediments (Pugh et al., 2016) in areas with extensive boulder habitat and high

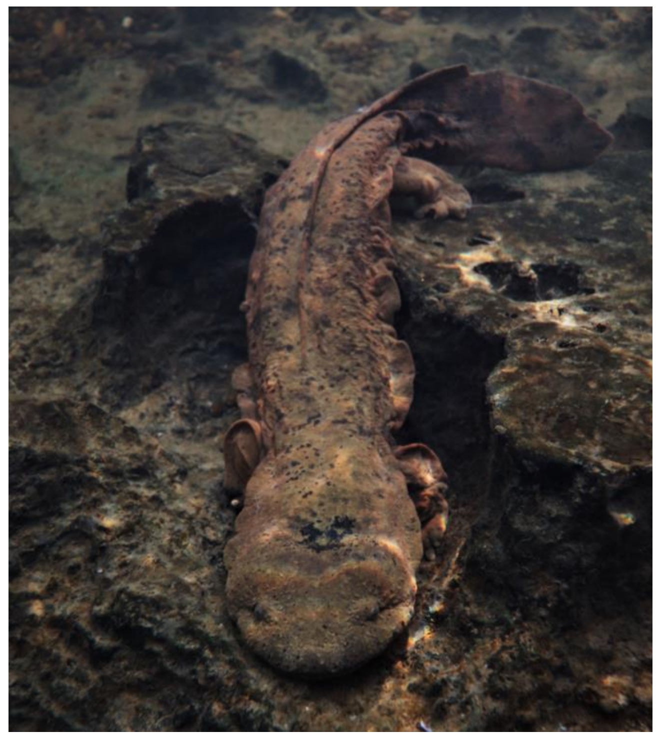


Figure 1. Image of adult eastern hellbender (*Cryptobranchus alleganiensis*). The photo was taken by J. Groffen in October 2020 at a study site in southwest Virginia.

upstream forest cover (Pugh et al., 2016; Jachowski and Hopkins, 2018) where they primarily rely on rock crevices for cover and reproduction (Nickerson and Mays, 1973).

Hellbenders engage in fully aquatic, external fertilization followed by obligate, solitary paternal care of their eggs and larvae (Bishop, 1941; Nickerson and Mays, 1973). In Virginia, male hellbenders are known to stay with their young for more than eight months (Hopkins et al., 2023). While the timing of the breeding season can vary depending on environmental factors and geographic location (Nickerson and Mays, 1973), in our study system, the timing of oviposition ranges from late August into the early weeks of September (O'Brien et al. 2024). Following a ~60-day period of embryonic development, larvae hatch from eggs and remain in the nest with the guarding male until the following April-May (Hopkins et al., 2023). Across the 8-month paternal care period, hellbenders rarely leave their nests (Settle et al., 2018; O'Brien, 2023). During this time, they will engage in defensive behavior in response to potential threats (Bishop, 1941; Nickerson and Mays, 1973; Settle et al., 2018; Unger et al., 2020), such as conspecific hellbenders (Unger et al., 2020) or predatory fish (Settle et al., 2018; Unger et al., 2020). In addition to their defensive behavior, hellbenders also exhibit behaviors such as nest cleaning, rocking, and tail-fanning (Settle et al., 2018; Unger et al., 2020; O'Brien, 2023), the latter of which has been documented in other cryptobranchids (Okada et al., 2015; Takahashi et al., 2017) and hypothesized to aid in increasing oxygen flow to embryos (Okada et al., 2015; Takahashi et al., 2017; Settle et al., 2018).

Hellbenders have undergone steep declines in populations since the 1970s, with some populations declining by 70-80% (Wheeler et al., 2003; Burgmeier et al., 2011; Graham et al., 2011) and others being completely extirpated or functionally extinct (USFWS, 2018). Due to rapid declines, the eastern hellbender is listed as a species of particular conservation concern in most states where it occurs (USFWS 2011). Factors driving population declines remain unclear but are suspected to include deforestation, increased fine sediments deposited in streams, pathogens and parasites, and degraded water quality (Williams et al., 1981; Wheeler et al., 2003; Briggler et al., 2007; Foster et al., 2009; Jachowski and Hopkins, 2018; USFWS, 2018; Hopkins et al., 2023). Recent evidence indicates that reduced forest cover near streams led to a higher incidence of nest failure and whole clutch filial cannibalism among hellbenders, ultimately contributing to their population declines (Hopkins et al., 2023; Brooks et al., 2024).

Parental cannibalism of offspring, also known as filial cannibalism, has been observed in a wide range of vertebrate taxa (Elwood, 1992; Stanback and Koenig, 1992; Manica, 2002; Gilbert et al., 2005; Klug and Bonsall, 2007; Huang, 2008; Culot et al., 2011; Okada et al., 2015) and occurs more prominently in species that rely on paternal care of offspring (Elgar and Crespi, 1992; Sargent, 1992; Manica, 2002; Klug and Bonsall, 2007) like the hellbender (Nickerson and Mays, 1973; O'Brien, 2023). The behavior is believed to be an adaptive decision to forego current reproduction if conditions are poor in favor of future opportunities (Klug and Bonsall, 2007). In other taxa, filial cannibalism increases with predation risk (Huang et al., 2008), resource competition (Klug and Bonsall, 2007), and energetic demands of the parent (Sargent, 1992; Manica, 2002), but the cues differ among species and circumstances (Belles-Isles and Fitzgerald, 1991; Lindstrom and Sargent, 1997; Klug and St. Mary, 2005; Klug et al., 2006). Prominent hypotheses to explain filial cannibalism, such as energy deficits (Klug and Bonsall, 2007), cannot explain the high frequency of cannibalism in hellbenders (Hopkins et al., 2023). While the increased occurrence of whole clutch cannibalism in hellbenders is associated with lower forest cover (Hopkins et al., 2023), the underlying mechanistic driver remains unclear.

Vector-parasite-host relationship

Several pathogens and parasites are known to infect hellbenders (Huang et al., 2010; Bodinof et al., 2011, 2012; Burgmeier et al., 2011; Gonynor et al., 2011; Regester et al., 2012; Souza et al., 2012; Tominaga et al., 2013). In our study system in VA, Hopkins et al. (2014) identified a new species of ectoparasitic leech (*Placobdella appalachiensis*) harbored by eastern hellbenders populations in southwest Virginia (Hopkins et al., 2014). Additionally, leeches are a vector of a newly identified species of endoparasite (*Trypanosoma sp*; Hopkins, unpublished), found in blood of hellbenders (Davis and Hopkins, 2013). Genetic analysis revealed that the species of Trypanosome infecting eastern hellbenders was previously undescribed (Hopkins, unpublished). Recent studies on wild hellbender populations in southwest Virginia have shown that trypanosomes, leeches, and coinfection are highly prevalent in stream reaches where they occur (Durant et al. 2015; Hopkins et al., 2016; Bodinof Jachowski et al., 2024).

Within the system, the cyclicity of leech reproduction, parental care, and delivery of offspring to the first blood meal (Blumenthal and Hopkins, unpublished) coincides with the onset of hellbender breeding season (Bishop, 1941; Peterson, 1988) in which interactions among conspecifics, and potential hosts, frequently occur (Nickerson & Mays, 1973; O'Brien et al., 2024). Leech offspring of the same genus require a rapid growth period, feeding 3-5 times, in order to survive over winter (Wilkialis, 1984; Moser et al., 2009). As a result, leech offspring may remain attached to their hellbender hosts for extended periods of time, which may increase the likelihood of trypanosome transmission from infected hosts to new vectors, as well as from infected vectors to hosts.

Respiration and Blood Physiology

The genus name "Cryptobranchus" translates to "hidden gills" and was applied to the genus after its distinct morphological characteristic observed in adults (Nickerson and Mays, 1973), which, despite being fully aquatic, lacks gills and do not typically use their lungs (Guimond and Hutchison, 1973, Coe et al., 2016). Hellbenders rely primarily on cutaneous respiration, utilizing highly vascularized lateral folds for gas exchange and requiring well-oxygenated aquatic environments (Nickerson and Mays, 1973; Guimond and Hutchison, 1973). Cutaneous respiration is constrained by multiple factors, requiring moist conditions, thin skin to maximize diffusion rate, and a high surface area to volume ratio required for efficient gas exchange (Vitt and Caldwell, 2013). Hellbenders overcome the limitations of cutaneous respiration by living in well-oxygenated habitats (Taketa and Nickerson, 1973; Ultsch, 2012), minimizing daily movements and associated oxygen demands (Ultsch, 2012), having extremely large red blood cells (Jerrett and Mays, 1973), and maximizing the surface area of thin vascularized skin through its lateral skin folds, where 90% of oxygen uptake and 98% of carbon dioxide release occurs (Guimond and Hutchison, 1973). During the process of cutaneous respiration, dissolved oxygen in the water initially enters highly vascularized capillaries in the skin of the lateral folds (Guimond and Hutchison, 1973; Boutilier and Toews, 1981). Red blood cells circulating in the capillaries bind oxygen using the oxygen-carrying protein, hemoglobin (Coates, 1975), which transports O^2 from the blood (high pO^2) to tissues (low pO^2) where the oxygen is used for aerobic respiration (Piiper, 1982). However, due to the near complete reliance on cutaneous respiration for gas exchange, hellbenders can not mechanically increase their respiration rate during times of increased demand. Thus, I hypothesized that hellbenders utilize similar mechanisms as other cutaneously respiring salamanders; the spleen acts as a compensatory respiratory organ where red blood cells are stored and rapidly released to meet energetic or respiratory demands (Frangioni and Borgioli, 1989; Frangioni and Borgioli, 1993). My thesis is the first research to test this idea.

Red blood cell metrics are a useful tool used to evaluate the aerobic capacity of an animal, with hematocrit and hemoglobin as the most common metrics (Johnstone et al., 2017). Hematocrit is the proportion of red blood cells found in the blood and can function as a determinant of blood-oxygen-carrying capacity in amphibians (Hillman, 1976). Hemoglobin is the protein found within the red blood cell that carries oxygen (Coates, 1975). Both measures are used to evaluate the health of an animal and can reveal underlying health conditions (Johnstone et al., 2017; Isbister, 1997). It is common for vertebrate blood to contain multiple hemoglobins, but hellbender blood contains a single type of hemoglobin (Taketa and Nickerson, 1973). As hemoglobin increases in the blood, the total amount of oxygen that can be transported increases, thus increasing oxygen-carrying capacity and delivery to tissues (Jensen et al., 1998). Animals can increase blood hemoglobin concentrations and thus blood oxygen carrying capacity by increasing the number of red blood cells in circulation (Hillman, 1976; Weber and Jensen, 1988), and/or packing more hemoglobin into each red blood cell (i.e., increasing mean corpuscular hemoglobin concentration (Hillman, 1976). However, there are costs associated with increasing red blood cells in circulation due to increased blood viscosity (Isbister, 1997, Hillman et al., 2010); therefore, animals should optimize their red blood cell physiology to maximize benefits in the face of such costs.

The proportion of immature red blood cells, known as polychromatic red blood cells, can help identify signs of anemia or other pathological conditions (Latimer et al. 2003; Campbell et al., 2004). Immature red blood cells can be distinguished from mature red blood cells by the characteristic blue-gray coloration which is due to residual RNA and a deficiency of hemoglobin (Latimer et al. 2003). An increase in immature red blood cells in circulation is indicative of increased erythropoietic activity and a regenerative response to anemia (Fallon et al. 2017; Fallon et al. 2020). This response is triggered by the loss or destruction of red blood cells due to hemorrhage or hemolysis (Latimer et al. 2003).

White blood cell differentials represent the relative proportion of major leukocyte cell types in circulation and are a common tool to assess animal health across vertebrate taxa which can be informative for identifying ongoing stressors and immune activity (Davis et al., 2008; Forzan et al., 2017). Neutrophils are among the first cells activated by the innate immune response, proliferating into circulation in response to stress, inflammation, and infection (Bain and Harr, 2022). Eosinophils and monocytes also proliferate in circulation during inflammatory responses, with eosinophils responsible for antiparasitic defenses and monocytes responsible for eliminating foreign substances (Latimer et al., 2003). Conversely, some cell types, such as lymphocytes and basophils, migrate out of circulation and into peripheral tissues upon activation (Latimer et al., 2003; Eberle and Voehringer, 2016; Bain and Harr, 2022). The migration of basophils occurs during allergic reactions and parasitic infection, and they also play a role in the inflammatory response as well as protective immunity against parasites (Eberle and Voehringer, 2016). Lymphocytes are the most abundant type of white blood cell in amphibians (Bain and Harr, 2022) and are involved in multiple immunological processes, including immunoglobin production and antibody-dependent cell-mediated cytotoxicity (Wright and Whitaker, 2001; Cambell, 2015). Notably, a paired migration of neutrophils into circulation and lymphocytes out of circulation into tissues occurs during the glucocorticoid stress response. This inverse physiological relationship is often represented as a ratio (i.e., N:L ratio) and has become a common metric used by researchers to evaluate stress across vertebrate taxa (Davis et al., 2008).

In addition to understanding quantitative shifts in white blood cell types, recognition of morphological changes in certain cell types is also important for diagnostic evaluations (Latimer et al., 2003). For example, evidence of toxic change and immature neutrophils are indicative of inflammatory disease states, prolonged immune demand, and insufficient neutrophil reserves (Latimer et al., 2003; Stacy et al., 2022). Accelerated production of neutrophils into circulation causes toxic morphological changes indicating abnormal or dyssynchronous maturation. Elevated proportions of immature neutrophils result when inflammatory demand exceeds storage pools. (Stacy et al., 2022).

Stress-Induced Hemoconcentration

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 evading a potential threat (Biron and Benfey, 1994; Brill et al., 2008). This process, referred to as hemoconcentration, is a physiological adaptation triggered by the release of catecholamines to meet sudden energy demands by increasing oxygen delivery to tissues for cellular respiration (Figure 2; Romero and Butler, 2007; Coles et al., 2009). Catecholamines activate a series of responses that increase heart rate and induce vasoconstriction of blood vessels (Romero and Butler, 2007), resulting in greater hydrostatic pressure exerted on capillaries (i.e., blood pressure; Patterson et al., 1995; Olson et al., 2003). Increased hydrostatic pressure causes an exchange of plasma from the vascular to interstitial space (Patterson et al., 1995; Olson et al., 2003; Hillman et al., 2010). The increase in heart rate and blood flow, in combination with plasma efflux, results in a greater proportion of red blood cells in the vascular space (i.e., hematocrit; Hillman et al., 2010). Catecholamines can also provoke the release of red blood cells from spleen storage into circulation maximizing gas exchange and delivery to tissues. The spleen serves as a primary erythropoietic organ in urodeles (Allender and Fry, 2008; Lopez et al., 2014; Udroiu and Sgura, 2017) which acts as a reservoir, where red blood cells are stored and quickly released in the case of variable respiratory conditions, hypoxia, stress, or hemorrhage (Frangioni and Borgioli, 1989; Frangioni and Borgioli, 1993; Isbister, 1997; Udroiu and Sgura, 2017).

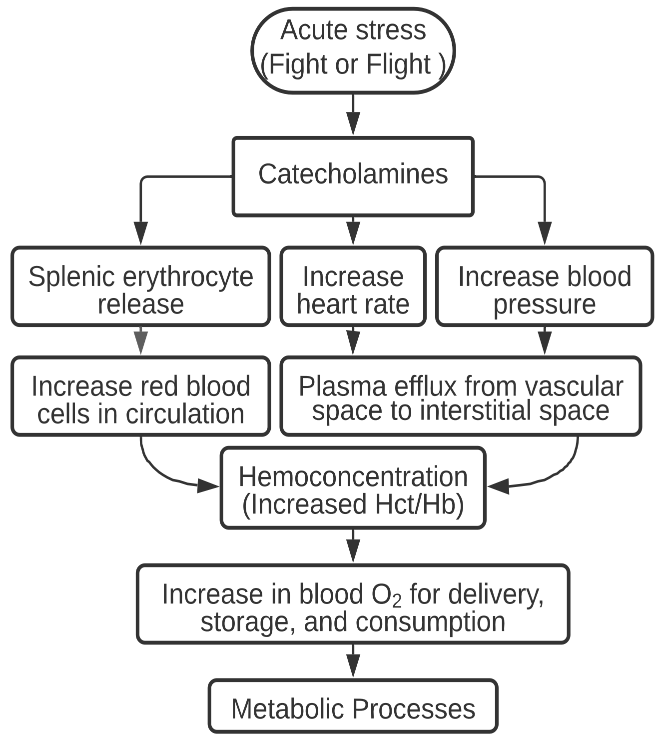


Figure 2. Conceptual diagram representing the physiological processes associated with stress-induced hemoconcentration. Acute stressors can result in an increase in hematocrit (Hct) and hemoglobin (Hb). The adrenal/interrenal glands release catecholamines, increasing heart rate and blood pressure within seconds, resulting in plasma efflux out of vascular space. Catecholamines can also provoke the release of red blood cells from spleen storage into circulation and notably vary across taxa. Both mechanisms result in hemoconcentration to maximize gas exchange and oxygen delivery to tissues during the fight or flight response.

Environmental Impacts on Physiology

Environmental temperatures overwhelmingly influence ectotherm physiology which mediates the effectiveness of metabolic and immune processes (da Silva et al., 2013). Available dissolved oxygen in aquatic habitats is inversely related to temperature (Li and Liu, 2019). Thus, temperature increases metabolic and respiratory demand while subsequently decreasing oxygen supply (Frangioni and Borgioli, 1993).

Conversely, low temperatures can have immunosuppressive effects on amphibian immune systems (Rollins-Smith and Woodhams, 2012). Multiple aspects of amphibian immune systems are known to be impaired by low temperatures, such as serum complement activity, lymphocyte proliferation (Maneiro and Carey, 1997), and antibody production (Cooper et al., 1992). However, ectotherms may rely on non-specific components of the innate immune response in low temperatures, such as phagocytic activity and neutrophil proliferation, to compensate for the suppression of other immune functions (Maneiro and Carey, 1997; Le Morvan et al., 1997; Raffel et al., 2006).

Additionally, ectotherm physiology can vary depending on circadian and circannual oscillations (Bridges et al., 1976; Frangioni and Borgioli, 1984). 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). Bodinof Jachowski et al. (2023) demonstrated that hellbender hematocrit fluctuates across season, but the seasonal trends differ between sexes. Additionally, male hellbenders exhibit seasonal peaks in testosterone during the onset of breeding (Galligan et al., 2021; Case et al., 2024), which is associated with reduced immune activity in some ectotherms (Zapata et al., 1992).

External factors that alter water chemistry could directly affect hellbender physiology (Harlan and Wilkinson, 1981). For example, as water temperature increases, oxygen solubility decreases (Li and Liu, 2019), thus reducing available dissolved oxygen in the aquatic environment for respiratory processes. In streams inhabited by hellbenders, dissolved oxygen increases in cooler months with colder and faster-flowing water. Seasonality of streamflow varies but typically increases following snow and ice melt and after precipitation, which are most common in spring (Dettinger and Diaz, 2000). Climate change threatens environmental oxygen availability as global temperatures continue to rise (Morrison et al., 2002). Deforestation also threatens oxygen availability as reduced canopy coverage increases sun exposure, increasing water temperatures (Caissei, 2006). Additionally, increased temperature variability associated with climate change also causes fluctuations in amphibian immune responses which could increase susceptibility to infectious diseases (Greenspan et al., 2017)

Sedimentation is considered to be the most impactful factor threatening eastern hellbenders (USFWS, 2018). Excessive sedimentation is thought to suffocate eggs (Nickerson and Mays 1973), fill interstitial spaces (Wood and Armitage, 1997), and bury shelter and nest rocks (Hopkins and Durant, 2011; Brooks et al., 2024), requiring greater parental investment to maintain the nests. Thus, sedimentation could influence hellbender physiology by decreasing interstitial space in shelter cavities used by hellbenders and reducing dissolved oxygen by promoting microbial activity in the benthos (Carling and McCahon, 1987), while simultaneously increasing energetic demands associated with cavity cleaning and maintenance (O'Brien, 2023).

Parasite Impacts on Physiology

Blood-feeding parasites could influence hellbender physiology due to the consumption or degradation of red blood cells and by eliciting immune responses. Leeches introduce a suite of biologically active compounds to the host through their saliva which optimizes conditions for blood meals and aids in evading host immune responses (Salzet et al., 2000; Hildebrandt and Lemke, 2011). Prior research has shown that leech infection can influence systemic neuroendocrine processes in hellbenders (Durant et al., 2015). Additionally, leech infection has been linked to increased total plasma proteins (Hopkins et al., 2016), and increased N:L ratios hellbenders (Durant et al., 2015; Hopkins et al., 2016). However, previous investigations could not determine whether leech infection could cause anemia in hellbenders (Hopkins et al., 2016). Even among a broad range of vertebrate taxa, literature on anemia caused by leeches is relatively scarce.

While the physiological effects of the trypanosomes on their aquatic hosts are poorly understood (Woo, 1994), like most blood-borne protozoa, they consume nutrients from red blood cells (Woo, 2006; Oladiran and Belosevic, 2012; Pishchany and Skaar, 2012) where trypanosomes secrete a lytic factor to erode the membrane of red blood cells in order to obtain nutrients (Islam and Woo, 1991). Ruszczyk et al. (2008) suggests one of the lytic factors utilized by trypanosomes infecting aquatic species is cysteine proteinase, which aids in the degradation and digestion of host hemoglobin for iron and protein (Pishchany and Skaar, 2012; Siqueira-Neto et al., 2018). While it varies by species and severity of infection, trypanosomes use hemolytic factors (Tizard et al., 1976) and immune-mediated mechanisms to damage and destroy host red blood cells (Murray, 1979). Trypanosome infection can cause a diversity of pathologies in ectothermic hosts, including anemia (Densmore and Green, 2007), and has been linked to increased proportions of immature red blood cells in both amphibians (Gonzolez et al., 2021) and fish (Laprirova and Zabotkina, 2018). However, most aquatic trypanosomes are thought to be nonpathogenic to their fish (Woo, 1994; Woo, 2006) and amphibian hosts (Omonona and Ekpenko, 2011).

Study System

I sampled male hellbenders from four different stream reaches along the upper Tennessee River Basin (Figure 3) that extend across Smyth and Washington County, Virginia. Each stream reach has a range of 30-35 concrete artificial shelters that have been deployed as part of a long-term study that has been ongoing for more than ten years (Hopkins et al., 2023). The four reaches were selected due to their stable populations of healthy hellbenders with consistent successful reproductive outcomes (Jachowski and Hopkins, 2018). In addition to healthy hellbender populations, sites were selected based on their similar site quality and upstream catchment-wide riparian forest cover (64.3-67.9%). I specifically chose to control for variation in forest cover because prior research demonstrated that it is related to hellbender hematological variables but is inherently confounded with other variables like hellbender population density and the presence of parasites (Bodinof Jachowski et al., 2024). Thus, my study design enabled me to disentangle the effects of parasites from other externalities that influence hellbender physiology.

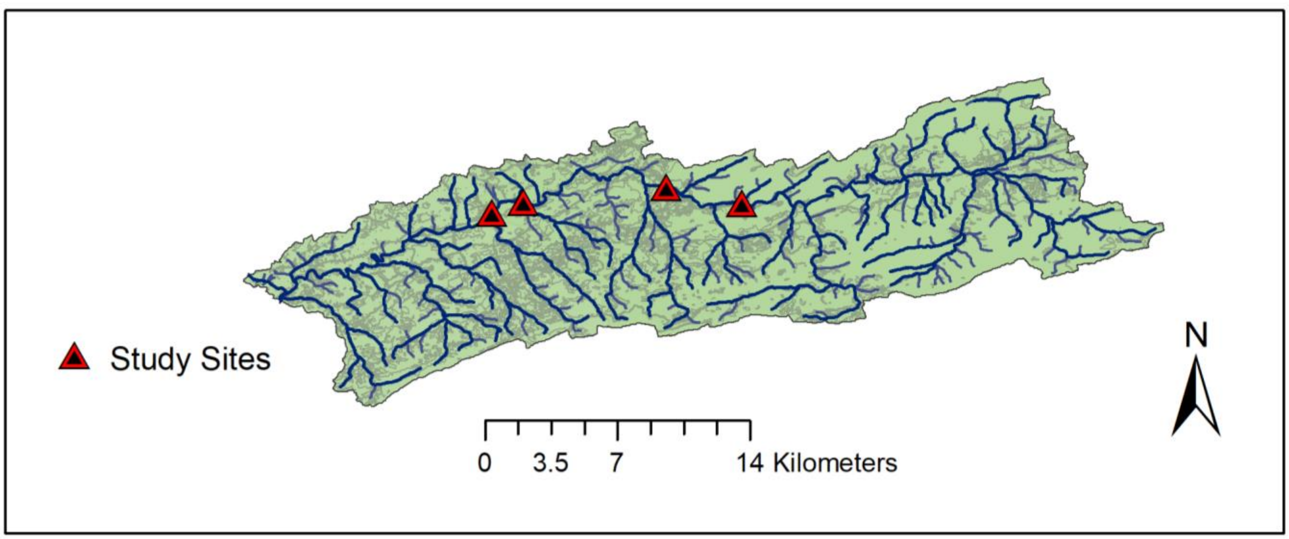


Figure 3. Geographic locations of the four streams reaches in a single river in the upper Tennessee River Basin in southwest Virginia, where hellbenders were sampled.

Overarching Research Objectives

The overarching goal of my thesis was to use hematological metrics as a diagnostic tool to gain mechanistic insight into the blood physiology of reproductive male hellbenders. Broadly, the goal of this research was to investigate parameters relative to metabolic, respiratory, and immune health while distinguishing between natural variation, the influence of extrinsic factors, and pathological impacts associated with parasites and coinfection. Further, I sought to determine if parasite infection was a proximate mechanism contributing to nest failure or filial cannibalism.

In the first chapter, I measured hematocrit, hemoglobin, and relative proportions of polychromatic red blood cells to understand how multiple factors influence metabolic and respiratory demands. I explore how hellbenders modulate circulating red blood cells in response to acute stress and fluctuations in environmental temperatures. I establish how red blood cell metrics vary with body size and investigate whether sedimentation impacts respiratory demands. Lastly, I monitor parasite infection across the parental care period and assess whether parasites or coinfection leads to anemia.

In the second chapter, I used white blood cell differentials and the presence of morphologically distinct cells that signal immune recruitment to monitor hellbender immune health across the parental care period. I sought to understand cellular adaptations to seasonal shifts in environmental temperatures. I aimed to evaluate how parasites and coinfection influence hellbender immune health and investigated whether there is a relationship between parasite abundance and the likelihood of filial cannibalism or nest failure.

References

Allender M.C. and M.M. Fry. 2008. Amphibian hematology. Vet Clin N Am Exot Anim Pract 11: 463–480

Bain P. and K.E. Harr. 2022. Hematology of Amphibians. Pp. 1228–1232 in Schalm's Veterinary Hematology, 7th ed. Wiley: Hoboken, New Jersey, USA

Biron M. and T.J. Benfey. 1994. Cortisol, glucose, and hematocrit changes during acute stress, cohort sampling, and the diel cycle in diploid and triploid brook trout (*Salvelinus fontinalis Mitchill*). Fish Physiol and Biochem 13:153–160.

Belles-Isles J.C. and G.J. Fitzgerald. 1991. Filial cannibalism in sticklebacks – a reproductive management strategy. Ethol Ecol Evol 3:49–62.

Bishop S.C. 1941. The Salamanders of New York. New York State Museum Bulletin. 324.

Blaustein, A.R. 1994. Chicken Little or Nero's fiddle? A perspective on declining amphibian populations. Herpetologica 50:85–97

Blaustein A.R., S.C. Walls, B.A. Bancroft, J.J. Lawler, C.L. Searle, and S.S. Gervasi. 2010. Direct and Indirect Effects of Climate Change on Amphibian Populations. Diversity 2: 281-313.

Bodinof C.M., J.T. Briggler, M.C. Duncan, J. Beringer, and J.J. Millspaugh. 2011. Historic occurrence of the amphibian chytrid fungus *Batrachochytrium dendrobatidis* in hellbender *Cryptobranchus alleganiensis* populations from Missouri. Dis Aquat Organ 96: 1–7

Bodinof C.M., J.T. Briggler, R.E. Junge, T. Mong, J. Beringer, M.D. Wanner, C.D. Schuette, J. Ettling, and J.J. Millspaugh. 2012. Survival and body condition of captive-reared juvenile Ozark hellbenders (*Cryptobranchus alleganiensis bishopi*) following translocation to the wild. Copeia 2012:150–159.

Bodinof Jachowski C.M., V. Alaasam, A. Blumenthal, A. Davis, and W.A. Hopkins. 2024. The habitat quality paradox: Loss of riparian forest cover leads to decreased risk of parasitism and improved body condition in an imperiled amphibian. Conserv Physiol 12:coad101

Boutilier R.G. and D.P. Toews. 1981. Respiratory properties of blood in a strictly aquatic and predominantly skin-breathing urodele, *Cryptobranchus alleganiensis*. Respir Physiol 46:161–176.

Bridges D.W., J.J. Cech, and D.N. Pedro. 1976. Seasonal Hematological Changes in Winter Flounder, *Pseudopleuronectes americanus*. Trans Am Fish Soc 105:596–600.

Brill R., P. Bushnell, S. Schroff, R. Seifert, and M. Galvin. 2008. Effects of anaerobic exercise accompanying catch-and-release fishing on blood–oxygen affinity of the sandbar shark (*Carcharhinus plumbeus*, Nardo). J Exp Mar Biol Ecol. 354:132–143.

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

Browne R.K., H. Li, Z. Wang, S. Okada, P.M. Hime, A. McMillan, M. Wu, R. Diaz, D. McGinnity, and J. T. Briggler. 2014. The giant salamanders (Cryptobranchidae): part B. biogeography, ecology, and reproduction. Amphib Reptile Conserv 5:30-50.

Brooks G.C., W.A. Hopkins, and H.K. Kindsvater. 2024. Concurrent threats and extinction risk in a long-lived, highly fecund vertebrate with parental care. Ecol Appl in press.

Burgmeier N.G., S.D. Unger, J.L. Meyer, T.M. Sutton, and R.N. Williams. 2011. Health and habitat quality assessment for the eastern hellbender (*Cryptobranchus alleganiensis alleganiensis*) in Indiana, USA. J Wildlife Dis 47: 836–848.

Caissie D. 2006. The thermal regime of rivers: A review. Freshw. Biol 51: 1389–1406.

Campbell T.W. 2015. Exotic Animal Hematology and Cytology, 4th ed. Blackwell Publishing, Ames, IA, USA.

Campbell T.W. 2004. Hematology of lower vertebrates. American College of Veterinary Pathologists & American Society for Veterinary Clinical Pathology, Middleton, WI, USA.

Campbell Grant E.H., D.A. Miller, and E. Muths. 2020. A synthesis of evidence of drivers of amphibian declines. Herpetologica 76:101-107.

Carling P.A. and C.P. McCahon. 1987. Natural Siltation of Brown Trout (*Salmo trutta L*.) Spawning Gravels During Low-Flow Conditions. Pp. 229–244 in J.F. Craig, J.B. Kemper, eds. Regulated Streams. Springer, Boston, MA.

Case B.F., J. Groffen, T.M. Galligan, C.M. Bodinof Jachowski, J.J. Hallagan, S.B. Hildreth, V. Alaasam, W.K. Ray, R.F. Helm, and W.A. Hopkins. 2024. Androgen and glucocorticoid profiles throughout extended uniparental paternal care in the eastern hellbender salamander (*Cryptobranchus a. alleganiensis*). Gen Comp Endocrinol. In Press.

Coates M.L. 1975. Hemoglobin function in vertebrates: An evolutionary model. J Mol Evol 6: 285–307.

Coe B.C., D.V. Drewett, C.M.B. Jachowski, and W.A. Hopkins. 2016. *Cryptobranchus alleganiensis alleganiensis* (Eastern Hellbender). Terrestrial movement. Herpetological Review 47:99-100.

Cohen J.M., D.J. Civitello, M.D. Venesky, T.A. McMahon, and J.R. Rohr. 2019. An interaction between climate change and infectious disease drove widespread amphibian declines. Glob. Chang Biol. 25:927-937.

Coles B.H., L. Crosta, L.A. Cruz-Martinez, P. De Herdt, G. Dorrestein, N. Harcourt-Brown, D.J. Harris, A.K. Jones, M. Krautwald-Junghanns, P. Macwhirter, G.H. Olsen, F. Pasmans, M. Pees, P.T. Redig, I. Robinson, A. Routh, S. Sanderson, L. Timossi, T.N. Tully, and A.B. Worell. 2009. Handbook of Avian Medicine. 2nd ed. Saunders-Elsevier, London, UK.

Collins J.P. and A. Storfer. 2003. Global amphibian declines: sorting the hypotheses. Divers. Distrib. 9:89-98.

Cooper E.L., R.K. Wright, A.E. Klembau, and C.T. Smith. 1992. Hibernation alters the frog's immune system. Cryobiology. 29: 616–631.

Culot L., Y. Lledo-Ferrer, O. Hoelscher, F.J.J. Muñoz Lazo, M.C. Huynen, and E.W. Heymann. 2011. Reproductive failure, possible maternal infanticide, and cannibalism in wild moustached tamarins, *Saguinus mystax*. Primates 52:179–186 da Silva G.S.F., M.L. Glass, and L.G.S. Branco. 2013. Temperature and respiratory function in ectothermic vertebrates. J Therm Biol 38:55–63.

Daszak P., A.A. Cunningham, and A.D. Hyatt. 2003. Infectious disease and amphibian population declines. Divers Distrib 9:141-150.

Davis A.K, D.L. Maney, and J.C. Maerz. 2008. The use of leukocyte profiles to measure stress in vertebrates: a review for ecologist. Funct Ecol 22:760-772.

Davis A.K. and W.A. Hopkin. 2013. Widespread trypanosome infections in a population of eastern hellbenders (*Cryptobranchus alleganiensis alleganiensis*) in Virginia, USA. Parasitol Res 112:453–456.

Densmore C. L. and D.E. Green. 2007. Diseases of Amphibians. ILAR J. 48:235-254

Dettinger M., and H. Diaz. 2000. Global Characteristics of Stream Flow Seasonality and Variability. J Hydrometeorol 1:289–310.

DuRant S.E., W.A. Hopkins, A.K. Davis, and L.M. Romero. 2015. Evidence of ectoparasite-induced endocrine disruption in an imperiled giant salamander, the eastern hellbender (*Cryptobranchus alleganiensis*). J Exp Biol 218:2297–2304.

Eberle J.U. and D. Voehringer. 2016. Role of basophils in protective immunity to parasitic infections. Semin Immunopathol 38:605–613.

Elgar M.A. and B.J. Crespi. 1992. Cannibalism: ecology and evolution among diverse taxa. Oxford University Press, Oxford.

Elwood R. 1992. Pup-cannibalism in rodents: causes and consequences. Pp. 299–322 in M. A. Elgar and B. J. Crespi, eds. Cannibalism: ecology and evolution in diverse taxa. Oxford University Press, Oxford

Fallon J.A., E.P. Smith, N. Schoch, J.D. Paruk, E.A. Adams, D.C. Evers, P.G.R. Jodice, C. Perkins, S. Schulte, and W.A. Hopkins. 2017. Hematological indices of injury to lightly oiled birds from the Deepwater Horizon oil spill. Environ Toxicol Chem 37:451–461.

Fallon J.A., E.P. Smith, N. Schoch, J.D. Paruk, E.A. Adams, D.C. Evers, P.G.R. Jodice, M. Perkins, D.E. Meattey, and W.A. Hopkins. 2020. Ultraviolet-assisted oiling assessment improves detection of oiled birds experiencing clinical signs of hemolytic anemia after exposure to the Deepwater Horizon oil spill. Ecotoxicology 29:1399–1408.

Fisher M.C. and T.W. Garner. 2020. Chytrid fungi and global amphibian declines. Nat Rev Microbiol 18:332-343.

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

Forzán M.J., J. Heatley, K.E. Russell, and B. Horney. 2017. Clinical pathology of amphibians: a review. Vet Clin Pathol 46:11-33.

Frangioni G. and G. Borgioli. 1984. Seasonal Erythrocyte Count Variations in Laboratory Raised Newts. Ital J Zool (Modena) 18:301–306.

Frangioni G. and G. Borgioli. 1989. Relationships Between Spleen and Respiration in the Newt. J Exp Zool 252:118–125.

Frangioni G. and G. Borgioli. 1993. Hematological changes in the newt during the process of splenic decongestion in reply to hypoxia. Ital J Zool (Modena) 60:267–270.

Galligan, T.M., R.F. Helm, B.F. Case, C.M.B. Jachowski, C.L. Frazier, V. Alaasam, and W.A. Hopkins. 2021. Pre-breeding androgen and glucocorticoid profiles in the eastern hellbender salamander (*Cryptobranchus alleganiensis alleganiensis*). Gen Comp Endocrinol 313:p.113899.

Gerlanc, N.M. and G.A. Kaufman. 2005. Habitat of origin and changes in water chemistry influence development of western chorus frogs. J Herpetol 39: 254-265.

Gilbert W.M., P.M. Nolan, A.M. Stoehr, and G.E. Hill. 2005. Filial cannibalism at a house finch nest. Wilson Bulletin 11:413–415.

Gonynor J.L., M.J. Yabsley, and J.B. Jensen. 2011. A preliminary survey of Batrachochytrium dendrobatidis exposure in hellbenders from a stream in Georgia, USA. Herpetol Rev 42:58–59.

