Summer 6-23-2023

Variation in Salamander Life History and Community Composition Across an Urban Gradient in Atlanta, Georgia, USA

Leah Rittenburg

VARIATION IN SALAMANDER LIFE HISTORY AND COMMUNITY COMPOSITION ACROSS AN URBAN GRADIENT IN ATLANTA, GEORGIA, USA

By

Leah Tamsin Rittenburg

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Integrative Biology

Department of Ecology, Evolution, and Organismal Biology

Kennesaw State University

Major Professor: Dr. Todd W. Pierson

Table of Contents

DEDICATION

I would like to dedicate this research to my father, Richard Rittenburg. I doubt he could have predicted that his daughter would leave Florida to do a masters project wrestling salamanders thirty minutes from his hometown. I wish I could have shared this with him, but I will forever be grateful for the years we had. This research is for you, Dad.

ACKNOWLEDGEMENTS

I have many people to thank for helping me complete this master's research. The greatest part of my master's was the incredible minds, spirits, and hearts of the people I met. You picked me up when I stumbled and celebrated with me when I succeeded. For that, I owe you everything.

I would like to extend only the biggest, most sincere "thank you" to my advisor, Dr. Todd Pierson. I am thoroughly convinced you have more than 24 hours in a day and have lived multiple lifetimes. You are one of the most brilliant, well-rounded, and kind people I have ever met. Thank you for helping me think deeper, communicate clearer, and sing louder in the field truck (though I think that part was against your will). I cannot and probably will not ever truly comprehend just how helpful you have been.

I would also like to thank my committee members, Dr. Nicholas Green and Dr. Sarah Guindre-Parker for helping this research get to where it needed to be. Nick, we joke in the MSIB program that you are single-handedly holding us together (statistics-wise). But I don't believe that's a joke. Plus, you make a mean chili, and I don't think I will ever forget that. Sarah, thank you for somehow serving the roles of committee member, scientist, and new mother all at once.

To my friends who have stuck with me for over a decade (Irene, Jaiya, Sam, Nalani, Tabby, Jeff): I owe you everything. Thank you for your beautiful kindness. To my new friends here in Georgia (Mariana, Micah, Seth, Ali, Chase, Cooper): you reminded me just how wonderful it can be to be yourself. Thank you.

This research would not have been possible without all the people who joined me in the field. Thank you, Yatin Kalki, for being the best, completely-opposite-to-me-in-everyway labmate I could ask for. I can't wait to see a dramatic increase in the number of snake papers coming out

of India. Additionally, I had the pleasure and honor of working closely with three undergraduate researchers (Devan, Emma, and Karam). You amazed me every day with your tenacity, curiosity, and good nature. Thank you for making these two years wonderful. I am so proud of you all.

To my mom: I would turn this 20,000-word thesis into a 40,000-word thesis if I wrote everything you have given me, taught me, and done for me. Thank you for showing me what the world can be if you share kindness, humility, good humor, and a cup of Milo with others. You always say how proud you are of me; I am glad that I can be a daughter you are proud of. Thank you and I love you.

To Nana and Poppa: thank you for allowing baby-Leah to boss you around for years. I have no idea where all your patience came from, but I owe you so much for it. Thank you for helping to raise me. I will always be incredibly grateful to have been so close with you. xoxo

To my dad: you were a kind, silly, wonderful father who deserved to be here to see this. To you, I dedicate this research. I love you and I miss you.

Lastly, I would like to thank the institutions that allowed me to sample at their sites. They showed me kindness and excitement for my work at a very uncertain time in my research. Thank you to the City of Atlanta, DeKalb County, Fernbank Museum, Emory University, and Druid Hills Golf Club. All salamanders were handled under KSU IACUC permit ACUP21-001.

ABSTRACT

Urbanization threatens to alter stream systems in watersheds in the eastern United States. These highly disturbed systems may result in many local extirpations of sensitive salamander species. One group of organisms that often persists in urban streams is the *Eurycea bislineata* (Two-lined Salamander) species complex. Many aspects of Two-lined Salamander life history are linked to environmental factors, particularly stream temperature, prey abundance, and stream hydrology. Urbanization threatens to alter these environmental factors, potentially influencing Two-lined Salamander life history. At 14 sites spanning an urban gradient in Atlanta, GA, I investigated the variation in life history and phenology in the Southern Two-lined Salamander, *E. cirrigera*. I aimed to investigate 1) the anthropogenic and natural factors influencing thermal profiles in urban streams; 2) the environmental drivers of larval period and size; 3) the relationship between clutch size, female size, and degree of urbanization; and 4) changes in plethodontid salamander species richness across an urban gradient, using environmental DNA. I found that 1) drainage basin area was a better predictor of stream temperature than impervious surface cover; 2) cooler August temperatures predicted a longer larval period and smaller larvae; 3) higher impervious surface cover predicted larger larvae and larger adult females; 4) larger females were associated with significantly larger clutches; and 5) plethodontid salamander species richness decreased with increasing impervious surface cover. The results of this research help to assess the ecological implications of urban development by expanding our understanding of the mechanisms underlying variation in *E. cirrigera* life history and the impacts of urbanization on salamander species richness.

BACKGROUND

Urban sprawl in the southeast United States is projected to more than double by 2060 (Terando et al., 2014), jeopardizing many terrestrial and aquatic ecosystems. In the Piedmont ecoregion of the United States, 62% of stream kilometers are expected to have >5% urbanized watersheds by 2060 while 25% of stream kilometers are expected to have >50% urbanized watersheds by 2060 (Van Metre et al., 2019). Stream health is at risk in developing areas, as urban factors upstream can have far-reaching effects within the stream. Stream degradation often drives declines in the biodiversity and abundance of native flora and fauna (McKinney & Lockwood, 1999; McKinney, 2002; Shochat et al., 2006). Increasing human land use has been tied to many detrimental effects to wildlife, including increased numbers and prevalence of emerging infectious diseases (Daszak et al., 2000), reduced gene flow due to physical man-made barriers (Fusco et al., 2021), and increased exposure to toxicants (Murray et al., 2019). Particularly at risk are species with limited dispersal ability, high habitat specificity, specialist diets, and higher sensitivity to environmental changes (McKinney, 2002; Scheffers & Paszkowski, 2011).

One important taxon that is threatened by stream degradation is salamanders. In the eastern United States, salamanders represent an important part of both terrestrial and aquatic ecosystems. Salamanders are important to the food chains of this region, acting as both predator and prey, and compose a significant proportion of the vertebrate biomass. For instance, salamander biomass in the Hubbard Brook Experimental Forest, New Hampshire was estimated to be double that of birds (Burton & Likens, 1975). Within the Appalachian Mountains, stream salamander biomass is three to six times that of headwater fish and is predicted to be greater than the biomass of both birds and small mammals (Peterman et al., 2008). The most diverse

group of salamanders in the southeast United States is the family Plethodontidae (Harding & Mifsud, 2017; Kozak, 2017). Plethodontids are lungless, respire cutaneously, and rely on moist habitats such as stream sides (Marshall & Camp, 2006; Harding & Mifsud, 2017). Because some plethodontids utilize both aquatic and terrestrial habitats in their lifetime, urban impacts may be especially profound.

Within the southeast United States, often only a few species from Plethodontidae remain in urban streams, particularly those from the *Eurycea bislineata* (Two-lined Salamander) species complex (Barrett & Guyer, 2008). This species complex is composed of six described species: the Northern Two-lined Salamander (*E. bislineata*; Green, 1818), the Southern Two-lined Salamander (*E. cirrigera*; Dunn, 1920), the Blue Ridge Two-lined Salamander (*E. wilderae*; Dunn, 1920), the Brown-backed Salamander (*E. aquatica*; Rose & Bush, 1963), the Junaluska Salamander (*E. junaluska*; Sever et al., 1976), and the Carolina Sandhills Salamander (*E. arenicola*; Stuart et al., 2020). Two-lined Salamanders inhabit the eastern United States and parts of eastern Canada, with some species having overlapping distributions (Kozak et al., 2006).

Despite the persistence of Two-lined Salamanders in urban streams, populations experience significant declines in urban waters (Willson & Dorcas, 2003; Miller et al., 2007; Price et al., 2011). All members of this species complex have a biphasic lifestyle; salamanders begin as eggs, then hatch into an aquatic larval stage, and finally undergo metamorphosis to become semi-aquatic adults (Bonett et al., 2014). Thus, larvae may be particularly susceptible to degraded stream habitats and adults may be impacted by both terrestrial and aquatic changes. Price et al. (2011) found that four years after urbanization, *E. cirrigera* larval abundance decreased by 60% and adult abundance decreased by 98% compared to pre-development abundance estimates.

Because the southeast United States is projected to be especially impacted by urban sprawl (Terando et al., 2014; Van Metre et al., 2019), Two-lined Salamander populations may experience marked declines and alterations to their life history in the near future.

Members of the Two-lined Salamander species complex are also great model organisms for studying life history in plethodontids due to the variation in life history traits across the complex's geographic range. Two-lined Salamanders display discrete variation in the length of the larval period—the time between hatching from an egg and undergoing metamorphosis. The larval period can range from one to three years, with metamorphosis occurring in warmer months (Duellman & Wood, 1954; Bruce, 1982a; Voss, 1993; Pierson & Miele, 2019). Two-lined Salamanders also display spatial variation in larval growth rates, size at metamorphosis, and oviposition timing (Bruce, 1982b; Murphy et al., 2016; Pierson et al., 2023). Males of the Two-lined Salamander species complex also display variation in courtship behavior and secondary sexual characters, with some populations including males of two discrete alternative reproductive tactics: 1) "searching" males with cirri and mental glands, thought to court on land, and 2) "guarding" males with enlarged jaw musculature, lacking cirri and mental glands, and thought to court in water (Sever, 1979; Pierson et al., 2019; Pierson et al., 2022).

Studying Two-lined Salamanders in urban environments is not only necessary in understanding threats to the species complex but offers a unique way to better understand the drivers of variation in life history. Due to factors associated with human land use, urban streams can display large differences in abiotic and biotic stream parameters over a relatively small geographic range. By studying Two-lined Salamanders in geographically close urban streams,

researchers can better understand environmental drivers of life history without sampling across populations, water basins, elevations, or large geographic distances.

In this study, I investigate how the life history of *E. cirrigera* (Southern Two-lined Salamander) and salamander community composition vary across an urban gradient in Atlanta, GA, USA. I aim to investigate 1) the anthropogenic and natural factors influencing thermal profiles in urban streams; 2) the environmental drivers of larval period and size; 3) the relationship between clutch size, female size, and degree of urbanization; and 4) changes in plethodontid salamander species richness across an urban gradient, using environmental DNA.

CHAPTER 1: ANTHROPOGENIC AND NATURAL FACTORS INFLUENCING THERMAL PROFILES IN URBAN STREAMS

1.1 BACKGROUND

The combination of changes associated with anthropogenic drivers of stream degradation has been named the "urban stream syndrome" (Meyer et al., 2005; Walsh et al., 2005). Affected streams tend to have more frequent and severe floods, changes in stream thermal profiles, an increase in nutrients and contaminants, and altered biological communities (Paul & Meyer, 2001; Walsh et al., 2005). Particularly relevant to stream salamander ecology are changes to the hydrological and thermal profiles of the stream.

1.1.a Hydrology

Urban streams undergo many notable changes to their hydrology. Headwater streams become less abundant as they are buried and converted into urban waterways such as gutters, storm drains, and culverts (Dunne & Leopold, 1978; Meyer & Wallace, 2001; Elmore & Kaushal, 2008). These alterations lead not only to a loss of small streams in urban systems, but also to increased impervious surface cover (ISC) surrounding waterways (Elmore & Kaushal, 2008). The remaining streams receive higher volumes of surface runoff from the increased ISC. Compared to runoff in forested catchments, runoff is twice the volume in disturbed catchments with 10 – 20% ISC, three times the volume with 35 – 50% ISC, and more than five times the volume with 75 – 100% ISC (Arnold & Gibbons, 1996). The increase in runoff associated with urban areas leads to

significant increases in the volume and frequency of flood events (Espey et al., 1966; Leopold, 1973; Booth & Jackson, 1997; Barrett et al., 2010a).

1.1.b Temperature

Stream temperature has important biological ramifications, including impacts to stream community composition, animal life histories (Anderson & Wallace, 1995; Beachy, 2018), leaf decomposition (Webster & Benfield, 1986; Boyero et al., 2014), and nutrient cycling (Demars et al., 2011). Thus, changes in stream temperatures can have cascading effects on the ecosystem. Natural variation in stream temperature is largely driven by stream structure (e.g., width, water volume, stream order, and distance from headwaters) and the environment (e.g., air temperature, canopy cover, and streambed composition) (reviewed in Leach et al., 2023). However, urban development can greatly impact stream temperatures. Changes to human energy-use, decreases in riparian vegetation, and runoff from heat-absorbing impervious surfaces can lead to higher stream temperatures in urban landscapes (Kinouchi, 2007; Nelson & Palmer, 2007; Rice et al., 2011). Runoff that reaches urban streams is often higher in volume due to increased impervious surface cover (i.e., water cannot permeate into the ground before reaching the stream) and drainage of city stormwater into streams (Espey et al., 1966; Arnold & Gibbons, 1996). Increased surface temperatures of impervious surfaces, relative to natural surfaces, warm runoff before it enters the stream (Thompson et al., 2008; Hester & Bauman, 2013). Surges in summer water temperatures due to thunderstorms in the Piedmont region of Maryland averaged +3.5°C in urban areas, with some surges increasing stream temperatures >7°C, which usually receded after 3 hours (Nelson & Palmer, 2007). With higher average

temperatures and more irregular thermal regimes, urban streams may experience alterations to temperature-dependent processes while also proving inhospitable to sensitive species.

Here, I investigate whether the effects of urbanization and drainage basin size on temperatures at my streams are similar to those reported in the literature. With accurate temperature data and an understanding of thermal regimes across an urban gradient I can better assess the environmental drivers of *Eurycea cirrigera* life history in urban streams.

1.2 METHODS

1.2.a Sample sites

All sample sites were within the Peachtree Creek watershed in Fulton County, GA and DeKalb County, GA. In 2011, the Peachtree Creek watershed covered 240 km^2 and was 83% developed with 32% ISC (U.S. Geological Survey, 2016). The watershed contains many first- and second-order streams running through easy-to-access public parks, making it an ideal location to study urban impacts on stream ecosystems. Additionally, this watershed is home to just one species in the *Eurycea bislineata* species complex (i.e., *E. cirrigera*), removing the challenge of identifying larvae in other stream systems where two or more species are sympatric.

To best study the impact of urbanization on streams and salamanders, I selected sample sites that 1) varied in stream order, amount of surrounding ISC, bank erosion, and stream bottom substrate composition and 2) contained *E. cirrigera* larvae. At each site, I established a 10 x 1 m transect that contained at least one riffle and followed the bank of the stream.

1.2.b Impervious surface cover and drainage basin area

To calculate the area (km^2) and the percent ISC of the surrounding drainage basin of each site, I used StreamStats v4.8.1 (U.S. Geological Survey, 2016). I selected a point at the downstream end of each 10 x 1 m transect to determine drainage basin area and percent ISC based on the NLCD 2011 impervious dataset (Homer et al., 2015).

1.2.c Stream temperature

At each site, I deployed one HOBO Pendant Temperature/Light 64K Data Logger (Onset; MA, USA). The logger was completely submerged and collected temperature and light intensity data every two hours from November 2021 to February 2023. For statistical analyses, I used 2022 temperatures from January and August, the coldest and warmest months in Atlanta, GA, respectively (see **1.2.d**).

For sites with missing data from January 2022 (due to lost temperature loggers), I interpolated data using data collected in January 2023. To do this, I retrieved temperature data from USGS stream gages using the package "dataRetrieval" v2.7.12 (De Cicco et al., 2022) in R v4.2.3 (R Core Team, 2023). To be included, a gage must have satisfied all of the following criteria: 1) had temperature data from 01 January 2022 to 31 January 2023; 2) was in the greater metro Atlanta area (using a rectangular boundary with diagonal corners of WGS 84 [33.63, −84.50] and [33.91, −84.24]); 3) was in the Upper Chattahoochee water basin; and 4) had a drainage basin area of less than 50 km^2. I compiled these data from USGS gages with data from my sites that had complete HOBO logger temperature data for both January 2022 and January 2023.

Using the package "caret" v6.0–94 (Kuhn, 2008) in R v.4.2.3 (R Core Team, 2023), I fit a linear regression model of mean January 2022 temperature as a function of mean January 2023 temperature using cross validation with 15 resample iterations. From this model, I interpolated mean January 2022 temperatures for all sites with missing data. I repeated this process to interpolate the mean daily range in temperatures for January 2022 for all sites with missing data.

1.2.d Statistical analyses

For each site, I calculated the mean temperature in January 2022 and August 2022. I also calculated mean January and August 2022 temperatures from the retrieved USGS gage data (see **1.2.c**) and compared these data visually with the mean temperatures at my sites to confirm that temperatures at my sites reflect patterns across urban streams in greater metro Atlanta.

I fit a linear regression model of mean temperature at my sites as a function of the interaction of ISC and month (January 2022 or August 2022) and the interaction of the natural logarithm (hereafter simply "log") of the drainage basin area and month using the function "lm()" in R v4.3.2 (R Core Team, 2023). I log-transformed drainage basin area to guard against high leverage of one of my sites that had a drainage basin area an order of magnitude greater than the second-largest drainage basin area. Drainage basin area was roughly log-normal.

In addition to mean temperature, the minimum and maximum temperatures at a site can impact organismal physiology and occupancy (Hutchison, 1961; Strickland et al., 2016). To account for this, I calculated the range of temperature for each day in January and August 2022. I then calculated the mean range in temperatures across all days in January and all days in August (hereafter referred to as "mean daily temperature range"). I fit a linear regression of the log of

the mean daily temperature range as a function of the interaction of ISC and month and the interaction of the log of the drainage basin area and month.

My models are based on the assumption that the most influential parameters affecting stream temperature at my sites are related to stream size and structure (drainage basin area used as a single metric to roughly capture these parameters) and runoff (ISC used to roughly capture the volume and temperature of runoff into streams) (Leach et al., 2023; see **1.1.b** for more details). This assumption is based on the relatively small sizes of my streams and is a necessary limitation due to my small sample size. In larger streams, additional energy exchange processes (e.g., buffering of temperatures with increased stream depth; direct solar radiation due to open canopy cover) would need to be considered.

To test for normality of the model residuals, I ran Shapiro-Wilk tests using the function "shapiro.test()" and inspected Q–Q plots in R. I tested for spatial autocorrelation in the model residuals using the "Moran.I()" function from the package "ape" v5.7–1 (Paradis & Schliep, 2019). I used an inverse distance matrix of latitude and longitude of each site to calculate a scaled Moran's I statistic for autocorrelation in the model residuals.

I set the significance threshold to $\alpha = 0.05$.

1.3 RESULTS

I surveyed streams at 14 sites in urban Atlanta: 9 public parks, 2 privately-owned forests, and 3 sites on 2 golf courses (Fig. 1.1). One golf course is hereafter referred to as "Golf Course A" for anonymity. Across my sites, percent impervious surface cover ranged from 2.9% – 38.6%

(mean = 21.87% ± 11.86 SD; Table S.1 in **Supplementary Material**). Drainage basin area ranged from 0.09 – 50.50 km^2 (mean = 4.78 ± 13.23 km^2; Table S.1 in **Supplementary Material**).

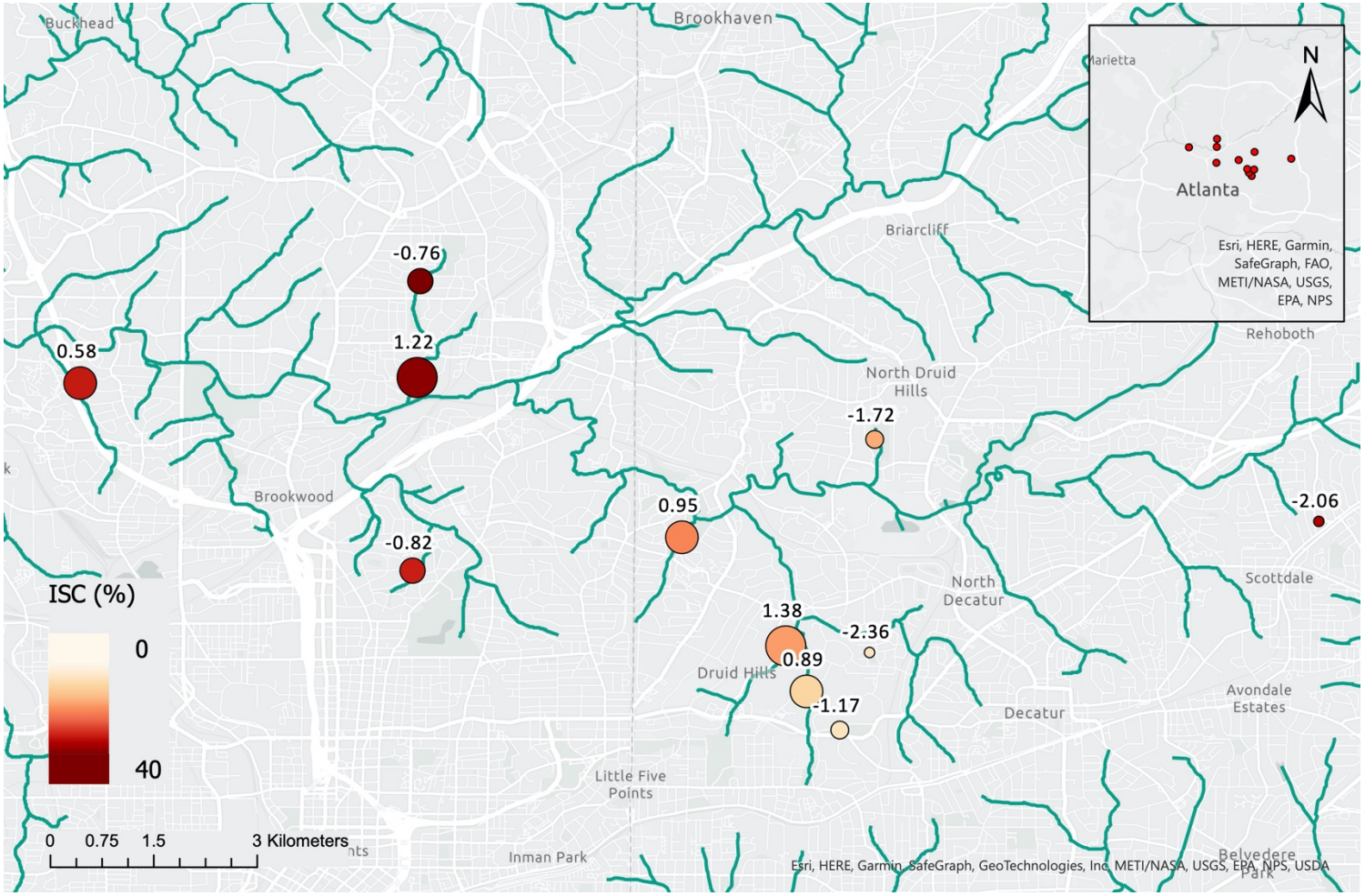


Fig.1.1: My fourteen sample sites in the Peachtree Creek watershed of urban Atlanta, GA, USA. Size of point scaled to the natural log of the drainage basin area (indicated above each point). Color of each point represents the percent impervious surface cover (ISC) of the drainage basin. Blue lines represent streams and are not scaled to stream width. Three sites ("Forest A," "Forest B," and "Golf Course A") not shown to preserve anonymity.

The cross-validated linear model used to interpolate mean January 2022 temperature using mean January 2023 temperature data was $y = 1.337(x) - 5.524$ (RMSE = 0.394, $R^2 = 0.968 \pm 0.122$). Interpolated mean January 2022 temperatures for the two sites missing data were 9.053°C (Beaverbrook Park) and 9.287°C (Sunnybrook Park). The cross-validated linear model used to interpolate mean daily temperature range in January 2022 using mean daily temperature range in January 2023 was $y = 0.995(x) + 0.063$ (RMSE = 0.084, $R^2 = 0.935 \pm 0.176$). Interpolated mean daily temperature ranges in January 2022 for the two sites missing data were 2.366°C (Beaverbrook Park) and 2.250°C (Sunnybrook Park).

The mean temperature across sites in January 2022 ranged 7.99 – 12.17°C (mean = 9.45°C ± 1.24 SD; Fig. S.2 and Table S.2 in **Supplementary Material**). The mean temperature across sites in August 2022 ranged 20.39 – 24.69°C (mean = 22.78°C ± 1.50 SD; Fig. S.3 and Table S.2 in **Supplementary Material**). The relationship between mean temperature and drainage basin area at my 14 sites reflect patterns across urban streams in greater metro Atlanta in August 2022 and roughly so in January 2022 (Fig. S.1 in **Supplementary Material**).

In the model of mean stream temperature, I found a significant interaction between month and drainage basin area (Table 1.1, Fig. 1.2). Drainage basin area (log-transformed) had a significant, negative effect on mean temperature in January (slope = −0.518, $t_{22} = -4.49$, $p < 0.001$), but a significant, positive effect on mean temperature in August (slope = 0.560, $t_{22} = 3.30$, $p = 0.003$). ISC had an insignificant, positive effect on mean stream temperature in January (slope = 0.018, $t_{22} = -0.40$, $p = 0.693$) and in August (slope = 0.032, $t_{22} = 1.30$, $p = 0.206$). This model explained much of the variation in mean stream temperatures (adjusted $R^2 = 0.98$).

