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Cover Photograph: A melanistic Eastern Gray Squirrel (*Sciurus carolinensis*) on the University of Guelph campus, Canada. Photograph © M.R. Stothart.

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Comfortably Numb? Regional Differences in the Relationship Between Indices of Urbanization and a Stress Indicator in Eastern Gray Squirrels

Rebecca Rimbach[1,2*], Alannah Grant[3], Pratik R. Gupte[4], Amy Newman[3], Mason R. Stothart[5], and Herman Pontzer[1,6]

Abstract - Wild animals face novel environmental challenges as natural habitats give way to urban areas, with numerous biotic and abiotic differences between the two. Urban 'stressors' may elicit a constant release of glucocorticoids via the hypothalamic–pituitary–adrenal (HPA) axis, and chronically elevated glucocorticoid levels can be associated with negative effects on health and reproduction. Reduced glucocorticoid secretion is proposed to facilitate adaptation to urban habitats by avoiding the negative health and reproductive effects of chronically elevated levels of circulating glucocorticoids. Here, we investigated this mechanism of adaptation to urban stress in a common species that occurs across the urban-rural gradient, and over a wide geographic range, *Sciurus carolinensis* (Eastern Gray Squirrel). We measured hair cortisol concentration (HCC), a long-term indicator of circulating glucocorticoids, of 192 squirrels from urbanized habitats and natural forest habitats in two study areas ~850 km apart, in the USA (N = 96 samples) and Canada (N = 96 samples). We examined the relationship between HCC and two correlated indices of urbanization, one reflecting vegetation (NDVI) and one reflecting human-made urban cover (NDBI). In the Canadian dataset, HCC showed quadratic relationships with NDVI and NDBI, indicating that squirrels have lower HCC in the most urbanized habitats (two university campuses). Males and females had similar HCC in the Canadian dataset. In the USA dataset, there was no relationship between HCC and either index, and males had higher HCC than females. These results suggest that urban habitats may be relatively benign for urban Eastern Gray Squirrels. Reduced glucocorticoid levels may represent a form of (phenotypic) plasticity that facilitates adaptation to and persistence in urban environments.

Introduction

Urban development is a major form of global change and presents wildlife with evolutionarily novel situations where environmental changes are more rapid than those experienced in their evolutionary past (Grimm et al. 2008, Palumbi 2001, Shochat et al. 2006). Urbanization causes the loss, fragmentation, and alteration of natural habitats via the replacement of natural vegetation with anthropogenic structures such as buildings and roads (Shochat et al. 2006). In addition, chemical, light and noise pollution, as well as human disturbance are increased in urban areas (Isaksson 2015, McKinney 2002, Shochat et al. 2006). Urban habitats can strongly filter for species, many species may go locally extinct as urban areas advance while synanthropic species thrive in anthropogenically modified habitats (Grimm et al. 2008, Grimmond 2007, McKinney 2002).

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Urban habitats can offer benefits to urban wildlife but can also expose them to detriments. For example, urban habitats often offer benefits in the form of predictable, abundant and easily accessible anthropogenic food sources (Oro et al. 2013, Plaza and Lambertucci 2017) and reduced predation rates from natural predators (Eötvös et al. 2018, McCleery 2009, Rodewald et al. 2011). But domestic animals and vehicle collisions can largely contribute to mortality of urban wildlife (Ditchkoff et al. 2006, Loss et al. 2013). Typically, urban habitats present urban wildlife with a variety of challenges, or 'stressors', that are specific to urban environments (Birnie-Gauvin et al. 2016, Isaksson 2015). Urban stressors include human disturbance (Fernández-Juricic 2002), vehicle traffic (Dowding et al. 2010), altered dietary quality (Isaksson 2015), and exposure to pollution (Chatelaina et al. 2021, Isaksson 2015). These ecological differences between urban and undeveloped habitats can lead to phenotypic differences between populations across the urban-natural gradient. These differences could arise because (i) urban colonizers might have had specific phenotypic traits, also termed exaptations (sensu Gould and Vrba 1982) that facilitated colonization of the urban habitat (e.g., lower sensitivity to urban stressors) and (ii) living in urban environments can lead to phenotypic, and ultimately evolutionary, changes which can lead to different phenotypes (Alberti et al. 2017, Ouyang et al. 2018, Shochat et al. 2006).

Exposure to a stressor elicits a physiological stress response in the form of a short-term secretion of glucocorticoids (cortisol or corticosterone) via the hypothalamic–pituitary–adrenal (HPA) axis. Secretion of glucocorticoids mediates adaptive responses such as stimulation of gluconeogenesis, inhibiting glucose utilization in nonessential tissues, mobilization of fat stores, and redirection of behavior (Sapolsky et al. 2000, Wingfield and Romero 2001). However, persistently elevated levels of circulating glucocorticoids can impair growth, and reproductive and immune functions (Boonstra 2013). Frequent exposure to urban stressors can result in elevated baseline levels of physiological stress which may result in chronic stress levels. Alternatively, individuals with a reduced sensitivity or 'desensitized' HPA axis, reflected in lower physiological stress levels compared to individuals from undisturbed areas, may be at an advantage in urban habitats. A desensitization of the HPA axis has been proposed to play a central role in the adaptation of animals to urban habitats (Bonier 2012).

