
Photo Credit: Corey Arnold, National Geographic

Cook County Animal and Rabies Control

Environmental Research Initiative Group Annual Report

2021

Dear Residents,

I am pleased to provide you with the annual summary of research conducted in partnership with the Cook County Department of Animal and Rabies Control (ARC). These reports and articles are a result of vital research funded by ARC to ensure that we understand the unique characteristics of urban wildlife in our region and ultimately protect our residents and companion animals from disease associated with urban wildlife in our ecosystem.

The County's long held partnerships with the Max McGraw Wildlife Foundation, University of Illinois Zoological Pathology Program and the Forest Preserves of Cook County Urban Otter Research Project provide a great deal of information about how our environment has evolved and alerts us to emerging trends and diseases in animal populations in northern Illinois and beyond.

These studies are utilized by researchers in Cook County, across the region and around the country. ARC's funding for these studies is derived from fees collected for rabies tags in Cook County. The studies are a crucial component of ARC's mission to ensure the safety of residents and companion animals in Cook County.

Respectfully,

Tanya S. Anthony

Chief Administrative Officer
Cook County Government

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ANNUAL REPORT 2021

RESEARCH PROJECTS SPONSORED BY THE ENVIRONMENTAL RESEARCH PROGRAM, COOK COUNTY ANIMAL & RABIES CONTROL

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Cook County Animal and Rabies Control

Environmental Research Program

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BRIEF INTRODUCTION

Wildlife disease is of great importance to the health and safety of humans and domestic animals with 73% of emerging and reemerging pathogens are known to be zoonotic (transmitted from animals to people). There is increasing evidence suggesting that urbanization and resultant land-use changes contribute to the emergence of wildlife diseases through multiple mechanisms, with consequences for human and pet health. In light of the increasingly close association between wildlife and humans in Cook County, the need for surveillance and proactive research is needed to guide and interlink human health and wildlife management programs with the goal of limiting the risk of human exposure to zoonotic diseases and other conflicts, such as attacks by coyotes.

The following provides a summary of surveillance and research conducted during 2021 and early 2022 on wildlife species in Cook County that pose important risks for people and pets. The following work represents collaborations between Cook County Animal and Rabies Control, the Forest Preserve District of Cook County, and the Max McGraw Wildlife Foundation, among other partners.

COYOTE MONITORING

Coyotes are important animals to monitor for diseases, as well as changes in behavior. This is because they range over most of the Chicago area, can host (or carry) a wide range of diseases, and are capable of (occasionally) attacking pets or (rarely) people. In 2021 and early 2022, we focused most of our activities on livetrapping and radiotracking coyotes for monitoring disease prevalence, movements, survival, and behavioral observations. We also continued efforts on capturing and monitoring white-tailed deer and river otters in Cook County.

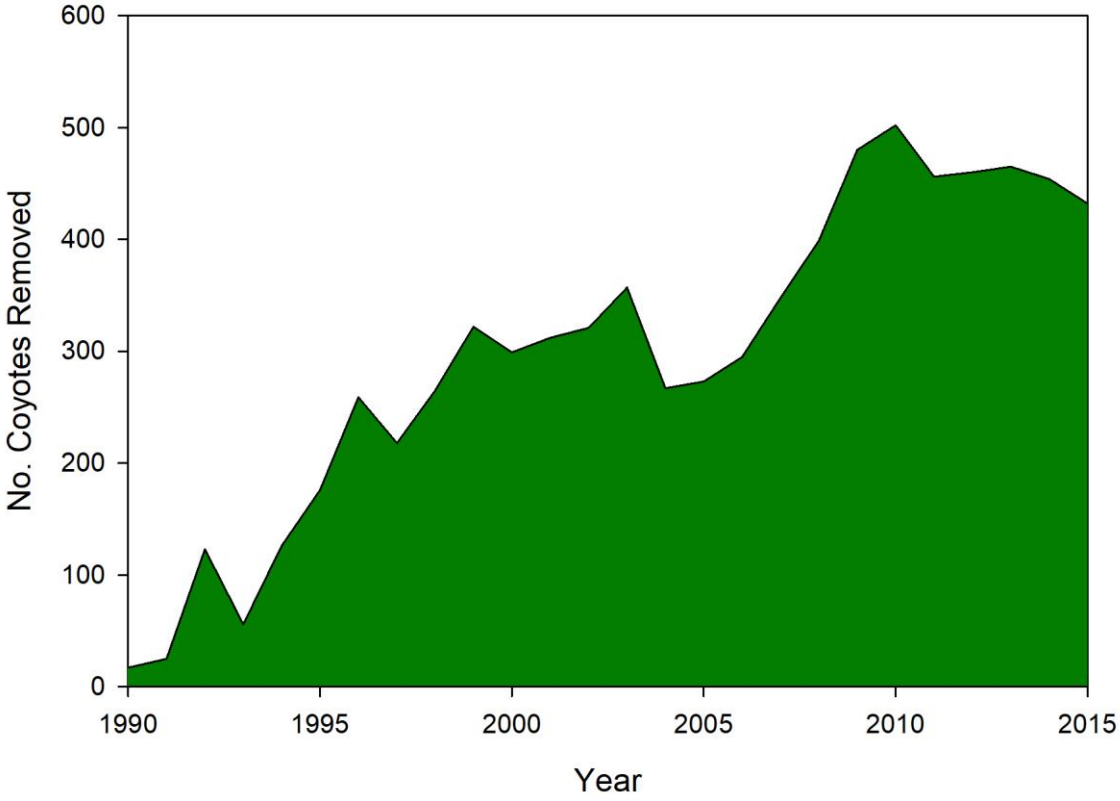
Background

Coyotes have greatly expanded their geographic range across North America, and have recently become top predators in many metropolitan areas. Their success in urban areas has important ecological implications, and is a potential threat to the health and well-being of people and their pets. Attacks on pets have occurred across the U.S., and attacks on people have become relatively more noticeable in the last 20 years. However, the actual risk posed by coyotes is poorly understood by the general public, and a lack of reliable information typically results in responses borne of emotion in homeowners and decision-makers. Understanding the ecology of coyotes in urban landscapes, and human responses to their presence, is crucial for developing effective responses on the threats coyotes pose to people and pets in metropolitan areas.

Capture of coyote as part of our long-term monitoring program. Courtesy of Jeff Nelson.

The number of human-coyote conflicts in the Cook County area increased dramatically during the 1990's, and has remained at a constant level since then, as indicated by the number of coyotes removed as nuisances from the Chicago metropolitan area.

Number of Coyotes Removed Annually from Northeastern Illinois by Nuisance Wildlife Control Permitees



We have been monitoring the Cook County coyote population since 2000 to determine population characteristics that may help minimize conflicts and guide management programs, in addition to surveillance for zoonotic diseases.

Capture Efforts

Each year we typically livetrap coyotes to collect blood and fecal samples and to individually mark and radiocollar them so we can follow their movements and observe their behavior. In 2021, we attempted to resume our standard trapping efforts that had been limited the previous year by Covid 19. During 2021 and early 2022, our livetrapping efforts yielded 33 captures and 3 recaptures of marked individuals. Newly-captured animals included 4 adults (1

F, 3 M), 16 subadults (8 F, 8 M), and 13 young of the year (6 F, 7 M). Blood samples were collected from each of these animals for disease monitoring. Since March 2000 to the current date (June, 2022), we have cumulatively captured and marked over 1,380 coyotes and radiocollared over 600 individuals.

Immobilized coyote about to receive a radiocollar. Courtesy Tom Uhlman, Scientists in the Field.

In addition to our trapping efforts, we also attempt to count and microchip pups at natal dens each year. Sampling and marking pups is important for estimating reproductive rate, among other aspects of the population. We have done this continuously each year since 2004; however, in 2020, we were unable to do this because of Covid 19 limitations. Thus, an objective for 2021 was to resume this activity. In 2021, we located 7 litters from radiocollared coyotes and subsequently microchipped 55 pups. This year, in 2022, we had a similar level of success, in which we located 8 litters and microchipped 51 pups. Average litter sizes were similar to previous years (7.9 foe 2021, and 6.4 for 2022).

Capture of coyote pups as part of our long-term monitoring program. Courtesy of Jeff Nelson.

Radiotracking Efforts

We record coyote locations and movements each year using a combination of VHF telemetry and GPS telemetry. In 2019, we radiotracked 68 radiocollared individuals throughout the year. Technicians recorded 4,962 coyote locations and we recorded over 10,000 GPS locations via satellites. Our radiotracking efforts declined during the Covid 19 shutdown in 2020, during which time we tracked 66 coyotes and recorded 3,486 VHF locations. In 2021, we tracked 73 coyotes and recorded 3,528 locations. A major objective of 2021 was to replace GPS collars that had been lost during 2020. These GPS-collared animals were used to facilitate outreach efforts such as a collaboration with National Geographic to illustrate movement patterns through the city (see below).

Technician radiotracking a VHF-collared coyote. Courtesy Tom Uhlman, Scientists in the Field.

Mortality Data

Another major objective of the study is to document fates of radiocollared coyotes, particularly with respect to disease and conflicts with people and pets. We recovered 14 mortalities during 2021, of which 7 were radiocollared (we also collect all coyotes found dead, regardless of whether they are marked). The causes of mortality for 2021 were vehicle collisions (29%), shot (21%), mange (21%), and unknown (29%). Unknown mortalities occasionally occur if a carcass is too decomposed for a full necropsy. Similar numbers have occurred to date in 2022, with 21 mortalities comprised of a greater proportion of animals trapped or shot.

It is important to note that the number of mortalities we recorded dropped substantially in 2020, likely due to our reduced monitoring effort; however, the number of recorded mortalities remained relatively low in 2021. For example, in previous years, we usually reported a consistent number of annual mortalities ranging in the low 40's. For example, during 2016-2018 they ranged from 42 to 43 mortalities each year. However, this dropped to 17 in 2020, and only 14 in 2021, one of the lowest numbers recorded in the study.

Radiocollared coyote during tracking in Chicago. Courtesy Alex Coombs, MMWF.

In the table below, we pool mortality data for long-term comparisons. The distribution of mortality causes was largely consistent across years until recently. Prior to covid, the primary cause of death was vehicle collisions, which was consistently higher than other causes during each period. However, most recently the frequency of vehicle-caused mortalities had dropped to a lower proportion than at any time in the study. However, any interpretation of these data are preliminary and more analysis, such as cause-specific mortality estimates, are needed. Also, the sample sizes are lower, which may influence these percentages. Notably, there is no appreciable change in the number of coyotes as part of conflicts with people (we do not include animals removed from airports in this case).

Causes of mortality for coyotes in Cook County.

Period	N	Vehicle	Shot	Mange	Unknown	Other	Conflict
2000-2011	182	47%	19%	12%	14%	7%	1%
2013-2014	38	37%	18%	21%	13%	8%	3%
2016-2017	48	54%	12%	4%	25%	4%	1%
2018-2019	72	54%	15%	11%	12%	1%	1%
2020-2021	31	26%	29%	16%	29%	0%	<1%

This is one line of evidence indicating that the level of conflict has remained about the same within the coyote population, and that there is no measurable shift in boldness or aggression. However, the public's perception may not reflect this because there may have been a numerical increase in nuisance coyotes if the coyote population has increased. In other words, the proportion of coyotes that creates a conflict has not changed, but as the population increases in overall size, this will result in a greater absolute number of coyotes in conflict with people.

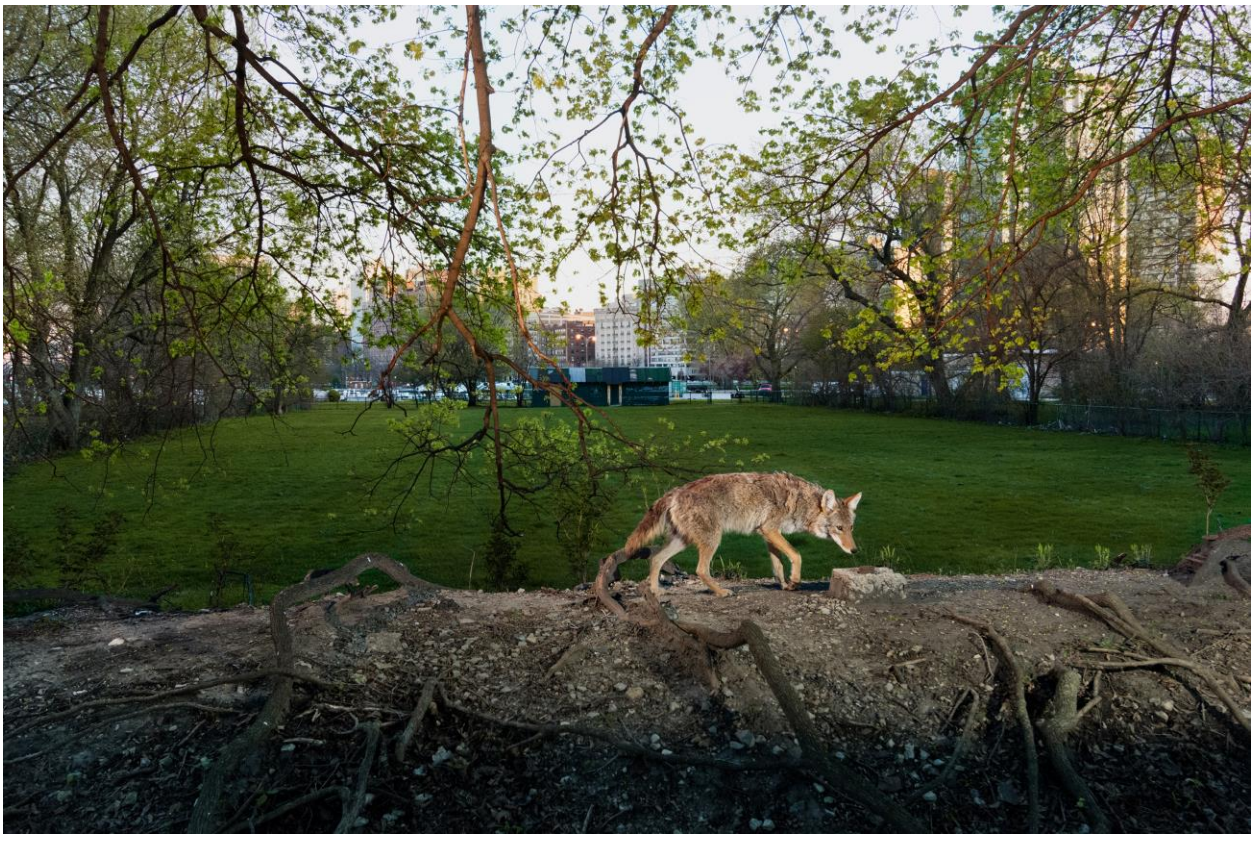
Movement Data

Using data from GPS collars, we have been able to document the ways in which people alter coyote behavior, often leading to conflicts. No other research programs have been able to follow coyotes from birth to their ultimate fate, and we have documented the few cases where coyotes have been fed by people which led to conflicts. Consequently this research has been used by various communities to establish or enforce no feeding ordinances.

A GPS-collared coyote in downtown Chicago. Image from Corey Arnold, National Geographic.

Another important aspect of the GPS technology is it allows us to monitor coyotes inhabiting the most urban parts of the landscape, which is important for us to determine if coyotes behave differently than our suburban study sites. To date, we have not seen an increase in aggressive behaviors exhibited by these coyotes toward people or their pets during the radiotracking, despite the proximity between the coyotes and people. However, the sample size is small, and we do not know if coyotes can continue to live downtown without eventually creating negative encounters with people. More research is needed in this area.

Of note, a main focus of our activities during the 2019-2020 winter was livetrapping and re-deploying GPS collars among coyotes located in the Chicago limits. Livetrapping in this area is always a complicated process that takes considerable time and effort.



A GPS-collared coyote located on the north side of Chicago. From Corey Arnold, National Geographic.

Multi-city novel object test

Last year we partnered with USDA Wildlife Research Center biologists to develop a design to conduct standardized novel object tests for coyotes residing in a variety of cities across North America. The objective is to determine if coyotes are behaving differently across cities, especially regarding boldness. This is a natural expansion of our recent work in Cook County where we conducted intesive novel object tests to determine pattens of boldness among coyotes in the county. As part of this large scale effort, we conducted multiple novel object tests in September-November. Results from this work will be compared to other cities and will help us better understand the variability among different coyote populations, and help us put our results in Cook County in context relative to the rest of the country.

We selected 25 sites for a camera and half also had a novel object. Cameras were maintained for at least 3 weeks. Coyotes were detected at least once for 18 of the sites. Overall visitation rates were higher for Cook County than for the other locations. Results from those tests are currently being tabulated and analyzed, and we will be able to interpret these results in a more meaningful way. At this point, the preliminary analysis suggests that coyotes in Cook County may be behaving differently than coyotes in some other cities.

Preliminary results of novel object tests across metropolitan areas.

Location	No. of Sites		Coyote Visitation Rate
Cook County	25		**72%**
Columbus	28		25%
Cleveland	39		26%

OUTREACH

A major activity each year is our professional and public outreach. Professional outreach activities include publishing our results in peer-reviewed literature, and presenting our results at scientific meetings. We maintain a website to make our results available to the general public and agencies, which is probably our most impactful outreach mechanism.

In 2021, we averaged >6k visits each week, resulting in >300k visits annually.

Google analytics for the website, urbancoyoteresearch.com, in terms of weekly visits.

Much of our outreach involves communicating with the media, including responses for information across North America. The following is a short list of 2021 media references to the project:

Media:

- Popular Science. February 9, 2021. Four wild animals that are thriving in cities.

- https://www.popsci.com/story/animals/urban-animal-populations-thrive/

- **New York Times.** February 18, 2021. Attacks by Urban Coyotes Are Rare, but Frightening
  - https://www.nytimes.com/2020/01/09/science/chicago-coyote-attacks.html

- **Vancouver Sun**. March 9, 2021. Urban wildlife expert links feeding coyotes to attacks on humans in Stanley Park
  - https://vancouversun.com/news/local-news/urban-wildlife-expert-links-feeding-coyotes-to-attacks-on-humans

- **CBC Radio, Quirks and Quarks**. April 9, 2021. How coyotes have managed to find success in the city like no other predator
  - https://www.cbc.ca/radio/quirks/coyotes-doing-well-in-the-city-asteroid-impact-created-rainforests-the-minimal-organism-and-more-1.5980412/how-coyotes-have-managed-to-find-success-in-the-city-like-no-other-predator-1.5980419

- **Toronto Star.** August 2, 2021. Coyotes are already among us. In fact, they may be Toronto's most successful urban invader since the squirrel.
  - https://www.thestar.com/news/gta/2021/08/02/coyotes-are-already-among-us-in-fact-they-may-be-torontos-most-successful-urban-invader-since-the-squirrel.html

- **CBC Kids News**. August 12, 2021. What to do if you meet a coyote in the city.
  - https://www.cbc.ca/kidsnews/post/what-to-do-if-you-meet-a-coyote-in-the-city

- **Minneapolis St Paul Magazine**. November 22, 2021. Tracking City Foxes and Coyotes.
  - https://mspmag.com/arts-and-culture/twin-cities-foxes-coyotes/

- **The Globe and Mail**. January 2, 2022. More coyotes are adapting to urban areas in Canada. Here's why they thrive in cities.
  - https://www.theglobeandmail.com/canada/article-more-coyotes-are-adapting-to-urban-areas-in-canada-heres-why-they/

- **PBS Overview**. November 4, 2021.
  - https://www.youtube.com/watch?v=OB7nomE1VL4&t=326s

Currently, the project is featured in the July issue of National Geographic. This story and accompanying photographs are the results of weeks of collaborative work during 2020 and

2021, and has resulted in photographs appearing in this report. Below is a photo of the photographer, Corey Arnold.

COLLABORATORS

An important aspect of this program is the collaborations and partnerships that form the foundation of much of this work. Partnerships increase our pool of expertise while simultaneously maximizing the financial support, since our partners typically invest their time and resources to individual projects. In addition to our primary agencies (Cook County Animal and Rabies Control, the Forest Preserve District of Cook County, and the Max McGraw Wildlife Foundation), our partners include Loyola University (Dr. Jean Dubach), University of Minnesota (Dr. Meggan Craft), University of New Mexico (Dr. Seth Newsome), University of Calgary (Dr. Ale Massolo), and USDA/APHIS National Wildlife Research Center (Dr. Julie Young).

PRODUCTS

PUBLICATIONS

1. Gehrt, S. 2021. Ghost Dogs and Their Unwitting Accomplices. Anthropology Now 13:41-53. https://doi.org/10.1080/19428200.2021.1982347

2. Worsley-Tonks K. E. L., Miller E. A., Anchor, C. L., Bender J. B., Gehrt S. D., McKenzie S. C., Singer, R. S., Johnson T. J., Craft M. E. 2021. Importance of anthropogenic sources at shaping the antimicrobial resistance profile of a peri-urban mesocarnivore. Science of the Total Environment 764:144166. https://doi.org/10.1016/j.scitotenv.2020.144166

3. Zepeda, Emily; Payne, Eric; Wurth, Ashley; Sih, Andrew; Gehrt, Stan. 2021. Early life experience with urbanization influences dispersal in coyotes (Canis latrans). Behavioral Ecology 32:728-737.

4. Jacob, Joanna; Kent, Molly; Benson-Amram, Sarah; Herculano-Houzel, Suzana; Raghanti, Mary Ann; Ploppert, Emily; Drake, Jack; Hindi, Bilal; Natale, Nick; Daniels, Sarah ; Fanelli, Rachel; Landis, Tim; Rzucidlo, Amanda; Gilbert, Amy; Johnson, Shylo; Lai, Annie; Hyer, Molly; Anchor, Chris; Gehrt, Stan; Lambert, Kelly. 2021. Cytoarchitectural Characteristics Associated with Cognitive Flexibility in Raccoons. Journal of Comparative Neurology, 529:3375-3388.

5. Worsley-Tonks; Katherine, Stanley Gehrt; Chris Anchor; Luis Escobar; Meggan E Craft. 2021. Infection risk varies within urbanized landscapes – the case of coyotes and heartworm. Parasites & Vectors, 14:1-13.

6. Worsley-Tonks, Katherine E. L., Stanley D. Gehrt, Elizabeth A. Miller, Randall S. Singer, Jeff B. Bender, James D. Forester, Shane C. McKenzie, Dominic A. Travis, Timothy J. Johnson, Meggan E. Craft. 2021. Comparison of antimicrobial-resistant Escherichia coli between urban raccoons and domestic dogs. Applied and Environmental Microbiology 87: DOI: https://doi.org/10.1128/AEM.00484-21

In Review:

1. Wilson, Evan C., Chris Anchor, Stanley D. Gehrt. In revision. What regulates urban coyotes? The role of sarcoptic mange in urban coyote population dynamics. Journal of Applied Ecology.

2. Gehrt SD, EM Muntz, EC Wilson, J Power, SD Newsome. In review. Severe environmental conditions create severe conflicts? Coyote attacks on humans in Cape Breton Highlands National Park, Nova Scotia. Journal of Applied Ecology.

In Preparation (near submission):

1. Milling, C.R., S. McKenzie, S.D. Gehrt. Evaluation of Iridium-transmitted GPS telemetry data for use in assessments of wildlife space use. In preparation for: *PLoS ONE*.

2. Milling, C.R., C. Anchor, S.D. Gehrt. Survival of translocated nuisance coyotes in the Chicago Metropolitan Area: implications for urban wildlife management. In preparation for: *Journal of Wildlife Management.*

3. Robinson, K, E Ellington, C Tonra, and S Gehrt. Stress in the city? Coyote cortisol varies with intrinsic and extrinsic factors within a heavily urbanized landscape. In preparation for: *Science of the Total Environment.*

4. Wurth, A, S Gehrt. Influence of urbanization on body size and condition of coyotes (Canis latrans) in the Chicago Metropolitan Area. In preparation for: *Journal of Mammalogy.*

PROFESSIONAL PRESENTATIONS

1. Milling, C. and S. Gehrt. (2021) Survival of translocated nuisance coyotes in the Chicago Metropolitan Area: implications for urban wildlife management. The Wildlife Society Annual Meeting. Virtual. November 1 – 5.

2. Milling, C., S. McKenzie, and S. Gehrt. (2021) Evaluating the quality and utility of Iridium-transmitted data for a free-ranging mesocarnivore. Montana Chapter of The Wildlife Society Annual Conference. Virtual. February 23 – 25.

3. Ellington, EH, Newsome, S, Gehrt, SD. (2021) How urbanization and diet influence coyote behavior. International Urban Wildlife Conference [Virtual].

4. Milling, CR, SD Gehrt, C Anchor. (2021) Do translocated coyotes live happily ever after? Managing urban canids and human expectations. Invited seminar at Gonzaga University, Spokane, WA. Oct. 22.

5. Zepeda, E., Payne, R., Wurth, A., Sih, A. and Gehrt, S. (2021) Early life experience with urbanization influences departure and transience behavior in coyotes. *The Wildlife Society, virtual*.

6. Gehrt, SD. 2021. Extreme Urban Ecology of an Apex Predator: Coyotes in Chicago. Invited Departmental Seminar, Queens College, New York. February 10.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PUBLIC AND ENVIRONMENTAL HEALTH MICROBIOLOGY

Comparison of Antimicrobial-Resistant *Escherichia coli* Isolates from Urban Raccoons and Domestic Dogs

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Timothy J. Johnson and Meggan E. Craft contributed equally to this work.

ABSTRACT Wildlife can be exposed to antimicrobial-resistant bacteria (ARB) via multiple pathways. Spatial overlap with domestic animals is a prominent exposure pathway. However, most studies of wildlife-domestic animal interfaces have focused on livestock and little is known about the wildlife-companion animal interface. Here, we investigated the prevalence and phylogenetic relatedness of extended-spectrum cephalosporin-resistant (ESC-R) *Escherichia coli* from raccoons (*Procyon lotor*) and domestic dogs (*Canis lupus familiaris*) in the metropolitan area of Chicago, IL, USA. To assess the potential importance of spatial overlap with dogs, we explored whether raccoons sampled at public parks (i.e., parks where people and dogs could enter) differed in prevalence and phylogenetic relatedness of ESC-R *E. coli* to raccoons sampled at private parks (i.e., parks where people and dogs could not enter). Raccoons had a significantly higher prevalence of ESC-R *E. coli* (56.9%) than dogs (16.5%). However, the richness of ESC-R *E. coli* did not vary by host species. Further, core single-nucleotide polymorphism (SNP)-based phylogenetic analyses revealed that isolates did not cluster by host species, and in some cases displayed a high degree of similarity (i.e., differed by less than 20 core SNPs). Spatial overlap analyses revealed that ESC-R *E. coli* were more likely to be isolated from raccoons at public parks than raccoons at private parks, but only for parks located in suburban areas of Chicago, not urban areas. That said, ESC-R *E. coli* isolated from raccoons did not genetically cluster by park of origin. Our findings suggest that domestic dogs and urban/suburban raccoons can have a diverse range of ARB, some of which display a high degree of genetic relatedness (i.e., differ by less than 20 core SNPs). Given the differences in prevalence, domestic dogs are unlikely to be an important source of exposure for mesocarnivores in urbanized areas.

IMPORTANCE Antimicrobial-resistant bacteria (ARB) have been detected in numerous wildlife species across the globe, which may have important implications for human and animal health. Wildlife can be exposed to ARB via numerous pathways, including via spatial overlap with domestic animals. However, the interface with domestic animals has mostly been explored for livestock and little is known about the interface between wild animals and companion animals. Our work suggests that urban and suburban wildlife can have similar ARB to local domestic dogs, but local dogs are unlikely to be a direct source of exposure for urban-adapted wildlife. This finding is important because it underscores the need to incorporate wildlife into antimicrobial

Citation Worsley-Tonks KEL, Gehrt SD, Miller EA, Singer RS, Bender JB, Forester JD, McKenzie SC, Travis DA, Johnson TJ, Craft ME. 2021. Comparison of antimicrobial-resistant *Escherichia coli* isolates from urban raccoons and domestic dogs. Appl Environ Microbiol 87:e00484-21. https://doi.org/10.1128/AEM.00484-21.

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resistance surveillance efforts, and to investigate whether certain urban wildlife species could act as additional epidemiological pathways of exposure for companion animals, and indirectly for humans.

KEYWORDS cephalosporin, dog, *Escherichia coli*, interface, phylogenetic, raccoon, urban

Human encroachment into natural habitats, urbanization, and wildlife adaptation to human activity have increased the extent to which humans and domestic animals interface with wildlife. Greater contact between humans, domestic animals, and wildlife increases the risk of infectious agent spillover (1–4). Our understanding of this phenomenon has mostly been driven by pathogen spillover from wildlife into human or domestic animal populations (e.g., Ebola virus, avian influenza virus, SARS-CoV, and SARS-CoV-2) (2, 5, 6). However, infectious agents can also spill over from human sources into wild animal populations through the environment, which can threaten public and domestic animal health if wildlife cause further spread and spillback into the human and/or domestic animal populations (1).

A quintessential example of spillover from human sources into wildlife is the dissemination of antimicrobial-resistant bacteria (ARB) (7–9). ARB that are typically associated with clinical settings have been detected in numerous wildlife species across the globe (9, 10). In general, wild animals are more likely to shed ARB if they are closer to human-dominated areas, such as livestock facilities, urban areas, landfills, and fish farms (8, 10–12). In some human-dominated settings, ARB prevalence in wildlife can be as high as 50% or more, such as in some bird, mesocarnivore, rodent, and ungulate populations (13–17). Further, wildlife present in these human-dominated areas tend to have ARB that are similar to those of local human and/or domestic animal populations, both in terms of genetic relatedness and the antimicrobial-resistance gene (ARG) profiles (18–20). Thus, it has become clear that many ARB detected in wildlife are of anthropogenic origin (10, 12). Further, because ARG can be horizontally transferred between bacteria via processes such as conjugation, there is a concern that AMR has the potential to spread in wildlife bacterial communities (21). Under this scenario, wildlife would not only act as vectors of AMR, but also as reservoirs (22, 23).

In urban settings, ARB have been detected in multiple wildlife species (e.g., rodent, gull, song bird species) (14, 23–25) and, in most cases, prevalence tends to be higher than in nonurban wildlife (14, 26). Urban wildlife can be exposed to ARB and associated ARG via multiple pathways, including contaminated waters, garbage or other food sources (9, 12, 23), and livestock manure (27). While ARB are unlikely to be directly transmitted between humans and wildlife, transmission could occur more readily via domestic animals. Companion animals are especially likely to be important because they frequently use the same green spaces as urban wildlife (28–30) and share several infectious agents with wildlife and humans (e.g., Hendra virus, *Salmonella* spp.) (31, 32), including ARB (32–34). Despite this potential risk, AMR research at the wildlife-companion animal interface has been explored infrequently and with conflicting results. In some cases, companion animals and wildlife have similar AMR profiles (15, 35), while in others there is less evidence of similarity (36), indicating that more research is needed.

Here, we compared ARB isolated from raccoons (*Procyon lotor*) and domestic dogs (*Canis lupus familiaris*) sampled in the metropolitan area of Chicago, IL, USA. We focused on raccoons and domestic dogs because they both frequently use urban green spaces (e.g., parks and backyards) (37), share several infectious agents (e.g., *Leptospira* spp., canine distemper virus), and can shed ARB (13, 34, 38). Further, our previous research revealed that raccoon and dog samples pooled by animal species had several ARG in common (39). In the present study, we explore the interface between raccoons and dogs in more detail by investigating the prevalence and phylogenetic relatedness of extended-spectrum cephalosporin-resistant *Escherichia coli* (ESC-R *E.*

coli) in 211 raccoons and 176 domestic dogs. ESC-R *E. coli* include both extended-spectrum beta-lactamase (ESBL) and AmpC beta-lactamase-producing *E. coli*, which are resistant to third generation cephalosporins (e.g., cefotaxime, ceftazidime). We focused on ESC-R *E. coli* because they are of increasing concern in human and veterinary medicine (40–43), and have been reported in healthy human (44–46), livestock (47, 48), and companion animal populations (41, 49–51), as well as in the environment (52–54). ESC-R *E. coli* has also been isolated from the feces of over 30 wildlife species (e.g., gulls, wild boar, mallard duck, rodent species) (8, 55), including over half of the 211 raccoons previously sampled in our system (13).

The specific objectives of this study were to (i) explore the extent to which raccoons and dogs have similar ESC-R *E. coli* profiles in terms of prevalence, phylogenetic relatedness, and number and types of ARG, and (ii) determine whether raccoons differed in ESC-R *E. coli* profile based on whether they were sampled at public parks (i.e., parks where people and dogs could enter) or at private parks (i.e., parks where people and dogs could not enter), and how this compared to domestic dogs. We hypothesized that raccoons would have a lower prevalence and diversity of ESC-R *E. coli* than dogs because of antimicrobial use in dogs, their intimate contact with humans (33, 34), and because of wildlife-domestic animal findings in other urban systems (e.g., reference 27). Additionally, we expected raccoons at public parks to have a higher prevalence of ESC-R *E. coli* than raccoons at private parks because of potentially higher contact rate with dog feces and human garbage. By extension, we also expected raccoons at private parks to have ESC-R *E. coli* that were more phylogenetically distinct to ESC-R *E. coli* isolated from dogs and raccoons at public parks.

RESULTS

Raccoon and domestic dog characteristics. Raccoons and dogs were sampled over the course of four seasons, from February to November 2018 in northwestern Chicago, IL, USA. Raccoons were captured and sampled from seven sites that differed based on whether they were urban or suburban and whether they were on private or public land (Fig. 1). Together, the seven sites covered a distance of ~40 km. At public sites, most dogs were required to be leashed by law. At private sites, dogs were not allowed to enter. Of the 211 raccoons sampled (17 of which were captured twice and one three times), 61.6% were sampled in suburban areas and 38.4% in urban areas, and 63.5% were sampled at public sites and 36.5% at private sites.

Domestic dogs were sampled at three of the seven sites where raccoons were sampled (two suburban and one urban) or at nearby dog parks (Fig. 1). Of the 176 dogs sampled, 12.5% were sampled from the same household as at least one other sampled dog. Based on dog owner survey results, 36.4% of dogs were ≤2 years of age, 42.6% were between 2 and 7, 19.3% were older than 7, and 1.7% had no age data. Stratified by sex, 56.3% dogs were males, 37.5% were females, and 6.2% had no data. In terms of antibiotic use, 30.1% of sampled dogs were on some form of antibiotic in the 12-months prior to sampling, 53.4% were not, 11.9% of owners were unsure, and 4.6% of owners did not respond. Based on where dogs were sampled, 48.3% of dogs were sampled at sites where raccoons were sampled and 51.7% at local dog parks. Most sampled dogs lived in the northwestern portion of the Chicago area (based on home ZIP code) (Fig. 1), and 64% of dogs had their home ZIP code that overlapped with at least one of the sites where raccoons were sampled (Fig. 1).

Domestic dogs had a lower prevalence of ESC-R *E. coli* than raccoons, but ESC-R *E. coli* bacteria isolated from dogs and raccoons were not genetically distinct and in some cases displayed a high degree of similarity and had multiple ARG in common. With a sample prevalence of 56.9% (95% confidence interval [CI] = 50.1% to 63.4%) and 16.5% (95% CI = 11.7% to 22.7%) for raccoons and dogs, respectively (Fig. 2A), there was a significantly higher odds of recovering at least one ESC-R *E. coli* isolate from raccoons than from dogs (Fisher's exact test; odds ratio [OR] = 3.44, 95% CI = 2.16 to 5.63; $P < 0.0001$). Whole-genome sequencing and multilocus sequence typing (MLST) revealed that of the 152 ESC-R *E. coli* isolates recovered from raccoons and

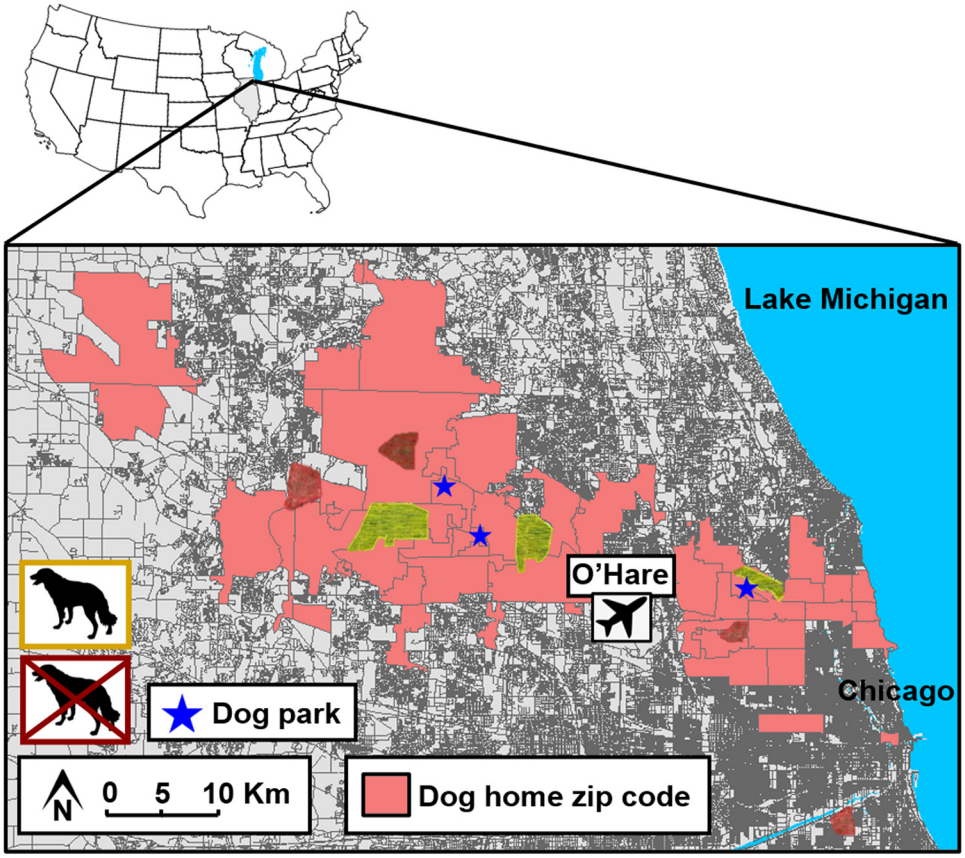

FIG 1 Sampling sites in the northwestern portion of the Chicago metropolitan area. Small dark red and yellow polygons depict sites where raccoons were sampled. The four small dark red polygons are private sites (i.e., sites where people and domestic dogs were not allowed to enter) and the three yellow polygons are public sites (i.e., sites where people and dogs were allowed to enter). Blue stars represent dog parks and pink polygons are dog home ZIP codes. Four shapefiles were used to create the map: (i) a street shapefile for Cook County (https://hub-cookcountyil.opendata.arcgis.com/datasets/4569d77e6d004c0ea5fada54640189cf_5), (ii) a street shapefile for DuPage County (https://gisdata-dupage.opendata.arcgis.com/datasets/roadtypecenterline?geometry=-89.010%2C41.659%2C-87.158%2C42.017), (iii) a Lake Michigan shapefile (https://gis-michigan.opendata.arcgis.com/datasets/5e2911231fe246128d0ff8495935ee85_12), and (iv) a U.S. shapefile (https://hub.arcgis.com/datasets/1b02c87f62d24508970dc1a6df80c98e_0?geometry=118.842%2C29.346%2C-4.029%2C67.392).

dogs (123 from raccoons and 29 from dogs), raccoons had a total of 55 unique sequence types (STs) and one unknown (the unknown ST closely resembled ST155, with variation in the *gyrB* allele only) and dogs had 20 unique STs and two unknown (one of the unknowns closely resembled ST58, with variation in the *parA* allele only, and the other was dissimilar to all STs) (Fig. 2B). Accounting for differences in samples sizes, bootstrapping the raccoon sample size to the dog sample size (i.e., from $n = 123$ to $n = 29$) revealed that the raccoon and dog populations likely shed a similar richness of STs (95% CI for raccoons = 16.1 to 23.8 using 1,000 bootstrap replicates). Of the STs detected, ST38 was most commonly detected in raccoon samples (8.8%), followed by ST973 (7.3%), and both ST68 and ST162 (4.8%) (Fig. 2B). For dogs, ST68 was most common (13.8%), followed by ST297 (10.3%) (Fig. 2B).

In terms of phylogenetic similarity, raccoons and dogs had 12 STs in common, including ST10, ST38, ST68, and ST131 (Fig. 2B). Core single-nucleotide polymorphism (SNP)-based phylogenetic analyses revealed that within-species average core SNP differences were similar to between-species average core SNP differences (raccoon to raccoon: 455.8 mean core SNP difference; dog to dog: 489.2; raccoon to dog: 480). Further, the maximum likelihood phylogenetic tree showed no clustering by species, with dog and raccoon samples randomly interspersed throughout the tree (Fig. 2C), which was supported

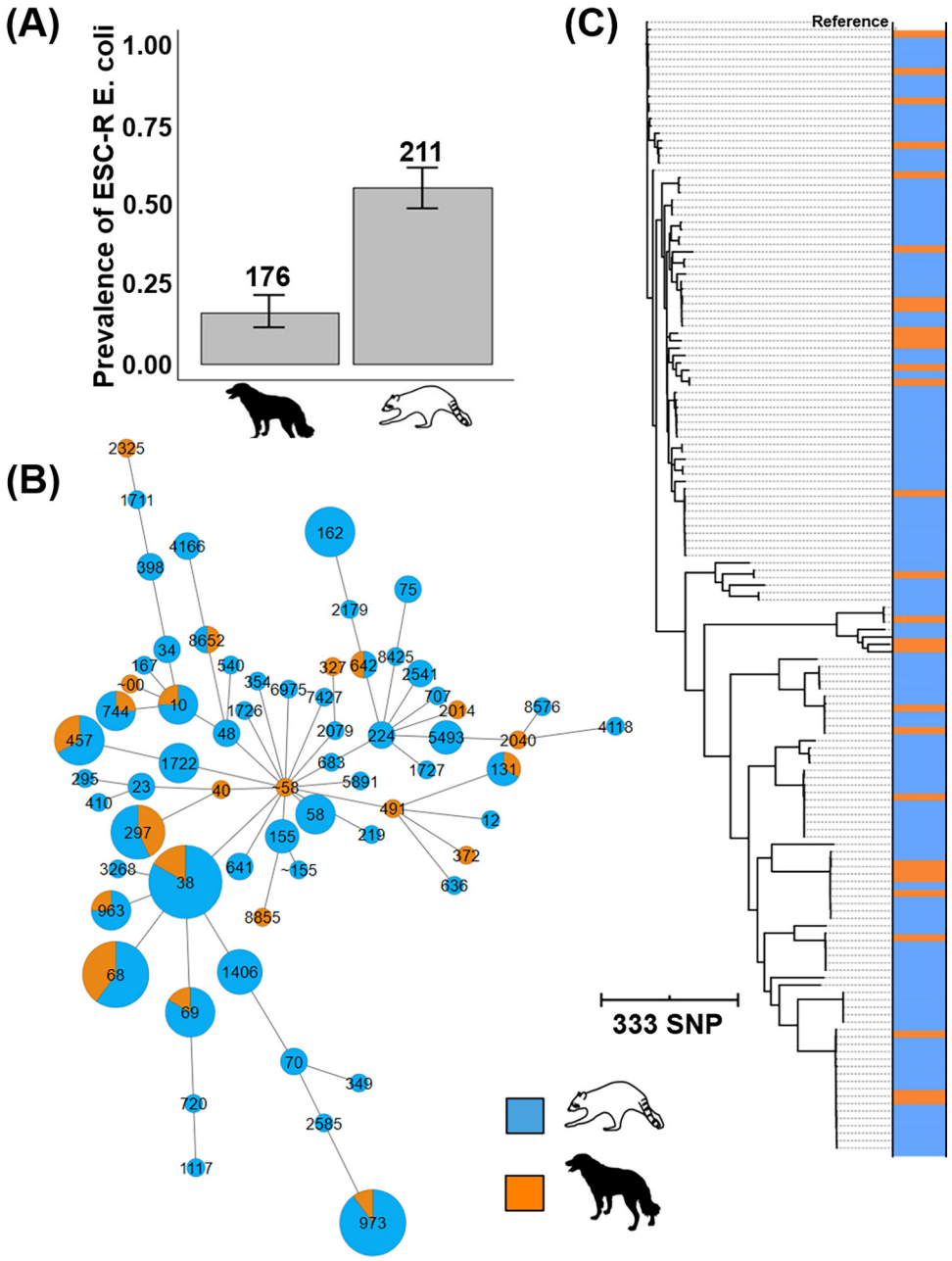

FIG 2 Prevalence and phylogenetic associations of ESC-R *E. coli* isolated from raccoons and domestic dogs. (A) Prevalence of ESC-R *E. coli*. Whiskers represent 95% confidence intervals and numbers above whiskers are sample sizes. (B) Minimum spanning tree of ESC-R *E. coli* sequence types (STs) detected in raccoons (blue) and domestic dogs (orange). The size of nodes represents the number of isolates and the length of lines connecting nodes represents the number of allelic differences. ST numbers preceded by a tilde were unknown. (C) Core SNP-based maximum likelihood phylogenetic tree of the 152 ESC-R *E. coli* and heatmap of isolates classified based on host species (i.e., raccoon, blue; dog, orange). The reference is *E. coli* K-12 strain MG1655.

by a lack of significant difference in the phylogenetic distance of ESC-R *E. coli* isolates by animal species (permutational multivariate analysis of variance [PERMANOVA]: $F_{1,\ 151} = 0.45$, $P = 0.85$). Focusing on isolates that belonged to one of the 12 STs shared between raccoons and dogs (19 isolates from dogs and 51 isolates from raccoons), pairs of isolates displayed a high degree of similarity, as they differed by less than 20 core SNPs in all cases and were similar both within and between animal species (Fig. 3).

