EMBRYONIC TEMPERATURE HAS CARRY-OVER EFFECTS ON BODY SIZE, DEVELOPMENT TIME, AND LARVAL SURVIVAL IN THE STREAMSIDE SALAMANDER (*AMBYSTOMA BARBOURI*)

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AN ABSTRACT OF A THESIS

EMBRYONIC TEMPERATURE HAS CARRY-OVER EFFECTS ON BODY SIZE, DEVELOPMENT TIME, AND LARVAL SURVIVAL IN THE STREAMSIDE SALAMANDER (*AMBYSTOMA BARBOURI*)

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Temperature is a critical ecological factor, influencing metabolism and, in turn, affecting many aspects of physiology, reproduction, behavior, and ultimately population growth. As temperatures continue to rise due to global change (i.e., climate change, urbanization), organisms will be exposed to novel temperature regimes. Global change will substantially impact embryo survival of ectotherms due to their relative lack of parental care, thermal sensitivity during development, and reduced capacity for thermoregulation. Although the effects of global change have been studied extensively using eggs of non-avian reptiles, much less is known about impacts on amphibian development and resultant phenotypes. Thus, I conducted an egg incubation study with *Ambystoma barbouri* (Streamside Salamander), to consider how incubation temperature impacts development. I collected eggs from across the Tennessee range, incubated them at various temperatures in the laboratory (5-25 °C), and measured resultant larval and metamorph phenotypes. Additionally, I monitored nest temperatures in disturbed and undisturbed habitats to assess the effects of urban development on stream temperature, as habitat loss due to urbanization is a major threat for this species. Pervasive and daily maximum temperatures were warmer in disturbed than undisturbed sites. Higher incubation temperature resulted in decreased egg survival, body size at hatching, and size at metamorphosis. I also describe the optimal thermal range (5-17 °C) and embryonic chronic heat tolerance (21 °C) of *A. barbouri.* Collectively, my results highlight the effects of global change on intermittent stream habitats and demonstrate that the embryonic thermal environment can have carry-over effects that persist through early life-stages.

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CERTIFICATE OF APPROVAL OF THESIS

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DEDICATION

This thesis is dedicated to my father, Gregory Thulander, who instilled a love of nature in me from a young age.

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CHAPTER 1: INTRODUCTION

Temperature is a critical ecological factor due to its universal effect on metabolism, thus influencing nearly all aspects of physiology, reproduction, behavior, and ultimately population viability and growth (Brett, 1971; Brown et al., 2004). As temperatures rise due to global change, organisms are exposed to novel climate regimes (IPCC, 2023), impacting all levels of biological organization (Sinervo et al., 2010; Vázquez et al., 2015). Climate change has received considerable attention due to its capacity to disrupt a diversity of ecosystems across the globe; however, habitat modification via urban or agrarian development is also a component of global change that increases the mean and variance of air, soil, and water temperatures (i.e., the urban heat island; Arnfield, 2003; Francis et al., 2019; Grey et al., 2023; Lauck et al., 2023). These modifications involve removal of trees and shrubs, which reduces shade cover and evapotranspiration, as well as the addition of impervious surfaces which collect and retain heat (Herb et al., 2008; Obiakor et al., 2012). Adjacent water bodies may also be warmed due to increased temperature of runoff and greater solar radiation as a consequence of canopy cover loss (Herb et al., 2008; Davis et al., 2019; Grey et al., 2023). Such changes can alter the physiology and survival of species that live in or near disturbed areas compared to more natural ecosystems (Ditchkoff et al., 2006; Macdonald et al., 2023). For example, threatened fish species in urban streams have reduced survival and smaller size compared to populations in non-urban areas (Burbank et al., 2021), and water fleas from urban areas have evolved a "fast" life history by maturing at a smaller

size and breeding at a younger age than those from rural habitats (Brans and Meester, 2018).

Although rising temperatures pose risks to many organisms, oviparous ectotherms (e.g. insects, reptiles, amphibians) may be particularly vulnerable. Their reproductive success is directly influenced by environmental temperature because females often leave eggs to develop under ambient conditions (Mainwaring et al., 2017), and climate change and urbanization increase nest temperatures, sometimes reducing egg survival or hatchling viability (Mui et al., 2016; Hall and Warner, 2018; Jackson et al., 2019; Tiatragul et al., 2020). Therefore, in the face of global change, species survival will depend on adjustments to nesting behavior, adaptation of embryo thermal tolerance, or some combination (Telemeco et al., 2009; Du and Shine, 2015; Du et al., 2019). For example, female three-lined skinks (*Bassiana duperreyi*) have adjusted to warming temperatures by nesting earlier in the year and in deeper cavities (Telemeco et al., 2009) while green sea turtles (*Chelonia mydas*) have adapted to warmer nesting beaches by producing eggs with greater heat tolerance (Weber et al., 2011). Although numerous studies demonstrate nesting and/or embryonic responses to global change, the majority of this work comes from studying non-avian reptiles (see Levy et al., 2015; Carlo et al., 2017, While et al., 2018; Sun et al., 2021). Conversely, our knowledge of developmental responses to change in incubation temperature of other vertebrate ectotherms, such as amphibians, is less developed (Orizaola et al., 2010; Hall, 2022; Massey and Hutchings, 2021; Oborová et al., 2024). This is particularly alarming given the dramatic, global decline of amphibians due to habitat degradation and disease (Collins, 2010; Fisher and Garner, 2020).

Many vertebrates, like reptiles, may adjust to rising temperatures via gradual changes in the timing or location of nesting (e.g., nesting earlier or at higher elevations; Du et al., 2023); however, amphibians often require shallow, intermittent waterbodies for reproduction that temporarily fill after discrete climatic events like rainstorms. Thus, nesting opportunities are punctuated over the landscape and through time, thereby limiting the ability to gradually shift nesting activity in response to environmental change. For example, small, stream-breeding species may be unable to traverse from one stream to another due to habitat fragmentation and, perhaps, the need to migrate across roads, leading to high mortality rates (Ward et al., 2008; Clipp and Anderson, 2014). Moreover, species that breed in ephemeral waters may respond more strongly to changes in rainfall than temperature which may lead to detrimental changes in nesting phenology (e.g. breeding later rather than earlier; Dalpasso et al., 2023). Once eggs are laid, they are sessile, unable to behaviorally thermoregulate, and shallow water exposes eggs to temperature swings that can influence embryo development in ways that impact fitness-relevant traits (Balshine, 2012; Brooks, 2004). In particular, stream temperature may influence egg survival, developmental rates, and body size at larval or metamorph stages.

Embryos of vertebrate ectotherms typically develop successfully over a relatively broad range of temperatures, known as the optimal thermal range (OTR) (Bachmann, 1969; van der Have, 2002; Sgrò et al., 2016; Hall and Sun, 2021). Knowing this range is critical to understand the ecophysiology of development and to determine how both current and future environmental temperatures influence egg survival and successful development in the wild. To describe the OTR, eggs are incubated across a range of constant temperatures to determine at what temperature survival is high and at what point

it declines (Andrews and Schwarzkopf, 2012; Hall and Sun, 2021). Once the OTR is described, it is assumed that when incubation temperatures rise above the species-specific threshold, even for a short period of time, abnormal development of body systems such as the cardiovascular or neural systems may occur and lead to eventual death of the embryo or reduce the viability of resultant hatchlings (Eme et al., 2015; Pype et al., 2015; Andrews and Schwarzkopf, 2012; Hall and Sun, 2021; Sanger et al., 2021). For example, the brown anole lizard (*Anolis sagrei;* Duméril and Bibron, 1837) has an OTR between 22 and 32 °C; however, current nest temperatures commonly fluctuate outside this range, indicating that physiological insult is common and will become more common with climate change (Hall and Warner, 2021). Laboratory studies demonstrate that temperatures above the OTR induce a mismatch between oxygen supply and demand (Hall and Warner, 2021), with long-term exposure resulting in the malformation of critical body systems (Sanger et al., 2021). In particular, even a few brief exposures (e.g., ½ or 1 hour) to these high temperatures can reduce egg survival as well as hatchling performance and hatchling survival (Hall and Warner, 2019; Hall and Warner, 2021).

Although extreme temperatures have many detrimental effects, moderate increases may be beneficial (Liu et al., 2022). Development proceeds more rapidly at warmer temperatures, leading to earlier hatching and, for amphibians, earlier metamorphosis (Angilletta, 2009; Ruthsatz et al., 2018). Since amphibian larvae are at high risk of predation and desiccation during the embryonic and larval stages (Marco and Blaustein, 2000; Relyea, 2007; Rudolf and Rödel, 2007; Richter-Boix et al., 2011), faster development may lead to higher survival rates to metamorphosis, at which point amphibian survival rates increase (Gamble et al., 2009; De Lisle and Grayson, 2011;

Homan et al., 2018; Messerman et al., 2020). However, developing at temperatures outside the OTR may lead to stress and result in energy being placed into body maintenance rather than growth (Metcalfe and Monaghan, 2001; Burraco et al., 2019). Warmer temperatures may reduce body size due to lower efficiency of converting yolk to tissue or increased metabolic rates during development (Herreid and Kinney, 1967; Kuramoto, 1978; Duellman and Trueb, 1994; Pauly and Lam, 2023). Smaller larvae or metamorphs may be preferentially preyed upon due to gape restrictions of predators, ease of capture, and reduced handling time (Brodie and Formanowicz, 1983; Gvoždík and Smolinský, 2015). Therefore, the effects of rising water temperatures may be highly complex and have differential effects across life stages; highlighting the need to enhance our understanding of the effects (long- and short-term) of thermal stress during embryonic development.

To consider the potential effects of rising temperatures on stream-breeding amphibian species, I collected *Ambystoma barbouri* (Streamside Salamander, Kraus and Petranka, 1989) eggs and incubated them across a range of temperatures that encompasses those experienced by eggs in local streams. *Ambystoma barbouri* nest in intermittent streams in a variety of habitats including critically rare cedar glades (Kraus and Petranka, 1989; Niemiller et al., 2011). In degraded habitats near suburban areas, *A. barbouri* will use streams where disturbance from mowing, reduced canopy cover, and human activity alter stream dynamics. This species is designated as state endangered in Tennessee, USA, largely due to habitat destruction caused by urban sprawl, indicating the need to understand the effects of habitat disturbance on reproduction and embryo development. Given the sensitivity of ectotherms to changes in mean and variance of

temperature (While et al., 2018; Massey and Hutchings, 2021) and the potential for urban development to alter thermal regimes (Arnfield, 2003), I aimed to determine 1) the effects of habitat disturbance on stream temperatures at *A. barbouri* breeding sites, and 2) the effects of incubation temperature on *A. barbouri* embryo development and resultant larval and metamorph phenotypes and survival. This information will improve our understanding of how rising temperatures due to global change impact fitness proxies of early life stages in ectotherms with complex life cycles.

CHAPTER 2: METHODS

Study Species

Mole salamanders (genus *Ambystoma*), spend most of their lives underground in proximity to fishless, intermittent wetlands but emerge to breed during fall or winter (Mitchell and Gibbons, 2010). Unlike other mole salamanders, *A. barbouri* relies on intermittent streams (Fig. B.1) for reproduction that depend on seasonal precipitation to maintain flow throughout the breeding, embryonic, and larval periods (Kraus and Petranka, 1989). Such streams are often in regions with karst topography, like cedar glades; however, they also occur in non-karst systems (Kraus and Petranka, 1989; Niemiller et al., 2011). Breeding phenology varies across latitudes, but in general, adults migrate to breeding streams between December and March, and females lay eggs on the underside of rocks or occasionally on submerged vegetation (Fig. B.2; Kraus and Petranka, 1989). The sticky, gelatinous coat around the embryo allows eggs to remain on substrates even in turbulent flow. Eggs usually hatch after 4-12 weeks (depending on temperature) and the larvae remain in the streams, feeding on various invertebrates (amphipods, isopods, worms, zooplankton) until metamorphosis, which takes 6-10 weeks (Petranka, 1998). After metamorphosis, little is known about juvenile and adult behavior due to their cryptic life history (Petranka, 1998).

Most of the continuous known range of *A. barbouri* is in Ohio, Kentucky, and Indiana but there is a disjunct population in Tennessee (Fig. B.3). In Tennessee, populations are restricted to the Central Basin, which is undergoing rapid development due to the spread of the Nashville metropolitan area (Fig. B.3). Notably, in 2021-2022 the

Nashville metropolitan area was among the top ten metropolitan areas for gaining new citizens (Wilder and Mackun, 2024) and Sumner County, part of the range of *A. barbouri,* has lost 16,000 acres of farmland to development (Zilber, 2024). Due to habitat loss, *A. barbouri* is listed as endangered at the state level (Tennessee Wildlife Resources Agency, 2018) and is being considered for federal protection under the Endangered Species Act. The impending threats to *A. barbouri* habitat may influence the thermal environment; however, little is known about temperature influences on egg survival and embryo development for the species. Moreover, because breeding streams can be relatively shallow, between 6-12 cm (Fig. B.1), they may exhibit important variation in temperature over space and time.

Egg Collection and Measurement of Stream Temperature

To characterize the thermal environment of eggs and larvae, I placed temperature loggers in and around *A. barbouri* nests across a 61.6 km span of the range in Tennessee (Fig. B.3). Field sites were based on historical home range data from Tennessee Wildlife Resource Agency (TWRA) and Tennessee Department of Environment and Conservation (TDEC). Sites were selected based on the feasibility of gaining access to streams (i.e., public lands, agreeable private landowners) and on historical data regarding population density based on conversations with TWRA and TDEC zoologists. A variety of habitat types (e.g. forested, cedar glade, open field) and levels of human activity, either disturbed or not disturbed sites, were included to assess effects of habitat type and canopy cover on stream temperature.

To record stream temperatures, I deployed temperature loggers (HOBO MX TidbiT 400, MX2203, manufactured by Onset Brands, Bourne, MA) at nine study sites, between 12/4/2022 and 2/2/2023 (Table A.1). At each site, I placed four total temperature loggers, set to record hourly: two at two separate locations in the stream. At each location, one logger was placed under a rock with a nest and the other under a rock without a nest but within 3 m of the nest. Protocols for locating nests are described below. The logger under a rock without a nest served to determine if nest temperatures differed from pervasive stream temperatures, and for redundancy in case of logger loss. This additional rock was selected by randomly choosing a direction through spinning a pencil: up- vs. down-stream from the nest, and then selecting the closest rock in that direction without a nest. I secured loggers using 100-lb fishing line and aluminum crimping sleeves. Temperature loggers were visited once between deployment and retrieval to check that they remained in place. At each visit, I recorded the presence of larvae and eggs and any visible changes in stream habitat such as algal growth or reduced water levels (Table A.2). Loggers were removed on 5/11/2023 and 5/15/2023, to capture data for egg and larval periods. Upon removal, I noted if eggs or larvae were present and recorded changes in stream habitat (Table A.2).

