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Detection of Streamside Plethodontid Salamanders using Traditional and Environmental DNA Surveys in an Urban Setting

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***Abstract.*—Urbanization threatens to alter many watersheds in the eastern U.S., often resulting in the degradation of smaller streams. These highly disturbed systems may result in local extirpations of sensitive species, especially those that use both terrestrial and aquatic habitats, such as lungless salamanders (family Plethodontidae). We present a case study using two methods to detect streamside plethodontid species presence in urban streams: repeated traditional field searches and single-visit collection of environmental DNA samples (eDNA). Across 19 sites in urban Atlanta, Georgia, USA, we found that these two methods did not significantly differ in mean number of plethodontid species detected. We uncovered a significant, negative relationship between plethodontid species richness and percentage impervious surface cover of the watershed, highlighting the negative effects of urbanization on biotic community health. With more streams impacted by urbanization, it is important to develop sound methods for inventory and monitoring efforts. We recommend a combination of both traditional and eDNA methods to capitalize upon the relative advantages of each.**

Key Words.—amphibian; community composition; *Desmognathus*; eDNA; *Eurycea*; *Gyrinophilus*; lungless; *Pseudotriton*

Introduction

Urban sprawl in the southeastern U.S. is projected to more than double by 2060 (Terando et al. 2014), jeopardizing many terrestrial and aquatic ecosystems. In the Piedmont ecoregion of the U.S., 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 infrastructure upstream can have far-reaching effects within the stream. Increasing human land use has been tied to many detrimental effects to wildlife, including habitat loss, increased numbers and prevalence of emerging infectious diseases, reduced gene flow due to physical human-made barriers, and increased exposure to toxicants (Daszak et al. 2000; Martinuzzi et al. 2015; Murray et al. 2019; Fusco et al. 2021), and effects often contribute to declines in the biodiversity and abundance of native flora and fauna (McKinney and Lockwood 1999; McKinney 2002; Shochat et al. 2006). Species with limited dispersal ability, specialized habitats or diets, and higher sensitivity to environmental change are of most concern (McKinney 2002; Scheffers and Paszkowski 2012).

Lungless salamanders (family Plethodontidae) are an important taxon that is threatened by stream degradation. The eastern U.S. is a hotspot for plethodontid diversity, particularly the Appalachian Mountains, which are home to over 125 species of this family of salamanders (https://amphibiaweb.org). Because many plethodontids use both aquatic and terrestrial habitats during their life, they are at risk from urban impacts to both the stream and the surrounding drainage basin. Many salamander species experience declines and local extirpations in highly disturbed systems, with salamander abundance negatively correlated with urban land cover (Orser and Shure 1972; Willson and Dorcas 2003a; Miller et al. 2007; Barrett and Guyer 2008). Salamander populations may be decreasing in urban landscapes due to the loss of stream-bottom heterogeneity, increased streamflow, loss of riparian buffers, buried headwaters, and altered stream temperature regimes (Espey et al. 1966; Hawkins et al. 1983; Barr and

Babbitt 2002; Barrett and Guyer 2008).

Developing fast, cheap, and accurate survey techniques to inventory and monitor stream communities is of high priority. Traditional survey methods for plethodontid salamanders include dip-netting, searching leaf packs, turning over cover objects, and setting out leaf-litter bags and pitfall traps (Willson and Dorcas 2003b; Strain et al. 2009). Over the past two decades, the use of environmental DNA (eDNA) has been investigated as a potential supplement to or substitute for traditional survey methods (Willerslev et al. 2003; Ficetola et al. 2008; Sun et al. 2024). Environmental DNA is DNA shed into the environment by an organism, including waste products, skin cells, mucous, and saliva (Rees et al. 2014). Survey methods using eDNA require the collection of an environmental sample (e.g., stream water), extraction of DNA from the sample, and analyses to determine the origin of the DNA. Using eDNA offers a less disruptive method of determining salamander species presence compared to traditional surveys and is particularly useful in detecting rare species and differentiating between morphologically similar species (e.g., Jerde et al. 2011; Davy et al. 2015; Ruppert et al. 2022; Brammell et al. 2023).

