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RESEARCH ARTICLE

Chronic stress and captivity alter the cloacal microbiome of a wild songbird

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ABSTRACT

There are complex interactions between an organism's microbiome and its response to stressors, often referred to as the 'gut–brain axis'; however, the ecological relevance of this axis in wild animals remains poorly understood. Here, we used a chronic mild stress protocol to induce stress in wild-caught house sparrows (*Passer domesticus*), and compared microbial communities among stressed animals, those recovering from stress, captive controls (unstressed) and a group not brought into captivity. We assessed changes in microbial communities and abundance of shed microbes by culturing cloacal samples on multiple media to select for aerobic and anaerobic bacteria and fungi. We complemented this with cultivation-independent 16S and ITS rRNA gene amplification and sequencing, pairing these results with host physiological and immune metrics, including body mass change, relative spleen mass and plasma corticosterone concentrations. We found significant effects of stress and captivity on the house sparrow microbiomes, with stress leading to an increased relative abundance of endotoxin-producing bacteria – a possible mechanism for the hyperinflammatory response observed in captive avians. While we found evidence that the microbiome community partially recovers after stress cessation, animals may lose key taxa, and the abundance of endotoxin-producing bacteria persists. Our results suggest an overall link between chronic stress, host immune system and the microbiome, with the loss of potentially beneficial taxa (e.g. lactic acid bacteria), and an increase in endotoxin-producing bacteria due to stress and captivity. Ultimately, consideration of the host's microbiome may be useful when evaluating the impact of stressors on individual and population health.

KEY WORDS: Microbiome, 16S rRNA gene, *Passer domesticus*, Gut–brain axis, Chronic stress, Hypothalamus–pituitary–adrenal axis

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INTRODUCTION

The microbiome, the group of microorganisms that live on or within a host, has received increased attention in recent years because of its importance in host health (Cho and Blaser, 2012). Often varying through both space and time (Caporaso et al., 2011a), the microbiome is commonly composed largely of bacteria, but also contains archaea, fungi, microeukaryotes and viruses. The microbiome affects host health by influencing development, behavior, metabolism, the inflammatory response and perhaps even cognitive function (Cho and Blaser, 2012; Slevin et al., 2020). In addition, the microbiome serves as a first line of defense against foreign microbes via interactions with the host (Lokmer and Mathias Wegner, 2015; Stothart et al., 2016) and potentially pathogenic foreign invaders (Kearns et al., 2017; Flechas et al., 2019).

Animals face stressful events across their lifespan. In vertebrates, stressors trigger activation of the hypothalamic–pituitary–adrenal (HPA) axis, a pathway responsible for the production and release of glucocorticoid hormones (Sapolsky et al., 2000; Boonstra, 2005). The gut microbiome has the potential to modulate the stress response, in particular the HPA axis, in a relationship commonly referred to as the 'gut–brain axis' or 'microbiota–gut–brain axis' (Foster and McVey Neufeld, 2013; De Palma et al., 2014; Slevin et al., 2020; Foster et al., 2017). The host's physiological stress response can also affect the microbiome (Bailey et al., 2010; Bailey et al., 2011). Via mechanisms that are not entirely resolved, chronic stress can result in greater susceptibility to infection (Glaser and Kiecolt-Glaser, 2005; Burkholder et al., 2008; Verbrugghe et al., 2012), while short bursts of acute stress may enhance immune function (Glaser and Kiecolt-Glaser, 2005; Dhabhar, 2009; Martin, 2009). Therefore, understanding the response of the microbiome to host stress can improve our overall understanding of the host's response and the role of the microbiome in host health, including resistance to potential infections and disease (Gross and Colmano, 1970; Murone et al., 2016; Stothart et al., 2016).

Although studies in humans and captive lab animals have revealed important relationships among the microbiome, the physiological stress response, and host behavior and health, the ecological relevance of the gut–brain axis in wild animals is poorly understood (Hird, 2017; Davidson et al., 2020). Compared with laboratory model species, wild animals have greater individual variation both in the magnitude of the glucocorticoid response to stressors (Kunzl and Sachser, 1999; Williams, 2008) and in the distinct microbiome taxa they harbor (Kreisinger et al., 2014), which could lead to different dynamics in the gut–brain axis. Recent work in free-living yellow-legged gull chicks (*Larus michahellis*) has revealed significant effects of exogenous glucocorticoids on the gut microbiome (Noguera et al., 2018). Specifically, corticosterone implants caused a decrease in both some potentially pathogenic and commensal microbial taxa. However, we know of no studies that

have specifically examined the effects of experimental stress – without exogenous corticosterone implants – on the microbiome of any wild avian species. Thus, at this point, we do not know whether environmental challenges that induce chronic stress might alter the microbiome of wild species in ways that could affect individual health and even population persistence.

As an additional concern, threatened and endangered wild species are also commonly brought into captivity for management purposes such as captive breeding and translocation (Griffith et al., 1989). Captivity can be a potent chronic stressor for wild species (Mason, 2010; Lattin et al., 2012), and often affects the microbiome (Xenoulis et al., 2010; McKenzie et al., 2017). These induced changes in the microbiome may arise partly because captivity modifies and standardizes factors that vary in the wild such as temperature, humidity, social interactions, housing and diet. Diet, in particular, has strong effects on the composition of the gut microbiome (Singh et al., 2017; Clayton et al., 2016; Martinez-Mota et al., 2019). Of note, and possibly related to these changes in the microbiome, captivity can also cause hyperinflammatory responses in captive animals (Martin et al., 2011), which can lead to an increase in reactive oxygen species (Mittal et al., 2013). This cascade can result in impaired cellular metabolism and cellular senescence (i.e. the negative impacts of aging) (Costantini, 2019). How chronic stress and captivity modulate the microbiome of wild animals, and the potential resilience of the microbiome following alleviation of chronic stress, remains unclear, yet is critical for effective conservation practices (Trevelline et al., 2019).

To address this knowledge gap and isolate the effect of an additional chronic stress treatment from the effect of captivity, we used cultivation-dependent and cultivation-independent methods to assess the cloacal bacterial, archaeal and fungal communities of wild-caught, captive house sparrows (*Passer domesticus*) exposed to a standardized chronic mild stress protocol. We then compared their microbiomes with those of unstressed reference (control) birds, birds recovering from chronic stress, and wild birds not brought into captivity. To further elucidate the relationships between the microbiome and host health, we further compared these microbial communities with the hosts' physiological responses to stress, including body mass change, relative spleen mass and plasma corticosterone levels.

MATERIALS AND METHODS

Animal collection

Forty-six adult house sparrows, *Passer domesticus* (Linnaeus 1758) (n=23 males, 23 females), were captured using mist nets and Potter traps at bird feeders in Arlington, Medford and Somerville, MA, USA, between 6 April and 22 May 2011 and housed in the animal facilities of Tufts University (Medford, MA, USA). Animals were maintained in an outdoor aviary until 2 weeks prior to the experiment, at which time they were brought into the lab and pair-housed (1 male and 1 female per cage), with a variety of perches available. *Ad libitum* mixed seed, grit and water were available throughout. Following the experiment, the birds were euthanized as part of a parallel study on the effects of chronic stress on corticosteroid receptor density in various tissues (Lattin and Romero, 2014). To compare the microbial communities of house sparrows that had been brought into captivity with those which had not, we collected an additional 11 house sparrows (n=5 males, 6 females) in May 2012 (matched for seasonal time of sampling with 2011 birds) from similar locations to the 2011 collections. These birds were caught in seed-baited Potter traps as part of an additional study on the effects of stress on host physiology (L.M.R., unpublished data). These samples were exclusively used for comparison with wild-caught, captive animals using cultivation-independent methods, not cultivation-dependent methods. Host physiological metrics were not obtained for wild, no-captivity hosts.

