**2. Materials and Methods**

**2.1 Sampling**

House sparrows were trapped at the Lincoln Park Zoo (Chicago, IL; urban site) and three private residence in Homewood, IL (suburban site); Hebron, IN (rural site) between 2017 and 2018. We used a combination of mist nests and metal potter traps to collect 65 house sparrows (n = 23 juvenile; n = 35 adults; n = 7 age unknown). House sparrows were surveyed in an urban area (n = 23), a suburban area (n = 32), and rural area (n = 7). Birds were immediately band with an U.S. Fish and Wildlife Service aluminum band (permit# 09924). Body feathers (~25) were plucked from each individual and stored at room temperature for glucocorticoid and testosterone analyses. We also collected morphological measurements (mm) using digital calipers (Mitutoyo, Japan), including the beak, tarsus, and wing. Finally, body mass (mm) was measured using a spring scale (Pesola Marque déposée).

**2.2 Feather Processing and Hormone Analyses**

**2.2.1 Corticosterone Assay**

We processed the feathers for glucocorticoid using a modified protocol from Bortolotti et al. 2008 Specifically, we washed the feathers in 5.0 ml of 90% methanol (methanol: distilled water) and agitated a mixer (Glas-col, Terra Haute, IN) for 1 min at setting 50. The methanol was poured off and an additional 5.0 ml were added to the feathers. This process was repeated for a total of three times, and then feather samples were placed on individual plastic trays to dry for 2-3 days to allow for drying. Once dry, we weighed out 0.002 ± 0.0002g feathers in 2.0 ml vials and pulverized them into a fine powder (Omni Bead Ruptor 24, setting 6.8 m/s, two 45 s cycles with a 15 s interval; Omni International, Kennesaw, GA). Pulverized feathers were then combined with 1 ml of 90% methanol, vortexed briefly, and then agitated on the Glas-col mixer for 4 hr (setting 50). Tubes were later centrifuged for 15 mins at 500 x*g* at 10°C, the supernatant was poured into clean plastic tubes, and then dried down under forced air and at 60°C in a hot-water bath. Once all samples were dried, we reconstituted samples in 250 μl of phosphate-buffered saline (0.2 M NaH2PO4, 0.2 M NaHPO4, NaCl) to produce a 4x concentrated extract. Samples were briefly vortex, sonicated for 20 min then agitated for 30 min on the Glas-col mixer before analysis.

A corticosterone enzyme immunoassay (EIA) was used to quantify the amount of glucocorticoids in the feathers. Specifically, we used an EIA that used corticosterone horseradish peroxidase (HRP) ligands and polyclonal antiserum (CJM006) provided by C. Munro (University of California, Davis, California, USA). Antiserum cross-reactivities for corticosterone were: corticosterone, 100%; desoxycorticosterone, 14.25%; tetrahydrocorticosterone, 0.9%; 11-deoxycortisol. 0.03%; prednisone, < 0.01%; prednisolone, 0.07%; cortisol, 0.23%; cortisone, < 0.01%; progesterone, 2.65%; testosterone 0.64% and estradiol 17β, < 0.01% (Santymire & Armstrong 2010; Narayan et al. 2011). We ran samples on immunoglobulin double antibody (goat-anti rabbit) coated plates. Corticosterone antiserum and HRP were used at dilutions of 1:225,000 and 1:200,000, respectively. The EIA was performed using modified methods from Munro & Stabenfeldt (1984). Each well was loaded with duplicates of 100 μl of EIA buffer solution (blank wells), and 50 μl of solutions in the following manner: triplicate of 1000 pg/well serially diluted to 3.9 pg/well corticosterone standards (q1550-020 Steraloids INC., New Port, Rhode Island); duplicates of 3x controls (african wild dog fecal extracts); duplicates of feather samples. Fifty microliters of HRP were added to each well followed by 50 μl of corticosterone antibody added to all but the blank wells. Plates were incubated on a plate shaker for 2 hours at room temperature and were washed 1x and left upside to dry for 20 min. We added 100 μl of Tetramethylbenzidine (TMB) peroxidase substrate ELISA (Moss, INC. Pasadena, MD) to each well and covered with a microplate sealer to incubate in artificial ceiling light at room temperature for 30 min to 1 hr before adding 1N hydrocholoric acid (Sigma Aldrich, St. Louis, MI) to stop reaction. Resulting optical density of plates was then measured at 450 nm and referenced at 620 nm in Gen5 program (version 3.02.2) on Epoch microplate spectrophotometer (BioTek Instrument INC., Winooski, VT).

The EIA was validated biochemically for house sparrows by demonstrating: 1) parallelism between binding inhibition curves of feather extract dilutions and the corticosterone standard (R2= ); and 2) significant recovery (>90%) of exogenous corticosterone (1.95 - 1000 pg/well) added to feather extracts. Assay sensitivity was 1.95 pg/ well and intra- and inter-assay coefficients of variation were <10%.

**2.2.2 Testosterone Assay**

We also analyzed feather testosterone. Polyclonal testosterone antiserum and HRP were also provided by C. Munro. Antiserum cross-reactivities for testosterone were: testosterone, 100%; 5a-dihydrotestosterone, 57.37%; androstenedione, 0.27%; androsterone and DHEA, 0.4%; cholesterol, 0.03%, b-estradiol, 0.02%, progesterone, pregnenolone, hydrocortisone, cholic acid, chenodeoxycholic acid, cholic acid methyl ester, dehydrocholic acid, deoxycholic acid, lithocholic acid, glycholic acid, taurodeoxycholic acid, taurocholic acid, taurochendeoxycholic acid and glycochenodeoxycholic acid, 0.02%. We ran samples on immunoglobulin double antibody (goat-anti rabbit) coated plates. Testosterone horseradish peroxidase and a polyclonal antiserum were used at dilution 1:375,000 and 1:750,000, respectively. Testosterone EIA was performed similar to the corticosterone EIA above. Each well was loaded with duplicates of 100 μl of EIA buffer solution (blank wells), and 50 μl of solutions in the following manner: triplicate of 300 pg/well serially diluted to 3.9 pg/well testosterone standards (COMPANY NAME); duplicates of 3x controls (TYPE); duplicates of feather samples. Fifty microliters of HRP were added to each well followed by 50 μl of corticosterone antibody added to all but the blank wells. Plates were incubated on a plate shaker for 2 hours at room temperature and were washed 1x and left upside to dry for 20 min. We added 100 μl of TMB peroxidase substrate ELISA (Moss, INC. Pasadena, MD) to each well and covered with a microplate sealer to incubate in artificial ceiling light at room temperature for 30 min to 1 hr before adding 1N hydrocholoric acid (Sigma Aldrich, St. Louis, MI) to stop reaction. Resulting optical density of plates was then measured at 450 nm and referenced at 620 nm in Gen5 program (version 3.02.2) on Epoch microplate spectrophotometer (BioTek Instrument INC., Winooski, VT).

The EIA was validated biochemically for house sparrows by demonstrating: 1) parallelism between binding inhibition curves of feather extract dilutions and the corticosterone standard (R2 = 0.999 ); and 2) significant recovery (>90%) of exogenous corticosterone (1.95 - 1000 pg/well) added to feather extracts. Assay sensitivity was 1.95 pg/ well and intra- and inter-assay coefficients of variation were <10%.

**2.3. Data analysis**