With regard to ARG, a total of 56 and 40 ARGs were identified in ESC-R *E. coli* isolated from raccoons and dogs, respectively, and most were found in isolates of both species

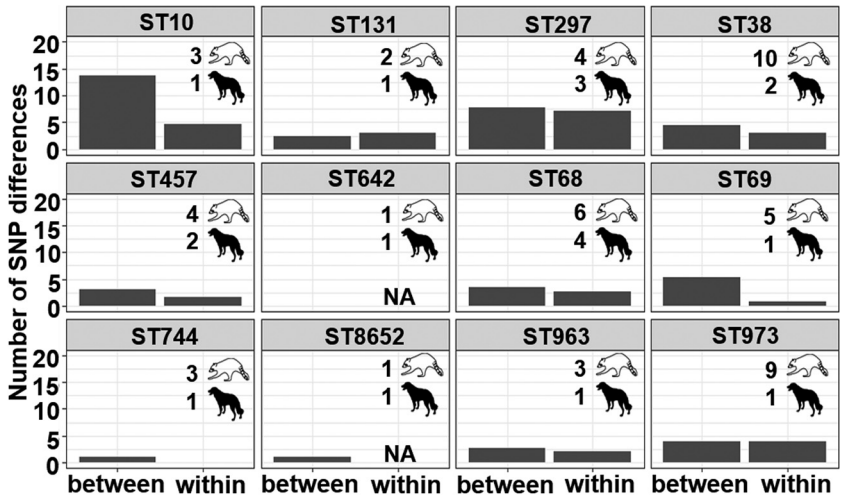

FIG 3 Mean number of core SNP differences between pairs of ESC-R *E. coli* isolates by sequence type (ST) based on whether pairs of isolates were from different animal species ("between") or the same animal species ("within"). Numbers next to raccoon and dog silhouettes are the number of isolates belonging to each animal species by ST. NA indicates that no comparison could be done.

(Fig. 4). Focusing on beta-lactam genes (i.e., *bla* genes), bla_{CMY-2} was the most prevalent in both raccoon and dog ESC-R *E. coli* isolates (54% and 62%, respectively), followed by bla_{TEM-1B} (26% and 21%, respectively). Further, 43.9% of beta-lactam genes detected in ESC-R *E. coli* isolated from raccoons were of bla_{CTX-M}-type, of which $bla_{CTX-M-15}$ was the most common, followed by $bla_{CTX-M-14}$ and $bla_{CTX-M-55}$. For dogs, bla_{CTX-M}-type genes accounted for 25% of beta-lactam genes, of which $bla_{CTX-M-1}$ and $bla_{CTX-M-55}$ were the most common. For non-beta-lactam genes, a greater proportion of ESC-R *E. coli* isolated from raccoons had fluoroquinolone and tetracycline ARGs than ESC-R *E. coli* isolated from dogs (Fig. 4).

Probability of isolating ESC-R *E. coli* from raccoons sampled at public parks was higher than for raccoons sampled at private parks, but only at suburban parks. After controlling for seasonal and urban-suburban context effects based on findings from previous work (13), binomial generalized linear mixed models (GLMMs) revealed that the odds of isolating ESC-R *E. coli* from raccoons varied significantly based on whether raccoons were sampled at public or private sites, with an interaction effect between whether a site was private or public and urban or suburban. Specifically, the odds of isolating ESC-R *E. coli* from raccoons was higher at public compared to private sites, but only in suburban sites and not urban sites (Table 1; Fig. 5).

ESC-R *E. coli* bacteria isolated from raccoons sampled at public parks were not phylogenetically distinct from those isolated from raccoons sampled at private parks or from those isolated from domestic dogs. There was no significant difference in the phylogenetic distance of ESC-R *E. coli* isolates recovered from raccoons sampled at public parks and raccoons sampled at private parks or from those recovered from domestic dogs (PERMANOVA: $F_{2, 151} = 0.43$, $P = 0.95$).

DISCUSSION

Wildlife can be exposed to ARB via multiple pathways, including through spatial overlap with domestic animals. However, in this study, we found no evidence that spatial overlap with domestic dogs acts as a major source of exposure for urban-adapted raccoons. ESC-R *E. coli* were three times more likely to be recovered from raccoons than domestic dogs, although isolates obtained from raccoons were not genetically distinct from those obtained from dogs and in some cases displayed a high degree of similarity (i.e., differed by less than 20 core SNPs). When exploring the importance of raccoon spatial overlap with dogs and people at parks, we found that the odds of isolating ESC-R *E. coli* from raccoons was higher when raccoons were sampled at public

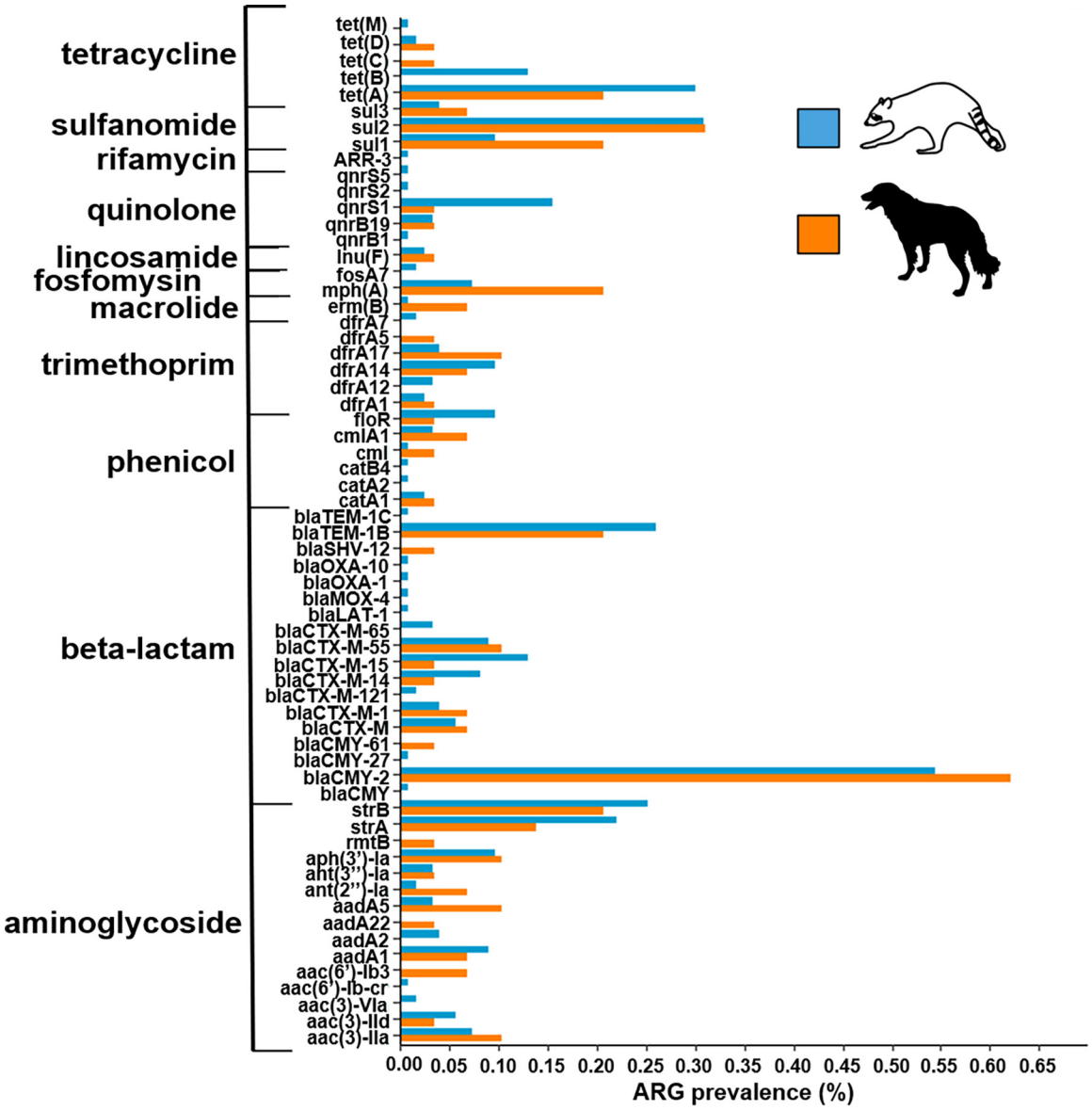

FIG 4 Prevalence of antimicrobial resistance genes (ARG) in ESC-R *E. coli* isolated from raccoons (blue) ($n = 123$) and domestic dogs (orange) ($n = 29$).

than at private parks, with this difference being only apparent at suburban and not urban parks. In terms of genetic relatedness of ESC-R *E. coli*, we found that ESC-R *E. coli* bacteria isolated from raccoons sampled at public parks were not distinct from those isolated from raccoons sampled at private parks or from those isolated from dogs.

It was surprising to find that raccoons had a higher prevalence of ESC-R *E. coli* than dogs, since dogs are considered reservoirs for AMR due to the use of antimicrobials in these animals and their close contact with humans and other animals in which antimicrobials are used (34). That said, wildlife could have higher AMR prevalence than dogs if they were exposed to ARB and ARG through pathways that dogs were less likely to be exposed to. For example, lakes and rivers are important pathways for the dissemination of ARB into the environment (52, 56, 57), and water-associated wildlife species are especially likely to be exposed (26, 58, 59). Raccoons select habitats with water bodies (60, 61) because a large proportion of their food is in or along rivers and lakes (62). Thus, it is possible that raccoons had a higher prevalence of ESC-R *E. coli* compared to

TABLE 1 Generalized linear mixed model results for isolating at least one ESC-R *E. coli* from raccoons[a]

Predictor variable	Odds ratio	95% CI	*P*
season (spring)	**8.05**	**(2.71–23.9)**	**<0.001**
season (summer)	**5.05**	**(2.03–12.59)**	**0.001**
season (winter)	0.49	(0.2–1.17)	0.11
urban-suburban context (urban)	**34.95**	**(5.42–225.39)**	**<0.001**
dog presence (yes)	**5.36**	**(1.26–22.83)**	**0.02**
urban context (urban) × dog presence (yes)	**0.07**	**(0.01–0.79)**	**0.03**

[a]Significant terms are depicted in boldface type (with 95% CI not overlapping with 1 and $P < 0.05$).

dogs because they were exposed to ARB via contaminated water sources. This is, however, speculative as no environmental samples were collected as part of this study. While previous work has suggested that differences in the prevalence of certain ARB between animals species could be attributed to differences in the host gut hospitability to certain bacteria (8, 55, 63), it is unlikely to be of importance here because ESC-R *E. coli* have previously been isolated from dogs in both clinical and community settings (64, 65). Further, work comparing the AMR profiles of owned and stray dogs and three mesocarnivore species supports the notion that environmental factors are more likely to be important than physiological ones (39). As such, differences in exposure risk are likely a more plausible explanation for the prevalence differences detected here than differences in host physiological characteristics. Differences in exposure risk may also explain differences in prevalence observed between urban and suburban raccoons, which are possibly due to variation in home range size and food availability, as discussed in reference 13.

While raccoons tended to have a higher sample prevalence of ESC-R *E. coli* than dogs, raccoons sampled at public parks were more likely to have ESC-R *E. coli* than raccoons sampled at private parks, but only in suburban areas and not urban areas. Previous work has shown the presence of domestic animals to be an important determinant of isolating ARB from wildlife (e.g., reference 66). However, since the dog population tended to have a low prevalence of ESC-R *E. coli* (16.5%), the presence of dogs themselves is unlikely to be the main factor associated with the differences detected at suburban sites. Instead, the difference in the number of people (with and without dogs) and the anthropogenic waste left at parks was potentially more influential. While water bodies are predicted to be the primary pathway of wildlife exposure to ARB, anthropogenic waste is also thought to be important (9). For example, wildlife can have a higher prevalence of ARB and ARG when using landfills (67), and similar ARB to those detected in landfills (59) or other wildlife sampled at landfills (68). Raccoons are

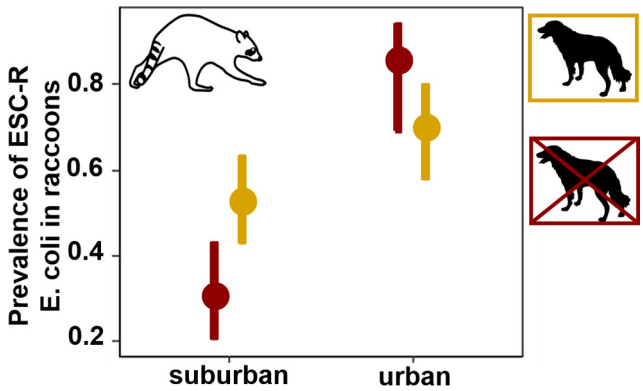

FIG 5 Raw prevalence of ESC-R *E. coli* in raccoons by urban-suburban context and dog presence (yellow, public park [i.e., people and domestic dogs can enter], red, private parks [i.e., people and domestic dogs cannot enter]). Whiskers are 95% confidence intervals. Raccoons were sampled in two public ($n = 78$) and two private ($n = 52$) suburban parks and one public ($n = 56$) and two private ($n = 25$) urban parks.

generalist and opportunistic feeders (69), and in urban and suburban areas they will feed on anthropogenic waste present in parks, either on the ground or in trash cans (70). Thus, raccoons sampled at public suburban parks may have had a higher prevalence than raccoons sampled at private suburban parks because of the higher exposure to people and anthropogenic waste. A lack of difference detected at urban parks could be because raccoons at both private and public parks were equally likely to be exposed to anthropogenic waste. However, because a small number of parks were examined (and only one urban public park), more work is needed to ascertain the importance of people and anthropogenic waste in influencing the prevalence of ARB in urban-adapted wildlife.

While dog and raccoon populations differed in ESC-R *E. coli* prevalence, ESC-R *E. coli* isolated from the two animal species were not genetically distinct. Further, in some cases raccoon and dog isolates differed by less than 20 core SNPs. Such a high degree of similarity could reflect transmission among dogs and raccoons sampled (71, 72). However, as well as having STs in common with dogs, raccoons also had several STs that were not detected in dogs and are typically associated with human sources, such as ST23, ST224, ST410, and ST167 (41). Further, ARB identified in wildlife have previously been attributed to human sources (18, 73), especially in urban areas (14, 25). This conforms to the general consensus that humans tend to play a more important role in the circulation of ARB and ARG in the community and the environment than companion animals (47). Hence, raccoons may have acquired ESC-R *E. coli* through exposure to human-derived sources of AMR rather than through contact with dog feces. Nevertheless, other human-associated STs, such as ST131 and ST10 (41), were found in both raccoons and dogs. Companion animals and people can have several ESC-R *E. coli* in common (74), either because of direct transmission or parallel microevolution (48). Thus, it is possible that dogs and raccoons had similar ESC-R *E. coli* because individuals of both species were exposed to human-associated AMR via different pathways. While the ESC-R *E. coli* isolated from raccoons could not be compared to those of people living in Chicago, work in other systems (e.g., reference 62) suggests that comparing the AMR profile of urban wildlife and coexisting human populations would be an important next step to take.

Finding no genetic distinction between ESC-R *E. coli* bacteria isolated from raccoons at public parks, raccoons at private parks, and dogs could indicate that raccoons and dogs have closely related ESC-R *E. coli* bacteria, or it could indicate that raccoons and dogs of Chicago present a diverse pool of ESC-R *E. coli* strains. Given that several ESC ARG tend to be transmitted horizontally via plasmids (42), we suspect the latter explanation is most likely. The fact that highly related ESC-R *E. coli* (i.e., differing by $<$20 core SNPs) were found between raccoons at private parks, raccoons at public parks, and dogs reinforces this point, and suggests that these bacteria are potentially being randomly disseminated to different hosts in the same environment. However, no firm conclusions can be made, partly because our study was limited by the number of isolates per sample (one per sample) and per host group (e.g., 29 for dogs versus 123 for raccoons). Given the number of ESC-R *E. coli* likely present per gram feces, examining a total of 152 isolates probably provided insufficient power to discern the diversity of ESC-R *E. coli* present in raccoons and dogs, and thus the degree of genetic relatedness. Further, other types of ARB and/or microbiology techniques may have provided better resolution for comparing AMR between raccoons and dogs. ESC-R *E. coli* were chosen because of their ease and frequency of isolation, and relevance to human medicine. Use of other ARB (e.g., methicillin-resistant *Staphylococcus aureus*) may or may not yield better resolution. Similarly, comparing the range of the resistance level between raccoon and dog samples using MICs may have provided more insight on the distribution of ARB in these two host species and should be explored in future studies. Thus, this study should be viewed as a first step toward understanding the ecology of AMR at the wildlife-companion animal interface.

In conclusion, an important finding of this study was the difference in prevalence of ESC-R *E. coli* between dogs and raccoons. We were over three times more likely to

recover ESC-R *E. coli* from raccoons than dogs. Raccoons have the potential to pose a risk to dogs if dogs come into contact with raccoon feces at parks or when raccoons visit residential backyards, especially if raccoon densities are high. Exploring whether AMR risk for dogs increases when dogs reside in areas where raccoons occur at high densities and have high prevalence of ARB would be a useful next step. Further, given the likely role of the environment for raccoon exposure to ARB, an important next step for studying AMR in companion animals would be to explore the importance of not only wildlife but also the environment. In previous work, we found that raccoons sampled in urban areas had a higher risk of exposure than raccoons sampled in suburban areas (13). Exploring whether similar patterns hold true for dogs while accounting for relevant epidemiological factors (e.g., dog diet, attendance at dog day care) (75, 76) would be insightful. In this way, we advocate that future work explore multiple AMR exposure pathways simultaneously (i.e., humans, domestic animals, wildlife, and the environment).

Environmental and wildlife AMR research has been grossly overlooked in understanding the epidemiology of ARB (9, 77), and our study highlights the need for continued research on wildlife AMR. To date, much wildlife AMR research has advocated targeting avian species (in particular gulls) as sentinels for AMR in the environment. We argue that mammalian species that reside in close proximity to humans, such as raccoons, could also be important targets. The fact that raccoons spend a large proportion of time in residential areas and along rivers and lakes (60, 78) makes them especially useful for understanding the spread and maintenance of ARB in urban and suburban environments. Since raccoons in many regions across the United States are tested for pathogens such as rabies virus, testing for the presence of clinically relevant ARB in feces and storing isolates for future genomics work would be a productive surveillance measure to initiate.

MATERIALS AND METHODS

Study site and design. In February to November 2018, raccoons were captured from seven sites in northwestern Chicago, IL, USA, of which four were suburban and three were urban (Fig. 1). Sites were classified as urban if the site and surrounding area (i.e., ~1 km buffer around each site) were composed of ≥80% impervious surface. Otherwise, sites were classified as suburban (for details see reference 13). Out of the seven sites, three were public sites (i.e., open to the public and domestic dogs) (Fig. 1), and four were private sites (i.e., inaccessible to the public and domestic dogs) (Fig. 1). Domestic dogs were sampled at each of the three public sites and at dog parks (park in which dogs mingle off leash) that were closest to three of the public sites (Fig. 1).

Raccoon and dog sampling. Raccoons were captured using box traps (Model 108, Tomahawk Live Trap Co., Tomahawk, WI, USA) (78) and immobilized with an injection of Telazol (Fort Dodge Animal Health, Fort Dodge, Iowa). Fecal samples were collected opportunistically from the rectum of each immobilized raccoon. After recovering from immobilization, all raccoons were released at the capture locations. Captures were approved by the University of Minnesota's Institutional Animal Care and Use Committee (protocol ID 1709-35105A) and by the Illinois Department of Natural Resources (permit number IDNR W17.0122).

Dogs were selected at random, but dogs less than 6 months of age were excluded. For every dog sampled, a standardized survey (Table S1 in the supplemental material) was given to dog owners detailing the age and sex of each dog, as well as history of antibiotic use in the past year. Dog owners were also asked for their home ZIP code. Dog fecal samples were collected by dog owners using their own dog waste bags or bags were provided by investigators. All dog and raccoon fecal samples were stored in brain heart infusion broth and 20% glycerol at −80°C until further analyses.

Phenotypic characterization of ESC-R *E. coli*. Presence of ESC-R *E. coli* was explored by testing *E. coli* susceptibility to cefotaxime, a third-generation cephalosporin. A detailed description of this procedure can be found in reference 13. Briefly, samples were enriched overnight in lauryl tryptose phosphate broth (Difco Laboratories, Detroit, MI, USA) and streaked onto CHROMagar ECC containing 2 μg/ml of cefotaxime (36, 79). If blue colonies (representative of *E. coli*) were obtained, one per sample was selected at random and restreaked on CHROMagar ECC containing 2 μg/ml of cefotaxime. All isolates were stored at −80°C until sequencing.

Sequencing, bioinformatics, and phylogenetic analyses. Whole-genome sequencing (WGS) was performed on all recovered ESC-R *E. coli* isolates. Details on DNA extraction, WGS, and quality check of raw reads can be found in reference 13.

Genetic associations among isolates were explored by first determining the multilocus sequence type (MLST) of each isolate. To do this, trimmed reads were assembled using SPAdes assembler (version 3.0) (80) with default parameters. The quality of assemblies was assessed by examining the N_{50} score of each isolate, which we calculated using QUAST (version 4.3) (81). Isolates were then classified into

TABLE 2 Description of statistical approaches[b]

Outcome variable	*n*	Analytical approach	Predictor variable	Random effect
Contingency table of ESC-R *E. coli* (presence/absence)	406	Fisher's exact test	species (dog/raccoon)	NA
ST richness	152	Bootstrapping	species	NA
Pairwise SNP distance of ESC-R *E. coli*	152	Univariable PERMANOVA	species	NA
ESC-R *E. coli* presence in raccoons (yes/no)	211	Multivariable binomial GLMM	private / public site, season (fall, winter, spring, summer), urban-suburban context (urban/suburban), urban context × private / public site	capture site, raccoon ID[a]
Pairwise SNP distance of ESC-R *E. coli*	152	Univariable PERMANOVA	host type (public park raccoon, private park raccoon, dog)	NA

[a]Variable was considered for inclusion as random effect in exploratory analyses but was found to contribute little to the overall variance ($p < 0.05$) and was thus excluded from analyses listed here.
[b]PERMANOVA, permutational multivariate analysis of variance; GLMM, generalized linear mixed model; ESC-R, extended-spectrum cephalosporin-resistant; ST, sequence type; SNP, single-nucleotide polymorphism; NA, not applicable.

different sequence types (STs) using mlst (https://github.com/tseemann/mlst) and the *in silico E. coli* PubMLST typing scheme. Associations between STs were visualized using minimum spanning trees, which were created in GrapeTree (82). To explore isolate similarity within STs, a core single-nucleotide polymorphism (SNP)-based phylogenetic analysis was performed. A detailed description can be found in reference 13. Briefly, trimmed reads were mapped to the *E. coli* K-12 laboratory strain MG1655 genome (accession number GCA_000005845.2), and recombinant regions were removed before generating a SNP distance matrix and constructing a maximum likelihood phylogenetic tree. The tree was visualized and annotated using the iTOL (Interactive Tree of Life) online software (83). Isolates that differed by less than 20 core SNPs were considered to be similar, as in references 71, 72, and 84–86.

The presence of ARG on assembled contigs was assessed using NCBI's BLASTn and the ResFinder database (88). An ARG was considered present if it had an identity of ≥90% and a coverage of ≥80%. For more information see reference 13.

Statistical analysis. (i) Objective 1: similarity of ESC-R *E. coli* isolated from raccoons and dogs based on prevalence, richness, and phylogenetic relatedness. The sample prevalence of ESC-R *E. coli* and 95% confidence intervals for raccoons and dogs were calculated using the "prevalence" package in R version 4.0.2 (87). Comparisons of the prevalence of ESC-R *E. coli* by species were performed using Fisher's exact test (Table 2). Using a similar approach to Mather et al. (89), the richness of ESC-R *E. coli* STs (number of unique STs found in raccoons and dogs) was compared between raccoon and dog populations by bootstrapping the raccoon sample ($n = 123$) to the size of the dog sample ($n = 29$) using 1,000 replicates. Deeper phylogenetic associations between ESC-R *E. coli* isolated from dogs and raccoons were explored by quantifying the pairwise SNP distance between isolates. Phylogenetic clustering by animal species (dog versus raccoon) was assessed by performing permutational multivariate analysis of variance (PERMANOVA) using the "adonis2" function in the "vegan" package (90) with the number of permutations set to 999. PERMANOVA can be used on any type of pairwise matrix (93) and can be used to identify factors shaping microbe phylogenetic associations (91). The assumption of homogeneity of variance was validated using the "betadisper" function in vegan.

(ii) Objective 2: difference in the probability of isolating ESC-R *E. coli* between raccoons sampled at public parks and raccoons sampled at private parks. The outcome variable for this analysis was presence of at least one ESC-R *E. coli* isolate in the feces of raccoons (yes or no) (Table 2). The interface of raccoons with dogs was quantified based on whether raccoons were sampled at private or public sites (private/public site). Associations were explored using a binomial generalized linear mixed model (GLMM) with a logit link function using the "lme4" package (92). Other predictors included season and urban-suburban context because previous work in this system found that both can influence the likelihood of isolating ESC-R *E. coli* from raccoons (13). We did not include raccoon age or sex as fixed effects because neither were expected to be important based on our previous work (13). The interaction between private/public site and urban-suburban context was also explored. Because 18 raccoons were captured more than once, we investigated the need for including "animal ID" as a random effect. To do this, we compared the Akaike information criterion (AIC) values between an intercept model with and without animal ID included as a random effect. There was no significant difference in AIC values between the two models (AIC = 319.49 and 317.9, $P = 0.52$), indicating that including animal ID as a random effect was not needed (Table 2). Capture site was included as a random effect to accommodate for any spatial autocorrelation in model residuals (Moran's I statistic post including capture site as a random effect: $z = -0.44$, $P = 0.67$).

(iii) Objective 3: phylogenetic similarity of ESC-R *E. coli* isolated from raccoons sampled at public parks, raccoons sampled at private parks, and dogs. The outcome variable in this analysis was pairwise SNP distance of ESC-R *E. coli*. The importance of the variable "host type" (i.e., public park raccoon, private park raccoon, or dog) at influencing the phylogenetic clustering of ESC-R *E. coli* was assessed by running a univariable PERMANOVA as in Objective 1 (Table 2).

Data availability. Raw reads were deposited in the National Center for Biotechnology Information's Sequence Read Archive (BioProject numbers PRJNA662117 and PRJNA671493). Isolates and accession numbers can be found in Table S2.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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Infection risk varies within urbanized landscapes: the case of coyotes and heartworm

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Abstract

Background: Urbanization can have profound effects on ecological interactions. For host–pathogen interactions, differences have been detected between urban and non-urban landscapes. However, host–pathogen interactions may also differ within highly heterogeneous, urbanized landscapes.

Methods: We investigated differences in infection risk (i.e., probability of infection) within urbanized landscapes using the coyote (*Canis latrans*) and mosquito-borne nematode, *Dirofilaria immitis* (the causative agent for canine heartworm), as a case study. We focused on a coyote population in Chicago for which extensive behavioral and heartworm infection data has been collected between 2001 and 2016. Our objectives were to: (i) determine how onset and duration of the heartworm transmission season varied over the 16-year period and across the urban–suburban gradient; and (ii) investigate how heartworm infection risk in coyotes varied over the years, across the urban–suburban gradient, by coyote characteristics (e.g., age, sex, resident status), and coyote use of the urbanized landscape (e.g., use of urban areas, mosquito habitats).

Results: While onset of the heartworm transmission season differed neither by year nor across the urban–suburban gradient, it was longer closer to the core of Chicago. Of the 315 coyotes sampled, 31.1% were infected with *D. immitis*. Older coyotes and coyotes sampled in later years (i.e., 2012–2016) were more likely to have heartworm. While coyote location in the urban–suburban gradient was not a significant predictor of infection, the proportion of urban land in coyote home ranges was. Importantly, the size and direction of this association varied by age class. For adults and pups, infection risk declined with urbanization, whereas for subadults it increased. Further, models had a higher predictive power when focusing on resident coyotes (and excluding transient coyotes). The proportion of mosquito habitat in coyote home ranges was not a significant predictor of infection.

Conclusions: Our findings suggest that urbanization may affect host exposure to vectors of *D. immitis*, that risk of infection can vary within urbanized landscapes, and that urbanization–wildlife infection associations may only be detected for animals with certain characteristics (e.g., age class and resident status).

Keywords: Age, Home range, Pathogen, Urban, Vector, Wildlife

Background

Urbanization causes a shift in climatic conditions and landscape structure and composition [1]. Temperatures tend to increase with urbanization due to pollution and impervious surfaces [2–4]. Vegetation becomes subdivided into patches surrounded by urban and suburban blocks. This shift in environmental context can have profound effects on processes unfolding in wildlife communities [5, 6]. For example, urban-induced fragmentation of the landscape and/or warming can alter animal behavior [7, 8] and species composition and abundance [9–12], which can in turn influence ecological relationships, such as predation and competition [13, 14].

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Host–pathogen interactions can also be affected by urbanization, although it may vary with context, and in many cases, depends on pathogen transmission mode (reviewed in [15–19]).

Vector-borne pathogens are prone to be affected by urbanization because of the insect vector's dependence on appropriate habitat, warm temperatures, and competent hosts [20, 21]. Whether we should expect an increase or decrease in vector-borne diseases with urbanization is a topic of intense debate, as opposing trends have been detected [16]. For example, the prevalence of both ticks and avian malaria was found to be higher in rural common blackbirds (*Turdus merula*) than in urban ones [22]. In contrast, West Nile virus seroprevalence was greater in urban than non-urban birds (e.g., [23–25]). Importantly, in addition to contrasting large-scale outcomes across landscapes (e.g., urban vs. rural), differences in infection risk (i.e., probability of infection) can also occur within urbanized landscapes [23, 26]. For instance, differences in the number and types of mosquito habitats (e.g., wetlands, artificial containers; [27]) across neighborhoods may lead to fine-scale differences in infection outcomes [28]. While differences in infection risk within urbanized landscapes have been detected in vector species [26, 28], whether we should expect to observe similar patterns in urban host populations is less well understood.

The mosquito-borne nematode *Dirofilaria immitis* is the causative agent for canine heartworm, which is one of the most important parasitic diseases of domestic dogs in North America [29, 30]. Successful development and transmission of *D. immitis* is dependent on warm, humid conditions [31, 32], along with the presence of competent vectors and hosts [33, 34]. Warm, humid conditions are important for both the onset and duration of the heartworm transmission season and mosquito survival and reproduction. While over 60 mosquito species are susceptible to *D. immitis*, only nine act as competent vectors [35] and differ in their ability to adapt to urban settings [27]. Competent hosts include domestic dogs and wild canids, in particular coyotes (*Canis latrans*). Coyotes in rural or natural areas are considered one of the primary reservoir hosts for *D. immitis* [36–38]. *D. immitis* prevalence can be as high as 37% in coyotes sampled in some rural areas [36, 39]. Despite an increasing presence of coyotes in many urban settings [40, 41], the distribution and prevalence of *D. immitis* in urban coyote populations is relatively unknown [41].

Here, we investigated how urbanization influences coyote risk of infection with *D. immitis*. To do this, we leveraged animal behavior and infection data from a well-studied coyote population sampled between 2001 and 2016 in the northwestern portion of the Chicago metropolitan area, which included both urban and suburban regions. Historically, coyotes were rare in the Chicago area, but they increased dramatically during the 1990s and are now common throughout the metropolitan area [42]. While *D. immitis* prevalence in Chicago is unknown, the number of domestic dog cases reported by veterinary clinics in the Chicago area has increased by over four-fold in the past decade [43]. Our objectives were to (i) determine how onset and duration of the heartworm transmission season varied over the 16-year period and across the urban–suburban gradient; and (ii) investigate how infection risk in coyotes varied over the years, across the urban–suburban gradient, by coyote characteristics (e.g., age, sex), and coyote use of the urbanized landscape (e.g., use of urban areas, mosquito habitats). Because the location and size of resident coyote home ranges vary less than those of transient coyotes [42, 44], we explored coyote use of the urbanized landscape for both resident and transient coyotes and for resident coyotes only.

Methods

Study area

The Chicago metropolitan area, with a human population of >9 million people, extends across six counties (i.e., Cook, DuPage, Kane, Lake, McHenry, Will) in northeastern Illinois, USA (41.88° N, 87.63° W). Chicago has a temperate climate, with mean summer and winter temperatures ranging from 26 to 33 °C and from −1 to 3 °C, respectively, and rainfall averaging ~845 mm per year [45]. Land cover in the region includes urban, suburban, natural areas, and agriculture. Landscapes in natural and urbanized areas include deciduous and coniferous forest, prairie, floodplain, wetland, open water, and managed green spaces (e.g., parks, greenways, golf courses).

The core of the metropolitan area, downtown Chicago, is situated on the edge of Lake Michigan. While proximity to a major water body can create a cooling effect and cause urban heat to shift westward [46], Chicago generally experiences only mild cooling that is most pronounced near the lakeshore during the summer [47] (although see [48]). This is in part due to Lake Michigan's downwind location from the southwest winds as well as warm water temperatures in late summer [47]. Further, any cooling effect of Lake Michigan in the core of Chicago is apparently counteracted by densely populated buildings, industrial zones, and train stations [49]. Thus, Chicago's heat island most likely occurs in the core of Chicago like traditional urban centers [1, 49], although low summer lake temperatures may push the heat island westerly.

Heartworm transmission season

Once infected, the temperature of the mosquito dictates the development of the microfilaria to the infective third

stage [50–52]. Microfilarial development occurs above a threshold temperature of 14 °C, the progress of which can be tracked through the accumulation of heartworm development units (HDUs; [53]) such that 1 HDU is equal to 1 day with an average temperature 1 °C above 14 °C [53]. Infective third-stage larvae will pass from the mosquito to the new host during blood meals only after enough HDUs have been accumulated throughout a given period [53]. The period during which infective larvae are transmitted is called the heartworm transmission season and can be constructed for any region given sufficient climatological data [50–52]. The heartworm transmission season is said to have begun when 30-day HDUs surpass 130 °C and ends when 30-day HDUs drop below 130 °C [53].

To investigate the influence of urbanization on the onset and duration of the heartworm transmission season, we created four zones, each 15 km wide, to characterize a gradient from urban to suburban landscapes. Zone 1 had an average housing density of ~3250/km^2, zone 2 of ~850/km^2, zone 3 of ~530/km^2, and zone 4 of ~400/km^2 (Fig. 1) based on the 2010 SILVIS housing density data set (SILVIS Lab Spatial Analysis for Conservation and Sustainability). We obtained daily mean temperature data for the years 2000–2015 from the PRISM Climate Group (PRISM Climate Group, Oregon State University, http://prism.oregonstate.edu). Daily mean temperatures are available as spatial grids of 4-km spatial resolution which are calculated by interpolating climate data obtained from weather monitoring networks. For each zone, we used three 4-km grids that were evenly distributed across the zone (Additional file 1: Figure S1). For each 4-km grid in each year, daily HDUs were calculated by subtracting the threshold temperature of 14 °C from the daily mean temperature [53]. Thirty-day HDUs were constructed by summing each daily HDU with the daily HDUs from the previous 29 days [52, 53,54]. The duration of each heartworm transmission season was determined by the number of months between the initiation and termination of the heartworm transmission season.

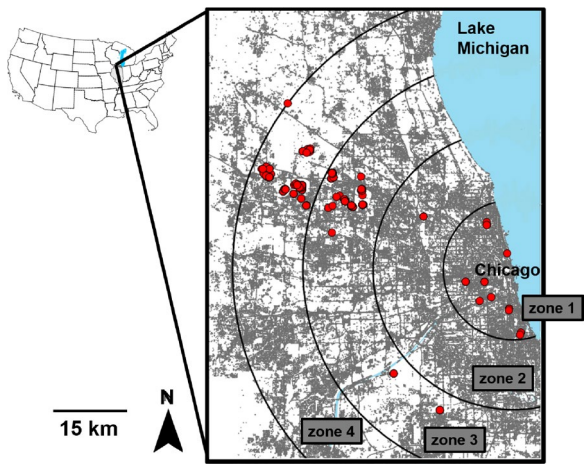

Fig. 1 Map of the Chicago metropolitan area. Grey land represents built-up/developed land and roads (i.e., impervious surfaces from the 2011 National Land Cover Database). Red circles are coyote capture locations ($n = 315$). Zones 1–4 delineated by black circular lines were generated to explore the relationship between urbanization and the onset and duration of the heartworm transmission season. The heartworm transmission season was estimated by collecting daily temperature data from each zone (see Additional file 1: Figure S1)

Coyote sampling

Coyotes were captured between February 2001 and December 2016 in the central and northwestern portion of the Chicago metropolitan area (Fig. 1). Most coyotes were captured in forest preserves, golf courses, small woodland parks, or in abandoned lots. Captures were performed opportunistically throughout the year, but primarily during winter and early spring. Coyotes were live-trapped with padded foothold traps and cable restraint devices [55]. With the exception of 19 coyotes, capture locations were recorded using a handheld GPS. For the 19 coyotes without specific location coordinates, we used the coordinates of the center of the park where trapping occurred. All captured individuals were transported in a metal dog carrier to a research laboratory. Each coyote was sedated while in the dog carrier with 2.5 mg/kg Telazol (Fort Dodge Animal Health, Fort Dodge, IA, USA), which was administered intramuscularly in the hind limb. After immobilization, each coyote was sexed, and aged based on reproductive condition and tooth wear [56, 57]. Pups were 6–12 months, subadults were between 1 and 2 years, and adults were >2 years. Pups less than 6 months were excluded since the prepatent period for heartworm is ~6 months [58]. Approximately 3 ml of blood was collected from each coyote and poured into serum separator tubes. Tubes were left for ~30 min in an upright position and allowed to clot before being centrifuged for 15 min at 1790×*g*. Serum was extracted and stored in cryovial tubes at −80 °C. Serum was left in the freezer for periods ranging from 6 months to 5 years prior to further analyses. After blood collection, all coyotes were ear tagged and fitted with very-high-frequency radio collars (Advanced Telemetry Systems, Isanti, MN, USA). After recovering from immobilization, animals were released at the capture locations.

Heartworm screening

Serum samples were submitted to the Veterinary Diagnostic Laboratory at the University of Illinois Urbana-Champaign for heartworm screening. Presence of heartworm antigen was assessed using a

membrane-bound ELISA test (SNAP® 4Dx® Plus Test, IDEXX Laboratories Inc.) following the manufacturer's instructions. SNAP® 4Dx® Plus Test detects proteins produced within the reproductive tract of adult female heartworms and has a sensitivity of 97.5% (95% CI 94.26–99.18) and specificity of 94.0% (95% CI 83.45–98.75) [59].

Home range analysis and resident status classification

Relocations of all radio-collared coyotes were recorded 2–3 times a week during the day, and once a week during the night [42]. Relocations were estimated using triangulation (with program LOCATE II; Pacer, Truro, Nova Scotia, Canada) with a truck-mounted antenna, or visual sightings. Relocations were used to estimate annual home ranges. We restricted annual home range estimates to individuals with a minimum of 30 relocations during at least 6 consecutive months. All relocations recorded beyond the 12-month period post capture were excluded. We assumed that the land cover types used by each coyote 6–12 months post capture were similar to those used when exposed to vectors of *D. immitis*.

We used two nonparametric methods to obtain home range estimates for each coyote: (1) we calculated and plotted 95% minimum convex polygons (MCPs); and (2) we used the adaptive local convex hull (a-LoCoH) method [59, 61]. For a-LoCoH, we calculated 95% contours and obtained the value of the adaptive sphere of influence "*a*" by calculating the maximum distance between two points [61]. We used MCP and a-LoCoH over other home range estimators (e.g., the kernel density estimator) because MCP is most frequently used for very-high-frequency data, and a-LoCoH minimizes the extent to which home ranges cross hard boundaries (e.g., highways, rivers) [42, 60]. Since MCP can overestimate home range size [61] and a-LoCoH underestimate home range size (particularly if the sample size of locations is relatively small) [61], we explored heartworm–land cover associations using both methods. Because results were similar across methods (see "Results"), we presented MCP results in the main text and a-LoCoH results in the supplementary materials. All home range analyses were performed using the "*adehabitatHR*" package [62] in the statistical program R version 4.0.2 [63].

Home ranges were imported into ArcGIS version 10.3 [64] and linked to land cover data to estimate the proportion of each land cover type present within each coyote's home range. We used the 2011 National Land Cover Database (https://www.mrlc.gov) (spatial resolution: 30 m) to subdivide the landscape into different land cover types. Fourteen land cover types were present in the area. We combined eleven of these into two categories: (1) "mosquito habitat" (open water, woody wetlands, and emergent herbaceous wetlands); and (2) "green spaces" (open developed space, mixed forest, evergreen forest, deciduous forest, cultivated crops, pasture/hay, grassland/herbaceous, shrub/scrub). The other three were urban land cover types (high, medium, and low developed urban land) and were examined as independent variables since we were interested in the degree of urbanization. High, medium, and low developed urban land indicates that 80–100%, 50–79%, and 20–49% of the land is impervious surface, respectively. Because all three urban variables were highly correlated with the "green spaces" variable and the "high developed urban land" variable with the "medium developed urban land" variable ($r^2 > 0.5$), we excluded the "green spaces" and "high developed urban land" variables from statistical analyses and focused on "mosquito habitat," "developed medium urban land," and "developed low urban land" variables.

We used multiple characteristics to discriminate resident and transient coyotes. Residents repeatedly used an explicit territory across two or more seasons, and transients shifted use areas across seasons and had larger home ranges that overlapped multiple territories [42, 65, 66]. Further, residents were often seen traveling with other coyotes, whereas transients did not, or residents shared the same territories with reproductive pairs occupying a territory.

Statistical analysis

We ran a linear regression to determine how the duration of the heartworm transmission season varied by year and urban zone (Table 1). The outcome variable in this model was duration of the heartworm transmission season (in months), and predictor variables were year (2001–2015) and urban zone (1–4). We also included latitude to account for any variation associated with collecting temperature data from three 4-km grids located at different latitudes within each zone (Additional file 1: Figure S1). Variation in the onset of the heartworm transmission season with year, urban zone, and latitude was not explored because there was little variation (i.e., onset of the heartworm transmission season occurred in June 97.9% of the time).

We ran a generalized linear model (GLM) to investigate how heartworm infection risk in coyotes varied across years (2001–2016), the urban–suburban gradient (urban zone 1–4), with coyote characteristics (e.g., age, sex), and coyote use of the urbanized landscape (i.e., mosquito habitat, medium developed urban land, and low developed urban land). However, we split the analysis into two because of differences in sample size for some of the fixed effects. One analysis included all coyotes tested for heartworm ($n = 315$ coyotes with 16 tested more than once) and the other included coyotes for which enough relocation data were obtained to estimate annual home

Table 1 Description of statistical approaches used

Analytical approach	*n*	Outcome variable	Fixed effects	Random effect(s)
Linear regression model	192	Duration of the heartworm transmission season (months)	Year (2000–2015) Urban zone (1–4) Latitude	NA
Binomial generalized linear mixed model	315[a]	Infection (yes/no)	Year (2001–2016; no heartworm data were collected in 2006 and 2007) Age class (pup (6–12 months), juvenile, adult) Sex Urban zone (1–4) Proportion of adults tested each year (as an offset)	Site Animal ID
Binomial generalized linear mixed model[b]	146	Infection (yes/no)	Year Age class Resident status (resident vs. transient)[c] Proportion low developed urban land in home range Proportion medium developed urban land in home range Proportion mosquito habitat in home range Proportion of adults tested each year (as an offset) Age class * proportion low developed Age class * proportion medium developed Age class * proportion mosquito habitat	Site

[a] Sixteen of the coyotes were captured more than once

[b] Four models were run using this model structure and composition: (1) for residents and transients using MCP; (2) for residents only using MCP; (3) for residents and transients using a-LoCoH; and (4) for residents only using a-LoCoH

[c] Variable was included only when both resident and transient coyotes were analyzed

ranges ($n=146$ coyotes, a subset of the 315 coyotes). The outcome variable was heartworm infection (yes/no), thus we ran binomial GLMs with logit link functions using the "*lme4*" R package [67]. Additionally, because we expected infection to increase with age [38, 39, 68], we also included the proportion of adults tested each year as an offset in both analyses (Table 1).

