Habitat quality influences gut microbiota via cortisol in Siberian flying squirrels (*Pteromys volans)*

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ABSTRACT

Interactions between the mammalian neuroendocrine system and the gut microbiota (i.e., the gut-brain axis) can shape how animals cope with environmental challenges. For example, both immune function and the extraction of energy from dietary items depend on microbiota, which can be influenced by the environment directly through transmission or indirectly through neuroendocrine responses. Environmental variation has been shown to translate into variation in microbiome profiles among wild animals, but whether this is due to different environmental microbial transmission or environmental conditions evoking microbiome-modulating physiological stress responses is not known. Here, we investigate covariation among habitat quality, cortisol levels and gut microbiota in adult and juvenile Siberian flying squirrels (*Pteromys volans*) inhabiting a region impacted by variable rates of human disturbance. We test competing hypotheses related to direct (e.g., via diet or transmission) versus indirect (e.g., via the gut-brain axis) effects of habitat on the gut microbiota. Overall, we found that adults had richer gut microbiota and higher concentrations of hair cortisol than juveniles. Our measures of habitat quality exerted direct effects on cortisol concentrations, but had no direct effects on microbiota. Regardless, cortisol predicted microbiota composition among adult squirrels. Finally, structural equation modeling revealed indirect effects of habitat quality on microbial diversity by way of elevations in cortisol among adults. In contrast, among juveniles, habitat-related cortisol variation had no downstream effects on microbial diversity. Together, these findings provide evidence for indirect effects of environmental quality on gut microbiota via the host endocrine system, and suggest these effects can be age-dependent.

INTRODUCTION

Anthropogenic change is rapidly reshaping ecological communities, with profound implications for both individual fitness and population resilience (Sih et al., 2011). Underlying these effects are the physiological systems that constrain or facilitate organismal responses to the broader environment (Todgham & Stillman, 2013), and identifying these systems has been a central focus in animal physiology (Fuller et al., 2010). Physiological systems that can coordinate phenotypic responses to these changes may be particularly powerful avenues through which organisms can adapt to environmental fluctuations. In vertebrates, the neuroendocrine system–

including the hypothalamic-pituitary- adrenal (HPA) axis–can transduce cues related to changing environmental conditions to coordinate phenotypic shifts that can maximize fitness (Dantzer, 2023; Martin et al., 2011). The HPA axis is an evolutionarily conserved regulator of the physiological stress response, and modulates host metabolism and energy status through a cascade of feedback interactions that culminates in the production of glucocorticoid hormones, including cortisol (Sapolsky et al., 2000). Because its rate of production is highly sensitive to a wide array of inputs, cortisol can index host physiological status in response to environment changes like shifts in diet, predation risk, population density, temperature, and the social environment (Sheriff et al., 2011; Wingfield et al., 1998).

Notably, the HPA axis can have both target and non-target effects on other physiological systems, including the symbiotic community of microorganisms that reside in the gastrointestinal tract [hereafter, gut microbiota, (Cryan & O'Mahony, 2011)]. While gut microbiota can be shaped by external factors like diet, environment, and host-to-host microbial transmission (Baniel et al., 2021; Orkin et al., 2019; Raulo et al., 2021; Ren et al., 2017; Sarkar et al., 2024), a "gut-brain axis" indicates that neuroendocrine function may be an internal proximate mechanism regulating host gut microbial variation (Cryan et al., 2019; Stothart et al., 2019). Elucidating potential neuroendocrine control over the gut microbiota is crucial because microbial variation, regardless of its drivers, can have profound impacts for host fitness. The gut microbiota can enhance pathogen protection (Spragge et al., 2023; Stecher & Hardt, 2011) and facilitate dietary flexibility (Cani et al., 2019; Kohl et al., 2016), but when imbalanced, can impair immune function and digestive efficiency (Pham & Lawley, 2014). Moreover, the gut-brain axis is inherently bidirectional such that microbiota can in turn influence the HPA axis (Burokas et al., 2015), and consequently, host behavior (Wu et al., 2021). Variation in the commensal gut microbiota can thus be driven by complex internal feedbacks between the microbiota and the brain, generating substantial covariation among host environment, the HPA axis, and gut microbiota with many causal linkages (**Figure 1**).

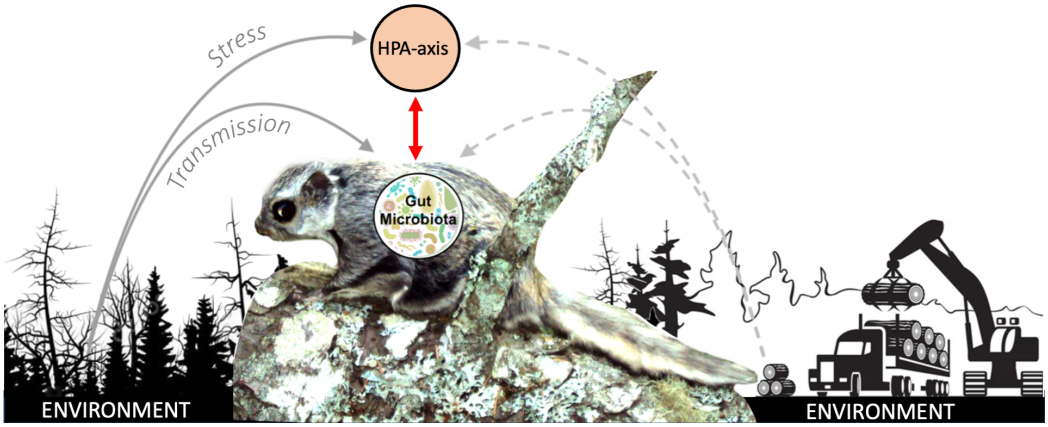

Figure 1. Conceptual depiction of potential causal relationships between the environment, gut microbiota, and hypothalamic-pituitary-adrenal (HPA) axis. The left and right side depict two different environments, one that is in a natural state and has low levels of disturbance and one that is structured by human action and has high levels of disturbance.

