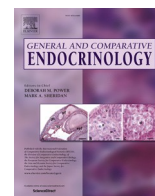




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The development of behavioral and endocrine coping styles in nestlings from urban and rural sites

Sarah Guindre-Parker^{*}, Denyelle A.V. Kilgour¹, Courtney R. Linkous¹

Department of Ecology, Evolution, & Organismal Biology, Kennesaw State University, Kennesaw, GA, United States

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ABSTRACT

Urbanization is increasing globally and altering the stressors that animals face in their everyday lives. Organisms often differ in their coping styles—both behavioral and endocrine—across urban to rural habitats. For example, urban animals are often bolder, more exploratory, and mount stronger glucocorticoid stress responses compared to their rural counterparts. While these coping styles are important in shaping fitness across the urban-to-rural gradient, it remains unclear when these differences arise in the life of organisms. We explore the development of coping styles in European starling nestlings (*Sturnus vulgaris*), an urban-adapted species. We test whether breathing rate, handling struggle rate, and bag struggle rate differ across sites and find no difference in the behavioral coping styles of nestlings raised in urban versus rural sites. We also explore differences in baseline and stress-induced glucocorticoids, finding that urban nestlings develop a stronger stress response than rural birds before fledging the nest. We find no significant correlations between behavioral and endocrine traits for urban or rural birds, which supports the two-tiered model of coping styles. One possibility is that behavioral and endocrine differences develop at different times over the lives of organisms. Our findings support prior work suggesting that behavioral and endocrine coping mechanisms act independently of one another, and suggests that endocrine coping mechanisms develop in early life and before differences in behavioral coping styles might arise. Future work on the mechanisms leading to early-life differences in coping styles—from genetics to maternal effects to environmental effects—is needed to best predict how urban-adapted organisms cope with environmental change. Studies across a greater number of sites will help disentangle site from urbanization effects.

1. Introduction

Over 55% of humans currently live in urban centers, and global urbanization is projected to continue to increase to 68% by 2050 (Nations, 2018). Understanding how organisms cope with life in urban areas is thus increasingly important to predict the future impacts of urbanization on biodiversity. An urban world is challenging to navigate, as urban centers present conditions that differ from the ones under which most organisms have evolved. For example, new challenges may include artificial light, noise pollution, toxins and endocrine disruptors, reduced access to natural resources (e.g., shelter or food), as well as increased human and predator encounters (Adams et al., 2005). The responses of organisms to urbanization have often been studied by categorizing species according to their abundance along urban to rural habitats (Injaian et al., 2020; Martin and Bonier, 2018; Palacio, 2020; Schoeman,

2016). While some species are commonly found in urban habitats—termed ‘urban-dwellers’ (Fischer et al., 2015) or ‘urban-adapters’ (Blair, 1996; McKinney, 2002)—many show significant intraspecific variation in their success in and responses to urbanization. For example, urban-dwelling species have shown recent population declines (Rosenberg et al., 2019; Stracey et al., 2010) and reduced fitness in cities compared to rural environments (Peach et al., 2008; Seress et al., 2012). Understanding responses to urbanization at the organismal level is thus critical to predict the future impacts of urbanization.

One multidimensional approach to understanding how organisms respond to novel environments has been from the perspective of studying their coping styles, as animals most likely rely on multiple correlated or complimentary coping mechanisms to maintain homeostasis under novel environmental conditions (Koolhaas et al., 2010; Taborsky et al., 2021; Taff and Vitousek, 2016). Coping styles originate

^{*} Corresponding author at: Kennesaw State University, Department of Ecology, Evolution, and Organismal Biology, 370 Paulding Ave NW, MD #1202, Kennesaw, GA 30144, United States.

E-mail address: sguindre@kennesaw.edu (S. Guindre-Parker).

¹ These authors contributed equally.

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from the psychology literature and characterize how organisms overcome stressors (Wechsler, 1995), though this framework is increasingly used in ecology and evolutionary biology (Monestier et al., 2015; Qu et al., 2018; Westrick et al., 2018). Coping styles can be defined as repeatable behavioral and neuroendocrine traits (Koolhaas et al., 1999) where individuals vary in their coping styles along a continuum ranging from proactive (more aggressive and active individuals) to reactive (more passive or inactive individuals). Theory suggests that proactive individuals may be more successful in urban areas where they face frequent and unfamiliar disturbances (Dammhahn et al., 2020; Hardman and Dalesman, 2018; Senar et al., 2017). Though the behavioral and endocrine components of coping styles may be causally related to one another—where glucocorticoid stress responses are correlated to aggression or activity behaviors (Creel, 2001)—hormones and behavior may also form two independent axes of coping styles. Koolhaas et al. (2010) differentiated between these ideas via the one-tiered and two-tiered models of coping styles. In the one-tiered model, behavioral and endocrine components of coping styles are correlated to one another and thus can be considered to vary along a single axis where one behavioral phenotype is typically associated with a specific endocrine phenotype (Farwell et al., 2014). In the two-tiered model, individuals can vary independently along a first axis of behavioral variation (proactive to reactive) and a second axis of endocrine reactivity (high to low reactivity), showing only weak or no correlation between the two (Koolhaas et al., 2010). Empirical work in free-living animals typically supports the two-tiered model (Farwell et al., 2014; Qu et al., 2018b; Sadoul et al., 2021; Santicchia et al., 2020; Westrick et al., 2018), though it remains unclear whether different populations vary in their coping style model. Environmental conditions or past exposure to stressors may strengthen correlations between behavior and hormones (Archard et al., 2012) since environmental selection may have pleiotropic effects in driving physiology and behavior simultaneously, resulting in correlations among these traits. For example, we might expect populations from urban habitats follow the one-tiered model—where urban environments select for specific behavioral and physiological traits that will end up correlated to one another—while rural ones follow the two-tiered model of coping styles due to weaker selection for specific coping styles in behavior and/or physiology.