Studies have found evidence supporting both, elevated (Beaugeard et al. 2019, Fokidis et al. 2009, Strasser and Heath 2013) and reduced levels of physiological stress in urban birds (e.g., Partecke et al. 2006, Atwell et al. 2012, Chávez-Zichinelli et al. 2013, Palma et al. 2020, Lane et al. 2021). Patterns reported in birds are species-specific and can vary depending on sex and life history stage considered (reviewed in Bonier 2012). Fewer studies have investigated changes in glucocorticoid levels in response to urbanization in groups other than birds (Murray et al. 2019). Urban reptiles show lower baseline and stress-induced corticosterone concentrations compared to rural individuals (Amdekar et al. 2018, French et al. 2008), whereas urban Jollyville Plateau salamanders (*Eurycea tonkawae* Chippindale, Price, Wiens and Hillis) show elevated baseline corticosterone levels compared to rural individuals (Gabor et al. 2018). Similar to the bird literature, the literature regarding urban mammals indicates that endocrine responses to urban habitats are species-specific, can differ between age classes (Price et al. 2018), and may even vary across a species' distribution range (Brunton et al. 2020). Urban and rural individuals of some species have similar glucocorticoid levels (Dowle et al. 2013, Potratz et al. 2019, Shimamoto et al. 2020), while urban individuals of other species have higher glucocorticoid levels compared to individuals living in more natural habitats (Brearley et al. 2012). Conversely, urban populations of other mammalian species show lower glucocorticoid levels compared to populations from more natural habitats (Łopucki et al. 2019, Lyons et al. 2017).

To date, our knowledge about the effects of urbanization on the endocrine system of mammals is still relatively limited (Murray et al. 2019), restricting our ability to assess the role of hormonal responses in mediating behavioral and physiological responses to the urban environment. Moreover, most studies have focused on a comparison between a single urban and a single less disturbed or natural site, but data from a variety of different habitats, collected at a broad spatial scale would allow more generalizable conclusions. Here, we assessed hair cortisol concentration (HCC) of *Sciurus carolinensis* Gmelin (Eastern Gray Squirrel) living in several different urbanized and forest habitats in the USA and Canada. Eastern Gray Squirrels are commonly found in urban habitats, often at high densities and have a wide geographic range (Koprowski 1994). The objective of this study was to assess how HCC of squirrels varies with two indices of urbanization, the normalized difference vegetation index (NDVI) and a normalized difference built-up index (NDBI).

Materials and Methods

Study Species

The Eastern Gray Squirrel is a medium sized (300–710 g) tree squirrel native throughout eastern North America (Koprowski 1994). It is commonly found in both deciduous forests and anthropogenic environments, such as parks and residential areas (Benson 2013, Engel et al. 2020). Population densities of more than 30 squirrels/ha have been reported in urban environments (Engel et al. 2020, Parker and Nilon 2008). Eastern Gray Squirrels frequently descend to the ground to forage and cache food (Koprowski 1994).

Study Sites

In the USA, we collected a total of 96 individual hair samples between July 2020 and June 2021 (Fig. 1, Table 1). We trapped 54 squirrels at two different locations, and opportunistically collected hair samples from 42 squirrels found as roadkill in and around Durham between