González L.P., C.M. Vargas-León, G.A. Fuentes-Rodríguez, M.L. Calderón-Espinosa, and N.E. Matta. 2021. Do blood parasites increase immature erythrocytes and mitosis in amphibians? Rev Biol Trop 69:615–625.

Graham S.P., E.C. Soehren, G.R. Cline, C.M. Schmidt, W.B. Sutton, J.R. Rayburn, S.H. Stiles, and J.A. Stiles. 2011. Conservation status of hellbenders (*Cryptobranchus alleganiensis*) in Alabama, USA. Herpetol Conserv Biol 6:242–249.

Green D.M., M.J. Lannoo, D. Lesbarrères, and E. Muths. 2020. Amphibian population declines: 30 years of progress in confronting a complex problem. Herpetol. 76:97-100.

Greenspan S.E., D.S. Bower, R.J. Webb, L. Berger, D.R. Rudd, L. Schwarzkopf, and R.A. Alford. 2017. White blood cell profiles in amphibians help to explain disease susceptibility following temperature shifts. Dev Comp Immunol 77:280–286.

Guimond R.W. and V.H. Hutchison. 1973. Aquatic respiration: an unusual strategy in the hellbender *Crytobranchus alleganiensis alleganiensis* (Daudin). Science 182:1263-1265.

Harlan R.A. and R.F. Wilkinson. 1981. The Effects of Progressive Hypoxia and Rocking Activity on Blood Oxygen Tension for Hellbenders, *Cryptobranchus alleganiensis*. J Herpetol 15:383.

Harvell C.D., C. Mitchell, J. Ward, S. Altizer, A. Dobson, R. Ostfeld, and M. Samuel. 2002. Climate warming and disease risks for terrestrial and marine biota. Science 296:2158–2162.

Hildebrandt J.P. and S. Lemke. 2011. Small bite, large impact–saliva and salivary molecules in the medicinal leech, *Hirudo medicinalis*. Naturwissenschaften 98:995–1008.

Hillman S.S. 1976. Cardiovascular correlates of maximal oxygen consumption rates in anuran amphibians. J. Comp. Phys. 109:199-207.

Hillman S.S., E.A. De Grauw, T. Hoagland, T. Hancock, and P. Withers. 2010. The role of vascular and interstitial compliance and vascular volume in the regulation of blood volume in two species of anuran. Physiol. Biochem. Physiol. 83:55-67.

Hof C., M.B. Araújo, W. Jetz, and C. Rahbek. 2011. Additive threats from pathogens, climate and land-use change for global amphibian diversity. Nature 480:516-521.

Hoffman M., C. Hilton-Taylor, A. Angulo, M. Böhm, T.M. Brooks, S.H.M. Butchart, K.E. Carpenter, J. Chanson, B. Collen, and N.A. Cox. 2010. The impact of conservation on the status of the world's vertebrates. Science 330:1503–1509.

Hopkins W.A. 2007. Amphibians as Models for Studying Environmental Change. ILAR J 48:270–277

Hopkins W.A., B.F. Case, J. Groffen, G.C. Brooks, C.M.B. Jachowski, S.T. Button, J.J. Hallagan, R.S.M. O'Brien, and H.K. Kindsvater. 2023. Filial Cannibalism Leads to Chronic Nest Failure of Eastern Hellbender Salamanders (*Cryptobranchus alleganiensis*). Am Nat 202:92–106.

Hopkins W.A. and S.E. DuRant. 2011. Innate immunity and stress physiology of Eastern Hellbenders (*Cryptobranchus alleganiensis*) from two stream reaches with differing habitat quality. Gen Comp Endocrinol 174:107-115.

Hopkins W.A., J.A. Fallon, M.L. Beck, B.H. Coe, and C.M.B. Jachowski. 2016. Haematological and immunological characteristics of eastern hellbenders (*Cryptobranchus alleganiensis alleganiensis*) infected and co-infected with endo- and ectoparasites. Conserv Physiol 4:cow002.

Hopkins W.A., W.E. Moser, D.W. Garst, D.J. Richardson, C.I. Hammond, and E.A. Lazo-Wasem. 2014. Morphological and molecular characterization of a new species of leech (Glossiphoniidae, Hirundinida): implications for the health of its imperiled amphibian host (*Cryptobranchus alleganiensis*). Zookeys 378:83–101.

Huang W.S. 2008. Predation risk of whole clutch filial cannibalism in a tropical skink with maternal care. Behav Ecol 19:1069–1074

Huang C.C., Y. Xu, J.T. Briggler, M. McKee, P. Nam, and Y.W. Huang. 2010. Heavy metals, hematology, plasma chemistry, and parasites in adult hellbenders (*Cryptobranchus alleganiensis*). Environ Toxicol Chem 29: 1132–1137.

Isbister J.P. 1997. Physiology and Pathophysiology of Blood Volume Regulation. Transfus. Sci. 18:409–423

Islam A. and P. Woo. 1991. Anemia and its mechanism in goldfish *Carassius auratus* infected with *Trypanosoma danilewskyi*. Dis Aquat Org 11:37–43.

Jachowski C.M.B. and W.A. Hopkins. 2018. Loss of catchment-wide riparian forest cover is associated with reduced recruitment in a long-lived amphibian. Biol Conserv 220:215–227.

Jensen F.B., A. Fago, and R.E. Weber. 1998. Hemoglobin Structure and Function. Fish Physiol 17:1–40.

Jerrett D.P. and C.E. Mays. 1973. Comparative hematology of the hellbender, *Cryptobranchus alleganiensis* in Missouri. Copeia 1973:331–337.

Johnstone C.P., A. Lill, and R.D. Reina. 2017. Use of erythrocyte indicators of health and condition in vertebrate ecophysiology: a review and appraisal. Biol. Rev. 92:150-168

Klug H. and M.B. Bonsall. 2007. When to care for, abandon, or eat your offspring: the evolution of parental care and filial cannibalism. Am Nat 170:886–901.

Klug H., K. Lindström, and C.M. St. Mary. 2006. Parents benefit from eating offspring: density-dependent egg survivorship compensates for filial cannibalism. Evolution 60:2087–2095.

Klug H. and C.M. St. Mary. 2005. Reproductive fitness consequences of filial cannibalism in the flagfish, *Jordanella floridae*. Anim Behav 70:685–691.

Lapirova T.B. and E.A. Zabotkina. 2018. Effect of trypanosomiasis on hematologic characteristics of bream (*Abramis brama*). Regul Mech Biosyst 9:309–314.

Latimer K.S., E.A. Mahaffey, and K.W. Prasse. 2003. Duncan & Prasse's Veterinary Laboratory Medicine: Clinical Pathology. Blackwell Publishing, Ames, IA.

Le Morvan C., P. Clerton, P. Deschaux, and D. Troutaud. 1997. Effects of environmental temperature on macrophage activities in carp. Fish Shellfish Immunol 7:209–212.

Lindström K. and R.C. Sargent. 1997. Food access, brood size, and filial cannibalism in the fantail darter, *Etheostoma flabellare*. Behav Ecol Sociobiol 60:46–51.

Lips K.R., J. Diffendorfer, J.R. Mendelson, and M.W. Sears. 2008. Riding the wave: Reconciling the roles of disease and climate change in amphibian declines. Plos Biol 6:441–454.

Li D. and S. Liu. 2019. Detection of Industrial Water Quality. Pp. 329–349 Li D. and S. Liu, eds. Water Quality Monitoring and Management. Academic Press.

Lopez D., L. Lin, J.R. Monaghan, C.R. Cogle, F.J. Bova, M. Maden, and E.W. Scott. 2014. Mapping hematopoiesis in a fully regenerative vertebrate: The axolotl. Blood 124:1232–1241.

Luedtke J.A., J. Chanson, K. Neam, L. Hobin, A.O. Maciel, A. Catenazzi, A. Borzée, A. Hamidy, A. Aowphol, and A. Jean. 2023. Ongoing declines for the world's amphibians in the face of emerging threats. Nature 622:308–314.

Manica A. 2002. Filial cannibalism in teleost fish. Biol Rev 77:261–277.

Maniero D. and C. Carey. 1997. Changes in selected aspects of immune function in the leopard frog, *Rana pipiens*, associated with exposure to cold. J Comp Physiol B: Physiol 167:256-263.

Morrison J., M.C. Quick, and M.G.G. Foreman. 2002. Climate change in the Fraser River Watershed: flow and temperature projections. J Hydrol 263:230–244.

Moser W.E., F.R. Govedich, and D.J. Klemm. 2009. Annelida, Hirudinida (leeches). Pp. 116–123 in G.E. Likens, ed. Encyclopedia of Inland Waters, vol. 2. Elsevier Press, Oxford, UK.

Murray M. 1979. The anemia of bovine African trypanosomiasis: an overview. In Pathogenicity of Trypanosomes. Pp. 121–127 in G. Losos and A. Chouinard, eds. International Research and Development Council, Ottawa. IDRC-132e.

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

O'Brien R.S.M., J. Groffen, A. Dayer, and W.A. Hopkins. 2024. Pre-breeding, Courtship, and Mating Behaviors of Wild Eastern Hellbenders (*Cryptobranchus alleganiensis alleganiensis*). Herpetologica. 80.

O'Brien R.S. 2023. Behavior and conservation: hellbender reproductive behavioral responses to poor water quality and the value of private lands research for conservation outreach. Doctoral dissertation, Virginia Tech.

Oladiran A. and M. Belosevic. 2012. Immune evasion strategies of trypanosomes: a review. J Parasitol 98: 284–292.

Olson K.R., D.W. McKinney, R.A. Dombkowski, and D.W. Duff. 2003. Transvascular and intravascular fluid transport in the rainbow trout: revisiting Starling's forces, the secondary circulation and interstitial compliance. J. Exp. Biol. 206, 457-467.

Okada S., Y. Fukuda, and M.K. Takahashi. 2015. Paternal care behaviors of Japanese giant salamander *Andrias japonicas* in natural populations. J Ethol 33:1–5.

Omonona A.O. and V. Ekpenko. 2011. Haematology and prevalence of blood parasites of the common frog (*Rana temporaria*) in the tropical environment. J Vet Med Anim Health 3:14–20.

Patterson S.M., D.S. Krantz, J.S. Gottdiener, G. Hecht, S. Vargot, and D.S. Goldstein. 1995. Prothrombotic effects of environmental stress: changes in platelet function, hematocrit, and total plasma protein. Psychosom Med 57:592–599.

Peterson C.L. 1988. Breeding activities of the hellbender in Missouri. Herpetol Rev 27:126-127.

Petranka J.W. 1998. Salamanders of the United States and Canada. Smithsonian Institution Press, Washington, D.C., USA 587p.

Piiper, J. 1982. Respiratory gas exchange at lungs, gills and tissues: mechanisms and adjustments. J Exp Biol 100:5–22.

Pishchany G. and E.P. Skaar. 2012. Taste for Blood: Hemoglobin as a Nutrient Source for Pathogens. PLoS Pathog 8:e1002535

Price S.J., T.W.J. Garner, R.A. Nichols, F. Balloux, C. Ayres, A. Mora-Cabello De Alba, and J. Bosch. 2014. Collapse of amphibian communities due to an introduced *Ranavirus*. Curr Biol 24:2586–2591.

Pugh M.W., M. Hutchins, M. Madritch, L. Siefferman, and M.M. Gangloff. 2016. Land-use and local physical and chemical habitat parameters predict site occupancy by hellbender salamanders. Hydrobiologia 770:105–116.

Pyron R.A. and J.J. Wiens. 2011. A large-scale phylogeny of Amphibia including over 2800 species, and a revised classification of extant frogs, salamanders, and caecilians. Mol Phylogen Evol 61:543–583.

Raffel T.R, J.R Rohr, J.M. Kiesecker, and P.J. Hudson. 2006. Negative effects of changing temperature on amphibian immunity under field conditions. Funct Ecol 20:819–828.

Regester K.J., H. Simpson, E.J. Chapman, and P.J. Petokas. 2012. Occurrence of the fungal pathogen *Batrachochytrium dendrobatidis* among eastern hellbender populations (*Cryptobranchus a. alleganiensis*) within the Allegheny-Ohio and Susquehanna River drainages, Pennsylvania, USA. Herpetol Rev 43: 90–93.

Rollins-Smith L.A. and D.C. Woodlands. 2012. Amphibian Immunity. Pp. 92-143 in G. Demas, R. Nelson, eds. Ecoimmunlogy. Oxford University Press, New York, NY.

Romero M.L. and L.K. Butler. 2007. Endocrinology of stress. Int J Comp Psychol 20:89–95.

Rome L.C., E.D. Stevens, and H.B. John-Alder. 1992. Temperature and thermal acclimation and physiological function. Pp. 183–205 in M.E. Feder, W.W. Burggren, eds. Environmental Physiology of the Amphibia. University of Chicago Press: London, UK,

Ruszczyk A., M. Forlenza, M. Joerink, C.M.S. Ribeiro, P. Jurecka, and G.F. Wiegertjes. 2008. *Trypanoplasma borreli* cysteine proteinase activities support a conservation of function with respect to digestion of host proteins in common carp. Dev Comp Immunol 32:1348–1361.

Salzet M., A. Capron, and G.B. Stefano. 2000. Molecular crosstalk in host-parasite relationships: Schistosome- and leech-host interactions. PT 16:536–540.

Sargent R.C. 1992. Ecology of filial cannibalism in fish: theoretical perspectives. Pp. 38–62 in M. A. Elgar and B. J. Crespi, eds. Cannibalism: ecology and evolution among diverse taxa. Oxford University Press, Oxford, UK

Settle R.A., J.T. Briggler, and A. Mathis. 2018. A quantitative field study of paternal care in Ozark hellbenders, North America's giant salamanders. J Ethol 36:235–242.

Siqueira-Neto J.L., A. Debnath, L.I. McCall, J.A. Bernatchez, M. Ndao, S.L Reed, and P.J. Rosenthal. 2018. Cysteine proteases in protozoan parasites. PLoS Negl Trop Dis 12:1–20.

Souza M.J., M.J. Gray, P. Colclough, and D.L. Miller. 2012. Prevalence of infection by *Batrachochytrium dendrobatidis* and *Ranavirus* in eastern hellbenders (*Cryptobranchus alleganiensis alleganiensis*) in eastern Tennessee. J Wildlife Dis 48:560–566.

Stacy N.I, C. Hollinger, J.E. Arnold, C. Cray, H. Pendl, P.J Nelson, and J.W. Harvey. 2022. Left shift and toxic change in heterophils and neutrophils of non-mammalian vertebrates: A comparative review, image atlas, and practical considerations. Vet Clin Pathol 51:18–44.

Stanback M.T. and W.D. Koenig. 1992. Pp. 277–298 in M. A. Elgar and B. J. Crespi, eds. Cannibalism: ecology and evolution in diverse taxa. Oxford University Press, Oxford.

Taber C.A., R.F. Wilkinson, and M.S. Topping. 1975. Age and growth of hellbenders in Niangua River, Missouri. Copeia 4:633–639.

Takahashi M.K. and T.K. Pauley. 2010. Resource allocation and life history traits of *Plethodon cinereus* at different elevations. Am Midl Nat 163:87–94.

Taketa F. and M.A. Nickerson. 1973. Hemoglobin of the aquatic salamander, Cryptobranchus. Comp Biochem Physiol 46A: 583–591.

Taylor B., D. Skelly, L.K. Demarchis, M.D. Slade, D. Galusha, and P.M. Rabinowitz. 2005. Proximity to Pollution Sources and Risk of Amphibian Limb Malformation. Environ Health Perspect 113:1497–1501.

Tizard I.R., W.L. Holmes, D.A. York, and A. Mellors. 1977. The generation and identification of the hemolysin of *Trypanosoma congolense*. Experientia 33:901–902.

Tominaga A., K.J. Irwin, M.J. Freake, K. Suzuki, and K. Goka. 2013. *Batrachochytrium dendrobatidis* haplotypes on the hellbender *Cryptobranchus alleganiensis* are identical to global strains. Dis Aquat Organ 102:181–186.

Udroiu I. and A. Sgura. 2017. The phylogeny of the spleen. Q Rev Biol 92:411–443.

Ultsch G.R. 2012. Metabolism, gas exchange, and acid-base balance of giant salamanders. Biol Rev 87:583–601.

Unger S., C.M. Bodinof Jachowski, L. Diaz, and L.A. Williams. 2020. Shelter Guarding Behavior of the Eastern Hellbender (*Cryptobranchus alleganiensis alleganiensis*) in North Carolina Streams. Southeast Nat 19:742–758.

United States Fish and Wildlife Service. 2011. Inclusion of the hellbender, including the eastern hellbender and the Ozark hellbender, in appendix III of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). Fed Regist 76: 61978–61978.

United States Fish and Wildlife Service. 2018. Species status assessment report for the eastern hellbender (*Cryptobranchus alleganiensis alleganiensis*). 104 pp.

Vitt L.J., and J.P. Caldwell. 2013. Water Balance and Gas Exchange. Pp. 181–202 in Herpetology: An Introductory Biology of Amphibians and Reptiles, 4th ed. Academic Press, San Diego, CA.

Vitt L.J., J.P. Caldwell, H.M. Wilbur, and D.C. Smith. 1990. Amphibians as harbingers of decay. BioScience 40:418.

Wake D.B. 1991. Declining amphibian populations. Science 253:860.

Weber R.E. and F.B. Jensen. 1988. Functional Adaptations in Hemoglobins from Ectothermic Vertebrates. Ann Rev Physiol 50:161–79

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

Williams R.D., J.E. Gates, C.H. Hocutt, and G.J. Taylor. 1981. The hellbender: a nongame species in need of management. Wildlife Society Bulletin, 9:94–100

Wilkialis J. 1984. The life history of *Haementeria costata* (Fr. Müller) (Glossiphonidae: Hirudinea). Hydrobiologia 109:219–227.

Woo P.T.K. 1994. Flagellate Parasites of Fish. Pp. 1–8 in J.P. Kreier, ed. Parasitic Protozoa, 2nd ed., Vol. VIII. Academic Press, New York, NY.

Woo P.T.K. 2006. Diplomonadida (Phylum Parabasalia) and Kinetoplastea (Phylum Euglenozoa). Pp. 46–114 in P.T.K. Woo ed. Fish Diseases and Disorders, Vol. 1: protozoan and metazoan infections. CABI, Wallingford, UK.

Wood P.J. and P.D. Armitage. 1997. Biological effects of fine sediment in the lotic environment. Environ Manag 21:203–217.

Wright K.M. and B.R. Whitaker. 2001. Amphibian Medicine and Captive Husbandry. Krieger Publishing Company, Malabar, FL, USA.

Yap T.A., M.S. Koo, R.F. Ambrose, D.B. Wake, and V.T. Vredenburg. 2015. Averting a North American biodiversity crisis: a newly described pathogen poses a major threat to salamanders via trade. Science 349:481-482.

Zapata A.G., A. Varas, and M. Torroba. 1992. Seasonal variations in the immune system of lower vertebrates. Immunol Today 13:142–147.

CHAPTER 1: The Influence of Environmental Conditions and Coinfection by Blood-feeding Parasites on Red Blood Cell Physiology of an Ectothermic Host.

Formatted for submission to Ecological and Evolutionary Physiology.

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

Katherine L. Slack[1], Jordy Groffen[1], William A. Hopkins[1]

[1]Department of Fish and Wildlife Conservation, Virginia Tech, Blacksburg, VA, USA, 24061

Corresponding Authors

Katherine L. Slack, 601-497-4813; kslack@vt.edu

Key Words: Ectotherm, hematology, parasite, coinfection, anemia, stress, season, temperature

What is already known: The pathologies associated with vector-borne blood parasites can prove fatal in endothermic species, yet their impact on ectothermic hosts remains grossly understudied, especially in wild populations. Additionally, the pathological response is typically attributed to parasites in the host blood rather than their vectors or simultaneous coinfection of both the parasite and the vector. Since the physiology of ectotherms fluctuates with environmental conditions like temperature, inferring ectothermic responses to parasites from endothermic host responses can be challenging.

What this study adds: Our study provides fundamental insight into seasonal differences in parasite dynamics and blood physiology of wild ectothermic hosts. Further, we found that symptoms of anemia in the ectothermic host was dependent on the attachment of the vector, rather than the blood parasite it transmits. We also highlighted multiple intrinsic and extrinsic factors that influence ectothermic red blood cell physiology which should be considered for monitoring the health of wild populations.

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 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 variation. 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 compared 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 like amphibians, our study provides timely foundational insights into multiple factors that influence their red blood cell physiology.

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

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 Trypanosomes that are vectored to their mammalian and avian hosts by biting arthropods (Tizard et al. 1977; Tuteja 2007; Brun et al. 2010; Perez-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; Hildebrant 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; Gonzalez 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 wild populations (Bricknell 2017). Thus, to elucidate the pathology of blood-feeding parasites on wild ectothermic hosts, we need a better understanding of the 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. 1975; Frangioni and Borgioli 1984) as well as temperature (da Silva et al. 2013), which needs 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+ years (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; Jachowski and Hopkins 2018). Hellbenders in our study system are parasitized by freshwater leeches (*Placobdella appalachiensis*; Hopkins et al. 2014; Hopkins et al. 2016), which serve as the vector for a newly identified species of trypanosome (Hopkins unpublished) which 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, USA the timing of oviposition ranged from August 30th to September 22nd. Following a ~60-day period of embryonic development, larvae hatch from eggs and remain 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, sedimentation, and blood-feeding endo- and ectoparasites would influence red blood cell physiology in hellbenders. We predicted that hellbenders infected with either or both parasites would have lower baseline hematocrit and hemoglobin due to 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 response to acute stress would be consistent with 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 a greater hemoconcentration response to stress, during colder months. Finally, we predicted that baseline hematocrit and hemoglobin concentrations would increase with siltation, one of the primary anthropogenic stressors believed to underlie hellbender population declines (USFWS 2018). We based this prediction on the possibility that silt in shelter cavities used by hellbenders might reduce dissolved oxygen (Carling and McCahon 1987) and/or increase their metabolic demands associated with cavity cleaning and maintenance (O'Brien 2023). 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.

Materials and Methods

Site description and sample collection

Our study was conducted in four stream reaches (each ~100-200 m long) along a ∼13km segment of a stream within the Upper Tennessee River Basin (VA, USA). 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 (Jachowski and Hopkins 2018; Hopkins et al. 2023). Underwater artificial shelters (N = 30 - 35/reach) were deployed up to 10 years prior to 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 are used to monitor population trends, annual reproduction, physiology, and behavior of hellbenders (Button et al. 2020*a,b*; Jachowski et al. 2020; Galligan et al., 2021; Hopkins et al. 2023; O'Brien et al., 2024; Brooks et al., 2024; Case et al., 2024). We monitored the hourly temperature of each stream reach using U24-001 HOBO® Conductivity Data Loggers 0-10,000 µS/cm (Onset Computer Corp, Bourne, MA, USA).

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 [Aug 30 – Sep22], N = 58) and attempted recapture at mid-embryonic development (~Day 30 [Oct 2 – Oct 17], N = 48), larval hatching (~Day 60 [Nov 5 – Nov 21], N = 44), and prior to 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 outgroup of non-nesting male hellbenders. Specifically, we sampled one group of unique non-nesting males at mid-embryonic 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 outgroup also provided the opportunity for a hematological comparison between males without nests and those 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 mins of capture [average time = 2:06 ± 0:06 min:s] hereafter time 0 [T0]) sample of whole blood (collecting an average of ~69µL/100g body mass) from the caudal vein (Hopkins and Durant 2011; Durant et al. 2015; Hopkins et al. 2016; Hopkins et al. 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 minutes 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 due to 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, prior to release 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; PCV). We determined hemoglobin in the field using a handheld hemoglobinometer (HemoCue® Hb 201+) 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® tri-level liquid controls (R&D Systems, Inc., Minneapolis, MN, USA). 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; Jachowski et al. 2024), as well as in other salamander research (Bazar et al. 2010). Alternative methods such as Sahli's hemoglobinometer have also been used for studying hellbenders (Jarrett and Mays 1973) and 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 hrs of sample collection, we centrifuged duplicate capillary tubes for each individual at 5g for 5 mins. We quantified hematocrit levels using a standard hematocrit capillary tube reader (McCormick Scientific, St Louis, MO, USA) 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 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. Slack) was blinded to the identity of each slide and examined fifty random fields of view in both whole blood and buffy coat smears. We used a light microscope at 400X 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 twenty whole blood smears in triplicate. We then calculated the ICC score for polychromasia and trypanosomes (ICC-polychromasia = 0.97; ICC-trypanosomes = 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, a nucleus occupying a greater portion of the cellular body, and more dispersed chromatin (Supplemental Fig. 1). 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 fifty, which was used as the standard denominator to quantify the proportion of polychromatic cells within fifty 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 fifty fields of view for fifteen slides with variable corresponding hematocrit (15-50%) and performed a linear regression ($Y = 3100.2 + 26.647*\text{Hematocrit}$; $R^2 = 0.70$) between these two parameters.

Based on this relationship, we were able to estimate the total number of red blood cells per fifty 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 if metrics differed following exposure to 60 minutes of acute stress. Polychromasia at T0 and T60 were highly correlated ($r = 0.92$) and did not change appreciably due to restraint. Therefore, we used the linear model describing this relationship (Supplemental Fig. 2; $Y = (0.015 + 1.064 * T0)$ 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.

Quantifying Sedimentation

To assess the impact of sedimentation on hematological parameters, we evaluated the amount of silt and organic matter in the artificial shelter using a qualitative scoring system (from 0-4). While in the field, an observer (J. Groffen) scored the state of each artificial shelter based on a predefined rubric (see Supplemental Table 1) at each recapture time from oviposition (day 0) through emergence (day 200). Silt was identified as the fine, powdery sediment, usually dark in color, that readily suspends in water when disturbed. Organic matter consisted of debris such as sticks, leaves, and tree nuts. This estimation technique was verified in a previous study by comparing the scoring from two independent observers and also by comparing this qualitative scoring to quantitative laboratory measurements of silt from sediment samples (O'Brien 2023).

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, sedimentation, and parasitic infection. For the red blood cell response variables, we chose a multivariate approach to reduce the dimensionality of the dataset and 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 components analysis (PCA) based on a correlation matrix to provide a response variable representing hematocrit, hemoglobin, and the 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/mid-embryonic development/hatching/emergence) and 24-hour median temperature (C°) prior to 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-hour median temperature (C°) 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 if temperature still had an effect. The results showed that temperature significantly affected 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 and sedimentation. 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) prior to 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) and sediment scores (silt and organic matter; scores 0-4). We used a Kruskal-Wallis test to assess how sediment scores varied across the paternal care period and a Dunn's test with a Bonferroni adjustment for post-hoc analysis. 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, capture restraint and body size, parasite infection intensity and temperature, silt scores and temperature, and organic matter scores and temperature.

We used the lme4 package for analyzing the principal components (PC1 and PC2) which were included as response variables with a normal distribution and 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.

Lastly, we compared male hellbenders with nests and the outgroup of non-nesting 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 only captures that occurred at mid-embryonic development and hatching, then performed a separate PCA which included both nesting males and non-nesting males from the outgroup. We used the resulting PC scores as the response variable. For the predictors, we included an interaction between reproductive status (nesting /non-nesting) 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 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 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; non-nesting outgroup, N = 15) in the years 2020 and 2021. Over the two years of the study which included multiple recaptures across the paternal care period, we obtained data from 190 capture events (nesting, N = 175; non-nesting outgroup, 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 concentration (T0, N = 190; T60, N = 189) as well as to create blood smears for microscopy and 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-hour median temperature prior to capture varied across season, ranging from 6.91 to 19.24 (C°). Temperatures were highest at oviposition, followed by a decline during mid-embryonic development, reaching their lowest levels at hatching and overwintering, and rising again during larval emergence in the spring (Fig. 1). Sediment scores also differed across season (Silt: Kruskal-Wallis $X^2 = 47.52$, d.f. = 3, $p < 0.001$; Organic Matter: Kruskal-Wallis $X^2 = 29.37$, d.f. = 3, $p < 0.001$). Silt scores were lowest at oviposition and increased as the season progressed. Organic matter scores were lowest at oviposition and emergence, but highest at mid-embryonic development and hatching. Results from the Dunn's test are included in Supplemental Figure 4, demonstrating statistical differences in sediment scores across the parental care period.

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%-89.6%, while coinfection decreased following mid-embryonic development (Fig. 2). Among infected individuals, the intensity of trypanosome infection varied significantly seasonally; it was highest at oviposition and similar at mid-embryonic development, but it then decreased at hatching and decreased further at emergence in the spring (Fig. 3A; Table 1; Supplemental Table 2). The intensity of leech infection was significantly greater at oviposition compared to the rest of the parental care period (Fig. 3B; Table 1; Supplemental Table 2). The number of leech bites within bitten individuals was significantly lower during spring emergence compared to the rest of the parental care period (Fig. 3C; Table 1; Supplemental Table 2). Parasite prevalence, the intensity of infection, and average red blood cell parameters within each sampling period are reported in Table 1.

The principal component analysis 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 was 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, hematocrit had a moderate influence, while hemoglobin had a weak influence on the principal component.

Among the candidate models for PC1, the two top-ranking models representing the effects of stress, temperature, total length, and parasitic infection accumulated all of the AICc weight (Table 3). The top-ranked model had an AICc weight of 0.93, while the second-ranked model had a weight of 0.07. The delta AICc 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 (stress: $\beta = 1.733$, SE = 0.355, t = 4.880, $p < 0.001$) and with temperature (temperature: $\beta = 0.266$, SE = 0.019, t = 13.688, $p < 0.001$; Fig. 5), but the interaction between stress and temperature was not statistically conclusive (stress X temperature: $\beta = -0.04$, SE = 0.025, t = -1.682, $p = 0.094$). PC1 decreased as total body length increased ($\beta = -0.094$, SE = 0.029, t = -3.254, $p = 0.002$) (Fig. 6) and as leech bites increased, but the latter was not statistically conclusive (leech bites: $\beta = -0.507$, SE = 0.308, t = -1.650, $p = 0.099$) regardless of the temperature (leech bites X temperature: $\beta = -0.001$, SE = 0.020, t = -0.040, $p = 0.968$). PC1 was negatively influenced by the number of leeches attached (leeches: $\beta = -3.249$, SE = 0.569, t = -5.708, $p < 0.001$), but the effect of leech infection was dependent on temperature (leeches X temperature: $\beta = 0.189$, 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. 5) and incorporated it into a 3D scatterplot (Fig. 7). Both figures (Supplemental Fig. 5 and Fig.7) demonstrate how PC1 values decrease with 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 delta AIC greater than 2, confirming it out-competed the lower-ranking candidate models (Table 4). PC2 increased in response to stress (stress: $\beta = 1.576$, SE = 0.271, t = 5.807, $p < 0.001$), and the magnitude of the stress response decreased as temperature increased (stress X temperature: $\beta = -0.049$, SE = 0.019, t = -2.597, p = 0.009) (Supplemental Fig. 6). Infection status influenced PC2; specifically, individuals with coinfection had much higher PC2 scores (coinfected: $\beta = 2.159$, SE = 0.486, t = 4.439, $p < 0.001$) compared to those with only trypanosomes present (only trypanosomes present: $\beta = 0.260$, SE = 0.486, t = 0.555, p = 0.580). As temperature decreased, PC2 increased in coinfected individuals (coinfected X temperature: $\beta = -0.140$, SE = 0.031, t = -4.546, $p < 0.001$). However, there was no statistical relationship between PC2 and individuals only infected with trypanosomes, even after accounting for temperature (only trypanosomes present X temperature: $\beta = -0.012$, SE = 0.030, t = -0.410, p = 0.682). Moreover, PC2 had strong positive loadings for the proportion of polychromasia (Table 2, Fig. 8A). The trends in Figure 8A 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 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 analysis that included both nesting males and the non-nesting outgroup at mid-embryonic development and hatching, the resulting loadings and proportion of variance for each principal component barely changed compared to the initial PCA that only included nesting males (Supplemental Table 3). 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 4. The effects of stress, temperature, and total length are similar to the effects previously stated regarding PC1. In contrast, the PC1 with the outgroup 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 outgroup similar to PC2 excluding the outgroup. Most importantly, individuals in the non-nesting outgroup had comparable polychromasia to the nesting males at mid-embryonic development and at hatching (Fig. 8A and B). Because the non-nesting 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 addition, nesting status did not influence red blood cell metrics for either PC1 or PC2, even when accounting for temperature (see Supplemental Table 4).

Discussion

Blood-feeding parasites have significant impacts on host physiology, but their effects on ectothermic hosts remain understudied compared to endotherms. Likewise, much less is known about the effects of blood-feeding vectors on their hosts compared to the deep knowledge on the blood parasites that they transmit. 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.

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 uninfected individuals and individuals only infected with trypanosomes. This increase in polychromatic cells is indicative of increased erythropoietic activity and a regenerative response to anemia (Fallon et al. 2017; Fallon et al. 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 compared to 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 singly infected 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 (Gonzolez et al. 2021) and freshwater fish (Laprirova 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 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), rather than biting vectors like leeches. In contrast to this expectation, the proportion of polychromasia was much higher in coinfected individuals, while uninfected individuals and those only infected with trypanosomes did not statistically differ (Fig. 8A). 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; Woo 2006) and amphibian hosts (Omonona and Ekpenko 2011). 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. 2023).

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. (2023) 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 multi-parasite 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. 8B). 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. 3B), supporting our previous work on leech-feeding ecology (Bodinof Jachowski et al. 2023). The intensity of trypanosome infection peaked at oviposition and mid-embryonic development, but it then decreased at hatching and decreased further at emergence (Fig. 3A). Leeches exhibit brooding and parental care of their offspring before leading them to their first blood meal (Blumenthal and Hopkins, unpublished) during the time when the hosts have their seasonally highest 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 3-5 times, in order to survive over winter (Wilkialis 1984; Moser et al. 2009) which means they not only repeatedly consume blood meals from the same host, but they are potentially reinfecting 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 hematocrit and hemoglobin was also most pronounced with peak leech attachment during oviposition (Fig. 3B). 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 through hatching (Fig. 3C; Fig. 8A). The timing of peak infection, as well as the lag time between 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, prior to when peak leech infection occurs.

Contrary to expectations based on the literature, hematocrit and hemoglobin decreased as total length increased (Fig. 6). Our findings contrast 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. (2023) 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 the bulk of existing literature on aquatic vertebrates to include fish and amphibians (Murachi 1959; Clark et al. 1979; Joshi and Tandon 1977; Davis 2008; Maerz et al. 2009; Anthony et al. 2010; Baghizadeh and Khara 2014; Fazio et al. 2017; Liu et al. 2023; but see Zhang et al. 2018). However, research in male-brooding 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 Ridgeway 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 compared to larger males. Across the 8-month 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 to care for by small males. Thus, we speculate that 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 future research explore the influence of body size on paternal care behaviors and red blood cell metrics in male hellbenders to determine if 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 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 cutaneous 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*,*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), when hellbenders are known to be most active (Humphries and Pauley 2000), and when available dissolved oxygen is at its lowest (Li and Liu 2019). Temperatures declined over mid-embryonic 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 was mobilized in response to stress. Variation in the magnitude of the hemoconcentration response has been observed in 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.

Contrary to our predictions, we did not identify a relationship between sedimentation in the nest cavity and red blood cell metrics. Sedimentation is considered to be the most impactful factor threatening eastern hellbenders (USFWS 2018). Excessive sedimentation is thought to suffocate eggs (Nickerson and Mays 1973), fill interstitial spaces (Wood and Armitage 1997), and bury shelter and nest rocks (Hopkins and Durant 2011; Brooks et al. 2024), requiring greater parental investment to maintain the nests. We had predicted that high amounts of sediment would increase energetic costs and respiratory demands associated with nest maintenance behaviors, resulting in elevated hematocrit and hemoglobin concentrations, but we found no such relationship. Because we focused our work in a relatively healthy stream with a robust hellbender population, it is possible that this lack of relationship stems from us using an inadequate range of microhabitat conditions to detect an effect. However, it is also possible that hellbenders don't modulate their care behaviors (and concomitant changes in physiology) in response to sedimentation, as was recently suggested by O'Brien (2023). We recommend future research that evaluates hellbender paternal care behaviors in more degraded stream reaches to fully understand the implications of sedimentation on their physiology.

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 co-infections.

Acknowledgments

The authors thank the Virginia Department of Wildlife Resources (EP2443089 and DWR-403-2023-0011) and the National Science Foundation (IOS-1755055) for funding our research. We also thank the Global Change Center at Virginia Tech and the Fralin Life Sciences Institute for maintaining the research infrastructure that made this project possible. We appreciate Dr. Ignacio Moore and Dr. Luis Escobar for their guidance and support, as well as their insightful comments that improved the manuscript. We are particularly grateful for J.D. Kleopfer's support of our research and for his efforts to protect hellbenders and other herpetofauna in Virginia. We appreciate the many individuals who assisted us in the field and the lab, including R. O'Brien, E. Fralin, K. Hahler, A. Patel, A. Holloway, J. Hargett, and E. Davis. All research was conducted under Virginia Department of Wildlife Resources collection permits and complied with Virginia Tech Institutional Animal Care and Use protocols 18-186, and 19-147.

Data Accessibility

The data included in this article will be shared on reasonable request to the corresponding author.

References

Anthony O., I. Kechukwu, and C.L. Obinnaya. 2010. Haematological profile of the African Lungfish, *Protopterus annectens* (Owen) of Anambra River, Nigeria. J Am Sci 6:123–130.