The distributions of model residuals did not show evidence of non-normality for mean January temperatures (W = 0.96, p = 0.72) nor for mean August temperatures (W = 0.88, p = 0.07). There was no significant spatial autocorrelation in the residuals of the linear regression predicting mean January 2022 temperature (p = 0.99). The linear regression predicting August 2022 mean temperature did show weak spatial autocorrelation in its residuals (p = 0.03), but this pattern was not concerning upon visual inspection of the spatial distribution of the residuals.

The mean daily temperature range across sites in January 2022 ranged 1.38 – 2.92°C (mean = 2.32°C ± 0.49 SD; Table S.2 in **Supplementary Material**). The mean daily temperature range across sites in August 2022 ranged 0.55 – 4.78°C (mean = 1.63°C ± 1.04 SD; Table S.2 in **Supplementary Material**). There were no significant additive or interactive terms in the linear model of the log of mean daily temperature range (Table 1.2, Fig. 1.3). The model explained a small amount of variance in mean range in temperature (adjusted R^2 = 0.11).

The distributions of model residuals did not show evidence of non-normality for the log of mean daily temperature range in January (W = 0.89, p = 0.08) nor in August (W = 0.95, p = 0.62). There was no significant spatial autocorrelation in the model residuals for January 2022 (p = 0.91) or August (p = 0.82).

Table 1.1: Model output for the linear regression of mean stream temperature as a function of the interaction of the percent impervious surface cover of the drainage basin (ISC) and month (August or January 2022) and the interaction of the log of the drainage basin area (km^2) and month. Asterisk indicates significance. Estimates are unstandardized.

Parameter	Estimate	Std. Error	t_{22}	p	
(Intercept)	22.173	0.615	36.025	< 2e−16	*
ISC	0.032	0.025	1.303	0.206	
Log(drainage area)	0.560	0.170	3.295	0.003	*
Month [January]	−13.210	0.870	−15.177	< 0.001	*
ISC * month [January]	-0.014	0.035	-0.399	0.693	
Log(drainage area) * month [January]	−1.078	0.240	−4.486	< 0.001	*

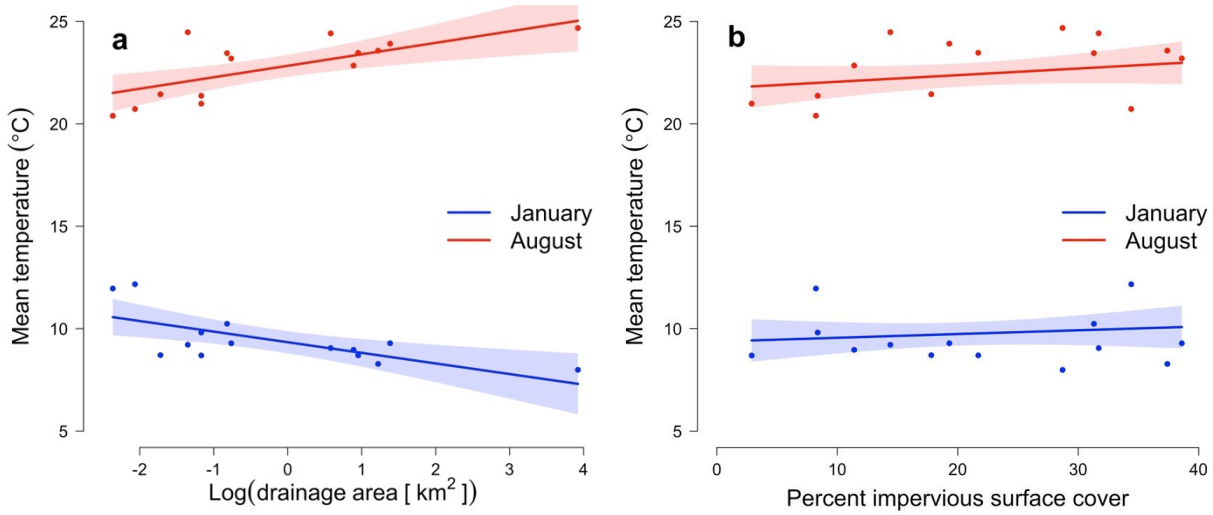


Fig 1.2: Mean stream temperature (°C) at 14 streams in urban Atlanta as a function of a) the log of the drainage basin area (km^2) and b) the percent impervious surface cover of the drainage basin. Points represent the mean temperature per site for January 2022 (blue) and August 2022 (red). Shaded regions represent 95% confidence intervals.

Table 1.2: Model output for the linear regression of the log of mean daily temperature range as a function of the interaction of the percent impervious surface cover of the drainage basin (ISC) and month (August or January 2022) and the interaction of the log of the drainage basin area (km^2) and month. Asterisk indicates significance. Estimates are unstandardized.

Parameter	Estimate	Std. Error	t_{22}	p	
(Intercept)	0.274	0.265	1.031	0.314	
ISC	0.003	0.011	0.295	0.771	
Log(drainage area)	−0.026	0.073	−0.360	0.722	
Month [January]	0.536	0.375	1.427	0.168	
ISC * month [January]	−0.003	0.015	−0.189	0.852	
Log(drainage area) * month [January]	0.002	0.104	0.021	0.984	

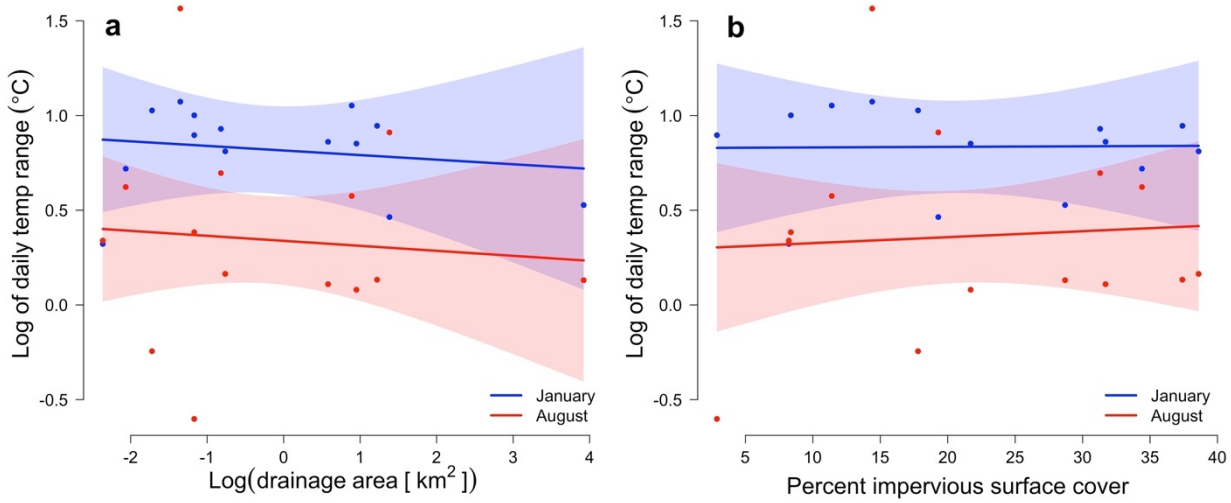


Fig 1.3: Log-transformed mean daily temperature range (°C) at 14 streams in urban Atlanta as a function of a) the log of the drainage basin area (km^2) and b) the percent impervious surface cover of the drainage basin. Points represent the mean temperature per site for January 2022 (blue) and August 2022 (red). Shaded regions represent 95% confidence intervals.

1.4 DISCUSSION

Temperature plays an important role in many processes relevant to salamander life history, including direct effects such as influencing the timing of metamorphosis (Voss, 1993; Beachy, 2018) and indirect effects such as influencing the chemical and nutrient cycles of the stream (Latimer & Quinn, 1998; USGS, 1999). Because stream thermal profiles impact salamander health and life history and can change with increasing urbanization (Kinouchi, 2007; Nelson & Palmer, 2007; Rice et al., 2011), I investigated the anthropogenic and natural factors influencing thermal profiles in urban streams.

At 14 streams in urban Atlanta, there was a positive but insignificant effect of ISC on mean stream temperature in August 2022. This trend is well-documented across different cities and scales (e.g., Galli, 1990; Kim, 2007; Rice et al., 2011). At my sites, a 1% increase in ISC led to a 0.03°C increase in mean temperature in August. This relationship is smaller than that estimated by Rice et al. (2011; 0.3°C), Galli (1990; 0.09°C), and Kim (2007; 0.07°C). There also was not a significant relationship between mean daily temperature range and ISC at my sites. This was unexpected, as urban streams often experience dramatic temperature surges during summer storms due to warm runoff from ISC (Nelson & Palmer, 2007; Hester & Bauman, 2013). The effect of impervious surface cover on stream temperature is mostly from warm runoff entering the stream. Runoff temperature is largely dependent on the temperature of the impervious surface before precipitation (Thompson et al., 2008). Many of my sites are in drainage basins with relatively high tree cover, potentially helping to keep runoff temperatures low. Additionally, my 14 sites spanned two counties that have historically differed in meeting runoff regulation goals

(Butler et al., 2018; DeKalb County, 2022) and may differ in how stormwater is managed. This may obscure the relationship between ISC and stream temperature across my sites.

In January 2022, mean stream temperature was negatively predicted by drainage basin area; larger streams were significantly colder than smaller streams. In August 2022, this relationship was inverted; larger streams were significantly warmer than smaller streams. Increasing seasonal variation in temperature further downstream from headwaters is a common pattern across studies (e.g., Galli, 1990; Webb & Zhang, 1999; Johnson et al., 2014). Headwaters arising from groundwater are relatively thermally buffered compared to water further downstream (Galli, 1990). As water moves away from the thermal stability of its groundwater source, it is more exposed to surface-level energy exchange (e.g., short-wave radiation from the sun, heat exchange with the air, and inflow from runoff) (Galli, 1990; Leach et al., 2023). The relationship between stream size and temperature is complex and shifts with increasing stream size (reviewed in Leach et al., 2023). However, my streams were small enough to maintain a roughly linear relationship with the log of the drainage basin area and followed the general relationship seen at USGS gages across the greater metro Atlanta area. The difference in seasonal variation in temperature across drainage basin areas has implications for life history, occupancy of thermally sensitive species, and nutrient cycling.

The goal of this Chapter was to confirm that my 14 streams had similar thermal profiles to what I would expect across gradients of urbanization and stream size. There are many other stream parameters that are both impacted by urbanization and biologically relevant, but due to the logistical, temporal, and statistical constraints of this study I do not include them here. However, it is worth noting that hydrological regimes (particularly peak flow), pH, dissolved

oxygen, and stream bottom composition are all worth exploring in future studies assessing salamander life history across an urban gradient (Barr & Babbitt, 2002; Smith & Grossman, 2003; Barrett et al., 2010a).

CHAPTER 2: ENVIRONMENTAL DRIVERS OF LARVAL PERIOD AND SIZE IN *EURYCEA CIRRIGERA*

2.1 BACKGROUND

2.1.a Larval period in the Eurycea bislineata *species complex*

Members of the *E. bislineata* species complex display varying larval periods, or the time the salamander spends as a larva before metamorphosing (Bruce, 1982a; Voss, 1993). Two-lined Salamander larval periods can range from one to three years (Duellman & Wood, 1954; Bruce, 1982a; Voss, 1993), and variation in larval period can be thought of as discrete. If a larva does not undergo metamorphosis during the summer, it will not undergo metamorphosis until the following summer (Bruce, 1982a; Pierson & Miele, 2019), resulting in the discrete variation in larval period (i.e., one, two, or three years long).

The length of the larval period varies geographically and is thought to be largely driven by stream temperature (Voss, 1993; Beachy, 2018). Voss (1993) found that in four Appalachian drainages *E. wilderae* from first-order streams had a one-year larval period while those from higher-order streams had a mix of one- and two-year larval periods. In the same study, higher-order streams were found to have warmer temperatures in the summer and cooler temperatures in the winter compared to first-order streams. Voss (1993) suggested that this wider thermal profile may affect the pituitary-thyroid regulation of metamorphosis. In lab experiments, warmer temperatures significantly predicted shorter larval periods in *E. wilderae* (Beachy, 2018). Thus, changing thermal profiles in urban streams, largely towards higher temperatures, may shorten larval periods of *E. cirrigera*.

2.1.b Growth rates in larval Eurycea cirrigera

Compared to other amphibian families, Plethodontidae experiences markedly slower growth rates (Beachy et al*.,* 2017). Plethodontid growth rates seem to be driven more by genetics than environmental conditions (Beachy et al., 2017). However, there is evidence that certain environmental factors influence growth rates. Murphy et al. (2016) found a significant, positive relationship between *E. cirrigera* larval size and stream temperature in a before-after control-impact study of urban streams. Larvae from urban streams were larger than those from control streams four years after land development had begun, perhaps due to increased metabolic rates in warmer streams (Murphy et al., 2016). However, in a controlled laboratory experiment, Beachy (2018) found that temperature did not have a significant impact on *E. wilderae* larval growth, but food availability did. Yet, Barrett et al. (2012) did not find a significant difference in the diets of *E. cirrigera* larvae in urban and forested streams, despite a significant difference in larval size.

The growth rate and size of an individual are also influenced by population and community dynamics. The growth rate of Two-lined Salamanders can be impacted by the presence of predators (Resetarits, 1991; Gustafson, 1993; Beachy, 1997); however, the interaction is complex. While Resetarits (1991) and Gustafson (1993) noted a decrease in *E. cirrigera* growth rates in the presence of a predator, Beachy (1997) observed that *E. wilderae* in high-predator trials grew significantly faster earlier in the larval period, then significantly slower later in the larval period. Beachy (1997) suggested that this initial increase was due to low conspecific density. Decreased conspecific density has been linked to increased overall body sizes in *E. cirrigera* (Murphy et al., 2016), a pattern common in other salamander species (Petranka & Sih, 1986; Petranka, 1989; but see Beachy, 1994). Urban streams typically have lower densities

of predator species (Morgan & Cushman, 2005; Barrett & Guyer, 2008; Clark & Landberg, unpublished data) and *E. cirrigera* (Willson & Dorcas, 2003; Miller et al., 2007; Price et al., 2011), potentially explaining the increase in size of *E. cirrigera* larvae in urban streams.

Here, I disentangle the effects of temperature and ISC on *E. cirrigera* larval period and size in order to better understand the environmental drivers of these life history traits.

2.2 METHODS

2.2.a Fieldwork

I surveyed each of my 14 sites (see **Chapter 1** for details on site selection) three times: September 2021, May 2022, and July 2022. Within each 10 x 1 m transect, I overturned all cover objects, searched leaf packs, and disturbed sediment and small rocks with a dip net downstream to catch any *E. cirrigera*. I classified individuals as larvae if they had external gills and no signs of adult coloration. I placed each larva inside a plastic bag with stream water and measured it from the tip of its snout to the distal end of the vent ("snout-vent length", SVL). Once I completed sampling inside the 10 x 1 m transect, I expanded sampling outside of the transect for roughly 20 – 30 minutes and recorded which individuals were found outside of the transect. I released all salamanders at their approximate capture location after collecting all measurements.

2.2.b Estimating larval period

First, I visually estimated whether each site had one or two size cohorts of larvae in September 2021 by comparing histograms of May 2022, July 2022, and September 2021 larval SVL at each site. By September, metamorphosis in *E. cirrigera* should have ceased, and first- and

second-year larvae are still distinct in size (Bruce, 1982a). Therefore, September is the ideal time to investigate whether the larvae at a site exhibit a one-year larval period or a mix of one- and two-year larval periods. If it was unclear whether September 2021 larvae displayed two size cohorts, I referred to May and July 2022 data. If these months had three distinct size cohorts (larvae hatched in 2022, larvae hatched in 2021, and larvae hatched in 2020 that are about to undergo metamorphosis), this suggested that September 2021 larvae had two size cohorts, but the first-year larvae overlapped the second-year larvae distribution by September.

To verify my visual estimates of larval period, I used the package "mclust" v6.0.0 (Scrucca et al., 2016) in R v4.2.3 (R Core Team, 2023). The "mclustBIC()" function from this package suggests the best finite Gaussian mixture model by weighing Bayesian information criterion (BIC) scores using a hierarchical clustering approach. Using the "mclustBIC()" function, I determined whether a unimodal distribution, bimodal distribution with equal variance between distributions, or bimodal distribution with unequal variance between distributions best fit the larval SVL data at each site. For sites in which "mclustBIC" suggested a distribution different from what I visually estimated, I revisited the May 2022, July 2022, and September 2021 histograms (Fig. S.7 in **Supplementary Material**) and determined whether the data supported my visual estimate or the conclusion reached by "mclustBIC()". I then fit the best model to each site's September 2021 larval SVL data using the function "Mclust()'' from the "mclust" package, which assigns each larva to the first or second distribution if the model is bimodal.

2.2.c Statistical analyses

I conducted a path analysis by fitting a piecewise structural equation model (SEM) to estimate the effects of drainage basin area, ISC, and temperature on larval period. I used the package "semEff" v0.6.1 (Murphy, 2022) to fit the SEM. The SEM included two regressions: 1) a logistic regression with a binary response of larval period (0 = absence of second-year larvae; 1 = presence of second-year larvae) as a function of mean August 2022 temperature, ISC, and the log of the drainage basin area, and 2) a linear regression of mean August 2022 temperature per site as a function of ISC and the log of the drainage basin area. I fit an additional SEM with the same structure, except replacing mean August 2022 temperature with mean January 2022 temperature in both regressions. To compare the fit of the SEMs, I created a receiver operating curve (ROC) for the logistic regression in each SEM then calculated the area under the curve (AUC) for each ROC using the "roc()" and "auc()" functions in the R package "pROC" v1.18.0 (Robin et al., 2011).

To investigate how urbanization might impact larval size, I calculated the mean SVL of larvae assigned to the first size cohort for each site and fit an SEM that included two linear regressions: 1) the average larval SVL per site as a function of mean August 2022 temperature, ISC, and the log of the drainage basin area, and 2) mean August 2022 temperature as a function of ISC and the log of the drainage basin area.

I estimated confidence intervals for all SEMs using the "bootEff()" function from the "semEff" package with 1000 bootstrap iterations and a seed of 13. To test for normality of the residuals of the larval size model, I ran Shapiro-Wilk tests using the function "shapiro.test()" and inspected Q–Q plots in R. I tested for spatial autocorrelation in the residuals of each regression

model in each SEM using the "Moran.I()" function from the package "ape" v5.7–1 (Paradis & Schliep, 2019). I used an inverse distance matrix of latitude and longitude of each site to calculate a scaled Moran's I statistic for autocorrelation in the model residuals. Using the "R2()" function from the "semEff" package, I calculated adjusted R^2 values to assess model fit.

2.3 RESULTS

2.3.a Larval Period

I visually determined that six of my fourteen sites in urban Atlanta had larvae with a two-year larval period (Table S.3, Fig. S.5, and Fig. S.6 in **Supplementary Material**). Two of my sites (Golf Course A and Eubanks Park) had evidence of a single-season larval period; larvae found in September had SVLs typical of metamorphs, both sites saw a dramatic decrease in numbers of first-year larvae from summer to fall, and two metamorphs were found at Eubanks Park in October 2023. To limit overfitting of the SEM, I grouped the sites with only a one-year larval period and the sites with what may be a single-season larval period into one group (binary response of 0 [absence of second-year larval period]).

The SEM with a direct effect of mean August temperature on larval period (Fig. 2.1, Fig. 2.2; AUC = 0.98) outperformed the SEM with a direct effect of mean January temperature (Fig. 2.3, Fig. 2.4; AUC = 0.71). Sites with warmer August temperatures were less likely to have larvae with two-year larval periods (Table 2.1, Fig. 2.1). Mean January temperature had a small, insignificant effect on whether a site had larvae with two-year larval periods (Table 2.3, Fig. 2.3). Neither SEM had significant spatial autocorrelation in the residuals of the logistic regression (p_{august} = 0.37; $p_{january}$ = 0.99).

Table 2.1: Model output from SEM of larval period as a function of mean August temperature. The standardized direct, indirect, and total effects of mean August 2022 stream temperature, impervious surface cover (ISC), and the log of drainage basin area on larval period. "Larval period" is a binary response (0 = absence of two-year larval period; 1 = presence of two-year larval period). Asterisk indicates significance. Std. Err. = standard error. "Lower CI" and "Upper CI" = lower and upper bounds to 95% confidence interval.

	Variable	Effect	Bias	Std. Err.	Lower CI	Upper CI	Significant
DIRECT	Aug mean temp	−0.244	−3.228	4.592	−11.520	−0.086	*
INDIRECT	ISC	−0.060	−0.779	1.521	−0.964	4.551	
	Log(drainage area)	−0.151	−1.918	2.923	−2.853	0.540	
TOTAL	ISC	−0.060	−0.779	1.521	−0.964	4.551	
	Log(drainage area)	−0.151	−1.918	2.923	−2.853	0.540	
	Aug mean temp	−0.244	−3.228	4.592	−11.520	−0.086	*
MEDIATORS	Aug mean temp	−0.211	−2.697	4.100	−2.858	0.032	

Table 2.2: Model output from linear regression modeling mean August temperatures. The standardized direct effects of impervious surface cover (ISC) and the log of drainage basin area on mean stream temperature in August 2022. Asterisk indicates significance. Std. Err. = standard error. "Lower CI" and "Upper CI" = lower and upper bounds to 95% confidence interval.

	Variable	Effect	Bias	Std. Err.	Lower CI	Upper CI	Significant
DIRECT	ISC	0.245	0	0.180	−0.248	0.516	
	Log(drainage area)	0.619	−0.028	0.172	0.244	0.885	*

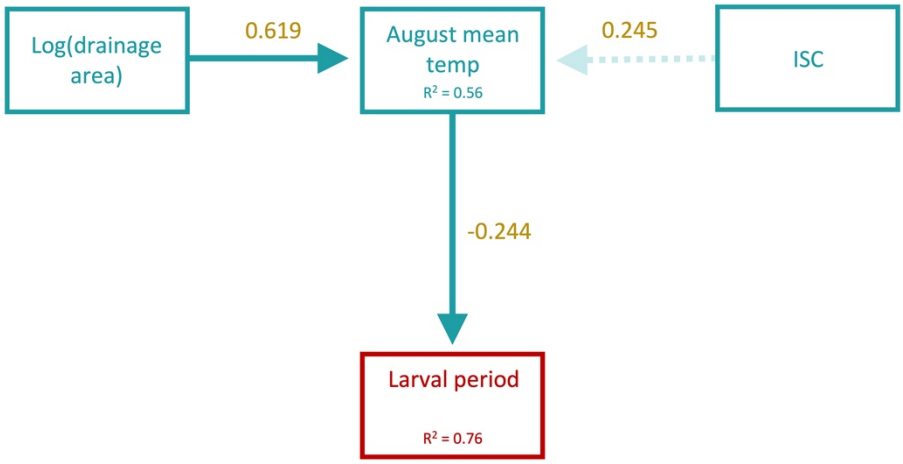


Fig. 2.1: SEM of larval period as a function of mean August 2022 stream temperature. SEM composed of two regressions: larval period as a function of mean August temperature; and mean August temperature as a function of impervious surface cover (ISC) and the log of drainage basin area. "Larval period" is a binary response (0 = absence of two-year larval period; 1 = presence of two-year larval period). Solid arrows indicate significance. Gold numbers above the arrows are standardized coefficients of effect. Red boxes and font indicate a salamander life history trait. Adjusted R^2 values shown.

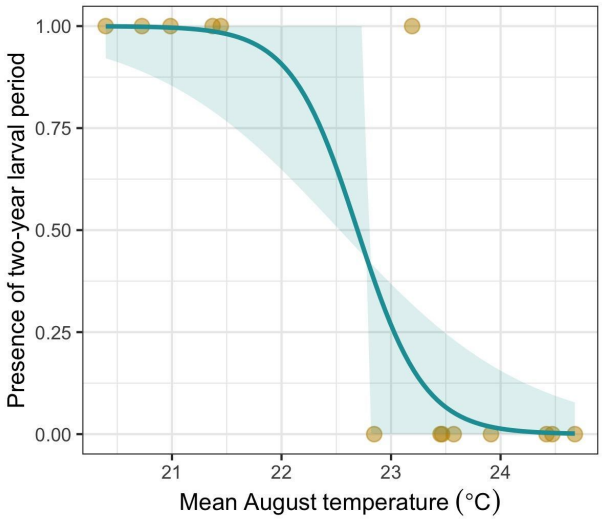


Fig. 2.2: The effect of mean August temperature (°C) on larval period length. I evaluated "larval period" as a binary response (0 = absence of two-year larval period; 1 = presence of two-year larval period) from larvae captured in September 2021, and I used temperatures from August 2022. Shaded regions show 95% confidence intervals from the logistic regression.

Table 2.3: Model output from SEM of larval period as a function of mean January temperatures. The standardized direct, indirect, and total effects of mean stream temperature in January 2022, impervious surface cover (ISC), and the log of drainage basin area on larval period. "Larval period" is a binary response (0 = absence of two-year larval period; 1 = presence of two-year larval period). Asterisk indicates significance. Std. Err. = standard error. "Lower CI" and "Upper CI" = lower and upper bounds to 95% confidence interval.

