# Host-microbe mutualism drives urea carbon salvage and acetogenesis during hibernation

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**Abstract**

Hibernation is a wintertime survival strategy employed by certain mammals that, through torpor use, reduces overall energy expenditure and permits long-term fasting. Although fasting resolves the challenge of winter food scarcity, it also removes dietary carbon, a critical biomolecular building block. Here, we demonstrate a process of urea carbon salvage (UCS) in the 13-lined ground squirrel, whereby urea carbon is reclaimed through gut microbial ureolysis and used in reductive acetogenesis, producing acetate—a short-chain fatty acid (SCFA) of major value to the host and its microbial community. We find that urea carbon incorporation into acetate is more efficient during hibernation than the summer active season, and that while both host and gut microbes oxidize acetate for energy throughout the year, the host's ability to absorb and oxidize acetate is highest during hibernation. Metagenomic data of the gut microbiome indicate retention during hibernation of genes involved in the degradation of gut mucin, an abundant endogenous nutrient. The hydrogen disposal associated with reductive acetogenesis from urea carbon provides a luminal environment that sustains fermentation of mucin oligosaccharides, thereby generating SCFAs and other metabolites usable by both the host and its gut microbes. Our findings introduce UCS as a novel mechanism that enables hibernating squirrels and their gut microbes to exploit two key endogenous nutrient sources – urea and mucins – in the resource-limited hibernation season.

## Introduction

Winter is a time of resource limitation for hibernating mammals and their gut microbes. This is especially true for species like the 13-lined ground squirrel (*Ictidomys tridecemlineatus*), which, thanks to their use of torpor, stop eating for approximately six months each year [1]. This effectively solves the animal's winter food scarcity problem, but it simultaneously deprives both the host and its gut microbes of exogenous nutrients. This can in turn jeopardize the dietary supply of valuable resources that are needed to sustain life throughout hibernation. However, mutualistic relationships exist between hosts and their microbial symbionts that allow the re-use of existing resources to meet this challenge.

One example of resource acquisition during fasting is urea nitrogen salvage (UNS), a gut microbe-dependent process that is used by ground squirrels to recycle urea nitrogen back into their tissue protein pools during hibernation [2], when they lack the typical dietary supply of nitrogen. This process, which peaks during late winter after months of fasting and just prior to the squirrel's emergence into the spring mating season, recycles nitrogen to support tissue protein synthesis throughout winter, and likely contributes to these hibernators' renowned

59 resistance to muscle atrophy [2,3]. Urea nitrogen is also incorporated into the microbial protein
60 pool, thus confirming that UNS benefits both symbiotic partners [2]. Thus, urea, which is
61 excreted as a waste product during summer, serves as an endogenous nitrogen source during
62 hibernation when the animals and their gut microbes lack an exogenous supply of this important
63 biomolecular building block.
64 In addition to nitrogen, urea contains carbon, another essential nutrient that is limited during
65 hibernation due to long-term fasting. Theoretically, the ability to capture this carbon via urea
66 carbon salvage (UCS) would also provide nutritional benefits to the microbiota and potentially to
67 the hibernator host. The existence of UCS has, to our knowledge, never been proposed in the
68 literature, perhaps because most animals do not experience extended periods with no carbon
69 intake where UCS could be beneficial. This is not the case for fasting hibernators, and
70 preliminary results from our previous study [2] suggested that urea carbon is indeed liberated by
71 microbial urea hydrolysis, biologically fixed, and incorporated into organic molecules. Thus, it is
72 possible that both host and microbes benefit from UCS during resource-limited hibernation.
73 In this study, we tested more rigorously the hypothesis that carbon liberated by urea
74 hydrolysis is incorporated into organic molecules, and that these molecules provide benefits for
75 ground squirrels and/or their microbiota during winter fasting. To accomplish this, we treated
76 squirrels with $^{13}C$,$^{15}N$-labeled urea and measured urea carbon (i.e., $^{13}C$) incorporation into the
77 microbiota, tissue metabolome and protein. We performed real-time in vivo $\delta^{13}C$ breath
78 experiments to estimate rates of microbial urea hydrolysis and absorption/metabolism of orally
79 gavaged acetate, as well as metagenomic analyses of bacterial genes involved in biological
80 carbon fixation and acetogenesis. In addition, we expanded the notion that fasted hibernators
81 and their gut microbes participate in mutualistic interactions that enable the use of host-secreted
82 compounds as nutrients by examining whether hibernator microbiomes are enriched in genes
83 coding for enzymes that degrade intestinal mucins, which are the most abundant endogenous
84 nutrients in the squirrel gut ecosystem during winter fasting.
85
86 **Materials & methods**
87 *Experimental animals*
88 From a total of 65 13-lined ground squirrels (39 females, 26 males; hereafter called squirrels),
89 39 were used for the doubly-labelled urea treatment experiments described in [2], 15 were
90 pretreated with oral gavages of $^{13}C$- acetate, and gut contents from 11 others were used for
91 mucin degradation metagenomics analyses. Squirrels were born in a vivarium on the University
92 of Wisconsin-Madison (UW-Madison) campus to wild-caught female squirrels that were

93 collected around Madison, Wisconsin in May 2018 and May 2019. The pregnant squirrels were
94 housed individually at 22°C with a 12:12 h light-dark cycle with ad libitum access to water and
95 rodent chow (Teklad no. 2020X, Envigo, Indianapolis, IN, USA) supplemented with fruit (apples)
96 and sunflower seeds once per week. After birth, pups remained with their mothers for 5 weeks
97 and then moved to individual cages. Following 2 weeks of ad libitum chow and fruit, food was
98 restricted to 12 g chow per day (supplemented with 1 g of sunflower seeds once per week) to
99 prevent excessive weight gain that occurs in captive-born squirrel pups. The consistent diet
100 minimized diet-induced effects on the gut microbiome and the molar ratio of $^{13}C$ to $^{12}C$ in
101 exhaled $CO_2$. Squirrels were held under these conditions until the day of experiment (Summer
102 group) or transferred to the cold room at the beginning of the hibernation season (Early and
103 Late winter groups).
104 In mid-September, squirrels in the Winter groups were moved to a 4°C environmental
105 chamber in the UW-Madison Biotron. The chamber was held in constant darkness except for ~5
106 min/day of dim light to enable activity checks. Food and water were removed after squirrels
107 began regular torpor-arousal cycles, which was 24–72 h after being moved into the chamber.
108 The 39 urea treatment squirrels were studied at in one of three time points: Summer (14
109 squirrels), Early Winter (12 squirrels), and Late Winter (13 squirrels). Each seasonal group was
110 divided into three treatment groups: $^{13}C$,$^{15}N$-urea with antibiotics, $^{13}C$,$^{15}N$-urea without
111 antibiotics, and unlabeled urea without antibiotics. Mean days in hibernation for the Early Winter
112 and Late Winter groups were 36 d (range 31–41 d) and 127 d (range 109–135 d), respectively.
113 Mean body masses on experiment days for Summer, Early Winter, and Late Winter groups
114 were 187.1 g (range 165.0–224.0 g), 155.6 g (range 123.4–187.2 g), and 134.1 g (range 105.5–
115 167.0 g), respectively.
116 The 15 acetate treatment squirrels were divided into four groups: Summer with antibiotics (4
117 squirrels), Summer without antibiotics (3 squirrels), Late Winter with antibiotics (3 squirrels), and
118 Late Winter without antibiotics (5 squirrels). Mean days in hibernation for the combined Late
119 Winter groups combined was 92 d (range 68–145 d). Mean body masses on experiment days
120 for Summer and Late Winter were 206.1 g (range 172.6–241.2 g) and 151.2 g (range 128.3–
121 174.0 g), respectively.
122 The 11 squirrels used for metagenomic analysis of mucin degradation genes were divided
123 into two groups: Summer without antibiotics (6 squirrels) and Late Winter without antibiotics (5
124 squirrels). Mean days in hibernation for the Late Winter group were 135 d (range 127–144 d).
125 Mean body masses on experiment days for Summer and Late Winter were 183.0 g (range
126 144.0–225.0 g) and 132.5 g (range 116.5–144.6 g)

