Experimental exposure to noise alters gut microbiota in a songbird.

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Abstract

Noise pollution is an unprecedented evolutionary pressure on wild animals that can lead to alteration of stress hormone levels and changes in foraging behavior. Both corticosterone and feeding behavior can have direct effects on gut bacteria, as well as indirect effects through changes in gut physiology. Therefore, we hypothesized that exposure to noise will alter gut microbial communities via indirect effects on stress hormones and foraging behaviors. We exposed captive white-crowned sparrows to city-like noise and measured each individuals' corticosterone level, food intake and gut microbial diversity at the end of four treatments (acclimation, noise, recovery, and control) using a balanced repeated measures design. We found evidence to support our prediction for a causal, positive relationship between noise exposure and gut microbiota. We also found evidence that noise acts to increase corticosterone and decrease food intake. However, noise appeared to act directly on the gut microbiome or, more likely, through an unmeasured variable, rather than through indirect effects via corticosterone and food intake. Our results help to explain previous findings that urban, free-living white-crowned sparrows have higher bacterial richness than rural sparrows. Our findings also add to a growing body of research indicating noise exposure affects stress hormone levels and foraging behaviors. Altogether, our study indicates that noise affects plasma corticosterone, feeding behavior, and the gut microbiome in a songbird and raises new questions as to the mechanism linking noise exposure to gut microbial diversity.

INTRODUCTION

Urbanization acts as an unprecedented evolutionary pressure on wild animals (Swaddle et al., 2015). Human-induced changes in the environment, such as noise and light pollution, can interfere with animal behaviors, such as foraging and communication (Purser & Radford, 2011; Slabbekoorn & den Boer-Visser, 2006). There can also be physiological consequences such as increased stress hormones (Chloupek et al., 2009), and differences in bacterial diversity between animals in urban and rural areas (Littleford-Colquhoun, Weyrich, Kent, & Frere, 2019; Phillips, Berlow, & Derryberry, 2018a; Teyssier et al., 2018). The mechanisms underlying these relationships are in many cases unknown, and we have yet to test some of the more complex interactions. For example, we know that cities often have higher levels of noise pollution, and noise levels can directly impact stress hormones (Blickley et al., 2012) and feeding behavior (Ware, McClure, Carlisle, & Barber, 2015) in animals. We also know that both stress and diet can impact gut physiology (Dinan & Cryan, 2013; Soderholm & Perdue, 2001). What is not known is the extent to which noise pollution alone affects gut bacterial communities, and how these effects might be mediated by feeding behavior and stress responses to noise. Addressing such gaps in knowledge will aid in furthering understanding of how urbanization affects wild animal populations.

Experimental manipulations of noise levels can lead to alteration of stress hormone levels and changes in foraging behavior. Short, high intensity noise elevated plasma corticosterone (CORT) levels in broiler chickens (*Gallus gallus*) (Chloupek et al., 2009). Likewise, in wild sage grouse (*Centrocercus urophasianus*) long exposure (chronic) to high noise levels elevated fecal CORT levels (Blickley et al., 2012), although

this effect is not seen with exposure to low chronic noise levels (in spotted owls (*Strix occidentalis*), see Tempel & Gutierrez 2003). Noise stress can also alter foraging behaviors. For example in three-spined sticklebacks (*Gasterosteus aculeatus*) noise shifted fishes' attention, resulting in decreased food-handling ability (Purser & Radford, 2011). Noise can also reduce foraging efficiency (Senzaki, Yamaura, Francis, & Nakamura, 2016) and increase predator vigilance behaviors in multiple species, including white-crowned sparrows (*Zonotrichia leucophrys*) and chaffinches (*Fringilla coelebs*) (L. Quinn, J. Whittingham, J. Butler, & Cresswell, 2006a; McClure, Ware, Carlisle, Kaltenecker, & Barber, 2013; Ware et al., 2015). These negative effects of noise on foraging behaviors do not seem to be via effects of noise stress on appetite, as in lizards (*Lacerta vivipara*) stress hormones (corticosterone) increase appetite (Cote *et al.* 2006, but see Saldanha *et al.* 2000). Together, these experimental studies suggest that exposure to noise can have both behavioral and physiological consequences in many animals, including birds.

Both corticosterone and feeding behavior can have direct effects on gut bacteria, as well as indirect effects through changes in gut physiology. Corticosterone and other stress hormones can induce changes in intestinal motility and intestinal permeability, as well as cause intestinal inflammation (Ait-Belgnaoui, Bradesi, Fioramonti, Theodorou, & Bueno, 2005; Amini-Khoei et al., 2019; Nakade et al., 2006). These alterations in gut physiology can have lasting effects on gut bacterial communities. For example, maternal separation stress can increase corticosterone, causing gut inflammation and changing gut bacterial communities (Amini-Khoei et al., 2019). Changes in feeding behavior, such as reduction in food intake, could also alter gut bacterial communities. For example, hibernation in ground squirrels (*Ictidomys tridecemlineatus*) and fasting in penguins (*Eudyptula minor, Aptenodytes patagonicus*) cause shifts in which bacterial taxa dominate gut microbial communities (Dewar et al., 2014; Dill-Mcfarland et al., 2014). Even small changes in food intake, like intermittent fasting schedules, can restructure the gut microbiome through effects on which bacteria are able to survive with fewer types or less regular food substrates (Beli et al., 2018). Irrespective of the external stimulus, a number of studies have demonstrated that changes in either corticosterone or feeding behavior can affect gut bacterial communities. Thus, overall, it is likely that noise pollution could have multiple direct and indirect effects on host gut microbiome; however, no study to our knowledge has experimentally manipulated noise levels and measured effects on feeding behavior, corticosterone and the composition and structure of gut microbial communities.

