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Serum biochemistry suggests a physiological response to environmental stress in a native urban Eastern Gray Squirrel (*Sciurus carolinensis*) population

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Abstract

Urban wildlife populations experience human-driven environmental changes that can be both beneficial and detrimental to individual health. We measured body condition and serum chemistry (electrolyte levels, markers of kidney and liver function, protein, glucose, and cholesterol) in a native urban and rural population of Eastern Gray Squirrel (*Sciurus carolinensis*) to assess whether we could detect physiological responses to environmental stressors or dietary differences. We found no differences in body condition between habitats and no evidence of malnutrition at either site. However, urban squirrels had higher blood glucose, lower potassium, phosphorus, chloride, and albumin:globulin ratios. These results align with previous findings of increased dietary sugar in cities and suggest that urban populations of gray squirrels are under greater environmental stress than rural populations, providing future directions for studying physiological responses to urbanization.

Key words: Eastern Gray Squirrel; metabolic disorder; nutrition; serum chemistry; urbanization

Introduction

Successful urban colonizing wildlife tends to be generalist, opportunistic omnivores that can thrive in a variety of habitats by quickly taking advantage of new resources (McKinney 2002). However, cities also pose new demands on urban animals. Relative to natural areas cities are generally hotter, noisier, more polluted, harbour denser populations of urban species, and have excesses of low-quality food in the form of human supplemental feeding and food waste (Alberti *et al.* 2016; Birnie-Gauvin *et al.* 2016). Such environmental stressors can have physiological repercussions for wildlife, including changes in immune function, oxidative stress levels, nutritional state, and metabolism (Bradley and Altizer 2007; Isaksson 2015; Birnie-Gauvin *et al.* 2016; Murray *et al.* 2019). However, the benefits of greater access to high-calorie human-derived foods year-round may outweigh the costs to individual health related to environmental stressors.

There is mounting evidence—largely among mammalian omnivores—of physiological responses to urban diets that mirror symptoms associated with diets high in carbohydrates and saturated fats in humans, such as increasing trends in obesity (Mendoza *et al.* 2007), elevated risk of cardiovascular disease, Type 2 diabetes, and other metabolic disorders (Heidemann *et al.* 2008; Eckel *et al.* 2010). For instance, greater body mass in urban-dwelling individuals has been reported in laboratory and feral rats (Klimentidis *et al.* 2011), foxes (Harrison 1997; Cypher and Frost 1999), Baboon (*Papio cynocephalus*; Bank *et al.* 2003), Florida Key Deer (*Odocoileus virginianus clavium*; Harveson *et al.* 2007), and Raccoon (*Procyon lotor*; Schulte-Hostedde *et al.* 2018). Urban American Crows (*Corvus brachyrhynchos*) have been shown to have elevated plasma cholesterol (Townsend *et al.* 2019), and Grizzly Bears (*Ursus arctos*) with access to human food subsidies have higher carbohydrate and lower protein levels than those in more natural habitats (Coogan *et al.* 2018). Whether these symptoms indicate poor health is uncertain. Thus, human food subsidies appear to represent a double-edged sword: increased calorie intake may positively influence body condition, survival, and reproductive success, but at the same time its limited nutrient quality may make it detrimental to animals' general health (Weaver *et al.* 2014).

Food subsidies are partly responsible for increased abundances and high population densities in successful urban species like rats, White-tailed Deer (*Odocoileus virginianus*), Raccoons, and Eastern Gray Squirrel (*Sciurus carolinensis*; Adams 1994; Parker and Nilon 2008). Higher than natural population densities of urban species in cities can increase the risk of disease and parasite transmission (Bradley and Alizer 2007). In addition, predator–prey dynamics are altered in cities due to both changes in community composition and because predators can subsidize their diet with human-derived food sources (Rodewald *et al.* 2011; Oro *et al.* 2013), allowing prey species to proliferate. The combination of heightened disease risk, increased stress, and exposure to pollutants has made urban wildlife generally less healthy than their rural counterparts (Murray *et al.* 2019).