In the analysis that included 315 coyotes, urban zone, age class, and sex were included as categorical fixed effects. Year was included as a continuous fixed effect, and nonlinear relationships with heartworm infection were examined using basis splines (Table 1) using the "*splines*" R package. Since 16 coyotes were tested more than once (Additional file 1: Table S1), we include "animal ID" as a random intercept and ran a generalized linear mixed model (GLMM) instead of a GLM using the "*lme4*" package. Further, we grouped observations into a "site" random intercept to account for any significant spatial autocorrelation in model residuals (Moran's *I* statistic after including site as a random effect: $z=0.52$, $P=0.3$). Coyotes were included in the site that was closest to their capture location except if there were man-made barriers (e.g., highways).

In the analysis that included 146 coyotes, we ran four models: (1) for residents and transients using MCP; (2) for residents only using MCP; (3) for residents and transients using a-LoCoH; and (4) for residents only using a-LoCoH. Fixed effects included the proportion of mosquito habitat, medium developed urban land, and low developed urban land in coyote home ranges. Age class and year were included as fixed effects because they were significant predictors in the analysis with 315 coyotes (see "Results"). Resident status was also included in models that examined both residents and transients. Since we expected age class to be an important predictor of infection, we also evaluated whether the association between infection and the proportion of mosquito habitat, medium developed urban land, and low developed urban land in coyote home ranges varied by age class by including interactions between these variables. We included site as a random intercept to account for any significant spatial autocorrelation (Moran's *I* statistic after including site as a random effect for residents and transients using MCP: $z=0.08$, $P=0.47$; for residents only using MCP: $z=-0.24$, $P=0.59$; for residents and transients using a-LoCoH: $z=-0.02$, $P=0.51$; for residents only using a-LoCoH: $z=-0.36$, $P=0.64$). Animal ID was not included as a random intercept because none of the coyotes in this second analysis were resampled more than once.

For all models, the most parsimonious model was identified using an information theory approach, comparing models with different variable combinations, and used the Akaike information criterion corrected for small sample size (AICc) to rank models [69, 70] using

the "*MuMIn*" R package [71]. If at least one model was within 2 ΔAICc values of the top-ranking model, model averaging was used to obtain mean effect sizes and 95% confidence intervals [69]. All continuous predictors were centered and standardized to facilitate interpretation of main effects and to perform model averaging [72]. Multicollinearity among continuous predictors was assessed using the variance inflation factor [73]. Scaled residuals of each model were examined for uniformity using the "*DHARMa*" package [74]. Model fit was assessed by calculating the marginal and conditional coefficients of determination (r_m^2 and r_c^2, respectively) [75]. r_m^2 is the variance explained by the fixed effects, and r_c^2 the variance explained by the fixed and random effects [75].

Results

Onset and duration of the heartworm transmission season

Across all urban zones (1–4) and years (2000–2015), the heartworm transmission season most often began in June (97.9% of the time) and lasted for a period of 2–5 months (mean of 3.56 months). For the duration of the heartworm transmission season, urban zone appeared in all three of the top-ranking models, and year and latitude in one (Additional file 1: Table S2). Model averaging of the top three models showed that urban zone was a significant predictor of infection (Table 2), while year and latitude were not (Table 2). The heartworm transmission season was significantly longer in zone 1 compared to zone 3 and 4 (Fig. 2). Pairwise comparisons revealed that the same was true for zone 2 compared to zone 3 and 4 (zone 2 vs. zone 3: $z=2.88$, $P=0.02$; zone 2 vs. zone 4: $z=2.89$, $P=0.02$).

Urbanization and coyote infection risk

Three hundred and fifteen coyotes were captured and tested for heartworm between 2001 and 2016 (16 were captured more than once; Additional file 1: Table S1). The number of animals captured and tested each year ranged from 5 in 2001 to 51 in 2014 (mean = 22.5 per year). Heartworm tests were performed on 94 pups (52 females and 42 males), 108 subadults (53 females and 55 males), and 113 adults (38 females and 75 males). Ninety-eight coyotes were positive for heartworm (31.1%). Prevalence ranged from 7.7% in 2011 ($n=13$) to 66.7% in 2016 ($n=21$) (Fig. 3a).

When examining infection risk for all captured coyotes ($n=315$), the best fit model contained only age class and year (Additional file 1: Table S3). Urban zone and sex were not important predictors of infection because they did not appear in the top-ranking model (Additional file 1: Table S3). A quadratic relation better explained the relationship between heartworm infection and year than a linear relationship (Table 3). Infection risk was lowest in 2008–2011 and increased in 2012–2016 (Fig. 3a). For age class, adults had a higher risk of infection than pups and subadults (Table 3; Fig. 3b).

Of the 315 coyotes tested for heartworm, 146 had enough relocations to estimate annual home ranges (mean number of relocations per animal = 163, range = 45–585). The 146 individuals comprised 37 pups (21 females and 16 males), 46 subadults (23 females and 23 males), and 63 adults (25 females and 38 males). In terms of resident status, this amounted to 107 residents and 39 transients. The years with the lowest number of coyotes tracked were 2005 and 2010 ($n=3$), and the years with the highest number of coyotes tracked were 2012 and 2013 ($n=22$) and 2014 ($n=23$). Association between heartworm infection and age class, year, resident status, and coyote use of the urbanized landscape tended to be

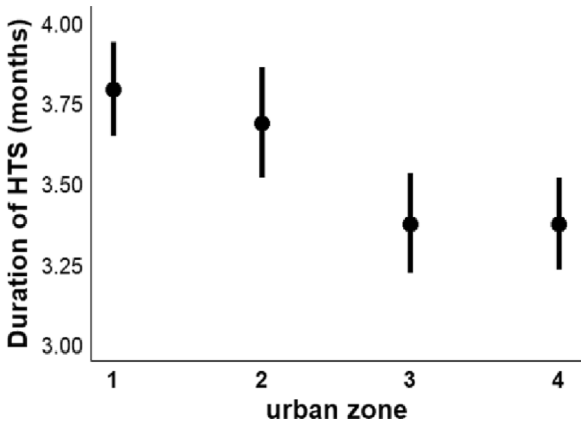

Fig. 2 Duration of the heartworm transmission season (in months) for each urban zone. Zone 1 is closest to the core of Chicago, and zone 4 is furthest away (Fig. 1, Additional file 1: Figure S1). Whiskers are 95% confidence intervals

Table. 2 Model averaging results from the linear regression model of the duration of the heartworm transmission season ($n=192$)

Predictors	Estimate	SE	*z*	Pr(>|*z*|)	95% CI
(Intercept)	3.79	0.08	48.79	< 0.001	3.63 to 3.93
Urban zone 2	−0.1	0.11	0.9	0.37	−0.31 to 0.12
Urban zone 3	−0.41	0.11	3.74	< 0.001	−0.63 to −0.2
Urban zone 4	−0.41	0.11	3.74	< 0.001	−0.63 to −0.2
Latitude	−0.04	0.04	0.94	0.35	−0.12 to 0.04
Year	0.03	0.04	0.84	0.4	−0.04 to 0.11

Predictors were obtained from the top-ranking models (ΔAICc < 2; Additional file 1: Table S2)

For urban zone, zone 1 is the reference level. Significant terms are those for which 95% confidence intervals [CI] do not overlap with 1 and P < 0.05.

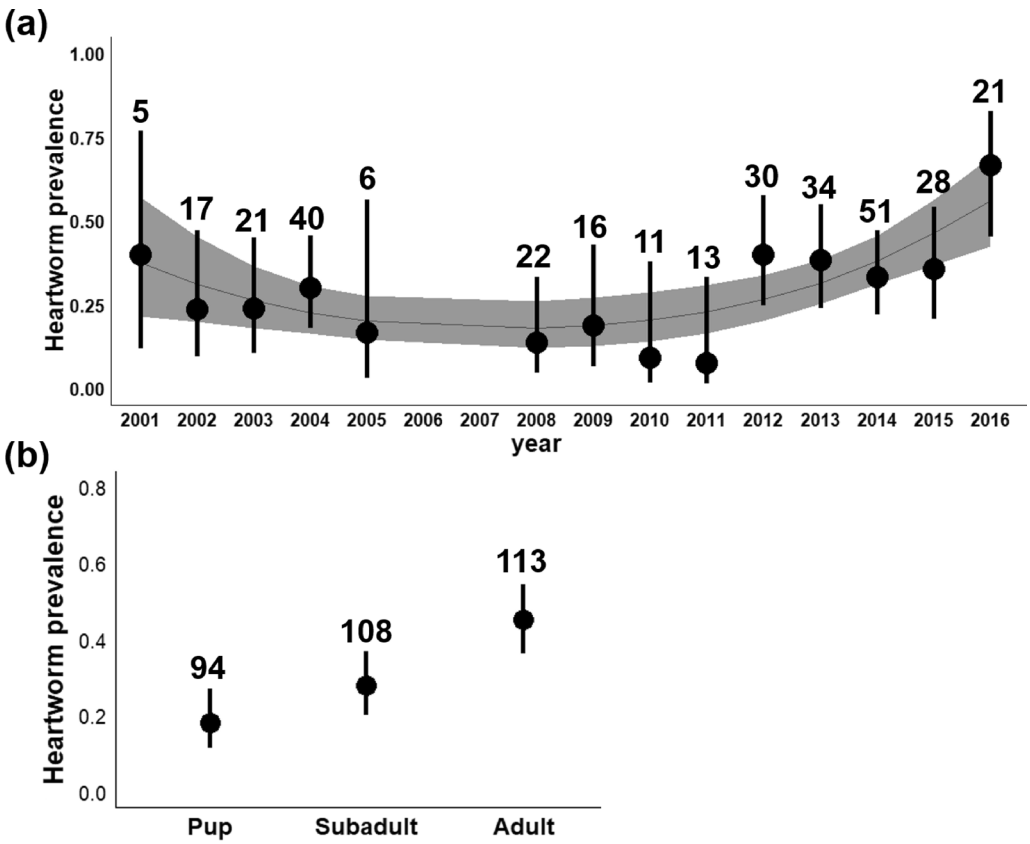

Fig. 3 Relationship between heartworm prevalence and **a** year and **b** age class. Whiskers are 95% confidence intervals. In panel **a**, the line denotes the mean infection risk by year based on a quadratic model. The shaded area denotes the 95% confidence interval, and numbers are sample sizes. No heartworm data were collected in 2006 and 2007.

Table. 3 Relationship between heartworm infection and coyote age class and year ($n = 315$)

Predictors	Estimate	SE	*z*	Pr(>|*z*|)	OR	95% CI
(Intercept)	−0.52	0.56	−0.93	0.35	0.6	(0.2–1.79)
Age class (subadult)	−1.005	0.35	−2.87	0.004	0.37	(0.18–0.73)
Age class (pup)	−1.74	0.41	−4.2	< 0.001	0.18	(0.08–0.4)
Year	−2.14	1.12	−1.9	0.06	0.12	(0.01–1.07)
Year (quadratic)	1.44	0.52	2.75	0.006	4.21	(1.5–11.9)

Predictors were obtained from the best fit GLMM (Additional file 1: Table S3)

For age class, adult is the reference level. Significant terms are those for which 95% confidence intervals [CI] do not overlap with 1 and $P < 0.05$. *SE* is the standard error, Pr(>|z|) the *P*-value associated with the *z* statistic, and *OR* the odds ratio

similar across all four models (i.e., resident and transient coyotes using MCP, resident coyotes only using MCP, resident and transient coyotes using a-LoCoH, and resident coyotes only using a-LoCoH). However, models with the greatest predictive power used MCP instead of a-LoCoH and focused on resident coyotes only. For top-ranking models using MCP (ΔAICc < 2), the largest r^2 value was 0.68 for the resident coyotes only analysis and 0.49 for the resident and transient coyote analysis. For top-ranking models using a-LoCoH, the largest r^2 value was 0.47 for the resident coyotes-only analysis and 0.5 for the resident and transient coyote analysis (Additional file 1: Table S3). Results using MCP are summarized in Table 4, Fig. 4, and Additional file 1: Table S4, and results using a-LoCoH are summarized in Additional file 1: Tables S4, S5, and Figure S2.

Table 4 Model averaging results from binomial generalized linear mixed models of heartworm infection risk in coyotes ($n = 146$)

Model	Predictor	Estimate	SE	z	Pr(>|z|)	Mean OR	95% CI
Resident and transient coy otes ($n = 146$)	(Intercept)	−0.59	0.45	1.33	0.18	0.55	(0.23–1.33)
	Age class (subadult)	−0.75	0.46	1.64	0.1	0.47	(0.19–1.16)
	Age class (pup)	−2.37	1.02	2.33	0.02	0.09	(0.01–0.69)
	Prop. low developed in home range	−0.22	0.27	0.82	0.41	0.8	(0.48–1.36)
	Prop. medium developed in home range	−0.63	0.33	1.9	0.06	0.53	(0.28–1.02)
	Prop. mosquito habitat in home range	0.33	0.23	1.46	0.15	1.4	(0.89–2.19)
	Year	−1.74	1.53	1.14	0.26	0.18	(0.01–3.54)
	Year (quadratic)	0.75	0.65	1.15	0.25	2.12	(0.59–7.64)
	Age class (subadult) * proportion medium developed	1.48	0.48	3.06	0.002	4.39	(1.7–11.3)
	Age class (pup) * proportion medium developed	−1.26	1.33	0.95	0.34	0.28	(0.02–3.84)
Resident coyotes only ($n = 107$)	(Intercept)	−0.49	0.3	1.62	0.1	0.61	(0.35–1.08)
	Age class (subadult)	−0.75	0.5	1.5	0.13	0.48	(0.18–1.27)
	Age class (pup)	−3.49	2.66	1.31	0.19	0.02	(0.00–4.48)
	Proportion medium developed in home range	−0.56	0.34	1.64	0.1	0.57	(0.29–1.12)
	Proportion mosquito habitat in home range	0.28	0.25	1.14	0.26	1.32	(0.82–2.14)
	Age class (subadult) * proportion medium developed	1.16	0.56	2.07	0.04	3.19	(1.06–9.53)
	Age class (pup) * proportion medium developed	−3.36	3.83	0.88	0.38	0.03	(0.00–62.73)

Predictors were obtained from the top-ranking models (ΔAICc < 2; Additional file 1: Table S4). Coyote home ranges were estimated by calculating and plotting 95% minimum convex polygons (MCPs). Results using 95% adaptive local convex hulls (a-LoCoH) are summarized in Additional file 1: Table S4 and S5)

SE is the standard error, Pr(>|z|) the *P*-value associated with the z statistic, and mean *OR* the mean odds ratio

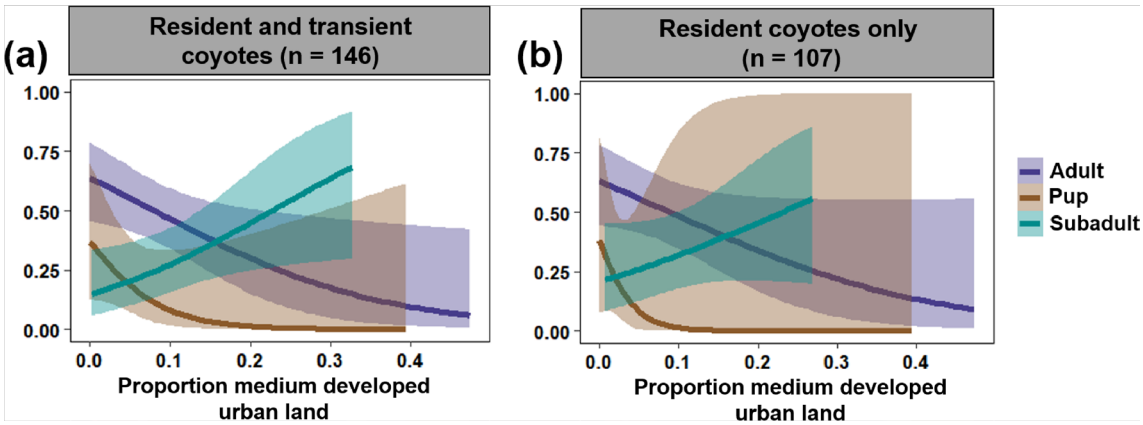

Fig. 4 Relationship between infection risk and the proportion of medium developed urban land in coyote home ranges by age class. Panel **a** includes both resident and transient coyotes ($n = 146$), and panel **b** resident coyotes only ($n = 107$). Lines are mean estimates of infection risk (i.e., probability of infection) by proportion of medium developed urban land in coyote home ranges. The shaded bands are 95% confidence intervals. Coyote home ranges were estimated using 95% minimum convex polygons (95% MCPs). For results using a-LoCoH, see Additional file 1: Figure S2

Age class, proportion of medium developed urban land in coyote home ranges, and the interaction between these two predictors appeared in all top-ranking models (Additional file 1: Table S4, except for resident coyotes only using MCP in which two out of three models had the two predictors and interaction) and thus were the most important predictors of heartworm infection. Further, for the resident and transient models, proportion of mosquito habitat and year were second most important, followed by proportion of low developed urban land in coyote home ranges and resident status (Additional file 1: Table S4). For the resident only models, year appeared in none of the top-ranking models (Additional file 1: Table S4). The interaction between age class and proportion of low developed urban land, and mosquito habitat in coyote home ranges were the least important as they

did not appear in any of the top-ranking models (Additional file 1: Table S4).

Model averaging revealed that when resident and transient coyotes were examined, pups had a lower infection risk than adults (Table 4 and Additional file 1: Table S5). Further, infection risk tended to decline as the proportion of medium developed urban land in coyote home ranges increased. However, this association varied by age class, where infection risk declined as the proportion of medium developed urban land in home ranges increased for adults but increased for subadults (Table 4, Fig. 4, and Additional file 1: Table S5 and Fig. S2). Further, changing the model reference level to pups revealed that, for the models that included both resident and transient coyotes, the relationship between infection and proportion of medium developed urban land in home ranges was significantly different between pups and subadults ($P < 0.05$ for all four model types), where pups, like adults, had a lower risk of infection with more medium developed urban land in their home ranges (Fig. 4 and Additional file 1: Table S2). No significant difference was detected between pups and subadults when focusing on resident coyotes only.

Discussion

Urbanization can have contrasting effects on host–pathogen interactions [15, 18 19]. Here, we found that urbanization influenced the duration of the heartworm transmission season and infection risk in coyotes. The heartworm transmission season was longer closer to the core of Chicago. Heartworm prevalence in coyotes increased during the study period and with coyote age. Further, the proportion of medium developed urban land in coyote home ranges was an important predictor of infection, but direction and size of the effect varied by age class and models had a higher predictive power when examining resident coyotes only. For adults and pups, infection risk declined with urbanization, whereas for subadults, it increased.

The Chicago coyote population had a heartworm prevalence of 31.1%. Previous studies performed in Madison, Wisconsin and Tucson, Arizona have found heartworm prevalence in urban coyotes to be 35.7% ($n = 14$) [76] and 0% ($n = 22$) [77], respectively. Importantly, we found that heartworm prevalence fluctuated yearly, with prevalence being as low as 8% in some years and as high as 67% in others, suggesting that there can be notable differences in prevalence across years. Further, heartworm prevalence tended to increase over the 16-year period, a finding that is in line with national trends observed in domestic dogs suggesting that heartworm prevalence is increasing over time across the USA [30]. For northern US states like Illinois, this increase may be associated with an increase in the number and density of mosquito vectors [78, 79], possibly due to the combined effect of shifting climate conditions and few mosquito abatement programs [30]. For Chicago, an increase in coyote numbers over the years [42] could also be an important factor.

The proportion of coyotes sampled closer to the core of Chicago also increased over the years, which may have contributed to an increase in heartworm prevalence over time. Twenty-six coyotes were sampled in the urban zone 1, of which 24 (92%) were sampled in 2013–2016. While urban zone was not a significant predictor of infection, coyote proximity to the core of Chicago may play a role because the heartworm transmission season tended to be longer closer to the core of Chicago. Additionally, heartworm prevalence in rural coyotes in Illinois is 16% [38], suggesting that urban coyotes might be at a higher risk of infection than non-urban coyotes. Further, mosquitoes sampled in urban areas can have a higher heartworm prevalence than rural mosquitoes [80]. This could be because one of the main vectors of *D. immitis*, *Aedes albopictus*, tends to thrive in urbanized landscapes owing to warmer conditions and the presence of natural and artificial water bodies and containers [35, 80, 81]. A non-significant effect of urban zone may be due to a smaller sample size closer to the core Chicago (i.e., only 29 coyotes were tested in zone 1 and 2 combined: 26 in zone 1 and three in zone 2).

Another potential reason for not detecting a significant effect of urban zone on coyote infection could be that measuring proximity of coyotes to the urban core simplifies or underestimates complex patterns occurring within urban patches. For example, mosquito abundance and richness, as well as infection, can vary across distances as small as neighborhoods [26], and the degree of landscape heterogeneity can influence mosquito diversity [82, 10]. Exploring land cover composition of coyote home ranges provided greater insight for associations with infection risk than proximity to the core of Chicago. The proportion of urban land in coyote home ranges was an important predictor of infection, but only when quantified as medium developed urban land and not low developed urban land. Since impervious surfaces account for 50–79% of total land cover for medium developed urban land and 20–49% for low developed urban land, impervious surfaces and built-up land may explain the observed association.

It was surprising that the proportion of mosquito habitats in coyote home ranges was a nonsignificant predictor of infection risk. In the case of domestic dogs, proximity to mosquito-bearing waters can be an important predictor of infection with *D. immitis* [83]. One reason for not detecting an association in our system could be that

there were other unaccounted for water bodies (e.g., man-made or temporary water bodies such as artificial containers, puddles, tires, trash cans) [84, 85]. Exploring whether the presence of man-made and temporary water bodies versus permanent/vegetated water bodies in coyote home ranges influences heartworm infection would be an important next step to take.

It is noteworthy that the relationship between infection risk and the proportion of medium developed urban land in coyote home ranges varied both in directionality and size by age class. The negative association detected for adults and pups is likely associated with built-up areas having lower mosquito abundance and mosquito species richness than green spaces (e.g., parks, forest preserves; [86, 87]). There are nine competent vectors of *D. immitis* [35], most of which breed in wetland and woodland areas [88]. For example, *Ae. vexans*, a floodwater mosquito, is most frequently found in riparian zones, roadside ditches, and wetlands [35, 89], and is therefore perhaps more commonly found in urban green spaces. Adults and pups may also be at a lower risk of infection with more medium developed urban land in their home range because there is perhaps more mosquito control than in green spaces. The small effect size detected for pups is probably because pups had a lower prevalence than adults. Indeed, heartworm infection risk tended to increase with age, a pattern that is consistent with previous work [38, 39, 68]. The positive association detected for subadults could be because most subadults are transitioning between their natal and new territories. During this dispersal period, subadults may be exposed to a broader range of microhabitats than adults and pups, and thus could have more opportunities to encounter environments with more mosquitoes. An important next step that would help disentangle the importance of these various potential explanations would be to explore whether there is a relationship between infection risk and habitat use within home ranges.

The fact that the directionality of the infection–urbanization association by age class remained the same regardless of the home range estimator used (MCP vs. a-LoCoH) highlights the strength of these associations. However, it is interesting that model predictive power increased when focusing on resident coyotes and excluding transients. Because transients tended to have larger and more complex home ranges than residents, we suspect that the infection–urbanization association might differ, or might be less apparent, if a larger number of transients were included in the analysis (and/or that transients were examined separately). More research is needed to determine whether transient coyotes likely would have the same infection–urbanization association as resident adult coyotes or as subadult coyotes.

We cannot infer that adult coyotes, particularly transients, were likely infected in the areas where they were sampled; however, we can for pups, as coyotes only tend to leave their natal territories as subadults [90]. The fact that the direction of the infection–urbanization association was the same in pups and adults suggests that coyotes from these two age classes may have been infected in the area (or similar environment) where they were sampled. This lends support for the notion that urban wildlife reflect their local environment [25, 91] and highlights the need to carefully consider which types of individuals should be examined to effectively capture any associations with local environments (e.g., pups and resident adults in our case study).

While this study provides insight on how urbanization might influence wildlife infection risk, there were a number of limitations. Firstly, coyote sampling varied across years and urban zones, which limited result interpretations in some cases. That said, to the best of our knowledge, this is one of the first + 15-year urban wildlife disease investigations and provides unique evidence that wildlife disease risk can vary over time in urbanized settings. Another broader limitation worth noting was the inability to account for the time lag between human-derived changes to the landscape, and vector and wildlife response to this change [16, 33]. This could be especially important for urban and suburban land covers, which tend to increase over time. Future work should focus on developing approaches that can approximate time lags between land-use change and host and pathogen response to such changes [16]. Another important limitation of this study was not accounting for socioeconomic factors. Recent studies suggest that a number of socioeconomic factors can influence the distribution of wildlife diseases in urbanized areas (e.g., household income) [92, 93]. Future vector-borne and wildlife disease studies should quantify socioeconomic factors alongside of structural and abiotic components when exploring effects of urbanization on infection risk [94].

Conclusions

Measuring the effects of urbanization on host–pathogen interactions is becoming an important area of research [15, 19], particularly as urbanized areas continue to expand [95]. Recent work has found differences in disease risk between urban and non-urban wildlife populations (reviewed in [18]). Here, we found that coyote infection with the vector-borne pathogen *D. immitis* can vary within urban and suburban areas, and that effects may only be detected for certain age classes, and when using certain metrics of urbanization. While we were not able to make comparisons with rural or wildland coyotes, the fact that we detected differences in infection risk among

coyotes residing in different urban and suburban areas highlights the complex way by which vector-borne diseases are transmitted in urbanized landscapes.

Abbreviations

HDU: Heartworm development unit; MCP: Minimum convex polygon; a-LoCoH: Adaptive local convex hull; GLM: Generalized linear model; GLMM: Generalized linear mixed model; AICc: Akaike information criterion corrected for small sample size.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13071-021-04958-1.

Additional file 1: Figure S1. Map of the Chicago metropolitan area. Black circular lines delineate the four urban zones. Red squares are grids where temperature data were gathered. Temperature data were obtained from the PRISM Climate Group (PRISM Climate Group, Oregon State University, http://prism.oregonstate.edu). **Figure S2.** Relationship between heartworm infection and the proportion of medium developed urban land in coyote home ranges by age class. Panel **a** includes both resident and transient coyotes ($n = 146$), and panel **b** resident coyotes only ($n = 107$). The lines are mean estimates of the probability of heartworm infection by proportion urban land in coyote home ranges. The shaded bands are 95% confidence intervals. Coyote home ranges were estimated using 95% adaptive local convex hulls (95% a-LoCoH). **Table S1.** Number of recaptured coyotes and years of captures ($n = 16$). Coyotes were grouped based on whether they tested positive or negative on the first and second occasion. **Table S2.** Linear regression models predicting the duration of the heartworm transmission season ($n = 192$). Models are ranked based on ΔAICc. **Table S3.** Generalized linear mixed models (GLMMs) predicting heartworm infection ($n = 315$). GLMMs are ranked based on ΔAICc. **Table S4.** Top twenty generalized linear mixed models predicting heartworm infection. Models within ΔAICc < 2 from the best fit model were included in model averaging. **Table S5.** Model averaging results from binomial generalized linear mixed models of the probability of heartworm infection in coyotes ($n = 146$) using the adaptive local convex hull (a-LoCoH). Predictors were obtained from the top-ranking models (ΔAICc < 2; Table S4).

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Authors' contributions

KELWT conducted data analysis and wrote the manuscript. SDG designed the study. CLA and SDG collected the data. SDG, LEE and MEC oversaw analyses. MEC and SDG secured the grants. MEC oversaw manuscript development. All authors provided comments to improve the manuscript and read and approved the final version.

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Availability of data and materials

Data are available on figshare (https://doi.org/10.6084/m9.figshare.15169146.v1).

Declarations

Ethics approval and consent to participate

Coyote captures were approved by the Ohio State University (IACUC ID: 2013A00000012-89 R1) and by Illinois Department of Natural Resources (permit: IDNR W17.0122).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Ghost Dogs and Their Unwitting Accomplices

Stanley Gehrt

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Ghost Dogs and Their Unwitting Accomplices

Stanley Gehrt

Foreword, by Rylan Higgins

Stan Gehrt and I "met" for the first time via Zoom in May of 2021. We talked for about an hour, and at some point, we learned that we grew up within about 45 minutes of each other in southeast Kansas. As an anthropologist, I have always found that such connections facilitate a level of comfort that makes for good conversation. Within minutes, furthermore, I was sure I was talking with not only an intelligent coyote researcher but also a compassionate human who had come to relate to animals in ways few people have. By the end of the conversation, biologist (Stan) and anthropologist (Rylan) had put heads together and arrived at a plan for a rather unique essay for *Anthropology Now*.

A week or so before my meeting with Stan, I had heard him talk on CBC Radio One while I drove home (in the Halifax region of Canada). He was being interviewed by *Quirks & Quarks* host Bob McDonald about his coyote research in Chicago, and several things about that conversation struck me. The general idea that North American cities are home to many, many coyotes was itself quite notable, as was Stan's in-depth knowledge about these urban-based creatures. The evolving story of how humans and coyotes have related to one another, and continue to, was also rather remarkable and revealed features of interaction that I had never considered, even though I, like nearly everyone in North America, live among coyotes. I also found Stan's relationship with coyotes both intriguing and endearing.

As I listened to Stan on the radio and later talked with him, I was certain that his research on coyotes in general, but especially on human-coyote relations, would make interesting content for *Anthropology Now*. Based on what he described, I came to think that Stan does something akin to an ethnography of coyotes. As of the writing of this Foreword, I am still not sure how fitting this comparison is. Does Stan's research with coyotes mimic in any significant way the work that anthropologists carry out? Perhaps it is a bit of a stretch with regard to some of his methods. Radio collars and the use of sedatives simply aren't part of the anthropology tool kit. But hanging out, communicating and forming relationships are. And this is what Stan does with coyotes. He knows individual animals in a way that is not entirely dissimilar from the human-to-human interactions that result from anthropological research.

Regardless, human/nonhuman relations are an increasingly common and important topic in anthropology. This vein of scholarship is producing a lot of compelling insights, including ideas about expanding our understanding of personhood to include nonhuman animals. Stan's research on coyotes weds very nicely with this trend and makes it clear that collaborations between biologists and anthropologists hold a lot of potential for developing in-depth knowledge about how our species relates to others. As

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Stan's essay below so nicely shows, a lot is being learned, but plenty remains unknown. I, for one, am glad that there are researchers like Stan forming relationships with animals, learning about their lives and addressing key questions about how people make meaning as we interact with the non-human world.

The Story of Human-Coyote Relations in North America

If you are reading this article from basically any place in North America, in all likelihood you have been an unwitting accomplice to one of the most amazing wildlife stories to take place in the last century. This story involves the coyote (*Canis latrans*) and its unqualified success at conquering the continent at least partially through its strange, paradoxical relationship with humans. To be clear, the consequence of the coyote's success is that most of you are living with coyotes whether you are aware of it or not, whether you are reading this from a rural farm, residential subdivision or even a downtown office.

There are many layers involved in the history of the coyote that combine to make it such a compelling wildlife story in North America. Two aspects form the underlying foundation. Firstly, during a period of extreme persecution and land conversion to primarily human use, the coyote has dramatically expanded its distribution and abundance across the continent. Secondly, in the last 20 to 30 years, coyotes have become residents in virtually all metropolitan areas in the United States and Canada, a truly remarkable process because it has involved establishing themselves as the apex predator in urban systems built and occupied by their most dangerous predator: humans. Consequently, many readers of this article are participants in this second part of the coyote's story, and this is also where my research comes in to play.

The coyote is an exclusive North American member of the Canidae family, which includes wolves, foxes, jackals and, of course, our domestic dogs. At the time of European expansion across the continent (1600–1850 AD), the coyote's range was mostly restricted to plains and deserts west of the Mississippi and from the Canadian border to parts of Central America.[1] On the open range of the West, the coyote occupied the mid-sized carnivore niche, hunting mostly small prey and scavenging off kills made by the larger predators, while at the same time avoiding those dangerous competitors. This lifestyle, hunting prey while avoiding larger predators, would serve the coyote well as the landscape shifted from one dangerous predator to another.

Although coyotes had a largely positive relationship with the first people to inhabit North America, and indeed figured prominently in western Native American culture, things would change with pioneer expansion by those with European ancestry and the landscape would become dangerous again. Intense persecution of mammalian predators took place with such effectiveness that larger predators mostly disappeared. As Native Americans experienced their own persecution in the face of white expansion, the relationship between coyotes and people shifted to one of hostility. However, while larger mammalian predators succumbed to persecution and were largely extirpated from their former ranges, coyotes responded to this pressure by dramatically expanding their range across all

conterminous states and Canadian provinces. They have also expanded southward, increasing their range in Central America, and by 2013 crossed the Panama Canal.[2]

The amazing aspect of this tremendous range expansion is that it has been accomplished in the face of intense human persecution. At no point have coyotes benefited from any sort of protection or conservation efforts by state or federal agencies. Throughout most of their range, coyotes are regulated as game animals, which allows people to harvest them but also to remove them as predators. In most states, harvest regulations are the most lenient for coyotes compared to other species. Unlike other game species, nearly all states allow year-round hunting of coyotes with no limits on the number that can be taken. Occasionally coyote hunting contests, bounties and other forms of incentives appear to increase this persecution. Currently, using reported harvest and predator control numbers, between 500,000 and 800,000 coyotes are harvested or killed as part of predator control measures in the United States each year. Of course, these are underestimates of the total number of animals killed, because coyotes are killed for other reasons as well, so the total number of animals removed may approach 1 million in some years. Yet, despite this pressure, despite the human efforts to control or remove them, the coyote not only persists but has more than doubled its range and increased in abundance. Put differently, after nearly two centuries of intensive "coyote control," there are more coyotes on the continent today than at any point since European colonization. No other wildlife species can claim that level of invulnerability to human persecution.

As remarkable as that success is, a more stunning aspect of the coyote's story is their amazing success populating all metropolitan areas in the United States and Canada.[3] If the coyote had an opposable digit, it is likely they would be using it to thumb their noses at our efforts to exterminate them by claiming residence in our own backyards. But is this perception true, and what does their perceived "success" in urban areas mean for us and our ever-evolving relationship to coyotes?

For the past 21 years, I have had the privilege of developing and supervising the largest study of coyotes to date within one of the largest urban centers in North America, the Chicago metropolitan area. Over the years, we have used various types of technology to peer into the hidden lives of these animals, lifestyles that remain largely hidden despite living within a landscape containing 9 million people. But even with the advantages of radiotelemetry, GPS (Global Positioning System) satellites, remote cameras, chemical analysis of tissue for diet, and the latest genetic tools, it never ceases to amaze me how difficult it is to study these animals, even in a system where these animals are living among millions of people. In many ways they are as mysterious to me as when we started.

The story of the emergence of the coyote in the Chicago system is emblematic of most major cities across the United States and Canada. Prior to the 1990s, coyotes were only found in the more remote areas of the Chicagoland area, and usually in low numbers. But at the close of the 20th century, it appeared that their numbers increased dramatically, such that coyotes began appearing in areas where they have never been seen before.[4] Animal control agencies began fielding

calls from concerned residents, and many communities demanded animals be removed because of the perceived risk. However, despite efforts to "depopulate" coyotes from the area, they persisted. This, in turn, led to the need for better understandings of how the coyote population in the Chicago area was functioning and of the real risks they posed to people and their pets.

At the turn of the 21st century, the phenomenon of the urban coyote was relatively new, and little information existed on which to direct management decisions and, perhaps more important, how to respond to the general public's increasing fear. This need for basic information was the initial motivation for our Chicago research. Our project began in March 2000, when we captured and radio-collared our first coyote, a subadult female, Coyote #1. I still remember the excitement of actually capturing a free-ranging, wild coyote just a few miles from O'Hare International Airport, with airliners flying overhead and thousands of cars passing by a few hundred meters away. Little did we know just how special that animal would become.

We also began the study by assigning each coyote the functional, if not creative, ID numbers corresponding to the order in which we capture them. Hence, Coyote #1 was the first coyote captured, and her mate, a handsome, large male, Coyote #115, was the 115th coyote captured; our most recently captured animal is Coyote #1376. Nevertheless, some individuals that we follow do earn nicknames that stick, such as #115's name "Mellonhead," because of his large head. Obviously, the rather boring number system helps to minimize anthropocentric

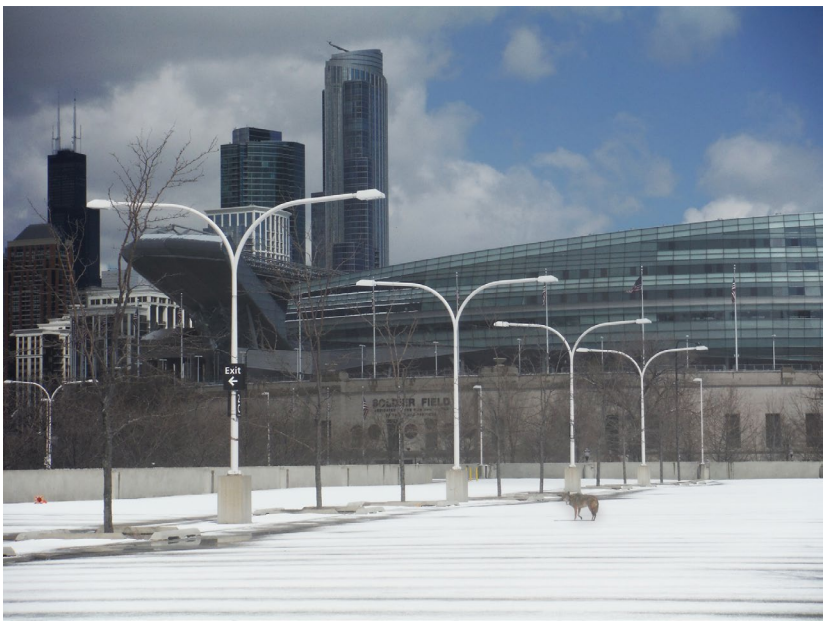

Image 1. An alpha male, Coyote #748, attending a den with his litter of newborn pups, on top of a parking garage across from Soldier Field, downtown Chicago, April 1, 2013. For more of his story, see: https://urbancoyoteresearch.com/coyote/748. Photo credit S. Gehrt.

influences in our science but also serves to help maintain some degree of impartiality as we are observers of their lives and try not to influence what transpires, which can be difficult. However, the radio collars serve as windows into their lives, and the process of spending countless hours observing certain animals naturally produces a relationship with them, even if they are unaware of it. The radio collars also allow us to document the end of their life, and the numbers help mitigate our loss to some degree, as we will inevitably record their death.

In my first night of tracking Coyote #1, she took me on a journey across five subdivisions and a tollway. This trek ended in a patch of weeds with my headlights shining on three men with dogs on leashes, completely unaware that a coyote was hiding only five meters away. In one night, that animal taught me the following: (1) we were underestimating their ability to move through a presumably challenging, urbanized landscape; (2) we were grossly underestimating the coexistence already occurring between people and coyotes; (3) we were likely underestimating the abundance of these animals in Cook County and, most important; (4) I definitely underestimated the budget for this research! She and her mate taught us many other things over a decade. Both lived for 12+ years and raised at least 38 offspring from seven litters. They spent every day of their lives living within a few meters of people and their pets, without conflict. One of their favorite hiding spots during the day was

Image 2. Graduate student Ashley Wurth and technician Abby-Gayle Prieur take measurements and samples from an immobilized coyote, adult male Coyote #1071. Photo credit: J. Nelson.

under a bush a couple meters from a post office, where hundreds of unsuspecting people walked past each day. To be fair, coyote pups sometimes destroyed Nerf footballs and stole chew bones from backyards, but these are hardly the behaviors worthy of human persecution.

Coyotes have highly structured social systems, in which family groups, or "packs," maintain exclusive territories that are defended from other coyotes. As the population grows, more of the landscape is filled with these territories, and young (and sometimes older) coyotes leaving their packs will attempt to create a territory in a new area. It is through this territorial system that the coyote population expanded across the Chicago region and into areas that had not previously experienced coyotes. Survival is relatively high and vacant territories are limited, so young coyotes are continually forced to explore and attempt to exploit strange, novel areas — which they do, quite well.

Using radiotelemetry and GPS technology to track over 1,300 marked and radiocollared animals, our research has shown that coyotes are capable of maintaining territories and raising litters in all parts of the Chicago area, even the most heavily developed regions we originally thought impossible.[5] For example, possibly the most urban of our coyotes, adult female #447, had a territory that encompassed all of downtown Chicago. Thus, she shared her territory with approximately 750,000 people, which does not include the commuters who worked downtown. She lived in that area for at least five years, without a conflict. Indeed, based on her location and the number of humans she shared space with, I would argue that she may have been the most "urban" coyote in the country.

To successfully live in the city, coyotes must avoid humans as much as possible. The vast majority of the coyote population goes about their daily lives largely unnoticed by people, even when they are living a few meters away. To do this, they may hide during the day and move at night. In fact, we have found that coyotes living in the most urbanized areas are exclusively nocturnal and travel further distances within larger territories than more suburban coyotes. They learn human traffic patterns and know the safest times and locations to cross roads. Coyotes learn when and where humans are most active, and they scale down their activities to avoid us. They spend a lot of time watching us and learning. Consequently, we are largely coexisting with them without knowing it. Indeed, they are so effective at avoiding us I have referred to them as Chicago's ghost dogs.[67]

Another important aspect of the relationship between urban coyotes and humans is food. Our initial assumption was that success in urban areas was likely because of a reliance on human-associated food. In other words, we assumed that coyotes in cities were living off of us. Starting in 2012, we began using stable isotopes to characterize individual coyote diets. We did so because traditional techniques, such as fecal analysis, tended to underestimate the use of human-processed foods. To do this, we collected a whisker from a captured coyote (so the individual information was known: sex, age, social status, location); the whisker was sectioned into multiple segments, and each segment was analyzed individually. This gave us a dietary profile for the animal over the

Image 3. Recapture of Coyote #967 on February 23, 2018. The red ear tags are slightly visible in the ears and radio collar peaks out under his chin. Photo credit J. Nelson.

weeks and months of whisker growth and allowed us to measure the variability of food items in their diet over time, as well as variability in diet across individuals in the population. The picture that emerged is that, much like us, coyotes are highly individualistic in their diets, even those within the same packs and living in the same areas. Most coyotes, moreover, have maintained diets largely dominated by natural foods, such as voles, mice, and rabbits, with only a minority heavily relying on human foods.[7] Basically, urban coyotes have a smorgasbord of natural and human-associated foods available to them, and unlike rural systems, food abundance is maintained across seasons and years.

Other lines of evidence support the conclusion that dietary resources are not limiting. Our study animals, on average, are in excellent health and body condition. There is a small trend for increasing size with urbanization among our population of coyotes, such that they tend to be heavier than rural animals. Another indicator of the benefits of city life is litter size. Each spring, we enter the dens of our study animals and microchip and measure neonate pups. We do this for a variety of reasons, but a primary one is to record litter size. Coyotes are able to scale their litter size relative to available resources, so when resources are abundant they may produce relatively large litters. We regularly record large litter sizes, at times averaging over 8 pups per litter, and sometimes exceeding 11 or more. Again, these lines of evidence reveal a picture of the metropolitan area as a type of hospitable refuge compared to more rural areas. As a kid born and raised in a small Kansas town, I would have never guessed

that an area with millions of people would be an oasis of sorts for coyotes.

There are, however, costs to living in the urban world. Coyotes in the core of the city must travel further and faster, within a reduced activity period, to obtain resources.[8] All coyotes must navigate roads, and for a transient, solitary coyote in a new part of town, a miscalculation means death. And if a coyote suddenly becomes too obvious to people, by, for example, engaging in regular daytime activity, there will inevitably be a call to lethally remove it. Although urban coyotes are relatively protected from hunting and trapping, human-caused mortalities are still the most common causes of death, either unintentional human-caused mortalities through vehicle collisions, which is by far the leading cause of mortality, or coyotes killed intentionally through removal efforts. A minority of these removals are the result of actual conflicts in the form of aggression or attacks on pets. Most coyote removals/killings are simply the result of animals becoming habituated to human activities.