	Variable	Effect	Bias	Std. Err.	Lower CI	Upper CI	Significant
DIRECT	Jan mean temp	0.063	0.437	3.357	−0.034	0.256	
INDIRECT	ISC	0.010	−0.009	0.524	−0.038	0.117	
	Log(drainage area)	−0.043	−0.326	2.571	−1.667	0.008	
TOTAL	ISC	0.010	−0.009	0.524	−0.038	0.117	
	Log(drainage area)	−0.043	−0.326	2.571	−1.667	0.008	
	Jan mean temp	0.063	0.437	3.357	−0.034	0.256	
MEDIATORS	Jan mean temp	−0.033	−0.336	2.749	−1.780	0.005	

Table 2.4: Model output from linear regression modeling mean January temperatures. The standardized direct effects of impervious surface cover (ISC) and the log of drainage basin area on mean stream temperature in January 2022. Asterisk indicates significance. Std. Err. = standard error. "Lower CI" and "Upper CI" = lower and upper bounds to 95% confidence interval.

	Variable	Effect	Bias	Std. Err.	Lower CI	Upper CI	Significant
DIRECT	ISC	0.167	−0.001	0.221	−0.254	0.597	
	Log(drainage area)	−0.689	0.044	0.145	−0.876	−0.382	*

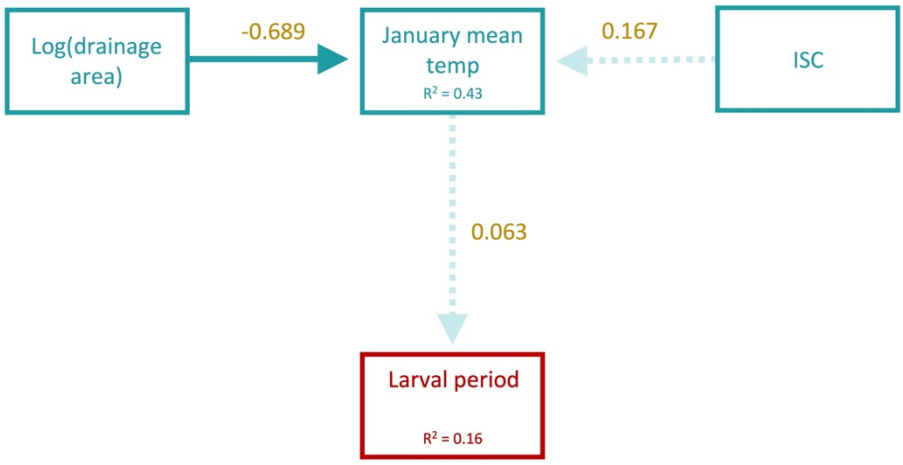


Fig. 2.3: SEM of larval period with January temperatures. SEM composed of two regressions: larval period as a function of mean stream temperature in January 2022; and mean stream temperature in January 2022 as a function of impervious surface cover (ISC) and the log of drainage basin area. "Larval period" is a binary response (0 = absence of two-year larval period; 1 = presence of two-year larval period). Solid arrows indicate significance. Gold numbers above the arrows are standardized coefficients of effect. Red boxes and font indicate a salamander life history trait. Adjusted R^2 values shown.

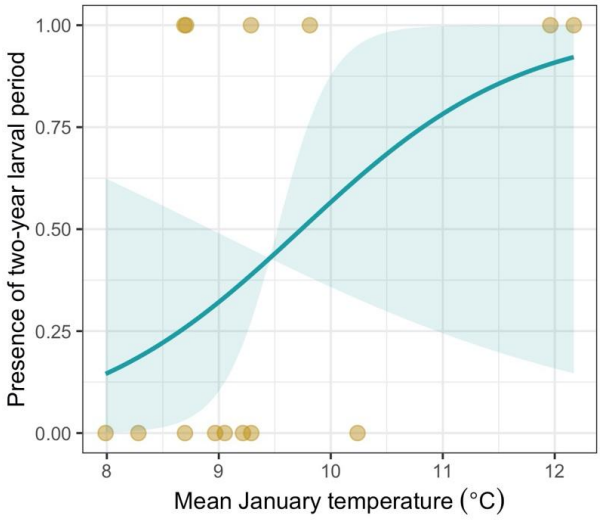


Fig. 2.4: The effect of mean January temperature (°C) on larval period length. I evaluated "larval period" as a binary response (0 = absence of two-year larval period; 1 = presence of two-year larval period) from larvae captured in September 2021, and I used temperatures from January 2022. Shaded regions show 95% confidence intervals from the logistic regression.

2.3.b Larval snout-vent length

Mean August 2022 temperature had a large, significant, and positive effect on larval SVL (Table 2.5, Fig. 2.5, Fig. 2.6). ISC had insignificant direct and indirect effects on larval SVL (Table 2.5). However, ISC had a significant, positive total effect on larval SVL, largely through the effect of ISC on mean August temperatures (Table 2.5, Fig. 2.5, Fig. 2.7). Drainage basin area (log-transformed) had a significant, negative effect on larval SVL, but a small total effect on larval SVL (Table 2.5, Fig. 2.5, Fig. 2.8). The linear regression predicting larval SVL did not have significant spatial autocorrelation in its residuals (p = 0.75). The distribution of model residuals did not show evidence of non-normality (W = 0.96, p = 0.74).

Table 2.5: Model output from SEM of larval snout-vent length (SVL). The standardized direct, indirect, and total effects of impervious surface cover (ISC), the log of drainage basin area, and mean August 2022 stream temperature on mean larval SVL of first-year larvae in September 2021. Asterisk indicates significance. Std. Err. = standard error. "Lower CI" and "Upper CI" = lower and upper bounds to 95% confidence interval.

		Effect	Bias	Std. Err.	Lower CI	Upper CI	Significant
DIRECT	ISC	0.116	−0.018	0.128	−0.113	0.453	
	Log(drainage area)	−0.436	0.056	0.173	−0.797	−0.123	*
	Aug mean temp	0.769	−0.086	0.211	0.331	0.980	*
INDIRECT	ISC	0.188	−0.038	0.124	−0.062	0.376	
	Log(drainage area)	0.476	−0.086	0.150	0.251	0.769	*
TOTAL	ISC	0.305	−0.056	0.125	0.098	0.517	*
	Log(drainage area)	0.041	−0.031	0.154	−0.291	0.266	
	Aug mean temp	0.769	−0.086	0.211	0.331	0.980	*
MEDIATORS	Aug mean temp	0.665	−0.124	0.151	0.564	0.875	*

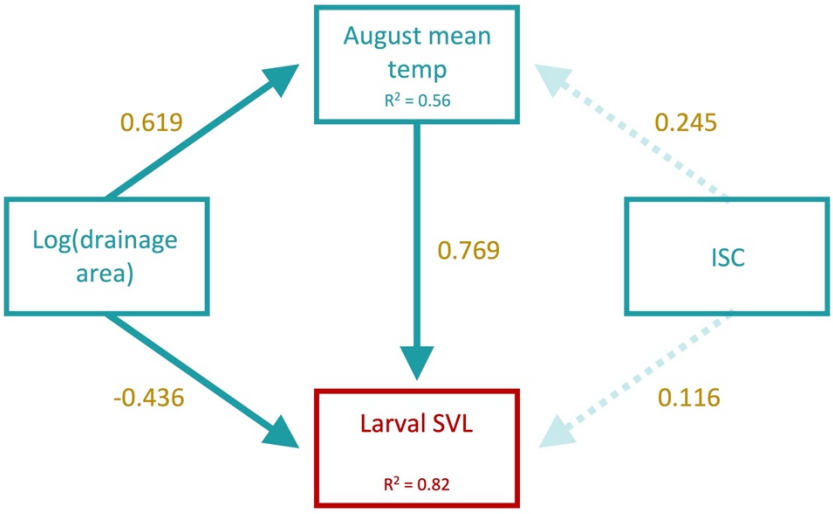


Fig. 2.5: SEM of larval snout-vent length (SVL). SEM composed of two regressions: SVL as a function of the log of the drainage basin area, mean August 2022 stream temperature, and impervious surface cover (ISC); and mean August 2022 stream temperature as a function of ISC and the log of the drainage basin area. Solid arrows indicate significance. Gold numbers above the arrows are standardized coefficients of effect. Red boxes and font indicate salamander life history trait. Adjusted R^2 values shown.

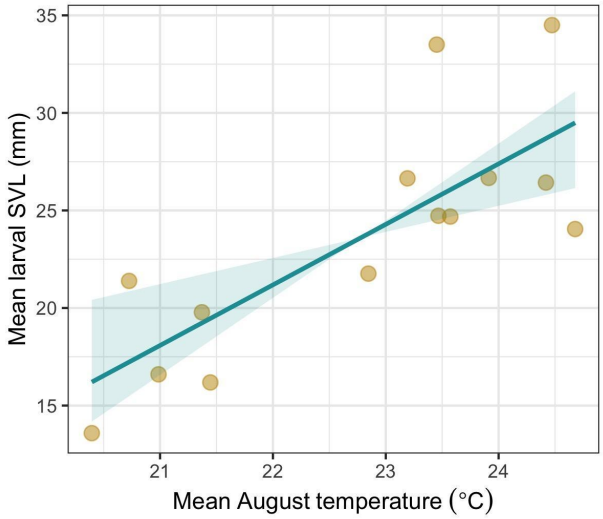


Fig. 2.6: Total effects of mean August temperature on mean larval snout-vent length (SVL) per site. 95% confidence interval of the regression shown.

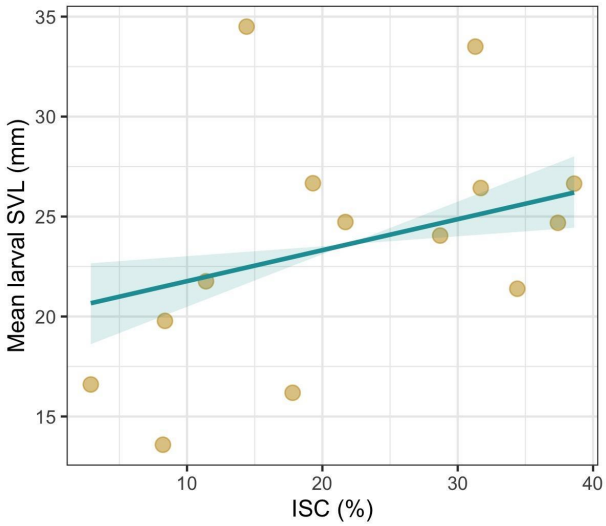


Fig. 2.7: Total effects of percent impervious surface cover (ISC) on mean larval snout-vent length (SVL) per site. 95% confidence interval of the regression shown.

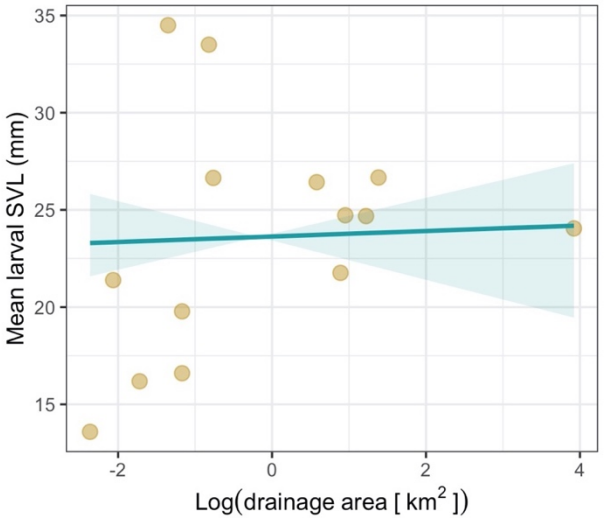


Fig. 2.8: Total effects of the log of the drainage basin area on mean larval snout-vent length (SVL) per site. 95% confidence interval of the regression shown.

2.4 DISCUSSION

Unlike other amphibians, members of the family Plethodontidae experience a relative decoupling of larval life history traits from the environment (Beachy, 2017). In other amphibians, a change in the surrounding environment such as shifting hydrology or temperatures can induce metamorphosis to maximize fitness (Werner, 1986; Denver, 1997, 2009; Beachy, 2018). This is particularly pronounced in species that inhabit ephemeral ponds. Here, timing of metamorphosis is a balance between maximizing growth as a larva, transitioning to a terrestrial adult before the pond dries, and maximizing survival (Werner, 1986; Denver, 1997, 2009). However, the plethodontid salamanders are largely released from these environmental pressures; species are either direct developing (skipping the aquatic larval phase altogether), have an aquatic larval phase in relatively permanent water bodies (mostly streams), or are paedomorphic (Bonett et al., 2014). Consequently, larval life history traits of plethodontid salamanders are thought to be driven mostly by genetics (Beachy, 2017), but with some plasticity across environmental gradients.

At 14 streams in urban Atlanta, I documented what appears to be three distinct larval periods: six sites with only a one-year larval period, six sites with a two-year larval period, and two sites with what may be a single-season larval period. To the best of my knowledge, this appears to be the first case of a single-season larval period in Two-lined Salamanders, though other species within the *Eurycea* genus can have single-season larval periods (e.g., *E. quadridigitata* [Semlitsch, 1980], *E. guttolineata* [Maldonado, 2022]).

Larval periods at my sites were almost perfectly segregated by mean August 2022 temperature, with all sites with only a one-year larval period having mean August temperatures

above 22.5°C. Five out of six sites with a two-year larval period had mean August temperatures below 22.5°C. This provides strong evidence that summer temperature predicts larval period length in this population of *E. cirrigera*.

The timing of metamorphosis in amphibians is controlled by the hypothalamic-pituitary-thyroid (HPT) axis. The HPT axis regulates the production of thyroid hormones associated with metamorphosis in amphibians, T_3 and T_4 (reviewed across clades and life histories in Bonett, 2016). However, as noted by Bonett (2016), the effect of temperature on the regulation of T_3 and T_4 is poorly understood in plethodontids with a biphasic life cycle. Induced metamorphosis in larvae from the family Hynobidae using thyroid hormones was impaired in low temperatures (Moriya, 1983). I speculate that the near perfect separation of the absence of a two-year larval period based on summer temperatures is the result of sustained higher temperatures influencing the regulation of thyroid hormones by the HPT axis. This would explain why summer temperature was a better predictor of larval period than winter temperature. This may also explain why the two sites with evidence of a single-season larval period were the only two sites to reach stream temperatures above 27°C in August 2022. Larvae at these sites may have experienced temperatures above some important temperature threshold of the endocrine regulation of metamorphosis. While I used two different cohorts (September 2021 and May/July 2022) to infer a single-season larval period at these two sites, my observation of two metamorphs in October 2022 at one of the sites supports a pattern that extends beyond a single year.

Larval size was directly affected by summer temperatures and drainage basin area. Warmer summer temperatures predicted significantly larger larval snout-vent lengths (SVL). While my streams never reached the critical thermal maximum typical of plethodontids of the

southeast US (Zweifel, 1957; Hutchison, 1961), higher temperatures within the optimal temperature range may have increased growth rates through increased metabolic rates (Ringia & Lips, 2007; Barrett et al., 2010b; Markle, 2015; but see Beachy, 2018). Alternatively, earlier oviposition allowing larvae more time to grow may explain larger larval sizes in September. Pierson et al. (2023) found that across the geographic range of the Two-lined Salamander species complex, oviposition occurred later in locations with lower mean annual temperatures. While warmer summer temperatures may drive earlier oviposition, the temperature during the winter breeding season is likely more influential. If stream temperature (versus air temperature) influences timing of oviposition, oviposition would occur later in colder streams in the winter. My sites that were the coldest in the winter were the warmest in the summer (see **Chapter 1**). The hypothesis that larval SVL and summer temperatures are positively correlated because oviposition occurred earlier in streams with warmer summer temperatures (i.e., cooler winter temperatures) is not supported.

An increase in stream temperature has many implications on abiotic and biotic processes relevant to salamander physiology and ecology. For example, temperature strongly impacts leaf litter breakdown (Webster & Benfield, 1986; Boyero et al., 2014), impacting microhabitat availability, macroinvertebrate communities, and stream carbon levels (Webster & Benfield, 1986; Meyer et al., 1998). Though temperature changes may alter macroinvertebrate communities, larval Two-lined Salamanders are rarely limited by food availability and their primary prey items (by biomass) are often clades that are relatively unaffected by urbanization (Petranka, 1984; Walsh et al., 2005). Additionally, Barrett et al. (2012) found that larval *E. cirrigera* had similar diets in streams differing in temperature by about 2 – 3°C (a range similar to

my sites; Barrett & Guyer, 2008). This does not support the hypothesis that higher temperatures at my sites increase larval SVL through shifts in diet composition and prey availability.

Alternatively, sites with warmer temperatures may have presented stressors that more sensitive salamander species could not tolerate. Decreased density of predatory salamanders is associated with increased larval growth rates (Resetarits, 1991; Gustafson, 1993; Beachy, 1997) and may explain my results. However, I did not measure density of *E. cirrigera* nor other salamander species; caution should be taken in interpreting differences in larval size as a result of differences in conspecific or predator density. Smaller SVLs of first-year larvae at sites with colder summer temperatures may be from competition with larger, second-year larvae (only present at sites with cooler summer temperatures). However, in a study of larval *E. bislineata* diet, Petranka (1984) found evidence of resource partitioning between hatchling and one-year old larvae, reducing competition for prey between age classes.

ISC did not have a significant direct effect on larval SVL but did have a significant total effect. This significant total effect was largely due to the indirect effect of ISC on SVL via the direct effect of ISC on mean August temperature. However, there was a small, insignificant direct effect of ISC on SVL that contributed to the significant total effect. In other words, increased ISC slightly increased SVL through a mechanism other than changes in stream temperature. This could possibly be explained by numerous "symptoms" of the urban stream syndrome, including increased severity of floods selecting for larger larvae (Espey et al., 1966; Leopold, 1973; Barrett & Guyer, 2008; Barrett et al., 2010a), changes to water chemistry (Paul & Meyer, 2001; Walsh et al., 2005), and changes to microhabitat availability (Hawkins et al., 1983; Finkenbine et al., 2000; Barr & Babbitt, 2002).

Drainage basin area, however, had a significant and negative direct effect but insignificant total effect on larval SVL. The negative direct effect was unexpected, as I expected either no effect or a positive effect. Previous literature on larval plethodontid salamanders supports the hypothesis of larger larvae being found further downstream due to larval drift (Bruce, 1985). It is possible that increased fish abundance and increased flow in larger streams select for smaller larvae that can better hide under cover objects (Barrett et al., 2010a). However, most studies on larval growth and size are either in laboratory experiments or undisturbed streams; urban streams experience different phenomena at different drainage basin sizes due to human land use patterns. My ability to explicitly model the effects of drainage basin area on larval SVL in urban streams is limited. Further studies on patterns of environmental change across stream sizes are needed to more accurately estimate salamander larval growth and to disentangle the effects of drainage basin, temperature, and changes associated with urbanization.

CHAPTER 3: THE RELATIONSHIP BETWEEN IMPERVIOUS SURFACE COVER, *EURYCEA CIRRIGERA* CLUTCH SIZE, AND FEMALE SIZE

3.1 BACKGROUND

Recent population declines in plethodontid salamanders have been linked to habitat degradation and fewer cover objects in urbanizing areas (Orser & Shure, 1972; Davic & Orr, 1987; Guy et al., 2004). Further, Macklem et al. (2022) found that *E. bislineata* egg production and brooding behavior decreased in watersheds with recent development. The combination of decreased cover object availability and shifts in thermal and hydrological regimes suggests that urbanization may impact *E. cirrigera* reproductive behavior and success.

Eurycea cirrigera typically oviposits between January and April. In the stream, females lay nests under cover objects, such as rocks, logs, and leaf litter, with preference for shallow sites with cobble and large substrata (Guy et al., 2004; Downing, unpublished data). Females stay to guard the nest (Bruce, 1982a; Brophy & Pauley, 2002). Clutch sizes range widely, but average roughly 20 – 60 eggs (Wood & McCutcheon, 1954; Brophy & Pauley, 2002). In Two-lined Salamanders, clutch size is positively correlated with female size at some sites (Wood & McCutcheon, 1954; Bruce, 1988) but not at others (Guy et al., 2004).

Here, I investigate the relationships between clutch size, female size, ISC, and temperature in order to better understand the impacts of urbanization on the reproductive output of Two-lined Salamander populations.

3.2 METHODS

3.2.a Fieldwork

I surveyed each of my 14 sites (see **Chapter 1** for sample site selection) in late January 2022, when nesting had begun. I established a 30 x 1 m transect that contained the original 10 x 1 m transect of September 2021 larvae sampling (See **Chapter 2** for larvae sampling) and roughly represented the stream bottom composition of the entire site. Starting downstream, I carefully overturned any cover object and sifted through leaf litter. Once I located an *E. cirrigera* nest, I quickly took a photograph of the entire nest out of the water and attempted to capture any adult associated with the nest. I then replaced the cover object with the nest to its original orientation in the water. If an adult was present and I successfully captured it, I placed it in an 11 x 18 x 5.75 cm rectangular plastic container with a ColorChecker Passport Photo color palette (MSCCPPCC0319; X-Rite Inc.; MI, USA) and roughly 3 cm of stream water. I took photographs using a cellphone camera (12.2MP, ƒ/1.8 aperture, field of view 76°) of each salamander inside the container, making sure the container was approximately level, glare was minimal, and the entire color palette and salamander were visible. I noted the sex and checked if females were gravid and/or had spermatophores in their cloacae. I considered any adult that was gravid and/or had a spermatophore in its cloaca to be female. I then immediately returned each adult to the nest it was found with.

3.2.b Data collection from photographs

From photographs, I categorized each clutch as one of the following three embryonic developmental stages: (1) a spherical, undifferentiated embryo; (2) at least some visible

differentiation; or (3) dark eye pigmentation, usually accompanied by other characteristics (e.g., dorsal pigmentation) typical of larvae near hatching (Pierson et al*.,* 2023). To approximate the ordinal date of oviposition for each nest, I used estimates of average developmental rates from Pierson et al. (2023) and subtracted 25 days from the observation date for stage 3 nests, 10 days for stage 2 nests, and 0 days for stage 1 nests.

To determine clutch size, an additional researcher and I separately and manually counted each egg from photographs. If our counts differed, we reviewed the photographs together and reached a consensus. I did not include in my analyses any clutch in which an accurate count could not be completed (e.g., due to poor photographs or inability to differentiate cloudy, inviable eggs from coloration on rock). If two different stages of development were documented on the same rock, I considered them separate nests. If two egg clusters were separated by a few centimeters or more, I also considered them separate nests.

In order to minimize the amount of time an adult salamander was being processed away from its associated nest, I did not measure SVL in the field but instead approximated length from photographs. I adapted the methods outlined by Aragón-Sánchez et al. (2017) to approximate the snout-girdle length (SGL) of adult female *E. cirrigera* from photographs using the ImageJ.JS online application v0.5.7 (Ouyang et al., 2019). I used SGL instead of snout-vent length (SVL) because the location of the distal end of the vent is hard to approximate from photographs of the dorsal side. SGL instead is the distance from the tip of the snout to the middle of the pelvic girdle, which is easy to consistently locate in dorsal photographs. I used the 50 mm ruler on the ColorChecker Passport Photo color palette (visible in each photo) as a standard for measuring SGL in ImageJ.JS.

3.2.c Statistical analyses

Because my objective is to investigate the relationship between clutch size, female size, and ISC, I only included in my model nests that fit the following criteria: 1) the nest was not found with another nest on the same rock, 2) the nest was found with only one female, 3) the female was not gravid, and 4) I had an accurate egg count for the nest.

I conducted a path analysis by fitting a piecewise structural equation model (SEM) to estimate the effects of drainage basin area, ISC, and temperature on female SGL and the effects of female SGL on clutch size. I used the package "semEff" v0.6.1 (Murphy, 2022) to fit the SEM. The SEM included three linear regressions: 1) clutch size of nests fitting the criteria described above as a function of the SGL of the associated female, 2) mean female SGL (using all females, regardless of association with a nest or whether she was gravid) per site as a function of mean August 2022 temperature, ISC, and the log of the drainage basin area, and 3) mean August 2022 temperature as a function of ISC and the log of the drainage basin area. I estimated confidence intervals using the "bootEff()" function from the package "semEff" v0.6.1 (Murphy, 2022) with 1000 bootstrap iterations and a seed of 13 in R v4.2.3 (R Core Team, 2023). I checked the fit of the model using the "fisherC()" function from the package "piecewiseSEM" v2.3.0 (Lefcheck, 2016), which calculates the Fisher's C statistic. Using the "R2()" function from the "semEff" package, I calculated adjusted R^2 values.

To test for normality of the model residuals, I ran Shapiro-Wilk tests using the function "shapiro.test()" and inspected Q–Q plots in R. I tested for spatial autocorrelation in the residuals of each regression in each SEM using the "Moran.I()" function from the package "ape" v5.7–1

(Paradis & Schliep, 2019). I used an inverse distance matrix of latitude and longitude of each site to calculate a scaled Moran's I statistic for autocorrelation in the model residuals.