127 The UW-Madison Institutional Animal Care and Use Committee approved all procedures.
128
129 *Animal treatments and stable isotope breath-testing*
130 For the 39 squirrels treated with doubly-labelled urea, the antibiotic treatments to deplete the gut
131 microbiota, the $^{13}C$,$^{15}N$-labeled urea treatments (200 mg urea $kg^{-1}$ body mass), the breath
132 testing experiments via cavity ring-down spectrometry (CRDS), and the euthanasia protocol
133 were all carried out as described in [2].
134 The 15 squirrels used to estimate host and microbe acetate oxidation capacity were treated
135 with $^{13}C$-labeled acetate (500 mg $kg^{-1}$ body mass) via oral gavage following light anesthesia (4%
136 isoflurane-$O_2$ mixture). Acetate oxidation capacity was measured using the CRDS-based breath
137 analysis, after which squirrels were euthanized. Antibiotics were administered as described in
138 [2].
139 The 11 squirrels used for metagenomic-based estimates of mucin glycan degradation were
140 administered saline via oral gavage following light anesthesia (4% isoflurane-$O_2$ mixture). On
141 the day of sampling, CRDS-based breath analysis was performed, after which they were
142 euthanized. There were no pretreatments for these squirrels.
143 In all cases, Early and Late Winter experiments were performed on euthermic squirrels
144 following forced arousal.
145
146 *Tissue harvesting and preparation*
147 Following euthanasia, we collected host tissues (liver, quadriceps) and cecal contents (a
148 proxy for the cecal microbiota) and prepared them for isotope ratio mass spectrometry (IRMS)
149 and nuclear magnetic resonance (NMR)-based metabolomics analyses as described in [2]. The
150 IRMS and NMR techniques were executed as described in [2]. All $^{1}H$ and $^{13}C$ spectra were
151 analyzed using the Chenomx software package (Version 8.5; Edmonton, AB, Canada).
152
153 *Metagenomics*
154 Generation of metagenomic data from cecal contents were performed as previously described
155 [2]. Briefly, genomic DNA was extracted using the Qiagen DNeasy PowerSoil Kit (Qiagen,
156 Hilden, Germany) according to manufacturer's specifications and quantified using a Qubit
157 Fluorometer (Invitrogen, San Diego, CA, USA). Sequencing was performed on an Illumina
158 NovaSeq6000 (Illumina, San Diego, CA, USA) using an S4 Reagent Kit v1.5 at 2x150bp PE.
159 Trimmomatic v0.38 [4] was used to remove sequencing adapters and low-quality reads, while
160 host DNA was filtered using bowtie2 v2.2.2 [5] against the 13-lined ground squirrel genome

161 (GenBank and RefSeq assembly accession = GCA_000236235.1). The remaining reads were
162 assembled using metaSPAdes v3.13.0 [6] and open reading frames (ORFs) were predicted
163 using prodigal v2.6.3 [7]
164 For the carbon fixation-related analyses, cecal content samples from three microbiota-
165 intact individuals were randomly chosen for Summer and Late Winter. Translated proteins from
166 the ORF predictions were compared against the Pfam [7] profiles for proteins involved in carbon
167 fixation and mucin degradation, as defined by KEGG using HMMER [8]. Identified sequences
168 were then mapped back to their contig of origin and taxonomically classified using centrifuge [9].
169 The percentage of target genes (*i.e.*, genes for carbon fixation or mucin degradation) in the
170 metagenomes were calculated by dividing the number of identified target genes by the total
171 number of predicted genes. The percentage of target genes classified to different taxa was
172 calculated by dividing the number of target genes classified to a specific taxon by the total
173 number of detected target genes.
174 For the mucin degradation analysis, we first identified putative enzymes thought to be
175 involved in mucin degradation from the Carbohydrate-Active enZYmes (CAZy) database [10],
176 including glycoside hydrolase (GH) 20 (exo-acting *β*-*N*-acetylglucosaminidases, *β*-*N*-
177 acetylgalactosamindase and *β*-6-$SO_3$-*N*-acetylglucosaminidases), GH33 (sialidase), GH84 ( *β*-
178 *N*-acetylglucosaminidases), GH85 (Endo-β-N-acetylglucosaminidases), and GH109 (α-*N*-
179 acetylgalactosaminidase). We then compared the translated proteins from the predicted ORFs
180 of the metagenomes against the CAZy database using dbCAN [11] and HMMER v3.3 [8].
181 To determine differences in mucin degradation genes between summer and late winter
182 metagenomes, we first normalized CAZyme mucin degradation genes by calculating reads per
183 kb per genome equivalent (RPKG) [12]. For each ORF, ORF length (kb) and the number of
184 mapped short reads were determined using bowtie2. Next, the number of mapped short reads
185 for each ORF was divided by ORF length and summed in each sample. This sum was then
186 divided by genome equivalents to calculate RPKG. RPKG seasonal differences were tested
187 using Kruskal-Wallis in R [13] (*kruskal.test*) and average genome size and genome equivalents
188 were tested using ANOVA (*aov*, stats package in R) to compare between summer and late
189 winter. Overrepresented CAZymes were identified with DESeq2 [14] and normalized counts
190 were transformed using the Hellinger method (*hellinger*, labdsv package in R).
191
192 *Statistical analyses*
193 Multivariate $^{13}$C-metabolomic data for cecal contents, liver and muscle were analyzed by
194 combining principal components analysis (PCA) for visualization and PERMANOVA analysis for

195 quantitation (MetaboAnalyst 6.0, www.metaboanalyst.ca). Effects of season and microbiota
196 presence on $^{13}C$-incorporation into identifiable metabolites (via NMR) and protein fraction of
197 tissue (via IRMS) were analyzed using 2-way ANOVA (GraphPad Prism v10.3). Breath $\delta^{13}C$
198 was plotted as a function of time following labeled urea or acetate treatment (2.5 to 3 h
199 depending on individual), and then area-under-the-curve (AUC) was calculated and expressed
200 relative to each individual's total breath analysis time. The data for urea-treated squirrels
201 originally reported in [2] were expressed here exponentially to eliminate negative values and
202 enable urea-to-acetate conversion efficiency calculations, which were expressed as the $^{13}C$-
203 acetate abundance relative to the exponentially converted breath $\delta^{13}C$ AUC for the urea-treated
204 squirrels. Time course traces for breath responses to doubly-labelled urea-treated squirrels can
205 be found in [2]. Seasonal differences in breath $\delta^{13}C$ AUC for urea-treated squirrels and relative
206 abundances of gut bacterial genes involved in biological carbon fixation (via Wood-Ljungdahl
207 reaction) and mucin degradation were analyzed using 1-way ANOVA with Tukey multiple
208 comparisons test (carbon fixation) and t-test (mucin degradation). Effects of season and
209 microbiota presence on breath $\delta^{13}C$ AUC for acetate-treated squirrels were analyzed using 2-
210 way ANOVA. Non-normally distributed data via Shapiro-Wilk test were log-transformed prior to
211 analysis. In cases where data remained non-normal, non-parametric tests were used. For data
212 presented in whisker plots, the upper and lower bounds of the box represent 75$^{th}$ and 25$^{th}$
213 percentiles, respectively; the horizontal line within the box represents the median value, and the
214 upper and lower whiskers represent the maximum and minimum values within the data set,
215 respectively. Statistical significance was set at $P<0.05$ for all comparisons.
216

217 **Results**

218 *Urea carbon ($^{13}C$) incorporation into host and microbe metabolome and protein*

219 We observed a total of 10, 12 and 15 metabolites enriched in urea-derived $^{13}C$ in cecal
220 contents, liver and muscle, respectively (Figs. 1, 2). The paired PCA-PERMANOVA multivariate
221 analysis revealed that, among the six treatment groups within each compartment, $^{13}C$-enriched
222 metabolite pools varied with season in cecal contents ($F=3.0992$, $R^2=0.4025$, $P=0.007$) and liver
223 ($F=2.7784$, $R^2=0.3766$, $P=0.009$), but not in muscle ($F=1.2785$, $R^2=0.2175$, $P=0.288$) (Fig. 1).
224 However, among all observable metabolites, only cecal content $^{13}C$-acetate was significantly
225 affected by the presence of a microbiota, whereby its concentrations in microbiota-intact
226 squirrels were 2- to 7-fold higher than those of microbiota-depleted squirrels (Fig. 2A; ANOVA
227 $P<0.0001$; original data were reported in Fig. S6 in [2] and expressed here in different units).
228 This microbiota effect confirmed that the incorporation of urea carbon into acetate molecules

