

1 **Glucocorticoids coordinate changes in gut microbiome composition in wild North**
2 **American red squirrels**

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4 Lauren Petruzzo¹, Tiantian Ren², Martin Wu², Rudy Boonstra³, Rupert Palme⁴, Stan
5 Boutin⁵, Andrew G. McAdam⁶, Ben Dantzer^{1,7}

6

7 1 Department of Psychology, University of Michigan, Ann Arbor, MI, USA, 48108

8 2 Department of Biology, University of Virginia, Charlottesville, VA, USA, 22904

9 3 Department of Biological Sciences, Centre for the Neurobiology of Stress, University
10 of Toronto Scarborough, Toronto, ON, Canada, M1C 1A6

11 4 Unit of Physiology, Pathophysiology and Experimental Endocrinology, Department of
12 Biomedical Sciences, University of Veterinary Medicine Vienna, Veterinärplatz 1,
13 Vienna, 1210, Austria

14 5 3 Department of Biological Sciences, University of Alberta, Edmonton, Alberta,
15 Canada, T6G 2E9

16 6 Department of Ecology and Evolutionary Biology, University of Colorado, Boulder,
17 CO, USA.

18 7 Department of Ecology and Evolutionary Biology, University of Michigan, Ann Arbor,
19 MI, USA 48108

20

21 Correspondence

22 Lauren Petruzzo (petruzzo@umich.edu)

23 Ben Dantzer (dantzer@umich.edu)

24

25 **Abstract**

26

27 Gut microbiome diversity plays an important role in host health and fitness, in part
28 through the diversification of gut metabolic function and pathogen protection. Elevations
29 in glucocorticoids (GCs) appear to reduce gut microbiome diversity in experimental
30 studies, suggesting that a loss of microbial diversity may be a negative consequence of
31 increased GCs. However, given that ecological factors like food availability and

32 population density may independently influence both GCs and microbial diversity,
33 understanding how these factors structure the GC-microbiome relationship is crucial to
34 interpreting its significance in wild populations. Here, we used an ecological framework
35 to investigate the relationship between GCs and gut microbiome diversity in wild North
36 American red squirrels (*Tamiasciurus hudsonicus*). We found that higher GCs predicted
37 lower gut microbiome diversity and an increase in metabolic taxa. In addition, we
38 identified a loss of potentially pathogenic bacteria with increasing GCs. Both dietary
39 heterogeneity and an upcoming masting event exhibited direct effects on gut
40 microbiome diversity, whereas conspecific density and host reproductive activity
41 impacted diversity indirectly via changes in GCs. Together, our results suggest that GCs
42 coordinate the effects of ecological change and host biology on gut microbiome
43 diversity, and highlight the importance of situating the GC-microbiome relationship
44 within an ecological framework.

45

46 **Introduction**

47

48 The intimate symbiosis between animals and their microbiomes has become a major
49 area of focus for animal behavior, ecology, and evolution research over the last decade.
50 In vertebrates, the gut microbiome in particular appears to interact strongly with other
51 host physiological systems [1]. Gut microbiota are sensitive to changes in host immune
52 function [2], brain development and behavior [3,4], circadian rhythms [5], and
53 metabolism [6]. Beyond these effects, the gut microbiome also responds to the host
54 endocrine system. In wild female primates, reproductive hormones like estrogen and
55 progesterone are associated with differences in gut microbiome composition [7,8]. In
56 humans, both androgens and estrogens, as well as metabolic hormones like insulin, are
57 linked to variation in gut microbiota [9,10]. Such connections reflect a larger “gut-brain
58 axis” through which the gut microbiota and nervous system communicate [11].

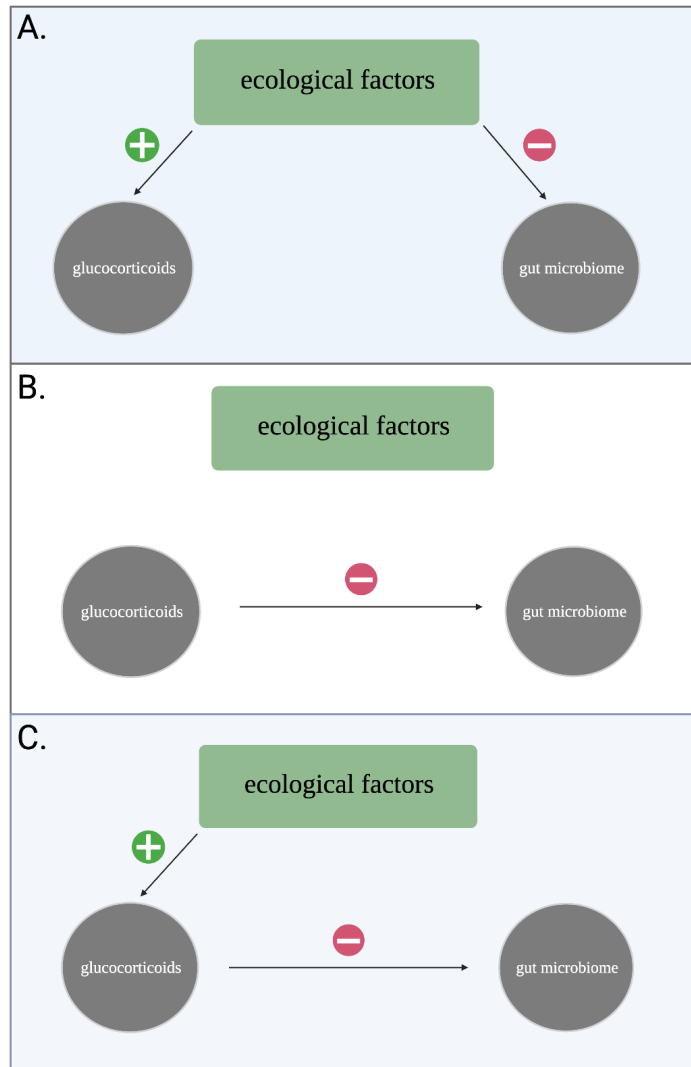
59

60 Recently, glucocorticoids (GCs) have emerged as a central component of the gut-brain
61 axis [12]. GCs are metabolic hormones produced via the activation of the hypothalamic-
62 pituitary-adrenal axis. They are involved in energy regulation and the physiological

63 stress response [13], and can induce adaptive phenotypic plasticity in response to
64 environmental change [14]. For example, elevated GCs can enhance fitness by
65 facilitating transitions between life history stages [15], supporting the energetic demands
66 of reproduction [16], and improving survival in response to fluctuating temperatures and
67 food availability [17]. GCs may also induce adaptive plasticity in the gut microbiome, a
68 host microbial community that responds rapidly to changes in ecology. Shifts in gut
69 microbiome composition can regulate energy balance as ambient temperatures rise and
70 fall [18], and can enhance digestion efficiency as an animal's energetic demands
71 increase (e.g., during reproduction) or as resource availability fluctuates [19–21].

72

73 One measure of gut microbiome composition - alpha diversity, a measure of the
74 taxonomic diversity within a community - appears particularly sensitive to changes in
75 host GCs. A taxonomically diverse microbiome confers community stability and
76 resilience, whereas a loss of diversity is presumed to have detrimental consequences
77 via increased host susceptibility to pathogenic infection [22,23]. Animal studies in which
78 GCs have been experimentally elevated have documented reduced gut microbiome
79 diversity in response to elevated GCs [24,25], while studies in unmanipulated
80 populations have found no relationship [26,27]. This inconsistency may indicate that the
81 link between GCs and gut microbiome diversity is modified by ecological factors (Figure
82 1), yet these are rarely included in such analyses (Table 1). For example, an increase in
83 food availability can cause transient elevations in GCs if conspecific density also
84 increases due to that elevation in food availability [28]. If elevated density results in
85 more frequent social interactions, it may enhance microbiome diversity directly via
86 increased microbial transmission among conspecifics [29]. Such environmental
87 covariance may drive the absence of a relationship between GCs and microbiome
88 diversity in unmanipulated populations (Figure 1) [30–32], necessitating a more
89 nuanced approach to determine how GCs impact the gut microbiome in wild animals.