Controlled experimental models in which gut microbiota respond to artificial stressors and/or synthetic hormone administration demonstrate that physiological stress can causally induce changes in the host gut microbiota. In yellow-legged gulls (*Larus michahellis),* synthetic corticosterone administration altered gut microbial communities by reducing gut microbial diversity, including both pathogenic and symbiotic taxa (Noguera et al., 2018). However, broader interpretations of these and similar studies [e.g., (Blanco et al., 2021)] remain limited in the absence of naturalistic stressors that can exert context-dependent effects on physiological stress responses (Crespi et al., 2013). Studies in wild populations can permit a more ecologically-relevant understanding of how gut microbiota respond to changes in the environment mediated by the host neuroendocrine function (Greyson-Gaito et al., 2020). Such studies can also provide insight into the potential of the gut-brain axis to serve as a mechanism of ecological adaptation (Alberdi et al., 2016). For instance, the integration of environmental cues by the HPA axis may induce shifts in microbial community composition that promote reconfiguration of gut microbiota to optimally track a changing dietary landscape (Baniel et al., 2021; Orkin et al., 2019). Habitat degradation and habitat loss may increase cortisol production to adaptively mobilize energy stores (Busch & Hayward, 2009), but exert collateral effects on gut microbiota by constraining taxonomic and/or functional diversity (Amato et al., 2013; Petrullo et al., 2022; Stothart et al., 2016). Parsing these effects requires data from unmanipulated populations to reveal indirect, and thus inconspicuous, connections between an organism's symbiotic microbiota and its broader ecology.

Here, we aim to disentangle covariation between ecological changes, HPA axis function, and the gut microbiota in a vulnerable population of Siberian flying squirrels (*Pteromys volans)*. Siberian flying squirrels are arboreal specialists adapted to live in natural state old-growth boreal forests, and are listed as vulnerable species within the European Union (Santangeli, Hanski, et al., 2013). Because their dietary niche is relatively narrow, they are especially susceptible to changes in forest structure. Habitat loss due to commercial logging is the primary threat to their populations (Santangeli, Wistbacka, et al., 2013; Wistbacka et al., 2018), resulting in the fragmentation of their habitat into variably-sized patches of suitable, old-growth forest separated by unsuitable habitat, including fields or young commercially-grown forest. In this study, we test the hypothesis that habitat quality (patch size, diversity, and suitability) influences gut microbiota by way of HPA axis activation (e.g., an indirect effect via the gut-brain axis). We take an integrative approach that includes structural equation modeling (Eisenhauer et al., 2015; Grace et al., 2010; Laughlin & Grace, 2019) to test alternative hypotheses related to direct versus indirect environmental effects without requiring experimental manipulation of this vulnerable species. We predict that individuals inhabiting low quality habitats (smaller patch size, lower habitat diversity, and higher rates of unsuitable habitat) will exhibit higher cortisol concentrations, indicative of HPA axis activation [(Sheriff et al., 2011), but see (Santangeli et al., 2019)]. We expect that elevated cortisol will predict lower gut microbial diversity, in line with other studies on wild vertebrates (Noguera et al., 2018; Petrullo et al., 2022; Stothart et al., 2016, 2019). Alternatively, gut microbiota may be influenced by environmental factors independently of HPA axis function (a direct effect), suggesting that gut-brain axis connections may be resistant to changes in habitat quality.

MATERIALS AND METHODS

Study species and field data collection

The Siberian flying squirrel (*Pteromys volans*) is an arboreal specialist rodent species adapted to climax-state spruce-dominated boreal forests. Within the European Union, most flying squirrels are found from Southern Finland, located in the western edge of their range across Eurasia. As old-growth forest specialists, flying squirrels are threatened by commercial forestry practices. In Finland boreal forests are typically cut before they reach the climax state optimal for flying squirrels. As Southern Finland is an area of intense commercial forestry, few disconnected patches of natural old-growth forest remain amidst younger commercial stands. As such, flying squirrel populations are scattered as a metapopulation across variably suitable and variably connected habitat patches.

In June 2015, we went through flying squirrel nest boxes, positioned across different habitat patches around Larsmo-Öja and Vaasa area in Finland [see (Santangeli et al., 2019)]. The study system consists of 69 study sites, each including 1-5 flying squirrel nest boxes. These sites are part of a long-term study on the flying squirrel ecology, and are deliberately positioned across a wide range of different flying squirrel habitats, ranging from urban park to natural old-growth boreal forests varying in their level of forestry-related disturbance (Santangeli et al. 2013b; Selonen et al. 2014; Wistbacka et al. 2018). We went through nest boxes during the first two weeks of June, at a time when flying squirrels are breeding and pups are on average ~6 weeks old. From nest boxes, we caught 79 individuals (48 adults and 31 pups). In most cases, nest boxes had either a solitary adult or one adult female and 1-4 pups. When caught, we collected data on the flying squirrels in situ, releasing them to their point of capture within 15 minutes of capture. Data collection included sexing and aging each individual, recording their body mass and femur length [see (Selonen et al. 2016)], and tagging each individual with a subcutaneous PIT-tag.