For most cities, coyotes are the largest predator in their midst, and attacks on people and pets do occur, albeit rarely. Thus, coyotes do represent a risk that was not present in most cities prior to their expansion, and part of our research is measuring that risk. Each year, 1 to 4 percent of the coyotes we monitored were removed as nuisances. In nearly all cases, the animal had not actually attacked or injured a pet or person but was becoming too obvious to people or may have conflicted with humas in other ways. For example, some "nuisance" coyotes are removed each year from airports, where there is understandably zero tolerance for disrupting flights. The large grasslands surrounding airports are unfortunately attractive for coyotes hunting rodents. Overall, of

Image 4. S. Gehrt holding a litter of seven pups from Coyote #581, an adult female living in the Chicago suburbs. Each year we enter dens once during the spring to count and microchip pups for population estimates and to record family relationships. Photo credit S. Eszterhas.

the animals we have marked, only a handful have attacked pets, and none have attacked or threatened a person.

The characteristics of conflicts often vary based on the quirky nature of coyotes. Coyote #748, an alpha male (meaning he was an adult with a mate), occupied a territory encompassing Lakeshore Drive and some of Chicago's most iconic sites, such as the Field Museum, Soldier Field and Sears Tower. He and his mate were "good" coyotes, in that they avoided people and their pets at all costs. This changed suddenly in April, when 748 suddenly became aggressive toward dogs, but in his own unique style. The pair had a newborn litter in a den at the top of a parking garage across from Soldier Field, a very popular dog-walking spot along the lakefront. During the first two weeks after the litter was born, he would sneak down from the garage and, ignoring the poor dog owner, "attack" a dog in an attempt to protect the den. Although there was a constant flow of dog walkers from early morning until late at night, 748 would "attack" only one dog each evening between 6:30 and 8:30 pm, and only between those hours. Equally as strange, he never injured a dog. He would jump on them and they would roll around with much yelping in front of their terrified owner, but then he would trot away, leaving the dog covered in saliva but otherwise unharmed. Fortunately, after the pair moved the litter to a different location away from dog walkers, 748 reverted to a "good" coyote again.

This case also illustrated a common human quirk that likely contributes perceptions of risk and trepidation regarding coyotes. People tend to exaggerate the size of animals, especially predators (no one ever reports encountering a "tiny" coyote, only the "big" ones). I became aware of 748's switch to "dog attacker" only an hour or two after his first attack, because the owner of the dog googled me and called my office while I was working late. While he was walking his dog on a leash near the stadium, a "huge" radio-collared coyote came "out of nowhere" and jumped on his dog. Luckily, his dog was not injured, but he described the coyote as over 100 lb. I asked him how he estimated the coyote to be that big, and he said that the coyote was at least as large as his dog, which was a 110-lb mastiff. When we captured 748 a month prior, he weighed a typical 29 lb. The heaviest animal we have captured to date was 42.4 lb. Somehow, with the animal right in front of him and even with his dog as a comparison, the owner managed to add 70 lb of imaginary size to 748. Our ability to unconsciously inflate size of animals we fear undoubtedly contributes to conflicts.

Although the actual risk of humans being attacked by coyotes is small, the *perceived* risk is often high. So, I'm regularly asked by members of the public and officials what good are coyotes? Why should people tolerate any risk, no matter how remote? Is there anything positive about coyotes in cities, or is the urban coyote story simply about managing risk? My answers to these questions likely are at least a bit surprising.

Predation is an important, even vital, function in ecosystems, and unfortunately the lack of predators in urban systems results in overpopulation of some prey species, often at the expense of habitats or damage to our property. For far too long, predation was absent or limited in our cities, such that urban

Image 5. S. Gehrt and Coyote #1, an adult female living in the suburbs near O'Hare International Airport. At the time of her recapture, she had been monitored continuously for nearly a decade and had outlived the battery life of her radio collar. Duct tape on the mouth was necessary because she was not immobilized. Photo credit S. Gehrt.

ecosystems were severely altered and some species (e.g., geese, deer, rodents) became artificially o verabundant. A s c oyotes h ave made their appearance onto the urban stage, it is possible that they have introduced predation to this severely altered ecosystem. Whether this is the case and to what extent became new research questions for us.

At different stages of our study, we were able to expand our objectives to explore the relationship between coyotes and two prey species that are known to become overabundant in urban areas: Canada geese and white-tailed deer. Using a variety of techniques and technologies, we documented that coyotes were responsible for taking the eggs from half of goose nests each year, thereby reducing the annual population growth rate from 14 percent to less than 2 percent. Regarding coyotes and deer, we found the predation rate of deer fawns ranges from 35 to 80 percent each year, with most years over 50 percent. In both cases, coyote predation has helped slow the population growth of the prey species at the local level. Deer are particularly problematic when overabundant, because they can cause ecological damage through herbivory, while also representing a threat to health and safety through collisions with automobiles.

A predation rate of more than 50 percent of fawns is sufficient to limit growth at the local level, and limiting the deer population has direct benefits for people. This is because the most dangerous wildlife species to people and their property in urban systems is not a predator but rather deer and their collisions with automobiles. Each year, tens of thousands of accidents occur with deer, especially in large urban centers, with injuries to people and occasionally fatalities. For example, during 2019 there were over 16,000 auto-deer accidents across Illinois, resulting in 604 injuries and four deaths. This is ac-

cording to data from Illinois Department of Transportation.[9] Only a few months prior to writing this essay, two people were tragically killed near our study area from a collision with a deer.[10] By comparison, there have only been two recorded human deaths from coyote attacks over the past 50 years across the United States and Canada.

There is a particular irony here. Through a largely unnoticed process, a predator that the public associates with risk actually helps to reduce the much more substantial risk posed by a prey species. When I talk to the public about urban coyotes, I often point out that it is very likely that coyotes actually save human lives regularly by reducing deer populations and the risk of deadly car accidents caused by deer.

An alpha predator can also impact the system by influencing the behavior of other, small predators. Outdoor domestic cats, or feral cats, are also a prominent feature of urban landscapes that can be a management dilemma. Over a four-year period, I created experimental feral cat shelters across parts of Chicagoland in areas where we also had coyotes radio-collared. We followed humane protocols and all cats were vaccinated; provided food, water and shelter; and radio-collared. We found a strong coyote effect on cats, but surprisingly it was primarily in the form of avoidance rather than predation. Despite establishing cat shelters in areas of high coyote densities, only 7 percent of 127 radio-collared cats were killed by coyotes. This is because nearly all cats avoided the green spaces and natural habitat fragments that were occupied by coyotes and restricted their movements to neighborhoods and yards. Essentially, coyotes served as buffers for green spaces that limited outdoor cat use, which benefits a variety of birds and small mammals that traditionally serve as prey for cats. Other studies have found that the diversity of native wildlife species in urban landscapes is higher where coyotes are present than where they are absent, largely due to their exclusion of outdoor cats.[11]

Although these positive aspects of coyotes in the system are important when trying to understand the various layers to this amazing coyote story, they are only the tip of the iceberg of our understanding how coyotes affect other wildlife species or whatever positive effects are associated with them. The studies mentioned above were incredibly challenging, took years of effort and required the best of our technologies to uncover coyote-related processes taking place among 9 million people. We are only scratching the surface of what coyotes bring to the urban ecosystem and, in fact, the roles they play across North America. It is an unfortunate fact that, by far, funding for research on coyote and other mammalian predators has been focused specifically on conflicts and ways to control or limit their populations. This leaves us with a limited understanding of how coyotes function ecologically or how we benefit from them.

Similarly, through their perseverance, coyotes are infusing themselves into our urban culture, as they did originally with Native Americans, and even western white America. Despite their best efforts, coyotes in the most urban areas have a difficult time avoiding people completely, especially in the most urban areas. It is these times when people encounter coyotes when they develop their own "coyote stories." When we began our

research two decades ago, urban coyote stories were rare, but now they are common and even transcend continental boundaries.

As an example, one of my favorite anecdotes comes courtesy of Coyote #447, an adult female I described earlier. Sometime in 2010, I received an email from a person from Switzerland who traveled to Chicago each year for a week of business meetings. He wondered whether he had encountered one of our study animals on his most recent trip. When he stayed in Chicago, his traditional routine was meetings all day and then a run at night in Grant Park. On this warm summer night, as he jogged, he was surprised by a dog-like animal passing him from behind on the path. It was notable because it was not leashed and was wearing this strange collar. She gave him a quick glance but never broke her effortless trot as she continued down the path. It happened so quickly, he wasn't sure if it was a coyote or a strange dog. However, as he continued his jog around the park, he kept an eye out for the animal. Sure enough, before he had completed his lap, she came from behind him again and, like before, barely acknowledged him as she casually lapped him, passing a few inches from his leg, as she did before. He thought her glance was mildly approving of his progress, and then she was gone.

When I responded that yes, this was Coyote #447, and she regularly used Grant Park, he was thrilled, using many exclamation points!!! He described his experience of being lapped by one of the famous "Chicago coyotes" in Grant Park as easily the most memorable experience from all his business trips, and he would remember it forever. Though this is a cute anecdote (it makes me smile each time I share it), it is worth noting that it is one of thousands of coyote encounters that take place each year that are not conflicts but rather a spice of life — memorable moments that are never reported in the media, unlike the rare cases of an attack on a dog. Much like their ecological effects on the urban ecosystem, coyotes are likely impacting human culture in subtle ways that have not yet been fully recognized.

So, how are you a participant in this coyote story, even if you do not have your own coyote story? With a rather high degree of certainty, most of you are living with coyotes. If you live or work within a metropolitan area, at some point you have passed within a few meters of a coyote. Some of you may pass them on foot or with your car on a regular basis. If you use a park, visit a cemetery, run an errand or play a round of golf, undoubtedly there is a coyote watching and learning from you. As you commute to work, a coyote is near the road or rail line, avoiding you. It is through your activity that you reinforce or, in some cases, change their behavior, and you are playing a role in one of the most amazing wildlife stories [1]in North America. More than anything else, the coyote's ability to live in an urban area and effectively coexist with us relies on its ability to avoid you. But I believe we can learn from coyotes as well, if we are willing. Coyotes teach us lessons in humility, whether that is scientists attempting (and often failing) to understand them or the many landowners, municipalities and government agencies attempting to exterminate them. They teach us every day that there is still much to learn about this world, even in our own backyards.

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Importance of anthropogenic sources at shaping the antimicrobial resistance profile of a peri-urban mesocarnivore

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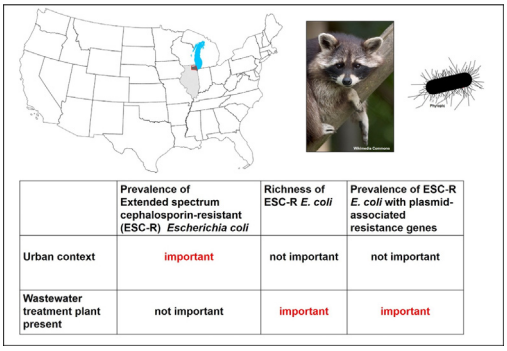
HIGHLIGHTS

- Wildlife exposed to anthropogenic sources can have antimicrobial resistant bacteria.
- Extended-spectrum cephalosporin-resistant (ESC-R) *Escherichia coli* were more common in urban than suburban raccoons.
- ESC-R *E. coli* richness was higher for raccoons sampled at sites with a wastewater treatment plant (WWTP).
- ESC-R *E coli* with plasmid-associated resistance genes were more common at sites with a WWTP.
- WWTP may increase the risk for antimicrobial resistance to spread in wildlife bacterial communities.

GRAPHICAL ABSTRACT



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ABSTRACT

Anthropogenically derived antimicrobial-resistant bacteria (ARB) and antimicrobial resistance genes (ARG) have been detected in wildlife. The likelihood of detecting ARB and ARG in wildlife increases with wildlife exposure to anthropogenic sources of antimicrobial resistance (AMR). Whether anthropogenic sources also increase the risk for AMR to spread in bacteria of wildlife is not well understood. The spread of AMR in bacteria of wildlife can be estimated by examining the richness of ARB and ARG, and the prevalence of ARB that have mobilizable ARG (i.e., ARG that can be transferred across bacteria via plasmids). Here, we investigated whether raccoons (*Procyon lotor*), with different exposures to anthropogenic sources, differed in prevalence and richness of extended-spectrum cephalosporin-resistant (ESC-R) *Escherichia coli*, richness of ARG present in ESC-R *E. coli*, and prevalence of ESC-R *E. coli* with plasmid-associated ARG. Sampling took place over the course of 10 months at seven sites in Chicago, USA. ESC-R *E. coli* were isolated from over half of the 211 raccoons sampled and were more likely to be isolated from urban than suburban raccoons. When examining the whole-genome sequences of ESC-R *E. coli*, 56 sequence types were identified, most of which were associated with the ARG bla_{CMY} and

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Wastewater treatment plant
Wildlife

bla_{CTX-M}. A greater richness of ESC-R *E. coli* sequence types was found at sites with a wastewater treatment plant (WWTP) than without, but no difference was detected based on urban context. ARG richness in ESC-R *E. coli* did not significantly vary by urban context nor with presence of a WWTP. Importantly, ESC-R *E. coli* carrying plasmid-associated bla_{CTX-M} and bla_{CMY} ARG were more likely to be isolated from raccoons sampled at sites with a WWTP than without. Our findings indicate that anthropogenic sources may shape the AMR profile of wildlife, reinforcing the need to prevent dissemination of AMR into the environment.

1. Introduction

Use of antibiotics in human and veterinary medicine has led to the emergence of many forms of antimicrobial resistant bacteria (ARB) (WHO, 2014). In addition to undermining the successful treatment of bacterial infections, intensive antimicrobial use has been linked to the widespread dissemination of ARB in the community and the wider environment (Laxminarayan et al., 2013; Radhouani et al., 2014). Hence, what was originally observed only in clinical and agricultural settings is now frequently reported in non-hospitalized people, animals, and the environment. ARB can enter the environment via multiple pathways, such as through rivers or streams receiving wastewater treatment plant (WWTP) effluent (Rizzo et al., 2013; Berendonk et al., 2015). These types of environments can be hotspots for horizontal gene transfer, and thus act as sources of antimicrobial resistance (AMR), and may also facilitate widespread dissemination of AMR in the environment (Wellington et al., 2013; Marti et al., 2014; Huijbers et al., 2015), directly exposing people, domestic animals, and wildlife to ARB (Berkner et al., 2014).

Wildlife exposure to ARB has been reported in a number of different species, from different ecosystems and continents (e.g., Cole et al., 2005; Jobbins and Alexander, 2015; Kipkorir et al., 2019). However, in most cases the risk of isolating ARB from wildlife tends to increase with wildlife proximity to human-dominated areas (Skurnik et al., 2006; Allen et al., 2010), or when wildlife come into contact with anthropogenic sources of AMR such as wastewater or landfills (Radhouani et al., 2014; Varela et al., 2015; Ahlstrom et al., 2018). For example, urban wildlife are more likely to have ARB than non-urban wildlife (e.g., Navarro-Gonzalez et al., 2018; but see Carter et al., 2018). Similarly, aquatic species present in waters contaminated with ARB, antimicrobial resistance genes (ARG), and/or antimicrobial residues are more likely to have ARB (e.g., Jobbins and Alexander, 2015). Because wildlife carriage of ARB is typically associated with human activity, wildlife are frequently used as sentinels for understanding the spread of AMR in the environment (Vittecoq et al., 2016; Furness et al., 2017; Torres et al., 2020; although see Swift et al., 2019).

Wildlife can also play a role in the dissemination of ARB to the wider environment (Cole et al., 2005; Vittecoq et al., 2016; Wang et al., 2017). This is especially true for migratory species, species that have large home ranges, or species that use multiple ecosystems (Arnold et al., 2016; Vittecoq et al., 2016). For example, aquatic avian species are predicted to play an important role in the dissemination of ARB to areas away from anthropogenic activity (Wang et al., 2017). Further, wildlife have the potential to act as secondary reservoirs or amplifiers of AMR (Radhouani et al., 2014; Carroll et al., 2015; Ramey and Ahlstrom, 2020; Vittecoq et al., 2016; Arnold et al., 2016; Dolejska and Literak, 2019), potentially facilitating the maintenance and spread of known ARB and emergence of novel ARB (Jones et al., 2008; Karesh et al., 2012; Ramey and Ahlstrom, 2020). However, the likelihood for this to occur depends on whether ARG can be transferred between bacteria of wildlife (Allen et al., 2010; Dolejska and Papagiannitsis, 2018). ARG can be transferred between bacteria if they are located on plasmids (a process known as conjugation), but are less likely to transfer if they are located on the chromosome of bacteria, unless they are transferred to plasmids via transposons or integrons. Wildlife that carry ARB with plasmid-associated ARG are more likely to act as secondary reservoirs of AMR (Dolejska and Papagiannitsis, 2018).

Most wildlife AMR research has focused on investigating the occurrence and similarity of ARB and ARG in relation to various anthropogenic sources or environments (e.g., urban vs. non-urban, presence of a WWTP; Carter et al., 2018; Swift et al., 2019). However, little is known about whether we should expect ARG to be plasmid- or chromosomally-associated (Dolejska and Literak, 2019). Environmental AMR research has shown that water and soil associated with anthropogenic sources of AMR (e.g., rivers connected to WWTPs) are not only more likely to have a greater richness of ARB and ARG (i.e., number of unique ARB and ARG), but also more plasmid-associated ARG, than environments that are not, or less associated with anthropogenic sources of AMR (Berendonk et al., 2015; Rizzo et al., 2013). Whether similar differences hold true for the wildlife in these environments is less well understood, yet it is essential for evaluating the importance of anthropogenic sources at shaping the AMR profile of wildlife.

Here, we used whole-genome sequencing and phylogenetic analyses to determine whether wildlife exposed to known anthropogenic sources of AMR were more likely to have a higher prevalence and richness of ARB and ARG and plasmid-associated ARG than wildlife that were not exposed. The study took place in the metropolitan area of Chicago over the course of ten months, and two anthropogenic sources were examined: 1) urban context (urban vs. suburban) (Parker et al., 2016); and 2) presence of a WWTP upstream of sampling sites (Marti et al., 2013; Rizzo et al., 2013; Wellington et al., 2013). The wildlife species of focus was the raccoon (*Procyon lotor*) because raccoons are widely distributed in urban and suburban areas (Gehrt et al., 2010; Bateman and Fleming, 2012), use multiple ecosystems (e.g., forage in aquatic and terrestrial systems), and are known to shed ARB and ARG (Bondo et al., 2016; Bondo et al., 2019; Worsley-Tonks et al., 2020). Further, raccoons have relatively small home ranges (<2 km in urban and suburban areas; Šálek et al., 2015) meaning that exposure to ARB is likely associated with the area in which each animal was sampled. The microorganism of focus was clinically relevant extended spectrum cephalosporin-resistant (ESC-R) *Escherichia coli*, which includes both extended-spectrum beta-lactamase (ESBL) and AmpC beta-lactamase producing *E. coli*. ESC-R *E. coli* are of increasing concern in human and veterinary medicine (Partridge, 2015; Woerther et al., 2013; Bezabih et al., 2020), and have been detected in the environment (e.g., Tacão et al., 2012; Egervärn et al., 2017; Fagerström et al., 2019) and in the feces of many wildlife species (Guenther et al., 2011).

Our objectives were to 1) describe the prevalence, richness, and characteristics of ESC-R *E. coli* and associated ARG in the sampled raccoon population; and 2) determine whether urban context and/or the presence of a WWTP upstream of capture sites influenced prevalence and richness of ESC-R *E. coli* sequence types, richness of ARG in ESC-R *E. coli*, and prevalence of ESC-R *E. coli* with plasmid-associated ARG. Hundreds of ARG can confer ESC resistance, but we focused on ARG from the bla_{CTX-M} and bla_{CMY} families because they are most commonly detected and are of clinical importance in the Chicago area and globally (Partridge, 2015; Bezabih et al., 2020; Logan et al., 2016, 2020). We predicted that raccoons sampled at urban sites and sites with a WWTP would have a higher prevalence of ESC-R *E. coli*, greater richness of ESC-R *E. coli* sequence types and associated ARG, and higher prevalence

of ESC-R *E. coli* with plasmid-associated ARG than raccoons sampled at suburban sites and sites without a WWTP.

2. Methods

2.1. Study site and design

From February–November 2018, raccoons were captured from seven sites in northwestern Chicago (Fig. 1). Sampling took place over the course of four seasons [winter (mid-December until end of March); spring (beginning of April until end of June); summer (beginning of July until mid-September); and fall (mid-September until mid-December)]. The seven sites differed in composition of natural vegetation, managed green spaces, and built-up land. Specifically, Crabtree, MMWF, PC, and Busse were mostly composed of a combination of green space and built-up land (built-up land made up 36–66%), while Edgebrook, DRCA, and Damen were mostly composed of built-up land (built-up land made up 86–97%). Sites were classified as urban if the mean number of households per square kilometer within a 1-km buffer around each site was greater than 1000 (Parsons et al., 2018). Otherwise, sites were classified as suburban. Data on household number per square kilometer were obtained from the 2010 SILVIS housing density dataset (SILVIS Lab Spatial Analysis for Conservation and Sustainability). Raccoons were sampled in urban and suburban sites and not rural sites to investigate the importance of wildlife proximity to human dominated areas, since proximity can be important for isolating ARB from wildlife (Skurnik et al., 2006; Allen et al., 2010; Furness et al., 2017). Raccoons were not sampled at rural sites because of the potential for raccoons to be exposed to ARB via agricultural sources in those settings. Additionally, four of the sites had rivers that were downstream from a WWTP (i.e., Busse, Damen, Edgebrook, and MMWF), and three were not downstream from a WWTP (i.e., CT, DRCA, and PC). Sites that were downstream from a WWTP were less than a kilometer from treatment outlets. Finally, since the home range of an urban and suburban raccoon tends to be less than 2 km^2 (Šálek et al., 2015; McClure et al., 2020), and the shortest distance between any of the seven sites was ~4 km (and often separated by interstates), raccoons most likely did not move between sites.

2.2. Raccoon handling

Raccoons were captured using box traps (Model 108, Tomahawk Live Trap Co., Tomahawk, WI, U.S.A.) (as in Prange and Gehrt, 2004) and immobilized with an injection of Telazol (Fort Dodge Animal Health, Fort Dodge, Iowa) (Gehrt et al., 2001). After collecting fecal samples opportunistically from each individual, all captured individuals were aged based on reproductive condition (adult or juvenile), sexed (male or female), and fitted with ear tags for identification. After recovering from immobilization, all animals were released at the capture locations. Fecal samples were stored in brain heart infusion broth and 20% glycerol at −80°C until processing. Captures were approved by the University of Minnesota's Institutional Animal Care and Use Committee (protocol ID: 1709-35105A) and by Illinois Department of Natural Resources (permit number: IDNR W17.0122).

2.3. Phenotypic characterization of ESC-R E. coli

To investigate the presence of ESC-R *E. coli*, we tested *E. coli* susceptibility to cefotaxime, a 3rd generation cephalosporin commonly used to test for the presence of ESC-R microbes (Gazin et al., 2012). More specifically, samples were enriched in Lauryl Tryptose Phosphate broth (Difco Laboratories, Detroit, MI, USA) overnight at 37°C and then streaked onto CHROMagar ECC (CHROMagar, Paris, France) containing 2 μg/mL of cefotaxime, a concentration that is typically used for environmental and wildlife research (e.g., Albrechtova et al., 2014; Furness et al., 2017) and is in accordance with the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines. If blue colonies were obtained (indicative of being *E. coli*), one colony was selected at random, restreaked on CHROMagar ECC containing 2 μg/mL of cefotaxime, and incubated overnight at 37°C. Isolates were then grown in 3 mL of LB broth overnight with shaking at 37°C and then stored at −80 °C until sequencing.

2.4. Genome assembly and gene content analysis

ESC-R *E. coli* isolates obtained from each sample were subjected to whole genome sequencing (WGS). DNA was extracted from an overnight growth of a single colony for each isolate using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, California) according to the manufacturer's instructions. WGS of isolates was performed using NovaSeq Illumina 150 bp paired-end sequencing and dual-indexed Nextera XT libraries (Illumina, USA) at the University of Minnesota Genomics Center (Saint Paul, Minnesota, USA). Raw reads were quality filtered and trimmed using Trimmomatic (version 0.33) (Bolger et al., 2014), which involved removing Illumina Nextera adapters, removing the bases off the start and end of reads if below a threshold quality of 3, having a sliding window of size 4 bp that removed bases if their phred score was <20, and having the minimum read length be 36 bp.

To identify ARG, trimmed reads were assembled using the SPAdes assembler (version 3.0) (Bankevich et al., 2012) with default

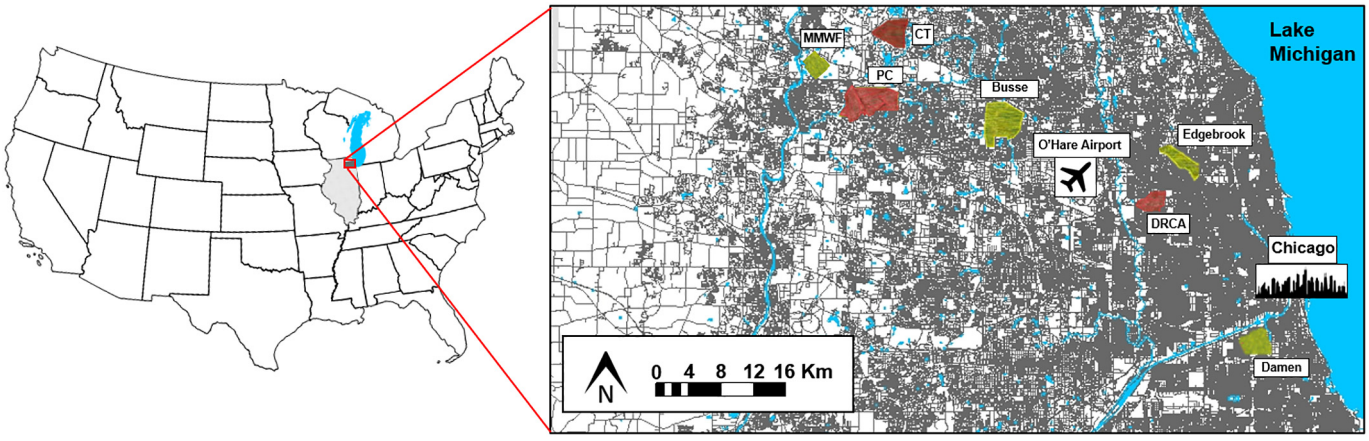

Fig. 1. Sampling sites in urban and suburban Chicago. The four yellow polygons are sites that had rivers that were downstream from a wastewater treatment plant (WWTP), and the three red polygons are sites not downstream from a WWTP. Damen, DRCA, and Edgebrook are urban sites, and Busse, CT, MMWF, and PC are suburban sites. Grey represents impervious surfaces.

parameters. The quality of assemblies was assessed by examining the N50 score of each isolate (mean = 85,675, range = 32,473-197,340), which was calculated using QUAST (version 4.3) (Gurevich et al., 2013). Presence of ARG on contigs was assessed using the Resfinder database (Zankari et al., 2012), which includes only acquired ARG, and not point mutations in chromosomal target genes. Open reading frames were identified using Prokka (version 0.7.17) (Seemann, 2014) and were then aligned to the Resfinder database using the NCBI BLASTn algorithm. ARG presence was based on an identity ≥90% and a coverage ≥80%. When multiple ARG alleles were identified on the same contig and at the same location (e.g., bla_{CMY-2} and $bla_{CMY-101}$), the allele with the highest identity and coverage was selected. In instances when alleles could not be differentiated, the ARG was not classified at the allele level but at the gene family level (e.g., described solely as bla_{CMY} and not bla_{CMY-2}).

2.5. Assessing the plasmid- vs. chromosomal-association of ARG conferring ESC-resistance

Given the challenges associated with identifying plasmids from short-read sequencing datasets (Arredondo-Alonso et al., 2017; Orlek et al., 2017), we opted to use two typing programs to classify ARG conferring ESC-resistance as plasmid or chromosomally-associated: 1) Mlplasmids (Arredondo-Alonso et al., 2018); and 2) MOB-suite (Robertson and Nash, 2018). Mlplasmids uses trained machine learning models (specifically Support Vector Machines), to predict whether contigs are plasmid- or chromosomally-associated using pentamer frequencies. Models in this typing program were trained using 583 *E. coli* genomes (168 chromosomal and 415 with plasmid entities) and have a sensitivity of 71% for detecting the plasmid class (Arredondo-Alonso et al., 2018). The default threshold for classifying contigs as plasmid or chromosome-associated is 50%, but we set the threshold to 70% to reduce the false positive error rate. MOB-suite types and reconstructs plasmids using publicly available Illumina short-read sequencing data. MOB-suite can classify contigs as plasmid-associated with a sensitivity and specificity of 95% and 88%, respectively. In instances where Mlplasmids and MOB-suite predicted different results (i.e., plasmid in one and chromosomal in the other), we classified contigs as unknown and did not include them in relevant downstream statistical analyses.

2.6. Phylogenetic analysis

We explored genetic associations among isolates by subjecting assembled contigs to traditional multilocus sequence typing (MLST) using mlst (https://github.com/tseemann/mlst) and the in silico *E. coli* PubMLST typing scheme. MLST classifies isolates into different sequence types (STs) by exploring the allelic profile of seven housekeeping genes (*adk*, *gyrB*, *fumC*, *icd*, *mdh*, *purA* and *recA*) unique to *E. coli* (Wirth et al., 2006). Associations between STs were visualized using minimum spanning trees, which were created in GrapeTree (Zhou et al., 2018).

Deeper phylogenetic associations were explored by performing single-nucleotide polymorphism (SNP)-based phylogenetic analysis from the core genomes of sequenced isolates. A core SNP alignment was created by mapping trimmed reads to the *E. coli* K-12 laboratory strain MG1655 genome (Accession number: GCA_000005845.2) using Snippy version 4.4.0 (https://github.com/tseeman/snippy). Recombinant regions were removed with Gubbins version 2.3.4 (Croucher et al., 2015). A SNP-distance matrix was created using snp-dist version 0.6.3 (https://github. com/tseemann/snp-dists). A maximum likelihood phylogenetic tree was constructed using IQ-TREE version 1.6.12 (Trifinopoulos et al., 2016), where model selection was performed using ModelFinder (Kalyaanamoorthy et al., 2017), and the tree was validated using 1000 ultrafast bootstrap repetitions (Hoang et al., 2017). The TVM + F + ASC + R3 model was identified as the best fit based on Bayesian Information Criterion. The resulting phylogenetic tree was visualized and annotated using the iTOL (Interactive Tree of Life) online software (Letunic and Bork, 2016).

2.7. Statistical analysis

The importance of urban context and presence of a WWTP at capture sites was examined firstly based on the probability of isolating at least one ESC-R *E. coli* from raccoons. To do this, we ran a binomial generalized linear mixed model (GLMM) with a logit link function using the '*lme4*' package (Bates et al., 2014) in R version 4.0.2 (R Development Core Team, 2020). Predictor variables included in the GLMM were urban context (urban vs. suburban) and presence of a WWTP at the site (yes vs. no) (Table 1). Additionally, we included season (fall, winter, spring, summer) because previous wildlife AMR research has found season to be important (Williams et al., 2011; Miller et al., 2020) (Table 1). We also included host age (juvenile vs. adult) and sex to control for their potential importance (Table 1). Sampling site was included as a random effect because model residuals were significantly spatially autocorrelated ($z = 2.83$, $p = 0.02$) (Table 1) (Dormann et al., 2007), which was tested using a permutation test (999 permutations) for Moran's I statistic from the '*spdep*' R package (Bivand et al., 2011). Spatial standardized weights were calculated using the 'dnearneigh' and 'nb2listw' functions in the '*spdep*' package. Because 18 raccoons were captured more than once, we also investigated the need for including 'animal ID' as a random effect. To do this, we compared the Akaike information criterion (AIC) values between an intercept model with and without animal ID included as a random effect. There was no significant difference in AIC values between the two models (AIC = 319.49 and 317.9, $p = 0.52$) indicating that including animal ID as a random effect was not needed (Table 1).

Secondly, we explored whether ESC-R *E. coli* sequence type (ST) richness varied with urban context and/or presence of a WWTP. ESC-R *E. coli* ST richness was defined as the number of unique STs by raccoon group (e.g., urban vs. suburban raccoons). Since richness measures can vary greatly with sample size, comparisons were made by subsampling the group with the larger sample size 10,000 times to the group with the smaller sample size (as in Mather et al., 2012).

Thirdly, the association between ARG richness in ESC-R *E. coli* and urban context, and presence of a WWTP was assessed by running a GLMM with a Poisson distribution and a log link function using the '*lme4*'package. ARG richness was defined as the number of unique ARG present in each ESC-R *E. coli* isolate. Season was also controlled for in this model. Capture site was not included as a random effect because model residuals were not significantly spatially autocorrelated (Moran's I statistic: $z = -1.79$, $p = 0.96$). In contrast, including animal ID as a random effect was necessary as it significantly improved model fit (AIC = 629.77 for generalized linear model (GLM) and 604.47 for GLMM, $p < 0.0001$) and controlled for overdispersion (before including animal ID as a random effect: $\chi^2 = 232.6$, $p < 0.001$; after: $\chi^2 = 73.84$, $p = 0.99$).

Finally, we explored whether urban context and presence of a WWTP influenced the probability of isolating ESC-R *E. coli* carrying plasmid-associated bla_{CTX-M} or bla_{CMY} from raccoons by running a binomial GLM. Season was also included as a predictor variable. Site was not included as a random effect as model residuals were not significantly spatial autocorrelated (Moran's I statistic: $z = -1.07$, $p = 0.93$). Because of the small sample size for this analysis ($n = 62$, Table 1), animal ID could not be included as a random effect.

For all models, predictor importance and model fit was performed by starting with a global model and subsequently identifying the most parsimonious model using model selection using the 'dredge' function in the '*MuMIn*' package (Barton, 2013). Models were ranked using AIC corrected for small sample size (AICc) (Burnham and Anderson, 2002; Johnson and Omland, 2004). If one or more models were within 2 AICc values of the highest-ranking model, model averaging was used to obtain standardized estimates and confidence intervals. Model fit

Table 1
Description of the four statistical approaches used to explore the importance of urban context and presence of a WWTP at influencing 1) the prevalence and 2) richness of ESC-R *E. coli*, 3) the richness of ARG, and 4) prevalence of ESC-R *E. coli* carrying plasmid-associated ARG.

Outcome variable	n	Predictor variables	Random effect(s)	Analytical approach
Isolation of at least one ESC-R *E. coli* in raccoon feces (yes/no)	230	• Urban context (urban/suburban) • Presence of a WWTP (yes/no) • Season (winter, spring, summer, fall) • Age (adult/juvenile) • Sex (male/female)	• Capture site • Animal ID[a]	Binomial GLMM
Richness of ESC-R *E. coli* sequence types	123	• Urban context • Presence of a WWTP	NA	Bootstrapping and subsampling
Richness of ARG present in ESC-R *E. coli*	123	• Urban context • Presence of a WWTP • Season	• Capture site[a] • Animal ID	Poisson GLMM
Isolation of at least one ESC-R *E. coli* carrying plasmid-associated bla_{CTX-M} or bla_{CMY} (yes/no)	62	• Urban context • Presence of a WWTP • Season	NA	Binomial GLM

n = sample size.
[a] Variable was considered for inclusion as random effect in exploratory analyses but was found to contribute very little to the overall variance ($p > 0.05$) and was thus excluded from analyses listed here.

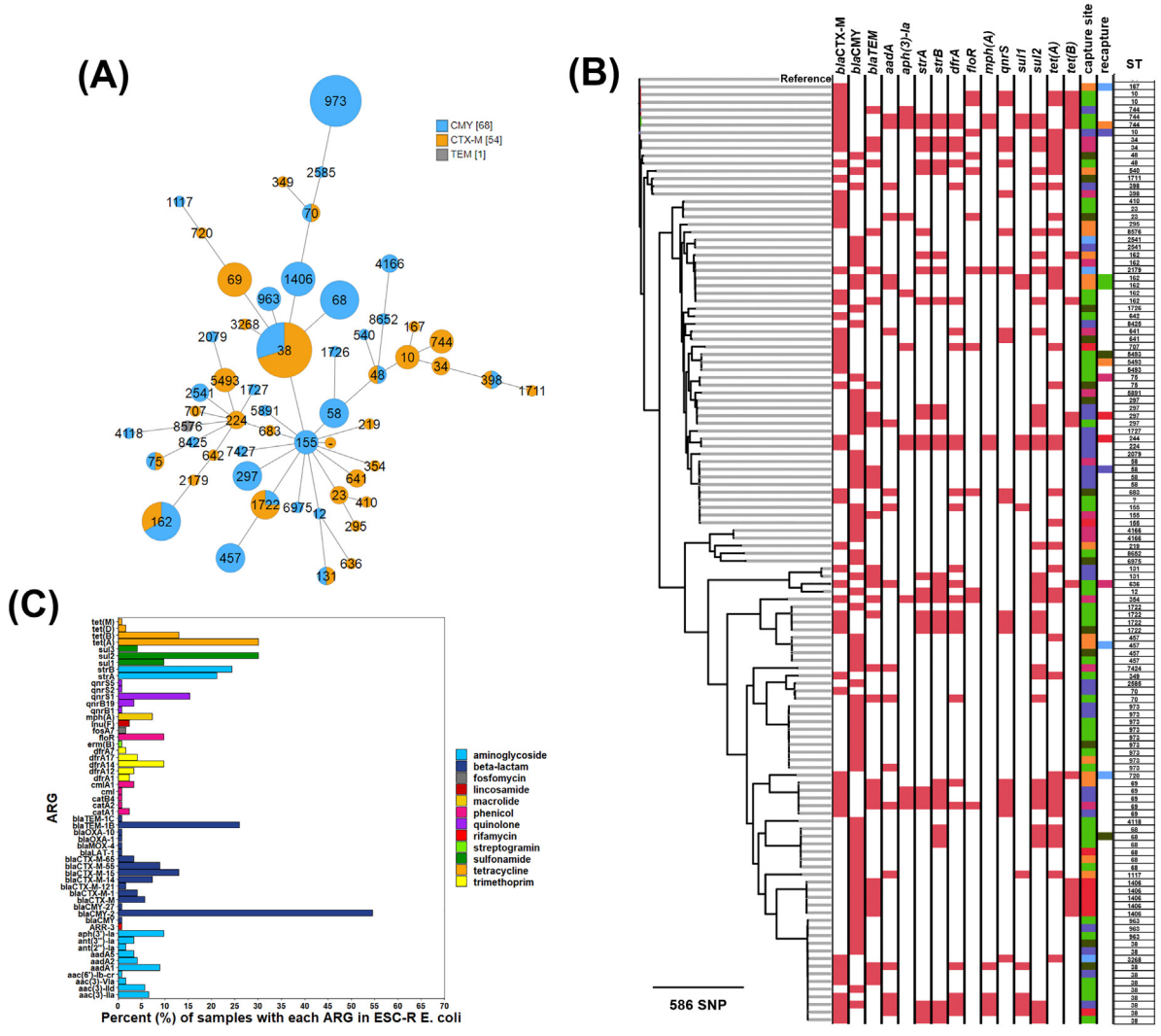

Fig. 2. Characteristics of ESC-R *E. coli* and associated antimicrobial resistance genes (ARG) detected in the feces of raccoons sampled in the metropolitan area of Chicago, USA. (**A**) Minimum spanning tree based on MLST allelic profiles of the 123 ESC-R *E. coli* isolates recovered from raccoons. Each node represents a unique sequence type (ST) and the size of the node represents the number of isolates classified as each ST. The length of lines connecting nodes represent the number of alleles that are found in common between STs. STs are divided into those that carry bla_{CTX-M} (orange), those that carry bla_{CMY} (blue), and those that carry neither bla_{CTX-M} nor bla_{CMY} but rather bla_{TEM} (grey). (**B**) core SNP-based maximum likelihood phylogenetic tree of the 123 ESC-R *E. coli* and heatmap of isolates classified based on ARG families (first 15 bands), capture site (16th band), and raccoon recapture (17th band). The ST of each isolate is also listed on the right side of the heatmap. For ARG families, only those that were detected in more than 10 raccoons are represented on the heatmap. The red color indicates that the ARG family is present. For capture site, each color represents a capture site (purple: Busse, light blue: CT, orange: Damen, red: DRCA, green: Edgebrook, black: MMWF, dark blue: PC). For raccoon recapture, each color represents an individual raccoon). The reference is the laboratory strain *E. coli* K-12 MG1655. (**C**) Percent of all samples for which ARG were detected in ESC-R *E. coli* of raccoons. Each color represents an antibiotic class.

was assessed by calculating the coefficient of determination (r^2) (Nakagawa and Schielzeth, 2013).

3. Results

3.1. Prevalence, richness, and characteristics of ESC-R E. coli and associated ARG isolated from raccoons

A total of 211 raccoons was sampled between February and November 2018, 17 of which were captured twice and one three times. At least one ESC-R *E. coli* colony was present in the fecal samples of 120 raccoons (sample prevalence = 56.9%). Of the 18 raccoons captured more than once, seven had at least one ESC-R *E. coli* present in feces on both capture events (Supplementary Table S1). Although ESC-R *E. coli* were recorded for 128 raccoon fecal samples (including recaptures), WGS was performed on 123 isolates only due to isolation issues and DNA concentration restrictions (isolate NCBI accession numbers can be found in Supplementary Table S2). MLST analysis revealed that the 123 isolated ESC-R *E. coli* belonged to 55 known STs and one unknown ST (Fig. 2A). The unknown ST closely resembled ST155 (with variation in the *gyrB* allele only). The most common STs included ST38, ST68, ST69, ST162, ST973, and ST1406 (Fig. 2A). The core-SNP based maximum likelihood phylogenetic tree indicated that when raccoons were sampled more than once, isolates did not cluster by raccoon ID (Fig. 2B). While isolates also did not cluster by capture site in general (Fig. 2B), over 50% of isolates obtained from raccoons sampled at DRCA ($n = 9$) were of the same sequence type (i.e., ST1406; Fig. 2A) and differed by 0 SNPs (Fig. 2B).

Fourteen unique beta-lactam resistance genes (which confer ESC-R) were detected in the isolates, most of which belonged to the $bla_{CTX\text{-}M}$ (prevalence = 43.9%), bla_{CMY} (prevalence = 56.1%), and bla_{TEM} (prevalence = 26.8%) ARG families (Fig. 2C). $bla_{CMY\text{-}2}$ and $bla_{TEM\text{-}1B}$ were the most prevalent beta-lactam resistance genes, followed by $bla_{CTX\text{-}M\text{-}15}$, $bla_{CTX\text{-}M\text{-}55}$, and $bla_{CTX\text{-}M\text{-}14}$ (Fig. 2C). Beta-lactam resistance genes of the $bla_{CTX\text{-}M}$ and bla_{CMY} families were distributed throughout the ESC-R *E. coli* population identified (Fig. 2B). However, in general, $bla_{CTX\text{-}M}$ and bla_{CMY} tended to cluster by ST (Fig. 2A).

The most prevalent non-beta-lactam resistance genes were from the aminoglycoside (e.g., 21.1% for *aph*(3″)-*Ib* and 24.4% for *aph*(6)-*Id*), tetracycline (e.g., 30.1% for *tet*(A) and 13% for *tet*(B)), and sulfonamide classes (e.g., 9.8% for *sul1* and 30.1% for *sul2*), followed by the quinolone class (e.g., 15.4% for *qnrS1*; Fig. 2C). The median number of ARG in an ESC-R *E. coli* isolate was 4, with over half of isolates having 2-4 ARG (53.7%).

When investigating whether contigs with beta-lactam resistance genes were more likely to be plasmid or chromosomally-associated, we were unable to classify 50% of contigs (77 out of 155 contigs in 52 out of 123 isolates). Mlplasmids predicted chromosomal association when MOB-suite predicted plasmid 11% of the time, and vice versa 39% of the time. In instances when Mlpasmids and MOB-suite predictions were the same, certain beta-lactam resistance genes were more likely to be found on chromosomal- or plasmid-associated contigs. In particular, $bla_{CMY\text{-}2}$, $bla_{TEM\text{-}1}$, $bla_{OXA\text{-}1}$, and $bla_{MOX\text{-}4}$ were typically found on contigs associated with plasmids, whereas $bla_{CTX\text{-}M\text{-}14}$, $bla_{CTX\text{-}M\text{-}15}$, and $bla_{CMY\text{-}27}$ were generally found on chromosomal contigs (Fig. 3).