Regardless of whether behavioral and endocrine coping styles are causally related to one another or just frequently correlated because of shared intrinsic or extrinsic drivers, these repeatable phenotypes play an important role in how organisms navigate their environments. Animal coping styles can even shape differences in fitness across individuals (Monestier et al., 2015; Twiss et al., 2020). Prior work in Indian rock agama (*Psammophilus dorsalis*) showed that males from suburban populations had elevated glucocorticoids and performed fewer behavioral displays of aggression compared to rural males (Carter et al., 2012). Striped field mice (*Apodemus agrarius*) (Dammhahn et al., 2020), sika deer (*Cervus nippon*) (Honda et al., 2018), water dragons (*Intellagama lesueurii*) (Baxter-Gilbert et al., 2019), song sparrows (*Melospiza melodia*) (Evans et al., 2010) and dark eyed juncos (*Junco hyemalis thurberi*) (Atwell et al., 2012) from urban habitats were also bolder than rural individuals. While differences in coping styles exist across urban and rural environments in a myriad of species, it remains unclear when and how these differences first arise.

While most research on coping styles has focused on adults, a few studies suggest that coping styles can emerge in early life. A study in house wrens (*Troglodytes aedon*) found that endocrine coping styles differ early in life along a gradient of urbanization, and are shaped both by genetic and environmental factors (Ouyang et al., 2019). In European blackbirds (*Turdus merula*), individuals born in urban habitats had lowered glucocorticoids (Partecke et al., 2006a) and were more neophobic (Miranda et al., 2013) than rural ones. Here, we investigated the development of coping style in a common urban-dweller—the European starling (*Sturnus vulgaris*)—to gain insight into when differences in coping styles emerge across urban versus rural habitats. Our study

focuses on the early-life nestling stage, which is an important developmental period of rapid growth for altricial birds. Because rapid growth is so critical for nestlings, this stage is typically characterized by a dampened response to stressors which could otherwise trade-off with or compromise growth (Bebus et al., 2020). We test the prediction that coping styles are similarly dampened in urban and rural nestlings during their peak of growth, but that differences in coping styles emerge as nestlings reach independence at fledging. We expect urban fledglings to show more proactive behavioral coping styles and mount stronger glucocorticoid stress-responses than their rural counterparts. Finally, we test the one- and two-tiered models of coping styles to explore whether correlations between behavioral and endocrine traits are similar for urban versus rural birds. We predict that correlations between behavioral and endocrine coping styles may be strongest in urban habitats, where the frequency and diversity of stressors encountered may be greater than rural ones.

2. Methods

2.1. Field data collection

We studied European starlings at two sites in Georgia which differed in their surrounding degree of urbanization. Both sites were farms where we mounted cedar nest boxes to fence posts (mean height \pm se = 66 ± 0.76 in. from the ground; mean distance between boxes \pm se = 22 ± 3.1 feet apart). The rural site was located in Taylorsville (latitude: 34.0914, longitude: -84.9079 ; average population density = $168.9/\text{mi}^2$) while the urban site was located in Acworth (latitude: 34.0621, longitude: -84.6038 ; average population density = $770.7/\text{mi}^2$). We used the ‘UrbanizationScore’ software (Lipovits et al., 2015)—an automatic urbanization scoring tool which yields a relative ranking of sites—to compare the two study locations. This tool confirmed that the rural site was indeed more rural and the urban site was indeed more urban, with these urbanization scores incorporating information about abundances of vegetation, buildings and paved roads and higher scores reflecting a higher degree of urbanization (rural site = -2.24 ; urban site = 2.24).

During the breeding season (March to June) in 2020 and 2021, we monitored nest boxes every few days to determine hatch date—defined as the first day one or more eggs hatched. Once nestlings were 9 or 10 days of age, we conducted the first sampling protocol which would be repeated exactly 7 days later (on day 16 or 17 of age). Nestlings aged 9 or 10 days are experiencing their peak rate of growth—the inflection point on a nestling growth curve (Ricklefs and Peters, 1979)—so samples from this stage should reflect peak somatic growth and thus capture possible trade-offs between growth and coping style. Conversely, we re-sampled nestlings exactly 7 days later (on day 16 or 17 of age) when nestlings are nearly ready to fledge the nest. Fledging usually occurs on day 21, though as early as day 17 in our population, so we picked a later developmental stage that was still early enough to prevent forced-fledging when we disturbed the nest.

During a sampling event, we started a stopwatch as soon as we opened the nest box and retrieved all nestlings; we collected a baseline blood sample from the brachial vein as quickly as possible for all individuals which would be used to measure baseline glucocorticoids (time to baseline sample \pm se = 124.7 ± 3.65 s). After the blood samples were completed for all individuals in a nest, we conducted three behavioral assays: we measured breathing rate, handling struggle rate, and bag struggle rate. These measures were selected as they represent the primary responses that altricial nestlings can mount towards a stressor (i.e., they cannot escape or injure a predator yet)—in prior studies, these measurements are positively correlated to overall activity and aggressiveness after animals gain independence (Boon et al., 2007; Charmantier et al., 2017) and can also be heritable (Brommer and Klunen, 2012). Breathing rate—which can indicate an animal’s response to acute stress—was measured by visually counting breath cycles during 30 s with the nestling held in a loose bander’s grip (belly up). Next, we

performed the handling struggle test by counting the number of struggles the nestling performed during 30 s with their legs, wings or head while held in the banders' grip on their back. Handling struggles may represent a nestling's boldness in the face of a stressful stimulus (the researcher) and may be akin to their response during a possible predation event. Finally, we placed the nestling in a bird bag and performed the bag struggle test by counting how many struggles or movements they made during 30 s. The bag struggle test, while still a stressful stimulus, may represent general nestling activity as the darker environment and distance from the researcher may resemble conditions in the nest (at least relative to the handling struggle test). After these behavioral assays, nestlings were left in the bird bag until 15 min from the initial nest disturbance. At this time, we collected a second small blood sample in order to measure stress-induced glucocorticoids (mean time to stress-induced sample \pm se = 17.1 ± 0.08 min since nest disturbance)—the 15 min restraint period was selected over the common 30 min restraint because nestling stress responses peak earlier and return to baseline more rapidly than adult birds (Bebus et al., 2020; Rensel et al., 2010). Once all nestlings had been sampled, we recorded their tarsus length, mass and banded them with a uniquely numbered metal band before returning them to their nest. An identical sampling protocol was repeated 7 days later, on day 16 or 17, in order to examine how behavioral and glucocorticoid coping styles changes during nestling development. In total, we sampled 97 nestlings at the rural site ($N = 31$ nests) and 16 nestlings at the urban site ($N = 5$ nests); 48 nestlings were sampled in 2020 and 65 nestlings were sampled in 2021.