We conducted a pilot observational study of serum biochemistry in Eastern Gray Squirrel aimed at detecting and identifying signs of physiological stress associated with reduced habitat quality or dietary differences in an urban environment. Serum biochemistry analysis is an effective way to measure population-level health, as it is a good indicator of disease state, nutritional status, and habitat quality (Hanks 1981). If we assume that rural individuals living in more natural environments generally have blood biochemistry parameters within normal ranges, then we can treat those results as a baseline from which deviations in urban individuals could point to health issues related to environmental stress. Eastern Gray Squirrels are opportunistic omnivores with a wide dietary breadth, typically consisting of the nuts, flowers, and buds of a variety of hardwood trees (such as oak, hickory, and beech), fungi, insects, cultivated crops, and, from time to time, small animals and bones (Koprowski 1994; McAllister *et al.* 2016). Eastern Gray Squirrels are a seasonal fat-accumulating, non-hibernating species that rely on lipid reserves and food caches while overwintering (Koprowski 1994). Their abundance is positively associated with human food provisioning (Parker and Nilon 2008; Bonnington *et al.* 2014), and they are frequently observed feeding on high-calorie human food waste (Vinopal 2017; Mahdawi 2018; https://www.reddit.com/r/SquirrelsEatingPizza/), suggesting their success in human-dominated habitats is partly associated with increased resources which allows them to accumulate more fat during autumn and have reliable access to food in winter apart from their cached reserves. We measured body condition and used serum chemistry analyses to assess electrolyte levels, markers of kidney and liver function, protein, glucose, and cholesterol, to get a general picture of population health in urban and rural squirrels. We hypothesized that different habitat qualities in urban and rural environments would produce different serum biochemistry profiles. We predicted profiles for urban Eastern Gray Squirrels would indicate physiological symptoms associated with environmental stress, such as abnormal liver, kidney, or immune functions. Additionally, similar to previous studies of urban mammals, we specifically expected to find higher glucose, cholesterol, and body condition in urban Eastern Gray Squirrels that would suggest increased consumption of anthropogenic food.

Methods

Sampling

Eastern Gray Squirrels were sampled from May to June 2019 at two sites with differing levels of human disturbance, near the northern limit of their native range. Here, squirrels typically breed twice a year (Koprowski *et al.* 2001), with breeding bouts typically beginning in mid-March and mid-July. The urban site was a ~10 ha park located on the University of Manitoba campus, Winnipeg, Manitoba (49.81°N, 97.14°W). Relative to natural forests it is sparsely treed and is adjacent to a river, a suburb, and bordered by major roads on two sides. This park experiences high human foot traffic. Squirrels at this site have easy access to birdfeeders and human food waste in trash cans and litter. Our second site was a rural hardwood forest patch of ~34 ha located in southern Manitoba (49.24°N, 98.01°W). The forest is bordered by agricultural land and a road (at a distance of 47 m), and has minimal human food waste. Crops surrounding this forest patch are corn, dry beans, soybeans, canola, and grains. These food items are not outside the Eastern Gray Squirrel's normal dietary range; however, gray squirrels typically preferably consume hardwood seeds when they are available, as they are in this forest stand (Korschgen 1981; Koprowski 1994).

Squirrels were trapped using Tomahawk live traps (Tomahawk Live Trap Co., Tomahawk, Wisconsin, USA) baited with peanut butter, then restrained in a capture bag (Koprowski 2002) without anesthetic. Sex, approximate age (adult or juvenile), weight (g), reproductive status (scrotal or non-scrotal for males; pregnant, lactating, or non-lactating for females), and body length (cm) from the base of the skull to the base of the tail was recorded for each individual. We implanted passive integrated transponder (PIT) tags for later identification. All animals were handled in a consistent order to minimize variation in serum analytes across individuals, recording routine measurements before taking blood samples. We measured body condition by taking the residuals from a linear regression of mass on body size (here, spine length). This method corrects for the effects of an individual's structural size on its mass (Schulte-Hostedde *et* *al.* 2001, 2005). We estimated body condition independently for the sexes within each location. Juveniles were excluded from body condition calculations (Table 1). Squirrels were released after capture.

