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Association Between Reduced Upstream Riparian Forest Cover and Impaired Development of Embryos From Eastern Hellbenders (*Cryptobranchus alleganiensis*)

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ABSTRACT

1. Populations of the Eastern Hellbender, (a large-bodied, fully aquatic salamander) inhabiting stream reaches with low catchment-wide riparian forest cover upstream, have experienced population declines and a shift toward a geriatric population age structure. These population declines and demographic shifts might be attributed to reduced embryo viability. Reduced egg quality/viability could negatively affect recruitment and has also been known to trigger filial cannibalism in other species. Therefore, we hypothesized that in comparison to high forest cover sites, hellbender eggs collected from low forest cover sites would have a greater incidence of developmental abnormalities and lower overall viability, and that this would predict whole-clutch cannibalism by the attending male.
2. We collected a subset of eggs (~20–35) from 99 clutches across sites with variable upstream riparian forest cover and reared these eggs through hatching in stream water under controlled laboratory conditions. At the same time, we monitored the fate of the remaining eggs from the same clutches in the field to document the frequency of whole-clutch filial cannibalism.
3. We found that eggs collected from sites with lower upstream forest cover had significantly shorter embryonic development times and produced a lower percentage of viable hatchlings (hatchlings with normal development times and morphology). The average modelled viability of hatchlings was 70% higher in sites with the highest forest cover compared to our sites with the lowest forest cover. In contrast to our predictions, we did not find evidence to suggest that egg viability in the lab predicted whole-clutch cannibalism in the field.
4. Although forest cover was a significant predictor of egg viability and underdevelopment, substantial variance in embryonic developmental traits was unaccounted for in our models suggesting that traits associated with adults (e.g., egg and/or sperm quality) may also play a role in determining developmental outcomes. Further experiments are needed to identify what factors (e.g., egg quality, water quality) disrupt the embryonic development of hellbenders as well as the proximate stimulus that causes adult male hellbenders to eat their young.
5. Our results emphasise the importance of restoring and protecting riparian forest cover to conserve sensitive stream species.

1 | Introduction

Declines in freshwater biodiversity are far greater than in terrestrial ecosystems and are attributed to a variety of anthropogenic global changes including habitat alteration and degradation (Albert et al. 2021; Dudgeon et al. 2006; Reid et al. 2019; Sala et al. 2000). For example, loss of forest cover in riparian zones around streams removes a buffer from solar radiation, runoff of dissolved and particulate matter, and nutrient inputs from agriculture and other land use (Graziano, Deguire, and Surasinghe 2022; Gregory et al. 1991). With climate change producing warmer conditions, as well as more intense and frequent precipitation events that promote runoff, the impacts of deforestation on freshwater ecosystems are expected to be magnified in the future (Capon et al. 2013).

One species threatened by riparian deforestation is the eastern hellbender (*Cryptobranchus alleganiensis alleganiensis*; Jachowski and Hopkins 2018). Hellbenders are a large-bodied, fully aquatic species of salamander. About 90% of their gas exchange is cutaneous (Nickerson and Mays 1973), making them reliant on cold, well-oxygenated rivers and streams for habitat, and thus vulnerable to the effects of deforestation. Eastern hellbender populations are rapidly declining across the species' range with almost 80% of their populations declining or functionally extirpated (USFWS 2018). Declining hellbender populations are often associated with a loss of upstream catchment-wide riparian forest cover and are characterised by a scarcity of young age classes (juveniles and subadults) and geriatric population age structure (Burgmeier et al. 2011; Jachowski and Hopkins 2018). A recent long-term study demonstrated that the underlying mechanism may stem from inadequate parental care, leading to exceptionally high rates of nest failure (Hopkins et al. 2023). Typically, male hellbenders care for their eggs and hatchlings for >8 months after oviposition in autumn (Hopkins et al. 2023). This involves tail fanning of eggs, selective hygienic cannibalism of dead eggs and defensive protection of the nest against predators (Bishop 1941; Settle, Briggler, and Mathis 2018; O'Brien 2023) until the larvae emerge from the nest in the spring. Occasionally, guarding hellbenders in habitats with high riparian forest cover will forgo paternal care and engage in whole-clutch filial cannibalism. In contrast, cannibalism rates of whole clutches at low forest cover sites can exceed 44% each year, which combined with other sources of nest failure is sufficient to cause population declines and the observed shift toward a geriatric population age structure (Brooks, Hopkins, and Kindsvater 2024; Hopkins et al. 2023).

In streams experiencing the most riparian deforestation, the proximate mechanism(s) causing high rates of whole-clutch cannibalism remain unknown but are critical to inform future conservation actions. Recent work indicates that high rates of cannibalism in areas with reduced forest cover are not caused by reduced male body condition or size (a surrogate for age; Taber, Wilkinson Jr., and Topping 1975; Hopkins et al. 2023), or depleted food (crayfish) resources, which are among the most commonly hypothesized triggers for whole-clutch cannibalism in other species (Manica 2002; Rohwer 1978). One alternative hypothesis for high rates of whole-clutch filial cannibalism is that low forest cover decreases the viability of hellbender eggs either through poor egg quality, poor fertilisation or degraded water quality that kills the embryos (Hopkins et al. 2023). Reduced egg quality or slow-developing embryos causes partial-clutch cannibalism in some fish species (Klug and Lindström 2008) and has recently been observed in hellbenders via underwater video recordings (O'Brien 2023). Therefore, it seems plausible that if a large proportion of hellbender eggs in a clutch are not developing properly, hellbenders may switch from caring for the eggs to whole-clutch cannibalism and use the clutch as a food resource. This decision would prevent the adult hellbender from wasting energy on parental care and minimise the possibility that the entire clutch decomposes, while the resulting energy gains would allow the adult to reinvest in future reproductive opportunities (Bose 2022; Klug and Bonsall 2007).