To understand the effects of incubation temperature on embryonic, larval, and metamorph survival and phenotypes, I collected eggs from the field for a laboratory incubation study. From 12/4/2022 to 01/19/2023, I collected 290 eggs from 19 clutches across field sites to ensure genetic diversity (Table A.1). To locate nests for egg collection and placement of temperature loggers, I conducted nest surveys at each site in places that were accessible and adjacent to a road or trail by walking upstream, turning every rock

that seemed suitable for nesting and returning rocks to their original position. I walked as far as necessary to locate three nests with freshly laid eggs, which I defined as embryos at Harrison stage 12 or earlier. Stage 12 is characterized by the formation of the blastopore and occurs prior to neurulation. This ensured that embryos traversed most of development in the laboratory and underwent major developmental events (gastrulation, neurulation, organogenesis) under experimental conditions. At each nest, I assessed Harrison stage using a jeweler's loupe and, using a laminated card, gently scraped 16 eggs from the underside of the rock and placed them into a 50 mL centrifuge tube rinsed and filled with stream water from the nest site. I took photos of each nest, surrounding habitat, and canopy cover.

Egg Incubation and Embryo Development

To determine the effects of water temperature on embryo development and egg survival, I incubated eggs across a range of temperatures in the laboratory (5 to 25 °C). Eggs were transported to Tennessee Tech University and placed individually in 2 oz glass jars filled with treated tap water (chlorine and chloramines removed). Water was treated using API® Tap Water Conditioner and warmed to room temperature. Each egg was assigned to a Harrison stage using a stereoscope (per Duellman and Trueb, 1994), photographed alongside a ruler (to nearest mm) for scale, and randomly assigned to one of four incubation treatments (5, 10, 20, and 25 °C; based on Hall, 2022) (n=4 eggs per treatment per clutch). I used Memmert brand IPP55plus incubators (Memmert USA, Eagle, WI) and independently measured the incubator temperatures every hour with HOBO TidbiT loggers that were housed in identical conditions to eggs. On 12/17/2022,

about two weeks after collecting the first eggs, I determined that the 25 °C treatment was too hot due to complete and rapid mortality. At this point, eggs from multiple clutches had been tested at 25 °C, and I decided it would not be informative to continue placing eggs in this treatment. Any eggs collected on 12/17/2022 and onwards were placed in one of the following treatments 5, 10, 20, and 22 °C (n=4 eggs per clutch). Therefore, eggs from across the Tennessee range were incubated at five temperatures (5, 10, 20, 22, 25 °C) in a split-clutch design. To account for potential variation across incubators, I used the average temperature recorded by the TidbiT loggers in each incubator for analysis of egg survival, specifically 5.3, 10.1, 20.0, 21.9, and 24.7 °C.

Throughout embryonic development, embryos were assigned a Harrison stage at the following intervals: the 22 and 25 °C treatment groups were assigned a stage three days per week (every MWF), the 10 and 20 °C groups twice per week (every MF), and the 5 °C group once weekly (every W) (Fig. B.4a, b). The variation in timing accounted for slower development at cooler temperatures. Periodically assigning embryos to a stage allowed me to calculate embryonic developmental rates for each treatment. I checked incubators daily for egg mortality based on fungal growth, collapse of embryo, or at later stages, the absence of a heartbeat or blood flow through the gills.

Larval Growth and Survival

To understand the effects of egg incubation temperature on larval survival and phenotypes, I maintained hatchlings in the laboratory under ambient room temperature conditions until metamorphosis. For each hatchling, I recorded the Harrison stage at hatching and took a photograph with a scale to later measure hatchling total length (Fig. B.4c). I housed hatchlings individually by randomly assigning them to one of twelve 20-

gallon aquaria, each holding 23 individual PVC tubes (23 cm long, 7 ¾ cm internal diameter) with mesh bottoms (20x20 fiberglass screen mesh) secured by silicon (Fig. B.5a). Each aquarium had one tube containing a bubbler for oxygenation and a Fluval® C2 30-gallon power filter with aquarium bio filter sponge to encourage microbial growth for biofiltration. Microbial growth was encouraged by inoculating tanks with another aquarium's filtrate and water was then cycled for 28 days prior to introduction of larvae. Filters were not changed during the experiment, but each tank had a 20% water change once per week. I performed pH, ammonium, nitrate, and nitrite tests weekly on one randomly chosen tank. If the test results showed increased levels of ammonium, nitrate, nitrite or altered pH, I ran water tests on all 12 tanks to minimize differences among tanks and changed water as needed. Uneaten food (see below) was vacuumed from the tanks once per month or as needed. All water added to aquaria was conditioned using API® Tap Water Conditioner.

Aquaria were maintained at room temperature, which fluctuated somewhat over time. To account for variation in temperature across aquaria due to spatial variation in room temperature, as well as among-larval variation due to differences in hatch dates produced by variation in collection date or treatment, I equipped each aquarium with a HOBO TidbiT temperature logger. These were set to record hourly, and I calculated the mean temperature experienced by each larva throughout its specific larval period based on individual dates of hatching and metamorphosis. Mean temperatures experienced by individual larvae were between 18.1 and 19.7 °C. These values were used as a covariate in analyses (see below). Moreover, these temperatures are relatively warm compared to

average stream temperatures at my field sites; however, they fall well within the range of temperatures experienced by larvae in the wild (see Chapter 3: Results).

Larvae were fed every MWF with increasing quantities of food: during the first thirty days, they were fed 2 mL of baby brine shrimp concentrate (average dry weight per feeding = 0.0163 g ± 0.000652 g), then for two weeks they were fed 1 mL of brine shrimp concentrate and 1 serving of blood worms (average dry weight per bloodworm serving = 0.0025 g ± 0.00062 g) to wean them onto blood worms. After weaning, they were fed two servings of blood worms. At 60 days they were fed 3 servings, and after 90 days they were fed 6 servings. *Ad libitum* feeding was not maintained due to concerns about water quality. Throughout the larval period, I checked for mortality and signs of metamorphosis (shortening of gills) daily. Once an individual showed signs of metamorphosis, they were monitored closely to determine the date that gills were fully absorbed, which I considered the completion of metamorphosis (Fig. B.4e). Metamorphs were photographed alongside a ruler, blotted dry and weighed (to nearest 0.001 g, Sartorius Lab Instruments mass balance BCE641-1S), and placed in terrestrial housing (described below). The date of metamorphosis was used to calculate the length of the larval period.

Juvenile Growth and Survival

To determine if the effects of egg incubation temperature persisted after metamorphosis, I monitored metamorphs' growth and survival for 90 days in ambient conditions. I placed metamorphs individually into a plastic container (Sterilite© 27.94 x 16.764 x 6.858cm), filled it with enough treated water to produce 1-2cm depth at one end

when tilted on a 1.6-cm wooden dowel, and then laid down a paper towel, a few pieces of soaked cardboard, and another moist paper towel (Fig. B.5b, c). Containers were randomly assigned a location on shelves and remained at those locations for 90 days. To account for variation in terrarium temperature due to differences in date of metamorphosis and potential fluctuations in room temperature, I equipped two mock containers holding one HOBO TidbiT logger each, placed in random locations. I then calculated the mean temperature experienced by each metamorph for 90-days post-metamorphosis. Mean temperatures were between 18.8 and 20.2 °C and these values were used as a covariate in analyses (see below). Juveniles (Fig. B.4f) were fed every MWF to create an *ad libitum* environment. They received a variety of invertebrates: dwarf white isopods (*Trichorhina tomentosa*), European nightcrawlers (*Dendrobaena hortensis*), and powder orange isopods (*Porcellionides pruinosus*). These feeders are relatively rich in vitamins A and B, and calcium, which are the primary limiting nutrients of captive amphibians (Wright and Whitaker, 2001). The Sterilite© containers were cleaned once per month or as needed by replacing all terrarium materials. Three individuals presented with an epidermal infection, were euthanized via submersion in Tricaine (MS-222), and subsequently excluded from analyses. Two of these infected individuals were examined by a veterinarian who specialized in amphibian disease to confirm this was not a chytrid infection (*Batrachochytrium dendrobatidis* or *B. salamandrivorans*). Finally, at the end of 90 days, each juvenile was weighed and photographed with a scale to estimate early-life growth rates. All surviving juveniles were maintained in captivity indefinitely to form a captive breeding assurance colony (data not reported here).

Statistical Analyses of Field Temperature and Habitat Data

Stream water temperatures were divided into embryonic vs larval periods so I could determine how environmental temperature differs across life stages. To divide the temperature logs between embryonic and larval periods, I calculated ecological developmental rates using laboratory data (see Chapter 3: Results). Ecological developmental rate reflects the time it takes an egg to hatch rather than the time to traverse a set of embryonic stages, accounting for the ability of eggs to hatch at different embryonic stages (see Chapter 3: Results). Ecological developmental rate was calculated by taking the inverse of the lab incubation period (i.e., days from egg collection until hatching) for each embryo and multiplying by 100 resulting in percent development completed per day (Taylor et al., 2021). Using a regression of ecological developmental rate on temperature, I estimated temperature-specific ecological developmental rates of embryos in the field for each hour recorded in each nest and, for each nest, determined the number of days until development equaled 100%. Additionally, the first hour and entire final day of each temperature log was removed to exclude temperatures recorded outside of the water. Temperatures during these days were considered embryonic temperatures and those after were considered larval temperatures.

To understand how habitat variation influences embryonic temperature and determine if nest temperatures differ from surrounding habitat, I ran a linear mixed-effects model with nest temperature as the response variable and habitat disturbance (y/n), open canopy (y/n), and specific location (rock with nest/ rock without nest) as fixed effects. Julian day (as a continuous variable) and hour (as a categorical variable) were

covariates to control for temperature changes over time and logger ID was a random effect. This model structure was also used to assess larval temperatures; however, specific location was excluded as a fixed effect because larvae can move throughout the habitat.

To understand if extreme embryonic and larval temperatures differed between disturbed and undisturbed sites, I analyzed the daily maximum and minimum temperatures with separate linear mixed-effects models with water temperature as the response variable, Julian day as a covariate, disturbance (y/n) as a fixed effect, and logger ID as a random effect. This structure was used on all four analyses: embryonic daily maximum and minimum and larval daily maximum and minimum.

Habitats were considered disturbed if the surrounding areas were altered by human activity, including presence of impervious surfaces and other human-made structures (Table A.1). Sites within preserved habitats, such as state protected lands, were considered undisturbed (Table A.1). I later quantified disturbance by using ArcGIS Pro Version 3.1.2 and the National Land Cover Database (NLCD; Dewitz, J. and U.S. Geological Survey, 2023) to calculate the land usage within 0.5-km radius of each site (Table A.3). Canopy cover was noted at original site visit and I utilized the NLCD and ArcGIS Pro to extract percent canopy cover for each temperature logger (Table A.4; Dewitz, J. and U.S. Geological Survey, 2023).

Statistical Analyses of Morphometric and Survival Data

I used ImageJ 1.53t (Schneider et al., 2012) to measure maximum width of embryos at time of collection (hereafter egg size), as egg size may influence larval and metamorph body size. I also measured the total body length (mm) of each individual at

hatching, metamorphosis, and ninety-days post-metamorphosis (Fig. B.6) because body size is an important predictor of performance and survival (Marvin, 2003; Hernández-Pacheco et al., 2020). If the spine was aligned linearly, I used straight-line measurements (Fig. B.6b), but if curved, I used the "Fit Spline" function (Fig. B.6c, d). For metamorphs and juveniles, I measured tail length (anterior of the hind legs to tip of tail) and head-trunk length (HTL; tip of nose to anterior of the hind legs; Pierce, 2022) (Fig. B.6c, d). Tail and head-trunk length may have important effects on performance such as swimming speed (Fitzpatrick et al., 2003; Landberg and Azizi, 2010). Using ImageJ was preferred to measuring body length with a ruler to reduce stress on the animals.

For egg, larval, and juvenile phenotypes, I fit linear mixed-effects (LME) models, and for survival analyses, I used generalized linear mixed effects (GLME) models with a binomial distribution. For egg survival, incubation temperature was a continuous variable as there were five treatment groups. Additionally, to estimate the embryonic chronic heat tolerance (ECHT) for the species, I followed the method of Hall and Sun (2021) and analyzed egg survival with a 3-parameter log logistic regression. This allows survival to be relatively high across a range of temperatures (the optimal thermal range [OTR]) and then rapidly decline toward zero. This pattern is typical for egg survival (van der Have, 2002; Hall and Sun, 2021) and allows estimation of a "lethal temperature 50" (LT50 or ECHT) which is the temperature at which survival is reduced by 50%. I bootstrapped the data to generate a 95% confidence interval of the ECHT (Hall and Sun, 2021). I used predictions from the log logistic model to estimate temperature at which survival began to rapidly decline to define the OTR for the species and determine which temperatures should be considered stressful. For larval survival and egg and hatchling phenotypes,

incubation temperature was a categorical variable because low egg survival at the warmest temperatures resulted in four treatment groups (5, 10, 20, 22 °C). All four groups had sufficient sample sizes for analysis, but the 22 °C group had a relatively low sample size (n=13). Juvenile and metamorph phenotypes were analyzed with only three treatment groups (5, 10, 20 °C) due to high mortality of larvae that were incubated at 22 °C (see results).

All models included incubation temperature as a fixed effect, covariates when appropriate (see results), and a random effect of clutch ID to account for maternal effects and avoid pseudo-replication. For larval survival, clutch ID was excluded as a random effect due to singularity. No analysis was conducted on juvenile survival due to high survival across all treatments (see results). Model residuals were visually inspected to assess statistical assumptions. All analyses were performed in R version 4.3.2 (R Core Team, 2023) using the *lmertest* and *lme4* packages for general and generalized linear mixed-effects models (Bates et al., 2015, Kuznetsova et al., 2017) and the *drc* package (Ritz et al., 2015) to perform 3-parameter logistic regression. Statistical significance of fixed effects was assessed with an ANOVA ($\alpha = 0.05$) and the *emmeans* package was used to perform post hoc comparisons and to adjust P-values using the false discovery rate correction (Lenth et al., 2018). The *sjPlot* package was used to calculate r^2, residual variance, variance of the random effect, and the intraclass correlation coefficient (Lüdecke, 2018). Figures were created using *ggplot2* (Wickham et al., 2011).