Captivity conditions and chronic stress treatment

Animals were allowed to acclimate to lab captivity conditions for 2 weeks prior to beginning the experimental chronic mild stress protocol. Although birds were randomly assigned to different treatment groups, we matched birds by initial capture date, so there were equal numbers of early caught, late caught, etc., birds in each treatment group. Twenty birds (n=10 males, 10 females) were randomly chosen and maintained as an unstressed reference (control) group in a separate room. All reference birds were housed in one room, and all stressed birds in another. The two rooms were part of the same animal facility, and were of similar size, with identical temperature, humidity, ventilation and husbandry conditions. Because birds exposed to stress were sampled at different time points throughout the experimental protocol, and because captivity itself can be a stressor, reference birds were sampled at the same times as stressed birds to control for the different periods of time held in captivity (Fig. 1). The 26 birds exposed to chronic stress (n=13 males, 13 females) were subjected to a standardized chronic mild stress protocol of five different rotating psychological stressors delivered at random times for 21 days (Rich and Romero, 2005) with a final 7 days of recovery as an additional 'recovery' group (Fig. 1). The psychological stressors used in this study (being rolled on a cart, radio, restraint, human voice/presence and cage disturbance) have all been shown individually to significantly increase plasma corticosterone in songbirds (Nephew and Romero, 2003; Rich and Romero, 2005). Chronic mild stress protocols like those used here have been used for >30 years in >1300 studies as a well-validated and reliable method to understand the effects of chronic stress on humans and laboratory animals, although their use in wildlife is much less common (Willner, 2017). More details on the effects of this protocol on glucocorticoid hormones and receptors of these host birds can be found in Lattin and Romero (2014). One bird from the recovery group was removed from the final analyses because antibiotics were administered during the course of the experiment to treat a minor abrasion (Table S1).

Each bird was sampled once during the experiment, at which time a cloacal swab and blood samples for baseline and stress-induced corticosterone were taken (Fig. 1). This was done prior to administration of mitotane, a drug that was necessary for the parallel study examining corticosteroid receptor density (Breuner and Orchinik, 2001; Lattin and Romero, 2014). To verify that the experimental treatment group experienced psychological chronic stress, body mass measurements were taken for each bird at the beginning of the experiment and at the time of cloacal swabbing (decreased body mass is one of the most robust measures of chronic stress) (Dickens and Romero, 2013). Blood was sampled from the brachial vein in heparinized microcapillary tubes within 3 min of entering the bird room (baseline samples) and after 30 min in a clean, breathable cloth bag (stress-induced samples). Blood corticosterone levels were determined using radioimmunoassay via the methods of Wingfield et al. (1992) using Esoterix antibody B3-163 (Calabasas, CA, USA). Spleen mass was measured as wet mass as in Lattin et al. (2013). Inter- and intra-assay coefficients of variation were determined by running standards in each assay. Intra-assay variation was 3.5%; inter-assay variation was 15.8%.

All procedures were performed according to the Association for Assessment and Accreditation of Laboratory Animal Care

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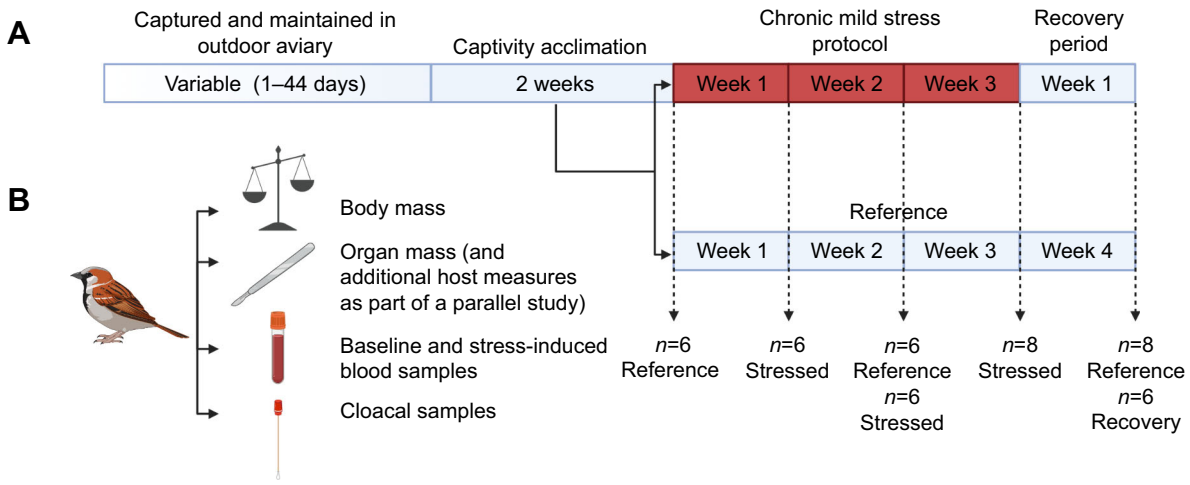

Fig. 1. Experimental design. (A) Schematic diagram of the sampling scheme used in this study. 'Reference' is wild-caught, no-treatment captive house sparrows (*Passer domesticus*). 'Stressed' is wild-caught, captive birds exposed to a chronic mild stress protocol. 'Recovery' is wild-caught, captive birds recovering from a chronic mild stress protocol. A fourth group of sparrows (n=11) that did not experience captivity were caught the following year and immediately sampled, and are not depicted in this diagram. (B) For all captive birds, at the time of sampling, each bird was weighed, and cloacal samples, and baseline and stress-induced blood samples were taken. Measurements of organ mass were made 36 h later (as well as additional measures taken as part of a parallel study; Lattin and Romero, 2014). See Table S1 for final sample sizes used in analyses. Image created with BioRender.com.

(AAALAC) guidelines and approved by the Tufts University Institutional Animal Care and Use Committee. Sparrows were collected under a Massachusetts state scientific collecting permit.

Microbial sampling

Animals experienced variable times of captivity in reference (control) and chronic mild stress treatments, as these birds were sampled on a schedule to assess the effects of chronic stress on corticosteroid receptors over time (Fig. 1) (Lattin and Romero, 2014). Cloacae were swabbed as per the methods of Stewart and Rambo (2000), as the cloacal community is generally reflective of a bird's urogenital and gastrointestinal microbial communities (Grond et al., 2018; Bodawatta et al., 2020) (Fig. S1). Briefly, a sterile 2 mm Dacron swab was inserted within the bird's cloaca and rotated for 5 s. The head of the swab was then aseptically removed and placed in an Eppendorf tube containing 1 ml of sterile thioglycolate transport medium (to protect anaerobic bacteria) and stored on ice until plating (<4 h). Birds that were captured the following year and which experienced no captivity had their cloacae swabbed immediately following capture using the same procedure as for the previous year's collection, except the swab heads were cut directly into the DNA extraction tubes. These non-captive host samples were only used for microbial community comparisons using cultivation-independent methods.

Microbial abundance

To determine the effect of chronic stress on the abundance of viable microorganisms shed from the cloaca – a proxy for species abundance within the microbiome – we plated diluted cloacal samples on various microbiological media. Unlike the cultivation-independent methods we employed later, this method provides an understanding of whether or not the cloacal microorganisms are alive, as well as an estimate of microbial cell abundance in the cloacal sample. This method further allowed us to directly compare our results with the relatively few microbial studies that have been conducted on the viable microbial community of wild house sparrows (e.g. Stewart and Rambo, 2000). Cloacal swab samples of captive birds (which included reference, stressed and recovery groups, N=45) were vortexed in thioglycolate transport broth with an initial volume of 1 ml of broth per swab (see Fig. S1 for a full schematic diagram of sampling and Table S1 for final sample sizes). These swab samples were diluted in the same transport broth and plated at 50 µl per plate on multiple microbiological media to select for various constituents of the microbial community as per the modified methods of Stewart and Rambo (2000) and Cordero et al. (2010). The minimum detection level of microbial abundance was calculated at approximately 20 colony forming units (CFUs, a proxy for viable microorganism cells) per bird sample. Specific media were used to detect aerobic bacteria [BBL Trypticase Soy Agar (TSA), Becton Dickinson, Sparks, MD, USA], enteric bacteria (Difco MacConkey Agar, Becton Dickinson), fastidious anaerobic microorganisms (Sheep's Blood Agar, Carolina Biological, Burlington, NC, USA), fungi [BBL Potato Dextrose Agar (PDA) Becton Dickinson] and fungal pathogens (BBL Mycosel™ Agar, Becton Dickinson). All plates were incubated at 37°C for the manufacturer's recommended amount of time: 48 h for fungi (PDA) and fungal pathogens (Mycosel), 24 h for bacteria (TSA), fastidious anaerobic microorganisms (Sheep's Blood Agar), and enteric bacteria (MacConkey Agar). The plates were incubated aerobically, except for the Sheep's Blood Agar plates, which were incubated anaerobically (using BBL GasPaks in GasPak jars, Becton Dickinson). Incubation conditions were consistent with those used by Stewart and Rambo (2000) and Lombardo et al. (1996) for *P. domesticus* cloacal samples.