Here, we exposed white-crowned sparrows to city-like noise and measured each individuals' corticosterone level, food intake and gut microbial diversity. Birds were acclimated for five days and then exposed to five days of noise or five days of no noise (control) in a balanced order design. The noise period was immediately followed by a five-day recovery period of no-noise. In other words, one set of birds had five days noise, five days recovery and five days control and a second set of birds had five days control, five days noise and five days recovery. We collected food intake data for each bird in the morning and in the afternoon on each day of the experiment. We collected plasma corticosterone levels and a cloacal swab to assay gut microbial diversity on the fifth (last) day of each of the four treatment periods (acclimation, noise, recovery and control) for each bird. We considered both average food intake and total food intake during each treatment. We predicted that gut microbial diversity and function (predicted using PICRUSt) would increase in noise based on our correlational data from free-living sparrows where birds in noisier, urban areas had higher alpha diversity (q0)(Berlow, Phillips, & Derryberry, 2020; Phillips, Berlow, & Derryberry, 2018b). We hypothesized that this effect of noise on the microbiome would be indirect and would occur through direct effects of corticosterone and food intake on gut microbial diversity. We predicted that corticosterone would increase in response to noise. If noise directly impacts feeding behavior, then food intake should decrease when noise is present. If noise instead affects feeding behavior through stress hormones, then food intake should increase during noise and highly correlate with corticosterone levels. Altogether, testing these predictions should provide insight into whether and how noise pollution affects the gut microbiome.

MATERIALS & METHODS

Study animal

Nuttall's white-crowned sparrows (*Zonotrichia leucophrys nuttalli)* are a useful system to test potential

mechanisms driving variation in gut microbial communities. They can be found breeding in both urban and rural habitats along the west coast of North America, on territories that vary in noise levels (Derryberry et al., 2016). They are also amenable to hand-rearing and experimental work in captive settings (Derryberry, 2007; Marler, 1970; Nelson, Marler, & Palleroni, 1995).

Our experimental subjects were collected as nestlings (day 2—4 of age from 12 nests, males = 14, females = 12, total subjects = 26) from territories in San Francisco, CA and then hand-reared in captivity. Importantly, all birds received the same diet, and the diet changed as appropriate between hand-rearing and after fledging. Briefly, we fed birds by hand at half hour intervals from dawn to dusk until 10–12 days post hatch, then at 1–hour intervals until 18 days post hatch, and thereafter at 3-hour intervals until the birds were feeding independently at about 4–5 weeks of age. Young birds were hand-reared using the Marler diet (Searcy, Peters, & Nowicki, 2004) delivered from 1–cc syringes. Older birds were fed dry seed and water ad libitum, along with greens, soaked seed, hard-boiled eggs and a vitamin supplement.

Once the noise exposure experiment started, all birds received only dry seed and water ad libitum. Birds were fed seed from automatic feeders with graduated marks to make food intake measurements unobtrusive. Grit and cuttlebone calcium supplement were also provided ad libitum. Diet was not otherwise supplemented during the experiment. Each bird received cage maintenance daily.

Birds were individually housed in sound attenuation chambers (Industrial Acoustics Model Mac-1). Chamber dimensions were 68.6cm wide x 53.3cm deep x 63.5cm high (outside) and 58.4cm x 40.6cm x 35.6cm (inside). Each chamber contained a light, a fan for ventilation, and a loudspeaker (Altec Lansing iM227 Orbit MP3). Birds were kept on a natural photoperiod for San Francisco, controlled by time clocks (Hydrofarm TM01715D). During the time of the experiment, lights came on at 6AM and went off at 9PM for a 15:9 light to dark schedule. The ambient temperature was maintained at 23°C. Within the chamber, males were housed in cages that measured 48.5 x 31 x 26 cm.

Experimental design

We used a repeated measures design. All birds received four treatments. The acclimation treatment was for five days of no noise and occurred at the start of the experiment for all birds. All birds also received a noise treatment of five days immediately followed by a recovery treatment of no noise for five days. All birds also received a control treatment of five days of no noise, with half of the birds receiving the control treatment before the noise+recovery treatments and half after the noise+recovery treatments. In other words, one set of birds had five days noise, five days recovery and five days control and a second set of birds had five days control, five days noise and five days recovery (Figure 1).

Noise exposure

We exposed birds to city-like noise, resulting in noise levels of 74—74.8 dBA within chambers. During 'no-noise' treatments, noise levels were 48.5—60 dBA (chambers varied in baseline ambient noise levels). A change of 6dBA is a doubling of sound pressure levels. Noise exposure started with lights on and lasted for six hours.

The 'city-like' noise playback was informed by noise recordings made on white-crowned sparrow breeding territories in San Francisco, CA. Briefly, we recorded two minutes of background noise using a Sennheiser ME62 omnidirectional microphone mounted facing upwards on a 1m tripod. We simultaneously measured the maximum sound pressure level every 10s using a tripod mounted 407736 Extech Sound Level Meter (response time = 125ms, accuracy = ±1.5dB, weighting = A). We calibrated the noise spectrum with the paired sound pressure levels using the Sound Level Meter function in SIGNAL, dropping outliers. We dropped outliers because the goal was to find the calibration constant for each background noise recording. Short temporal events (e.g., a dog bark or a person shout) can bias calibration. We determined outliers using a standard method based on quartiles. This was Q2 ± 1.5 * (Q3-Q1). To limit any bias in the calibration, we dropped identified outliers from both the recording and the SPL estimates for the calibration. We then averaged these 16 noise spectra and generated a noise file in Reaper 4.76 (Eksteins, 2012) to mimic this noise spectrum by applying an FFT filter to white noise, which decreased the spectral energy by 6 dB per octave up to 2.5 kHz

and 9 dB per octave above 2.5kHz. This was the noise file that was used during the noise treatments.