CHAPTER 3: RESULTS

Effects of Habitat Conditions on Stream Water Temperatures

Field sites categorized as disturbed had a mean of 64.7% developed landcover (± 20.3% SD) while undisturbed sites had a mean of 2.9% (± 3.8% SD) (Table A.3). Nest locations designated as closed canopy had a mean of 61.8% canopy cover (± 22.1% SD) while open canopy sites had a mean of 10.3% (± 15.2%) (Table A.4). Temperatures during the embryonic period were 1.5 °C warmer (± 0.4 SE) in disturbed vs undisturbed sites (Table A.5; Fig. B.7a) but canopy cover had no statistically clear effect nor was there any temperature difference between locations with and without nests (Table A.5). Maximum daily temperatures were 2.0 °C (± 0.4 SE) warmer in disturbed sites but minimum daily temperatures did not differ based on disturbance level (Table A.5; Fig. B.7b, c). Embryonic and larval maximum temperatures increased slightly across Julian days, but pervasive and minimum temperatures showed no clear change (Table A.5). Based on laboratory data for egg survival, temperatures were more likely to fluctuate above the OTR (see Chapter 3: Results; Survival) in disturbed vs. undisturbed sites, with 1.9% and 0.5% of hours spent above the OTR at disturbed and undisturbed sites, respectively (Fig. B.7b).

Water temperatures during the larval period were 0.7 °C warmer (±0.3 SE) at disturbed sites and 0.7 °C warmer (±0.3 SE) at open canopy sites and increased across Julian day (Table A.5; Fig. B.8a). Maximum daily temperatures were 1.8 °C (±0.9 SE) warmer at disturbed sites but minimum daily temperatures did not differ between disturbance levels (Table A.5; Fig. B.8b, c). Maximum and minimum temperatures

increased slightly (≤ 0.1 °C per day) across Julian days (Table A.5). Finally, all water temperatures exhibited expected daily variation across both embryonic and larval periods with the warmest temperatures between 12:00 – 16:00 and coolest temperatures from 3:00 – 7:00 (Table A.6; Fig. B.7, 8).

Effects of Incubation Temperature on Survival

In the laboratory, incubation temperature had a large effect on egg and larval survival such that individuals incubated at 5 and 10 °C had greater survival across both life stages than those incubated at warmer temperatures. Specifically, egg survival decreased with temperature, with 84.7%, 88.1%, 60.8%, 26.1%, and 0% survival at 5, 10, 20, 22, and 25 °C, respectively (Table A.7). The ECHT was 21.0 °C (20.4 - 21.6 95% CI) and the egg survival curve begins to rapidly decline above 17 °C, indicating the OTR for development is between 5 and 17 °C (Fig. B.9). Larval percent survival was 88.5%, 89.8%, 57.8%, and 25% for the 5, 10, 20, and 22 °C incubation treatments, respectively (Table A.7; Fig. B.10a). Notably, the number of larvae remaining for survival analysis varied by treatment due to egg mortality: 61, 59, 45, and 12 eggs hatched from 5, 10, 20 and 22 °C, respectively (Fig. B.10a). Effects of incubation temperature on survival did not persist past metamorphosis. Juvenile survival was high for all treatments; only one juvenile from the 10 °C treatment group died (Fig. B.10b).

Effects of Incubation Temperature on Developmental Rate and Hatchling Phenotypes

Ecological developmental rate increased with temperature but did not covary with initial egg size (Table A.8; Fig. B.11a). Developmental rate was slowest at 5 °C, 1% per day (±0.8% SE), and fastest at 22 °C, 8% per day (±0.2% SE) (Table A.8). Harrison stage at hatching was affected by temperature and generally increased with egg size such that larger eggs hatched at later stages (Table A.8; Fig. B.11b). For every 1 mm increase in egg size, Harrison stage at hatching increased by 0.88 (±0.28 SD). Eggs hatched at the latest stage when incubated at 10 °C (42.5±1.11 SD) and at slightly earlier stages across the remaining temperatures (41.28±0.61 SD at 5 °C; 41.87±1.34 SD at 20 °C; 41.53±1.05 SD at 22 °C) (Fig. B.11b).

Body length at hatching varied according to incubation treatment and positively covaried with egg size (Table A.8). The 20 and 22 °C treatments resulted in significantly lower body size than at 10 or 5 °C; however, larvae from the 10 °C incubation had the largest body length of 15. 3mm (±0.179 SE), which was 0.98 (±0.175 SE), 2.15 (±0.205 SE), and 2.70 mm (±0.378 SE) larger than those from 5, 20, and 22 °C, respectively (Fig. B.11c).

Effects of Incubation Temperature on Phenotypes at Metamorphosis

Time to metamorphosis was shorter for eggs incubated at warmer temperatures and for individuals that were larger at hatching, but variation in aquarium temperature had no effect (Table A.9). Specifically, days to metamorphosis was longest for embryos incubated at 5 °C (128 days ±4 SE), but there was no statistical difference between those incubated at 10 °C (103 days ±4 SE) or 20 °C (106 days ±5 SE) (Table A.9; Fig. B.12a). For every 1 mm increase in size at hatching, individuals took 2.9 fewer days (±1.4 SE) to reach metamorphosis. Thus, the smallest individuals at hatching (10.4 mm ± 0.6 SD) took

19.7 days longer to reach metamorphosis than those that were largest at hatching (17.2 mm ± 0.4 SD).

Body length, tail length, head-trunk length (HTL), and mass at metamorphosis varied according to incubation treatment but did not covary with either aquarium temperature or length at hatching (Table A.9). Metamorphs were largest in both mass and length when they were incubated at 5 °C (0.647 g ±0.02 SE; 47 mm ±0.8 SE) and smallest from 10 °C (0.561 g ± 0.03 SE; 44.6 mm ± 0.8 SE); however, neither treatment was statistically different from metamorphs that were incubated at 20 °C (Table A.9; Fig. B.12b, c). HTL followed a similar trend as body mass and length so that metamorphs had the largest HTL from 5 °C (24.6mm ± 0.4 SE) and smallest HTL from 10 °C (23.2mm ± 0.4 SE) but neither were statistically different from those incubated at 20 °C (Table A.9; Fig. B.12d). Tail length was statistically larger for individuals from 5 than 10 °C; however, pairwise comparisons after a false discovery rate correction show no statistical difference between the treatments (Table A.9; Fig. B.12e).

Effects of Incubation Temperature on Juvenile Phenotypes

Incubation temperature affected body length, tail length, head-trunk length (HTL), and mass at 90 days post-metamorphosis when size at metamorphosis was used as a covariate (i.e., estimate of growth) but variation in terrarium temperature had no clear effect (Table A.10). Juveniles had the most growth when they were incubated as embryos at 10 °C but were only statistically different than those from the 20 °C incubation (Table A.10; Fig. B.13). Juveniles were largest in both body length and mass from 10 °C group (66.5mm ± 1.6 SE; 1.96g ± 0.09 SE) and smallest from 20 °C group (60.8mm ± 1.9 SE;

1.63g ± 0.1 SE) (Table A.10; Fig. B.13a, b). Tail length and HTL followed the same trend whereas individuals from the 10 °C incubation temperature had the largest tails (33.3mm ± 0.7 SE) and HTL (33.3mm ± 1.0 SE) and 20 °C had the smallest tails (30.3mm ± 1.3 SE) and HTL (30.6mm ± 0.8 SE) (table A.10; Fig. B.13c, d).

CHAPTER 4: DISCUSSION

Global change affects mean and variance of temperature, exposing organisms to novel thermal regimes (Arnfield, 2003; IPCC, 2023). Animals with complex life cycles (e.g. amphibians) are at increased risk from global change due to dependencies on coinciding hydroperiods and specific temperatures ranges as well as a greater opportunity for disturbance to disrupt one of multiple required habitats (Carey & Alexander, 2003; Crozier et al., 2008; Blaustein et al., 2010; Nolan et al., 2023). Embryos are particularly sensitive as they cannot behaviorally adjust to novel environments and rely on immediate physiological adjustments (Du et al., 2019).

Although amphibians and their eggs are known to be sensitive to temperature, few studies have investigated how ecologically relevant changes in incubation temperature influence survival of later stages or other such carry-over effects (Blaustein et al., 2010; Li et al., 2013). My study demonstrates that there are increased water temperatures at disturbed sites throughout the embryonic and larval period for a stream-breeding amphibian, with disturbed sites more often fluctuating above the OTR. Incubation within the OTR, and specifically at 10 °C, has long-term fitness benefits such as high embryonic and larval survival, later developmental stage at hatching, larger size at hatching and at the juvenile stage, and a relatively short time to metamorphosis. Although incubation at 5 °C resulted in similar rates of egg and larval survival, lengthened time to hatch and to complete metamorphosis, along with smaller body size at the juvenile stage, are potentially detrimental. Therefore, not all temperatures within the OTR (between 5 and 17°C) maximize fitness proxies. Finally, the negative effects of warm incubation

temperatures were manifested in both survival and body size across life stages. Thus, the effects of rising temperatures during embryonic development have potential to carry over into later stages, demonstrating the complex effects of the thermal environment on species with multiple, distinct life stages.

Environmental Temperatures

To my knowledge, this study is first to report continuous stream water temperatures for the extent of the embryonic and larval periods of a salamander, allowing for new insight on habitat characteristics of breeding areas. Pervasive and maximum daily stream temperatures were significantly warmer at disturbed sites than undisturbed sites; a trend that has been previously documented through efforts to understand the effects of urbanization on freshwater ecosystems. Specifically, increased temperatures near urban areas have been observed in low-order streams (Paul and Meyer, 2001; Rice et al., 2011), which are similar to ephemeral streams occupied by *A. barbouri* but differ in their biotic make-up (Meyer et al., 2007). This is likely due to runoff from relatively warm, impervious surfaces and decreased canopy cover (Nelson and Palmer, 2007; Herb et al., 2008; Sudduth et al., 2011; Grey et al., 2023). However, intermittent stream temperatures are also influenced by ground-water inputs such that shallower ground water sources are usually warmer than deeper sources (Anderson, 2004; Environmental Protection Agency, 2015). Additionally, if water enters a stream directly from overland flow of impervious surfaces through runoff, surges in stream temperature result (Somers et al., 2013), but percolation through soil reduces such thermal pollution (Long, 2011).

As the current study was not designed to address all the potential causes of temperature differences between disturbed and undisturbed sites, I am unable to describe the underlying causes of increased temperature at disturbed sites. Regardless, temperatures at disturbed sites rarely reached the estimated ECHT of *A. barbouri* embryos; only three of nine sites, two of which were disturbed sites, reached 21 °C. Notably, mean temperatures across all but one site were between 8-10 °C, indicating that there is no immediate threat of rising mean stream temperatures. This indicates there is not likely any direct increase in egg mortality at disturbed sites due to mean temperature; however, there is potential for sublethal effects of temperature, such as decreased size at hatching.

Sublethal effects are more likely consequences at my study sites, given that four of nine sites (n = 2 disturbed & 2 undisturbed) experienced temperatures above the estimated OTR (>17 °C). For example, studies conducted on lizard eggs exposed to repetitive, sublethal temperatures demonstrate reduced egg survival and body size at hatching (Hall and Warner 2021; Carlo et al., 2017). Similarly, when newt eggs are exposed to wide daily fluctuating temperatures, resultant larvae have decreased burst speed at moderate temperatures when compared to lower amplitude fluctuation treatments (Měráková and Gvoždík, 2009). Brief exposure to extreme temperatures affects muscle fiber type and quantity, heart size, and developmental of the nervous system (Alsop, 1919; Booth, 2017 and 2018; Assersohn et al., 2021). It is plausible that although temperatures experienced by *A. barbouri* embryos in the wild are not directly lethal, temporary exposure to sublethal temperatures, as was observed at disturbed sites, may lead to eventual mortality or reduced fitness.

Temperatures throughout the larval period were higher than those of the embryonic period, with hourly temperatures reaching above 30 °C, and two sites, only one of which was disturbed, consistently reaching > 34 °C, which exceeds the preferred temperature and critical thermal maximum of other larval *Ambystoma* (Keen & Schroeder, 1975). Temperature tolerance of amphibians increases over the early life-stages and is generally higher for larvae than embryos (Turriago et al., 2015). Notably, warmer larval acclimation temperatures increase the critical thermal maximum of larvae (Keen and Schroeder, 1975), suggesting that larvae at disturbed sites may be more tolerant to high temperatures due to the overall warmer conditions. Furthermore, disturbed sites were only 0.7 °C warmer than undisturbed sites during the larval period compared to 1.5 °C warmer during the embryonic period. Because larvae are able to behaviorally thermoregulate, they may be less at risk to temperature stress compared to embryos. Currently, water temperatures are unlikely to pose an immediate lethal threat to *A. barbouri*; however, the effects of ongoing suburban, urban, and agrarian development and climate change may compound to create unsuitable conditions in the future (Maloney et al., 2020).

Survival

Embryo survival is typically high across a range of constant temperatures (OTR) with a steep decline at the extremes (van der Have, 2002; Sgrò et al., 2016; Hall and Sun, 2021). *Ambystoma barbouri* embryos had high survival at the coolest temperatures (5 °C); consequently, I was unable to determine the lower extent of the OTR, but the lower extent has been described to be around 3 °C for other ambystomatids (Anderson, 1972).

The OTR of *A. barbouri* therefore probably ranges from about 3 °C to 17 °C. Outside of the OTR, I also described the LT50 (ECHT– see Hall and Sun, 2021) for *A. barbouri* at 21 °C, which is the point at which egg survival is reduced by 50%. This is comparable to other reported lethal temperatures in the genus *Ambystoma*; *A. trigrinium* at 20 °C, *A. macrodactylum singillatum* at 22 °C, *A. gracile gracile* at 25 °C (Anderson, 1972); however, it is much lower than other amphibians such as many anurans (Moore, 1939; Turriago et al., 2015). It is likely that within the OTR, there is an optimal temperature for overall fitness. Though egg survival is high at a range of temperatures, many hatchling phenotypes exhibit an optimum (e.g. swimming speed in turtles; Mueller et al., 2019). Across the tested temperatures, 10 °C appears to be optimal for *A. barbouri* fitness, as survival was high across each early life-stage, Harrison developmental stages were advanced at hatching, time to metamorphosis was short, and body size was largest at the juvenile stages. Moreover, field temperatures were close to 10 °C, alluding to embryo adaptation to a similar thermal regime.