3.3 RESULTS

From 22 January to 31 January 2022, I collected data from 92 *E. cirrigera* nests and 219 adult *E. cirrigera*. I found 67 nests in developmental stage 1, 17 in stage 2, and 8 in stage 3. Clutch size ranged 2 – 118 eggs (mean = 60.36 eggs ± 25.46 SD; Table S.3 in **Supplementary Material**). I found evidence of communal nesting at four of my sites, with the following nests found together on the same rock (Table S.4 in **Supplementary Material**): nests 25 and 26 with a single guarding male, nests 38 and 39 with three females (two of which were gravid), nests 65 and 66 with a gravid female and an adult of undetermined sex, nests 74 and 75 with a gravid female and two adults of undetermined sex, nests 78 and 79 with two females (neither gravid), and nests 81, 82, and 83 with three females (none gravid).

Twenty-nine nests fulfilling the criteria described in section **3.2.c** were included in the SEM. The snout-girdle length (SGL) of the 29 females associated with these nests ranged 34.2 – 42.4 mm (mean = 39.30 mm ± 2.37 SD). SGL of all measured females (N = 127) ranged 33.57 – 43.62 mm (mean = 38.44 mm ± 2.41 SD). 84/127 females were gravid and 0/127 had spermatophores in their cloacae.

The SEM fit the observed data relatively well (Fisher's C statistic = 9.5, $p = 0.147$). Female SGL had a significant, positive direct effect on clutch size (Fig 3.1, Fig 3.2). ISC had a significant, positive indirect effect on clutch size (Table 3.1, Fig 3.3). Neither the log of the drainage basin area nor mean August 2022 temperature had significant effects on clutch size (Table 3.1). ISC had

significant, positive direct and total effects on mean female SGL (Table 3.2, Fig. 3.1, Fig. 3.4). Neither the log of the drainage basin area nor mean August 2022 temperature had significant effects on mean female SGL (Table 3.2, Fig. 3.1). I did not detect significant spatial autocorrelation in the residuals of the model of mean SGL (p = 0.22). The distributions of model residuals did not show evidence of non-normality for clutch size (W = 0.95, p = 0.20) nor mean female SGL (W = 0.92, p = 0.31).

Table 3.1: SEM output for clutch size. The standardized direct effect of female snout-girdle length (SGL) on clutch size and the standardized indirect and total effects of impervious surface cover (ISC), the log of the drainage basin area, and mean August 2022 stream temperature on clutch size. Asterisk indicates significance. Std. Err. = standard error. "Lower CI" and "Upper CI" = lower and upper bounds to 95% confidence interval.

	Variable	Effect	Bias	Std. Err.	Lower CI	Upper CI	Significant
DIRECT	Female SGL	0.620	0.001	0.102	0.355	0.791	*
INDIRECT	ISC	0.395	−0.066	0.141	0.184	0.691	*
	Log(drainage area)	0.041	−0.030	0.124	−0.218	0.290	
	Aug mean temp	−0.163	0.028	0.159	−0.445	0.199	
TOTAL	ISC	0.395	−0.066	0.141	0.184	0.691	*
	Log(drainage area)	0.041	−0.030	0.124	−0.218	0.290	
	Female SGL	0.620	0.001	0.102	0.355	0.791	*
	Aug mean temp	−0.163	0.028	0.159	−0.445	0.199	
MEDIATORS	Female SGL	0.273	−0.068	0.194	−0.017	0.678	
	Aug mean temp	−0.141	0.029	0.141	−0.475	0.130	

Table 3.2: SEM output for mean female snout-girdle length (SGL). The standardized direct, indirect, and total effects of impervious surface cover (ISC), the log of the drainage basin area, and mean August 2022 stream temperature on mean female SGL per site. Asterisk indicates significance. Std. Err. = standard error. "Lower CI" and "Upper CI" = lower and upper bounds to 95% confidence interval.

	Variable	Effect	Bias	Std. Err.	Lower CI	Upper CI	Significant
DIRECT	ISC	0.702	−0.119	0.229	0.346	0.966	*
	Log(drainage area)	0.229	−0.080	0.264	−0.226	0.769	
	Aug mean temp	−0.263	0.045	0.253	−0.641	0.366	
INDIRECT	ISC	−0.065	0.013	0.085	−0.287	0.066	
	Log(drainage area)	−0.163	0.033	0.161	−0.523	0.163	
TOTAL	ISC	0.637	−0.106	0.209	0.323	0.976	*
	Log(drainage area)	0.066	−0.048	0.197	−0.259	0.559	
	Aug mean temp	−0.263	0.045	0.253	−0.641	0.366	
MEDIATORS	Aug mean temp	−0.228	0.046	0.224	−0.719	0.217	

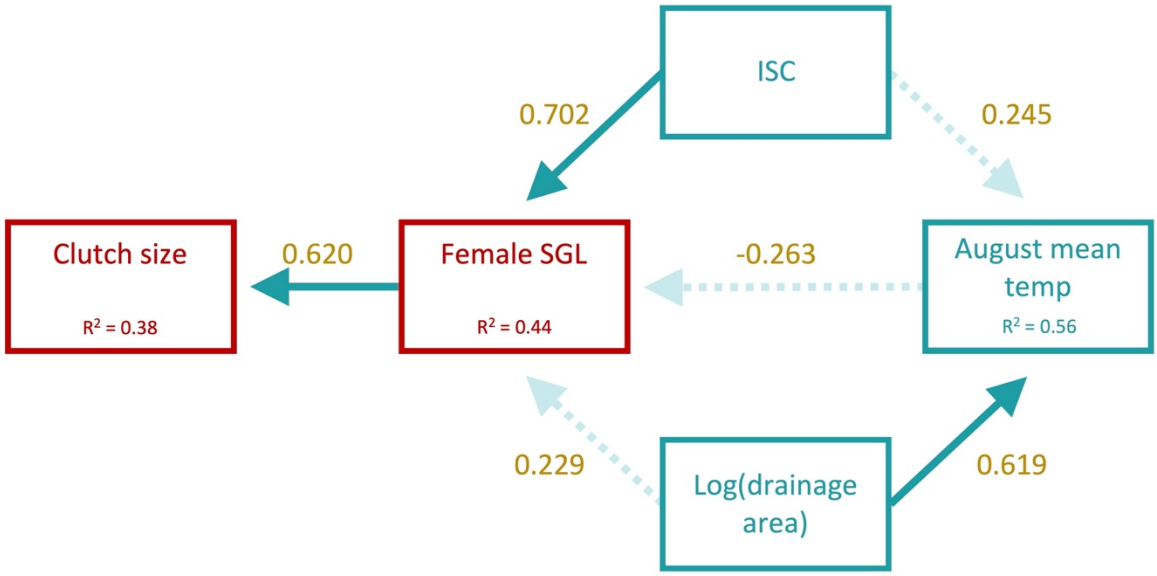


Fig. 3.1: Model output from the SEM of clutch size and female snout-girdle length (SGL). SEM composed of three regressions: clutch size as a function of female SGL; mean SGL for all females per site as a function of impervious surface cover (ISC), mean August 2022 stream temperature, and the log of drainage basin area; and mean August 2022 stream temperature as a function of ISC and the log of the drainage basin area. Solid arrows indicate significance. Gold numbers above the arrows are standardized coefficients of effect. Red boxes and font indicate salamander life history traits. Adjusted R^2 values shown.

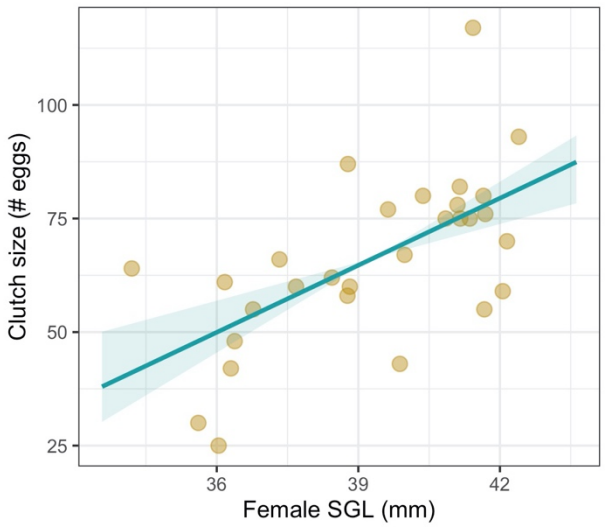


Fig. 3.2: Total effects of female snout-girdle length (SGL) on clutch size of each nest. 95% confidence interval of the regression shown.

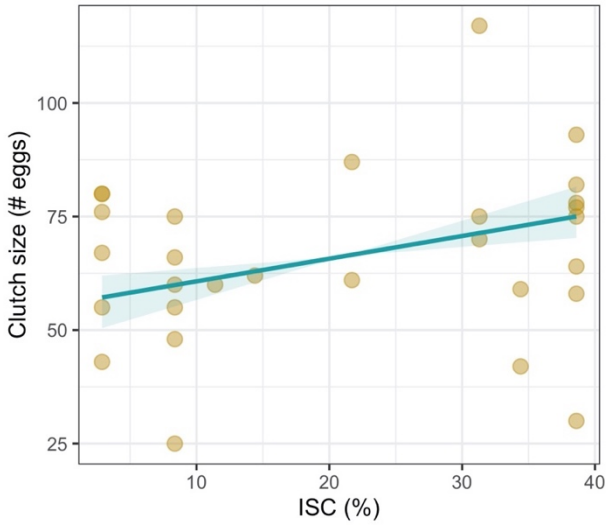


Fig. 3.3: Total effects of percent impervious surface cover (ISC) on clutch size of each nest. 95% confidence interval of the regression shown.

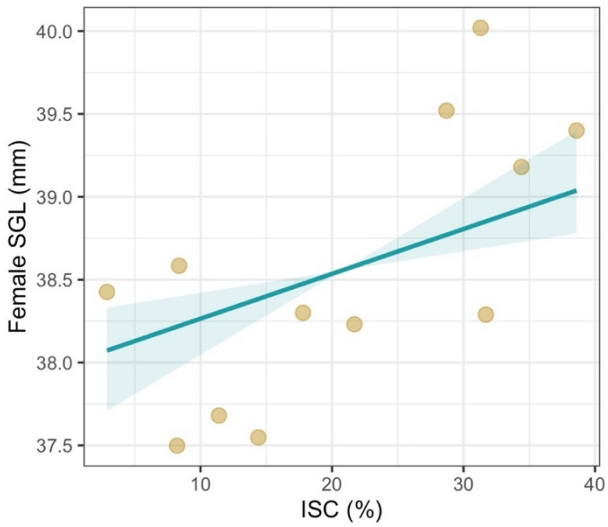


Fig. 3.4: Total effects of percent impervious surface cover (ISC) on mean snout-girdle length (SGL) of all females found at each site. 95% confidence interval of the regression shown.

3.4 DISCUSSION

The relationship between female size and clutch size in plethodontid salamanders varies by study and species (e.g., Bruce, 1969; Hom, 1987; Taylor et al., 1990; Beachy & Bruce, 2003; Milanovich et al., 2006; Howard & Maerz, 2022). Previous studies on Two-lined Salamanders differ on the degree of impact that female size has on clutch size, with some studies finding a significant positive relationship (Wood & McCutcheon, 1954; Bruce, 1988) and some finding no relationship (Guy et al., 2004). I found a significant, positive effect of female snout-girdle length (SGL) on clutch size, with an increase in SGL by 1 mm associated with an increase in clutch size by approximately 5 eggs. Larger females may have more energy to allocate to egg production or may be able to store more eggs in her body before oviposition. Alternatively, larger females may be better at guarding their nests from predators, resulting in fewer lost eggs and thus larger observed clutch sizes (Hom et al., 1990).

Increased impervious surface cover (ISC) of the drainage basin predicted significantly larger females, with females being roughly 0.5 mm larger for every 10% increase in ISC. In the Peachtree Creek watershed, small streams can have drainage basins with up to 40 – 50% impervious surface cover, with females in these streams predicted to be about 2 – 2.5 mm larger than streams with minimal surrounding ISC. Increased ISC is driving larger females through mechanisms other than temperature. Human land use can greatly alter stream productivity and macroinvertebrate communities (reviewed in Walsh et al., 2005). Stream macroinvertebrate communities tend to decrease in sensitive species and shift towards higher abundance of certain worms and midges, both of which are prey items to Two-lined Salamanders (Walsh et al., 2005). This in turn may impact adult salamander growth. Alternatively, increased urbanization is consistently tied to decreased salamander abundance and richness (Orser & Shure, 1972; Willson & Dorcas, 2003; Miller et al., 2007; Barrett & Guyer, 2008; Barrett & Price, 2014)—a trend I detected across sites in the Peachtree Creek watershed (see **Chapter 4** for results). This decrease in both intraspecific and interspecific competition may be driving the increased size in adult female salamanders—a pattern well-supported in larval salamander growth, but understudied in adult plethodontid growth (Resetarits, 1991; Gustafson, 1993; Beachy, 1997; Davis & Maerz, 2009).

Additionally, the indirect effect of ISC on clutch size, through female SGL, is significant. While these results suggest that increased ISC results in higher reproductive fitness, many other variables would need to be investigated to better understand the impact of ISC on site-level reproductive output. Survival to sexual maturity, age at sexual maturity, clutch success, nest abandonment, nest predation levels, length of time between nesting attempts, and adult

population size all impact the reproductive output of a population and may be impacted by disturbance to streams. Future studies should continue to investigate how urbanization impacts reproductive behavior and fitness.

While larval SVL may be predicted by mean August 2022 stream temperatures (see **Chapter 2**), female SGL was not. In a lab experiment by Beachy (2018), *E. wilderae* larvae metamorphosed at a significantly smaller size in high temperature treatments compared to low temperature treatments due to a shorter larval period. Females in warmer streams may be larger as larvae but undergo metamorphosis earlier and at a smaller size (see **Chapter 2** for larval period and size). However, warm summer temperatures may promote adult growth indirectly through increased metabolism or resource availability (Ringia & Lips, 2007; Markle, 2015), counteracting the negative relationship between temperature and metamorphic size.

However, as cautioned by Connette et al. (2015), trends in mean adult plethodontid size should be interpreted carefully. Connette et al. (2015) found significant variation in reported body sizes between years and across surveys. This may be caused by differences in annual weather patterns that influence growth and size, or detection biases from factors like rainfall before a survey. Because I calculated mean female SGL using data from only a single survey per site, trends in female SGL might be artifacts of detection bias or variation in annual weather patterns. However, Connette et al. (2015) surveyed a terrestrial salamander, while I surveyed females in the stream. This may help to limit the biases encountered while searching for fossorial salamanders (e.g., rainfall increasing surface activity). Regardless, my results should be interpreted carefully due to small sample sizes and limited survey efforts. While not practical for

this study, multi-year data collection across more sites would help to support (or refute) my findings.

CHAPTER 4: PLETHODONTID SALAMANDER COMMUNITY COMPOSITION ACROSS AN URBAN GRADIENT USING eDNA

4.1 BACKGROUND

Many salamander species experience declines and local extirpations in highly disturbed systems (Barrett & Guyer, 2008; Barrett & Price, 2014), with salamander abundance negatively correlated with urban land-cover (Orser & Shure, 1972; Willson & Dorcas, 2003; Miller et al., 2007). Salamander populations may be decreasing in urban landscapes due to the loss of stream bottom heterogeneity (Hawkins et al., 1983; Finkenbine et al., 2000; Barr & Babbitt, 2002), increased streamflow (Espey et al., 1966; Leopold, 1973; Barrett & Guyer, 2008), loss of riparian buffers (Willson & Dorcas, 2003), and altered temperature regimes (Barr & Babbitt, 2002). Salamanders thus face numerous stressors in urban landscapes that often result in decreased biodiversity (Barrett & Guyer, 2008).

Survey efforts can help determine the extent of urban impact on local salamanders by monitoring biodiversity and characterizing community composition. A relatively new alternative to traditional sampling offers a way to test for species presence using environmental DNA (Willerslev et al., 2003; Ficetola et al., 2008). Environmental DNA (eDNA) is DNA shed into the environment by an organism from sources such as waste products, skin cells, mucous, and saliva (Rees et al., 2014). Survey methods using eDNA require the collection of an environmental sample, usually water, which is then filtered and processed in the lab to determine what organisms the DNA belongs to. Using eDNA offers a less disruptive method of determining species presence/ absence and is particularly useful in detecting cryptic species and

differentiating between morphologically similar species (Jerde et al., 2011; Davy et al., 2015). However, few studies have utilized eDNA to investigate vertebrate community changes across an urban gradient (e.g., Kelly et al., 2016; Charvoz et al., 2021).

Despite the promises of eDNA sampling, results can be difficult to interpret due to the biotic and abiotic factors impacting detection probability. Detection probability can be contingent on the habitat use, density, and biomass of the target species (Pilliod et al., 2013, 2014; Mize et al., 2019; Yates et al., 2019). Detection can also be impacted greatly by environmental factors that influence the movement and degradation of eDNA, such as flow, pH, sedimentation, UV exposure, and temperature (Pilliod et al., 2014; Jane et al., 2015; Stricklet et al., 2015; Stoeckle et al., 2017). Additionally, polymerase chain reaction (PCR) inhibitors, downstream analysis errors, and the stochasticity of PCR contribute to potential issues in detection accuracy using eDNA (Kebschull & Zador, 2015; McKee et al., 2015). Together, these factors may place limits on the accuracy of results.

Here, I investigate plethodontid salamander species richness across an urban gradient. I use eDNA sampling and a metabarcoding approach to avoid the multiple limitations of traditional survey methods, including logistical constraints, multiple cryptic species, and health and safety risks to researchers in urban streams.

4.2 METHODS

4.2.a Sample collection and filtration

I sampled 25 sites along tributaries leading to Peachtree Creek, North Fork Peachtree Creek, and South Fork Peachtree Creek in Fulton County, GA and DeKalb County, GA. These sites

included the 14 sample sites I surveyed for **Chapters 1 – 3**. I selected sites to cover a range of habitat disturbance, ISC, and drainage basin areas.

At each site, I entered the stream downstream of where I collected the water sample. With gloves on, I filled a 1 L plastic bottle with stream water, moved upstream roughly 2 m and repeated this process with a new bottle until I filled three 1 L bottles. If the stream was too shallow, I used a fresh paper cup to transfer water into the 1 L bottle. I filled an additional 1 L bottle with distilled water at each site using a new paper cup and gloves to act as a negative control. I stored samples in a cooler with ice packs until filtration, which occurred within 10 hours. I collected all eDNA samples between 20 and 24 November 2021.

I filtered each 1L sample through a 0.45 µm cellulose nitrate filter (Thermo Fisher Scientific; MA, USA) using a vacuum filtration system. I ended filtration if a sample was taking longer than one hour to filter, and recorded the total volume filtered. Using bleach-sterilized scissors and forceps between filters, I cut each filter in half and stored it at –20°C until DNA extraction, roughly one month later.

4.2.b DNA extraction and PCR

I incubated one half of each filter overnight at 55°C with 180 µL Buffer ATL and 20 µL Proteinase K (Qiagen; Hilden, Germany), vortexing occasionally. After incubation, I moved both the filter and the solution to a QIAshredder spin column (Qiagen; Hilden, Germany) and centrifuged the column for 2 min at 11,000 rpm. I then added 200 µL Buffer AL (Qiagen; Hilden, Germany) to the resulting flow-through and incubated for 10 min at 55°C. Next, I added Sera-Mag SpeedBeads solution (Faircloth & Glenn, 2011; Thermo Fisher Scientific; MA, USA) in a 1:2

ratio of flow-through to SpeedBeads solution and let the samples sit for 15 min. Using a magnet, I separated the SpeedBeads from the solution, discarded the solution, washed the SpeedBeads with 80% ethanol, and suspended the SpeedBeads in 100 µL of molecular-grade water (Faircloth & Glenn, 2011; Rohland & Reich, 2012; Shahraki et al., 2019). I stored the final product at –20°C.

I amplified extracted DNA using the plethodontid salamander metabarcoding protocol described by Glenn et al. (2019). This assay targets the 12S region of the mitochondrial genome, includes a two-step PCR protocol, and results in quadruple-indexed amplicons. For each PCR run, I included a PCR negative control (5 µL of water instead of DNA template) and a PCR positive control (DNA extracted from a *Plethodon shermani* x *P. teyahalee* hybrid from Macon County, NC). I included a PCR inhibitor removal step between the first and second PCR using SpeedBeads in a 2:1 ratio of SpeedBeads to PCR product. All PCRs were run on a T100 Thermo Cycler (Bio-Rad; CA, USA). I verified successful PCR amplification of the target amplicon using 1% agarose gel electrophoresis.

I pooled final PCR products from all 100 field samples (three stream samples and one negative control per site x 25 sites). For each field sample that produced a clear target band in the gel, I added 3 µL of PCR product into the pool. For each field sample that did not produce a clear target band, I added 8 µL of PCR product into the pool. I added SpeedBeads in a 9:10 ratio of SpeedBeads to pooled PCR product and let this sit for 10 minutes. I then put the SpeedBead/ pooled PCR product mix on a magnet, discarded the solution, washed the SpeedBeads with 80% ethanol, and suspended the SpeedBeads in 30 µL of molecular-grade water (Faircloth & Glenn, 2011; Rohland & Reich, 2012; Shahraki et al., 2019). After confirming the presence of the target

amplicon via gel electrophoresis, I added 5.4 µL of the final eluate (with SpeedBeads removed via magnet) to 25 µL of molecular-grade water to create the final eDNA library.

I sent the eDNA library to the Clinical Genomics Center at the Oklahoma Medical Research Foundation for PE150 sequencing on the Illumina MiSeq 300v2 platform. I then demultiplexed and denoised reads with DADA2 (Callahan et al., 2016) in QIIME 2 v2022.2.1 (Bolyen et al., 2019). Before denoising reads, I trimmed off the first 18 bp and truncated to 100 bp in both forward and reverse reads. I then assembled reads against a custom reference database at 85% similarity using QIIME 2 v2022.2.1 (Bolyen et al., 2019). The reference database included sequences from six species of plethodontid salamander known to occur regionally (Table S.5 in **Supplementary Material**). I considered a species present at a site if at least one of the three site replicates contained DNA of that species.

4.2.c Statistical analyses

To evaluate the effect of urbanization on stream salamander species richness, I fit a quasi-Poisson generalized linear model (GLM) of species richness per site as a function of ISC and the log of the drainage basin area using the "glm()" function in R v4.2.3 (R Core Team, 2023). I chose a quasi-Poisson distribution as I expected my data to be overdispersed and discrete (i.e., many sites with low species richness) based on previous literature suggesting low salamander species richness in urban habitats (e.g., Willson & Dorcas, 2003; Miller et al., 2007; Barrett & Guyer, 2008). Because the data are discrete counts, I checked for overdispersion visually using a histogram of estimated species richness per site to support my model structure.

To test for normality of the model residuals, I ran a Shapiro-Wilk test using the function "shapiro.test()" and inspected Q–Q plots in R. I tested for spatial autocorrelation in the residuals of the model using the "Moran.I()" function from the package "ape" v5.7–1 (Paradis & Schliep, 2019). I used an inverse distance matrix of latitude and longitude of each site to calculate a scaled Moran's I statistic for autocorrelation in the model residuals.

4.3 RESULTS

Of my 75 stream samples, 42 returned at least one read with 85% similarity or more to my reference database of six plethodontid species. Five out of the 25 sampled sites did not return any reads across all site replicates (Fig. 4.1; Table S.6 in **Supplementary Material**). None of the 25 sampling negative controls returned reads (Table S.6 in **Supplementary Material**). The mean number of reads per sample across all species was 4541 ± 7184 SD.

Across 25 sites in urban Atlanta, I identified six species of plethodontid salamanders: *Eurycea cirrigera* (Southern Two-lined Salamander; detected at 19/25 sites), *E. guttolineata* (Three-lined Salamander; detected at 3/25 sites), *Desmognathus perlapsus* (Chattooga Dusky Salamander; detected at 13/25 sites), *D. cheaha* (Talladega Seal Salamander; detected at 3/25 sites), *Pseudotriton ruber* (Red Salamander; detected at 5/25 sites), and *Gyrinophilus porphyriticus* (Spring Salamander; detected at 2/25 sites) (Fig. 4.1).

Because five of my twenty-five sites returned zero sequence reads (despite visual confirmation of salamander presence at these sites), I fit an additional quasi-Poisson GLM of species richness as a function of ISC only including the 20 sites with successful reads. This allowed

me to investigate the effect of urbanization on species richness with minimized bias of likely PCR inhibition (false negatives).

The mean species richness per site for all 25 sites was 1.80 species ± 1.41 SD. The mean species richness per site for only the 20 sites that returned reads was 2.25 species ± 1.21 SD. ISC was a significant predictor of species richness in the 20 sites with reads (B = –0.019 ± 0.008 , t_{17} = –2.52, p = 0.022), but not when all 25 sites were included in the model (B = –0.017 ± 0.011, t_{22} = –1.46, p = 0.158) (Fig. 4.2). The log of the drainage basin area was not significant in either the 20-site model (B = –0.032 ± 0.095, t_{17} = –0.34, p =0.742) nor the 25-site model (B = –0.067 ± 0.111, t_{22} = –0.61, p = 0.549). I did not detect significant spatial autocorrelation in the residuals of the model with the 20 sites with reads (p = 0.103) nor in the residuals of the model with all 25 sites (p = 0.053). The distribution of model residuals did not show evidence of non-normality for the model with 20 sites (W = 0.96, p = 0.46) nor the model with 25 sites (W = 0.92, p = 0.06).