Plasma corticosterone sampling

Blood was collected on the 5th (last) day of each treatment period between 10am and 12pm (noon), in capillary tubes after pricking the brachial artery with a 26G $\frac{1}{2}$ Precision Glide needle. All blood samples were collected within 3 minutes of opening the chamber door to avoid the effects of handling on plasma corticosterone levels. Samples were then spun in a microcentrifuge to separate plasma from other blood components. Plasma corticosterone levels were determined using commercial corticosterone enzyme immunoassay kits (Enzo Life Sciences, cat no. ADI-900-097). This assay was optimized previously for zebra finch plasma (Wada, Hahn, & Breuner, 2007). Following the same procedure, samples were diluted 1:40 and 1% plasma volume of steroid displacement buffer was added. Samples from each individual were run on the same plate while samples within each plate were randomized within the plate. Out of 104 samples, 21 samples fell under the detection limit, thus the detection limit for the particular plate was assigned for those samples. Inter- and intra-plate coefficient of variations were 4.9% and 1.3%, respectively.

Food intake

To collect food data with minimal interruption of normal behavior, we pre-labeled the automatic food dispensers so that food consumption could be recorded without disturbing the birds. We did this by weighing each food cylinder on a balance and making a mark on the cylinder with the addition of 5 grams of seed. Thus, each cylinder had a series of graduated marks per 5 grams of food. Each day, we recorded the level of food in the dispenser and calculated food intake. These data were collected at noon (when sound ended) and just before lights off (~8:30pm) each day.

Gut bacterial sampling

Cloacal swabs were collected on the 5th (last) day of each treatment period directly after blood was collected. The outside of the cloaca was cleaned with an alcohol swab, and sterile water was used to ease the swab into the cloaca. Once fully inserted, the swab was turned gently for 3-5 seconds. Swabs were stored in RNA later (Invitrogen; Carlsbad, CA USA) and frozen at -20 °C. Our work in another passerine has shown that cloacal swabs capture information about gut bacterial communities in the large intestine (Berlow, Kohl, & Derryberry, 2019).

DNA was extracted from cloacal swabs using the Qiagen PowerSoil DNA isolation kit (Qiagen; Hilden, Germany) following the provided protocol, with some modifications to the standard protocol as suggested by Vo and Jedlicka (2014). To further increase DNA yield, the two steps (solutions C2 and C3) which precipitate non-DNA substances were combined per the recommendation of a Qiagen technician (pers. commun.).

We amplified the v4 region of the 16s rRNA bacterial gene using 515F/806R universal primers (~292 bp amplicon) in a 25 µL final volume (Integrated DNA Technologies; Coralville IA, USA) (Caporaso et al., 2012). Each PCR reaction contained: 12 µL sterile, molecular grade water, 1 µL bovine serum albumin, 10 µL 5' Hot Mastermix (Thermo Fisher; Waltham MA, USA), 0.5 µL of each primer (at 100 µM conc.) and 2 µL of DNA template. Each reaction was carried out three times to reduce PCR bias. Water was used as a negative control for each set of reactions. Denaturation of DNA was performed initially at 94 °C for 2 minutes, then the following program was cycled 35 times: 94 °C for 8 s, annealing at 50 °C for 20 s, extension at 72 °C for 30 s. A final elongation was performed at 72 °C for 10 minutes. PCR success was verified with gel electrophoresis.

Samples with fewer than two successful amplifications were re-amplified, and two or three successful PCR products were pooled for each sample in preparation of Illumina tag addition. Samples with fewer than two successful amplifications were not included in sequencing and were not considered in our results. After sequence ng we had 68 samples from 19 birds; 15 acclimation, 18 control, 18 noise, 17 recovery. Dual-end barcodes in the style of TruSeq HT primers were used to provide a unique combination for each sample (Integrated DNA Technologies). Successful tag addition was confirmed using gel electrophoresis wherein tagged samples were compared to untagged samples to ensure the amplicon was longer. Samples then had

their concentrations normalized using a SequalPrep normalization kit (Thermo Fisher). The resulting PCR product was pooled and purified using Agencort AmPure magnetic beads (Beckman Coulter; Brea CA, USA), then sequenced at the University of Tennessee Genomics Core on an Illumina MiSeq platform with v2 reagent kit and paired-end 250-bp protocol.

16S sequences were processed using the QIIME2 pipeline version 2019.10 (Bolyen et al., 2019). To remove sequence errors and trim primers from sequences we used the Divisive Amplicon Denoising Algorithm (DADA) (Rosen, Callahan, Fisher, & Holmes, 2012). Then we aligned sequences, and generated a phylogeny using FastTree, rooting at the midpoint (Price, Dehal, & Arkin, 2010). We used amplicon sequence variants to group sequences (100% similarity). We used the Silva database to assign taxonomy (Quast et al., 2013). Lastly, we removed all sequences matching mitochondria, chloroplast, or archaea. We obtained a total of 1,429,415 sequences (mean=21,020, SD=13.944, see Table S1 for sequence and OTU counts for each sample).

Bacterial community metrics

Gut bacterial alpha diversity was measured using hill numbers, which were calculated from an ASV table after rarefying samples to a depth of 1000 sequences. Hill numbers provide multiple measures of alpha diversity using the same units (effective number of species). Hill number transformations are calculated as orders of q, written as ${}^{q}D$, with q of 0 (${}^{0}D$) representing bacterial richness, q of 1 (${}^{1}D$) representing exponential of Shannon entropy, including both richness and evenness, and q of 2 (${}^{2}D$) representing the inverse of Simpson's index wherein species are weighted according to their abundance (Chao, Chiu, & Jost, 2014). Essentially, the effective number of species is less sensitive to rare bacteria as q increases. We calculated hill numbers using the 'd' function in the R package 'vegetarian' (Jost, 2009). We also measured alpha diversity using Faith's phylogenetic diversity, calculated in Qiime2 (Bolyen et al., 2019).

Gut bacterial beta diversity was calculated in Qiime2 using Jaccard, unweighted UniFrac, Bray-Curtis, and Weighted Unifrac. The former two include information about presence/absence of bacterial taxa and the latter two account for relative abundances of bacterial taxa. UniFrac distances account for phylogenetic relatedness.