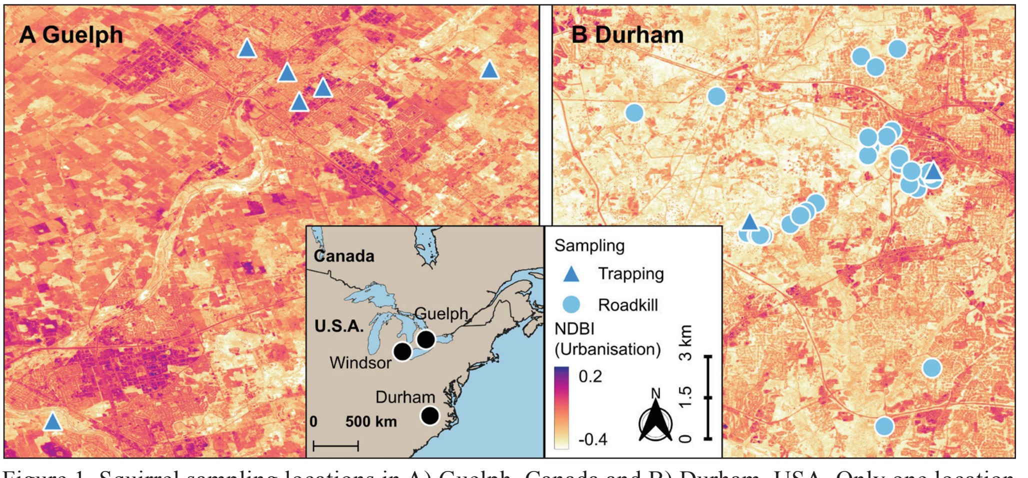

Figure 1. Squirrel sampling locations in A) Guelph, Canada and B) Durham, USA. Only one location was sampled in Windsor, Canada and its location is indicated in the inset in Panel A). Blue triangles show locations in Guelph and Durham where we collected hair samples during live-trapping, while light blue circles represent hair samples collected from roadkill (several samples were collected at most locations). The color (yellow to purple) reflects the normalized difference built-up index (NDBI), an index of urbanization, where darker colors indicate more urbanized areas and lighter colors indicate less urbanized areas. The primarily urban and agricultural areas around Guelph make it more urbanized than the more localized urban development of Durham.

November 2020 and June 2021. Duke University West campus (36.0026 N, 78.9408 W; 33 squirrels), 4 km from Durham, North Carolina, USA served as an urban site, and the Korstian Division of Duke Forest (35.9810 N, 79.0168 W; 21 squirrels), Chapel Hill, North Carolina, USA served as a rural forest site. Duke West campus (2.9 km^2) contains a heterogenous mixture of buildings, sparsely treed grass lawns and several small forested areas that contain several species of oak, maple, hickory and pine, offering natural food sources to squirrel populations, along with numerous trash cans. The Duke campus population comprised ~16,780 students in 2021 and Duke West campus contains the majority of residential quads. The Korstian Division consists of a mix of conifer and hardwood forest (7.4 km^2) and contains 11.7 km of hiking trails accessible to recreational users. There are no trash bins in the Korstian Division and although people have access to the site, anthropogenic food sources are not available for wildlife (no trash or litter was observed in the trapping area, Rebecca Rimbach, pers. obs.).

In Canada, we collected a total of 67 individual hair samples between June 2016 and August 2017 (Fig. 1, Table 1). We collected 53 samples in and around Guelph and 14 samples in Windsor, and bolstered our dataset with previously published HCC data from 29 samples (14 from a forest site and 15 from the urban University of Guelph campus) collected in Canada (Stothart et al. 2019). This dataset comprises a total of 96 individuals sampled across two cities and universities, namely the University of Windsor campus (42.30696, -83.0673; 14 squirrels) and the University of Guelph campus (43.53101, -80.2265; 15 squirrels). The University of Windsor campus population comprised ~16,000 students in 2017 and has a 0.5 km^2 campus comprised of dense clusters of buildings separated by treed courtyards with trash cans. The University of Windsor is bordered on all side by dense residential housing subdivisions. The University of Guelph campus population comprised ~29,507 students in 2017. The 3 km^2 University of Guelph campus contains a heterogenous mixture of buildings, and sparsely treed grass lawns dominated by mixtures of conifers, honey locust, black walnut and several species of maple offering natural food sources to squirrel populations, along with scattered trash cans. It is bordered on 3 sides by 1–2 storey residential properties, and on its fourth side by a 1.65 km^2 arboretum. Three urban parks in the city of Guelph were also sampled (Dairy Bush: 43.52465, -80.2372; Exhibition Park: 43.5488, -80.2607; Royal City Park: 43.5379, -80.2426; 39 squirrels). The Dairy Bush is an unmaintained 0.07 km^2 deciduous forest which lacks trash bins and is bordered by residential subdivisions. Exhibition Park (0.12 km^2) and Royal City Park (0.2 km^2) are city parks with walking trails, playgrounds, sports fields, grass lawns, and sparse deciduous tree cover. Both have trash bins throughout and are surrounded by 1–2 storey residential properties. Canadian samples were also taken from two forest sites, the rare Charitable Research Reserve (43.3811, -80.3479; 14 squirrels), a 0.7 km^2 forest including fragmented wooded lots and conifer plantations sur-

Table 1. Overview of hair cortisol samples of Eastern Gray Squirrels (*Sciurus carolinensis*) living in forest habitats and urbanized habitats, showing sample sizes, sex and age ratios, and average (± SD) NDVI and NDBI. Samples from Durham include samples obtained during trapping and from roadkill.

	Habitat type	Sample size	Sex (F:M)[a]	Age (A:J)[b]	NDVI	NDBI
Durham	Forest	27	14:13	17:10	0.660 ± 0.083	-0.369 ± 0.028
	Urbanized	69	32:37	56:13	0.356 ± 0.057	-0.200 ± 0.041
Guelph	Forest	28	13:15	28:0	0.426 ± 0.074	-0.311 ± 0.024
	Urbanized	54	33:21	54:0	0.267 ± 0.081	-0.165 ± 0.108
Windsor	Urbanized	14	7:7	14:0	0.370 ± 0.076	-0.208 ± 0.050

[a]F = female, M = male; [b]A = adult, J = juvenile.

rounded by agricultural developments and the Starkey Hill Conservation Area (43.53923, -80.1517; 14 squirrels), a 0.4 km^2 mixed conifer and hardwood forest situated 8 km outside the city of Guelph containing 4 km of walking trails open for public access. It should be noted that both rare and Starkey hill do not contain anthropogenic food sources available to wildlife (no trash bins available on site, Mason R. Stothart, pers. obs.).