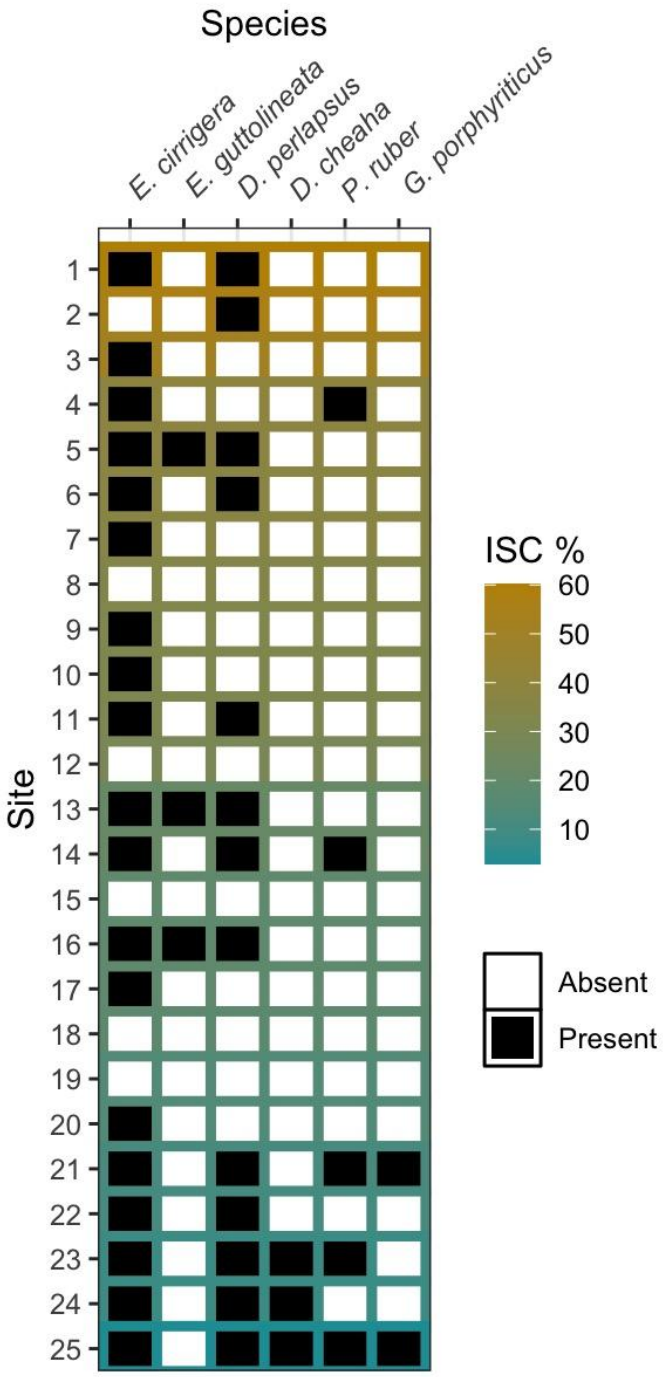


Fig. 4.1: Detection of plethodontid salamanders across 25 urban streams using eDNA. Black boxes indicate successful detection of the species in at least one of three eDNA samples per site. The percent impervious surface cover (ISC) for each site is indicated by the box outline color (blue = low; yellow = high).

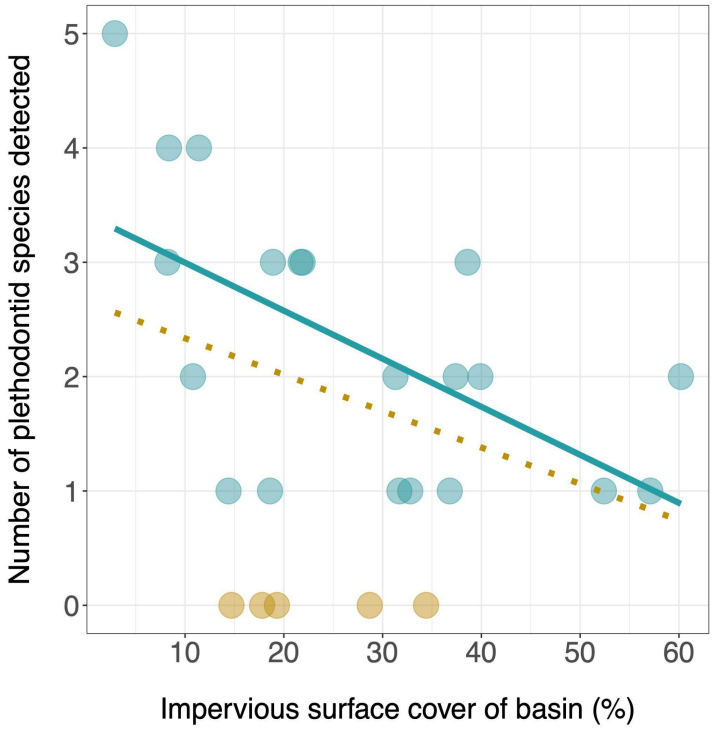


Fig. 4.2: The number of plethodontid salamander species detected as a function of the percent impervious surface cover (ISC) of each site. Yellow dots represent sites with zero species detected due to potential PCR inhibition. Blue dots represent sites with at least one species detected. Lines represent linear regressions of the number of species detected using eDNA as a function of ISC for all sites (yellow, dotted line) and only sites with at least one species detected (blue line).

4.4 DISCUSSION

Five of my twenty-five sites returned zero sequence reads despite confirmed salamander presence at these sites. These results are likely due to PCR inhibition, a common problem in environmental samples (Schrader et al., 2012). I collected samples in November 2021 when leaf litter was abundant in the streams. Humic and tannic acids from leaf litter are well-known PCR inhibitors (Abbaszadegan et al., 1993; Opel et al., 2010) and may explain my five site-level false negatives. Additionally, Atlanta has historically struggled to keep sewage and treated wastewater

out of streams (Butler et al., 2018; DeKalb County, 2022). Contaminants such as metal ions and proteins from wastewater or surrounding human land use may also have inhibited PCR (Shieh et al., 1995; Rock et al., 2010). Site-level false negatives can decrease the accuracy of eDNA and thus steps should be taken to reduce inhibition and results should be interpreted carefully. I included an inhibitor-reducing step in my protocol, though future studies should include an internal positive control to determine if a negative site is truly negative for all target species or experienced PCR inhibition (Hoorfar et al., 2004). Additionally, I suspect I underestimated species richness at some sites using eDNA. For example, all samples in which I detected *E. guttolineata* returned fewer than 50 reads. Sites that were deemed negative (zero reads) may actually have had *E. guttolineata* in the stream, but DNA concentrations below the limit of detection. Alternatively, *E. guttolineata* may have been entirely terrestrial or have relatively few larvae during my November eDNA sampling (Bruce, 1982b; Maldonado, 2022). Site-level negatives may be validated using conventional surveys and/or repeated eDNA sampling over seasons.

The decrease in plethodontid richness and abundance in urban versus rural streams is well-documented (Orser & Shure, 1972; Willson & Dorcas, 2003; Miller et al., 2007; Barrett & Guyer, 2008; Barrett & Price, 2014). My results provide a finer-scale resolution by investigating plethodontid species richness across an urban gradient. There was a significant negative trend between the percent impervious surface cover of the drainage basin (ISC) and estimated plethodontid species richness at my sites when only the 20 sites with reads were included in the model. When all 25 sites were included in the model, there was still a negative trend between ISC and species richness, though insignificant. Salamander populations may experience declines and local extirpations due to a combination of changes typical of urban streams (Paul & Meyer,

2001; Walsh et al., 2005). The loss of stream bottom heterogeneity and degradation of upland habitat may reduce habitat availability for larval and adult salamanders (Hawkins et al., 1983; Finkenbine et al., 2000; Barr & Babbitt, 2002; Willson & Dorcas, 2003). Increased frequency and severity of flood events can wash away larvae (Espey et al., 1966; Leopold, 1973; Barrett & Guyer, 2008; Barrett et al., 2010a) while altered stream temperature regimes can cause stress or death of larval and adult salamanders (Barr & Babbitt, 2002). My results support the use of ISC as a single metric for urbanization that captures a variety of potential drivers of salamander species loss.

The relationship between species richness and drainage basin area was not significant in either model. Likely, my sites did not span a wide enough range in drainage basin area to see an effect; only 2 of my 25 sites had a drainage basin area larger than 6 km^2. I suspect if I expanded survey efforts to larger streams, I would see a decrease in plethodontid species richness, as these species are usually associated with headwaters and small streams (Petranka, 1998).

While my results detected a general negative trend between ISC and species richness, some species may be more sensitive to environmental changes associated with urbanization than others. To test how urbanization affects the likelihood of a species occupying a site, I would need to collect data from natural streams of similar sizes to my urban streams and in close proximity to urban Atlanta. Without these data (or many more sample sites across an urban gradient), I cannot address how urbanization individually impacts each observed plethodontid species. The occupancy of one species also does not occur independently of other species. It is possibly that with the local extirpation of a sensitive salamander species, a more tolerant species could then

occupy the open niche. Understanding these finer scale shifts in community composition across an urban gradient is important in understanding urban impacts on plethodontids.

CONCLUSION

As more stream kilometers are impacted by urbanization, understanding shifts in life history, fitness, and abundance of stream-reliant species becomes increasingly important. My results contribute to the growing body of literature investigating the effects of urbanization on salamander populations. Additionally, my research helps to disentangle the environmental drivers of multiple life history traits in *Eurycea cirrigera* (Southern Two-lined Salamander). I found that 1) drainage basin area was a better predictor of stream temperature than impervious surface cover, 2) cooler summer temperatures predicted the presence of a two-year larval period and smaller larvae, 3) higher impervious surface cover predicted larger larvae and adult females, 4) larger females were associated with significantly larger clutches, and 5) plethodontid salamander species richness decreased with increasing impervious surface cover.

However, a study far larger than mine would need to be conducted to further disentangle urban-associated environmental changes impacting salamander life history and species richness. Here, I used a single metric of urbanization (ISC) to account for a conglomerate of potential changes to urban streams (primarily hydrology and temperature). With a larger study, "urbanization" could be further broken down into multiple variables of interest (e.g.: changes to peak flow, canopy cover, and vegetative buffer width). I encourage future researchers to continue to explore different ways in which urbanization may impact life history traits of stream biota.

STATEMENT OF INTEGRATION

My thesis had two main objectives: 1) employing traditional field methods to investigate how *Eurycea cirrigera* life history and phenology vary across an urban gradient, and 2) utilizing environmental DNA (eDNA) to assess salamander community composition across an urban gradient.

For my first objective, I integrated multiple disciplines to more robustly assess environmental drivers of salamander life history in urban streams. In exploring the relationships between impervious surface cover (ISC), stream temperature, and salamander life history, I had to draw from chemistry (e.g., specific heat of water, energy exchange processes), hydrology (e.g., changes to flow and stream morphology), social sciences and urban planning (e.g., channelization of stormwater, management of land, human impacts to land), physiology (e.g., endocrinology, metabolic rates, critical thermal maxima), natural history (e.g., habitat use, phenology, fitness), and statistics (e.g., using a path analysis approach to better disentangle environmental drivers of life history).

My second objective utilized a relatively new approach to species monitoring—environmental DNA (eDNA)—as well as a new method of eDNA extraction—SpeedBeads. Similar to my first objective, this objective required the integration of techniques and concepts from multiple disciples across multiple levels of biological organization. These techniques and concepts included molecular lab techniques (e.g., DNA extraction, PCR, sequencing), chemistry (e.g., PCR inhibition, DNA degradation in the environment), natural history (e.g., habitat use, phenology), and ecology (e.g., abundance).

REFERENCES

Abbaszadegan, M., Huber, M. S., Gerba, C. P., & Pepper, I. L. (1993). Detection of enteroviruses in groundwater with the polymerase chain reaction. *Applied and Environmental Microbiology* 59, 1318–1324.

Anderson, N. H., & Wallace, J. B. (1995). Habitat, Life History, and Behavioral Adaptations of Aquatic Insects. In R. W. Merritt & K. W. Cummins (Eds.), *An Introduction to the Aquatic Insects of North America* (3rd ed., pp. 38–58). Kendall Hunt Publishing Company.

Aragón-Sánchez, J., Quintana-Marrero, Y., Aragón-Hernández, C., & Hernández-Herero, M. J. (2017). ImageJ: A free, easy, and reliable method to measure leg ulcers using digital pictures. *International Journal of Lower Extremity Wounds* 16, 269–273.

Arnold, C. L., & Gibbons, J. C. (1996). Impervious surface coverage: the emergence of a key environmental indicator. *Journal of the American Planning Association* 62, 243–258.

Barr, G. E., & Babbitt, K. J. (2002). Effects of biotic and abiotic factors on the distribution and abundance of larval Two-lined Salamanders (*Eurycea bislineata*) across spatial scales. *Oecologia* 133, 176–185.

Barrett, K., & Guyer, C. (2008). Differential responses of amphibians and reptiles in riparian and stream habitats to land use disturbances in western Georgia, USA. *Biological Conservation* 141, 2290–2300.

Barrett, K., & Price, S. J. (2014). Urbanization and stream salamanders: a review, conservation options, and research needs. *Freshwater Science* 33, 927–940.

Barrett, K., Helms, B. S., Guyer, C., & Schoonover, J. E. (2010a). Linking process to pattern: Causes of stream-breeding amphibian decline in urbanized watersheds. *Biological Conservation* 143, 1998–2005.

Barrett, K., Helms, B. S., Samoray, S. T., & Guyer, C. (2010b). Growth patterns of a stream vertebrate differ between urban and forested catchments. *Freshwater Biology* 55, 1628–1635.

Barrett, K., Samoray, S. T., Helms, B. S., & Guyer, C. (2012). Southern Two-lined Salamander diets in urban and forested streams in western Georgia. *Southeastern Naturalist* 11, 287–296.

Beachy, C. K. (1994). Community ecology in streams: Effects of two species of predatory salamanders on a prey species of salamander. *Herpetologica* 50, 129–136.

Beachy, C. K. (1997). Effect of predatory larval *Desmognathus quadramaculatus* on growth, survival, and metamorphosis of larval *Eurycea wilderae*. *Copeia* 1997, 131–137.

Beachy, C. K. (2018). Effects of growth rate and temperature on metamorphosis in *Eurycea wilderae* (Caudata, Plethodontidae, Hemidactyliinae, Spelerpini; Blue Ridge Two-lined Salamander). *Southeastern Naturalist* 17, 423–432.

Beachy, C. K., & Bruce, R. C. (2003). Life history of a small form of the plethodontid salamander *Desmognathus quadramaculatus*. *Amphibia-Reptilia* 24, 13–26.

Beachy, C. K., Ryan, T. J., & Bonett, R. M. (2017). How metamorphosis is different in plethodontids: Larval life history perspectives on life-cycle evolution. *Herpetologica* 73, 252–258.

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., Alexander, H., Alm, E. J., Arumugam, M., Asnicar, F., Bai, Y., Bisanz, J. E., Bittinger, K., Brejnrod, A.,

Brislawn, C. J., Brown, C. T., Callahan, B. J., Caraballo-Rodríguez, A. M., Chase, J., … Caporaso, J. G. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nature Biotechnology* 37, 852–857.

Bonett, R. M. (2016). An integrative endocrine model for the evolution of developmental timing and life history of plethodontids and other salamanders. *Copeia* 104, 209–221.

Bonett, R. M., Steffen, M. A., & Robison, G. A. (2014). Heterochrony repolarized: a phylogenetic analysis of developmental timing in plethodontid salamanders. *EvoDevo* 5.

Booth, D. B., & Jackson, C. R. (1997). Urbanization of aquatic systems: degradation thresholds, stormwater detection, and the limits of mitigation. *Journal of the American Water Resources Association* 33, 1077–1090.

Boyero, L., Cardinale, B. J., Bastian, M., & Pearson, R. G. (2014). Biotic vs. abiotic control of decomposition: a comparison of the effects of simulated extinctions and changes in temperature. *PLoS ONE* 9, Article e87426.

Brophy, T. R. & Pauley, T. K. (2002). Reproduction in West Virginia populations of the southern two-lined salamander (*Eurycea cirrigera*). *The Maryland Naturalist* 45, 13– 22.

Bruce, R. C. (1969). Fecundity in primitive plethodontid salamanders. *Evolution* 23, 50–54.

Bruce, R. C. (1982a). Egg-laying, larval periods and metamorphosis of *Eurycea bislineata* and *E. junaluska* at Santeetlah Creek, North Carolina. *Copeia* 1982, 755–762.

Bruce, R. C. (1982b). Larval periods and metamorphosis in two species of salamanders of the genus *Eurycea*. *Copeia* 1982, 117–127.

Bruce, R. C. (1985). Larval periods, population structure and the effects of stream drift in larvae of the salamanders *Desmognathus quadramaculatus* and *Leurognathus marmoratus* in a southern Appalachian stream. *Copeia* 1985, 847–854.

Bruce, R. C. (1988). An ecological life table for the salamander *Eurycea wilderae*. *Copeia* 1988, 15–26.

Burton, T. M., & Likens, G. E. (1975). Salamander populations and biomass in the Hubbard Brook Experimental Forest, New Hampshire. *Copeia* 1975, 541–546.

Butler, K., Hamann, J., Roach, T., & Ross, J. (2018). *Atlanta is largely in compliance with its combined sewer overflow consent decree, but has not yet met all requirements*. United States Environmental Protection Agency: Washington, DC.

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., & Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods* 13, 581–583.

Charvoz, L., Apothéloz-Perret-Gentil, L., Reo, E., Thiébaud, J., & Pawlowski, J. (2021). Monitoring newt communities in urban area using eDNA metabarcoding. *PeerJ* 9, Article e12357.

Connette, G. M., Crawford, J. A., & Peterman, W. E. (2015). Climate change and shrinking salamanders: Alternative mechanisms for changes in plethodontid salamander body size. *Global Change Biology* 21, 2834–2843.

Daszak, P., Cunningham, A. A., & Hyatt, A. D. (2000). Emerging infectious diseases of wildlife-threats to biodiversity and human health. *Science* 287, 443–449.

Davic, R. D., & Orr, L. P. (1987). The relationship between rock density and salamander density in a mountain stream. *Herpetologica* 43, 357–361.

Davis, A. K., & Maerz, J. C. (2009). Effects of larval density on hematological stress indices in salamanders. *Journal of Experimental Zoology* 311A, 697–704.

Davy, C. M., Kidd, A. G., & Wilson, C. C. (2015). Development and validation of environmental DNA (eDNA) markers for detection of freshwater turtles. *PLoS ONE* 10, Article e0130965.

De Cicco, L.A., Hirsch, R.M., Lorenz, D., Watkins, W.D., & Johnson, M. (2022). dataRetrieval: R packages for discovering and retrieving water data available from Federal hydrologic web services, v.2.7.12. *U.S. Geological Survey*.

DeKalb County. (2021). *Annual Report No. 10*. DeKalb County Department of Watershed Management: Decatur, GA.

Demars, B. O. L., Manson, J. R., Ólafsson, J. S., Gíslason, G. M., Gudmundsdóttir, R., Woodward, G., Reiss, J., Pichler, D. E., Rasmussen, J. J., & Friberg, N. (2011). Temperature and the metabolic balance of streams. *Freshwater Biology* 56, 1106–1121.

Denver, R. J. (1997). Environmental stress as a developmental cue: Corticotropin-releasing hormone is a proximate mediator of adaptive phenotypic plasticity in amphibian metamorphosis. *Hormones and Behavior* 31, 169–179.

Denver, R. J. (2009). Stress hormones mediate environment-genotype interactions during amphibian development. *General and Comparative Endocrinology* 164, 20–31.

Duellman, W. E., & Wood, J. T. (1954). Size and growth of the Two-lined Salamander, *Eurycea bislineata rivicola*. *Copeia* 1954, 92–96.

Dunn, E. R. (1920). Some reptiles and amphibians from Virginia, North Carolina, Tennessee, and Alabama. *Proceedings of the Biological Society of Washington* 33, 129–138.

Dunne, T., & Leopold, L. B. (1978). *Water in Environmental Planning* (1st ed.). W.H. Freeman and Company.

Elmore, A. J., & Kaushal, S. S. (2008). Disappearing headwaters: Patterns of stream burial due to urbanization. *Frontiers in Ecology and the Environment* 6, 308–312.

Espey, W. H. Jr., Morgan, C. W., & Masch, F. D. (1966). Study of some effects of urbanization on storm runoff from a small watershed. In *Report 44D 07–6501 CRWR-2.*

Faircloth, B., & Glenn, T. (2011). *Serapure*. University of California, Los Angeles. https://ethanomics.files.wordpress.com/2012/08/serapure_v2-2.pdf

Ficetola, G. F., Miaud, C., Pompanon, F., & Taberlet, P. (2008). Species detection using environmental DNA from water samples. *Biology Letters* 4, 423–425.

Finkenbine, J. K., Atwater, J. W., & Mavinic, D. S. (2000). Stream health after urbanization. *Journal of the American Water Resources Association* 36, 1149–1160.

Fusco, N. A., Pehek, E., & Munshi-South, J. (2021). Urbanization reduces gene flow but not genetic diversity of stream salamander populations in the New York City metropolitan area. *Evolutionary Applications* 14, 99–116.

Galli, J. (1990). *Thermal impacts associated with urbanization and stormwater best management practices*. Metropolitan Washington Council of Governments: Washington, DC.

Glenn, T. C., Nilsen, R. A., Kieran, T. J., Sanders, J. G., Bayona-Vásquez, N. J., Finger, J. W., Pierson, T. W., Bentley, K. E., Hoffberg, S. L., Louha, S., Garcia-De Leon, F. J., del Rio Portilla, M. A., Reed, K. D., Anderson, J. L., Meece, J. K., Aggrey, S. E., Rekaya, R., Alabady, M., Belanger, M. … Faircloth, B. C. (2019). Adapterama I: Universal stubs and primers for 384 unique

dual-indexed or 147,456 combinatorially-indexed Illumina libraries (iTru & iNext). *PeerJ* 7, Article e7755.

Green, J. (1818). Descriptions of several species of North American Amphibia, accompanied with observations. *Journal of the Academy of Natural Sciences of Philadelphia* 1, 348–359.

Gustafson, M. P. (1993). Intraguild predation among larval plethodontid salamanders: A field experiment in artificial stream pools. *Oecologia* 96, 271–275.

Guy, C. J., Ratajczak, R. E., & Grossman, G. D. (2004). Nest-site selection by Southern Two-lined Salamanders (*Eurycea cirrigera*) in the Georgia Piedmont. *Southeastern Naturalist* 3, 75–88.

Harding, J. H., & Mifsud, D. A. (2017). *Amphibians and Reptiles of the Great Lakes Region*. University of Michigan Press, USA.

Hawkins, C. P., Murphy, M. L., Anderson, N. H., & Wilzbach, M. A. (1983). Density of fish and salamanders in relation to riparian canopy and physical habitat in streams of the Northwestern United States. *Canadian Journal of Fisheries and Aquatic Sciences* 40, 1173–1185.

Hester, E. T., & Bauman, K. S. (2013). Stream and retention pond thermal response to heated summer runoff from urban impervious surfaces. *Journal of the American Water Resources Association* 49, 328–342.

Hom, C. L. (1987). Reproductive ecology of female Dusky Salamanders, *Desmognathus fuscus* (Plethodontidae), in the Southern Appalachians. *Copeia* 1987, 768–777.

Hom, C. L., Willits, N. H., & Clark, C. W. (1990). Fitness consequences of nest defense in plethodontid salamanders: Predictions of a dynamic optimization model. *Herpetologica* 46, 304–319.

Homer, C., Dewitz, J., Yang, L., Jin, S., Danielson, P., Xian, G., Coulston, J., Herold, N., Wickham, J., & Megown, K. (2015). Completion of the 2011 National Land Cover Database for the conterminous United States- representing a decade of land cover change information. *Photogrammetric Engineering and Remote Sensing* 81, 346–354.

Hoorfar, J., Malorny, B., Abdulmawjood, A., Cook, N., Wagner, M., & Fach, P. (2004). Practical considerations in design of internal amplification controls for diagnostic PCR assays. *Journal of Clinical Microbiology* 42, 1863–1868.

Howard, J. S., & Maerz, J. C. (2022). A model for estimating final clutch size from follicle counts in plethodontid salamanders. *Journal of Herpetology* 56, 191–195.

Hutchison, V. H. (1961). Critical thermal maxima in salamanders. *Physiological Zoology* 34, 92–125.

Jane, S. F., Wilcox, T. M., McKelvey, K. S., Young, M. K., Schwartz, M. K., Lowe, W. H., Letcher, B. H., & Whiteley, A. R. (2015). Distance, flow and PCR inhibition: eDNA dynamics in two headwater streams. *Molecular Ecology Resources* 15, 216–227.

Jerde, C. L., Mahon, A. R., Chadderton, W. L., & Lodge, D. M. (2011). “Sight-unseen” detection of rare aquatic species using environmental DNA. *Conservation Letters* 4, 150–157.

Johnson, M. F., Wilby, R. L., & Toone, J. A. (2014). Inferring air-water temperature relationships from river and catchment properties. *Hydrological Processes* 28, 2912–2928.