A small drop of whole blood was preserved on FTA cards to genetically sex individuals (Kilgour et al. *under review*), and remaining samples were centrifuged in the field and plasma was stored on ice until we could return to campus. Next, plasma samples were stored at -80°C until we could perform physiological assays (within 6 months).

2.2. Lab analyses

We used a commercially available kit from Arbor Assays to measure glucocorticoids (DetectX Corticosterone Enzyme Immunoassay Kit, K014-H5) according to the manufacturer's protocol for small volumes. This protocol was validated for our samples (Kilgour et al. *under review*) and we selected a 1:25 dilution to ensure that baseline samples with low corticosterone concentrations still fell within the range of the standard curve. We combined 10 μL of plasma with 10 μL of dissociation reagent for 5 min at room temperature. Next, we added 230 μL of assay buffer before 50 μL of each diluted sample was added to the 96-well plate. Samples were run in duplicate, and we randomized which individuals were included on each plate as well as the position of each sample on the plate; paired baseline and stress-induced samples from the same individual and sampling event were always run on the same plate. All wells received 25 μL of conjugate and 25 μL of antibody reagents before incubating at room temperature for 1 h while shaking at 500 rpm. The plate content was then emptied and washed 4 times with wash buffer before 100 μL of TMB substrate was added. The plate was allowed to develop for 30 mins in the dark (no shaking) before 50 μL of stop solution was added. Absorbance was read using a BioTek plate reader (ELX808) at 450 nm and corticosterone concentrations were calculated based on the standard curve included on every plate (range from 78 pg/ml to 10,000 pg/ml). We calculated the intra-assay coefficient of variation (CV) by comparing hormone concentrations from duplicates of the same sample, where the mean intra-assay CV across all samples was 4.56%. We also assessed the inter-assay CV from a pooled plasma sample with an aliquot run on every plate; the mean inter-assay CV was 10.3%. Baseline glucocorticoids and stress-induced glucocorticoids were each log transformed for analyses. We also calculated the stress response as the difference between stress-induced and baseline glucocorticoids (which did not need to be transformed).

3. Statistical analyses

We used a series of general and generalized linear mixed models to assess how behavioral and endocrine coping styles (1) changed during nestling development across sites, (2) differed at fledging age across sites, and (3) covaried with one another across sites. First, we used generalized linear mixed models (GLMMs) with a poisson distribution to examine how behavioral coping styles developed in starling nestlings since breathing rate, handling struggle rate and bag struggle rate were poisson distributed count data. We built three GLMMs—one for each of the behavioral coping styles as the dependent variable—with identical fixed and random effect structures. The fixed effects included sex, year, the Julian date at sampling, the body condition of the nestling (residuals of mass on tarsus), the age of the nestling, the site ID, and the interaction between age and site. These models also included a nested random effect of chick ID nested within nest ID. We used three additional similar linear mixed models (LMMs) to examine how glucocorticoids changed during nestling development. We used three models with baseline glucocorticoids, stress-induced glucocorticoids, or the glucocorticoid stress response as the dependent variable, along with identical fixed and random effects as the behavioral models (i.e., sex, year, date, body condition, age, site, interaction of age by site, and chick ID nested within nest ID). In the baseline glucocorticoid model, we added a fixed effect for the time it took to collect the baseline blood sample, while for the stress-induced and stress response models we added a fixed effect for the time at which the second blood sample was collected.

In order to examine whether European starling nestlings differed in their coping styles at fledging, when their phenotype may play an important role in shaping survival during their first year of life, we built additional LMMs and GLMMs to examine predictors of coping styles for day 16 or 17 nestlings only. We used three GLMMs—one for breathing rate, one for handling struggles rate and one for bag struggle rate—with sex, year, body condition and site as the fixed effects as well as nest ID as the random effect. We also built three similar LMMs—one for baseline glucocorticoids, one for stress-induced glucocorticoids, and one for the glucocorticoid stress response—with sex, year, body condition, site and the time it took to collect the respective blood sample as the fixed effects as well as nest ID as the random effect. Finally, we also examine pairwise correlations between the behavioral coping style measurements and the endocrine coping styles measurements for rural and urban nestlings separately. We used pair-wise Spearman's rank correlations to identify how the three behavioral and three endocrine variables in our study covaried for nestlings at the rural site and for nestlings at the urban site on days 16 or 17 of age.

All analyses were performed in RStudio version 1.3.1073. We used package nlme (version 3.1–152) to perform all LMMs and package lme4 (version 1.1–27.1) to perform all GLMMs. Pair-wise Spearman's rank correlations were performed in package corplot (version 0.90). We checked that all model variance inflation factors were below 3, and residuals for LMMs appeared normally distributed and not heteroskedastic.

4. Results

We first examined how behavioral and endocrine coping styles developed in nestlings that transitioned from their peak growth rate to independence at fledging age. We found that breathing rates differed across years, decreased with body condition, increased with nestling age and were elevated in nestlings from the urban site compared to the rural one. However, there was no significant interaction of age and site that would indicate that breathing rate changed with age differently in nestlings from one site compared to the other (Table 1). Similarly, breathing rate was not correlated to nestling sex, or date of sampling. We found that the handling struggle rate of nestlings differed across years, was lower in males compared to females, and increased slightly with nestling body condition. Handling struggle rates were uncorrelated to

Table 1

We explored the development of behavioral coping styles by sampling 122 nestlings across 38 nests during their peak of growth (days 9 or 10) and re-sampling each nestling again near fledging (days 16 or 17; a total of 222 behavioral measurements). We present results of generalized linear mixed models with a poisson distribution for nestling (A) breathing rates, (B) handling struggle rates, and (C) bag struggle rates. Asterisks and bolding denote significant predictor variables where the P-value was below or equal to 0.05. No residual is shown for the random effects, as this is a generalized linear mixed model.