229 was dependent upon microbial activity, most likely reflecting reductive acetogenesis that utilized
230 $^{13}CO_2$ generated by ureolytic bacteria after administration of $^{13}C$,$^{15}N$-urea. Microbial involvement
231 in $^{13}C$-acetate formation was not observed in liver despite $^{13}C$-acetate concentrations being
232 slightly higher in Late Winter than Summer or Early Winter (Fig. 2B; ANOVA P>0.05), or in
233 muscle where concentrations of $^{13}C$-acetate were below detectable limits (Fig. 2C).
234 In contrast to our previous observations [2] that urea-derived $^{15}N$ is incorporated into host
235 (liver, muscle) and microbiota (cecal contents) protein pools, we found no evidence of
236 microbiota-dependent incorporation of $^{13}C$ into protein fractions of the liver, muscle, or
237 microbiota after $^{13}C$,$^{15}N$-urea administration (Fig. S1). The small sample volumes of cecal
238 contents in the microbiota-intact squirrels required pooling all samples into a single aliquot for
239 IRMS analysis, which ultimately precluded statistical testing. Sample volumes for the
240 microbiota-depleted squirrels were also too small for analysis, likely because the protein fraction
241 of the cecal contentss was largely bacterial and thus absent from the microbiota-depleted
242 squirrels. However, we observed a trend for greater incorporation of $^{13}C$ into protein fractions of
243 Summer cecal contents compared with Early or Late Winter fractions (Fig. S1).
244
245 *Seasonal effect on ureolytic activity and urea-to-acetate efficiency*
246 The CRDS-based measurements of breath $\delta^{13}C$ reported in [2] for estimation of in vivo gut
247 microbial ureolytic activity were transformed here to express the $\delta^{13}C$ values as positive
248 numbers, thus enabling efficiency measurements (see Materials and Methods). This analysis
249 revealed that, as reported in [2], microbial ureolytic activity was higher in Summer than in Early
250 or Late Winter squirrels, which were not different (Fig. 3A; ANOVA P=0.0017).
251 Each squirrel's urea-to-acetate efficiency was expressed as the ratio of its cecal urea-
252 derived $^{13}C$-acetate concentration to its total $^{13}CO_2$ abundance, represented as its transformed
253 breath $\delta^{13}C$ value shown in Fig. 3A. This efficiency measure reflects the amount of liberated
254 urea carbon that is directed towards acetogenesis. We found that urea-to-acetate efficiency is
255 generally higher during hibernation than summer, with peak efficiency in Early Winter (Fig. 3B;
256 ANOVA P=0.0135; t-test P=0.0112 when grouping Early and Late Winter into a single
257 hibernation group).
258
259 *Seasonal effect on microbial biological carbon fixation capacity*
260 To estimate the capacity of the squirrel gut microbiome to biologically fix carbon and enable
261 acetogenesis, we analyzed the microbial metagenome for genes coding for enzymes involved in
262 the Wood-Ljungdahl (W-L) pathway of $CO_2$ fixation. The relative abundances of W-L-related

263 genes did not change from Summer to Late Winter (Fig. 4A; t-test P=0.2882) despite the ~90%
264 reduction in bacterial density by Late Winter [15]. Analysis of the top ten bacterial taxa from
265 taxonomic classification of W-L genes, as determined by sequence abundance, revealed a
266 seasonal shift from Summer to Late Winter in the bacterial potential for reductive acetogenesis
267 that could include use of urea carbon (Fig. 4B). Most notable was the finding that the greatest
268 percentage of W-L genes in Summer metagenomes (1.74%) belonged to the *Bacteroides*,
269 whereas over twice that amount (3.63%) was classified to *Blautia* in Late Winter. The
270 percentage of W-L gene sequences represented in *Blautia* spp. in Late Winter metagenomes
271 was over 5-fold than in Summer metagenomes.
272
273 *Capacities for acetate oxidation*
274 An increase in $\delta^{13}$C breath after administration of $^{13}$C,$^{15}$N-urea indicates that oxidation of one or
275 more bioenergetic metabolites by the microbiota, by squirrel cells, or both is a consequence of
276 ureolysis in the gut lumen. Our identification of acetate molecules in the microbial metabolome
277 that contain urea-derived carbon [2] implicated acetate as one of those metabolites. To explore
278 the site of acetate oxidation in the squirrel holobiont, and whether there is a seasonal difference
279 in capacity for acetate oxidation by the microbiota and/or host, we orally gavaged microbiota-
280 intact and -depleted squirrels from Summer and Late Winter with a body mass-specific dose of
281 $^{13}$C-labeled acetate, and then measured their breath $\delta^{13}$C using CRDS. None had been treated
282 with labeled urea. We found that squirrels from all groups exhibited rapid and large increases in
283 breath $\delta^{13}$C (Fig. 5A), indicative of acetate oxidation and subsequent release of $^{13}CO_2$ in breath.
284 Squirrels in Late Winter generally exhibited greater breath $\delta^{13}$C responses than squirrels in
285 Summer (Fig. 5B; ANOVA P=0.0003), but there were seasonal differences in how the presence
286 of the gut microbiota influenced the responses. In Summer, breath $\delta^{13}$C as measured by AUC
287 was highest in squirrels with intact microbiotas, whereas in Late Winter, breath $\delta^{13}$C was highest
288 in squirrels with depleted microbiotas (Fig. 5B). Consequently, microbiota-depleted squirrels
289 exhibited both the lowest (Summer) and highest (Late Winter) responses, with a >3-fold
290 difference between the two (Fig. 5B; ANOVA P=0.001). Season also influenced the time
291 required to reach peak breath $\delta^{13}$C, with Summer squirrels reaching peak values early (~30 min
292 post-gavage) relative to Late Winter squirrels (~75 min post-gavage) (Fig. 5A).
293
294 *Mucin degrading capacity in the squirrel microbiome*
295 Like urea, mucin glycoproteins are host-derived compounds that provide benefits to gut
296 microbes and their hosts. Mucins are secreted from goblet cells in the gut epithelial layer and

297 are either bound to epithelial cell membranes or secreted directly into the lumen. The glycan
298 component of these molecules are complex carbohydrates whose degradation releases a
299 variety of smaller oligosaccharides that are fermented anaerobically, producing energy-rich
300 metabolites including SCFAs that can be oxidized for ATP generation, used as signaling
301 molecules, or other purposes.
302 We initially planned to examine seasonal variation in recycling of mucin carbon from the
303 microbiota back into squirrel tissues using isotope labeling methods similar to those used for
304 urea [2], but were unable to do so due to a lack of commercially available $^{13}C$-labeled mucin
305 oligosaccharides that are resistant to metabolism by mammalian enzymes and could be
306 gavaged into squirrel guts. Therefore, we used metagenomics to test the hypothesis that the
307 seasonal shifts in gut microbiota composition we previously identified [16,17] is associated with
308 a change in the metagenome from Summer to Winter that favors retention of genes capable of
309 degrading mucins. We focused on gene families that encode CAZymes with the capacity to
310 degrade dietary polysaccharides, mucin glycans, or both, and identified 17 that were
311 overrepresented in either Summer or Winter metagenomes. Of the 17, we found five CAZymes
312 that were enriched in Winter vs. Summer, all of which are capable of degrading intestinal
313 mucins (Fig. 6).
314
315 **Discussion**
316 This study was designed to provide functional context for our observation that, in 13-lined
317 ground squirrels, carbon contained in urea molecules that enter the gut lumen is incorporated
318 into acetate within the microbial metabolome [2]. Here we show that this effect is restricted to
319 acetate among a suite of metabolites we examined and is more efficient during winter fasting
320 than the summer fed season. These and other findings discussed below support the idea that
321 through the symbiotic relationship between hibernators and their gut microbes, urea is
322 repurposed as a dual-use endogenous nutrient source during hibernation, with both its carbon
323 and nitrogen salvaged and used for synthesis of important biomolecules. Moreover, our findings
324 reveal a previously undescribed pathway for acetate production in the squirrel gut that uses $CO_2$
325 released by ureolytic microbes for hydrogen disposal via reductive acetogenesis. This helps to
326 maintain an efficient fermentative environment for the degradation of mucin glycoproteins, the
327 most abundant endogenous nutrients in the gut in fasted hibernators.
328
329 *Urea carbon salvage*