Notably, nest temperatures dropped below freezing throughout the embryonic period, but lethal consequences of cold temperatures have only been described for terrestrial nesting *Ambystoma opacum* and are otherwise undescribed (Graham, 1971; Hall, 2022). Development moves at a slower pace at cool temperatures due to reduced enzymatic function, but slower developmental rates at cold temperatures do not necessarily reduce fitness. At near freezing temperatures, amphibian embryos have been recorded to survive short periods of exposure to freezing temperatures (Buchanan, 1938; Licht, 1971); however, short periods of exposure to high temperatures decreased embryonic survival (Hall and Sun, 2021). As temperatures above the OTR and ECHT of

A. barbouri were occasionally observed in breeding streams, some developmental abnormalities would be expected after short exposure to high temperatures. For example, when *Anolis sagrei* (Brown Anole) eggs were exposed to acute heat shock shortly after oviposition, craniofacial malformations were more common when compared to eggs that did not experience acute heat shock (Sanger et al., 2021). Acute heat shock has not been thoroughly studied in *Ambystoma*, yet I documented their exposure to such events in the wild, highlighting the need to further understand the embryonic developmental limits with respect to acute heat stress.

Interestingly, I observed carry-over effects of incubation temperature on larval survival, where larvae from eggs incubated at 20 and 22 °C had significantly lower survival than those from 5 and 10 °C. These results indicate that warmer temperatures induced abnormalities that did not prevent embryo development, *per se*, but were detrimental to larval physiology. Explanations may include premature death of particular cell types or cell lines or the malformation of organs or whole-body systems (Edwards et al., 2003; Eme et al., 2015; Pype et al., 2015; Sanger et al., 2021). For example, shifts in temperature after gastrulation but before organogenesis in *Coregonus clupeaformis* (Lake Whitefish) led to reduced heart rate and oxygen consumption throughout the remaining embryonic period and at hatching, suggesting lasting effects of temperature on cardiovascular physiology (Eme et al., 2015). High temperatures during the embryonic period of the lizard *Anolis sagrei* led to cell death in the developing forebrain, most likely leading to irregular movement patterns and, as a results, reduced feeding efficiency (Sanger et al., 2021). Another possible explanation for the reduction in hatchling survival from the warmest treatment groups is that these embryos had a lower rate of conversion

of yolk to body tissue (Jonsson et al., 2022) and therefore had less muscle mass (Johnston, 1983) or altered enzymatic activity (Schnurr et al., 2014) which would in turn influence swimming speed, feeding ability, and digestive efficiency (Wilson et al., 2005; Volkoff and Rønnestad, 2020). Additionally, abnormal development of critical organ systems may have been sublethal to embryos but lethal to larvae that require more oxygen and controlled movements for feeding. An embryo's energy demand is met via yolk provided from the mother, but larvae require the coordinated function of multiple body systems to capture and digest food. Specifically, abnormal development of neural pathways governing chemoreception, the primary method by which amphibian larvae detect prey (Veeranagoudar et al., 2004), would lead to an inability to find and feed on provided blood worms, leading to death through starvation. Although swimming speed was not considered in this study, as all larvae were raised individually, in their natural environment reduced swimming speed may affect the larva's ability to escape predators and compete with conspecifics, thereby leading to lower survival rates.

Juvenile survival was high across all treatments, indicating that beyond metamorphosis, incubation temperature has little impact on long-term survival. The exact cause of this is unclear but may relate to the reduction in energetic needs after metamorphosis. Prior to metamorphosis, larvae feed at higher rates to attain necessary nutrients (Crump, 1981; Duellman and Trueb, 1994; Kirschman et al., 2017). Furthermore, juvenile and adult survival of amphibians is reported to be relatively high compared to egg and larval stages (Gamble et al., 2009; De Lisle and Grayson, 2011; Homan et al., 2018; Messerman et al., 2020), and my results further support this, as embryonic thermal stress does not appear to negatively influence survival once an

individual has reached the juvenile stage. Alternatively, it could simply emerge from among-individual variation in thermal tolerance (Drown et al., 2021; Nati et al., 2021). Indeed, the ICC of embryo survival was low to moderate (0.35) which indicates meaningful effects of clutch ID, a proxy for genotype, on survival in response to temperature. Ultimately these carry-over effects on survival highlight the vulnerability of embryos and larvae to thermal stress and that the effects of incubation temperature may not present across all life-stages. Yet, for those individuals incubated at 20 or 22 °C that survived to the juvenile stage, their survival may represent selection for individuals with a higher heat tolerance.

Developmental Rates

Development rate is tightly correlated with temperature. Generally, cellular characteristics (i.e., membrane viscosity, enzyme function) have a 10-15 °C range of proper function and proceed at higher rates with warmer temperatures across that range; however, at extremes, development theoretically slows due to irreparable damage to enzymes and changes in fluidity of cellular membranes that alter rates of reactions due to ion leakage (Angilletta, 2009). The relationship between developmental rate and temperature has been studied at length in amphibians (Lillie and Knowlton, 1897; Moore, 1939; Anderson, 1972); however, my research goes a step further as I also describe the impact of egg temperature on development of later life stages, such as the time to metamorphosis (Semlitsch et al., 1988).

Among ectotherms there is a trade-off between hatching time and size at hatching (Jonsson et al., 2011; Warkentin, 2011a). Hatching earlier may benefit an individual

through increased food availability due to reduced competition and therefore larger size when is greater intraspecific competition (Warner and Shine, 2007; Uller and Olsson, 2010). *Anolis sagrei* incubated at warmer temperatures hatched earlier and at a smaller size than those incubated at lower temperatures but had higher survival to adulthood, likely due to reduced competition caused by early hatching (Pruett and Warner, 2021). A similar trend may occur in natural environments for *A. barbouri,* as there may be a competitive advantage to hatching earlier and therefore being among the largest body-size class, which is less likely to be depredated and has a higher variety of food options (Smith and Petranka, 1987; McWilliams and Bachmann, 1989; Urban, 2007; Gvoždík and Smolinský, 2015). Yet, hatching at a smaller and earlier developmental stage may be disadvantageous through predator preference for small larvae and overall reduced survival (Warkentin, 1995; Gvoždík and Smolinský, 2015). Decreased developmental rates at cooler temperatures is advantageous given that being further developed and therefore larger in size at hatching allows better predator evasion (Sih and Moore, 1993; Voss, 1993; Warkentin, 2011b). However, slower developmental rates, and therefore a lengthened amount of time in the egg, increases the possibility of egg predation or desiccation. Although developmental rate is heavily studied in amphibians, the ecological relevance is not thoroughly understood and changes in hatching time may have intra- or inter-specific consequences that are not yet described.

Additionally, in response to stressful environments (e.g. hypoxia, air exposure, egg predator presence) amphibian eggs experience accelerated hatching and subsequently hatch at earlier stages and smaller size (Warkentin, 2011a; Warkentin et al., 2017). Both hypoxic conditions and air exposure can coincide with reduced water levels and therefore

increased temperature could be an additional environment cue for accelerated hatching. Since environmentally cued hatching can lead to hatching at an earlier developmental stage, it is likely that 20 and 22 °C were stressful temperatures potentially causing earlier hatching when compared with 10 °C, which was well within the OTR.

Incubation temperature also influenced time to metamorphosis for individuals incubated at 5 °C but not 10 or 20 °C. This is notable because larvae from 5 °C were not the smallest at hatching and were not expected to require extra time to reach minimum size for metamorphosis. This result is in contrast with Orizaola et al. (2010), who found that *Rana arvalis* (Moore Frog) when incubated at 4 °C metamorphosized earlier than individuals incubated at 15 °C. When individuals take longer to metamorphosize, risk desiccation and predation risks increase (Relyea, 2007; Rudolf and Rödel, 2007; Richter-Boix et al., 2011); however, they would be expected to be larger at metamorphosis due to the extended period of larval growth (Semlitsch et al., 1988). *Ambystoma barbouri* incubated at 5 °C took an average of 138 days (±4 SE) to reach metamorphosis which may push larvae into the drying period of ephemeral streams in Tennessee (Table A.2). To my knowledge, an extended larval period in response to cold embryonic temperatures has not been reported and the mechanisms behind it are unclear. Metamorphosis is induced by thyroid hormone, production of which is assisted by thyroid glands that develop during late embryogenesis (Dodd and Dodd, 1976). Abnormal development of the thyroid glands in larvae incubated at 5 °C could have led to a longer larval period, because without thyroid hormone, metamorphosis cannot proceed (Bonnet, 2016). Alternatively, variation in post-hatching metabolic rate (i.e., growth) in response to incubation temperature has been observed in *Danio rerio* (Zebrafish) and was attributed

to differences in gene expression among treatments, specifically genes involved in metabolism, apoptosis, and cell stress were recorded to have differential expression (Scott and Johnston, 2012). The differences across treatments in time to metamorphosis suggest that incubation temperature affects larval metabolism, potentially through gene expression, further highlighting the complex relationship between embryonic temperature and its effects across life stages.

Body Size

Larval and metamorph body size can influence feeding rates and growth, and thus survival to adulthood as well as mating success of males and fecundity of females (Semlitsch, 1987; Urban, 2007; Gvoždík and Smolinský, 2015). Thus, it is critical to understand how environmental factors, such as temperature, influence morphology. *Ambystoma barbouri* hatchlings were smallest when incubated at the warmest temperatures and largest from the 10 °C incubation treatment. The smaller size observed at hatching for the 20 and 22 °C incubation treatment is likely due to reduced efficiency in converting yolk to body tissue and decreased time to hatching caused by higher rates of the enzymatic processes that dissolve the egg membranes (Herreid and Kinney, 1967; Kuramoto, 1978; Duellman and Trueb, 1994; Wu et al., 2023). However, individuals from the 5 °C incubation also hatched at a smaller size, a potential side-effect of hatching at an earlier Harrison stage than those incubated at 10 °C. Smaller size at hatching may increase the likelihood of being depredated by common predators such as dragonfly

larvae (Urban, 2007; Gvoždík and Smolinský, 2015; but see Oswald et al., 2020). Predation is a leading cause of mortality for salamander larvae and size plays an important role in intra- and inter-guild predation rates (Werner, 1986; Mott and Sparling, 2016). Head size, which is correlated to body size, is particularly important for ambystomatids as they are gape-restricted predators such that smaller larvae, which have smaller heads, have fewer prey options than bigger larvae, which have larger heads (Smith and Petranka, 1987; McWilliams and Bachmann, 1989).

Larval body size has been linked to size at metamorphosis and adulthood (Kaplan, 1985; Semlitsch, 1987). Therefore, it would be expected that the smallest larvae, which were incubated at 20 and 22 °C, would be the smallest throughout the experiment. My results do not fully support this prediction, as *A. barbouri* metamorphs from the 20 °C treatment, which were smallest upon hatching, were not smaller than the 5 or 10 °C treatments at metamorphosis. This indicates that stressful embryonic temperatures might make *A. barbouri* larvae more susceptible to predation early in the larval period, but compensatory growth is possible. Notably, smaller larvae were more likely to die than larger ones leaving more larvae of larger size reached metamorphosis and may be a different explanation for similar sizes across treatments at metamorphosis.

Compensatory growth has been observed in anurans and some salamanders whereby eggs raised in stressful environments that hatch at reduced size may grow at a faster rate during larval or juvenile life stages, typically due to increased feeding rates (Nicieza and Metcalfe, 1997; Räsänen et al., 2002; Hurst et al., 2005; Orizaola et al., 2010; Landberg, 2013; Krause and Caspers, 2016; Charbonnier et al., 2018; Burraco et al., 2021). This suggests that compensatory growth may be an important survival tactic

for *A. barbouri* larvae and positively influence the likelihood of reaching metamorphosis, at which point mortality rates are dramatically reduced (Kissel et al., 2019). To my knowledge, the mechanisms behind compensatory larval growth in response to warm incubation temperatures, such as was observed here, have not been described or reported prior to this study. The larvae in this experiment were all fed the same amounts; however, those that hatched at a smaller size may have consumed higher amounts of the provided food, allowing for increased growth.

At 90 days post-metamorphosis individuals from the 10 °C treatment, which were similarly sized at metamorphosis when compared to the other treatments, were statistically larger than those from 20 °C but statistically similar to 5 °C individuals. These results demonstrate the continued benefit of embryonic temperatures being well within the OTR as growth rates were highest for 10 °C, likely leading to larger adults and therefore higher fecundity (Semlitsch, 1987; Wise and Jaeger, 2021). Tail length and HTL were tightly correlated to total length, however, their significance differs across life stages. The importance of tail length is more pronounced in the larval stage when predator evasion depends on burst speed or endurance, which is correlated to tail morphology in salamanders (Fitzpatrick et al., 2003; Yurewicz, 2004; Landberg and Azizi, 2010). In the terrestrial stages, the tail is commonly used for fat storage (Wake and Dresner, 1967). HTL, which is similar to snout-to-vent-length (SVL), is positively correlated to clutch size and therefore higher fecundity (Semlitsch, 1987; Wise and Jaeger, 2021). As with total length at 90 days post-metamorphosis, HTL and tail length were largest when incubated at 10 °C indicating that if size differences continue into adulthood, those individuals would be the most fecund. Hero et al. (2005) suggested that

low fecundity in amphibians can be a predictor of susceptibility to population decline and an indication of a reduction of population resilience. This illustrates that the ongoing threats of habitat destruction, urbanization, and climate change may impact multiple stages of the *A. barbouri* life cycle in Tennessee.