Baghizadeh E. and H. Khara. 2015. Variability in hematology and plasma indices of common carp, *Cyprinus carpio*, associated with age, sex, and hormonal treatment. Iran J Fish Sci 14: 99–111

Bazar M.A., M.J. Quinn, K. Mozzachio, J.A. Bleiler, C.R. Archer, C.T. Phillips, and M.S. Johnson. 2009. Toxicological responses of red-backed salamanders (*Plethodon cinereus*) to soil exposures of copper. Arch Environ Contam Toxicol 57: 116–122.

Biron M. and T.J. Benfey. 1994. Cortisol, glucose, and hematocrit changes during acute stress, cohort sampling, and the diel cycle in diploid and triploid brook trout (*Salvelinus fontinalis Mitchill*). Fish Physiol and Biochem 13:153–160.

Birchard G.F. 1997. Optimal hematocrit. Theory, regulation, and implications. Am Zool 37:65–72

Bishop S.C. 1941. The Salamanders of New York. New York State Museum Bulletin. 324.

Bodinof Jachowski C.M., V. Alaasam, A. Blumenthal, A. Davis, and W.A. Hopkins. 2023. The habitat quality paradox: Loss of riparian forest cover leads to decreased risk of parasitism and improved body condition in an imperiled amphibian. Conserv Physiol. in press.

Bricknell I. 2017. Types of pathogens in fish, waterborne diseases. Pp. 53–80 in Fish Diseases Prevention and Control Strategies. Academic Press.

Bridges D.W., J.J. Cech, and D.N. Pedro. 1976. Seasonal Hematological Changes in Winter Flounder, *Pseudopleuronectes americanus*. Trans Am Fish Soc 105:596–600.

Brill R., P. Bushnell, S. Schroff, R. Seifert, and M. Galvin. 2008. Effects of anaerobic exercise accompanying catch-and-release fishing on blood–oxygen affinity of the sandbar shark (*Carcharhinus plumbeus*, Nardo). J Exp Mar Biol Ecol. 354:132–143.

Brooks G.C., W.A. Hopkins, and H.K. Kindsvater. 2024. Concurrent threats and extinction risk in a long-lived, highly fecund vertebrate with parental care. Ecol Appl 34: e2946.

Brun R., J. Blum, F. Chappuis, and C. Burri. 2010. Human African trypanosomiasis. Lancet 375:148–59.

Budischak S.A., A.E. Wiria, F. Hamid, L.J. Wammes, M.M. Kaisar, L. van Lieshout, E. Sartono, T. Supali, M. Yazdanbakhsh, and A. Graham. 2018. Competing for blood: the ecology of parasite resource competition in human malaria–helminth co-infections. Ecol Lett 21:536–545.

Burnham K.P. and D. R. Anderson. 2002. Model Selection and Multimodel Interface, A Practical Information-Theoretic Approach, 2nd ed. Springer-Verlag, New York

Button S.T., J.J. Hallagan, C.M.B. Jachowski, B.F. Case, J. Groffen, and W.A. Hopkins. 2020a. Weathering the storm: Improving the availability and stability of artificial shelters for hellbender salamanders. River Res Appl 36:1944–1953.

Button S.T., C.M.B. Jachowski, B.F. Case, J. Groffen, and W.A. Hopkins. 2020b. The influence of multiscale habitat variables and population density on artificial shelter use by hellbenders (*Cryptobranchus alleganiensis*). Herpetologica 76:355–365.

Caljon G., J. Van Den Abbeele, B. Stijlemans, M. Coosemans, P. De Baetselier, and S. Magez. 2006. Tsetse Fly Saliva Accelerates the Onset of *Trypanosoma brucei* Infection in a Mouse Model Associated with a Reduced Host Inflammatory Response. Infect Immun 74:6324-6330.

Carling P.A. and C.P. McCahon. 1987. Natural Siltation of Brown Trout (*Salmo trutta L*.) Spawning Gravels During Low-Flow Conditions. Pp. 229–244 in J.F. Craig, J.B. Kemper, eds. Regulated Streams. Springer, Boston, MA.

Case B.F., J. Groffen, T.M. Galligan, C.M. Bodinof Jachowski, J.J. Hallagan, S.B. Hildreth, V. Alaasam, W.K. Ray, R.F. Helm, and W.A. Hopkins. 2024. Androgen and glucocorticoid profiles throughout extended uniparental paternal care in the eastern hellbender salamander (*Cryptobranchus a. alleganiensis*). Gen Comp Endocrinol. 355:114547

Clark S., D.H. Whitmore, and R.F. McMahon. 1979. Considerations of blood parameters of largemouth bass, *Micropterus salmoides*. J Fish Biol 14:147–158.

da Silva G.S.F., M.L. Glass, and L.G.S. Branco. 2013. Temperature and respiratory function in ectothermic vertebrates. J Therm Biol 38:55–63.

Danylchuk A.J. and M.G. Fox. 1994. Age and size-dependent variation in the seasonal timing and probability of reproduction among mature female pumpkinseed, *Lepomis gibbosus*. Environ Biol Fish 39:119–127.

Davies A.J. and M.R.L. Johnston. 2000. The biology of some intraerythrocytic parasites of fishes, amphibians, and reptiles. Adv Parasitol 45:1–107.

Davis A.K. 2008. Ontogenetic changes in erythrocyte morphology in larval mole salamanders, *Ambystoma talpoideum*, measured with image analysis. Comp Clin Path 17:23–28

Davis A.K. and W.A. Hopkin. 2013. Widespread trypanosome infections in a population of eastern hellbenders (*Cryptobranchus alleganiensis alleganiensis*) in Virginia, USA. Parasitol Res 112:453–456.

Densmore C. L. and D.E. Green. 2007. Diseases of Amphibians. ILAR J. 48:235-254

Descamps S., J. Bêty, O.P. Love, and H.G. Gilchrist. 2011. Individual optimization of reproduction in a long-lived migratory bird: a test of the condition-dependent model of laying date and clutch size. Funct Ecol 25:671–681.

Desser S.S., M.E. Siddall, and J.R. Barta. 1990. Ultrastructural observations on the developmental stages of *Lankesterella minima* (Apicomplexa) in experimentally infected *Rana catesbeiana* tadpoles. J Parasitol 76:97–103.

DuRant S.E., W.A. Hopkins, A.K. Davis, and L.M. Romero. 2015. Evidence of ectoparasite-induced endocrine disruption in an imperiled giant salamander, the eastern hellbender (*Cryptobranchus alleganiensis*). J Exp Biol 218:2297–2304.

Edwards M.J., B. Canon, J. Albertson, and R.H. Bigley. 1971. Mean Red Cell Age as a Determinant of Blood Oxygen Affinity. Nature 230:583–584.

Fallon J.A., E.P. Smith, N. Schoch, J.D. Paruk, E.A. Adams, D.C. Evers, P.G.R. Jodice, C. Perkins, S. Schulte, and W.A. Hopkins. 2017. Hematological indices of injury to lightly oiled birds from the Deepwater Horizon oil spill. Environ Toxicol Chem 37:451–461.

Fallon J.A., E.P. Smith, N. Schoch, J.D. Paruk, E.A. Adams, D.C. Evers, P.G.R. Jodice, M. Perkins, D.E. Meattey, and W.A. Hopkins. 2020. Ultraviolet-assisted oiling assessment improves detection of oiled birds experiencing clinical signs of hemolytic anemia after exposure to the Deepwater Horizon oil spill. Ecotoxicology 29:1399–1408.

Fazio F., C. Saoca, I. Vazzana, and G. Piccione. 2017. Influence of body size on blood hemogram in rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792). Vet Med Open J 2:91–94.

Frangioni G. and G. Borgioli. 1984. Seasonal Erythrocyte Count Variations in Laboratory Raised Newts. Ital J Zool (Modena) 18:301–306.

Frangioni G. and G. Borgioli. 1989. Relationships Between Spleen and Respiration in the Newt. J Exp Zool 252:118–125.

Frangioni G. and G. Borgioli. 1993a. Hematological changes in the newt during the process of splenic decongestion in reply to hypoxia. Ital J Zool (Modena) 60:267–270.

Frangioni G. and G. Borgioli. 1993b. Splenic respiratory compensation and blood volume in the newt. J Zool Lond 229:505–514.

Galligan, T.M., R.F. Helm, B.F. Case, C.M.B. Jachowski, C.L. Frazier, V. Alaasam, and W.A. Hopkins. 2021. Pre-breeding androgen and glucocorticoid profiles in the eastern hellbender salamander (*Cryptobranchus alleganiensis alleganiensis*). Gen Comp Endocrinol 313:p.113899.

González L.P., C.M. Vargas-León, G.A. Fuentes-Rodríguez, M.L. Calderón-Espinosa, and N.E. Matta. 2021. Do blood parasites increase immature erythrocytes and mitosis in amphibians? Rev Biol Trop 69:615–625.

Greenwood B.M., D.A. Fidock, D.E. Kyle, S.H. Kappe, P.L. Alonso, F.H. Collins, and P.E. Duffy. 2008. Malaria: progress, perils, and prospects for eradication. J Clin Invest 118:1266–76.

Guimond R.W. and V.H. Hutchison. 1973. Aquatic respiration: an unusual strategy in the hellbender *Crytobranchus alleganiensis alleganiensis* (Daudin). Science 182:1263-1265.

Hildebrandt J.P. and S. Lemke. 2011. Small bite, large impact–saliva and salivary molecules in the medicinal leech, *Hirudo medicinalis*. Naturwissenschaften 98:995–1008.

Hopkins W.A., B.F. Case, J. Groffen, G.C. Brooks, C.M.B. Jachowski, S.T. Button, J.J. Hallagan, R.S.M. O'Brien, and H.K. Kindsvater. 2023. Filial Cannibalism Leads to Chronic Nest Failure of Eastern Hellbender Salamanders (*Cryptobranchus alleganiensis*). Am Nat 202:92–106.

Hopkins W.A. and S.E. DuRant. 2011. Innate immunity and stress physiology of Eastern Hellbenders (*Cryptobranchus alleganiensis*) from two stream reaches with differing habitat quality. Gen Comp Endocrinol 174:107-115.

Hopkins W.A., J.A. Fallon, M.L. Beck, B.H. Coe, and C.M.B. Jachowski. 2016. Haematological and immunological characteristics of eastern hellbenders (*Cryptobranchus alleganiensis alleganiensis*) infected and co-infected with endo- and ectoparasites. Conserv Physiol 4:cow002.

Hopkins W.A., W.E. Moser, D.W. Garst, D.J. Richardson, C.I. Hammond, and E.A. Lazo-Wasem. 2014. Morphological and molecular characterization of a new species of leech (Glossiphoniidae, Hirundinida): implications for the health of its imperiled amphibian host (*Cryptobranchus alleganiensis*). Zookeys 378:83–101.

Hopkins W.A., S.E. DuRant, M.L. Beck, W.K. Ray, R.F. Helm, and L.M. Romero. 2020. Cortisol is the predominant glucocorticoid in the giant paedomorphic hellbender salamander (*Cryptobranchus alleganiensis*). Gen Comp Endocrinol, 285:e113267.

Humphries W.J. and T.K. Pauley. 2000. Seasonal changes in nocturnal activity of the hellbender, *Cryptobranchus alleganiensis*, in West Virginia. J Herpetol 34:604-607.

Jachowski C.M.B., B.E. Ross, and W.A. Hopkins. 2020. Evaluating artificial shelter arrays as a minimally invasive monitoring tool for the hellbender *Cryptobranchus alleganiensis*. Endanger Species Res 41:167–181

Jachowski C.M.B. and W.A. Hopkins. 2018. Loss of catchment-wide riparian forest cover is associated with reduced recruitment in a long-lived amphibian. Biol Conserv 220:215–227.

Jerrett D.P. and C.E. Mays. 1973. Comparative hematology of the hellbender, *Cryptobranchus alleganiensis* in Missouri. Copeia 1973:331–337.

Johnstone C.P., A. Lill, and R.D. Reina. 2017. Use of erythrocyte indicators of health and condition in vertebrate ecophysiology: a review and appraisal. Biol. Rev. 92:150-168

Joshi B.D. and R.S. Tandon. 1977. Seasonal variations in the haematologic values of freshwater fishes. *I. Heteropneustes fossilis* and *Mystus vittatus*. Comp Physiol Ecol 2:22–26.

Kaufman W.R. 1989. Tick-host interaction: a synthesis of current concepts. PT 5:47–56.

Kose A., S. Zengin, B. Kose, N. Gunay, C. Yildirim, H. Kılınc, and I. Togun. 2008. Leech bites: massive bleeding, coagulation profile disorders, and severe anemia. Am J Emerg Med 26:1067.e3–1067.e6.

Lainson R. and I. Paperna. 1995. Light and electron microscopic studies of *Lankesterella petiti* n. sp. (Apicomplexa: Lankesterellidae) infecting *Bufo marinus* (Amphibia: Anura) in Pará, North Brazil. Parasite 2:307–313.

Lapirova T.B. and E.A. Zabotkina. 2018. Effect of trypanosomiasis on hematologic characteristics of bream (*Abramis brama*). Regul Mech Biosyst 9:309–314.

Laroche R.A.S., K.L. Weinersmith, M.L. Davis, L. Angeloni, J.R. Baylis, S.P. Newman, S.P. Egan, and D.D. Wiegmann. 2023. Size-associated energetic constraints on the seasonal onset of reproduction in a species with indeterminate growth. Oikos 2023:e09739.

Latimer K.S., E.A. Mahaffey, and K.W. Prasse. 2003. Duncan & Prasse’s Veterinary Laboratory Medicine: Clinical Pathology. Blackwell Publishing, Ames, IA.

Li D. and S. Liu. 2019. Detection of Industrial Water Quality. Pp. 329–349 Li D. and S. Liu, eds. Water Quality Monitoring and Management. Academic Press.

Liu X., Z. You, W. Luo, J. Xiong, and G. Wang. 2023. Blood cells and hematological parameters of Chiala Mountain Salamander, *Batrachuperus karlschmidti* (Urodela, Hynobiidae). PeerJ 11:e15446.

Maerz J., J. Milanovich, A. Davis, and J. DeVore. 2009. An investigation of factors influencing erythrocyte morphology of red-backed salamanders (*Plethodon cinereus*). Anim Biol 59:201–209.

Makowsky R., L.A. Wilson, and T.K. Pauley. 2010. Sexual dimorphism in the Eastern Hellbender (*Cryptobranchus alleganiensis alleganiensis*). Herpetol Conserv Biol 5: 44–48.

Martínez-García G., R.M. Santamaría-Espinosa, J.J. Lira-Amaya, and J.V. Figueroa. 2021. Challenges in Tick-Borne Pathogen Detection: The Case for *Babesia spp*. Identification in the Tick Vector. Pathogens 10:92.

Moser W.E., F.R. Govedich, and D.J. Klemm. 2009. Annelida, Hirudinida (leeches). Pp. 116–123 in G.E. Likens, ed. Encyclopedia of Inland Waters, vol. 2. Elsevier Press, Oxford, UK.

Murachi S. 1959. Haemoglobin contents, erythrocyte sedimentation rate, and haematocrit of the blood in young of the carp, *Cyprinus carpio*. J Fac Fish Anim Husb Hiroshima Univ 2:241–247.

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

Nuttall P. 1998. Displaced tick-parasite interactions at the host interface. Parasitology 116:S65–S72.

O'Brien R.S.M., J. Groffen, A. Dayer, and W.A. Hopkins. 2024. Pre-breeding, Courtship, and Mating Behaviors of Wild Eastern Hellbenders (*Cryptobranchus alleganiensis alleganiensis*). Herpetologica. 80.

Omonona A.O. and V. Ekpenko. 2011. Haematology and prevalence of blood parasites of the common frog (*Rana temporaria*) in the tropical environment. J Vet Med Anim Health 3:14–20.

Pérez-Molina J.A. and I. Molina. 2018. Chagas Disease. The Lancet 391:82–94.

Petranka J.W. 1998. Salamanders of the United States and Canada. Smithsonian Institution Press, Washington, D.C., USA 587p.

Pishchany G. and E.P. Skaar. 2012. Taste for Blood: Hemoglobin as a Nutrient Source for Pathogens. PLoS Pathog 8:e1002535.

Pugh M.W., M. Hutchins, M. Madritch, L. Siefferman, and M.M. Gangloff. 2016. Land-use and local physical and chemical habitat parameters predict site occupancy by hellbender salamanders. Hydrobiologia 770:105–116.

Pyron R.A. and J.J. Wiens. 2011. A large-scale phylogeny of Amphibia including over 2800 species, and a revised classification of extant frogs, salamanders, and caecilians. Mol Phylogen Evol 61:543–583.

R Core Team 2022. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna. http://www.R-project.org/.

Rajesh M, Rehana S. 2022. Impact of climate change on river water temperature and dissolved oxygen: Indian riverine thermal regimes. Sci Rep 12:1–12.

Nature Publishing Group UK.Ridgway M.S., B.J. Shuter, and E.E. Post. 1991. The relative influence of body size and territorial behaviour on nesting asynchrony in male smallmouth bass, *Micropterus dolomieui* (Pisces: Centrarchidae). J Anim Ecol 60:665–681.

Romero M.L. and L.K. Butler. 2007. Endocrinology of stress. Int J Comp Psychol 20 :89–95.

Salzet M., A. Capron, and G.B. Stefano. 2000. Molecular crosstalk in host-parasite relationships: Schistosome- and leech-host interactions. PT 16:536–540.

Schielzeth H. 2010. Simple means to improve the interpretability of regression coefficients. Methods Ecol Evol 1:103–113.

Schmunis G.A. and Z.E. Yadon. 2010. Chagas disease: A Latin American health problem becoming a world health problem. Acta Tropica 115:14–21.

Settle R.A., J.T. Briggler, and A. Mathis. 2018. A quantitative field study of paternal care in Ozark hellbenders, North America’s giant salamanders. J Ethol 36:235–242.

Siqueira-Neto J.L., A. Debnath, L.I. McCall, J.A. Bernatchez, M. Ndao, S.L Reed, and P.J. Rosenthal. 2018. Cysteine proteases in protozoan parasites. PLoS Negl Trop Dis 12:1–20.

Sokal R.R. and F.J. Rohlf. 1995. Biometry: The Principles and Practice of Statistics in Biological Research. 3rd ed. W.H. Freeman, New York, NY 887 pp.

Suski C.D. and M.S. Ridgway. 2007. Climate and body size influence nest survival in a fish with parental care. J Anim Ecol 76:730–739.

Taber C. A., R.F. Wilkinson, and M.S. Topping. 1975. Age and growth of hellbenders in Niangua river, Missouri. Copeia 4:633–639.

Tejedo M. 1992. Effects of body size and timing of reproduction on reproductive success in female natterjack toads (*Bufo calamita*). J Zool 228:545–555.

Tizard I.R., W.L. Holmes, D.A. York, and A. Mellors. 1977. The generation and identification of the hemolysin of *Trypanosoma congolense*. Experientia 33:901–902.

Tuteja R. 2007. Malaria−an overview. FEBS J 274:4670–4679.

Unger S., C.M. Bodinof Jachowski, L. Diaz, and L.A. Williams. 2020. Shelter Guarding Behavior of the Eastern Hellbender (*Cryptobranchus alleganiensis alleganiensis*) in North Carolina Streams. Southeast Nat 19:742–758.

USFWS. 2018. Species status assessment report for the Eastern Hellbender (*Cryptobranchus alleganiensis alleganiensis*). 104 pp.

Velguth K.E., M.E Payton, and J.P. Hoover. 2010. Relationship of hemoglobin concentration to packed cell volume in avian blood samples. J Avian Med Surg 24:115–121.

Wilkialis J. 1984. The life history of *Haementeria costata* (Fr. Müller) (Glossiphonidae: Hirudinea). Hydrobiologia 109:219–227.

Witeska M. 2015. Review: Anemia in teleost fish. Bull Eur Ass Fish Pathol 35:148-160

Woo P.T.K. 1994. Flagellate Parasites of Fish. Pp. 1–8 in J.P. Kreier, ed. Parasitic Protozoa, 2nd ed., Vol. VIII. Academic Press, New York, NY.

Woo P.T.K. 2006. Diplomonadida (Phylum Parabasalia) and Kinetoplastea (Phylum Euglenozoa). Pp. 46–114 in P.T.K. Woo ed. Fish Diseases and Disorders, Vol. 1: protozoan and metazoan infections. CABI, Wallingford, UK.

Wood P.J. and P.D. Armitage. 1997. Biological effects of fine sediment in the lotic environment. Environ Manag 21:203–217.

Zhang Y.N., J.L. Xiong, Q.Q. Liu, Y. Min, J.P. Gou, and J.R. Li. 2018. Blood cell composition and hemoglobin concentration in *Batrachuperus londongensis*. Chin J Zool 53:75–81.

Tables

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, USA. Also displayed are parameter values from non-nesting males (outgroup) who were only sampled once during the study.

	Sampling day					
Parameter	**Oviposition**	**Mid-Embryonic Development**		**Hatching**		**Emergence**
	N=58	N=48	N=8 (outgroup)	N=44	N=7 (outgroup)	N=25
T0 Hct %	38.1 ± 0.94	36.1 ± 0.99	35.6 ± 2.02	27.0 ± 1.10	22.8 ± 1.88	33.6 ± 1.70
T60 Hct %	48.3 ± 0.69	44.3 ± 0.88	47.9 ± 1.07	41.1 ± 0.97	33.6 ± 1.40	44.0 ± 1.59
T0 Hb (g/dL)	9.9 ± 0.25	8.7 ± 0.26	9.0 ± 0.41	6.4 ± 0.27	5.1 ± 0.47	8.0 ± 0.42
T60 Hb (g/dL)	12.0 ± 0.21	10.3 ± 0.26	10.5 ± 0.30	9.1 ± 0.21	7.6 ± 0.36	9.6 ± 0.45
T0 MCHC (g/dL)	26.0 ± 0.27	24.1 ± 0.28	25.4 ± 0.72	23.9 ± 0.44	22.2 ± 0.67	23.9 ± 0.61
T60 MCHC (g/dL)	24.8 ± 0.28	23.3 ± 0.33	21.9 ± 0.43	22.3 ± 0.36	22.6 ± 0.90	21.8 ± 0.45
Polychromasia (%)	1.6 ± 0.24	5.4 ± 0.93	1.0 ± 0.35	6.7 ± 0.91	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 ± 0.22	5.5 ±4.50	2.2 ± 0.44	2.2 ± 0.490	1.7 ± 0.42
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

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. Abbreviations: T0, baseline sample; T60, sample following 60 minutes of acute stress; Hct, hematocrit (also referred to as packed cell volume); Hb, blood hemoglobin concentration.

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

Parameters	**PC1**	**PC2**	**PC3**
Hemoglobin	0.659	0.185	0.729
Hematocrit	0.626	0.402	-0.669
Polychromasia	-0.417	0.897	0.149
Eigenvalue	2.148	0.777	0.074
Proportion of Variance	0.716	0.259	0.025
Cumulative Variance	0.716	0.975	1.000

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

Linear Mixed Effects Models	K	AICc	Delta AICc	AICc Weight	Cumulative Weight
PC1~Time*Temp + TLcm + Leeches*Temp + Bites*Temp	11	879.83	0	0.93	0.92
PC1~Time*Temp + TLcm + Leeches*Temp + Bites*Temp + Tryps	12	884.89	5.06	0.07	1.00
PC1~Time*Temp + TLcm + Infection*Temp	11	910.71	30.88	0	1.00
PC1~Time*Temp + TLcm	7	917.57	37.74	0	1.00
PC1~Time*Temp + TLcm + Leeches*Temp	9	917.97	38.14	0	1.00
PC1~Time*Temp + Leeches*Temp	8	918.63	38.80	0	1.00
PC1~Time*Temp	6	918.82	38.99	0	1.00
PC1~Time*Temp + TLcm + Tryps	8	921.80	41.97	0	1.00
PC1~Time*Temp + Leeches*Temp + Tryps	9	923.58	43.75	0	1.00
PC1~Time*Temp + TLcm + Organic*Temp	15	935.76	55.93	0	1.00
PC1~Time*Temp + TLcm + Silt*Temp	15	936.78	56.96	0	1.00
PC1~Time*Temp + TLcm + Organic*Temp + Silt*Temp	23	958.74	78.91	0	1.00
PC1~Time*TLcm	6	1084.09	204.26	0	1.00
PC1~Time	4	1090.33	210.50	0	1.00
PC1~1	3	1173.50	293.67	0	1.00

The response variable (PC1) represents the first principal component scores after performing the principal component analysis on hematocrit, hemoglobin, and polychromasia. The predictor variables included in the model represent exposure to acute handling stress (Time; T0 vs T60), 24-hour median temperature prior to capture (Temp), total length in centimeters (TLcm), the abundance of leeches, the abundance of leech bites (Bites), the abundance of trypanosomes (Tryps), infection status (Infection; Uninfected, Coinfected, Only Trypanosomes Present), and sediment scores for the amount of silt and organic matter (Organic) inside the artificial shelter.

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 Models	K	AICc	Delta AICc	AICc Weight	Cumulative Weight
PC2~Time*Temp + Infection*Temp	10	674.51	0.0	0.99	0.99
PC2~Time*Temp + Leeches*Temp + Bites*Temp	10	684.64	10.13	0.01	1.00
PC2~Time*Temp	6	687.83	13.32	0.00	1.00
PC2~Time*Temp + Leeches*Temp	8	689.69	15.18	0.00	1.00
PC2~Time*Temp + Leeches*Temp + Bites*Temp + Tryps	11	690.92	16.41	0.00	1.00
PC2~Time*Temp + Tryps	7	693.93	19.42	0.00	1.00
PC2~Time	4	694.16	19.65	0.00	1.00
PC2~Time*Temp + Leeches*Temp + Tryps	9	695.71	21.20	0.00	1.00
PC2~Time*Temp + Bites*Temp	8	702.40	27.89	0.00	1.00
PC2~1	3	833.60	159.09	0.00	1.00

The response variable (PC2) represents the second principal component scores after performing the principal component analysis on hematocrit, hemoglobin, and polychromasia. The predictor variables included in the model represent exposure to acute handling stress (Time; T0 vs T60), 24-hour median temperature prior to capture (Temp), the abundance of leeches, the abundance of leech bites (Bites), the abundance of trypanosomes (Tryps), and infection status (Infection; Uninfected, Coinfected, Only Trypanosomes Present).

Supplemental Table 1 Rubric used for evaluating sedimentation in artificial shelters of nesting male hellbenders.

Score	Silt	Organic Matter
0	No silt	No organic material
1	Almost no silt	Minimal organic material. (Covering less than 10% of the box floor, no deep piles.)
2	Dusting of fine silt. Does not cover underlying substrate.	Some organic material (Covering10-30% of the box floor, no deep piles)
3	Layer of fine silt fully covering underlying substrate. Less than ½ mm thick	Lots of organic material (Covering 30-70% of the floor or a deep pile that fills less than 50% of head space between the top of the box and the sediment)
4	Thick layer of fine silt at least ½ mm thick. (Box is muddy)	Full of organic material (Covering all of the box floor or pile of organic material filling more than 50% of head space between the top of the box and the sediment)

Supplemental Table 2 Statistical outcomes from pairwise comparisons of parasite intensity between sampling days.

Parasite Intensity	Contrast	Ratio	SE	p value
Trypanosomes	Oviposition / Mid-embryonic	0.976	0.0263	0.8042
	Oviposition / Hatching	1.297	0.0388	<.0001
	Oviposition / Emergence	1.73	0.0672	<.0001
	Mid-embryonic / Hatching	1.329	0.0376	<.0001
	Mid-embryonic / Emergence	1.773	0.0701	<.0001
	Hatching / Emergence	1.334	0.0549	<.0001
Leeches	Oviposition / Mid-embryonic	4.227	0.588	<.0001
	Oviposition / Hatching	3.349	0.535	<.0001
	Oviposition / Emergence	6.978	1.503	<.0001
	Mid-embryonic / Hatching	0.792	0.15	0.6105
	Mid-embryonic / Emergence	1.651	0.408	0.178
	Hatching / Emergence	2.084	0.541	0.0242
Leech Bites	Oviposition / Mid-embryonic	0.826	0.107	0.4516
	Oviposition / Hatching	0.808	0.109	0.3904
	Oviposition / Emergence	4.934	0.769	<.0001
	Mid-embryonic / Hatching	0.978	0.107	0.9969
	Mid-embryonic / Emergence	5.972	1.009	<.0001
	Hatching / Emergence	6.109	1.055	<.0001

Intensity of infection is determined by the number of parasites present in an infected individual and the intensity of leech bites represents the number of leech bites within individuals with bites (at least one leech bite present). Mid-embryonic represents Mid-embryonic development. Ratio represents the odds ratio on the response scale.

Supplemental Table 3 Results from principal component analysis of red blood cell metrics including both the nesting and non-nesting outgroup of male eastern hellbenders (*Cryptobranchus alleganiensis*)

Parameters	**PC1**	**PC2**	**PC3**
Hemoglobin	0.651	0.214	0.728
Hematocrit	0.624	0.396	-0.674
Polychromasia	-0.432	0.893	0.125
Eigenvalue	2.192	0.738	0.068
Proportion of Variance	0.731	0.246	0.022
Cumulative Variance	0.731	0.977	1.000

Supplemental Table 4 Statistical Outcomes from top-ranked models.

Response	Predictors	Estimate	SE	t-value	Pr(>|t|)
PC1	Intercept	-0.212	1.365	-0.155	0.877
	Time 60	1.733	0.355	4.880	<0.001
	Temperature	0.267	0.019	13.688	<0.001
	Total length	-0.095	0.029	-3.254	0.0019
	Leeches	-3.250	0.569	-5.708	<0.001
	Bites	-0.508	0.308	-1.650	0.099
	Time60*Temperature	-0.041	0.025	-1.682	0.094
	Leeches*Temperature	0.189	0.033	5.746	<0.001
	Bites*Temperature	-0.001	0.020	-0.040	0.968
PC1 non-nesting outgroup	Intercept	-0.418	1.676	-0.250	0.803
	Time 60	2.884	0.471	6.121	<0.001
	Temperature	0.297	0.030	9.780	<0.001
	Total length	-0.089	0.036	-2.495	0.015
	Non-nesting	-0.899	1.199	-0.750	0.456
	Leeches	-0.429	0.377	-1.136	0.257
	Bites	0.021	0.308	0.069	0.945
	Time60*Temperature	-0.122	0.037	-3.288	0.0012
	Non-nesting*Temperature	0.153	0.106	1.438	0.156
	Leeches*Temperature	0.015	0.032	0.470	0.638
	Bites*Temperature	-0.029	0.026	-1.082	0.280
PC2	Intercept	-1.088	0.439	-2.481	0.014
	Time 60	1.576	0.271	5.807	<0.001
	Temperature	0.037	0.028	1.340	0.181
	Only Trypanosomes Present	0.260	0.469	0.555	0.579
	Coinfected (leeches attached)	2.159	0.486	4.439	<0.001
	Time60*Temperature	-0.049	0.019	-2.597	0.010
	Only Trypanosomes Present *Temperature	-0.012	0.030	-0.410	0.682
	Coinfected (leeches attached)*Temperature	-0.140	0.031	-4.546	<0.001
PC2 non-nesting outgroup	Intercept	-2.791	0.651	-4.290	<0.001
	Time 60	1.357	0.330	4.111	<0.001
	Temperature	0.163	0.047	3.496	0.0006
	Non-nesting	-0.229	0.776	-0.295	0.769
	Only Trypanosomes Present	1.013	0.694	1.461	0.146
	Coinfected (leeches attached)	2.621	0.696	3.764	0.0002
	Time60*Temperature	-0.054	0.026	-2.084	0.0387
	Non-nesting*Temperature	-0.005	0.069	-0.073	0.941
	Only Trypanosomes Present *Temperature	-0.061	0.051	-1.240	0.230
	Coinfected (leeches attached)*Temperature	-0.151	0.050	-3.006	0.0031

Figures

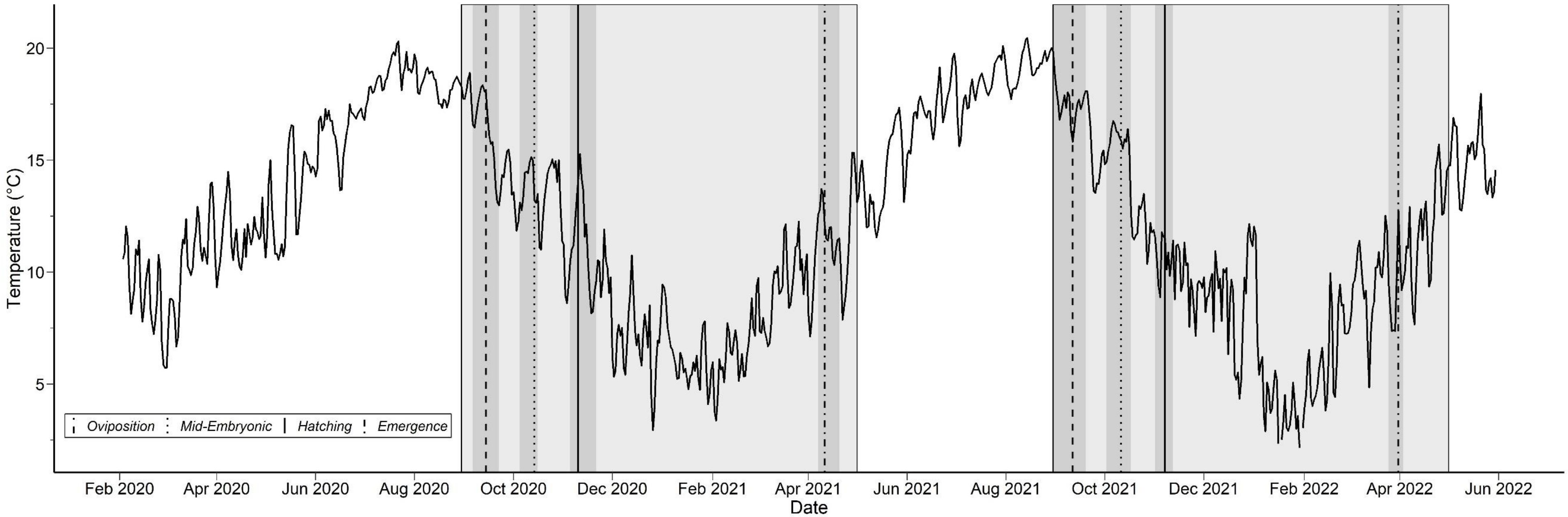


Figure 1 Seasonal variation in stream temperature across the paternal care period of eastern hellbenders (*Cryptobranchus alleganiensis*) in 2020 and 2021. Stream temperature (°C) is presented as the 24-hour daily median stream temperature averaged across four stream reaches. The two enclosed boxes indicate the range of the 8-month-long paternal care periods in 2020 and 2021. Vertical lines within the enclosed boxes indicated the average date of oviposition (dashed lines), and sampling at mid-embryonic development (dotted lines), larval hatching (solid line), and spring emergence (dot-dashed lines).

