1. TITLE PAGE

Host-microbiome mutualism drives urea carbon salvage and acetogenesis during hibernation

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2. ABSTRACT

Hibernation is a seasonal survival strategy employed by certain mammals that, through torpor use, reduces overall energy expenditure and permits long-term fasting. Although fasting solves 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 hibernating 13-lined ground squirrels, whereby urea carbon is reclaimed through gut microbial ureolysis and used in reductive acetogenesis to produce acetate, a short-chain fatty acid (SCFA) of major value to the host and its gut microbiota. 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 supply throughout the year, the host's ability to absorb and oxidize acetate is highest during hibernation. Metagenomic analysis of the gut microbiome indicates that genes involved in the degradation of gut mucins, an abundant endogenous nutrient, are retained during hibernation. The hydrogen disposal associated with reductive acetogenesis from urea carbon helps facilitate this mucin degradation by providing a luminal environment that sustains fermentation, thereby generating SCFAs and other metabolites usable by both the host and its gut microbes. Our findings introduce UCS as a 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.

3. SIGNIFICANCE STATEMENT

When food becomes scarce during winter, hibernating mammals induce torpor to minimize energy demands and enable monthslong fasting. However, fasting eliminates the intake of essential nutrients such as carbon. We identified a two-step microbial-host interaction in ground squirrels – urea carbon salvage (UCS) – which counters carbon limitation by salvaging carbon from waste urea. Through activities of ureolytic and acetogenic bacteria, urea-derived CO_2 is reduced by free hydrogen to form acetate, whose oxidation provides energy for gut microbes and the host. This process also helps maintain a permissive environment for fermentation of other host-derived, energy-dense compounds such as mucins. UCS broadens our understanding of host-microbe mutualism under extreme nutritional constraints and may represent a widespread adaptation among fasting mammals.

4. MAIN TEXT

Introduction

For most mammals and their gut microbes, winter is a time of resource limitation. Some species survive by storing extra fat reserves and finding other, less preferred food items. In contrast, hibernators like the 13-lined ground squirrel (*Ictidomys tridecemlineatus*) survive by entering torpor for weeks at a time, which allows them to stop eating for approximately six months each year [1]. Winter fasting solves the hibernator's food scarcity problem, but it deprives the animal – and its gut microbes – of exogenous nutrients that are crucial for sustaining life. This challenge could be met in part by mutualistic relationships between hosts and their microbial symbionts that transform endogenous compounds into valuable nutrient resources.

One example of resource reclamation during fasting is urea nitrogen salvage (UNS), a gut microbe-dependent process 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 resistance to muscle atrophy [2,3]. Urea nitrogen is also incorporated into the microbial protein pool, thereby confirming that UNS benefits both symbiotic partners [2]. Thus, urea, which is excreted as a waste product during summer, serves as an endogenous nitrogen source during hibernation when these animals and their gut microbes lack an exogenous supply of this important biomolecular building block.

In addition to nitrogen, urea also contains carbon, another essential nutrient that is limited during hibernation due to long-term fasting. Theoretically, the ability to capture this carbon via urea carbon salvage (UCS) would also provide nutritional benefits to the microbiota and potentially to the hibernator host. Although various animals are known to use UNS as a nitrogen conservation system [4], to our knowledge UCS has never been proposed as a mechanism to maintain carbon balance in species that undergo extended fasting, which is the case for most hibernators. But preliminary results from our previous study [2] support the existence of UCS and suggest that urea carbon is liberated by microbial urea hydrolysis, biologically fixed, and incorporated into organic molecules. Thus, it is possible that both host and microbes benefit from UCS during resource-limited hibernation.

In this study, we tested more rigorously the hypothesis that carbon liberated by urea hydrolysis is incorporated into organic molecules, and that these molecules provide benefits for ground squirrels and/or their microbiota during winter fasting. To accomplish this, we treated

106 squirrels with ^{13}C,^{15}N-labeled urea and measured urea carbon (i.e., ^{13}C) incorporation into the
107 microbiota as well as tissue metabolome and protein. We performed real-time in vivo $\delta^{13}C$
108 breath experiments to estimate rates of microbial urea hydrolysis and absorption/metabolism of
109 orally gavaged acetate, as well as metagenomic analyses of bacterial genes involved in carbon
110 fixation and acetogenesis. In addition, we expanded the notion that fasted hibernators and their
111 gut microbes participate in mutualistic interactions that enable the use of host-secreted
112 compounds as nutrients by examining whether hibernator microbiomes are enriched in genes
113 encoding enzymes that degrade intestinal mucins, which are the most abundant endogenous
114 nutrients in the squirrel gut ecosystem during winter fasting.
115
116 **Materials & methods**
117 *Experimental animals*
118 From a total of 65 13-lined ground squirrels (39 females, 26 males; hereafter called squirrels),
119 39 were used for the doubly labelled urea treatment experiments described in [2], 15 were
120 pretreated with oral gavages of ^{13}C-acetate, and gut contents from 11 others were used for
121 carbon fixation and mucin degradation metagenomics analyses. All squirrels were born in a
122 vivarium on the University of Wisconsin-Madison (UW-Madison) campus to wild-caught female
123 squirrels that were collected around Madison, Wisconsin, USA in May 2018 and May 2019. The
124 pregnant squirrels were housed individually at 22°C with a 12:12 h light-dark cycle with ad
125 libitum access to water and rodent chow (Teklad no. 2020X, Envigo, Indianapolis, IN, USA)
126 supplemented with fruit (apples) and sunflower seeds once per week. After birth, pups remained
127 with their mothers for 5 weeks and then moved to individual cages. Following 2 weeks of ad
128 libitum chow and fruit, food was restricted to 12 g chow per day (supplemented with 1 g of
129 sunflower seeds once per week) to prevent excessive weight gain that occurs in captive-born
130 squirrel pups. The consistent diet minimized diet-induced effects on the gut microbiota and the
131 breath $\delta^{13}C$ value. Squirrels were held under these conditions until the day of experiment
132 (Summer group) or transferred to the cold room at the beginning of the hibernation season
133 (Early and Late Winter groups).
134 In mid-September, squirrels in the Winter groups were moved to a 4°C environmental
135 chamber in the UW-Madison Biotron. The chamber was held in constant darkness except for ~5
136 min/day of dim light to enable activity checks. Food and water were removed after squirrels
137 began regular torpor-arousal cycles, which was 24–72 h after being moved into the chamber.
138 Each of the 39 urea-treatment squirrels were studied at one of three time points: Summer
139 (14 squirrels), Early Winter (12 squirrels), and Late Winter (13 squirrels). Each seasonal group

140 was divided into three treatment groups: $^{13}C,^{15}N$-urea with antibiotics, $^{13}C,^{15}N$-urea without
141 antibiotics, and unlabeled urea without antibiotics. Mean days in hibernation for the Early Winter
142 and Late Winter groups were 36 d (range 31–41 d) and 127 d (range 109–135 d), respectively.
143 Mean body masses on experiment days for Summer, Early Winter, and Late Winter groups
144 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–
145 167.0 g), respectively.
146 The 15 acetate-treatment squirrels were divided into four groups: Summer with antibiotics (4
147 squirrels), Summer without antibiotics (3 squirrels), Late Winter with antibiotics (3 squirrels), and
148 Late Winter without antibiotics (5 squirrels). Mean days in hibernation for the combined Late
149 Winter groups was 92 d (range 68–145 d). Mean body masses on experiment days for Summer
150 and Late Winter were 206.1 g (range 172.6–241.2 g) and 151.2 g (range 128.3–174.0 g),
151 respectively.
152 The 11 microbiota-intact squirrels used for metagenomic analysis of carbon fixation and
153 mucin degradation genes included 6 Summer and 5 Late Winter squirrels. Mean days in
154 hibernation for the Late Winter group were 135 d (range 127–144 d). Mean body masses on
155 experiment days for Summer and Late Winter were 183.0 g (range 144.0–225.0 g) and 132.5 g
156 (range 116.5–144.6 g)
157 The UW-Madison Institutional Animal Care and Use Committee approved all procedures.
158
159 *Animal treatments and stable isotope breath-testing*
160 For the 39 squirrels treated with $^{13}C,^{15}N$-labeled urea, the antibiotic treatments to deplete the gut
161 microbiota, the $^{13}C,^{15}N$-labeled urea treatments (200 mg urea kg^{-1} body mass), the breath
162 testing experiments via cavity ring-down spectrometry (CRDS), and the euthanasia protocol
163 were all carried out as described in [2].
164 The 15 squirrels used to estimate host and microbe acetate oxidation capacity were treated
165 with ^{13}C-labeled acetate (500 mg kg^{-1} body mass) via oral gavage following light anesthesia (4%
166 isoflurane-O_2 mixture). Acetate oxidation capacity was measured using the CRDS-based breath
167 analysis, after which squirrels were euthanized. Antibiotics were administered as described in
168 [2].
169 The 11 squirrels used for metagenomic-based estimates of mucin glycan degradation were
170 administered saline via oral gavage following light anesthesia (4% isoflurane-O_2 mixture). On
171 the day of sampling CRDS-based breath analysis was performed, after which squirrels were
172 euthanized.