In this study, we aimed to assess the influence of riparian forest cover on hellbender egg viability using a combination of laboratory and field observations across 3 years of study. We hypothesized that lower riparian forest cover reduces the viability of hellbender eggs. Because reduced offspring viability and improper development of eggs can be a trigger for partial-clutch cannibalism (Bandoli 2016; Klug and Lindström 2008; Vallon and Heubel 2016; Vallon, Anthes, and Heubel 2016; Takahashi, Okada, and Fukuda 2017) and has been a hypothesized but unexplored trigger for whole-clutch filial cannibalism in other species (Bose 2022), we predicted that eggs from clutches with low viability would be more likely to be completely cannibalised by the attending nesting male in the field.

2 | Methods

2.1 | Study System in Virginia

We conducted our research in a long-term study system in the upper Tennessee River Basin in southwestern Virginia, USA. The system is composed of three rivers; however, the exact names and locations of these rivers cannot be disclosed to prevent disturbance of vulnerable hellbender populations. Generally, sites in this system with less than ~63% upstream catchment-wide riparian forest cover tend to have degraded water quality, reduced hellbender population density, a geriatric population age structure (Jachowski and Hopkins 2018) and unsustainably high frequencies of whole-clutch filial cannibalism (Hopkins et al. 2023). Within each of our three rivers, we previously designated 2–5 stream reaches as long-term study sites ($N = 10$ total reaches; Hopkins et al. 2023), all of which have been monitored continuously for the last 8–16 years. Stream reaches span a range in upstream forest cover (% upstream catchment-wide riparian forest = 54.1%–67.9%; based on a 50 m buffer on both sides of the stream throughout the upstream catchment as calculated in Hopkins et al. 2023) and associated changes to water quality. While the range of percent forest cover in our system is not large, it is sufficient to capture significant differences in hellbender nest fate, population age structure, population density and evidence of declines, and water quality (Jachowski and Hopkins 2018; Hopkins et al. 2023). Additionally, in other systems, small reductions in forest cover percentage have been found to significantly influence headwater streams (England and Rosemond 2004). During initial site selection, we were mindful of natural variation in water quality (e.g., specific conductivity) given underlying physiography and thus we ensured that varying water quality among sites could not be solely attributed to the geology of the surrounding area (Bodinof Jachowski, Millspaugh, and Hopkins 2016; Griffith 2014; Jachowski and Hopkins 2018).

Over the last decade, we designed, constructed and installed approximately 350 artificial underwater shelters for studying hellbenders in the 10 stream reaches (~30–38/reach; Button, Hallagan, et al. 2020; Button, Bodinof Jachowski, et al. 2020; Hopkins et al. 2023; Figure S1). These artificial shelters have proven to be valuable monitoring (Button, Bodinof Jachowski, et al. 2020; Jachowski, Ross, and Hopkins 2020) and research tool for studying hellbender reproductive behaviour (O'Brien et al. 2024), physiology (Galligan et al. 2021; Case et al. 2024; Slack, Groffen, and Hopkins 2024) and ecology (Hopkins et al. 2023).

Artificial shelters also allow for the safe and relatively easy observation of hellbender nesting behaviour and success in situ. For example, over the course of an 8-year study, Hopkins et al. (2023) found that hellbenders established 182 nests in artificial shelters. During the duration of that study, 101 unique males established nests, with 44 males nesting in more than 1 year, and 64% of these using the same artificial shelter for multiple years (Hopkins et al. 2023). Since that study's conclusion, an additional 214 nests have been established in shelters in our study system. Thus, hellbenders successfully and repeatedly use artificial shelters for nesting in their natural environment, allowing for safe and convenient data collection of the attending parental males and their clutches.

2.2 | Study Design

Sampling took place during the hellbender nesting season (late August–late April) of 2018, 2019, and 2021. We surveyed artifical shelters at the beginning of each hellbender nesting season (late August) and continued to check them every 24–72 h thereafter until nesting activity in each stream reach was complete. After identifying freshly laid hellbender eggs (within 48–72 h of oviposition), we waited 24 h to collect eggs to ensure that reproductive activity had ceased within the shelter. At the time of egg collection, we obtained quantitative and qualitative data on the nest and the attending nesting male. Using previously described methods, we enumerated each nest's exact clutch size and obtained morphological characteristics of each nesting male (Hopkins et al. 2023). We then collected a random subsample of the eggs (equivalent to ~10% of an average clutch). Once eggs were collected, they were immediately transported back to the laboratory in secure, plastic containers filled with their respective site water. Plastic containers were transported in a cooler with ice, and the temperature of the eggs' water was monitored with digital thermometers while in transport to ensure that the eggs did not experience substantial change in temperature (≤ 2°C–3°C) during transit.

In the field, we determined hellbender nest fate based on observations from four sampling events throughout the hellbender nesting season (oviposition [day 0], mid-embryonic development [day 30], hatching [day 60] and near larval emergence in the spring [day 200]). Nest fates (success, whole-clutch cannibalism and other failure types) were determined using the same diagnostic criteria as outlined in Hopkins et al. (2023). Given the research objectives of this study, we were specifically interested in nest failures caused by whole-clutch filial cannibalism (for a full classification scheme, see Hopkins et al. 2023).

In addition to biological data, we collected water chemistry data in the field using a YSI 556 (Yellow Springs, OH) handheld multiparameter instrument during each sampling event. Water chemistry data included: dissolved oxygen (mg/L and % saturation), specific conductivity (μS/cm) and pH.

2.3 | Captive Rearing

Once the eggs were transported to Virginia Tech, we transferred them into our captive-rearing system. We designed this system to allow us to raise hellbender eggs in their own site water maintained at environmentally relevant temperatures. The system consists of a large recirculating water bath that maintains the desired water temperature using EcoPlus Commercial Grade Water Chillers 1–1/2 HP (Vancouver, WA). Glass aquaria filled with appropriate site water are immersed in this water bath to enable isolation of clutches and their respective site water from one another. In total, the captive-rearing system can hold up to 48, 38-L glass aquariums that are filled with respective site water and chilled by the surrounding water bath. The individual aquaria also contain plastic netting in the shape of a basket and an automated aeration system that turns on every 3 min. When on, the aeration system agitates eggs with bubbles for 30 s from submerged air stones underlying the clutch which causes eggs to float and rotate in the water column before settling back onto the plastic netting at the end of each aeration interval (Video S1). This periodic movement of eggs prevents embryonic adhesions (the embryos sticking to the jelly envelopes; Nussbaum 1985) and is intended to mimic the benefits of physical agitation of hellbender parental care behaviours such as tail fanning (O'Brien 2023).

