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Does cholesterol differ in urban and rural European Starlings (*Sturnus vulgaris*) across different developmental stages?

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ABSTRACT—Urban areas—characterized by high human densities, buildings, and impermeable surfaces—are increasing globally and represent a leading threat to wildlife by drastically altering the natural resources wildlife species are accustomed to. Prior work suggests that living in urban habitats can cause wildlife to show increased cholesterol levels. In biomedical research, elevated cholesterol is linked to disease, but the consequence of elevated cholesterol in wildlife remains unclear. We measured total cholesterol in European Starlings (*Sturnus vulgaris*), an urban-adapted species, across an urban and a rural site. We asked: (1) Do urban starlings have elevated cholesterol? and (2) Is elevated cholesterol correlated to negative physiological outcomes in starlings? We found that nestlings from the urban ($N = 16$) and rural ($N = 98$) sites showed similar cholesterol, but adult starlings from the urban ($N = 5$) habitat showed elevated cholesterol compared to rural ($N = 36$) birds. However, elevated cholesterol was not correlated with increased oxidative damage to DNA, lowered body condition, or increased baseline glucocorticoids across any age class, suggesting that elevated cholesterol did not come at a physiological cost to starlings. Future work is needed to explore what mechanism(s) drive variation in cholesterol across urban and rural birds, as well as whether any long-term consequences of elevated cholesterol exist. *Received 25 July 2023. Accepted 5 January 2024.*

Key words: body condition, DNA damage, glucocorticoids, urbanization.

Le cholestérol diffère-t-il entre les etourneaux sansonnets (*Sturnus vulgaris*) urbains et ruraux selon les différents stades de développement?

RÉSUMÉ (French)—Les zones urbaines, caractérisées par de densités humaines élevées et des bâtiments et des surfaces imperméables, augmentent au niveau mondial et représentent une menace majeure pour la faune sauvage en modifiant radicalement les ressources naturelles auxquelles les espèces sont habituées. Des travaux antérieurs suggèrent que vivre dans des habitats urbains peut entraîner une augmentation des taux de cholestérol chez la faune sauvage. Dans la recherche biomédicale, un taux de cholestérol élevé est lié à la maladie, mais les conséquences d'un taux de cholestérol élevé chez la faune sauvage ne sont pas claires. Nous mesurâmes le cholestérol total chez l'étourneau sansonnet (*Sturnus vulgaris*), une espèce adaptée à la ville, sur un site urbain et un site rural. Nous demandâmes: (1) est-ce que les étourneaux sansonnets urbains ont un taux de cholestérol élevé? et (2) est-ce qu'un taux de cholestérol élevé est corrélé à des résultats physiologiques négatifs chez les étourneaux? Nous constatâmes que les oisillons des sites urbains ($N = 16$) et ruraux ($N = 98$) présentaient un taux de cholestérol similaire, mais que les étourneaux adultes de l'habitat urbain ($N = 5$) présentaient un taux de cholestérol élevé par rapport aux étourneaux adultes de l'habitat rural ($N = 36$). Cependant, un taux de cholestérol élevé n'était pas corrélé à une augmentation des dommages oxydatifs de l'ADN, à une condition corporelle réduite ou à une augmentation des glucocorticoïdes de base dans toutes les classes d'âge, ce qui suggère qu'un taux de cholestérol élevé n'a pas eu de coût physiologique pour les étourneaux. Des travaux futurs sont nécessaires pour explorer le(s) mécanisme(s) qui déterminent la variation du cholestérol chez les oiseaux urbains et ruraux, ainsi que pour déterminer s'il existe des conséquences à long terme d'un taux de cholestérol élevé.

Mots-clés: condition corporelle, dommage à l'ADN, glucocorticoïdes, urbanisation.

Intraspecific comparative studies provide a useful framework for understanding how urbanization affects birds. Among wildlife, birds are commonly used as study systems to understand the impacts of urbanization on wildlife (McKinney 2008) because there exists large variation within and among species in the ability of individuals to tolerate or thrive in urban environments (Fanelli et al. 2022). By comparing individuals of the same species experiencing more- versus less-urbanized habitats, we can gain an understanding of how urbanization can affect individuals and populations. Prior work in House Sparrows (*Passer domesticus*) showed that birds captured in highly urbanized areas weighed less, had shorter tarsi, and were in lower body condition than birds found in neighborhoods with a low human density (Liker et al. 2008). In addition to influencing the growth or body condition of individual birds, which are often correlated with survival and reproduction (Bailey et al. 2016), urban habitats can also impact fitness directly. Bailey et al. (2016) found that breeding success in urban environments was lower, where urban birds had smaller clutches and higher clutch failures as well as reduced growth rates and body condition prior to fledging. These differences were linked to long-term fitness, where nestlings in urban habitats

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had lower overall survival rates compared to rural nestlings (Bailey et al. 2016).

Living in urban environments may also impact birds in less direct ways that do not immediately alter reproduction or survival but can nevertheless have long-term impacts on organismal fitness. For example, birds from urban environments often show differences in their physiology—including hormones (Sinclair et al. 2022), gut microbiome (Murray et al. 2020), DNA methylation (Watson et al. 2020), and cholesterol (Townsend et al. 2019)—compared to ones from rural habitats. Cholesterol is a type of dietary fat that is most commonly acquired from fatty foods in the diet. Cholesterol is an essential lipid for many critical functions including the production of cell membranes and being a precursor for the synthesis of vital substances including steroid hormones, bile acids, and vitamin D in vertebrates. However, cholesterol can also have harmful effects on health if circulating concentrations become too elevated (Zampelas and Magriplis 2019).

A recent study found that cholesterol differed in a wild bird across urban and rural populations (Townsend et al. 2019): American Crows (*Corvus brachyrhynchos*) had higher plasma cholesterol levels in urban habitats while rural crows had lower plasma cholesterol. The results of this study were replicated in 2 regions and yielded the same pattern (California and New York; Townsend et al. 2019). Similarly, Gambel's Quail (*Callipepla gambelii*) found in areas with more grass had lower circulation plasma concentrations of cholesterol, compared to quail in urban areas with access to more dietary fats (Funk et al. 2020). Marteinson and Verreault (2020) also found that Ring-billed Gulls (*Larus delawarensis*) spending more time foraging in urbanized areas showed elevated plasma cholesterol levels. Together, these studies suggest that one impact of living in urban habitats may be that birds experience elevated cholesterol levels compared to their rural counterparts. These patterns may arise because animals in urban habitats have reduced access to natural prey or increased access to anthropogenic foods. Unfortunately, our understanding of how urbanization affects animal physiology remains limited, and in the case of cholesterol it remains unclear whether elevated levels resulting in urban habitats come at any costs as would be expected based on the biomedical literature.