Serum samples and tests

Blood samples were collected from six urban and nine rural individuals (Table 1). Samples were taken at the urban site between 13 and 15 May 2019 and the rural site between 7 and 14 June 2019. Blood (≤1 mL) was drawn on-site from the femoral vein using a syringe without anticoagulant and stored on ice until delivery to the Manitoba Veterinary Diagnostics Services lab (Department of Manitoba Agriculture Food and Rural Development, Winnipeg, Manitoba). Samples were processed within 12 h of capture. Serum biochemistry profiles were run for each sample, consisting of the following tests: sodium, potassium, chloride, urea, creatinine, calcium, phosphorus, magnesium, amylase, lipase, alkaline phosphatase (ALKP), gamma-glutamyl transferase (GGT), bilirubin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatine kinase, glucose, cholesterol, total protein, albumin, and globulin. Some tests were omitted when sample volume was lacking; thus, we do not report results for amylase, lipase, or creatine kinase. Sample sizes for each comparison are given in Table 1. Because this is the first serum biochemical comparison between urban and rural gray squirrel populations and such comparisons in wildlife, in general, are still rare, our strategy was to quantify as broad a range of blood markers as possible, including those that required rapid testing, within the tight early summer non-reproductive period to provide a baseline status upon which we could build future work.

Statistical analysis

All analyses were done in R version 3.6.1 (R Core Team 2013). We first plotted and visually inspected the data using boxplots, then tested differences in serum variables between sites using non-parametric Kruskal-Wallis tests due to small sample size and unbalanced groups. We found no sex differences for serum measurements (Figures 1–3), thus did not include sex as a factor in our analyses.

Results

Body mass for adults were similar between urban and rural study sites (Table 1). There were no detectable differences in body condition between sexes.

Table 1. Body condition and serum parameters in urban and rural Eastern Gray Squirrel (*Sciurus carolinensis*), Manitoba, Canada. Sample sizes differ when individuals were excluded due to age (mass and body condition) or low serum sample volume. Significant differences were found in serum parameters of urban and rural squirrels shown in bold.

	Urban		Rural		χ^2	*P*
	n	Median (range)	*n*	Median (range)		
Mass (g)	6	709.75 (577.00–737.50)	7	611.00 (570.00–785.00)	—	—
Body condition	6	36.73 (−85.35–54.84)	7	−21.84 (−65.79–95.91)	0.02	0.89
Sodium (mmol/L)	6	144.50 (142.00–149.00)	9	146.00 (141.00–150.00)	0.80	0.37
Potassium (mmol/L)	**6**	**5.15 (4.30–10.30)**	**9**	**8.70 (5.80–14.40)**	**5.57**	**0.02**
Chloride (mmol/L)	**6**	**113.00 (112.00–116.00)**	**9**	**117.00 (113.00–120.00)**	**5.95**	**0.01**
Calcium (mmol/L)	6	2.34 (2.12–2.41)	8	2.23 (2.04–2.31)	2.40	0.12
Phosphorus (mmol/L)	**6**	**1.79 (1.34–2.43)**	**8**	**3.20 (2.62–4.12)**	**9.60**	**<0.01**
Magnesium (mmol/L)	6	1.23 (1.03–1.32)	8	1.25 (1.00–1.60)	0.10	0.75
Urea (mmol/L)	6	7.65 (5.00–13.30)	9	8.70 (7.60–13.40)	3.37	0.07
Creatinine (mmol/L)	6	46.50 (40.00–50.00)	9	50.00 (41.00–91.00)	1.01	0.31
Total bilirubin (µmol/L)	6	0.00 (0.00–0.00)	8	0.00 (0.00–2.00)	1.62	0.20
ALKP (U/L)	6	622.00 (271.00–1010.00)	8	924.00 (180.00–1385.00)	2.02	0.16
GGT (U/L)	6	10.00 (5.00–15.00)	8	10.50 (7.00–46.00)	0.71	0.40
ALT (U/L)	**6**	**3.00 (3.00–14.00)**	**8**	**10.00 (3.00–51.00)**	**3.73**	**0.05**
AST (U/L)	6	128.00 (69.00–246.00)	8	175.50 (92.00–394.00)	1.67	0.20
Glucose (mmol/L)	**6**	**7.45 (6.10–10.90)**	**9**	**5.00 (2.50–6.70)**	**8.70**	**<0.01**
Cholesterol (mmol/L)	6	5.35 (4.45–6.78)	8	4.84 (3.19–5.76)	1.07	0.30
Total protein (g/L)	6	68.50 (61.00–85.00)	9	66.00 (59.00–85.00)	0.28	0.59
Albumin (g/L)	6	38.00 (31.00–44.00)	8	36.50 (32.00–53.00)	0.07	0.80
Globulin (g/L)	6	31.50 (29.00–41.00)	8	29.50 (24.00–32.00)	3.10	0.08
Albumin:Globulin	**6**	**1.15 (1.00–1.30)**	**8**	**1.30 (1.10–1.70)**	**3.91**	**0.05**