Fixed effects	A) Breathing rate			B) Handling struggle rate			C) Bag struggle rate		
	estimate ± se	z-value	P-value	estimate ± se	z-value	P-value	estimate ± se	z-value	P-value
Intercept	3.14 ± 0.04	95.6	<0.001*	1.63 ± 0.10	16.4	<0.001*	0.93 ± 0.11	8.20	<0.001*
Sex (male)	0.02 ± 0.03	0.49	0.62	-0.40 ± 0.13	-3.18	0.001*	-0.03 ± 0.15	-0.19	0.85
Year	0.10 ± 0.03	3.41	<0.001*	0.19 ± 0.08	2.24	0.03*	-0.12 ± 0.09	-1.33	0.19
Body condition	-0.004 ± 0.002	-1.97	0.049*	0.01 ± 0.006	2.16	0.03*	-0.01 ± 0.008	-0.87	0.39
Julian date	0.02 ± 0.03	0.57	0.57	-0.14 ± 0.08	-1.66	0.10	-0.04 ± 0.09	-0.48	0.63
Age	0.04 ± 0.02	2.14	0.03*	0.03 ± 0.04	0.79	0.43	-0.20 ± 0.05	-4.09	<0.001*
Site (urban)	0.31 ± 0.09	3.57	<0.001*	-0.22 ± 0.26	-0.83	0.40	-0.03 ± 0.27	-0.12	0.91
Age × Site	-0.002 ± 0.04	-0.05	0.96	0.07 ± 0.10	0.64	0.52	-0.20 ± 0.12	-1.66	0.10
Random effects	variance	sd		variance	sd		variance	sd	
Nest ID	0.02	0.13		0.08	0.29		0.06	0.25	
Chick ID/Nest ID	0.001	0.03		0.29	0.54		0.40	0.64	

date, age, site, or the interaction between age and site. Nestling bag struggle rates were uncorrelated to sex, body condition, year, date, site, or the interaction between site and age. Bag struggle rates decreased with nestling age (Fig. 1). We found that all three glucocorticoid measurements examined in our study were uncorrelated to nestling sex, body condition, or date of sampling (Table 2). Baseline glucocorticoids and the glucocorticoid stress response (but not stress-induced glucocorticoids) differed across years. Baseline glucocorticoids were positively correlated to the number of seconds it took to sample a nestling,

though stress-induced glucocorticoids and the stress response were uncorrelated to the timing of the second blood sample. Finally, baseline glucocorticoids, stress-induced glucocorticoids, and the stress response were significantly correlated with age, site and the interaction between age and site. In other words, all three glucocorticoid traits increased more quickly during development in nestlings at the urban site whereas they increased more slowly with age in nestlings at the rural site (Fig. 1).

Next, we explored whether fledglings had similar behavioral and endocrine coping styles across the two sites. This developmental stage