To predict the functional role played by bacterial taxa present in the gut, we used Phylogenetic Investigation of Communities by Reconstruction of Observed States (PICRUSt)(Langille et al., 2013). This analysis predicts abundances of gene families from 16s using the Kyoto Encyclopedia of Genes and Genomes (KEGG). Only OTUs that are present in the GreenGenes database (version 13.5) were included, as required by PICRUSt. To assess how well represented our samples were by the reference genome, we used weighted Nearest Sequence Taxon Index (NSTI). To determine which predicted metabolic gene abundances differed between treatment groups, we used Linear Discriminant Analysis Effect Size (LEfSe).

Data analysis

To determine whether treatment had an effect on gut bacterial diversity (${}^{0\text{-}2}D$ and Faith's pd), food intake, and plasma corticosterone levels, we ran mixed linear models using the packages "lme4" and "nlme" in R (Bates, Mächler, Bolker, & Walker, 2015; Pinheiro J, Bates D, DebRoy S, 2017). We performed ANOVAs on our models to determine model significance. To determine specific significant relationships and their directions, we used a Tukey post-hoc test from the package "TukeyC" in R (Faria, Jelihovschi, & Allaman, 2015).

Then, to determine the relative impact of noise, stress hormones, and food intake on gut bacterial communities, we conducted a path analysis with the specific predictions that exposure to noise would increase alpha diversity, either directly, or indirectly through corticosterone and/or food intake. Path analysis is a form of structural equation modelling that is useful for comparing complex models and evaluating hypothesis that include causality (Streiner, 2005). For each order of q (${}^{q}D$) we ran the full model with no interaction terms and included models for indirect relationships (${}^{q}D$ ~ noise + cort + food intake, cort ~ noise, food intake ~ noise). In all models we included the order of treatments and bird ID nested within sex as random effects.

Last, in order to determine whether beta diversity was different between treatment groups, we used the adonis function in vegan to perform a PERMANOVA on the four measures of beta diversity mentioned above (Oksanen, 2015).

RESULTS

Some bacterial taxa were shared by a majority of birds

We found that the most common phyla among white-crowned sparrow individuals were proteobacteria, actinobacteria, firmicutes, and bacteroidota. These four phyla were the only ones present in more than 50% of samples (Table 1; Table S2). The most prevalent (found in the highest number of samples) genera of bacteria were Staphylococcus (76% of samples), Rothia (71%), Pantoea (62%), Acinetobacter (60%), and Corynebacterium (54%). These genera also had some of the highest average abundances, although the highest average abundance was less than 10% (Table S3; Figure 2).

Gut bacterial communities varied across noise exposure treatments

We found that noise exposure treatment (i.e. acclimation, noise, recovery, and control) explained variation in ^{1}D and Faith's phylogenetic diversity (ANOVA, ^{1}D F=3.2,P =0.03; faith pd F=4.4, P =0.007; Table 2, Table S4), and was close to significant for ^{2}D (ANOVA,^{2}D F= 2.7, P =0.06; Table 2, Table S4). A post-hoc comparison of alpha diversity across treatments revealed that the recovery period had the highest alpha diversity and was significantly higher than control for most measures of alpha diversity (Tukey post-hoc; Table 2; Table S4). To remind, our prediction was that alpha diversity would be highest during the noise treatment, and although alpha diversity was higher in noise than in control, it was highest during the recovery treatment (Figure 3), which is the period always immediately following noise exposure. We did not find a difference in beta diversity between treatments (PERMANOVA, Table S5).

Predicted gut bacterial function also differed between treatment groups (LDA $>$ 3; Figure 4). Consistent with alpha diversity findings, the largest difference was seen between the control and recovery periods (11 genes different), suggesting a delayed effect of noise on the gut microbiome. However, an NSTI analysis showed that many of our samples had poor representation in the reference genome (average NSTI = 0.17 $\pm$ 0.14; Table S6).

Noise exposure had direct effects on the microbiome

We predicted there would be indirect effects of noise exposure on alpha diversity via corticosterone and food intake. Because feeding behavior varied between the morning (when birds were exposed to noise) and the afternoon (when they were not), we examined this prediction considering total food intake and then morning and afternoon food intake separately. In most of our models, we did not find support for our prediction that noise exposure would have an indirect effect on alpha diversity via corticosterone and/or feeding behavior. Instead, we found evidence for direct effects of noise exposure on alpha diversity, particularly when considering total food intake or afternoon food intake (Table S7, Figure 5). In the case of afternoon food intake, noise exposure treatment had direct effects on most measures of alpha diversity, except for^{0}D (path analysis; Table S7; Figure 5). In the case of overall food intake, noise exposure treatment did have a direct effect, but only on Faith's phylogenetic diversity (Table S7, Figure 5).

When we examined the predicted effects of corticosterone and feeding behavior on alpha diversity, we did not find direct effects of corticosterone or food intake (afternoon or overall) on alpha diversity. The only model in which food intake was important was when noise exposure had an indirect effect on ^{0}D (richness) via morning food intake; however, this was not the case for any other measure of alpha diversity (Table S7, Figure 5).

Noise exposure had effects on corticosterone *and food intake*

As predicted, we found that noise exposure had direct effects on corticosterone (path analysis; Table S7, Figure 5). corticosterone levels varied across treatments, such that corticosterone levels tended to be lowest during acclimation (the first treatment period for all birds) as compared to all other treatments (ANOVA,

Tukey post-hoc, A vs R P =0.04, A vs C P =0.03; Table S8; Figure 6). Although corticosterone levels were not at their highest during noise exposure (as we predicted) they did increase with noise exposure and stayed elevated (Figure 5.6).

We also found evidence for direct effects of noise exposure on feeding behavior (path analysis; P <0.001; Table S7; Figure 5). We had predicted that if noise exposure directly affected feeding behavior, then food intake should go down during noise treatments. Consistent with this prediction, we found that food intake varied with treatment and tended to be highest during acclimation (at the start of the experiment) and lowest during the noise treatment (ANOVA, Tukey post-hoc, all acclimation comparisons P <0.001; Table S9; Figure 7). However, inconsistent with this prediction was our finding that during the noise treatment, food intake was actually higher in the morning (when the birds were exposed to noise) than in the afternoon (when they were not). However, food intake in the mornings during noise treatment was still less than morning food intake during acclimation (Table S7, Fig 6.7).