Physiological sample collection

We clipped a small amount (~10 mg) of hair from the side of the tail of each individual squirrel to measure hair cortisol content as a proxy for long-term stress exposure of our study subjects (Heimbürge et al., 2019). Hair was chosen because it is a minimally invasive sample medium that can index long-term stress exposure across time periods spanning weeks to a few months [i.e., during the period of hair growth, (Gormally & Romero, 2020)]. As a proxy for environmental stress, this is a more realistic measure than blood samples, as plasma cortisol can be affected by short-term stressors like handling at the time of collection (Desantis et al., 2016; Sheriff et al., 2011). Hair sampling was done in a systematic and consistent way for all individuals: samples were cut as close as possible to the base of the hair and all samples were cut in the same exact way, from the same part of the tail, by the same person. This ensured that the cortisol concentrations measured from the hair were as comparable as possible between individuals, as they would all relate to a similar time span of hair growth, during the same time of the year. Collected hair samples were stored in a dry place at room temperature before being sent off for cortisol content analysis in the Berlin Zoo. Fecal samples were collected directly and opportunistically during animal handling and transferred into a clean labeled Eppendorf tube

with 1 ml of 98% ethanol as a preservative. To avoid contamination, tubes and fecal pellets were handled with gloves, which were changed between every individual. Fecal samples were stored in a fridge for the duration of the field work (2 weeks) and subsequently in a -80°C freezer, until they were shipped frozen to the Earth Microbiome Project at the University of Colorado for sequencing.

Habitat quality measures

We used data from yearly habitat surveys [see (Wistbacka et al., 2018)] and Geographic Information System (GIS) to classify habitats across the study sites into three discrete classes following Wistbacka et al. (2018). Suitable habitat refers to breeding habitat for females, defined as layered spruce dominated mixed forests, containing trees of different size and age (Table 1). Semi-suitable habitat is defined as mono specific (typically pine plantations) forests where flying squirrels can move but cannot breed because of lack of resources (such as food provided by deciduous trees or shelter provided by spruce trees (Wistbacka et al., 2018). Unsuitable habitat is represented by open areas covered by young sapling stands or clear-cuts, roads, fields, water, or built up areas (i.e., areas unusable for flying squirrels to move, feed, and/or breed). We then calculated three measures of habitat quality: 1) patch size (area in m), 2) patch diversity (different types of habitats present within 300 m of an individual's nest box) and 3) proportion of suitable area within a 100 m radius from the center of each nesting site, which is where the nest boxes are located, following Santangeli et al. (2013b) and Wistbacka et al. (2018). The amount of unsuitable habitat as well as size of the patch have been found to capture relevant habitat variation within the range typically used by flying squirrels (Hanski et al., 2000; Santangeli, Hanski, et al., 2013; Wistbacka et al., 2018).

Measurement of hair cortisol concentrations

Hair samples were washed with 100% methanol for 2 min. Methanol was then decanted and the dried samples were powdered in a bead mill. Next, hairs were extracted with 400 µl 90% methanol and aliquots of 20 µl, diluted 1:2 with water, and analyzed in the enzyme immunoassay (EIA). Hydroxycorticosterone was quantified using a polyclonal in-house antibody against cortisol-21-hemisuccinate (HS), coupled with bovine serum albumin (BSA) raised in rabbits, used together with the corresponding 21-HS-steroid coupled to peroxidase (HRP) as label for the EIA. Antibody cross-reactivity with different glucocorticoids was as follows: 4-pregnen-11a,17,21-triol-3,20-dione (cortisol) 100%, 4-pregnen-11ß,21-diol-3,20-dione (corticosterone) 13.2%, and 4-Pregnen-21-ol-3,20-dione (desoxycorticosterone) < 0.1%, respectively [see (Voigt et al., 2004) for more details].

Cortisol standards were prepared, in duplicates of 20 µl, by 1:1 dilutions in assay buffer ranging from 0.2 to 100 pg/20 µl. All the duplicates, together with 100 µL cortisol-HRP conjugate in assay buffer (50 mM Na_2HPO_4/Na_2HPO_4, 0.15 M NaCl, 0.1% BSA, pH 7.4), were added to a plate, which was then incubated overnight at 4 °C and then washed four times with washing solution. One hundred fifty microliter substrate solution was added per well (1.2 mM H_2O_2, 0.4 mM 3,3′,5, 5′-tetramethylbenzidine in 10 mM sodium acetate, pH 5.5), and the plates were incubated in the dark for 40 min. Fifty microliters of 4 N H_2SO_4 were then added to stop the reaction and the samples were measured at 450 nm with a 12-channel microtiter plate reader

(Infinite M 200; Tecan). Hormone concentrations were calculated using the Magellan software [Tecan; see (Finkenwirth et al., 2010)]. The sensitivity of the assay was defined as two standard deviations from the signal given by the zero blank and thus was 0.2 pg per well.

Microbial DNA sequencing and bioinformatics

DNA extraction, amplification, and sequencing was performed in the Knight Laboratory at the University of Colorado in 2016, following Earth Microbiome Project protocols (see http://www.earthmicrobiome.org/emp-standard-protocols/16s/). In short, DNA extraction was done using MO Bio PowerSoil kits and PCR was run with primers 515F/806R, targeting the V4 region of the 16S rRNA bacterial gene (Caporaso et al., 2012). Resulting amplicons were sequenced using an Illumina MiSeq. Sequences were clustered into OTUs using the UPARSE pipeline (with de novo clustering using 97% similarity threshold and maximum expected error ≤2). Further preprocessing was done with the phyloseq package in R (McMurdie & Holmes, 2013). Based on sample completeness curves produced with the iNext package (Hsieh et al., 2016), we chose to filter out samples with < 10,000 reads as unrepresentative of the community diversity. Using the same package, we also derived asymptotic alpha-diversity estimates for each sample. Furthermore, we removed from the data all OTUs assigned to chloroplasts/Cyanobacteria, as we assumed these sequences do not represent gut microbes. Finally, taxon abundances were normalized as proportions within their sample. Microbiome variation was then described through two set of metrics: richness (number of unique taxa) and Shannon diversity (an abundance-weighted measure of diversity) as measures of alpha diversity, and Jaccard similarity (proportion of shared taxa between two samples) as a measure of beta-diversity.