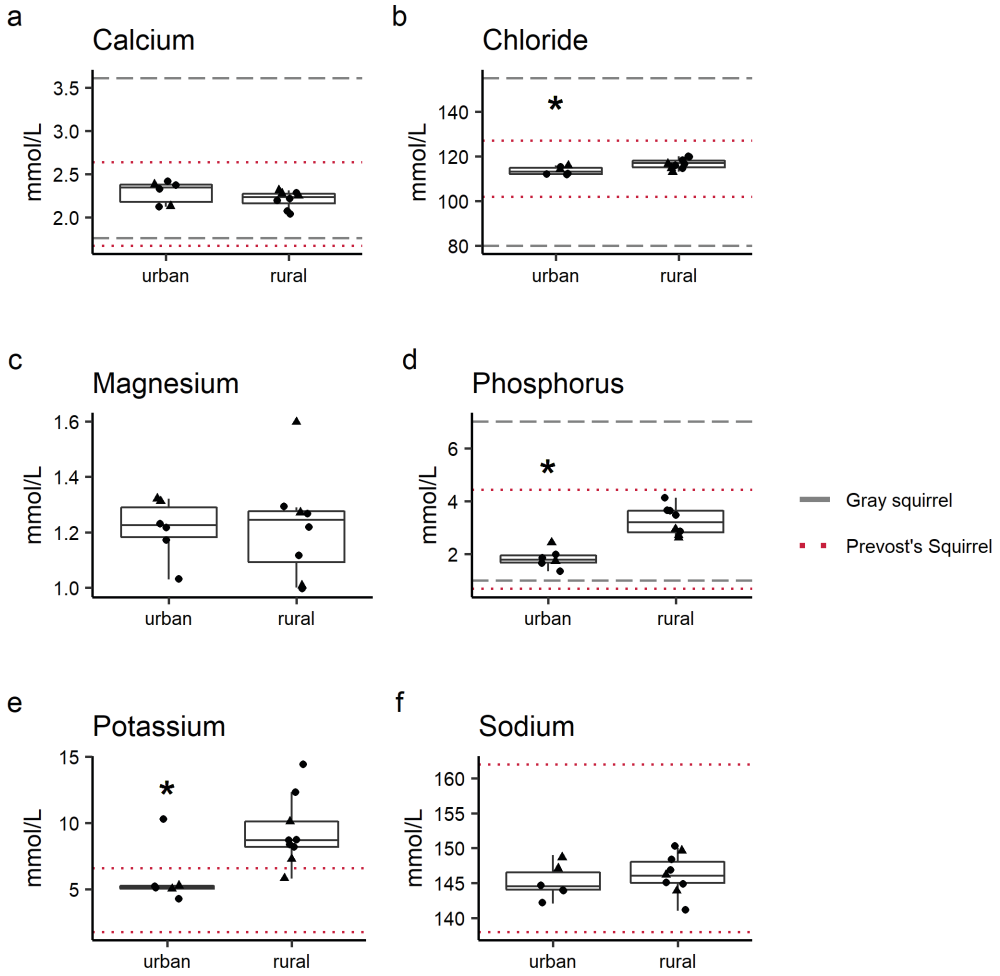

FIGURE 1. Boxplots for serum electrolytes in urban and rural Eastern Gray Squirrel (*Sciurus carolinensis*), Manitoba, Canada. Circles are females, triangles are males. Available reference ranges for gray squirrel (Hoff *et al.* 1976; Koprowski 1994) and for Prevost's Squirrel (*Callosciurus prevostii*; Teare 2013) are shown. Asteriks (*) indicate significant differences between urban and rural populations ($P < 0.05$).