Kebschull, J. M., & Zador, A. M. (2015). Sources of PCR-induced distortions in high-throughput sequencing data sets. *Nucleic Acids Research* 43, Article e143.

Kelly, R. P., O'Donnell, J. L., Lowell, N. C., Shelton, A. O., Samhouri, J. F., Hennessey, S. M., Feist, B. E., & Williams, G. D. (2016). Genetic signatures of ecological diversity along an urbanization gradient. *PeerJ* 4, Article e2444.

Kim, H. J. (2007). *Temperatures of urban streams: Impervious surface cover, runoff, and the importance of spatial and temporal variations*. [Master's thesis, University of Maryland].

Kinouchi, T. (2007). Impact of long-term water and energy consumption in Tokyo on wastewater effluent: Implications for the thermal degradation of urban streams. *Hydrological Processes* 21, 1207–1216.

Kozak, K. H. (2017). What drives variation in plethodontid salamander species richness over space and time? *Herpetologica* 73, 220–228.

Kozak, K. H., Blaine, R. A. & Larson, A. (2006). Gene lineages and eastern North American palaeodrainage basins: phylogeography and speciation in salamanders of the *Eurycea bislineata* species complex. *Molecular Ecology* 15, 191– 207.

Kuhn, M. (2008). Building predictive models in R using the caret package. *Journal of Statistical Software* 28, 1–26.

Latimer, J. S., & Quinn James G. (1998). Aliphatic petroleum and biogenic hydrocarbons entering Narragansett Bay from tributaries under dry weather conditions. *Estuaries* 21, 91–107.

Leach, J. A., Kelleher, C., Kurylyk, B. L., Moore, R. D., & Neilson, B. T. (2023). A primer on stream temperature processes. *WIREs Water*, Article e1643.

Lefcheck, J. S. (2016). PiecewiseSEM: Piecewise structural equation modeling in R for ecology, evolution, and systematics. *Methods in Ecology and Evolution* 7, 573–579.

Leopold, L. B. (1973). River channel change with time: An example. *Geological Society of America Bulletin* 84, 1845–1860.

Maldonado, B. R. (2022). *Larval life history of the Three-lined Salamander* Eurycea guttolineata *(Caudata: Plethodontidae)*. [Master's thesis, Southeastern Louisiana University].

Markle, T. M. (2015). *Ecology and evolution of geographic range size variation in North American plethodontid salamanders: Perspectives from thermal physiology*. [Doctoral dissertation, University of Minnesota].

Marshall, J. L., & Camp, C. D. (2006). Environmental correlates of species and genetic richness in lungless salamanders (family Plethodontidae). *Acta Oecologica* 29, 33–44.

McKee, A. M., Spear, S. F., & Pierson, T. W. (2015). The effect of dilution and the use of a post-extraction nucleic acid purification column on the accuracy, precision, and inhibition of environmental DNA samples. *Biological Conservation* 183, 70–76.

McKinney, M. L. (2002). Urbanization, biodiversity, and conservation. *BioScience* 52, 883–890.

McKinney, M. L., & Lockwood, J. L. (1999). Biotic homogenization: A few winners replacing many losers in the next mass extinction. *Trends in Ecology & Evolution* 14, 450–453.

Meyer, J. L., & Wallace, J. B. (2001). Lost linkages and lotic ecology: Rediscovering small streams. In M. C. Press, N. J. Huntly, & S. Levin (Eds.), *The 41st Symposium of the British Ecological Society* (pp. 295–317). Blackwell Science.

Meyer, J. L., Paul, M. J., & Taulbee, W. K. (2005). Stream ecosystem function in urbanizing landscapes. *Journal of the North American Benthological Society* 24, 602–612.

Meyer, J. L., Wallace, J. B., & Eggert, S. L. (1998). Leaf litter as a source of dissolved organic carbon in streams. *Ecosystems* 1, 240–249.

Milanovich, J. R., Trauth, S. E., Saugey, D. A., & Jordan, R. R. (2006). Fecundity, reproductive ecology, and influence of precipitation on clutch size in the Western Slimy Salamander (*Plethodon albagula*). *Herpetologica* 62, 292–301.

Miller, J. E., Hess, G. R., & Moorman, C. E. (2007). Southern Two-lined Salamanders in urbanizing watersheds. *Urban Ecosystems* 10, 73–85.

Mize, E. L., Erickson, R. A., Merkes, C. M., Berndt, N., Bockrath, K., Credico, J., Grueneis, N., Merry, J., Mosel, K., Tuttle-Lau, M., von Ruden, K., Woiak, Z., Amberg, J. J., Baerwaldt, K., Finney, S., & Monroe, E. (2019). Refinement of eDNA as an early monitoring tool at the landscape-level: Study design considerations. *Ecological Applications* 29, 1374–1388.

Morgan, R. P., & Cushman, S. F. (2005). Urbanization effects on stream fish assemblages in Maryland, USA. *Freshwater Science* 24, 643–655.

Moriya, T. (1983). The effect of temperature on the action of thyroid hormone and prolactin in larvae of the salamander *Hynobius retardatus*. *General and Comparative Endocrinology* 49, 1–7.

Murphy, M. V. (2022). SemEff: Automatic calculation of effects for piecewise structural equation models. v 0.6.1.

Murphy, M. O., Agha, M., Maigret, T. A., Price, S. J., & Dorcas, M. E. (2016). The effects of urbanization on body size of larval stream salamanders. *Urban Ecosystems* 19, 275–286.

Murray, M. H., Sánchez, C. A., Becker, D. J., Byers, K. A., Worsley-Tonks, K. E. L., & Craft, M. E. (2019). City sicker? A meta-analysis of wildlife health and urbanization. *Frontiers in Ecology and the Environment* 17, 575–583.

Nelson, K. C., & Palmer, M. A. (2007). Stream temperature surges under urbanization and climate change: Data, models, and responses. *Journal of the American Water Resources Association* 43, 440–452.

Opel, K. L., Chung, D., & McCord, B. R. (2010). A study of PCR inhibition mechanisms using real time PCR. *Journal of Forensic Sciences* 55, 25–33.

Orser, P. N., & Shure, D. J. (1972). Effects of urbanization on the salamander *Desmognathus fuscus fuscus*. *Ecology* 53, 1148–1154.

Ouyang, W., Mueller, F., Hjelmare, M., Lundberg, E., & Zimmer, C. (2019). ImJoy: An open-source computational platform for the deep learning era. *Nature Methods* 16, 1199–1200.

Paradis, E., & Schliep, K. (2019). Ape 5.0: An environment for modern phylogenetics and evolutionary analyses in R. *Bioinformatics* 35, 526–528.

Paul, M. J., & Meyer, J. L. (2001). Streams in the urban landscape. *Annual Review of Ecology and Systematics* 32, 333–365.

Peterman, W. E., Crawford, J. A., & Semlitsch, R. D. (2008). Productivity and significance of headwater streams: Population structure and biomass of the Black-bellied Salamander (*Desmognathus quadramaculatus*). *Freshwater Biology* 53, 347–357.

Petranka, J. W. (1984). Ontogeny of the diet and feeding behavior of *Eurycea bislineata* larvae. *Journal of Herpetology* 18, 48–55.

Petranka, J. W. (1989). Density-dependent growth and survival of larval *Ambystoma*: Evidence from whole-pond manipulations. *Ecology* 70, 1752–1767.

Petranka, J. W., & Sih, A. (1986). Environmental instability, competition, and density-dependent growth and survivorship of a stream-dwelling salamander. *Ecology* 67, 729–736.

Petranka, J. W. (1998). Salamanders of the United States and Canada. Smithsonian Institution Press, USA.

Pierson, T. W., & Miele, A. (2019). Reproduction and life history of Two-lined Salamanders (*Eurycea* cf. *aquatica*) from the upper Tennessee River Valley, USA. *Herpetological Conservation and Biology* 14, 111–118.

Pierson, T. W., Deitloff, J., Sessions, S. K., Kozak, K. H., & Fitzpatrick, B. M. (2019). Morphological polymorphism associated with alternative reproductive tactics in a plethodontid salamander. *The American Naturalist* 193, 608–618.

Pierson, T. W., Rittenburg, L. T., Kalki, Y., Fields, N., & Hutcheson, K. G. (2022). *Eurycea cirrigera* (Southern Two-lined Salamander). Reproductive Morphology. *Herpetological Review* 53, 457–458.

Pierson, T. W., Blake-Sinclair, J., Rittenburg, L. T., & Kalki, Y. (2023). Geographic variation in the reproductive phenology of a widespread amphibian. *Journal of Herpetology* 57, 125–132.

Pilliod, D. S., Goldberg, C. S., Arkle, R. S., & Waits, L. P. (2013). Estimating occupancy and abundance of stream amphibians using environmental DNA from filtered water samples. *Canadian Journal of Fisheries and Aquatic Sciences* 70, 1123–1130.

Pilliod, D. S., Goldberg, C. S., Arkle, R. S., & Waits, L. P. (2014). Factors influencing detection of eDNA from a stream-dwelling amphibian. *Molecular Ecology Resources* 14, 109–116.

Price, S. J., Browne, R. A., & Dorcas, M. E. (2011). Evaluating the effects of urbanisation on salamander abundances using a before-after control-impact design. *Freshwater Biology* 57, 193–203.

R Core Team (2023). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/

Rees, H. C., Maddison, B. C., Middleditch, D. J., Patmore, J. R. M., & Gough, K. C. (2014). The detection of aquatic animal species using environmental DNA - a review of eDNA as a survey tool in ecology. *Journal of Applied Ecology* 51, 1450–1459.

Resetarits, W. J. (1991). Ecological interactions among predators in experimental stream communities. *Ecology* 72, 1782–1793.

Rice, J. S., Anderson, W. P., & Thaxton, C. S. (2011). Urbanization influences on stream temperature behavior within low-discharge headwater streams. *Hydrological Research Letters* 5, 27–31.

Ringia, A. M., & Lips, K. R. (2007). Oviposition, early development and growth of the Cave Salamader, *Eurycea lucifuga*: Surface and subterranean influences on a troglophilic species. *Herpetologica* 63, 258–268.

Robin, X., Turck, N., Hainard, A., Tiberti, N., Lisacek, F., Sanchez, J., & Müller, M. (2010). pROC: an open-source package for R and S+ to analyze and compare ROC curves. *BMC Bioinformatics* 12.

Rock, C., Alum, A., & Abbaszadegan, M. (2010). PCR inhibitor levels in concentrates of biosolid samples predicted by a new method based on excitation-emission matrix spectroscopy. *Applied and Environmental Microbiology* 76, 8102–8109.

Rohland, N., & Reich, D. (2012). Cost-effective, high-throughput DNA sequencing libraries for multiplexed target capture. *Genome Research* 22, 939–946.

Rose, C. S. (1995). Skeletal morphogenesis in the urodele skull: II. Effect of developmental stage in thyroid hormone-induced remodeling. *Journal of Morphology* 223, 149–166.

Rose, F. L., & Bush, F. M. (1963). A new species of *Eurycea* (Amphibia: Caudata) from the Southeastern United States. *Tulane Studies in Ecology* 10, 121–128.

Scheffers, B. R., & Paszkowski, C. A. (2012). The effects of urbanization on North American amphibian species: Identifying new directions for urban conservation. *Urban Ecosystems* 15, 133–147.

Schrader, C., Schielke, A., Ellerbroek, L., & Johne, R. (2012). PCR inhibitors - occurrence, properties and removal. *Journal of Applied Microbiology* 113, 1014–1026.

Scrucca, L., Fop, M., Murphy, T. B., & Raftery, A. E. (2016). mclust 5: clustering, classification and density estimation using Gaussian finite mixture models. *The R Journal* 8, 289–317.

Semlitsch, R. D. (1980). Growth and metamorphosis of larval Dwarf Salamanders (*Eurycea quadridigitata*). *Herpetologica* 36, 138–140.

Sever, D. M. (1979). Male secondary sexual characters of the *Eurycea bislineata* (Amphibia, Urodela, Plethodontidae) complex in the sourthern Appalachian Mountains. *Journal of Herpetology* 13, 245–253.

Sever, D. M., Dundee, H. A., & Sullivan, C. D. (1976). A new *Eurycea* (Amphibia: Plethodontidae) from southwestern North Carolina. *Herpetologica* 32, 26–29.

Shahraki, A. H., Chaganti, S. R., & Heath, D. (2019). Assessing high-throughput environmental DNA extraction methods for meta-barcode characterization of aquatic microbial communities. *Journal of Water and Health* 17, 37–49.

Shieh, Y. S., Wait, D., Tai, L., & Sobsey, M. D. (1995). Methods to remove inhibitors in sewage and other fecal wastes for enterovirus detection by the polymerase chain reaction. *Journal of Virological Methods* 54, 51–66.

Shochat, E., Warren, P. S., Faeth, S. H., McIntyre, N. E., & Hope, D. (2006). From patterns to emerging processes in mechanistic urban ecology. *Trends in Ecology & Evolution* 21, 186–191.

Smith, S., & Grossman, G. D. (2003). Stream microhabitat use by larval Southern Two-lined Salamanders (*Eurycea cirrigera*) in the Georgia Piedmont. *Copeia* 2003, 531–543.

Stoeckle, B. C., Beggel, S., Cerwenka, A. F., Motivans, E., Kuehn, R., & Geist, J. (2017). A systematic approach to evaluate the influence of environmental conditions on eDNA detection success in aquatic ecosystems. *PLoS ONE* 12, Article e0189119.

Strickland, J. C., Pinheiro, A. P., Cecala, K. K., & Dorcas, M. E. (2016). Relationship between behavioral thermoregulation and physiological function in larval stream salamanders. *Journal of Herpetology* 50, 239–244.

Strickler, K. M., Fremier, A. K., & Goldberg, C. S. (2015). Quantifying effects of UV-B, temperature, and pH on eDNA degradation in aquatic microcosms. *Biological Conservation* 183, 85–92.

Stuart, B. L., Beamer, D. A., Farrington, H. L., Beane, J. C., Chek, D. L., Pusser, L. T., Som, H. E., Stephan, D. L., Sever, D. M., & Braswell, A. L. (2020). A new Two-lined Salamander

(*Eurycea bislineata* complex) from the Sandhills of North Carolina. *Herpetologica* 76, 423–444.

Taylor, C. L., Wilkinson Jr., R. F., & Peterson, C. L. (1990). Reproductive patterns of five plethodontid salamanders from the Ouachita Mountains. *The Southwestern Naturalist* 35, 468–472.

Terando, A. J., Costanza, J., Belyea, C., Dunn, R. R., McKerrow, A., & Collazo, J. A. (2014). The southern megalopolis: Using the past to predict the future of urban sprawl in the Southeast U.S. *PLoS ONE* 9, Article e102261.

Thompson, A. M., Kim, K., & Vandermuss, A. J. (2008). Thermal characteristics of stormwater runoff from asphalt and sod surfaces. *Journal of the American Water Resources Association* 44, 1325–1336.

U.S. Geological Survey. (1999). The quality of our nation's waters: nutrients and pesticides. *U.S. Geological Survey Circular* 1225.

U.S. Geological Survey. (2016). The StreamStats program, online at http://streamstats.usgs.gov

Van Metre, P. C., Waite, I. R., Qi, S., Mahler, B., Terando, A., Wieczorek, M., Meador, M., Bradley, P., Journey, C., Schmidt, T., & Carlisle, D. (2019). Projected urban growth in the southeastern USA puts small streams at risk. *PLoS ONE* 14. Article e0222714.

Voss, R. S. (1993). Relationship between stream order and length of larval period in the salamander *Eurycea wilderae*. *Copeia* 1993, 736–742.

Walsh, C. J., Roy, A. H., Feminella, J. W., Cottingham, P. D., Groffman, P. M.. & Morgan, R. P. (2005). The urban stream syndrome: Current knowledge and the search for a cure. *Journal of the North American Benthological Society* 24, 706–723.

Webb, B. W., & Zhang, Y. (1999). Water temperatures and heat budgets in Dorset chalk water courses. *Hydrological Processes* 13, 309–321.

Webster, J. R., & Benfield, E. F. (1986). Vascular plant breakdown in freshwater ecosystems. *Annual Review of Ecology and Systematics* 17, 567–594.

Werner, E. E. (1986). Amphibian metamorphosis: Growth rate, predation risk, and the optimal size at transformation. *The American Naturalist* 128, 319–341.

Willerslev, E., Hansen, A. J., Binladen, J., Brand, T. B., Gilbert, M. T. P., Shapiro, B., Bunce, M., Wiuf, C., Gilichinsky, D. A., & Cooper, A. (2003). Diverse plant and animal genetic records from Holocene and Pleistocene sediments. *Science* 300, 791–795.

Willson, J. D. & Dorcas, M. E. (2003). Effects of habitat disturbance on stream salamanders: Implications for buffer zones and watershed management. *Conservation Biology* 17, 763–771.

Wood, J. T., & McCutcheon, H. N. (1954). Ovarian egg complements and nests of the Two-lined Salamander, *Eurycea b. bislineata* X *cirrigera*, from southeastern Virginia. *The American Midland Naturalist* 52, 433–436.

Yates, M. C., Fraser, D. J., & Derry, A. M. (2019). Meta-analysis supports further refinement of eDNA for monitoring aquatic species-specific abundance in nature. *Environmental DNA* 1, 5–13.

Zweifel, R. G. (1957). Studies on the critical thermal maxima of salamanders. *Ecology* 38, 64–69.

SUPPLEMENTARY MATERIAL

Table S.1: Latitude (WGS 84), longitude (WGS 84), percent impervious surface cover (ISC), and drainage basin area for my 14 sample sites. DJNP = Daniel Johnson Nature Preserve. DHGC = Druid Hills Golf Club. DHGC "Fernbank" and "Lullwater" refer to properties adjacent to each site.

Site	Latitude	Longitude	ISC (%)	Drainage basin area (km^2)
Beaverbrook Park	33.81757	−84.42092	31.7	1.7871
DJNP	33.79746	−84.34249	21.7	2.5900
Deepdene Park	33.77232	−84.32191	8.4	0.3108
DHGC Fernbank	33.77736	−84.32625	11.4	2.4346
DHGC Lullwater	33.78329	−84.32895	19.3	3.9886
Eubanks Park	33.79311	−84.37760	31.3	0.4403
Forest A	33.77733	−84.32369	2.9	0.3108
Forest B	33.80417	−84.32288	28.7	50.5048
Golf Course A	33.80210	−84.37549	14.4	0.2590
Heaton Park	33.78243	−84.31803	8.2	0.0940
Needham Park	33.79953	−84.25947	34.4	0.1269
Peachtree Hills Park	33.81830	−84.37700	37.4	3.3929
Sunnybrook Park	33.83087	−84.37659	38.6	0.4662
WD Thomson Park	33.81023	−84.31736	17.8	0.1790

Table S.2: Mean temperature, mean daily temperature range, maximum HOBO logger reading, and minimum HOBO logger reading for January and August 2022 for my 14 sample sites. Mean temperature and mean daily temperature range in January 2022 for Beaverbrook Park and Sunnybrook Park based on interpolation from model. DJNP = Daniel Johnson Nature Preserve. DHGC = Druid Hills Golf Club.

Site	Jan. mean temp (°C)	Jan. range in temps (°C)	Jan. max temp (°C)	Jan. min temp (°C)	Aug. mean temp (°C)	Aug. range in temps (°C)	Aug. max temp (°C)	Aug. min temp (°C)
Beaverbrook Park	9.05	2.37	-	-	24.42	1.12	26.29	23.00
DJNP	8.70	2.34	18.43	4.21	23.47	1.08	25.71	22.14
Deepdene Park	9.81	2.72	18.81	5.14	21.37	1.47	25.22	19.95
DHGC Fernbank	8.97	2.86	18.43	3.89	22.85	1.78	25.13	21.09
DHGC Lullwater	9.29	1.59	17.67	5.45	23.91	2.49	26.68	21.95
Eubanks Park	10.24	2.53	17.76	4.52	23.45	2.01	27.86	21.76
Forest A	8.69	2.45	17.67	4.21	20.99	0.55	21.86	20.23
Forest B	7.99	1.69	18.05	3.47	24.68	1.14	26.29	23.00
Golf Course A	9.22	2.92	18.71	3.79	24.47	4.78	29.95	21.57
Heaton Park	11.96	1.38	17.86	5.96	20.39	1.40	25.81	19.47
Needham Park	12.17	2.05	19.09	4.62	20.73	1.86	26.59	19.47
Peachtree Hills Park	8.28	2.57	18.33	3.05	23.57	1.14	25.71	22.14
Sunnybrook Park	9.29	2.25	-	-	23.19	1.18	25.32	21.66
WD Thomson Park	8.71	2.79	18.05	3.68	21.45	0.78	24.26	20.71

Table S.3: The presence (1) or absence (0) of a two-year larval period based on visual assessment of histograms as well as Gaussian mixture models by the package "mclust" (Scrucca et al., 2016), mean snout-vent length (SVL) of first-year larvae, number of nests, mean clutch size, mean snout-girdle length (SGL) of all females, number of females captured (number of escaped females in parentheses), and mean estimated ordinal date of oviposition per site. DJNP = Daniel Johnson Nature Preserve. DHGC = Druid Hills Golf Club.

Site	Visual larval period	Mclust larval period	Mean larval SVL (mm)	Number of nests	Mean clutch size (# eggs)	Mean female SGL (mm)	Number of females	Ordinal date of ovi-position
Beaverbrook Park	0	0	26.43	4	49.00	38.29	15	16
DJNP	0	0	24.73	6	68.50	38.23	31 (1)	22
Deepdene Park	1	1	19.78	18	54.78	38.58	21 (1)	25.47
DHGC Fernbank	0	0	21.76	1	60.00	37.68	1	30
DHGC Lullwater	0	0	26.67	0	-	-	0	NA
Eubanks Park	0	1	33.50	13	72.54	40.02	6 (1)	16.56
Forest A	1	1	16.60	10	60.40	38.43	13	28
Forest B	0	0	24.05	0	-	39.52	1	NA
Golf Course A	0	0	34.50	6	59.83	37.55	6	30
Heaton Park	1	1	13.59	8	64.50	37.50	8	24
Needham Park	1	1	21.39	7	53.71	42.06	2	16.33
Peachtree Hills Park	0	0	24.69	0	-	-	0	NA
Sunnybrook Park	1	0	26.65	11	66.18	39.40	18 (5)	14.89
WD Thomson Park	1	1	16.19	8	46.75	38.30	15	22

Table S.4: Nest ID, date of observation, site name, encounter IDs (EIDs) for adults associated with the nest, clutch size, development stage, and overturned status (1 = nest site found overturned) of each nest observed in January 2022. DJNP = Daniel Johnson Nature Preserve. DHGC = Druid Hills Golf Club.