173 All experiments were performed on squirrels whose body temperatures (T_b) were in the
174 euthermic range (above 30ºC) so that the physiological and biochemical processes we
175 examined could proceed at optimal temperatures. The T_b range of Summer squirrels when
176 euthanized was 36-38 ºC. Hibernating squirrels were induced to arouse from a torpor bout (thus
177 simulating the natural interbout arousal periods that last 12-24 h) by transferring the animal
178 cage from the 4°C cold room to the ~22°C lab. Experiments commenced when T_b was at least
179 36°C.
180
181 *Tissue harvesting and preparation for analyses*
182 Following euthanasia, we collected host tissues (liver, quadriceps) and cecal contents (a proxy
183 for the cecal microbiota) and prepared them for isotope ratio mass spectrometry (IRMS) and
184 nuclear magnetic resonance (NMR)-based metabolomics analyses as described in [2]. The
185 IRMS and NMR techniques were executed as described in [2]. All ^{1}H and ^{13}C spectra were
186 analyzed using the Chenomx software package (Version 8.5; Edmonton, AB, Canada).
187
188 *Metagenomics*
189 Generation of metagenomic data from cecal contents was performed as previously described
190 [2]. Briefly, genomic DNA was extracted using the Qiagen DNeasy PowerSoil Kit (Qiagen,
191 Hilden, Germany) according to manufacturer's specifications and quantified using a Qubit
192 Fluorometer (Invitrogen, San Diego, CA, USA). Sequencing was performed on an Illumina
193 NovaSeq6000 (Illumina, San Diego, CA, USA) using an S4 Reagent Kit v1.5 at 2x150bp PE.
194 Trimmomatic v0.38 [5] was used to remove sequencing adapters and low-quality reads, while
195 host DNA was filtered using bowtie2 v2.2.2 [6] against the 13-lined ground squirrel genome
196 (GenBank and RefSeq assembly accession = GCA_000236235.1). The remaining reads were
197 assembled using metaSPAdes v3.13.0 [7] and open reading frames (ORFs) were predicted
198 using prodigal v2.6.3 [8]
199 For the carbon fixation-related analyses, translated proteins from the ORF predictions were
200 compared against the Pfam [8] profiles for enzymes involved in Wood-Ljungdahl pathway (WLP)
201 carbon fixation, as defined by KEGG [9–11] using HMMER [12]. These enzymes included fdhF
202 (formate dehydrogenase), fhs1 (formate tetrahydrofolate ligase), fchA (methenyltetrahydrofolate
203 cyclohydrolase), foID (methylenetetrahydrofolate dehydrogenase (NADP+) /
204 methenyltetrahydrofolate cyclohydrolase), metF MTHRF (methylenetetrahydrofolate reductase
205 (NADH)), cdhE acsC (acetyl-CoA decarbonylase/synthase, CODH/ACS complex subunit
206 gamma), cdhD acsD (acetyl-CoA decarbonylase/synthase, CODH/ACS complex subunit delta),

207 cdhC (acetyl-CoA decarbonylase/synthase subunit beta), and cooS acsA (anaerobic carbon-
208 monoxide dehydrogenase catalytic subunit). Identified sequences were then mapped back to
209 their contig of origin and taxonomically classified using centrifuge [13]. The percentage of target
210 genes in the metagenomes were calculated by dividing the number of identified target genes by
211 the total number of predicted genes. The percentage of target genes classified to different taxa
212 was calculated by dividing the number of target genes classified to a specific taxon by the total
213 number of detected target genes.
214 For the mucin degradation analysis, we first identified putative enzymes thought to be
215 involved in mucin degradation from the Carbohydrate-Active enZymes (CAZy) database [14],
216 including glycoside hydrolase (GH) 20 (exo-acting *β*-*N*-acetylglucosaminidases, *β*-*N*-
217 acetylgalactosamindase and *β*-6-SO_3-*N*-acetylglucosaminidases), GH33 (sialidase), GH84 (*β*-
218 *N*-acetylglucosaminidases), GH85 (Endo-β-N-acetylglucosaminidases), and GH109 (α-*N*-
219 acetylgalactosaminidase). We then compared the translated proteins from the predicted ORFs
220 of the metagenomes against the CAZy database using dbCAN [15] and HMMER v3.3 [12].
221 To determine differences in mucin degradation genes between Summer and Late Winter
222 metagenomes, we first normalized CAZyme mucin degradation genes by calculating reads per
223 kb per genome equivalent (RPKG) [16]. For each ORF, ORF length (kb) and the number of
224 mapped short reads were determined using bowtie2 [5]. Next, the number of mapped short
225 reads for each ORF was divided by ORF length and summed in each sample. This sum was
226 then divided by genome equivalents to calculate RPKG. Genome equivalents was determined
227 using MicrobeCensus [16] with default parameters. Seasonal differences in CAZyme normalized
228 counts were analyzed in R [17] using an ANOVA (*aov*) and Tukey's HSD (*TukeyHSD*) if they
229 were normally distributed, or with the Kruskal-Wallis test (*kruskal.test*) and Dunn's test
230 (*dunn.test*) if they were not normally distributed. P-values were adjusted for false discovery rate
231 using the Benjamini-Hochberg procedure (*p.adjust*). Results were considered significant if adj P
232 < 0.05, except for Dunn's test where results were considered significant if adj $P \leq 0.025$.
233 Overrepresented CAZymes were identified with DESeq2 [18] and normalized counts were
234 transformed using the Hellinger method (*hellinger*, labdsv package in R).
235
236 *Statistical analyses*
237 Multivariate ^{13}C-metabolomic data for cecal contents, liver and muscle were analyzed by
238 combining principal components analysis (PCA) for visualization and PERMANOVA analysis for
239 quantitation (MetaboAnalyst 6.0, www.metaboanalyst.ca). Effects of season and microbiota
240 presence on ^{13}C-incorporation into identifiable metabolites (via NMR) and protein fraction of

241 tissue (via IRMS) were analyzed using 2-way ANOVA (GraphPad Prism v10.3). Breath $\delta^{13}C$
242 was plotted as a function of the 3 h following labeled urea or acetate treatment, and then area-
243 under-the-curve (AUC) was calculated and expressed relative to each individual's total breath
244 analysis time [19]. The data for urea-treated squirrels originally reported in [2] were expressed
245 here as an exponential function of base *e* to eliminate negative values and enable urea-to-
246 acetate conversion efficiency calculations, which were expressed as the ^{13}C-acetate abundance
247 relative to the exponentially converted breath $\delta^{13}C$ AUC for the urea-treated squirrels. Time
248 course traces for breath responses to doubly labeled urea-treated squirrels can be found in [2].
249 Seasonal differences in breath $\delta^{13}C$ AUC for urea-treated squirrels and relative abundances of
250 gut bacterial genes involved in carbon fixation (via WLP) and mucin degradation were analyzed
251 using Student's t-test. Effects of season and microbiota presence on breath $\delta^{13}C$ AUC for
252 acetate-treated squirrels were analyzed using 2-way ANOVA. Non-normally distributed data via
253 Shapiro-Wilk test were log-transformed prior to analysis. In cases where data remained non-
254 normal, non-parametric tests were used. For data presented in whisker plots, the upper and
255 lower bounds of the box represent 75th and 25th percentiles, respectively; the horizontal line
256 within the box represents the median value, and the upper and lower whiskers represent the
257 maximum and minimum values within the data set, respectively. Statistical significance was set
258 at $P<0.05$ for all comparisons.
259
260 **Results**
261 *Urea carbon (^{13}C) incorporation into host and microbe metabolome and protein*
262 We observed a total of 10, 12 and 15 metabolites enriched in urea-derived ^{13}C in cecal
263 contents, liver and muscle, respectively (Figs. 1, 2). The paired PCA-PERMANOVA multivariate
264 analysis revealed that, among the six treatment groups (Summer, Early Winter, Late Winter;
265 with and without microbiotas) within each compartment, ^{13}C-enriched metabolite pools varied
266 with season in cecal contents ($F=3.0992$, $R^2=0.4025$, $P=0.007$) and liver ($F=2.7784$, $R^2=0.3766$,
267 $P=0.009$), but not in muscle ($F=1.2785$, $R^2=0.2175$, $P=0.288$) (Fig. S1). However, among all
268 observable metabolites, only cecal content ^{13}C-acetate was significantly affected by the
269 presence of a microbiota, whereby its concentrations in microbiota-intact squirrels were 2- to 7-
270 fold higher than those of microbiota-depleted squirrels (Fig. 1A; ANOVA $P<0.0001$; original data
271 were reported in Fig. S6 in [2] and expressed here in concentration). This microbiota effect
272 confirmed that the incorporation of urea carbon into acetate molecules was dependent upon
273 microbial activity, most likely reflecting reductive acetogenesis that used $^{13}CO_2$ generated by
274 ureolytic bacteria after administration of ^{13}C,^{15}N-urea. ^{13}C-acetate levels in liver were low and