330 Like UNS, UCS begins with the movement of urea molecules from the blood into the gut lumen,
331 mediated by urea transporters (UT-B), where it is hydrolyzed by ureolytic gut microbes. We
332 recently showed that the abundance of UT-B in the squirrel cecum increases from Summer to
333 Late Winter, suggesting a greater capacity to transport urea into the lumen during the
334 hibernation season [2]. We also showed that ureolytic bacteria are found in the squirrel
335 microbiota and that rates of ureolysis are higher in Summer than in Early or Late Winter, likely
336 due to the reduction in total bacterial abundance that occurs in ground squirrels from summer to
337 winter [15,16].
338 Following urea hydrolysis, the next step in UCS involves urea carbon incorporation into
339 organic compounds (metabolites), a process carried out by gut microbes. These metabolites
340 would then be available for use by microbes and/or absorption and processing by the host. To
341 investigate this, we screened the metabolomes of the luminal cecal contents (a proxy for the
342 microbiota) and the squirrels' liver and skeletal muscle specifically for $^{13}C$-enriched metabolites
343 which, because our squirrels were treated with $^{13}C$,$^{15}N$-labeled urea, would indicate urea
344 carbon-containing metabolites. Looking broadly across the multivariate data via paired PCA and
345 PERMANOVA analyses, we found no significant effect of gut microbiota presence/absence on
346 the $^{13}C$-metabolomes of these three compartments (Fig. 1), suggesting that at this level of
347 analysis there is minimal incorporation of urea carbon into organic compounds. However, after
348 analyzing individual metabolites, we observed a significant gut microbe-dependent incorporation
349 of urea carbon into acetate (Fig. 2).
350 Our finding that urea carbon-derived acetogenesis occurs in the gut microbiota of
351 squirrels was notable for several reasons. First, although cecal acetogenesis by the reduction of
352 $CO_2$ with $H_2$ was first reported in 1977 [18] and replicated subsequently in the hindgut of many
353 monogastric species, the carbon source in those studies was not identified and was generally
354 assumed to be oligosaccharides produced by fermentation of dietary carbohydrates. Thus, our
355 study is the first to report incorporation of urea carbon in acetogenic reactions in the hindgut.
356 Also notable is that the cecal $^{13}C$-acetate concentrations are similar throughout the year
357 despite the decrease in bacterial abundance during hibernation [15,16]. This is coupled with the
358 sustained percentage from summer to winter of bacterial genes that code for enzymes in the
359 Wood-Ljungdahl pathway (Fig. 4). In this pathway, $CO_2$ is reduced by $H_2$ to form acetate and
360 water, thus linking the $CO_2$ produced by urea hydrolysis with acetogenesis. Taken together,
361 these findings suggest that the chronic lack of dietary substrates during hibernation may induce
362 a compensatory response in the squirrel microbiota that enhances metabolic functions and
363 survival in those bacteria that persist during winter fasting. This would include those genes that

364 code for carbon fixation and acetogenesis, facilitating the production of bioenergetic substrates
365 such as acetate. This is consistent with the stringent response, whereby bacteria under nutrient
366 stress such as carbon starvation reallocate resources from growth towards the upregulation of
367 survival-related genes until conditions improve [19–21].
368 Another example of molecular shifts activated by changing nutrients is the canonical
369 glucose-acetate transition in *Escherichia coli*, whereby cells excrete acetate while growing on
370 glucose and later absorb and catabolize it for energy when glucose is depleted. This is
371 facilitated by rapid gene expression modifications that suppress biosynthetic pathways and
372 upregulate catabolic ones [22,23]. Here, we posit that a similar situation may exist for the
373 microbiomes of fasting hibernators. When typical carbon sources in the gut such as glucose
374 derived from dietary polysaccharides are depleted over the winter fast, bacteria likely scavenge
375 for other sources [22]. Urea-derived acetogenesis via biological carbon fixation appears to be
376 one such pathway that persists throughout hibernation, possibly enabled by altered expression
377 of carbon fixation and acetogenesis genes in certain bacterial species, despite an overall
378 reduction of bacterial density due to winter fasting [15,16]. In the case of fasted hibernators, the
379 contributions of pathways that recycle endogenous host-derived compounds such as urea and
380 mucins may play significant roles in energy-sparing, thus conserving stored lipids that are the
381 major fuel source during torpor and arousals [24]. Although we detected no seasonal changes in
382 the proportion of genes for carbon fixation (Fig. 4A), transcriptomic or proteomic analyses might
383 reveal that ground squirrel microbiomes upregulate these enzymatic pathways from summer
384 feeding to winter fasting.
385 Interestingly, more of the urea carbon liberated by microbial ureolysis appears to be
386 used for acetogenesis during hibernation than during the summer active season. Specifically,
387 the ratio of $^{13}C$-acetate to total exhaled $^{13}CO_2$ was higher for hibernating squirrels than for
388 Summer squirrels (Fig. 3B). This suggests that less urea carbon is lost as $CO_2$ in breath during
389 winter, when exogenous carbon intake has ceased and when urea carbon, via carbon fixation
390 and subsequent acetogenesis, would most benefit the hibernator holobiont.
391
392 *Potential benefits of urea carbon-derived acetate for hibernator host and microbiota*
393 We perceive at least four benefits that UCS-mediated acetogenesis may offer hibernating
394 species during winter fasting. First, acetate can serve as a direct energy source for host cells,
395 with intestinal epithelial cells (IECs) being most likely to benefit given their dependence on
396 luminal SCFAs such as butyrate to meet energy needs [25]. Under normal fed conditions,
397 butyrate is the preferred energetic substrate for IEC; however, when butyrate availability is

398 limited, acetate oxidation can supply ATP [26]. A similar shift in the nutritional landscape of the
399 cecal lumen occurs during hibernation, where concentrations of all SCFAs, including butyrate
400 concentrations, fall to 3% of Summer levels. Importantly, acetate concentrations remain at 30-
401 40% [15,16] and it is therefore possible that UCS-derived acetogenesis contributes to this
402 energy supply for the host, particularly at the level of IECs.
403 To lend empirical support for acetate's role as an energy source for hibernators and their
404 gut microbes, we orally gavaged $^{13}C$-acetate into Summer and Winter squirrels. We then used
405 CRDS to measure breath $\delta^{13}C$, whereby elevated values above baseline indicate oxidation of
406 $^{13}C$-acetate to $^{13}CO_2$. Breath $\delta^{13}C$ rapidly increased following $^{13}C$-acetate gavage in microbiota-
407 intact Summer and Late Winter squirrels, resulting in similar AUC values for the 3 h post-gavage
408 data collection period (Fig. 5). To distinguish host acetate oxidation from potential microbial
409 acetate oxidation, we repeated the experiments on microbiota-depleted squirrels in both
410 seasons. In Summer squirrels, depleting the microbiota halved the breath $\delta^{13}C$ AUC (suggesting
411 a microbial contribution to acetate oxidation), whereas in Late Winter squirrels it had no effect
412 on breath $\delta^{13}C$ AUC but produced a higher peak in $\delta^{13}C$ that was more quickly attained. Overall,
413 the Late Winter microbiota-depleted squirrels had AUC values 3-fold higher than Summer
414 microbiota-depleted squirrels. These results suggest two things about acetate use during
415 hibernation. First, during summer, gut microbes oxidize much of the acetate they produce,
416 whereas during hibernation, a greater proportion of the acetate is absorbed and oxidized by the
417 host. Thus, the host appears to benefit most from the bioenergetic contributions of acetate
418 during hibernation. We suspect that the primary site of oxidation for microbially generated
419 acetate in squirrels, especially during hibernation, is within IECs. This is consistent with the lack
420 of observable $^{13}C$-acetate in liver and muscle, and with the generally deficient energy state of
421 IECs during long periods of food deprivation [27]. Second, the 3-fold higher breath $\delta^{13}C$
422 responses after $^{13}C$-acetate gavage in Late Winter compared with Summer microbiota-depleted
423 squirrels indicate that the capacity to absorb and oxidize acetate increases during hibernation,
424 when resources are limited and organic compounds such as acetate are especially valuable.
425 Although we have not directly measured rates of intestinal acetate absorption, these results are
426 consistent with our previous work showing higher normalized rates of $Na^+$, glucose, and amino
427 acid absorption by the small intestinal epithelium in hibernating than summer squirrels [28,29].
428 Similar results were observed for nutrient uptake into brush border membrane vesicles [30], and
429 it is plausible that similar seasonal effects on intestinal acetate absorption via monocarboxylate
430 transporters underlie the results seen here. A second, indirect way that urea carbon-derived
431 acetate may influence IEC metabolism during winter fasting is through its conversion to their

432 preferred fuel source by acetate-consuming, butyrate-producing bacteria within the microbiome
433 [31]. Together, these results suggest that the hibernator's physiology is seasonally optimized to
434 benefit from UCS-derived acetogenesis, which may suggest positive selection on this
435 mechanism.