Statistical analyses

Identifying drivers of variation in gut microbiota

To identify the factors that predicted variation in host gut microbiota, we performed a first marginal permutational analysis of variance (PERMANOVA) on microbiome beta-diversity (Jaccard similarity values) using the *adonis2* function in the *vegan* package (Oksanen et al., 2007). We first tested the effects of host age, sex, date, and family membership on microbiome composition in the full dataset (N=78). We then ran a second marginal PERMANOVA with family set as a blocking factor, controlling for age and sample date, to assess effects of the three habitat measures (habitat diversity, patch size, and amount of unsuitable area) on gut microbiota composition. We additionally tested for variation in homogeneity of dispersion as a function of host age using the *betadisper()* function and a generalized linear mixed-effects model (family = beta).

Differential abundance of microbial taxa

To identify the taxonomic differences between the gut microbial communities of adults and juveniles, we constructed a series of linear mixed effects-models using lme4 (Brooks et al., 2017). Models test how age (fixed factor) predicted the relative abundance of bacterial families (arcsine square root transformed), controlling for host sex, date of sample collection, and nest box/family membership (random effects). We accounted for multiple comparisons and an

inflated false discovery rate for multiple hypothesis testing by adjusting all P values using the Benjamini-Hochberg correction (Benjamini & Hochberg, 2000).

Dyadic models

To test whether variation in cortisol predicted variation in gut microbiota, we built dyadic Bayesian beta regression models using the *brms* package in R (R Core team, 2015), as previously validated and described in (Raulo et al., 2021; see also: https://github.com/nuorenarra/Analysing-dyadic-data-with-brms). We used pairwise Jaccard similarity values as our response variable and predicted it with pairwise measures for hair cortisol similarity, with sex similarity (0/1=same/different sex), age similarity (0/1=same/different age category), spatial distance (metric distance between nest boxes) and nest sharing (0/1=same/different nest) added as covariates. All covariates either naturally ranged from 0 to 1 or were scaled to do so, to make model estimates for all terms comparable. As Jaccard similarity is a proportional metric bound between 0 and 1, a logit link function was used. As the modeled values are pairwise measures, and as such not independent of each other, a multi-membership random effect structure was built into the model to account for the effect of identity of both individuals in each pairwise comparison. The model was run first for all individual pairs in the data and subsequently separately for adults and pups, omitting age similarity as a covariate.

Investigating effects of host traits and habitat quality on gut microbiota and cortisol

We used linear mixed-effects models to examine gut microbial alpha-diversity (richness, Shannon) as a function of host traits and habitat quality. Shannon indices were tukey transformed to achieve normality of residuals. A first set of models included host age and sex as fixed covariates, and family membership as a random effect predicting gut microbial alpha-diversity with hair cortisol and measures of habitat quality (patch size, patch diversity, and amount of unsuitable area). For models investigating the effects of habitat quality on gut microbiota, we included sex as a fixed factor, and sampling date and time as random factors given work suggesting diurnal patterns of cortisol secretion from circulation into hair (Sharpley et al., 2010, 2011).

Testing for direct versus indirect effects using structural equation modeling

We constructed structural equation models (SEMs) to simultaneously test for direct and indirect (via cortisol) effects of habitat quality on gut microbiota using *piecewiseSEM* (Lefcheck, 2016). Structural equation modeling is a quantitative approach that allows for the testing of a priori causal hypotheses in complex ecological systems through the partitioning of direct and indirect (mediator) effects of multiple variables nested within one or more mixed-effects component models (Laughlin & Grace, 2019). The *piecewiseSEM* package specifically improves upon other more traditional SEM approaches because it accounts for smaller sample sizes through Akaike Information Criterion (AIC) model comparison and permits the inclusion of random effects and thus repeated sampling (Lefcheck, 2016). Given the appreciable differences in both cortisol concentrations and gut microbial diversity between adults and juveniles, we built separate SEMs for each age class and tested only the measures of habitat quality that were found to be significant predictors of cortisol in our previous models. Thus each SEM was composed of two component models to retain power and avoid overfitting with interaction terms. For each SEM,

the first component model included hair cortisol concentrations (log-transformed) as the dependent variable, habitat quality (patch size, diversity or amount of unsuitable habitat) and sex as fixed effects, and family membership as a random effect. The second component model included gut microbial alpha-diversity (richness or Shannon diversity) as the dependent variable, hair cortisol as the fixed effect and family membership as a random effect (sex was not included as it had no effect on microbial variation in previous models). We determined the overall fit of the SEM using Fisher's C statistic.

RESULTS

1. Intrinsic and extrinsic drivers of gut microbial variation in flying squirrels

The gut microbiota of flying squirrels (N = 78) contained microbiota from across 14 phyla, with most microbiota from Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria (Figure 2A). Within these phyla, samples contained microbiota from 97 families (Figure S1). Specifically, *Lachnospiraceae* (mean relative abundance = 39.4% across samples), *Ruminococcaceae* (21%), and *Bacteroidaceae* (20.4%) were the most highly abundant families, with all other families contributing less than 5% to the total gut microbial community (Figure S1).