275 unaffected by the gut microbiota, though concentrations were slightly higher in Late Winter than
276 Summer or Early Winter (Fig. 1B; ANOVA P>0.05). In muscle, ^{13}C-acetate levels were below
277 detectable limits.
278 In contrast to our previous observations [2] that urea-derived ^{15}N is incorporated into host
279 (liver, muscle) and microbiota (cecal contents) protein pools, we found no evidence of
280 microbiota-dependent incorporation of ^{13}C into protein fractions of the liver, muscle, or
281 microbiota after ^{13}C,^{15}N-urea administration (Fig. S2). For the microbiota-intact squirrels, the
282 small volumes of cecal content samples required pooling all samples into a single aliquot for
283 IRMS analysis, which ultimately precluded statistical testing. For microbiota-depleted squirrels,
284 the samples volumes were too small for analysis. However, we observed a trend for greater
285 incorporation of ^{13}C into protein fractions of Summer cecal contents compared with Early or Late
286 Winter fractions (Fig. S2).
287
288 *Seasonal effect on ureolytic activity and urea-to-acetate efficiency*
289 The CRDS-based measurements of breath $\delta^{13}C$ reported in [2] for estimation of in vivo gut
290 microbial ureolytic activity were transformed here to express the $\delta^{13}C$ values as positive
291 numbers, thus enabling efficiency measurements (see Materials and Methods). This analysis
292 revealed that, as reported in [2], microbial ureolytic activity was higher in Summer than in Early
293 or Late Winter squirrels, which were not different (Fig. 2A; ANOVA P=0.0017).
294 Each squirrel's urea-to-acetate efficiency was expressed as the ratio of its cecal urea-
295 derived ^{13}C-acetate concentration to its total $^{13}CO_2$ abundance, represented as its transformed
296 breath $\delta^{13}C$ value shown in Fig. 2A. This efficiency measure reflects the amount of liberated
297 urea carbon that is directed towards acetogenesis. We found that urea-to-acetate efficiency is
298 generally higher during hibernation than summer (Fig. 2B; ANOVA P=0.0135; t-test P=0.0112
299 when grouping Early and Late Winter into a single hibernation group).
300
301 *Seasonal effect on microbial carbon fixation capacity*
302 To estimate the capacity of the squirrel gut microbiome to fix carbon and enable acetogenesis,
303 we analyzed Summer and Late Winter microbial metagenomes for nine genes encoding for
304 WLP-related enzymes. The relative abundances of eight out of nine genes were sustained from
305 Summer to Late Winter (Fig. 3A) despite the ~90% reduction in bacterial density by Late Winter
306 [20], while the ninth fchA (methenyltetrahydrofolate cyclohydrolase) increased from Summer to
307 Late Winter (Fig. 3A; t-test P=0.003). Analysis of the top 10 bacterial genera from taxonomic
308 classification of WLP genes, as determined by sequence relative abundance, revealed a

309 seasonal shift from Summer to Late Winter in the bacterial potential for reductive acetogenesis
310 that could include use of urea carbon (Fig. 3B). Most notable was the finding that the greatest
311 percentage of WLP genes in Summer metagenomes (1.74%) belonged to *Bacteroides*, whereas
312 over twice that amount (3.63%) was classified to *Blautia* in Late Winter. The percentage of WLP
313 gene sequences represented in *Blautia* spp. in Late Winter metagenomes was over 5-fold than
314 in Summer metagenomes.
315
316 *Capacities for acetate oxidation*
317 An increase in $\delta^{13}C$ breath after administration of ^{13}C,^{15}N-urea indicates that oxidation of one or
318 more bioenergetic metabolites by the microbiota, by squirrel cells, or both is a consequence of
319 ureolysis in the gut lumen. Our identification of ^{13}C-acetate molecules in the microbial
320 metabolome that contain urea-derived carbon [2] implicated acetate as one of those
321 metabolites. To explore the site of acetate oxidation in the squirrel holobiont, and whether there
322 is a seasonal difference in capacity for acetate oxidation by the microbiota and/or host, we orally
323 gavaged microbiota-intact and -depleted squirrels from Summer and Late Winter with a body
324 mass-specific dose of ^{13}C-labeled acetate and then measured their breath $\delta^{13}C$ using CRDS.
325 None had been treated with labeled urea. We found that squirrels from all groups exhibited
326 rapid and large increases in breath $\delta^{13}C$ (Fig. 2C), indicative of acetate oxidation and
327 subsequent release of $^{13}CO_2$ in breath. Squirrels in Late Winter generally exhibited greater
328 breath $\delta^{13}C$ responses than squirrels in Summer (Fig. 2D; ANOVA P=0.0003), but there were
329 seasonal differences in how the presence of the gut microbiota influenced the responses. In
330 Summer, breath $\delta^{13}C$ as measured by AUC was highest in squirrels with intact microbiotas,
331 whereas in Late Winter, breath $\delta^{13}C$ was highest in squirrels with depleted microbiotas (Fig. 2D).
332 Consequently, microbiota-depleted squirrels exhibited both the lowest (Summer) and highest
333 (Late Winter) responses, with a >3-fold difference between the two (Fig. 2D; ANOVA P=0.001)
334 suggesting that the host may be especially primed for acetate absorption and oxidation during
335 the winter fast. Season also influenced the time required to reach peak breath $\delta^{13}C$, with
336 Summer squirrels reaching peak values Early (~30 min post-gavage) relative to Late Winter
337 squirrels (~75 min post-gavage) (Fig. 2C).
338
339 *Mucin degrading capacity in the squirrel microbiome*
340 Like urea, mucin glycoproteins are host-derived compounds that provide benefits to gut
341 microbes and their hosts. Mucins are secreted from goblet cells in the gut epithelial layer and
342 are either bound to epithelial cell membranes or secreted directly into the lumen. Mucin glycans

343 are complex carbohydrates whose degradation releases a variety of smaller oligosaccharides
344 that are fermented anaerobically, producing energy-rich metabolites including SCFAs that can
345 be oxidized for ATP generation or serve other roles such as signalling molecules.
346 Owing to a lack of commercially available ^{13}C-labeled mucins that are resistant to
347 breakdown by mammalian enzymes, we used metagenomics to test the hypothesis that the
348 seasonal shifts in gut microbiota composition we previously identified [21,22] are associated
349 with an enhanced capacity to degrade mucins. We focused on gene families that encode
350 CAZymes with the capacity to degrade dietary polysaccharides, mucin glycans, or both, and
351 identified 17 that were overrepresented in either Summer or Late Winter metagenomes. Of the
352 17, we found five CAZymes that were enriched in Late Winter vs. Summer, all of which are
353 capable of degrading intestinal mucins (Fig. 4).
354
355 **Discussion**
356 This study was designed to provide functional context for our previous observation that in 13-
357 lined ground squirrels, carbon contained in urea molecules that enter the gut lumen is
358 incorporated into acetate within the microbial metabolome [2]. Here we show that this effect is
359 restricted to acetate among a suite of metabolites we examined and is more efficient during
360 winter fasting than the summer fed season. Together, our findings support the idea that through
361 the symbiotic relationship between hibernators and their gut microbes, urea is repurposed as a
362 dual-use endogenous nutrient source during hibernation, with both its carbon and nitrogen
363 salvaged and used for synthesis of important biomolecules. This previously undescribed
364 pathway for acetate production in the ground squirrel gut uses CO_2 released by ureolytic
365 microbes for hydrogen disposal via reductive acetogenesis, thereby contributing to maintenance
366 of an efficient fermentative environment for the degradation and use of endogenous host
367 compounds, such as mucins, that benefit the hibernator and its microbiota during the long winter
368 fast.
369
370 *Urea carbon salvage*
371 Like UNS, UCS begins with the movement of urea molecules from the blood into the gut lumen,
372 mediated by urea transporters (UT-B), where it is hydrolyzed by ureolytic gut microbes. We
373 recently showed that the abundance of UT-B in the squirrel cecum increases from Summer to
374 Late Winter, suggesting a greater capacity to transport urea into the lumen during the
375 hibernation season [2]. We also showed that ureolytic bacteria are found in the squirrel
376 microbiota and that rates of ureolysis are higher in Summer than in Early or Late Winter, likely