In humans, we know that elevated cholesterol can lead to the thickening and hardening of arterial walls and blood vessels, leading to cardiovascular disease (Jauho 2019). Similarly, Meek et al. (2014) found that lab rats (*Rattus norvegicus*) consuming high-fat western diets led to an overall increase in cholesterol levels, which is similar to that seen in humans consuming high-fat diets. However, we know relatively little about the consequences of elevated cholesterol in birds or in free-living animals.

In this study we examine the relationship between urbanization and physiology of free-living European Starlings (*Sturnus vulgaris*) using an integrative approach. We assess whether birds from an urban and a rural site differed in their cholesterol, as well as whether elevated cholesterol levels are correlated with being in a worse physiological state as predicted based on biomedical work in other taxa. We explore these questions at different developmental time points, including in nestlings at their peak of growth, in nestlings about to fledge, and in breeding adult birds.

We use a multi-year dataset to address 2 main questions. First, how does urbanization shape the cholesterol of European Starlings? We predicted that starlings, both nestlings and adults, from the urban site would show higher blood cholesterol levels compared to rural ones as has been shown for other species of birds previously. Second, we ask whether elevated cholesterol is correlated with being in worse physiological state in adult or nestling birds. To explore such possible costs of elevated cholesterol, we investigated the correlation between cholesterol and the following indices of health: body condition, DNA damage from oxidative stress, and glucocorticoid physiology. We predicted that birds with elevated cholesterol titers would show altered indices of health—for example, elevated cholesterol may be linked with higher body condition (as shown in American Crows; Townsend et al. 2019), while elevated cholesterol is positively correlated with elevated oxidative damage and glucocorticoids (as shown in chickens; Latour et al. 1996, Boostani et al. 2015). Thus, we tested whether cholesterol was correlated to body condition (using an index of the residuals of mass on skeletal size).

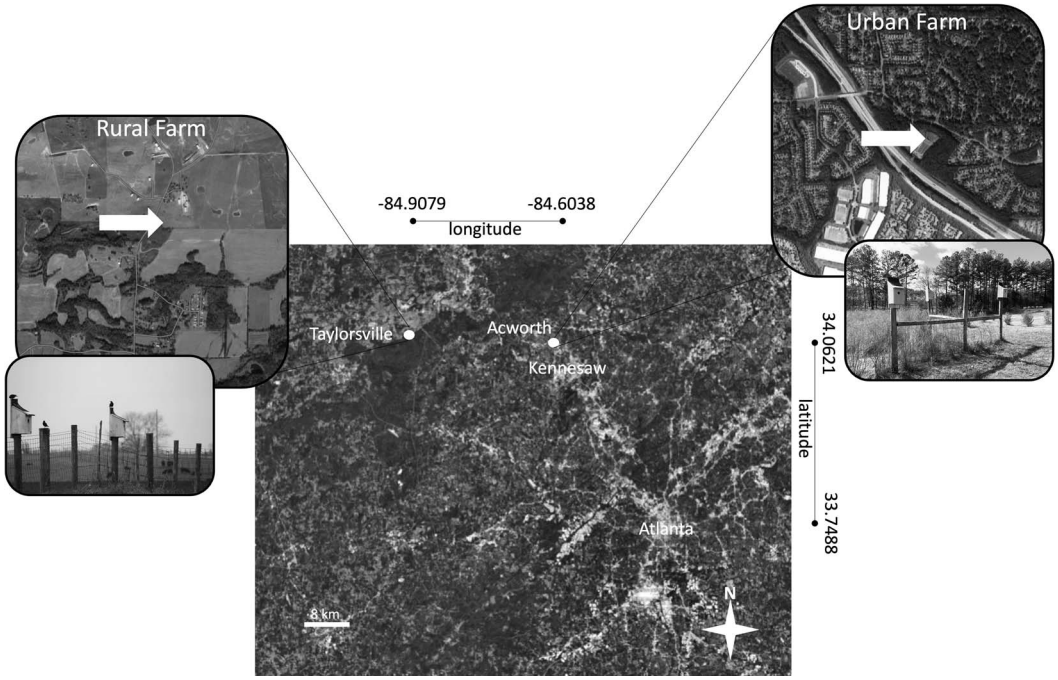

Figure 1. A satellite map of the study area, depicting the rural and urban farm site locations relative to the city of Atlanta. For each site, we include a more detailed satellite image where a white arrow indicates the specific location where nest boxes were monitored and showing greater detail of the surrounding habitat. The images below each site's insert show an example of nest box location and surrounding vegetation for the 2 study sites.

We measured DNA damage resulting from oxidative stress to test whether high cholesterol diets are associated with elevated oxidative damage as shown in prior biomedical studies (Tomofuji et al. 2006). Finally, we examined whether cholesterol and glucocorticoids were positively correlated, since cholesterol is a precursor to glucocorticoid synthesis and high cholesterol diets can lead to elevated glucocorticoids (Anderson et al. 2019). Overall, this study contributes to our understanding of how urbanization shapes bird cholesterol titers in 2 new ways: by assessing age-related changes in cholesterol across urban and rural starlings, and by exploring correlations between cholesterol and other indices of physiological condition.

Methods

Study system

We studied free-living European Starlings—a widespread cavity-nesting songbird that is considered an urban-adapted species (Cabe 2021)—because they reside and breed in both urban and rural environments. Starlings are social birds that feed and flock together during winter months and are usually socially monogamous during the breeding season (Kessel 1957). Since these birds readily use nest boxes across a gradient of urban-to-rural conditions, they make an excellent model for this study.