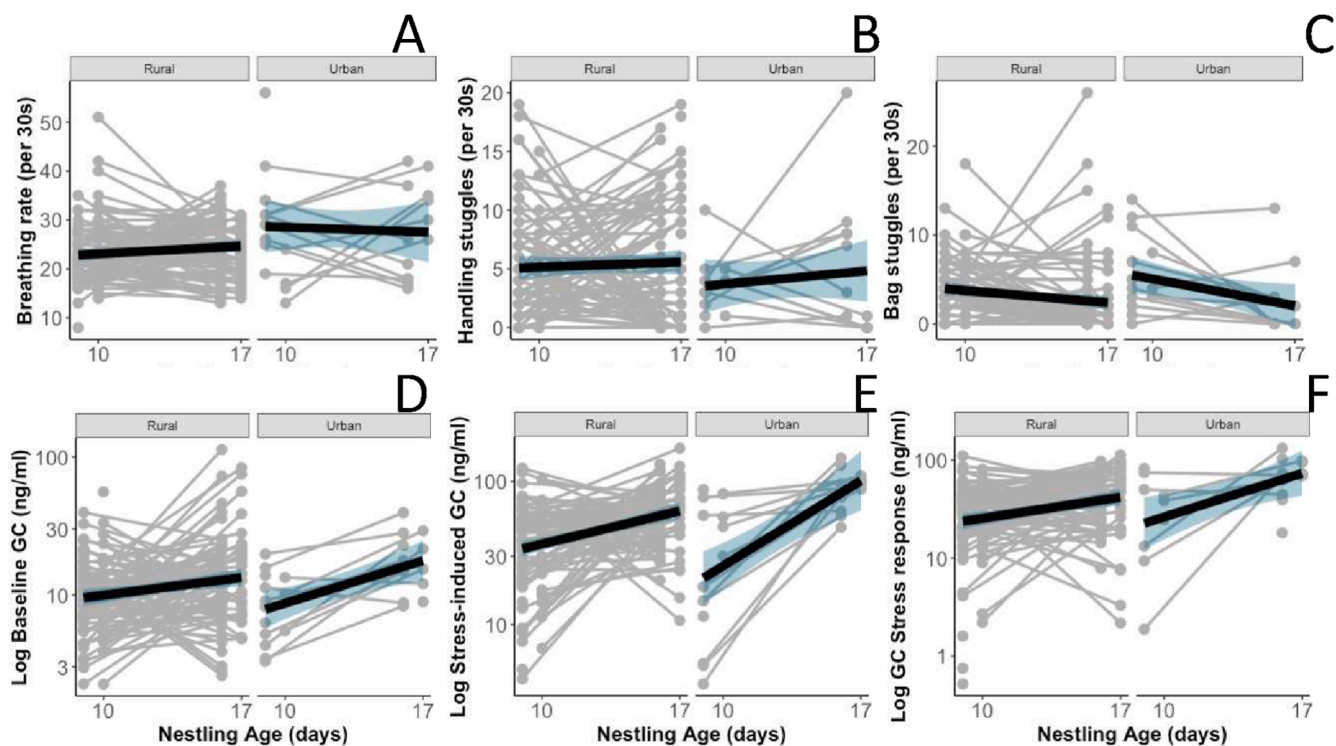


Fig. 1. The development of coping styles as starling nestlings transition from peak growth rates (days 9 or 10) to preparing to fledge the nest (days 16 or 17) is displayed using a reaction norm approach. In each panel, grey symbols depict individual data points and grey lines depict the change in phenotype of one individual nestling as they grew (i.e., a reaction norm). The thick black line represents the average change in phenotype with age for each site, and the blue shading shows the 95% confidence interval. We found that nestling (A) breathing rate was higher in urban compared to rural nestlings. While it tended to increase slightly with age, breathing rate did not develop differently over time in urban or rural nestlings. Nestling (B) handling struggle rate was unrelated to site, age or the interaction between the two. Nestling (C) bag struggle rates decreased with age, but was unrelated to site. Similarly, bag struggle rates did not develop differently in urban and rural nestlings as they aged. Finally, we found that all glucocorticoid traits—including (D) baseline glucocorticoids, (E) stress-induced glucocorticoids, and (F) the glucocorticoid stress response—were higher in urban nestlings than rural ones, were positively correlated to nestling age, and there was a significant interaction between age and site. In other words, all three glucocorticoid measurements increased with age more rapidly in urban nestlings than they did in rural nestlings (i.e., the slope of the black line is steeper for urban birds). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2

We explored the development of endocrine coping styles by sampling 121 nestlings across 38 nests during their peak of growth (days 9 or 10) and re-sampling each nestling again near fledging (days 16 or 17; a total of 218 behavioral measurements). We present results of general linear mixed models for nestling (A) baseline glucocorticoids, (B) stress-induced glucocorticoids following a 15 min restraint, and (C) the glucocorticoid stress response as measured by the difference between the two prior variables. Asterisks and bolding denote significant predictor variables where the P-value was below or equal to 0.05.

Fixed effects	A) Baseline GC			B) Stress-induced GC			C) GC Stress response		
	estimate ± se	t-value	P-value	estimate ± se	t-value	P-value	estimate ± se	t-value	P-value
Intercept	813 ± 232	3.50	<0.001*	−391 ± 260	−1.50	0.14	−27455 ± 10579	−2.60	0.01
Sex (male)	0.02 ± 0.09	0.26	0.80	−0.01 ± 0.1	−0.12	0.90	1.89 ± 3.51	0.54	0.59
Time to sample	0.004 ± 0.001	4.79	<0.001*	−0.001 ± 0.001	−1.31	0.19	−0.03 ± 0.03	−1.35	0.18
Year	−0.40 ± 0.12	−3.49	0.001*	0.20 ± 0.13	1.52	0.14	13.6 ± 5.24	2.60	0.01*
Body condition	−0.004 ± 0.003	−0.62	0.53	0.005 ± 0.004	−0.06	0.95	0.03 ± 0.24	0.13	0.90
Julian date	−0.0003 ± 0.003	−0.08	0.94	−0.001 ± 0.004	1.14	0.89	0.02 ± 0.20	0.15	0.88
Age	0.04 ± 0.01	2.90	0.005*	0.06 ± 0.01	4.41	<0.001*	1.87 ± 0.52	3.62	0.001*
Site (urban)	−1.29 ± 0.48	−2.68	0.01*	−2.06 ± 0.54	−3.81	<0.001*	−46.2 ± 21.1	−2.19	0.04*
Age × Site	0.09 ± 0.04	2.54	0.01*	0.16 ± 0.04	4.25	<0.001*	4.85 ± 1.46	3.32	0.001*
Random effects	sd			sd			sd		
Nest ID	0.18			0.21			9.48		
Chick ID/Nest ID	0.0001			0.0002			0.003		
Residual	0.60			0.60			23.1		

corresponds to when these starlings first leave the nest and experience the environment beyond the nest box where coping styles are likely to be critical for dealing with stressors they encounter. We found only one behavioral measurement—breathing rates—was correlated to site (Table 3). While handling and bag struggle rates were similar for urban and rural fledglings, urban fledglings had elevated breathing rates. Breathing rate was correlated with year, but uncorrelated to sex, and body condition. Handling struggle rates were elevated in female nestlings and decreased with improving body condition, but unrelated to year. Bag struggle rates were uncorrelated with sex or year but decreased with body condition (Fig. 2). We found that fledging starlings from the urban site did not differ in their baseline glucocorticoids or stress-induced glucocorticoids, but they mounted stronger glucocorticoid stress responses compared to rural nestlings (Table 3). All three glucocorticoid traits were uncorrelated to sex and body condition for fledging-aged starlings. Baseline glucocorticoids increased the longer it took to collect the initial blood sample and varied across years, but stress-induced glucocorticoids and the stress response were uncorrelated to sampling time or year (Fig. 2).

Table 3

We compared the behavioral and endocrine coping styles of 101 nestlings across 35 nests at fledging age (days 16 or 17). We present results of generalized linear mixed models with a poisson distribution for nestling (A) breathing rates, (B) handling struggle rates, and (C) bag struggle rates. We also present results of general linear mixed models for nestling (D) baseline glucocorticoids, (E) stress-induced glucocorticoids following a 15 min restraint, and (F) the glucocorticoid stress response as measured by the difference between the two prior variables. Asterisks and bolding denote significant predictor variables where the P-value was below or equal to 0.05. Residuals are only shown for the random effects of linear mixed models, while no residual can be calculated for the generalized linear mixed models.