DISCUSSION

In our study we experimentally manipulated noise levels to examine potential causal relationships between noise exposure and gut microbiota, as well as potential mechanisms that might mediate this relationship, including stress hormones and food intake. We found evidence to support our prediction for a causal, positive relationship between noise and gut microbiota. We also found evidence that noise acts to increase corticosterone and decrease food intake. However, we did not find support for our prediction of an indirect effect of noise on gut microbial diversity via corticosterone and/or food intake; instead, noise appeared to act directly on the gut microbiome or, more likely, through an unmeasured variable. The timing of these effects was different as well, with the greatest effects of noise on gut microbial diversity, function and food intake being seen not during noise exposure but afterwards, in recovery periods.

These results help to explain our previous findings that urban white-crowned sparrows have higher bacterial richness than rural sparrows. In our previous work, we found that noise levels were higher in urban areas, suggesting that birds in areas of higher noise levels have higher alpha diversity (Phillips et al., 2018a). However, in follow up work, we did not find a strong correlation between territory noise levels and alpha diversity; instead, habitat and morphological traits were more important in explaining variation in gut microbial diversity (Berlow et al., 2020). The influence of these other aspects of a bird's environment could obscure the role of individual variables such as noise levels. Experimentally testing the effect of individual aspects of urbanization on the gut microbiome should lead to a better understanding of what shapes gut microbial communities, particularly as the relationship between urbanization and gut microbial diversity appears to vary across systems (Berlow et al., 2019; Littleford-Colquhoun et al., 2019; Teyssier et al., 2018). Here, our experiment isolated noise from other variables associated with the urban-rural gradient, such as diet (Teyssier et al 2020), and showed that noise alone does explain variation in gut microbiota, specifically with exposure to noise increasing alpha diversity and shifting bacterial function (however, high NSTI values indicate that accuracy of functional results are limited, and thus our interpretation is restricted). This experimental finding is consistent with our original work with wild birds (Phillips*et al* . 2018), that suggested a positive relationship between noise levels and alpha diversity. Our work highlights the importance of considering noise levels when investigating variation in gut microbial communities across urbanization gradients.

Noise exposure increased plasma corticosterone levels; and this effect had residual consequences in that corticosterone levels remained elevated even after noise playback stopped. A study on wild white-crowned sparrows found that male birds had higher baseline corticosterone levels in urban areas as compared to nearby rural areas (Bonier et al., 2007). In fact, the Bonier *et al* . (2007) study was conducted in the same locations and on same species as our own work (Berlow et al., 2020), and captive birds used for this study were also collected from locations in the same urban populations. Although the Bonier *et al* . (2007) study did not explore possible mechanisms underlying the relationship between urbanization and corticosterone levels, our results suggest that as there is higher background noise in urban areas (Derryberry et al., 2016). Thus noise may be one of the factors contributing to higher baseline corticosterone in some urban birds.

Our study also adds to general knowledge of the relationship between noise and corticosterone, with some studies showing that noise increases corticosterone levels (Blickley et al., 2012; Chloupek et al., 2009), and others showing there is no relationship (Tempel & Gutierrez, 2003) depending on the duration and intensity of the noise exposure. These studies examined a range of noise amplitudes and durations, from 10 minutes to 24hrs/day for weeks at a time. Noise levels have generally been chosen according to biological relevance in each system, for example 24/7 drilling sounds on a sage-grouse lek (Blickley et al., 2012). We chose relatively long exposure times in the morning and a noise profile to mimic traffic patterns. However, had our treatment periods been longer than five days it may have better reflected life on an urban territory, and we may have observed corticosterone levels peak during noise treatment with a return to lower baseline corticosterone during recovery. The question of how noise affects stress hormones would benefit from an in-depth examination of what duration and intensity of noise triggers a glucocorticoid response, as this would guide experimental design in studies examining down-stream effects of noise stress. What is clear is that noise can trigger a hormonal stress response, and thus is likely to be involved in the physiological consequences of urbanization.

Our finding that food intake was reduced during periods of noise playback supported our prediction that noise would affect feeding behavior. This finding is consistent with studies in other systems that find various measures of foraging behavior are impacted by noise exposure (L. Quinn, J. Whittingham, J. Butler, & Cresswell, 2006b; McClure et al., 2013; Purser & Radford, 2011; Senzaki et al., 2016). Specifically, white-crowned sparrows have been experimentally shown to decrease foraging duration during short (8 minutes) noise playbacks at amplitudes lower than our experiment (61 and 55 dbA)(Ware et al., 2015). Our alternative prediction was that noise might affect food intake indirectly through direct effects of corticosterone on feeding behavior. In that case, we would have expected a positive relationship between corticosterone levels and food intake. Our work suggests that noise exposure affects food intake most likely through effects on feeding behavior (consistent with previous work on this species) but we cannot rule out an effect of corticosterone on appetite also influencing food intake.

Counter to our predictions, we did not find support for the hypothesis that noise indirectly impacts the gut microbiome through corticosterone or food intake, and there are a couple of possible explanations for this result. Because of the apparent delayed response of corticosterone and food intake to noise exposure as seen in this study, longer treatment periods may be needed to capture the indirect effects of noise on the gut microbiome via stress hormones or feeding behavior. The delayed response we observed may be due to a delay in the physiological response of the digestive tract to elevated stress hormone levels or decrease in food intake, in which case a longer noise exposure period might have resulted in a clearer relationship between noise, stress hormones or food intake, and the gut microbiome. Alternatively, there may be a variable responsible for the observed relationship between noise exposure and gut microbial diversity which has not been measured in this experiment. For example, perhaps a hormone other than corticosterone such as catecholamines which impacts gut physiology is affected by noise (Gesi et al., 2002; Mittal et al., 2017). It is hard to imagine a direct effect of noise exposure internally on gut microbial community composition. Although diet is a relevant factor in the differences between urban and rural birds (Teyssier et al., 2020), we do not think diet drove differences observed before and after noise treatments, as birds were provided the same diet. It is possible that noise may change habitat usage or food choices in wild populations, thus affecting what surfaces a bird interacts with and therefore what bacteria are available to colonize the gut. In our study birds were confined to a small cage with homogenous surface types, therefore it is unlikely that this potential relationship between habitat use and noise level would explain the effect of noise on gut microbial diversity. However, it could be that noise exposure altered their use of materials in the environment, such as their cuttlebone or shredding of newspaper, that in turn changed microbial exposure. These are empirical questions which bear consideration in the design of future studies examining how noise may affect animal gut microbial communities.