Control swabs that had not come into contact with birds were run in parallel for all parts of the assay on all media, and for each day of sampling. Samples were plated in duplicate at 1:1, 1:10 and 1:100 dilution on all media, except Sheep's Blood Agar, for which samples were plated singly at 1:10 and 1:100 dilution because of biomass limitations. The remainder of each cloacal swab sample and medium (~100 µl, or 1/10 the original sample) was stored at −80°C for cultivation-independent assessment of diversity.

Identity of isolated putative fungal pathogens

Five birds had cloacal samples that resulted in growth on the Mycosel™ Agar. This medium is used to select for fungal

pathogens, some of which can present public health concerns and which are also detected occasionally in house sparrows (Dulisz et al., 2021). To confirm that these isolates were indeed putative pathogens, one colony originating from each bird was selected for species identification via the methods of Madden et al. (2017). Briefly, DNA was extracted from each isolate using the MoBio PowerSoil DNA isolation kit (Carlsbad, CA, USA) as per the manufacturer's instructions. A fragment of the ITS1-5.8S-ITS2 rRNA gene was amplified using the universal fungal primers Pn3 and Pn34, following the amplification protocol of Viaud et al. (2000). Each 50 µl PCR cocktail contained 10 µl of GoTaq reaction buffer (Promega Corporation, Madison, WI, USA), 0.5 µl of GoTaq polymerase (Promega Corporation), 3 µl of 25 mmol l^{-1} $MgCl_2$, 2 µl of 10 mmol l^{-1} dNTPs (Promega Corporation), 0.5 µl of 20 µmol l^{-1} of each primer, 5 µl of template DNA and water. Successfully amplified fragments were sequenced via Sanger sequencing by MacrogenUSA (Rockland, MD, USA) using the amplification primers. Following sequencing, contiguous sequences were trimmed and constructed using DNA Baser software (Heracle BioSoft, Mioveni, Romania). Resulting sequences were compared with those in the GenBank database via BLAST (Altschul et al., 1990) to determine putative species identity.

Community diversity via 16S and ITS rRNA gene sequencing

Cloacal swab DNA from birds that did and did not experience captivity was extracted, amplified and sequenced using previously published methods for bacteria, archaea and fungi (Barberán et al., 2014; Madden et al., 2017). Briefly, the remainder of the transport medium and swab was used to extract DNA from samples of birds that experienced captivity, while the entire swab sample was used for DNA extraction from birds that had not experienced captivity (see Fig. S1 for schematic diagram). Extracted DNA samples were amplified in triplicate using 5 PRIME Hot Master Mix (5 PRIME, Gaithersburg, MD, USA). For bacterial community assessment, the 16S rRNA gene primers 515f and 805r were used, with attached Illumina adapters and 12 bp error-correcting barcodes (Caporaso et al., 2011b; Caporaso et al., 2012). PCR cycling parameters were 94°C for 5 min, followed by 40 cycles of 94°C for 45 s, 50°C for 30 s, and 72°C for 90 s, with a final extension at 72°C for 10 min. For fungal diversity measures, the ITS gene primers ITS1-F and ITS2 were used, with attached Illumina adapters and 12 bp error-correcting barcodes (McGuire et al., 2013). PCR cycling parameters were 94°C for 5 min, followed by 40 cycles of 94°C for 45 s, 50°C for 30 s, and 72°C for 90 s, with a final extension at 72°C for 10 min. No-template controls and DNA extraction 'blanks' were amplified alongside all samples to check for contamination; because these samples failed to amplify, they were not sequenced. While this does not preclude low-level sequence contamination, samples from two birds failed to have more than a few hundred quality sequences following our processing steps (while three were ultimately dropped from analysis as a result of low sequencing depths), suggesting the assigned sequences used in these analyses are not the result of contaminants.

Successful amplicons were cleaned using the MoBio Ultra-Clean™ PCR clean-up kit (see Table S1 for samples that successfully amplified and were used in later analyses). We assessed DNA concentration via fluorescence using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA). DNA concentrations were normalized across samples and pooled separately for the 16S and ITS rRNA gene amplicons. Sequencing was performed on an Illumina MiSeq using 300-cycle Paired End kits at the University of Colorado BioFrontiers Institute Next-Gen Sequencing Core Facility.

Bioinformatic and statistical analyses

Raw 16S rRNA gene sequences (n=4.3 million) were processed and quality filtered using the UPARSE pipeline (Edgar, 2013) and clustered into operational taxonomic units (OTUs) following previously outlined protocols (Rideout et al., 2014). Quality filtered reads (n=3.7 million) were first clustered into OTUs against the SILVA database (v.1.2.3) (Quast et al., 2013) at 97% identity, and reads that did not hit the SILVA database were clustered in *de novo* mode. Taxonomy was assigned to each OTU with the Ribosomal Database Project (RDP) classifier (Cole et al., 2014) using the SILVA database (Quast et al., 2013) as a reference. We first filtered out OTUs that were unassigned at the domain and phylum levels, and then rarefied samples to 4608 sequences per sample to control for uneven sequencing depths. Three samples were dropped as a result of read counts that were below this threshold. Our final sample N for 16S rRNA gene analyses was therefore 48 (see Table S1 for details).

All downstream analyses were conducted in R (http://www.R-project.org/). Comparisons among treatment groups always included pooled samples of hosts across sampling time points. Beta diversity was calculated using Bray–Curtis dissimilarities with the vegan package (https://CRAN.R-project.org/package=vegan), and visualized with a principal coordinate analysis (PCoA). We assessed significant differences in community composition between categories with a non-parametric PERMANOVA (adonis) with 10,000 permutations (Anderson, 2001). We next compared Bray–Curtis similarity values between categories and assessed significance with Welch's t-tests after applying Benjamini–Hochberg corrections. To further examine the association between the host's physiological response and microbial community structure and diversity, we calculated Spearman's correlations between the first PCoA axis values (a proxy for community composition) and different host traits (days in captivity, defined as the total number of days the birds spent in the lab and aviary settings, baseline and stress-induced corticosterone, spleen mass standardized by body mass, and fraction mass change).

We calculated Shannon Diversity and Pielou's Evenness and assessed significant differences with Welch's t-tests using Benjamini–Hochberg corrections for multiple comparisons. To identify the OTUs most important for distinguishing the different groups of birds (wild, stressed, recovery, reference), we used a random forest model from the randomForest R package (Liaw and Wiener, 2002), using 10,000 trees on an OTU table filtered of low abundance (<0.1%) taxa. We confirmed the results by examining the out-of-bag error rate and leave-one-out cross-validations with 1000 permutations in the caret R package (https://CRAN.R-project.org/package=caret). Finally, we calculated significant differences in the abundance of culturable microorganisms with Welch's t-tests after applying Benjamini–Hochberg corrections.

To determine the effect of chronic stress on the relative abundance of lipopolysaccharide (LPS)-producing taxa – pro-inflammatory taxa capable of producing the endotoxins that are elevated in captive birds (Martin et al., 2011) – we manually annotated the list of bacterial taxa identified based on putative LPS production capability as per Sutcliffe (2010) (with additional information from Antunes et al., 2016; Boedeker et al., 2017; Hu et al., 2014; Salguero et al., 2019; Shimizu, 2016; Waite et al., 2017; see Supplementary Materials and Methods for the fully annotated list of taxa). The mean relative sequence abundance of probable LPS

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producers were compared among treatment groups using pairwise Welch's *t*-tests, with Benjamini–Hochberg corrections.

Raw ITS rRNA gene sequences (n=1.2 million) were processed similarly to the 16S rRNA gene reads. Low-quality reads (n=705,926) were first removed based on poor quality scores using the UPARSE pipeline (Edgar, 2013). ITS rRNA gene reads were checked for ribosomal insertions with ITSx (Bengtsson-Palme et al., 2013) and any ribosomal insertions were removed. Reads were next clustered at 97% sequence identity against the UNITE database (Abarenkov et al., 2010) and any reads that did not hit the database were clustered in *de novo* mode. Because so few samples amplified successfully (n=3, average sequencing depth per sample 27,543) and all were from birds that experienced no captivity (Table S1), we did not analyze these communities beyond noting the taxa present.