436 A second potential role for acetate produced from urea carbon for the hibernating host is
437 its role as a ketogenic substrate. The ketone β-hydroxybutyrate is the preferred fuel source for
438 the heart and brain of ground squirrels during hibernation [32]. Ketogenesis, which takes place
439 primarily in the liver but can occur in other sites including the gut [33,34], is regulated by the
440 rate-limiting enzyme (3-hydroxy-3-methylglutaryl-CoA synthetase 2; HMGCS2). We showed
441 previously that HMGCS2 is expressed in 13-lined ground squirrel intestine and its protein
442 abundance is 2-fold higher during hibernation than summer [35]. This finding is consistent with
443 those from mice and rats whereby short-term fasting elevates both intestinal gene expression of
444 HMGCS2 [36] and the conversion of acetate to ketone bodies in isolated colonocytes [26].
445 Furthermore, a role for the microbiota in stimulating hepatic ketogenesis in mice under nutrient
446 deprivation was shown when mice with intact gut microbiotas elevated hepatic ketogenesis
447 following a 24 h fast, but the response was reduced in mice lacking a microbiota [37]. Given that
448 levels of fatty acids, which are the most common substrates for ketogenesis, are likely reduced
449 in hibernator IECs due to the lack of dietary lipid intake, acetate may be an effective ketogenic
450 precursor for the gut during hibernation.

451 A third potential way that UCS-derived acetate benefits the hibernating host is through
452 its effects on secretory IgA (sIgA), the most abundant immunoglobulin in mammals, and one
453 that provides the first line of defense at the epithelial surface. We showed previously that small
454 intestinal expression of sIgA is greater in torpor phases of hibernation compared with summer
455 levels, part of a suite of changes in the intestinal immune system that appears to strengthen
456 anti-microbial defenses and thereby homeostasis of the host with its gut microbiota [38]. More
457 recent studies have implicated microbial-derived acetate, but not butyrate, in playing an
458 essential role in regulation of sIgA production that helps shape commensal microbiota
459 composition and minimize microbial pathogen colonization [39,40]. Thus, maintenance of host-
460 microbiota mutualism during winter fasting may require alternative means to generate acetate
461 for immune protection such as UCS-derived acetogenesis.

462 Finally, gut microbial acetogenesis plays a key role in maintaining an environment
463 conducive to ongoing fermentation. The fermentative breakdown of complex carbohydrates
464 produces $H_2$ which, if not removed or metabolized, will eventually inhibit fermentative reactions
465 by inhibiting the regeneration of the coenzyme $NAD^+$ from NADH, thus slowing the

466 thermodynamic favourability of SCFA production and hindering bacterial growth [41].
467 Acetogenic bacteria help to keep $H_2$ partial pressure at an optimal level by reducing $CO_2$ with
468 $H_2$, producing acetate and $H_2O$. Methanogenesis plays a similar role in many animals (but
469 without formation of a bioenergetic compound like acetate), though there is no evidence for
470 hydrogenotrophic methanogens (Archaea) in the microbiotas of hibernating ground squirrels
471 [15–17] or bats [42]. Thus, acetogenesis is likely the primary $H_2$ disposal mechanism used in the
472 hindgut of mammalian hibernators. This is useful for the holobiont during hibernation because,
473 while there is a lack of exogenous complex carbohydrates during the winter fast, there is a
474 continuous supply of mucin glycoproteins in the intestinal lumen that, as described in the
475 following section, can serve as endogenous source of fermentable carbohydrates.
476
477 *Seasonal changes in mucin degradation capacity in the ground squirrel microbiota*
478 Mucins are complex glycoproteins that are secreted from goblet cells in the gut epithelium that,
479 upon hydration in the lumen, form mucus, a complex viscoelastic matrix that protects the
480 epithelium and serves as a habitat for a diverse assemblage of microbes [43]. Mucins also
481 provide nutritional benefits to gut microbes and their hosts [43], which are particularly beneficial
482 during winter fasting. In the absence of dietary (typically plant) glycans, host-derived mucins
483 become the major substrates that support microbial growth and proliferation, and contribute to
484 host energetic needs by producing SCFAs that fuel IECs [44]. Coupled with our previous
485 observation that mucin (MUC2) secretion continues throughout the hibernation season [17],
486 these molecules, like urea, may serve as valuable sources of carbon, nitrogen and energy
487 during the winter fast when exogenous nutrients are absent. Intact mucins are not digestible by
488 mammalian enzymes, but bacteria that express certain glycosyl hydrolases (GHs) can cleave
489 and metabolize glycan linkages, releasing oligosaccharides that are further fermented by the
490 same or other bacteria for energy, and in so doing produce beneficial metabolites for the host
491 [45]. There are two features of the gut ecosystem that must be in place for mucin glycan
492 degradation to proceed during hibernation: a thermally permissive environment for reactions
493 catalyzed by GHs and other enzymes, and a mechanism to dispose of $H_2$ whose accumulation
494 in the lumen slows fermentation. The thermal requirement is met by the ~12-24 h IBA periods
495 when host body temperature is above 30 ºC. The requirement for $H_2$ disposal, which is likely
496 compromised in hibernators by reduced luminal $CO_2$ generation due to the absence of
497 fermentable dietary substrates, can be met by the dual actions of $CO_2$ production by ureolytic
498 bacteria and its subsequent use in hydrogen disposal by acetogens.

499 We initially planned to examine seasonal variation in recycling of mucin-derived carbon
500 from the microbiota into squirrel tissues using isotope labeling methods similar to those used for
501 urea [2], but were unable to do so due to a lack of commercially available $^{13}C$-labeled mucin
502 oligosaccharides that are resistant to breakdown by mammalian enzymes. We therefore
503 investigated whether the capacity for mucin degradation in squirrel microbiotas changes over
504 the annual hibernation cycle. Our metagenomic analysis suggests an important role for bacteria
505 with mucin degrading capacity during winter fasting, as the five CAZymes gene families that are
506 enriched in Winter compared with Summer microbiomes all have mucin degradation capacity,
507 whereas those enriched in Summer microbiomes include a mix of mucin and non-mucin
508 degraders. This is consistent with our previous findings that several of the bacteria whose
509 relative abundances increase in Winter are known mucin degraders [17,28]. Moreover, all five
510 genes enriched in the Winter microbiomes were classified to at least one member of the phylum
511 Bacteroidota, whose relative abundance in the squirrel microbiota increases over 2-fold from
512 Summer to Winter [16,17]. Noteworthy genera to which winter-enriched gene families are
513 classified include *Akkermansia* and *Alistipes spp*. *Akkermansia muciniphila*, which is the sole
514 member of the phylum Verrucomicrobiota present in the ground squirrel microbiota [15,16], can
515 subsist on mucin alone as its sole carbon and nitrogen source [46]. This property may be
516 responsible for the over 4-fold increase in relative abundance of Verrucomicrobiota during
517 hibernation [16,17]. The genus *Alistipes*, a member of Bacteroidota and another prominent
518 mucin degrader [45], also increases during hibernation [16,17]. Interestingly, beyond its role as
519 a mucin degrader, *Alistipes* has the highest percentage of urease genes in the ground squirrel
520 microbiome during hibernation, increasing nearly two-fold in abundance from Summer to Winter
521 [2]. It therefore plays dual roles in processing of host endogenous nutrients with potential
522 benefits to the host and its microbiota.
523
524 *Conclusion*
525 Collectively, these findings introduce UCS as a novel, mutually beneficial mechanism through
526 which hibernating mammals and their gut microbes meet metabolic demands during prolonged
527 fasting. We propose a process whereby carbon limitation in the gut of fasted hibernators is
528 ameliorated by a sequential set of host-microbe interactions involving first ureolytic and then
529 acetogenic bacteria. This two-step mutualism, initiated by urea hydrolysis, first generates
530 acetate that is avidly absorbed and oxidized by the hibernator host at levels that exceed
531 summer values and may participate in other host functions including immunity and intestinal
532 barrier function. Next, via hydrogen disposal, it helps sustain the gut ecosystem as an efficient

533 environment for microbial fermentation of host compounds such as mucin glycoproteins, thus
534 unlocking the value of endogenous nutrient sources for both the microbiota and host. In
535 conjunction with UNS — which enables sustained protein synthesis in host tissues during
536 hibernation [2] — UCS establishes urea as a valuable endogenous nutrient source throughout
537 the winter fast and underscores the integral role that gut microbiota play in hibernation's
538 metabolic phenotype. Consistent shifts in gut microbial composition observed across various
539 hibernating mammals [47,48] and the finding that UCS also takes place in summer squirrels
540 suggest that urea's dual role in enabling its nitrogen and carbon to be recycled into molecules
541 with nutritional value may be widespread among mammals. Taken together, these insights
542 deepen our understanding of the host–microbe interactions that underlie metabolic resilience
543 and highlight the vital roles gut microbiota play as animals endure extended periods without
544 external nutrient sources.
545
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681

682 **Figure captions**

683 **Figure 1.** Principal components analyses (PCA) for $^{13}$C-enriched metabolomes from 13-lined
684 ground squirrel cecal contents (A), liver (B), and quadriceps muscle (C). Treatment groups
685 include gut microbiota-intact and -depleted groups from Summer (sum), early winter (EW), and
686 late winter (LW) squirrels. PERMANOVA results for each tissue are presented next to its
687 respective score plot.