Nest ID	Date	Site	Adult 1 EID	Adult 2 EID	Adult 3 EID	Female SGL	Clutch size (# eggs)	Develop-mental stage	Overturned
1	1/22/22	Eubanks Park	564				7	2	0
2	1/22/22	Eubanks Park					84	2	0
3	1/22/22	Eubanks Park					80	1	1
4	1/22/22	Eubanks Park					107	1	0
5	1/22/22	Eubanks Park					57	1	1
6	1/22/22	Eubanks Park	565			41.43	117	2	0
7	1/22/22	Eubanks Park					74	1	1
8	1/22/22	Eubanks Park					24	2	0
9	1/22/22	Eubanks Park					51	1	0
10	1/22/22	Eubanks Park	566			42.15	70	1	0
11	1/22/22	Eubanks Park	567				98	1	0
12	1/22/22	Eubanks Park					99	1	1
13	1/22/22	Eubanks Park	571			40.85	75	1	0
14	1/22/22	Sunnybrook Park	576			42.40	93	2	0
15	1/22/22	Sunnybrook Park	577			41.15	82	1	0
16	1/22/22	Sunnybrook Park	578				69	1	0
17	1/22/22	Sunnybrook Park	580			41.10	78	1	0
18	1/22/22	Sunnybrook Park	581			35.61	30	1	0
19	1/22/22	Sunnybrook Park	583			38.77	58	3	0
20	1/22/22	Sunnybrook Park	584			41.16	75	2	0
21	1/22/22	Sunnybrook Park					28	1	0
22	1/22/22	Sunnybrook Park	589			34.20	64	2	0
23	1/22/22	Sunnybrook Park					74	2	0
24	1/22/22	Sunnybrook Park	591			39.63	77	1	0
25	1/22/22	Beaverbrook Park	599				19	2	0
26	1/22/22	Beaverbrook Park	599				81	1	0
27	1/22/22	Beaverbrook Park					26	2	0
28	1/22/22	Beaverbrook Park					70	2	0
29	1/23/22	WD Thomson Park					24	1	0
30	1/23/22	WD Thomson Park					38	1	0
31	1/23/22	WD Thomson Park	627	628			2	1	0
32	1/23/22	WD Thomson Park	630				59	1	0

33	1/23/22	WD Thomson Park	633				84	1	0
34	1/23/22	WD Thomson Park	635				87	1	0
35	1/23/22	WD Thomson Park					22	1	1
36	1/23/22	WD Thomson Park	636				58	1	0
37	1/23/22	DJNP	658	659			77	1	0
38	1/23/22	DJNP	660	661	662		83	1	0
39	1/23/22	DJNP	660	661	662		82	1	0
40	1/23/22	DJNP	680			36.17	61	1	0
41	1/23/22	DJNP	683				21	1	0
42	1/23/22	DJNP	684			38.78	87	1	0
43	1/29/22	Needham Park					32	3	0
44	1/29/22	Needham Park	690			42.06	59	3	0
45	1/29/22	Needham Park					53	1	0
46	1/29/22	Needham Park	691			36.30	42	1	0
47	1/29/22	Needham Park					54	2	0
48	1/29/22	Needham Park	692				74	2	0
49	1/29/22	Needham Park					62	3	0
50	1/30/22	Forest A	693			39.98	67	1	0
51	1/30/22	Forest A	694			41.65	80	2	0
52	1/30/22	Forest A	695			40.37	80	1	0
53	1/30/22	Forest A	696			36.77	55	1	0
54	1/30/22	Forest A	697	698			40	1	0
55	1/30/22	Forest A	702			39.88	43	1	0
56	1/30/22	Forest A	703	704			62	1	0
57	1/30/22	Forest A					72	1	0
58	1/30/22	Forest A	706			41.69	76	1	0
59	1/30/22	Forest A	707	708			29	1	0
60	1/30/22	Deepdene Park	713				76	1	0
61	1/30/22	Deepdene Park	715	716	717		66	1	0
62	1/30/22	Deepdene Park	718			36.04	25	1	0
63	1/30/22	Deepdene Park	719				49	3	0
64	1/30/22	Deepdene Park	720			38.82	60	1	0
65	1/30/22	Deepdene Park	722	723			66	1	0
66	1/30/22	Deepdene Park	722	723			34	1	0
67	1/30/22	Deepdene Park	724				92	1	0
68	1/30/22	Deepdene Park	726			41.67	55	1	0
69	1/30/22	Deepdene Park	735				5	1	0
70	1/30/22	Deepdene Park					84	1	0
71	1/30/22	Deepdene Park					81	3	0

72	1/30/22	Deepdene Park	740				5	1	0
73	1/30/22	Deepdene Park	741			36.38	48	1	0
74	1/30/22	Deepdene Park	742	743	744		19	3	0
75	1/30/22	Deepdene Park	742	743	744		80	2	0
76	1/30/22	Deepdene Park	746			37.33	66	1	0
77	1/30/22	Deepdene Park	750			41.36	75	1	0
78	1/30/22	Heaton Park	754	755			56	1	0
79	1/30/22	Heaton Park	754	755			48	1	0
80	1/30/22	Heaton Park					24	2	0
81	1/30/22	Heaton Park	759	760	761		60	3	0
82	1/30/22	Heaton Park	759	760	761		118	2	0
83	1/30/22	Heaton Park	759	760	761		68	1	0
84	1/30/22	Heaton Park	763				65	1	0
85	1/30/22	Heaton Park					77	1	0
86	1/31/22	Golf Course A					86	1	0
87	1/31/22	Golf Course A					63	1	0
88	1/31/22	Golf Course A	768				79	1	0
89	1/31/22	Golf Course A					52	1	0
90	1/31/22	Golf Course A	773			38.44	62	1	0
91	1/31/22	Golf Course A	775				17	1	0
92	1/31/22	DHGC Fernbank	777			37.68	60	1	0

Table S.5: Species, collection location and date, GenBank accession number (if applicable), and researchers for each 12S sequence included in eDNA sequence reference database. *Desmognathus perlapsus* identity uncertain; specimen collected from *D. perlapsus* X *D. ocoee* hybrid zone and may be possible mtDNA haplotype from *D. perlapsus.*

Species	Location	Collection date	GenBank accession number	Researchers
Eurycea cirrigera	29.957472, −90.142833		KY752074	Xue,S.-Q. & Han,Y.
Eurycea guttolineata	34.99153, −83.55660	26 Oct. 2013		Pierson, T.W
Desmognathus perlapsus	34.98963, −83.55568	26 Oct. 2013		Pierson, T.W
Desmognathus cheaha	Alabama		AY549668	Rissler et al. 2004
Pseudotriton ruber	34.95406, −83.55270	26 Oct. 2013		Pierson, T.W
Gyrinophilus porphyriticus	34.98963, −83.55568	26 Oct. 2013		Pierson, T.W

Table S.6: Sample ID by site number and replicate letter (N = distilled water sampling negative), number of sequencing reads per species, µL pooled during library preparation, and percent impervious surface cover (%) of the site.

Sample	*Eurycea cirrigera*	*Eurycea guttolineata*	*Desmognathus perlapsus*	*Desmognathus cheaha*	*Pseudotriton ruber*	*Gyrinophilus porphyriticus*	µL pooled	ISC (%)
01_A	10044	0	338	0	0	0	8	60.2
01_B	14777	0	583	0	0	0	3	60.2
01_C	16642	0	1580	0	0	0	3	60.2
01_N	0	0	0	0	0	0	3	60.2
02_A	0	0	0	0	0	0	8	57.1
02_B	0	0	8	0	0	0	8	57.1
02_C	0	0	0	0	0	0	8	57.1
02_N	0	0	0	0	0	0	8	57.1
03_A	0	0	0	0	0	0	8	52.4
03_B	8	0	0	0	0	0	8	52.4
03_C	0	0	0	0	0	0	8	52.4
03_N	0	0	0	0	0	0	8	52.4
04_A	20580	0	0	0	179	0	8	39.9
04_B	15367	0	0	0	77	0	8	39.9
04_C	21302	0	0	0	244	0	8	39.9
04_N	0	0	0	0	0	0	8	39.9
05_A	7072	0	654	0	0	0	8	38.6
05_B	29971	0	690	0	0	0	8	38.6
05_C	17691	36	1135	0	0	0	8	38.6
05_N	0	0	0	0	0	0	8	38.6
06_A	785	0	11	0	0	0	8	37.4
06_B	0	0	0	0	0	0	8	37.4
06_C	341	0	0	0	0	0	8	37.4
06_N	0	0	0	0	0	0	8	37.4
07_A	0	0	0	0	0	0	8	36.8
07_B	205	0	0	0	0	0	8	36.8
07_C	0	0	0	0	0	0	8	36.8
07_N	0	0	0	0	0	0	8	36.8
08_A	0	0	0	0	0	0	8	34.4
08_B	0	0	0	0	0	0	8	34.4
08_C	0	0	0	0	0	0	8	34.4
08_N	0	0	0	0	0	0	8	34.4
09_A	33	0	0	0	0	0	8	32.8
09_B	5951	0	0	0	0	0	8	32.8
09_C	8972	0	0	0	0	0	8	32.8
09_N	0	0	0	0	0	0	8	32.8
10_A	345	0	0	0	0	0	8	31.7
10_B	548	0	0	0	0	0	3	31.7
10_C	243	0	0	0	0	0	3	31.7
10_N	0	0	0	0	0	0	3	31.7
11_A	102	0	5	0	0	0	3	31.3
11_B	542	0	0	0	0	0	8	31.3
11_C	45	0	0	0	0	0	8	31.3
11_N	0	0	0	0	0	0	8	31.3
12_A	0	0	0	0	0	0	8	28.7
12_B	0	0	0	0	0	0	8	28.7
12_C	0	0	0	0	0	0	8	28.7
12_N	0	0	0	0	0	0	8	28.7
13_A	13416	26	0	0	0	0	8	21.9
13_B	8738	0	27	0	0	0	8	21.9
13_C	7836	0	214	0	0	0	8	21.9
13_N	0	0	0	0	0	0	8	21.9
14_A	2109	0	0	0	0	0	8	21.7

14_B	2977	0	7	0	54	0	3	21.7
14_C	2982	0	44	0	0	0	8	21.7
14_N	0	0	0	0	0	0	3	21.7
15_A	0	0	0	0	0	0	8	19.3
15_B	0	0	0	0	0	0	8	19.3
15_C	0	0	0	0	0	0	8	19.3
15_N	0	0	0	0	0	0	8	19.3
16_A	13293	0	24	0	0	0	8	18.9
16_B	25021	9	53	0	0	0	8	18.9
16_C	12551	0	310	0	0	0	8	18.9
16_N	0	0	0	0	0	0	8	18.9
17_A	12824	0	0	0	0	0	8	18.6
17_B	16842	0	0	0	0	0	8	18.6
17_C	22596	0	0	0	0	0	8	18.6
17_N	0	0	0	0	0	0	8	18.6
18_A	0	0	0	0	0	0	8	17.8
18_B	0	0	0	0	0	0	8	17.8
18_C	0	0	0	0	0	0	8	17.8
18_N	0	0	0	0	0	0	8	17.8
19_A	0	0	0	0	0	0	3	14.7
19_B	0	0	0	0	0	0	8	14.7
19_C	0	0	0	0	0	0	8	14.7
19_N	0	0	0	0	0	0	8	14.7
20_A	0	0	0	0	0	0	8	14.4
20_B	0	0	0	0	0	0	8	14.4
20_C	23	0	0	0	0	0	8	14.4
20_N	0	0	0	0	0	0	8	14.4
21_A	2033	0	0	0	72	26	3	11.4
21_B	9870	0	37	0	302	0	8	11.4
21_C	4313	0	85	0	481	0	8	11.4
21_N	0	0	0	0	0	0	8	11.4
22_A	10384	0	92	0	0	0	8	10.8
22_B	6455	0	15	0	0	0	8	10.8
22_C	8877	0	228	0	0	0	8	10.8
22_N	0	0	0	0	0	0	8	10.8
23_A	2729	0	38	0	392	0	8	8.37
23_B	1563	0	0	17	153	0	3	8.37
23_C	3623	0	21	0	797	0	3	8.37
23_N	0	0	0	0	0	0	3	8.37
24_A	13784	0	920	545	0	0	8	8.22
24_B	0	0	0	0	0	0	8	8.22
24_C	15826	0	1173	1840	0	0	8	8.22
24_N	0	0	0	0	0	0	8	8.22
25_A	3789	0	14	43	85	404	8	2.89
25_B	3192	0	5	14	180	171	8	2.89
25_C	3527	0	0	99	160	345	8	2.89
25_N	0	0	0	0	0	0	8	2.89

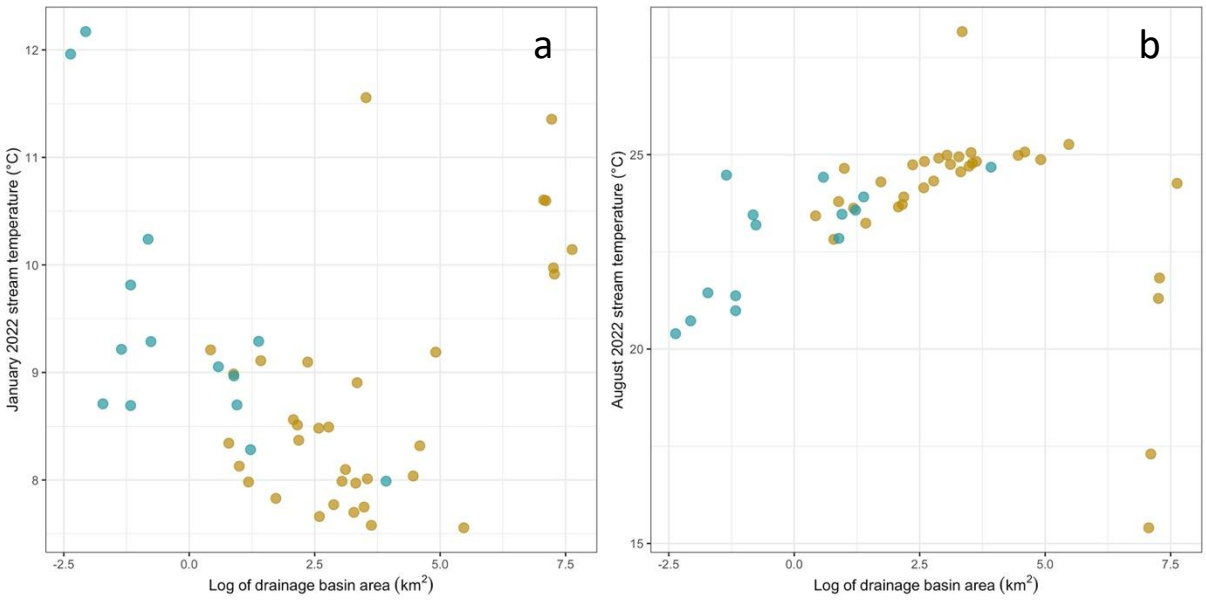


Fig. S.1: Mean stream temperatures in a) January 2022 and b) August 2022 across different drainage basin Temperature data from USGS stream gages located in the greater metro Atlanta area (yellow dots) and the 14 streams included in this study (blue dots).

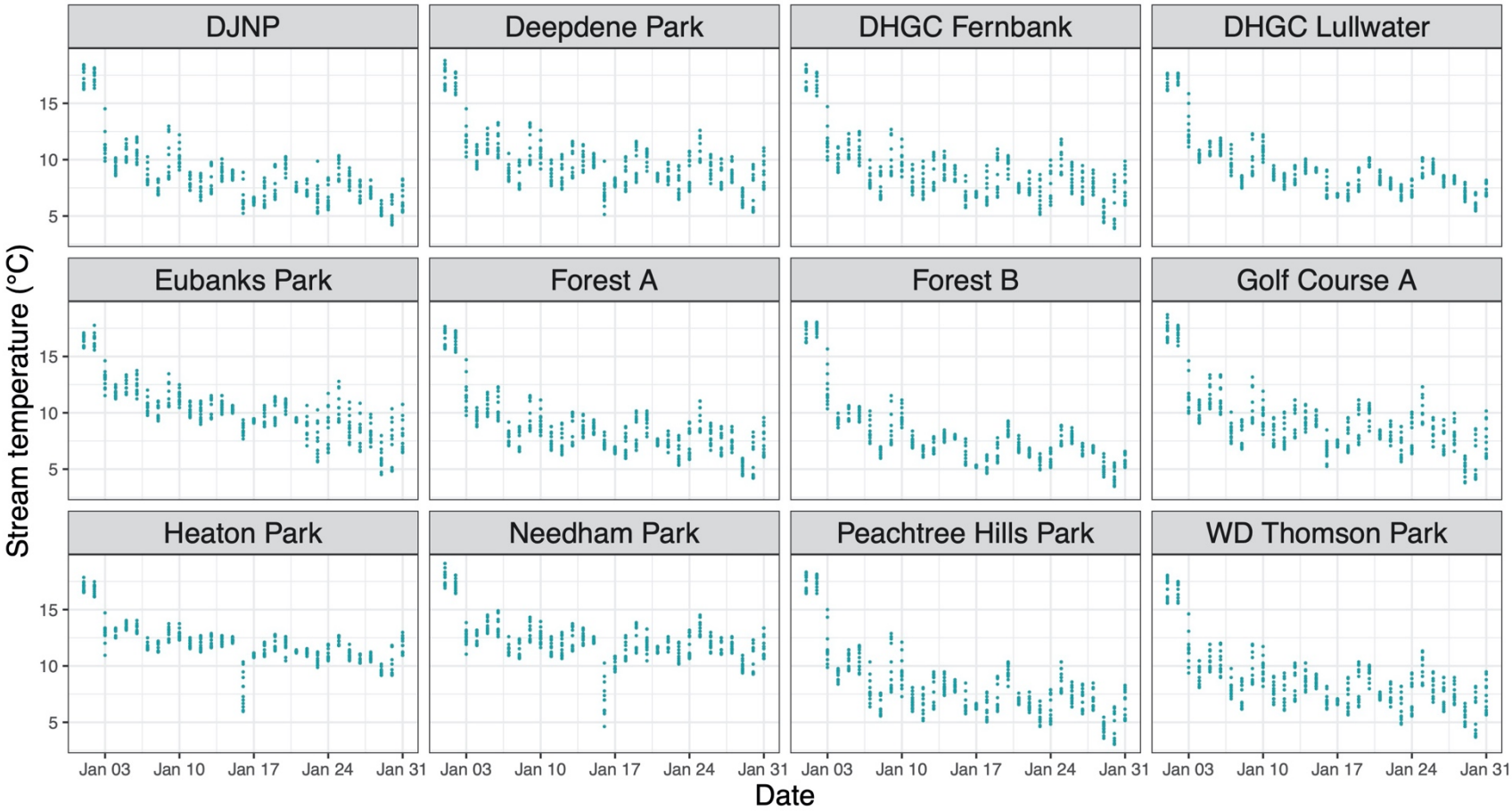


Fig. S.2: Temperature readings taken every two hours in January 2022. Beaverbrook Park and Sunnybrook Park are missing data for January 2022 (not included in this figure). DJNP = Daniel Johnson Nature Preserve. DHGC = Druid Hills Golf Club.

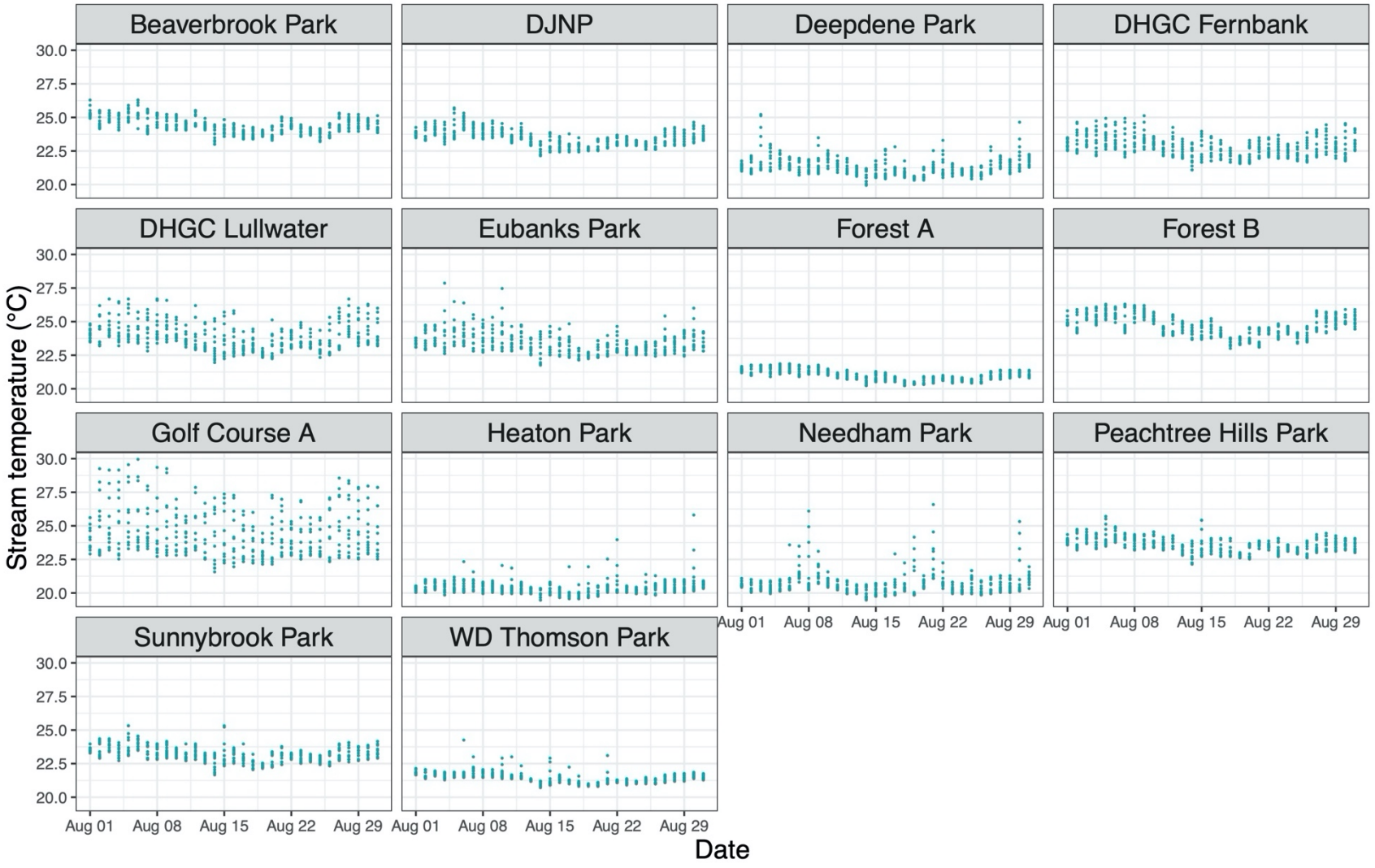


Fig. S.3: Temperature readings taken every two hours in August 2022. DJNP = Daniel Johnson Nature Preserve. DHGC = Druid Hills Golf Club.

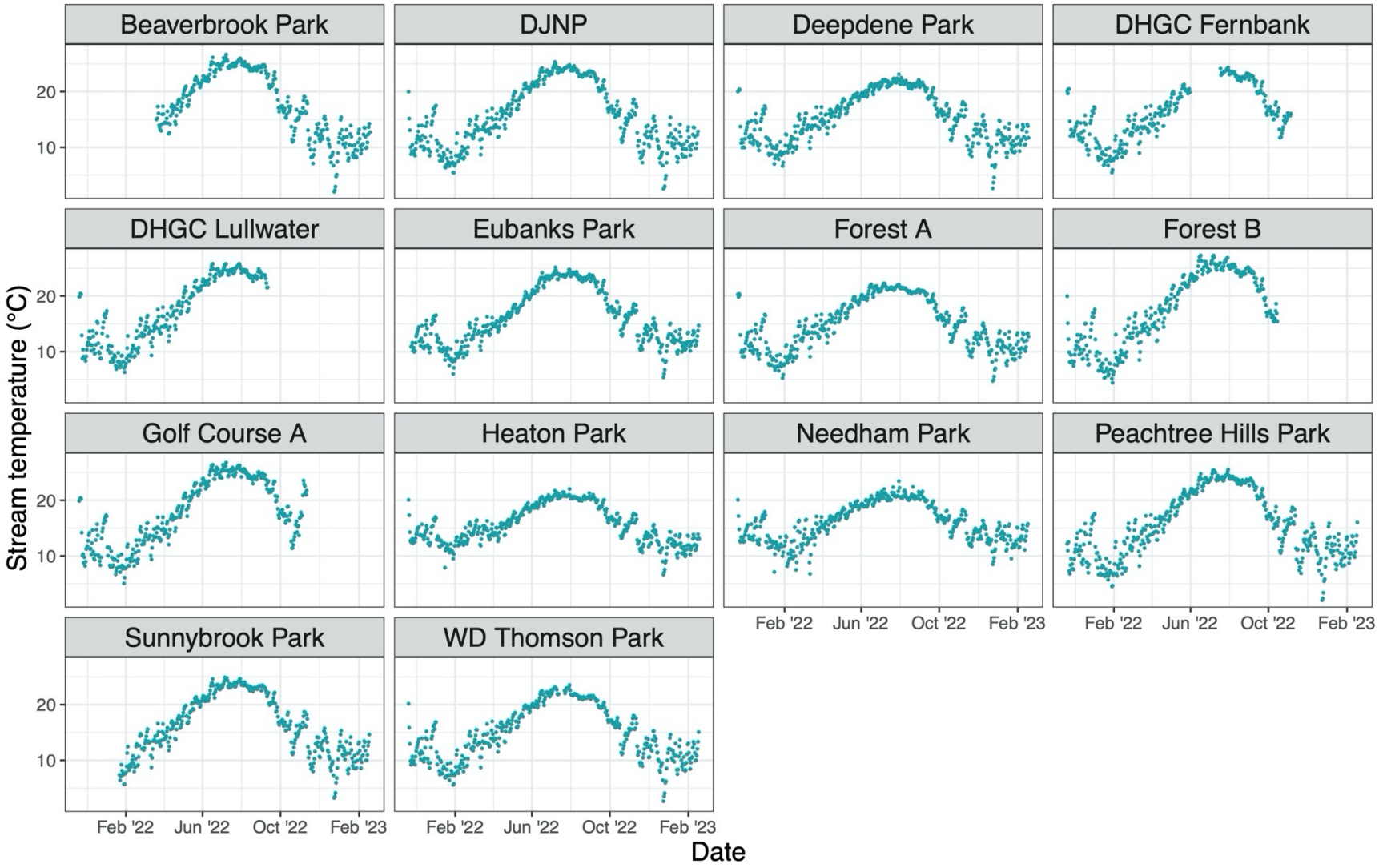


Fig. S.4: Mean temperature per day from January 2022 to February 2023. DJNP = Daniel Johnson Nature Preserve. DHGC = Druid Hills Golf Club.