377 due to the reduction in total bacterial abundance that occurs in ground squirrels from summer to
378 winter [20,21].
379 Urea carbon is next incorporated into organic compounds, a process carried out by
380 acetogenic microbes. These metabolites then become available for use by microbes and/or
381 absorption and processing by the host. To investigate this, we screened metabolomes of cecal
382 contents (a proxy for the microbiota) and the squirrels' liver and skeletal muscle specifically for
383 ^{13}C-enriched metabolites which, because our squirrels were treated with ^{13}C,^{15}N-labeled urea,
384 would indicate urea carbon-containing metabolites. Looking broadly across the multivariate data
385 via paired PCA and PERMANOVA analyses, we found no significant effect of gut microbiota
386 presence/absence on the ^{13}C-metabolomes of these three compartments (Fig. S1), suggesting
387 that at this level of analysis there is minimal incorporation of urea carbon into organic
388 compounds. However, analysis of individual metabolites revealed a significant gut microbe-
389 dependent incorporation of urea carbon into the SCFA acetate (Fig. 1).
390 Our finding that urea carbon-derived acetogenesis occurs in months-long fasted
391 hibernating mammals was notable for several reasons. First, although cecal acetogenesis by
392 the reduction of CO_2 with H_2 was first reported in 1977 [23] and replicated subsequently in the
393 hindgut of other monogastric species, the carbon source in those studies was not identified and
394 generally assumed to be oligosaccharides produced by fermentation of dietary carbohydrates.
395 Recently, Firth et al. [24] reported that colonization of mouse and human microbial communities
396 with a ureolytic strain of the gut microbe *Blautia* leads to urea-dependent acetogenesis and
397 cross-feeding of urea carbon from acetate to other SCFAs. Our study demonstrates urea
398 carbon-incorporation into acetate in a wild species, extends its physiological relevance by
399 initiating the experiments with circulating urea, and proposes that the host-microbe interactions
400 that enable urea carbon-derived acetogenesis are regulated in part by the nutritional status of
401 the host animal.
402 Also notable is the similarity of cecal ^{13}C-acetate concentrations throughout the year
403 despite the decrease in bacterial abundance during hibernation [20]. This is consistent with the
404 sustained percentage from Summer to Winter of bacterial genes that encode for enzymes in the
405 Wood-Ljungdahl pathway (WLP; Fig. 3A), though the taxa assigned to those genes differ (Fig.
406 3B). In this pathway, CO_2 is reduced by H_2 to form acetate and water, thus linking the CO_2
407 produced by urea hydrolysis with acetogenesis (Fig. 5). Taken together, these findings suggest
408 that the chronic lack of dietary substrates during hibernation induces a compensatory response
409 in the squirrel microbiome that expands metabolic function and enhances survival in those
410 bacteria that persist during winter fasting. Genes encoding carbon fixation- and acetogenesis-

411 related enzymes would facilitate the production of bioenergetic substrates such as acetate that
412 could positively impact both microbes and the hibernator host. This is consistent with the
413 stringent response, whereby bacteria under nutrient stress such as carbon starvation reallocate
414 resources from growth towards the upregulation of survival-related genes until conditions
415 improve [25–27].
416 Another example of molecular shifts activated by changing nutrient supply is the
417 canonical glucose-acetate transition in *Escherichia coli*, whereby cells excrete acetate while
418 growing on glucose and later absorb and catabolize it for energy when glucose is depleted. This
419 is facilitated by rapid changes in gene expression that suppress biosynthetic pathways and
420 upregulate catabolic ones [28,29]. Here, we posit that a similar strategy may exist for the
421 microbiomes of fasting hibernators; when typical carbon sources in the gut such as glucose
422 derived from dietary polysaccharides are depleted over the winter fast, bacteria scavenge for
423 other carbon sources [28]. Urea carbon-derived acetogenesis appears to be one such pathway
424 that persists throughout hibernation, enabled in part by a sustained (or slightly elevated)
425 proportion of bacterial genes involved in carbon fixation and acetogenesis (Fig. 3A). In the case
426 of fasting hibernators, the contributions of pathways that recycle endogenous host-derived
427 compounds such as urea and mucins may play significant roles in energy-sparing, thus
428 conserving stored lipids that are the major fuel source during torpor and arousals [30].
429 Interestingly, more of the urea carbon liberated by microbial ureolysis appears to be
430 used for acetogenesis during hibernation than during the summer active season. Specifically,
431 the ratio of ^{13}C-acetate to total exhaled $^{13}CO_2$ was higher in hibernating than in summer
432 squirrels (Fig. 2B). It is possible that urea carbon is incorporated into compounds other than
433 acetate, but our ^{13}C-metabolomics results show that this incorporation is not appreciable, at
434 least in the tissues we analyzed. This suggests that less urea carbon is lost as CO_2 in breath
435 during winter, when exogenous carbon intake has ceased and when urea carbon, via carbon
436 fixation and subsequent acetogenesis, would most benefit the hibernator holobiont.
437
438 *Potential benefits of urea carbon-derived acetate for hibernator host and microbiota*
439 We perceive several benefits of urea carbon-derived acetogenesis for hibernating species
440 during winter fasting. The most direct is acetate's value as an energy source for host cells, with
441 intestinal epithelial cells (IECs) the most likely to benefit given their dependence on luminal
442 SCFAs such as butyrate to meet energy needs [31]. Under normal fed conditions, butyrate is
443 the preferred energetic substrate for IECs; however, when butyrate availability is limited, acetate
444 oxidation can supply them with ATP [32]. A similar shift in the nutritional landscape occurs

445 during hibernation, when cecal concentrations of all SCFAs decrease relative to Summer levels,
446 though to different extents. For example, cecal butyrate concentrations fall to as low as 3% of
447 Summer levels, whereas acetate concentrations remain at 30-40% of Summer levels [20,21]. It
448 is therefore possible that urea carbon-derived acetogenesis contributes to this energy supply for
449 the host, particularly at the level of IECs.
450 To lend empirical support for acetate's role as an energy source for hibernators and their
451 gut microbes, we orally gavaged Summer and Late Winter squirrels with ^{13}C-acetate. We then
452 used CRDS to measure breath $\delta^{13}C$, whereby elevated values above baseline indicate oxidation
453 of ^{13}C-acetate to $^{13}CO_2$. Breath $\delta^{13}C$ rapidly increased following ^{13}C-acetate gavage in
454 microbiota-intact Summer and Late Winter squirrels, resulting in similar AUC values for the 3 h
455 post-gavage collection period (Fig. 2D) despite the lower total bacterial abundance of Late
456 Winter relative to Summer squirrels [20]. To distinguish host acetate oxidation from potential
457 microbial acetate oxidation, we repeated the experiments on microbiota-depleted squirrels in
458 both seasons. In Summer squirrels, depleting the microbiota halved the breath $\delta^{13}C$ AUC
459 (suggesting a strong microbial contribution to acetate oxidation); whereas in Late Winter
460 squirrels, microbiota depletion had no effect on breath $\delta^{13}C$ AUC but produced a higher $\delta^{13}C$
461 peak that was attained more quickly. Overall, the Late Winter microbiota-depleted squirrels had
462 AUC values 3-fold higher than Summer microbiota-depleted squirrels (Fig. 2D). These results
463 suggest two things about acetate use in hibernating squirrels. First, during summer, gut
464 microbes oxidize much of the acetate they produce (perhaps contributing to greater ^{13}C
465 incorporation into cecal content protein; Fig. S2), whereas during hibernation, a greater
466 proportion of the acetate is absorbed and oxidized by the host. Thus, the squirrel host appears
467 to benefit most from the bioenergetic contributions of acetate during hibernation. We suspect
468 that the primary site of oxidation for microbially generated acetate in squirrels, especially during
469 hibernation, is within IECs. This is consistent with the lack of observable ^{13}C-acetate in liver and
470 muscle, and with the generally deficient energy state of IECs during long periods of food
471 deprivation [33]. Second, the 3-fold higher breath $\delta^{13}C$ responses after ^{13}C-acetate gavage in
472 Late Winter compared with Summer microbiota-depleted squirrels indicate that the capacity to
473 absorb and oxidize acetate increases during hibernation, when resources are limited and
474 organic compounds such as acetate are especially valuable. Although we have not directly
475 measured rates of intestinal acetate absorption, these results are consistent with our previous
476 work showing higher normalized rates of Na^+, glucose, and amino acid absorption by the small
477 intestinal epithelium in hibernating than in summer squirrels [34,35]. Similar results were
478 observed for nutrient uptake into brush border membrane vesicles [36], and it is plausible that