European Starlings were sampled from March to June in 2020 and 2021 across 2 sites that differ in their degree of urbanization: 1 urban farm (34.0621 N, −84.6038 W) and 1 rural farm (34.0914 N, −84.9079 W) in the greater Atlanta area (Georgia, USA; Fig. 1). To quantify the degree of urbanization across sites, we used the UrbanizationScore software (Lipovits et al. 2015) that automatically generates an urbanization score to rank sites in a relative manner, where a higher positive score is more urban. The UrbanizationScore tool uses information about vegetation, paved roads, buildings, and forested areas to calculate degree of urbanization. This score supported our classification that the urban farm is more urban than the rural farm (urban site = 2.236; rural site =

−2.236). This score was also supported by differences in human population size and density based on the zip code in which the property belongs.

The rural site was located in Taylorsville (population size = 3,226; density ≥ 388/km^2) while the urban site was located in Acworth (population size = 45,698; density ≥ 3,885/km^2). Finally, as additional support for a difference in urbanization across these sites, we measured natural variation in the availability of insect prey at each site. Insect abundance is a critical component of a starling's environment, since starling parents use insects almost exclusively to provision their young, including ground larva and flying insects (Mennechez and Clergeau 2001). In addition, we found anecdotal data from provisioning videos that adult starlings in our population provision nestlings with various types of insect prey, including ground larva and winged insects.

We sampled insect communities at each study site in 2021 only, where we assessed flying insect abundance using commercially available sticky paper traps that capture flying insects that come into contact with the paper (Hall 2009). New sticky paper traps were mounted to the bottom of 2 randomly selected nest boxes at each site every 2 weeks throughout the breeding season (late Mar–early Jun) and were then stored in ziplock bags at −80°C until analysis. Sticky traps were then photographed, and ImageJ software (Schneider et al. 2012) detected the surface area of the trap that was covered by insects. We visually checked each ImageJ output to ensure twigs or non-insect material were excluded from the area covered by insects.

Field sampling

We monitored nest boxes beginning in mid-February until breeding began. Nests were monitored until clutches were complete then checked every 2–3 d for predation, abandonment, hatching, and fledging. In total we monitored 91 nesting attempts, with 78 at the rural site and 13 at the urban site. We defined hatch day as the date when the first nestling (or more) hatched from their egg. Nestlings were sampled twice; the first sampling event occurred at 9 or 10 d of age, which corresponds to the inflection point of nestling growth curves (their peak of growth). Each nestling was resampled 7 d later at 16 or 17 d of age, which represents a state where they are nearly ready to fledge the nest.

Prior studies suggest that condition at fledging is a good predictor of survival chances for young passerines (Naef-Daenzer et al. 2001). In total, we sampled 114 nestlings from 37 nests during their peak growth days (N_{rural} = 98, N_{urban} = 16) and 110 nestlings from 35 nests at fledging age (N_{rural} = 97, N_{urban} = 13). At the first capture, chicks were banded with a metal U.S. Geological Survey Bird Banding Lab band and a baseline blood sample was collected in under 3 min after nest disturbance to measure cholesterol, oxidative damage to DNA, and baseline corticosterone (CORT; the primary glucocorticoid in birds). Blood samples were collected via brachial venipuncture with a 26-gage needle and collected into a heparinized capillary tube. We then collected morphometric measurements including tarsus length (in mm) and mass (in g) to calculate an index of body condition. The second sampling event followed the same procedure as the first capture, taking place exactly 1 week later.

In addition to sampling nestlings, we sampled 41 adult starlings that were captured across 29 nests in 2021 only (N_{rural} = 36, N_{urban} = 5). Parents were caught using nest box Van Ert traps that were triggered closed when the parent landed on the nest inside the nest box. Nest boxes with traps were watched closely and trapped adults were immediately retrieved from the nest. Parents received a uniquely numbered metal band along with a unique color band combination for resighting. Like nestlings, adult starlings were sampled following the sampling procedure outlined above, which included a baseline blood sample collected in under 3 min of capture followed by taking morphometrics. If parents had a second brood (N = 7 in our dataset), the same procedures were conducted on these adults again during their second brood number.

Blood samples from nestlings and adults were used to measure cholesterol using 15 μL of whole blood and a point of care device in the field immediately following sampling (CardioChek, PTS Diagnostics, Whitestown, Indiana, USA, previously validated for use in passerine birds; Morales et al. 2020). This device detects total

cholesterol concentrations in whole blood ranging between 100 and 400 mg/dL. None of the birds sampled in our study exceeded the maximum detection limit. However, when individuals had total cholesterol titers below the minimum threshold, we assigned them a value of 100 mg/dL, which represents the maximum cholesterol for these animals ($N = 58$ individuals, primarily nestlings, fell below the minimum threshold). We also placed a small volume of whole blood on FTA cards for molecular sexing (Kilgour et al. 2022) as female and male nestlings look very similar, and the rest of the blood sample was centrifuged to separate red blood cells from plasma. Plasma was stored on wet ice while out in the field and transferred to a −80°C freezer at Kennesaw State University by the end of the day and until lab analyses could be conducted (i.e., DNA damage, corticosterone).

DNA damage assay

We measured DNA damage resulting from oxidative stress, since prior biomedical work has shown that high cholesterol diets are associated with elevated oxidative damage (Tomofuji et al. 2006). We used a commercially available kit (DetectX DNA Damage kit; Arbor Assays Inc., Ann Arbor, Michigan, USA) according to the manufacturer's protocol to measure 8-hydroxy-2-deoxyguanosine (8-OHdG), which is a marker of oxidized guanine from DNA and RNA that indicates existing oxidative damage to genetic material. A standard curve of 8-hydroxy-2-deoxyguanosine stock solution (concentration range = 62.6–8,000 pg/mL) was included on each plate as well as 2 blank controls. We diluted 1 µL of plasma with 200 µL of assay buffer, and 50 µL of the diluted sample was added to duplicate wells of the assay plate. Next, we added 25 µL of the conjugate reagent and 25 µL of the antibody reagent to each well before shaking the plate at 500 rpm at room temperature for 2 h. Wells were then emptied and washed 4 times with 200 µL of wash buffer, then tapped clean on absorbent towels before adding 100 µL of TMB substrate to every well. Plates were then incubated at room temperature for an additional 30 min before adding 50 µL of stop solution to all wells. Plates were shaken for 15 min before being read in a plate reader at optical density of 450 nm. The mean intra-assay coefficient of variation calculated from the duplicate samples was 8.33% and the inter-assay coefficient of variation calculated from a pooled sample run on every plate was 16.3%. The inter-assay coefficient of variation may be higher in our study as it was completed over multiple years, although the manufacturer reports variability between 8.1% and 13.4% for the same sample run across several days.