RESULTS

Microbial abundance and diversity

Microbial abundance was determined by dilution plating on non-selective media (TSA and PDA) and selective media (MacConkey and Mycosel™), and an enriched medium incubated under selective conditions (Sheep's Blood Agar incubated anaerobically). This classical method allows for an estimate of viable microbes within a sample, even though the interpretation of these results is limited by the potential differences in biomass collected across birds. By using different media, we also gained some insight into the composition of the structure of the viable community (e.g. detection of specific pathogenic organisms), while the cultivation-independent methods we used provided a more complete picture of the total community regardless of total abundance, viability or 'culturability'. The dilution plating revealed a highly variable microbial abundance among birds with no significant effect of the stress treatment detected (Fig. S2). Samples incubated anaerobically on Sheep's Blood Agar (Fig. S2) had the highest counts of CFUs. This variation among birds, as well as the total viable abundance observed in each cloacal sample across media, is in alignment with past research conducted on wild, non-captive house sparrow cloacae using similar methods (Stewart and Rambo, 2000). This suggests our sampled population is representative of the greater wild house sparrow population. Across captive treatment groups and sex, there were five birds with cloacal isolates that grew on the medium that selects for fungal pathogens (Mycosel™) (Table S2). These isolates were confirmed to be the fungal pathogen *Candida albicans* based on sequence comparisons (Table S2).

Using cultivation-independent methods, we recovered a total of 813 bacterial OTUs and 129 fungal OTUs across all birds. Analysis of bacterial Shannon Diversity (Fig. S3A) and community evenness (Fig. S3B) revealed a significant difference in diversity between the reference and stressed groups (P<0.01) and reference versus recovery birds (P≤0.05). No other significant differences were detected for richness or evenness in the pairwise comparisons between wild-caught captive and wild birds or between wild, stressed, recovery or reference captive birds.

Microbial community composition

We detected significant effects of captivity on the cloacal microbiome (Fig. 2A; PERMANOVA, P≤0.001, R^2=0.30). In addition, exposure to a standardized chronic mild stress protocol significantly altered cloacal bacterial community composition in captive stressed birds relative to captive reference birds (P≤0.001, R^2=0.26). Furthermore, birds recovering from chronic mild stress displayed significantly different bacterial communities as compared with the reference birds (P≤0.001, R^2=0.14). The altered bacterial community of the birds that experienced chronic stress was not associated with a significant reduction in body mass in the treatment group, but was associated with a significant decrease in blood stress-induced corticosterone levels (ANOVA, P=0.02; final N=31 because one host was not measured for stress-induced corticosterone levels). This significant difference in plasma hormone levels between treatment groups confirmed the hosts' dampened response to stressors and thus the general effectiveness of the mild chronic stress protocol (Lattin et al., 2012) (see Lattin and Romero, 2014, for additional evidence in support of the effectiveness of this treatment on these hosts).

We performed pairwise comparisons of Bray–Curtis similarity values among bird microbial communities (Fig. 2B), which compared the community composition within a sample group (e.g. reference versus reference birds) or between groups (e.g. reference versus stressed birds). This analysis revealed significant differences in Bray–Curtis similarity values between reference: stressed and all other group comparisons, supporting the non-parametric PERMANOVA results. Furthermore, pairwise comparisons between the groups of recovery and reference birds were significantly higher than between recovery and stressed groups, suggesting that following the end of a period of chronic stress, cloacal microbial communities may partially recover to reference levels; however, after 1 week, they still resembled the stressed microbial community more than the reference community.

We further investigated the role of the host physiological response in structuring cloacal bacterial composition and diversity by comparing Shannon Diversity and the PCoA first axis values (a proxy for community composition) with host physiological metrics (e.g. change in body mass, relative spleen mass, and baseline and stress-induced blood corticosterone concentrations). There were no significant correlations between host traits and bacterial diversity. However, we found a significant, if moderate, correlation between bacterial community composition (including hosts from across the three captivity treatment groups: reference, stressed and recovery) and stress-induced corticosterone levels (Rho=−0.42, P≤0.05) (Fig. 3). No other host physiological metrics (including days in captivity, relative spleen mass, fractional body mass change and baseline corticosterone plasma levels) were significantly correlated with bacterial community composition across these treatment groups.

Microbial taxonomic composition: random forest analysis

Bacterial communities from wild-caught, captive hosts (including reference, stressed and recovery treatments) were composed largely of taxa within the phyla Firmicutes (mean relative abundance 53%) and Proteobacteria (20%) (Fig. 4), as assessed via cultivation-independent amplicon sequencing. At the family level, reference birds were dominated by Enterococcaceae (64%) and Lactobacillaceae (20%), which were only present in appreciable abundance in recovery birds (28% and 9%, respectively, in comparison to 3% and 1% in chronically stressed birds) (Fig. 4). The bacterial communities of birds that experienced the chronic mild stress treatment were dominated by taxa in the families Helicobacteraceae, Enterobacteriaceae and Burkholderiaceae, which had the highest relative abundance (29%, 20% and 11%, respectively). Finally, samples from birds that had not experienced captivity were largely composed of taxa from the Campylobacteraceae family (69%) with a small contribution from Enterococcaceae (6%) and Lactobacillaceae (4%). ITS rRNA gene sequences were only detected in three hosts, all

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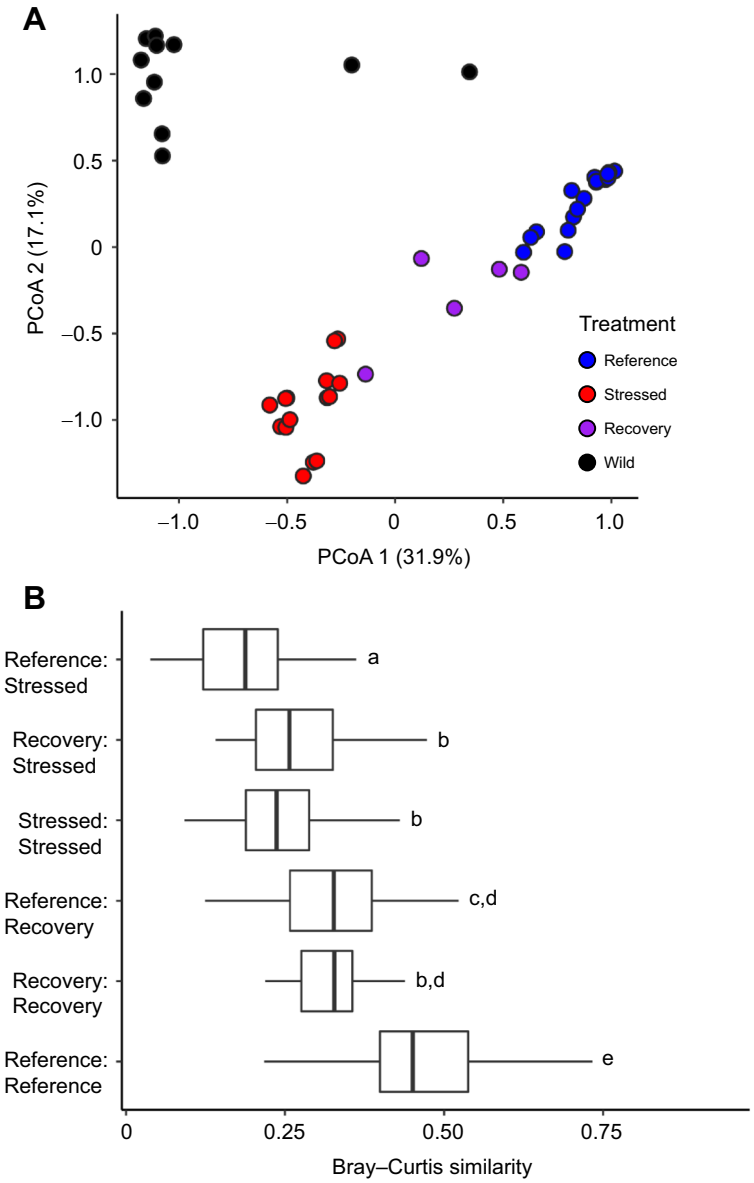

Fig. 2. Microbial community composition. (A) Principal coordinates analysis (PCoA) based on pairwise Bray–Curtis dissimilarity values of bacterial community structure in house sparrow cloacae. (B) Boxplots (median, upper and lower quartiles and 1.5× the interquartile range) showing the Bray–Curtis similarities of individual hosts within and across categories. Different lowercase letters indicate categories that significantly vary as determined by Welch's *t*-tests with Benjamini–Hochberg corrected *P*-values. Comparisons such as 'Reference: Reference' refer to the similarity values between birds within the same treatment group, while comparisons such as 'Reference:Recovery' refer to the similarity values between birds across groups. See Fig. 1 and Table S1 legends for descriptions and sample sizes of different groups. Note that higher Bray–Curtis similarity values indicate that the composition of the bacterial communities is more similar between any two categories of samples.

of which were wild birds. The sequences were primarily from the phylum Ascomycota (~95%) from the families Mycosphaerellaceae and Pleosporaceae.