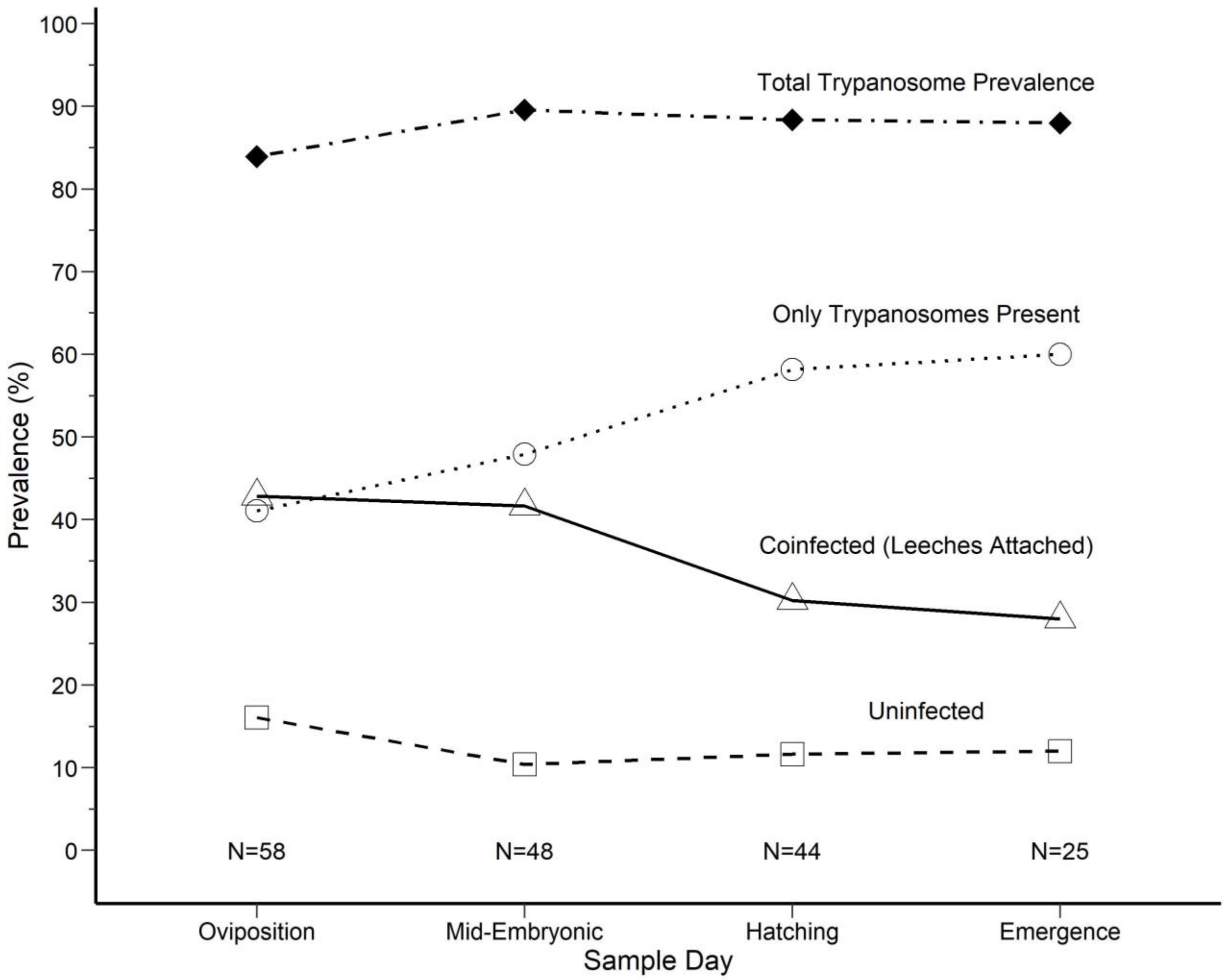


Figure 2 Prevalence of parasite infection in male eastern hellbenders (*Cryptobranchus alleganiensis*) with nests across the paternal care period. Prevalence is the proportion of individuals within a given infection status category out of the sample size at each capture period (labeled on the graph above 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 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 there were no instances in which an individual was infected with leeches only. Also, present on the graph is the total proportion of hellbenders infected with trypanosomes regardless of leech attachment, which is represented as '*Total Trypanosome Prevalence.'*

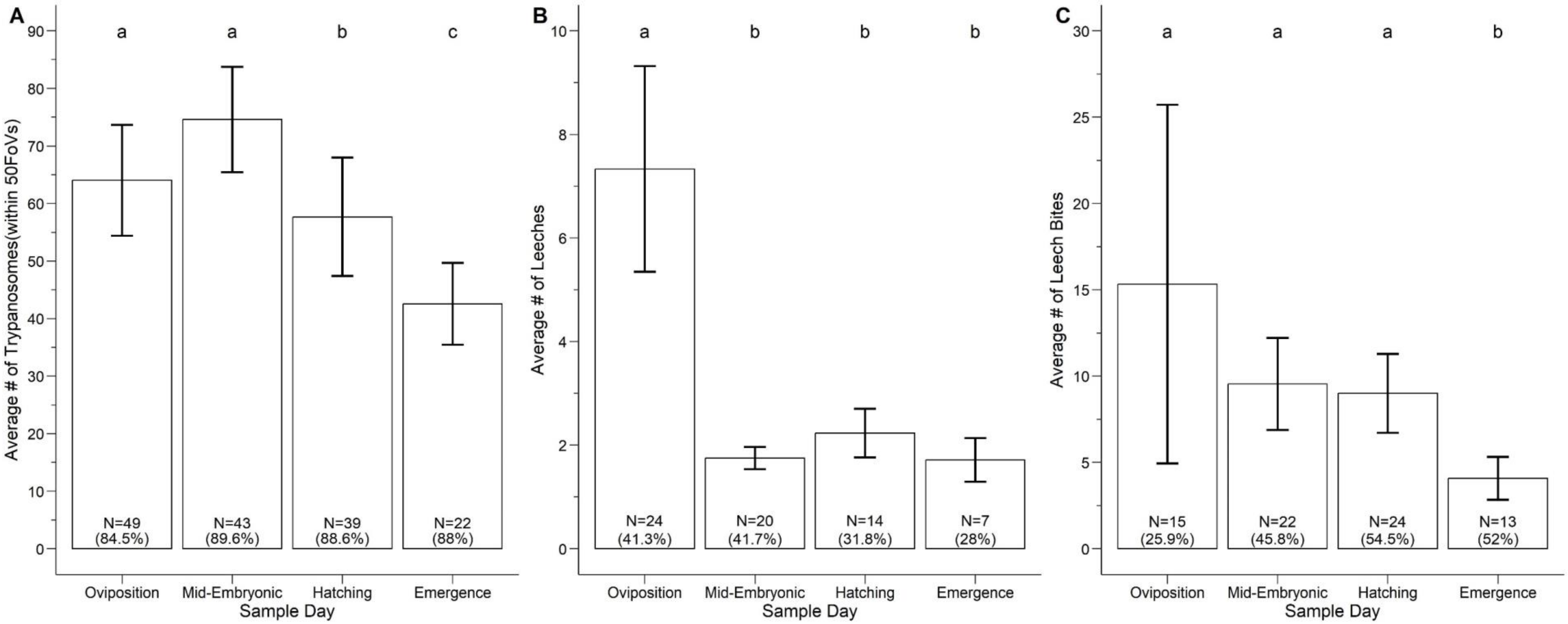


Figure 3 Intensity of parasitic infection among infected eastern hellbenders (*Cryptobranchus alleganiensis*) with nests across the paternal care period. Columns represent arithmetic means, and error bars represent ±1 SEM. The sample size and the parasite prevalence for each sampling day are noted at the bottom of each column. **(A)** The average number of trypanosomes within infected individuals (at least one trypanosome present) across the paternal care period. **(B)** The average number of leeches attached at capture among infected individuals (at least one leech attached) across the paternal care period. **(C)** The average number of leech bites within individuals with bites (at least one leech bite present). Superscripts 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 $\alpha = 0.05$.

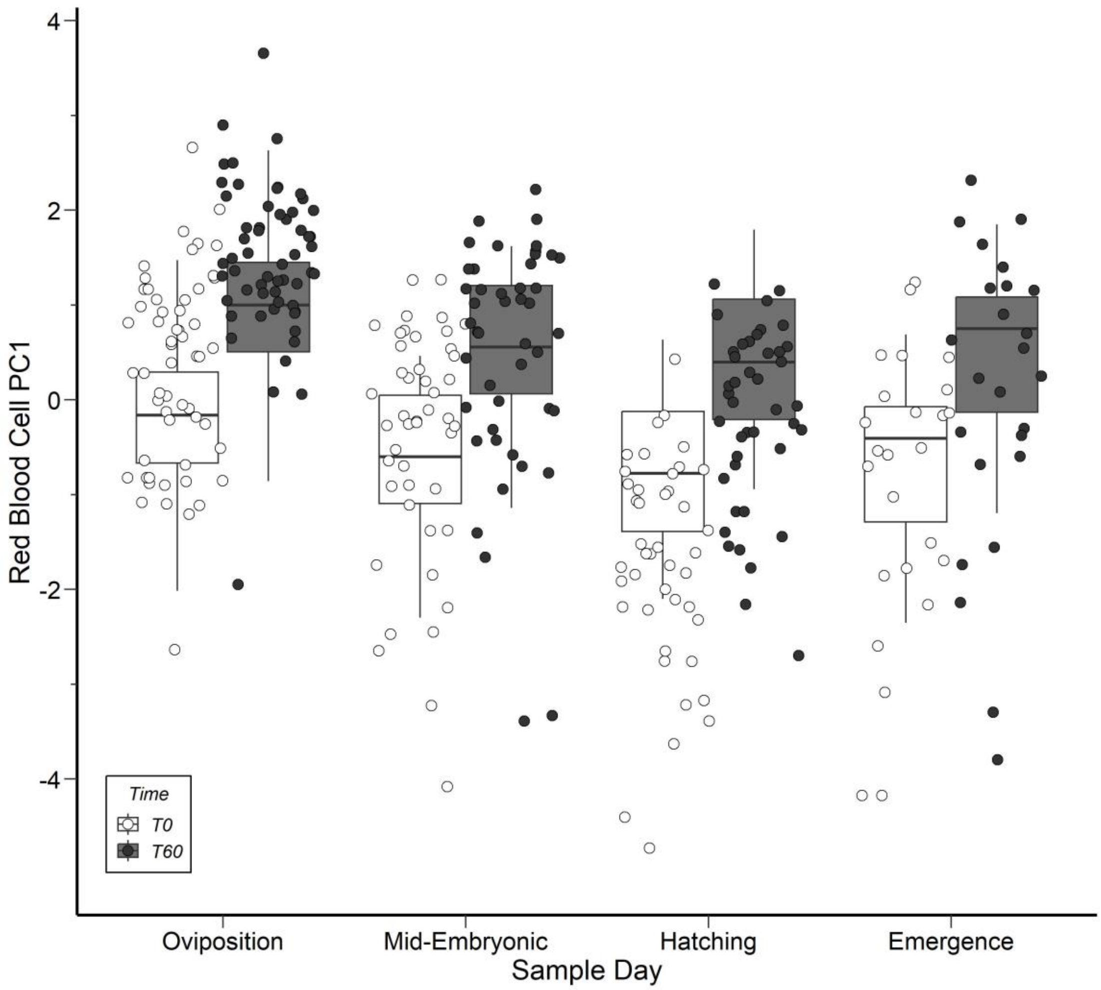


Figure 4 Model predictions from top ranking AICc model (boxplots) overlaid with raw data (points), predicting T0 and T60 PC1 scores across the paternal care period of hellbenders (*Cryptobranchus alleganiensis*). AICc rankings suggest that stress, total length, temperature, leeches, and leech bites influence red blood cell metrics from nesting male hellbenders.

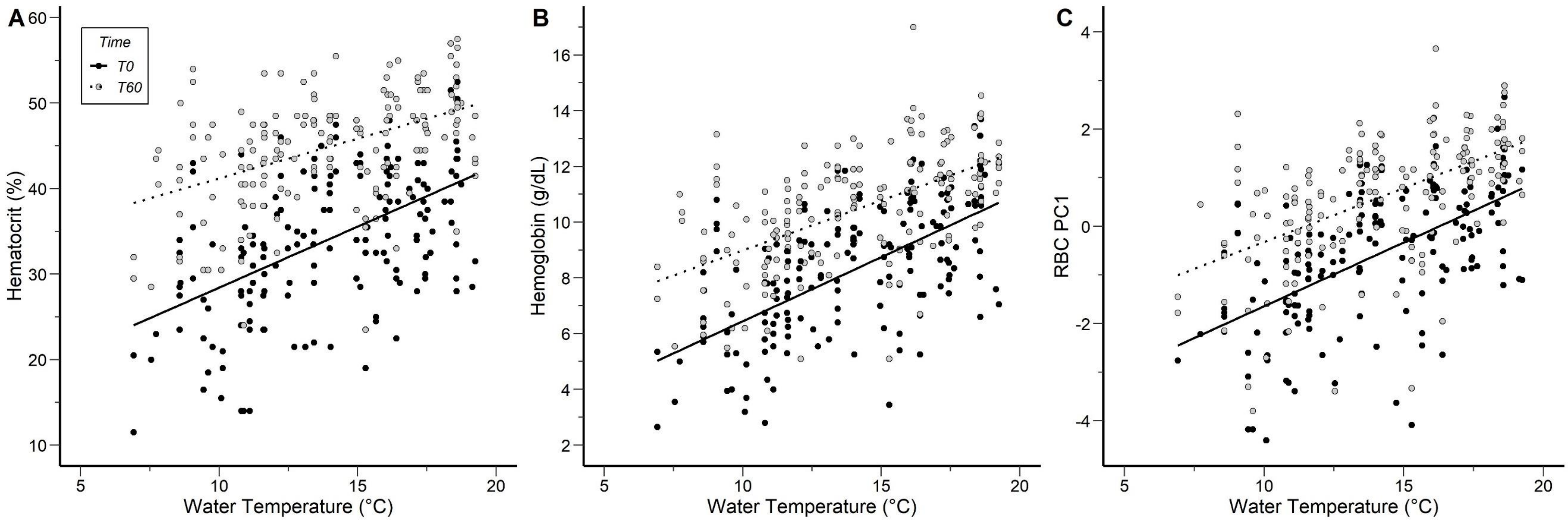


Figure 5 The relationship between temperature and red blood cell metrics in nesting male eastern hellbenders. **(A)** The relationship between stream temperature (°C) on hematocrit (%) in blood collected at capture (T0) and after one hour of restraint (T60). **(B)** The relationship between stream temperature and hemoglobin concentrations (g/dL) at T0 and T60. **(C)** The relationship between stream temperature and PC1 scores from the principal components analysis conducted on red blood cell metrics, hematocrit, hemoglobin, and polychromasia. Lines represent model predictions, while points represent raw data. While hematocrit and hemoglobin are presented here for visual purposes, the final statistical analysis only used PC scores.

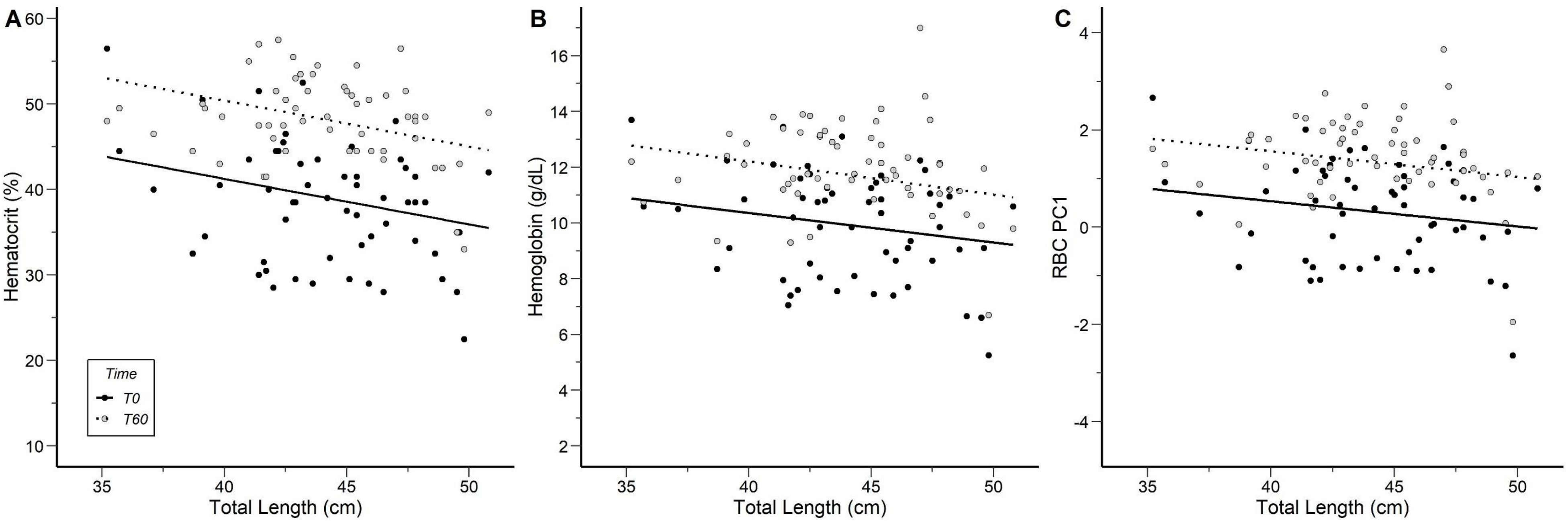


Figure 6 The relationship between total length (cm) and red blood cell metrics at oviposition in nesting male eastern hellbenders. **(A)** The relationship between total body length hematocrit (%) in blood collected at capture (T0) and after one hour of restraint (T60). **(B)** The relationship between total body length and hemoglobin concentrations (g/dL) at T0 and T60. **(C)** The relationship between total body length and PC1 scores from the principal components analysis conducted on red blood cell metrics, hematocrit, hemoglobin, and polychromasia. Lines represent model predictions, while points represent raw data. While hematocrit and hemoglobin are presented here for visual purposes, the final statistical analysis only used PC scores. For ease of visualization, data only shows the relationship between total length and red blood cell metrics during the oviposition capture period, but this pattern is maintained across all sampling periods..

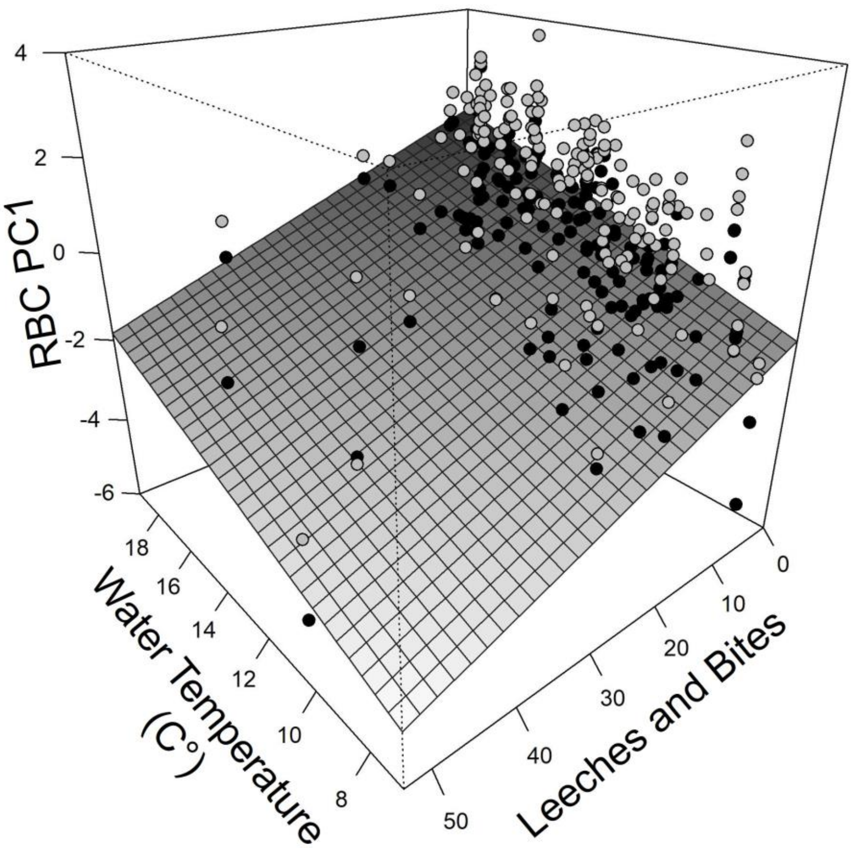


Figure 7 The relationship between temperature, the combined total of leeches and bites, and PC1 in nesting male eastern hellbenders. Note that RBCPC1 had positive loadings for hematocrit (0.624) and hemoglobin (0.651) but negative loadings for polychromasia (-0.432). The raw values are plotted for clarity, and the plane was generated from simple linear regressions. Black points indicate baseline (T0) values, while gray points indicate stress-induced (T60) values. The single plane represents the effects of temperature, leeches, and bites on all PC1 values. See Supplemental Figure 5 to visualize the trends for both T0 and T60. For visualization, we excluded one outlier from an individual during oviposition with 204 combined leeches and bites. This figure is for visualization purposes only. Leeches and bites were evaluated as separate predictor variables for statistical analysis.

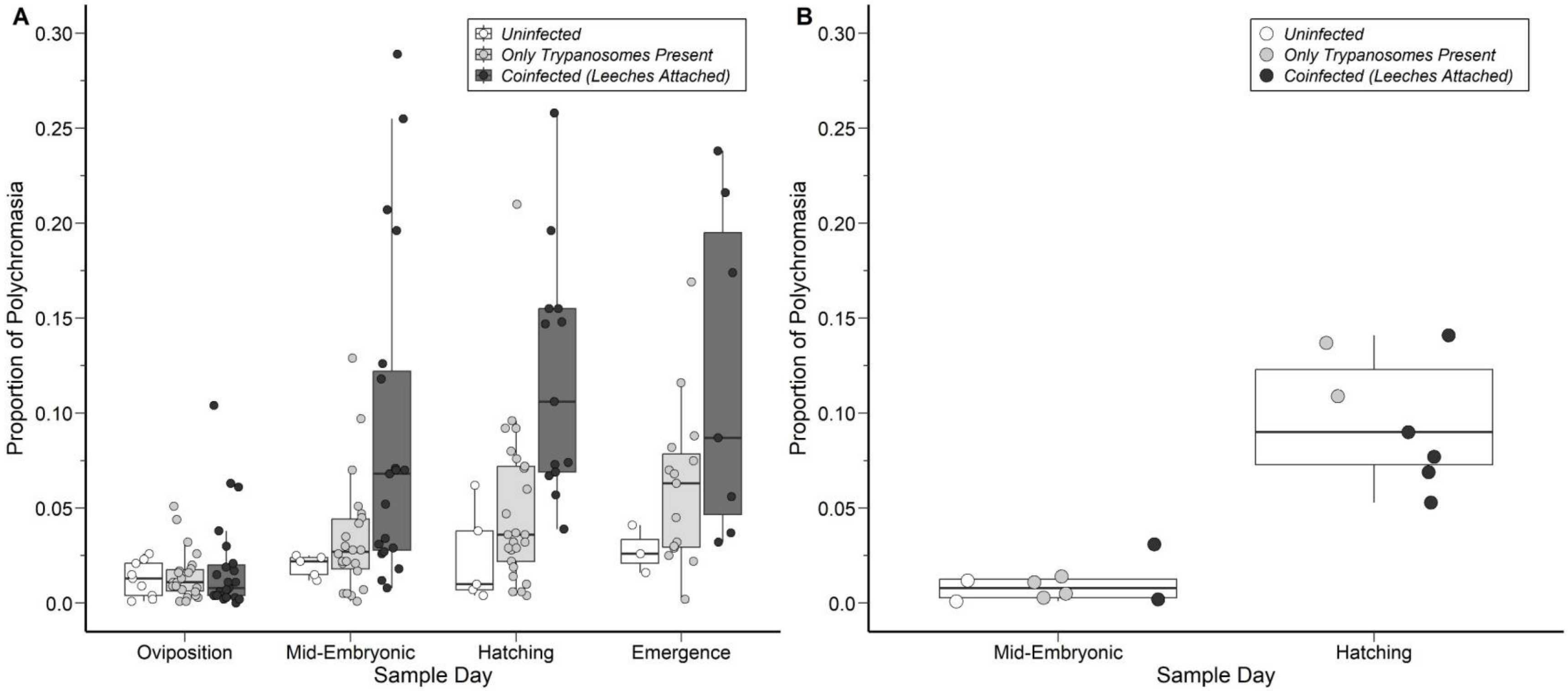


Figure 8 Proportion of polychromatic red blood cells and parasite prevalence across the parental care period. **(A)** The variation in the proportion of polychromatic red blood cells at capture (T0) grouped by infection status in nesting male eastern hellbenders (*Cryptobranchus alleganiensis*) across the paternal care period. **(B)** The variation in the proportion of polychromatic red blood cells at T0 in the non-nesting outgroup of male eastern hellbenders. The males in the outgroup were only captured and bled for one capture period during mid-incubation or hatching in 2021. The out-group was used to assess whether repeated bleeds influenced red blood cell metrics. Since individuals in the non-nesting outgroup (8B) had comparable polychromasia to individuals with nests (8A), we conclude that red blood cell regeneration is not a response to repeated bleeds across the parental care period.

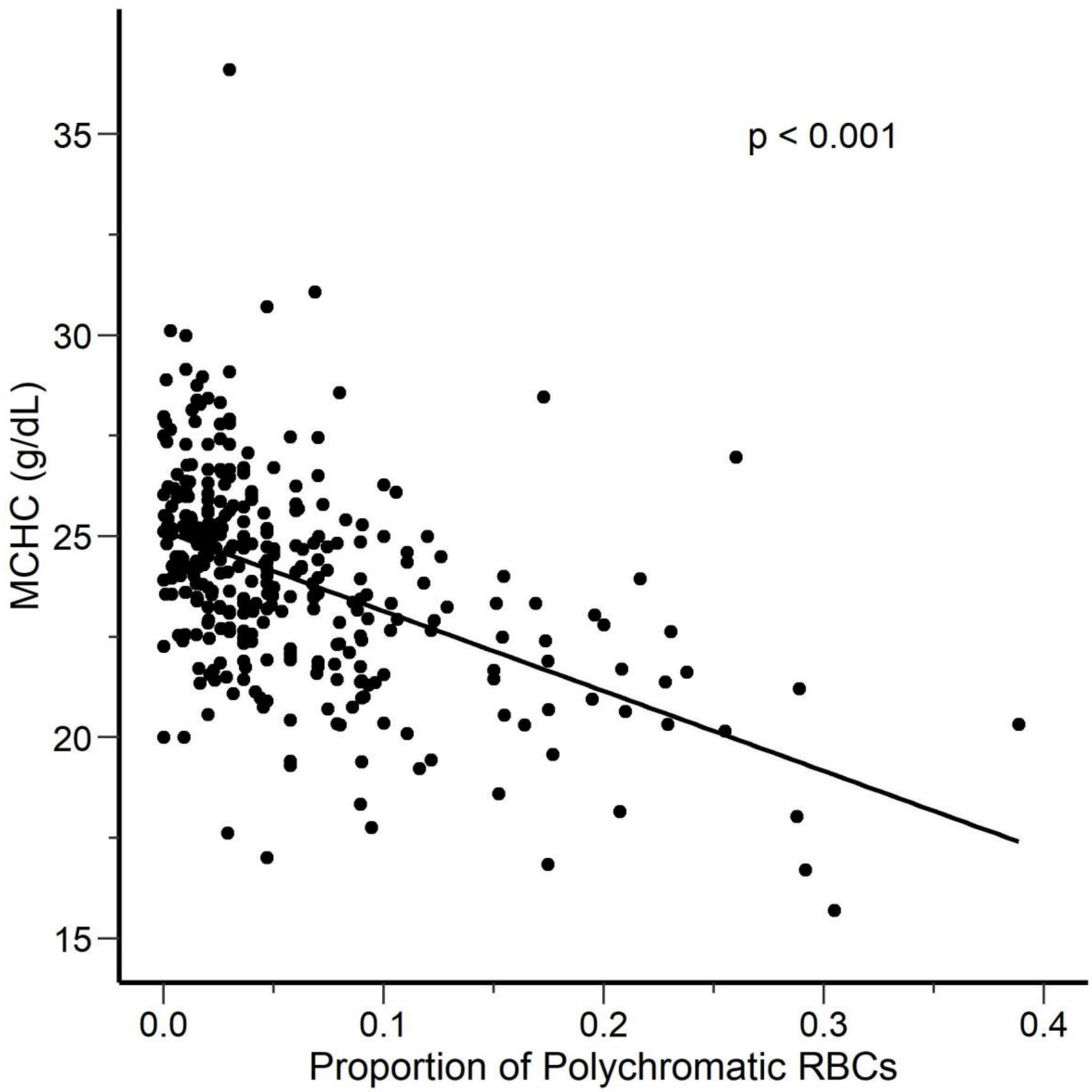
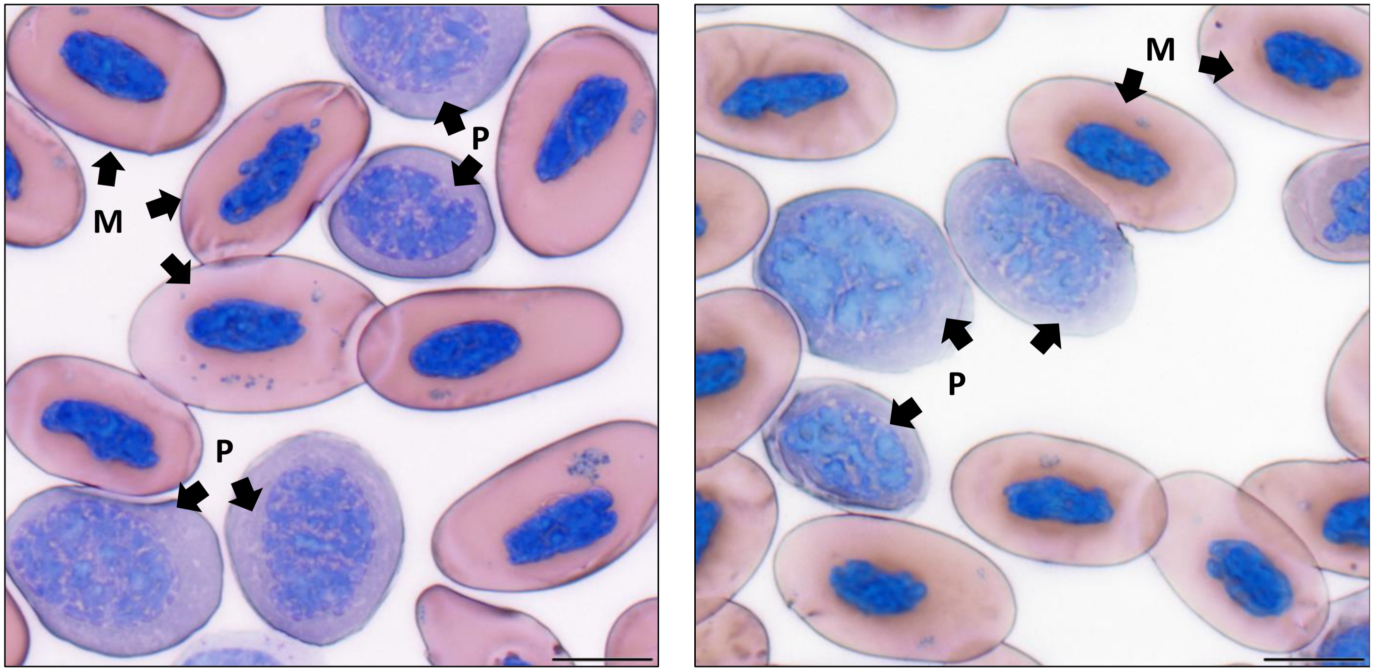
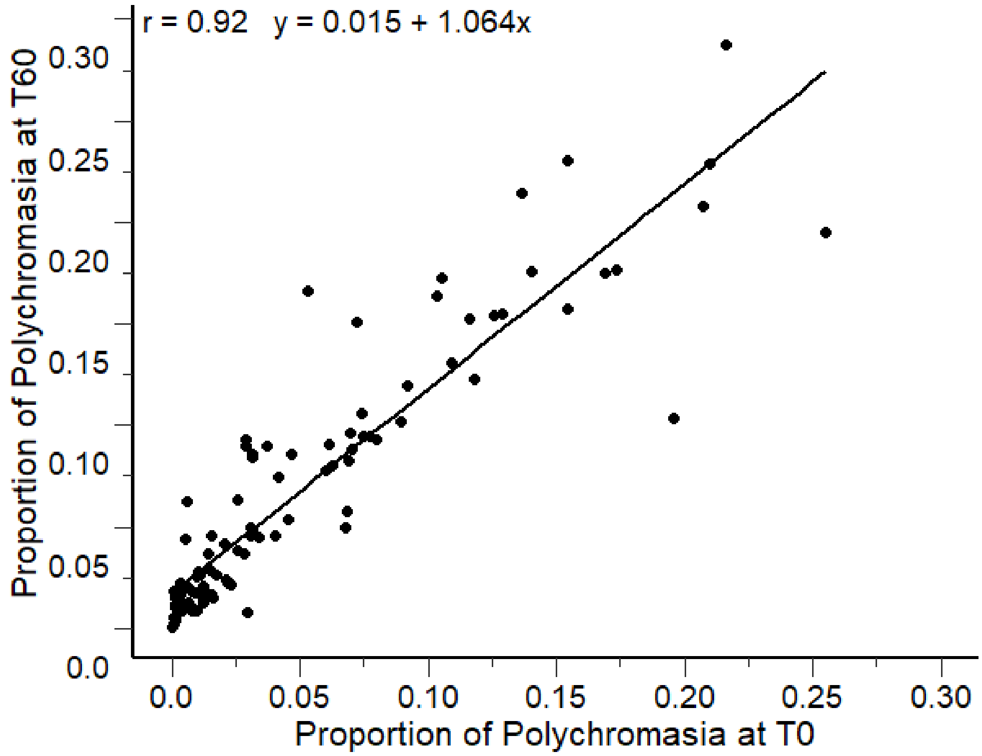


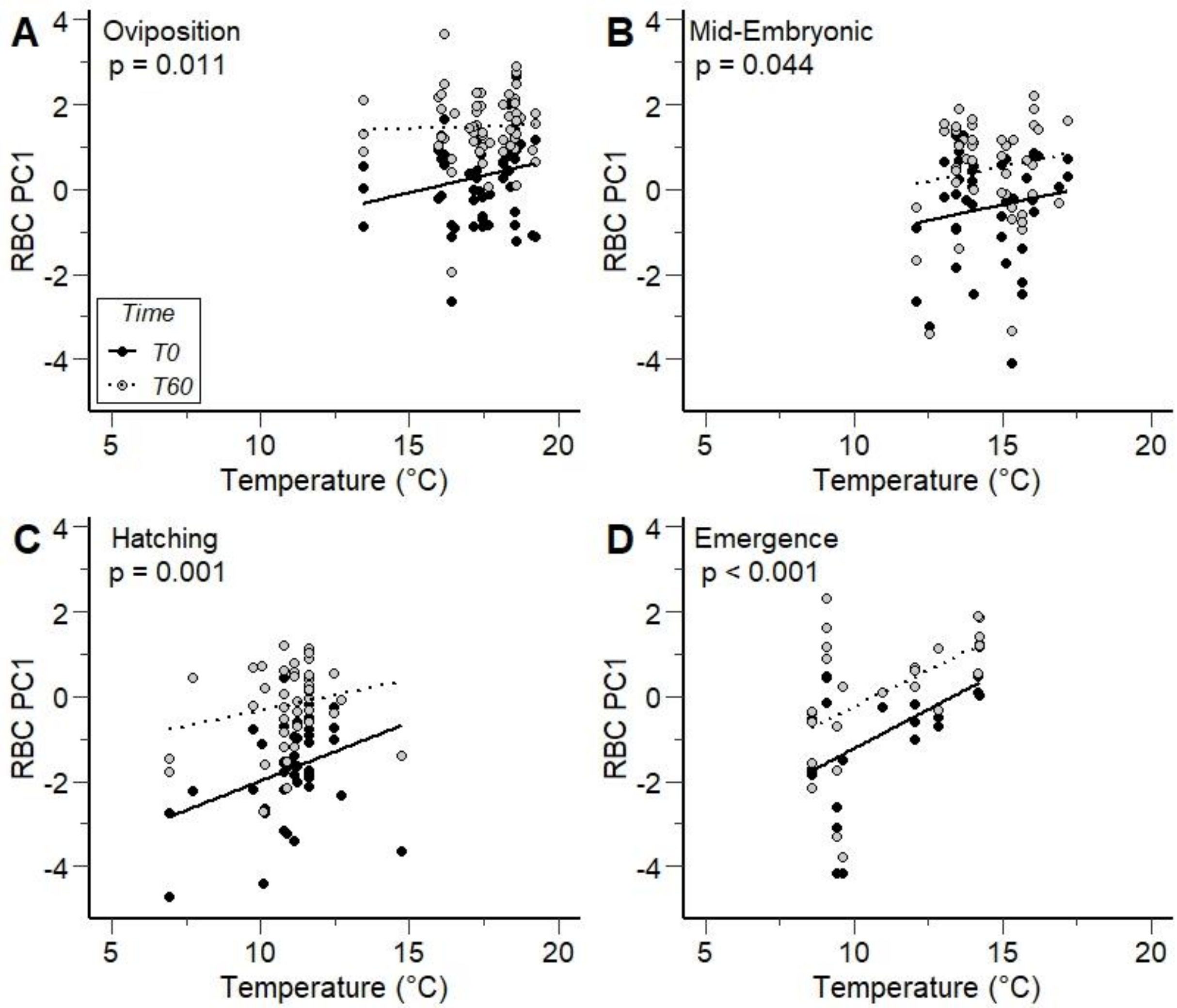
Figure 9 The relationship between the proportion of polychromatic red blood cells and mean corpuscular hemoglobin concentration (MCHC; g/dL). Note, that the figure reflects the results from the linear mixed effects model from a secondary analysis and that the MCHC values were not included in the principal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



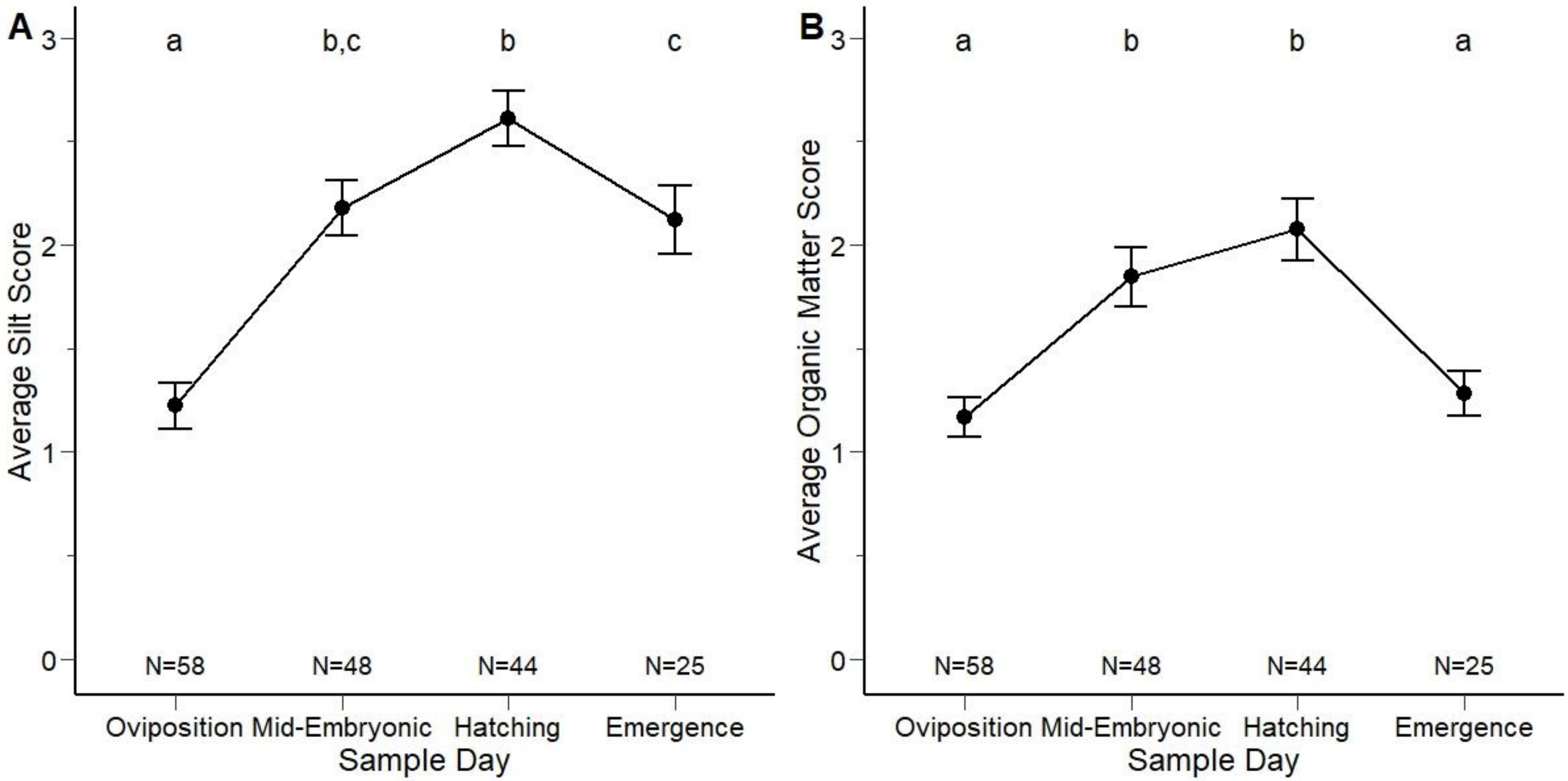
Supplemental Figure 1 Microscopic images of red blood cells from eastern hellbenders. Images show the identifiable differences between mature red blood cells (labeled as M) and polychromatic red blood cells (labeled as P). The black scale bar represents 20 µm.