Collection of Hair Samples in the Field

In the Durham area, we captured squirrels using Tomahawk Model 102 traps (Tomahawk Live Trap Co., WI, USA) baited with peanuts and peanut butter. We trapped squirrels between 7:00 AM and 12:00 noon, and checked traps at 45–60 minute intervals. We anesthetized trapped squirrels using isoflurane as part of the data collection for another study (Rebecca Rimbach, unpubl. data). We tagged individuals with a passive integrated transponder (Avid2024, Avid Identification Systems Inc., Norco, USA) and recorded sex, age class (adult vs juvenile) and reproductive status. We determined the age class by using a combination of body size, toothwear and fur condition. For males, we recorded whether they were scrotal (testes descended) or not (testes in the abdominal cavity), and for females, we recorded whether nipples were visible or not. We also palpated their abdomen to check for fetuses. We excluded pregnant females from data the collection to avoid potentially negative effects of anesthesia to the female and her developing young. While individuals were anesthetized, we collected a hair sample from their tail. Using clean scissors, we cut hair as close to the tail as possible, ~2 cm along the tail, in the middle of the tail. In addition to samples from trapped squirrels, we opportunistically collected hair samples from 42 squirrels (15 adult and 4 juvenile females, 18 adult and 5 juvenile males) found as roadkill in and around Durham between November 2020 and June 2021. Upon encountering a dead squirrel on the road, we recorded its sex, age and location, and collected a hair sample using the same methods described for trapped squirrels. We only collected hair samples from fresh roadkill, i.e., not showing signs of decomposition. We collected hair samples in different habitat types, which can be categorized into four broad categories in descending order of urbanization: urban (Duke University campus; most disturbed), residential area, residential forest and forest (least disturbed type). Squirrels were trapped and handled with approval by the Duke University Institutional Animal Care and Use Committee (Protocol number: A057-20-03 8N), North Carolina Wildlife Resources Commission (Permit number: 20-SC01363, 21-SC01363), Duke University and Duke Forest (R1920-492).

In Canada, we collected hair samples by shaving a 3 cm x 3 cm patch from the outer thigh of live captured but unanesthetized squirrels. Animals were trapped and handled with approval by the University of Guelph Animal Care Committee (AUP no. 3506) and the Ontario Ministry of Natural Resources (WSCA no. 1087323).

Laboratory Analysis—Hormone Extraction

We determined HCC as a measure of physiological stress because HCC provides a reliable representation of long-term cortisol secretion on the scale of several weeks or even years (Gormally and Romero 2020, Heimbürge et al. 2019). Moreover, sampling of hair is easy, less invasive than blood sampling and HCC is stable over months and years of storage (Gormally and Romero 2020, Heimbürge et al. 2019).

For hair samples from Durham squirrels, we extracted hair cortisol following the methods detailed by Davenport and colleagues (2006). In short, we cut 1 cm of hair that was most proximal to the tail (i.e., the youngest hair) and then cut samples into 2–3 mm segments with fine, clean scissors. We cleaned hair samples in 2 ml isopropanol, which we incubated on a shaker for 3 minutes at room temperature. Then we decanted the liquid and dried hair samples for 24 hours. We weighed an average of 0.016 ± 0.004 mg of hair using a laboratory scale (± 0.0001 g, Ohaus Pioneer scale), added 1 ml of methanol per 10 mg hair to each sample, and incubated the tubes at room temperature for 24 hours with slow rotation on a shaker to extract the steroids. Following extraction, we dried 0.7 ml of each extract at 38 °C under a stream of nitrogen gas. The next day, we reconstituted the dried extract with 0.1 ml of buffer provided with the assay kit.

For samples collected from Canadian squirrels (from Guelph and Windsor areas), we again extracted hair cortisol following the methods of Davenport and colleagues (2006). We weighed 0.05 g (± 0.0001 g, Fisher Scientific scale) of each sample into a 2 ml screw-cap tube. To clean hair, we added 0.5 ml of 100% ethanol to each tube and then immediately vortexed before briefly centrifuging samples (10 rmp x 5 minutes, Spectrafuge, mo: 16M). Following centrifuging, we discarded supernatant and repeated cleaning process 3 times before drying samples under fume hood for 36 hours. We weighed 0.02 g of washed hair, placed samples into new 2 ml tubes along with 6 ceramic beads and then placed tubes into a bead mill homogenizer for 4 repetitions of 3 by 30 second intervals at 6 m/s (Benchmark bead homogenizer, mo: d2400). We added 1.5 ml of methanol to each homogenized sample and incubated samples in a shaking water bath for 20 hours at 200 rpm. We then dried and subsequently reconstituted extracted cortisol as detailed above.