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 probability can also be greatly impacted by environmental factors that influence the movement and degradation of eDNA, such as flow, pH, sediment type, UV exposure, and temperature (Pilliod et al. 2014; Jane et al. 2015; Strickler et al. 2015; Stoeckle et al. 2017). Additionally, polymerase chain reaction (PCR) inhibitors, downstream analysis errors (e.g., sample or reagent contamination), and the stochasticity of PCR contribute to potential issues in detection accuracy using eDNA (Kebschull and Zador 2015; McKee et al. 2015). Together, these factors may place limits on the accuracy of results.

In a meta-analysis of 194 studies comparing eDNA to traditional methods, eDNA had equivalent or better overall performance in terms of sensitivity, cost, and the number of detectable species (Fediajevaite et al. 2021). Fediajevaite et al. (2021) found that eDNA sampling is more sensitive than traditional survey methods for amphibians and invertebrates, less sensitive for annelids and reptiles, and roughly equal for mammals and molluscs. Studies comparing the use of traditional surveys and eDNA surveys to estimate salamander species richness, however, are often conducted in relatively pristine systems or controlled experiments (e.g., Pilliod et al. 2013; Katano et al. 2017; Plante et al. 2021). Although eDNA surveys have been successfully implemented in many urban streams (e.g., Bagley et al. 2019; Charvoz et al. 2021; Zhang et al. 2022), these systems may still have some properties that decrease detection and warrant special consideration, including PCR-inhibiting pollutants, increased streamflow, increased UV exposure due to decreased canopy cover, and increased sedimentation (Leopold 1973; Finkenbine et al. 2000; Meyer et al. 2005; Walsh et al. 2005; Nelson and Palmer 2007).

Here, we compared the results of traditional field surveys and eDNA surveys targeting plethodontid salamanders in urban streams in Atlanta, Georgia, USA. We also evaluated whether the percentage impervious surface cover of the surrounding drainage basin (a common proxy for urbanization) impacts the plethodontid salamander species richness. We expected that eDNA sampling would produce similar or slightly higher estimates of plethodontid species richness than traditional field methods. We also expected that plethodontid species richness would have a negative relationship with percentage impervious surface cover.

Materials and Methods

Study sites.—We sampled 19 sites along the tributaries leading to Peachtree Creek, North Fork Peachtree Creek, and South Fork Peachtree Creek within metro Atlanta, Georgia, USA (Fig. 1). As of 2011, the Peachtree Creek water basin spanned 240 km^2 of the Atlanta region and was 83% developed with 32% impervious surface cover (http://streamstats.usgs.gov/ss). We attempted to choose streams that varied in size, local habitat degradation, and in the percentage impervious surface cover (ISC) of the surrounding drainage basin. Sites were publicly accessible, included 17 public parks and two privately owned forests, and ranged from relatively high-quality, forested headwater streams to open canopy, channelized streams. Eleven of our 19 sites had completely independent drainage basins (i.e., separate headwater tributaries). Of the eight sites with overlapping drainage basins, six sites were farther than 1.5 stream km away from the next closest site and had an additional confluence between them. Two sites were approximately 0.5 stream km apart and were situated on the same stream reach without