90

91 **Figure 1. Conceptual model demonstrating how ecological factors may structure**
 92 **the relationship between glucocorticoids and the gut microbiome. (A)**

93 Environmental covariance results in direct effects on both variables and diminishes a
 94 detectable effect of GCs on gut microbiome diversity. (B) Ecology does not influence

95 either variable and a direct effect of GCs on gut microbiome diversity is preserved. (C)

96 Ecological factors influence gut microbiome diversity indirectly via host GCs. Note that

97 the three scenarios are not mutually exclusive, such that a combination of direct (A) and

98 indirect (B) effects may result in the appearance or absence of a relationship between

99 GCs and gut microbiome diversity.

100

Study	Species	N	GCs	Effect	Ecological factors included?
Noguera et al., 2018	Gulls (<i>Larus michahellis</i>)	29	Experimental	↓ α -diversity	No
Stothart et al., 2019	gray squirrels (<i>Sciurus carolinensis</i>)	29	Natural	No effect	No
Uren Webster et al., 2020	Atlantic salmon (<i>Salmo salar</i>)	168	Experimental	↓ α -diversity	No
Vlčková et al., 2018	Gorillas (<i>Gorilla gorilla gorilla</i>)	42	Natural	No effect	No

102

103 **Table 1.** Prior experimental and correlational studies on the relationship between
 104 glucocorticoids and gut microbiome alpha diversity in captive and wild vertebrates.

105

106 In this study, we test the hypothesis that ecological factors structure the relationship
 107 between GCs and gut microbiome diversity in wild North American red squirrels
 108 (*Tamiasciurus hudsonicus*) living in the Yukon, Canada. Red squirrels are highly
 109 territorial animals that experience dramatic shifts in food availability and population
 110 density as a result of fluctuations in their preferred food source, seeds from white
 111 spruce trees (*Picea glauca*) [33]. Squirrels incorporate other food sources into their diet
 112 when seasonally available (e.g., fungi, bark, leaves, flowers) [34], resulting in changes
 113 in dietary diversity that may directly impact gut microbiome diversity. However, spruce
 114 seeds comprise the majority of their diet [34] despite their episodic availability. Masting
 115 events occur every 4-6 years in white spruce, resulting in the production of a
 116 superabundance of cones containing seeds that become available in the autumn. By
 117 contrast, few to no cones are available in non-mast years [33,35]. In anticipation of an
 118 upcoming spruce mast, squirrels exhibit an extended breeding season and concomitant

119 behavior changes: territoriality breaks down and conspecific interactions are expected
120 to increase due to increased breeding frequency and infanticidal behavior [36,37]. An
121 upcoming spruce mast may thus exert direct positive effects on gut microbiome diversity
122 via more frequent social interactions, which leads to greater horizontal microbial
123 transmission [29].

124

125 Squirrel densities also fluctuate in parallel with food pulses, with densities at their lowest
126 in the months prior to a mast and highest in the spring following a mast [28,38].

127 Although sociality is expected to increase gut microbiome diversity in group-living
128 animals [29], this effect may not occur in territorial species [39]. For example, elevated
129 conspecific densities result in increased frequency of long-range territorial vocalizations
130 emitted by red squirrels in our study population [40], which can in turn reduce interaction
131 frequency by deterring territorial intrusions [39,41]. Indeed, conspecific interactions in
132 squirrels do not appear to vary with density, and the number of territorial intruders has
133 been both negatively correlated [40] and unrelated [41] to density. Given that both
134 actual and perceived increases in density cause GC elevations in red squirrels [28,42],
135 density may have indirect rather than direct effects on microbiome diversity due to the
136 psychosocial stress of anticipating greater competition [28].

137

138 In line with prior studies (Table 1), we predicted to find an overall negative association
139 between GCs and gut microbiome alpha diversity, along with an increase in pathogenic
140 taxa and taxa involved in host metabolism with increasing GCs. We then used a
141 multivariate structural equation modeling approach [43,44] to integrate GCs, gut
142 microbiome diversity, and ecological variables into a single causal network [45]. We
143 tested a set of *a priori* hypothesized relationships related to the direct and indirect
144 effects of dietary heterogeneity, an upcoming spruce mast, and conspecific density on
145 both GCs and gut microbiome diversity (Figure S1). We expected to find that dietary
146 heterogeneity and an upcoming spruce mast would have direct positive effects on gut
147 microbiome diversity. Conversely, we predicted that density would have an indirect
148 negative effect on diversity by way of GC elevations. We additionally included biological
149 factors (reproductive activity, sex, age) in our analysis, given their potential effects on

150 GCs and microbiome composition [46–48]. We predicted that reproductive activity had
151 positive direct effects on both GCs and gut microbiome diversity, as a result of
152 increased energetic demands [49,50] and conspecific interactions, respectively. In line
153 with prior studies, we expected that older age would predict lower microbiome diversity,
154 and that males would exhibit greater microbiome diversity due to travel across territories
155 for multiple mating in the breeding season [51].

156

157 **Results**

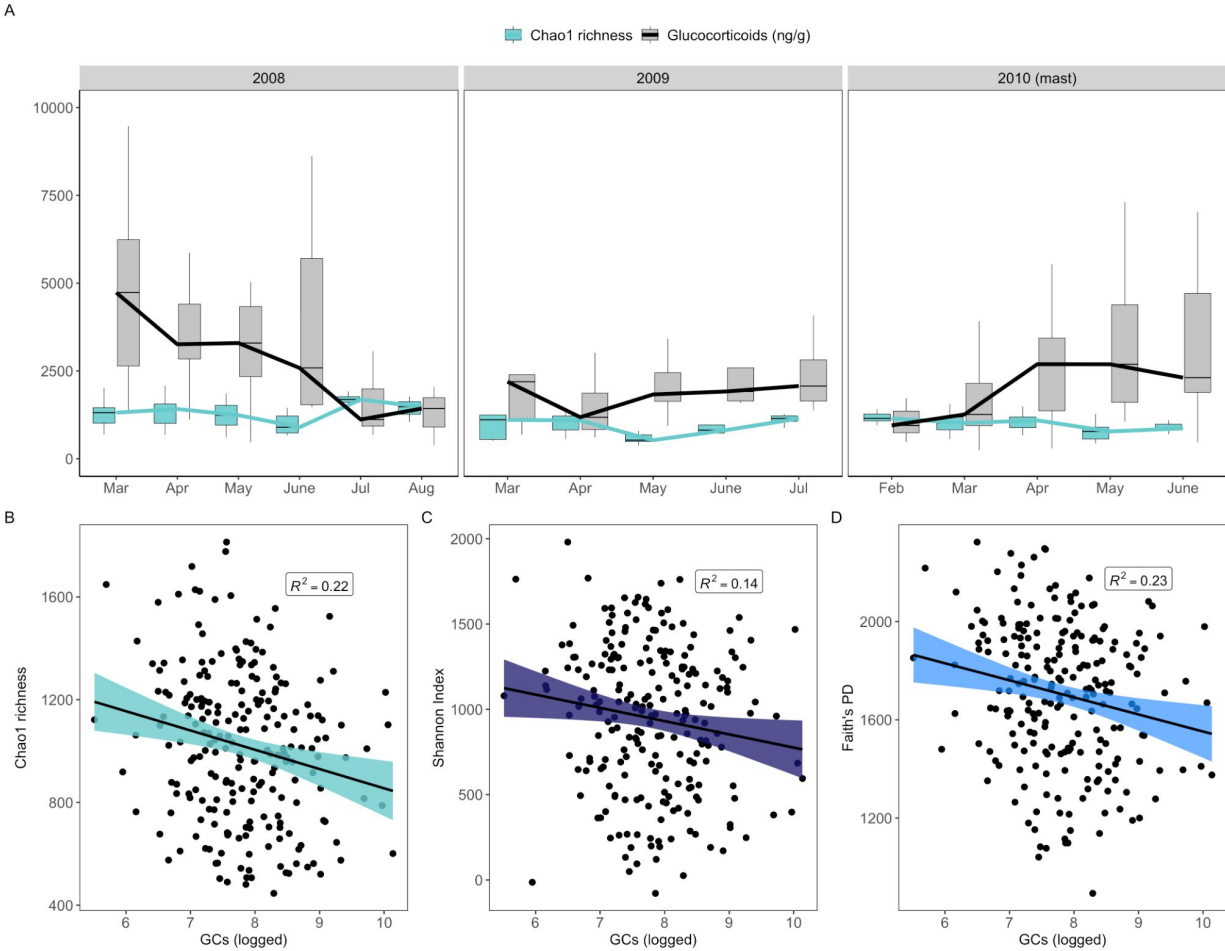
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159 **1. Gut microbiome diversity is negatively associated with glucocorticoids**