Laboratory Analysis—EIA Analysis

We assayed all samples in duplicate using a sensitive (0.007 μg dl−1 detection limit) commercially available salivary cortisol EIA kit previously used to quantify hair cortisol (Cat. No. 1-3002, Expanded Range High Sensitivity EIA kit, Salimetrics, PA, USA), including in Eastern Gray Squirrels (Stothart et al. 2019). Serial dilution curves closely followed the standard curve. Inter- and intra-assay coefficients of variation were 3.8% and 1.7%, respectively.

Landscape Analysis

We obtained two ecologically relevant metrics of landscape characteristics for our study sites, the NDVI (normalized difference vegetation index; [Pettorelli 2013]) and NDBI (normalized difference built-up index; [Zha et al. 2003]). While NDVI measures vegetation growth, it is a commonly used and widely informative metric of natural resource availability and ecological integrity (Pettorelli 2013). In contrast, NDBI relies on the specific spectral reflectance characteristics of human-made materials such as concrete to indicate the level of urbanization (Zha et al. 2003). We used Google Earth Engine (Gorelick et al. 2017) to process Sentinel-2, Level-1C snapshots taken between April 01, 2020 and October 01, 2020 into NDVI and NDBI values, using band 8 (near infra-red; 835.1 or 833 nm) and band 4 (red; 664.5 nm or 665 nm) to calculate NDVI, and band 11 (short-wave infra-red 1613.7 nm or 1610.4 nm) and band 8 to calculate NDBI, translating Zha and colleagues' (2003) approach with LANDSAT snapshots for use with Sentinel 2 data. The snapshots comprised of data from the individual satellites S2A and S2B, hence the minor differences in band wavelengths. Eastern Gray Squirrel home ranges are usually smaller than 5 ha (Koprowski 1994), and therefore, we extracted the mean and standard deviation of summer NDVI, and NDBI, for an area of 4 ha around the trapping location or the location of the roadkill.

Statistical Analysis

We analyzed all data in R v.4.0.5 (R Core Team 2021). Due to differences in the body region chosen for hair sample collection (thigh vs tail) and differences in hormone extraction methods, we analyzed data from Canada and from the USA separately. Prior to all analysis, we Box-Cox transformed HCC to achieve normality (Canada: λ = -0.1; USA: λ = -0.5). We assessed the relationship between HCC and two indices of urbanization: NDVI and NDBI. Both indices are strongly correlated (Pearson's product-moment correlations: Canada: $t = -20.21$, $df = 94$, $P < 0.0001$, $r = -0.90$; USA: $t = -30.11$, $df = 94$, $P < 0.0001$, $r = -0.95$)' and thus, should not be included in the same model. We fitted a total of four linear mixed models (LMMs), one per index and per study area. We used Box-Cox transformed HCC as the response variable in all models. Because the relationships between HCC and either index (NDVI or NDBI) might be non-linear (Price et al. 2018), we included either index and its polynomial term ($NDVI^2$ or $NDBI^2$) in the models. For the models fit on the Canadian samples, we further included sex, and sampling location (Guelph and Windsor) as fixed effects, and sample collection month as a random factor. We did not include age as a factor because only adults were sampled. For the models fit on the samples from the USA, we further included sex and age (juvenile vs adult) as fixed effects, and sample collection month and trapping type (trapped alive vs roadkill) as random factors. We did not include reproductive status in any model because it was largely confounded with the variable age and inclusion of reproductive status resulted in multicollinearity.

We tested for one-way interactions between fixed effects, and determined the contribution of interaction terms using likelihood ratio tests (LRT) by comparing the model with the interaction term to the model without. We excluded interaction terms if the explanatory power of the model was not improved by the inclusion of the interaction, based on the LRT (Pinheiro and Bates 2000). For all models, we checked that model assumptions were met using the 'performance' package (Lüdecke et al. 2021). We checked variance inflation factors (Zuur et al. 2010) using the 'vif' function in the car package (Fox and Weisberg 2011), which did not indicate collinearity (all vifs < 2). We plotted model estimates using the 'effects', 'sjPlot' and 'ggplot' packages (Fox and Weisberg 2018, 2019; Lüdecke 2021; Wickham 2016).

Results

HCC varied between sampling areas and was 68.9 ± 70.6 pg/mg (mean ± SD) in Durham, 106.3 ± 70.1 pg/mg in Guelph and 94.9 ± 44.7 pg/mg in Windsor. HCC of samples collected in Canada showed quadratic relationships with NDVI and NDBI (Table 2, Fig. 2A+B). HCC was similar between Guelph and Windsor and between males and females (Table 2). No one-way interaction term between fixed effects was retained in either model.

HCC of samples collected in the USA were not associated with NDVI or with NDBI (Table 3, Fig. 2C+D). HCC was similar between adults and juveniles, and males had higher HCC compared to females (Table 3, Fig. 2C+D). No one-way interaction term between fixed effects was retained in either model.