A marginal PERMANOVA on Jaccard similarity revealed that an individual's nest box, which reflects family membership, explained a substantial amount of variation in gut microbiota composition (R^2 = 0.47, F = 1.17, p = 0.001). Additionally, host age additionally explained some of the variance (R^2 = 0.03, F = 1.89, p = 0.001, Table S1, Figure 2B). There was no effect of host sex on gut microbial composition (Table S1). Compared to adults, juvenile squirrels exhibited significantly greater compositional variability (greater distances to group centroid in Jaccard compositional space; estimate ± SE: 0.14 ± 0.03, z = 4.84, $p < 0.001$, Figure S2, Table S3), demonstrating greater inter-individual differences in microbial composition during early development compared to later life. At the taxonomic level, juveniles housed significantly higher relative abundances of gut microbiota from the families *Bacteroidaceae*, *Rikenellaceae*, and *Erysipelotrichaceae* (Figure 2C, Table S4). By contrast, adults housed greater relative abundances of *Coriobacteriaceae, Syntrophomonadaceae, and Eubacteriaceae* (Figure 2C, Table S4).

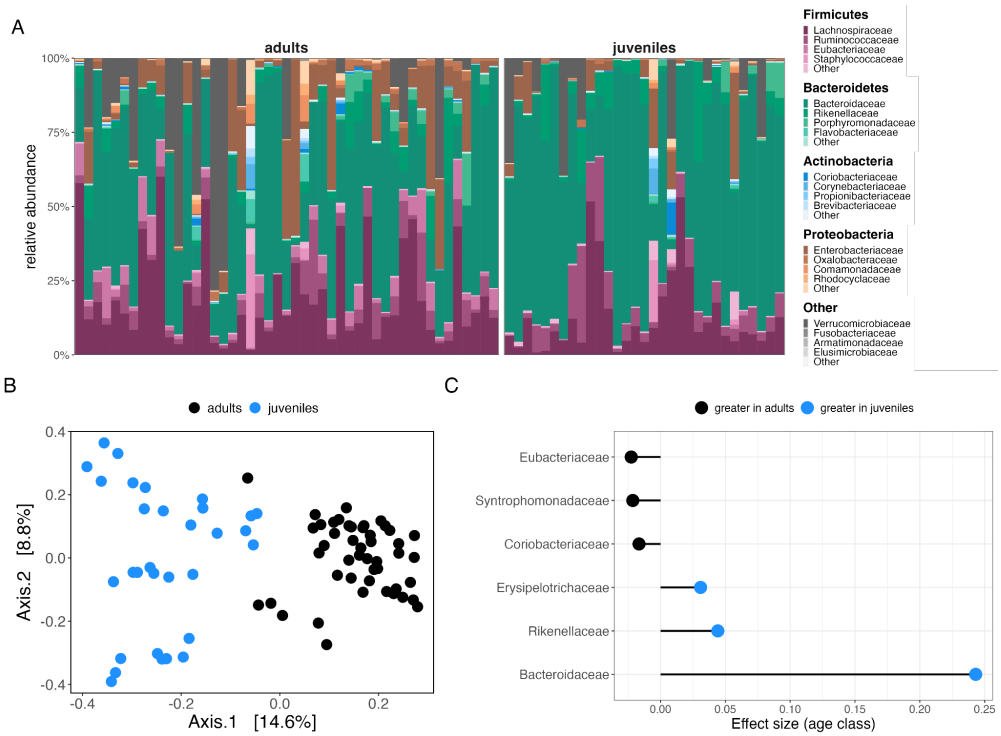

Figure 2. Strong effects of age class on microbial community composition. (**A)** The flying squirrel gut microbiota was dominated by microbiota from four main phyla. The most abundant microbial families (ranked) are shown below each phyla in the legend. **(B)** Principal coordinates analysis of Jaccard similarity across all fecal samples (N =78) by age class. **C)** Differential relative abundances of bacterial families (P_{FDR} > 0.05) as a function of age class. Lollipop plot depicts effect sizes from linear mixed-effects models testing the differential relative abundance of bacterial families (relative abundance > 0.05%) as a function of age class.

2. Cortisol predicts within- and among-individual microbial diversity

For individuals from which we had hair cortisol concentrations (N =43), we found that juveniles exhibited substantially lower cortisol than adults (linear mixed-effects model predicting hair cortisol concentrations, estimate ± SE: -0.79 ± 0.13, t = -6.24, p < 0.0001, Table S5, Figure S3) as well as lower gut microbial alpha-diversity (linear mixed-effects model predicting microbial richness (number of OTUs), estimate ± SE: -327.81 ± 36.99, t = -8.86, p < 0.0001; predicting Shannon Index, -40.87 ± 4.76, t = -8.59, p < 0.0001; Table S6, Figure S3). Covariation between cortisol and microbial diversity was also age-dependent: among adults, higher cortisol predicted greater gut microbial Shannon diversity (linear mixed-effects model, estimate ± SE: 0.48 ± 0.02, t = 28.61, p < 0.0001), with a marginal effect on microbial richness (estimate ± SE: 26.82 ± 14.02, t = 1.91, p = 0.07, Table S7, Figure 3A). However, there was no relationship between cortisol and either measure of microbial alpha-diversity among juveniles (Table S7, Figure 3B).

To further parse the relationship between cortisol and gut microbial composition, we modeled microbial beta-diversity (Jaccard similarity) as a function of similarity in hair cortisol concentrations, controlling for spatial distance, sex, and nest sharing (Raulo et al., 2021, 2024). Among adults, individuals with more similar cortisol concentrations shared a greater proportion

of microbial taxa than those with more divergent cortisol concentrations (dyadic Bayesian generalized linear-mixed effects model, posterior mean = -0.36, CI = -0.60 to -0.12; Figure 3C, Table S8). Additionally, adults in closer spatial proximity to one another also had more similar gut microbiota (posterior mean = -0.23, CI = -0.36 to -0.11; Figure 3C, Table S8). By contrast, among juveniles, neither spatial distance nor differences in hair cortisol concentrations predicted gut microbiome similarity (Table S6). Instead, the only significant predictor of juvenile microbiota similarity was nest (family-membership), such that juveniles shared a greater proportion of their gut microbial taxa with their siblings/nest-mates (posterior mean = 1.47, CI = 0.89 to 2.07; Figure 3D, Table S9). Sex (same sex versus different sexes) did not predict microbial similarity in either age class.