Fixed effects	A) Breathing rate			B) Handling struggle rate			C) Bag struggle rate		
	estimate ± se	z-value	P-value	estimate ± se	z-value	P-value	estimate ± se	z-value	P-value
Intercept	3.2 ± 0.03	98.1	<0.001*	1.57 ± 0.20	7.91	<0.001*	0.47 ± 0.23	2.02	0.04*
Sex (male)	−0.03 ± 0.04	−0.85	0.40	−0.48 ± 0.11	−4.52	<0.001*	−0.12 ± 0.15	−0.80	0.41
Year	0.19 ± 0.03	6.81	<0.001*	0.22 ± 0.19	1.16	0.24	−0.02 ± 0.21	−0.10	0.92
Body condition	−0.002 ± 0.002	−1.05	0.30	−0.02 ± 0.007	−3.79	<0.001*	−0.03 ± 0.009	−2.65	0.008*
Site (urban)	0.29 ± 0.08	3.74	<0.001*	−0.47 ± 0.63	−0.74	0.49	−0.36 ± 0.69	−0.53	0.60
Random effects	variance	sd		variance	sd		variance	sd	
Nest ID	0.007	0.08		1.02	1.01		1.19	1.09	

Fixed effects	D) Baseline GC			E) Stress-induced GC			F) GC Stress response		
	estimate ± se	t-value	P-value	estimate ± se	t-value	P-value	estimate ± se	t-value	P-value
Intercept	1391 ± 332	4.18	<0.001*	7.17 ± 297	0.02	0.98	−28347 ± 14329	−1.97	0.052
Sex (male)	−0.04 ± 0.11	−0.41	0.68	0.03 ± 0.08	0.34	0.73	5.40 ± 4.99	1.08	0.28
Time to sample	0.006 ± 0.001	5.60	<0.001*	−0.001 ± 0.001	−0.77	0.45	−0.03 ± 0.04	−0.75	0.46
Year	−0.69 ± 0.16	−4.18	<0.001*	−0.001 ± 0.14	−0.01	0.99	14.1 ± 7.1	1.98	0.056
Body condition	−0.01 ± 0.007	−0.87	0.39	−0.004 ± 0.005	−0.75	0.45	−0.17 ± 0.30	−0.57	0.57
Site (urban)	0.04 ± 0.24	0.18	0.85	0.41 ± 0.21	1.93	0.06	30.4 ± 10.0	3.04	0.005*
Random effects	sd			sd			sd		
Nest ID	0.32			0.31			12.1		
Residual	0.50			0.38			23.0		

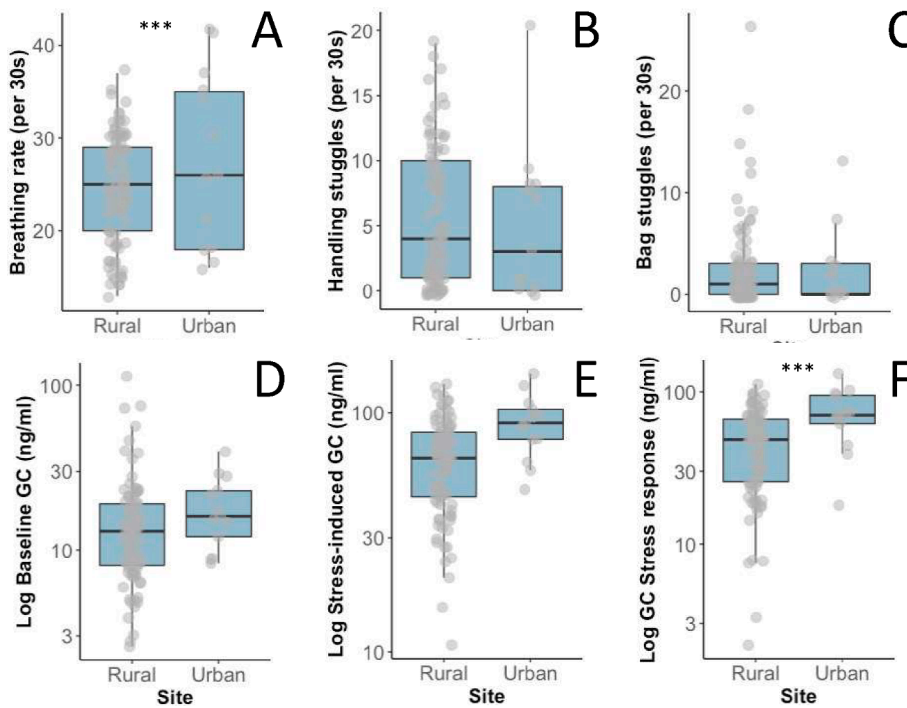


Fig. 2. Starling fledglings from rural and urban sites did not differ in their (A) breathing rates, (B) handling struggle rates, (C) bag struggle rates, or (D) baseline glucocorticoids. Urban fledglings had (E) elevated stress-induced glucocorticoids and (F) mounted stronger glucocorticoids stress response when compared to starlings from the rural site. Figures depict box and whisker plots, and grey circles represent individual data points. Asterisks indicate statistically significant differences across sites as identified in our LMMs and GLMMs.