Discussion

The results of this study show that the relationship between HCC and NDVI and NDBI is not consistent across the extensive distribution range of Eastern Gray Squirrels. HCC of squirrels showed a quadratic relationship with NDVI and NDBI in the Canadian samples, but not in the samples from the USA. In Canada, HCC decreased at low NDVI and at high NDBI values, thus HCC was lower in areas with relatively little vegetation and in areas with more human-made materials and structures.

The regional differences in HCC, where HCC was higher in the Canadian samples compared to the USA samples, and the absence of a relationship between HCC and the examined indices in the Durham area may stem from overall differences in the level of urbanization between the sampling areas. NDVI and NDBI present a fine scale of habitat heterogeneity and an objective measure of urbanization, compared to a more subjective categorization of habitat types as either urban or rural, and show that the Durham area was generally less urbanized when compared with the Guelph area (Table 1, Fig. 1). Forest and urbanized habitats in Durham had higher NDVI and lower NDBI values, respectively, on average compared to forest and urbanized habitats in Guelph (Table 1), and even the most urbanized habitats (highest NDBI value) in the Durham area fell within the middle range of NDBI values recorded in the Guelph area (Fig. 2). This middle range of NDBI values was also the area where HCC began to decrease in the Canadian dataset. Thus, it is possible that we simply lack hair samples from more urbanized areas in Durham and that the addition of such samples would result in a similar relationship. However, regional differences in the effect of urbanization on the prevalence of specific phenotypes (here urban-rural clines of melanism) have also been observed in grey squirrels (Cosentino and Gibbs 2022). Moreover, it has also been reported that the relationship between glucocorticoid levels and urbanization can differ across a species' distribution range. For example, a recent study on *Macropus giganteus* Shaw (Eastern Grey Kangaroos) reported that kangaroos sampled in urban sites in southern Australia had lower FGM levels compared to non-urban counterparts from the same region, whereas the opposite pattern was found for urban kangaroos and their non-urban counterparts in northern Australia (Brunton et al. 2020). These results are consistent with the persistence patterns of kangaroo populations, where urban populations in the northern study sites are more fragmented and declining, whereas urban populations in the southern sites persist often at high population densities (Brunton et al. 2020). Together, the study by Brunton and colleagues (2020) and our results highlight that studies across large geographic ranges may offer important insights into species' responses to urbanization.

HCC of squirrels showed quadratic relationships with NDVI and NDBI in the Canadian samples. This indicates that squirrels sampled in areas with low NDVI and high NDBI values had lower HCC. Thus, squirrels living in areas with relatively little vegetation and more hu-

Table 2. Results of linear mixed models to test for an association between hair cortisol concentration of 96 Eastern Gray Squirrels (*Sciurus carolinensis*) sampled in Canada and mean NDVI and mean NDBI. Factors in bold type indicate significant predictors of hair cortisol concentration.

Predictors	*Estimates*	*CI*	*p*	*Predictors*	*Estimates*	*CI*	*p*
(Intercept)	-6.36	-7.03 – -5.69	<0.001	(Intercept)	-5.75	-5.92 – -5.57	<0.001
NDVI	5.35	1.13 – 9.56	**0.013**	NDBI	-3.60	-5.75 – -1.44	**0.001**
$NDVI^2$	-6.63	-12.91 – -0.36	**0.038**	$NDBI^2$	-7.28	-13.89 – -0.66	**0.031**
Area[Guelph vs. Windsor]	0.26	-0.06 – 0.58	0.117	Area[Guelph vs. Windsor]	0.10	-0.15 – 0.34	0.443
Sex[male]	0.04	-0.09 – 0.17	0.539	Sex[male]	0.01	-0.12 – 0.13	0.937
Random Effects				Random Effects			
σ2	0.096			σ2	0.091		
ICC	0.171			ICC	0.070		
N_{month}	5			N_{month}	5		
Marginal R^2	0.125			Marginal R^2	0.210		
Conditional R^2	0.275			Conditional R^2	0.265		

man-made materials and structures, here the University of Guelph campus and the University of Windsor campus, had lower HCC compared to those living in less urbanized areas such as urban parks and forest sites. Eastern Gray Squirrels commonly occur on university campuses, often at high densities (Hein 1997). University campuses can harbor high plant biodiversity (Liu et al. 2021), potentially offering abundant tree cover and natural food resources. Eastern Gray Squirrels make regular use of backyard resources and feeders (Hansen et al. 2020), and are also frequently observed feeding in dumpsters and trash bins in cities and on university campuses. Some mammals benefit from living in urban areas due to the increased availability of anthropogenic resources in the urban environment (Dowle et al. 2013, Lyons et al. 2017, Oro et al. 2013, Plaza and Lambertucci 2017). Access to anthropogenic food sources can improve body condition (Kaneko and Maruyama 2005, Otali and Gilchrist 2004, Townsend et al. 2019, Wilcoxen et al. 2015), and increase reproductive success in mammals (Beckmann