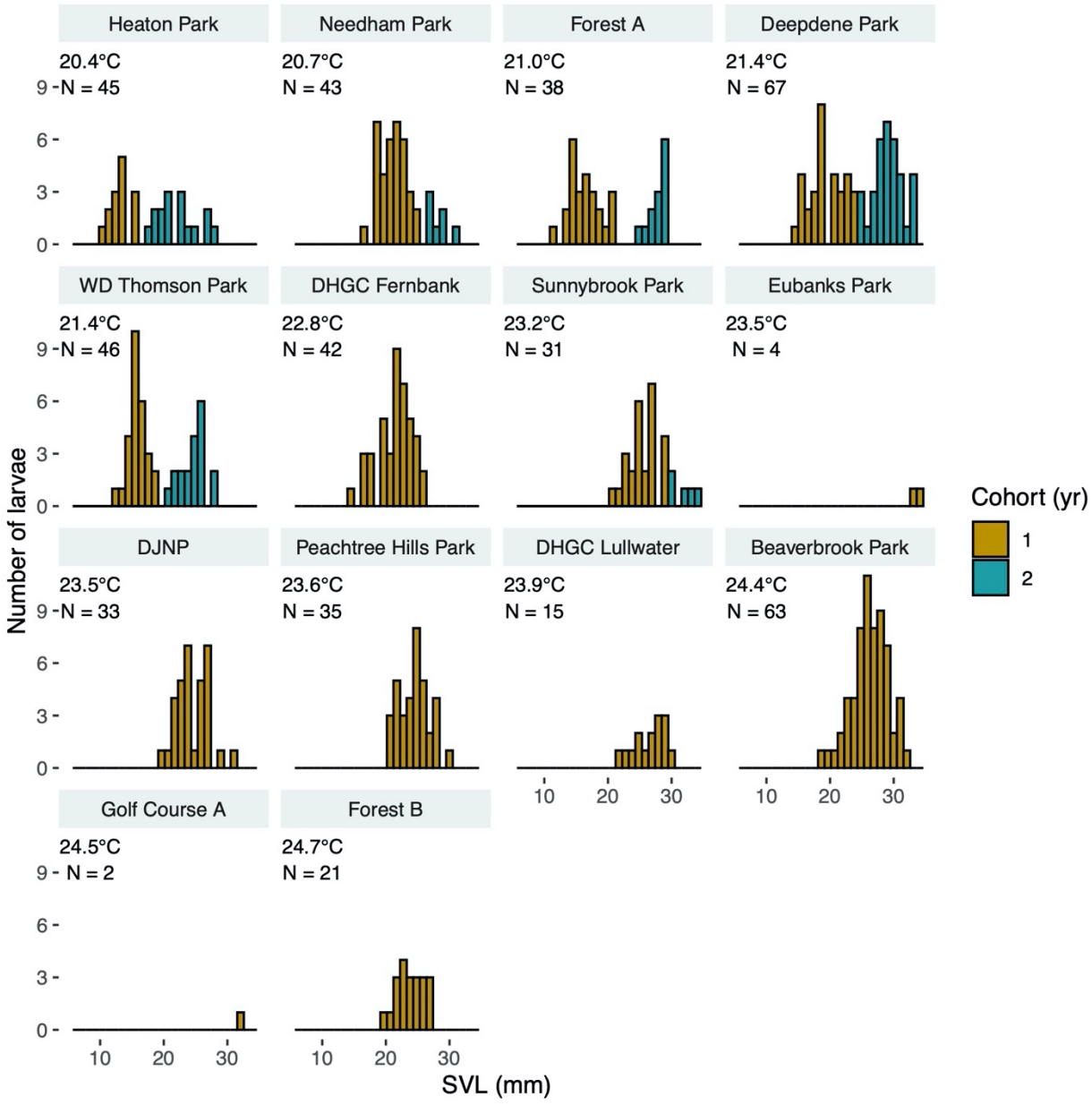


Fig. S.5: Larval period of each site arranged by mean August 2022 temperature. Snout-vent length (SVL) of *E. cirrigera* larvae measured in September 2021. Each larva assigned to either first-year cohort (yellow) or second-year cohort (blue). Mean August 2022 temperature indicated in top left corner of each panel. Number of larvae measured per site indicated beneath temperature. DJNP = Daniel Johnson Nature Preserve. DHGC = Druid Hills Golf Club.

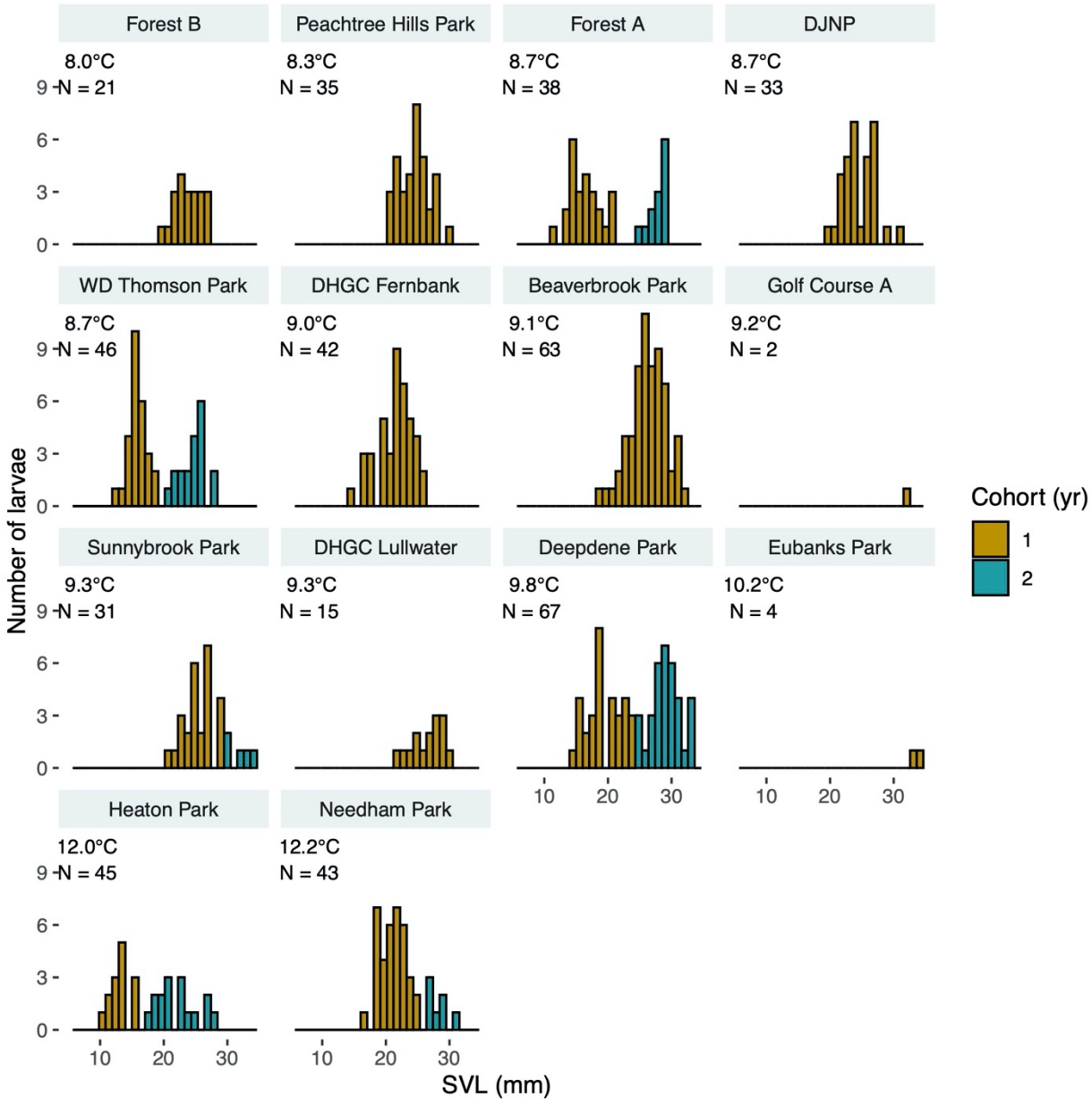
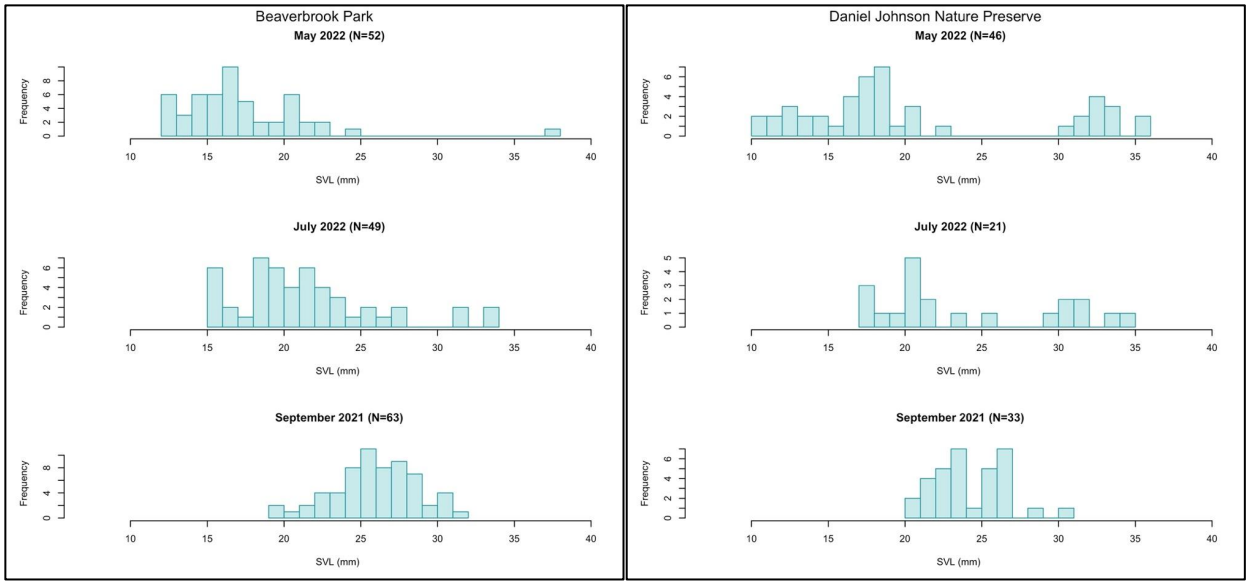
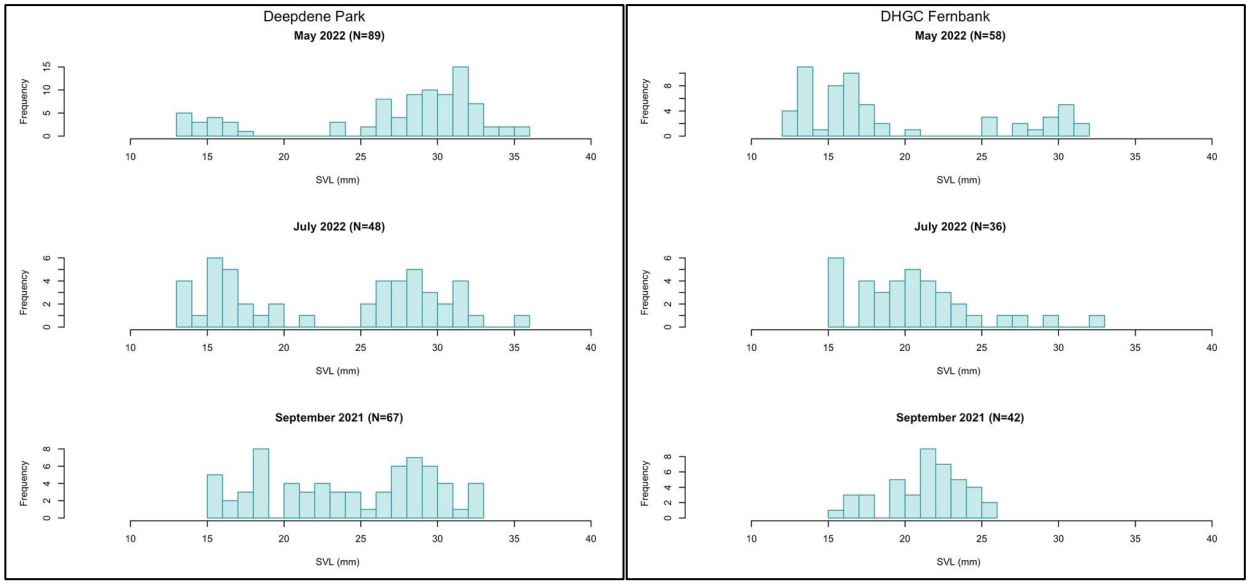
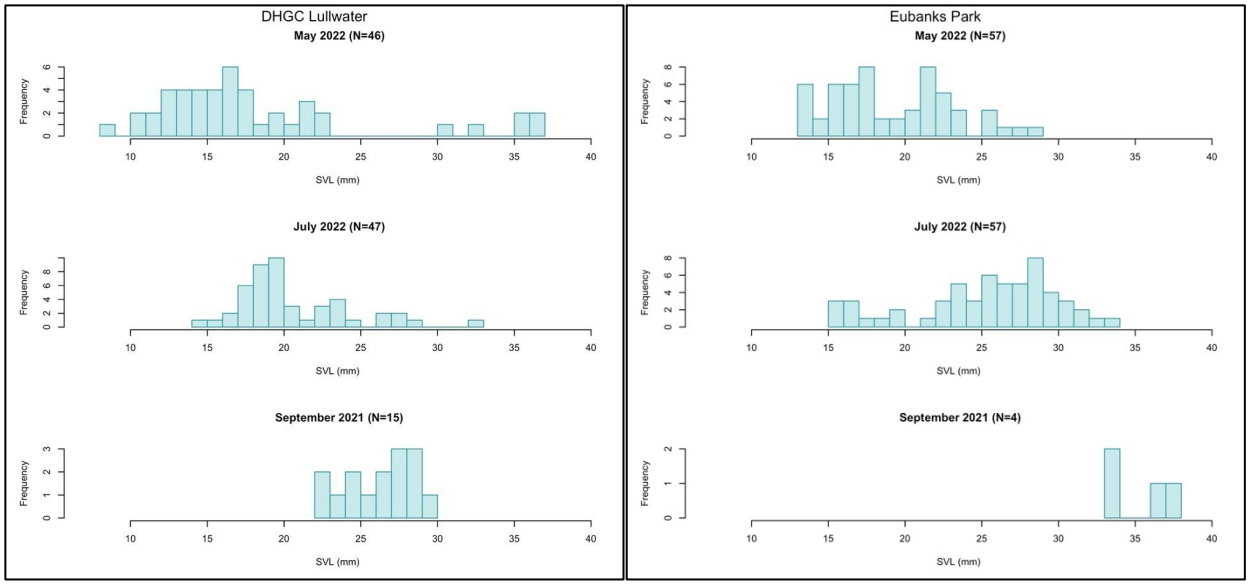
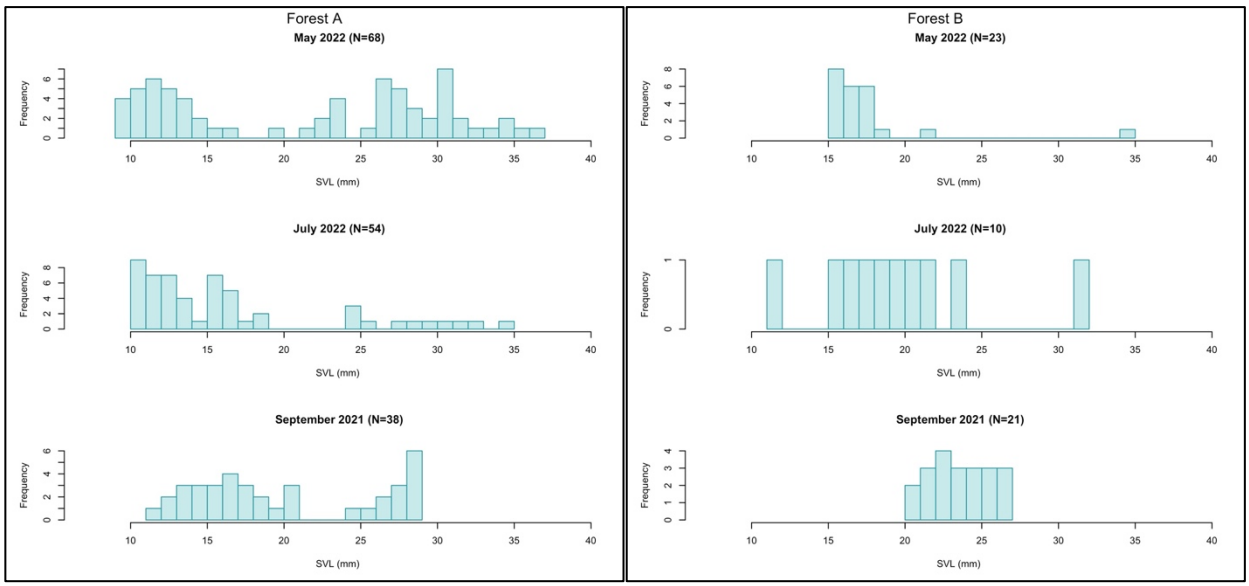


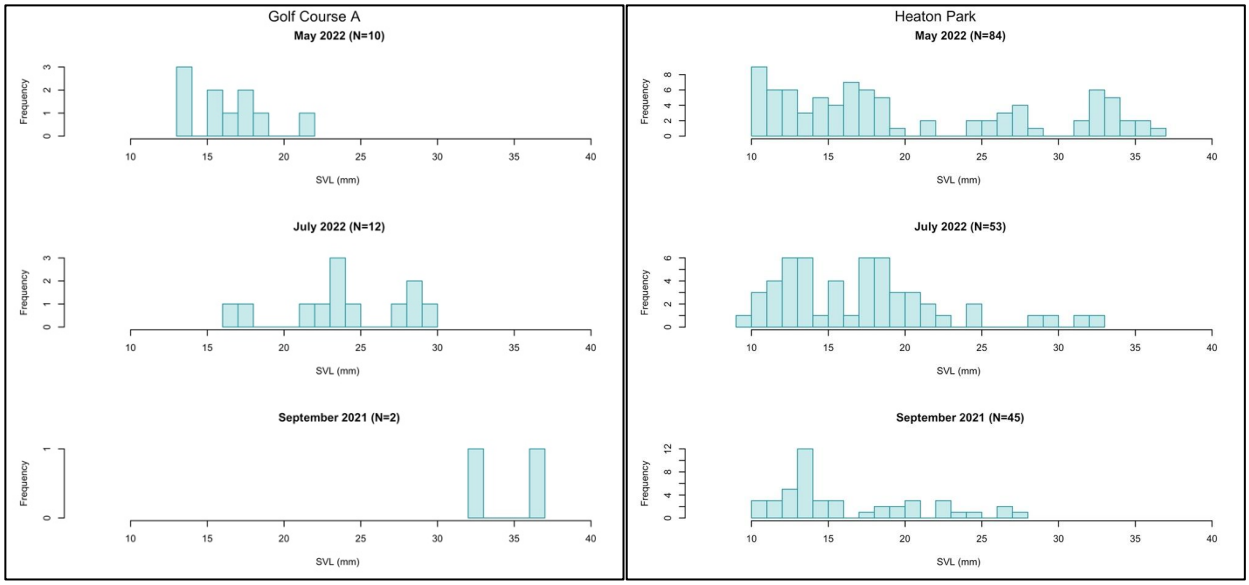
Fig. S.6: Larval period of each site arranged by mean January 2022 temperature. Snout-vent length (SVL) of *E. cirrigera* larvae measured in September 2021. Each larva assigned to either first-year cohort (yellow) or second-year cohort (blue). Mean January 2022 temperature indicated in top left corner of each panel. Number of larvae measured per site indicated beneath temperature. DJNP = Daniel Johnson Nature Preserve. DHGC = Druid Hills Golf Club.

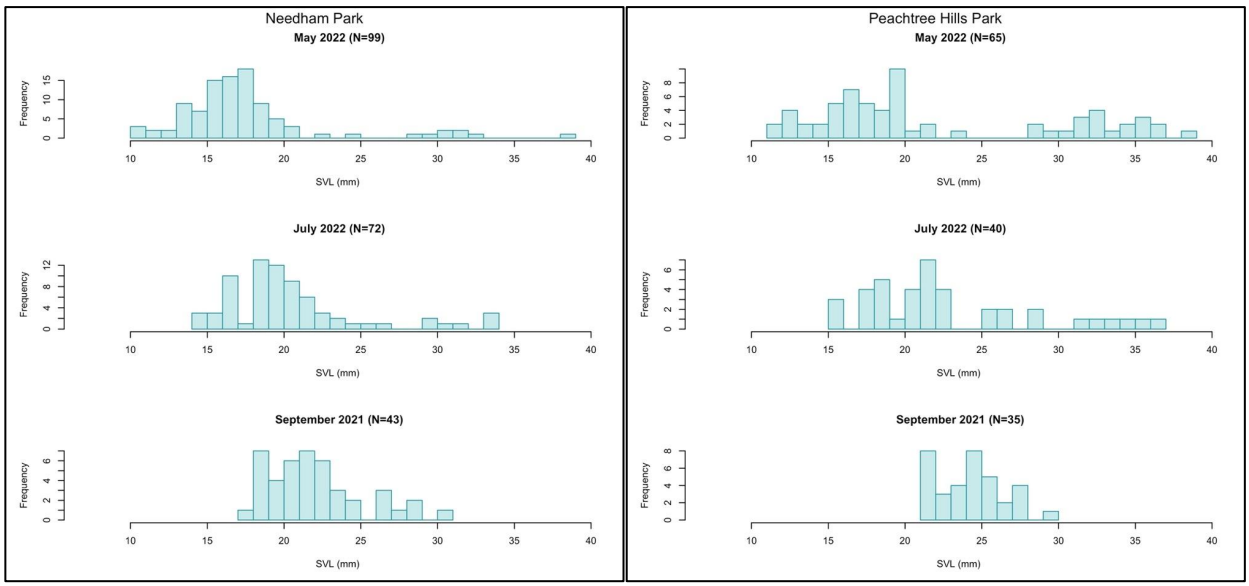

Beaverbrook Park
May 2022 (N=52)
July 2022 (N=49)
September 2021 (N=63)
Daniel Johnson Nature Preserve
May 2022 (N=46)
July 2022 (N=21)
September 2021 (N=33)
Frequency
SVL (mm)


Deepdene Park
May 2022 (N=89)
July 2022 (N=48)
September 2021 (N=67)
DHGC Fernbank
May 2022 (N=58)
July 2022 (N=36)
September 2021 (N=42)
Frequency
SVL (mm)


DHGC Lullwater
May 2022 (N=46)
July 2022 (N=47)
September 2021 (N=15)
Eubanks Park
May 2022 (N=57)
July 2022 (N=57)
September 2021 (N=4)
Frequency
SVL (mm)


Forest A
May 2022 (N=68)
July 2022 (N=54)
September 2021 (N=38)
Forest B
May 2022 (N=23)
July 2022 (N=10)
September 2021 (N=21)
Frequency
SVL (mm)


Golf Course A
May 2022 (N=10)
July 2022 (N=12)
September 2021 (N=2)
Heaton Park
May 2022 (N=84)
July 2022 (N=53)
September 2021 (N=45)
Frequency
SVL (mm)


Needham Park
May 2022 (N=99)
July 2022 (N=72)
September 2021 (N=43)
Peachtree Hills Park
May 2022 (N=65)
July 2022 (N=40)
September 2021 (N=35)
Frequency
SVL (mm)

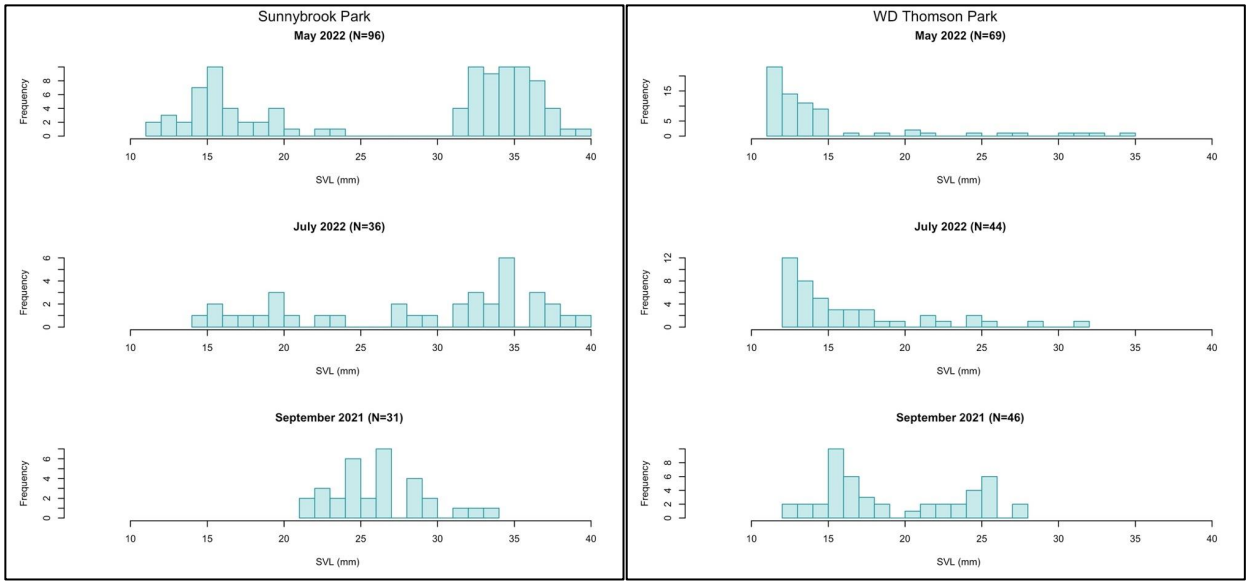


Fig. S.7: Histograms of larval snout-vent length (SVL) in 14 urban streams for May 2022, July 2022, and September 2021 surveys. Number of larvae measured per survey indicated in parentheses.