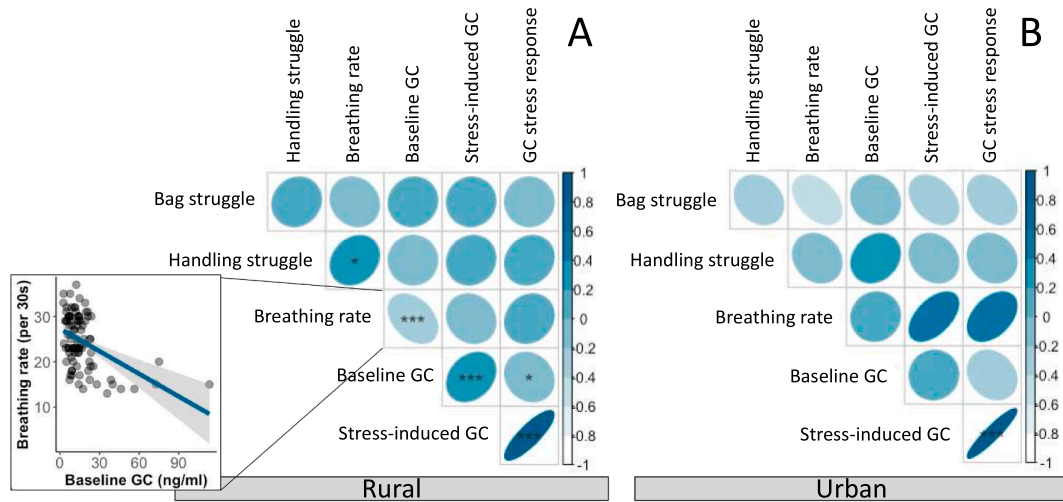


Fig. 3. A correlation plot depicting pair-wise spearman rank correlations among components of the behavioral and endocrine coping styles of starling nestlings. The shading and shape of ellipses represent parametrically scaled to the correlation value, with corresponding numerical scale on the right (rho ranges from -1 to 1 , with coefficients closer to 0 suggesting a lack of correlation). Asterisks depict significant pair-wise spearman rank correlations, where one asterisk corresponds to P-value < 0.05 , two asterisks correspond to P-value < 0.01 and three asterisks correspond to P-value < 0.001 . Panel (A) shows correlations among coping style variables for fledglings (day 16 or 17) at the rural site only ($N = 94$), while panel (B) shows correlations for fledglings from the urban site ($N = 13$). We found that a single behavioral trait was correlated to one of the glucocorticoid traits for rural nestlings only: this negative correlation between breathing rate and baseline glucocorticoids is shown as an inset in panel A.

developed at different rates in nestlings from urban versus rural sites, though measures of behavioral coping styles did not. All measures of glucocorticoids—including baseline, stress-induced, and the stress response—increased more rapidly as nestlings aged for chicks in urban nest boxes compared to the increase observed in nestlings from rural ones. In contrast, though some of the behavioral phenotypes were correlated with age (i.e., breathing rate increased with nestling age while bag struggle rate decreased with nestling age), the development of behavioral coping styles did not differ across urban and rural nestlings. Our results suggest that differences in endocrine coping styles emerge

prior to any potential differences in behavioral coping styles in urban versus rural starlings. Prior work with European blackbirds from urban and rural sites found that differences in behavioral (Miranda et al., 2013) and endocrine coping styles (Partecke et al., 2006a) were already present in the first year of life: differences in behavioral coping styles were present in November whereas differences in endocrine coping styles were present in December for nestlings hatched during the prior March to June period. Though the authors did not sample birds during the nestling phase, they did perform a common garden experiment where urban and rural nestlings were reared under common

conditions—these results suggest that genetic rather than early-life environmental conditions shape differences in coping styles across urban and rural habitats. A study in the house wren explored endocrine coping styles in rural and urban nestlings; nestlings from urban habitats already had elevated baseline glucocorticoids compared to rural ones on the day of hatching (Ouyang et al., 2019), supporting our findings that endocrine coping styles can emerge early for altricial nestlings. The authors went on to conduct a cross-fostering experiment which revealed that both genetic and early-life environmental factors shaped endocrine coping styles (Ouyang et al., 2019). The early-life environment in urban areas can clearly impact animals during their development—some of the early-life environmental factors that could lead to differences in coping styles range from differential exposure to noise, light or chemical pollution, to differences in parental care, to differences in the rate of nest disturbances. While our study does not allow us to disentangle the roles of genetic versus early-life effects on coping styles, we do show that differences between urban and rural animals emerge before young even leave the nest. Our study worked across two sites, so it remains possible that these patterns are shaped by site differences that are unrelated to urbanization—future work across a larger gradient of conditions will be necessary to expand upon our results.

In light of differences in the development of endocrine coping styles across urban and rural sites, we found that starling fledglings from urban habitats had elevated breathing rates and mounted a stronger stress response than rural ones. This result supports the idea that breathing rate represents a measure of acute stress. As in a prior study, we found that nestling glucocorticoids were dampened at days 9/10 (Bebus et al., 2020), which supported our prediction that the stress response is dampened during an animal's peak of growth so as not to cause a trade-off with somatic growth. Differences in endocrine coping styles emerged only in day 16 or 17 starlings, when the rate of growth has slowed considerably and when nestlings will soon leave the nest. These findings add to several intraspecific studies examining the relationship between the glucocorticoid stress response and urbanization. Results of such studies are equivocal (Bonier, 2012; Iglesias-Carrasco et al., 2020), where some species show stronger stress responses in urban populations (Fokidis et al., 2009), others show stronger stress responses in rural populations (Huang et al., 2020; Partecke et al., 2006b), and yet others find no difference in glucocorticoids across populations that span the urban to rural gradient (Angelier et al., 2016; Heiss et al., 2009). The *urban endocrine hypothesis* (Bonier, 2012) suggests that urban-dwellers and urban-avoiders should differ in their endocrine traits which could explain why different relationships have been found between endocrine coping styles and urbanization across species. However, a recent meta-analysis failed to find consistent interspecific differences in glucocorticoids according to a species' abundance in urban habitats for birds and reptiles (Injaian et al., 2020). Our results suggest that at least for one urban-dwelling species—the European starling—life in urban environments was associated with an elevated stress response. An explanation for this pattern is that urban living exposes animals to frequent and diverse disturbances where responding quickly and appropriately is critical. This is in contrast to the idea that animals in urban habitats should produce a dampened stress response as they become habituated to frequent stressors or disturbances. It remains possible that the stress response of urban adult birds would dampen over time with exposure to disturbances. The long-term costs or benefits of differences in glucocorticoid physiology of starling nestlings remains to be explored.