Infrastructure issues caused some variations over the years in water temperature in the laboratory that were beyond our control. For the first 2 years of the study, the rearing system was in an off-campus facility that experienced substantial fluctuations in ambient air temperature due to inadequate building controls. Although this never caused temperatures in the rearing system to rise, in some instances, the ambient air temperature would drop low enough to cause water temperatures in our recirculating water bath to decrease below their desired setpoints. Thus, in 2020, we moved the entire rearing system into an on-campus animal facility with greater air temperature control, which allowed better control of water temperature in 2021.

On the day of egg collection, we filled an aquarium with respective site water for each subset of eggs and floated the vessel from the field containing the eggs in the aquarium, allowing for a gradual equilibration of water temperature.

Once temperatures equilibrated, eggs were released into the aquarium. We monitored the eggs every day from intake into the lab until embryonic death or hatching. On a weekly basis, we changed 30% of the aquarium water with fresh water from each clutch's respective field site. We collected site water biweekly and ensured the water received constant aeration in between collection and use, to allow for gas exchange, maintain high DO and preserve freshness (Roy et al. 2021). We monitored husbandry conditions with biweekly water chemistry analyses using a HACH colorimeter kit (Loveland, CO; nitrate (NO_3^-), nitrite (NO_2^-), ammonia (NH_3), phosphate (PO_4^{3-})) and YSI sonde (temperature, dissolved oxygen, specific conductivity, pH, total dissolved solids and salinity). On a daily basis, we ensured that all the eggs were fully rotating in each aquarium. We noted the number of dead embryos each day, and promptly removed them to prevent microbial contamination of the remaining eggs.

Once an egg hatched, we removed the hatchling and euthanized it by submerging it in a 0.2% solution of tricaine methane sulfonate (MS222; buffered to a pH of 7.0). We blotted all excess water from each hatchling's exterior and obtained its wet mass with a Mettler Toledo XS205 (Columbus, OH) dual range balance (nearest 0.01 mg). We then measured the total length of each hatchling with a Fowler Sylvac (Canton, MA) electronic calliper (nearest 0.01 mm). During processing, we also recorded any hatchling abnormalities, including axial malformations (Bantle et al. 1991; Unger and Mathis 2013), oedema (Bantle et al. 1991; Henle and Dubois 2017) or distended yolk sac, obvious underdevelopment (compared to sibling hatchlings) or lack of movement prior to euthanasia. Hatchlings were categorised as underdeveloped based on several factors. These included both the timing of hatching (i.e., generally < 45 days post-oviposition) and morphological observations such as embryonic stage, fragility and/or abnormally large size of the yolk sac, premature tissue development and/or the absence of movement prior to euthanasia (Figure S2). Our criteria are also consistent with a previous study on developmental stages of the closely related Chinese giant salamander (*Andrias davidianus*) which documented premature hatching (i.e., hatching before the tail circulation developmental phase) and concluded that these were less likely to survive than hatchlings emerging after this phase (Jian et al. 2007). Further, a follow-up study of ours demonstrated that underdeveloped hatchlings are not viable and typically die within days of premature hatching. Following processing, hatchlings were frozen individually at −80°C and were later used for a companion study on the frequency of multiple paternity in hellbenders and its possible relationship to filial cannibalism (as in fish; Neff 2003).

At the end of our experiment, we calculated the percentage of the clutch that produced viable hatchlings (hatchlings without abnormalities). This metric provides a more comprehensive assessment of early development than hatching success because it accounts for only individuals that successfully hatch and have the capacity to survive in the days following hatching (Hopkins et al. 2006). Hatchlings were considered viable if they did not have any abnormalities such as oedema, axial malformations or underdevelopment. We observed several instances of polyembryony (our study was the first known recording of polyembryony in hellbenders; Groffen et al. 2022). Double embryos ($N = 8$ instances) encased in a single jelly coat were counted as two eggs for our total number of eggs collected from each clutch.

2.4 | Data Analysis

All analyses were conducted in R version 4.3.2. (R Core Team 2023). We used the lme4 package (Bates et al. 2015) in R to run all models and ggplot2 (Wickham 2016) to construct figures.

In our analyses, our response variables consisted of proportional data (i.e., % viability, % hatching success, % underdeveloped hatchlings, etc.), continuous data (i.e., morphometrics, time to hatch, and water quality variables) and binomial data (i.e., cannibalism [yes/no]). In each of our models with proportional data, we used a generalised linear model with a binomial distribution. Using binomial distributions for proportional response variables is accepted in biological and ecological data analyses (Warton and Hui 2011; Quinn and Keough 2002; Zuur et al. 2009) because the proportions are derived from count data (i.e., number of viable hatchlings) and the raw data represent a binomial response (i.e., yes, an egg was viable or no, an egg was not viable). Additionally, for several of our models with continuous data, we scaled the variables to aid in model convergence and normality assumptions. Variables were scaled with a mean of zero and a standard deviation of one (or the *z*-scores of the variable; Quinn and Keough 2002). For each of our models, we conducted diagnostic plots using the DHARMa package (Hartig 2022) and compared R^2 values of other possible data transformations (i.e., log or arcsine $\sqrt{\%}$) using the MuMIn package (Bartoń 2023). Our scaled models consistently outperformed transformed data based on DHARMa diagnostics and also had higher R^2 values compared to other data transformations.

To examine the influence of forest cover on the percentage of eggs that were viable, we constructed a generalised linear model with a binomial distribution with percent viability as the response variable, forest cover and year as fixed effects and shelter ID as a random effect. We calculated percent hatchling viability as the proportion of viable hatchlings out of the total number of eggs collected from each clutch. To ensure correct model fit, we also included the number of eggs collected from each nest as weights (Dunn and Smyth 2018). We then conducted a follow-up analysis to determine how forest cover influences hatching success and each type of abnormality. We excluded two conjoined (fused) embryos from the follow-up analysis that were considered not viable but did not reasonably fit into any of our three abnormality categories.