Corticosterone assay

We examined whether elevated cholesterol is associated with glucocorticoid physiology since prior work suggests that high cholesterol diets can lead to elevated glucocorticoids (Anderson et al. 2019). Baseline corticosterone, which is a measurement of metabolic demand and balance often used as an index of health (Madliger and Love 2016), was measured using a commercially available enzyme immunoassay kit (DetectX Corticosterone Kit, Arbor Assays Inc.) according to the company's protocol that was previously validated in starlings (Guindre-Parker et al. 2022, Kilgour et al. 2022). Assays were conducted within 6 months of plasma collection. We combined 10 µL of plasma with 10 µL of dissociation reagent, incubating at room temperature for 5 min. Samples were then diluted 1:25 in assay buffer. A standard curve of stock solution (concentration range 78.125–10,000 pg/mL) was included on each plate as well as 2 blank controls. The assay was completed in duplicate wells of a flat-bottomed 96-well plate with 25 µL of the conjugate reagent and 25 µL of the antibody reagent added to each well before shaking the plate at 500 rpm at room temperature for 1 h. Plate contents were then emptied and washed 4 times with 200 µL of wash buffer, then tapped clean on absorbent towels before adding 100 µL of TMB substrate to every well. Plates were then incubated at room temperature for an additional 30 min before adding 50 µL of stop solution to all wells. Plates were read in a plate reader at optical density of 450 nm using a 4-parameter logistic curve to calculate sample corticosterone concentrations. The intra-assay coefficient of variation (from duplicate samples) was 4.56% and the inter-assay coefficient of variation (from a pooled sample with an aliquot run on every plate) was 10.3%.

Statistical analyses

We examined the effects of urbanization on cholesterol using multiple linear mixed-effects models where we conducted separate models for nestlings and adults. Our first nestling model was a repeated-measures analysis that assessed the change in nestling cholesterol over the development of individual chicks (as they aged from days 9/10 to days 16/17) via an interaction between nestling age and site to determine whether cholesterol changed more rapidly during development at the urban versus rural site. This repeated-measures model included cholesterol as the dependent variable, along with the following independent variables: chick age, chick sex, brood number (since some parents attempted multiple broods in one season), nestling body condition (residuals of a linear regression between mass and tarsus length), site (urban vs. rural), and the interaction between site and age (days since hatch).

Preliminary analyses revelated that Julian date of breeding did not vary by site (2-sample *t*-test: $t = 1.77$, $P = 0.10$) and was not correlated to cholesterol titers (estimate ± SE = 0.25 ± 0.35, *t*-value = 0.73, $P = 0.47$). In addition, since hatch date was redundant with brood number—where first broods were necessarily earlier in the season and subsequent broods occurred later in the season—we did not include Julian hatch date in our models. Because we sampled each nestling twice, and we sampled multiple nestlings at each nest, nest ID and chick ID were used as random effects to account for nonindependence of samples and nestlings (chick ID nested within nest ID).

In addition to this repeated-measures approach, we also assessed whether cholesterol differed with site in a cross-sectional analysis across 3 developmental stages: nestlings at their peak of growth (day 9/10), nestlings about to fledge (day 16/17), and breeding adults. For the 2 nestling age classes, we created 2 separate models that included cholesterol as the dependent variable along with sex, brood number, site, and body condition as independent variables and nest ID as the random effect. For the adult model (the third model), we included site, sex, and brood number as independent variables, and nest ID as a random effect because some parents in our dataset were sampled more than once when undertaking multiple breeding attempts and since we sometimes sampled both members of a breeding pair, which may not represent independent samples.

Finally, to investigate whether elevated cholesterol was negatively correlated to physiological state in adults and nestlings, we used 2 additional linear mixed-effects models (1 for adults and 1 for nestlings at 16/17 d of age); each model included DNA damage, baseline corticosterone, body condition, time to collecting the blood sample (in seconds), and sex as independent variables. We also included nest ID as the random effect for nestlings and adults (since multiple nestlings or both parents were usually sampled at each nest). All analyses were performed in RStudio 1.4.1106 (RStudio Team 2020, R Core Team 2021) using the *nlme* package (3.1-155). Model residuals were normally distributed, so cholesterol levels did not need to be transformed to meet the assumptions of linear models.

Results

We found that the mean percentage of the sticky trap covered in insects was significantly lower at the urban farm (mean = 12.3%) compared to the rural farm (mean = 41.1%; unpaired *t*-test: $t = 3.77$, df = 32, $P < 0.001$). Together with the UrbanizationScore tool, our measure of flying insect availability supports a significant difference in the extent of urbanization across our 2 study sites, where increased urbanization is associated with decreased availability of flying insect prey.

We examined if over the course of nestling development (from days 9/10 to days 16/17) cholesterol levels increased more quickly within individuals at the urban versus rural site (Table 1). We found that site was not correlated to nestling cholesterol, nor was the interaction of site and chick age (Table 1). We also found that sex, brood number, and body condition were not correlated to cholesterol (Table 1). However, we did find that nestling age was positively correlated to cholesterol levels, where individual nestlings increased in cholesterol as they got older regardless of the site where they were being raised (Table 1).

Next, we used cross-sectional data to compare cholesterol across the urban and rural sites for nestlings at their peak of growth, nestlings about to fledge, and in adults. We found that site, sex,

Table 1. Differences in cholesterol in urban and rural European Starlings. Results from a repeated-measures linear-mixed model are presented here where bolded variables with an asterisk denote significant predictors (with a *P*-value equal to or below 0.05). Cholesterol was examined over the course of nestling development to test whether cholesterol changed more rapidly at one site or the other. We sampled 120 nestlings ($N_{rural} = 104$, $N_{urban} = 16$) across 37 nests (some nestlings could not be sampled a second time due to death/disappearance).