Two females at each site were lactating, however, no squirrels were detectably pregnant. Serum profiles for lactating females were not outliers, therefore they were retained for further analysis.

Urban squirrels had significantly higher glucose levels than those from the rural site ($P < 0.01$, $\chi^2_1 = 8.70$; Table 1; Figure 1), as we expected. Cholesterol levels did not detectably differ (Table 1; Figure 1). Among serum ions, potassium ($P = 0.02$, $\chi^2_1 = 5.57$), phosphorus ($P < 0.01$, $\chi^2_1 = 9.6$), and chloride ($P = 0.01$, $\chi^2_1 = 5.95$) were significantly lower in urban squirrels (Table 1; Figure 1). Sodium, calcium, and magnesium levels were comparable at both sites (Table 1; Figure 1).

ALT was significantly lower at the urban site ($P = 0.05$, $\chi^2_1 = 3.73$; Table 1; Figure 2). A majority (5/6) of urban squirrels had concentrations below the detection limit of 3 U/L (Figure 2). We did not detect any differences in other serum enzymes (ALKP, GGT, AST; Figure 2). Total bilirubin, creatinine, urea, total protein levels, as well as albumin and globulin were similar at both sites (Table 1; Figures 2 and 3). The ratio of albumin to globulin (A:G ratio) was significantly higher in rural squirrels ($P = 0.05$, $\chi^2_1 = 3.91$; Table 1; Figure 3).

Discussion

In general, serum parameters at both sites were within published ranges for Eastern Gray Squirrels (Hoff *et al.* 1976; Koprowski 1994) and reference