To better understand the taxonomic drivers of bacterial community differences across treatment groups, we constructed a random forest model (Fig. 5) based on the four community types (reference, stressed, recovery and wild birds) with a 25% out-of-bag error rate and a Cohen's kappa statistic of 0.64 ($P \leq 0.0001$), which confirms that the observed accuracy is significantly higher than expected due to random chance. The top 20 most discriminating OTUs accounted for ~62% of all sequences and included taxa primarily from Firmicutes and the Gammaproteobacteria groups (Table S3). Reference birds were distinguished by bacteria from the genera *Streptococcus*, *Lactobacillus* and *Catellicoccus*. Stressed and recovery birds were distinguished by taxa from the genera *Acinetobacter*, *Pseudomonas* and *Massilia*. Finally, wild birds were most distinguished by taxa from the genus *Campylobacter*.

Certain bacterial taxa are more likely than others to produce the immunogenic endotoxin LPS, which has been found to be elevated in captive house sparrows (Martin et al., 2011). We therefore investigated whether the abundance of these pro-inflammatory taxa increased in response to chronic stress. The relative abundance of sequences from probable LPS-producing taxa was significantly higher in stressed and recovery birds than in reference (captive control) birds (Fig. 6) (Welch's *t*-test, Benjamini–Hochberg corrected $P<0.01$). Birds experiencing the chronic stress treatment had a more than 15-fold increase in the percentage relative abundance of sequences from probable LPS-producing taxa in comparison to captive reference hosts. These increases were driven by a reduction in LPS-negative taxa such as lactic acid bacteria, and a converse increase in members of the families Enterobacteriaceae, Pseudomonadaceae and Helicobacteraceae. Recovery birds, too, displayed significantly elevated levels of probable LPS-producing bacteria with a 7-fold increase over reference birds ($P<0.05$). Interestingly, wild birds, which experienced no captivity, also had elevated levels of LPS-producing bacteria, but this was likely due to the high levels of *Campylobacter* found within this sampled population (Figs 4, 5 and 6). The results of this analysis for the wild treatment group should therefore be interpreted with caution.

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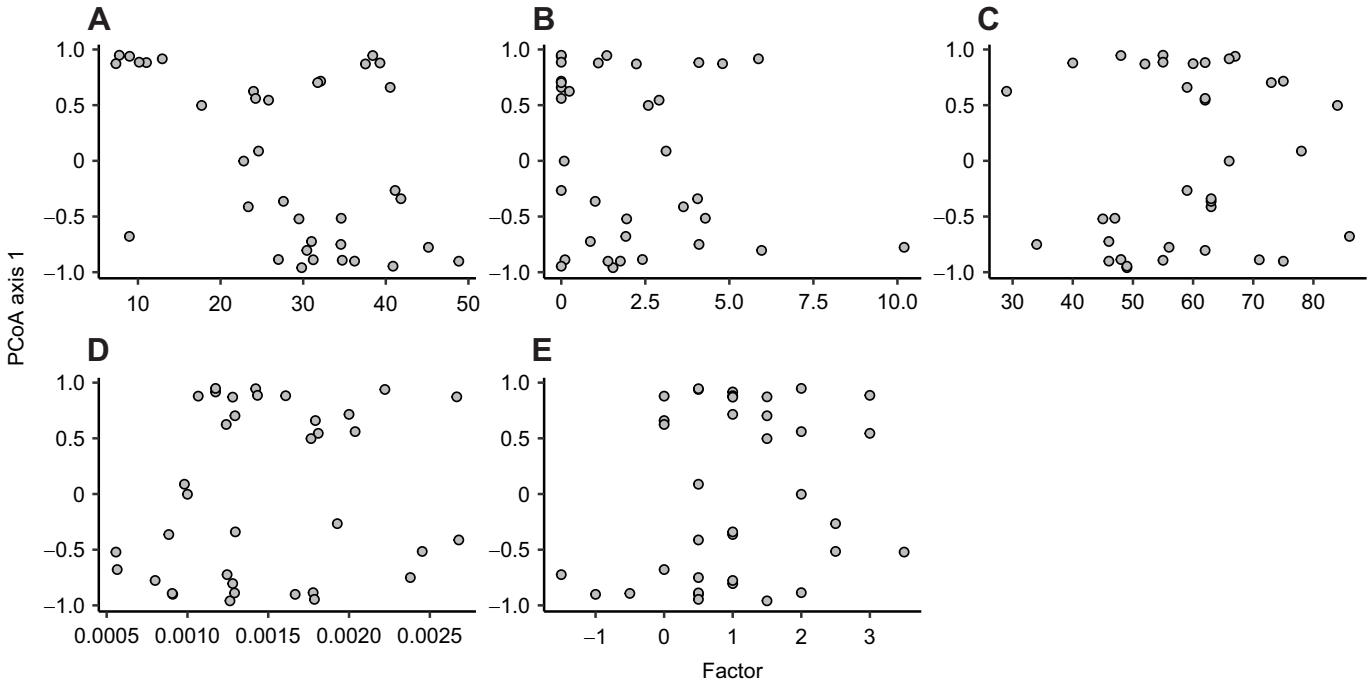

Fig. 3. Correlation between bacterial community composition and different host factors in wild-caught captive house sparrows. Scatterplots of Spearman's correlations between PCoA axis 1 values, a proxy for bacterial community composition as determined by 16S rRNA gene sequencing, and (A) stress-induced corticosterone, (B) baseline corticosterone, (C) total number of days in captivity, (D) relative spleen mass and (E) body mass change in wild house sparrows brought into captivity. Stress-induced corticosterone (A) was significantly correlated to bacterial community composition (Rho=−0.42, P=0.01, sample N=36). No other factors were correlated.

DISCUSSION

The relationship between an animal's physiology, stress response and microbiome – the gut–brain axis – remains poorly understood in wild species, despite having important ramifications for animal health and population management. Here, we investigated the independent effects of chronic stress and captivity on the

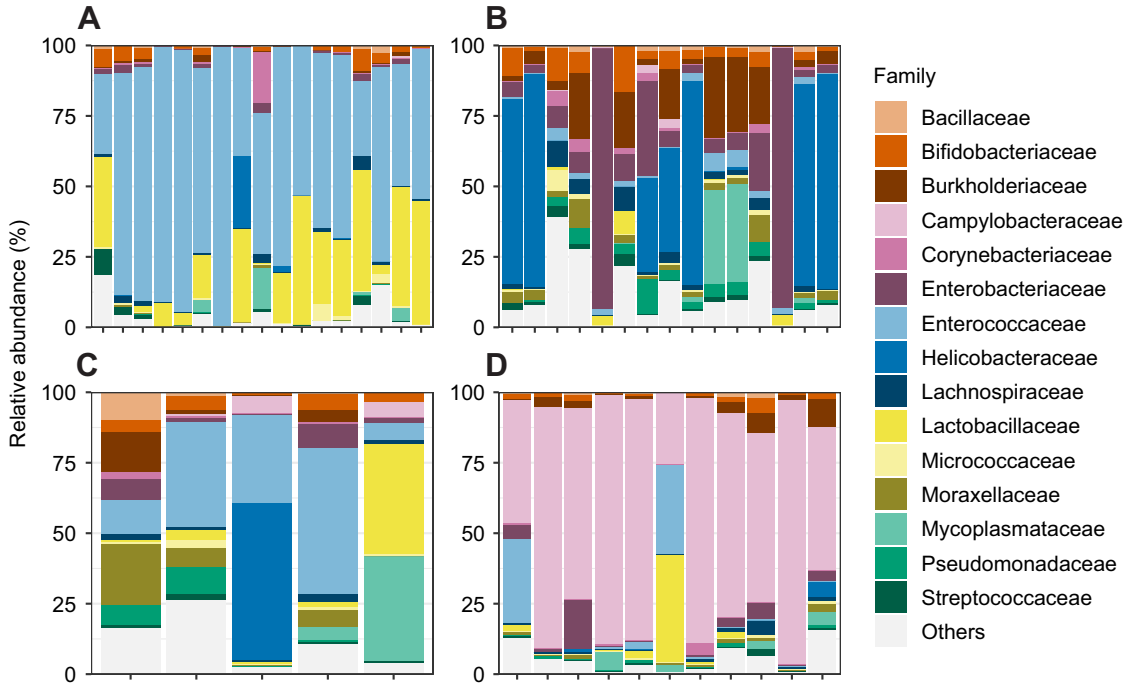

Fig. 4. Microbial community taxonomic composition. Stacked bar plots of the top 15 most abundant bacterial families in house sparrow cloacae as determined by 16S rRNA gene amplification and sequencing. The category 'Others' includes all families not in the top 15 and is colored in light gray. Individuals were exposed to a chronic mild stress protocol (B; wild-caught, stressed, n=15), recovering from chronic mild stress exposure (C; wild-caught, recovery, n=5), captive but not stressed (A; wild-caught, reference, n=17), or caught and immediately sampled without captivity (D; wild, n=11).