Our study indicates that noise affects plasma corticosterone, feeding behavior, and the gut microbiome in a songbird. Our finding that noise increases corticosterone helps to clarify a complicated body of research with conflicting findings about the effect of various types of noise exposure on stress hormones. Although noise has

previously been shown to impact many aspects of foraging behavior such as time spent foraging and foraging efficiency, this study adds volume of food consumed to the myriad ways in which noise can impact feeding behavior. Finally, we found support for an impact of noise on alpha diversity of gut bacterial communities and found that after 5 days of noise exposure we were not able to determine whether corticosterone and food intake were the mechanisms underlying this relationship. In the future, research at the intersection of urban ecology and microbiology would benefit from more experimental research to complement findings in the field. This would help us better understand the contribution of specific variables on the gut microbiome, as well as what mechanisms are responsible for those relationships. Integration of functional research such as a multi-omics approach would pair well with these experiments, and provide a next step in understanding the consequences of environmental disturbance for wild animals.

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DATA ACCESSIBILITY

All files necessary for replicating our processing and analysis are available on GitHub (https://github.com/mBerlow/WCSPnoise2020) and sequences are available through the Sequence Read Archive (accession number SUB8852031).

AUTHOR CONTRIBUTIONS

MB and EPD designed the experiment, MB conducted the experiment. HW processed and analyzed blood samples for hormones, MB processed all other samples and conducted data analysis with consultation from EPD and HW. MB wrote manuscript with editing by EPD and HW.

TABLES & FIGURES

Table 1 – Prevalence and average abundance of common phyla.

Phylum	prevalence (% of samples occurring in)	average abundance
Actinobacteriota	94%	29%
Bacteroidota	57%	1%
Firmicutes	94%	27%
Proteobacteria	97%	41%

Table 2 – Mixed linear model results assessing the effect of treatment on alpha diversity. All models included treatment order, and individual bird nested within sex as random effects (+ 1|order + 1|sex/bird). P-values for all post-hoc comparisons can be found in Table S4

	lm anova	lm anova		Tukey post-hoc	Tukey post-hoc	
Alpha diversity measure	model F	*P*		significant comparisons	*P*	
^{0}D	1.9449	0.131		n/a		
^{1}D	3.2003	0.029	*	Recovery - Control	0.029	*
^{2}D	2.6617	0.056	.	Recovery - Control	0.06	.
faith's pd	4.3798	0.007	*	Recovery - Control	0.02	*
				Recovery - Noise	0.016	*

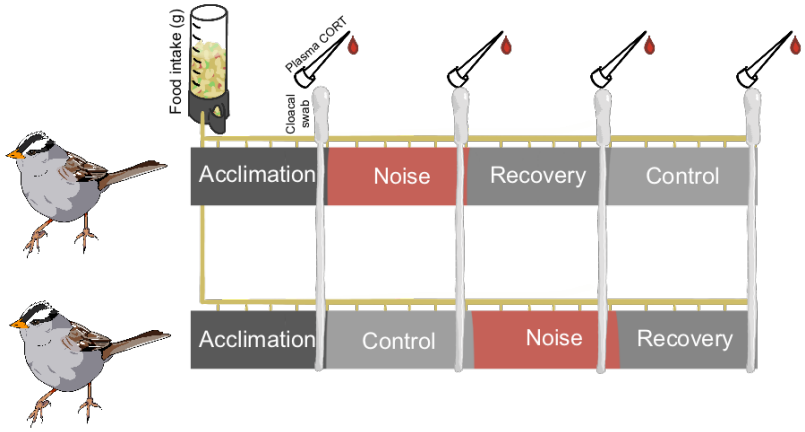


Figure 1: Experimental design diagram. Each treatment group had 13 birds, with one group receiving noise and recovery first, and the other group receiving control first. Blood for plasma cort and cloacal swabs were collected on the last (5^{th}) day of each treatment period, and food intake was recorded twice daily.