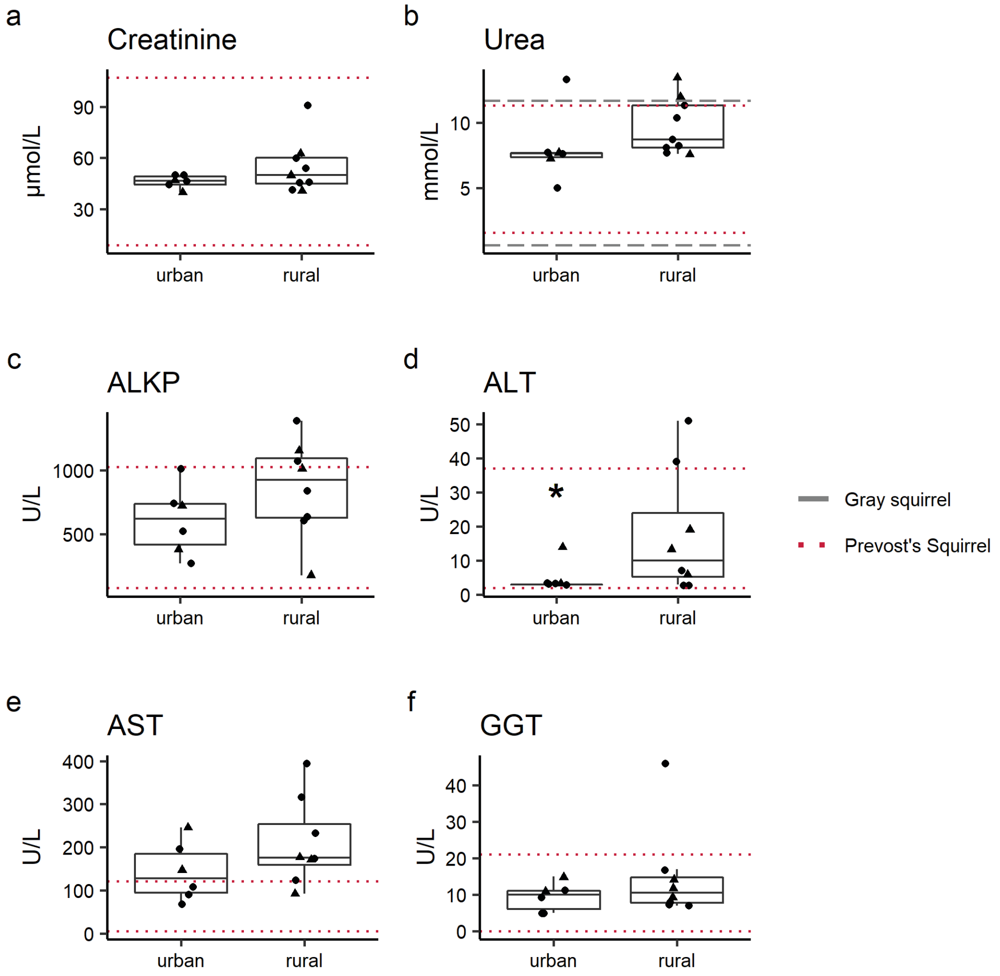

FIGURE 2. Boxplots for creatinine, urea, and enzymatic markers for liver and kidney function in urban and rural Eastern Gray Squirrel (*Sciurus carolinensis*). Circles are females, triangles are males. Available reference ranges for gray squirrel (Hoff *et al.* 1976; Koprowski 1994) and for Prevost's Squirrel (*Callosciurus prevostii*; Teare 2013) are shown. ALKP = Alkaline phosphatase; ALT = alanine aminotransferase (note: 5/ 6 urban grey squirrels had levels below the detectable limit of 3 U/L); AST = aspartate aminotransferase; GGT = gamma-glutamyl transferase. Asteriks (*) indicate significant differences between urban and rural populations ($P < 0.05$).

ranges for another member of Sciuridae, Prevost's Squirrel (*Callosciurus prevostii*; Teare 2013; Figures 1–3). We found no difference in body condition between sites, therefore the chemistry value differences were not likely to be strongly related to body condition as measured by size-corrected weight. Although serum analytes were not correlated with body condition, they were suggestive of differences in habitat quality and nutritional status between our urban and rural sites.

We found elevated blood glucose in urban squirrels, supporting our expectation that individuals with easy access to human food waste have higher sugar intake. This result was consistent with previous findings in Raccoons and Baboons (Banks *et al.* 2003; Schulte-Hostedde *et al.* 2018), potentially reflecting a more general trend among urban mammalian omnivores. In addition to increased blood sugar, chloride, phosphorus, and potassium concentrations differed across urban and rural populations. Electrolyte abnormalities frequently co-occur with metabolic disorders in humans (Liamis *et al.* 2014), however, these results might reflect differing compositions of anthropogenic versus natural foods rather than physiological responses to a change in diet (Choi *et al.* 2002).