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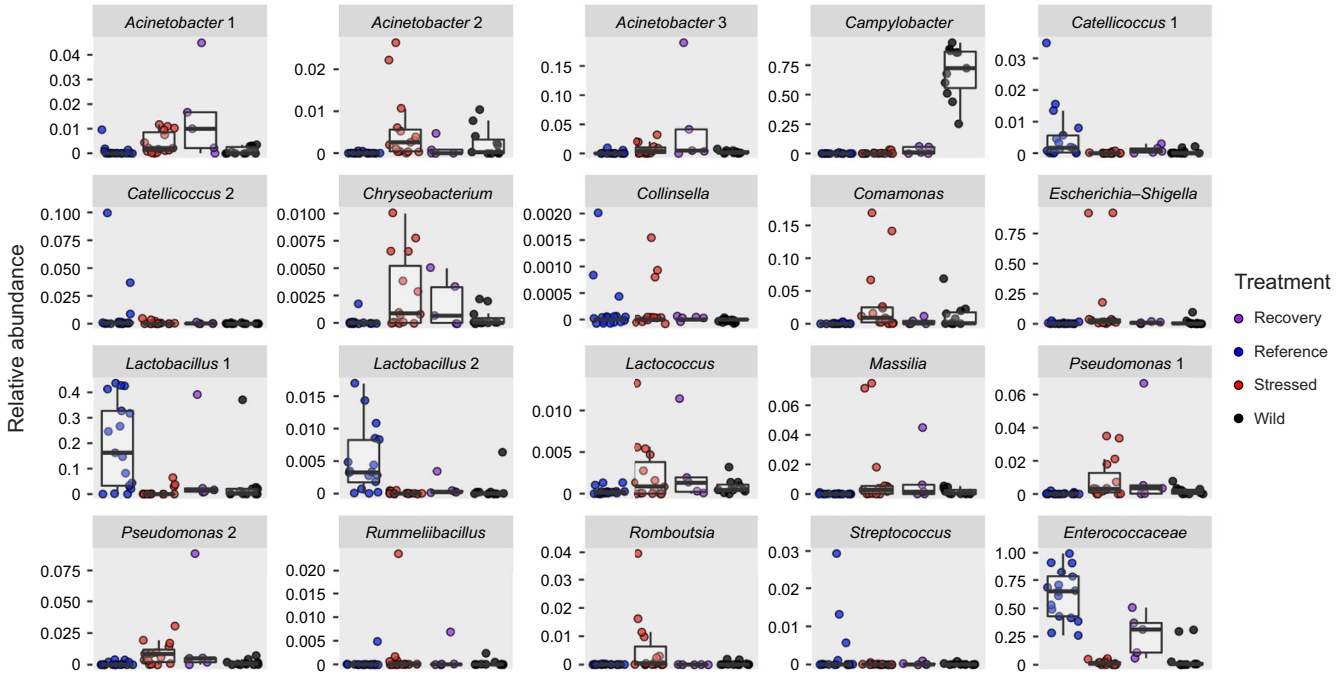

Fig. 5. Relative abundance of key bacterial taxa. Box plots of the relative abundance of the top 20 operational taxonomic units (OTUs) in house sparrow cloacae that were most important for discriminating between categories as determined by the random forest model, plotted by treatment. For each OTU-level plot (labeled with the most resolved taxonomic assignment of that OTU), points are colored according to treatment. See Fig. 1 and Table S1 legends for descriptions and sample sizes of different groups. See Table S3 for the corresponding OTU IDs.

microbiome of a wild songbird. Overall, our data revealed significant effects of a standardized chronic mild stress protocol on the cloacal microbiome of wild-caught, captive house sparrows (Fig. 7). This is consistent with these repeated mild stressors introducing a form of ecological disturbance in the microbiome that resulted in a modified bacterial community (Zaneveld et al., 2017; Karl et al., 2018). We found that changes in bacterial community composition due to stress were significantly correlated with restraint-induced corticosterone levels (regardless of whether the individual had been exposed to the chronic mild stress protocol), suggesting changes in host physiology are associated with corresponding changes in bacterial communities. Our work builds upon previous studies showing a strong connection between the gut microbiota and the HPA axis (Grenham et al., 2011; Stothart et al., 2016, 2019; Vodička et al., 2018; Webster et al., 2020; Xu et al., 2020), and suggests that the gut–brain axis has similar dynamics in wild sparrows to those in humans and laboratory models. Interestingly, our results indicate a partial resilience of cloacal microbial communities, where they can potentially recover to near-reference levels following the alleviation of chronic stress; however, an important caveat to this is that our work only sampled 5 recovering individuals, so more research is needed to understand how host-associated microbial communities recover (Reid et al., 2011) as well as the potentially beneficial and immunogenic traits associated with different microbial taxa of these communities (Mackos et al., 2017; Kearns and Shade, 2018).

Captivity presents a number of shifts in a host's behavior (e.g. changes in contact with conspecifics), environment (e.g. new inoculation sources and diet) and physiology (e.g. chronic or acute stress) that can each impact the microbiome (van Veelen et al., 2020; Singh et al., 2017; Clayton et al., 2016; Martinez-Mota et al., 2019; Bailey et al., 2010, 2011). Therefore, it was critical in this study of the microbiome to isolate the effects of chronic stress from those of captivity by comparing wild-caught reference birds with wild-caught birds experiencing a standardized chronic stress protocol. When controlling for captivity, a number of dominant bacterial taxa in reference birds dramatically decreased in relative abundance in response to chronic stress. These taxa included those assigned to the families Lactobacillaceae and Enterococcaceae. These bacterial taxa, which collectively belong to the lactic acid bacteria functional group, have shown probiotic capacity in mammals (Rinkinen et al., 2003; Ljungh and Wadström, 2006) and birds (Kurzak et al., 1998; Aliakbarpour et al., 2012). The mechanism proposed for this probiotic effect is often ascribed to the capacity of lactic acid bacteria to directly promote host health as well as indirectly promoting health by inhibiting the growth of potentially pathogenic microorganisms (Douillard and de Vos, 2014; Gómez et al., 2016; Jeong et al., 2016; Taroub et al., 2018). While it is unclear what role lactic acid bacteria play in the avian cloaca, their relative decrease in abundance in the microbiomes of stressed birds and their continued relative decreased abundance in recovery birds may suggest the loss of beneficial functions provided by the gut and associated microbial community.