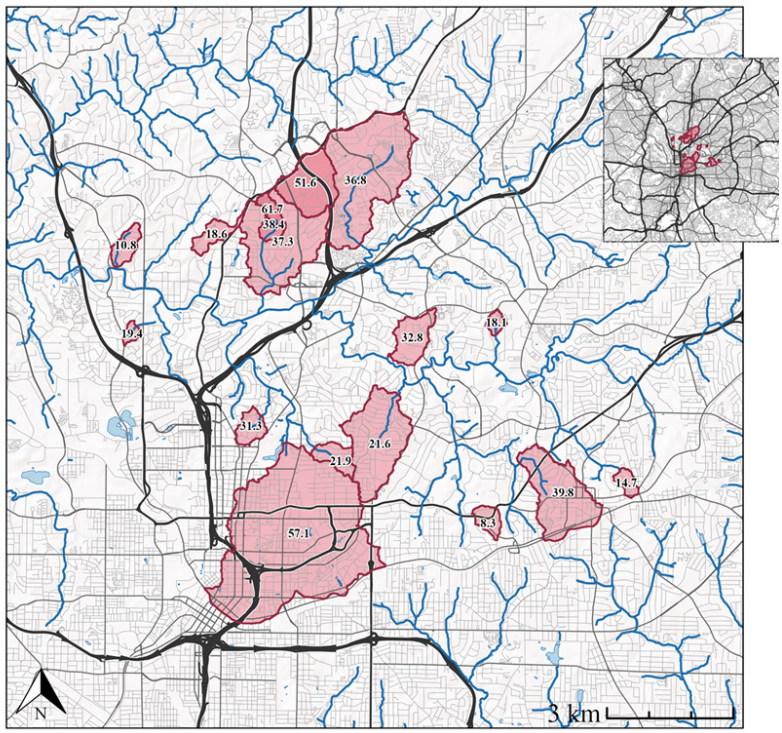

Figure 1. Drainage basins for 17 of 19 sample sites across Atlanta, Georgia, USA. The percentage impervious surface cover of each basin is indicated at the center of each basin polygon. We estimated drainage basins and percentage impervious surface cover using StreamStats v4.8.1 (http://streamstats.usgs.gov/ss). To preserve anonymity, drainage basins for Sites 5 and 8 are not shown.

an additional confluence between them (Sites 6 and 17). We assumed spatial independence for eDNA sampling (Pilliod et al. 2014; Shogren et al. 2017; Jo and Yamanaka 2022; Pont 2024).

To characterize stream size and urbanization, we used StreamStats v4.8.1 (http://streamstats.usgs.gov/ss). StreamStats is an online application that uses data from the 3D Elevation Program of the U.S. Geological Survey, the National Hydrology Dataset, and the Watershed Boundary Dataset to delineate streams and estimate multiple land use and streamflow parameters. We selected the point in the stream where we collected eDNA to determine percentage ISC based on the National Land Cover Database 2011 30-m resolution impervious dataset (Homer et al. 2015). Across our 19 survey sites, percentage impervious surface cover (ISC) of the drainage basin ranged from 2.9–61.7% (mean = 29.0 ± [standard deviation] 16.3%) and drainage basin area ranged from 0.1–50.5 km^2 (mean = 4.3 ± 11.5 km^2; see additional details in Supplemental Information Table S1).

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

We filtered each 1 L sample through a 0.45 µm cellulose nitrate filter (Thermo Fisher Scientific, Waltham, Massachusetts, USA) using a vacuum filtration system. We ended filtration if a sample took longer than 1 h to filter, and we recorded the total volume filtered. Using bleach-sterilized scissors and forceps, we cut each filter in half and stored it at -20° C until DNA extraction, roughly one month later.

DNA extraction and PCR.—We conducted all pre-PCR steps in a dedicated dead air box, and with pipettors used only for eDNA work. We used a modified bead extraction protocol to isolate environmental DNA from filters, which can be an inexpensive and effective method (e.g., Shahraki

et al. 2019). We incubated one half of each filter overnight at 55° C with 180 µL Buffer ATL and 20 µL Proteinase K (Qiagen N.V., Hilden, Germany), agitating occasionally with a vortex mixer. After incubation, we moved both the filter and the solution to a QIAshredder spin column (Qiagen N.V.) and centrifuged the column for 2 min at 11,000 rpm. We then added 200 µL Buffer AL (Qiagen N.V.) to the resulting flow-through and incubated for 10 min at 55° C. Next, we added Sera-Mag SpeedBeads solution (Thermo Fisher Scientific; Rohland and Reich 2012) in a 2:1 volumetric ratio of SpeedBeads solution to flow-through and let the samples sit for 15 min. Using a magnet, we separated the SpeedBeads from the solution, discarded the solution, washed the SpeedBeads with 80% ethanol, and resuspended in 100 µL molecular-grade water. We stored the final product at -20° C.