Dependent variable	Fixed effects	ß ± SE	*t*-value	*P*-value
Cholesterol	Site (Urban)	−14.39 ± 24.98	−0.58	0.5682
	Chick age	**1.34 ± 0.66**	**2.30**	**0.0452***
	Sex (Male)	2.23 ± 4.66	0.48	0.6324
	Brood number	−1.28 ± 8.70	−0.15	0.8836
	Body condition	−0.39 ± 0.31	−1.27	0.2087
	Site (Urban): Chick age	1.93 ± 1.82	1.06	0.2901
	Random effects	Standard deviation		
	Bird ID in Nest ID	16.85		
	Bird ID	0.003		
	Residuals	31.34		

body condition, and brood number were not related to cholesterol for nestlings at days 9/10 or at days 16/17 (Table 2). In young nestlings experiencing their peak growth rate, we found that mean cholesterol was similar across sites (Fig. 2). However, for older nestlings about to fledge the nest, we found that urban nestlings appear to show marginally higher mean cholesterol than rural ones (although this effect was not statistically significant; Fig. 2). In contrast, we found that adults breeding at the urban site had significantly higher cholesterol than adults breeding at the rural site (Table 3, Fig. 2). Adult males had higher cholesterol compared to females. Similar to our findings in nestlings, we found that brood number and body condition were not significant predictors of adult cholesterol (Table 3).

Finally, we explored possible correlations between elevated cholesterol and physiological condition to test the hypothesis that elevated cholesterol in wild birds was associated with lowered physiological state as expected from biomedical studies. Contrary to our prediction, we found that cholesterol was not correlated to DNA damage, to baseline CORT, nor to body condition (Table 4) for nestling (Fig. 3) or adult European Starlings (Fig. 4).

Discussion

Cholesterol is an essential lipid with important physiological functions, including in the production of critical hormones (e.g., testosterone) and the production of bile. However, excessive cholesterol is linked to an array of health issues in the biomedical literature, such as cardiovascular disease and the hardening of arterial walls and blood vessels (Jauho 2019). Our work adds to scarce literature on cholesterol in free-living birds, exploring whether cholesterol differed for birds living in an urban versus rural habitat. We found that adult starlings from an urban habitat showed elevated cholesterol compared to rural birds. Similarly, while young nestlings at their peak of growth (days 9/10) did not differ in mean cholesterol across the urban and rural sites, we found that urban nestlings approaching fledging age (days 16/17) showed marginally elevated cholesterol relative to rural ones. This cross-sectional pattern, where cholesterol increases with age, is also seen in our repeated measures analysis where individual nestlings increased in cholesterol as they got older. While few studies have explored cholesterol in nestling birds making it difficult to tell whether this pattern is supported in other species, our results in adult starlings mirror those previously reported for American Crows (Townsend et al. 2019), Gambel's Quail (Funk et al. 2020), and Ring-billed Gulls (Marteinson and Verreault 2020), where urban birds have elevated cholesterol concentrations compared to more rural ones.

Prior work on cholesterol in birds suggests that differences in diet may shape variation in cholesterol along an urban to rural gradient (Townsend et al. 2019). The most thorough study to date showed that,

Table 2. Differences in cholesterol in nestling urban and rural European Starlings. Results from 2 separate linear-mixed models are presented here. Drivers of nestling cholesterol were explored at 2 developmental stages: (A) during the peak of growth (days 9/10) and (B) near fledging (days 16/17). This included 114 nestlings ($N_{rural} = 98$, $N_{urban} = 16$) from 37 nests during their peak growth days and 107 nestlings ($N_{rural} = 94$, $N_{urban} = 13$) from 35 nests at fledging age.

		(A) Nestlings aged 9/10 d			(B) Nestlings aged 16/17 d		
Dependent variable	Fixed effects	ß ± SE	*t*-value	*P*-value	ß ± SE	*t*-value	*P*-value
Cholesterol	Site (Urban)	−1.22 ± 17.87	−0.07	0.9460	25.80 ± 13.50	1.91	0.0650
	Brood number	−5.56 ± 14.13	−0.39	0.6967	7.13 ± 11.18	0.64	0.5279
	Sex (Male)	1.68 ± 4.23	0.40	0.6925	−5.00 ± 6.16	−0.81	0.4193
	Body condition	−0.14 ± 0.46	−0.30	0.7625	−0.19 ± 0.37	−0.51	0.6138
	Random effect	Standard deviation			Standard deviation		
	Nest ID	34.58			17.94		
	Residuals	18.99			29.27		

in American Crows, plasma cholesterol increased as urbanization increased. The authors performed an experimental food supplementation where they provided anthropogenic food to the animals, which caused an increase in mean cholesterol levels (Townsend et al. 2019). The leading explanation for their results is that access to anthropogenic foods in urban habitats may lead to increased cholesterol levels under these conditions (Townsend et al. 2019).

Across other vertebrate taxa, anthropogenic foods appear to affect cholesterol levels similarly in urban habitats. A study in wild boar (*Sus scrofa*) found that urban-living individuals ate more anthropogenic foods and had elevated triglyceride levels, which are another type of lipid that play a role in storing unused calories (Castillo-Contreras et al. 2021). Another study looking at kit foxes (*Vulpes macrotis*) found that foxes residing in urban habitats used more anthropogenic food sources leading to elevated cholesterol levels (Cypher and Frost 1999). Our study also supports this idea: we know that adult starlings residing in urban areas had increased cholesterol while also having reduced access to flying insect prey—this would suggest that eating other food types including possible anthropogenic foods could explain differences in cholesterol levels across our study sites.

However, we cannot conclude that dietary differences led to cholesterol differences across sites since we were not able to measure or manipulate starling diet in our study. Anecdotally, we have observed starlings eating anthropogenic foods previously and many photographs of European Starlings eating out of garbage cans across the world exist to support this possibility. It remains likely that urban starlings may be more likely to consume anthropogenic foods than rural ones, causing their cholesterol to rise, but future work is needed to test this hypothesis.