Chronic stress can cause immunosuppression in vertebrate animals (Spencer et al., 2001), and one component of the immune system that has come under scrutiny in both captive and chronically stressed animals is the inflammatory response. Captivity induces a hyper-inflammatory response in house sparrows, where captive birds express greater levels of pro-inflammatory cytokines (Martin et al., 2011). This hyper-inflammatory response can have negative effects on host physiology and fitness (Costantini, 2019). While the mechanism for this hyper-inflammatory response remains unknown, in captive birds it has been correlated with higher levels of circulating pro-inflammatory endotoxins (Martin et al., 2011). Endotoxins, such as LPS, are pathogen-associated molecular patterns (PAMPs), which induce a pro-inflammatory immune response in

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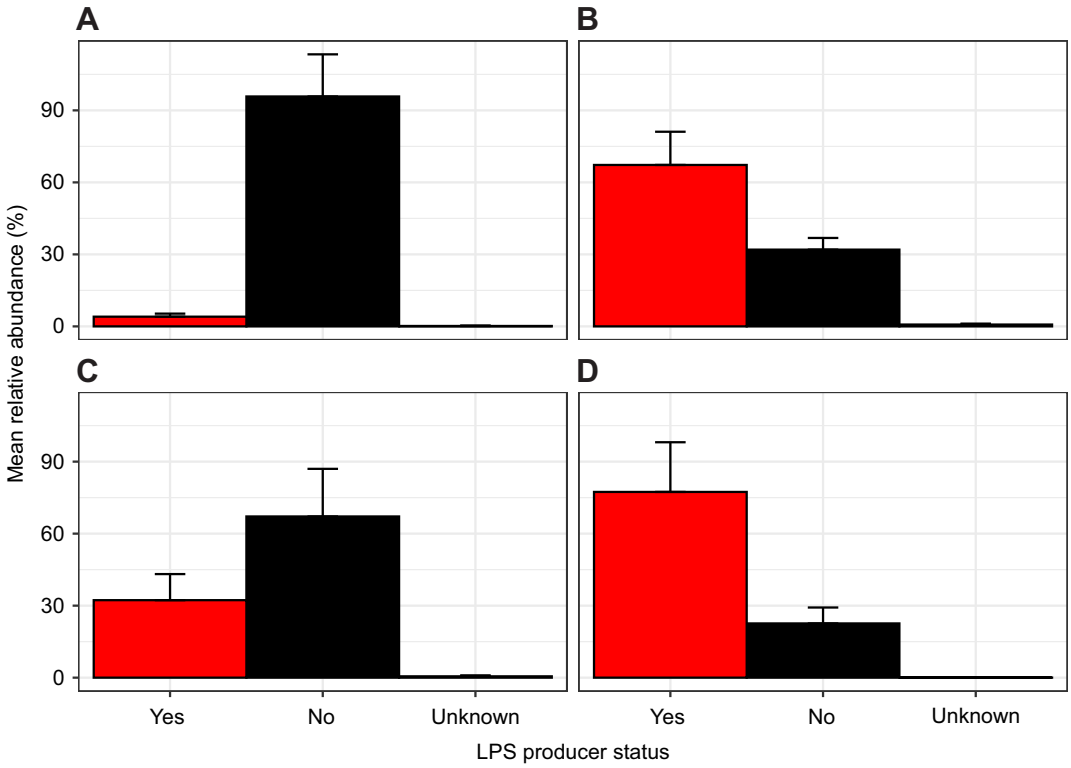

Fig. 6. Relative abundance of sequences from putative lipopolysaccharide (LPS)-producing bacterial taxa. Mean (±s.e.m.) relative abundance in house sparrow cloacae across treatments as determined by 16S rRNA gene sequencing. For each of the four treatment categories (A, reference; B, stressed; C, recovery; D, wild), <1% of taxa could not be assigned to a known LPS status; those taxa are represented as 'Unknown' (see Supplementary Materials and Methods for more details on assignments). LPS-producing (endotoxin-producing) taxa were more abundant in wild-caught hosts experiencing (B), or recovering from (C), chronic stress than in wild-caught control ('reference'; A) hosts. The high relative abundance of LPS-producing bacteria in the wild, no-captivity hosts (D) likely reflects a *Campylobacter* infection in this population (*Campylobacter* is an LPS-producing bacterial taxon). See Fig. 1 and Table S1 legends for sample sizes.

animal hosts. An increase in circulating endotoxin levels and the subsequent hyper-inflammatory response in captive birds could result from stress-induced changes to the gut integrity and the resultant increased translocation of LPS from gram-negative enterobacteria (i.e. the 'leaky gut syndrome' observed in both human and non-human study organisms) (Camilleri, 2019; Alhasson et al., 2017). This increase could also, and not independently, result from stress-induced changes to the microbiome that increase the abundance of endotoxin-producing bacteria, as captivity alone can cause significant stress in wild house sparrows (Fischer et al., 2017; Lattin et al., 2017; Love et al., 2017). In our study, we noted a substantial increase in the relative abundance of putative LPS-producing bacteria in the microbiomes of stressed birds, with the changes in the community associated with changes in blood stress-induced corticosterone levels. This community shift to a higher relative abundance of endotoxin-producing taxa, potentially in conjunction with host factors related to the integrity of the gut (Hollander and Kaunitz, 2020), may be contributing to the hyperinflammatory response observed in captive birds. This may warrant concern, as the relative abundance of LPS-producing taxa remained elevated even 7 days after the chronic stress treatment had ceased. While further studies will be needed to examine the consequences of these microbial composition shifts, this presents a future opportunity to investigate whether microbial intervention via the targeted reduction of pro-inflammatory bacteria, or the addition of lactic acid probiotics (Foster et al., 2017), could effectively mitigate some of the negative effects of chronic stress observed in captive wild animals.

An established limitation of assessing microbial community differences through marker gene sequencing – such as the analyses we did here – is that it fails to distinguish live cells from dead cells and does not disambiguate changes in relative abundance from changes in absolute abundance. Therefore, these methods cannot determine whether the chronic mild stress treatment produced a 'bloom' of LPS-producing taxa, or a population 'crash' of LPS-negative, lactic acid bacteria (or some combination of the two). This pattern, it should be noted – as well as the similar methodological limitations – has been encountered by others investigating the effect of a mild stressor on the gut microbiome of captive Atlantic salmon (Webster et al., 2020), hinting that this may be a broader stress response pattern experienced across divergent species. The cultivation-dependent methods we used do not suffer from these same limitations; however, the media used in this study were not targeted for cultivating lactic acid bacteria, and the swab method for collecting samples is only semi-quantitative; therefore, such a change in total abundance would not likely be captured with these methods. Additional studies that measure the absolute abundance of lactic acid and endotoxin-producing bacteria in the gut will be required to better determine the nature and consequences of relationships among host physiology, chronic stress and the microbiome. However, regardless of viability or absolute changes in cell counts, there was a clear stress treatment signal in the microbiome of these birds. As one example, Enterococcaceae, which constituted more than half the average bacterial community of reference birds, shrunk to only 3% of the average community in

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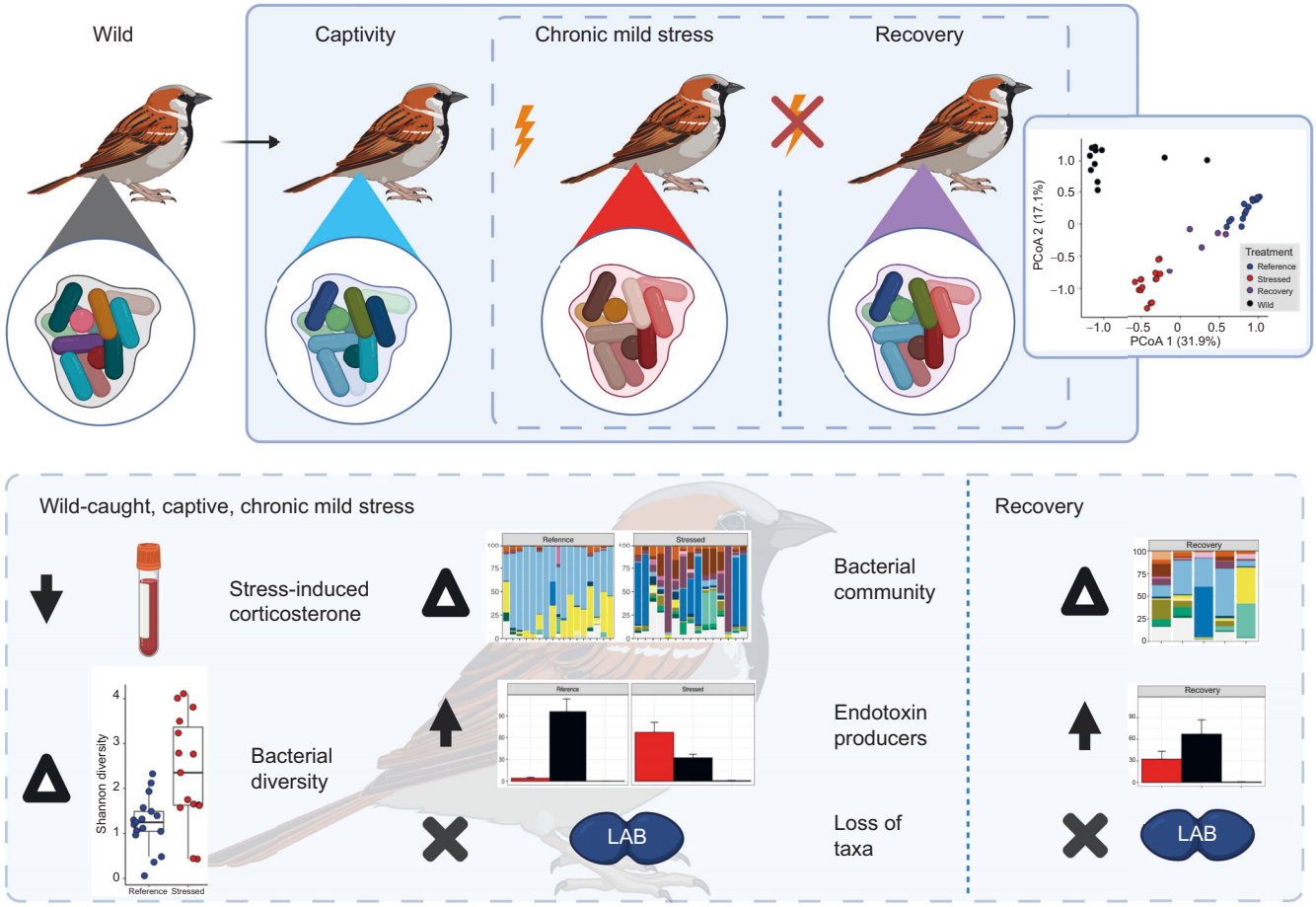