We 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 indexed amplicons. We re-evaluated these primers to assess whether they would be appropriate for our study system using the R package PrimerMiner v0.22 (Elbrecht and Leese 2017). To do this, we produced a sequence alignment of species in our study region from publicly available data, including both mitochondrial amplicons and mitochondrial sequences derived from whole-genome sequencing (Supplemental Information Table S2). The six species in our study area are the Southern Two-lined Salamander (*Eurycea cirrigera*), Three-lined Salamander (*E. guttolineata*), Chattooga Dusky Salamander (*Desmognathus perlapsus*), Talladega Seal Salamander (*D. cheaha*), Red Salamander (*Pseudotriton ruber*), and Spring Salamander (*Gyrinophilus porphyriticus*). We used the function evaluate_primer() to evaluate non-fusion versions of the forward and reverse primers and the function primer_threshold() to combine these results. Most species had no mismatches in the forward primer binding sites, with the exceptions of *E. cirrigera* (one mismatch; PrimerMiner score = 31.4) and *E. guttolineata* (two mismatches; PrimerMiner score = 74.7). All species had no mismatches in the reverse primer binding sites. Thus, we determined that our primers were suitable for all species using the default PrimerMiner score threshold (100).

We prepared genomic libraries using fusion versions of these primers. For each PCR run, we included a PCR negative control (5 µL of water instead of DNA template) and a PCR positive control using a plethodontid species not found in our study region (i.e., DNA extracted from a *Plethodon shermani* x *P. teyahalee* hybrid from Macon County, North Carolina, USA). We used three replicates per sample for the first round of PCR. Between the first and second PCR, we pooled together sample replicates (but kept site replicates separate) and included a cleanup with SpeedBeads in a 2:1 volumetric ratio. We conducted all PCRs on a T100 Thermo Cycler (Bio-Rad, Hercules, California, USA) following the protocols in Glenn et al. (2019). We verified successful PCR amplification of the target amplicon using 1% agarose gel electrophoresis.

We pooled final PCR products from all 76 field samples (three stream samples and one field negative control per site × 19 sites) and seven PCR controls. For each field sample that produced a clear target band in the gel, we added 3 µL of PCR product into the pool. For each field sample that did not produce a clear target band, we added 8 µL of PCR product into the pool. We added 8 µL of PCR product from each negative control (n = 19 field controls + seven PCR controls). We purified this pool with SpeedBeads, using a 9:10 volumetric ratio of SpeedBeads to pooled PCR product and resuspending in 30 µL molecular-grade water. After confirming the presence of the target amplicon via gel electrophoresis, we added 5.4 µL of the final eluate (with SpeedBeads removed via magnet) to 25 µL molecular-grade water to create the final pool of eDNA libraries.

We sent this pool to the Clinical Genomics Center at the Oklahoma Medical Research Foundation for PE150 sequencing on the Illumina MiSeq 300v2 platform, targeting 100K reads per sample. We demultiplexed and denoised reads with DADA2 (Callahan et al. 2016) in QIIME 2 v2025.7.0 (Bolyen et al. 2019). Before denoising reads, we trimmed off the first 18 bp and truncated forward and reverse reads to 110 bp. We then assembled reads against a custom reference database consisting of sequences from the same sources used to evaluate the primers with PrimerMiner (Supplemental Information Table S2). We assembled reads against this database at 95% similarity, and we considered a species present at a site if at least one of the three site replicates contained eDNA of that species. To evaluate non-target amplification, we used BLAST v2.16.0 to determine the source of the 100 most abundant amplicon sequence variants (ASVs) that did not map to our reference database (Camacho et al. 2009).

Traditional field surveys.—As part of a separate, larger survey for herpetofauna in metro Atlanta conducted by the Amphibian Foundation, we completed traditional field surveys at the same sites from which we collected eDNA. We used a dip net to search wetlands, creeks, springs, and seepage areas for larval salamanders, following standard field survey methods (e.g., Bruce 1982; Willson and Dorcas 2003b). For adult salamanders, we lifted cover objects such as rocks, logs, moss, leaf litter, and human-made objects. We placed captured salamanders in an acrylic box with a scale bar and took standard dorsal and lateral photos to emphasize key characteristics that aid in verifying identification. At each site, we recorded all observations of eggs, larvae, and adult plethodontid salamanders.

Following a common sampling scheme used in traditional field surveys, we visited each site at least three times (winter/spring, summer, and fall). We did not survey during or directly after heavy rain events. We conducted all surveys between 0900–1800 and calculated survey time as the number of surveyors multiplied by survey minutes. For each site, we aimed for a survey time of 40 min/ha, with a minimum survey time of 15 min; however, this included time spent searching for other terrestrial amphibians (e.g., direct-developing woodland salamanders of the genus *Plethodon*) and reptiles.