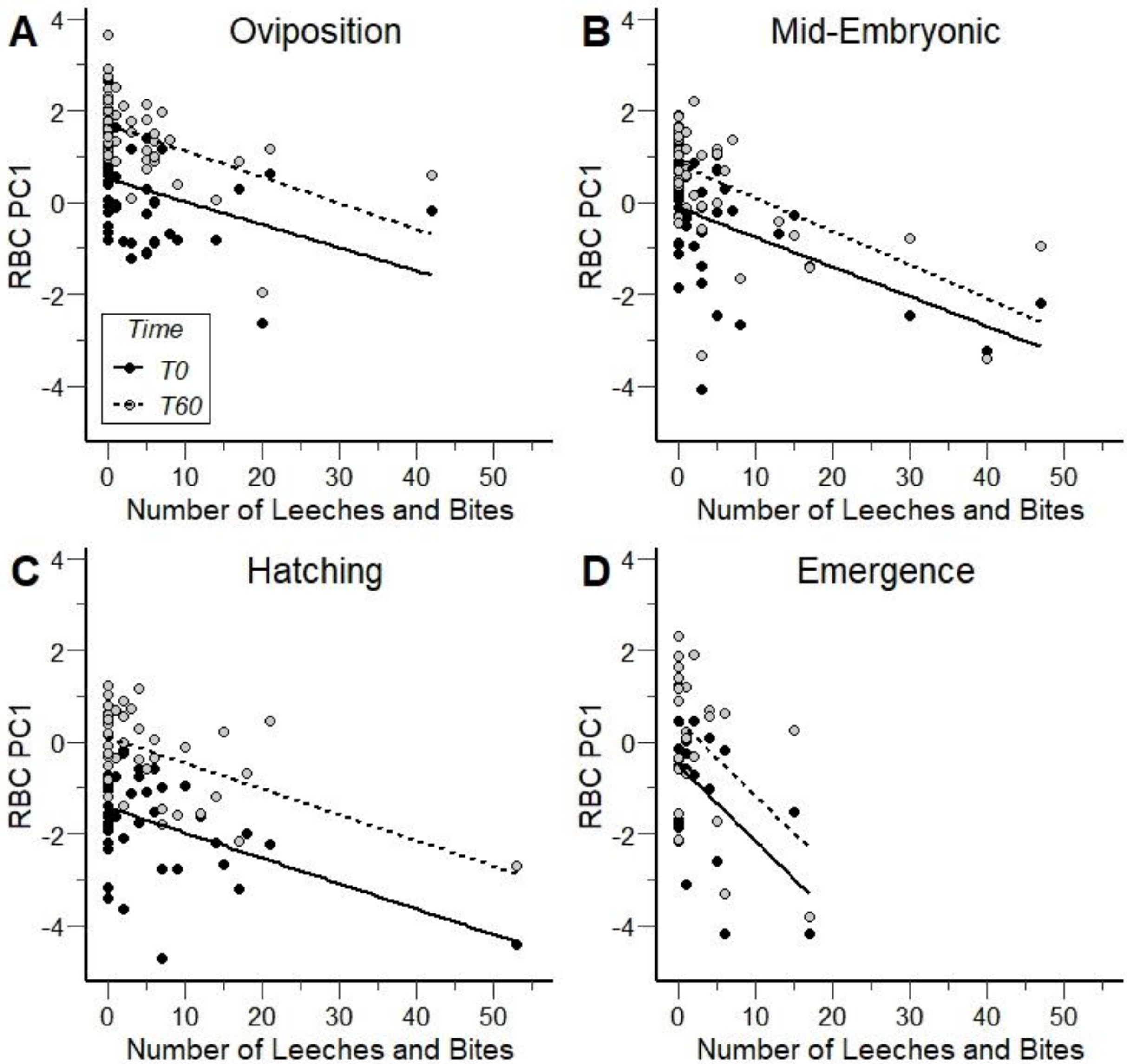
Supplemental Figure 2 Linear regression showing the relationship between the proportion of polychromatic red blood cells at T0 and T60 during the 2021 paternal care period. This was done to determine if there was a difference in the proportion of polychromatic red blood cells between individuals sampled immediately upon capture and following exposure to 60 minutes of acute stress. The was used to address missing data for the 2020 paternal care period in which only T0 was collected. Due to the high correlation between T0 and T60 (Pearson's correlation coefficient; r = 0.92), we concluded that the proportion of polychromatic red blood cells does not significantly change after 60 minutes of acute stress.



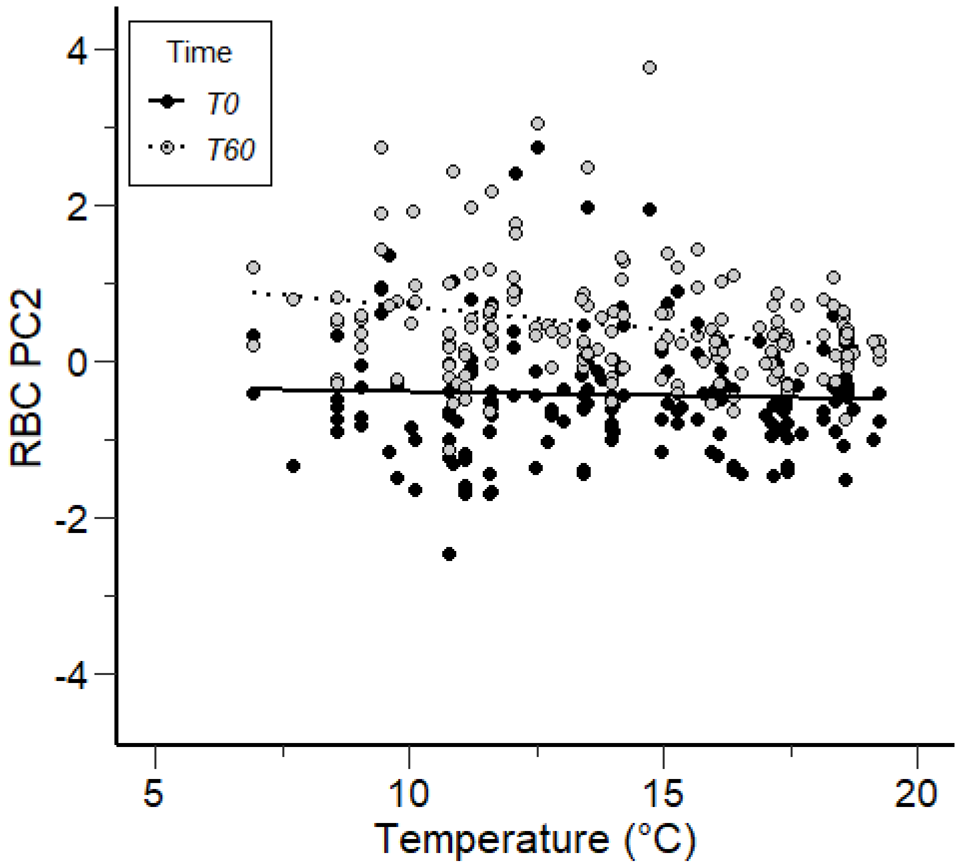
Supplemental Figure 3 Results from the exploratory analysis evaluating the effects of temperature within each sample day. This was used to determine which variable (sampling day or temperature) would be the most appropriate predictor to represent seasonal changes in the model. The resulting p values from the individual models are reported with the corresponding sampling day. Temperature significantly affected PC1 within each sampling day, indicating that temperature is still an overarching driver of red blood cell metrics and was a more appropriate predictor than sample day to represent seasonal changes.



Supplemental Figure 4 Changes in sediment scores of artificial nesting shelters across the paternal care period of eastern hellbenders (*Cryptobranchus alleganiensis*) in 2020 and 2021. The proportion of artificial shelters with each **(A)** silt score and **(B)** organic matter score out of the sample size of each capture day (labeled above the corresponding sample day). Letters in superscript indicate statistical differences in scores across the parental care period after using a Bonferroni adjustment on the p-values.



Supplemental Figure 5 The relationship between the combined total number of leeches and bites and PC1 during **(A)** Oviposition, **(B)** Mid-Embryonic Development, **(C)** Hatching, and **(D)** Emergence. The raw values are plotted for clarity, and the predictions are from simple linear regressions. We excluded one outlier from an individual during oviposition with 204 combined leeches and bites. This figure is for visualization purposes only. Leeches and bites were evaluated as separate predictor variables for statistical analysis.



Supplemental Figure 6 The relationship between stream temperature and PC2 scores from the principal components analysis conducted on red blood cell metrics, hematocrit, hemoglobin, and polychromasia. Lines represent model predictions, while points represent raw data.

CHAPTER 2: Parasites influence immunological responses but not reproductive success of hellbender salamanders (*Cryptobranchus alleganiensis)*.

Formatted for Submission to Integrative Organismal Biology

Parasite infections influence immunological responses but not reproductive success of male hellbender salamanders (*Cryptobranchus alleganiensis*).

Katherine L. Slack[1], Jordy Groffen[1], Andrew K. Davis[2], William A. Hopkins[1]

[1]Department of Fish and Wildlife Conservation, Virginia Tech, Blacksburg, VA, USA, 24061

[2]Odum School of Ecology, University of Georgia, Athens, GA 30602, USA.

Corresponding Authors

Katherine L. Slack, 601-497-4813; kslack@vt.edu

William A. Hopkins, 540-553-1031; hopkinsw@vt.edu

Key Words: Amphibian, cannibalism, coinfection, hellbender, hematology, immune indices, parasite, temperature

Parasite infections influence immunological responses but not reproductive success of hellbender salamanders (*Cryptobranchus alleganiensis*).

Abstract

The emergence and spread of infectious diseases is a significant contributor to global amphibian declines, requiring increased surveillance and research. We assessed host-vector-parasite dynamics using a population of eastern hellbender salamanders (*Cryptobranchus alleganiensis*) that harbor leeches (*Placobdella appalachiensis*) that transmit endoparasitic blood parasites (*Trypanosoma* spp.) to the host, with coinfection frequently occurring. We centered our study on adult males throughout their extended 8-month paternal care period because recent research indicates that nest failure caused by lack of paternal care and filial cannibalism is contributing to hellbender population declines. Recognizing the potential for parasites to modulate host physiology and behavior, we explored how infection severity influences paternal health and reproductive success. We assessed white blood cell profiles of adult male hellbenders in response to parasites, coinfection, and seasonal temperature fluctuations, while also investigating the predictive value of parasite infection on nest success. We found that hellbenders exhibited seasonal shifts in white blood cell indices; as temperatures increased across seasons (from 5° C to 20° C), the proportion of neutrophils and eosinophils decreased (by 14% and 46%, respectively) in circulation while the proportion of lymphocytes and basophils increased (by 8% and 101%, respectively). Moreover, the proportion of neutrophil precursors increased by 80% under colder temperatures, which signifies seasonal immune cell recruitment. We demonstrated that neutrophils and eosinophils increased while lymphocytes decreased in response to leech infection. However, as leech and trypanosome infection intensity increased together the proportion of lymphocytes increased while neutrophils and eosinophils decreased, underscoring

the complex interactions between coinfection and immune responses of hellbenders that warrant future research. Despite the influence of coinfection on hellbender physiology, we detected no evidence to support the hypothesis that parasites influence the likelihood of nest failure or whole clutch filial cannibalism. In light of amphibian declines being exacerbated by climate change and disease, our study emphasizes the need to establish hematological reference values that account for physiological adaptations to seasonal fluctuations in temperature and different life history stages and to study the physiological responses of imperiled amphibian species to parasites.

Introduction

Amphibians are among the most endangered vertebrates, with 41% of species threatened with extinction, predominantly due to climate change, habitat loss and degradation, and emerging infectious diseases (Hoffman et al., 2010; Luedtke et al., 2023). Due to their physiology and specialized habitat requirements, amphibians are particularly susceptible to subtle perturbations in the environment (Vitt et al., 1990; Wake, 1991; Blaustein et al., 1994; Hopkins, 2007), which makes them valuable indicators of environmental conditions (Blaustein, 1994; Blaustein and Wake, 1995; Hopkins, 2007). Therefore, investigating aspects of amphibian physiology can provide insight into how environmental factors impact individual health and even population dynamics. However, the scale at which anthropogenic disturbances impact amphibian populations is context-dependent, varying among species and even sub-populations (Blaustein and Kiesecker, 2012; Green et al., 2020). Thus, investigations into the general threats to imperiled amphibians must also consider the complex, localized environmental conditions involved. For example, nearly all aspects of the physiology of wild amphibians fluctuate with environmental temperatures (da Silva et al., 2013; Herczeg et al., 2021). As such, recognizing seasonal patterns in physiological indices is essential to distinguish between natural variation and the impact of other factors such as disease or anthropogenic disturbance.

Shifts in the proportions of various white blood cell types in the circulation can provide valuable insights into the status of amphibian immune systems and how they are influenced by parasites and other extrinsic factors (Allender and Fry, 2008; Campbell, 2015; Forzan et al., 2017; Davis and Golladay, 2019; Bain and Harr, 2022; Davis and Maerz, 2023). Each type of white blood cell performs distinct immune functions, offering information that can aid in identifying different immune responses (Campbell, 2015; Forzan et al., 2017; Bain and Harr, 2022). In addition to quantitative assessments of white blood cell types, examining the morphological features of some white blood cell types can identify stages of cellular development and abnormalities, which serve as informative diagnostic tools (Bain and Harr, 2022; Stacy et al., 2022). For example, the presence of neutrophil precursors and neutrophils with toxic change can signal immune recruitment and a response to increased inflammation (Stacy et al., 2022). However, to maximize the interpretability of white blood cell types and cellular abnormalities in relation to parasitic infection, it is important to establish species-specific reference values that account for seasonal fluctuations in immune function. For example, temperature affects the immune performance of amphibians, and prolonged exposure to cold temperatures may suppress components of the adaptive immune response (Maneiro and Carey, 1997). Furthermore, fluctuations in temperature could leave amphibians more susceptible to infection because of the suppressive effects of temperature fluctuations on the immune system (Raffel et al., 2006*a*). While an increase in neutrophil precursors has been observed in frogs following long-term exposure to cold temperatures (Maneiro and Carey, 1997), these metrics are not widely applied as diagnostic tools and have yet to be applied to wild herpetofauna. Given the seasonal suppression of critical immune functions in amphibians, it is essential to investigate seasonal profiles of white blood cell composition in amphibians in order to effectively interpret their immune responses to parasites or other environmental factors.

The eastern hellbender (*Cryptobranchus alleganiensis*) is an imperiled amphibian species whose physiology is understudied but is infected with multiple types of parasites and is thus well suited for studies seeking to disentangle natural fluctuations in white blood cell counts from variability caused by parasitic infection. This giant, fully aquatic salamander is also an indicator species of good water quality due to its dependency on cutaneous respiration for gas exchange (Nickerson and Mays, 1973; Guimond and Hutchison, 1973; Coe et al., 2016). Hellbender populations have undergone steep declines in population abundance since the 1970s, with some declining by 70-80% (Wheeler et al., 2003; Burgmeier et al., 2011; Graham et al., 2011) and other populations being extirpated or functionally extinct (USFWS, 2018). The factors causing their population declines are multifaceted, and habitat degradation and disease are among the most common factors implicated (USFWS, 2018; Hopkins et al., 2023). Thus, hellbenders are a good species for investigating factors that can compromise amphibian health and survival. In some streams in southwest Virginia, hellbenders are exposed to sanguivorous leeches, *Placobdella appalachiensis* (Hopkins et al., 2014), which are the vectors for a newly discovered but unnamed trypanosome species, *Trypanosoma* spp. (Davis and Hopkins, 2013). Available evidence suggests that both parasites, leech and trypanosome, are most prevalent in stream reaches with the highest density of hellbenders (Jachowski and Hopkins, 2018; Bodinof Jachowski et al., 2024), suggesting the importance of host density to the parasites' population dynamics. The parasites are known to elicit immune responses (Hopkins et al., 2016; Bodinof Jachowski et al., 2024), cause symptoms of anemia (Slack et al, 2024), and the leech is implicated in endocrine disruption (DuRant et al., 2015) in the hosts. However, whether these parasites affect any fitness-related traits of the hosts remains unknown. Because recent investigations have revealed that high rates of nest failure caused by inadequate paternal care and whole clutch filial cannibalism may be contributing to population declines (Hopkins et al., 2023; Brooks et al., 2024), understanding whether parasitic infection influences paternal care and resulting reproductive success is a high priority.

In this study, we assessed the effects of parasitism on white blood cell profiles of male hellbenders guarding nests with eggs across their prolonged 8-month parental care period. The primary objectives were to determine whether white blood cell profiles fluctuate in response to seasonal changes in environmental temperature, investigate the influence of parasitic infection on these immune indices, and ascertain if parasitic infection is associated with nest failure or filial cannibalism in hellbenders. We focused on males guarding their nests because the parental care period is known to be a particularly sensitive time for vertebrates that exhibit care (Smith and Wootton, 1995; Hershman and Zera, 2007), including hellbenders (Hopkins et al., 2023), and some blood parasites are thought to exert their greatest effect on hosts during demanding or strenuous life history stages (Nordling et al., 1998; Norte et al., 2009). We hypothesized that hellbenders would exhibit seasonal shifts in white blood cell profiles correlated to environmental temperatures. We predicted that the proportion of circulating lymphocytes and basophils would decrease with temperature while the proportion of eosinophils and neutrophils would increase as temperature decreases (Maneiro and Carey, 1997; Raffel et al., 2006*a*; Bodinof Jachowski et al., 2024). We also predicted that as temperatures decrease, hellbenders would exhibit signs of immune recruitment through increased proportions of band and toxic neutrophils. We also hypothesized that parasitic infection would influence white blood cell profiles. Under this hypothesis, we predicted that the proportions of eosinophils and neutrophils in circulation would increase in response to parasitic infection, while the proportion of lymphocytes and basophils would decrease as they migrate out of the vascular space into tissues (Latimer et al., 2003; Eberle and Voehringer, 2016). We also predicted that the proportion of band and toxic neutrophils would increase with parasite infection intensity due to the prolonged demand for inflammatory and innate immune responses (Stacy et al., 2022). Lastly, we predicted that the likelihood of whole-clutch cannibalism and/or nest failure would increase as parasite infection intensity increases in male hellbenders.

Materials and Methods

Species Description

The eastern hellbender (*Cryptobranchus alleganiensis alleganiensis*) is a fully aquatic giant salamander of the family Cryptobranchidae and 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+ years (Taber et al., 1975; Nickerson and Mays, 1973). Hellbenders 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) in areas with extensive boulder habitat and high upstream forest cover (Pugh et al., 2016; Jachowski and Hopkins, 2018). As a notoriously cryptic salamander, hellbenders primarily rely on rock crevices for cover and reproduction (Nickerson and Mays, 1973).

Hellbender reproductive ecology differs from most other North American salamanders. While the timing of the breeding season can vary depending on environmental factors and geographic location (Nickerson and Mays, 1973), in our study, the timing of oviposition ranged from August 22th to September 22nd. After securing a suitable crevice under a large boulder to breed, male hellbenders engage in fully aquatic, external fertilization followed by prolonged, solitary, obligate paternal care of their eggs and larvae (Bishop, 1941; Nickerson and Mays, 1973; Hopkins et al., 2023). In Virginia, male hellbenders are known to stay with their young for more than eight months (Hopkins et al., 2023). Following a ~60-day embryonic developmental period, larvae hatch from eggs and remain in the nest with the guarding male until the following April-May (Hopkins et al., 2023).

Hellbenders in our study population are host to sanguivorous leeches (*P. appalachiensis)* and a trypanosome (*Trypanosoma* spp.) that is vectored by the leech (Davis and Hopkins, 2013; Hopkins et al. 2014; Jachowski and Hopkins, 2018; Bodinof Jachowski et al., 2024; Hopkins et al., unpublished). These leeches are known to inhabit hellbender nest cavities and boulders used for shelter year-round and exhibit brooding and parental care behaviors in the summer months (Blumenthal and Hopkins, unpublished). Following the leech brooding period, the parent delivers the leech offspring to its first blood meal (Blumenthal and Hopkins, unpublished), the timing of which is synchronous with the beginning of the hellbender breeding season (Hopkins et al., 2023; O'Brien et al., 2024) when multiple hellbender hosts are entering nest cavities and establishing territories (O'Brien et al., 2024). Experimental infection confirmed that the leech is an effective vector for the trypanosomes (Hopkins et al., unpublished), a hematophagous endoparasite that is highly prevalent in local populations of hellbenders (DuRant et al., 2015; Hopkins et al., 2016; Bodinof Jachowski et al., 2024; Slack et al., 2024). Notably, recent research suggests that peak trypanosome transmission likely occurs during the simultaneous reproductive periods of the hellbender host and leech vector (Slack et al., 2024).

Site description and sample collection

Our study was conducted in four stream reaches (each ~100-200 m long) along a ∼13km segment of a single stream within the Upper Tennessee River Basin (VA, USA). 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 (Jachowski and Hopkins, 2018; Hopkins et al., 2023). Based on eight years of monitoring nests, annual nest success in these four reaches averaged 49%, and rates of whole clutch filial cannibalism were 14 % (Hopkins et al., 2023). Underwater artificial shelters (N = 30 – 35/reach) were deployed up to 10 years prior to 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 are used by our research team to monitor population dynamics, behavioral ecology, physiology, and annual reproduction (Button et al., 2020 *a*, *b*; Jachowski et al., 2020; Hopkins et al., 2023; O'Brien, 2023; O'Brien et al., 2024). We monitored the hourly temperature of each stream reach using U24-001 HOBO® Conductivity Data Loggers 0-10,000 µS/cm (Onset Computer Corp, Bourne, MA, USA).

We captured reproductive male hellbenders nesting in our artificial shelters at four time intervals across the paternal care period in 2020 and 2021. Sex was confirmed based on external cloacal morphology (Makowsky et al., 2010). We sampled each individual at oviposition (Day 0 [Aug 30 - Sep22], N=58) and attempted recapture at mid-embryonic development (~Day 30 [Oct 2 – Oct 17], N=48), larval hatching (~Day 60 [Nov 5 – Nov 21], N=44), and just prior to juvenile emergence from the nest (~Day 200 [March 25 – April 20], N=25) or until nest failure (Table 1). Our prior work demonstrated that adult male hellbenders are tolerant of repeated capture and blood collection and that repeated handling does not affect their parental care or nesting success (Hopkins et al., 2023; O'Brien, 2023).

Blood Processing and Hematology

At each time interval we removed a male hellbender from its artificial shelter and used a heparinized syringe to obtain a baseline (within 3 mins of capture) sample of whole blood (collecting an average of ~69µL/100g body mass), following the methods outlined in Hopkins and DuRant (2011). 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 and the number of leech bite wounds on each individual, which are easily identified and distinct from any other markings on hellbenders. 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, prior to release back to its artificial shelter (Hopkins and DuRant, 2011).

At each time interval where a baseline blood sample was collected, we made duplicate blood smears with fresh whole blood using a standard two-slide wedge technique. We also injected ~75 µL of whole blood into two heparinized capillary tubes and transported them on ice to the laboratory. Within 8 hours of sample collection, we centrifuged duplicate capillary tubes for each individual at 5g for 5 mins. 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 removed the buffy coat, the thin white layer of white blood cells on top of 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 (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. Slack) was blinded to the identity of each slide and examined fifty random fields of view in both whole blood and buffy coat smears. We used a light microscope at 400X magnification to quantify white blood cell differentials in whole blood smears and the number of trypanosomes in buffy coat smears. We quantified the proportion of eosinophils, basophils, neutrophils, lymphocytes, and monocytes, which based on their specific function can be informative for identifying ongoing stressors and immune activity (Davis et al., 2008; Forzan et al., 2017). Eosinophils (Fig. 1.A) play a role in the inflammation process and antiparasitic defense (Latimer et al., 2003). Basophils (Fig. 1.B) migrate out of circulation and into peripheral tissues during allergic reactions and parasitic infection and are also important in the inflammatory response as well as protective immunity against parasites (Eberle and Voehringer, 2016). The proliferation of neutrophils (Fig. 1.C) into circulation occurs in response to stress, inflammation, and infection (Latimer et al., 2003). Lymphocytes (Fig. 1.D) are the predominant leukocyte type in amphibians (Bain and Harr, 2022) and are involved in multiple immunological processes in which they migrate out of peripheral circulation into tissues upon stimulation (Latimer et al., 2003). Notably, the glucocorticoid stress response promotes the paired migration of neutrophils into circulation and lymphocytes out of circulation into tissues.

As a result, the relationship between the two cell types is often represented as a ratio (i.e., N:L ratio) and has become a common metric used by researchers to evaluate the vertebrate stress response (Davis et al., 2008). Monocytes also play a role in mediating inflammatory responses and eliminating foreign substances (Latimer et al., 2003); however, hellbenders have very few in circulation (Hopkins et al., 2016; Bodinof Jachowski et al., 2024).

In addition to quantifying these standard types of white blood cells, we went further to distinguish different types of neutrophils. We calculated the proportion of neutrophils with toxic change (hereafter referred to as toxic neutrophils; Fig. 1.E-F) and neutrophil precursors (hereafter referred to as band neutrophils; Fig. 1.G-H) out of the total number of circulating white blood cells. Toxic neutrophils occur in response to rapid demand for increases in neutrophils, causing accelerated production, resulting in abnormal maturation (Stacy et al., 2022). While the morphological appearance of toxic neutrophils varies across taxa, distinctive features used to recognize them include degranulation, cytoplasmic vacuolation or foaminess, and variation in the shape and color of cytoplasm granules (Fig. 1 E-F; Stacy et al., 2022). Elevated proportions of band neutrophils result when inflammatory demand exceeds neutrophil storage pools. Characteristics used to identify band neutrophils include a band-, bean-, or round-shaped non-segmented nuclei and a higher nucleus-to-cytoplasm ratio (Fig. 1 G-H; Stacy et al., 2022; Latimer et al., 2003). To our knowledge, our study is the first to quantify neutrophil precursors and toxic change in a wild amphibian.

Trypanosomes (Fig. 1, I-L) were identified using morphological descriptions (Davis and Hopkins, 2013) that have been used previously (DuRant et al., 2015; Hopkins et al., 2016; Bodinof Jachowski et al., 2024). We used the combined total of trypanosomes counted on both of the duplicate buffy coat smears to represent parasite relative abundance. Before the study started, we confirmed the reliability and reproducibility of the observer by blindly quantifying twenty whole blood smears in triplicate. We then used the intraclass correlation coefficient (ICC; Sokal and Rohlf, 1995) and calculated the ICC score for leukocytes and trypanosomes (ICC-Neutrophils = 0.93; ICC-Lymphocytes = 0.95; ICC-Eosinophils = 0.92; ICC-Basophils = 0.85; ICC-Monocytes = 0.88; ICC-Trypanosomes = 0.98) to demonstrate a high degree of reproducibility. Our method of measuring trypanosome relative abundance is semi-quantitative and has yet to be validated with qPCR, so we also calculated the ICC score between the two duplicate buffy coat smears to confirm that our semi-quantitative metric is consistently repeatable (ICC [duplicates] -Trypanosomes = 0.92).

Nest fate

We determined ultimate nest outcomes following methods outlined in Hopkins et al. (2023). We considered nests to have failed if they no longer contained eggs or larvae. Conversely, we considered a nest to be successful if at least one larva was observed in the nest in the spring and the attending male was present during at least three of the four recapture attempts across the paternal care period. We determined if nest failure was due to whole clutch cannibalism if males were observed regurgitating eggs during capture or at least two of the following characteristics were observed: the clutch was torn apart with eggs missing, extreme bloating (or an obviously distended abdomen), and significant, abnormal weight gain since the prior capture (diagnostic characteristics described in detail in Hopkins et al. 2023).

Statistical Analysis

We used R (R Core Team 2022) to conduct all statistical analyses. Prior to analysis, we used the Shapiro-Wilks test for normality and applied a square root transformation on the proportion of eosinophils, basophils, band neutrophils, and toxic neutrophils, then scaled all white blood cell variables to have a mean of 0 and a standard deviation of 1. Given our white blood cell variables were quantified as proportions, we chose a multivariate approach to reduce the dimensionality of the dataset and account for their interdependence. We performed a principal components analysis (PCA) based on a correlation matrix to retain the first principal component as a proxy of the proportion of total neutrophils (the sum of normal, band, and toxic neutrophils), lymphocytes, eosinophils, and basophils. On average, the proportion of monocytes did not exceed 2%, so they were excluded from the PCA analyses and are not discussed further.

Sampling day (oviposition/mid-embryonic development/hatching/emergence) and 24-hour median temperature (C°) prior to capture were strongly confounded predictors. Thus, both variables could not be included within the same model. However, they did not produce consistently comparable model outputs. To address this, we performed preliminary model comparisons for all white blood cell response variables to determine which predictor was the best fit for each analysis. We found that temperature overwhelmingly outcompeted sampling day for all white blood cell variables except for the proportion of toxic neutrophils. Based on those results, we chose to use the 24-hour median temperature (C°) as a continuous variable to represent seasonal effects when analyzing principal components and band neutrophils, while including sampling day as a categorical predictor for toxic neutrophils. We used the median temperature that hellbenders experienced 24 hours prior to sampling for simplicity and because preliminary models indicated that it performed similarly to shorter and longer time intervals.

The abundance of leeches, leech bites, and trypanosomes varied widely among individuals, so we scaled each variable representing relative parasite abundance to have a mean of 0 and a standard deviation of 1 (Schielzeth, 2010) prior to using them as predictors in the models. Additionally, predictors related to the potential interactive effects of leeches and trypanosomes on the host and the interaction between parasite infection status and temperature on immune indices were included in the analysis.

We created linear mixed-effects models to assess how immune indices responded to parasites and seasonal changes in environmental conditions. Principal Component scores of white blood cells were used as response variables, while predictors included water temperature and parasite abundance as continuous variables, and infection status as a categorical variable. Infection status represented whether hellbenders were uninfected, coinfected, or only infected with trypanosomes (we found no individuals only infected with leeches, see results). However, we also considered whether the effect of parasites depended on the severity of infection which is why we also used parasite abundance in our initial model selection. However, we did not include both infection variables in the same models. We also included interactions between the abundance of leeches and trypanosomes as well as the interaction between infection status and temperature. Additionally, we performed separate analyses using the transformed and scaled values of band neutrophils and toxic neutrophils as response variables. We used the same predictors and interactions for these models but used sampling day instead of temperature in the model for toxic neutrophils because it outperformed temperature in preliminary models. We used the lme4 package for analyzing the principal components (WBC PC1 and PC2), band neutrophils, and toxic neutrophils with a normal distribution and identity link function. The individual PIT-tag ID was included as a random effect in all linear mixed-effects models to account for repeated measures on the same individuals.

We used generalized linear models to assess whether parasitic infection influenced nest outcomes. Prior research found that the majority of instances of whole-clutch cannibalism and nest failure can be confirmed within the first two months of embryonic development, prior to eggs hatching (Hopkins et al., 2023). Thus, we used parasite infection status or parasite abundance at oviposition in our models to determine whether the severity of infection directly following nest establishment could predict ultimate nest outcomes. Using infection data at mid-embryonic development produced comparable results (analysis not shown). We performed two separate analyses and used the binary outcomes of nest success/nest failure and nest success/complete clutch cannibalism as the response variables. We used the glm function in the base package for analyzing nest outcomes with a binomial distribution. For our predictors, we included the abundance of leeches, leech bites, and trypanosomes as well as infection status. We also included interactions between the abundance of leeches and the abundance of trypanosomes. We then used Akaike's Information Criterion corrected for small sample sizes (AICc) (Burnham and Anderson, 2002) to determine the best-fitting model.

Results

Over the two years of the study and multiple recaptures across the parental care period, we had 172 capture events and collected data from 58 individual adult male hellbenders with nests. We obtained a complete dataset including morphometrics, white blood cell profiles, and metrics to evaluate parasitic infection (leeches, leech bites, and trypanosomes) for 56 out of 58 individuals (Table 1, Table 2), and partial data for the remaining two individuals. Of the 58 nesting males, 25 were successful and 33 were failures. Of the 33 failures, 10 were confirmed whole clutch cannibals. White blood cell profiles of hellbenders varied depending on cell type and sampling interval (Table 1) and all fell within the range previously documented for this species (Hopkins et al., 2016; Bodinof Jachowski et al., 2024) and expected of amphibians more broadly (Davis and Maerz, 2023). Among the white blood cell differentials, lymphocytes were the most abundant cell type (median = 57%, range = 33 – 77%) followed by total neutrophils (normal, toxic, and band combined; median = 30%, range = 14 – 52%), eosinophils (median = 9%, range = 0.4 – 25%), basophils (median = 4%, range = 0 – 13%), and monocytes (median = 0.4%, range = 0 – 5%). Of their total white blood cell count, hellbenders also had considerable variation in the proportions of toxic (median = 15%, range = 3 – 40%) and band neutrophils (median = 5%, range = 0 – 27%). Likewise, we observed substantial variability in the abundance of leeches (range = 0 – 45), leech bites (range = 0 – 159), and trypanosomes (range = 0 – 323). Moreover, parasite prevalence and intensity of infection were variable across the paternal care period, which is demonstrated in Table 2.

Principal components 1 and 2 explained 81% of the variance (Table 3). White blood cell PC1 explained 50.4% of the variance and was positively loaded with neutrophils and eosinophils, but was negatively loaded with lymphocytes (Table 3). Given the inverse relationship between neutrophils and lymphocytes found in PC1, we consider the N:L ratio to be represented in this principal component score. White blood cell PC2 explained 30.7% of the variance and was positively loaded with basophils and neutrophils but was negatively loaded with eosinophils and lymphocytes.

Among the candidate models for WBC PC1, the top-ranking model representing the effects of water temperature and parasitic infection accumulated 0.63 of the AICc weight (Table 4). The delta AICc between the first and second-ranked 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. As water temperature increased, PC1 decreased ($\beta = -0.095$, $SE = 0.028$, $t = -3.397$, $p < 0.001$) (Fig. 2.A). Considering the correlations between the PC scores and white blood cell profiles (Supplemental Table 1), we interpret this statistical outcome to signify that the proportion of lymphocytes in circulation was greatest during warm temperatures. In contrast, the proportions of neutrophils and eosinophils were lowest during warm temperatures. Post-hoc visualization of the raw cell differentials against temperature confirmed these patterns (Supplemental Fig. 1). WBC PC1 was positively influenced by the number of leeches attached ($\beta = 0.708$, SE = 0.167, $t = 4.243$, $p < 0.001$). While trypanosome abundance alone did not significantly influence WBC PC1 ($\beta = 0.099$, SE = 0.108, $t = 0.914$, $p = 0.362$), as the abundance of trypanosomes and leeches increased together in co-infected individuals, there was a negative effect on PC1 ($\beta = -0.403$, SE = 0.137, $t = -2.940$, $p = 0.004$) (Fig. 3). The positive influence of leeches on PC1 values indicates that leech infection resulted in elevated neutrophils and eosinophils and lower lymphocytes. Interestingly, as the abundance of both leeches and trypanosomes increased in coinfected individuals, a negative shift occurred in PC1 values, indicating a greater number of lymphocytes in circulation and fewer eosinophils and neutrophils (Fig. 3).

The top-ranked model for WBC PC2, which only included temperature as a predictor, had an AICc weight of 0.83 and a delta AIC greater than 2, confirming it out-competed the lower-ranking candidate models (Table 5). WBC PC2 increased as temperature increased ($\beta = 0.100$, SE = 0.022, $t = 4.511$, $p < 0.001$) (Fig. 2.B). After evaluating the correlations between white blood cell profiles and PC2, we found that eosinophils and basophils had strong relationships with the PC scores while neutrophils and lymphocytes were not well represented in the PC2 scores (Supplemental Table 1). Thus, we interpret our model to indicate that as temperature increased, basophils increased while eosinophils decreased in circulation. Post-hoc visualization of the raw cell differentials against temperature confirmed these patterns (Supplemental Fig. 1).