Environmental Temperature and the Optimal Thermal Range

Describing the OTR of a species is a fundamental step toward characterizing ecologically relevant thermal physiology and understanding the evolution of thermal phenotypes (Andrews and Schwarzkopf, 2012; Hall and Sun, 2021). However, some temperatures within the OTR may maximize fitness-relevant traits more than others (Mueller et al., 2019). Of the experimentally tested temperatures, 10 °C was the optimum constant incubation temperature for *A. barbouri*. Across each early life stage, survival was high for those incubated at 10 °C, unlike those incubated at 20 and 22 °C that experienced a significant decrease in larval survival. Furthermore, individuals from 10 °C were largest at both hatching and 90 days post-metamorphosis at which stages body size is important phenotypes for predator avoidance and reproduction, respectively. Although *A. barbouri* were incubated at 5 °C also maintained a high survival rate and large size through metamorphosis, their embryonic and larval periods were extensive and therefore in natural environments may have higher predation rates. Notably, mean stream temperatures were between 8-10 °C, suggesting that Tennessee *A. barbouri* may be adapted to this range. This could explain high survival and body size for eggs incubated at 10 °C. However, stream temperatures also reached above the OTR and ECHT (21 °C), possibly leading to disruptions in development such as neural malformation or reduced

muscle mass even after a brief exposure (Johnston, 1983; Sanger et al., 2021). Describing the OTR and ECHT for *A. barbouri* now allows for further studies into the tolerance of temperature variability, a more ecologically relevant trait for a state endangered species whose range is undergoing rapid human and changes to influences intermittent stream habitats.

Conclusions

The impacts of temperature on ectothermic embryos and subsequent life stages are becoming increasingly important to understand as factors of global change alter thermal regimes (Herb et al., 2008; Obiakor et al., 2012; Davis et al., 2019; Grey et al., 2023; Stark et al., 2023). Oviparous ectotherms are at particular risk due to the vulnerability of the sessile embryonic stage that is unable to behaviorally thermoregulate and receives little parental care. The current study further highlights the threats of global change through describing temperature differences between disturbed and undisturbed breeding streams of an amphibian, characterizing the impact of urbanization on ephemeral stream habitat. I also demonstrate that temperatures experienced during the embryonic period can have carry-over effects throughout early life stages by leading to reduced body size and reduced survival. For many ectothermic species, body size is directly correlated to fecundity and therefore reduced overall adult size within populations may lead to reduced reproductive effort. Embryonic incubation temperature not only influenced hatching success but also influenced larval survival, such that larvae from warm incubations had reduced survival to metamorphosis. This finding suggests that studies that predict survival probability based only on embryonic survival are highly

conservative, as death due to stressful incubation temperatures leads to larval mortality as well. Furthermore, exposing only the embryos and not larvae to different temperatures allowed for targeted understanding of the effects of stressful temperatures on early life stages that is not possible when larvae remain at experimental temperatures as has been done in past studies (Moriya, 1980; Arrighi et al., 2013; Smith et al., 2015). Further study of the carry-over effects of embryonic temperature on later life stages and the impact of urbanization on ephemeral waterways is needed to better understand how ectotherms such as *A. barbouri* will respond to ongoing global change.

APPENDIX A: TABLES

Table A.1

Study sites and corresponding egg, clutch, and habitat characteristics of Ambystoma barbouri

Note. Harrison Stages are provided for eggs at the time of collection and temperatures are for the embryonic period (see methods). Date - date site visited; Clutches – number of clutches from which eggs were collected; Mean H.S. – mean Harrison stage; H.S. range – Harrison stage range of eggs upon collection. StDev – standard deviation of stream temperatures. Disturbed – Y – disturbed site, N – undisturbed site.

Table A.1 (continued)

Site	Date	Clutches	Mean H.S.	H.S. Range	Max Temp °C	Min Temp °C	Mean Temp °C	StDev °C	Disturbed
Cedars of Lebanon	12/4/2022	1	7.43	7-8	14.3	1.2	9.1	2.6	N
Lebanon, Site 2	12/6/2022	3	8.98	4-12	21.5	-0.9	8.7	2.8	N
Gallatin, Site 1	12/9/2022	3	5.63	1-8	15.8	-6.6	9.3	3.9	Y
Couchville State Natural Area	12/17/2022	3	1.92	1-6	16.4	0.5	8.3	2.5	N
Flat Rock State Natural Area	12/20/2022	3	8.45	6-12	16.2	3.3	9.3	2.2	N
Private Landowner	1/6/2023	3	1.18	1-2	18.1	4.2	9.1	2.2	N
Hendersonville	1/10/2023	2	9.74	8-11	14.7	5.1	9.4	1.7	Y
Gallatin, Site 2	1/19/2023	1	1	1	-	-	-		Y
Madison	1/22/2023	0	-	-	23.5	2.5	9.7	3.8	Y
Lebanon, Site 1	2/2/2023	0	-	-	21.2	5.7	12.5	2.9	Y

Table A.2

Mid- and end-season field site observations

Note. StDev – standard deviation. Algae – large amounts of algae were observed in the stream.

Site	Date	Eggs present	Larvae present	StDev	Stream condition	Algae
Gallatin, site 1	1/19/2023	Yes	Yes	-	Flowing	No
	5/15/2023	No	No	-	Flowing	Yes
Hendersonville	3/11/2023	Yes	Yes	-	Flowing	No
	5/15/2023	No	No	-	Flowing	No
Madison	4/16/2023	No	Yes	-	Partially Dry	No
	5/11/2023	No	Yes	0.102	Partially Dry	No
Lebanon, site 1	3/11/2023	No	Yes	-	Flowing	Yes
	5/15/2023	No	No	-	Flowing	Yes
Lebanon, site 2	1/5/2023	Yes	No	-	Flowing	No
	5/11/2023	No	Yes	0.300	Flowing	No
Cedars of Lebanon	1/5/2023	Yes	No	-	Flowing	No
	5/15/2023	No	Yes	0.298	Flowing	No
Couchville State Natural Area	3/11/2023	Yes	Yes	-	Flowing	Yes
	5/15/2023	No	Yes	0.159	Flowing	Yes
Private Landowner	3/11/2023	No	Yes	-	Flowing	Yes
	5/15/2023	No	No	-	Flowing	Yes
Flat Rock State Natural Area	5/11/2023	No	Yes	0.321	Flowing	No

Table A.3

Land use of area surrounding field sites

Note. Percent land use within a 0.5-km radius of field sites, generalized to be within five categories per the National Land Cover Database, 2021. (*) Denotes that a temperature logger was not placed at a site.

	Site	% developed	% forested	% wetland	% farmland	% other
Disturbed	Gallatin, site 1	48.5	2.9	0	43.5	5.2
	Gallatin, site 2*	52.8	3.3	0	43.9	0
	Hendersonville	49.0	25.3	0	20.9	4.8
	Madison	89.4	9.0	0	1.6	0
	Lebanon, site 1	90.0	0.6	0	9.0	0.5
Not Disturbed	Lebanon, site 2	3.9	78.2	0.6	17.3	0
	Cedars of Lebanon	0	99.7	0	0.3	0
	Couchville State Natural Area	1.4	82.1	0	16.3	0.2
	Private Landowner	9.2	48.3	0	42.5	0
	Flat Rock State Natural Area	0	81.2	0	18.8	0

Table A.4

Canopy cover of nest locations

Note. Percent canopy cover at specific temperature logger locations at each site. Canopy cover percentage was extracted from National Land Cover Database canopy cover 2021 using ArcGIS Pro. Open canopy represents categories as determined in the field based on observations.

	Site	% Canopy Cover	Open Canopy
Disturbed	Gallatin, site 1	0	Yes
		0	Yes
	Hendersonville	39	No
		0	Yes
	Madison	15	Yes
		19	Yes
	Lebanon, site 1	11	No
		14	Yes
Not Disturbed	Lebanon, site 2	74	No
		74	No
	Cedars of Lebanon	68	No
		74	No
	Couchville State Natural Area	0	Yes
		45	Yes
	Private Landowner	70	No
		72	No
	Flat Rock State Natural Area	74	No
		0	Yes

Table A.5

Model summary results for effects of habitat characteristics on field temperatures

Note. Results from linear mixed effects models analyzing the effects of disturbance and canopy cover on water temperature during the embryonic and larval periods. Bold text denotes statistical significance ($\alpha = 0.05$). (*) Denotes that hour was included in model as a categorical variable and results for each hour are reported in Table A.6. N – sample size, σ^2 – residual variance, τ_{00} – variance of the random effect, ICC – intraclass correlation coefficient.

Response	Fixed Effect	*df*	Estimate	*SE*	t-value	p-value
Embryonic Temperature						
N=32244	Intercept	108	8.239	0.534	15.438	**<0.0001**
conditional r^2=0.243	Disturbed (Y)	24	1.513	0.400	3.784	**0.001**
marginal r2=0.138	Open Canopy (Y)	24	-0.817	0.403	-2.025	0.054
	Specific Location (No Nest)	24	-0.119	0.382	-0.313	0.757
	Julian Day	31375	0.001	0.001	0.891	0.373
	Random effect (σ^2 = 6.89, τ_{00} = 0.96, ICC = 0.12, N = 28)					
Embryonic Daily Maximum Temperature						
N=1371	Intercept	866	5.026	1.663	3.023	**0.003**
conditional r^2=0.259	Disturbed (Y)	25	2.007	0.404	4.973	**<0.0001**
marginal r^2=0.147	Julian Day	1027	0.014	0.004	3.271	**0.001**
	Random effect (σ^2 = 6.35, τ_{00} = 0.96, ICC = 0.13, N = 28)					
Embryonic Daily Minimum Temperature						
N=1371	Intercept	842	8.519	1.745	4.883	**<0.0001**
conditional r^2=0.134	Disturbed (Y)	26	0.724	0.409	1.770	0.088
marginal r^2=0.014	Julian Day	987	-0.003	0.005	-0.552	0.581
	Random effect (σ^2 = 7.06, τ_{00} = 0.98, ICC = 0.12, N = 28)					
**Larval Temperature*						
N=55667	Intercept	89	22.629	0.276	-81.911	**<0.0001**
conditional r^2=0.552	Disturbed (Y)	23	0.663	0.280	2.366	**0.027**
marginal r^2=0.522	Open Canopy (Y)	23	0.719	0.278	2.590	**0.016**
	Julian Day	55639	0.075	0.001	180.411	**<0.0001**
	Random effect (σ^2 = 6.71, τ_{00} = 0.45, ICC = 0.06, N = 26)					
Larval Daily Maximum Temperature						
N=2333	Intercept	414	-31.473	1.167	-26.972	**<0.0001**
conditional r^2=0.621	Disturbed (Y)	24	1.776	0.854	2.079	**0.048**
marginal r^2=0.422	Julian Day	2310	0.105	0.002	46.080	**<0.0001**
	Random effect (σ^2 = 8.64, τ_{00} = 4.53, ICC = 0.34, N = 26)					
Larval Daily Minimum Temperature						
N=2333	Intercept	2093	-13.927	0.816	-17.074	**<0.0001**
conditional r^2=0.324	Disturbed (Y)	24	0.204	0.241	0.847	0.405
marginal r^2=0.285	Julian Day	2328	0.054	0.002	30.291	**<0.0001**
	Random effect (σ^2 = 5.30, τ_{00} = 0.31, ICC = 0.05, N = 26)					

Table A.6

Model summary results for effects of hour of day on field temperatures

Note. Hour results from linear mixed-effects models analyzing the effects of disturbance and canopy cover on water temperature during the embryonic and larval periods. Bold text denotes statistical significance ($\alpha = 0.05$). *N* – sample size, σ^2 – residual variance, τ_{00} – variance of the random effect, ICC – intraclass correlation coefficient.

Response	Fixed Effect	*df*	Estimate	*SE*	t-value	p-value
Embryonic Temperature						
N=32244	Hour 1	32192	-0.067	0.101	-0.659	0.510
conditional r^2=0.243	Hour 2	32192	-0.120	0.101	-1.183	0.237
marginal r2=0.138	Hour 3	32192	-0.172	0.101	-1.699	0.089
	Hour 4	32192	-0.221	0.101	-2.177	**0.030**
	Hour 5	32192	-0.263	0.101	-2.589	**0.010**
	Hour 6	32192	-0.302	0.101	-2.975	**0.003**
	Hour 7	32192	-0.257	0.101	-2.535	**0.011**
	Hour 8	32192	-0.024	0.101	-0.232	0.817
	Hour 9	32192	0.442	0.101	4.357	**<0.0001**
	Hour 10	32192	1.041	0.101	10.255	**<0.0001**
	Hour 11	32192	1.629	0.101	16.074	**<0.0001**
	Hour 12	32192	2.094	0.101	20.687	**<0.0001**
	Hour 13	32192	2.291	0.101	22.667	**<0.0001**
	Hour 14	32192	2.216	0.101	21.928	**<0.0001**
	Hour 15	32192	1.966	0.101	19.448	**<0.0001**
	Hour 16	32192	1.592	0.101	15.750	**<0.0001**
	Hour 17	32192	1.200	0.101	11.859	**<0.0001**
	Hour 18	32192	0.897	0.101	8.870	**<0.0001**
	Hour 19	32192	0.691	0.101	6.827	**<0.0001**
	Hour 20	32192	0.508	0.101	5.021	**<0.0001**
	Hour 21	32192	0.340	0.101	3.365	**0.001**
	Hour 22	32192	0.204	0.101	2.012	**0.044**
	Hour 23	32192	0.094	0.101	0.926	0.354
	Random effect (σ^2 = 6.89, τ_{00} = 0.96, ICC = 0.12, N = 28)					
Larval Temperature						
N=55667	Hour 1	55617	-0.160	0.076	-2.103	**0.035**
conditional r^2=0.552	Hour 2	55617	-0.310	0.076	-4.054	**<0.0001**
marginal r^2=0.522	Hour 3	55617	-0.432	0.076	-5.671	**<0.0001**
	Hour 4	55617	-0.548	0.076	-7.189	**<0.0001**
	Hour 5	55617	-0.654	0.076	-8.591	**<0.0001**
	Hour 6	55617	-0.718	0.076	-9.428	**<0.0001**
	Hour 7	55617	-0.519	0.076	-6.807	**<0.0001**
	Hour 8	55617	0.063	0.076	0.823	0.410
	Hour 9	55617	0.925	0.076	12.151	**<0.0001**
	Hour 10	55617	2.025	0.076	26.582	**<0.0001**

Table A.6 (continued)

Response	Fixed Effect	*df*	Estimate	*SE*	t-value	p-value
Larval Temperature						
N=55667	Hour 11	55617	3.029	0.076	39.770	**<0.0001**
conditional r^2=0.552	Hour 12	55617	3.893	0.076	51.113	**<0.0001**
marginal r^2=0.522	Hour 13	55617	4.418	0.076	58.023	**<0.0001**
	Hour 14	55617	4.559	0.076	59.888	**<0.0001**
	Hour 15	55617	4.273	0.076	56.143	**<0.0001**
	Hour 16	55617	3.761	0.076	49.408	**<0.0001**
	Hour 17	55617	3.088	0.076	40.569	**<0.0001**
	Hour 18	55617	2.362	0.076	31.046	**<0.0001**
	Hour 19	55617	1.720	0.076	22.608	**<0.0001**
	Hour 20	55617	1.224	0.076	16.086	**<0.0001**
	Hour 21	55617	0.854	0.076	11.222	**<0.0001**
	Hour 22	55617	0.554	0.076	7.284	**<0.0001**
	Hour 23	55617	0.307	0.076	4.041	**<0.0001**
	Random effect (σ^2 = 6.71, τ_{00} = 0.45, ICC = 0.06, N = 26)					

Table A.7

Model summary results for effects of incubation temperature on Ambystoma barbouri embryonic and larval survival

Note. Results from generalized linear mixed effects models describing the effects of embryonic temperature on survival of *Ambystoma barbouri* for the embryonic and larval periods. Bold text denotes statistical significance (α = 0.05). The 5 °C treatment is the reference. *N* – sample size, σ^2 – residual variance, τ_{00} – variance of the random effect, ICC – intraclass correlation coefficient.