Given similar body conditions between urban and

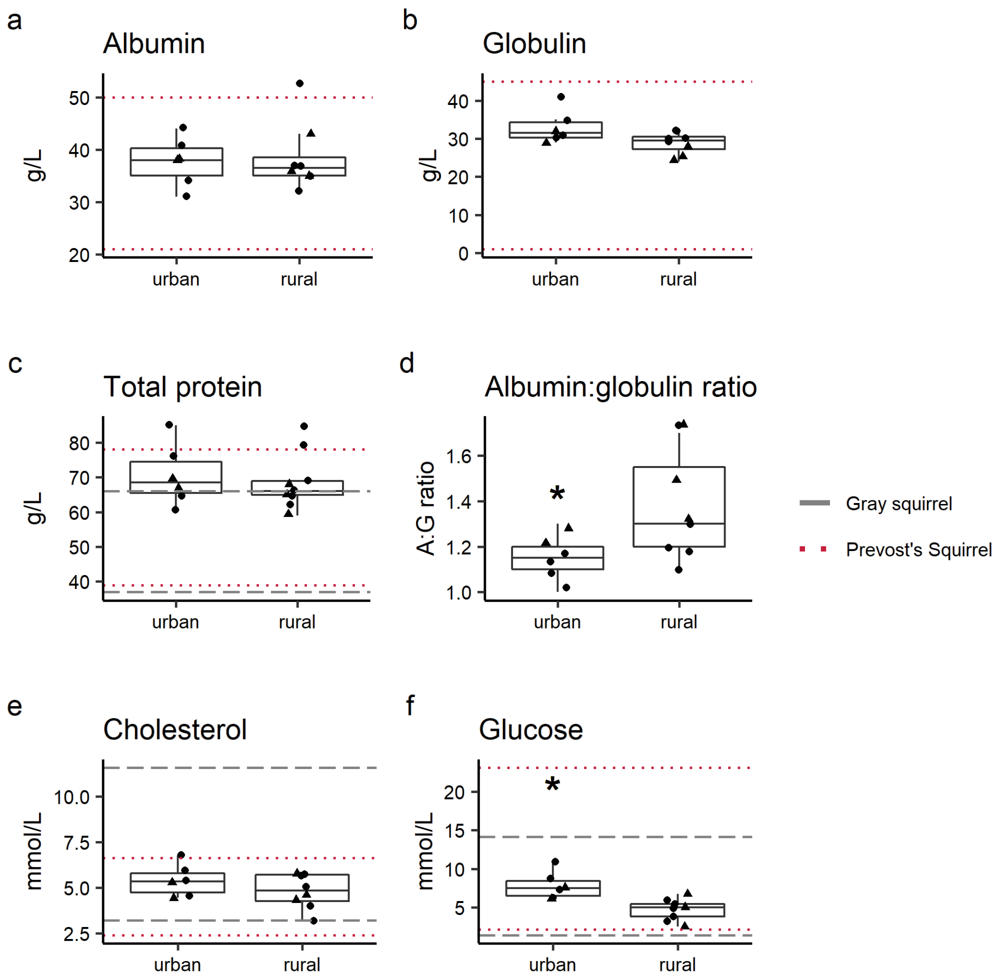

FIGURE 3. Boxplots for serum protein, cholesterol, and glucose in urban and rural Eastern Gray Squirrel (*Sciurus carolinensis*), Manitoba, Canada. Circles are females, triangles are males. Available reference ranges for gray squirrel (Hoff *et al.* 1976; Koprowski 1994) and for Prevost's Squirrel (*Callosciurus prevostii*; Teare 2013) are shown. Asteriks (*) indicate significant differences between urban and rural populations ($P < 0.05$).

rural sites, and that glucose values were within previously published ranges, it is unclear if these squirrels are at risk of developing metabolic disorders. Due to their ecology and physiology, Eastern Gray Squirrels may have mechanisms in place for regulating body mass and fat accumulation that reduces the risk of metabolic disturbance while accumulating lipid reserves. Indeed, increased access to supplemental food may help autumn weight gain in gray squirrels—similar to Grizzly Bears, which are better able to optimize food intake to maximize weight gain before hibernation when they have access to anthropogenic food (Coogan and Raubenheimer 2016; Coogan *et al.* 2018). These characteristics suggest that Eastern Gray Squirrels may be pre-adapted to exploit human food wastes, and are perhaps robust to major health consequences of urban diets.

We also note interesting patterns concerning serum protein markers. Serum albumin reflects long-term protein status, while urea levels shift in response to short-term protein availability (Caldeira *et al.* 2007). Creatinine is reduced when muscle mass decreases due to protein catabolism in the body (Caldeira *et al.* 2007; Robert and Schwanz 2013). Although we did not find statistically detectable differences between populations in creatinine and urea, in both cases they tended to be lower in urban populations. Total protein was not different between sites and was on the high margin compared to previously published ranges (Hoff *et al.* 1976; Koprowski 1994), indicating that neither population was malnourished with respect to protein intake. However, we found significantly lower ratios of albumin to globulin (A:G ratios) in urban squirrels. Globulin is expected to increase after infection and is also positively associated with inflammatory responses and nutritional stress (Kilgas *et al.* 2006). Individuals in better physiological condition have higher A:G ratios. This result is interesting but difficult to interpret at this stage because inflammation is typically an early physiological response to many kinds of stressors. Previous studies using transcriptomic data report candidate genes associated with metabolism and immunity that are under selection in cities, and greater expression of genes involved in the inflammatory response in urban populations (Harris *et al.* 2013; Watson *et al.* 2017). This result aligns with general trends of greater physiological stress associated with environmental stressors in urban populations, but the underlying causes remain to be explored.