Statistical analyses.—To evaluate the relative performance of our two methods, we ran a Two-tailed, Paired *t*-test on the number of plethodontid species detected at each site using eDNA sampling and traditional field survey methods. To evaluate the effect of urbanization on plethodontid species richness, we fit a Generalized Linear Model (GLM) of species richness per site (combining results of both survey methods) as a function of ISC using the glm() function in R v4.4.2 (R Development Core Team 2024). Because we had a well-defined community from which we were sampling (i.e., six species), we treated species richness as a binomial response. We evaluated our model for over- or underdispersion, zero-inflation, and uniformity of residuals using the package DHARMa v0.4.7 (Hartig 2024). We tested for spatial autocorrelation in the residuals of the model using the Moran.I() function from the package ape v5.7–1 (Paradis and Schliep 2019). We 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. We set the significance threshold to $\alpha = 0.05$ for all analyses. All raw sequence data are available from the NCBI SRA (PRJNA1321338), and all code is available from Zenodo (doi.org/10.5281/zenodo.17117512).

Results

We recovered a mean of 77K reads per eDNA sample (standard deviation [SD] = 143K), with > 99% of reads passing quality-filtering. After denoising, we retained a mean of 54K reads per eDNA sample (SD = 133K), with a mean of 6.5K reads mapping to the reference database (SD = 8.0K; Supplemental Information Table S3). Across all sites, these mapped reads included five of the six species in our reference database; the most ubiquitous species was *Eurycea cirrigera* (14/19 sites), and the only undetected species was *E. guttolineata* (0/19 sites). Four sites returned zero sequence reads mapping to our reference database. From the 100 most abundant ASVs that did not map to our reference database, the only BLAST matches to vertebrates were three species of local ranid frogs. None of the field or laboratory negative controls produced visible bands during gel electrophoresis. From these controls, we recovered a mean of 35K reads per sample (SD = 49K), with > 99% of reads passing quality-filtering. After denoising, we retained a mean of only 5.0 reads per sample from these controls (SD = 11.2); none of these reads mapped to our reference database, and none matched the vertebrates among the top 100 ASVs in unmapped reads.

Cumulatively across all traditional field surveys, we detected all six species of streamside plethodontid salamanders in the community. The most ubiquitous species was *E. cirrigera* (19/19 sites), and the least common were *E. guttolineata* (1/19 sites) and *Gyrinophilus porphyriticus* (1/19 sites). Thus, with both survey methods combined, we detected six species of streamside plethodontid salamanders: *E. cirrigera* (19/19 sites), *E. guttolineata* (1/19 sites), *Desmognathus perlapsus* (14/19 sites), *D. cheaha* (3/19 sites), *Pseudotriton ruber* (5/19 sites), and *Gyrinophilus porphyriticus* (1/19 sites; Fig. 2). We collected species presence data for these six species of plethodontid salamanders across 19 sites, for a total of 114 estimations of species presence across all sites. Of these 114 estimations, eDNA and traditional field survey methods agreed on the presence or absence of a species in 98 estimations (86%; Fig. 2).

The number of plethodontid salamander species detected per site in our single-visit eDNA sampling (mean = 1.68 ± 1.34 species; range of values, 0–5