Response	Fixed Effect	Estimate	*SE*	z-value	p-value
Embryonic Survival					
N=290	Intercept	0.877	0.357	2.455	**0.01**
conditional r^2=0.592	Temperature	-0.238	0.031	-7.758	**<0.0001**
marginal r^2=0.373	*Random effect (σ^2 = 3.29, τ_{00} = 1.77, ICC = 0.35, N = 19)*				
Larval Survival					
N=176	Intercept	0.083	0.286	0.292	0.77
R^2=0.259	10 °C Treatment	-0.061	0.070	-0.870	0.39
	20 °C Treatment	-0.249	0.076	-3.261	**0.001**
	22 °C Treatment	-0.540	0.122	-4.436	**<0.0001**
	Hatching Total Length	0.057	0.020	2.899	**0.004**

Table A.8

Model summary results for effects of incubation temperature on Ambystoma barbouri embryonic and hatchling phenotypes

Note. Results from linear mixed effects models examining the effects of temperature on embryonic and hatchling phenotypes of *Ambystoma barbouri.* Bold text denotes statistical significance (α = 0.05). The 5 °C treatment is the reference. *N* – sample size, σ^2 – residual variance, τ_{00} – variance of the random effect, ICC – intraclass correlation coefficient.

Response	Fixed Effect	*df*	Estimate	*SE*	t-value	p-value
Developmental Rate						
N=178	Intercept	83	1.005	0.468	2.150	**0.035**
conditional r^2=0.956	10 °C Treatment	162	1.407	0.113	12.438	**<0.0001**
marginal r^2=0.955	20 °C Treatment	170	6.572	0.122	53.849	**<0.0001**
	22 °C Treatment	173	7.002	0.191	36.622	**<0.0001**
	Egg Width	82	0.007	0.163	0.041	0.967
	Random effect (σ^2 = 0.38, τ_{00} = 0.01, ICC = 0.03, N = 19)					
Harrison Stage at Hatching						
N=176	Intercept	130	38.787	0.790	49.128	**<0.0001**
conditional r^2=0.365	10 °C Treatment	158	1.212	0.171	7.080	**<0.0001**
marginal r^2=0.261	20 °C Treatment	163	0.660	0.185	3.559	**0.0005**
	22 °C Treatment	167	0.317	0.294	1.079	0.282
	Egg Width	133	0.886	0.275	3.222	**0.002**
	Random effect (σ^2 = 0.85, τ_{00} = 0.14, ICC = 0.14, N = 19)					
Length at Hatching						
N=178	Intercept	47	9.261	0.988	9.375	**<0.0001**
conditional r^2=0.447	10 °C Treatment	147	0.983	0.238	4.136	**0.0001**
marginal r^2=0.429	20 °C Treatment	164	-1.168	0.256	-4.556	**<0.0001**
	22 °C Treatment	173	-1.722	0.402	-4.282	**<0.0001**
	Egg Width	46	1.793	0.345	5.174	**<0.0001**
	Random effect (σ^2 = 1.67, τ_{00} = 0.05, ICC = 0.03, N = 19)					

Table A.9

Model summary results for effects of incubation temperature on Ambystoma barbouri phenotypes at metamorphosis

Note. Results from linear mixed effects models examining effects of incubation temperature on phenotypes of *Ambystoma barbouri* at metamorphosis. Bold text denotes statistical significance (α = 0.05). The 5 °C treatment is the reference. *N* – sample size, σ^2 – residual variance, τ_{00} – variance of the random effect, ICC – intraclass correlation coefficient.

Response	Fixed Effect	*df*	Estimate	*SE*	t-value	p-value
Days to Metamorphosis						
N=132	Intercept	119	233.280	136.254	1.712	0.089
conditional r^2=0.591	10 °C Treatment	113	-35.024	3.812	-9.188	**<0.0001**
marginal r^2=0.391	20 °C Treatment	116	-31.872	4.900	-6.505	**<0.0001**
	Length at Hatching	126	-2.877	1.360	-2.116	**0.036**
	Aquarium Temperature	118	-2.763	6.997	-0.395	0.694
	Random effect (σ^2 = 336.93, τ_{00} = 164.61, ICC = 0.33, N = 18)					
Mass at Metamorphosis						
N=132	Intercept	119	-0.234	0.836	-0.280	0.780
conditional r^2=0.384	10 °C Treatment	112	-0.086	0.023	-3.668	**0.0004**
marginal r^2=0.086	20 °C Treatment	116	-0.033	0.030	-1.111	0.269
	Length at Hatching	126	-0.003	0.008	-0.302	0.763
	Aquarium Temperature	117	0.048	0.043	1.119	0.265
	Random effect (σ^2 = 0.01 τ_{00} = 0.01, ICC = 0.33, N = 18)					
Total Length at Metamorphosis						
N=132	Intercept	118	33.098	27.329	1.211	0.228
conditional r^2=0.384	10 °C Treatment	112	-2.447	0.764	-3.204	**0.002**
marginal r^2=0.052	20 °C Treatment	115	-0.991	0.982	-1.009	0.315
	Length at Hatching	125	0.128	0.273	0.469	0.640
	Aquarium Temperature	117	0.629	1.403	0.448	0.655
	Random effect (σ^2 = 13.51, τ_{00} = 7.27, ICC = 0.35, N = 18)					
Tail Length at Metamorphosis						
N=132	Intercept	119	23.356	16.320	1.431	0.16
conditional r^2=0.314	10 °C Treatment	112	-1.092	0.458	-2.387	**0.019**
marginal r^2=0.037	20 °C Treatment	117	-0.633	0.588	-1.078	0.28
	Length at Hatching	127	-0.037	0.162	-0.227	0.82
	Aquarium Temperature	118	-0.020	0.839	-0.024	0.98
	Random effect (σ^2 = 4.86, τ_{00} = 1.97, ICC = 0.29, N = 18)					
Head-trunk Length at Metamorphosis						
N=132	Intercept	117	8.924	13.282	0.672	0.50
conditional r^2=0.427	10 °C Treatment	112	-1.355	0.370	-3.659	**0.0004**
marginal r^2=0.066	20 °C Treatment	115	-0.362	0.477	-0.758	0.45
	Length at Hatching	125	0.167	0.133	1.252	0.213
	Aquarium Temperature	116	0.690	0.682	1.013	0.31
	Random effect (σ^2 = 3.18, τ_{00} = 2.00, ICC = 0.39, N = 18)					

Table A.10

Model summary results for effects of incubation temperature on Ambystoma barbouri phenotypes at 90 days post-metamorphosis

Note. Results from linear mixed effects models examining the effects of embryonic temperature on phenotypes of *Ambystoma barbouri* at 90 days post-metamorphosis. Bold text denotes statistical significance ($\alpha = 0.05$). The 5 °C treatment is the reference. *N* – sample size, σ^2 – residual variance, τ_{00} – variance of the random effect, ICC – intraclass correlation coefficient, HTL – head-trunk length.

Response	Fixed Effect	*df*	Estimate	*SE*	t-value	p-value
Mass at 90 days Post-Metamorphosis						
N=123	Intercept	117	7.327	3.468	2.112	**0.037**
conditional r^2=0.421	10 °C Treatment	117	0.206	0.164	1.258	0.211
marginal r^2=0.348	20 °C Treatment	115	-0.121	0.174	-0.692	0.490
	Mass at Metamorphosis	104	1.653	0.263	6.273	**<0.0001**
	Terrarium Temperature	117	-0.337	0.174	-1.932	0.056
	Random effect (σ^2 = 0.13, τ_{00} = 0.02, ICC = 0.11, N = 18)					
Total Length at 90 days Post-Metamorphosis						
N=123	Intercept	115	101.462	63.569	1.596	0.113
conditional r^2=0.415	10 °C Treatment	114	3.379	3.003	1.125	0.263
marginal r^2=0.304	20 °C Treatment	112	-2.334	3.196	-0.730	0.467
	Total Length at Metamorphosis	109	0.919	0.152	6.049	**<0.0001**
	Terrarium Temperature	114	-4.115	3.227	-1.275	0.205
	Random effect (σ^2 = 42.02, τ_{00} = 7.94, ICC = 0.16, N = 18)					
Tail Length at 90 days Post-Metamorphosis						
N=123	Intercept	115	52.415	42.316	1.239	0.22
conditional r^2=0.330	10 °C Treatment	114	1.843	1.994	0.924	0.36
marginal r^2=0.221	20 °C Treatment	113	-1.152	2.125	-0.542	0.59
	Tail Length at Metamorphosis	113	0.840	0.173	4.854	**<0.0001**
	Terrarium Temperature	115	-2.010	2.157	-0.932	0.35
	Random effect (σ^2 = 18.35, τ_{00} = 2.97, ICC = 0.14, N = 18)					
Head-trunk Length at 90 days Post-Metamorphosis						
N=123	Intercept	117	45.859	27.130	1.690	0.093
conditional r^2=0.444	10 °C Treatment	116	1.664	1.285	1.295	0.198
marginal r^2=0.355	20 °C Treatment	114	-0.961	1.362	-0.706	0.48
	HTL at Metamorphosis	102	0.870	0.128	6.804	**<0.0001**
	Terrarium Temperature	116	-1.797	1.362	-1.319	0.19
	Random effect (σ^2 = 7.77, τ_{00} = 1.26, ICC = 0.14, N = 18)					

APPENDIX B: FIGURES

Figure B.1

Intermittent streams used by Ambystoma barbouri for breeding

Note. (a) Cedar glades and (b) forested karst systems were considered undisturbed sites and (c) and (d) show examples of sites disturbed by land development. Photography by Julia Thulander (a, c, d) and Joshua Hall (b).

Figure B.2.

Ambystoma barbouri nest and adult during the breeding season

Note. (a) Eggs of *A. barbouri* on the underside of a rock in an intermittent stream. (b) Adult *A. barbouri* on the stream bottom. Photography by Julia Thulander.

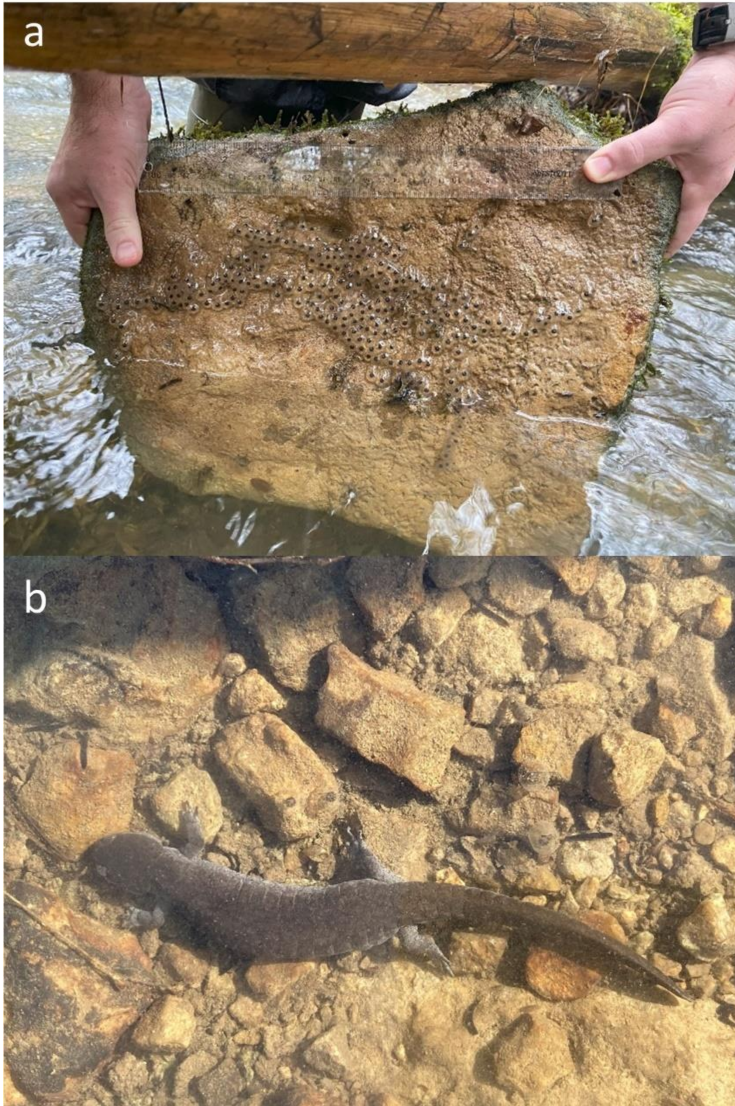


Figure B.3

Known range of Ambystoma barbouri by county

Note. Blue counties have a record on the Global Biodiversity Information Facility (GBIF). Counties with hatch marks are the metropolitan areas of Nashville, TN; Lexington, KY, and Cincinnati, OH. Red circles are egg collection sites. GBIF data were downloaded on 3/7/2024.