479 similar seasonal effects on intestinal acetate absorption via monocarboxylate transporters
480 underlie the results seen here. Another indirect way that urea carbon-derived acetate may
481 influence IEC metabolism during winter fasting is through its conversion to their preferred fuel
482 source by acetate-consuming, butyrate-producing bacteria within the microbiota [37]. Together,
483 these results suggest that the hibernator's physiology is seasonally optimized to benefit from
484 urea carbon-derived acetogenesis, which may suggest positive selection on this mechanism.
485 Another potential role for urea carbon-derived acetate in the hibernating host is its role
486 as a ketogenic substrate. The ketone β-hydroxybutyrate is the preferred fuel source for the heart
487 and brain of ground squirrels during hibernation [38]. Ketogenesis, which takes place primarily
488 in the liver but can occur in other sites including the gut [39,40], is regulated by the rate-limiting
489 enzyme (3-hydroxy-3-methylglutaryl-CoA synthetase 2; HMGCS2). We showed previously that
490 HMGCS2 is expressed in the 13-lined ground squirrel intestine and its protein abundance is 2-
491 fold higher during hibernation than in summer [41]. This finding is consistent with those from
492 mice and rats whereby short-term fasting elevates both intestinal gene expression of HMGCS2
493 [42] and the conversion of acetate to ketone bodies in isolated colonocytes [32]. Furthermore, a
494 role for the microbiota in stimulating hepatic ketogenesis in mice under nutrient deprivation was
495 shown when mice with intact gut microbiotas elevated hepatic ketogenesis following a 24 h fast,
496 but the response was reduced in mice lacking a microbiota [43]. Given that levels of fatty acids,
497 which are the most common substrates for ketogenesis, are likely reduced in hibernator IECs
498 due to the lack of dietary lipid intake, acetate may be an effective ketogenic precursor for the gut
499 during hibernation.
500 In addition to providing acetate as an energy source, acetogenesis also contributes to
501 maintenance of a gut environment that is conducive to microbial breakdown of dietary
502 substrates and host-secreted compounds. Pathways associated with microbial fermentation
503 require electron sinks to dispose of free elections, and production of H_2 is a major electron sink.
504 However, high H_2 partial pressure in the lumen can be harmful to bacterial and host cells and
505 can slow key microbial reactions that favor substrate fermentation and the production of SCFA,
506 such as the regeneration of the coenzyme NAD^+ from NADH [44]. Microbes whose enzymatic
507 repertoire include H_2 consumption – such as acetogens and methanogens – thus play essential
508 roles in maintaining the gut lumen in a condition permissive to ongoing fermentation of
509 substrates whilst minimizing damage to microbes and their hosts. A second possible role for
510 UCS could thus be to sustain the fermentation of endogenous host compounds. The most
511 abundant of these are secreted mucins, especially during hibernation when IEC proliferation
512 and sloughing are suppressed [45]. Although methanogenesis plays a similar role (but without

513 formation of a bioenergetic compound like acetate), there is no evidence for hydrogenotrophic
514 methanogens (Archaea) in the microbiotas of hibernating mammals including ground squirrels
515 [20–22] and bats [46]. Thus, acetogenesis is likely the primary H_2 disposal mechanism used in
516 the hindgut of mammalian hibernators.
517
518 *Seasonal changes in mucin degradation capacity in the ground squirrel microbiota*
519 Mucins are complex glycoproteins secreted from goblet cells in the gut epithelium that, upon
520 hydration in the lumen, form mucus, a complex viscoelastic matrix that protects the epithelium
521 and serves as a habitat for a diverse assemblage of microbes [47]. Mucins also provide
522 nutritional benefits to gut microbes and their hosts [47], which would be particularly beneficial
523 during winter fasting. In the absence of dietary (typically plant) glycans, host-derived mucins
524 become major substrates that support microbial growth and proliferation, and they contribute to
525 host energetic needs by producing SCFAs that fuel IECs [48]. Coupled with our previous
526 observation that cecal mucin (MUC2) expression continues during hibernation [22], these
527 molecules, like urea, can serve as valuable sources of carbon, nitrogen and energy during the
528 winter fast when exogenous nutrients are absent. Intact mucins are not digestible by
529 mammalian enzymes, but bacteria that express certain glycosyl hydrolases (GHs) can cleave
530 and metabolize glycan linkages, releasing oligosaccharides that are further utilized by the same
531 or other bacteria for energy, and in so doing produce beneficial metabolites for the host [49].
532 Our metagenomics analysis revealed a strong seasonal influence on the capacity of the
533 ground squirrel microbiome to degrade mucins during winter fasting. The five CAZyme gene
534 families that are enriched in Late Winter compared with Summer microbiomes all have mucin
535 degradation capacity, whereas those enriched in Summer microbiomes include a mix of mucin
536 and non-mucin degraders. This is consistent with our previous findings that several of the
537 bacteria whose relative abundances increase in Winter are known mucin degraders [22,34].
538 Moreover, all five genes enriched in the Late Winter microbiomes were classified to at least one
539 member of the Bacteroidota; this phylum, whose relative abundance in hibernating squirrels
540 increases over 2-fold from Summer to Winter [21,22], includes the highest number of species
541 with mucin-degrading capacity in mammals. Other noteworthy genera to which winter-enriched
542 gene families are classified include *Akkermansia, Alistipes* and *Blautia*. *Akkermansia*
543 *muciniphila*, which is the sole member of the phylum Verrucomicrobiota present in the ground
544 squirrel microbiota [20,21], can subsist on mucin alone as its sole carbon and nitrogen source
545 [50]. This property may be responsible for the over 4-fold increase in relative abundance of
546 Verrucomicrobiota during hibernation [21,22]. The genus *Alistipes*, a member of Bacteroidota

547 and another prominent mucin degrader [49], also increases during hibernation [21,22]. Beyond
548 its role as a mucin degrader, *Alistipes* has the highest percentage of urease genes in the ground
549 squirrel microbiome during hibernation, increasing nearly two-fold in abundance from summer to
550 late winter [2]. *Blautia*, which has the highest percentage of WLP genes in the Late Winter
551 metagenome (Fig. 4B), is also a mucin degrader [49] and contains four of the five mucin-
552 degrading GHs that we found to be elevated in Late Winter vs. Summer microbiomes (Fig 4).
553 Moreover, like *Alistipes*, *Blautia* also contains genes that encode for urease in ground squirrels
554 [2]. Taken together with our previous findings [2], the results presented here suggest that
555 several bacterial genera play multiple roles in the processing of the endogenous nutrients urea
556 and mucin that yield potential benefits to the hibernator host and its microbiota in the absence of
557 food intake.
558
559 *Conclusion*
560 Collectively, these findings introduce UCS as a mutually beneficial mechanism through which
561 hibernating mammals and their gut microbes meet metabolic demands during prolonged fasting.
562 We propose a process whereby carbon limitation in the gut of fasted hibernators is ameliorated
563 by a sequential set of host-microbe interactions involving ureolytic and acetogenic bacteria (Fig.
564 5). This two-step mutualism – which could be carried out by the same microbe (e.g., *Blautia*
565 [24]) or by different ones (e.g., *Blautia* and *Alistipes*) – links urea hydrolysis with generation of
566 acetate, providing an oxidizable substrate for gut microbes and the hibernator host. And via H_2
567 disposal, it helps sustain the gut ecosystem as an efficient environment for microbial
568 fermentation of host compounds such as mucin glycoproteins, thus unlocking the value of
569 endogenous nutrient sources for both the microbiota and its host. In conjunction with UNS —
570 which facilitates sustained protein synthesis in host tissues during hibernation [2] — UCS
571 establishes urea as a valuable endogenous nutrient source throughout the winter fast and
572 underscores the integral role that gut microbes play in hibernation's metabolic phenotype.
573 Consistent shifts in gut microbial composition observed across various hibernating mammals
574 [51,52] and the finding that UCS also takes place in summer squirrels suggest that the dual role
575 for urea in enabling its nitrogen and carbon to be recycled into molecules with nutritional value
576 may be widespread among hibernating and non-hibernating mammals, including humans [24].
577 Taken together, these insights deepen our understanding of the host–microbiome interactions
578 that underlie metabolic resilience and further highlight the vital roles that the gut microbiota
579 plays for animals enduring extended periods without external nutrient sources.
580

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739

740 **7. FIGURE LEGENDS**

741 **Figure 1**. ^{13}C-urea carbon incorporation into metabolomes for cecal contents (A), liver (B), and
742 quadriceps muscle (C) of microbiota-intact (filled bars) and microbiota-depleted (open bars) 13-
743 lined ground squirrels from Summer (S), Early Winter (EW) and Late Winter (LW). For each
744 panel, the plots portray ^{13}C-incorporation into the metabolite named above the plot. Asterisks
745 indicate a significant effect of season (Season) or gut microbiota presence/absence (ABX),
746 where * means P<0.05, ** means P<0.01, and *** means P<0.001. n=5 for all data.