160

161 Both gut microbiome alpha diversity and GCs were highly variable across seasons in
162 each of our sampling years, with gut microbial diversity reaching its maxima during the
163 summer months of July and August (Figure 2A), coinciding with increased dietary
164 diversity [34,52]. GCs were highest in early spring (March), with the exception of the
165 mast year of 2010 in which GCs steadily increased across the first part of the year
166 (Figure 2A). Consistent with our predictions and in line with prior studies in which GCs
167 were experimentally manipulated (Table 1), GCs were negatively associated with gut
168 microbiome alpha diversity. Individuals with greater GC concentrations exhibited
169 relatively lower taxonomic diversity (i.e. species richness, Chao1: estimate \pm SE: -75.05
170 ± 25.91 , $t = -2.90$, $P < 0.01$; Figure 2B). Greater GCs were also associated with lower
171 Shannon Indices, a composite measure of species richness and evenness (Shannon: $-$
172 77.64 ± 36.00 , $t = -2.16$, $P < 0.05$, Figure 2C), as well as lower phylogenetic diversity in
173 the gut microbial community (Faith's PD: -69.51 ± 26.59 , $t = -2.61$, $P < 0.01$, Figure 2D).
174 The negative relationship between GCs and gut microbiome alpha diversity was robust
175 to individual variation in GC production, with higher individually-averaged GCs similarly
176 predicting lower species richness (estimate \pm SE: -0.027 ± 0.011 , $t = -2.45$, $P < 0.05$).

177



178

179 **Figure 2. Host production of glucocorticoids is negatively associated with gut**
 180 **microbiome alpha diversity.** (A) Boxplot and line graph showing the opposing
 181 relationship between mean fecal glucocorticoid metabolites (GCs, scaled) and median
 182 gut microbiome diversity (Chao1 richness) across each month of the three sampling
 183 years. Outliers removed from plot for visualization purposes. (B) Partial residual plots
 184 (points represent individual samples) showing the relationship between gut microbiome
 185 taxonomic richness (Chao1), (C) taxonomic richness and evenness (Shannon Index),
 186 and (D) phylogenetic diversity (Faith's Phylogenetic Diversity) and matched fecal
 187 glucocorticoid concentrations (GCs) (N = 227).

188

189

190 **2. Glucocorticoids predict variation in gut microbiome composition at the**
191 **taxonomic level**

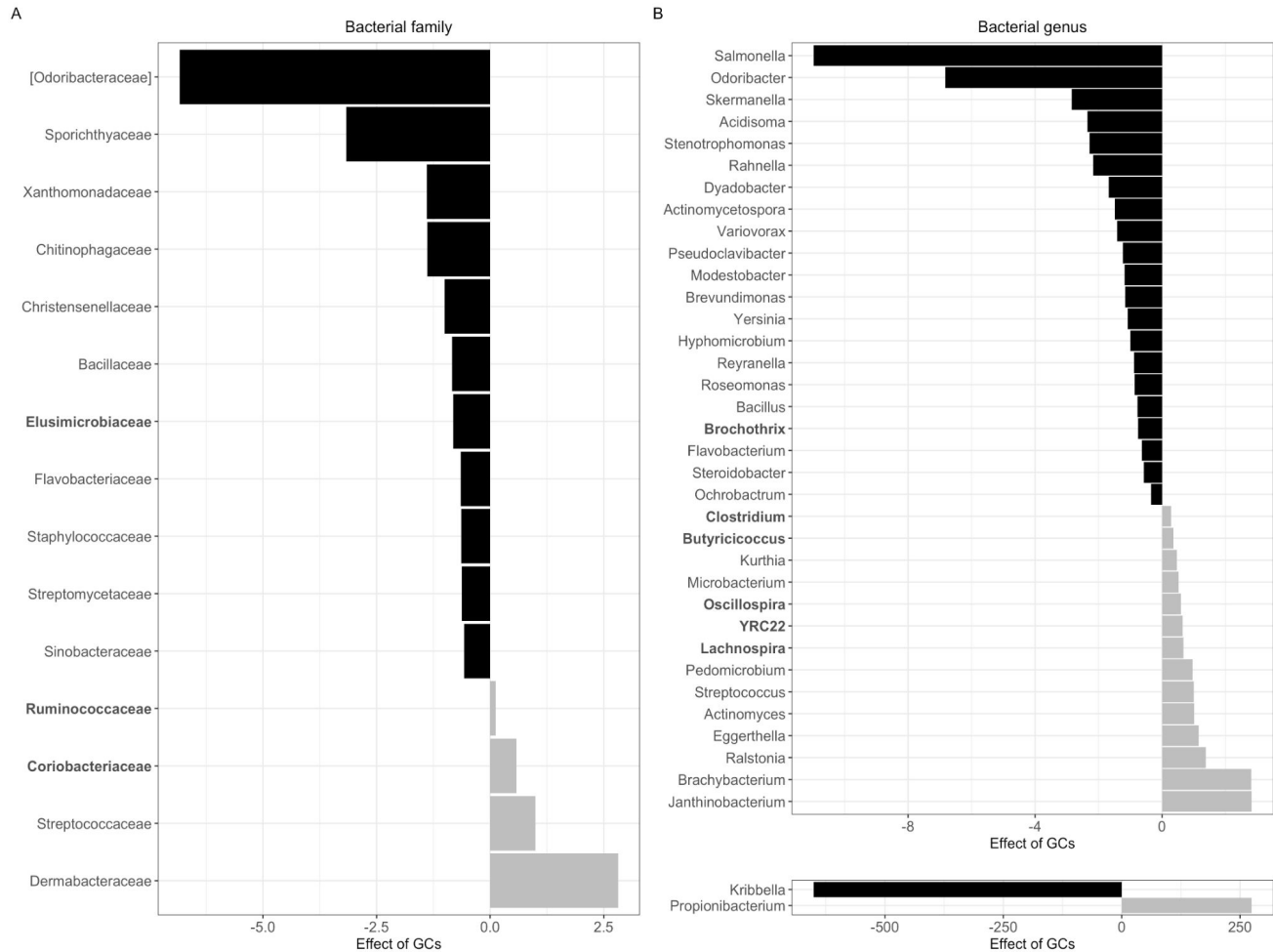
192

193 We constructed a series of negative binomial linear mixed-effects models to determine
194 how reduced gut microbiome alpha diversity is reflected in changes at the taxonomic
195 level and identify taxa whose relative abundances changed with increasing GCs. We
196 found that elevated GCs were associated with changes in gut microbiome composition
197 at both the family (Figure 3A) and genus (Figure 3B) levels. Increased GCs predicted
198 shifts in the relative abundance of 15 bacterial families, predominantly a decrease in
199 rare bacterial families (i.e. taxa that contribute < 0.01% relative abundance to the
200 microbial community) (Figure 3A; Table S1). An exception was a reduction in
201 *Elusimicrobiaceae*, which contributed an average of 0.13% relative abundance to the
202 gut microbiome community (estimate = -0.81, $P_{\text{FDR}} < 0.0001$). By contrast, elevated
203 GCs were associated with an increase in *Coriobacteriaceae* (estimate = 0.57, $P_{\text{FDR}} <$
204 0.01), *Streptococcaceae* (estimate = 1.0, $P_{\text{FDR}} < 0.0001$), and *Dermabacteraceae*
205 (estimate = 2.81, $P_{\text{FDR}} < 0.0001$), and *Ruminococcaceae* (estimate = 0.12, $P_{\text{FDR}} < 0.02$)
206 (Figure 3A). *Ruminococcaceae*, a family of largely cellulolytic and fibrolytic bacteria, is
207 an abundant (~25% relative abundance) and core taxa in the red squirrel gut
208 microbiome (Ren et al., 2017).