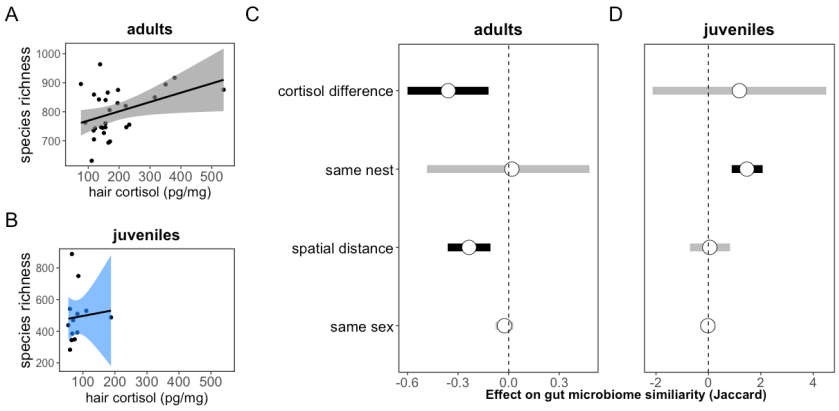

Figure 3. Among adults, higher cortisol predicts greater microbial richness, while similar hormone profiles predict a more similar microbiota. (A) Among adults, hair cortisol concentrations were positively associated with microbial richness, while **(B)** among juveniles, there was no significant relationship between hair cortisol and microbial richness. **(C)** Adult squirrels with less similar hair cortisol concentrations and larger spatial distances between them had less similar microbiota (i.e., effect on the negative side, credible intervals do not overlap 0). **(D)** Among juveniles, differences in cortisol did not predict gut microbiome similarity but juveniles residing in the same nests shared significantly more gut microbial taxa than juveniles residing in separate nests. Posterior means (points) and their 95% credible intervals (lines) are plotted from dyadic Bayesian GLMM with pairwise microbiota similarity among squirrels (Jaccard similarity) as the response.

3. Habitat quality influences cortisol without direct effects on the gut microbiota

Habitat quality was associated with variation in hair cortisol concentrations, but the magnitude and directionality differed depending on age class and habitat measure. Broadly, patch size alone was negatively associated with hair cortisol concentrations, with the relationship largely being driven by adults (animals inhabiting smaller patches had higher cortisol, estimate ± SE: -0.15 ± -0.07, t = -2.16, P = 0.038, Figure 4A, Table S10). Habitat diversity positively predicted hair cortisol, but only in juveniles (estimate ± SE: 0.34 ± 0.14, t = 2.34, P = 0.026), but not in adults (Figure 4B, Table S10). Amount of suitable habitat had no effect on cortisol concentrations in adults or juveniles (Figure 4C, Table S10). Moreover, none of the three habitat measures had direct effects on either measure of gut microbial diversity (richness or abundance-weighted Shannon diversity) in either age class (Table S11).

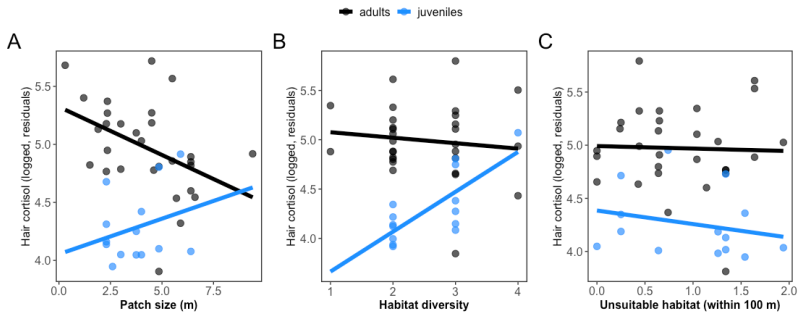

Figure 4. Age-dependent patterns of cortisol production and gut microbial diversity as a function of habitat quality. (A) Patch size and **(B)** habitat diversity explain variation in hair cortisol in an age-dependent manner, but there are no effects of **(C)** unsuitable habitat on hair cortisol concentrations in either adults or juveniles. Additionally, there are no direct effects of any of the three habitat measures on gut microbial richness. Scatterplots show results from linear mixed-effects models. Points reflect samples (partial residuals), lines indicate regression lines from generalized linear-mixed effects models.

4. Indirect effects of habitat quality on gut microbiota via cortisol

Because habitat quality predicted hair cortisol concentrations (see section 3) and cortisol had a positive effect on gut microbial richness among adults (see section 2), we used structural equation models to test whether variation in hair cortisol as a result of habitat quality mediated downstream effects of cortisol on gut microbial diversity. In the adult model, patch size was the only measure of habitat quality to predict downstream effects on the gut microbiota through hair cortisol: smaller patches increased hair cortisol (standardized β = -0.39, P = 0.15), which in turn increased microbial diversity (standardized β = 0.39, P = 0.46, Figure 5A, Table S12). Among juveniles, there was no significant relationship between cortisol and microbial diversity. The only significant path was the path linking habitat diversity to hair cortisol: a more diverse habitat increased hair cortisol (standardized β = 0.84, P = 0.001), but unlike adults, there was no downstream effect of elevated cortisol on microbial diversity (Table S12, Figure 5B).