The top-ranked model for band neutrophils also only included temperature as a predictor and had an AICc weight of 0.55. However, the second-ranked model was the null model which had a weight of 0.35, and the delta AICc between the two models was less than 2 (Table 6), indicating that temperature provided a modest improvement in model fit compared to the null model, but there remains considerable variation in the data that our more complex models were unable to explain. The proportion of band neutrophils increased in circulation as temperature decreased ($\beta = -0.065$, SE = 0.022, $t = -2.999$, $p = 0.003$) (Fig. 4).

To qualitatively summarize the effects of water temperature on various cell types, we found that as temperatures increased from approximately 5° C to 20° C, the proportion (on average) of neutrophils decreased by 14% and the proportion of eosinophils decreased by 46%. Conversely, the proportion (on average) of lymphocytes increased by 8% and the proportion of basophils increased by 101%. Lastly, the proportion of band neutrophils decreased by 80% (Supplemental Fig. 1).

The top-ranked model for toxic neutrophils only included sample day as a predictor and had an AICc weight of 0.86 with a delta AIC greater than 2, confirming it out-competed the lower-ranking candidate models (Table 7). Compared to values during oviposition, the proportion of toxic neutrophils did not significantly differ at mid-embryonic development ($\beta = 0.271$, SE = 0.173, $t = 1.570$, $p = 0.119$) or at hatching ($\beta = 0.254$, SE = 0.179, $t = 1.417$, $p = 0.159$). The proportion of toxic neutrophils was significantly lower at spring emergence compared to the rest of the parental care period ($\beta = -0.574$, SE = 0.213, $t = -2.688$, $p = 0.008$) (Fig. 5, Supplemental Table 2).

For the candidate models predicting nest success, the null was the top-ranking model with an AICc weight of 0.26. The following three candidate models accumulated similar AICc weights. The low delta AICc values (Supplemental Table 3) indicate that there was considerable uncertainty in the model selection and suggest that the predictors representing parasite infection are unlikely to explain the likelihood of nest success.

For the candidate models predicting the likelihood of whole clutch cannibalism, the top-ranking model included the abundance of parasites and the interaction between the leeches and trypanosomes as the predictors which had an AICc weight of 0.54. The second-ranked model was the null with a weight of 0.20 and delta AICc of less than 2 (Supplemental Table 4), indicating that predictors included in the top model might be relevant but did not substantially improve the overall model fit. The likelihood of whole clutch cannibalism was not significantly influenced by the abundance of trypanosomes ($\beta = -0.954$, SE = 0.981, $z = -0.972$, $p = 0.331$), leeches ($\beta = 1.230$, SE = 1.046, $z = 1.176$, $p = 0.240$), or the interaction between the two parasites ($\beta = -4.140$, SE = 2.500, $z = -1.656$, $p = 0.098$). Considering the results from both analyses exploring nest fate, we found no evidence to indicate that parasite infection influences the likelihood of nest success or whole clutch cannibalism (Fig. 6 A,B).

Discussion

Parasites and pathogens contribute to global amphibian declines by impacting host survival and fitness-related traits, but parasites that are not directly linked to high morbidities or mortality in amphibians draw comparably less attention than these more dramatic examples (Bower et al., 2019). Additionally, infectious diseases tend to cause greater impacts on small populations of threatened species than more abundant species (May, 1988), emphasizing the need to understand the sublethal effects of parasites in species of conservation concern. To effectively do so in wild amphibian populations, however, it is crucial to understand how immune indices shift with environmental conditions to distinguish pathological effects from natural variation. Here, we used white blood cell profiles to monitor the health of male eastern hellbender salamanders across their ~8-month-long paternal care period to evaluate the direct impacts of parasites and coinfection on host physiology and investigate if parasite infection negatively influences nest success. We demonstrated that reference values for white blood cell profiles of hellbenders from healthy populations vary with seasonal temperatures. We found that coinfection of leeches and trypanosomes is associated with immune responses in hellbenders, but we found no evidence to suggest that parasite infection or coinfection affects nest success.

We observed changes in hellbender white blood cell profiles that correlated with seasonal fluctuations in environmental temperatures. As predicted, the proportion of circulating basophils and lymphocytes was highest during warmer periods of the paternal care period. Basophils are considered short-lived cells and migrate out of circulation and into tissues upon activation (Eberle and Voehringer, 2016) similar to how lymphocytes typically respond (Latimer et al., 2003). The seasonal dynamics of basophil proliferation in amphibians, however, remain underexplored in the literature and our identification of a correlation between temperature and circulating basophils represents a novel contribution to our understanding of amphibian immunology. In contrast to basophils, hellbenders had lower proportions of circulating neutrophils and eosinophils in warmer periods of the paternal care period. Similar fluctuations in white blood cells in response to temperature have been observed in other amphibians (Maniero and Carey, 1997; Raffel et al., 2006*a*). Our findings also align with prior research on hellbenders where N:L ratios and eosinophils were lower in warmer compared to colder temperatures (Bodinof Jachowski et al., 2024).

Although cold temperatures can exert immunosuppressive effects on some amphibians (Rollins-Smith and Woodhams, 2012; Herczeg et al., 2021), our study highlights the potential for physiological adaptations to cold conditions, which may serve to prime the cellular immune system when disease susceptibility is heightened (Greenspan et al., 2017). Characteristics of the adaptive immune system in ectotherms, such as lymphocyte production, antibody responses, and complement activity, are reduced in cold temperatures, likely because they are metabolically expensive (Rijkers et al., 1980; Cooper et al., 1992; Maniero and Carey, 1997; Rollins-Smith and Woodhams, 2012). To overcome this, ectotherms rely on elements of their innate immune responses to reinforce their immune defenses by increasing circulating neutrophils (Maniero and Carey, 1997; Raffel et al., 2006*a*) and phagocytic activity (Pxytycz and Jozkowicz, 1994). Considering the phagocytic capabilities of neutrophils, and to a lesser extent, eosinophils (Latimer et al., 2003), the observed increase in the proportion of circulating neutrophils and eosinophils in colder temperatures could be an adaptative strategy that protects hellbenders when other immune defenses are less active (Maniero and Carey, 1997), or to prepare them for an increased risk of encountering pathogens in spring (Pxytycz and Jozkowicz, 1994).

The significance of neutrophils in seasonal protective immunity is further emphasized by our identification of seasonal shifts in the morphological characteristics of neutrophils, signifying seasonal differences in immune cell recruitment. Toxic neutrophils are mature cells that exhibit toxic changes due to accelerated production, causing abnormal maturation, while band neutrophils are immature cells that increase in circulation when immune demand exceeds neutrophil storage pools (Stacy et al., 2022). The proportion of band neutrophils in circulation increased as temperature decreased, which has been observed in other amphibian species in response to long-term laboratory exposure to low temperatures (Maniero and Carey, 1997). Moreover, we found that the proportion of toxic neutrophils in circulation remained the same across oviposition, mid-embryonic development, and hatching but significantly decreased during larval emergence. This observation suggests that rapid demand for circulating neutrophils subsides in the spring as water is warming. When considering the concurrent trends of both band and toxic neutrophils, our results imply a prolonged demand for neutrophils across the paternal care period, with a notable increase during the coldest period prior to overwintering, that may serve to maintain immunocompetence throughout the year. Taken together, these results underscore the possible critical role of neutrophils in seasonal protective immunity in hellbenders and suggests further work is needed to understand their seasonal immunological role in other amphibian species.

It is plausible that factors other than temperature may have contributed to some of the seasonal effects that we observed, and the nature of our descriptive field study prevents us from identifying possible contributions of other seasonal factors. Raffel et al. (2006*a*) suggested that there may be seasonal variations in circulating white blood cells that are independent of temperature. Our findings on toxic neutrophils underscore a seasonal trend that supports this possibility. Furthermore, seasonal fluctuations in behavior and physiology linked to reproduction could impact immune indices at different times of the year. For example, circannual rhythms in hormones such as androgens and glucocorticoids, known to modulate immune function, might contribute to seasonal variations in white blood cells (Zapata et al., 1992). Notably, seasonal variations in these hormones have been observed in male hellbenders (Case et al., 2024), providing support for this possibility. Future experimental work is required to disentangle the relative influence of temperature and other seasonally variable factors on white blood cells.

Hellbenders exhibited notable shifts in white blood cell profiles in response to parasitic infection, but this response depended on which parasites infected the host. As predicted, when the abundance of leeches increased, the proportion of neutrophils and eosinophils increased, and lymphocytes decreased. These patterns were similar to those observed in two prior studies when samples were collected in the summer prior to the breeding season (DuRant et al., 2015; Hopkins et al., 2016). Our findings also suggest the likelihood that parasites caused the increase in N:L ratios and eosinophils compared to uninfected individuals observed in a previous landscape-scale study, which could not disentangle the effects of habitat quality from parasitic infection because they were confounded (Bodinof Jachowski et al., 2024). By focusing on a narrow range of habitat quality in a single stream in our study, we were able to isolate the impact of parasites on white blood cell counts from other habitat-related factors. In contrast to the results for leeches, we did not find evidence that trypanosomes alone altered white blood cell profiles. Our results suggest that in the absence of leech infection, trypanosome infection was not associated with a strong immune response in hellbenders, regardless of infection intensity. This could be because most aquatic trypanosomes are nonpathogenic to fish (Woo, 1994; Woo, 2006) and amphibian hosts (Omonona and Ekpenko, 2011). Alternatively, other species of trypanosomes have well-documented strategies for evading the hosts' immune responses (Oladiran and Belosevic 2012), which is an equally plausible explanation. In notable contrast to our findings, Hopkins et al. (2016), found a strong relationship between trypanosome presence and increased circulating eosinophils and N:L ratios in hellbenders. The difference between our findings and theirs could be attributed to the method used to represent trypanosome infections. Whereas we used a semi-quantitative measure of parasite abundance to assess the severity of infection, they used a presence/absence metric to indicate the incidence of infection. However, our metric of trypanosome infection severity could be even more useful if validated against qPCR in future studies.

We detected an unexpected interaction between leeches and trypanosomes in coinfected hellbenders. That is, as the abundance of both leeches and trypanosomes increased concurrently, the proportion of lymphocytes increased in circulation while eosinophils and neutrophils decreased. In other host-parasite systems, the temporal sequence of parasite exposure can strongly influence the host's response and disease progression (Herczeg et al., 2021), which might explain the difference in white blood cell profiles when evaluating the effect of leech abundance alone versus the interaction between leech and trypanosome abundance. Leeches, acting as vectors for trypanosomes in hellbenders, engage in active parental care behaviors, delivering their offspring to their first blood meal (Blumenthal and Hopkins, unpublished). Consequently, hellbender hosts likely mount an immune response to multiple biting leech vectors during the initial transmission of the trypanosome parasites. Repeated exposure to salivary antigens from leeches may prompt the host to develop cellular and humoral reactions, altering the site of leech attachment, which may promote rejection of the ectoparasite (Wikel, 1982). Leeches have been linked to other immunological responses in hellbenders indicative of inflammation and infection such as increased total plasma proteins as well as increased bactericidal capacity of plasma (Hopkins et al., 2016). While elevated eosinophils, in particular, are often an indicator of parasitic disease states (Allender and Fry, 2007; Bain and Harr, 2022), both neutrophils and eosinophils are recruited into circulation in response to inflammation and infection (Latimer et al., 2003; Campbell, 2015). Thus, the observed shifts in neutrophils, eosinophils, and lymphocytes potentially indicate an active innate immune response to leeches, recruiting inflammatory cells to the site of ectoparasite exposure (Andrade et al., 2005). After the initial leech infection, trypanosomes likely proliferate within coinfected hellbenders and the host's immune response undergoes a shift, which we hypothesize contributes to the observed interaction with leeches. The reduction in neutrophils and eosinophils might suggest an attempt to mitigate the inflammatory response influenced by the leech vector, possibly to prevent excessive tissue damage. Alternatively, it could indicate immune exhaustion where the emigration of neutrophils and eosinophils from the vasculature to the tissues exceeds the replacement rate in the blood (Latimer et al., 2003) and could be a sign of severe, acute inflammatory disease (Bain and Harr, 2022). Lymphocytes are the primary cells in the host's adaptive immune response to infection (Janeway et al., 2001) and perform a variety of immune functions, including immunoglobin production and antibody-dependent cell-mediated cytotoxicity (Cambell, 2015; Wright and Whitaker, 2001). Thus, the observed increase in circulating lymphocytes might suggest acquired immunity as the severity of leech and trypanosome infection increases concurrently. In mammalian systems that have been studied more extensively, hosts rely on B and T lymphocytes for protection against trypanosomes and eventual elimination of the parasites, with B cells particularly responsible for clearing the parasites from blood during infection (Mansfield and Paulnock, 2005). Therefore, coinfected hellbender hosts may exhibit a similar immune response to trypanosome infection, though it is unclear as to why this response would depend on the presence of the leech vector. Our reliance on white blood cell differentials alone limited our ability to draw conclusions regarding the observed immunological patterns, but future studies that include complete white blood cell counts and cytokines could enhance our understanding of the underlying mechanism.

Contrary to our predictions, we did not identify relationships between parasite infection and basophils, band neutrophils, or toxic neutrophils. Basophils offer protective immunity against both ecto- and endoparasites (Eberle and Voehringer, 2016; Karasuyama et al., 2021) and may play a surveillance role by recruiting eosinophils during parasite infection as they do in other vertebrates (Wright and Whitaker, 2001; Campbell, 2015). Basophils are short-lived cells that migrate out of circulation when activated (Eberle and Voehringer, 2016), but the proportion of circulating basophils differs among species (Megarani et al., 2020; Davis and Maerz, 2023). Because basophils make up a relatively small proportion of circulating white blood cells in hellbenders (Hopkins et al., 2016; Bodinof Jachowski et al., 2024), we may have failed to capture sufficient variation in basophil proportions for a detectable response to parasite infection against the backdrop of substantial seasonal variation in basophils. Similarly, we were unable to directly link elevated band and toxic neutrophils to parasitic infection, possibly due to a lag time in immune responses. For example, Slack et al., 2024, demonstrated that coinfected hellbenders do not exhibit symptoms of regenerative anemia until ~30 days following peak leech attachment. Additionally, hellbenders may exhibit signs of immune recruitment in response to other infectious pathogens that we did not evaluate; leeches may transmit viruses, fungi, and bacteria to hellbenders, as they do in other systems (Sawyer, 1986; Mock, 1987; Barta and Desser, 1989; Raffel et al., 2006*b*). Toxic neutrophils are known to occur as part of the immune response of amphibians to gram-negative bacteria found in aquatic environments, such as *Vibrio* and *Aeromonas* (Bain and Harr, 2022). We encourage future research to determine whether leeches transmit other pathogens to hellbenders and whether this has the potential to contribute to some of the immune responses that we observed.

While parasitic coinfection influenced hellbender physiology, we found no evidence relating parasites to nest failure or whole-clutch cannibalism. In other systems, parasites can modulate the behavior and metabolic demands of the host (Haye and Ojeda, 1998; Dick et al., 2010) even leading to increased rates of cannibalism (Bunke et al., 2015). Additionally, parasites can reduce mating success, fecundity, and offspring viability in other hosts (Lehmann, 1993; Hasik and Siepielski, 2022). While our research found no evidence to indicate that parasites are a direct catalyst for increased cannibalism or nest failure in hellbenders, the impacts of infection on other fitness-related traits and behaviors are complex and worthy of future investigation. For example, parasite infection in other vertebrates is associated with reduced reproductive and paternal behaviors (Saumier et al., 1986; Henderson et al., 1995; but see Klein, 2003). Given that we recently found coinfection with leeches and trypanosomes was associated with multiple symptoms of anemia in hellbenders (Slack et al., 2024), it seems possible that parasites could modulate a shift from paternal care (e.g., tail fanning of eggs; O'Brien et al., 2024) to self-maintenance behaviors in hellbenders (e.g., side to side rocking to increase skin oxygenation; O'Brien et al., 2024) which could affect hatching success of eggs. Therefore, future studies that simultaneously examine the effect of leech and trypanosome infection on red blood cell physiology, immune responses, and parental care behaviors would be valuable.

By investigating the impacts of parasitic infection and environmental conditions on hellbender immunology, our research offers valuable insights into the basic physiology of this imperiled species. Our findings reveal seasonal shifts in hellbender white blood cell profiles, including indicators of immune cell recruitment that may reflect immunological adaptations to fluctuating temperatures. Thus, our work provides valuable reference values for the species. Additionally, we demonstrated that coinfecting parasites influence hellbender physiology, but their immunological responses were complex and depended on infection intensity of both parasites. Future experimental research could help disentangle some of these complexities, and help isolate the effect of temperature from other seasonal factors that could affect hellbender immunology. More broadly, our research addresses fundamental knowledge gaps related to amphibian immunology and host-parasite interactions, with broader implications for understanding amphibian population declines in the Anthropocene.

Acknowledgments

This work was supported by the Virginia Department of Wildlife Resources [EP2443089 and DWR-403-2023-0011] and the National Science Foundation [IOS-1755055]. We thank the Global Change Center at Virginia Tech and the Fralin Life Sciences Institute for maintaining the research infrastructure that made this project possible. We appreciate Dr. Ignacio Moore and Dr. Luis Escobar for their guidance and support, as well as their insightful comments that improved the manuscript. We are particularly grateful for J.D. Kleopfer's support of our research and for his efforts to protect hellbenders and other herpetofauna in Virginia. We appreciate the many individuals who assisted us in the field and the lab, including R. O'Brien, E. Fralin, K. Hahler, A. Patel, A. Holloway, J. Hargett, and E. Davis. We are thankful for the assistance of Dr. Thomas Cecere who assisted with capturing cellular images in Figure 1. All research was conducted under Virginia Department of Wildlife Resources collection permits and complied with Virginia Tech Institutional Animal Care and Use protocols 18-186 and 19-147.

Data Availability

The data underlying this article will be shared on reasonable request to the corresponding author.

References

Allender M.C. and M.M. Fry. 2008. Amphibian hematology. Vet Clin N Am Exot Anim Pract 11: 463–480

Andrade B.B., C.R. Teixeira, A. Barral, and M. Barral-Netto. 2005. Haematophagous arthropod saliva and host defense system: a tale of tear and blood. An Acad Bras Cienc 77:665–693

Bain P. and K.E. Harr. 2022. Hematology of Amphibians. Pp. 1228–1232 in Schalm's Veterinary Hematology, 7th ed. Wiley: Hoboken, New Jersey, USA

Barta J.R. and S.S Desser. 1989. Development of *Babesiosoma stableri* (Dactylosomatidae; Adeleina; Apicomplexa) in its leech vector (*Batracobdella picta*) and the relationship of the dactylosomatids to piroplasms of higher vertebrates. J Protozool 36: 241–253.

Bishop S.C. 1941. The Salamanders of New York. New York State Museum Bulletin. 324.

Blaustein A.R., D.B. Wake, and W.P Sousa. 1994.Amphibian declines: Judging stability, persistence, and susceptibility or populations to local and global extinctions. Conserv Biol 8:60–71

Blaustein A.R. 1994. Chicken Little or Nero's fiddle? A perspective on declining amphibian populations. Herpetologica 50:85–97

Blaustein A.R. and D.B. Wake. 1995. The puzzle of declining amphibian populations. Sci Am 272:52–57

Blaustein A.R. and J.M. Kiesecker. 2012. Complexity in conservation: lessons from the global decline of amphibian populations. Ecology Letters 5:597–608

Blaustein A.R., S.C. Walls, B.A. Bancroft, J.J. Lawler, C.L. Searle, and S.S. Gervasi. 2010. Direct and Indirect Effects of Climate Change on Amphibian Populations. *Diversity* 2: 281-313.

Bodinof Jachowski C.M., V. Alaasam, A. Blumenthal, A. Davis, and W.A. Hopkins. 2024. The habitat quality paradox: Loss of riparian forest cover leads to decreased risk of parasitism and improved body condition in an imperiled amphibian. Conserv Physiol 12:coad101

Bower D.S., L.A Brannelly, C.A. McDonald, R.J. Webb, S. E. Greenspan, M. Vickers, M.G. Gardner, and M.J. Greenlees. 2019. A review of the role of parasites in the ecology of reptiles and amphibians. Austral Ecol 44: 433-448.

Brooks G.C., W.A. Hopkins, and H.K. Kindsvater. 2024. Concurrent threats and extinction risk in a long-lived, highly fecund vertebrate with parental care. Ecol Appl in press.

Bunke M., M.E. Alexander, J.T.A. Dick, M.J. Hatcher, R. Paterson, and A.M. Dunn. 2015. Eaten alive: cannibalism is enhanced by parasites. R. Soc. open sci 2:e140369

Burgmeier N.G., S.D. Unger, J.L. Meyer, T.M. Sutton, and R.N. Williams. 2011. Health and habitat quality assessment for the eastern hellbender (*Cryptobranchus alleganiensis alleganiensis*) in Indiana, USA. J Wildlife Dis 47: 836–848.

Burnham K.P. and D. R. Anderson. 2002. Model Selection and Multimodel Interface, A Practical Information-Theoretic Approach, 2nd ed. Springer-Verlag, New York, USA

Button S.T., J.J. Hallagan, C.M.B. Jachowski, B.F. Case, J. Groffen, and W.A. Hopkins. 2020a. Weathering the storm: Improving the availability and stability of artificial shelters for hellbender salamanders. River Res Appl 36:1944–1953.

Button S.T., C.M.B. Jachowski, B.F. Case, J. Groffen, and W.A. Hopkins. 2020b. The influence of multiscale habitat variables and population density on artificial shelter use by hellbenders (*Cryptobranchus alleganiensis*). Herpetologica 76:355–365.

Campbell T.W. 2015. Exotic Animal Hematology and Cytology, 4th ed. Blackwell Publishing, Ames, IA, USA

Coe B.C., D.V. Drewett, C.M.B. Jachowski, and W.A. Hopkins. 2016. *Cryptobranchus alleganiensis alleganiensis* (Eastern Hellbender). Terrestrial movement. Herpetological Review 47:99-100.

Cohen J.M., D.J. Civitello, M.D. Venesky, T.A. McMahon, and J.R. Rohr. 2019. An interaction between climate change and infectious disease drove widespread amphibian declines. Glob. Chang Biol. 25:927-937.

Cooper E.L., R.K. Wright, A.E. Klembau, and C.T. Smith. 1992. Hibernation alters the frog's immune system. Cryobiology. 29: 616–631.

da Silva G.S.F., M.L. Glass, and L.G.S. Branco. 2013. Temperature and respiratory function in ectothermic vertebrates. J Therm Biol 38:55–63.

Davis A. K., and C. Golladay. 2019. A survey of leukocyte profiles of red-backed salamanders from Mountain Lake, Virginia, and associations with host-parasite types. Comp Clin Path 28: 1743-1750.

Davis A.K. and J.C. Maerz. 2023. Assessing Leukocyte Profiles of Salamanders and Other Amphibians: A Herpetologists' Guide. Methods Mol Biol. 2562:443-458.

Davis A.K, D.L. Maney, and J.C. Maerz. 2008. The use of leukocyte profiles to measure stress in vertebrates: a review for ecologist. Funct Ecol 22:760-772.

Davis A.K. and W.A. Hopkin. 2013. Widespread trypanosome infections in a population of eastern hellbenders (*Cryptobranchus alleganiensis alleganiensis*) in Virginia, USA. Parasitol Res 112:453–456.

Dick J.T.A, M. Armstrong, H.C. Clarke, K.D. Farnsworth, M.J. Hatcher, M. Ennis, A. Kelly, and A.M Dunn. 2010. Parasitism may enhance rather than reduce the predatory impact of an invader. Biol Lett 6:636–638.

DuRant S.E., W.A. Hopkins, A.K. Davis, and L.M. Romero. 2015. Evidence of ectoparasite-induced endocrine disruption in an imperiled giant salamander, the eastern hellbender (*Cryptobranchus alleganiensis*). J Exp Biol 218:2297–2304.

Eberle J.U. and D. Voehringer. 2016. Role of basophils in protective immunity to parasitic infections. Semin Immunopathol 38:605–613.

Forzán M.J., J. Heatley, K.E. Russell, and B. Horney. 2017. Clinical pathology of amphibians: a review. Vet Clin Pathol 46:11-33.

Graham S.P., E.C. Soehren, G.R. Cline, C.M. Schmidt, W.B. Sutton, J.R. Rayburn, S.H. Stiles, and J.A. Stiles. 2011. Conservation status of hellbenders (*Cryptobranchus alleganiensis*) in Alabama, USA. Herpetol Conserv Biol 6:242–249.

Green D.M., M.J. Lannoo, D. Lesbarrères, and E. Muths. 2020. Amphibian population declines: 30 years of progress in confronting a complex problem. Herpetol. 76:97-100.

Greenspan S.E., D.S. Bower, R.J. Webb, L. Berger, D.R. Rudd, L. Schwarzkopf, and R.A. Alford. 2017. White blood cell profiles in amphibians help to explain disease susceptibility following temperature shifts. Dev Comp Immunol 77:280–286.

Guimond R.W. and V.H. Hutchison. 1973. Aquatic respiration: an unusual strategy in the hellbender *Crytobranchus alleganiensis alleganiensis* (Daudin). Science 182:1263-1265.

Harshman L.G. and A.J. Zera. 2007. The cost of reproduction: The devil in the details. Trends Ecol Evol 22:80-86.

Hasik A.Z. and A. M. Siepielski. 2022.Parasitism shapes selection by drastically reducing host fitness and increasing host fitness variation. Biol Lett 8:20220323

Haye P.A and F.P. Ojeda. 1998. Metabolic and behavioral alterations in the crab *Hemigrapsus crenulatus* (Milne-Edwards 1837) induced by its acanthocephalan parasite *Profilicollis antarcticus* (Zdzitowiecki 1985). J Exp Mar Biol Ecol 228:73–82

Herczeg D., J. Ujszegi, A. Kásler, D. Holly, and A. Hettyey. 2021. Host–multiparasite interactions in amphibians: a review. Parasites Vectors 14:296

Henderson D., D.M. Bird, M.E. Rau, and JJ. Negro. 1995. Mate choice in captive American kestrels, *Falco sparverius* parasitized by a nematode, *Trichinella pseudospiralis*. Ethol;101:112–120.

Hoffman M., C. Hilton-Taylor, A. Angulo, M. Böhm, T.M. Brooks, S.H.M. Butchart, K.E. Carpenter, J. Chanson, B. Collen, and N.A. Cox. 2010. The impact of conservation on the status of the world’s vertebrates. Science 330:1503–1509.

Hopkins W.A. 2007. Amphibians as Models for Studying Environmental Change. ILAR J 48:270–277

Hopkins W.A. and S.E. DuRant. 2011. Innate immunity and stress physiology of Eastern Hellbenders (*Cryptobranchus alleganiensis*) from two stream reaches with differing habitat quality. Gen Comp Endocrinol 174:107–115.

Hopkins W.A., W.E. Moser, D.W. Garst, D.J. Richardson, C.I. Hammond, and E.A. Lazo-Wasem. 2014. Morphological and molecular characterization of a new species of leech (Glossiphoniidae, Hirundinida): implications for the health of its imperiled amphibian host (*Cryptobranchus alleganiensis*). Zookeys 378:83–101.

Hopkins W.A., J.A. Fallon, M.L. Beck, B.H. Coe, and C.M.B. Jachowski. 2016. Haematological and immunological characteristics of eastern hellbenders (*Cryptobranchus alleganiensis alleganiensis*) infected and co-infected with endo- and ectoparasites. Conserv Physiol 4:cow002.

Hopkins W.A., B.F. Case, J. Groffen, G.C. Brooks, C.M. Bodinof Jachowski, S.T. Button, J.J. Hallagan, R.S.M. O'Brien, and H.K. Kindsvater. 2023. Filial Cannibalism Leads to Chronic Nest Failure of Eastern Hellbender Salamanders (*Cryptobranchus alleganiensis*). Am Nat 202:92–106.

Huang W.S. 2008. Predation risk of whole clutch filial cannibalism in a tropical skink with maternal care. Behav Ecol 19:1069–1074

Jachowski C.B. and W.A. Hopkins. 2018. Loss of catchment-wide riparian forest cover is associated with reduced recruitment in a long-lived amphibian. Biol Conserv. 220:215–227.

Jachowski C.M.B., B.E. Ross, and W.A. Hopkins. 2020. Evaluating artificial shelter arrays as a minimally invasive monitoring tool for the hellbender *Cryptobranchus alleganiensis*. Endanger Species Res 41:167–181

Janeway C.A., P. Travers, M. Walport, and M.J. Schlomchik. 2001. Immunobiology. The Immune System in Health and Disease, 5th ed. Garland Publishing, New York, USA

Karasuyama H., S. Shibata, S. Yoshikawa, and K. Miyake. 2021. Basophils, a neglected minority in the immune system, have come into the limelight at last. Int Immunol 33:809–813.

Klein S.L. 2003. Parasite manipulation of the proximate mechanisms that mediate social behavior in vertebrates. Physiol Behav 79:441–449

Latimer K.S., E.A. Mahaffey, and K.W. Prasse. 2003. Duncan & Prasse's Veterinary Laboratory Medicine: Clinical Pathology. Blackwell Publishing, Ames, IA.

Lehmann T. 1993. Ectoparasites: direct impact on host fitness. Parasitol. Today 9, 8-13.

Makowsky R., L.A. Wilson, T.K. Pauley. 2010. Sexual dimorphism in the Eastern Hellbender (*Cryptobranchus alleganiensis alleganiensis*). Herpetol Conserv Biol 5: 44–48.

Maniero D. and C. Carey. 1997. Changes in selected aspects of immune function in the leopard frog, Rana pipiens, associated with exposure to cold. J Comp Physiol B: Physiol 167:256-263.

Mansfield J.M and D.M. Paulnock. 2005. Regulation of innate and acquired immunity in African trypanosomiasis. Parasite Immunol 27: 361–371.

May R.M. 1988. Conservation and Disease. Conserv Biol 2: 28-30.

Megarani D.V., A.B. Hardian, D. Arifianto, C.M. Santosa, and S.I.O Salasia. 2020. Comparative Morphology and Morphometry of Blood Cells in Zebrafish (*Danio rerio*), Common Carp (*Cyprinus carpio carpio*), and Tilapia (*Oreochromis niloticus*). J Am Assoc Lab Anim Sci 59 67:3-680.

Mock B.A. 1987. Longitudinal patterns of trypanosome infections in red spotted newts. J Parasitol 73: 730–737

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

Nordling D., M. Andersson, S. Zohari, and L. Gustafsson. 1998. Reproductive effort reduces specific immune response and parasite resistance, Proc Biol Sci 265:1291-1298.

Norte A.C., P.M. Araújo, H.L. Sampaio, J.P. Sousa, and J.A. Ramos .2009. Haematozoa infections in a Great Tit Parus major population in Central Portugal: Relationships with breeding effort and health. Ibis 151:677-688.

O'Brien R.S. 2023. Behavior and conservation: hellbender reproductive behavioral responses to poor water quality and the value of private lands research for conservation outreach. Doctoral dissertation, Virginia Tech.

O'Brien R.S.M., J. Groffen, A. Dayer, and W.A. Hopkins. 2024. Pre-breeding, Courtship, and Mating Behaviors of Wild Eastern Hellbenders (*Cryptobranchus alleganiensis alleganiensis*). Herpetologica 80.

Oladrian A. and M. Belosevic. 2012. Immune Evasion Strategies of Trypanosomes: A Review. J Parasitol 98:284-292.

Omonona A.O. and V. Ekpenko. 2011. Haematology and prevalence of blood parasites of the common frog (*Rana temporaria*) in the tropical environment. J Vet Med Anim Health 3:14–20.

Petranka J.W. 1998. Salamanders of the United States and Canada. Smithsonian Institution Press, Washington, D.C., USA 587p.

Pugh M.W., M. Hutchins, M. Madritch, L. Siefferman, and M.M. Gangloff. 2016. Land-use and local physical and chemical habitat parameters predict site occupancy by hellbender salamanders. Hydrobiologia 770:105–116.

Pxytycz B. and A. Jozkowicz. 1994. Differential effects of temperature on macrophages of ectothermic vertebrates. J Leukoc Biol 56:729–731

Pyron R.A. and J.J. Wiens. 2011. A large-scale phylogeny of Amphibia including over 2800 species, and a revised classification of extant frogs, salamanders, and caecilians. Mol Phylogen Evol 61:543–583.

R Core Team 2022. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna. http://www.R-project.org/.

Raffel T.R, J.R Rohr, J.M. Kiesecker, and P.J. Hudson. 2006a. Negative effects of changing temperature on amphibian immunity under field conditions. Funct Ecol 20:819–828.

Raffel T.R., J.R. Dillard, and P.J. Hudson. 2006b. Field evidence for leech-borne transmission of amphibian *Ichthyophonus sp.* J Parasitol 92:1256–1264.

Rijkers T., E.M.H. Frederix-Wolters, and W.B. Van Muiswinkel. 1980. The immune system of cyprinid fish. Kinetics and temperature dependence of antibody-producing cells in carp (*Cyprinus carpio*) Immunol 41: 91–97

Rollins-Smith L.A. and D.C. Woodhams. 2012. Amphibian Immunity. In: G. Demas, R. Nelson (Eds.) Ecoimmunlogy. Oxford University Press, New York, NY, pp. 92-143.

Saumier M.D., M.E. Rau, and D.M. Bird. 1986. The effect of *Trichinella pseudospiralis* infection on the reproductive success of captive American kestrels (*Falco sparverius*). Can J Zool 64:2123 –2128.

Sawyer R.T. 1986. Leech Biology and Behavior, Vol II, Feeding Biology, Ecology, and Systematics. Clarendon Press, Oxford, UK, pp 419–793.

Schielzeth H. 2010. Simple means to improve the interpretability of regression coefficients. Methods Ecol Evol 1:103–113.

Slack K.L., J. Groffen, and W.A. Hopkins. 2024. The Influence of Environmental Conditions and Coinfection by Blood-feeding Parasites on Red Blood Cell Physiology of an Ectothermic Host. Ecol Evol Phys. In Press.

Smith C., and R.J. Wootton. 1995. The costs of parental care in teleost fishes. Rev Fish Biol Fisheries 5:7–22.

Sokal R.R. and F.J. Rohlf. 1995. Biometry: The Principles and Practice of Statistics in Biological Research. 3rd ed. W.H. Freeman, New York, NY 887 pp.

Stacy N.I, C. Hollinger, J.E. Arnold, C. Cray, H. Pendl, P.J Nelson, and J.W. Harvey. 2022. Left shift and toxic change in heterophils and neutrophils of non-mammalian vertebrates: A comparative review, image atlas, and practical considerations. Vet Clin Pathol 51:18–44.

Taber C. A., R.F. Wilkinson, and M.S. Topping. 1975. Age and growth of hellbenders in Niangua river, Missouri. Copeia 4:633–639.

United States Fish and Wildlife Service. 2018. Species status assessment report for the eastern hellbender (*Cryptobranchus alleganiensis alleganiensis*). 104 pp.

Vitt L.J., J.P. Caldwell, H.M. Wilbur, and D.C. Smith. 1990. Amphibians as harbingers of decay. BioScience 40:418.

Wake D.B. 1991. Declining amphibian populations. Science 253:860.

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

Wikel S.K. 1982. Immune responses to arthropods and their products. Ann Rev Entomol 27:21–48

Wilber M.Q., C.J. Briggs, and P.T. Johnson. 2020. Disease's hidden death toll: Using parasite aggregation patterns to quantify landscape-level host mortality in a wildlife system. J Anim Ecol 89:2876–2887

Woo P.T.K. 1994. Flagellate Parasites of Fish. Pp. 1–8 in J.P. Kreier, ed. Parasitic Protozoa, 2nd ed., Vol. VIII. Academic Press, New York, NY.

Woo P.T.K. 2006. Diplomonadida (Phylum Parabasalia) and Kinetoplastea (Phylum Euglenozoa). Pp. 46–114 in P.T.K. Woo ed. Fish Diseases and Disorders, Vol. 1: protozoan and metazoan infections. CABI, Wallingford, UK.

Wright K.M. and B.R. Whitaker. 2001. Amphibian Medicine and Captive Husbandry. Krieger Publishing Company, Malabar, FL, USA.

Zapata A.G., A. Varas, and M. Torroba. 1992. Seasonal variations in the immune system of lower vertebrates. Immunol Today 13:142-147

Tables

Table 1 Characteristics of white blood cell parameters of adult male eastern hellbenders (*Cryptobranchus alleganiensis*) across the 8-month parental care period from a population in southwest Virginia, USA.