For our second set of analyses, we examined whether forest cover impacted hatchling morphometrics. Because developmental time is closely related to size at hatching (Warkentin 1999; Warne, Kardon, and Crespi 2013), we began by modelling the relationship between time to hatch and hatchlings' morphometrics. We constructed two linear mixed-effects models with scaled variables to ensure model convergence. For these models, we included the hatchling mass and length as our response variables, with time to hatch and sampling year as additive fixed effects, and shelter ID as a random effect. With these initial models, we found a strong relationship between the time to hatch and our morphometric data (see Results). Therefore, we also included developmental time as a covariate in models that also considered the relationship between forest cover and hatchling morphometrics. We constructed two linear mixed models (one for total length and one for total mass) with the scaled fixed effect of forest cover, additive effects of year and developmental time, and shelter ID as a random effect. We scaled the data to ensure appropriate model fit and convergence.

We used a linear mixed model to examine whether forest cover impacts the time to egg hatching. To accomplish this, we included forest cover and year as predictor variables, time to hatch as the response and shelter ID as a random effect. We scaled both forest cover and days to hatch to ensure model fit and convergence.

To determine whether nests with a lower percent viability in the laboratory were more likely to fail due to cannibalism of the remaining clutch in the field, we constructed a generalised linear model with a binomial distribution. We included whole-clutch cannibalism as the response variable and percent viability of hatchlings and year as fixed effects. Shelter ID was not included as a random effect in our cannibalism models because there were not enough replicates of shelter ID for the model to converge. We explored how the models might change with shelter ID as a fixed effect, but it did not significantly change our results or model significance, indicating that its exclusion from the models would not change our interpretation of the results.

Finally, we constructed several models to determine how forest cover and year of study influenced water chemistry variables. Nitrogen and phosphorous compounds (nitrate (NO_3^-), nitrite (NO_2^-), ammonia (NH_3) and phosphate (PO_4^{3-})) in individual aquaria were recorded to ensure suitable husbandry conditions for hellbenders. Water concentrations of these compounds remained low in all aquaria over the course of the 3-year study (see Results) and were not analysed statistically beyond determining how they differed across sampling years through linear models for all four compounds. In each model, we included scaled nitrogen or phosphorous compounds as the response variable and year as a fixed effect. We constructed two generalised linear models with a gamma distribution to examine how forest cover affects specific conductivity (μS/cm) and pH from our field YSI measurements collected over the three sampling years. Specific conductivity or pH were response variables, and forest cover and year were predictor variables in these models.

3 | Results

Over 3 years of study, we collected 387–1427 eggs per year ($n = 3090$ total eggs). Eggs were obtained from 99 total nests (range of 15–42 nests per year). From each nest, we collected an average of 11% ± 0.004 SE of nests' initial clutch size in the field. In total, our analysis consisted of 35 hellbender nests from our declining populations (which have < 63% forest cover and ~85% nest failure; Hopkins et al. 2023) and 64 nests from our higher quality populations. Each of our sampling years had a similar distribution of nests from higher to lower quality sites (range each year ~59%–69% for higher quality sites and ~31%–41% for our lower quality sites).

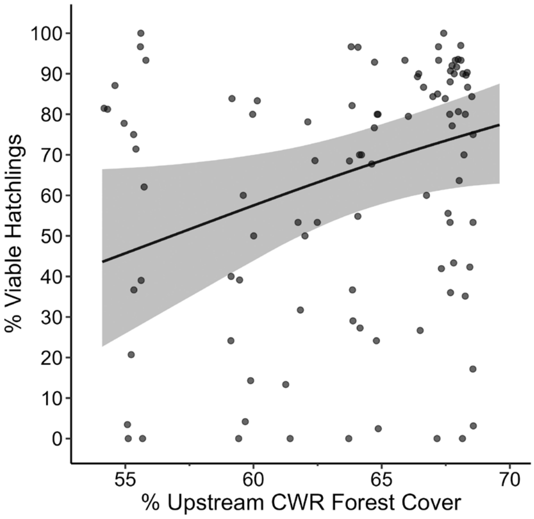

FIGURE 1 | Upstream catchment-wide riparian (CWR) forest cover had a significant effect on the percent viability of successful hellbender (*Cryptobranchus alleganiensis*) hatchlings from eggs that were reared in the laboratory in water from their natal site. The figure depicts the significant relationship between percent viability and forest cover with the grey area representing prediction intervals for our model ($p = 0.028$). The model includes the fixed effect of sampling year and the random effect of artificial shelter ID, which are not visibly evident in the raw data displayed on the graph. Points are offset slightly on the x-axis to provide clarity.

We found that the overall viability of hellbender eggs in our study was high (~71%), but that forest cover ($\beta = 0.102 \pm 0.046$ SE, $p = 0.028$; Figure 1) and year ($p < 0.001$, Table S1) were significant predictors of the percentage of viable hatchlings produced from clutches (conditional R^2 for the full model = 0.502; marginal R^2 for fixed effects = 0.067). The modelled average viability at our lowest forest cover site was 43.6% and at our highest forest cover site, it was 74.3%, equating to a ~70% increase in hatchling viability from low to high forest cover (Figure 1). Percent viability was significantly different among sampling years, with 2021 having the lowest percentage of viable hatchlings and 2018 having the highest.