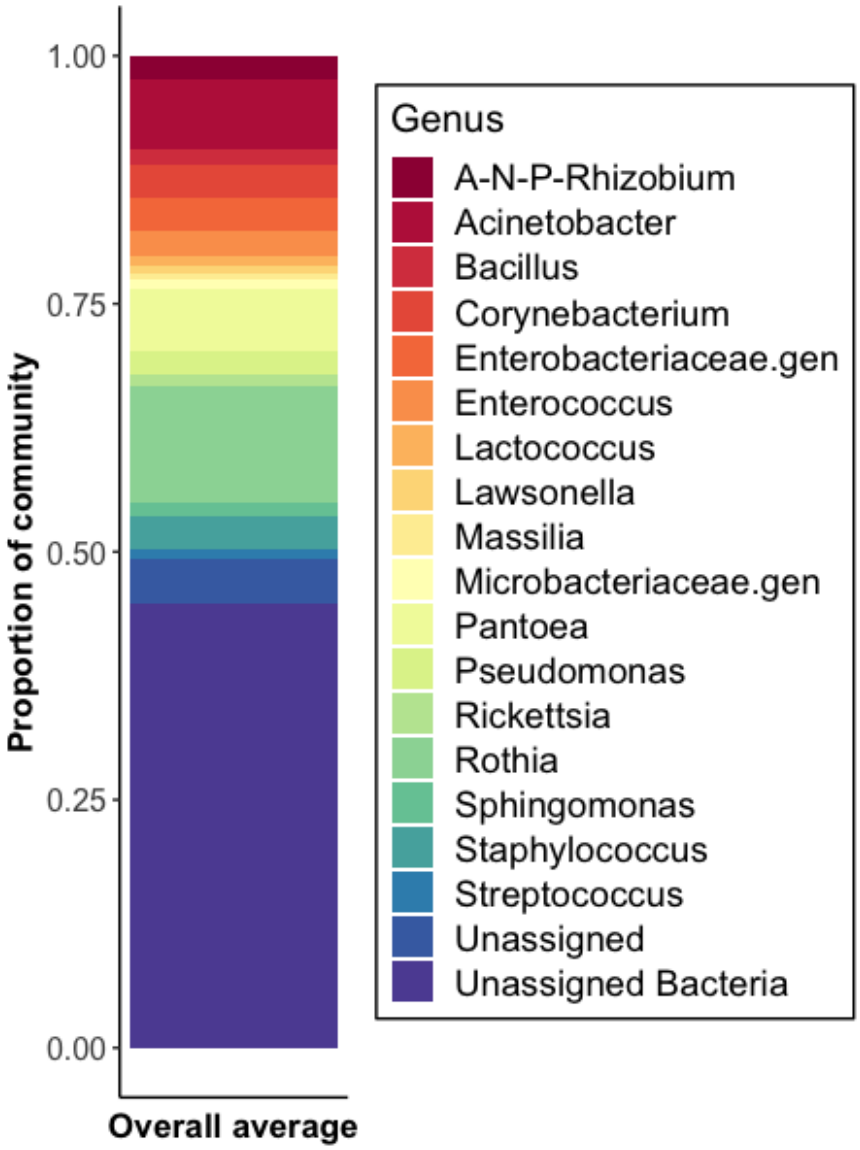


Figure 2 – Relative abundances of bacterial genera in captive white-crowned sparrows combined from samples across all treatments. Figure of relative abundances of bacterial phyla can be found in the supplemental figures (Figure S1).

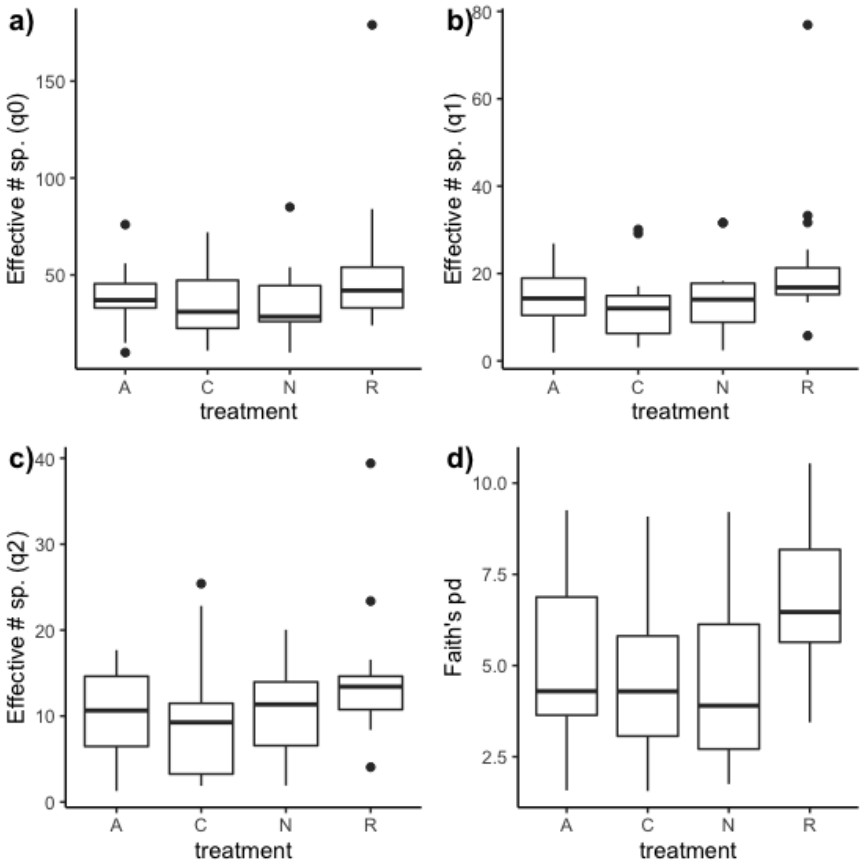
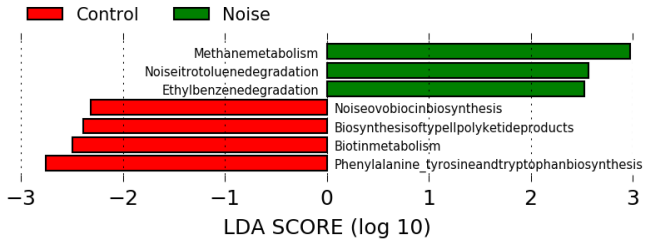


Figure 3 – Alpha diversity in response to noise treatment by order of treatment received (A=acclimation, C=control, N=noise, R=Recovery). Noise exposure has an effect on alpha diversity.



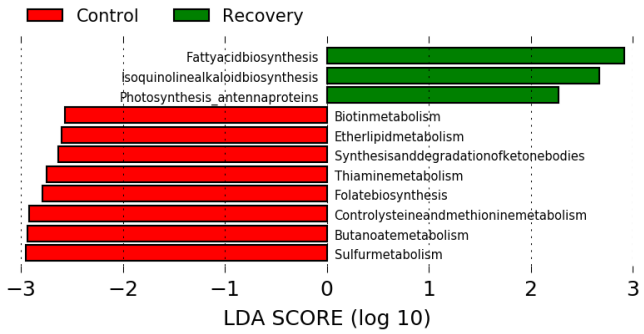


Figure 4 — Linear discriminant analysis effect size (LEfSe) comparing predicted gene abundances (PICRUSt) between control and noise treatments (top), and control and recovery treatments (bottom). Colors correspond to which treatment was found to have disproportionately more abundance of that gene. More predicted genes differed between control and recovery than between control and noise, suggesting a delayed effect of noise on the gut microbiome.