Parameter	Sampling day											
	Oviposition N=56			**Mid-Embryonic** N=48			**Hatching** N=44			**Emergence** N=25		
	Uninfected N=9	Tryp Only N=23	Coinfected N=24	Uninfected N=5	Tryp Only N=23	Coinfected N=20	Uninfected N=5	Tryp Only N=25	Coinfected N=13	Uninfected N=3	Tryp Only N=15	Coinfected N=7
Neutrophils %	25.9 ± 2.2	26.1 ± 1.7	32.2 ± 1.7	27.3 ± 2.0	31.1 ± 1.7	32.9 ± 1.6	23.9 ± 2.8	29.6 ± 1.5	34.0 ± 2.1	27.8 ± 2.6	27.9 ± 2.1	25.2 ± 2.1
Lymphocytes %	61.2 ± 2.9	60.9 ± 2.0	51.9 ± 2.2	61.0 ± 1.5	54.9 ± 1.6	53.5 ± 1.9	66. ± 3.7	58.1 ± 1.8	52.7 ± 2.2	55.5 ± 5.8	57.5 ± 1.8	55.3 ± 2.4
Eosinophils %	5.0 ± 1.2	7.3 ± 0.9	9.9 ± 1.0	6.9 ± 1.4	9.8 ± 1.0	9.7 ± 0.7	6.5 ± 1.5	8.6 ± 0.8	9.0 ± 1.2	14.2 ± 5.5	10.4 ± 1.0	12.7 ± 1.2
Basophils %	7.0 ± 1.2	5.4 ± 0.6	5.4 ± 0.5	4.5 ± 0.7	3.82 ± 0.5	3.6 ± 0.5	2.3 ± 0.6	3.1 ± 0.3	3.8 ± 0.4	2.3 ± 0.2	3.9 ± 0.2	6.3 ± 1.1
Band Neutrophils %	4.8 ± 0.9	5.3 ± 0.8	6.3 ± 1.2	5.5 ± 2.2	5.1 ± 0.8	5.6 ± 1.0	8.0 ± 4.0	8.1 ± 1.4	8.7 ± 2.0	4.3 ± 0.4	5.3 ± 0.9	8.4 ± 2.4
Toxic Neutrophils %	14.5 ± 2.0	16.7 ± 1.5	15.8 ± 1.7	18.3 ± 2.2	17.0 ± 1.8	18.8 ± 1.8	12.6 ± 2.6	18.6 ± 1.7	18.1 ± 2.0	8.1 ± 0.5	11.9 ± 1.9	12.5 ± 1.5
N:L ratio	0.44 ± .05	0.46 ± .05	0.68 ± .06	0.45 ± .04	0.59 ± .05	0.65 ± .06	0.37 ± .07	0.54 ± .04	0.68 ± .07	0.51 ± 0.08	0.51 ± .05	0.47 ± .05

Arithmetic means (±1 SEM) are presented for white blood cell parameters in adult male hellbenders across the parental care period and infection status. Sampling day represents repeated capture intervals corresponding to offspring development in which oviposition represents nest initiation [day ~0], mid-embryonic represents mid-embryonic development [day ~30], hatching represents larval hatching [day ~60], and emergence represents spring larval emergence [day ~200]. Parameters represent the proportion of each cell type out of the total counted within 50 fields of view on whole blood smears. N:L ratio represents the proportion of neutrophils divided by the proportion of lymphocytes.

Table 2 Characteristics of parasitic infection of adult male eastern hellbenders (*Cryptobranchus alleganiensis*) across the 8-month parental care period from a population in southwest Virginia, USA.

Parameter	**Sampling Day**			
	Oviposition	**Mid-Embryonic**	**Hatching**	**Emergence**
	N=56	N=48	N=44	N=25
Leech bites	15.3 ± 10.39	9.6 ± 2.66	9.0 ± 2.29	4.1 ± 1.24
Leech prevalence (%)	42.8%	41.7%	30.2%	28.0%
Leech intensity	7.3 ± 1.98	1.8 ± 0.22	2.2 ± 0.45	1.7 ± 0.42
Trypanosome prevalence (%)	83.9%	89.6%	88.4%	88.0%
Trypanosome intensity	64.0 ± 9.6	74.6 ± 9.15	57.7 ± 10.29	42.5 ± 7.1

Parasite prevalence represents the proportion of the population infected, and intensity is determined by the number of parasites present in an infected individual. Leech bites are represented as the arithmetic mean (±1 SEM).

Table 3 Results from principal component analysis on the proportions of white blood cells in male eastern hellbender (*Cryptobranchus alleganiensis)* blood and parameter loadings for each principal component (PC)

White Blood Cell PCA				
Parameters	**PC1**	**PC2**	**PC3**	**PC4**
Neutrophils	0.616	0.279	0.426	0.601
Lymphocytes	-0.687	-0.166	0.117	0.698
Eosinophils	0.374	-0.578	-0.641	0.339
Basophils		0.749	-0.627	0.192
Eigenvalue	2.016	1.229	0.744	0.011
Proportion of Variance	0.504	0.307	0.186	0.003
Cumulative Variance	0.504	0.811	0.997	1.000

Table 4 Results from linear mixed effects model selection examining the effects of seasonal variation in temperature and parasites on white blood cell parameters in eastern hellbenders.

Linear Mixed Effects Models	**K**	**AICc**	**Delta AICc**	**AICc Weight**	**Cumulative Weight**
PC1~Temperature + Trypanosomes*Leeches	7	578.78	0	0.63	0.63
PC1~Temperature + Trypanosomes*Leeches + Leech bites	8	581.76	2.98	0.14	0.77
PC1~Temperature + Trypanosomes + Leeches	6	582.61	3.82	0.09	0.87
PC1~1	3	582.97	4.18	0.08	0.94
PC1~Temperature	4	584.52	5.74	0.04	0.98
PC1~Temperature + Trypanosomes	5	586.97	8.18	0.01	0.99
PC1~Temperature + Trypanosomes+ Leeches+ Leech bites	7	587.01	8.23	0.01	1.00
PC1~Infection Status*Temperature	8	592.84	14.06	0.00	1.00

Table 5 Results from linear mixed effects model selection examining the effects of seasonal variation in temperature and parasites on white blood cell parameters in eastern hellbenders.

Linear Mixed Effects Models	**K**	**AICc**	**Delta AICc**	**AICc Weight**	**Cumulative Weight**
PC2~ Temperature	4	510.06	0.00	0.83	0.83
PC2~ Temperature + Trypanosomes	5	513.59	3.53	0.14	0.97
PC2~Temperature + Trypanosomes + Leeches	6	518.55	8.49	0.01	0.98
PC2~Temperature + Trypanosomes*Leeches	7	518.78	8.72	0.01	0.99
PC2~Infection Status*Temperature	8	520.30	10.42	0.00	0.99
PC2~1	3	521.25	11.19	0.00	1.00
PC2~Temperature + Trypanosomes*Leeches + Leech Bites	8	523.32	13.25	0.00	1.00
PC2~Temperature + Trypanosomes + Leeches + Leech Bites	7	523.40	13.33	0.00	1.00

Table 6 Results from linear mixed effects model selection examining the effects of seasonal variation in temperature and parasites on band neutrophils in eastern hellbenders.

Linear Mixed Effects Models	**K**	**AICc**	**Delta AICc**	**AICc Weight**	**Cumulative Weight**
Band Neutrophils ~ Temperature	4	483.83	0.00	0.55	0.55
Band Neutrophils ~ 1	3	484.71	0.88	0.35	0.90
Band Neutrophils ~ Temperature + Trypanosomes	5	489.01	5.18	0.04	0.95
Band Neutrophils ~ Temperature + Leeches*Trypanosomes	7	489.44	5.60	0.03	0.98
Band Neutrophils ~ Temperature + Leeches*Trypanosomes + Leech bites	8	490.91	7.07	0.02	1.00
Band Neutrophils ~ Temperature + Leeches + Trypanosomes	6	493.48	9.64	0.00	1.00
Band Neutrophils ~ Temperature + Leeches + Trypanosomes + Leech bites	7	497.90	14.06	0.00	1.00
Band Neutrophils ~ Infection Status*Temperature	8	502.34	18.51	0.00	1.00

Table 7 Results from linear mixed effects model selection examining the effects of seasonal variation and parasites on toxic neutrophils in eastern hellbenders.

Linear Mixed Effects Models	**K**	**AICc**	**Delta AICc**	**AICc Weight**	**Cumulative Weight**
Toxic Neutrophils ~ Sample Day	6	477.29	0.00	0.86	0.86
Toxic Neutrophils ~ Sample Day + Trypanosomes	7	482.04	4.75	0.08	0.94
Toxic Neutrophils ~ 1	3	483.04	5.75	0.05	0.99
Toxic Neutrophils ~ Sample Day + Trypanosomes + Leeches	8	487.53	10.24	0.01	0.99
Toxic Neutrophils ~ Sample Day + Trypanosomes + Leeches + Leech bites	9	489.90	12.61	0.00	1.00
Toxic Neutrophils ~ Sample Day* Infection Status	14	490.97	13.68	0.00	1.00
Toxic Neutrophils ~ Sample Day + Trypanosomes*Leeches	9	492.40	15.11	0.00	1.00
Toxic Neutrophils ~ Sample Day + Trypanosomes*Leeches + Leech bites	10	494.44	17.15	0.00	1.00

Supplemental Table 1 Pearson's correlation coefficients between the proportion of white blood cells in male hellbenders and the resulting principal component scores.

Parameters	**PC1**	**PC2**
Neutrophils	0.875	0.309
Lymphocytes	-0.975	-0.184
Eosinophils	0.531	-0.641
Basophils	-0.131	0.830

Supplemental Table 2 Statistical outcomes from pairwise comparisons of toxic neutrophils between sampling days.

Contrast	Estimate	SE	p value
Oviposition / Mid-Embryonic	-0.271	0.173	0.400
Oviposition / Hatching	-0.254	0.179	0.492
Oviposition / Emergence	0.574	0.214	0.041
Mid-Embryonic / Hatching	0.018	0.184	0.999
Mid-Embryonic / Emergence	0.845	0.219	0.001
Hatching / Emergence	0.828	0.223	0.002

Statistical outcomes correspond to data shown in Figure 5. Estimates and standard error are on the response scale which was transformed, scaled, and centered prior to analysis. Sampling day represents repeated capture intervals corresponding to offspring development in which oviposition represents nest initiation [day ~0], mid-embryonic represents mid-embryonic development [day ~30], hatching represents larval hatching [day ~60], and emergence represents spring larval emergence [day ~200].

Supplemental Table 3 Results from generalized linear model selection examining the effects of parasites on nest fate (success vs. all failures) in eastern hellbenders.

Linear Mixed Effects Models	**K**	**AICc**	**Delta AICc**	**AICc Weight**	**Cumulative Weight**
Success ~ 1	1	81.37	0.00	0.26	0.26
Success ~ Trypanosomes	2	81.94	0.57	0.19	0.45
Success ~ Infection status + Leech bites	4	82.03	0.66	0.19	0.64
Success ~ Leech bites + Trypanosomes + Leeches	4	82.28	0.91	0.16	0.80
Success ~ Infection status	3	83.55	2.18	0.09	0.89
Success ~ Leeches + Trypanosomes	3	83.83	2.46	0.08	0.96
Success ~ Trypanosomes*Leeches	4	85.28	3.91	0.04	1.00

Supplemental Table 4 Results from generalized linear model selection examining the effects of parasites on nest failure resulting from whole clutch cannibalism in eastern hellbenders.

Linear Mixed Effects Models	**K**	**AICc**	**Delta AICc**	**AICc Weight**	**Cumulative Weight**
Cannibal ~ Leeches*Trypanosomes	4	42.05	0.00	0.54	0.54
Cannibal ~ 1	1	44.00	1.95	0.20	0.75
Cannibal ~ Trypanosomes	2	45.51	3.46	0.10	0.84
Cannibal ~ Leeches + Trypanosomes	3	46.26	4.20	0.07	0.91
Cannibal ~ Infection status	3	47.25	5.19	0.04	0.95
Cannibal ~ Leech bites + Trypanosomes + Leeches	4	48.12	6.07	0.03	0.97
Cannibal ~ Infection status + Leech bites	4	48.17	6.12	0.03	1.00

Figures

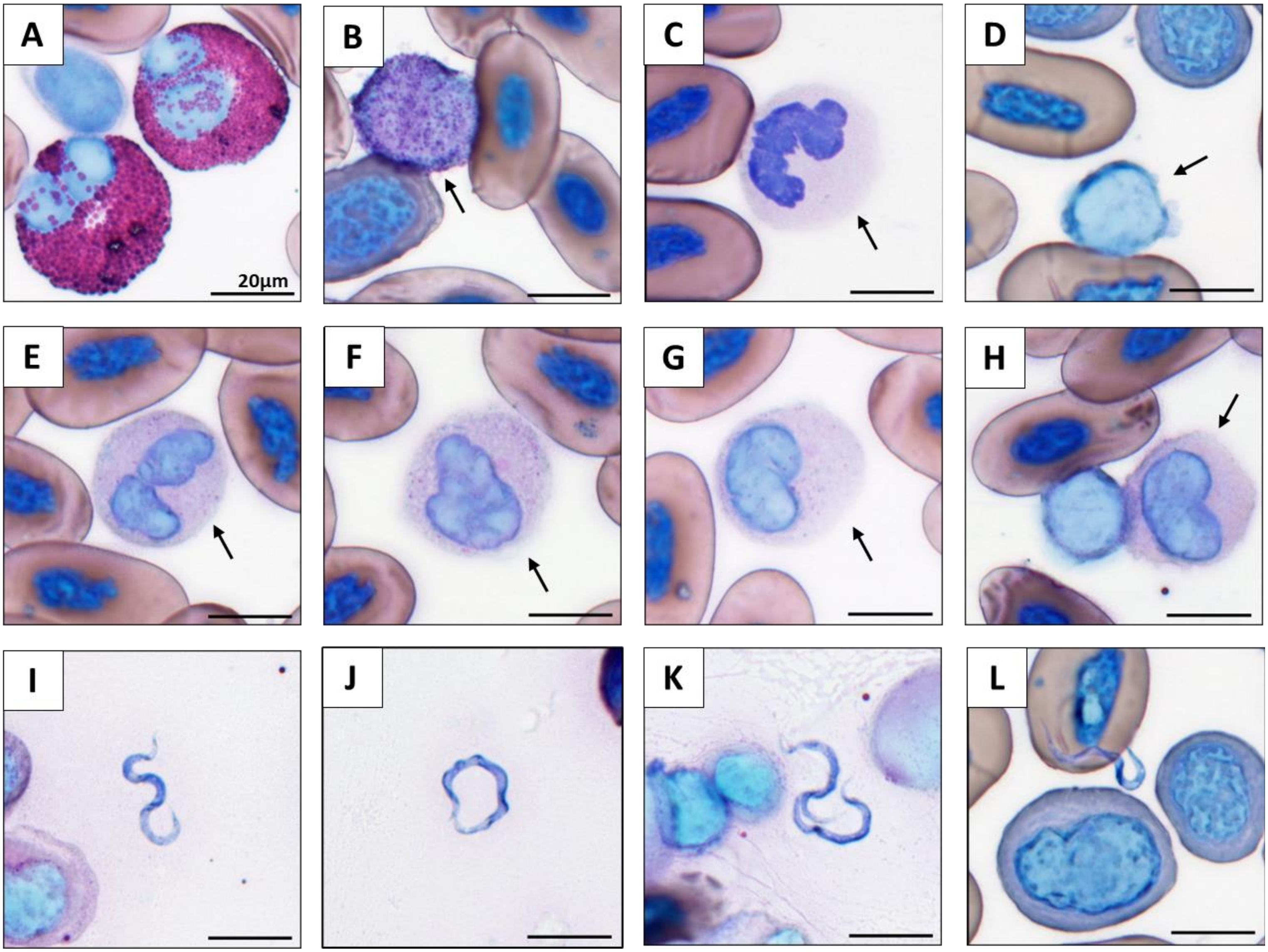


Figure 1. Microscopic images of hellbender white blood cells and trypanosomes. Images include **(A)** two eosinophils alongside a thrombocyte, **(B)** a basophil (arrow) next to a polychromatic red blood cell and mature red blood cells, **(C)** a healthy mature neutrophil (arrow) next to partial mature red blood cells, **(D)** a lymphocyte (arrow) adjacent to partial mature red blood cells and polychromatic red blood cells, **(E-F)** mature neutrophils (arrow) with toxic granulation with partial mature red blood cells, **(G)** band neutrophil (arrow) with visible secondary granules, **(H)** band neutrophil (arrow) with visible secondary granules, alongside a lymphocyte and mature red blood cells, **(I-K)** trypanosomes from buffy coat smears, **(L)** and a trypanosome from whole blood smear alongside mature and polychromatic (young) red blood cells. Images were taken on a compound light microscope at 400X magnification. The black scale bar represents 20 µm.

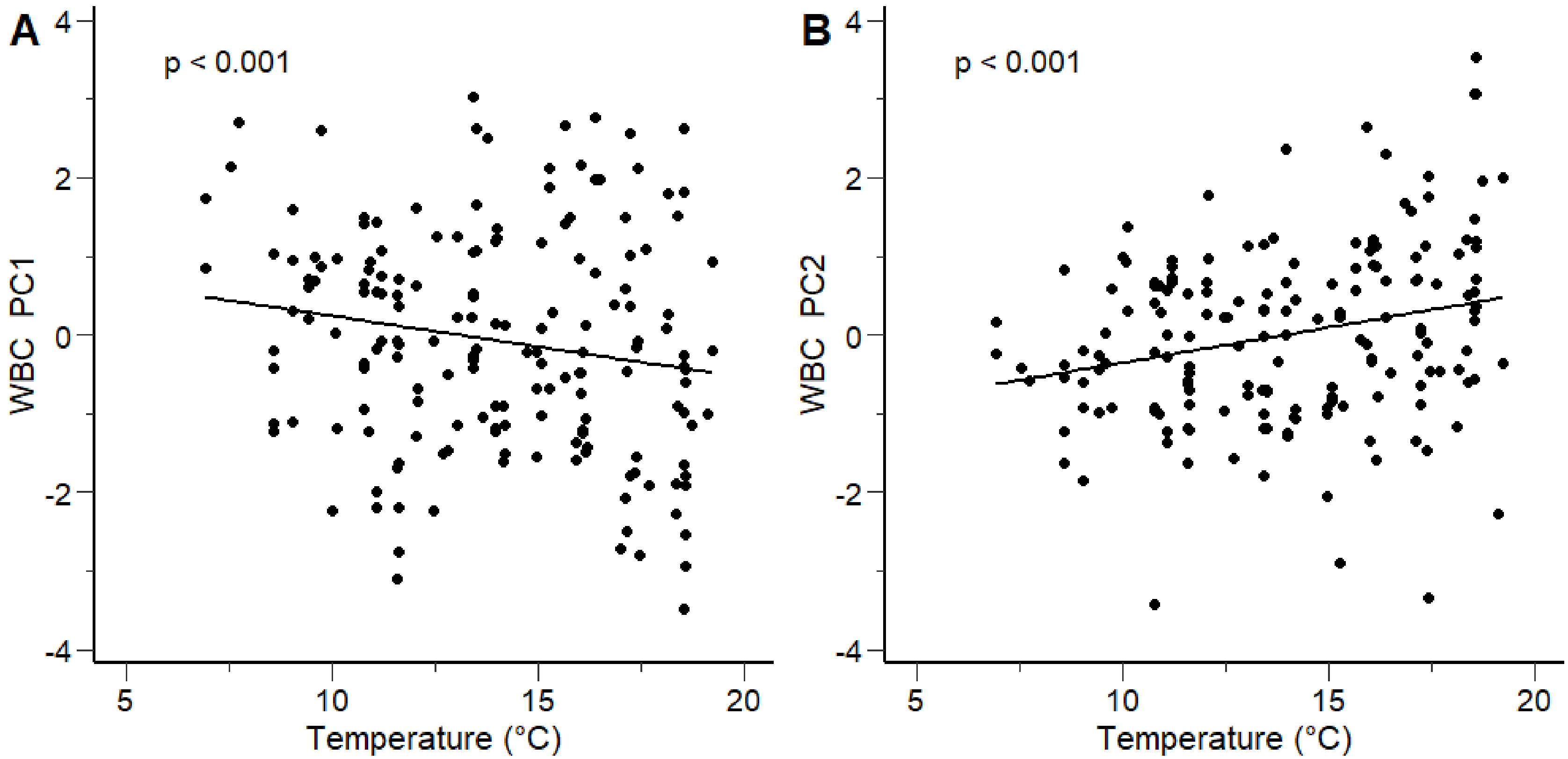


Figure 2. The relationship between temperature and the principal component scores (PC) for white blood cell (WBC) profiles. **(A)** The relationship between stream temperature (°C) and WBC PC1 which explained 50.3% of the variance and was positively loaded with neutrophils (0.616) and eosinophils (0.371) but was negatively loaded with lymphocytes (-0.689) **(B)** The relationship between stream temperature (°C) on WBC PC2 which explained 30.6% of the variance and was positively loaded with neutrophils (0.284) and basophils (0.744) but was negatively loaded with lymphocytes (-0.163) and eosinophils (-0.583). Lines represent model predictions from the top-ranking models using AICc, while points represent principal component scores.

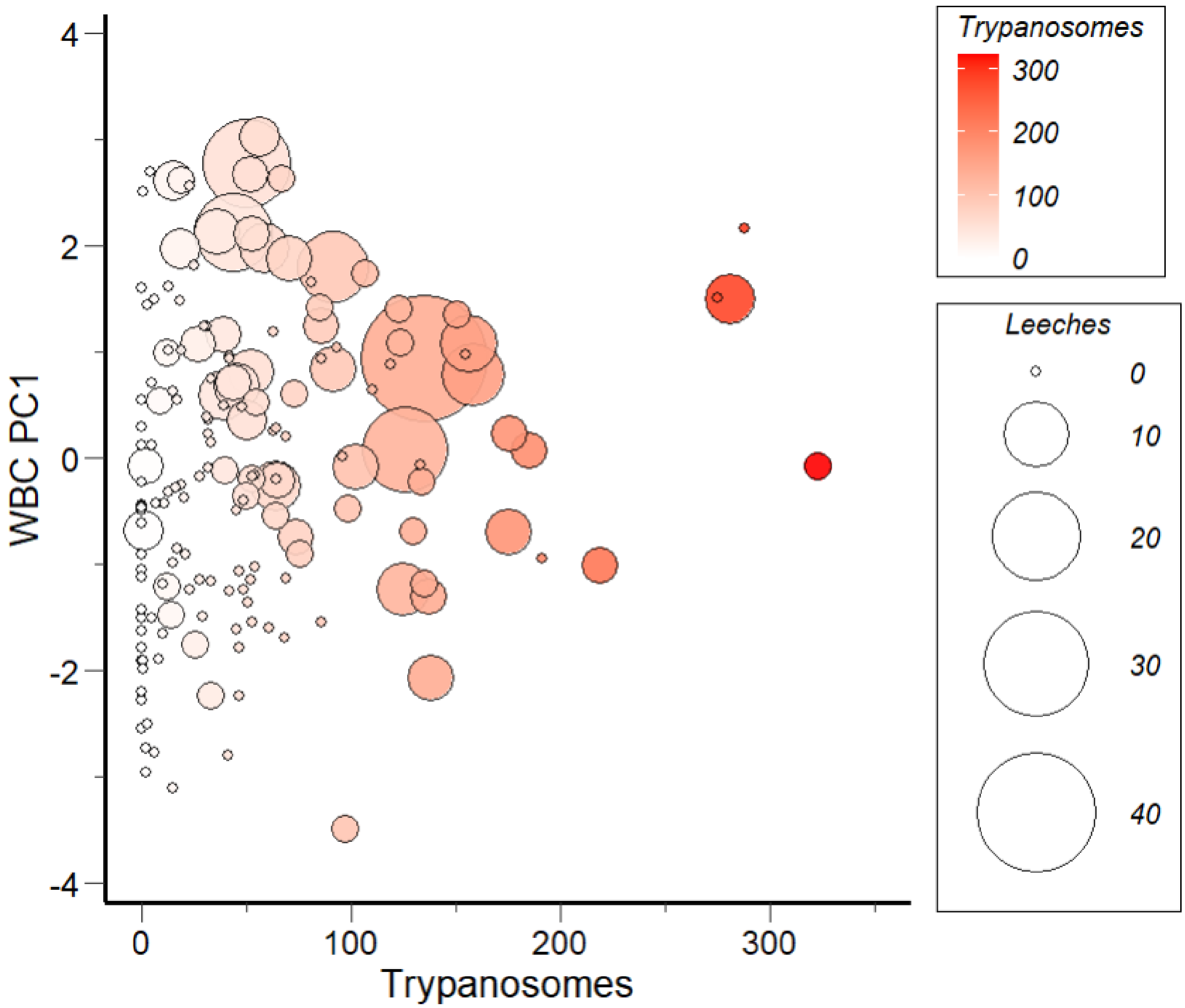


Figure 3. The relationship between the abundance of parasites and white blood cell (WBC) principal component (PC) 1, demonstrating the interactive effects between leeches and trypanosomes. Trypanosomes represent the total number of trypanosomes counted within 50 random fields of view across duplicate buffy coat slides while leeches represent the number of leeches attached to the individual at the time of capture. Corresponding to our results from the top-ranked model using AICc, as the size of the circles (leech abundance) increases, PC1 values exhibit a positive shift. However, as the size of the circles increases and increases in color (trypanosome abundance), PC1 values exhibit a negative shift.

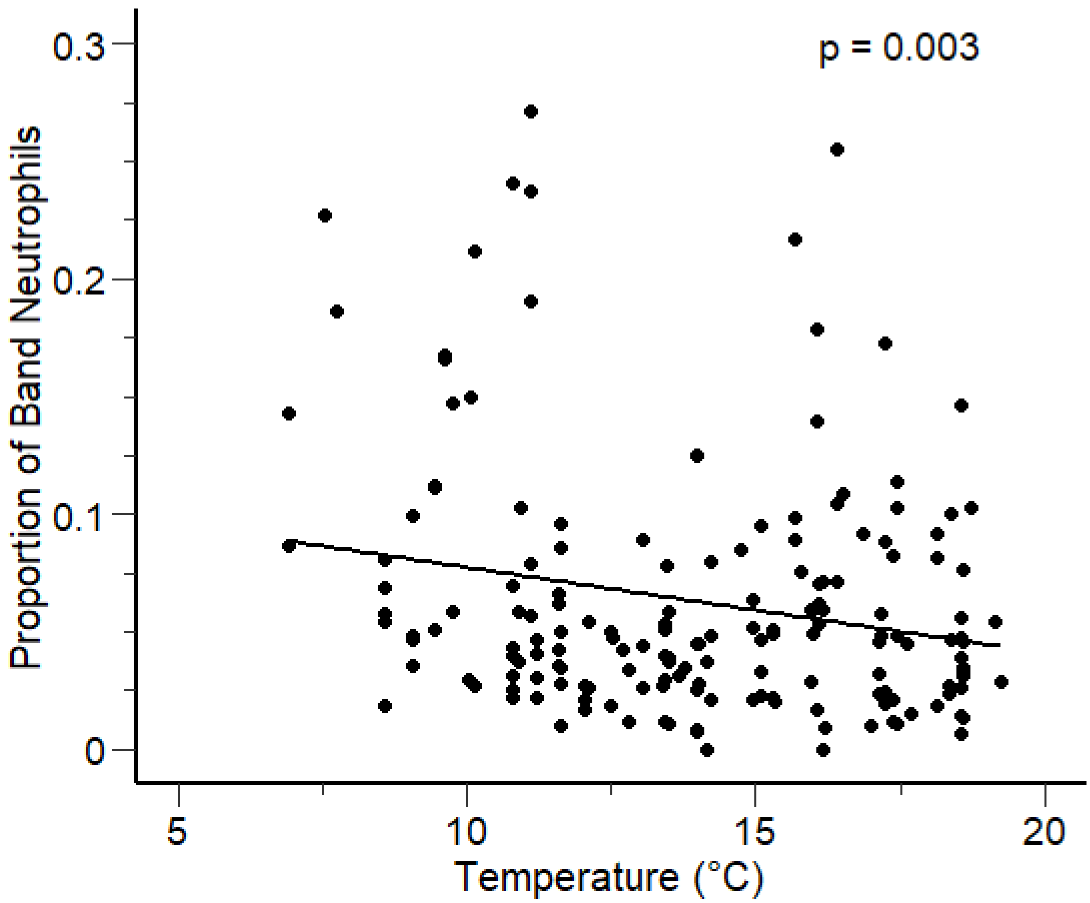


Figure 4. The relationship between temperature and the proportion of band neutrophils in circulation. Lines represent model predictions from the top-ranking models using AICc, while points represent principal component scores.

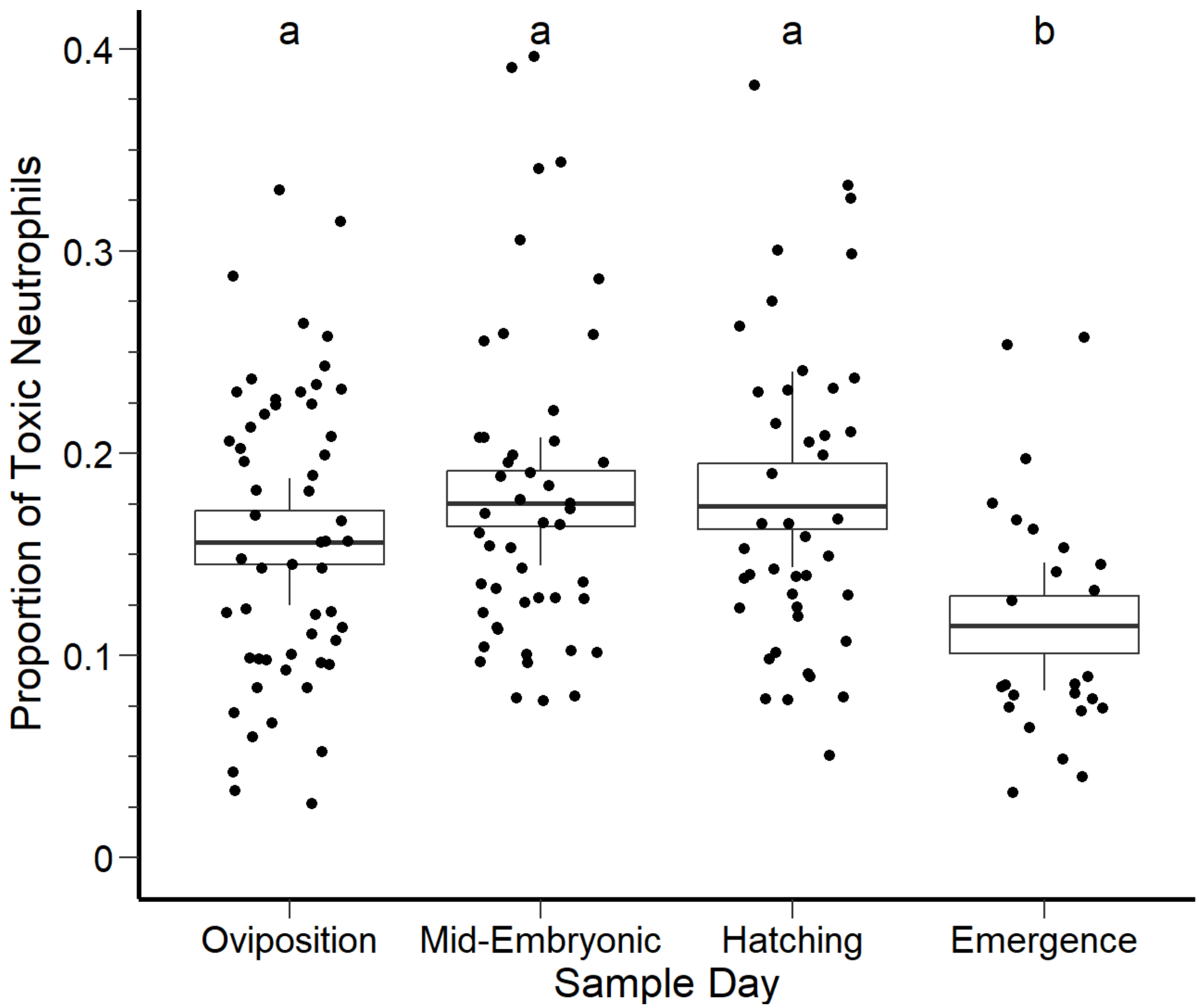


Figure 5. Seasonal differences in the proportion of toxic neutrophils across the parental care period. Box plots represent the distribution of model predictions from the top-ranking models using AICc while the points represent raw data. Letters in superscript indicate statistical differences in the proportion of toxic neutrophils across the parental care period (Supplemental Table 2). Sampling day represents repeated capture intervals corresponding to offspring development in which oviposition represents nest initiation [day ~0], mid-embryonic represents mid-embryonic development [day ~30], hatching represents larval hatching [day ~60], and emergence represents spring larval emergence [day ~200].

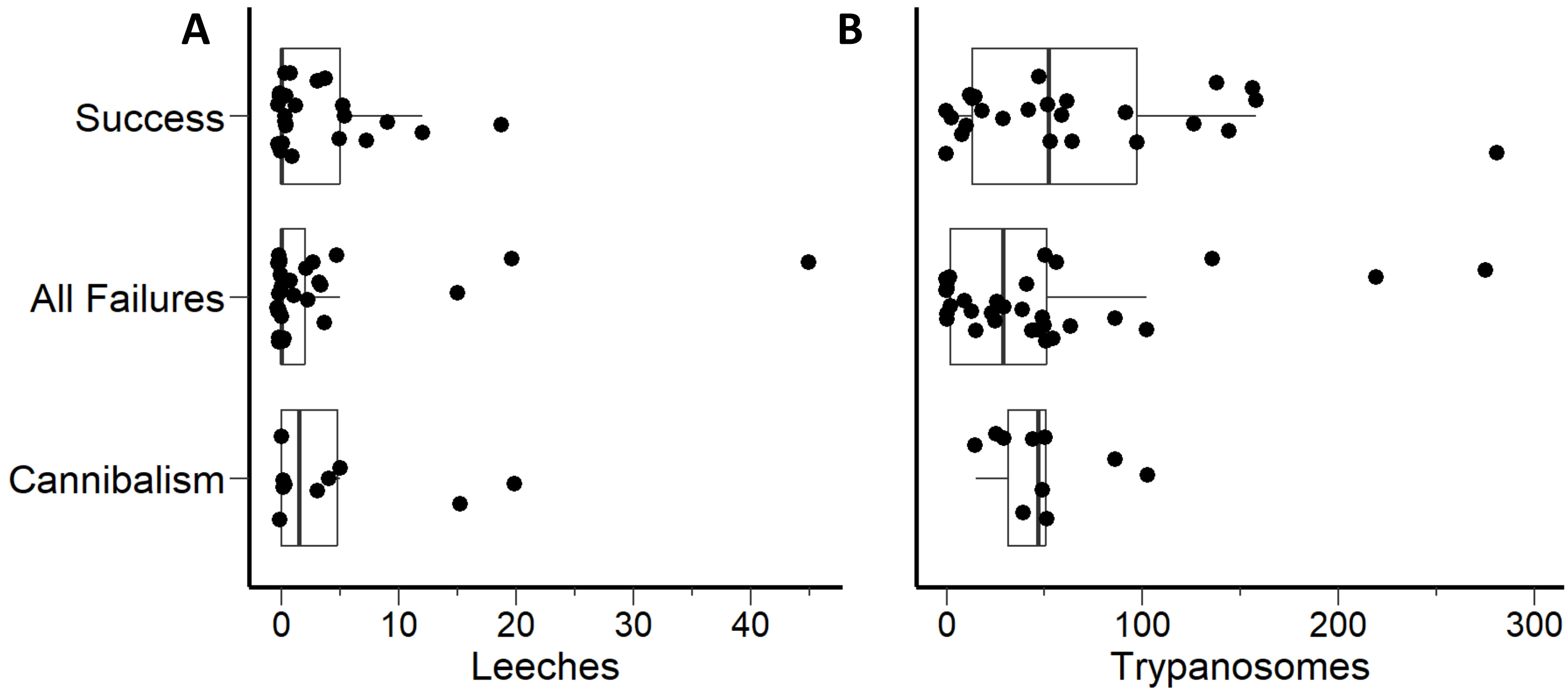


Figure 6. The relationship between parasitic infection and nest fate. **(A)** The distribution of nest outcomes in response to the abundance of leeches during oviposition. **(B)** The distribution of nest outcomes in response to the abundance of trypanosomes during oviposition. Box plots represent the relative distribution of parasite abundance within the categorized nest outcome and points indicate raw data. Cannibalism represents the nest fate category in which nest failure was confirmed as whole clutch cannibalism. While all categories of nest outcomes are expressed in the figure, statistical analyses were performed separately to evaluate if parasite infection influenced the likelihood of success vs. failure and success vs. whole clutch cannibal.

CONCLUSION

My research provides fundamental insight into the dynamic blood physiology of an imperiled amphibian. I identified various intrinsic and extrinsic factors that influence hellbender blood physiology, including seasonal temperature fluctuations, which must be considered when monitoring the health of wild populations. I uncovered the impact of parasites on immune responses and found that coinfected hellbenders exhibited symptoms of regenerative anemia. Further, I determined that the occurrence of anemia in hellbenders was not solely dependent on the trypanosome transmitted by the leech vector, but rather on the vector's attachment. Together, my findings shed light on the intricate relationships between host physiology, multiple parasites, and environmental factors, highlighting the pathologies associated with coinfections involving the vector. As with all research, my studies raise questions for further exploration.

Coinfection

One noteworthy revelation stemming from my research pertains to the remarkably high prevalence of trypanosome infection within the system, with all hosts harboring leeches being infected with trypanosome parasites. The feeding behavior of leeches in our system is yet to be fully understood, although related species exhibit a slow metabolism and feed only a few times annually (Sawyer, 1986; Phillips et al., 2020). This raises an intriguing question - how do trypanosomes sustain such high prevalence in hellbenders, given that their vector may interact with a host only sporadically? Our findings, coupled with prior laboratory research, lead us to hypothesize that parasites strategically exploit the convergence of reproductive phenology, behavior, and physiology of the host to optimize trypanosome transmission and persistence.