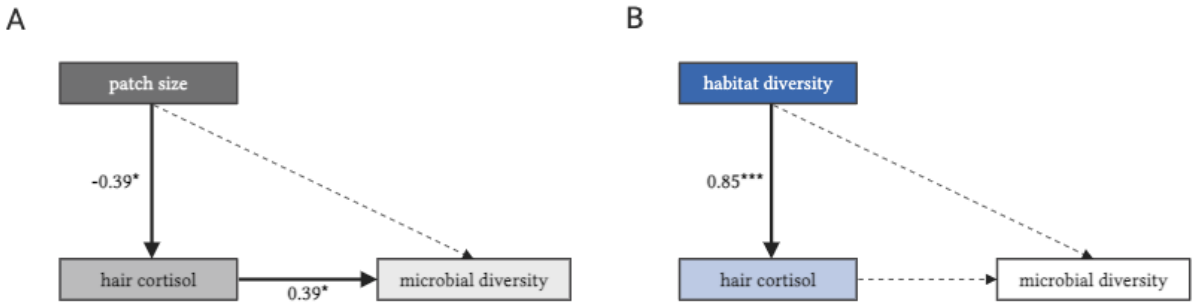

Figure 5. Age-dependent indirect effects of habitat quality on microbial richness. (A) Smaller patch sizes increased richness via elevations in hair cortisol in adult squirrels, but **(B)** elevations in cortisol induced by habitat diversity had no downstream effects on richness in pups . Plots A & C depict raw data to facilitate visualization of differences in magnitude of cortisol production by age class. Solid arrows indicate significant paths, dashed arrows indicate non-

414 significant paths. Covariates not shown for visualization purposes (see Table SX for details.
415 Model estimates for significant paths are shown with significance level (*P < 0.05, ** P < 0.01,
416 ***P < 0.001) and reflect standardized beta weights from mixed-effects component models
417 controlling for sex, sample date, time, and nest box. Tests of directional separation indicated
418 that models were not missing any significant paths and fit the data well (adult SEM: Fisher's C =
419 0.52, df = 2, P = 0.77; juvenile SEM: Fisher's C = 1.77, df = 2, P = 0.41).
420
421 **DISCUSSION**
422 To date, most work aimed at identifying drivers of microbial variation has focused on direct
423 effects of extrinsic environmental variables, with less focus on the intrinsic physiological
424 mediators of microbial variation in wild animals. Yet, robust connections between the
425 neuroendocrine system and host microbiota exist, and because the neuroendocrine system is
426 responsive to environmental conditions, gut-brain connections may contribute to organismal
427 adaptation to environmental change. Here, we present evidence for indirect effects of the
428 environment on the gut microbiota via the HPA axis response in a vulnerable small mammal
429 population inhabiting a landscape with high rates of anthropogenic disturbance. We found age-
430 dependency in these patterns, with adult squirrels exhibiting greater variation in cortisol
431 concentrations, with robust covariation in cortisol and gut microbial community composition.No
432 such connections were present among the nest-living juveniles, whose cortisol concentrations
433 varied only within the lower end of adult concentrations. Together, this work contributes to a
434 broader understanding of the central role of glucocorticoid hormones in the intimate connection
435 between the gut and brain in free-living vertebrates.
436
437 Habitat disturbance can be a major source of environmental stress for affected animals, with the
438 potential to activate the physiological stress response in both adaptive and maladaptive ways
439 (Dantzer et al., 2014; Jaimez et al., 2012; Romero, 2004; Sheriff et al., 2011). Here, we found
440 that adult squirrels responded hormonally to variation in habitat size, such that hair cortisol
441 concentrations were higher among squirrels inhabiting smaller patches. Many arboreal animals,
442 including tree squirrels and flying squirrels, are adapted to mature forest habitat (Selonen &
443 Mäkeläinen, 2017). Changes in forest structure can reduce the size of usable habitat, causing
444 habitat fragmentation, and subsequent lack of resources which can in turn elevate cortisol
445 concentrations [small mammals: (Boyle et al., 2021), nonhuman primates: (Carlitz et al., 2016;
446 Rangel-Negrín et al., 2009)]. Smaller patches have less resources and inevitably place
447 individuals in closer proximity to habitat edges, which can be dangerous. Indeed, closeness to
448 habitat edge has been shown to increase cortisol production in another arboreal mammal [e.g.,
449 squirrel gliders (*Petaurus norfolcensis*), (Brearley et al., 2010, 2012)]. When habitat sizes shrink,
450 adults may need to mobilize energy stores to cope with the increased metabolic burden
451 associated with having to travel greater distances for food or mates (Crespi & Denver, 2005). If
452 smaller habitat patches result in higher rates of exposure to stressful stimuli common at edges
453 (e.g., humans, predators), elevated cortisol among adults inhabiting smaller patches may also
454 reflect increased psychological stress, vigilance, and/or anti-predator behaviors (Creel et al.,
455 2009; Voellmy et al., 2014). Earlier work in the same study population found no relationship
456 between patch size and hair cortisol but did not test for age-dependence in these patterns

(Santangeli et al., 2019). Coupled with our findings, these data suggest opposing HPA axis responses to variation in habitat size depending on age-class.