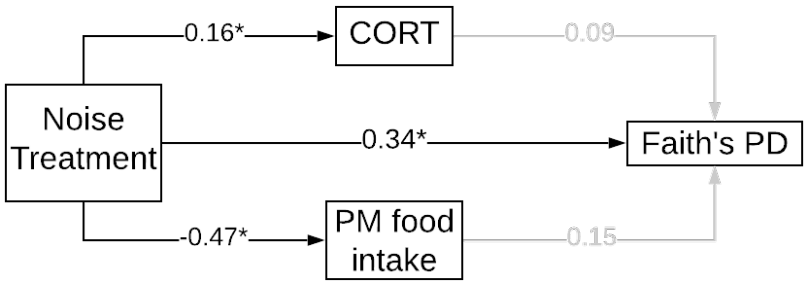


Figure 5 – Path analysis results assessing relative contributions of noise treatment, corticosterone (CORT), and food intake to Faith's phylogenetic diversity. Path analysis figures for other measures of food intake and alpha diversity can be found in the supplemental materials (Figure S2). * indicated significant relationships, gray arrows indicate non-significant relationships. Order of treatment and individual bird nested within sex were included as random effects.

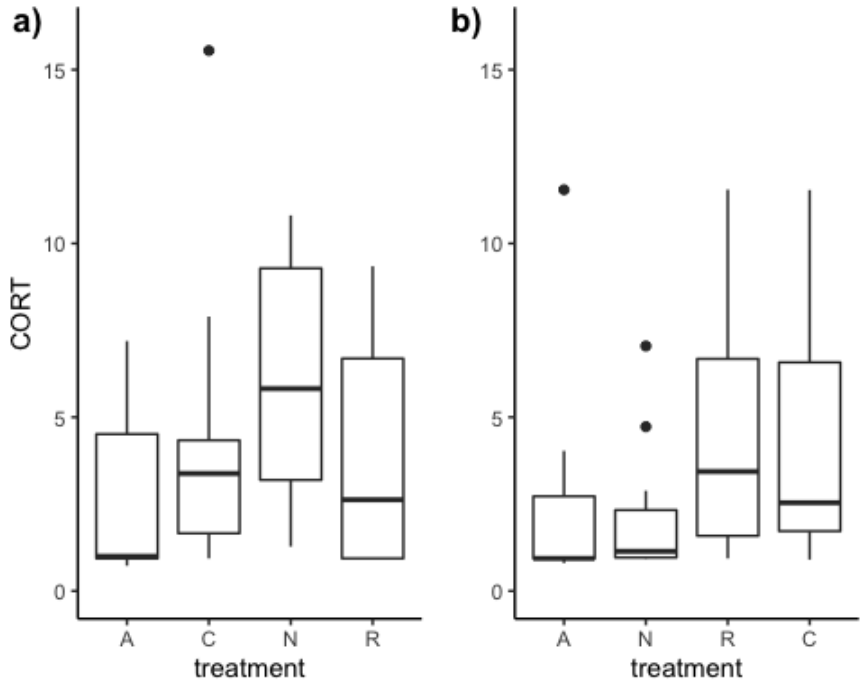


Figure 6 – CORT response to noise treatment by order of treatment received (A=acclimation, C=control, N=noise, R=Recovery). Noise exposure has an effect on corticosterone levels and does not return to normal after noise playback has stopped.

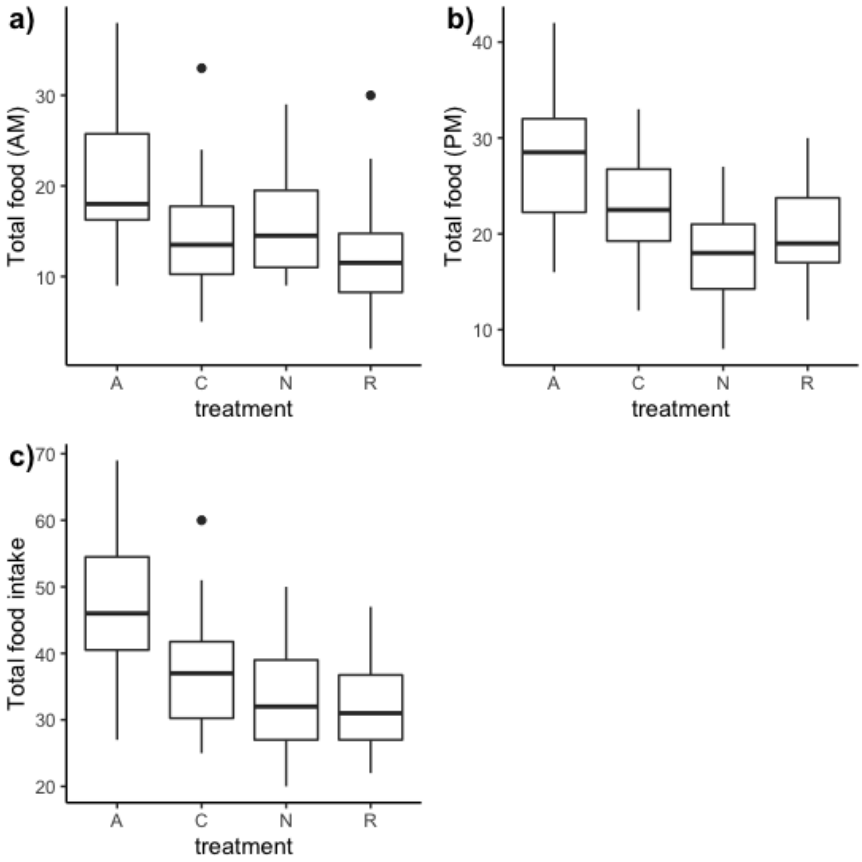


Figure 7 – Food intake for each treatment a) in the morning during noise playback for noise treatment, b) in the afternoon after noise playback for noise treatment, c) all day food intake. Black points indicate outliers.