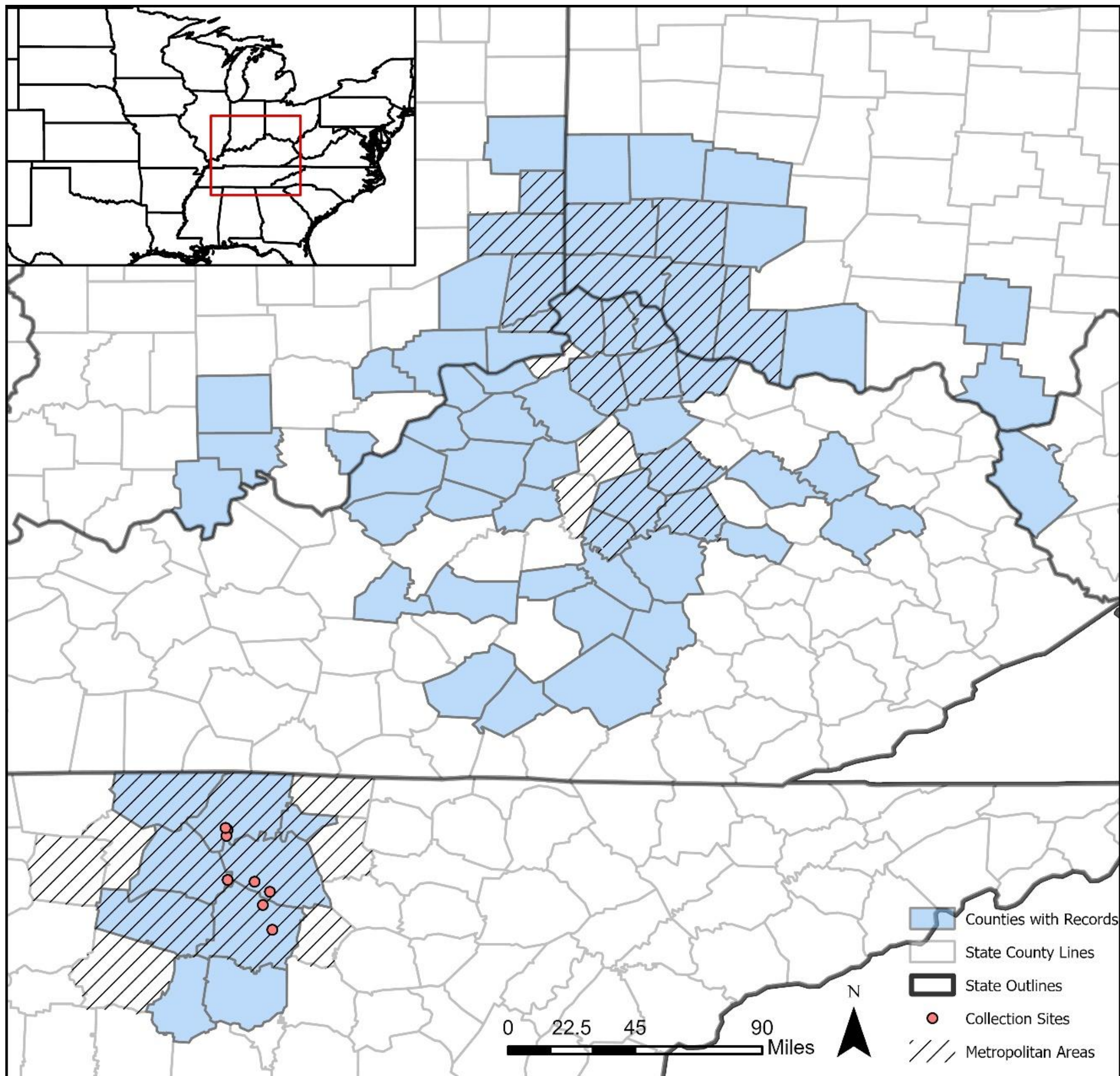


Figure B.4

Development of Ambystoma barbouri

Note. (a) Embryo at Harrison stage 6, magnification 40x (b) embryo at stage 36 (c) hatchling at stage 43 (d) 60-day old larvae, stage 18 (e) metamorph (f) juvenile at two months post-metamorphosis. Photography by Julia Thulander.

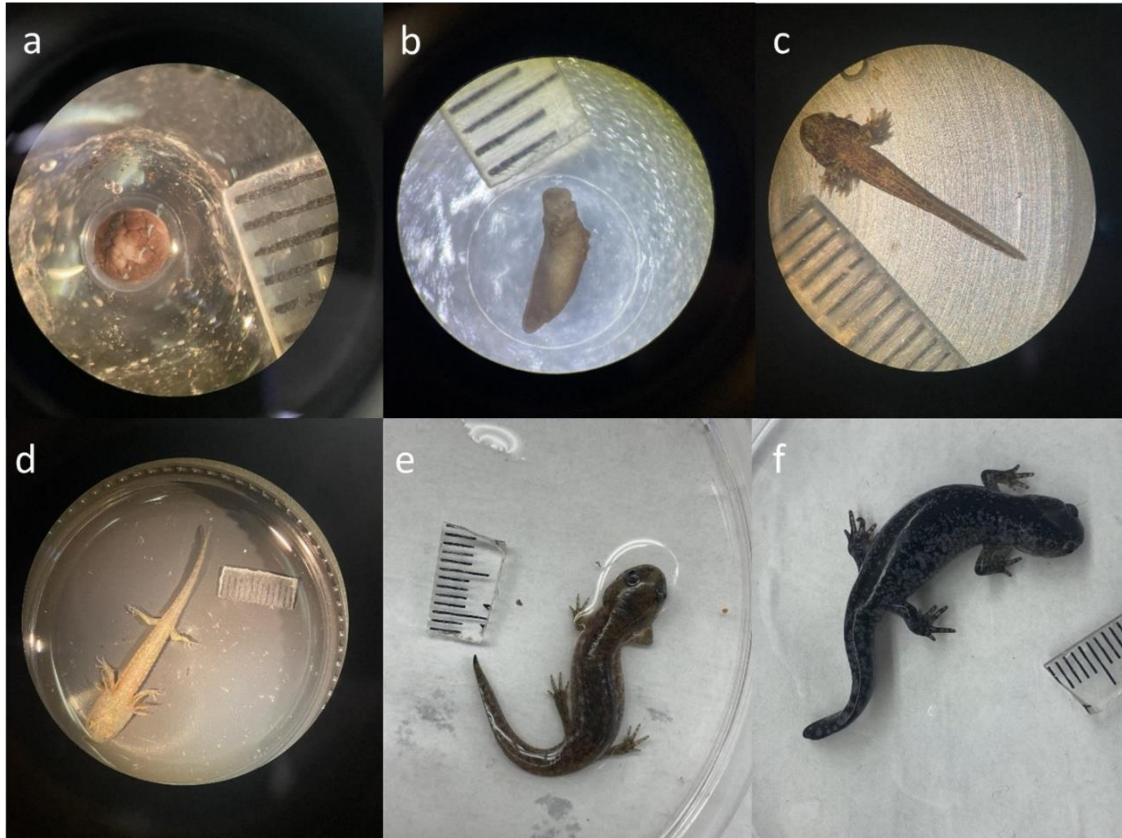


Figure B.5

Enclosures for Ambystoma barbouri larvae and juveniles

Note. Housing units for (a) larvae, and (b, c) juveniles, in the laboratory. Photography by Julia Thulander.

Figure B.6

Measurement of egg size and body size of Ambystoma barbouri using ImageJ

Note. (a) Egg size was measured at the widest point of the embryo. Body size was measured as total body length of (b) hatchlings, (c) metamorphs, and (d) juveniles. For metamorphs and juveniles, a line was drawn between the anterior attachment of hind limbs (yellow line) and a measurement was taken of head-trunk length (red line) and tail length (pink line). Photography by Julia Thulander.

Figure B.7

Ambystoma barbouri embryonic field temperatures

Note. (a) Red and blue lines represent mean hourly water temperatures throughout the embryonic period at sites that were or were not disturbed, respectively; grey circles are raw data, and bars represent standard deviation. (b) Maximum and (c) minimum daily water temperature throughout the embryonic period by site for disturbed (red) and undisturbed (blue) sites. Shaded area denotes temperatures greater than the estimated optimal thermal range (17 °C; see main text); dashed line at 10 °C denotes optimal temperature based on laboratory treatments; dotted line denotes freezing point of water.

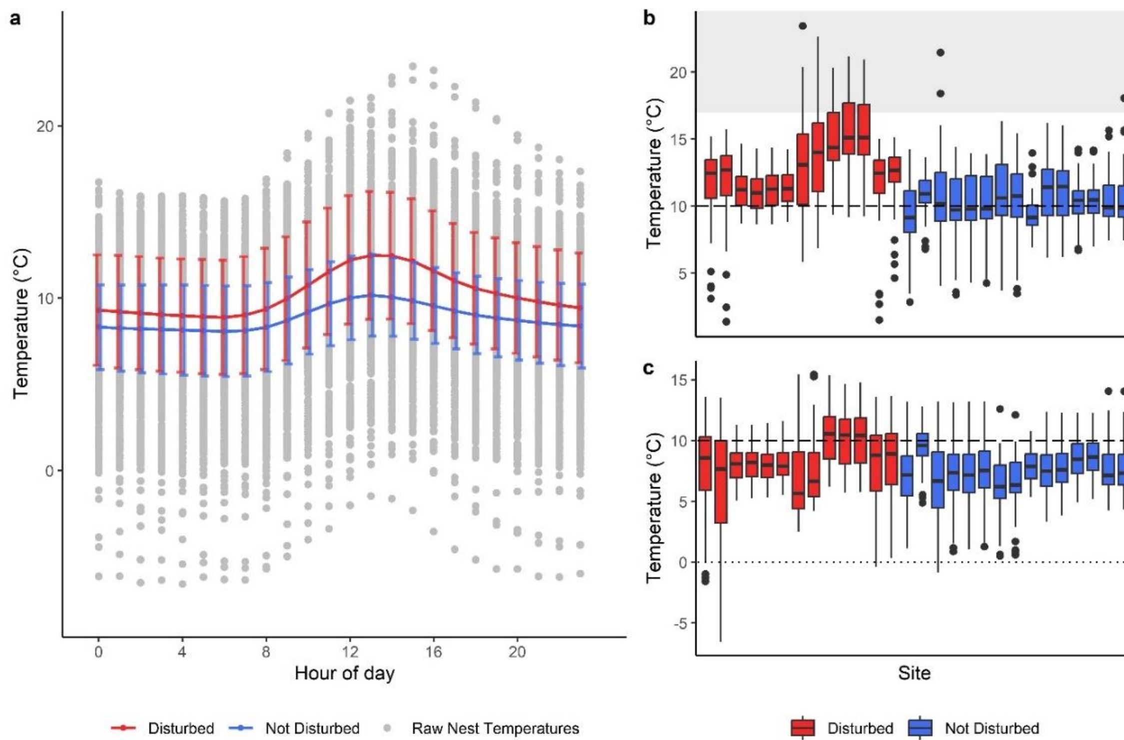


Figure B.8

Ambystoma barbouri larval field water temperatures

Note. (a) Red and blue lines represent average hourly water temperature at sites that had high or low levels of disturbance, respectively; grey circles are raw data and bars represent standard deviation. (b) Maximum and (c) minimum daily water temperature throughout the larval period by site for disturbed (red) and undisturbed (blue) sites.

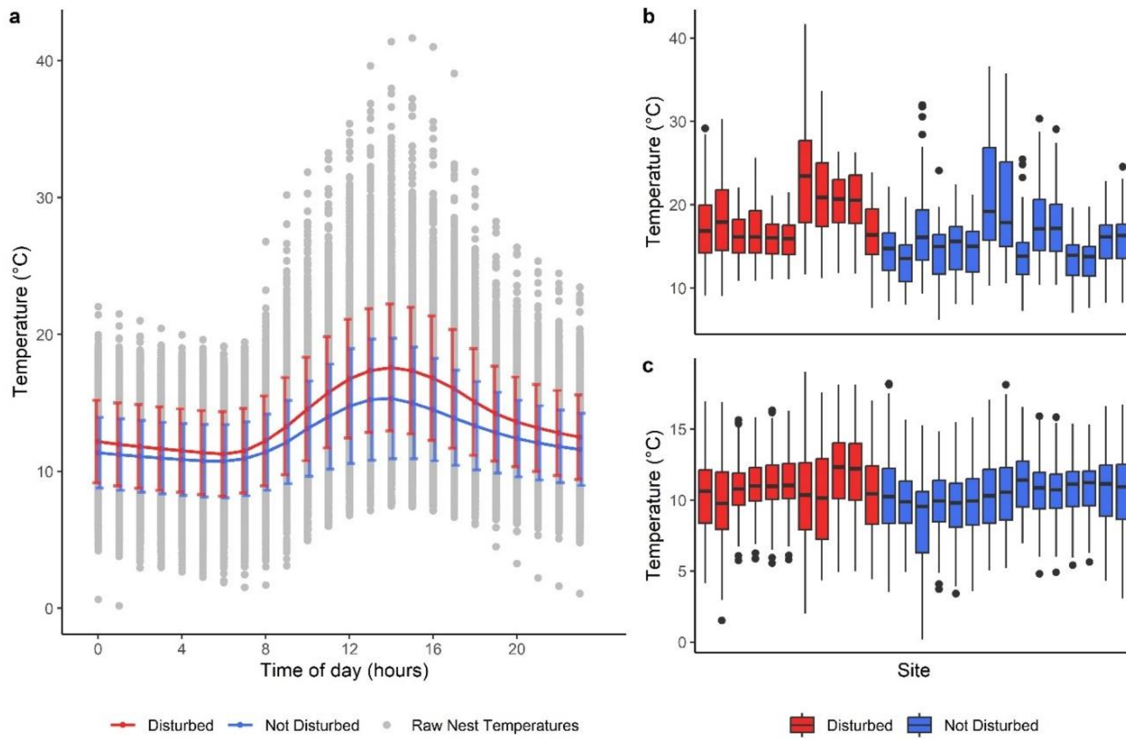


Figure B.9

Egg survival of Ambystoma barbouri across incubation temperature

Note. Black circles denote raw data of individual embryo survival and are jittered to avoid overplotting. The solid black line is the model estimate of survival across temperature. Gray shaded area denotes temperatures greater than the optimal thermal range (> 17 °C). Vertical black solid and gray dotted lines denote the embryo chronic heat tolerance (21.0 °C) and 95% confidence interval (20.4 °C, 21.6 °C), respectively.