Among juveniles, hair cortisol was predicted by a different habitat quality measure: habitat diversity. The relationship was positive such that juvenile squirrels inhabiting more diverse habitats also exhibited higher cortisol. High habitat diversity is typically viewed as an indicator of a healthy ecosystem (Rapport, 1989), which may translate into higher cortisol concentrations among juveniles if they are exposed to a greater diversity of predators, competitors, or nest parasites. Overall, that the hormonal response was divergent between adults and juveniles suggests differential sensitivity to environmental stress depending on life history stage (Crespi et al., 2013). Indeed, while habitat disturbance appears to increase host glucocorticoids consistently in adult mammals (Dantzer et al., 2014), this relationship becomes inconsistent when juveniles are included (Pérez-Ortega & Hendry, 2023). In our study system adult flying squirrels interact with various dimensions of their habitat more directly than juveniles that are bound to their nests and exposed to local phenomena and sibling competition.

Previous work has documented direct effects of temperature and vegetation on the gut microbiota of a different population of flying squirrels (Liu et al., 2019). Here, however, we found no evidence for direct effects of habitat quality (by any of our measures) on gut microbiota. Instead, our results suggest indirect–but age-dependent–effects of habitat quality on gut microbiota, which run through activation of the HPA axis and the production of endogenous cortisol. While most studies document a decrease in microbial alpha-diversity with increasing host cortisol concentrations [e.g., (Petrullo et al., 2022; Stothart et al., 2016)], we find the opposite: adults with higher hair cortisol concentrations exhibited greater, rather than reduced, gut microbial alpha-diversity. While microbial diversity may be enhanced in highly social taxa like red bellied lemurs via stress-related behaviors like social grooming (Raulo et al., 2018), this is an unlikely explanation in less social Siberian flying squirrels. However, a similar behavioral mechanism may exist: higher cortisol can promote increased activity (e.g., foraging), which may in turn increase opportunities for social and/or environmental microbial transmission that increases microbial diversity. It is also possible, and perhaps even likely, that the gut microbiota can be modified by cortisol-induced changes in the gut (e.g., pH), and that the directionality of these cortisol-induced microbiota modifications may vary widely between species. Indeed, as a diverse microbiota is typically viewed as beneficial for hosts, cortisol-induced increases in microbial richness may reflect microbial diversification as an adaptive response to environmental stress. Alternatively, it may reflect a loss of the functional homogeneity that results from canalized microbial responses to ecological challenges (e.g., specialized diets), with potentially deleterious effects for hosts.

Both gut microbiota and the HPA axis can exhibit age-dependent sensitivities to a wide array of intrinsic and extrinsic inputs (Sapolsky & Meaney, 1986). It is thus perhaps unsurprising that their coordinated function as an integrated axis can also vary with host age (Scott et al., 2017). Because HPA axis responses are typically attenuated during early development in young mammals (Sapolsky & Meaney, 1986), juvenile cortisol production may not reach high enough concentrations to influence microbial composition at the community level (i.e., a dose-

dependent effect). Indeed, we found that juveniles exhibited significantly lower cortisol concentrations compared to adults (Santangeli et al., 2019). Juveniles also spend a substantial amount of time in their nests with littermates: they are continually exposed to conspecific microbiota. Indeed, we found that nest box was the only significant predictor of pairwise microbial similarity among juveniles. Persistent social microbial transmission across littermates may therefore eclipse cortisol-induced differences in gut microbiota across individuals, particularly if the pathways linking microbiota to the host endocrine system have not yet been sufficiently primed (Burokas et al., 2015). Finally, because cortisol accumulation in the hair shaft represents an aggregate measure of host cortisol production over an extended period of time, it may be too coarse a measure to capture brief but pronounced hormonal responses if these responses were more variable in juveniles than in adults (Gormally & Romero, 2020). Because developmental decoupling of the HPA axis and gut microbiota during early life may explain, at least in part, age-related differences in gut microbiota (Heitlinger et al., 2017), we encourage future research to consider potential modifying effects of host age on gut-brain axis function.

As interest in how gut-brain axis connections contribute to host ecology and evolution continues to grow, so does the number of outstanding questions. Structural equation modeling offers one approach for assessing the gut-brain axis in natural and/or vulnerable populations in which experimental manipulations are unethical or impossible (Lane, 2006). Structural equation modeling improves over traditional multivariate modeling approaches because it can quantify in relative terms direct and indirect relationships between correlated variables (Grace et al., 2010). However, it cannot account for the bidirectionality inherent to the gut-brain axis (Burokas et al., 2015). Because gut microbiota can produce hormones and neurotransmitters that influence central nervous system function (Carabotti et al., 2015), determining the directionality of gut-brain connections in the wild will inevitably require the application of controlled experimental manipulations in field settings. Moreover, uncovering the capacity of gut-brain axis connections to promote ecological adaptation will necessarily rely on the inclusion of fitness and fitness-related measures in such studies. For example, what dimensions of host reproduction and longevity are impacted by microbial responses to ecologically-triggered host hormone production? Broadly, advancing our understanding of the gut-brain axis in nature will therefore require a delicate balance of field-based experiments in response to naturalistic stressors, and assessment of fitness costs and benefits within an ecological context (Davidson et al., 2020; Greyson-Gaito et al., 2020). More specifically, conservation efforts may benefit by considering the potential for indirect ecophysiological effects of habitat degradation in the management of vulnerable species populations.

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DATA AVAILABILITY STATEMENT

All data and code for this project are available at the following figshare repository: https://figshare.com/s/fdb14e7ca9ca6f269f42. A tutorial on the Bayesian dyadic models can be found at AR's github page: https://github.com/nuorenarra.

AUTHOR CONTRIBUTIONS

LP carried out the statistical analyses of the data and led writing of the manuscript. AR conceptualised the hypotheses herein, led the data collection, developed statistical methods for analysis of the data and helped writing the manuscript. AS participated in the data collection, derived the habitat diversity measures from GIS data and provided feedback on the manuscript. RW developed the field system, participated in the data collection and provided feedback on the manuscript. AH helped develop the analysis methods and provided feedback on the manuscript.

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