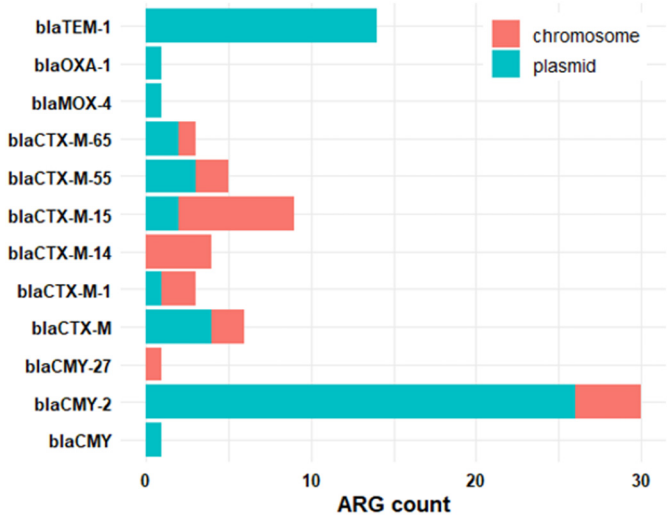

Fig. 3. Predictive association of contigs carrying beta-lactam resistance genes with chromosome or plasmid location. Predictions were performed using Mlplasmids and MOB-suite. Only contigs that were classified as plasmid- or chromosomally-associated by both Mlplasmids and MOB-suite are presented (78 out of 155 contigs. In terms of number of isolates: 71 isolates out of 123 isolates).

3.2. Importance of anthropogenic sources at influencing the ESC-R E. coli profile of raccoons

3.2.1. Urban context and season were important predictors for isolating ESC-R E. coli from raccoons

Season and urban context appeared in all top-ranking models (ΔAICc < 2, Supplementary Table S3), and were therefore the most important predictors for isolating ESC-R *E. coli* from raccoons. Presence of a WWTP and raccoon sex and age each appeared in one of the top four ranking models (Supplementary Table S3). In general, the top GLMMs explained 39% of the variance for the fixed effects and 55% with site included as a random effect (Supplementary Table S3). Model averaging revealed that there was a significantly higher probability of isolating ESC-R *E. coli* from raccoons if they were sampled in the summer and spring than in the winter and fall (Table 2; Fig. 4A) and if raccoons were sampled at urban rather than suburban sites (Table 2; Fig. 4B). Raccoon age, sex, and the presence of a WWTP did not significantly influence the likelihood of isolating ESC-R *E. coli* from raccoons (Table 2).

3.2.2. Presence of a WWTP at sampling sites was an important predictor of ESC-R E. coli ST richness, but not ARG richness

Raccoons sampled at sites that were downstream from a WWTP had a greater richness of ESC-R *E. coli* STs than raccoons sampled at sites that were not downstream from a WWTP (Table 3). This finding remained consistent after sub-sampling the larger group (i.e., WWTP present) to the smaller group (i.e., WWTP not present; Table 3). In contrast, ST richness was similar between raccoons sampled at urban and suburban sites (Table 3), even after sub-sampling the larger group (i.e., urban) to the smaller group (i.e., suburban; Table 3).

In terms of ARG richness among isolates, model selection revealed that the top two ranking models were the intercept model and a model that only included presence of a WWTP (Supplementary

Table 2
Model averaging results from binomial generalized linear mixed models of the probability of isolating at least one ESC-R *E. coli* from raccoons ($n = 230$). Predictors were obtained from the top ranking models (ΔAICc < 2; Supplementary Table S3). Significant terms are depicted in bold. The reference level for "Season" is "fall", for "Urban context" and "WWTP present" is "no", for "Age" is "juvenile", and for "Sex" is "female".

Predictor	Mean OR	95% CI
Season (spring)	**7.68**	**(2.59–22.72)**
Season (summer)	**5.2**	**(2.09–12.94)**
Season (winter)	0.46	(0.19–1.11)
Urban context (urban)	**10.48**	**(1.45–75.93)**
WWTP present (yes)	1.64	(0.3–9.16)
Age (juvenile)	1.34	(0.63–2.85)
Sex (male)	1.18	(0.59–2.35)

Mean OR represents the mean odds ratio and 95% CI the 95% confidence intervals for each mean OR.

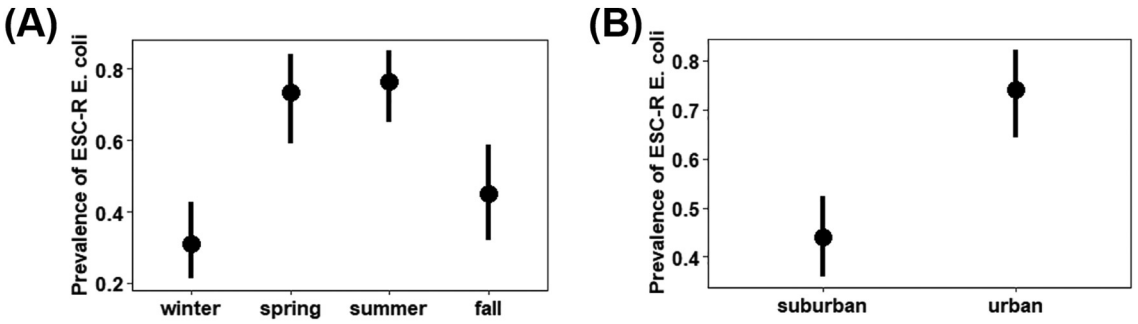

Fig. 4. Raw prevalence of ESC-R *E. coli* in raccoons based on (**A**) season; and (**B**) urban context. Whiskers are 95% confidence intervals.

Table S4). For the intercept model, animal ID explained 44% of the overall variance (Supplementary Table S4). In the WWTP model, WWTP explained only 0.3% of the overall variance and thus was not a significant predictor of ARG richness among isolates ($p > 0.05$).

3.2.3. Presence of a WWTP influenced the probability of isolating ESC-R E. coli carrying plasmid-associated $bla_{CTX\text{-}M}$ or bla_{CMY} from raccoons

Presence of a WWTP at sampling sites appeared in the two top ranking models (ΔAICc < 2, Supplementary Table S5), and was therefore the most important predictor for isolating ESC-R *E. coli* carrying $bla_{CTX\text{-}M}$ or bla_{CMY} from raccoons. Season appeared in one of the two top models, and urban context appeared in none (Supplementary Table S5). In general, the top GLMs explained a maximum of 22% of the overall variance (Supplementary Table S5). Model averaging revealed that ESC-R *E. coli* carrying plasmid-associated $bla_{CTX\text{-}M}$ or bla_{CMY} were more likely to be isolated from raccoons if raccoons were sampled at sites with a WWTP than without (Table 4; Fig. 5). Season had no significant effect on the probability of isolating ESC-R *E. coli* carrying plasmid-associated $bla_{CTX\text{-}M}$ or bla_{CMY} from raccoons (Table 4).

4. Discussion

Our understanding of the importance of anthropogenic sources at shaping the AMR profile of wildlife is in its infancy. Here we show that urban context is an important predictor for isolating ESC-R *E. coli* from raccoons, with ESC-R *E. coli* more likely to be identified in raccoons from urban sites than from suburban sites. While the presence of a WWTP at sampling sites did not influence the probability of isolating ESC-R *E. coli*, it was an important predictor of both ESC-R *E. coli* ST richness and the probability of isolating ESC-R *E. coli* carrying plasmid-associated $bla_{CTX\text{-}M}$ or bla_{CMY}. Season also had an impact on the likelihood of isolating ESC-R *E. coli* from raccoons, with higher probability in the spring and summer than the fall and winter. Our findings show that anthropogenic factors are important at influencing the AMR profile of wildlife.

Detecting ESC-R *E. coli* in the feces of raccoons is not surprising as ESC-R *E. coli* have been detected in raccoons and other wildlife species previously (Guenther et al., 2011). However, it is noteworthy that over half of raccoons had ESC-R *E. coli* and that many were classified as STs of clinical relevance (i.e., commonly identified among infections in humans). In fact, the detection of typically human-associated sequence types such as ST131, ST410, ST10, ST69, and ST23, along with ARG such as $bla_{CTX\text{-}M\text{-}15}$ and $bla_{CTX\text{-}M\text{-}14}$, in raccoon feces reinforces the concern that clinically relevant isolates are present in the environment (Woodford et al., 2011; Wang et al., 2017) and ARB and ARG detected in wildlife are of anthropogenic origin (Vittecoq et al., 2016; Wellington et al., 2013). Further, our classifications of beta-lactam ARG as either plasmid- or chromosomally-associated tended to be similar to those described in human and domestic animal (Partridge, 2015; Hamamoto et al., 2016, 2020; Zurfluh et al., 2015) and wildlife isolates (Guenther et al., 2010, 2017; Tausova et al., 2012; Poirel et al., 2012). Taken together, these findings suggest that the AMR situation unfolding in wildlife might mirror that observed in human and domestic animal communities (Guenther et al., 2011; Wang et al., 2017). This is important because it suggests that ARB that circulate in human and domestic animal populations also have the potential to circulate in wildlife populations.

Our result that ESC-R *E. coli* were more likely to be isolated from raccoons sampled in urban sites than suburban sites is in line with general trends that wildlife that reside close to human-dominated areas are more likely to have a higher prevalence of anthropogenically-derived ARB than wildlife sampled at further distances (Vittecoq et al., 2016; Skurnik et al., 2006; Furness et al., 2017; Dolejska et al., 2007). This finding is important because it lends support for the hypothesis that certain wildlife populations and/or species can be used as sentinels for understanding the dissemination of AMR in the environment (Vittecoq et al., 2016). What specific factors present in urban areas and absent in suburban areas are driving these differences is unclear and could not be determined in this study. However, several factors are likely to be involved and are probably additive, such as a higher concentration of heavy metals in urban rivers or greater contact with human waste in urban areas (Almakki et al., 2019; Baker-Austin et al., 2006; Wright and Mason, 1999). Additionally, urban and suburban raccoons can differ in their feeding habits and population densities, which could be contributing to the urban context effect. For example, raccoons are generalist mesocarnivores and will exploit anthropogenic food resources when available (Prange et al., 2003; Bateman and Fleming, 2012). It is possible

Table 3
Richness of ESC-R *E. coli* sequence types (STs) by urban context and presence of a wastewater treatment plant (WWTP) at sampling sites. Bootstrap 95% confidence intervals were not presented for predictor levels with the lowest sample sizes.

Predictor	Level	*n*	ST richness	Bootstrap 95% CI
Urban context	Suburban	57	37	–
	Urban	66	34	(34.01–42.30)
Presence of WWTP	No	39	24	–
	Yes	84	44	(30.59–39.24)

Table 4
Model averaging results from binomial generalized linear models for the probability of isolating at least one ESC-R *E. coli* carrying plasmid-associated $bla_{CTX\text{-}M}$ or bla_{CMY} from raccoons ($n = 62$). Predictors were obtained from the top ranking models (ΔAICc <2; Supplementary Table S5). Significant terms are depicted in bold. Note that the variable 'urban context' was dropped during model selection. The reference level for "Season" is "fall" and for "WWTP present" is "no".

Predictor	Mean OR	95% CI
Season (spring)	0.27	(0.03–2.82)
Season (summer)	0.55	(0.05–6.4)
Season (winter)	0.12	(0.01–1.46)
WWTP present (yes)	**4.1**	(**1.21–13.94**)

Mean OR represents the mean odds ratio and 95% CI the 95% confidence intervals.

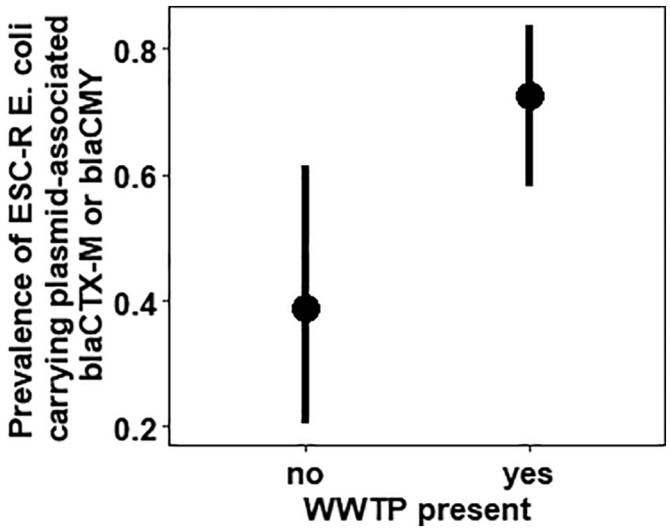

Fig. 5. Raw prevalence of ESC-R *E. coli* carrying plasmid-associated bla_{CTX-M} or bla_{CMY} ARG based on the presence of WWTP at sampling sites. Whiskers are 95% confidence intervals.

that urban raccoons had greater access to anthropogenic food sources than suburban raccoons, which may have resulted in urban raccoons having a higher exposure risk to ARB than suburban raccoons. Similarly, raccoon population densities tend to increase along the rural-urban gradient (Šálek et al., 2015; Slate et al., 2020), which may mean that urban raccoons have higher contact rates than suburban raccoons and thus more potential for ARB transmission among raccoons. Combining tools from landscape ecology and wildlife movement ecology could help tease apart the importance of various environmental and host factors (Singer et al., 2006; Arnold et al., 2016) and would be an important next step to take.

It was surprising that no association was found between the probability of isolating ESC-R *E. coli* and presence of a WWTP at sampling sites, given WWTPs are one primary pathway by which ARB and ARG are disseminated to the environment (Wellington et al., 2013). Further, wildlife that use or reside close to WWTPs are expected to have a higher risk of exposure to ARB and ARG (Arnold et al., 2016). In fact, rivers that are downstream of a WWTP are posited to be important AMR exposure pathways for wildlife (Nelson et al., 2008; Radhouani et al., 2014). A lack of association detected in this study could be because sites differed not only in the presence of a WWTP, but also in urban context. Since other wildlife research has detected an association with WWTPs (e.g., Dolejska et al., 2007; Swift et al., 2019), we suspect that WWTPs likely played a role in this study, but that the urban context effect masked the association with WWTP. However, the presence of a WWTP at sampling sites was a significant predictor of isolating ESC-R *E. coli* carrying plasmid-associated bla_{CTX-M} or bla_{CMY}. As well as facilitating the dissemination of ARB and ARG to the environment (Wellington et al., 2013), WWTPs can act as hotspots for the horizontal transfer of ARG among bacteria (Berendonk et al., 2015; Rizzo et al., 2013). Thus, it is possible that the importance of WWTPs at facilitating the dissemination of anthropogenically-derived ARB and ARG to the environment was only apparent when focusing on those ARB that carry plasmid-associated ARG. Our results therefore suggest that the association with WWTPs might be more complex than a simple presence-absence association, and suggests that the risk for AMR to spread in wildlife bacterial communities is higher when wildlife reside downstream from a WWTP than when they do not.

The finding that raccoons sampled at sites with a WWTP had a greater richness of ESC-R *E. coli* STs than raccoons sampled at sites without a WWTP is in line with previous environmental and wildlife AMR research (e.g., Akiyama and Savin, 2010; Furness et al., 2017), and further supports that raccoons at WWTP sites are more likely to display an anthropogenic source profile of AMR. While several factors could be influencing this finding, it highlights a need to better understand the association between AMR in wildlife and their exposure to waters derived from WWTPs. Freshwater studies have shown that the prevalence, richness, and abundance of ARB and ARG in river systems tend to be higher downstream than upstream of WWTPs (e.g., Marti et al., 2013; Bueno et al., 2020). Exploring whether similar associations hold true for wildlife sampled up and downstream from a WWTP would help tease apart the role of WWTPs in shaping the AMR profile of wildlife.

While not the main focus of this study, season was an important predictor for isolating ESC-R *E. coli* from raccoons. The risk of isolating ESC-R *E. coli* was higher for raccoons sampled during the spring and summer than for raccoons sampled during the fall and winter. While this finding should be considered with caution because raccoons were only sampled for a 10-month period, a seasonal effect is likely to exist as it has been observed in other wildlife AMR studies (e.g., Williams et al., 2011; Miller et al., 2020) and in aquatic environmental research (e.g., Keen et al., 2018). Factors that could be contributing to this potential seasonal effect include ice melt or rainfall increasing the risk of heavy metals and ARB entering river systems in the spring, and/or bacterial growth and proliferation in the summer. Alternatively, the potential seasonal effect could be associated with differences in raccoon diet, which is thought to be influential for AMR in other wildlife systems (e.g., Jobbins and Alexander, 2015). Seasonal differences could also have been caused by differences in raccoon demographic or social dynamics (e.g., Prange et al., 2011). More work is needed to ascertain any seasonal effect and we recommend that differences in animal behavior across seasons (e.g., Hirsch et al., 2016) be explored alongside the AMR-season relationship. Further, exploring behavioral differences among individuals may help better understand the importance of individual ID in explaining the variance of certain AMR components, such as ARG richness within ARB.

A potential limitation of our work is that only two anthropogenic sources of AMR were explored, but there are others that could be important at influencing the AMR profile of raccoons (e.g., urban runoff; Almakki et al., 2019). Indeed, most of our statistical models explained <40% of the overall variance. It is possible that a large portion of the unexplained variance was attributed to other anthropogenic factors not accounted for in this study. Alternatively, it could be because we did not explore the interface with domestic animals. In urban settings, pets can be important reservoirs and sources of AMR for humans (Guardabassi et al., 2004), and may also be reservoirs for wildlife. Accounting for all potential sources of AMR is a common challenge for environmental AMR research (Bueno et al., 2018), and we advocate that future work develop approaches to fill this methodological gap. Another important limitation worth attention is the lack of comparison with the anthropogenic source sites themselves. The assumption in this study was that the AMR profile of raccoons was associated with urban context and the presence of a WWTP. However, no soil or water samples were collected from sites. A final limitation worth mentioning is the chromosomal versus plasmid classification. Predictions made should be taken with a degree of caution because the classification programs Mlpasmids and MOB-suite only agreed 50% of the time. Nevertheless, the fact that several of the predicted plasmid- and chromosomally-associations have been reported in other research, suggests that when Mlpasmids and MOB-suite agreed, the plasmid/chromosomal classification was likely to be robust.

5. Conclusions

Associations detected with urban context and the presence of a WWTP lends support to the hypothesis that some AMR in wildlife is derived from anthropogenic sources (Vittecoq et al., 2016). However, differences in the importance of each factor at different biological levels of resistance (i.e., prevalence of ARB for urban context versus prevalence of

ARB with plasmid-associated ARGs for WWTP) highlights the complex ways in which anthropogenic sources may influence the AMR profile of wildlife. Importantly, several wildlife AMR studies stress that isolation of ARB and detection of ARG differs by wildlife species, and thus different species may play different roles in the dissemination of AMR in the environment (Williams et al., 2011; Jobbins and Alexander, 2015; Vittecoq et al., 2016; Torres et al., 2020). Here we show that there can even be great variation among individuals of the same species. Whether raccoons act as reservoirs or sentinels of AMR remains unknown, but given their association with both terrestrial and aquatic systems (Gehrt and Fritzell, 1998; Henner et al., 2004), raccoons may act as important conduits for the introduction of ARB disseminated via rivers into terrestrial systems. More generally, our results lend support for the hypothesis that wildlife exposed to anthropogenic sources of AMR (in our case urban sites and sites with a WWTP) are more likely to have an anthropogenic source profile of AMR. This may indicate that wildlife at these sites act as secondary reservoirs of AMR. However, studies exploring how control of AMR at anthropogenic sources influences the AMR profile of local wildlife would be needed to confirm this speculation. While several investigations have shown that wildlife sampled at sites with anthropogenic sources of AMR tend to have a higher prevalence of ARB, few have explored differences based on plasmid vs. chromosomal association of ARGs. Comparing the chromosomal vs. plasmid association of ARG using Mlpasmids and MOB-suite was insightful, and we recommend this approach be used in future wildlife and environmental AMR research.

Data accessibility

Raw reads were deposited in the *National Center for Biotechnology Information*'s Short Read Archive (BioProject number PRJNA662117). Isolate accession numbers can be found in Supplementary Table S2.

Author contributions

Conceptualization: KELW-T, EAM, CLA, JBB, TJJ, and MEC.
Data curation: KELW-T and SCM.
Formal analysis: KELW-T and EAM.
Funding acquisition: KELW-T, JBB, SDG, TJJ, and MEC.
Investigation: KELW-T, EAM, and SCM.
Methodology: KELW-T, EAM, SDG, RSS, TJJ, and MEC.
Project administration: KELW-T, SCM, TJJ, and MEC.
Supervision: EAM, SDG, RSS, TJJ, and MEC.
Validation: KELW-T.
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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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RESEARCH ARTICLE

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Cytoarchitectural characteristics associated with cognitive flexibility in raccoons

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Abstract

With rates of psychiatric illnesses such as depression continuing to rise, additional preclinical models are needed to facilitate translational neuroscience research. In the current study, the raccoon (*Procyon lotor*) was investigated due to its similarities with primate brains, including comparable proportional neuronal densities, cortical magnification of the forepaw area, and cortical gyrification. Specifically, we report on the cytoarchitectural characteristics of raccoons profiled as high, intermediate, or low solvers in a multiaccess problem-solving task. Isotropic fractionation indicated that high-solvers had significantly more cells in the hippocampus (HC) than the other solving groups; further, a nonsignificant trend suggested that this increase in cell profile density was due to increased nonneuronal (e.g., glial) cells. Group differences were not observed in the cellular density of the somatosensory cortex. Thionin-based staining confirmed the presence of von Economo neurons (VENs) in the frontoinsular cortex, although no impact of solving ability on VEN cell profile density levels was observed. Elongated fusiform cells were quantified in the HC dentate gyrus where high-solvers were observed to have higher levels of this cell type than the other solving groups. In sum, the current findings suggest that varying cytoarchitectural phenotypes contribute to cognitive flexibility. Additional research is necessary to determine the translational value of cytoarchitectural distribution patterns on adaptive behavioral outcomes associated with cognitive performance and mental health.

KEYWORDS
cognitive flexibility, cytoarchitecture, raccoon, von Economo neurons

1 | INTRODUCTION

Given the current challenges associated with the identification of appropriate preclinical models for psychiatric illnesses (Denayer et al., 2014), the raccoon (*Procyon lotor*), a species virtually ubiquitous in North America, offers potential as an informative translational model for primate brains (Hamir, 2011). Specifically, primates and raccoons share similar cortical neuron density profiles, a high

degree of cortical gyrification, and corresponding cortical magnification of forepaw/hand areas (Jardim-Messeder et al., 2017; Krubitzer, 2007; Pubols et al., 1965). Anecdotally known for their intelligence and advanced problem-solving abilities (Zeveloff, 2002), raccoons appear to also share behavioral and cognitive characteristics such as cognitive flexibility with humans and other primates. Their behavioral adaptability is evident by their infiltration of a wide variety of habitats including areas with vastly different temperature ranges, levels of urbanization, vegetation, and elevation (Gienapp et al., 2008).

Although the natural history, anatomy, and roles of disease transmission of the Northern raccoon have been thoroughly investigated (Hirsch et al., 2013; Iwaniuk & Whishaw, 1999; MacClintock, 2002; Prange et al., 2004), few studies have systematically explored cognitive and behavioral strategies in these animals. Early 20th century studies patterned after experimental psychologist Edward Thorndike's puzzle box approach suggested that raccoons were curious and innovative, with their putative intelligence level on a "higher-order" than cats and dogs and more in line with nonhuman primates (Cole, 1907; Davis, 1907). Subsequent research provided evidence that raccoons retained information in a delayed reaction task more efficiently than dogs and rats (Hunter, 1913; Hunter, 1915). After this time, however, rats ascended to the research scene in psychology laboratories, as well as other biomedical laboratories, diminishing interest in the raccoon as a viable research model for cognitive functions (Lambert et al., 2019; Pettit, 2010). After nearly a century-long hiatus from research focused on raccoon cognition and neurobiological functions, more recent studies have corroborated earlier conclusions about their unique problem-solving abilities. Specifically, when presented with an Aesop's Fable type of task designed to assess an understanding of water displacement, raccoons used innovative approaches to solving the task such as using their bodies to upend the apparatus as opposed to the more predictable strategy of adjusting the water level with strategic pebble placement (Stanton et al., 2020). When exposed to multi-access puzzle boxes similar to the initial laboratory problem-solving assessments of raccoons, both success and innovation were observed, as well as pronounced individual differences (Daniels et al., 2019). Cognitive flexibility of raccoons was recently assessed in a reversal-learning task and the animals exhibited rapid associative learning by successfully completing reversals with observed trends in improvement over time (Stanton et al., 2020). Interestingly, in a task requiring the use of a stick to push desired food through a pipe, wild raccoons exhibited a dependence on olfaction and somatosensory exploration instead of manipulating the stick to obtain the food reward (Morton, 2020).

A potential underlying neural mechanism of the observed cognitive efficacy of raccoons in natural habitats is the presence and distribution of von Economo neurons (VENs). Although previously described by neuroanatomists including Santiago Ramón y Cajal, Constantin von Economo provided a thorough description of these rod and corkscrew-shaped neurons and their typical locations in anterior cingulate and frontoinsular (FI) cortical areas (von Economo, 1926; as described in Allman et al., 2010). Subsequent computer-assisted and immunohistological analyses confirmed these distinctive cell types in Layer Vb of the human anterior cingulate cortex (Nimchinsky et al., 1995). Initial comparative analyses suggested that the cells were unique to humans and great apes, with higher VEN densities in human primates (Allman et al., 2011; Nimchinsky et al., 1999). Subsequent research, however, has confirmed the presence of VENs in other mammalian species including cows, sheep, deer, horses, pigs, Nile hippopotami, elephants, macaques, and whales (Butti et al., 2013; Butti et al., 2014; Butti et al., 2015; Evrard et al., 2012; Hakeem et al., 2009; Hof & Van Der Gucht, 2007; Raghanti et al., 2015; Raghanti et al., 2019), as well as in avian species (i.e., parrots (Shubha & Suchi, 2017)). It has been proposed that the VENs arose independently in cetaceans, elephants, and hominids and may be necessary to overcome the geometric challenges associated with rapid neural processing in large brains, especially considering that VENs are not reliably present in the elephant's closest living relative that is comparatively much smaller, the rock hyrax (Hakeem et al., 2009; Raghanti et al., 2015).

Characterized as fast projecting cells, VENs in the insular area are speculated to be associated with interoception, emotional processing, and task-directed responses (Seeley et al., 2006; Ibegbu et al., 2015). Hominoid VENs exhibit immunoreactivity to proteins such as interleukin 4 receptor, neuromedin B, and activating-transcription factor 3 (Sherwood et al., 2006; Stimpson et al., 2011). In unpublished findings in our laboratory, a stereological investigation of three wild adult raccoon brains confirmed the presence of VENs in the FI (see Figure 1). The percentage of VENs in raccoons was observed to be approximately 6% of the neuronal cellular population, compared to previously reported 11% in the human anterior insular cortex and 8 and 5% in the cow and horse, respectively (Raghanti et al., 2015). In this initial unpublished analysis, VENs were not observed in the raccoon ACC as they have been observed in other species, a finding that deserves further investigation.

Morphologically, VENs are defined by their thin, elongated cell body and long dendrites projecting from apical and basal neural processes (Correa-Júnior et al., 2020). They are often found in proximity to large neurons with a bifurcated apical dendrite known as fork cells (Dijkstra et al., 2018; Raghanti et al., 2015). Once thought to be specific to large-brained animals exhibiting complex social-cognitive behavior (e.g., hominoid primates and cetaceans), as previously mentioned, VENs have been observed in perissodactyls, artiodactyls, afrotherians, and other primates (Raghanti et al., 2015). Although the existence of VENs in such diverse species supports the suggestion that VENs are the result of convergent evolutionary processes facilitating varying adaptive specializations, it is also a possibility that VENs are associated with the mechanical challenges associated with larger, gyrencephalic brains. Consequently, more research is necessary to elucidate evolutionary adaptations associated with these neurons.

Another likely neuroanatomical determinant of problem-solving abilities of raccoons is the neurophysiological processing of the hippocampus (HC), a brain structure known for its role in cognitive and emotional processing (Bird & Burgess, 2008; Hartley et al., 2007;

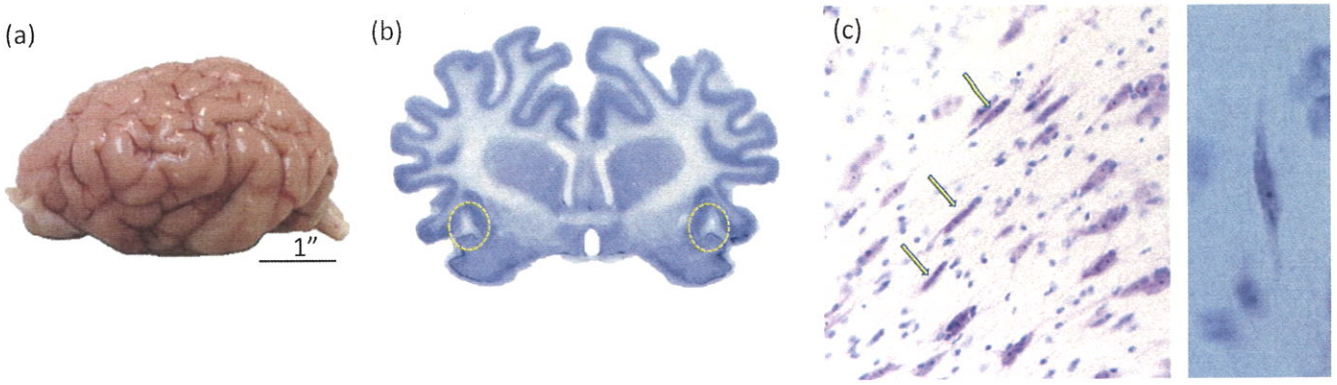

FIGURE 1 Representative images of neuroanatomical characteristics in the raccoon. Cortical gyrification is observed in the intact raccoon brain (a) and in an anterior coronal thionin-stained section, the frontoinsula region (b) is identified (dashed circles). A few representative von Economo neurons are identified in the frontoinsular cortical region (c) at 5× (left) 40× (right) magnification [Color figure can be viewed at wileyonlinelibrary.com]

Maguire et al., 2003; Tyng et al., 2017). Activation of the HC in humans, especially in the right hemisphere, has been implicated in insight learning related to unique problem-solving tasks (Luo & Niki, 2003). Hippocampal volume has been associated with enhanced spatial abilities related to foraging in rodents, caching and spatial memory in birds, and navigational skills in London taxi drivers (Krebs et al., 1989; Pravosudov & Clayton, 2002; Maguire et al., 2006). Also critical for learning processes, the HC serves as a hub for neuroplasticity with neural circuits exhibiting the ability to modify functional capabilities quite rapidly for long-term periods (Lynch et al., 1978). The dentate gyrus, embedded in the HC, is recognized as a neurogenic zone, producing new neurons throughout an animal's life (Kempermann and Gage, 1999; Kempermann, 2002) that contribute to the malleability of the brain's synaptic wiring and functional adaptations (Citri et al., 2008). Within the dentate gyrus, the hilar area has been associated with the identification of relevant changes in external stimuli (i.e., pattern separation), an important element of effective cognitive strategies (Myers & Scharfman, 2009; Yassa & Stark, 2011).

The distributions of different cellular phenotypes within the cortex and HC (e.g., percentages of various glial and neuronal cells) may also be important contributors to cognitive abilities (Dallérac & Rouach, 2016). Glial to neuronal ratios, for example, have been explored across species with interesting results suggesting that increased glial cell densities do not appear to be dependent on increased metabolic needs for neurons (Herculano-Houzel, 2011; Herculano-Houzel, 2014), opening the possibility for alternative interpretations such as an impact on synaptic processes or cognitive abilities (Vasile et al., 2017). In primate investigations, humans have been shown to have higher glial to neuron ratios in the dorsolateral prefrontal cortex compared to 18 species of anthropoids (Sherwood et al., 2006). Focusing on astrocytes' influence on neuronal functions, these specific glial cells are known to fully participate in neural processes and have been implicated in facilitating the integration of neural networks (Poskanzer & Yuste, 2016; Santello et al., 2019). Thus, the distribution of specific cellular phenotypes may translate to neural efficiency and cognitive variations.

In the current study, we explored potential neuroanatomical determinants of problem-solving ability in wild-caught, captive raccoons assessed behaviorally in a previously published study (see Daniels et al., 2019). After the animals were profiled into high, intermediate, and low problem-solving ability categories, the number of neural cells (neurons and nonneurons) in the HC and somatosensory cortex (SSCX; a cortical area associated with forepaw use in the raccoon; Krubitzer, 2007) was determined using isotropic fractionation (IF; Herculano-Houzel & Lent, 2005). Further, to determine cellular phenotypes in the HC and FI cortex, thionin-based histological assessments were conducted to determine the presence of VENs and non-VENs (i.e., glial cells and other neurons) in the FI cortex for the three groups of animals. It was hypothesized that high-performing raccoons would have altered distributions of hippocampal cells, as well as a higher number of VENs in the FI cortex, than the other groups. Although the SSCX was hypothesized to play an integral role in interacting with the task, this area was not hypothesized to be directly implicated in cognitive flexibility; consequently, no cellular differences were hypothesized across the problem-solving groups.

2 | MATERIALS AND METHODS

2.1 | Animals

Eighteen wild raccoons (*P. lotor*) were live-trapped in Larimer County, CO, near the National Wildlife Research Center (NWRC). The animals were maintained in captivity for up to 2 years prior to behavioral assessments. One female gave birth while housed at the NWRC. Two of the offspring were included in the behavioral study, bringing the subject total to 20 raccoons (male $N = 8$; female $N = 12$; estimated average age = 2.9 years, estimated age range 2–5 years). All raccoons were individually housed in an outdoor enclosure (3 × 3 × 2.5 m).

The study was approved by the Animal Review Board at the USDA NWRC (QA-2492).

As described in a previous publication (Daniels et al., 2019), these animals were assessed for cognitive flexibility using a multiaccess problem-solving box prior to the histological analysis in the current study to explore neural contributions to problem-solving abilities in this species Briefly, each raccoon was presented with a puzzle box apparatus containing a food reward (see Figure 2 for graphic description of this task). The puzzle box could be opened via three solution types: a window, a side latch, and a door. To move forward in the trials, the animal had to successfully open a solution type and retrieve the reward three times. Raccoons that failed to do so were recorded as *nonsolvers* ($n = 6$) and further testing ceased. Raccoons that successfully solved the same solution three times were tested on subsequent nights, with the initial preferred access method locked. The puzzle box was presented again but with only two remaining solution types unlocked and available. The same procedure was followed where animals needed to solve one of the remaining solution types at least three times to move on to the next night of testing. On the third and final night of testing, successful individuals were presented with the puzzle box with the two previously opened solution types locked and one remaining solution type unlocked and available. Those raccoons that solved one or two solution types were labeled *intermediate solvers* ($n = 5$). In this study, there were no raccoons that only solved one solution type, so all of the intermediate solvers opened two solution types. Finally, the raccoons that successfully solved all three solution types three times each were labeled *high-solvers* ($n = 7$), as they were representative of the most cognitively flexible animals. Due to tissue loss in the preparation and processing of the brains, two were not suitable for further processing, leading to lower numbers than used for the behavioral assessment. As previously mentioned, the full behavioral results are reported as a separate manuscript (see Daniels et al., 2019). Following the completion of additional in vivo studies approved at the NWRC and at the time of tissue sampling for those studies, the animals were humanely euthanized by inducing anesthesia with 5% isoflurane at 5 L/min, followed by an intermuscular injection

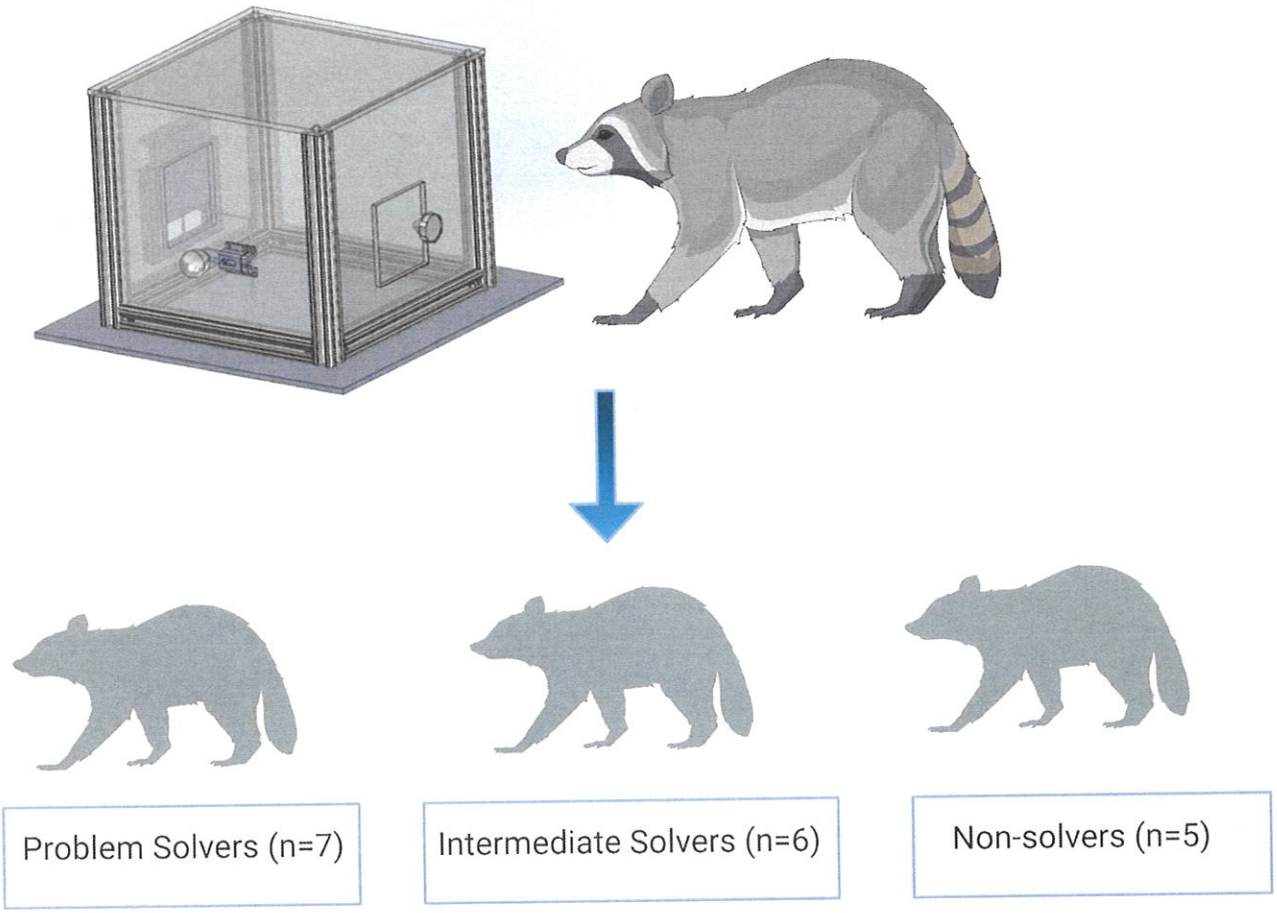

FIGURE 2 Representation of the puzzle box behavioral task to assess cognitive flexibility. Raccoons were introduced to the puzzle box apparatus containing a food reward inside, obscured by three different locking mechanisms, each one outfitted on a different access window. After raccoons demonstrated they could obtain the reward via one locking mechanism on three independent trials, the initial locking option was locked to force the animal to solve a second locking mechanism, and subsequently, a third locking mechanism. *Nonsolvers* included animals that did not successfully access the reward through any of the available windows on first exposure, and were not allowed to advance in the task. *Intermediates* included animals that solved up to two of the three access mechanisms correctly, and *solvers* (also referred to as high-solvers) included animals that successfully opened all three locking mechanisms, three times each (see Daniels et al., 2019) [Color figure can be viewed at wileyonlinelibrary.com]

of ketamine-xylazine mix (10 mg/kg ketamine [100 mg/ml, Zetamine, VetOne, Boise, Idaho] and 2 mg/kg xylazine [100 mg/ml, XylaMed, VetOne]) and then a 2–4 ml cardiac injection of pentobarbital and phenytoin sodium (Euthanasia Solution, VetOne).

2.2 | General histological procedures

Intact brains postfixed in 4% paraformaldehyde were received in our laboratory and stored at 4°C. Once the brains were weighed, the dura mater was removed so they could be separated along the longitudinal fissure into the left and right hemispheres that were subsequently weighed once again. The right hemisphere was assigned to IF analyses and the left hemisphere to thionin-based histological analyses. In a few cases, however, the right cortical tissue was slightly compromised with an occasional nick in the cortex, making it inappropriate for IF histology; in those cases, the left hemisphere was assigned to the IF group. For cryoprotection prior to sectioning, the hemispheres dedicated to thionin-based histology were transferred to a series of 10, 20, and 30% sucrose solutions, with 1 week between each interval. The remaining hemisphere was maintained in 4% paraformaldehyde and was further dissected and processed regionally for IF assessment. All ingredients of the fixative solution were purchased from Sigma Aldrich, St. Louis, MO. The brains were coded to ensure experimenters were blind to the animal's group assignment based on the behavioral task described above.

2.3 | IF histological procedures

The HC and SSCX from one hemisphere of each brain were carefully dissected from the tissue and processed for IF, a method used to determine cell density of specific brain areas (Herculano-Houzel & Lent, 2005). For each dissected hemisphere, half of the HC was removed in its entirety in the curved bean-shaped form of the hippocampal formation (CA fields and dentate gyrus), encapsulated in white matter. The SSCX for that same hemisphere was collected by removing the entirety of the subcortical white and gray matter by peeling the cortex from the striatum (Bandeira et al., 2009). Specifically, the SSCX was defined as the cortical area caudal to the sulcus cruciatus, anterior to the sulcus ansatus, and dorsal to the sulcus sylvius (Herron, 1978; Krubitzer & Dooley, 2013; Schober, 1991; Welker & Seidenstein, 1959). Each targeted region was weighed (see Table 1 for brain section weights for each group), then the entire dissected region of interest (ROI) was sectioned into small pieces of tissue comprised of all gray and white matter and placed in a handheld glass Dounce 40 ml homogenizer (Kontes Glass Co., Vineland, NJ). A minimum of 1 ml chilled dissociation solution (40 mM sodium citrate and 1% Triton-X100; Sigma Aldrich) per 100 mg of tissue was utilized for the mechanical disruption process. Tissue pieces were ground until no longer visible, adding dissociation solution as needed, to ensure that a sufficient nuclei suspension had been obtained. Following homogenization, the suspension was poured into a graduated cylinder where the fluorescent marker, 4,6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma Aldrich) (10 mg/ml), was added to visualize cell nuclei. The final volume was recorded before the homogenates were transferred to a new conical tube, which was stored at 4°C and protected from light. Because the brain sections were removed in their entirety, including gray and white matter, cellular numbers were expected to be higher than previous reports exclusively using gray matter.

To count total nuclei, 1 ml aliquots were homogenized using a handheld cordless Bel-Art homogenizer and a clean plastic pestle (Cole-Parmer, Vernon Hills, IL). A total of 10 μl was loaded onto a Neubauer chamber (i.e., hemocytometer) and placed under a *Zeiss Axio* microscope for visualization. Nuclei were initially visualized under the DAPI filter at 10× magnification for proper positioning, before moving to 40× for counting. Utilizing the centered 1 × 1 mm^2 square etched into the hemocytometer as the counting field, experimenters selected 10 of the 25 squares along an "X" pattern, summing the number of nuclei present in each small square, which each held a volume of 4 nl (Figure 3(b)). To obtain a representative sample count, this process was repeated four times. The cell counts were summed, averaged, and a coefficient of variation was calculated, which had to be lower than the 15% predetermined criterion to be acceptable. The following equation was utilized to calculate the total nuclei present in the original sample and, subsequently, extrapolate the cell count for the particular ROI from each animal:

$$\text{Average nuclei count} \times (1{,}000{,}000/\text{volume [nl] counted}) \times \text{total sample volume (ml)}$$

Since the initial counts consisted only of DAPI-positive nuclei, a fluorescent molecule that binds to DNA regions rich with adenine-thymine interactions, there was no molecular differentiation between neurons and nonneurons at this stage. To evaluate the neuronal percentage present in each sample, an aliquot (1 ml) of homogenates for each animal and ROI was co-stained for the neuronal nuclear protein marker, NeuN, using a Cy3 labeled primary anti-NeuN

TABLE 1 Hemisphere and brain area weights

Solver type	Hemisphere weight (g)	Hippocampus (mg)	Somatosensory cortex (g)
Nonsolver ($N = 6$)	19.18 ± 0.99	381.39 ± 100.87	5.32 ± 0.55[a]
Intermediate ($N = 5$)	19.16 ± 0.82	473.60 ± 120.99	4.56 ± 0.46
Solver ($N = 7$)	18.52 ± 0.82[a]	553.69 ± 116.48	5.22 ± 0.13[a]

Note: Values represent averages ± *SEM*.
[a]*N* - 1, value missing for one animal.