There are few published studies of serum biochemistry in gray squirrels, making inferences about individual health difficult. Our values were well within reported ranges in Hoff *et al.* (1976; Figures 1–3). We note, however, that those ranges were obtained from samples taken year-round, and thus might obscure potential seasonal variation in analyte levels. Seasonal variation in diet and food intake in Eastern Gray Squirrels (Koprowski 1994) would also drive seasonal differences in blood chemistry, making the comparison of our values to annual averages in serum parameters not ideal. We also note sampling dates across these urban and rural sites differed by roughly a month, but it seems unlikely that this gap would affect food availability as vegetation phenology appears similar for these sites at this temporal scale. Trees in southern Manitoba begin producing leaves and bloom in early April (e.g., Bur Oak [*Quercus macrocarpa* Michaux], Red Pine [*Pinus resinosa* Aiton], Eastern White Pine [*Pinus strobus* L.], White Spruce [*Picea glauca* (Moench) Voss], and Black Spruce [*Picea mariana* (Miller) Britton, Sterns & Poggenburgh]); Ahlgren 1957; Lechowicz 1984) and both sites are fully leafed by mid-May.

Our main findings—higher glucose, shifts in electrolyte balance, and lower A:G ratio—suggest that this urban squirrel population is consuming anthropogenic food and is exhibiting a physiological stress response to poorer habitat quality compared to the rural site. We note that this was an exploratory comparison of serum biochemistry between urban and rural Eastern Gray Squirrel populations, and results should be interpreted as hypothesis generating. Sample sizes were small in terms of the number of individuals captured and broad in terms of the number of blood markers analyzed. Many of the variables examined here can vary based on several factors such as age, individual health, time since last meal, and stress associated with capture and handling. Here, all squirrels were baited, captured, and handled in the same way, in essence controlling for these effects on serum biochemistry. However, in future the effects of stress and food intake on glucose might be minimized by measuring glycated serum proteins (as in Schulte-Hostedde *et al.* 2018), which is an indicator of circulating blood sugar integrated over a longer period rather than instantaneous measurements of serum glucose concentrations. Future studies examining the risk of metabolic disorders in Eastern Gray Squirrels could additionally measure circulating hormone levels, such as insulin, and urine glucose to encompass the main symptoms used to diagnose metabolic disorders in other species (Heidt *et al.* 1984; Greco 2001; Ciobotaru 2013). Quantifying corticosteroid levels from plasma or fur would provide an indication of physiological stress over short and long periods, respectively. Furthermore, pinpointing causes of inflammation would be informative for identifying specific features of urbanization that have physiological effects on wildlife. Potential sources of inflammation could be untangled using hematological tests to distinguish between infection (parasitic, viral, or bacterial), or other chronic influences (e.g., diet, pollution). We note that we obtained blood smears for urban individuals ($n = 6$) and a small subset of the rural individuals presented here ($n = 2$), but due to lack of rural samples were unable to make a formal comparison. However, among those individuals for which we did obtain samples, no blood parasites were detected.

The effects of urban environmental stressors on individual health have important impacts on population and evolutionary dynamics. Although increased resources allow synanthropic species to reach much higher densities in cities, as our results demonstrate, greater abundance does not always correspond to good overall health (Fedriani *et al.* 2001; Murray *et al.* 2015; Schulte-Hostedde *et al*. 2018), meaning large urban populations may suffer higher mortality. Food provisioning can have indirect consequences on survival by promoting the spread of disease and parasites in denser populations (Orams 2002; Oro *et al.* 2013) that are weakened by poor nutrition and stress. In this way, supplemental food may be an important selection pressure in cities shaping evolution in successful urban species within and outside their native range. What this means for population demographics in the long-term is unclear, even for species that do well in cities. The consequences of human food waste reverberate throughout levels of the urban ecosystem, and understanding these complex relationships is important for wildlife health and our ability to control populations of synanthropic species in cities.

Author Contributions

Writing – Original Draft: C.S.; Writing – Review & Editing: C.S., R.P.K., J.R.T., and C.J.G.; Conceptualization: C.S., R.P.K., J.R.T., and C.J.G.; Investigation: C.S. and R.P.K.; Methodology: C.S., J.R.T., and C.J.G.; Formal Analysis: C.S. and C.J.G.; Funding Acquisition: C.S., R.P.K., and C.J.G.

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SUPPLEMENTARY MATERIAL:

Appendix S1. Raw data used in analyses.