Our research showed that differences in cholesterol across sites were present in adults but not in nestlings, and that nestlings increased in cholesterol as they aged and approached fledging. These patterns could suggest that these differences in cholesterol in adults from urban versus rural sites are accumulated over time and over the life of an individual. Living in an urban habitat (or even

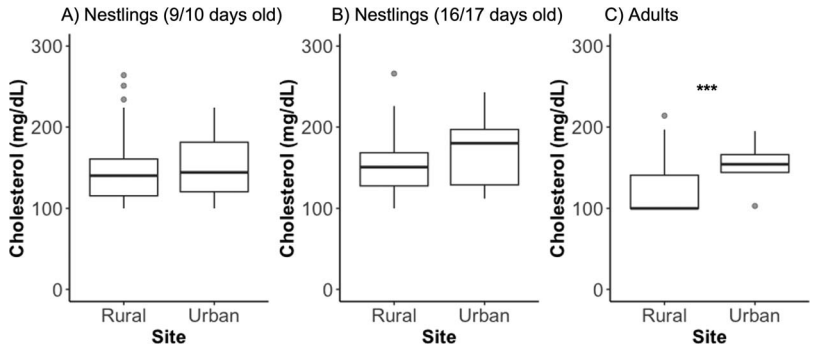

Figure 2. We found that European Starlings from the urban site showed elevated cholesterol compared to birds from the rural site in Georgia. This pattern became more accentuated with age. (A) Young nestlings at their peak of growth (days 9/10) did not differ in mean cholesterol across urban and rural sites, (B) urban fledglings (days 16/17) showed a slightly elevated mean cholesterol compared to rural ones although it was not a significant difference, and (C) adult birds have cholesterol that is significantly higher in urban than rural habitats. Boxplots show the median (middle line), the first and third quartiles (box), the minimum and maximum values in the data (whiskers) and potential outliers (gray circles). Three asterisks denote significantly different means as detected in our mixed models.

consuming different diets) over prolonged periods of time may be necessary before cholesterol differences can be measured across populations experiencing differences in their environments. It is possible that starling parents provision their young differently across urban and rural sites, which could lead older nestlings from urban sites to show slightly elevated cholesterol compared to their rural counterparts (although this difference was not statistically significant). Despite studies showing variation in cholesterol levels across species and populations (Morales et al. 2020), few longitudinal studies exist to identify what factors contribute to driving cholesterol levels within an individual. Thus, more work is needed to understand how cholesterol variation is shaped by ecological conditions and across the life of an individual bird.

We found no correlations between cholesterol and indices of physiological condition including DNA damage, body condition, and CORT. While little is known about how cholesterol may affect physiology or condition in wildlife, previous research by Bavelaar and Beynen (2003) found that high-fat diets were not a direct cause of weight gain in Gray Parrots (*Psittacus erithacus*), which would support a lack of correlation between body condition and cholesterol in starlings. Another study by Freeman (1971) showed a direct link between elevated cholesterol in domestic chickens (*Gallus gallus*) and elevated glucocorticoid levels, similar to that of humans, which contradicts our finding that cholesterol and CORT are not correlated in starlings. Despite being a precursor to glucocorticoid synthesis, it is possible that cholesterol

Table 3. Differences in cholesterol in adult urban and rural European Starlings. Results from a linear-mixed model exploring predictors of adult cholesterol levels are presented here where bolded variables with an asterisk denote significant predictors (with a *P*-value equal to or below 0.05). This included 41 adults ($N_{rural} = 36$, $N_{urban} = 5$) sampled across 29 nests.

Dependent variable	Fixed effects	ß ± SE	*t*-value	*P*-value
Cholesterol	**Site (Urban)**	**30.84 ± 12.57**	**2.26**	**0.030***
	Sex (Male)	**20.59 ± 8.44**	**2.44**	**0.020***
	Brood number	−11.84 ± 10.07	−1.18	0.262
	Body condition	0.23 ± 0.50	0.52	0.615
	Random effect	Standard deviation		
	Bird ID	0.01		
	Residuals	31.81		

Table 4. Differences in cholesterol in nestling and adult urban and rural European Starlings. Results from 2 separate linear-mixed models exploring correlations between cholesterol and physiological state for (A) nestlings and (B) adults are presented here. This included 98 nestlings ($N_{rural} = 86$, $N_{urban} = 12$) sampled from 33 nests, and 36 adults ($N_{rural} = 31$, $N_{urban} = 5$) sampled across 25 nests.

		(A) Nestlings 16/17 days			(B) Adults		
Dependent variable	Fixed effects	ß ± SE	*t*-value	*P*-value	ß ± SE	*t*-value	*P*-value
Cholesterol	DNA damage	−0.13 ± 0.11	−1.14	0.2585	−0.03 ± 0.12	−0.26	0.7987
	Baseline CORT	−0.15 ± 0.21	−0.69	0.4932	−0.74 ± 0.69	−1.07	0.3054
	Body condition	−0.04 ± 0.38	−0.12	0.9081	0.60 ± 0.53	1.13	0.2820
	Time base bleed	−0.08 ± 0.06	−1.35	0.1833	0.25 ± 0.12	2.02	0.0658
	Sex (Male)	−2.97 ± 6.36	−0.47	0.6417	17.2 ± 11.1	1.54	0.1497
	Random effect	Standard deviation			Standard deviation		
	Nest ID	17.87			0.0023		
	Residuals	28.92			30.10		

is not limiting in the regulation of baseline glucocorticoids and thus the 2 are not correlated under the conditions of our study. Since DNA damage, body condition, and CORT can all be predictors of overall condition, we predicted we would see declines in these health indicators with elevated cholesterol levels in starlings.

A common theme in prior studies linking oxidative damage, glucocorticoids, and cholesterol is an experimental stressor (e.g., Latour et al. 1996, Boostani et al. 2015), and it is possible that birds in our study were not coping with similar stressors that altered these physiological traits simultaneously. One possibility for our results is that our sample size is too small and sampling a larger number of sites that capture greater variability in cholesterol across starlings is needed to see a correlative cost of elevated cholesterol. While our study sites differed in the degree of urbanization they experienced as measured via the UrbanizationScore tool, additional sampling at more urban sites could help reveal possible costs of elevated cholesterol in future studies.