A follow-up analysis of hatching success and the three abnormality categories (oedema, underdevelopment and/or axial malformations) revealed that underdevelopment and hatching success were the primary contributors to the relationship between forest cover and percent viability (Figure 2A,B). Overall average hatching success was high (~80%) but highly variable in our study, and the prevalence of developmental abnormalities was low (overall average for underdevelopment = 6%, oedema = 0.3% and axial malformations = 1%). Forest cover was a significant predictor of underdevelopment ($\beta = -0.244 \pm 0.077$ SE, $p = 0.002$), but not hatching success ($p = 0.576$; Figure 2A), oedema ($p = 0.609$; Figure 2D), or axial malformations ($p = 0.227$; Figure 2C). We found that the conditional R^2 for the full underdevelopment model was 0.741 and the marginal R^2 for the fixed effects was 0.323. Additionally, we found an effect of year on underdevelopment ($p < 0.001$), hatching success ($p < 0.001$) and axial malformations ($p < 0.001$), with 2021 having the highest probability of underdevelopment and axial malformations and 2019 having the lowest hatching success

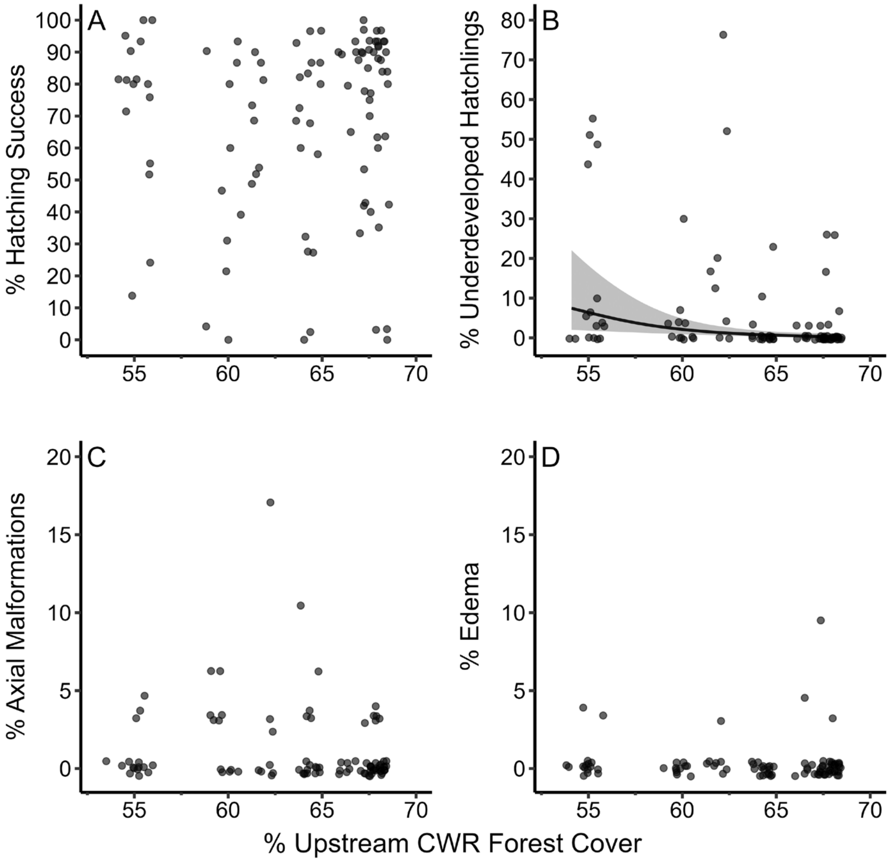

FIGURE 2 | The four variables used to calculate the percent viability of hatchlings from 99 clutches of eggs from hellbenders (*Cryptobranchus alleganiensis*) collected from sites along a gradient of upstream catchment-wide riparian (CWR) forest cover. Eggs were reared in captivity in their own site water. Note that the scale of y-axes varies among panels. Also, note that raw data are plotted, but each of the four models also included the fixed effect of sampling year and the random effect of shelter ID. Panel (A) depicts hatching success ($p = 0.576$); Panel (B) depicts the significant relationship between (CWR) forest cover and the percentage of underdeveloped hatchlings ($p = 0.002$, conditional $R^2 = 0.741$) with the grey area representing prediction intervals; Panel (C) depicts the percentage of hatchlings with axial malformations ($p = 0.227$); Panel (D) depicts the percentage of hatchlings with oedema ($p = 0.609$). Note that hatching success and the percentage of underdeveloped hatchlings were the dominant contributors to the significant relationship between forest cover and percent viability presented in Figure 1. Points are offset slightly on the x-axis to provide clarity.

(Table S1). In total, ~10% of hatchings were considered abnormal (219/2105 total hatchlings), but most of this was attributable to underdevelopment (179/219 abnormal hatchlings) which was more frequently observed at our low forest cover sites causing their viability to be reduced relative to high forest cover sites (Figure 2B).

For our morphometric and developmental data, we found that time to egg hatching had a significant positive relationship with total mass (conditional $R^2 = 0.801$; marginal $R^2 = 0.591$) and length (conditional $R^2 = 0.754$; marginal $R^2 = 0.575$). As developmental time increased so did both the mass (g) ($\beta = 0.871 \pm 0.015$ SE, $p < 0.001$) and length (mm) ($\beta = 0.760 \pm 0.015$ SE, $p < 0.001$) of hatchlings (Figure 3). We also found that developmental time was significantly correlated with forest cover ($\beta = 0.234 \pm 0.087$ SE, $p = 0.007$). As forest cover increased, the time to hatch increased as well (Figure 4A; conditional $R^2 = 0.589$; marginal $R^2 = 0.169$). In a follow-up analysis, we found that the significant, positive relationship between forest cover and time to hatch persisted even if premature (i.e., underdeveloped) hatchlings were excluded from the model ($p < 0.001$). However, upstream forest cover did not influence the total mass ($p = 0.104$) or total length ($p = 0.072$) of hatchlings once we corrected for time to hatch (Figure 4B,C). Time to egg hatching was also influenced by year, with the hatchlings in 2018 and 2021 hatching out 11 and 12% faster than in 2019 (Table S1). Likewise, we also found a significant effect of year on both length ($p < 0.001$) and mass ($p < 0.001$), but the biological effect size among years was small. Specifically, pairwise comparisons of body size measured across years revealed that hatchlings in 2019 were ~5% and 8% lighter than 2018 and 2021, respectively and hatchlings in 2021 were ~1% and 4% shorter than 2019 and 2018, respectively (Table S1).

When integrating our field observations with those in the lab, we found no significant relationship between percent viability in the lab and cannibalism of the remaining whole clutch by males in the field ($p = 0.137$; Figure 5). Further, we did not detect a significant effect of year on the incidence of wholeclutch cannibalism ($p = 0.232$).