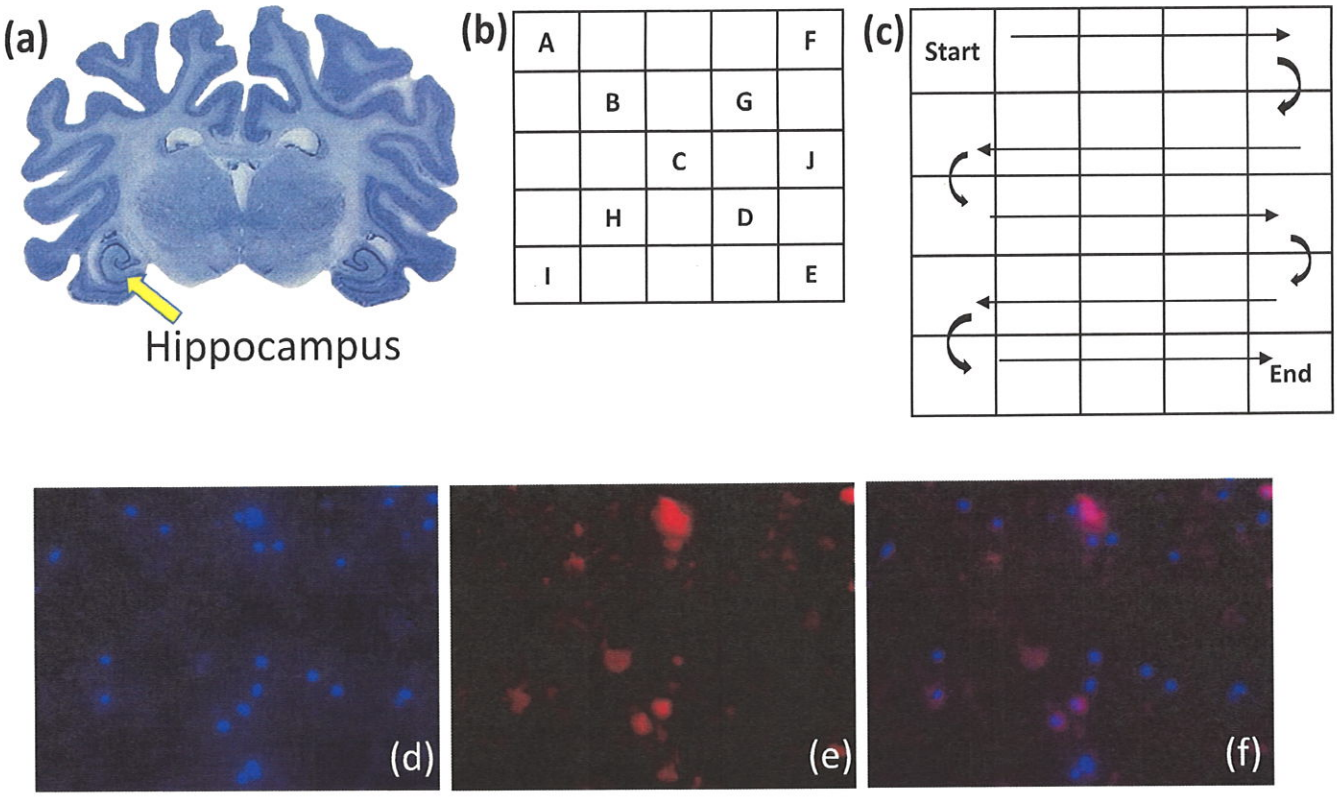

FIGURE 3 Isotropic fractionation. The somatosensory cortex (not shown) and hippocampus (a) were dissected from one hemisphere and evaluated for cellular density using isotropic fractionation. Cells were stained with DAPI to visualize individual nuclei, then loaded onto a hemocytometer and counted in an X pattern (b) under a light microscope to determine cell densities. Following 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) counting, the cell homogenates were treated with anti-NeuN and visualized at 40×, again on a hemocytometer, where all cells in the 25 × 25 grid pattern were counted (c) and characterized as neuronal or nonneuronal. Cells visible under the DAPI channel (d) that were also visible under the TRITC channel (e) were determined to be neurons, further confirmed by the merged image showing colocalization (f). Cells that were only visible under the DAPI channel were characterized as nonneuronal [Color figure can be viewed at wileyonlinelibrary.com]

antibody, ABN78C3 [1:300] (Millipore Sigma, Burlington, MA). The aliquots were processed initially with an antigen-retrieval step (1 h at 75°C in 0.2 M boric acid, pH 9) to improve access to available binding sites. Following the antigen retrieval step, the samples were stained with anti-NeuN in PBS-BSA (5%) and incubated for 24–48 h at 4°C, protected from light. The stained homogenates were then collected in a tabletop centrifuge (5 min, 1500*g*), the supernatant was decanted, and the sample pellet was resuspended in up to 1 ml PBS and protected from light with foil.

To determine the neuronal percentage, 10 μl samples were loaded onto the hemocytometer and 100 nl volumes were counted until a minimum of 500 cells were identified under the DAPI filter (Figure 3(c)). Cy3 fluorescence (~550 nm excitation, ~570 nm emission) was visualized under the standard TRITC filter. Photomicrographs of cell nuclei were captured under each filter to assess those that exhibited DAPI only, or DAPI and NeuN staining, using the Zeiss Zen software. Cell counts were determined by observers who were blind to group conditions; further, an interobserver reliability agreement threshold of 90% was established prior to cell quantification to assure that variation among observers did not exceed 10%. The number of co-stained nuclei was divided by the total number of DAPI-stained nuclei, and multiplied by 100 to determine the percent of neurons in the sample. See Figure 3 for IF procedures.

2.4 | Thionin-staining histological procedures

The FI cortex and the ventral HC from one hemisphere of each raccoon brain were processed for morphological cellular analysis using thionin-stained sections. Because the anterior portion of the HC has a large ventral presence, similar to humans and foxes, thought to be due to the increased neocortical tissue (Kempermann, 2012), the ventral hippocampal area was investigated in the current study. Each hemisphere was cryopreserved by transferring the tissue into 20% and then 30% sucrose prior to sectioning; subsequently, the tissue was blocked in appropriate sections to reveal the FI in one section and, more posterior, the HC section. Segments were mounted using Fisher Healthcare Tissue Plus O.C.T compound (Wilmington, MA), and placed in an M525 Microm microtome cryostat (Thermo Fisher Scientific) at −20°C for 20 min, or until fully frozen, prior to sectioning. The tissue was then sectioned at a thickness of 50 μm, thaw-mounted

onto charged microscope slides (2 × 3 in) (Thermo Fisher Scientific), and allowed to dry for 48 h before staining with thionin (0.1% dilution) (Allied Chemical Corporation, Morristown, NJ) and coverslipping with permount (Electron Microscopy Sciences, Hatfield, PA). To generate average cell profile density measures for individual sampling areas, an average of three sections was quantified per animal for the HC samples. For the FI analysis, an average of nine brain sections was quantified per animal. The comparatively low number of HC sections was due to more frequent tissue damage in this area compared to the FI sections, likely due to the postfixed, rather than perfused, tissue used in this study. Tissue was imaged on a Zeiss Axioscope M.2 light microscope (Carl Zeiss, Oberkochen, Germany) equipped with Neurolucida software version 2020 3.1 (Microbrightfield, Inc., Williston, VT). The area of interest (either dentate gyrus of the HC or FI cortex) was first visualized at 5× magnification and, using consistent visual landmarks, each area was marked with three (HC) or four (Layer V FI) computer-generated markers to identify the specific counting locations within each region (see Figure 5). Once the targeted area was identified at low magnification to assure consistent placement of the software generated reference marker, the objective was then moved to 40× magnification for quantification within a 200 × 300 μm^2 perimeter box. All visible neurons and glial cells were quantified using this two-dimensional (2D) assessment. Considering the relatively large sampling window, consistent sampling locations, interobserver reliability agreement thresholds set at 90%, and the ample distance between sections (150 μm) to avoid double counting cells, the 2D use of the x and y planes was determined to be appropriate to generate accurate estimates (Benes & Lange, 2001). In the FI, neurons were categorized as VENs, (defined as an elongated bipolar cell body), forked cell (large cell body with bifurcating apical dendrite), or non-VENs (e.g., pyramidal cells); glial cells were categorized using criteria previously outlined (see Garcia-Cabezas et al., 2016). In the HC, the same categories were used with the exception of the VEN category. Since to our knowledge VENs have not been identified in the HC of any species, fusiform cells were assessed due to their similar elongated phenotype as observed in the VENs (Dickerson et al., 2007; Schwerdtfeger & Buhl, 1986). Within each thionin-stained section, average cell profile density levels were obtained by counting three ROI perimeter boxes per HC tissue section and four ROI boxes for each FI tissue section. The average density counts for each ROI box in the targeted brain area was determined for each solving group and subsequently used for further data analyses. See Figure 5 for consistent placement of sampling boxes for the thionin quantification procedures.

2.5 | Statistical methods

In all comparisons of the three solving groups, data were assessed via one-way analysis of variance (ANOVA) assessments determining the effects of three levels of task performance ability on the various cytoarchitectural measures in SPSS Statistics software, version 27. When appropriate, Tukey's post hoc tests were performed to detect group differences (with an alpha value of .05). One histology assessment did not include intermediate solvers due to tissue loss; in that case, the two remaining groups were compared using Student's two-tailed *t* test, with an alpha value of .05. Descriptive statistics were calculated through SPSS or Excel. All data are shown as mean ± *SEM* for each group unless otherwise noted. Graphs and figures were generated using *GraphPad Prism* software, version 6.0 and BioRender.com, respectively.

3 | RESULTS

3.1 | IF-determined cell density

Based on prior evidence that raccoons exhibit cortical magnification of their forepaw/hand areas, we investigated the SSCX since it is an area essential to interactions with physical stimuli but was not expected to impact cognitive functions. High-solvers ($x\bar{c}$ = 270,594,097) did not differ from either intermediate solvers ($x\bar{c}$ = 214,821,875) or nonsolvers ($x\bar{c}$ = 242,337,500) in total nuclei. Further, intermediates and nonsolvers were also not found to differ from one another. Although no effect of task performance was observed for total cell density in the SSCX, a significant overall effect was observed for the total HC nuclei and total nonneuronal HC nuclei ($F(2,15) = 6.067$, $p = .0117$, $\eta_p^2 = 0.447$; $F(2,14) = 3.847$, $p = .0466$, $\eta_p^2 = 0.355$, respectively). Post hoc tests indicated that the high solvers had significantly more cells ($x\bar{c}$ = 51,472,917) than the nonsolver group ($x\bar{c}$ = 35,237,500; $p = .012$; see Figure 4(a)). A similar trend was observed for total cell count between high solvers and the intermediate group ($x\bar{c}$ = 39,017,500), but did not reach statistical significance ($p = .0663$). Additionally, high solvers were found to have more nonneuronal nuclei on average ($x\bar{c}$ = 43,959, 439) compared to nonsolvers ($x\bar{c}$ = 31,523, 979) and intermediates ($x\bar{c}$ = 32,545,144); however, this observation is most accurately described as a nonsignificant trend since the post hoc tests failed to reach a statistically significant threshold for either comparison ($p = .071$ and $p = .101$, respectively; see Figure 4(b)). One intermediate solver could not be analyzed for NeuN staining due to poor sample quality. No group differences in NeuN+ cells were observed.

For additional characterization of the brain areas used for the IF analyses, a one-way ANOVA was used to determine differences among hemisphere, HC, and somatosensory cortical weights. No statistical differences were observed in these measures (see Table 1 for group means for each brain area).

3.2 | Morphology-based cellular categories in dentate gyrus and FI cortex

IF provides an accurate assessment of total regional cell counts, but it does not provide specific information about morphological characteristics

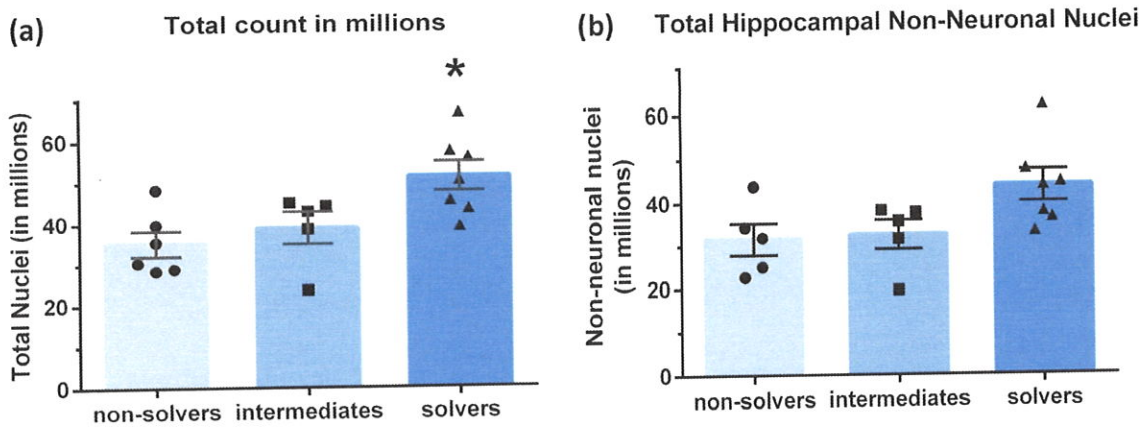

FIGURE 4 Isotropic fractionation-based cellular profiles in the raccoon hippocampus. (a) High-problem solving raccoons ($N = 7$) were found to have significantly higher total cell counts (i.e., nuclei) in the hippocampus compared to nonsolvers ($N = 6$) (*$p = .0117$) as determined by one-way analysis of variance (ANOVA) with Tukey's post hoc tests. (b) Isotropic homogenates stained with anti-NeuN revealed a greater proportion of nonneuronal cells present in high-problem solving raccoons following a one-way ANOVA ($p = .0466$), though post hoc tests failed to demonstrate significant group differences (solvers vs. nonsolvers: $p = .0706$; solvers vs. intermediates: $p = .1006$) [Color figure can be viewed at wileyonlinelibrary.com]

of the cellular components in the brain. As previously described, to further investigate the cellular phenotypic distribution in the brains of these raccoons, one hemisphere from each animal was devoted to cryopreservation and sections containing either the FI cortex or HC were assessed for the presence of neurons, nonneurons (i.e., glial cells), fork cells, or VENs (FI)/fusiform cells (DG; see Figure 5). Thionin-stained tissue containing the FI cortex was unavailable for intermediate solvers; therefore, group comparisons were between high solvers and nonsolvers only. In the FI cortex, high solvers had fewer average total cell density levels in the sampled visual fields; however, no statistically significant differences were observed compared to nonsolvers ($p = .2829$; see Table 2). Further, no significant differences were observed in nonneuron average counts ($p = .1503$; Welch's corrected t test used due to heterogeneity of variance for this measure). Fork neurons and von Economos were observed throughout the FI in all problem-solving types (see Figure 6), yet no differences in cell profile density levels were detected among groups for total VENs or fork neurons or for percentage levels of VENs or neurons ($p > .05$ in all comparisons; see Table 2). Although thionin-stained cell sampling via ROI cellular counts data fall short of the accuracy of a full stereological analysis, the percent of VENs observed in the sampled FI areas of these raccoons (approximately 8%) was higher, yet similar to the previous stereological assessment (6.33%) shown in Figure 1.

Cellular distribution was also assessed in the HC in thionin-stained sections; specifically, in the hilus of the dentate gyrus. Unlike the FI cortex, tissue was available for all three solving groups. Focusing on total neuron and nonneuron averages, high solvers did not differ significantly from either intermediates or nonsolvers; however, high solvers were found to contain higher numbers of fusiform-shaped neuronal cells ($x\bar{c} = 1.42$) in targeted sampled areas compared to intermediates ($x\bar{c} = 0.61$, $p = .0495$) and nearly significantly more than nonsolvers ($x\bar{c} = 0.79$, $p = .0692$; $F(2,11) = 4.640$, $p = .035$, $\eta_p^2 = 0.458$). See Figure 7 for graphs and representative photomicrographs and Table 2 for relevant group averages. Note that the SSCX was not examined further in these thionin-based cytology assessments since differences were not observed following IF analysis and VENs have not been observed in this area.

4 | DISCUSSION

The findings of the current study provide insights into the neurobiological mechanisms of problem-solving abilities in raccoons. Corroborating previous research emphasizing the influence of hippocampal plasticity in altered learning and memory outcomes (Guigueno & Sherry, 2017; Maguire et al., 2006; Sherry & Hoshooley, 2010; Sobrero et al., 2016), hippocampal cellular counts were associated with performance proficiency in the multiaccess problem-solving task. Specifically, the increased number of nonneuronal cells detected in the homogenized hippocampal tissue suggests that glial cells contribute to behavioral outcomes in this particular task. As predicted, no differences in cellular distributions among the three levels of performance were observed in the SSCX. Focusing on neuronal cytoarchitectural profiles in the thionin-stained tissue, the high-solving group had significantly more elongated fusiform cells in the dentate gyrus. Further, the existence of VENs (determined via morphology characteristics) in the FI cortex confirmed previous unpublished evidence of VENs in the raccoon. Differences in VEN counts in the FI, however, were not observed among the three solving groups. To our knowledge, these results represent the first functional neuroanatomical findings associated with cognitive ability in the raccoon.

As evident in their proficiency in obtaining anthropogenic sources of food in urban habitats (Prange et al., 2004), raccoons are known to approach novel challenges in innovative ways (Daniels et al., 2019; Morton, 2020; Stanton et al., 2017). The reported problem-solving flexibility in raccoons may be influenced by their primate-like cortical

neuronal density (Herculano-Houzel et al., 2006; Jardim-Messeder et al., 2017). Raccoons have approximately 400 million cortical neurons, a significant increase from the rat preclinical model that possesses approximately 32 million cortical neurons (Herculano-Houzel, 2009). Considering that humans possess approximately 16 billion cortical neurons (Herculano-Houzel, 2009; Herculano-Houzel, 2012), the complexity of the raccoon's cerebral cortex, coupled with the magnification of the forepaw/hand area, make it an attractive model for translational neuroscience research.

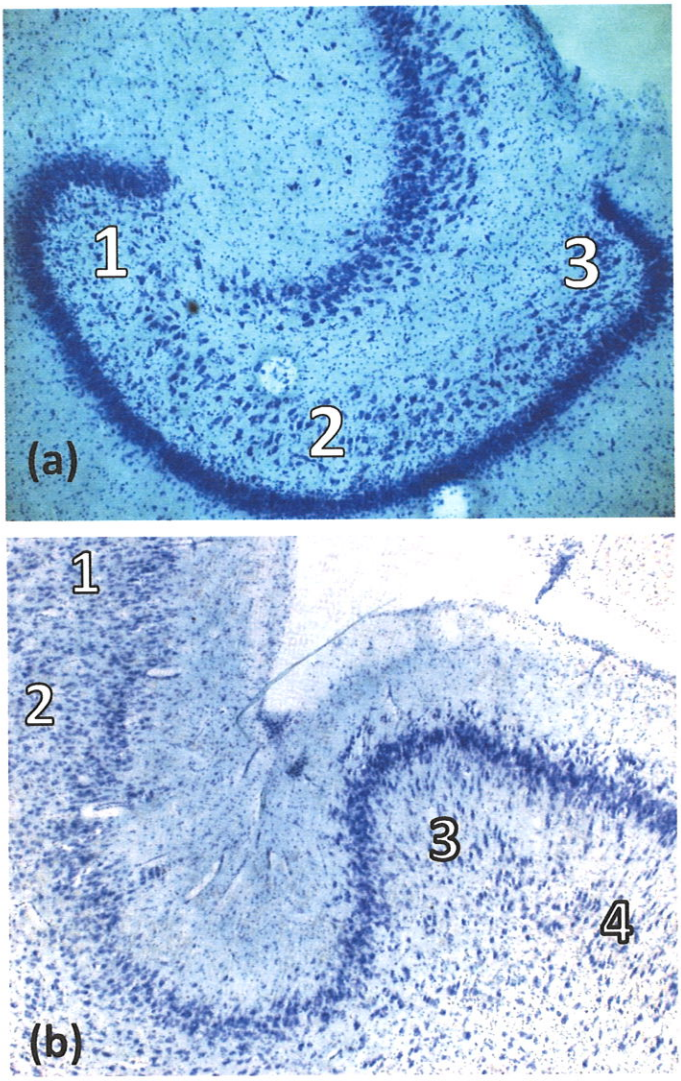

FIGURE 5 Representative images and sampling locations for cell counting in thionin-stained tissue. (a) The dentate gyrus (hilus) of the hippocampus shown at 2.5×, with numbers representing the three sampling regions collected within each tissue section for each animal. (b) The anterior frontoinsular region shown at 5×, with numbers outlining the four general sampling locations for all tissue sections [Color figure can be viewed at wileyonlinelibrary.com]

Focusing on hippocampal cytological parameters determined by IF, the high-performing raccoons possessed a higher number of non-neuronal cells than the lower performing animals. As observed with neurons, glial complexity can contribute to neuroplasticity and adaptive behavioral outcomes (Hoogland & Parpura, 2015). The brain's glial cells, including astrocytes, microglia, and oligodendroglia, differentially contribute to neural functions (Fields et al., 2014). Myelination provided by oligodendroglia facilitate the velocity of neuronal conductance (Nave, 2010); astrocytes regulate local blood flow that facilitates the movement of neurochemicals and nutrients to the neurons (MacVicar & Newman, 2015), and microglia maintain neural circuitry by surveying potential threats and influencing neuroplasticity (Wake et al., 2013). Additional functional roles of astrocytes have been identified in the facilitation of synaptic plasticity and regulation of neuronal network oscillations processes that influence cognitive functions (Santello et al., 2019). Thus, the increased number of glial cells in the hippocampal area of the raccoons may have contributed to the efficient problem-solving ability of the high-performing animals in multiple ways. Caution should be taken when interpreting these findings; however, since specific glial markers were not used in the IF assays. These findings are also limited by task exposure duration considering that the nonsolvers had less time interacting with the puzzle box due to their dismissal from the trials after failing to solve the initial locking task panel. Subsequent investigations controlling for the duration of task interactions would further clarify these initial findings to discern predisposed versus training-induced influences on the hippocampal cellular counts.

Following the report of lower neuronal to glial ratios in specific cortical areas of noted "genius" Albert Einstein, neuron to glia ratios have also been associated with cognitive complexity (Diamond et al., 1985; Tien et al., 2019). Now that it is known that glial cell density varies across different brain areas (Herculano-Houzel, 2014; Herculano-Houzel et al., 2006), it is important to investigate the neuron vs. glial densities to learn more about its contribution to functional output. For example, compared to other primates, a lower neuron to glial ratio is observed in the human frontal cortex (Sherwood et al., 2006). Even so, little evidence of function-related intraspecies

TABLE 2 Cytoarchitecture analysis of sampled regions from the anterior frontoinsular region in raccoons

Solver type	Nonneurons	Neuron total	VENs	Fork neurons	% VENs[a]	% All neurons[b]
Nonsolver ($N = 5$)	88.84 ± 8.17	34.11 ± 3.31	2.30 ± 0.62	0.26 ± 0.13	6.88 ± 1.80	27.75 ± 0.84
Solver ($N = 7$)	74.25 ± 1.96	30.55 ± 1.27	2.82 ± 0.36	0.18 ± 0.08	9.51 ± 1.62	29.12 ± 0.77

Note: Values (mean ± *SEM*) represent averages from the field of vision (200 × 300 μm), and are not cumulative counts.
Abbreviation: VEN, von Economo neuron.
[a]% VEN represents the proportion of neurons characterized as VENs.
[b]% All neurons represent the proportion of total cells that were characterized as VEN, fork, or pyramidal neurons.

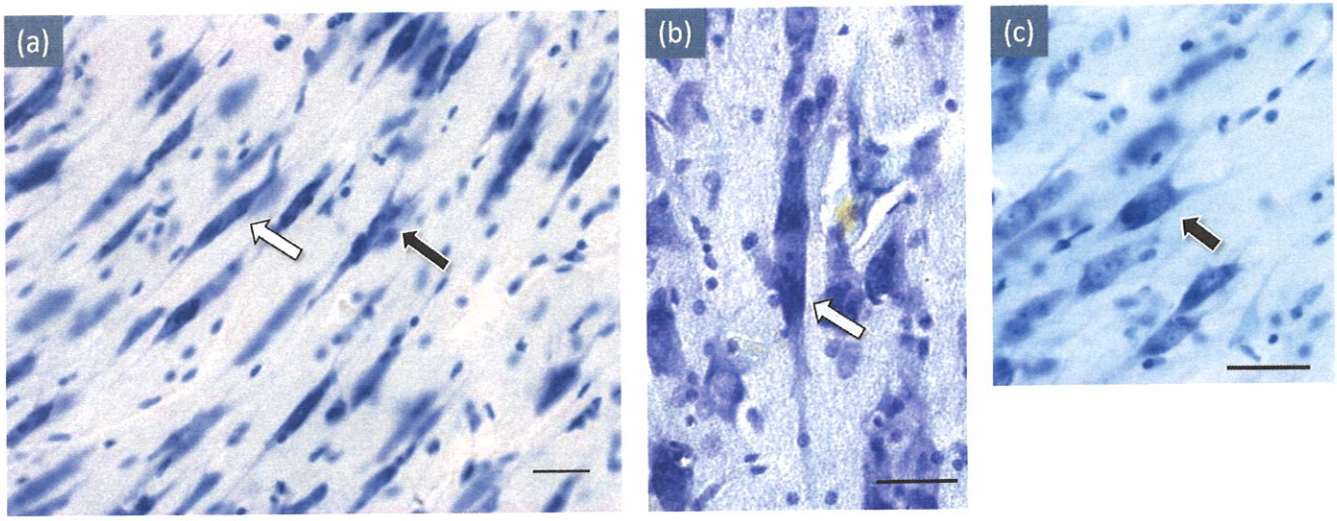

FIGURE 6 Frontoinsular cellular phenotypes. Photomicrographs of thionin-stained cells imaged at 40×, located in the frontoinsular region. Among nonneurons and pyramidal neurons (a), the presence of both von Economo neurons (a,b) and fork neurons (a,c) were observed. White arrows designate von Economo cells, and black arrows designate fork cells. All scale bars represent 30 μm. A summary of all other cellular profiling measures can be found in Table 2 [Color figure can be viewed at wileyonlinelibrary.com]

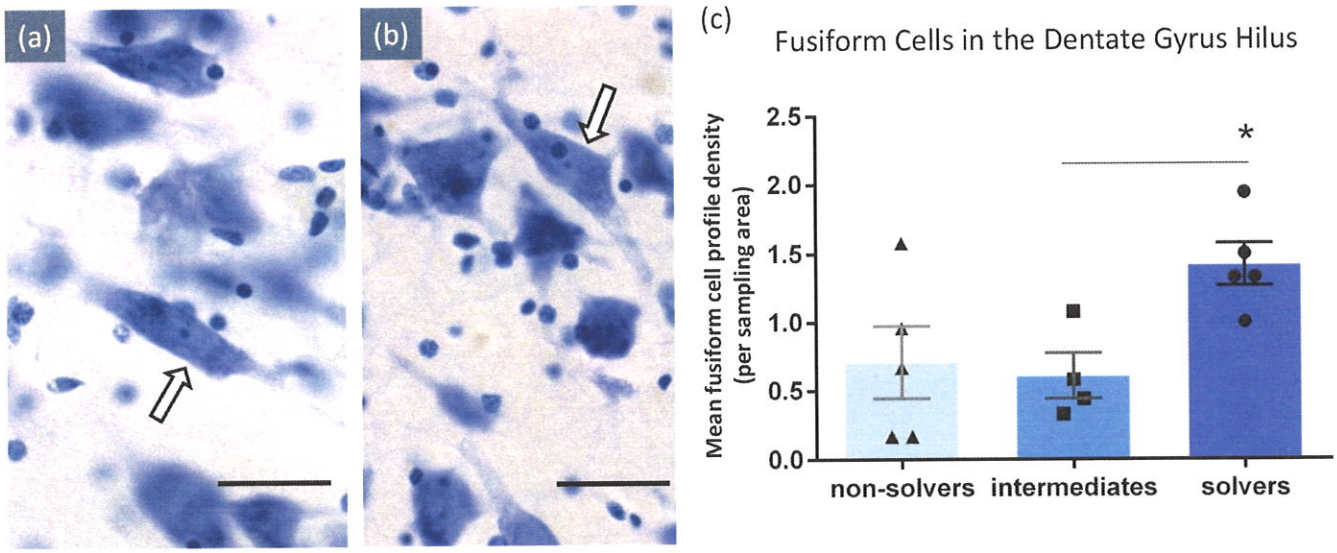

FIGURE 7 Fusiform cells in the raccoon dentate gyrus hilus. Representative photomicrographs of thionin-stained fusiform cells observed in the hilus at 40× (a,b). These cells were found to be more prevalent in high-problem solving raccoons, compared to intermediate or nonsolving raccoons (c). Significant differences were detected between solvers and intermediates via a one-way analysis of variance (ANOVA), with Tukey's post hoc (*$p = .0495$). All other cell-profiling measures for the hippocampus cells can be found in Table 3. All scale bars represent 30 μm [Color figure can be viewed at wileyonlinelibrary.com]

neuron to glia ratio differences have been observed in preclinical models (Bardi et al., 2016). Although no differences in percentages of neurons in sampled areas were observed in the hippocampal hilus or FI area in the current study, it is important to continue to investigate these cellular profiles in functional neuroscience explorations. Considering that the use of thionin-based morphology to identify nonneuronal cells such as glial cells (García-Cabezas et al., 2016) is less reliable than more specific cellular profiling (e.g., neurochemical or transcriptomic markers) and stereological-based estimations, additional histological analyses would be informative.

Described as homologous to extratelencephalic excitatory projecting neurons (Hodge et al., 2020), VENs may facilitate the rapid processing of neural information in targeted brain areas (Hakeem et al., 2009; Stimpson et al., 2011). In the neuron-dense human cortex, the *connectomic hypothesis* suggests that the presence of VENs leads to more rapid cellular projections among specialized neural modules that facilitate sustained neural activation for advanced cognitive

TABLE 3 Cytoarchitecture analysis of the hilus of the dentate gyrus region in raccoons

Solver type	Nonneurons	Neuron total	Fusiform neurons	% Fusiform neurons[a]	% All neurons[b]
Nonsolver ($N = 5$)	120.0 ± 9.15	27.80 ± 3.41	0.71 ± 0.27	2.73 ± 0.52	18.74 ± 1.73
Intermediate ($N = 4$)	119.05 ± 5.17	27.09 ± 1.99	0.61 ± 0.17	2.31 ± 0.65	18.64 ± 1.43
Solver ($N = 5$)	105.6 ± 9.99	29.60 ± 4.61	1.42 ± 0.15*	5.08 ± 0.61	21.93 ± 3.06

Note: Values (mean ± *SEM*) represent averages from the field of vision (200 × 300 μm), and are not cumulative counts.

[a]% Fusiform neurons represent the proportion of neurons characterized as fusiform cells.

[b]% Neurons represents the proportion of total cells that were characterized as either fusiform cells or other neurons.

functions—a neural model also referred to as the *global neuronal workspace* (Mashour et al., 2020). Although no differences in number of VENs in the FI were observed among the three groups of raccoons, once again, VENs were confirmed to be present in the FI cortex. Considering the absence of these neurons in rodent models, this finding provides further support for the translational value of the raccoon model. Even in the absence of differential contributions of VENs to problem-solving ability, their presence provides potential insights into the cognitive abilities of raccoons. For example, the postnatal emergence of VENs suggests that an animal's experiences, broadly defined, influence VEN development (Allman et al., 2011), providing evidence of their putative role in the formation of specialized neural circuits that facilitate an animal's unique and adaptive behavioral responses (Stimpson et al., 2011). Further, the fourfold increase of VENs in "super-agers" who show little evidence of age-typical cognitive decline provides evidence of the potential facilitatory role of VENs in cognitive functions (Rogalski et al., 2013). Additionally, the increased density of VENS in the crest, as opposed to the walls, in the human medial frontopolar cortex suggests that VENs may also serve a mechanical function in cortical gyrification (Gonzalez-Acosta et al., 2018).

Considering the similarity between the morphology of VENs and bipolar fusiform cells, it is interesting that more fusiform cells were observed in the dentate gyrus of the high-performing raccoons. Individual differences in dentate gyrus cell numbers were also observed in degus (*Octodon degus*), suggesting that this area is likely responsive to individual environmental and social experiences (Sobrero et al., 2016). Additionally, bipolar fusiform cells increase processing speed within the HC and extrahippocampal areas in a similar, but less rapid, fashion as the cortical VENs (Scharfman, 2011; Schwerdtfeger & Buhl, 1986). In the absence of VENs in the ACC (as observed in previously unpublished findings in our laboratory), these subcortical fusiform cells may facilitate emotional and cognitive processing in the raccoon. Interestingly, VENs were also observed to be scarce in the elephant ACC, providing additional evidence that the cortical landscape of VENs has evolved independently for species-specific adaptive functions (Hakeem et al., 2009). For example, the existence of VENs in the posterior insular cortex may enhance transmission of auditory and visceral autonomic functions leading to increased survival in prey species that have VENs, such as deer and sheep (Raghanti et al., 2015). The rich network of interoceptive afferents in the macaque insular cortex that facilitates the perceived physiological status of the body's organs provides additional support for the VENs in visceral functions (Evrard, 2019). Further, VEN expression of FE2FZ and CTIP2, transcription factors known to regulate subcerebral projections, likely facilitate the linkage of cortical autonomic target sites to downstream brainstem and spinal cord sites (Cobos & Seeley, 2015). Thus, compared to species with VENs in both the FI and ACC, unique VEN distributions in the raccoon may result in varied functional outcomes; consequently, the specific distribution patterns on VENS should be more thoroughly investigated.

The convergence of findings in the current study suggests that the raccoon model is valuable for neuroscience research. Although housing raccoons in traditional laboratories is not a practical option, it is important to explore various field techniques that provide relevant neurobiological data in a diverse array of species to contribute to the growing body of rodent neurobiological findings. For example, the IF technique provides an opportunity to investigate intraspecies cellular densities in post-fixed tissue extracted from wild-caught animals. Such investigations will increase our knowledge of functional neuroscience influences (e.g., cellular profiles of animals with varying cognitive abilities) in animals living inside and outside of laboratory conditions. Further, the presence of VENs in the raccoon FI offers an opportunity to explore how these rapidly processing neurons facilitate complex cognitive functions. Considering that alterations in VEN populations have been associated with several neuropsychiatric conditions including autism, schizophrenia, and dementia (Allman et al., 2005; Cauda et al., 2014; Seeley et al., 2006), the translational value of preclinical models that possess these neuron types increases the likelihood of identifying key mechanisms of these challenging and threatening conditions. Additional research confirming the role of varied cytoarchitectural landscapes on behavioral outcomes will contribute valuable information to emerging neuroethological and psychiatric knowledge bases.

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AUTHOR CONTRIBUTIONS

Joanna Jacob, **Molly Kent**, and **Kelly Lambert**: Participated in data collection, data analysis, and manuscript preparation. **Sarah Benson-Amram**, **Suzana Herculano-Houzel**, and **Mary Ann Raghanti**:

Participated in data collection and manuscript preparation. **Emily Ploppert, Jack Drake, Bilal Hindi, Nick R. Natale, Sarah Daniels, Rachel Fanelli, Anderson Miller, Tim Landis, Amy Gilbert, Shylo Johnson, Annie Lai, Molly Hyer, Amanda Rzucidlo, Chris Anchor**, and **Stan Gehrt**: Participated in data collection.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

Raw data included in this manuscript were generated at the University of Richmond and are available from the corresponding author (K. L.) upon request.

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Original Article

Early life experience influences dispersal in coyotes (*Canis latrans*)

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Natal dispersal plays an important role in connecting individual animal behavior with ecological processes at all levels of biological organization. As urban environments are rapidly increasing in extent and intensity, understanding how urbanization influences these long distance movements is critical for predicting the persistence of species and communities. There is considerable variation in the movement responses of individuals within a species, some of which is attributed to behavioral plasticity which interacts with experience to produce interindividual differences in behavior. For natal dispersers, much of this experience occurs in the natal home range. Using data collected from VHF collared coyotes (*Canis latrans*) in the Chicago Metropolitan Area we explored the relationship between early life experience with urbanization and departure, transience, and settlement behavior. Additionally, we looked at how early life experience with urbanization influenced survival to adulthood and the likelihood of experiencing a vehicle related mortality. We found that coyotes with more developed habitat in their natal home range were more likely to disperse and tended to disperse farther than individuals with more natural habitat in their natal home range. Interestingly, our analysis produced mixed results for the relationship between natal habitat and habitat selection during settlement. Finally, we found no evidence that early life experience with urbanization influenced survival to adulthood or the likelihood of experiencing vehicular mortality. Our study provides evidence that early life exposure influences dispersal behavior; however, it remains unclear how these differences ultimately affect fitness.

Key words: ***Canis latrans*, dispersal, natal habitat preference induction, urban ecology**

INTRODUCTION

Natal dispersal makes up the bulk of most species' long distance movements (Studds et al. 2008). These movements influence ecological processes at multiple levels of biological organization. Natal dispersal affects individual fitness (Clobert et al. 2012), population extinctions and colonizations (Bowler and Benton 2009), gene flow for dispersers, and the codispersers that move with them (Trakhtenbrot 2005; Cowie and Holland 2006), and species invasions (Shigesada and Kawasaki 2002).

Natal dispersal consists of three stages: departure from the natal home range, transience through the matrix environment, and settlement in the new home range (Ronce 2007). For many species, dispersal is a plastic, condition dependent behavior. Due to the high cost of dispersal, the development of an adaptive dispersal strategy is essential to the survival and fitness of the animal (Bonte et al. 2012). For behaviorally flexible natal dispersers, the natal habitat is thought to play a significant role in shaping this behavior (Davis 2008).

The natal habitat influences departure in myriad ways. Most obvious is the relationship between departure and the quality of the natal habitat: departure rates tend to increase as habitat quality decreases (Lurz et al. 1997; Lin and Batzli 2001; Baguette et al. 2011; Legrand et al. 2015; but see Baines and McCauley 2018). Changing environmental conditions, including resource availability, competition, and predation rates, require that the animal assesses the relative qualities of the natal and matrix habitats to make an adaptive decision about whether it should stay or go (Schtickzelle and Baguette 2003; McCauley and Rowe 2010). The natal habitat can also influence departure by influencing how the animal assesses habitat quality (Stamps et al. 2009). Natal habitat preference induction (NHPI) describes the process in which an animal develops a preference for habitat features experienced in the natal home range (Davis and Stamps 2004). Although it is usually studied in the context of settlement, this induced habitat preference is likely to influence each stage of the dispersal process. For instance, NHPI

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may discourage an animal from departing the natal home range if it does not perceive the matrix environment as suitable habitat, whether or not it is (Benard and McCauley 2008; Piper et al. 2013).

Transience is a particularly risky stage of dispersal. During transience, the animal moves through unfamiliar matrix habitat where it is vulnerable to predation, depletion, and injury (Bonte et al. 2012). Similarly to departure, NHPI can influence how an animal perceives various habitats and environmental features during transience, where it decides to go, and how long it will search for suitable habitat. This is critical because transience length is positively associated with mortality rate (Johnson et al. 2009; Cox and Kesler 2012). Importantly, early life experiences in the natal habitat can act as a primer, influencing an animal's ability to navigate the challenges of the matrix environment (Clobert et al. 2009; Sih 2011; Frankenhuis and Del Giudice 2012). For example, coral reef damselfish (*Pomacentrus wardi*) who were exposed to predator cues as fry had higher survival rates as adults than fish with no early life predator experience (Lönnstedt et al. 2012).

Finally, NHPI has been shown to have an effect on settlement behavior across taxa (Selonen et al. 2007; Mabry and Stamps 2008; Dixson et al. 2014; Camacho et al. 2016; Sanz-Pérez et al. 2018). Species that experience NHPI tend to select habitats similar to those found in their natal home range, consequently they are more likely to settle in that type of habitat. This can be adaptive when it allows individuals to more easily identify suitable habitat in a heterogeneous landscape. Additionally, early experience with certain habitat features can result in the development of phenotypes that are best suited for those habitats (Stamps and Davis 2006). Therefore, NHPI can confer an adaptive advantage on animals who choose habitats for which their phenotype is best suited.

Like other types of behavioral plasticity, the plastic dispersal behavior discussed above should be particularly adaptive in heterogeneous environments (Snell-Rood 2013). This is the case in many urban areas, where habitat fragments are interspersed between developed areas of different intensities. Increases in human activity and the rapid loss and fragmentation of habitat resulting from urbanization can have profound effects on animal movement, including dispersal (Ricketts 2001; van der Ree et al. 2015; Tucker et al. 2018). While dispersal is costly no matter the environment, increased detection by humans and collisions with vehicles can make urban areas particularly dangerous for dispersers (Baker and Harris 2007). However, not all individuals' or species' dispersal behavior is negatively impacted by urbanization. Fey et al. (2016) demonstrated that during dispersal, red squirrels (*Sciurus vulgaris*) cross roads with high traffic volume with little risk of mortality.

Coyotes are an ideal animal for studying dispersal behavior and NHPI in urban environments. They exhibit high levels of behavioral plasticity and are one of the few large carnivores to establish populations in almost every major city in North America (Poessel et al. 2017). In urban areas, they decrease risks associated with travelling through the environment by avoiding humans spatially and temporally (Murray and St. Clair 2015; Ellington and Gehrt 2019). In addition to their behavioral flexibility, coyotes are ideal for studying the influence of natal experience on dispersal because of their strong tendency to disperse. A study conducted by Harrison (1992) found that of the coyotes collared, 80% dispersed within their first year of life. In natural systems, departure from the natal home range often occurs in response to social pressure from parents which is influenced by aspects of habitat quality (Bekoff and Wells 1986, Gese et al. 1996). Finally, earlier research suggests coyotes may experience NHPI. Studies by Sacks et al. (2004; 2008) revealed that habitat type is a strong predictor of the genetic structure of the population of coyotes in central California which the authors suggest is a result of natal habitat biased dispersal.

Given the rapid rate of urbanization and the critical role dispersal plays in individual, population, and community processes, understanding how urbanization impacts dispersal behavior is important in predicting species and community responses. Despite the potential importance of NHPI in shaping adaptive responses to urbanization, to our knowledge there are few studies that explore the phenomenon in these environments and even fewer that study the behavioral pattern in carnivores (but see Mannan et al. 2007 and Milleret et al. 2019).

To understand the effects of natal habitat on dispersal in urban environments, we studied the departure, transience, and settlement behavior of coyotes in the Chicago metropolitan area. The heterogeneous landscape of the area is made of diverse land use types, including nature preserves and high density urban development. We predicted that: 1) high habitat quality in natural areas would result in lower departure rates from these areas; 2) due to NHPI, coyotes who did disperse from natural areas would travel farther during transience in pursuit of natural habitat; 3) during settlement, coyotes would select habitats similar to those experienced in their natal home range; and 4) due to lack of early life experience with humans and vehicles, coyotes dispersing from natural areas would be less likely to survive to adulthood and suffer from higher rates of vehicle related mortality. To address these predictions, we looked at the relationship between proportion of developed habitat in the natal home range and the likelihood an animal would leave its natal home range, how far it traveled during transience and where it settled. We also evaluated the relationship between proportion developed habitat in the natal home range, survival to adulthood, and vehicle-related mortality to address the hypothesis that early-life experience with developed habitat better prepares coyotes to navigate this habitat.

METHODS

Coyotes included in this analysis were part of a long term study exploring the behavioral ecology, disease ecology, and management of urban coyotes (Gehrt et al. 2009; Newsome et al. 2015; Worsley-Tonks et al. 2020). Each coyote included in the study met two requirements: their parents were collared with very high frequency (VHF) transmitters in the year the coyote was born and they were VHF collared after leaving the natal den. Because juvenile movement tends to be restricted to the den site and rendezvous sites (areas frequented by members of a pack after pups have left the den) within the parents' home range in the first few months of life (Harrison et al. 1991), we estimated the natal home range using the parents' location data and the post-departure home range (hereinafter referred to as the "adult home range") using the offspring's location data (see Figure 1 for examples). Using these home ranges, we quantified characteristics of each stage of the dispersal process. This included departure from the natal home range, transience distance, and habitat type pre- and post-dispersal. Using data collected postmortem, we explored how natal habitat type influenced the likelihood a coyote survived to adulthood and the likelihood a coyote experienced a vehicle related mortality.

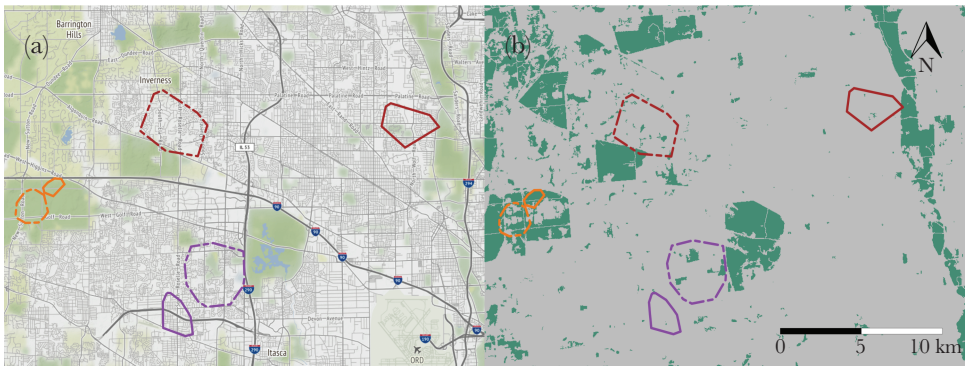

Figure 1
Maps showing the natal (dotted line) and adult (solid line) 95% MCP home ranges of three dispersed coyotes. Each focal coyote is represented with a different color. The hybrid map (a) is a combination of street maps and satellite maps and depicts the various levels of development coyotes might traverse during dispersal. The raster map (b) was generated using the NLCD landcover classifications and depicts "natural" (green) and "developed" (gray) habitat. This raster was used to determine the proportion of developed habitat in the natal home range, the adult home range, and in available habitat.