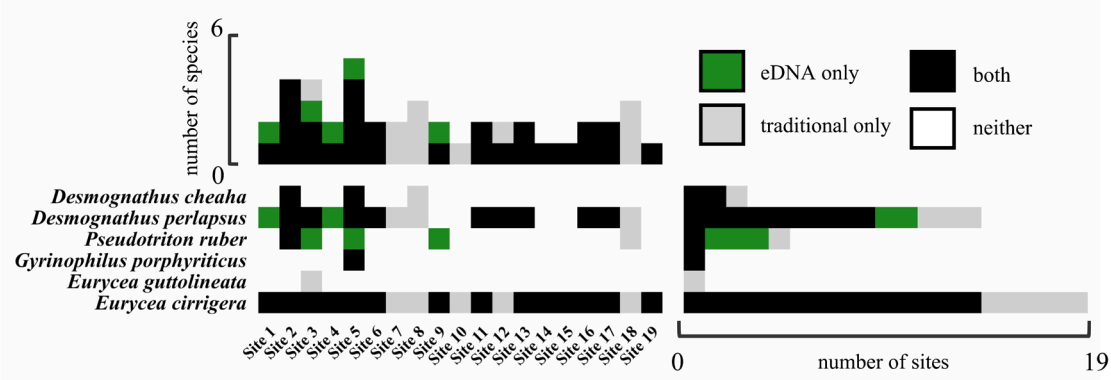

Figure 2. Presence/absence data for the Southern Two-lined Salamander (*Eurycea cirrigera*), Three-lined Salamander (*E. guttolineata*), Spring Salamander (*Gyrinophilus porphyriticus*), Red Salamander (*Pseudotriton ruber*), Chattooga Dusky Salamander (*Desmognathus perlapsus*), and the Talladega Seal Salamander (*D. cheaha*) across 19 sites in Atlanta, Georgia, USA. Square color represents which method(s) successfully detected a species at a site: neither method (white), traditional field surveys only (gray), environmental DNA (eDNA) sampling only (green), or both methods (black). Stacked squares at the end of each row and column show cumulative results for each species and site, respectively.

species) and the number detected with our repeated traditional field survey methods (mean = 2.00 ± 1.00 species; range of values, 1–4 species; Fig. 3) did not differ significantly (t = -1.14, df = 18, P = 0.268). Impervious surface cover (ISC) had a negative association (Fig. 4) with species richness (B = -0.03 ± [standard error] 0.01; $\boldsymbol{z}$ = -2.03, P = 0.042). We found no evidence of over- or under-dispersion (0.69; P = 0.318), zero-inflation (0; P = 0.338), or non-uniformity of residuals (D = 0.14; P = 0.774), and we did not detect significant spatial autocorrelation in the residuals of the GLM (P = 0.274).

Discussion

Monitoring changes to plethodontid salamander community composition in response to urbanization can provide valuable data about the abiotic limitations to species distributions and the drivers of population declines, and it can aid in the prioritization of habitat for protection and restoration. In our survey of 19 sites in Atlanta, we found a significant negative relationship between the percentage impervious surface cover (ISC) of the drainage basin, a common measure of urbanization, and species richness. Some species (e.g., *Eurycea cirrigera*) were ubiquitous, while others (e.g., *Gyrinophilus porphyriticus*) were restricted to the least urbanized streams. Salamander populations may experience declines and local extirpations due to a combination of changes typical of urban streams, including the loss of stream-bottom heterogeneity, degradation of upland habitat, increased frequency and severity of flood events, and altered stream temperature regimes (Hawkins et al.

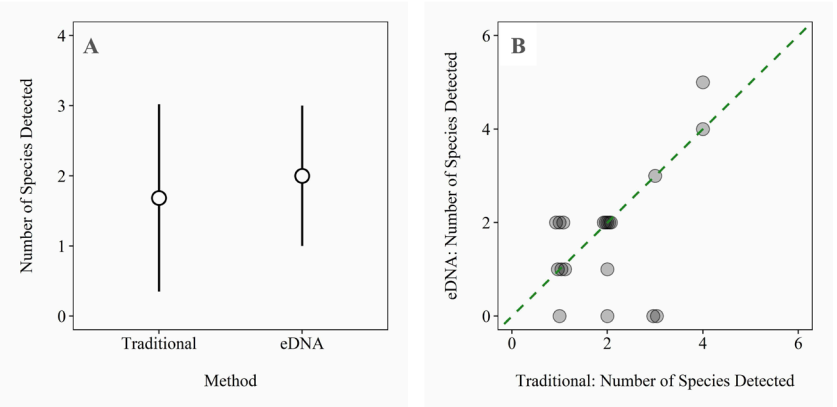

Figure 3. (A) Mean estimated plethodontid salamander species richness across all 19 urban Atlanta, Georgia, USA sites for traditional field survey methods (Traditional) and environmental DNA surveys (eDNA). Error bars indicate one standard deviation. (B) Observed streamside plethodontid salamander species richness per site for traditional field survey (Traditional) and environmental DNA (eDNA) survey methods. Each dot represents one site. Dashed line represents the line of equality (eDNA = Traditional).