We found that the point measurements of specific conductivity taken with the YSI instrument ($p < 0.001$; Figure 6) had a significant inverse relationship with forest cover ($R^2 = 0.798$). The year of sampling also significantly affected the specific conductivity collected with the YSI instrument ($p < 0.001$; Table S2), with 2019 having the highest specific conductivity and 2021 having

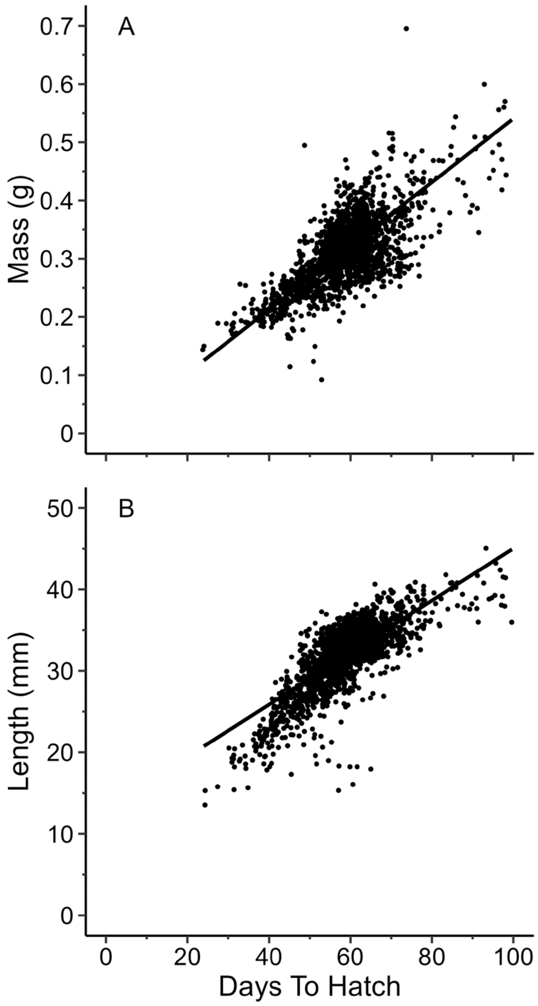

FIGURE 3 | The mass ($p < 0.001$) and length ($p < 0.001$) of hellbender (*Cryptobranchus alleganiensis*) hatchlings, raised in their natal site water in a captive rearing facility, was positively correlated with the time it takes for the embryo to hatch. Each model also included the fixed effect of year and random effect of shelter ID. Panel (A) depicts the linear model for hatchling mass where the line = 0.018 + 0.480 + 0.871 *Day Hatched − 0.242 *Year 2019 + 0.131*Year 2021. Panel (B) depicts the linear model for hatchling length where the line = 0.169 + 0.409 + 0.760 *Day Hatched − 0.248 *Year 2019 − 0.324*Year 2021.

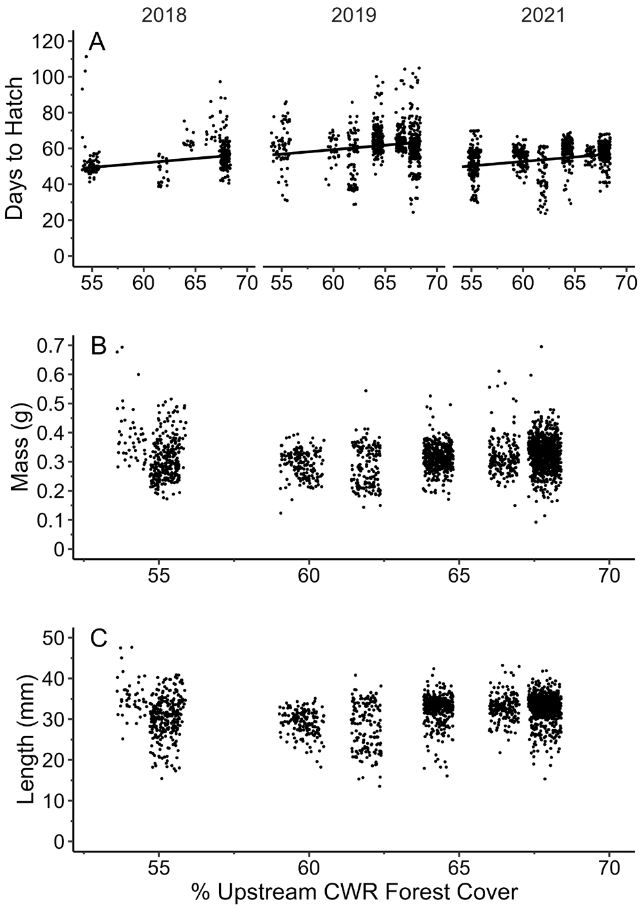

FIGURE 4 | The time to hatching for captive-reared hellbender (*Cryptobranchus alleganiensis*) eggs increased with upstream catchment-wide riparian (CWR) forest cover but had no impact on the mass or length of hellbenders at hatching. Panel (A) depicts the significant relationship between time to hatching and forest cover ($p = 0.007$, conditional $R^2 = 0.589$), as well as the differences in sampling years. Panels (B) ($p = 0.104$) and (C) ($p = 0.072$) depict the lack of a relationship between forest cover and morphometrics when the time to hatch is considered in the analysis. Each model also included the fixed effect of year and random effect of shelter ID. Points are offset on the x-axis to provide clarity.

the lowest. In contrast, we did not detect a significant relationship between forest cover and the point measurements of pH taken with the YSI instrument in the field ($p = 0.857$), but there was an effect of year ($p < 0.001$), with 2018 having the highest pH and 2021 having the lowest (Table S2).