747

748 **Figure 2.** (A) Breath δ^{13}C values for 13-lined ground squirrels from Summer (S), Early Winter
749 (EW) and Late Winter (LW) treated with ^{13}C-labelled urea. The data represent total area under
750 the curve (AUC) for the 3 h period following urea treatment, as first reported in [2]. The data are
751 expressed here exponentially to eliminate negative values and thus enable the urea-to-acetate
752 efficiency calculations. (B) Urea-to-acetate efficiency, which is the ratio of an individual squirrel's
753 ^{13}C-enriched cecal acetate concentration to its breath δ^{13}C AUC value. (C) Mean breath δ^{13}C for
754 Summer (green) and Late Winter (blue) 13-lined ground squirrels treated with ^{13}C-labeled
755 acetate via oral gavage at time 0. Horizontal and vertical error bars represent SEM; closed and
756 open symbols represent squirrels with intact and depleted microbiotas. (D) Mean area under the
757 curve (AUC) values for data in (C), with 2-way ANOVA results presented on the graph and
758 asterisks indicating significant differences as determined by post-hoc test, where * indicates
759 P<0.05, ** indicates P<0.01, and *** indicates P<0.001. n=5 for A and B, n=3-4 for C and D.

760

761 **Figure 3.** Seasonal effect on (A) the percentage of nine Wood-Ljungdahl pathway (WLP)-
762 related gut bacterial genes for carbon fixation (see text for abbreviation definitions) in the 13-
763 lined ground squirrel gut microbiome, as well as (B) the top 10 bacterial genera from taxonomic
764 classification of WLP genes as determined by sequence abundance.

765

766 **Figure 4.** Glycoside hydrolase (GH) carbohydrate-active enzymes (CAZymes) involved in mucin
767 degradation that are seasonally enriched in Late Winter compared with Summer squirrels.
768 Displayed counts represent DESeq2-normalized counts that were Hellinger-transformed. Darker
769 colors indicate higher counts and lighter colors indicate lower counts. T-test results: GH20
770 P=0.0043; GH33 P<0.0001; GH84 P=0.0048; GH85 P=0.0027; GH109 P=0.0051. N=6 for
771 Summer, N=5 for Late Winter.
772
773 **Figure 5**. Proposed mechanism for urea carbon salvage in hibernating mammals during
774 euthermic interbout arousals. Urea, endogenously produced in the liver, is transported by
775 epithelial urea transporters from the blood into the cecal lumen, where it is hydrolyzed by
776 ureolytic bacteria into NH_3 and CO_2. NH_3 is subjected to different possible fates, some resulting
777 in urea nitrogen incorporation into microbial and host tissue protein pools via microbial and host
778 amino acid (AA) synthesis, a process called urea nitrogen salvage [2]. CO_2 serves as the
779 substrate for bacterial Wood-Ljungdahl pathway (WLP) carbon fixation producing acetate, a
780 short chain fatty acid (SCFA) that is oxidized by gut microbes and host cells for energy, as well
781 as serving other potential roles in the host (see text). The WLP reactions consume H_2, which
782 sustains an efficient fermentation environment for degradation of host compounds such as
783 mucin glycoproteins. Together, this 2-step mutualism transforms urea from a waste molecule
784 into a useful nutrient source and unlocks the value of other endogenous nutrient sources such
785 as mucins for both host and microbe during the resource-limited hibernation season.
786

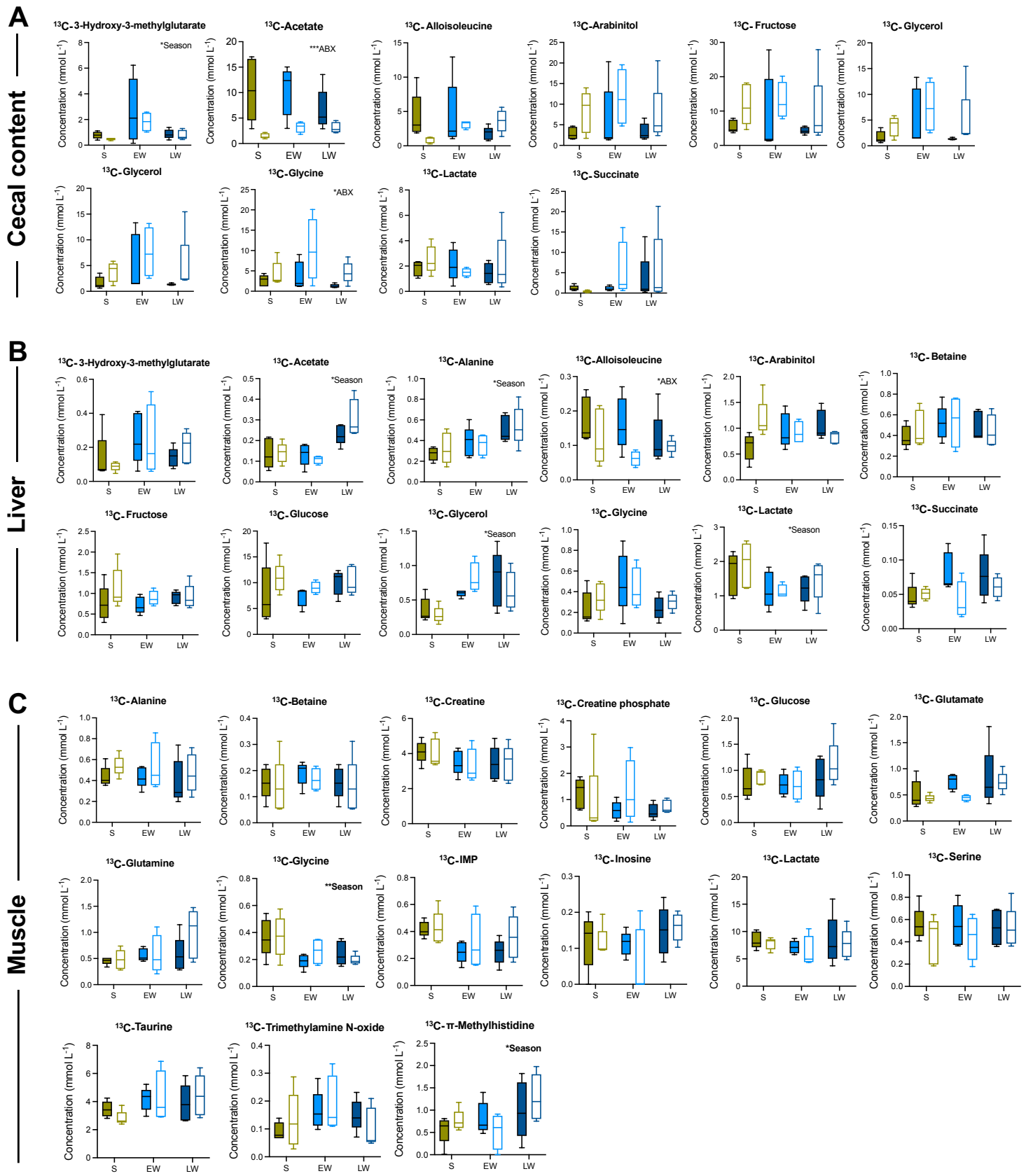


Figure 1. ^{13}C-urea carbon incorporation into metabolomes for cecal content (A), liver (B), and quadriceps muscle (C) of microbiome-intact (filled bars) and microbiome-depleted (open bars) 13-lined ground squirrels from Summer (S), Early Winter (EW) and Late Winter (LW). For each panel, the plots portray ^{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.