Study area

The Chicago metropolitan area includes Chicago and its surrounding suburbs which make up one of the largest urban centers in North America. Consequently, the region is made up of mostly developed land uses including commercial, residential and industrial areas. Notably, the Forest Preserve District of Cook County maintains protected areas which amount to 70,000 acres or 11% of land cover in the county (Wang and Moskovits 2001). The preserves are patchily distributed throughout the landscape providing habitat for wild flora and fauna.

We characterized the landscape into habitat types using the 2016 National Land Cover Database (United States Geological Survey). This database classifies the landscape into 16 land cover categories at a 30 m resolution. We reclassified these categories into two groupings, "natural" and "developed." Natural habitat included forest, shrubland, grassland, wetlands, and water. Developed habitat includes areas with more than 20% impervious surface cover. We included cultivated land in developed habitat due to its relatively high level of human disturbance.

Live captures and telemetry

Coyotes used in this study were captured year round between 2000 and 2018. Trapping was done opportunistically in nature preserves and on private properties across the Chicago metropolitan area using foot-hold traps or cable restraints. After animals were captured, they were transported to a laboratory where they were immobilized with Telazol (Zoetis Manufacturing & Research) and fitted with VHF radiocollars (Advanced Telemetry Systems and Lotek Wireless). Each coyote was weighed and sexed. Blood samples were collected and later used to determine parent–offspring relationships. All procedures were approved by Ohio State University's Institutional Animal Care and Use Committee (Protocol Nos. 2006A0245, 2010A00000113, 2013A00000012).

Coyotes were located using triangulation with a truck mounted antenna or by visual observations. Triangulations were recorded using a minimum of three bearings with a maximum of twenty minutes between first and final bearings. Coordinates were recorded with the program LOCATE II (Pacer). Coyotes were located once during the day, typically two or three times per week, and at night during tracking shifts in which we focused on a group of coyotes and obtained sequential locations at 60–120 minute intervals for 5–6 hours during the night. When radiocollared coyotes could not be located by vehicle, we conducted flights with a helicopter or fixed wing aircraft to locate signals and then confirmed their location on the ground. Such flights were deployed opportunistically in most years, and covered northeastern Illinois and parts of Wisconsin and Indiana.

Determining parent–offspring relationships

To confirm that the correct parent location data were being used to generate each focal coyote's natal home range, parent–offspring relationships were established using blood or tissue samples collected at the time of capture. Individuals were genotyped using 12 microsatellite markers and scored using Genetic Analysis System Software (version 8.0, Beckman-Coulter, Inc., Fullerton, California). Genotypes of pups were matched with parents using the programs CERVUS and PASOS (version 1.0; Duchesne et al. 2005). Further details can be found in Hennessy et al. (2012).

Calculation and analysis of home range, dispersal, and mortality characteristics

Departure

The departure analysis examined the relationship between natal home range habitat type and the likelihood of departing from the natal home range. Eighty-five offspring from 44 parent coyotes were used in this analysis. Coyotes were categorized as dispersers if their adult home range had no overlap with their natal home range. Natal home ranges were calculated using the location data of parents from the year the focal coyote was born (number of locations used to calculate natal home ranges: mean = 306 ± 231). We only included offspring whose parents had at least 60 location observations recorded in the year the offspring was born. We calculated 95% minimum convex polygons (MCP) for each focal coyote's parent in the package adehabitatHR (*v 0.4.18*; Calenge 2006) in R 4.0.2 (R Core Team 2020). Due to the high degree of overlap between the space use of mated pairs, when location data were available for both parents, we combined those data and calculated one MCP (Chamberlain et al. 2000). The focal coyote's adult home ranges were calculated in the same way using the last six months or less of its location data (number of locations used to calculate adult home ranges: mean = 60 ± 31; duration of tracking for adult home ranges in days: mean = 160 ± 42). Subsetting the offspring location data was necessary because some of the individuals were collared

prior to departure resulting in MCPs that spanned natal and adult home ranges. Previous studies indicate that coyote dispersal begins when coyotes are 6 months old and that most coyotes disperse within their first year of life (Harrison 1992). By focusing on the last 6 months of data, we ensured that all of the coyotes experienced this typical dispersal window, in other words, all coyotes were at least 1 year old by the end of the 6 month tracking period. Due to the challenges associated with collecting dispersal data, we included all offspring regardless of the number of recorded locations.

The proportions of developed habitat in the natal home range and the adult home range were calculated using the *raster* package in R and the NLCD raster (*v3.0–12*; Hijmans 2015). We evaluated the relationship between departure and proportion of developed habitat in the natal home range by constructing a generalized linear model (GLM) with the binomial outcome variable, dispersed or not dispersed. Proportion developed habitat in natal home range and sex were included as fixed effects.

Transience distance

The transience analysis assessed the relationship between natal home range habitat type and dispersal distance. First, we had to determine which coyotes were residents who had completed dispersal and which coyotes were transients who had yet to establish a home range. To determine whether a dispersed coyote was a resident, we calculated the area of the minimum convex polygon for all of the offspring using successively greater proportions of their location data, starting with the location data collected in their first 2 weeks of tracking and increasing the time frame by 2 weeks until their entire tracking period was included. We looked for individuals whose home range size reached an asymptote (Supplementary Appendix 1). We interpreted the asymptote as an indication that an animal was using the same areas throughout its tracking period and was a resident animal. Once animals were determined to be residents, their transience distances were calculated by measuring the distance between the centroid of the natal and adult home ranges using the package *rgeos* in R (*v0.5.5*, Bivand et al. 2017).

To test the relationship between dispersal distance and proportion of developed habitat in the natal home range, we constructed a GLM using a gamma distribution for the outcome variable, dispersal distance. Proportion developed habitat in natal home range, proportion developed habitat in available habitat, and sex were included as fixed effects.

We used three alternative methods to identify the area (and thus proportion of developed habitat in that area) available to coyotes during dispersal and settlement (Supplementary Appendix 2). The first, the dispersal habitat method, is best suited for coyotes with sufficient location data during dispersal (i.e., post-departure and pre-settlement). This method allowed us to evaluate the actual habitat experienced by the coyote during dispersal. For the dispersal method, we combined the location data of offspring (this included data prior to the 6 month subset period) and the location data of their parents from the year the focal coyote was born. We calculated the 100% MCPs using this combined dataset and then removed the natal home range since this area was not available to the coyotes for dispersal (our definition of dispersal excludes the natal home range). This method, however, is not informative in cases where a coyote had no or few location data collected during this time. The individualized dispersal distance method was ideal for individuals who dispersed intermediate distances because it incorporates the habitat along the direct path from the focal coyote's natal home range to their adult home range, while also including areas where the coyote may have made exploratory bouts within a radius defined by its actual dispersal distance. For the individualized dispersal distance method, we drew a circle of available habitat around the centroid of the natal home range with a radius equal to the dispersal distance of the focal coyote. However, particularly for individuals that settled long distances from their natal home range, the individualized dispersal distance method likely included areas that the animal did not actually experience before settling. Finally, the median dispersal distance method was useful for coyotes who dispersed short distances because it included more habitat than the dispersal habitat and individualized dispersal distance methods, accounting for exploratory bouts the coyotes likely made. For the median dispersal distance method, we drew a circle of available habitat around the centroid of the natal home range with a radius equal to the median dispersal distance of all dispersal distances in the sample. While each of these methods has weaknesses, our confidence in results is enhanced if the different methods produce the same qualitative results. We ran three versions of the dispersal distance model, one for each of the three different habitat availability metrics.

Settlement: natal habitat preference induction

To determine if coyotes from the Chicago metropolitan area experience NHPI, we examined the relationship between the proportion of developed habitat in the natal home range, the adult home range, and in available habitats that the individual coyote could have potentially settled in. The latter is important for testing whether the habitat type in the offspring's adult home range resembles its natal home range more than we would expect by random chance. This analysis included 19 resident dispersers.

We examined the relationship between proportion of developed habitat in the natal and adult home ranges by constructing a linear model using the Manly-Chesson index α as the outcome variable (Chesson 1978; Minder and Pyron 2018). The Manly-Chesson index α is calculated as follows:

$$\alpha = \frac{r_i/p_i}{\sum r_i/p_i}, i = 1, ..., m$$

Where r_i = the proportion of used habitat type i, p_i = the proportion of available habitat type i, and m = the number of habitat types. Here, we simplified habitat types into two types: developed versus natural, that is, $m = 2$. We calculated the Manly-Chesson index α for developed habitat. If $\alpha = 0.5$ then habitat is used randomly; there is no preference. If $\alpha > 0.5$ developed habitat is selected for and if $\alpha < 0.5$, developed habitat is avoided. We included the proportion of developed habitat in natal home range and sex as fixed effects in the model. We ran three separate NHPI models, one for each of the different availability metrics. NHPI is supported if there is a positive relationship between developed habitat in the individual's natal home range and α, its preference for developed habitat.

Mortality

Of the 85 coyotes in the original dispersal analysis, 48 were recovered postmortem. Mortality data for these coyotes included approximate date of death and the suspected cause of death. In particular, we were able to identify mortality due to vehicle collisions with high confidence. With these 48 coyotes, we conducted two mortality analyses. The first assessed the relationship between survival to adulthood, that is, 2 years, and developed habitat in the natal home range. We constructed a Cox proportional

hazards regression model, including the proportion of developed habitat in the natal and adult home ranges and sex as fixed effects. The second analysis assessed the relationship between mortality due to vehicle collision and developed habitat in the natal home range. We ran a GLM with the binomial outcome variable, death by vehicle or by other cause. Proportion developed habitat in natal and adult home ranges were included as fixed effects.

Statistical analysis

Statistical analyses using linear models were performed using the *stats* package (*v 3.6.2;* R Core Team 2020). We report parameter estimates, standard errors, *t*-values, and *P*-values for parameters in these models. GLMs were formatted with the package glmmTMB (*v 0.2.3*; Brooks et al. 2017). The Cox proportional hazards regression model was performed using the *survival* package (Therneau 2020). We report parameter estimates, standard errors, *z*-values, and *P*-values for parameters in these models.

RESULTS

Departure

To assess the influence of experience in the natal home range on the propensity of coyotes to disperse, we analyzed the relationship between the proportion of developed habitat in the natal home range and departure. Of the 85 coyotes included in the analysis, 22 had no overlap between their adult and natal home ranges, satisfying our criteria for dispersal (see Table 1 for the dispersal status of all coyotes). Of those 22 coyotes, 14 had natal home ranges that consisted of more than 50% developed habitat. According to our model, proportion of developed habitat in the natal home range was positively associated with dispersal tendency (estimate = 2.591 ± 1.130, z = 2.292, P = 0.022). Coyotes with the largest proportion of developed habitat in their natal home range (0.97) were 2.7 times more likely to disperse than coyotes with the smallest proportion of developed habitat in their natal home range (0.22; Figure 2). Sex was not a significant predictor of dispersal (estimate = −0.705 ± 0.522, z = −1.350, P = 0.177).

Transience

We analyzed the influence of experience in the natal home range on the transience behavior of coyotes by examining the relationship between the proportion of developed habitat in the natal home range and dispersal distance. Dispersal distances in this study ranged from 1.7 to 60.0 km (mean = 18.1 ± 3.7 km; median = 8.1 km). The proportion of developed habitat in the natal home range had a significant and positive relationship with dispersal distance in models using each of the three availability methods (Table 2). The model using the dispersal habitat method predicted that coyotes with the highest proportions of developed habitat in their natal home range would travel 3.9 times as far as coyotes with the lowest proportions of developed habitat in their natal home range (Figure 3). The proportion of developed habitat in habitat available while in transience was only a significant predictor in the model using the median dispersal distance method for determining habitat availability. Based on this model, the proportion of developed habitat in both the coyotes' natal habitat and in available habitat during transience tended to be associated with longer dispersal distances. Sex was not a significant predictor in any of the models.

Table 1

The dispersal status of the 85 coyotes included in the departure analysis. Coyotes categorized as "successfully dispersed" were those who exhibited no natal home range overlap. Animals categorized as "dispersal incomplete" exhibited natal home range overlap but were recovered postmortem outside of their natal home range. Animals who were categorized as "did not disperse" were animals who exhibited overlap with their natal home range and who died in the natal home range. Finally, animals with an "unknown" dispersal status were those who exhibited natal home range overlap and who were not recovered postmortem

Dispersal Status	Number of coyotes
Successfully dispersed	22
Dispersal incomplete	24
Did not disperse	12
Unknown	27

Settlement

To understand if experience with the natal home range habitat influences preference for that habitat type during settlement, we analyzed the relationship between natal home range habitat type and the selection for developed habitat in the adult coyotes. Interestingly, the proportion of developed habitat in the natal home range was only a significant predictor of selection for developed habitat in one of the models (Table 3). With this model, a higher proportion of developed habitat in the natal home range was associated with a stronger preference for developed habitat in the adult home range; however, overall selection for developed habitat was weak. Individuals with the lowest levels of developed habitat in their natal home range were predicted to exhibit a strong avoidance of developed habitat (α = 0.10) while animals with an average proportion of developed habitat in their natal home range still slightly avoided developed habitat (α = 0.44). Animals with the highest levels of developed habitat in the natal home range were still predicted to exhibit only a weak preference for developed habitat (α = 0.58). Sex was not a significant predictor in any of the models.

Mortality

We analyzed the relationship between survival to adulthood and natal habitat type. Survival to adulthood (age 2) was quite high with only 11

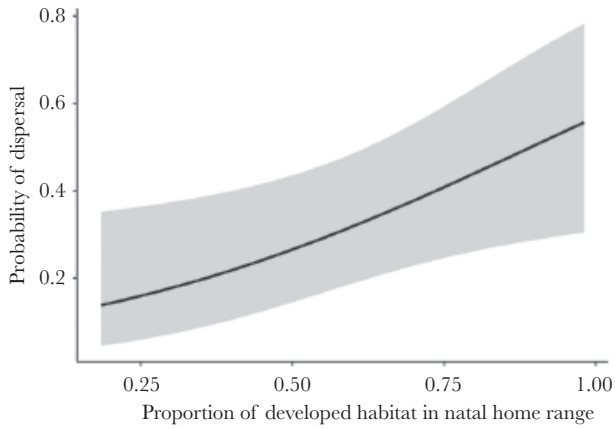

Figure 2

Model predicted effect of the proportion of developed habitat in the natal home range on departure. The model included sex as a fixed effect. Shaded region is the 95% confidence interval.

Table 2

Parameter estimates for the dispersal distance analysis. The model (distance ~ 1 + natalDeveloped + availableDeveloped + sex) included distance dispersed (distance; km) as the outcome variable. The proportion of developed habitat in the natal home range (natalDeveloped) and in the available habitat (availableDeveloped) and sex (sex) were included as fixed effects. The analysis was conducted three times, each with a different method for determining habitat availability

Availability metric	Predictor	Estimate	Std. error	*z* value	*P* value
Dispersal habitat method	(Intercept)	−0.098	0.575	−0.171	0.865
	natalDeveloped	1.819	0.659	2.761	0.006**
	availableDeveloped	1.832	0.740	2.474	0.013*
	sex (m)	−0.073	0.336	−0.219	0.827
Individualized dispersal distance method	(Intercept)	0.164	0.989	0.166	0.868
	natalDeveloped	2.095	0.696	3.011	0.003**
	availableDeveloped	1.065	1.190	0.895	0.371
	sex (m)	0.019	0.435	0.045	0.964
Median dispersal distance method	(Intercept)	1.145	2.910	0.394	0.694
	natalDeveloped	2.297	0.787	2.917	0.004**
	availableDeveloped	−0.279	3.690	−0.076	0.940
	sex (m)	−0.180	0.380	−0.474	0.636

*<0.05; **<0.01.

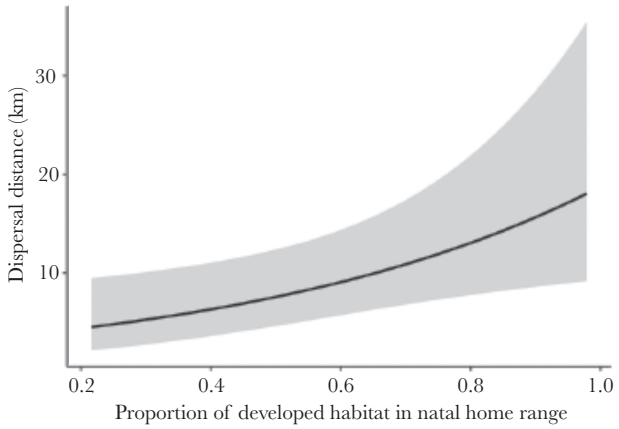

Figure 3

Model predicted effect of proportion of developed habitat in the natal home range on dispersal distance. The model included proportion of developed habitat in available habitat as a fixed effect. Predictions depicted here were generated using the dispersal habitat method for determining available habitat. Shaded region is the 95% confidence interval.

of 48 coyotes dying before reaching adulthood; however, survival (or not) to adulthood was not explained by either the proportion of developed habitat in the natal home range (estimate = −0.779 ± 0.773, z = −1.007, P = 0.314), the proportion of developed habitat in the adult home range (estimate = 1.414 ± 0.761, z = 1.859, P = 0.063), or sex (estimate = −0.222 ± 0.272, z = −0.816, P = 0.414).

To understand if experience with developed habitat in the natal home range influenced the likelihood a coyote experienced a vehicle related death, we analyzed the relationship between natal home range habitat type and vehicle related mortality. Twenty-eight of the 48 coyotes included in the mortality analysis died after being hit by a vehicle. Neither the proportion of developed habitat in the natal home range (estimate = −0.529 ± 1.519, z = −0.349, P = 0.727) nor the proportion of developed habitat in the adult home range (estimate = −0.789 ± 1.633, z = −0.484, P = 0.629) had a significant effect on the probability of a coyote experiencing a vehicle related death.

DISCUSSION

We used VHF data from a long term study of urban coyotes to explore the relationship between early life experience with urbanization and dispersal and mortality. We found evidence that coyotes from natal home ranges with more developed habitat were more likely to disperse than animals from primarily natural habitats. Contrary to our prediction, of the dispersing coyotes, coyotes with more developed habitat in their natal home range dispersed farther. Models using different habitat availability metrics to explore the relationship between natal habitat type and adult habitat selection produced mixed results; however, we found some evidence that coyotes experience NHPI. Finally, we found no evidence that experience with developed habitat in the natal home range influences survival to adulthood or the likelihood of experiencing a vehicle-related mortality.

Early life experience may shape juvenile coyotes' perceptions of habitat quality and their willingness to depart from the natal home range. While urban wildlife generally avoid humans and their associated landscapes, there is evidence that avoidance is plastic and varies with experience (Kitchen et al. 2011; Vincze et al. 2016). Studies comparing the behavioral traits of urban and rural coyotes found that urban coyotes tend to be bolder and more exploratory toward humans and novel objects (Breck et al. 2019; Brooks et al. 2020). Additionally, Schell et al. (2018) identified a flexible, transgenerational mechanism for human tolerance in coyotes where successive litters born to the same mated pair exhibited increased habituation towards humans as the parents' became more habituated . In addition to responses to individual environmental features, larger scale changes in preference for human altered habitats have been observed in other mammalian species (Knopff et al. 2014; Scrafford et al. 2017). Raccoons in more highly urbanized areas show increased selection for human-use areas, which may be a response to their experience using anthropogenic resources in these areas (Bozek et al. 2007). Because the decision to leave the natal home range is dependent on perceptions of habitat quality, experience with more or less urbanization in the natal home range is likely influencing what the animal perceives as the optimal decision (Bowler and Benton 2009). Animals who primarily experience natural habitat in their early life might perceive the surrounding matrix as more hostile decreasing their motivation to initiate dispersal relative to those with more experience in urban habitats.

In addition to environmental factors, social interactions play an important role in dispersal (Wey et al. 2015). For coyotes, intrapack interactions are particularly important in determining departure behavior (Bekoff 1977). Mated pairs will often engage

Table 3

Parameter estimates for the natal habitat preference induction analysis. The model (selectionDeveloped ~ 1 + natalDeveloped + sex) included the Manly-Chesson index α (selectionDeveloped) as the outcome variable. The proportion of developed habitat in the natal home range (natalDeveloped) and sex (sex) were included as fixed effects. The analysis was conducted three times, each with a different method for determining habitat availability

Availability metric	Predictor	Estimate	Std. error	*t* value	*P* value
Dispersal habitat method	(Intercept)	0.365	0.174	2.104	0.052
	natalDeveloped	0.219	0.223	0.985	0.340
	sex (m)	0.012	0.117	0.106	0.917
Individualized dispersal distance method	(Intercept)	0.045	0.247	0.181	0.859
	natalDeveloped	0.578	0.317	1.825	0.087
	sex (m)	0.147	0.166	0.886	0.389
Median dispersal distance method	(Intercept)	−0.021	0.227	−0.092	0.928
	natalDeveloped	0.620	0.292	2.127	0.049*
	sex (m)	0.055	0.153	0.357	0.726

*<0.05; **<0.01.

in antagonistic interactions with yearlings or older offspring during the mating season, driving them out of the natal home range. However, increased resource availability in natural areas may reduce social pressure to disperse. Interestingly, anecdotal evidence for alternative "dispersal" behavior has been observed in this system. In nature preserves, some coyotes have been observed to take over part of their natal home range causing shifts in their parents' home range away from that area (Gehrt, unpublished data). This behavior is likely less frequent or absent in developed habitat particularly if lower resource availability results in increased competition and social pressure to disperse (Gese et al. 1996).

Transience is risky (Bowler and Benton 2009). In undisturbed areas, dispersers are more likely to die during transience than their nondispersing counterparts and this increases with distance travelled during transience (Bekoff and Wells 1986; Bonnet et al. 1999; Letty et al. 2000; Meek et al. 2003; Moehrenschlager and McDonald 2003). In urban areas, risk may be enhanced as animals are required to navigate through unfamiliar matrix habitat where various human and vehicle related dangers are common. Dispersal distances in this study (mean: 18.1 ± 3.7 km) were substantially shorter than mean distances observed in less disturbed areas, which range from 51 to 310 km (Harrison 1992; Kolbe and Squires 2004; Sasmal et al. 2019). In our study, data collection methods bias the sample toward individuals who disperse within the area of high tracking effort. However, other studies indicating that habitat fragmentation inhibits animals' movement suggest that the patchy, developed landscape of the Chicago metropolitan area may also contribute to shorter dispersal distances (Tucker et al. 2018). Shorter dispersal distances in response to human activity or development could reduce gene flow and thus facilitate evolutionary adaptation to urbanization. In particular, if human altered habitats act as a barrier to wildlife dispersing from more remote habitats in the same way that they inhibit the dispersal movements of animals in our study, urban populations may undergo microevolution at a more rapid rate than would be expected if immigration rates remained at undisturbed levels (Sol et al. 2013; Miranda 2017; Adducci et al. 2020). Although for a behaviorally flexible generalist like the coyote, phenotypic plasticity is generally considered the most salient response to human disturbance, in many taxa, genetic adaptations are also important (Atwell et al. 2012; Miranda et al. 2013; Mueller et al. 2013; Alberti et al. 2020; Lambert et al. 2020).

We found that of the coyotes who did disperse, those with more urban development in their natal home range tended to disperse farther. Larger home ranges in developed habitat may force coyotes from these areas to disperse farther to find suitable, unoccupied habitat (Gehrt et al. 2009). While this may put developed coyotes at a disadvantage due to the risks they might face travelling long distances through the urban matrix, given coyotes' high degree of behavioral plasticity and their early life experience with developed habitat we hypothesized that these coyotes are better at navigating these risks. Studies examining the relationship between behavioral responses to human disturbance and previous experience have found that experience with humans and human altered habitats plays an important role in shaping adaptive behavior (Zaccaroni et al. 2007; Thurfjell et al. 2017). Despite this evidence, our mortality analyses did not indicate that this phenomenon occurs in these coyotes. This might be attributed to our limited sample size. However, it is possible that early life experience with anthropogenic risks and increased exposure to anthropogenic risks during dispersal have additive effects in developed coyotes resulting in no difference in the likelihood that they and their natural counterparts will survive to adulthood or experience a vehicle related mortality.

While previous studies have suggested coyotes may experience NHPI (Sacks et al. 2004, Sacks et al. 2008), the mixed results in our study are open to multiple interpretations. If the median dispersal distance method for establishing availability is most accurate, coyotes may exhibit NHPI. In that case, habitat preferences formed in the natal home range could have important population-level effects. For instance, if only coyotes with the highest proportion of developed habitat are settling in highly urbanized areas and coyotes born in more natural versus more developed habitat within the Chicago metropolitan area are dispersing to and mating with individuals from the same habitats, this assortative mating could change the scale at which microevolution might act as an adaptive mechanism (Richardson et al. 2014). Instead of interbreeding between all coyotes in the region, genetically distinct subpopulations within highly urbanized areas may be undergoing selection specific to that habitat. In addition to its effects on individual fitness and population dynamics, it could also have implications for human–coyote interactions. Many of the behavioral traits that allow animals to take advantage of novel opportunities and cope with novel challenges in urban environments are also traits that increase the likelihood an animal interacts with humans (Barrett et al. 2019). Studies comparing traits including neophobilia and boldness in urban and rural passerines found evidence that some of the differences in behavior between populations can be attributed to

microevolution (Atwell et al. 2012; Miranda et al. 2013; Müller et al. 2013). Associative breeding among the least neophobic and most bold coyotes has the potential to enhance human-coyote conflict in these areas.

Despite some evidence for NHPI, the lack of a significant relationship between natal habitat type and selection during settlement in our other two model suggests that in these coyotes, the phenomenon may not be occurring or may at best, be weak. There are a number of explanations for an absence of NHPI. Coyotes may have an innate preference for natural areas that outweighs early life experiences. Again, this is supported by previous research that indicates coyotes prefer natural or rural areas to urban areas (Tigas et al. 2002; Gehrt et al. 2009; Gese et al. 2012; Poessel et al. 2016; Wang et al. 2017, Ellington and Gehrt 2019). Alternatively, given the high costs of searching for and establishing a new home range, other intrinsic characteristics like condition or extrinsic factors like competition may drive settlement decisions rather than previous experience with a given habitat type (Clobert et al. 2009; Rémy et al. 2014; Wey et al. 2015). Finally, for animals that exhibit high levels of plasticity, if early life experience is not reinforced, its effects on behavior may be updated to reflect more recent experiences. This has been shown in rats (*Rattus spp.)* whose behavioral reactivity to stress, which increases after experiencing early life maternal separation, can be reversed with environmental enrichment (Francis et al. 2002). The results of these two models agree with a number of studies that found no evidence of NHPI suggesting innate preferences or experiences other than the natal habitat may be more important for shaping dispersal behavior in some species (Reiskind and Zarrabi 2013; Ousterhout 2014).

CONCLUSION

Despite dispersal's importance at various levels of biological organization, few wildlife studies have examined the stages of dispersal beyond departure and even fewer have looked at this behavior in an urban setting. We show that early life experience with urbanization influences departure, transience, and potentially settlement behavior. While we did not find that these differences resulted in changes to survival to adulthood, we suspect that they may have implications for individual fitness, population structure, and human–wildlife interactions.

Future studies should focus on fine scale heterogeneity in intrinsic and extrinsic factors associated with dispersal. Intrinsic traits like condition and behavioral type are important factors that interact with the environment to produce variation in dispersal behavior. Additionally, more detailed information about the natal and matrix environments should be considered. While we were constrained to two habitat types based on our sample size, considering multiple dimensions of environmental variability like vegetation density and type, level of impervious surface cover, patch size, human population density, road density, and traffic rate will allow the identification of specific urban environmental factors that drive differences in dispersal behavior. These are lost when the landscape is dichotomized into categories like developed and natural or urban and rural. Combining detailed features about the animal and its environment may help identify how they interact to produce dispersal behavior. Finally, gaining a deeper understanding of how various dispersal strategies are associated with survival and fitness will be crucial in identifying which dispersal responses are truly adaptive and how those strategies influences dynamics at the various levels of biological organization.

SUPPLEMENTARY MATERIAL

Supplementary data are available at *Behavioral Ecology* online.

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Data availability: Analyses reported in this article can be reproduced using the data provided by Zepeda et al. (2021).

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Cook County Department of Animal and Rabies Control Environmental Impact Program Annual Report 2021

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Disease surveillance is a critical component of ongoing and effective wildlife management and has benefits for public health. Surveillance improves understanding of wildlife disease dynamics, the effect of disease on pass-through migratory species and permanent resident animals, and, probably of utmost importance, facilitates monitoring for emergence/re-emergence of novel diseases of concern to wildlife, domestic animal, and public health communities in Cook County. The majority of infectious diseases of concern for public (human) health have wildlife reservoirs. As the current coronavirus (COVID-19) pandemic highlights, overlooking disease in animals that could spill over to humans has enormous personal and economic costs. Notably, University of Illinois Zoological Pathology Program (ZPP) has been on the frontlines of surveillance for SARS-CoV-2 spillback to wildlife and zoo animals throughout the country. Monitoring for spillback is critical as mutation and/or recombination with other viruses could result in a variant that has even more severe consequences for public health.

The ZPP, in cooperation with personnel from Cook County Animal and Rabies Control and the Forest Preserve District of Cook County (FPDCC), has been monitoring wildlife diseases in Cook County since 1993. The multidisciplinary knowledge accumulated through decades of wildlife and habitat study has allowed for major advances in evaluation of ecosystem health and recognition of diseases of concern to public health.

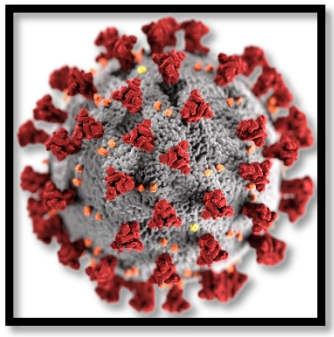
In 2021, ZPP evaluated carcasses from 202 animals of the following species and groups:

Canids (coyote, fox and dog)	11
Mesocarnivores (raccoon, skunk, otter, mink)	52
Cervids (deer and elk)	1
Fish	85
Reptile/amphibian	27
Avian	21
Other Mammal (rodent, marsupial)	5

Continued surveillance endeavors are important given disease presence and risk of spread in the County. Pathogens of particular concern, potentially affecting humans, domestic animals and wildlife, notably include rabies, chronic wasting disease (CWD), influenza, leptospirosis, West Nile Virus, SARS-CoV-2, and tick-borne diseases. Surveillance is also important to assess the health of ecosystems utilized for public recreation. In 2021, ZPP finalized a study looking at environmental contaminants (PCBs and Mercury) in fish from lakes in Cook County (see attached manuscript).

The following cases from the past year further exemplify the approach and application of results for the betterment of human and animal health in Cook County.

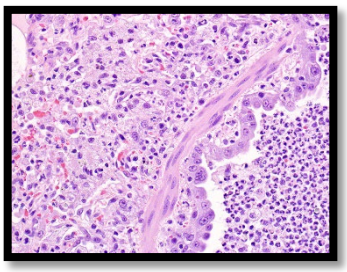
SARS-CoV-2, the virus that causes COVID-19 in humans, is a known zoonotic agent having likely originated in animals. For much of the pandemic it has been known that cats, mink and dogs could be infected, and surveillance has been targeted towards these species. Deer have also now been shown to be able to get infected and shed the virus representing a possible reservoir for the virus in local wildlife. In November 2021, SARS-CoV-2 infection hit one of the zoos within Cook County and infected coatis, a procyonid native to South America that is closely related to raccoons. Given the close relatedness of the coati to raccoons and the large numbers of raccoons in the region, surveillance was initiated to screen raccoons for infection. To date, there are no confirmed cases of SARS-CoV-2 infection in raccoons.



Chronic wasting disease (CWD) is a debilitating neurological disease of free-ranging elk and deer caused by a prion (infectious protein) similar to bovine spongiform encephalopathy ("mad cow" disease). All deer are specifically evaluated for CWD. Statewide in FY 2021 there were 1,165 CWD-positive deer from both hunter-killed and intensive culling/surveillance programs (IDNR CWD Annual Report FY 2021). This represents an almost *10-fold increase from FY 2020*. In Cook County, 3 positive deer were identified through the IDNR surveillance **program**. Targeted surveillance of deer in collaboration with the FPDCC to further assess the prevalence of CWD in the county could not be performed due to COVID but is planned for 2022.

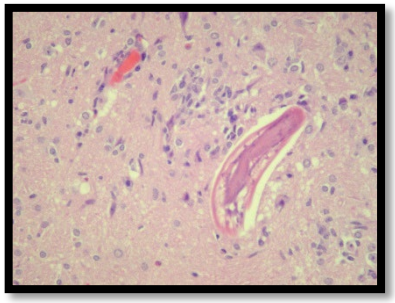
Leptospirosis is a disease caused by the bacteria *Leptospira interrogans*. The bacteria can survive within water and soil for weeks and can be shed into the environment by infected wildlife species. People and companion animals can be infected directly from other animals or by ingestion of contaminated water or soil in the environment. In both people and companion animals, infection can result in kidney and liver damage, even failure. ZPP has been conducting a multi-year study into leptospirosis in Cook County to better understand the role of wildlife in disease ecology. Our surveillance has identified squirrels as a new species of concern for leptospirosis monitoring for and another potential vector for transmission. There were no identified cases of leptospirosis in submitted wildlife in 2021.

Canine distemper virus (CDV) is a virus that affects carnivores causing pneumonia (image on left) and encephalitis. The virus is normally present in Cook County carnivores and causes sporadic deaths of raccoons, foxes, skunks and coyotes. However, in 2004, there was a large CDV outbreak that spilled over into domestic dogs both in shelters and those that were privately owned. Any disease that causes encephalitis can alter an animal's behavior and increase the risk of unprovoked bites and attacks warranting public health concern. In 2021 there were 38 cases of CDV primarily in raccoons and skunks. Many of the affected animals had neurologic signs ("zombie raccoons"). Timely reporting and confirmation of the



cause to CCARC allowed messaging to the public. With the westward geographic progression of raccoon rabies, differentiation between rabies and CDV will be critical. The ZPP laboratory has developed multiple tests for rapid diagnosis of CDV, and our parent lab, the VDL, is an approved testing site for rabies in Illinois.

In collaboration with EIRG partners, ZPP is monitoring raccoon populations for the parasite ***Baylisascaris procyonis***. The parasite is a normal inhabitant of the raccoon gastrointestinal tract.



Eggs are shed in the feces of raccoons and can develop into infective larvae in the environment. These larvae can infect humans. Following ingestion, larvae migrate through the gut wall into various tissues. Most concerning is that the larvae often migrate to the brain (image on left) where they cause encephalitis and extensive damage despite treatment. Infections can be fatal or cause profound neurologic disabilities. Children are at increased risk of infection when they place potentially contaminated objects and fingers into their mouths. Ongoing research has been studying the distribution and seasonality of infections in raccoons to determine risk factors for human infection.

2021 marked the final year in a 5 year active surveillance aimed at assessing **general fish health** in a selection of Cook County inland lakes regularly used for recreational fishing by the public.

This work is in collaboration with the Forest Preserves of Cook County Fisheries Management Division. All collected fish received full postmortem examinations including gross necropsy, wet-mount cytologic assessment for gill and skin parasites, and complete histopathology. Examination has revealed varying levels of parasitism in the fish (wet mount image on left), however, no changes to indicate an associated health detriment have been noted. Importantly, no lesions considered to pose risk for human consumption were detected in any fish species, from any lake, at any time point during the survey. In all examined fish, skeletal muscle (flesh) was free of inflammation and other tissue damage. Results of this research are currently being collated and analyzed for publication.

The fish health surveillance program has also served as the basis for a Master's student project correlating levels of tissue contaminants on fish health. In the Forest Preserves of Cook County, all of the lakes are currently under state-issued fish consumption advisories due to high levels of tissue toxicants including mercury (Hg) and polychlorinated biphenyls (PCBs). This manuscript was published in 2021 and is attached to this report.

Other ZPP activities have focused on surveillance for diseases of concern including rabies, West Nile Virus, avian influenza (bird flu), SARS CoV-2 spillover in wildlife, tularemia, yersiniosis, and epizootic hemorrhagic disease in deer.

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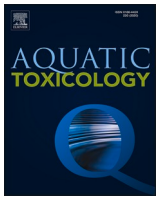

Pathologic impacts of contaminants in freshwater fish of Cook County IL

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Lake

ABSTRACT

Mercury (Hg) and polychlorinated biphenyls (PCBs) are widespread environmental toxicants in urban environments with negative impacts to fish health. The present study evaluated the potential association between muscle tissue contaminant (total Hg and total PCB) concentrations and indicators of health in benthic and predatory fish collected from four Forest Preserves of Cook County lakes in the Chicago metropolitan area. Common carp (carp; *Cyprinus carpio*) and largemouth bass (LMB; *Micropterus salmoides*) were sampled three times a year (spring, summer, fall) during 2019 and 2020. Water quality analyses (temperature, color, turbidity, dissolved oxygen, total alkalinity, pH, chloride, nitrate, phosphate, ammonium, and pH) were performed concurrently with fish collections. Tissue (skin-on fillet) contaminant concentrations were compared between lake types and fish species and assessed for any relationship with fish morphometric data and pathologic lesions. Main health indicator endpoints included muscle lipid content, parasite burden, and pathologic lesions. Mean total PCB concentrations were greater in carp (203.1 ± 152 μg/kg, wet weight), and mean Hg concentrations were greater in LMB (0.11 ± 0.1 mg/kg, wet weight). In most fish, concentrations of both toxicants surpassed the EPA's lowest threshold to restrict fish consumption for sensitive cohorts (0.029 mg/kg for Hg and 1.5 μg/kg for PCBs). In both species, Hg positively correlated with splenic pigmented macrophage aggregate area ($P < 0.001$). In carp, Hg also positively correlated with hepatocellular pigmentation ($P < 0.01$). Mercury correlated with standard length in both species (LMB: $P < 0.001$, carp: $P = 0.95$), but polychlorinated biphenyls only correlated with standard length in carp ($P < 0.001$). No association was found between intraspecific contaminant concentrations and parasite burden, year, or lake type, though differences were noted among individual lakes. The contaminant burden appeared well-tolerated with only mild Hg-associated and no appreciable PCB-associated lesions. However, possible effects on reproduction or behavior were not fully assessed, and future studies are warranted.

1. Introduction

Widespread contamination of freshwater fish with mercury (Hg) and polychlorinated biphenyls (PCBs) is a global issue with known harmful effects on human and environmental health (US Environmental Protection Agency [USEPA], 2000). Investigations into aquatic toxicants are often focused on human health, and more research is necessary to better understand implications to fish health, particularly in the heavily industrialized Chicago area. The Forest Preserves of Cook County (FPCC), Chicago, Illinois, contains over 40 bodies of water, all of which are currently under state-issued fish consumption advisories due to high concentrations of tissue toxicants (Illinois Department of Public Health [IDPH], 2021).

Within the FPCC, there are two lake types, seepage and impoundment. Seepage-type lakes have no inlet or outlet and derive most of their water from precipitation and runoff, whereas impoundment-type lakes, (formed by damming a slow-moving body of water), have an inlet and outlet and derive most of their water from stream drainage. Because of differences in hydrology, these two lake types can have differences in total surface area for run-off, an important source of aquatic pollution. Additionally, variation in pH and other water chemistry parameters among water bodies could affect contaminant availability. Previous

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studies have noted associations between water chemistry and watershed influxes and tissue contaminant concentrations in fish, though the importance of each of these factors may have wide regional variation (Wiener et al., 1990).

The major source of environmental Hg in the Chicago area is from coal-burning power plants (Gratz et al., 2016). Nationwide, power plants contribute approximately 40% of atmospheric mercury (Olson et al., 2020). In aquatic environments, inorganic mercury can be converted in the water column and in sediment by sulfate-reducing bacteria to a more bioavailable and toxic form, methylmercury (MeHg), which is the predominant form in fish tissues (Olson et al., 2020). Mercury bioavailability is influenced by several water quality parameters, and is negatively correlated with pH, dissolved oxygen (DO), calcium, alkalinity, total hardness, conductance, total nitrogen, and total phosphorous (Lange et al., 1993). Due to its high affinity binding to proteins, MeHg crosses gastrointestinal and blood-brain barriers, has a very low excretion rate, and thus results in both bioaccumulation and biomagnification (Bjørklund et al., 2017). The highest concentrations of MeHg are found in renal, neural, and hepatic tissue (Bjørklund et al., 2017). Though not fully elucidated, the predominant mechanism of MeHg toxicity is believed to be due to enzyme inhibition through high affinity binding to protein sulfhydryl groups (Tchounwou et al., 2012). Methylmercury is also pro-oxidative with the potential to manifest in neurotoxicity, hepatotoxicity, gastrointestinal toxicity, and nephrotoxicity (Tchounwou et al., 2012). In humans, MeHg toxicity leads to neurologic symptoms, primarily in developing fetuses and children (Trasande et al., 2005). In fish, high dietary exposure in experimental studies have been associated with metabolic, necrotizing, and/or reparative lesions involving the liver and/or kidney (Mela et al., 2007; Oliveira Ribeiro et al., 2002). Both laboratory- and field-based studies have associated tissue Hg concentrations with increased hepatic pigment and/or increased pigmented macrophage aggregates (PMAs; Barst et al., 2016, Barst et al., 2011; Drevnick et al., 2008; Meinelt et al., 1997; Mubarokah et al., 2018; Müller et al., 2015)

Polychlorinated biphenyls are a group of over 200 related, highly stable, synthetic chemicals individually termed “congeners”. Congener mixtures were produced and sold under the trade name Aroclor and had widespread industrial uses in dielectric and coolant fluid (US Dept of Health and Human Services [HHS], 2000). Despite a 1978 production ban due to their recognition as persistent environmental pollutants, PCBs continue to be a widespread environmental contaminant of concern (HHS, 2000). Toxicity of individual PCB congeners is influenced by the position (coplanar or non-coplanar) of chlorine atoms on the biphenyl group. Coplanar PCBs may cause immunotoxicity, endocrine disruption, and mutagenic effects in humans through agonism of the aryl hydrocarbon receptor (Faroon and Ruiz, 2016; Takeuchi et al., 2017). Non-coplanar PCBs may impair thyroid function, resulting in decreased immune function and survival (Faroon and Ruiz, 2016). In fish, PCB toxicity resulting from environmental exposure has been primarily associated with reproductive lesions such as follicular atresia and hepatic lesions such as foci of cellular alteration (a putative preneoplastic lesion) and neoplasia (Barron et al., 2000; Dey et al., 1993). Although most experimental exposure studies have been mainly performed using select PCB congeners at high concentrations, an experimental study mimicking environmental situations (in terms of doses, composition, and congener distribution) had similar reproductive lesions (Daouk et al., 2011). This study is the first environmental study investigating the effects of these contaminants on fish health in the Chicago metropolitan area.

1.1. Objectives

The primary objective of this study was to investigate associations between tissue contaminant (total Hg and PCB) concentrations and indicators of health in common carp (carp; *Cyprinus carpio*, a benthic omnivorous species) and largemouth bass (LMB; *Micropterus salmoides*, a highly predatory species) within the FPCC inland lakes. Secondary objectives of this study were to compare fish tissue contaminant concentrations by lake type (impoundment vs. seepage), species (trophic level), and morphometric data.

2. Materials and methods

2.1. Fish and water sampling

Carp and LMB were sampled in May, August, and October 2019–2020 from four locations (Fig. 1). Fish were collected from both seepage (Axehead and Powderhorn) and impoundment (Busse and Tampier) lakes. The fish and water quality data used in this study were collected by FPCC fisheries biologists as part of the FPCC’s ongoing Fish Health Surveillance Program (FHSP). Fish were presented euthanized to the University of Illinois Zoological Pathology Program for diagnostic necropsy. Water samples were collected from each lake at the time of fish collection, and FPCC fisheries biologists reported results for the following indicators: temperature, color, turbidity, dissolved oxygen (DO), total alkalinity, chloride (Cl^-), nitrate (NO_3), phosphate (PO_4), ammonium (NH_4) and pH.