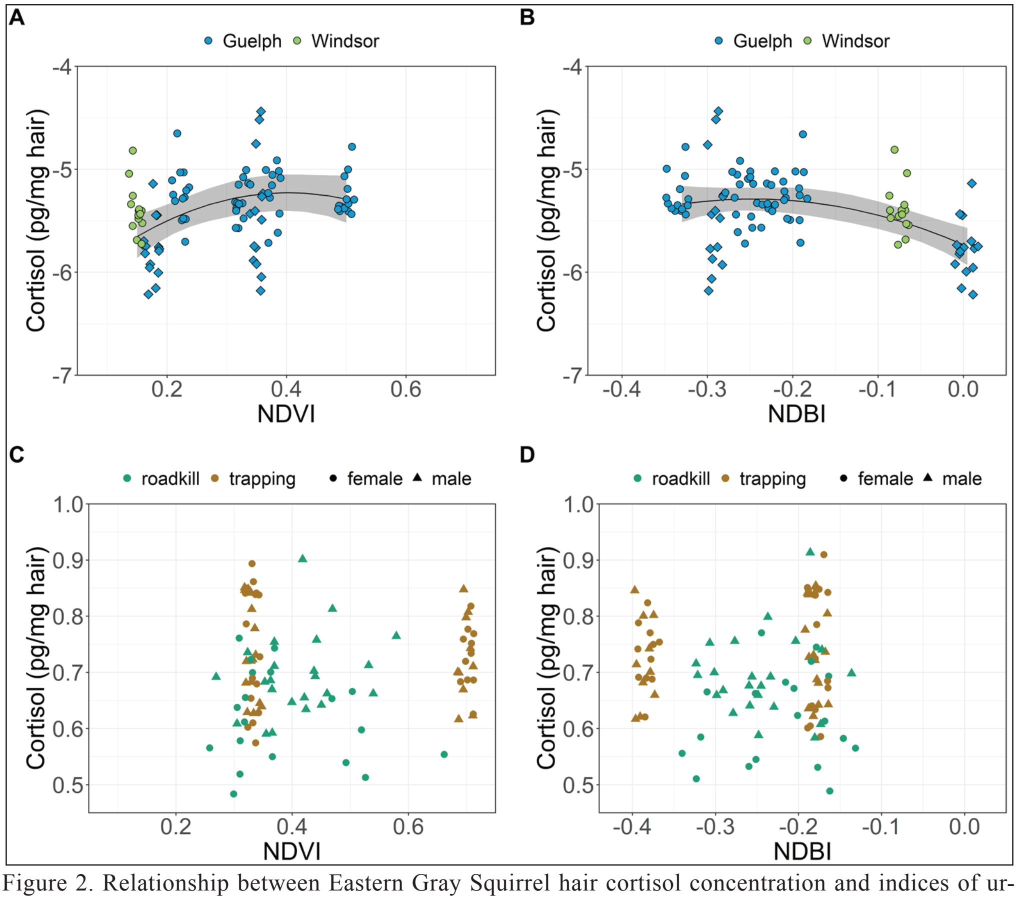

Figure 2. Relationship between Eastern Gray Squirrel hair cortisol concentration and indices of urbanization in samples from Canada (A+B) and the USA (C+D). Hair cortisol concentration showed quadratic relationships with (A) NDVI and (B) with NDBI in the samples from Canada. Hair cortisol concentration was not related to (C) NDVI or (D) NDBI in the samples from the Durham area. Model estimates (lines) and confidence bands (shaded areas) for the fitted values based on standard errors computed from the covariance matrix of the fitted regression coefficients are shown. Box-Cox transformed (Canada: λ = -0.1; Durham: = λ = -0.5) hair cortisol values are presented as points, in (A) and (B) colors indicate the different sampling areas (Guelph and Windsor, diamonds indicate previously published data by Stothart et al. 2019), and in (C) and (D) colors indicate if samples were collected from alive squirrels or from roadkill (circles represent females and triangles represent males).

and Berger 2003, Prange et al. 2004). In addition to natural food sources, university campuses likely offer ample anthropogenic food sources via trash bins and dumpsters near student accommodation and canteens, likely also via gardens and feeders found in adjacent residential areas. Moreover, predation pressure is often altered in urban habitats and urban predators broaden their diet via inclusion of anthropogenic food sources (Eötvös et al. 2018, Gámez et al. 2022, Rodewald et al. 2011). Predation pressure on urban tree squirrels tends to be relaxed (Bowers and Breland 1996, McCleery et al. 2008), and urban squirrels are less wary of humans compared to squirrels from rural habitats (Bateman and Fleming 2014, Cooper et al. 2008, Engel et al. 2020, Parker and Nilon 2008). Access to abundant natural and anthropogenic food sources, together with an altered predation pressure, may explain why squirrels had lower HCC in the two most urbanized habitats included in this study.

Similar to the results reported here for squirrels sampled in Canada, a parabolic relationship between fecal glucocorticoid metabolite (FGM) levels and urbanization has been reported in *Marmota flaviventris* Audubon and Bachman (Yellow-bellied Marmots; Price et al. 2018). Marmots living at intermediate levels of urbanization had the highest FGM levels, suggesting that urban stressors are present at areas of intermediate urbanization which result in elevated FGM levels (Price et al. 2018). Such parabolic relationships between urbanization and glucocorticoid levels are likely shaped by trade-offs between many several factors, such as resource availability, predation pressure and human presence and activities.