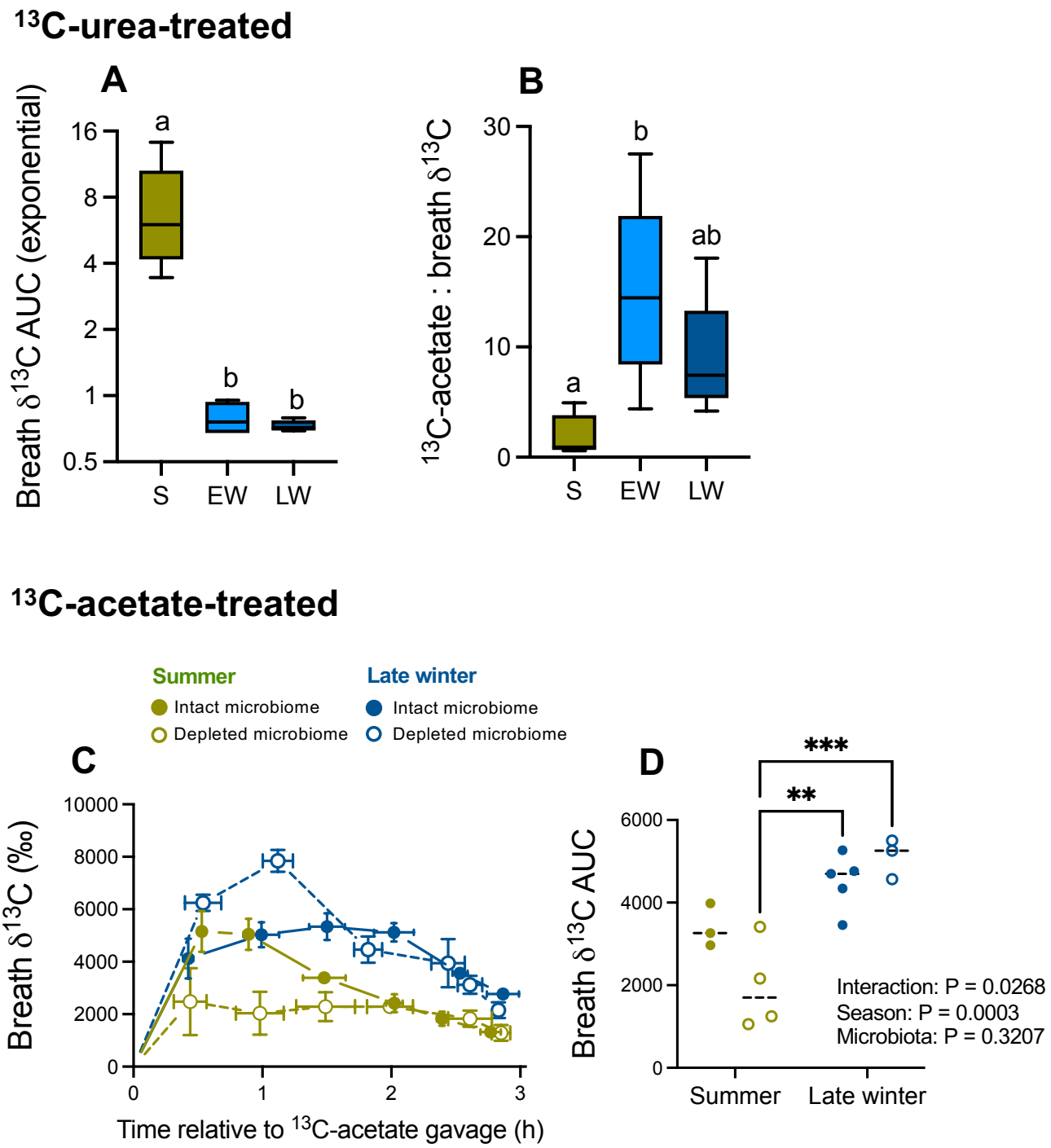
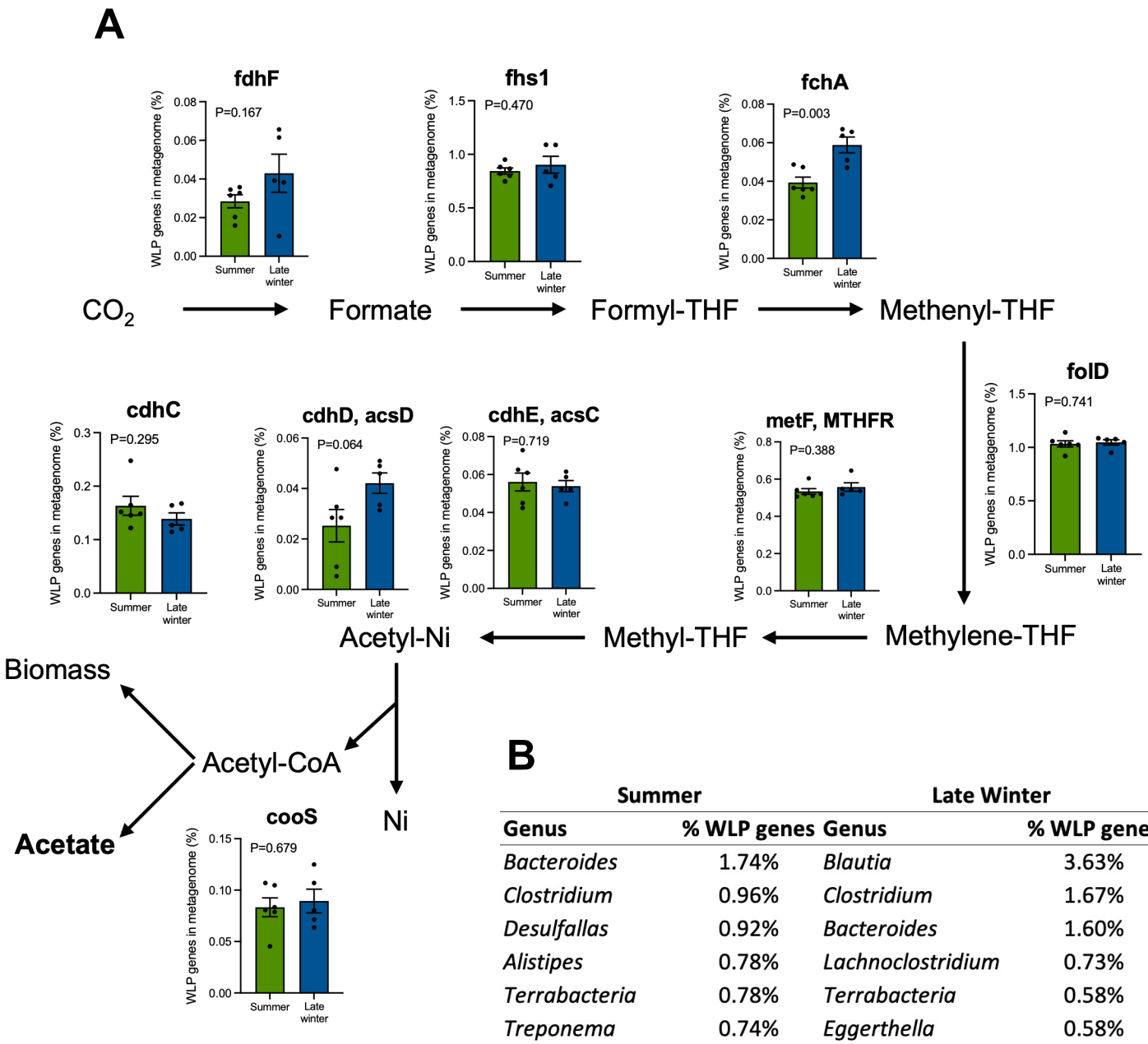


Figure 2. (A) Breath $\delta^{13}C$ values for 13-lined ground squirrels from Summer (S), Early Winter (EW) and Late Winter (LW) treated with ^{13}C-labelled urea. The data represent total area under the curve (AUC) for the 3 h period following urea treatment, as first reported in [2]. The data are expressed 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 ^{13}C-enriched cecal acetate concentration to its breath $\delta^{13}C$ AUC value. (C) Mean breath $\delta^{13}C$ for Summer (green) and Late Winter (blue) 13-lined ground squirrels treated with ^{13}C-labeled acetate via oral gavage at time 0. Horizontal and vertical error bars represent SEM; closed and open symbols represent squirrels with intact and depleted microbiotas. (D) Mean area under the curve (AUC) values for data in (C), 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 $P<0.001$. n=5 for A and B, n=3-4 for C and D.



B

Summer		Late Winter	
Genus	**% WLP genes**	**Genus**	**% WLP genes**
Bacteroides	1.74%	*Blautia*	3.63%
Clostridium	0.96%	*Clostridium*	1.67%
Desulfallas	0.92%	*Bacteroides*	1.60%
Alistipes	0.78%	*Lachnoclostridium*	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%
Corynebacterium	0.37%	*Desulfallas*	0.44%

Figure 3. Seasonal effect on (A) the percentage of nine Wood-Ljungdahl pathway (WLP)-related gut bacterial genes for carbon fixation (see text for abbreviation definitions) in the 13-lined ground squirrel gut microbiome, as well as (B) the top 10 bacterial genera from taxonomic classification of WLP genes as determined by sequence abundance.

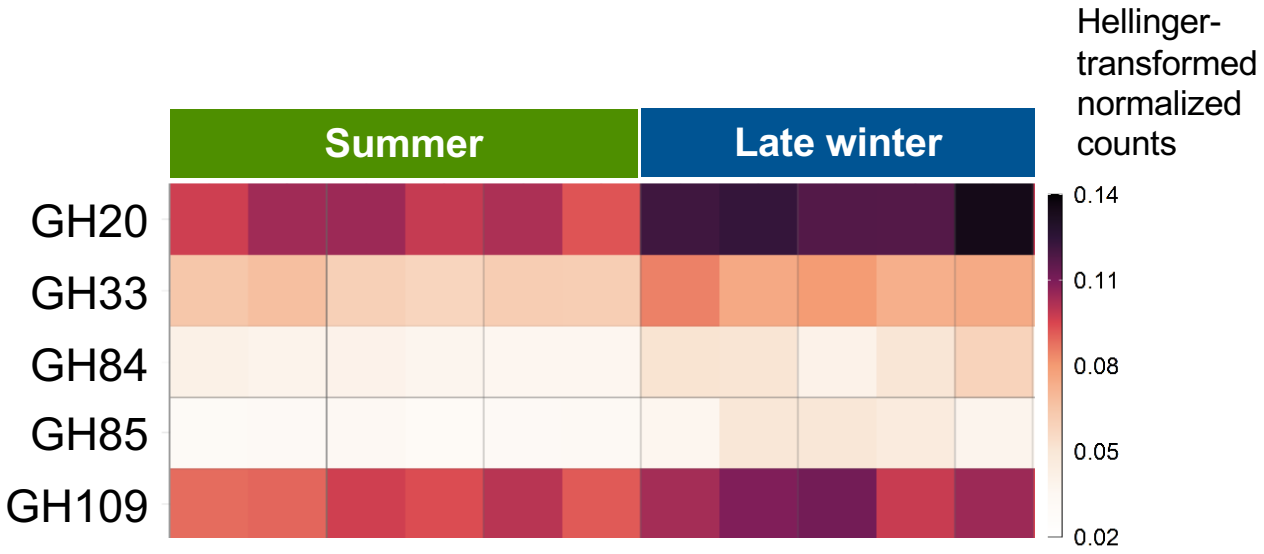


Figure 4. Glycoside hydrolase (GH) carbohydrate-active enzymes (CAZymes) involved in mucin degradation that are seasonally enriched in Late Winter compared with Summer squirrels. Displayed counts represent DESeq2-normalized counts that were Hellinger-transformed. Darker colors indicate higher counts 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.