Fig. 7. Graphical abstract summarizing the major findings of this study. Chronic stress induced significant changes in the microbiome of a wild-caught avian host, including the loss of potentially beneficial bacterial taxa and an increase in potentially inflammatory endotoxin-producing taxa. The top right inset is from Fig. 2A; the bottom left inset is from Fig. S3. LAB, lactic acid bacteria. Figure created with BioRender.com.

the stressed birds. Exploring the utility of the microbiome as a potential indicator of chronic stress in wild songbirds may merit future research (as has been tested in plant systems by Zolti et al., 2020), because identifying chronically stressed animals remains remarkably difficult (Dickens and Romero, 2013).

While our primary goal was to investigate the effects of chronic stress, a secondary goal was to understand how captivity changes the microbiome of wild birds. Broadly, the microbiomes of all of the house sparrows sampled were dominated by the bacterial phyla Firmicutes and Proteobacteria. These results are similar to previously published work on the gastrointestinal tract of other bird species (Hird et al., 2015; Noguera et al., 2018), including house sparrows (Mirón et al., 2014; Kohl et al., 2019, 2018). Birds maintained in captivity displayed significantly altered microbiomes relative to their wild counterparts. Captivity has been shown to alter the microbiome of other wild animals (e.g. Wienemann et al., 2011; Alfano et al., 2015; Clayton et al., 2016). However, while we found that overall composition varied between wild-caught captive and wild sparrows, our results stand in contrast to previous studies showing reduced microbial diversity in captive animals relative to wild animals (Becker et al., 2014; Kohl et al., 2014; Schmidt et al., 2019). While wild birds were sampled the following year relative to captive birds, a potential confounding factor in our analyses, other work has shown recurring seasonal or annual patterns in microbial communities of environmental (Fortunato et al., 2012; Kearns et al., 2016) and host-associated systems (Davenport et al., 2014; Copeland et al., 2015; Erwin et al., 2015; Kearns et al., 2017; Novakova et al., 2017). A greater obstacle for comparing these treatment groups may be the high relative abundance of *Campylobacter* in the non-captive population. This bacterial genus, a potential concern to public health as it can cause gastroenteritis, is often carried by house sparrows (Benskin et al., 2009; Kelly et al., 2022). Wild songbirds are a suspected environmental reservoir of this pathogen, and often are colonized by feeding on environmental sources such as manure (Benskin et al., 2009; Hald et al., 2016). This dominance of *Campylobacter* in the microbiomes of wild versus wild-caught captive birds likely reflects microbial inoculation differences, rather than treatment effects per se, and possibly limits the extent to which we can interpret our captivity comparisons.

Our analyses have focused on the bacterial component of the microbiome as our results revealed that the microbiome of the house sparrow, both wild and wild-caught captive, contains negligible contributions from archaea and fungi. The similar colony counts observed on both TSA and PDA media, along with the few cloacal samples that amplified using fungus-specific primers, suggest this community is predominantly bacterial in both viable cell abundance and taxonomic diversity. A notable exception was five birds across captive treatment groups and sex that were shedding high levels of viable *Candida albicans*. This indicates these birds had candidiasis,

which, similar to *Campylobacter*, is a frequent infection in urban birds. This yeast can present a concern to public health, as this fungus can infect humans, but many avian hosts shed the yeast asymptomatically (Lord et al., 2010; Dulisz et al., 2021). Interestingly, we did not detect the presence of *Candida* in the same cloacal samples via ITS rRNA gene sequencing (although we did detect these taxa in non-captive hosts). The predominance of bacteria within the community, and the variable presence of *Candida* across hosts, is consistent with other studies on the microbiomes of wild house sparrows (Dulisz et al., 2021; Stewart and Rambo, 2000). The fact that a few of these birds were shedding *Candida* likely has no implications for the treatment effects we observed on the microbiome; however, it highlights that understanding the microbiomes of wild-caught, captive animals can help us understand the contribution of these microbes to the individual host's health, and – in their possible role as vectored pathogens – contributors to the health of other hosts.

In conclusion, we demonstrated significant effects of both chronic stress and captivity on the cloacal microbiome of wild-caught house sparrows. Changes in bacterial community composition were correlated with changes in host physiology regardless of experimental stress treatment, indicating a strong link between gut microbiota and the HPA axis, and building upon previous work on this topic. We found the cloacal microbiome to be somewhat resilient to stress at the bacterial community level following the cessation of chronic stress; however, our results suggest an altered function of gut microbial communities and the loss of potentially beneficial taxa (e.g. lactic acid bacteria) may contribute to longer-term host physiological change and the potential for reduced host immune capacity. Furthermore, our results showing that captive sparrows do not regain the microbiome of wild sparrows suggest that caution is needed in extrapolating microbiome studies from captive to wild animals. Future research is needed to examine whether probiotics, or a more diverse and naturalistic diet, could help restore some of the bacterial taxa lost or reduced in captive birds. The functional role of different bacterial taxa found in songbirds, and their effects on host health, also needs to be further clarified to determine whether some captivity-induced changes in the microbiome could be neutral or even positive. Perhaps most importantly, we found the microbial community responded to host stress with an increased relative abundance of endotoxin-producing bacteria, supporting a microbial mechanism for the increased inflammatory response observed in avians experiencing captivity and associated stressors. Overall, these results clarify the relationship between stress and the host physiological response of wild avians, and highlight the importance of considering the host's microbiome when evaluating the impact of stressors on the health of individuals and populations.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: A.A.M., L.M.R., C.R.L.; Methodology: A.A.M., A.M.O., J.B.H., P.T.S., C.R.L.; Formal analysis: A.A.M., A.M.O., P.J.K., B.E.W.; Investigation: A.A.M., C.R.L.; Resources: A.A.M., N.F., P.T.S., L.M.R.; Data curation: A.A.M., A.M.O.; Writing - original draft: A.A.M., P.J.K., J.B.H., N.F., P.T.S., B.E.W., L.M.R., C.R.L.; Writing - review & editing: A.A.M., A.M.O., P.J.K., J.B.H., N.F., P.T.S., B.E.W., L.M.R., C.R.L.; Visualization: A.A.M., A.M.O., P.J.K.; Supervision: J.B.H., N.F., P.T.S., B.E.W., L.M.R.; Funding acquisition: A.A.M., A.M.O., P.T.S., L.M.R., C.R.L.

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Data availability
16S rRNA gene Illumina short read sequencing data can be found in the NCBI Sequence Read Archive under accession number: PRJNA695275. Sanger sequencing data can be found in the NCBI GenBank database under accession numbers: MW408203, MW408205, MW408206, MW408208 and MW408209. Metadata and analysis code and files are available from GitHub: https://github.com/amoliverio/birdstress.

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