209

210 At the genus level, the relative abundance of 22 bacterial genera was significantly
211 reduced with increasing GCs. Similar to changes at the family level, the majority of
212 bacterial genera reductions were rare taxa (Figure 3B) with the exception of *Brochothrix*
213 (mean 0.02% relative abundance). Contrary to our predictions but in line with a prior
214 study on birds (Noguera et al., 2018), we found that two potentially pathogenic genera --
215 *Yersinia* (estimate = -1.09, $P_{\text{FDR}} < 0.001$) [53] and *Salmonella* (estimate = -10.98, $P_{\text{FDR}} <$
216 0.0001) [54] -- decreased in relative abundance with increasing host GCs. Conversely,
217 a greater proportion of abundant taxa were found to increase with increasing GCs
218 (Figure 3B). Greater host GCs predicted greater relative abundances of *Clostridium*
219 (estimate = 0.28, $P_{\text{FDR}} < 0.01$), *Butyrivicoccus* (estimate = 0.35, $P_{\text{FDR}} < 0.01$),

220 *Oscillospira* (estimate = 0.60, $P_{FDR} < 0.0001$), *YRC22* (estimate = 0.65, $P_{FDR} < 0.01$), and
 221 *Lachnospira* (estimate = 0.67, $P_{FDR} < 0.01$).
 222



223
 224
 225

Figure 3.

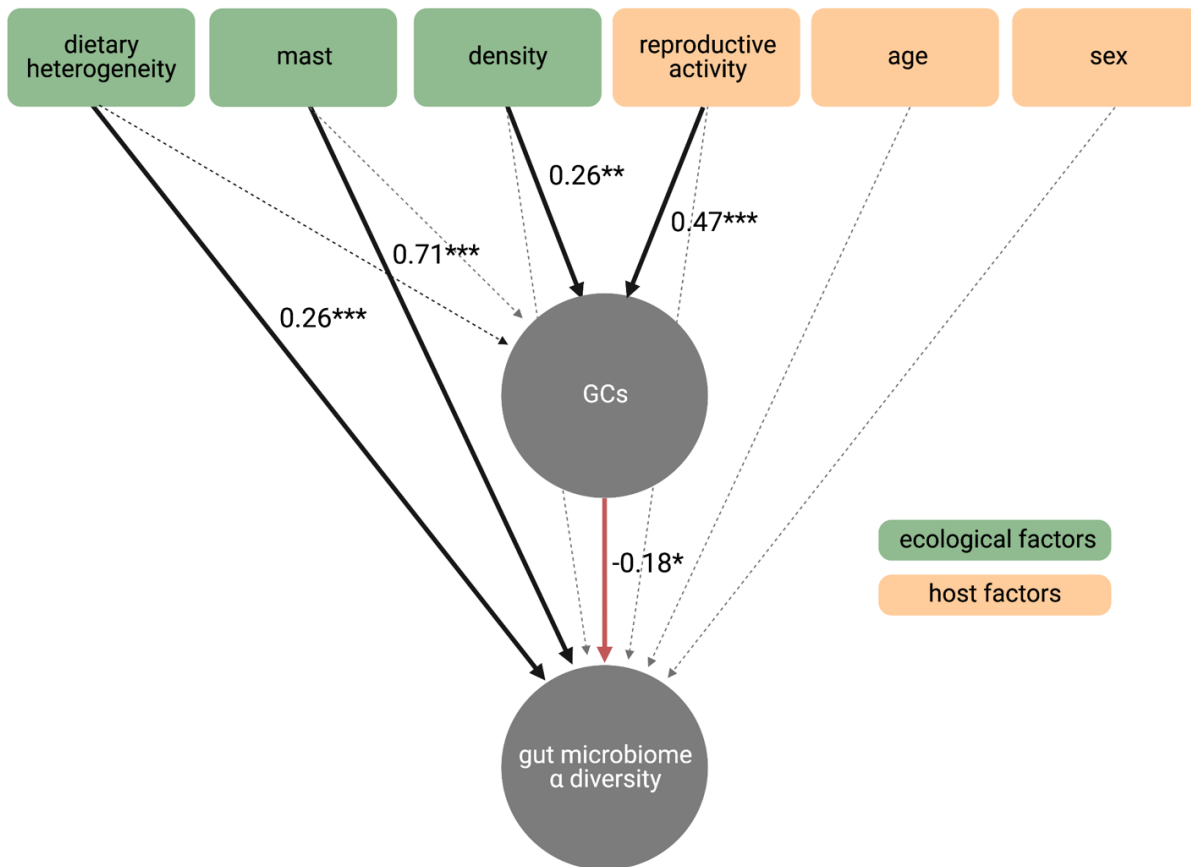
226 **Figure 3. Increased glucocorticoids predict the loss and gain of bacterial taxa in**
 227 **the red squirrel gut microbiome.** Barplots depict bacterial families (A) and genera (B)
 228 whose relative abundance was significantly (Benjamini-Hochberg adjusted $P < 0.05$)
 229 predicted by changes in host glucocorticoid concentrations. Bold taxa exhibited a mean
 230 relative abundance $> 0.01\%$. Effects of GCs reflect model estimates generated by
 231 negative binomial mixed models testing the effect of GCs on the relative abundance of
 232 each bacterial taxa, controlling for collection date, food supplementation status, and
 233 individual ID. Black bars represent a decrease in relative abundance with increasing

234 GCs; grey bars represent an increase with increasing GCs. Taxa depicted at the bottom
235 of Panel B (*Kribbella*, *Propionibacterium*) exhibited model estimates ~10x larger than
236 the rest of the taxa and are therefore separated from the main plot solely for
237 visualization purposes.

238

239 To determine how ecological and host factors contributed to the effects of GCs on gut
240 microbiome alpha diversity, we fit a structural equation model (SEM) based on a set of *a*
241 *priori* hypothesized pathways (Figure S1). The SEM was constructed to test the relative
242 direct and indirect effects of three ecological factors (dietary heterogeneity, an
243 upcoming spruce mast, and conspecific density) and three host factors (reproductive
244 activity, age, and sex) on GCs and gut microbiome diversity. Overall, the SEM revealed
245 direct and indirect pathways by which ecological and host factors exert cascading
246 effects on microbial diversity. In line with our predictions, dietary heterogeneity and the
247 presence of an upcoming spruce mast both exhibited direct effects on gut microbiome
248 diversity, such that a more heterogeneous diet (standardized $\beta = 0.26$, $P < 0.001$) and
249 an upcoming spruce masting event (standardized $\beta = 0.71$, $P < 0.001$) led to greater
250 microbial diversity. Tests of directed separation revealed that there was no effect of
251 either dietary heterogeneity or an upcoming spruce mast on host GCs. By contrast,
252 conspecific density and reproductive activity indirectly, but not directly, affected gut
253 microbiome diversity via changes to host GCs. Higher densities (standardized $\beta = 0.26$,
254 $P < 0.01$) and reproductive activity (standardized $\beta = 0.47$, $P < 0.001$) predicted greater
255 GC concentrations, which in turn reduced microbial diversity (standardized $\beta = -0.18$, P
256 < 0.05). There was no effect of age or sex on gut microbiome diversity, and tests of
257 directed separation similarly found no effect of age or sex on host GCs.