Another alternative explanation is that while all of the traits we measured may be individual indicators of overall health, cholesterol instead influences more strongly other physiological systems that were not measured in our study. A previous study in pet Gray Parrots found that increased cholesterol led to cardiovascular disease, although cholesterol was able to be mitigated by changing the parrots' diets—this is similar to patterns seen in humans (Bavelaar and Beynen 2003). It appears that cholesterol titers above 200 mg/dL increase heart disease risk, and although mean cholesterol for urban and rural starlings across age groups were always below this threshold, some individuals sampled in our study did have titers above 200 mg/dL. We were not able to biopsy for cardiovascular disease in our study to test this possibility in starlings. While our study documents clear variation in cholesterol levels across starlings from an urban and a rural site, it remains unclear what cholesterol levels would be classified as "excessive" in starlings before adverse effects would begin to occur. It is also possible that even within the range of cholesterol concentrations measured in our study, "high cholesterol" starlings

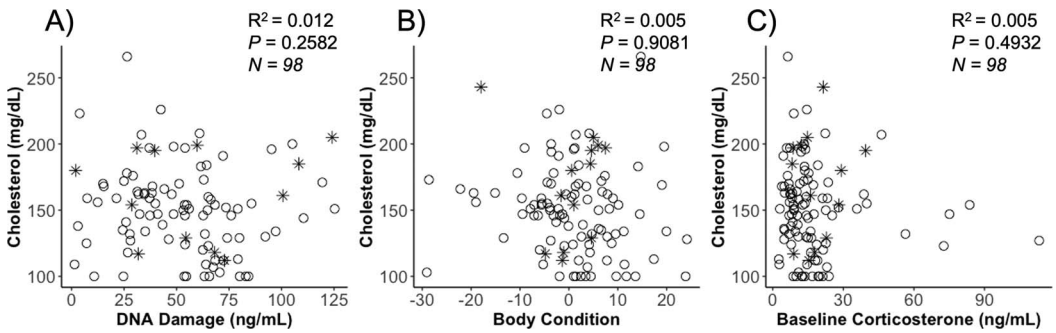

Figure 3. We found no relationships between European Starling nestling cholesterol and (A) DNA damage (ng/mL), (B) body condition (residuals of mass on tarsus), and (C) baseline corticosterone (ng/mL). Asterisks indicate samples from urban site, while circles indicate samples from rural site. All tests are 2-tailed; the model-wide R^2 (calculated via package *r2glmm*) was 0.042 whereas we report effect-specific R^2, *P*-values, and sample size (*N*) in the figure.

remain below the threshold for costs of high cholesterol or cardiovascular disease.

A final possibility for our finding that cholesterol was not related to other measures of condition is due to the correlative nature of our study. Experimental work supplementing starlings with cholesterol would be needed to conclude whether there are physiological costs of elevated cholesterol in this species. Overall, more work is needed to understand the consequences of elevated cholesterol in songbirds, and to bridge biomedical research in humans and ecological research in a broader number of taxa experiencing increasing urbanization. Perhaps in longer-lived animals, older individuals, or captive birds, the impacts of elevated cholesterol could be measured more easily in future studies.

We find that although cholesterol levels are similar across habitat types in developing nestlings, adults from our urban study site have significantly higher cholesterol compared to birds from the rural site. Future work should examine whether these differences in cholesterol are shaped by variation in diet across sites, as has been shown for other studies in birds and mammals. Despite higher cholesterol concentrations in urban birds, we found no evidence that elevated cholesterol negatively impacted an individual's condition. Elevated cholesterol may have affected other indices of condition not measured in our study, and we do not know the long-term effects of elevated cholesterol across multiple years. Our results nevertheless highlight important variation in cholesterol across populations of birds exposed to urbanization. Future work is needed to

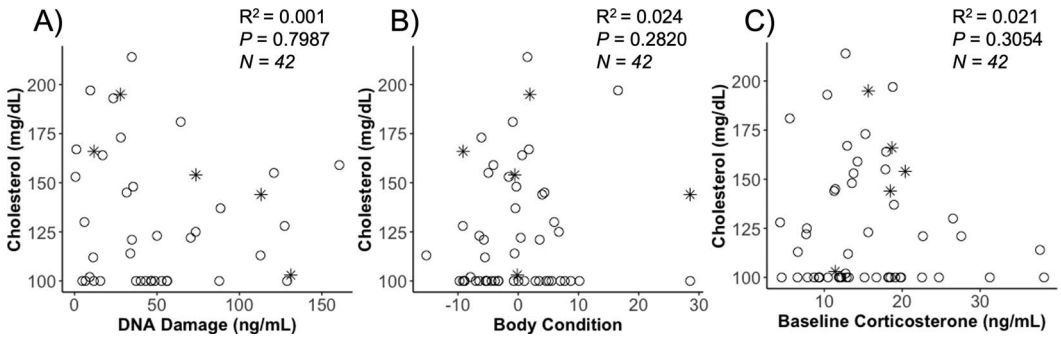

Figure 4. Differences in cholesterol in urban and rural European Starlings. We found no relationships between adult cholesterol and (A) DNA damage (ng/mL), (B) body condition (residuals of mass on tarsus), and (C) baseline corticosterone (ng/mL). Asterisks indicate samples from urban site, while circles indicate samples from rural site. All tests are 2-tailed; the model-wide R^2 (calculated via package *r2glmm*) was 0.104 whereas we report effect-specific R^2, *P*-values, and sample size (*N*) in the figure.

build on our results to better understand what factors drive variation in cholesterol across urban and rural birds, as well as what the consequences of elevated cholesterol in urban habitats may be in the long term.

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Literature cited

Anderson GW, Kenyon CJ, Al-Dujaili EA. 2019. Postprandial changes in salivary glucocorticoids: Effects of dietary cholesterol and associations with bile acid excretion. Nutrients. 11:360.

Bailey J, Scheifler R, Berthe S, Clément-Demange V-A, Leblond M, et al. 2016. From eggs to fledging: Negative impact of urban habitat on reproduction in two tit species. Journal of Ornithology. 157:377–392.

Bavelaar FJ, Beynen AC. 2003. Influence of amount and type of dietary fat on plasma cholesterol concentrations in African grey parrots. Journal of Applied Research in Veterinary Medicine. 1:1–8.