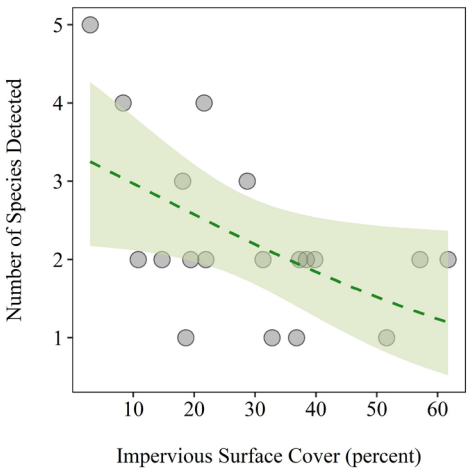

Figure 4. Generalized Linear Model regression line (dotted) of the number of streamside plethodontid salamander species detected per site as a function of percentage impervious surface cover. The number of species detected includes results from both eDNA and traditional field surveys. The 95% confidence interval is shown with green shading.

1983; Finkenbine et al. 2000; Barr and Babbitt 2002; Willson and Dorcas 2003a; Barrett and Guyer 2008). That plethodontid richness and abundance are lower in urban streams than in what can be called rural or natural streams is well documented (e.g., Orser and Shure 1972; Willson and Dorcas 2003a; Miller et al. 2007; Barrett and Guyer 2008; Barrett and Price 2014), but few studies investigate plethodontid species richness and abundance across an urban gradient. Our results suggest that urban streams exhibit finer-scale patterns that can be masked when streams are simply labeled urban or rural, and the use of ISC as a single metric for urbanization may be a good predictor of salamander species loss.

Despite their importance, urban streams present challenges that may restrict or bias survey efforts. Traditional field surveys at urban sites may present a safety risk to researchers, including pollutants and contaminants, sharp objects (e.g., broken glass hidden in stream beds), and crime (Ousey 2000; Paul and Meyer 2001; Bradley et al. 2021). On the other hand, eDNA detection probability may be jeopardized by changes associated with urban streams, including increased flooding, changes in the thermal profile, changes to streambed composition, and an increase in contaminants and PCR inhibitors (Leopold 1973; Finkenbine et al. 2000; Paul and Meyer 2001; Nelson and Palmer 2007).

Across 19 sites in urban Atlanta, Georgia, USA, we did not find a significant difference between eDNA-based surveys and traditional field surveys in the number of plethodontid salamander species detected. However, our results suggest differences between the two methods that may reflect relative advantages and disadvantages. For example, we detected *Pseudotriton ruber* at a total of five sites, three of which were by eDNA only. This is a large, but secretive, species with a long larval period, and our results may be consistent with higher detection probability via aquatic eDNA than via traditional search methods. Additionally, plethodontid larvae are notoriously difficult to identify using traditional field methods, whereas eDNA is able to distinguish species regardless of life stage. Compared to traditional survey methods, eDNA may provide an advantage for detecting species with low abundance or in habitats that traditional surveys may be biased against (e.g., deep pools), as well as species that may be difficult to identify visually.

Our eDNA sampling, however, also highlighted a weakness of this method: its susceptibility to PCR inhibition. Four of our sites returned zero sequence reads mapping to our reference database despite confirmed salamander presence at these sites. We suspect that these results may be due to PCR inhibition or environmental factors that bind or degrade DNA, common problems in eDNA sampling (Schrader et al. 2012), that can be caused by factors including but not limited to humic and tannic acids from leaf litter or contaminants such as metal ions and proteins from wastewater or surrounding human land use (Abbaszadegan et al. 1993; Shieh et al. 1995; Opel et al. 2010; Rock et al. 2010; Butler et al. 2018). Indeed, partial inhibition may have also caused our failure to detect individual species at additional sites (e.g., *E. cirrigera* at Site 12, from which we recovered very few reads). We extracted DNA using a method that can reduce inhibition (i.e., SpeedBeads), but we did not explicitly test for residual inhibition. Testing for inhibition is common and recommended in eDNA studies targeting individual species but not yet widespread in metabarcoding studies (Klymus et al. 2024). It certainly would have improved the interpretability of our data, however, and we strongly recommend this practice in future work like ours. Other technical challenges with eDNA include potential false positives through downstream drift (Pilliod et al. 2014; Shogren et al. 2017) and field or laboratory contamination (Sepulveda et al. 2020). Downstream drift of eDNA should be an especially large challenge for the interpretation of data from sampling sites located directly in-line in a single stream; this is the case for our Sites 6 and 17,

but we found perfect concordance between eDNA and traditional surveys at these two locations. We acknowledge that, although our positive detections are valid, the absence of inhibition testing prevents us from distinguishing true negatives from PCR inhibition-induced false negatives.