Fisher's C: 9.61; P > 0.05; AICc: 50.24



258

259 **Figure 4. Ecology and host biology influence gut microbial diversity via**
260 **glucocorticoids.** Structural equation model assessing direct and indirect effects of
261 ecological and host factors on glucocorticoids (GCs) and gut microbiome alpha diversity
262 (Chao1 richness). Solid black arrows represent significant positive paths; solid red
263 arrows represent significant negative paths; dotted arrows represent non-significant
264 paths. Text labels indicate standardized beta estimates (i.e., effect sizes) and
265 significance (P < 0.05 *, P < 0.01 **, P < 0.001 ***) for each of the predicted pathways
266 tested in the SEM.

267

268 Discussion

269

270 Determining if ecological factors structure the relationship between glucocorticoids
271 (GCs) and gut microbiome alpha diversity is crucial to interpreting the adaptive value of
272 the GC-microbiome connection in wild animals. We show that elevated GCs robustly

273 predict reduced gut microbiome diversity in wild red squirrels living in an ecosystem
274 characterized by fluctuations in food and conspecific density. Greater host GCs were
275 associated with reductions in rare and pathogenic taxa, and a gain of commensal
276 bacteria involved in butyrate production and cellulose metabolism, suggesting reduced
277 diversity may reflect reorganization of gut metabolic function. Incorporating ecological
278 and host factors into a single causal network revealed a cascade of direct and indirect
279 effects on gut microbiome diversity, and the retention of a significant negative
280 relationship between diversity and GCs. Together, our results demonstrate that the link
281 between GCs and gut microbiome alpha diversity is robust to ecological factors that
282 directly influence the gut microbiome in wild populations. Further, our findings suggest
283 that GCs may integrate changes in ecology and host biology to induce gut microbiome
284 plasticity.

285

286 Red squirrel gut microbiome alpha diversity varied across seasons and peaked in
287 summer, coinciding with the period of greatest dietary heterogeneity [34,52] (Figure 2A).
288 GCs exhibited greater variability overall: GCs reached their peak in the early spring in
289 non-mast years (2008, 2009), but in summer of the mast year (2010) (Figure 2A).
290 Despite this variability, there was a significant negative association between GCs and
291 gut microbiome diversity such that individuals with greater GCs exhibited lower alpha
292 diversity across three separate measures (species richness, evenness, and
293 phylogenetic diversity) (Figure 2B-D). Though prior studies in unmanipulated
294 populations did not detect a relationship between GCs and gut microbiome alpha
295 diversity (Table 1), but see [55] for oral microbiome), our results align with experimental
296 studies in which GCs were manipulated. This suggests that gut-brain axis
297 communication, particularly between GCs and gut microbiome composition, is under
298 strong selection in our population.

299

300 As GCs increased, the taxa that decreased in the gut microbiome were overwhelmingly
301 rare taxa that contributed < 0.01% in relative abundance to the overall community (e.g.,
302 *Odoribacteriaceae*, *Sporichthyaceae*) (Figure 3). This finding aligns with expectations
303 about the effects of disturbances on gut microbiome composition from an ecological

304 perspective [56]. Resilience to microbial disturbances is greater among abundant taxa
305 than non-abundant taxa [57], likely due to divergent patterns of colonization and
306 succession. Abundant taxa retain their position in microbial communities via selective
307 filtering and by occupying core niches [56]. By contrast, rare taxa are incorporated into
308 the microbiome largely via stochastic processes, though they contribute significantly to
309 community alpha diversity measures [58]. A reduction in rare taxa with increasing GCs
310 in our population may therefore indicate that the effects of elevated GCs on rare
311 bacteria in the gut mimic those expected by broader microbial disturbances (e.g.,
312 antibiotics, infection) [59].

313

314 A decrease in the relative abundance of rare bacteria may also serve to reorganize host
315 metabolic priorities through replacement by core taxa that can better support changes in
316 host energetic demands. Overall, we found that increases in host GCs were
317 accompanied by increases in microbial taxa involved in host metabolism (Figure 3).
318 Both *Oscillospira*, which correlates with the consumption of spruce buds in the late
319 spring [52], and *Coriobacteriaceae*, a common rodent gut microbe involved in energy
320 metabolism [60], increased in relative abundance with increasing GCs. In experimental
321 settings, housing stress caused an increase in *Coriobacteriaceae* [61], suggesting that it
322 may similarly contribute to maintaining energy balance in wild rodents facing
323 challenging environmental conditions. We additionally found that individuals with
324 elevated GCs exhibited greater relative abundances of *Ruminococcaceae*, a bacterial
325 family of cellulolytic and fibrolytic bacteria involved in acclimating to dietary changes in
326 wild animals [20,62]. Together, our results suggest that in our study population, GCs
327 may coordinate adaptive shifts in gut microbiome composition in response to increased
328 energetic demands, seasonal changes in diet, or both.

329

330 Resistance to pathogens has been proposed as one of the major evolutionary
331 advantages conferred by host microbial communities [63]. Butyrates, compounds
332 produced via fermentation by microbiota in the large intestine [64], are particularly
333 critical to preventing intestinal pathogen invasion [65]. We found that the butyrate-
334 producing bacteria *Butyricicoccus* (family *Ruminococcaceae*) and *Clostridium* were

335 elevated in the red squirrel gut microbiome as GCs increased (Figure 3). Moreover,
336 elevated GCs were associated with lower relative abundances of two potentially
337 pathogenic genera: *Salmonella* and *Yersinia*. *Salmonella* are rod-shaped, Gram-
338 negative bacteria that can cause gastroenteritis in both rodents and humans upon
339 infection [66]. Similarly, *Yersinia*, which includes *Y. pestis*, *Y. pseudotuberculosis*, and
340 *Y. enterocolitica*, are commonly harbored in the gut microbiota of wild rodents and lead
341 to enteric and systemic disease [54,67], though we note there is currently no evidence
342 of *Yersinia* disease in our population. While a loss of these taxa with increasing GCs
343 contradicts theoretical expectations of pathogen susceptibility as microbial diversity
344 decreases, our findings align with a prior study in free-living birds in which elevated GCs
345 similarly reduced the relative abundance of intestinal pathogens [24], and in piglets in
346 which GCs reduced *Salmonella*, specifically [68]. Together, these data suggest that
347 elevations in GCs may confer short-term protection against gastrointestinal pathogens,
348 potentially through transient increases in gut immune function or butyrate production.
349 However, the pathogenicity of these taxa can only be confirmed by a strain-level
350 genomic analysis beyond the analysis performed in this study, and determining
351 differences in production of butyrates with variation in host GCs requires gene functional
352 data. We thus encourage future studies to implement high resolution bioinformatic
353 approaches (e.g., shotgun sequencing, metabolomics) whenever possible to better
354 understand these patterns.

355

356 To disentangle the effects of ecological and host factors on the relationship between
357 GCs and the gut microbiome, we constructed a structural equation model (SEM) based
358 on a set of causal *a priori* hypothesized pathways (Figure S1) [43,69]. As predicted, an
359 upcoming spruce mast had a direct positive effect on gut microbiome diversity, and this
360 path was the strongest path in the SEM (standardized $\beta = 0.71$; Figure 4). An increase
361 in gut microbiome diversity in the mast year of 2010, compared to the non-mast years of
362 2008 and 2009, aligns with our expectation of territorial breakdown and increased social
363 interactions due to an extended breeding season in the months leading up to a masting
364 event [36,37]. Given that the positive link between sociality and gut microbiome diversity
365 is well-supported at least in some taxa [8,29,70], squirrels may exhibit increased gut

366 microbiome diversity due to greater horizontal transmission of microbes between
367 conspecifics as social interactions become more frequent.

368

369 Similar to the effects of an upcoming spruce mast, dietary heterogeneity had a direct
370 positive effect on microbial diversity, though this effect was approximately 2.5x weaker
371 than that of the upcoming mast (standardized $\beta = 0.26$). Gut microbiome alpha diversity
372 was greatest in the months in which the food available to red squirrels was most
373 heterogeneous (e.g., fungi, buds, and seeds) [34]. Indeed, a varied diet is expected to
374 increase microbiome diversity through greater substrate selection for diverse ecological
375 niches [71]. This effect of dietary heterogeneity on gut microbiome diversity, coupled
376 with prior work on the relationship between diet and gut microbiome composition in this
377 population [52], suggests that the red squirrel gut microbiome responds rapidly to shifts
378 in food availability.