Despite the lack of differences in the behavioral coping styles of urban and rural nestlings in our study, differences in behavioral coping styles have been found for diverse taxa across a gradient of urbanization. Striped field mice (*Apodemus agrarius*) (Dammhahn et al., 2020), sika deer (*Cervus nippon*) (Honda et al., 2018), water dragons (*Intellagama lesueurii*) (Baxter-Gilbert et al., 2019), song sparrows (*Melospiza melodia*) (Evans et al., 2010) and dark eyed juncos (*Junco hyemalis thurberi*) (Atwell et al., 2012) from urban habitats are bolder than rural individuals. Similarly, individuals from more urban populations are less

neophobic (Huang et al., 2020), more active (Moule et al., 2016), and faster to explore novel environments (Charmantier et al., 2017; Thompson et al., 2018) compared to individuals of the same species from rural populations in birds and reptiles. The behavioral measurements we selected are repeatable and heritable traits for other species (Boon et al., 2007; Brommer and Klun, 2012; Charmantier et al., 2017), suggesting that even measurements in the nest may be informative about an individual's behavior after fledging. One possible explanation for the results of our study is that the behavioral assays in our study may have been performed too early in the development of these animals to capture differences across individuals or sites. In the nest box, young starlings may face limited benefits from being bolder, more exploratory, or less neophobic such that behavioral coping styles are not apparent until after fledging has occurred. For example, a starling nestling would be unlikely to discover a new food source or to evade a predator in the nest such that exploration or shyness would not benefit them. It is also possible behavioral coping styles arise from experience, which nestlings lack. Prior work has shown that urban great tit (*Parus major*) nestlings were more exploratory, had faster breathing rates, and performed more struggles during handling (Charmantier et al., 2017; Senar et al., 2017), however, suggesting that at least in some systems behavioral coping styles can emerge in altricial nestlings. Acquiring differences in behavioral coping styles in the nest may be too early for species where young may disperse to different habitat types—it remains unclear whether nestlings born in urban habitats are more likely to remain and breed in urban areas too. Finally, an alternative explanation for the results of our study is that unlike other species where differences in the behavior of urban and rural individuals have been shown, European starlings may simply not exhibit differences in behavioral coping styles across a gradient of urbanization. As an urban-dwelling species thought to thrive across diverse conditions, behavior may play a limited role in coping with environmental differences across habitat types.

Though our study is correlative—and thus it is not possible to imply whether endocrine and behavioral coping styles are causally related—we examined pair-wise correlations between the behavioral and the endocrine traits measured in our study to test for the one- versus two-tiered models of coping style for rural and urban nestlings. We found few correlations among behavioral and endocrine traits at either of our study sites, which supports the two-tiered model of coping styles in European starlings. For example, breathing rate was negatively correlated to baseline glucocorticoids but in rural nestlings only. Breathing rate is often thought of as a measure of acute stress (Torné-Noguera et al., 2014) though in our study it was uncorrelated to stress-induced glucocorticoids. Differences in microclimate across the study sites may shape this correlation: for example, warmer temperatures at one site may favor reduced breathing rates and elevated baseline glucocorticoids to save energy. Our results are similar to other empirical studies that suggest the two-tiered model of coping styles is best supported for free-living animals (Farwell et al., 2014; Qu et al., 2018; Sadoul et al., 2021; Santicchia et al., 2020; Westrick et al., 2018). While we found a few more correlations among traits in the rural relative to urban site, one possible explanation for this difference is the lower number of urban nestlings sampled in our study. Nestlings from the urban were less likely to survive to fledging age (*unpublished data*, Guindre-Parker), which resulted in fewer nestlings sampled at the urban site on days 16 or 17. We also note that variability in behavioral and glucocorticoid traits appears greater in rural nestlings compared to urban ones. Once again, this could be due to differences in sample size, but future efforts to explore whether variability in phenotype differs along an urban to rural gradient are needed—individual variation in a trait is required, along with heritability, for selection to act on said trait. Differences in variability in coping styles would thus be a precursor required for differences in evolution under urban versus rural environments (Szulkin et al., 2020). Our study's support for the two-tiered model of coping styles is perhaps not surprising, given that differences in endocrine coping styles emerged rapidly during nestling development

whereas we did not find differences in behavioral coping styles at fledging age across sites. This raises an interesting possibility: that endocrine and behavioral coping styles may develop on different timeframes during an animal's life. There is evidence that coping styles change with age in humans (Wingo et al., 2015) and in North American red squirrels (*Tamiasciurus hudsonicus*) (Kelley et al., 2015; Westrick et al., 2018), though more work is needed to better understand the exact timeline(s) over which endocrine and behavioral coping styles emerge. If endocrine and behavioral coping styles developed at different speeds, it would make the two-tiered model of coping styles more likely to be supported for animals sampled earlier in life like in our study. An alternative possibility is that endocrine and behavioral coping styles are simply not causally linked or are only causally linked under a restricted set of conditions (i.e., such as laboratory condition) which would explain why support for the two-tiered model of coping styles is most common for wildlife.

6. Conclusion

We find that differences in endocrine coping styles across urban and rural environments emerge in early life—at fledging—where urban animals mount stronger stress responses compared to rural ones. These differences between urban and rural nestlings arise rapidly following their peak of growth, where urban nestlings show rapid increases in the stress response as they approach fledging whereas rural ones show slower increases in glucocorticoids with age. In contrast, only breathing rates differed across sites for nestlings at fledging age, and urban versus rural nestlings did not differ in the rate of development of behavioral coping styles during nestling development. Thus, it appears that endocrine and behavioral differences in coping styles documented across gradients of urbanization in prior studies may arise at different times in the life of an animal, with differences in endocrine coping styles arising earlier than differences in behavioral coping styles. Perhaps unsurprisingly then, our work supports the two-tiered model of coping styles; we find only weak support for correlations between behavioral and endocrine components of coping styles in rural animals and no correlations for urban ones.

CRedit authorship contribution statement

Sarah Guindre-Parker: Conceptualization, Supervision, Investigation, Writing – original draft. **Denyelle A.V. Kilgour:** Investigation, Writing – review & editing. **Courtney R. Linkous:** Investigation, Writing – review & editing.

Declaration of Competing Interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ygcen.2022.114091>.

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