688

689 **Figure 2**. $^{13}$C-urea carbon incorporation into metabolomes for cecal content (A), liver (B), and
690 quadriceps muscle (C) of microbiome-intact (filled bars) and microbiome-depleted (open bars)
691 13-lined ground squirrels. For each panel, the plots portray $^{13}$C-incorporation into the metabolite
692 named above the plot. Asterisks indicate a significant effect of season (Season) or gut
693 microbiota presence/absence (ABX), where * means P<0.05, ** means P<0.01, and *** means
694 P<0.001. n = 5 for all data.

695

696 **Figure 3.** (A) Breath $\delta^{13}$C values for 13-lined ground squirrels treated with $^{13}$C-labelled urea.
697 The data represent total area under the curve (AUC) for the 3 h period following urea treatment,
698 as first reported in [2]. The data are expressed here exponentially to eliminate negative values
699 and thus enable the urea-to-acetate efficiency calculations. (B) Urea-to-acetate efficiency, which
700 is the ratio of an individual squirrel's $^{13}$C-enriched cecal acetate concentration to its breath $\delta^{13}$C
701 AUC value.

702

703 **Figure 4.** Seasonal effect on (A) the percentage of gut bacterial genes in the 13-lined ground
704 squirrel gut microbiome related to the Wood-Ljungdahl pathway for biological carbon fixation, as
705 well as (B) the top 10 bacterial taxa from taxonomic classification of Wood-Ljungdahl genes as
706 determined by sequence abundance.

707

708 **Figure 5.** (A) Mean breath $\delta^{13}$C for Summer (green) and Late Winter (blue) 13-lined ground
709 squirrels treated with $^{13}$C-labeled acetate via oral gavage at time 0. Horizontal and vertical error
710 bars represent SEM, and closed and open symbols represent squirrels with intact and depleted
711 microbiotas. (B) Mean area under the curve (AUC) values for data in (A), with 2-way ANOVA
712 results presented on the graph and asterisks indicating significant differences as determined by
713 post-hoc test, where * indicates P<0.05, ** indicates P<0.01, and *** indicates P<0.001.

714

715 **Figure 6.** Glycoside hydrolase (GH) carbohydrate-active enzymes (CAZymes) involved in mucin
716 degradation that are seasonally enriched in Late Winter compared with Summer squirrels.
717 Displayed counts represent DESeq2-normalized counts that were Hellinger-transformed. Darker
718 colors indicate higher counts and lighter colors indicate lower counts. T-test results: GH20
719 P=0.0043; GH33 P<0.0001; GH84 P=0.0048; GH85 P=0.0027; GH109 P=0.0051. N=6 for
720 Summer, N=5 for Later Winter.
721

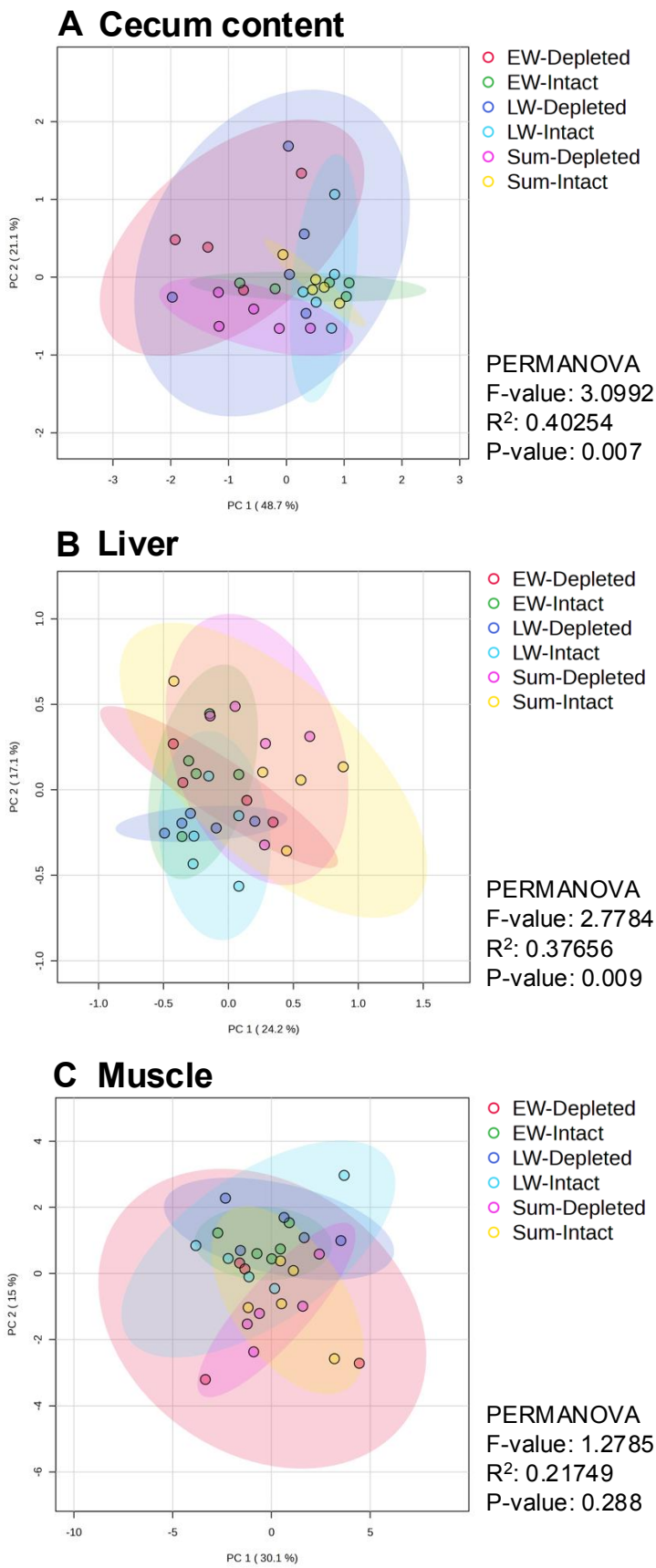



**Figure 1.** Principal components analyses (PCA) for 13C-enriched metabolomes from 13-lined ground squirrel cecal contents (A), liver (B), and quadriceps muscle (C). Treatment groups include gut microbiota-intact and -depleted groups from Summer (sum), early winter (EW), and late winter (LW) squirrels. PERMANOVA results for each tissue are presented next to its respective score plot.