379

380 By contrast, conspecific density indirectly, but not directly, impacted gut microbiome
381 diversity by way of GCs, lending support to previous findings that the frequency of social
382 interactions (and thus horizontal transmission) is not related to squirrel density in this
383 population [40]. In line with our expectations, elevated conspecific densities predicted
384 increased host production of GCs (standardized $\beta = 0.26$) [28], which in turn predicted
385 reduced gut microbiome diversity (standardized $\beta = -0.18$) (Figure 4). Red squirrels are
386 highly sensitive to changes in density, and signals of both actual and perceived elevated
387 density lead to GC increases independent of other ecological factors that covary with it
388 (e.g., food) [28]. That elevated density reduced gut microbiome diversity via increasing
389 GCs aligns with our understanding of the regulation of the gut-brain axis by the social
390 environment, stress, and psychological state in laboratory rodents [72]. In the wild,
391 vocalizations can buffer individuals from physical interactions with conspecifics even in
392 times of high densities in highly territorial animals like red squirrels. Our results suggest
393 that the indirect effects of increased density on gut microbiome diversity likely reflect the
394 psychosocial stress of increased competition, demonstrating a novel link between social
395 stress and the gut-brain axis in a wild mammal.

396

397 Reproductive state was the only host factor to exhibit an effect on gut microbiome
398 diversity, and the effect was indirect and the strongest path to GCs in the model
399 (standardized $\beta = 0.47$). As predicted, being reproductively active (e.g., scrotal for
400 males, and breeding, gestating, or lactating for females) predicted greater GCs, and in
401 turn a reduction in microbial diversity (standardized $\beta = -0.18$). In both males and
402 females, reproduction increases host metabolic demands broadly [50]and, in females in
403 our population, GCs specifically [49]. A consequent reduction in microbiome diversity
404 may therefore better support host energy balance by increasing the relative abundance
405 of core microbiota at the expense of rare taxa that contribute less to the metabolic
406 functions of the community. Contrary to our predictions, we found no effect of sex or
407 age on microbial diversity, and the SEM did not identify an effect of either on GCs via
408 tests of directed separation [69]. Studies in humans and other mammals have found
409 mixed effects of age on both GCs and microbial diversity [48,73,74]. Sex effects on
410 microbiome composition related to hormones and behavior have been documented in
411 experimental rodent models [47,75], but studies in wild populations have not typically
412 found sex differences in gut microbiome composition [76]. Our results, coupled with
413 inconsistent findings in other animals, suggest that the effects of age and sex on gut
414 microbiome alpha diversity are likely species-specific, and/or that the effects of other
415 factors (e.g., reproductive state, diet) may overwhelm the effects age and sex on
416 microbial diversity.

417

418 While the mechanisms by which GCs impact gut microbiome diversity are not well
419 understood, a number of potential pathways may explain how host GCs impact gut
420 microbiota. First, GCs can alter lipid metabolism, leading to lipid accumulation in the gut
421 [77]. Increased lipid metabolism reduces taxonomic diversity in the gut microbiome of
422 laboratory rodents as some bacteria exhibit sensitivity to lipid accumulation [78].
423 Second, GCs may impact microbial diversity via circadian rhythm dysregulation.
424 Elevated GCs can disrupt host circadian rhythms in laboratory settings [78], and
425 circadian disruption directly reduces gut microbiome alpha diversity in mice [79]. Finally,
426 increased GCs can result in a decrease in the synthesis of mucins, proteins that make
427 up the mucosal layer of the gut in which microbiota live. The mucosal layer is integral to

428 the stability of the gut microbiome and largely determines its composition [80]. A down-
429 regulation of mucin synthesis as a result of elevated GCs may therefore disrupt gut
430 microbiome stability by reducing the resilience of the mucosal layer, leading to a
431 reduction in non-core bacteria and an overall loss of community diversity [81].

432

433 Of note is the bidirectionality of the gut-brain axis demonstrated in laboratory rodent
434 studies [82,83], and thus the potential bidirectionality of the GC-microbiome relationship
435 in wild animals. Here, we focused on the unidirectional effects of GCs on gut
436 microbiome diversity similar to prior studies (Table 1). However, gut microbiota can
437 themselves regulate the hypothalamic-pituitary-adrenal axis, such that shifts in gut
438 microbiome composition may directly modulate host production of GCs [84] and
439 contribute to a feedback loop between the two systems [85]. Statistical constraints
440 inherent to structural equation modeling prevented us from incorporating a bidirectional
441 relationship between GCs and microbial diversity into this study [69]. Nonetheless, the
442 bidirectionality of the gut-brain axis has important implications for the evolution of the
443 relationship between GCs and microbial diversity in wild mammals [11]. We encourage
444 future research on wild populations to implement experimental frameworks when
445 possible to better characterize the complexity and adaptive value of the GC-gut
446 microbiome relationship.

447

448 **Methods**

449

450 Ethics Statement

451 All methods were carried out in accordance with relevant guidelines and regulations. All
452 research methods and protocols were conducted under animal ethics approvals from
453 Michigan State University (AUF#04/08-046-00), University of Guelph (AUP#09R006),
454 and University of Michigan (PRO00005866). All authors complied with the ARRIVE
455 guidelines.

456

457 Study population

458 Subjects for this study were wild North American red squirrels (*Tamiasciurus*
459 *hudsonicus*) inhabiting a natural environment in southwest Yukon, Canada (61°N,
460 138°W). All subjects were continuously monitored as part of the Kluane Red Squirrel
461 Project, a long-term field study that has been conducting a combination of live-trapping,
462 focal behavioral observations, sampling, and experimental manipulations in the area
463 since 1987 [38,86]. Individual squirrels are marked with small metal ear tags and unique
464 combinations of colored wire threaded through the ear tags. Individuals are monitored
465 from birth to death in each year of study, roughly from March to October, using live-
466 trapping and behavioral observations [38]. Individuals included in our study lived on one
467 of three grids (Agnes or AG, Kloo or KL, Sulphur or SU). On AG, individuals were
468 supplemented with peanut butter from October to May in each year for a separate
469 experiment focused on experimentally increasing squirrel population density [28,38]. On
470 KL and SU, no food supplementation was provided. All models controlled for food
471 supplementation status given its potential impacts on gut microbiome composition.

472

473 Sampling

474 We collected 227 samples from 88 individuals across three years (2008-2010). When
475 individuals were captured, they were handled such that their unique identity could be
476 determined (by reading ear tags), sexed, and their reproductive condition could be
477 recorded. Fecal samples were collected opportunistically during live-trapping from
478 underneath the traps using forceps. Following capture and handling of squirrels, fresh
479 fecal samples were collected from underneath the traps, kept on wet ice until they could
480 be frozen at -20 C within 5 hrs of collection in the field. Samples contaminated with
481 urine were not collected and all samples were kept at -20 C until analysis. We removed
482 one fecal pellet from each sample using sterilized forceps for microbiome sequencing
483 and then used the rest of the sample to measure fecal GC metabolites.

484

485 Age

486 The age of each squirrel was known as individual squirrels were uniquely tagged in their
487 natal nest when they were ~25 days of age and age is accordingly recorded at each
488 trapping event [38].

489

490 Reproductive activity

491 Squirrels were live-trapped regularly and handled using visualization to determine
492 reproduction state. During each trapping event simultaneous with the collection of
493 microbiome and hormone samples, the reproductive state of the individual was
494 determined via abdominal palpation [86]. Males were considered reproductively active if
495 their testes were scrotal, and not reproductively active if testes were abdominal. For
496 females, pregnancy status was assessed via abdominal palpation for fetuses as well as
497 by examining nipple condition. Females were determined to be reproductively active if
498 they were gestating, lactating, or breeding based on nipple condition. We have
499 previously found that females that are reproductively active (pregnant or lactating) have
500 higher fecal GC metabolites than those that were not reproductively active whereas
501 there were no differences in the effects of reproductive activity (presence or absence of
502 scrotal testes) in males [49].