In the laboratory, nitrogenous and phosphorus compounds remained well below the concentrations used as benchmarks for captive husbandry conditions of adult hellbenders and other species of larval amphibians (Earl and Whiteman 2010; Ettling et al. 2013; Garriga, Montori, and Llorente 2017; Griffis-Kyle 2005; Jofre and Karasov 1999). However, each of the nitrogenous and phosphorous compounds differed significantly among sampling years: nitrate ($p = 0.002$), nitrite ($p < 0.001$), ammonia ($p < 0.001$) and phosphate ($p = 0.021$). We also found a significant difference in water temperatures among sampling years ($p < 0.001$). The average water temperature in our rearing system was about 13%–16% higher in 2021 than the other 2 years (Table S2) due to the upgrades we made that provided better temperature control (see Methods). Although we found a year effect for all our water quality metrics, there were no obvious consistent patterns among the years (Table S2).

4 | Discussion

We demonstrated that loss of upstream riparian forest cover is associated with reduced viability of developing hellbender embryos. Although embryo viability was highly variable even within a stream reach, clutches from sites with the highest forest cover had modelled average viability 70% higher than clutches from the site with the lowest forest cover. Despite this observation, we found no evidence to indicate that adult male hellbenders are cannibalising their entire clutches in the field due to reduced embryo viability.

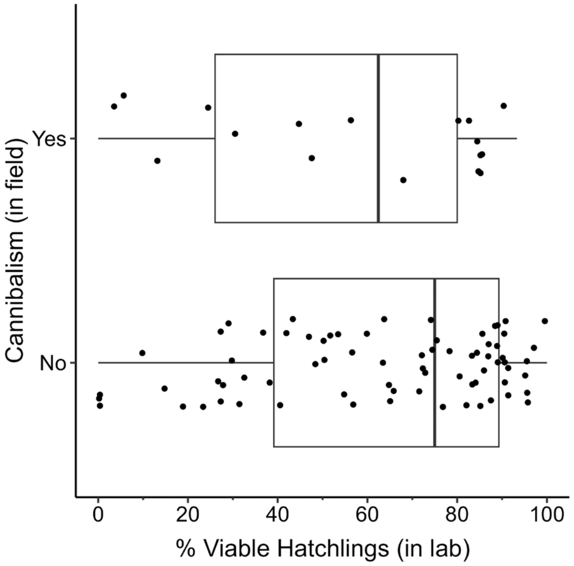

FIGURE 5 | The percent of viable hatchlings of captive-reared hellbender (*Cryptobranchus alleganiensis*) eggs had no significant effect ($p = 0.137$) on whether their nest of origin failed due to whole-clutch cannibalism in the field. The model also included the fixed effect of year.

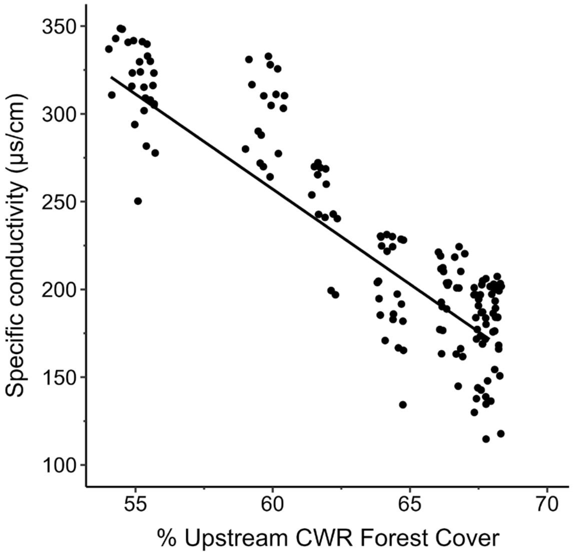

FIGURE 6 | Specific conductivity (µS/cm) point measurements collected with an YSI handheld instrument from hellbender (*Cryptobranchus alleganiensis*) nesting sites over 3 years of study had a negative relationship with upstream catchment-wide riparian (CWR) forest cover ($p < 0.001$). Points are offset on the x-axis to provide clarity. The line depicts the modelled relationship between specific conductivity and forest cover where the line = −7.402e-3 + 1.925e-04 * Forest Cover − 5.157e-04 * Year 2019 + 1.862e-05 * Year 2021. The model also included the fixed effect of year.

In combination with hatching success, premature or underdeveloped hatchlings were the primary hatchling abnormality driving the association between hatchling viability and upstream riparian forest cover. Although we can only speculate as to what factor(s) might cause the elevated incidence of premature hatching at low forest cover sites, one possibility is that it is caused by the higher specific conductivity of water at our lower forest cover sites. Specific conductivity (Dyck et al. 2021; Coughlan, Waters, and Touchon 2021) and increased salinity (Haramura 2016; Hopkins, French, and Brodie Jr. 2013; Tornabene, Breuner, and Hossack 2021) have been associated with premature hatching in other amphibian species. Premature hatching is problematic because it leads to smaller, less developed larvae which has been shown to decrease the growth and survival of amphibian larvae by delaying the onset of feeding (Warkentin 1999) and limiting their early ability to evade predators (Warkentin 1995; Boone, Scott, and Niewiarowski 2002).

We also found that embryonic development time (time to hatch) was longer at sites with higher forest cover, which resulted in hatchlings from higher forest cover being slightly longer. Importantly, these differences in embryonic development time were not driven by differences in the frequency of premature hatching across forest cover, indicating other mechanisms must be in play. Amphibians often display incredible plasticity in their early development (Warkentin 2011). Environmental and physiological factors such as temperature (Smith et al. 2015; Shadle et al. 2023), dissolved oxygen (Seymour et al. 2000; Warkentin 2002; Mills and Ward 2015), chemical predator cues (Saenz et al. 2003; Chivers et al. 2001), low water conditions (Denver, Mirhadi, and Phillips 1998) and growth rates in combination with body size (Wilbur and Collins 1973) can influence time to hatching or other developmental milestones (e.g., time to metamorphosis). However, these variables were controlled in our laboratory-rearing system. Similar to what we hypothesized for hatchling underdevelopment, differences in specific conductivity among sites could be a possible mechanism underlying developmental rate more generally. Aligned with our findings, other studies have found that amphibian eggs or larvae reared in water with higher dissolved ion concentrations hatched out sooner (Tornabene, Breuner, and Hossack 2021; Hopkins, French, and Brodie Jr. 2013; Haramura 2016), were smaller at hatching (Hopkins, French, and Brodie Jr. 2013; Hopkins, Brodie Jr., and French 2014; Haramura 2016) and were smaller at metamorphosis (Chinathamby et al. 2006; Snodgrass et al. 2008). In contrast, other studies have found that higher dissolved ion concentrations led to delayed hatching (Ocampo, Chuirazzi, and Takahashi 2022; Yaghobi et al. 2018) and slower pre-metamorphic larval development (Kearney et al. 2014; Gomez-Mestre et al. 2004), suggesting that the effect of specific conductivity on early developmental rate may be species- or context- specific. Because our study was not designed to disentangle the effects of water quality variables from other parental factors like egg quality, future cross-fostering experiments (raising eggs from different sites in water from different sites) are needed to determine whether conductivity or other factors explain our findings.