2.2. Necropsy procedures

Total length (TL, cm), standard length (SL, cm), and weight (g) were recorded. Wet mount preparations of skin scrapes and gill clips were evaluated cytologically for lesions and ectoparasites using light microscopy. External and internal examinations were performed, and sections of gill, heart, brain, liver, spleen, stomach (for LMB), intestine, gonad, body wall, skin, and eye were preserved in 10% neutral buffered formalin (NBF). Sex was determined for mature fish. Fresh skin-on fillets were collected, placed in individual sterile lab-grade plastic bags (Whirl-Pak), and frozen at -20 °C. A separate sample of skin-on fillet was preserved in 10% NBF for histopathology.

2.3. Contaminant analysis

Fillet samples (>10 g) from each fish ($N = 86$) were transferred frozen to Pace Analytical Services, LLC (Green Bay, Wisconsin, USA) for tissue homogenization and contaminant analysis. Total Hg analysis was performed by thermal decomposition, amalgamation, and atomic absorption spectrophotometry according to the USEPA Method 7473 (USEPA, 2007a). Analysis of total Hg instead of MeHg in fish tissue is recommended by the USEPA due to the cost-prohibitive nature of MeHg analysis and because the large proportion (variable, though typically 95–99% of Hg in tissues of larger fish is present as MeHg (USEPA, 2007a; Lescord et al., 2018). Polychlorinated biphenyl (total and by Aroclor) analysis was performed by gas chromatography and high-resolution mass spectrometry (Method 8082A; USEPA, 2007b). Undetectable concentrations were estimated at half of the detection limit, which was the same for all PCB congeners. Mercury concentration values above the calibration curve were extrapolated using the lower calibration curve. Percent lipid was reported with contaminant data and was used as a measure of body proximate condition (Kaufman et al., 2007).

All PCB method blanks and 61% of Hg method blanks were beneath the detection limit, and all were beneath the reporting limit. Method blank values were subtracted from their respective analyte values prior to analysis. Most (99%) matrix spike recoveries and matrix spike duplicate recoveries ranged from 75 to 105%. Certified reference material (raw chicken breast) recoveries were all within quality control limits (75–105%). Polychlorinated biphenyl and Hg data were reported as wet weight. Lipid normalization was not performed due to the lack of a significant relationship between toxicant concentration and percent lipid (Hebert and Keenleyside, 1995).

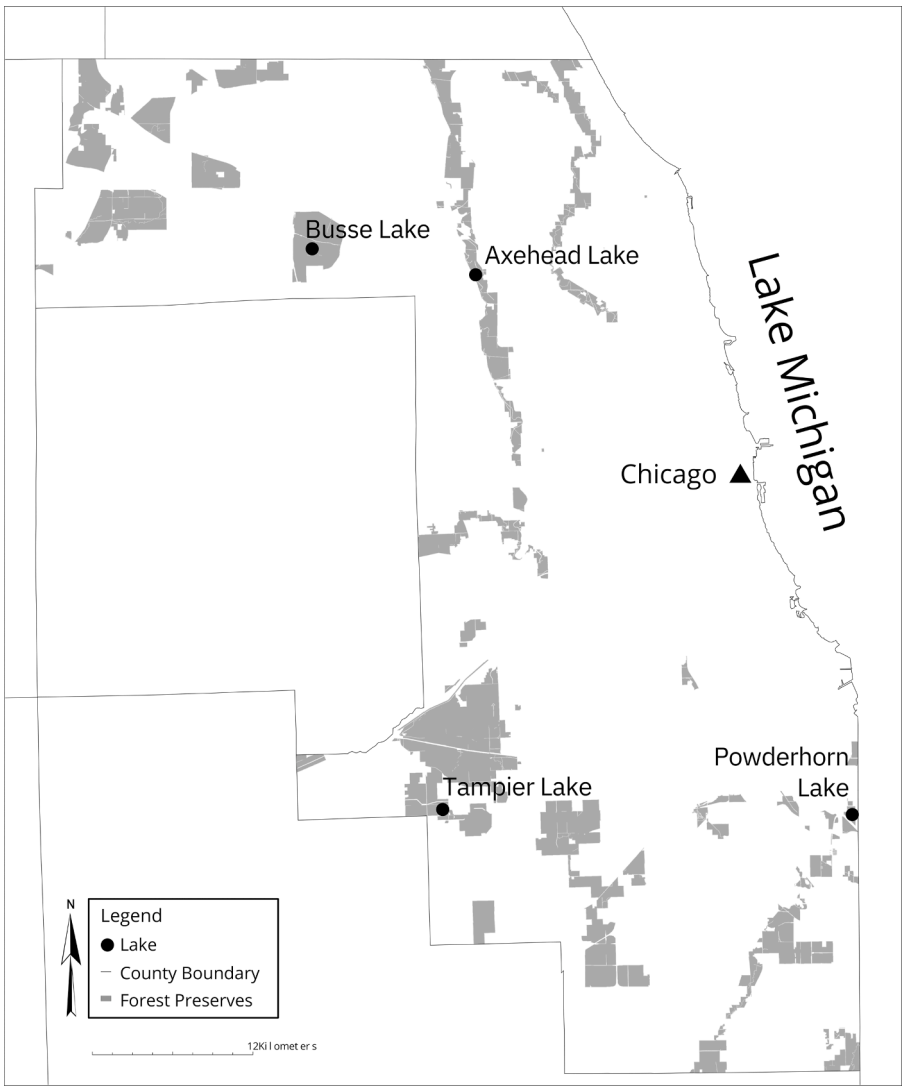


Fig. 1. Map of the Forest Preserves of Cook County (FPCC), Illinois, highlighting the four lakes sampled in 2019 and 2020: Busse (impoundment-type), Axehead (seepage-type), Tampier (impoundment-type), and Powderhorn (seepage-type).

2.4. Histopathologic analysis

A histologic review was performed on a full set of tissues including liver, gonad, brain, spleen, cranial kidney, caudal kidney, gill, stomach (LMB), intestine, swim bladder, axial skeletal muscle, and skin. Formalin-fixed samples were processed routinely, paraffin-embedded, and cut at 4–5 μm thickness on a rotary microtome. Tissue sections were mounted onto glass slides, stained with hematoxylin and eosin (H&E), and analyzed by light microscopy with an Olympus CX41 microscope (Olympus, Shinjuki, Tokyo, Japan). After each seasonal collection, individual cases were assigned a random number using an online list randomizer for blinded review (Haahr and Haahr, 1998). Prior to analysis, a literature review was performed for familiarization with histologic lesions associated with lipophilic toxicant exposure (Pinkney et al., 2017; Wolf and Wolfe, 2005; Wolf et al., 2015; Wolf and Wheeler, 2018). Most lesions were assessed semi-quantitatively using previously published generic grading scale criteria, with grade increasing with percentage of tissue affected (Schafer et al., 2018). Diffuse changes (e.g., hepatocellular pigment and protein accumulation) were assessed by level of severity. Parasite burden, distribution, and type (to phylum or class level) were also evaluated. For more comprehensive estimation of parasitic burden, histologic lesions were later combined with gross and cytologic findings. Composite parasite scores were created for encysted larval metazoans (trematodes and cestodes) and gastrointestinal luminal helminths by addition of the scores assigned to individual organs.

Gonadal staging was performed using the following scheme adapted from Blazer (2002). Male gonads were categorized as pre-spermatogenic/regressed, early spermatogenic, mid-spermatogenic, late spermatogenic, or post-spermatogenic/spent. Female gonads were given the following designations: Stage 0 – follicles undeveloped with only pre-vitellogenic oocytes; Stage 1 – early development; $\geq$ 90% follicles pre-vitellogenic and others early to mid-vitellogenic; Stage 2 – mid-development; majority of follicles are early and mid-vitellogenic with larger oocytes containing peripheral yolk vesicles; Stage 3 – late development; majority of follicles are late vitellogenic with eosinophilic yolk globules distributed throughout ooplasm.

2.5. Image analysis: average hepatocellular size and splenic pigmented macrophage aggregate (PMA) area

Photomicrographs of the liver and spleen were captured using an Olympus BX43 microscope, a DP73 digital camera, and cellSens Entry software (Olympus Corporation, Tokyo, Japan). For the liver, average hepatocyte area (μm^2) was calculated by taking ten consecutive, non-overlapping digital images (352 $\times$ 264 μm) systematically at 400x

magnification, with a standardized starting point for each tissue section. Areas of parasitism or associated granulomatous inflammation were avoided. A 700 μm^2 grid overlay was placed, and a single hepatocyte within every 10th box manually outlined and measured using Fiji software until a total of 100 hepatocytes were measured per fish (Schindelin et al., 2012). For the spleen, the average PMA area per 200x field was calculated by taking five consecutive, non-overlapping digital images (704 × 528 μm) systemically at 200x magnification with a consistent starting point and also avoiding areas of parasitism/granulomatous inflammation. Pigmented macrophage aggregates, identified by pigment color and appearance (golden-brown to tan, coarsely granular) were manually outlined and measured. Measured PMAs were marked to avoid duplicate measurements.

2.6. Statistical analyses

Statistical analyses were performed using R version 4.0.3 (R Core Team, 2017) and Rstudio software (R Studio Team, 2020). A Shapiro-Wilk test was used to test for data normality, and appropriate log transformations were applied if needed. A series of T-tests (student's and Welch's) were performed to identify significant differences between the following predictor variables: year, species, sex and the following outcome variables: morphometric data (weight, SL), tissue contaminant concentration (total PCB, total Hg), lipid content, image analysis data (splenic PMA area, hepatocellular size), and parasite scores. The same statistical method was used to identify significant differences in water chemistry parameters (temperature, DO, total alkalinity, Cl-, NO_3, PO_4, NH_4, and pH; surface and 1 m depth) by year and lake type. Seasonal variation was not statistically assessed due to low sample size. A two-way analysis of variance (ANOVA) was used to compare toxicologic data by year and lake type. Fish collected from all years, seasons, and lake types were pooled for statistical comparisons between species, by pathologic lesions, by morphometric data, and by parasite burden. Simple linear regressions were performed to identify significant relationships between the above water quality parameters and tissue contaminant concentrations. Multivariate linear regressions were performed to account for the effect of covariates. Simple and multivariate linear regressions were performed to identify significant relationships and account for the effect of covariates between variables in each of the following categories: tissue contaminant concentrations, morphometric data, image analysis data, and parasite score. The overwhelming majority of histologic lesions were scored as minimal and were thus analyzed categorically by presence/absence. *T*-tests (student's and Welch's) were used to identify significant relationships between histologic lesions and tissue contaminant concentrations. Binomial logistic regressions were performed to identify significant relationships between morphometric data, toxicologic data, and histologic lesions. An alpha level of 0.05 was considered statistically significant in all tests.

3. Results

A total of 40 carp (21 females, 19 males) and 46 LMB (20 females, 23 males, 3 sex undetermined) were collected with approximately equal distribution by year, season, and lake type (Tables S1a–d). The average weight and SL for carp were 2043 ± 1495 g and 44 ± 8 cm, respectively, and the average weight and SL for LMB were 520 ± 262 g and 28 ± 5 cm, respectively. Weight and SL did not vary significantly by year or lake type ($P > 0.20$).

There was no significant association ($P > 0.05$) between year, lake type and measured outcome variables (tissue contaminant concentrations, pathologic lesions, parasite burden) after removing one carp outlier. Only one carp was collected from Axehead lake during the second year, and Hg concentrations in this individual fish (0.12 μg/kg) were higher than the average Hg concentrations of carp collected from Axehead lake the previous year (0.05 ± 0.02 μg/kg, $N = 3$).

Total Hg concentrations in LMB (0.11 ± 0.1; range 0.009–0.38 mg/kg) were significantly higher ($P < 0.001$) than in carp (0.05 ± 0.03; range 0.004–0.12 mg/kg). Total Hg concentrations significantly and positively correlated with splenic PMA area in both species ($R^2_{adj} = 0.43$ [LMB], 0.28 [carp]; $P < 0.001$; Fig. 2). Photomicrographs exhibiting the range of splenic PMA severity are illustrated in Fig. 3. In carp, total Hg concentrations also significantly and positively correlated with hepatocellular cytoplasmic pigment ($P < 0.01$; photomicrographs illustrated in Fig. 4). Standard length was included as a covariate due to its positive correlation with splenic PMAs in LMB ($R^2 = 0.3$; $P < 0.001$; Fig. 5a), though this relationship was not significant in carp ($P = 0.95$; Fig. 5b). There was no significant difference in SL between carp with or without hepatocellular pigment ($P = 0.32$) or between tissue Hg concentrations and other pathologic lesions in either fish species.

Mean total PCB concentrations in carp (203.1 ± 152; range 11.8–505 μg/kg) were significantly higher ($P < 0.001$) than in LMB (33.2 ± 35.5; range 4.1–121 μg/kg). In carp, total PCB concentrations significantly and positively correlated with SL ($R^2 = 0.32$; $P < 0.001$; Fig. 6a). There was no association between SL and total PCB concentrations in LMB ($P > 0.05$; Fig. 6b) or between tissue PCB concentrations and other pathologic lesions or parasite burden.

Although contaminant concentrations did not vary significantly by lake type, there was notable variation in tissue PCB concentrations by individual lake (Table 1). Impoundment-type lakes had higher concentrations of Cl^- than seepage-type lakes, though this difference was not statistically significant ($P = 0.20$; Table S2). In carp, tissue Hg concentrations positively correlated with Cl^- ($R^2_{adj} = 0.23$, $P = 0.03$) and negatively correlated with pH and DO ($R^2_{adj} = 0.23$; pH: $P = 0.04$; DO: $P = 0.01$). In LMB, tissue Hg concentrations negatively correlated with DO, NO_3, and total alkalinity ($R^2_{adj} = 0.58$; DO: $P = 0.01$; NO_3: $P = 0.03$; total alkalinity: $P = 0.03$). PCB concentrations in carp positively correlated with NH_4^+ ($R^2_{adj} = 0.4$; $P = 0.02$) and negatively correlated with DO ($R^2_{adj} = 0.4$; $P = 0.02$). In LMB, PCB concentrations negatively correlated with NO_3^- ($R^2_{adj} = 0.25$; $P = 0.02$).

Parasite burden and diversity were not associated with contaminant concentrations in either species, however there were notable differences between carp and LMB (Table S3). All LMB had larval metazoan endoparasitism (mean score = 6.95, range 2–14); 100% had larval trematodes (metacercariae) and 61% had larval cestodes (plerocercoids). The majority (65%) of LMB also had encysted nematodes. Larval metazoan endoparasitism was not detected in carp. Gastrointestinal helminths were more prevalent in LMB (75%) than carp (20%), and the average gastrointestinal helminth score in LMB (1.86 ± 1.43) was significantly greater ($P < 0.001$) than in carp (0.2 ± 0.4). Branchial ectoparasitism was noted in both species of fish; carp and LMB had the same prevalence of branchial ciliates (15%), whereas LMB had a greater prevalence of monogeneans (96%) than carp (30%). Cutaneous ectoparasitism was not detected in either species. Renal myxozoan plasmodia were also noted exclusively in LMB (43.4% of individuals). Tissue granulomas were detected in 100% of LMB and 72.5% of carp; no intralesional bacteria were detected, and granulomas were considered parasite associated in LMB. Granulomas identified in carp were generally rare, small, quiescent, and of undetermined etiology.

Stages of gonadogenesis (vitellogenesis and spermatogenesis) varied by species, though low sample sizes precluded statistical evaluation. Largemouth bass (synchronous spawners) had greater seasonal variation compared to carp (asynchronous spawners). No ovotestis were detected in any fish, and primary inflammation (i.e., not considered to be associated with parasitism) was detected at minimal concentrations with no evident association with variables of interest. Many histologic changes previously reported to be associated with lipophilic contaminant intoxication were either not detected or were present at minimal levels lacking association with tissue contaminant concentrations (Table S4, Fig. S1 [examples]). Neoplastic disease was not observed in any of the fish examined.

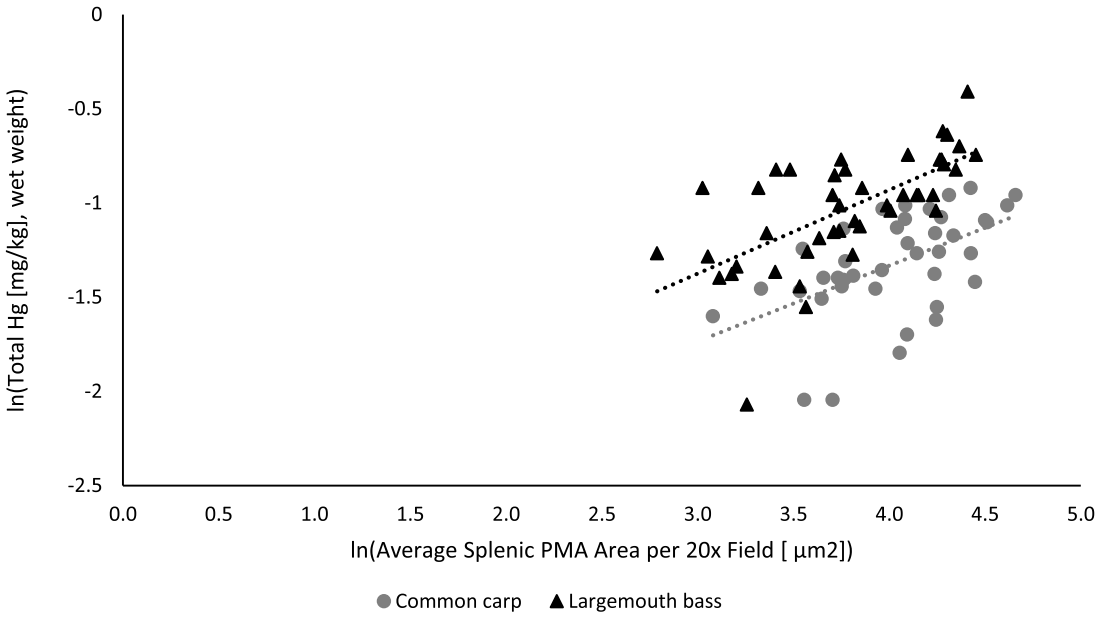


Fig. 2. Correlation between fish total Hg (mg/kg) in fillets and average splenic PMA area (μm^2) in largemouth bass (black triangle; $R^2 = 0.42$; $P < 0.001$; $y = 0.44x - 2.7$) and common carp (gray circle; $R^2 = 0.28$; $P < 0.001$; $y = 0.40x - 2.94$) collected from lakes in Cook County, Illinois, 2019–2020.

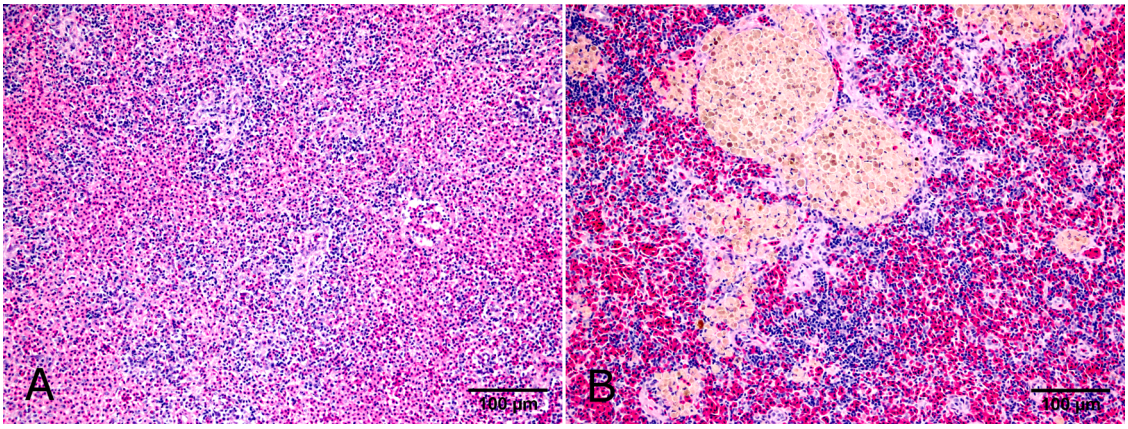


Fig. 3. Photomicrographs of spleen of fish collected from lakes in Cook County, Illinois, 2019–2020 with varying degrees of pigmented macrophage aggregate (PMA) coverage. (A) Example of largemouth bass with no appreciable splenic PMAs. (B) Example of common carp with increased splenic PMA area (hyperplasia).

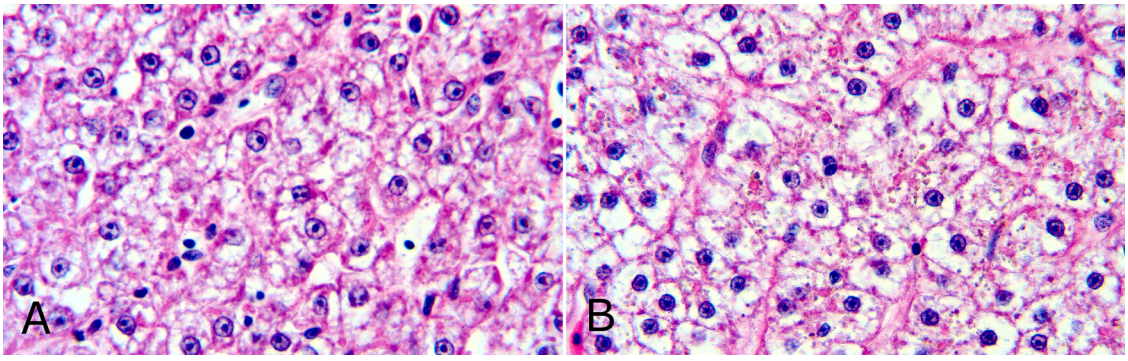


Fig. 4. Photomicrographs of hepatic parenchyma of common carp collected from lakes in Cook County, Illinois, 2019–2020 with (A) no appreciable hepatocellular cytoplasmic pigment and (B) hepatocellular cytoplasmic tan granular pigment.

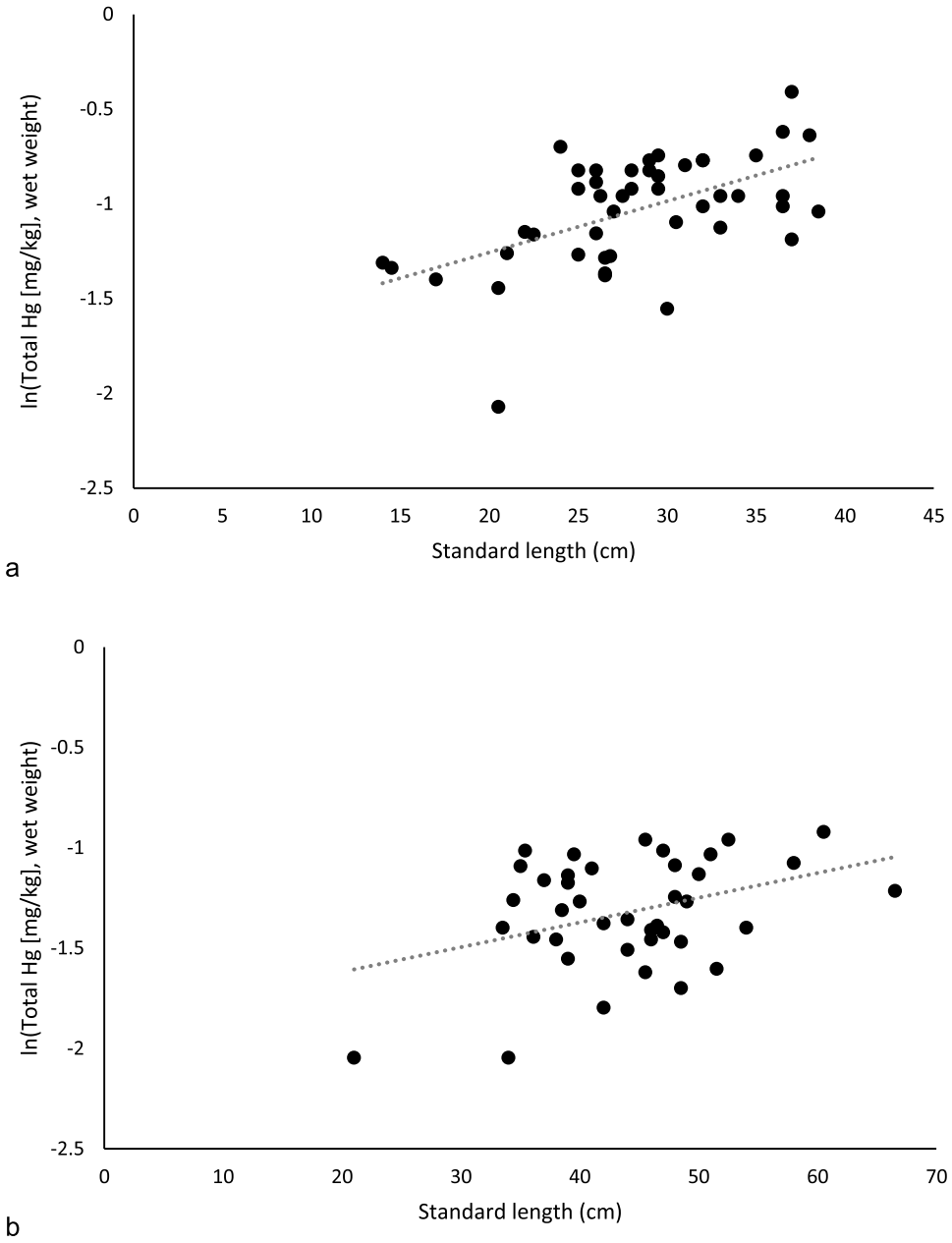


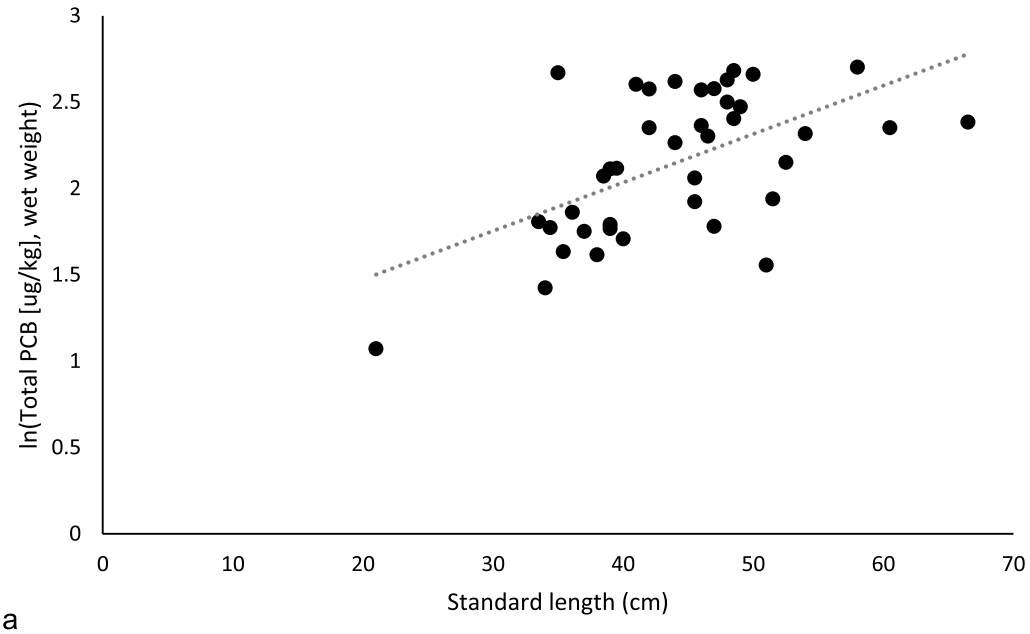
Fig. 5.. a. Correlation between standard length and total Hg concentrations in fillets from largemouth bass collected from lakes in Cook County, Illinois, 2019–2020 ($R^2 = 0.3$; $P < 0.001$; $y = 0.03x - 1.8$). Fig. 5b. Correlation between standard length and total Hg concentrations in fillets from common carp collected from lakes in Cook County, Illinois, 2019–2020 ($R^2 = 0.14$; $P = 0.28$; $y = 0.01x - 1.87$).

4. Discussion

4.1. Mercury

Mild Hg-associated pathologic changes were detected in both species of fish. Total Hg concentrations positively correlated with splenic PMA area in both carp and LMB. Pigmented macrophage aggregates are focal accumulations of macrophages containing pigments such as lipofuscin, ceroid, melanin, and/or hemosiderin (Wolke, 1992). Pigmented macrophage hyperplasia generally indicates increased cellular breakdown and can be influenced by toxic, metabolic, nutritional, infectious, and/or environmental factors (Wolf and Wheeler, 2018). Several previous studies have positively associated tissue Hg concentrations with hepatic PMAs in fish (Barst et al., 2016, 2011; Drevnick et al., 2008; Meinelt et al., 1997; Mubarokah et al., 2018; Müller et al., 2015). Using laser ablation-inductively coupled plasma-mass spectrometry (ICP-MS), Barst et al. (2011) localized Hg at the cellular level in spotted gar (*Lepisosteus oculatus*) and determined that hepatic PMAs had twice as many Hg counts as the adjacent parenchyma. In yellow perch (*Perca flavescens*), Hg has been localized to both hepatic PMAs as well as hepatocellular lysozymes (Müller et al., 2015). Findings from these studies imply that Hg sequestration by tissue PMAs may act as a "sink" that could increase fish tolerance to high Hg levels.

Fish tissue Hg concentrations in this study (range 0.004–0.38 mg/kg wet weight, skin-on fillet) were generally lower than previously reported concentrations associated with similar lesions in wild fish (range 0.04–0.7 mg/kg wet weight, muscle or skin-on fillet, Barst et al., 2016; Drevnick et al., 2008; Müller et al., 2015), and well below the threshold level experimentally associated with clinically significant pathology (0.5 mg/kg wet weight, muscle, equivalent whole body threshold of 0.2 mg/kg, wet weight; Dillon et al., 2010; Sandheinrich and Wiener, 2011). Only mild Hg-associated pathologic changes were noted here, though



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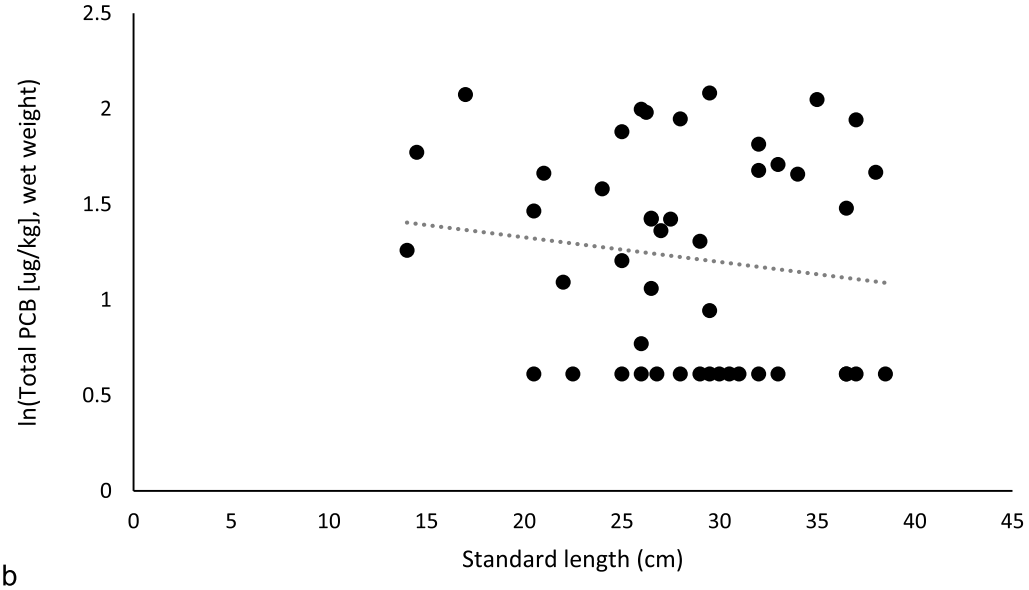

b

Fig. 6.. a. Correlation between standard length and total PCB concentrations in fillets from common carp collected from lakes in Cook County, Illinois, 2019–2020 (R^2 = 0.32; $P < 0.001$; y = 0.03x + 0.91). Fig. 6b. Correlation between standard length and total PCB concentrations in fillets from largemouth bass collected from lakes in Cook County, Illinois, 2019–2020 (R^2 = 0.02; P = 0.43; y = -0.01x + 1.58). Points below the method detection limit were estimated at half the detection limit (0.6 ug/kg) and included in the analyses.

effects on fitness remain undetermined and are likely minimal. Despite the low Hg concentrations in reference to fish pathogenicity, the majority of fish (91% LMB, 73% carp) had tissue Hg concentrations at or above 0.029 mg/kg, the lowest threshold concentration for restriction of fish consumption determined by the EPA (i.e., consumption of > 16 meals/month; USEPA 2000). Current local fish consumption guidelines recommend limiting predator fish consumption to 1 meal/week or 1 meal/month for sensitive cohorts (pregnant women, children) to minimize Hg exposure (IDPH, 2021).

Largemouth bass, a highly predatory species, had significantly higher concentrations of tissue Hg than carp (omnivorous species), indicating biomagnification had occurred. The consideration of standard length (a surrogate for age) in evaluating lesion data is also important because many toxicant-associated lesions are nonspecific, and occurrence and severity increase with age (Bermejo, 2007). Tissue Hg bioaccumulation was also evident by the positive correlation between length and Hg concentration in both species of fish; however, this relationship was only statistically significant for LMB. Mercury bioaccumulation and biomagnification are well-documented in fish and occurs with such consistency that Hg stable isotope analysis can be performed to determine species trophic level when unknown (Al-Reasi et al., 2007). Largemouth bass tissue Hg concentrations correlated with few water chemistry parameters (negative correlation with pH and DO, positive correlation with Cl^-) and were generally supportive of previous reports associating acidic, anoxic environments with greater Hg bioavailability (Lange et al., 1993).

4.2. Polychlorinated biphenyls

In contrast to Hg, no pathologic changes associated with PCB contamination were detected in either fish species. Tissue total PCB concentrations fell well below those associated with decreased survival and reproduction (30,000 μg/kg; Freeman and Idler, 1975; Hansen et al., 1974). Barron et al. (2000) found that hepatic foci of cellular

Table 1
Contaminant concentrations (wet weight) in fillets from common carp (carp) and largemouth bass (LMB) collected from lakes in Cook County, Illinois, 2019–2020, by location and lake type.

Lake type	Sample location	Carp		LMB	
		Total PCB (μg/kg)	Total Hg (mg/kg)	Total PCB (μg/kg)	Total Hg (mg/kg)
Seepage	Axehead lake	174.28 ± 63.12 ($n = 4$)	0.07 ± 0.04 ($n = 4$)	6.99 ± 6.66 ($n = 10$)	0.13 ± 0.02 ($n = 10$)
	Powderhorn lake	286.13 ± 144.59 ($n = 12$)	0.04 ± 0.02 ($n = 12$)	64.05 ± 33.64 ($n = 12$)	0.08 ± 0.04 ($n = 12$)
Impoundment	Busse lake	240.53 ± 174.95 ($n = 12$)	0.04 ± 0.02 ($n = 12$)	35.36 ± 30.52 ($n = 12$)	0.16 ± 0.09 ($n = 12$)
	Tampier lake	92.24 ± 57.42 ($n = 12$)	0.06 ± 0.03 ($n = 12$)	21.88 ± 32.64 ($n = 12$)	0.07 ± 0.04 ($n = 12$)

alteration (FCA) and neoplasia in walleye were associated with liver total PCB concentrations ranging from 400 to 900 μg/kg in assessment areas. No association was observed between tissue PCB concentrations and hepatocellular FCA, though only 8% of the fish (7 carp) had concentrations surpassing 400 μg/kg. Of these carp, only 1 had hepatocellular FCA, and this individual had a tissue PCB concentration of 459 μg/kg. The method detection limit (8.2 μg/kg) in the study was above the EPA's lowest threshold for fish consumption (> 16 meals/month, 1.5 μg/kg), thus the true risk to human health was equivocal. Recommendations for consumption of common carp in the Cook County lakes sampled ranged from 1 meal per week to 1 meal per month, depending on the size of the fish (less or greater than 23 inches, respectively; IDPH, 2021).

Carp had significantly higher concentrations of tissue PCBs than LMB, suggesting that bioaccumulation played a more important role in PCB acquisition than biomagnification. Bioaccumulation in carp was further evidenced by the significant positive correlation between PCB concentration and length, whereas this association was not evident in LMB. Sediment mineral solids and organic matter are important binding sites for PCBs, and carp may have had higher exposure than LMB as a result of their benthivore status, allowing for contaminated sediment particles to be ingested with food items (Eggleton and Thomas, 2004). Carp tissue PCB concentrations positively correlated with water $NH_4^+$and negatively correlated with DO, which is consistent with previous reports indicating that eutrophication (increased nutrient and mineral enrichment) increases the association of PCBs to dissolved organic matter (Gunnarsson and Rosenberg, 1996).

4.3. Lake type

No association between lake type (seepage vs. impoundment) and tissue contaminant concentrations in either fish species was found. However, there was notable variation in carp tissue PCB concentrations between individual lakes, with carp from Powderhorn and Busse having the highest concentrations. This finding may be due to the proximity of these lakes to industrialized areas or superfund sites. Powderhorn Lake is located in an industrialized area 3.7 miles from the Lake Calumet Cluster (LCC) superfund site, which is an 87-acre group of land, waste storage, and disposal facilities in southeastern Chicago. An ecological risk assessment of the LCC site conducted by the USEPA revealed high concentrations of sediment contaminants, including PCBs (Soucek et al., 2013; Sprenger et al., 2001). Similarly, Busse Lake is near a heavily industrialized area. Previous studies have found that differences in hydrology between lake types may be overshadowed by regional influences (e.g., geology, atmospheric pollution; Lange et al., 1993). In the Chicago area, lake proximity to highly industrialized and/or superfund sites may have overshadowed any effect of lake type. Additionally, no significant differences in water quality parameters between the two lake types were noted, which may have partially accounted for the lack of significant variation in tissue contaminant concentrations.

4.4. Parasitism

Parasitism was the most common pathologic finding in this study with many individual fish colonized by multiple parasites. Although parasites are common in wild fish, overall parasite burden can be influenced by tissue contaminants (Booton et al., 2018). In this study, parasite burden was not significantly associated with tissue contaminant concentrations, lake type, size, or condition. Largemouth bass had a greater parasite burden and diversity when compared to carp, which was likely reflective of their higher trophic level and carnivorous diet. Despite the relatively high parasite burden in some LMB, there was no histologic evidence of organ dysfunction or indication of decreased overall health.

4.5. Limitations and future directions

Effects of contaminants on reproduction were not fully examined in this study due to a few limitations. Fish were collected multiple times during the year according to previously determined protocols for the FHSP, rather than at times corresponding with spawning. Thus, seasonal effects on gonadogenesis impacted sample sizes for some gonadal stages and the low sample sizes precluded statistical evaluation. This precluded assessment of contaminant-associated effects on reproductive maturity. Changes in gonad cell proportions, which are best evaluated by rigorously standardized gonad collection and analytical techniques (e.g., preservation in Bouin's solution, quantitative analysis), were also not assessed in the present study (Wolf et al., 2004). A future study using fish collected at spawning would allow evaluation of reproductive parameters (i.e., gonadosomatic index, serum vitellogenin, stage gonadogenesis, and gonad cell proportions) to investigate possible effects of contaminants on fish reproductive health.

Tissues utilized for the toxicologic review in this study were originally collected for diagnostic purposes in accordance with the FHSP protocols. Therefore, the comparative sensitivity in detecting subtle lesions (e.g., hepatic foci of cellular alteration) may have been decreased compared to more focused toxicologic studies that evaluate greater numbers of tissue sections in fewer targeted organs. This investigation was also limited to two species of fish representing different trophic levels, and evaluation of greater numbers of species may be valuable in further elucidating the relationship between tissue contaminant concentrations and fish trophic level.

5. Conclusion

Illinois fish consumption advisories reported high concentrations of tissue contaminants (Hg and PCBs) in FPCC inland lake fish, warranting this investigation of possible impacts on fish health (IDPH, 2021). In this study, mild pathologic changes associated with total Hg, and no lesions associated with total PCB concentrations in carp or LMB from four FPCC lakes were documented. Mercury-associated lesions were considered secondary to chronic, low-level oxidative damage and was similar to previous studies investigating Hg in wild fishes. These results indicate that, although FPCC fish tissue contaminant concentrations remain a concern for human consumption, the concentrations were below the threshold level for adverse effects (0.5 mg/kg, muscle, wet weight) in the fishes examined and appeared to be well-tolerated. Additional studies are warranted to evaluate the effects of contaminants on fish reproduction, behavior, and recruitment in this region.

This study highlighted the importance of trophic level on the selective bioaccumulation and biomagnification of Hg and PCBs in FPCC lakes. Contaminant concentration and parasite burden varied

significantly between benthic (carp) and predatory (LMB) species. Lake type (seepage vs. impoundment) was not a significant determinant of fish tissue contamination or pathology, and regional influences (e.g., proximity to a superfund site) may play a greater role than individual lake hydrology in the Chicagoland area. These findings emphasize the value of performing ecotoxicologic research at a local level for better appreciation of local variables and risk factors for toxicity.

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CRediT authorship contribution statement

Sierra M. Imanse: Conceptualization, Methodology, Investigation, Writing – original draft, Writing – review & editing. **Chris L. Anchor:** Conceptualization, Methodology, Resources. **Gretchen C. Anchor:** Conceptualization, Methodology, Investigation, Writing – review & editing. **Jennifer A. Landolfi:** Investigation, Writing – review & editing, Supervision. **Michael J. Kinsel:** Investigation, Writing – review & editing, Supervision. **Jeffrey M. Levengood:** Writing – review & editing. **Martha A. Delaney:** Conceptualization, Methodology, Investigation, Writing – review & editing, Supervision. **Karen A. Terio:** Conceptualization, Methodology, Resources, Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2021.106043.

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OTTER BEHAVIORAL, DISEASE, AND TOXICOLOGY WORK

THE URBAN RIVER OTTER RESEARCH PROJECT

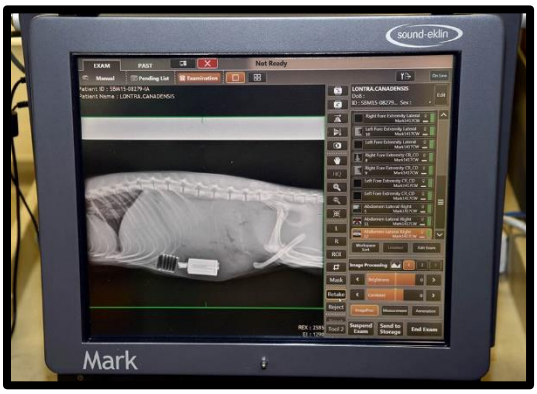
The first modern otter sign documented in Cook County occurred in the early 1990s.

The observational study detection was part of a statewide Illinois Department of Natural Resources (IDNR) effort to document water loving mammals. This observational effort was prior to the formal river otter, state-wide reintroduction which took place from 1994-1997. The river otter release occurred at fifteen sites, throughout Illinois: the closest to CHICAGO was over one hundred and fifty miles to the south.

The Forest Preserve District of Cook County (FPDCC) initiated a preliminary otter telemetry study in 2015.The purpose of the preliminary work was to:

- Test the efficacy of trapping in the urban environment.
- Test the efficacy of various trapping modalities.
- Test the efficacy of radiotracking a water endemic animal in the urban environment.
- Begin the process of understanding home range and land use in an urban setting.
- Begin to understand mortality and foraging issues in the urban setting.

The FPDCC enlisted the assistance of staff from the Brookfield Zoo; this ensured expert veterinary surgical protocol during the radio implant procedure. The initial group of otters consisted of six individuals. The successful implementation of this first cohort of otters, stimulated the beginning the Urban Otter Research Project.



The Urban Otter Research Project (URORP) has been modeled after the Urban Coyote Study. The otter study is partnered and funded by:

- Cook County Animal and Rabies Control.
- Forest Preserve District of Cook County.
- Ohio State University (OSU).
- Max McGraw Wildlife.
- Brookfield Zoo.
- USDA.

Principle investigators Dr. Stan Gehrt and Chris Anchor are directing the otter study.

Blood is monitored for disease, adipose tissue for toxicology, and whiskers for stable isotope food Studies.

Early results include:

• Remarkable ability to move and navigate urban settings.

• Exploitation of varied food types (invertebrate, large turtles…...).

• Apparent ease of overland movement.

River otters disappeared from the Chicago landscape for one hundred years. The otter's re-introduction to this urban landscape affords the opportunity; to study an apex predator in a situation that few would have predicted. The initiation of the URORP is made possible with funding from Cook County Rabies and Animal Control. An OSU graduate student (Zack Hahn) will work full time evaluating some of the many findings yet to come. Pathology will be performed by the University of Illinois

Photo Credit: Corey Arnold, National Geographic