503

504 Dietary heterogeneity

505 Although red squirrels consume primarily white spruce seeds, they also consume a
506 number of other foods (e.g., spruce bark and needles, willow leaves and buds, fungi,
507 and bearberry flowers) and thus experience varying levels of seasonal dietary
508 heterogeneity [34]. We coded dietary heterogeneity by ranking the availability of these
509 different foods across seasons from greatest (3) to least (1). Samples collected prior to
510 June of each year were ranked as 1, while samples collected in the month of June and
511 late summer (July-August) were ranked as 2 and 3, respectively.

512

513 Conspecific density

514 Densities (expressed as squirrels per hectare) for each grid of the study (KL, SU, AG)
515 were calculated separately for each year (2008, 2009, 2010) across the dataset using
516 census data. In May of each year, we determined the number of squirrels owning a
517 territory on our study areas using a combination of live-trapping and behavioral
518 observations. Because squirrels are diurnal, regularly exhibit territorial calls, and their

519 territories are visually conspicuous, we were able to completely enumerate all squirrels
520 living in our study areas.

521

522 Sequencing and bioinformatics

523 Microbiome data used in this study are a subset of previously published data [52]. DNA
524 extraction and sequencing was performed as described in Ren et al. 2017. Briefly, the
525 V1-V3 hypervariable region of the 16S rRNA bacterial gene was amplified using two
526 universal primers: 27F (5'-ARGGTTTGATCMTGGCTCAG-3') and 534R (5'-
527 TTACCGCGGCTGCTGGCAC-3'). Samples were barcoded for PCR amplification,
528 pooled, gel purified, and then sequenced on an Illumina MiSeq using 300 bp paired-end
529 sequences. Sequences were then filtered, quality controlled, and reads were
530 successfully merged using QIIME [87]. Chimeras were removed using USEARCH [88]
531 and sequences determined to be non-chimeric by both de novo and reference-based
532 algorithms were retained. Reads were clustered to OTUs using UCLUST [89] with an
533 identity threshold of 97% (genus-level). Mitochondria and chloroplast were removed,
534 and samples were rarefied to 4000 reads per sample.

535

536 Hormone metabolite analysis

537 The time period from collection in the field to freezing (~5 hrs) did not impact fecal GC
538 metabolites [49]. We measured fecal GC metabolites using previously validated
539 protocols [49,90]. Briefly, samples were lyophilized for 14-16 hrs, bathed in liquid
540 nitrogen, and pulverized using a mortar and pestle. A subsample (0.05 g) was then
541 extracted using 80% methanol where the samples were vortexed at 1450 RPM for 30
542 min followed by centrifuging for 15 min at 2500 g [49]. The supernatant was then used
543 in an enzyme-immunoassay that employed an antibody that measures GC metabolites
544 with a 5 α -3 β ,11 β -diol structure [91]. We have previously validated this assay and shown
545 that the antibody can accurately measure increases in adrenal production of GCs [49].
546 We have also shown that our measures of fecal GC metabolites are comparable across
547 assays [92]. Using pooled samples that were run repeatedly on different plates (n =
548 115) in our laboratory show that the estimates of optical density for these pooled
549 samples were highly repeatable ($R = 0.851$, 95% CI = 0.543-0.925). Using a linear

550 mixed-effects model, we partitioned the variance in the optical density recorded for the
551 pooled samples that were run across these different plates and found that most of the
552 variance was due to the sample itself (85.1%) with little of it being explained by intra-
553 assay variation as all samples were run in duplicate (4.9%) or by inter-assay variation
554 (9.9%).

555

556 Statistical analysis

557 All statistical analyses were conducted in R (v. 3.5.2.) (R. Core Team, 2015). OTU and
558 taxonomy tables were imported into R and merged into a phyloseq object for
559 downstream analyses using the ape [93] and phyloseq [94] packages. All figures were
560 created in R, with the exception of the conceptual model (Figure 1) and the structural
561 equation model figures (Figures 4 and S1), which were created in bioRender
562 (www.biorender.com).

563

564 Alpha diversity

565 The estimate_richness() function in the phyloseq package was used to calculate the
566 observed richness (Chao1) and Shannon Index of alpha diversity. Faith's Phylogenetic
567 Distances were calculated using the pd() function on the phyloseq object in picante [95].
568 Linear mixed-effects models were used to assess the relationship between bacterial
569 diversity and fecal glucocorticoid metabolites (GCs), including individual ID as a random
570 intercept, and collection date and food supplementation as fixed effects. GCs
571 concentrations were log-transformed to improve model fit. Shannon Indices were Tukey
572 transformed prior to analysis to achieve residual normality. All models were assessed
573 for multicollinearity among predictor variables by calculating variance inflation factors
574 (VIF < 5).

575

576 Differential abundance testing

577 To identify the bacterial taxa whose relative abundances were significantly associated
578 with changes in host GCs, we constructed negative binomial mixed models and
579 implemented our analysis using the NBZIMM package [96]. Negative binomial models
580 outperform other traditional differential abundance methods (e.g. DESeq) because they

581 are better equipped to handle the zero-inflation and sparsity common to microbiome
582 count data [96]. Taxa included in differential abundance testing were filtered with a
583 liberal threshold of $> 0.001\%$ relative abundance to the overall microbiome community
584 to avoid excluding rare taxa as they contribute substantially to measures of community
585 diversity [58,97]. Models included the read count of each bacterial taxa as the
586 dependent variable, GCs (logged) as a fixed effect, controlling for collection date (fixed),
587 food supplementation (fixed), and individual id (random). Taxa whose negative binomial
588 models did not converge due to a high presence of zeroes were modeled instead with
589 zero-inflated negative binomial models using the `glmer.zinb()` function in the same
590 package (NBZIMM). We controlled the false discovery rate by applying a Benjamini-
591 Hochberg FDR correction to all p-values. Adjusted p-values < 0.05 were considered
592 statistically significant.

593

594 Structural Equation Modelling

595 To integrate ecological and host variables into our model framework investigating the
596 relationship between GCs and gut microbiome diversity, we constructed a structural
597 equation model using (SEM) using the `piecewiseSEM` package [69]. SEM is an effective
598 way to evaluate direct and indirect effects of multiple variables within complex
599 ecological systems [43]. Unlike traditional variance covariance-based SEM, piecewise
600 SEM approaches allow for the inclusion of random effects, the construction of a single
601 causal network from multiple separate models, and the ability to handle small sample
602 sizes and compare models using Akaike information criterion (AIC) [69].

603

604 Using `piecewiseSEM`, we investigated whether the relationship between GCs and gut
605 microbiome diversity (endogenous variables, i.e., variables of interest) was moderated
606 by host and/or ecological factors (exogenous variables, i.e., variance outside of the
607 model structure). All categorical variables were converted to numeric variables prior to
608 modeling. To build the SEM, we first constructed two component linear mixed models.
609 The first model tested the effects of conspecific density, reproductive activity, dietary
610 heterogeneity, and an upcoming mast on GCs. The second model tested the effects of
611 reproductive activity, dietary heterogeneity, an upcoming mast, age, and GCs on gut

612 microbiome diversity (Chao1 richness). Both component models included sample
613 collection date and food supplementation status as a fixed effect and individual ID as a
614 random effect. However, food supplementation did not affect GCs or gut microbiome
615 diversity in either of the component models (effect on GCs: estimate \pm SE -4.86 ± 3.08 , t
616 = -1.58 , P = 0.12; effect on gut microbiome alpha diversity: estimate \pm SE $-29.14 \pm$
617 79.13 , t = -0.37 , P = 0.71), and was therefore removed from the SEM to improve model
618 fit (AICc) and refine the standardized beta estimates. The overall fit of the SEM was
619 evaluated using Shipley's test of d-separation Fisher's C statistic and AICc.

620

621

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888

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893

894 **Author Contributions**

895 L.P. and B.D. conceived and designed the study. Microbiome data were generated by
896 T.R. and M.W. Hormone data were generated by R.P., R.B., and B.D. Data analysis
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900

901 **Competing Interests**

902 The authors declare no competing interests.

903

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909

910 **Data Availability**

911 All sequences, hormone data, and R code related to this manuscript are available at
912 figshare (<https://figshare.com/s/1d848d6c1e4550acdabf>).