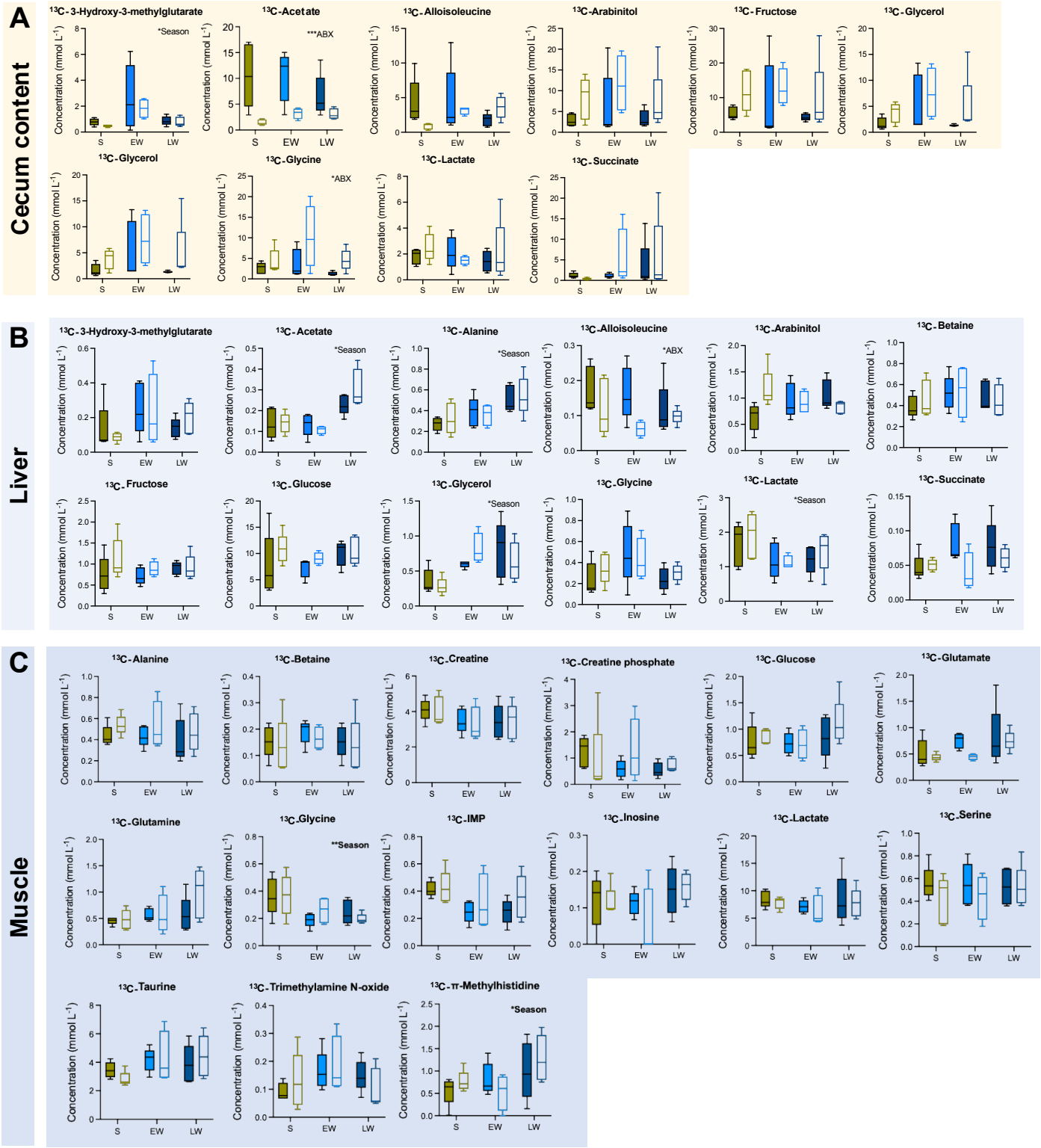



**Figure 2**. $^{13}$C-urea carbon incorporation into metabolomes for cecal content (A), liver (B), and quadriceps muscle (C) of microbiome-intact (filled bars) and microbiome-depleted (open bars) 13-lined ground squirrels. For each panel, the plots portray $^{13}$C-incorporation into the metabolite named above the plot. Asterisks indicate a significant effect of season (Season) or gut microbiota presence/absence (ABX), where * means P<0.05, ** means P<0.01, and *** means P<0.001. n = 5 for all data.

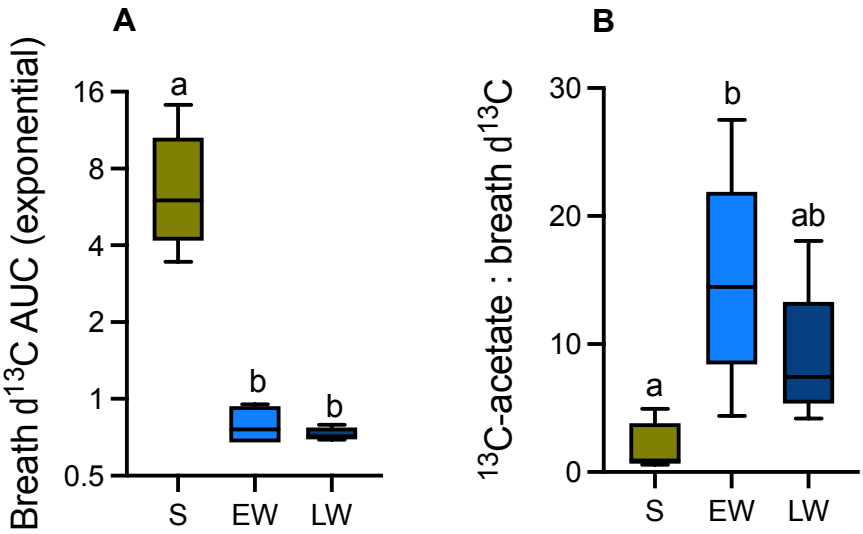
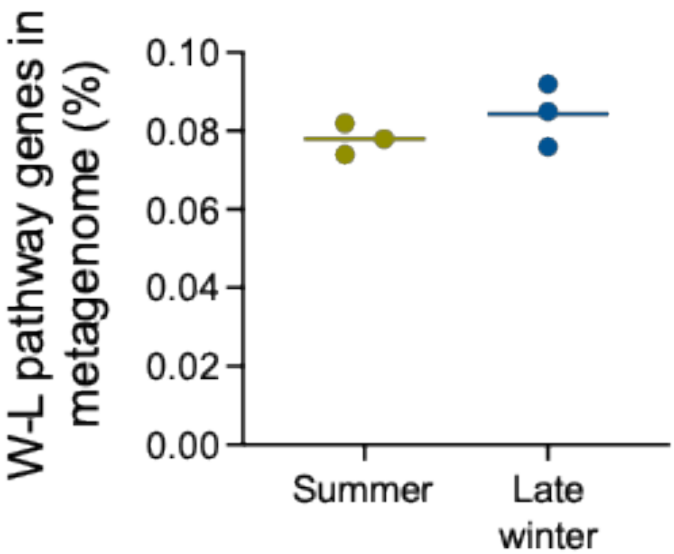



**Figure 3**. (A) Breath $\delta^{13}C$ values for 13-lined ground squirrels treated with $^{13}C$-labelled urea. The data represent total area under the curve (AUC) for the 3 h period following urea treatment, as first reported in [2]. The data are expressed here exponentially to eliminate negative values and thus enable the urea-to-acetate efficiency calculations. (B) Urea-to-acetate efficiency, which is the ratio of an individual squirrel's 13C-enriched cecal acetate concentration to its breath $\delta^{13}C$ AUC value.

**A**




**B**

| Summer | | Late Winter | |
|---|---|---|---|
| **Taxon** | **% W-L Genes** | **Taxon** | **% W-L Genes** |
| *Bacteroides* | 1.74% | *Blautia* | 3.63% |
| *Clostridium* | 0.96% | *Clostridium* | 1.67% |
| *Desulfallas* | 0.92% | *Bacteroides* | 1.60% |
| *Alistipes* | 0.78% | *Lachnoclostri* | 0.73% |
| *Terrabacteria* | 0.78% | *Terrabacteria* | 0.58% |
| *Treponema* | 0.74% | *Eggerthella* | 0.58% |
| *Blautia* | 0.67% | *Treponema* | 0.51% |
| *Lactobacillus* | 0.63% | *Oscillibacter* | 0.51% |
| *Neisseria* | 0.55% | *Olsenella* | 0.44% |
| *Corynebacter* | 0.37% | *Desulfallas* | 0.44% |

**Figure 4.** Seasonal effect on (A) the percentage of gut bacterial genes in the 13-lined ground squirrel gut microbiome related to the Wood-Ljungdahl pathway for biological carbon fixation, as well as (B) the top 10 bacterial taxa from taxonomic classification of Wood-Ljungdahl genes as determined by sequence abundance.