We found that the year of sampling had a significant influence on percent viability, time to hatch, and the length and mass of hatchlings. The year effects we observed are likely due, at least in part, to significant differences in rearing temperatures among sampling years. Temperature can fundamentally change most aspects of amphibian development, including hatching success (Jonsson, Jonsson, and Hansen 2022), metamorphosis (Sinai et al. 2022; Ruthsatz et al. 2018), and body size (Smith et al. 2015; Kaplan and Phillips 2006, but see Watkins and Vraspir 2006). Warmer temperatures can also induce faster hatching (Smith et al. 2015), which may explain why the percentage of underdeveloped hatchlings was greater in 2021, our warmest year. It is important to note that temperature fluctuations also happen in the field and that we found variation across sampling years in several other water quality variables, preventing us from isolating temperature as the definitive mechanism underlying some of our observations. Future experimental work could resolve these uncertainties.

We did not find evidence to support the hypothesis that reduced egg viability promotes filial cannibalism by nesting hellbenders in the field. Although our experimental approach allowed us to monitor development much more closely than would be possible in the field and to eliminate numerous potential confounds (e.g., possible variation in parental care, predation risk, siltation, water temperature, and dissolved oxygen among streams and stream reaches), it prohibited us from evaluating whether the complex environmental challenges that hellbender eggs face in the field affect embryo viability and the likelihood of cannibalism. For example, dissolved oxygen is a notable water quality metric that we held constantly high (~10 mg/L) in the laboratory through scheduled aeration but can fluctuate substantially in the field, especially with large clutches that fill the majority of artificial shelters or nest cavities (Hopkins et al. 2023). Dissolved oxygen plays a crucial role in the hatching success and post-hatching larval survival of many aquatic salamander and fish species (Gilmore, Doubleday, and Gillanders 2018; Mills and Ward 2015; Mills and Barnhart 1999). Additionally, recent behavioural studies have found that dissolved oxygen can influence hellbender parental care decisions that could adversely affect embryo development (i.e., possible trade-offs in self-maintenance and parental care behaviours; O'Brien 2023). Likewise, sedimentation was absent in our captive rearing system but can also differ greatly among artificial shelters, within natural nesting microhabitats in a stream reach, among reaches with different upstream forest cover, and with precipitation events. Sedimentation can contain accumulated trace metal contaminants that can increase amphibian embryonic mortality (Birge et al. 1977) or influence developmental rate (Peltzer et al. 2013). For some fish species, sedimentation has also been known to smother eggs and/or promote microbial processes that deoxygenate microhabitats (Henley et al. 2000; Poole et al. 2020).

It is important to note that our study design also controlled the spread of harmful microbes from dead embryos to living embryos in the laboratory by the removal of dead embryos each day, which would need to be achieved by the hellbender father in the field. Hellbenders and closely related *Andrias* salamanders engage in hygienic filial cannibalism where they selectively ingest dead eggs, which is thought to prevent the spread of pathogen infection through the clutch (O'Brien 2023; Okada, Fukuda, and Takahashi 2015; Takahashi, Okada, and Fukuda 2017). In the field, the average clutch size is ~300 eggs (and can exceed 1200; Hopkins et al. 2023) and eggs are sometimes densely compacted in natural or artificial nest cavities. Therefore, it seems plausible that reductions in embryo viability in the field could cross a threshold beyond which hellbenders are incapable of efficient removal of dead embryos. Crossing this hypothetical threshold could lead to accelerated mortality of other eggs in the clutch due to harmful microbes, which could trigger clutch failure, nest abandonment and/or whole-clutch filial cannibalism by the father. It remains unknown whether the lower viability observed in clutches from sites with low forest cover in our study was sufficient to cause microbial proliferation through a clutch. This and other hypothetical mechanisms will require additional research in the laboratory and field to fully understand how the loss of forest cover may be impacting hellbender development and parental care decisions.

Salamanders, like the hellbender, are good models for understanding anthropogenic influences on freshwater biodiversity because of their permeable skin and other physiological and life history traits (Hopkins 2007). Past research has shown that riparian forest habitat modulates water quality variables that affect aquatic salamanders (Clipp and Anderson 2014). Our study complements prior work by suggesting that reduced riparian forest cover affects the early development of hellbender embryos developing downstream, but experimental work is required to pinpoint what water quality variables, or other factors (e.g., egg quality) contribute to these developmental disruptions. In addition, further work is needed to determine what factors contribute to the high variability we observed in hatching success and percent viability across a forest cover gradient. Such variance makes it challenging to detect associations with environmental conditions without robust sampling, so understanding contributing factors will improve future study designs and conservation efforts. Nevertheless, our results in combination with past work (Hanna, Raudsepp-Hearne, and Bennett 2020; Hopkins et al. 2023; Hutton et al. 2021; Jachowski and Hopkins 2018; Pitt et al. 2017; Surasinghe and Baldwin 2015) indicate that riparian habitat protection and re-forestation are crucial conservation aims for the proliferation of hellbender populations and other sensitive stream biodiversity.

Author Contributions

Conceptualization: H.A.F., B.F.C., J.G., W.A.H. Developing methods: B.F.C., J.G., W.A.H. Data analysis: H.A.F. Preparation of figures and tables: H.A.F. Conducting the research, data interpretation, writing: H.A.F., B.F.C., J.G., A.H., W.A.H.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Data are available upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.