Leeches exhibit brooding and parental care of their offspring and utilize hellbender shelters and nest rocks (Blumenthal and Hopkins, unpublished; Bodinof Jachowski et al. 2023) for refuge in the fast-flowing freshwater streams. Coincidentally, the hellbender breeding season, characterized by multiple hosts entering nest sites, establishing territories, and engaging in breeding interactions (O'Brien et al., 2024), aligns with the time when leeches lead their offspring to their first blood meal (Blumenthal and Hopkins, unpublished). This synchronization maximizes the frequency and number of host-host interactions and likely host-parasite interactions during this crucial period in the leech's lifecycle.

Because leech infection peaks during the hellbender breeding season and nest establishment, I hypothesize that this is also the peak time for trypanosome transmission to the host. Notably, it coincided with the period when the host exhibited the highest hematocrit and hemoglobin concentrations, thus optimizing the availability of food resources for both the trypanosomes and the leech vector. Furthermore, the leech, while delivering its offspring to the hellbender host, potentially introduces its progeny to trypanosomes for the first time. This transmission to young leeches can occur if the host already carries the blood parasites, or if the adult leech transmits trypanosomes to the host that are subsequently transmitted to the young leeches.

Following nest establishment, a hellbender host will remain at the nest for at least 8 months (Hopkins et al., 2023), presenting ample opportunities for blood meals and an extended period for vector-host contact. Leech offspring of a similar species undergo rapid growth, requiring 3-5 blood meals to survive over winter (Wilkialis, 1984; Moser et al., 2009). This not only involves repeated consumption of blood meals from the same host but also potentially reintroduces trypanosomes into the host's bloodstream. Even if the hellbender host's immune system effectively eliminates trypanosomes from the blood, they might still face continuous reintroduction of parasites from the leech offspring during this time.

As temperatures decrease in the fall, trypanosome infection intensity decreases, and host hematocrit and hemoglobin reach seasonal lows. But despite reduced food resources for the blood parasites, the established trypanosome infection persists. Our results indirectly indicate that hellbender host metabolism decreases at overwintering temperatures, and the immune system undergoes a shift where the proportion of circulating neutrophils and eosinophils increases while the proportion of circulating lymphocytes decreases. In other taxa infected with trypanosomes, hosts rely on lymphocytes and complement activity to target and eliminate trypanosomes (Mansfield and Paulnock, 2005; Onyilagha and Uzonna, 2019). The observed decrease in the proportion of circulating lymphocytes in hellbender hosts during colder periods could compromise their defenses overwinter and contribute to the persistence of trypanosome infection.

Whether hellbenders effectively eliminate the trypanosome parasite over winter and the following spring remains uncertain but seems unlikely for at least two additional reasons. First, hellbenders in the spring and summer harbor leeches, albeit at a lower frequency and intensity than in the late summer and fall, indicating that re-infection with trypanosomes is likely. Second, the leech's life cycle restarts in the summer as infected leech offspring from the previous year, now adults, begin reproducing and preparing their offspring for their initial blood meals. The convergence of life cycles between the host and vector implies the potential for the leech to remain at hellbender nesting locations year-round, awaiting the hosts' return for breeding. This scenario creates an optimal system for trypanosome transmission and likely contributes to the high prevalence of infection observed among hellbenders in our system.

Trypanosomes may also leverage the host's immune response to the leech vector to maximize transmission opportunities. For instance, biting vectors prompt host immune responses, which optimize the invasion and proliferation of transmitted parasites (Nuttall, 1998; Salzet et al., 2000; Caljon et al., 2006). Our findings revealed a correlation between leeches and altered white blood cell differentials, indicative of an inflammatory response, consistent with prior research (Hopkins et al., 2016; Bodinof Jachowski et al., 2024). However, the interaction between the abundance of trypanosomes and leeches had an inverse impact on white blood cells, suggesting that the types of cellular immune responses differ in the host when trypanosome and leech abundance increases concurrently. This raises the question: what implications does this shifting immune response hold for the host?

Our interpretations are constrained by our reliance on momentary snapshots of white blood cell profiles, hindering a comprehensive understanding of disease progression and parasite elimination. A complementary metric for assessment is circulating cytokines, which offer specificity regarding immune system triggers, shedding light on different types of responses (Liu et al., 2021). Cytokine profiles can distinguish between proinflammatory, anti-inflammatory, allergic, and anti-parasite responses and, therefore, serve as valuable indicators of infection (Spellberg and Edwards, 2001; Graham et al., 2007; Liu et al., 2021). In the context of parasite transmission, research on tsetse flies, sand flies, and ticks have utilized cytokine profiles to demonstrate that vector saliva promotes Th2 polarization in the host (Kopecky et al., 1999; Kovar et al., 2001; Norsworthy et al., 2004; Caljon et al., 2006). This polarization benefits transmitted parasites, as the effective elimination of trypanosomes and other blood parasites relies on a Th1 response early in infection followed by a Th2 response during chronic infection (Baetselier et al., 2001; Namangala et al., 2001). An imbalance in Th1 and Th2 responses may result in the failure to control early parasite replication (Baetselier et al., 2001; Wiegertjes et al., 2005).

Leech saliva, akin to other biting vectors, contains multiple immunomodulatory compounds that could impact the host's physiology (Hildebrandt and Lemke, 2011; Salzet et al., 2000). In some leech species, their saliva contains histamine, which can act as a vasodilator and increase the permeability of host endothelium, which is beneficial to blood-feeding leeches (Baskova et al., 2008). However, histamine can act as a pro-inflammatory and regulatory compound that can elicit a suite of physiological responses, including Th1/Th2 polarization as well as the modulation of neutrophils, eosinophils, and basophils (Castelo Branco et al., 2018). Plausibly, compounds in leech saliva may redirect hellbender immune responses away from trypanosomes, providing the parasites with a kick-start in proliferation before the host's immune response recognizes the foreign invader. As trypanosome infection intensifies and leeches are detached, hellbenders may subsequently modify their immune responses to eliminate the blood parasites.

My findings revealed something intriguing about the hellbenders' response to leech parasitism - the leech bites on their bodies lasted for weeks, despite the relatively fast wound healing we observe in hellbenders during the paternal care period. This led me to wonder if the leech bites were more than just residual scars - could they be an indicator of trypanosomiasis? In mammalian hosts, an early indication of trypanosomiasis is the development of firm, red, or violet lesions at the site of the tsetse fly bite, known as "trypanosomal chancres" (Figure 1; Barry and Emery, 1984; Bittencourt and Ashworth, 1995; Baral, 2010). These chancres occur due to the proliferation of trypanosomes in the host tissues at the bite wound before undergoing morphological changes and migrating to the blood (Bittencourt and Ashworth, 1995; Baral, 2010). Therefore, the prolonged presence of a morphologically distinct lesion remaining at the site of the leech bite could signify inoculation and proliferation of the trypanosomes in the hellbender hosts.

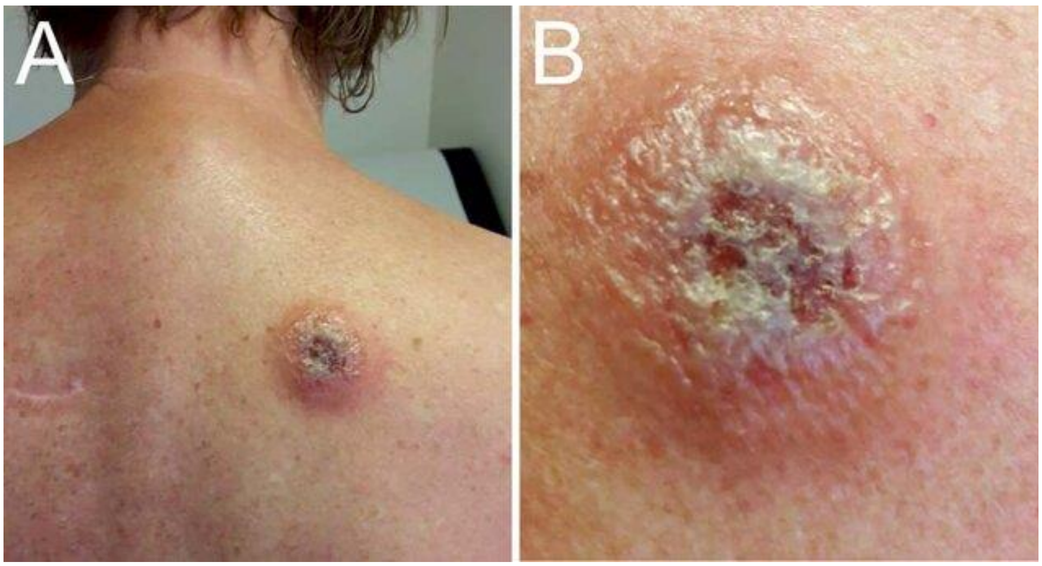


Figure 1. Trypanosome chancre on human host (van Genderen et al., 2021). **(A)** Image of trypanosomal chancre on the back of a human host and was approximately 5cm in diameter. **(B)** Image of chancre at a higher magnification.

It remains unclear if the trypanosomes in our system undergo different developmental stages between the vector and host. While all trypanosomes we observed had similar long and slender forms, some appeared to vary in length (Figure 2), and we observed multiple instances of what I suspect to be binary fission (Figure 3). To gain a more thorough comprehension of their life cycle and expansion within both the host and vector, I suggest that future research focus on examining trypanosomes with scanning electron microscopy. This method would enable high-resolution images of the parasite's distinct physiological structures.

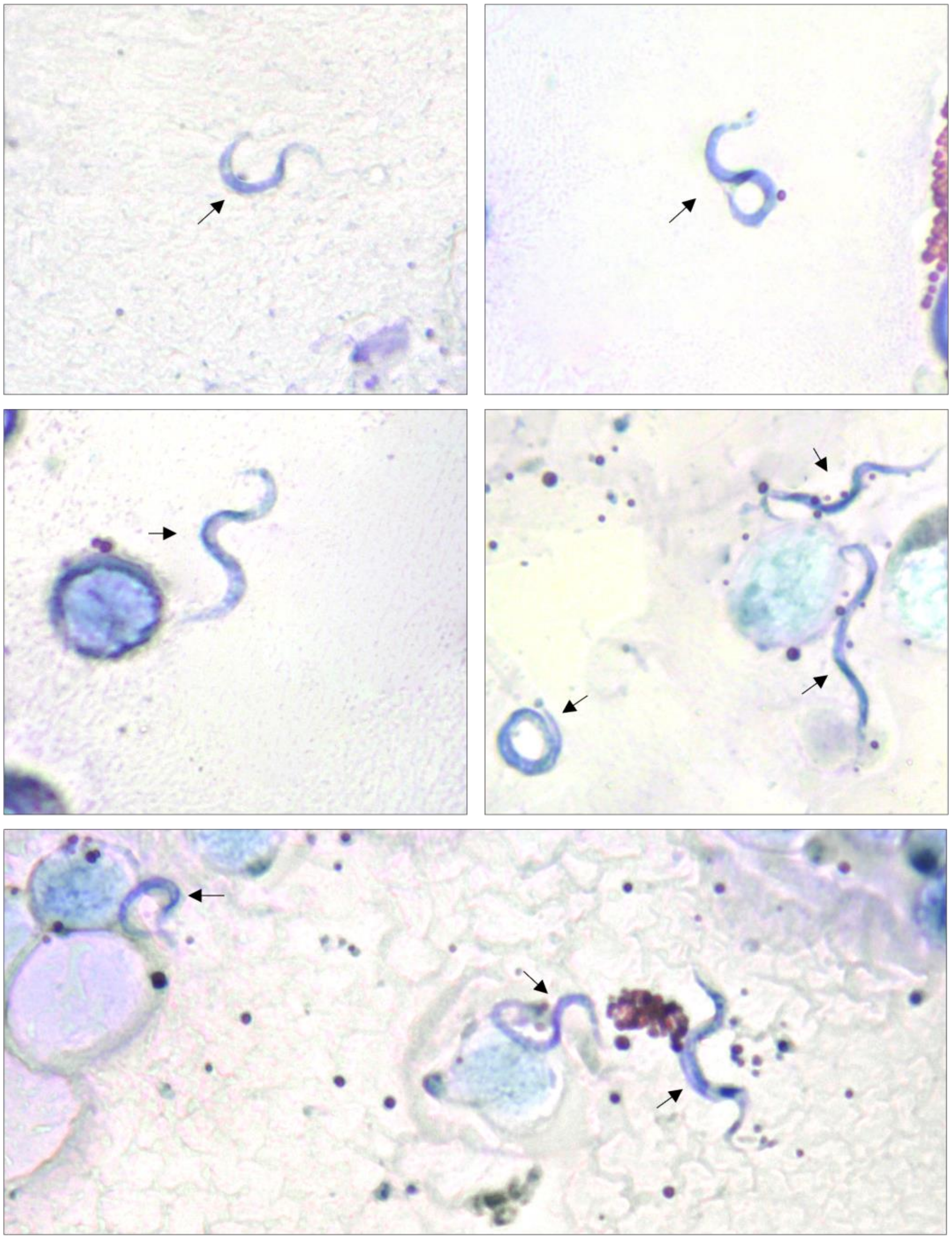


Figure 2. Microscope images of trypanosomes (arrows) with variable lengths on a buffy coat smear. Images were captured on a compound light microscope at X400 magnification. Total length of trypanosome parasites averages at 40.7µm but can range between 30.5µm and 49.5µm (Davis and Hopkins, 2013).

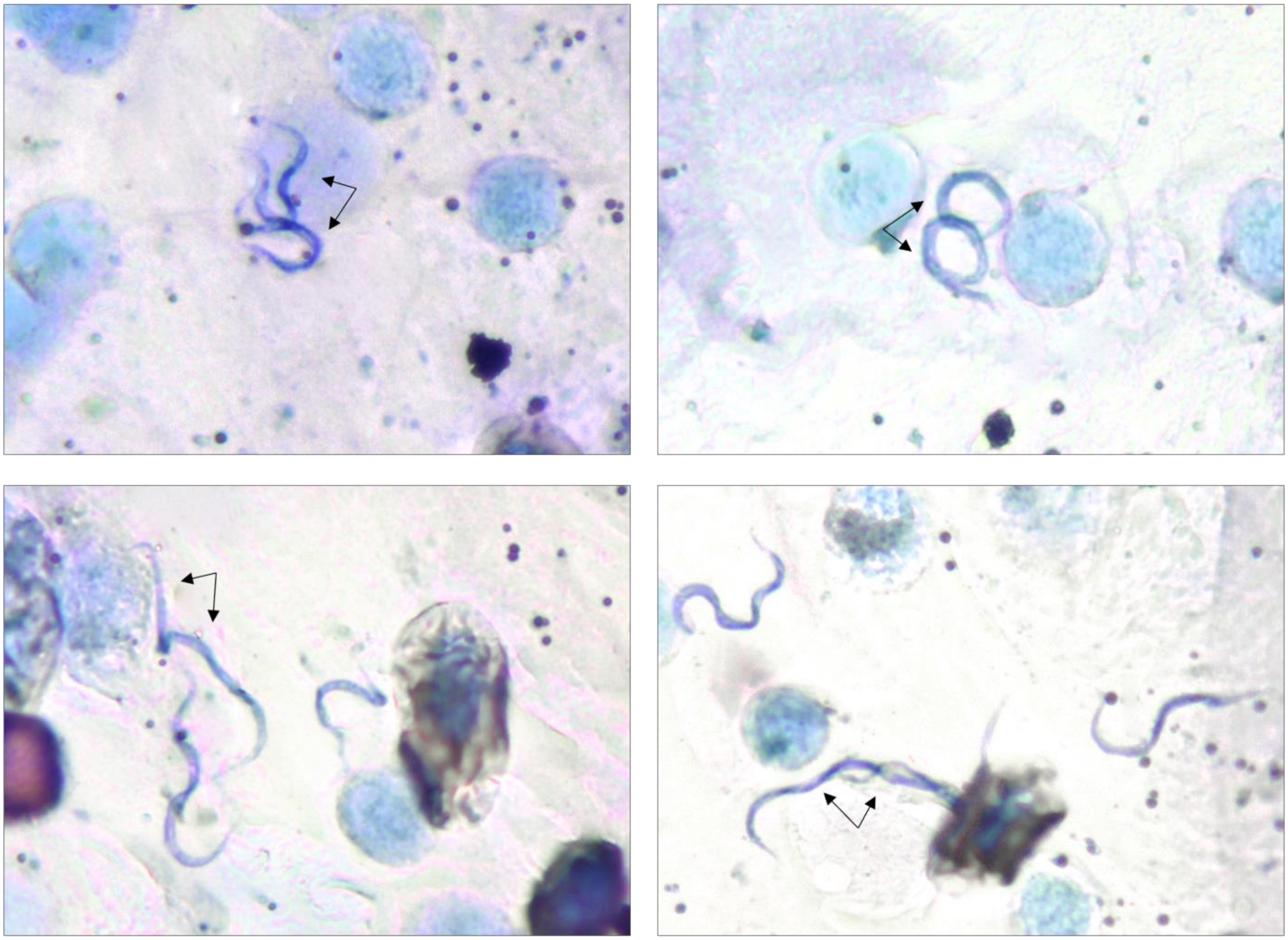


Figure 3. Microscope images of trypanosomes (arrows) which led me to hypothesize that asexual duplication may occur in the circulation of the hellbender host. Images were captured on a compound light microscope at X400 magnification. Total length of trypanosome parasites averages at 40.7µm but can range between 30.5µm and 49.5µm (Davis and Hopkins, 2013).

Our findings also raise questions about the conclusions drawn by Durant et al. (2015) in their study, which associated leech infection, but not trypanosomes, with disruptions in circadian rhythms and endocrine processes in hellbenders. They proposed that compounds in leech saliva might be responsible for systemic endocrine disruption. However, I contend that the potential impact of trypanosomes might have been overlooked due to the methodologies employed in that earlier study. Durant et al. (2015) quantified trypanosome infection using standard whole blood smears, a method later found by Hopkins et al. (2016) to have only a quarter of the efficacy of buffy coat smears for detecting trypanosomes. My personal observations revealed an exceedingly greater abundance of trypanosomes in buffy coat smears (within 50 fields of view) compared to whole blood smears, substantiating the findings of Hopkins et al. (2016). Given the high prevalence of trypanosome infection and the cooccurrence of leech and trypanosome infections in all individuals, it is likely that Durant et al. (2015) considerably underestimated the frequency and intensity of trypanosome infections in their study.

This prompts a reevaluation of the observed endocrine disruption. Was it truly caused by leeches, as suggested by Durant et al. (2015), or could it be attributed to the effects of trypanosomes on the central nervous system? In various systems, trypanosomes infiltrate the skin, tissues, and blood before invading the central nervous system (Bittencourt and Ashworth, 1995; Onyilagha and Uzonna, 2019). Disturbed circadian rhythms, especially in sleeping patterns, are well-established hallmarks of trypanosomiasis in mammalian hosts (Bittencourt and Ashworth, 1995; Onyilagha and Uzonna, 2019). Furthermore, endocrine abnormalities, possibly influenced by trypanosomes on the host's pituitary gland, are known to occur in the later stages of trypanosomiasis (Bittencourt and Ashworth, 1995). Thus, a reexamination of the relationship between trypanosome infection, disrupted circadian rhythms, and endocrine processes in hellbenders emerges as a compelling avenue for further investigation.

Mechanisms of Anemia

While my study demonstrates that the leech vector is the primary cause of anemia in hellbenders, it remains unclear as to how they elicit a regenerative response, especially given their small size relative to their host's large body size. Typically, in endotherms, regenerative anemia from hemolysis elicits a more intense response than hemorrhagic anemia (Latimer et al. 2003) which is why we initially suspected trypanosomes as the primary cause of anemia (Stijlemans et al. 2018). Despite this, our evidence shows that the attachment of the leeches is the key indicator of red blood cell regeneration. The leeches in our system are relatively small, with an average size of 5mm in length (Hopkins et al. 2014), which calls into question whether the observed regenerative response is a consequence of excessive blood loss (i.e., hemorrhage). However, one would expect that if blood meals by leeches led to regenerative anemia, repeated phlebotomy would exacerbate the effects. By comparing the results to the outgroup that had not been previously bled, we confirmed that repeated phlebotomy did not cause anemia. The question then arises: if repeated bleeding does not elicit regeneration, how does the attachment of small leeches cause such a dramatic response? A possible explanation is that hellbender hosts may indeed experience excessive blood loss due to leeches. Leech saliva contains a suite of chemical compounds, including vasodilators and anticoagulants (Hildebrandt and Lemke 2011; Salzet et al. 2000), which may leave an open wound at the site of the bite following the departure of the leech potentially leading to blood loss beyond that ingested by the leech. Perhaps it could be from the cumulative effects of multiple leeches attached to the host for an extended period of time. It could also be from the rate of consumption associated with the rapid growth period required by leech offspring prior to overwintering (Wilkialis 1984; Moser et al., 2009). The precise amount of blood consumed by leeches in a single blood meal and the rate at which leech offspring metabolize their blood meal remain unknown and more research is required to quantify blood loss in hellbender hosts.

Additionally, red blood cell regeneration could be from a combination of hemorrhage and hemolysis attributed to the leech. Leeches elicit immune responses beyond marked shifts in white blood cells, including increased total plasma proteins (Hopkins et al. 2016). Some plasma proteins, such as immunoglobins, play an essential role in the immune-mediated lysis of red blood cells which could result in regenerative anemia (Flegel, 2015). Thus, the combination of minor blood loss over extended periods of time, along with lysis of red blood cells, could be the cause of such a dramatic response. Literature on aquatic leech vectors and their pathological impacts on wild hosts is scarce. While my research identifies the contribution of the vector to regenerative anemia in hellbenders, I encourage researchers to explore whether this phenomenon is present in other aquatic systems and investigate the underlying mechanisms causing anemia.

Conservation Implications

In my research, I used hematological metrics commonly used in veterinary medicine that can be easily adapted for outdoor conditions, and are resilient to transport, interpretable, and applicable for ecologists and wildlife managers. Among endothermic vertebrates, blood parameters are used to recognize the influence of habitat degradation, physiological costs of breeding, exposure to pollutants, and disease (Maceda-Veiga et al., 2015; Kloskowski et al., 2017; Fallon et al., 2017) but such applications have yet to extend to imperiled ectotherms like amphibians. Additionally, the rapid decline of amphibian populations emphasizes the need for monitoring in light of epidemics like those caused by ranavirus and chytrid fungus (Lips et al., 2008, Price et al., 2014) which have contributed to the mass mortality of some amphibian species.

Given the significant variations in the anatomy and physiology of amphibians across different taxa, reproductive stages, and sexes (Allender and Fry, 2008) it is important to develop species-specific reference values for monitoring amphibian health in both captive and wild systems. This should also take into account the impact of environmental conditions and seasonality on the physiology of ectothermic species, in order to accurately identify any fluctuations in reference values and differentiate them from pathological effects of diseases. Thus, my research established hematological reference values from a healthy population of hellbenders, recognizing how they shift in response to extrinsic factors, and monitoring disease progression. Moreover, our findings regarding the ~30-day lag time for identifying red blood cell regeneration emphasize the benefits of repeated monitoring and show that the pathologies at the point of capture may reflect prior interactions with parasites or accumulations over time.

Researchers are interested in how animals respond to stressors to evaluate the physiological response to existing threats, but not all tools are applicable to ectothermic species. Common metrics used to evaluate the stress response in other vertebrates, such as N:L ratios, behave differently in ectotherms because the leukocyte response is much slower compared to endothermic species (Davis et al. 2008). For example, Durant et al. (2015) showed that marked shifts in N:L ratios do not occur in hellbenders until 28 hours after the initial stressor which shows that some common metrics are not always applicable to field studies with brief periods of handling. Thus, my research helped to establish hemoconcentration as an alternative metric to evaluate the rapid response to stress, which could be an effective tool for monitoring other ectotherm populations.

The metric of hemoconcentration holds significant potential in wildlife science for assessing oxygen demands under various stressors like predator encounters, increased resource competition, and environmental changes. In the realm of fish physiology, researchers have explored the influence of stressors such as handling, confinement, and pollution on hemoconcentration (Biron and Benfey, 1984; Mazon et al., 2002; Brill et al., 2008). However, the interpretive value of hemoconcentration measurements hinges on contextualizing them against baseline values. If baseline values fall within the normal reference range and the degree of hemoconcentration is low, it may signify a dampened response to a specific stressor. For example, in aquaculture settings, researchers assessed the influence of light spectrum in rearing facilities on the stress response to confinement. Their results indicate that fish reared under white light exhibited a greater hemoconcentration response compared to those under blue light, suggesting the potential use of blue light to mitigate acute stress responses to confinement (Karakatsouli et al., 2012). Conversely, elevated baseline values could indicate an existing condition affecting blood, such as dehydration or hypoxic conditions (Frangioni and Borgioli, 1993; Veldhuijzen Van Zanten et al., 2005), leading to a lower magnitude of hemoconcentration compared to healthy individuals with normal baseline values. This nuanced understanding of hemoconcentration in relation to baseline values emphasizes the need for robust, species-specific reference values to enhance its utility in gauging stress responses in wildlife.

Conclusion

My research fills fundamental knowledge gaps regarding amphibian physiology and provides novel contributions to the fields of disease ecology, ecophysiology, and wildlife conservation. My findings revealed that the leech vector is responsible for regenerative anemia in hellbenders, rather than the trypanosome blood parasite that it transmits. Although leeches are present in both terrestrial and aquatic environments, and interact with various wildlife species

(Phillips et al., 2020), pathologies associated with leeches are rarely studied, particularly in ectotherms. I suspect that this phenomenon is not unique to hellbenders and that leeches could be affecting other aquatic species as well, but they have gone unnoticed thus far. My study is the first to utilize hemoconcentration as a method to evaluate the acute stress response in amphibians. By implementing its use to monitor a wild population of hellbender salamanders across a critical ~8-month period of their life history (parental acre of young), my findings show that seasonal fluctuations in red blood cell metrics reflect the state of energetic demands relative to environmental temperatures. But when temperatures are the lowest of the season (winter), hellbenders can still rapidly release red blood cells into circulation in response to a potential threat. Finally, my research has highlighted the importance of establishing reference values for red blood cell and immune indices and recognizing how they shift in response to extrinsic factors in order to identify pathologies associated with infectious disease and monitor disease progression in endangered amphibians. It is my hope that these scientific contributions will aid other researchers in their exploration of amphibian physiological adaptations and their efforts to develop and implement conservation strategies.

References

Allender M.C. and M.M. Fry. 2008. Amphibian hematology. Vet Clin N Am Exot Anim Pract 11: 463–480

Baetselier P., B. Namangala, W. Noël, L. Brys, E. Pays, and A. Beschin. 2001. Alternative versus classical macrophage activation during experimental African trypanosomosis. Int J Parasitol 31:575-587.

Baral T.N. 2010. Immunobiology of African Trypanosomes: Need of Alternative Interventions. BioMed Res. Int. 2010:e389153

Barry J.D. and D.L. Emery. 1984. Parasite development and host responses during the establishment of Trypanosoma brucei infection transmitted by tsetse fly. Parasitol. 88:67-84.

Baskova I.P., Z. Ferner, A.S. Balkina, S. Kozin, O.V. Kharitonova, L.L. Zavalova, and V.G. Zgoda. 2008. Steroids, histamine and serotonin in medicinal leech salivary gland secretions. Biomed Khim 54:127–139

Biron M. and T.J. Benfey. 1994. Cortisol, glucose, and hematocrit changes during acute stress, cohort sampling, and the diel cycle in diploid and triploid brook trout (*Salvelinus fontinalis Mitchill*). Fish Physiol and Biochem 13:153–160.

Bittencourt A.L. and T.G. Ashworth. 1995. Trypanosomiasis. pp 653–704 in: Tropical Pathology. Spezielle pathologische Anatomie, vol. 8. Springer, Berlin, Heidelberg.

Blaustein A.R., S.C. Walls, B.A. Bancroft, J.J. Lawler, C.L. Searle, and S.S. Gervasi. 2010. Direct and Indirect Effects of Climate Change on Amphibian Populations. *Diversity* 2: 281-313.

Bodinof Jachowski C.M., V. Alaasam, A. Blumenthal, A. Davis, and W.A. Hopkins. 2024. The habitat quality paradox: Loss of riparian forest cover leads to decreased risk of parasitism and improved body condition in an imperiled amphibian. Conserv Physiol 12:coad101

Brill R., P. Bushnell, S. Schroff, R. Seifert, and M. Galvin. 2008. Effects of anaerobic exercise accompanying catch-and-release fishing on blood–oxygen affinity of the sandbar shark (*Carcharhinus plumbeus*, Nardo). J Exp Mar Biol Ecol. 354:132–143.

Caljon G., J. Van Den Abbeele, B. Stijlemans, M. Coosemans, P. De Baetselier, and S. Magez. 2006. Tsetse Fly Saliva Accelerates the Onset of *Trypanosoma brucei* Infection in a Mouse Model Associated with a Reduced Host Inflammatory Response. Infect Immun 74:6324-6330.

Castelo Branco A.C.C., F. S. Yamada Yoshikawa, A.J. Pietrobon, and M.N. Sato. 2018. Role of Histamine in Modulating the Immune Response and Inflammation. Mediators Inflamm 2018:e9524075

Davis A.K., D.L. Maney, and J.C. Maerz. 2008. The use of leukocyte profiles to measure stress in vertebrates: a review for ecologist. Funct Ecol 22:760-772.

Davis A.K. and W.A. Hopkin. 2013. Widespread trypanosome infections in a population of eastern hellbenders (*Cryptobranchus alleganiensis alleganiensis*) in Virginia, USA. Parasitol Res 112:453–456.

DuRant S.E., W.A. Hopkins, A.K. Davis, and L.M. Romero. 2015. Evidence of ectoparasite-induced endocrine disruption in an imperiled giant salamander, the eastern hellbender (*Cryptobranchus alleganiensis*). J Exp Biol 218:2297–2304.

Fallon J.A., E.P. Smith, N. Schoch, J.D. Paruk, E.A. Adams, D.C. Evers, P.G.R. Jodice, C. Perkins, S. Schulte, and W.A. Hopkins. 2017. Hematological indices of injury to lightly oiled birds from the Deepwater Horizon oil spill. Environ Toxicol Chem 37:451–461.

Flegel W.A. 2015. Pathogenesis and mechanisms of antibody-mediated hemolysis. Transfusion 55:S47-S58.

Frangioni G. and G. Borgioli. 1993. Hematological changes in the newt during the process of splenic decongestion in reply to hypoxia. Ital J Zool (Modena) 60:267–270.

Graham A.L., I.M. Cattadori, J.O. Lloyd-Smith, M.J. Ferrari, and O.N. Bjørnstad. 2007. Transmission consequences of coinfection: cytokines writ large? Trends Parasitol 23: 284–291.

Hildebrandt J.P. and S. Lemke. 2011. Small bite, large impact–saliva and salivary molecules in the medicinal leech, *Hirudo medicinalis*. Naturwissenschaften 98:995–1008.

Hopkins W.A., W.E. Moser, D.W. Garst, D.J. Richardson, C.I. Hammond, and E.A. Lazo-Wasem. 2014. Morphological and molecular characterization of a new species of leech (Glossiphoniidae, Hirundinida): implications for the health of its imperiled amphibian host (*Cryptobranchus alleganiensis*). Zookeys 378:83–101.

Hopkins W.A., J.A. Fallon, M.L. Beck, B.H. Coe, and C.M.B. Jachowski. 2016. Haematological and immunological characteristics of eastern hellbenders (*Cryptobranchus alleganiensis*

alleganiensis) infected and co-infected with endo- and ectoparasites. Conserv Physiol 4:cow002.

Hopkins W.A., B.F. Case, J. Groffen, G.C. Brooks, C.M.B. Jachowski, S.T. Button, J.J. Hallagan, R.S.M. O'Brien, and H.K. Kindsvater. 2023. Filial Cannibalism Leads to Chronic Nest Failure of Eastern Hellbender Salamanders (*Cryptobranchus alleganiensis*). Am Nat 202:92–106.

Karakatsouli N., P. Katsakoulis, G. Leondaritis, D. Kalogiannis, S.E. Papoutsoglou, S. Chadio, and N. Sakellaridis. 2012. Acute stress response of European sea bass *Dicentrarchus labrax* under blue and white light. Aquaculture 364-365:48-52.

Kloskowski J., E. Kaczanowska, J. Krogulec, and P. Grela. 2017. Hematological indicators of habitat quality: Erythrocyte parameters reflect greater parental effort of Red-necked Grebes under ecological trap conditions. The Condor *119*:239-250.

Kopecky J., M. Kuthejlova, and J. Pechova. 1999. Salivary gland extract from *Ixodes ricinus* ticks inhibits production of interferon-gamma by the upregulation of interleukin-10. Parasite Immunol 21:351–356.

Kovar L., J. Kopecky, and B. Rihova. 2001. Salivary gland extract from *Ixodes ricinus* tick polarizes the cytokine profile toward Th2 and suppresses proliferation of T lymphocytes in human PBMC culture. J. Parasitol. 87:1342–1348.

Latimer K.S., E.A. Mahaffey, and K.W. Prasse. 2003. Duncan & Prasse's Veterinary Laboratory Medicine: Clinical Pathology. Blackwell Publishing, Ames, IA.

Lips K.R., J. Diffendorfer, J.R. Mendelson, and M.W. Sears. 2008. Riding the wave: Reconciling the roles of disease and climate change in amphibian declines. Plos Biol 6:441–454.

Liu C., D. Chu, J. George, H.A. Young, and G. Liu. 2021. Cytokines: From Clinical Significance to Quantification. Advanced Science. 8:e2004433

Maceda-Veiga A., J. Figuerola, A. Martínez-Silvestre, G. Viscor, N. Ferrari, and M. Pacheco. 2015. Inside the Redbox: Applications of haematology in wildlife monitoring and ecosystem health assessment. Sci Total Environ 514:322-332.

Mansfield J.M and D.M. Paulnock. 2005. Regulation of innate and acquired immunity in African trypanosomiasis. Parasite Immunol 27: 361–371.

Mazon A.F., E.A.S. Monteiro, G.H.D. Pinheiro, and M.N. Fernadez. 2002. Hematological and physiological changes induced by short-term exposure to copper in the freshwater fish, *Prochilodus scrofa*. Brazil J Biol 62:621-631

Moser W.E., F.R. Govedich, and D.J. Klemm. 2009. Annelida, Hirudinida (leeches). Pp. 116–123 in G.E. Likens, ed. Encyclopedia of Inland Waters, vol. 2. Elsevier Press, Oxford, UK.

Namangala B., W. Noel, P. De Baetselier, L. Brys, and A. Beschin. 2001. Relative contribution of interferon-gamma and interleukin-10 to resistance to murine African trypanosomosis. J Infect Dis 183:1794–1800.

Norsworthy N.B., J. Sun, D. Elnaiem, G. Lanzaro, and L. Soong. 2004. Sand fly saliva enhances *Leishmania amazonensis* infection by modulating interleukin-10 production. Infect Immun 72:1240–1247.

Nuttall P. 1998. Displaced tick-parasite interactions at the host interface. Parasitology 116:S65–S72.

O'Brien R.S.M., J. Groffen, A. Dayer, and W.A. Hopkins. 2024. Pre-breeding, Courtship, and Mating Behaviors of Wild Eastern Hellbenders (*Cryptobranchus alleganiensis alleganiensis*). Herpetologica. 80.

Onyilagha C. and J.E. Uzonna. 2019. Host Immune Responses and Immune Evasion Strategies in African Trypanosomiasis. Front Immunol 10:e478699

Phillips A.J., F.R. Govedich, and W.E. Moser. 2020. Leeches in the extreme: Morphological, physiological, and behavioral adaptations to inhospitable habitats. Int J Parasitol Parasites Wildl 12:318-325

Price S.J., T.W.J. Garner, R.A. Nichols, F. Balloux, C. Ayres, A. Mora-Cabello De Alba, and J. Bosch. 2014. Collapse of amphibian communities due to an introduced *Ranavirus*. Curr Biol 24:2586–2591.

Salzet M., A. Capron, and G.B. Stefano. 2000. Molecular crosstalk in host-parasite relationships: Schistosome- and leech-host interactions. PT 16:536–540.

Sawyer R.T. 1986. Leech Biology and Behaviour, vols. I–III, Clarendon Press, Oxford.

Spellberg B. and J.E. Edwards. 2001. Type 1/Type 2 immunity in infectious diseases. Clinical infectious diseases: an official publication of the Infectious Diseases Society of America 32:76–102.

Stijlemans B., P.D. Baetselier, S. Magez, J.A. Van Ginderachter, and C.D. Trez. 2018. African Trypanosomiasis-Associated Anemia: The Contribution of the Interplay between Parasites and the Mononuclear Phagocyte System. Front Immunol 9:e316111

van Genderen P.J.J., J.L. Nouwen, M. De Mendonça Melo, B.J.A. Rijnders, and J.J. van Hellemond. 2021. Single-Dose Pentamidine Substantially Reduces Viability of Trypanosomes in Human East African Trypanosomiasis. J Travel Med 28:taab080.

Veldhuijzen Van Zanten J.J.C.S., G. Thrall, D. Wasche, D. Carroll, and C. Ring. 2005. The influence of hydration status on stress-induced hemoconcentration. Psychophysiol 42:98-107

Wiegertjes G.F., M. Forlenza, M. Joerink, and J.P. Scharsack. 2005. Parasite infections revisited. Dev Comp Immunol 29:749–758.

Wilkialis J. 1984. The life history of *Haementeria costata* (Fr. Müller) (Glossiphonidae: Hirudinea). Hydrobiologia 109:219–227.