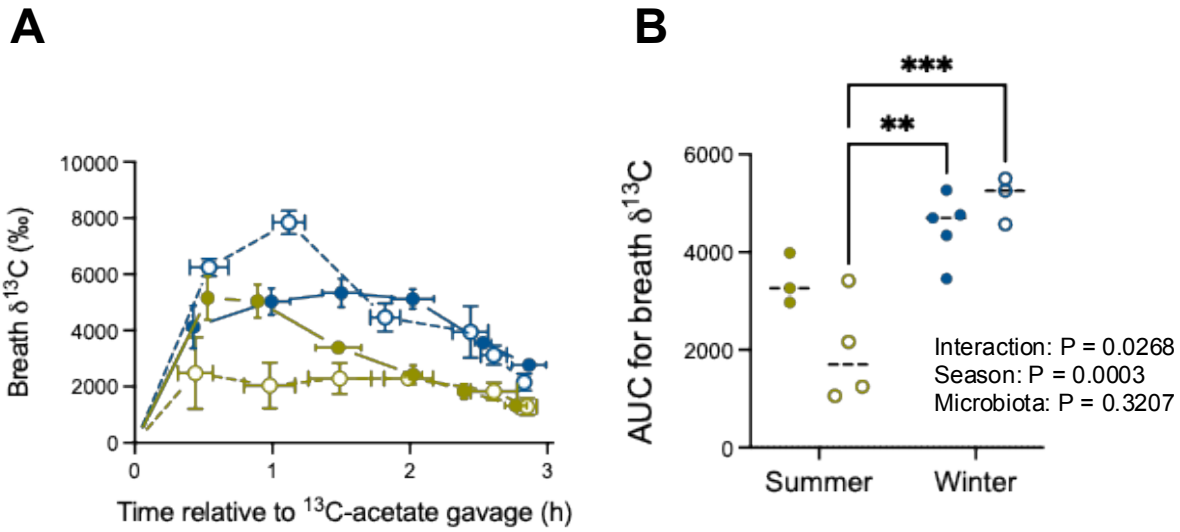



**Figure 5.** (A) Mean breath $\delta^{13}C$ for Summer (green) and Late Winter (blue) 13-lined ground squirrels treated with 13C-labeled acetate via oral gavage at time 0. Horizontal and vertical error bars represent SEM, and closed and open symbols represent squirrels with intact and depleted microbiotas. (B) Mean area under the curve (AUC) values for data in (A), with 2-way ANOVA results presented on the graph and asterisks indicating significant differences as determined by post-hoc test, where * indicates $P<0.05$, ** indicates $P<0.01$, and *** indicates $P<0.001$.

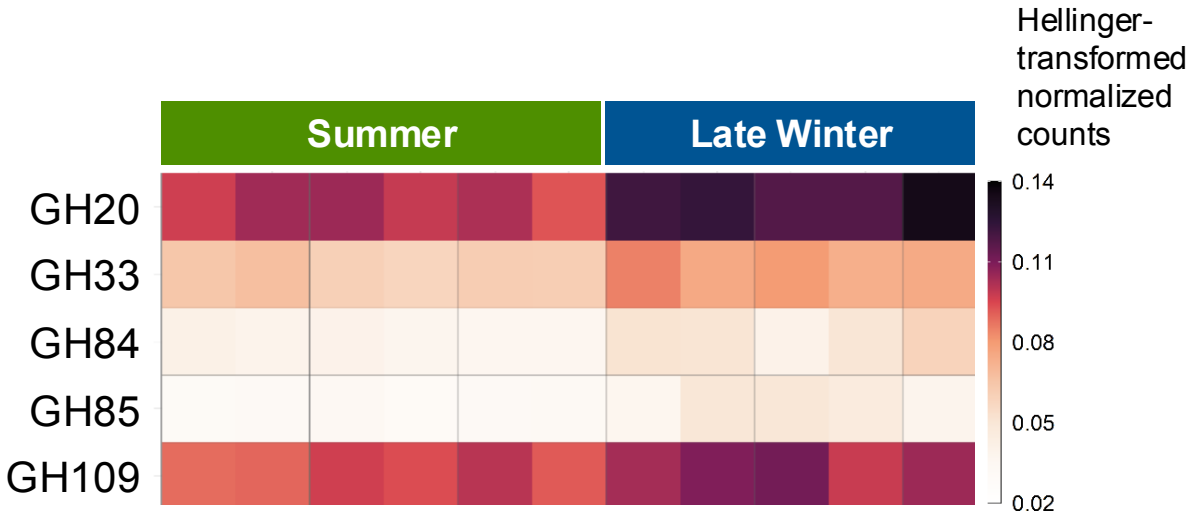



**Figure 6.** Glycoside hydrolase (GH) carbohydrate-active enzymes (CAZymes) involved in mucin degradation that are seasonally enriched in Late Winter compared with Summer squirrels. Displayed counts represent DESeq2-normalized counts that were Hellinger-transformed. Darker colors indicate higher counts and lighter colors indicate lower counts. T-test results: GH20 P=0.0043; GH33 P<0.0001; GH84 P=0.0048; GH85 P=0.0027; GH109 P=0.0051. N=6 for Summer, N=5 for Later Winter.

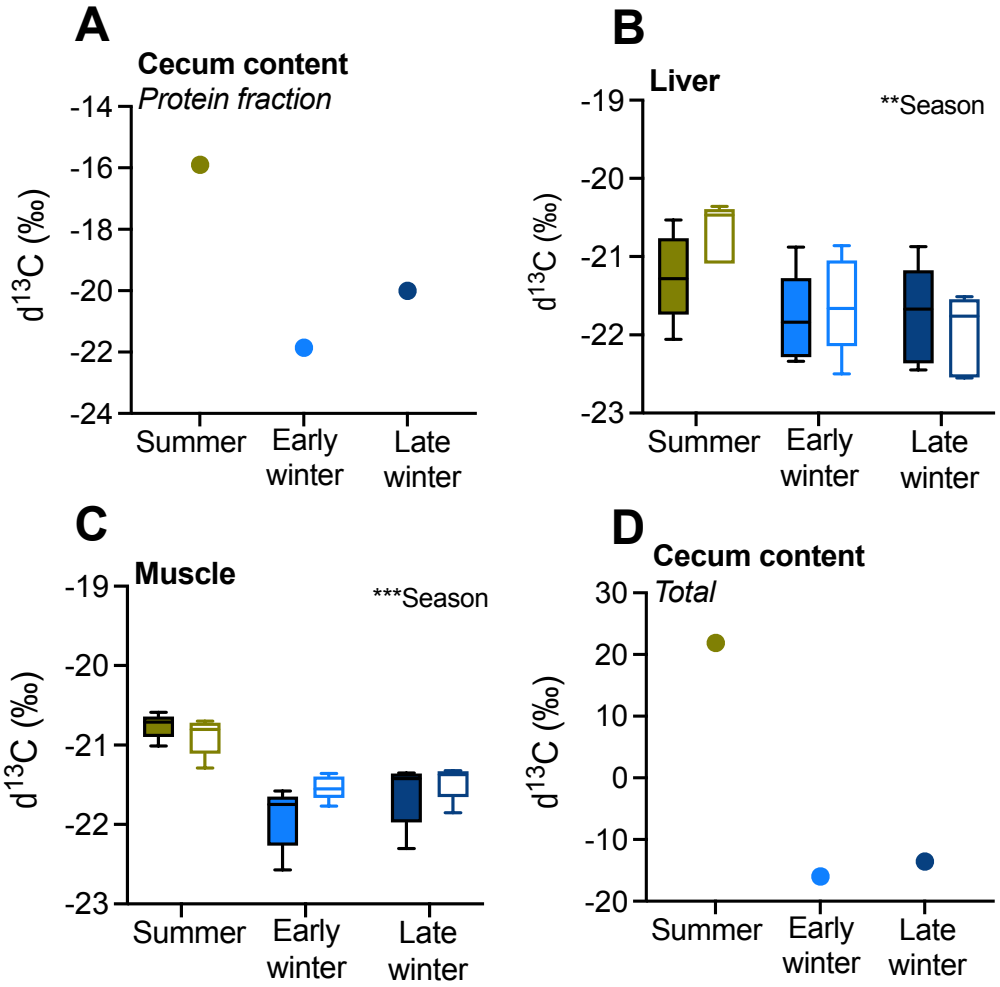



**Figure S1.** Isotope ratio mass spectrometry-based measurements of $^{13}$C-urea carbon incorporation into the protein fractions of (A) cecum content, (B) liver and (C) quadriceps muscle. $^{13}$C-urea carbon incorporation into "total" cecum content is also shown (D), which is an analyses run on the total cecal content milieu, not just the protein fraction. Cecal content panels (A, D) lack microbiota-depleted data because ABX-treated animals had insufficient content for analyses. Asterisks indicate a significant effect of season (Season) or gut microbiota presence/absence (ABX), where ** indicates P<0.01 and *** indicates P<0.001. n = 5 for all data except for cecum content (A, D; n=1, pooled samples).