Boostani A, Sadeghi AA, Mousavi SN, Chamani M, Kashan N. 2015. The effects of organic, inorganic, and nanoselenium on blood attributes in broiler chickens exposed to oxidative stress. Acta Scientiae Veterinariae. 43:1–6.

Cabe PR. 2021. European Starlings (*Sturnus vulgaris*) as vectors and reservoirs of pathogens affecting humans and domestic livestock. Animals. 11:466.

Castillo-Contreras R, Mentaberre G, Fernandez Aguilar X, Conejero C, Colom-Cadena A, et al. 2021. Wild boar in the city: Phenotypic responses to urbanization. Science of the Total Environment. 773:145593.

Cypher BL, Frost N. 1999. Condition of San Joaquin kit foxes in urban and exurban habitats. Journal of Wildlife Management. 63:930–938.

Fanelli RE, Martin PR, Robinson OJ, Bonier F. 2022. Estimates of species-level tolerance of urban habitat in North American birds. Ecology. 103:e3821.

Freeman BM. 1971. Stress and the domestic fowl: A physiological re-appraisal. World's Poultry Science Journal. 32:249–256.

Funk A, Hutton P, Earl S, Deviche P, Sweazea K. 2020. Levels of land use and land cover in Phoenix, Arizona are associated with elevated plasma triglycerides in the Gambel's Quail, *Callipepla gambelii*. Comparative Biochemistry and Physiology Part A: Molecular and Integrative Physiology. 247:110730.

Guindre-Parker S, Kilgour DAV, Linkous CR. 2022. The development of behavioral and endocrine coping styles in nestlings from urban and rural sites. General and Comparative Endocrinology. 327:114091.

Hall DG. 2009. An assessment of yellow sticky card traps as indicators of the abundance of adult *Diaphorina citri* (Hemiptera: Psyllidae) in citrus. Journal of Economic Entomology. 102:446–452.

Jauho M. 2019. Patients-in-waiting or chronically healthy individuals? People with elevated cholesterol talk about risk. Sociology of Health and Illness. 41:867–881.

Kessel B. 1957. A study of the breeding biology of the European Starling (*Sturnus vulgaris*) in North America. American Midland Naturalist. 58:257–331.

Kilgour DAV, Linkous CR, Pierson TW, Guindre-Parker S. 2022. Sex ratios and the city: Secondary offspring sex ratios, parental corticosterone, and parental body condition in an urban-adapted bird. Frontiers in Ecology and Evolution. 10:894583.

Latour MA, Laiche SA, Thompson JR, Pond AL, Peebles ED. 1996. Continuous infusion of adrenocorticotropin elevates circulating lipoprotein cholesterol and corticosterone concentrations in chickens. Poultry Science. 75:1428–1432.

Liker A, Papp Z, Bókony V, Lendvai ÁZ. 2008. Lean birds in the city: Body size and condition of House Sparrows along the urbanization gradient. Journal of Animal Ecology. 77:789–795.

Lipovits Á, Czúni L, Seress G. 2015. A tool for quantifying the urban gradient. ATINER'S Conference Paper Series. No: PLA2015-1709, 1–10.

Madliger CL, Love OP. 2016. Employing individual measures of baseline glucocorticoids as population-level conservation biomarkers: Considering within-individual variation in a breeding passerine. Conservation Physiology. 4: cow048.

Marteinson SC, Verreault J. 2020. Changes in plasma biochemistry in breeding Ring-billed Gulls: Effects of anthropogenic habitat use and contaminant exposure. Environment International. 135:105416.

McKinney ML. 2008. Effects of urbanization on species richness: A review of plants and animals. Urban Ecosystems. 11:161–176.

Meek TH, Eisenmann JC, Keeney BK, Hannon RM, Dlugosz EM, Garland T Jr. 2014. Effects of early-life exposure to Western diet and wheel access on metabolic syndrome profiles in mice bred for high voluntary exercise. Genes, Brain and Behavior. 13:322–332.

Mennechez G, Clergeau P. 2001. Settlement of breeding European Starlings in urban areas: Importance of lawns vs. anthropogenic wastes. In: Marzluff JM, Bowman R, Donnelly R, editors. Avian ecology and conservation in an urbanizing world. New York (NY): Springer Science+Business Media; p. 275–287.

Morales A, Frei B, Leung C, Titman R, Whelan S, et al. 2020. Point-of-care blood analyzers measure the nutritional state of eighteen free-living bird species. Comparative Biochemistry and Physiology Part A: Molecular and Integrative Physiology. 240:110594.

Murray MH, Lankau EW, Kidd AD, Welch CN, Ellison T, et al. 2020. Gut microbiome shifts with urbanization and potentially facilitates a zoonotic pathogen in a wading bird. PLOS One. 15:e0220926.

Naef-Daenzer B, Widmer F, Nuber M. 2001. Differential post-fledging survival of Great and Coal tits in relation to their condition and fledging date. Journal of Animal Ecology. 70:730–738.

R Core Team. 2021. R: A language and environment for statistical computing. 4.1.1. Vienna (Austria): R Foundation for Statistical Computing.

RStudio Team. 2020. RStudio: Integrated development for R. Boston (MA): RStudio, PBC.

Schneider CA, Rasband WS, Eliceiri KW. 2012. NIH Image to ImageJ: 25 years of image analysis. Nature Methods. 9:671–675.

Sinclair EC, Martin PR, Bonier F. 2022. Among-species variation in hormone concentrations is associated with urban tolerance in birds. Proceedings of the Royal Society B. 289:2022160020221600.

Tomofuji T, Azuma T, Kusano H, Sanbe T, Ekuni D, et al. 2006. Oxidative damage of periodontal tissue in the rat periodontitis model: Effects of a high-cholesterol diet. FEBS Letters. 580:3601–3604.

Townsend AK, Staab HA, Barker CM. 2019. Urbanization and elevated cholesterol in American Crows. Condor. 121(3):duz040.

Watson H, Powell D, Salmón P, Jacobs A, Isaksson C. 2020. Urbanization is associated with modifications in DNA methylation in a small passerine bird. Evolutionary Applications. 14:85–98.

Zampelas A, Magriplis E. 2019. New insights into cholesterol functions: A friend or an enemy? Nutrients. 11:1645.