A shared limitation of both traditional and eDNA sampling is the sensitivity of results to the timing and intensity of the surveys. For example, we collected eDNA with a single visit to each site in November 2021, which is a time of year during which every target species would have either been actively courting or have aquatic larvae in the stream. By comparison, our traditional surveys included site visits during three different seasons. We emphasize that our two sampling methods do not represent equal effort or equivalent time periods. Instead, they compare a rapid eDNA assessment conducted on a single visit with a repeated field survey. Thus, readers should not interpret our results as generally characteristic of the value of eDNA versus traditional field methods, but rather a single case study. Our eDNA sampling approach made for efficient and scalable field sampling (i.e., approximately 5 min per site for field collection), but our cumulative detection probabilities likely would have been greater had we surveyed again at another time of year. For example, we failed to detect *Eurycea guttolineata* where we found them with traditional surveys, but this species may have been easier to detect with eDNA in the spring, when it has aquatic larvae in the stream (Bruce 1970; Maldonado 2022). Additionally, adding eDNA sampling at different times of year may take advantage of environmental conditions during which PCR inhibition is weaker (e.g., with fewer decomposing leaves in the stream).

When determining which survey methods to use, monitoring programs across urban streams should carefully consider the unique biotic and abiotic challenges associated with their sites, the scope and goals of the program, and the available resources (e.g., money, trained personnel, access to lab instruments, time). Environmental DNA sample collection is faster and less disruptive than methods often used in traditional surveys (e.g., lifting cover objects, searching leaf packs), which may be beneficial when working in fragile or rare systems or when time is a limiting factor. Traditional surveys may be the most cost-effective option for studies with few sampling events or target species, but eDNA is often cheaper, especially when the necessary eDNA assay is already developed (Smart et al. 2016; Bálint et al. 2018; Plante et al. 2021). Environmental DNA sampling becomes an especially cost- and time-effective option when projects focus on multiple, distantly related taxa (Thomsen et al. 2012; Valentini et al. 2016). For example, we could investigate fish species richness across our sites using our remaining DNA eluate without returning to the field or hiring taxonomic experts. In a separate unpublished study, we detected a common amphibian pathogen, *Batrachochytrium dendrobatidis* (*Bd*), in many of our eDNA samples, a promising outlook for the use of eDNA in monitoring pathogens in urban streams.

Traditional survey methods, however, have distinct advantages over the use of eDNA in ecological surveys. Traditional survey methods can produce data on life history, demographic structure, and unique observations that eDNA currently cannot. Because eDNA metabarcoding typically requires a known reference sequence to match reads against, traditional surveys may be superior for detecting undescribed or unexpected species. Although standardizing and optimizing eDNA protocols will improve the accuracy and precision of estimating abundance in the future, current practice restricts the use of eDNA as a reliable substitution for traditional survey methods in determining species abundance (Yates et al. 2019; Plante et al. 2021). Similarly, the successful use of eDNA as a substitute for tissue sampling to monitor genetic diversity is currently limited (e.g., Weitemier et al. 2021; Zanovello et al. 2023).

In summary, we found that stream salamander communities became less diverse with urbanization across 19 streams in Atlanta, Georgia, USA. We found no significant difference between our traditional and environmental DNA surveys in estimating species richness, but we uncovered challenges unique to each method. By further understanding how urbanization alters community composition of wildlife, decision-makers and researchers can better allocate resources to improve conservation outcomes. Studies collecting data on species presence in urban settings are especially useful in assessing changes to biotic communities, drivers of local extirpations, and identification of habitats that would benefit most from restoration or protection. Thus, selecting the best survey method(s) based on resources and goals will help improve inventory and monitoring efforts and conservation outcomes.

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