Our results show that males tend to have higher HCC compared to females in the Durham area, but not in Canada. It is currently unclear why we did not find a sex-difference in the Canadian samples, but sex-specific differences have also been reported in birds (reviewed in Bonier 2012) and other mammals. For example, male chipmunks have higher HCC than females (Lyons et al. 2017) and female southern brown bandicoots have higher FGCM levels compared to males (Dowle et al. 2013). A meta-analysis assessing how anthropogenic disturbances affect vertebrate glucocorticoid levels found that in general, males respond more strongly to human disturbance than females (Dantzer et al. 2014).

Table 3. Results of linear mixed models to test for an association between hair cortisol concentration of 96 eastern Eastern Gray Squirrels (*Sciurus carolinensis*) sampled in the Durham area, USA and mean NDVI and mean NDBI. Factors in bold type indicate significant predictors of hair cortisol concentration.

Predictors	*Estimates*	*CI*	*p*	*Predictors*	*Estimates*	*CI*	*p*
(Intercept)	0.62	0.26 – 0.98	0.001	(Intercept)	0.55	0.21 – 0.89	0.001
NDVI	0.25	-1.33 – 1.82	0.760	NDBI	-1.06	-3.78 – 1.66	0.446
$NDVI^2$	-0.25	-1.86 – 1.36	0.762	$NDBI^2$	-1.99	-7.15 – 3.17	0.450
Age[juvenile]	-0.02	-0.06 – 0.02	0.409		-0.02	-0.05 – 0.02	0.453
Sex[male]	0.04	0.01 – 0.07	**0.022**	Sex[male]	0.04	0.00 – 0.07	**0.027**
Random Effects				Random Effects			
σ2	0.006			σ2	0.006		
ICC	0.427			ICC	0.467		
N_{month}	10			N_{month}	10		
N_{method}	2			N_{method}	2		
Marginal R^2	0.044			Marginal R^2	0.049		
Conditional R^2	0.453			Conditional R^2	0.493		

One limitation of our study is the fact that hair samples from the Canadian sampling sites were collected from the outer thigh whereas hair samples in Durham were collected from the tail. Several studies have reported that HCC varies between body regions in wild and domesticated animals (e.g., in caribou, reindeer, chimpanzees, marmots, kangaroos, Canada lynx, cattle, pigs and horses), whereas these differences were not reported in other species (rabbits, bears, reindeer and coyotes) (reviewed in Heimbürge et al. 2019). Whether such differences also occur in Eastern Gray Squirrels is currently unclear. Thus, we cannot discern whether the regional differences in hair cortisol levels are due to differences between body regions used for hair sampling or extrinsic factors, such as variation in ambient temperature. In addition, we cannot rule out that some individuals sampled here were dispersing individuals because our study animals were not radio-collared and data does not stem from long-term study sites but represent opportunistic sampling. In the case of dispersing individuals, NDBI and NDVI values might not reflect the environmental conditions that individuals lived in when cortisol was incorporated into the growing hair. Another limitation of the study is that we did not collect hair samples from squirrels living in very urbanized areas such as city centers. Notably, both Durham and Guelph are smaller cities (269,700 and 131,800 inhabitants, respectively) and it remains to be studied whether the patterns we report here for the Canadian samples would also be observed when studying squirrels living in more urbanized major cities.

In the future, common garden experiments or cross-fostering of individuals should be used to assess if the observed relationship between HCC and indices of urbanization are driven by (non-genetic) plasticity or evolutionary change. As urbanization continues to expand further into undisturbed habitats, understanding how wildlife copes and adapts to urban habitats and their specific stressors will become increasingly important in the context of conservation and management strategies. Reduced glucocorticoid levels may represent a form of adaptive (phenotypic) plasticity that facilitates adaptation to and persistence in urban environments. Plasticity in traits such as stress-sensitivity may characterize urban wildlife populations and affect community assembly in urban habitats.

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Author Contributions

R.R., A.G. and M.R.S. designed the study and collected the data, R.R., A.G. and M.R.S. prepared hair samples and performed the hormone assays, P.R.G. performed the landscape analysis, and R.R. wrote the first manuscript draft. All authors contributed critically to the drafts and gave final approval of publication.

Competing Interests

We declare we have no competing interests.

Code Availability

Python code for the calculations of NDVI and NDBI can be found on the GitHub repository (https://github.com/pratikunterwegs/stress-squirrels). The dataset and R code used in formal analysis are available as supplemental files (available online at https://www.eaglehill.us/URNAonline2/suppl-files/urna-203-Rimbach-S1-Analysis_USA.Rmd and https://www.eaglehill.us/URNAonline2/suppl-files/urna-203-Rimbach-S2-Analysis_Canada.Rmd).

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