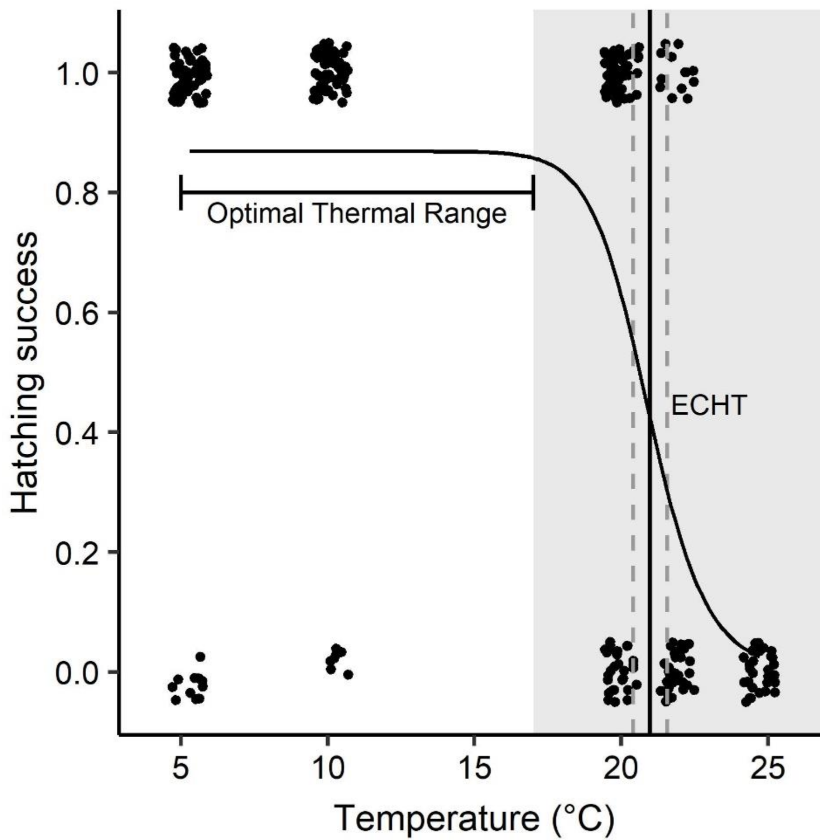


Figure B.10

Larval and juvenile survival of Ambystoma barbouri by egg incubation temperature

Note. (a) Percent survival of larvae to metamorphosis. (b) Percent survival of juveniles to 90 days post-metamorphosis. Letters denote statistical significance of pairwise comparisons after false discovery rate correction. No statistical test was conducted on juvenile survival due to low mortality.

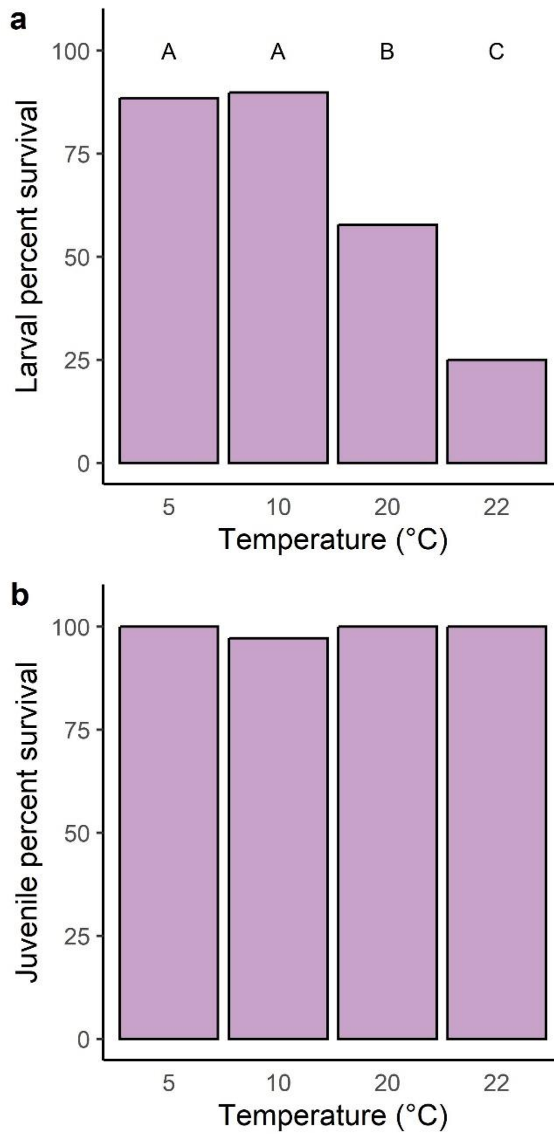


Figure B.11

Embryonic and hatchling phenotypes of Ambystoma barbouri across incubation temperature

Note. (a) Ecological developmental rate (percent development per day from collection to hatching), (b) Harrison stage at hatching, and (c) total length at hatching. Letters denote statistical significance of pairwise comparisons after false discovery rate correction.

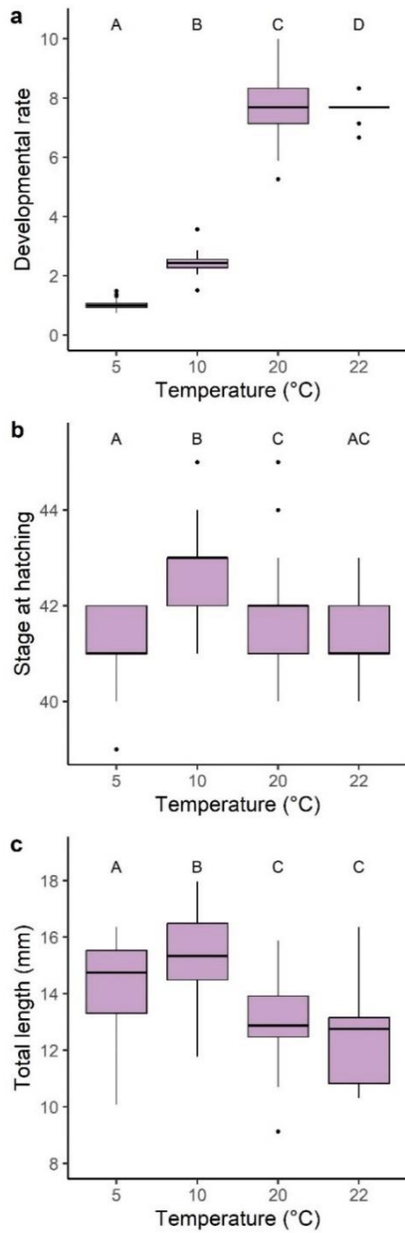


Figure B.12

Phenotypes at metamorphosis of Ambystoma barbouri across incubation temperature

Note. (a) Days to metamorphosis, (b) body mass, (c) total length, (d) head-trunk length (HTL), and (e) tail length. Letters denote statistical significance of pairwise comparisons after false discovery rate correction. Only 3 larvae from the 22 °C treatment survived to metamorphosis and was therefore excluded from these analyses.

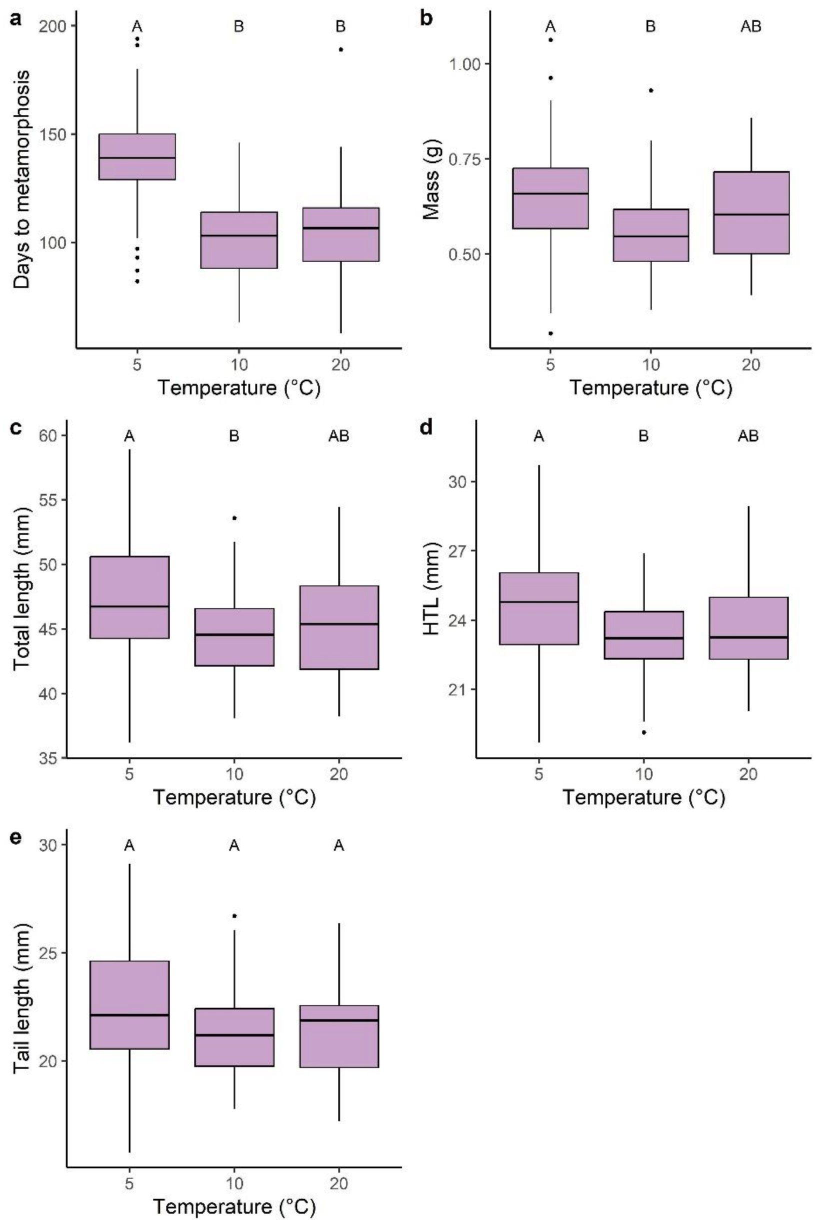
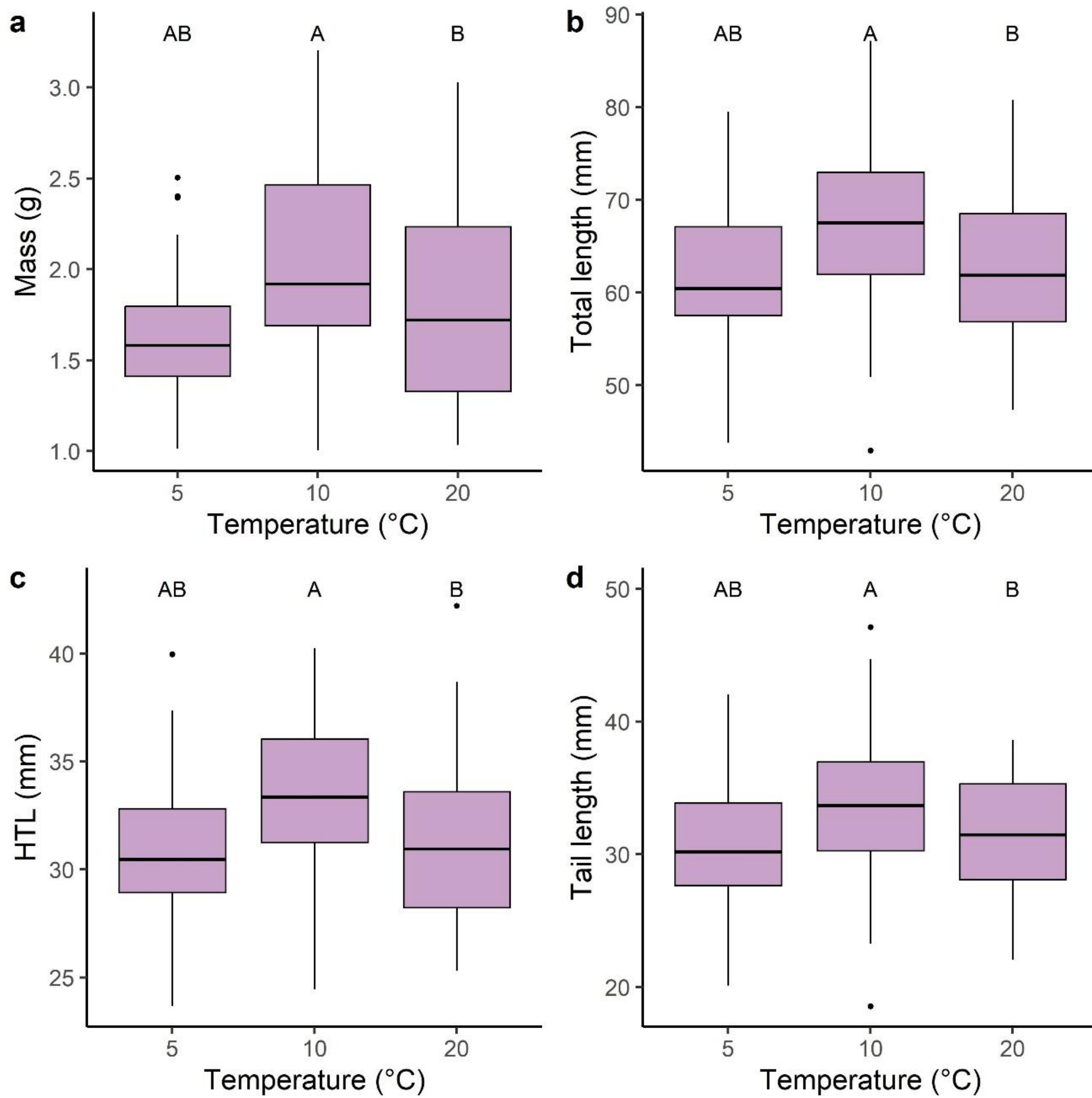


Figure B.13

Phenotypes at 90 days post-metamorphosis of Ambystoma barbouri across incubation temperature

Note. (a) Body mass, (b) total length, (c) head-trunk length (HTL) and (d) tail length. Letters denote statistical significance of pairwise comparisons after false discovery rate correction.



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VITA

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