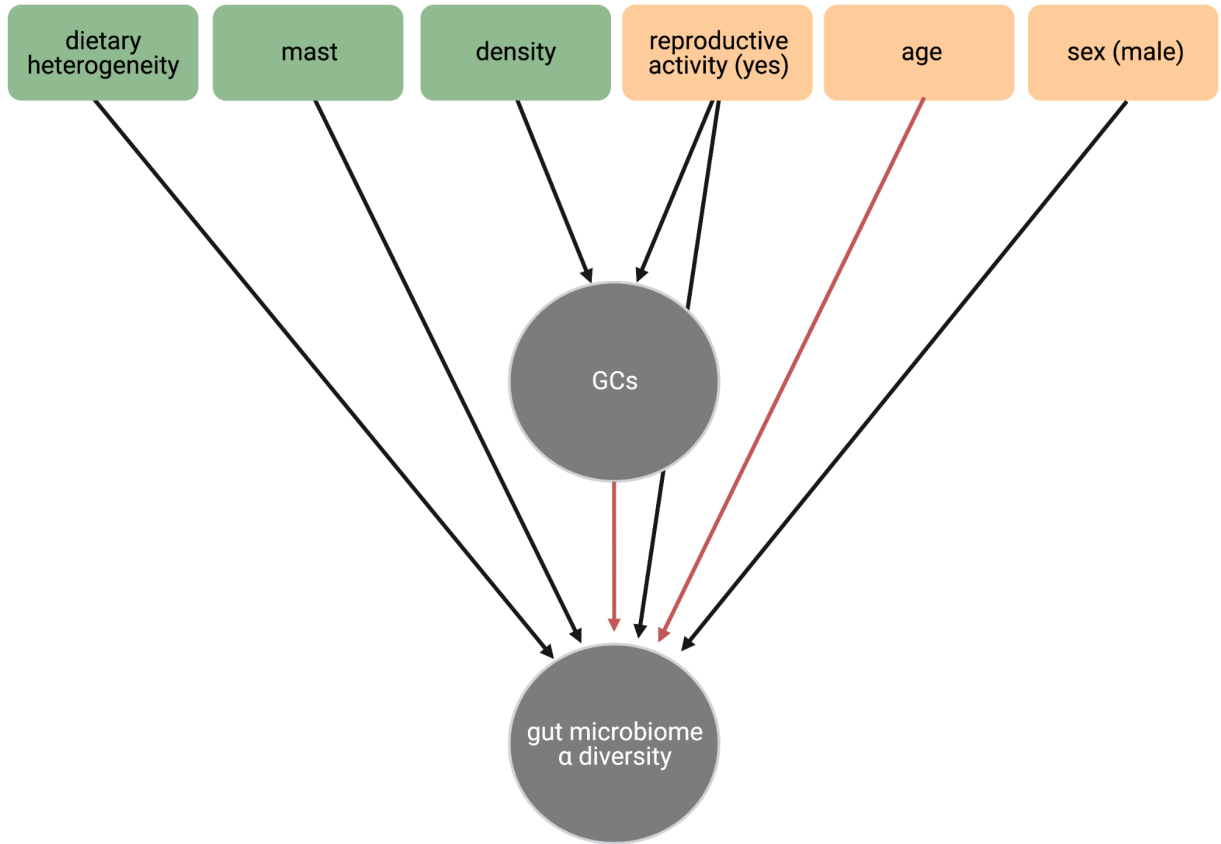
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Supplemental materials

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916 **Figure S1. A *priori* structural equation model depicting hypothesized pathways**
917 **and directionality among variables.** Black solid lines represent hypothesized
918 significant positive relationships; red solid lines represent hypothesized significant
919 negative relationships. Dotted line represents hypothesized non-significant relationship.



920

921 **Table S1. Differentially abundant taxa at the family level.** Significant (FDR-adjusted
 922 P value < 0.05) results from negative binomial mixed models testing the effect of GCs
 923 on each bacterial family controlling for collection date and food supplementation status.

bacterial-family	model-estimate	p-value	fdr-adjusted-p	change-with-GCs
[Odoribacteraceae]	-6.83	0.00	0.00	DECREASE
Sporichthyaceae	-3.17	0.00	0.00	DECREASE
Xanthomonadaceae	-1.39	0.00	0.00	DECREASE
Chitinophagaceae	-1.38	0.00	0.00	DECREASE
Christensenellaceae	-1.01	0.00	0.00	DECREASE
Bacillaceae	-0.84	0.00	0.01	DECREASE
Elusimicrobiaceae	-0.81	0.00	0.00	DECREASE
Flavobacteriaceae	-0.64	0.00	0.00	DECREASE
Staphylococcaceae	-0.64	0.01	0.05	DECREASE
Streptomycetaceae	-0.63	0.00	0.00	DECREASE
Sinobacteraceae	-0.57	0.00	0.00	DECREASE
Ruminococcaceae	0.12	0.00	0.02	INCREASE
Coriobacteriaceae	0.57	0.00	0.01	INCREASE
Streptococcaceae	1.00	0.00	0.00	INCREASE
Dermabacteraceae	2.81	0.00	0.00	INCREASE

924

925 **Table S2. Differentially abundant taxa at the genus level.** Significant (FDR-adjusted
 926 P value < 0.05) results from negative binomial mixed models testing the effect of GCs
 927 on each bacterial genus controlling for collection date and food supplementation status.

bacterial-genus	model-estimate	p-value	fdr-adjusted-p	change-with-GCs
Kribbella	-650.76	0.00	0.00	DECREASE
Salmonella	-10.98	0.00	0.00	DECREASE
Odoribacter	-6.83	0.00	0.00	DECREASE
Skermanella	-2.85	0.00	0.00	DECREASE
Acidisoma	-2.35	0.00	0.00	DECREASE
Stenotrophomonas	-2.29	0.00	0.00	DECREASE
Rahnella	-2.17	0.00	0.00	DECREASE
Dyadobacter	-1.68	0.00	0.00	DECREASE
Actinomycetospora	-1.49	0.00	0.00	DECREASE
Variovorax	-1.42	0.00	0.00	DECREASE
Pseudoclavibacter	-1.24	0.00	0.00	DECREASE
Modestobacter	-1.19	0.00	0.00	DECREASE
Brevundimonas	-1.17	0.01	0.02	DECREASE
Yersinia	-1.08	0.00	0.00	DECREASE
Hyphomicrobium	-1.00	0.00	0.01	DECREASE
Reyranelia	-0.88	0.00	0.00	DECREASE
Roseomonas	-0.87	0.00	0.00	DECREASE
Bacillus	-0.77	0.00	0.02	DECREASE
Brochothrix	-0.76	0.00	0.00	DECREASE
Flavobacterium	-0.64	0.00	0.00	DECREASE
Steroidobacter	-0.57	0.00	0.00	DECREASE
Ochrobactrum	-0.35	0.01	0.04	DECREASE
Clostridium	0.28	0.00	0.01	INCREASE
Butyricoccus	0.35	0.00	0.00	INCREASE
Kurthia	0.46	0.00	0.00	INCREASE
Microbacterium	0.52	0.01	0.04	INCREASE
Oscillospira	0.60	0.00	0.00	INCREASE
YRC22	0.65	0.00	0.00	INCREASE
Lachnospira	0.67	0.00	0.02	INCREASE
Pedomicrobium	0.96	0.00	0.00	INCREASE
Streptococcus	1.00	0.00	0.00	INCREASE
Actinomyces	1.01	0.00	0.00	INCREASE
Eggerthella	1.15	0.00	0.00	INCREASE
Ralstonia	1.38	0.00	0.00	INCREASE
Brachybacterium	2.81	0.00	0.00	INCREASE
Janthinobacterium	2.82	0.00	0.00	INCREASE
Propionibacterium	274.64	0.00	0.00	INCREASE

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