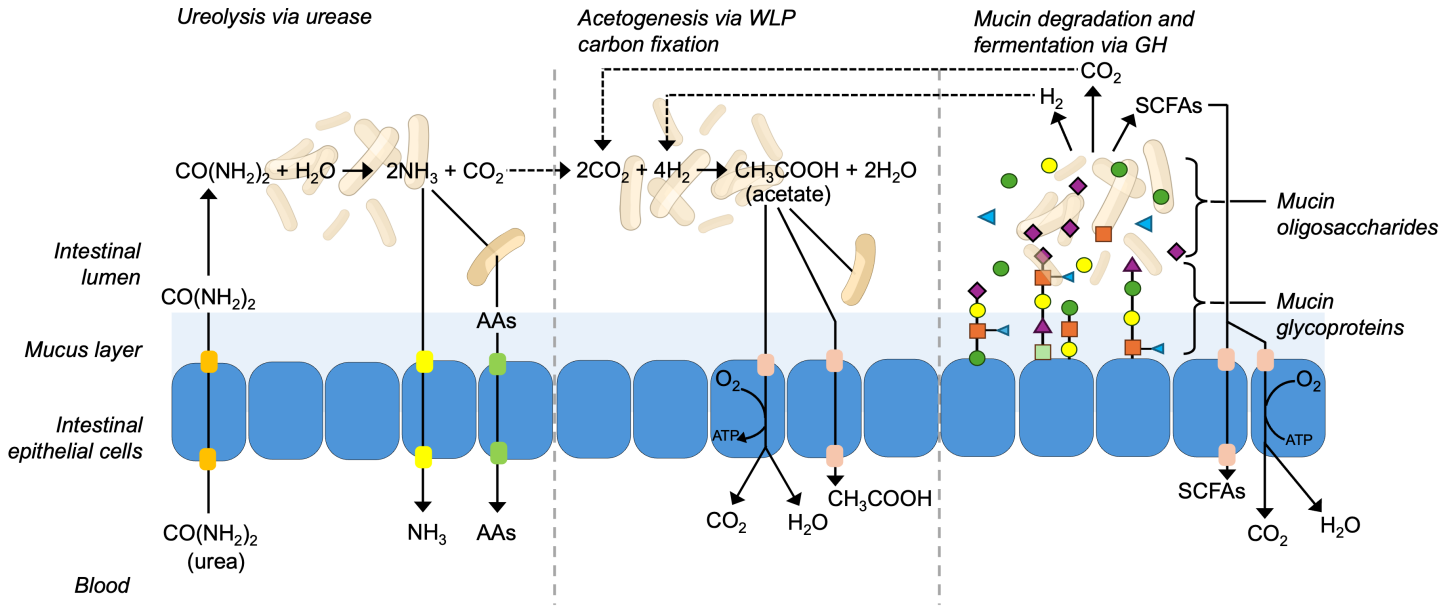


Figure 5. Proposed mechanism for urea carbon salvage in hibernating mammals during euthermic interbout arousals. After synthesis in the liver, circulating urea enters the cecal lumen via urea transporters where it is hydrolyzed by ureolytic bacteria into NH_3 and CO_2. NH_3 is subjected to different possible fates, some resulting in urea nitrogen incorporation into microbial and host protein pools via microbial and host amino acid (AA) synthesis, a process called urea nitrogen salvage [2]. CO2 serves as the substrate for bacterial Wood-Ljungdahl pathway (WLP) carbon fixation producing acetate, a short chain fatty acid (SCFA) that is oxidized by gut microbes and host cells for energy, as well as serving other potential roles in the host (see text). The WLP reactions consume H2, which sustains an efficient fermentation environment for degradation of host compounds such as mucin glycoproteins. Together, this 2-step mutualism transforms urea from a waste molecule into a useful nutrient source and unlocks the value of other endogenous nutrient sources such as mucins for both host and microbe during the resource-limited hibernation season.

Supplemental figures

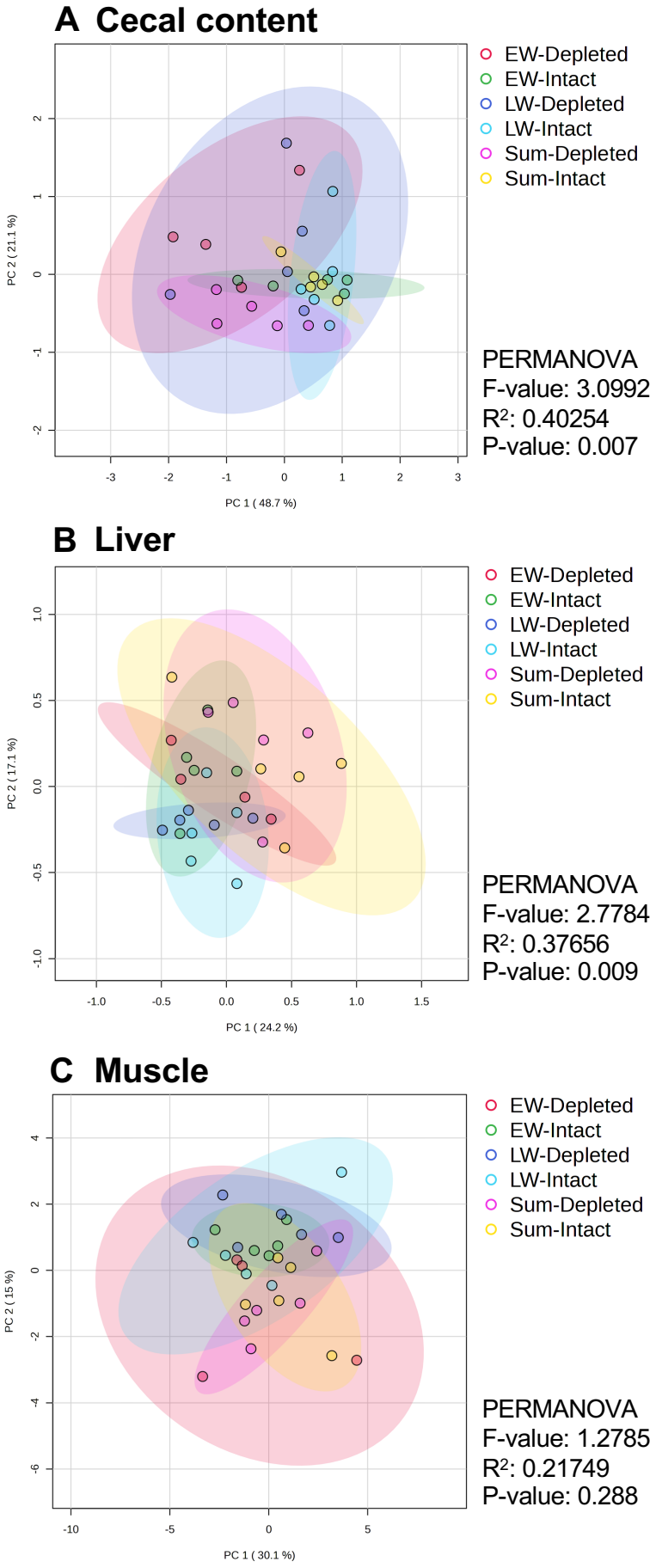


Figure S1. Principal components analyses (PCA) for ^{13}C-enriched metabolomes from 13-lined ground squirrel cecal contents (A), liver (B), and quadriceps muscle (C). Treatment groups include gut microbiota-intact and -depleted groups from Summer (sum), Early Winter (EW), and Late Winter (LW) squirrels. PERMANOVA results for each tissue are presented next to its respective score plot.

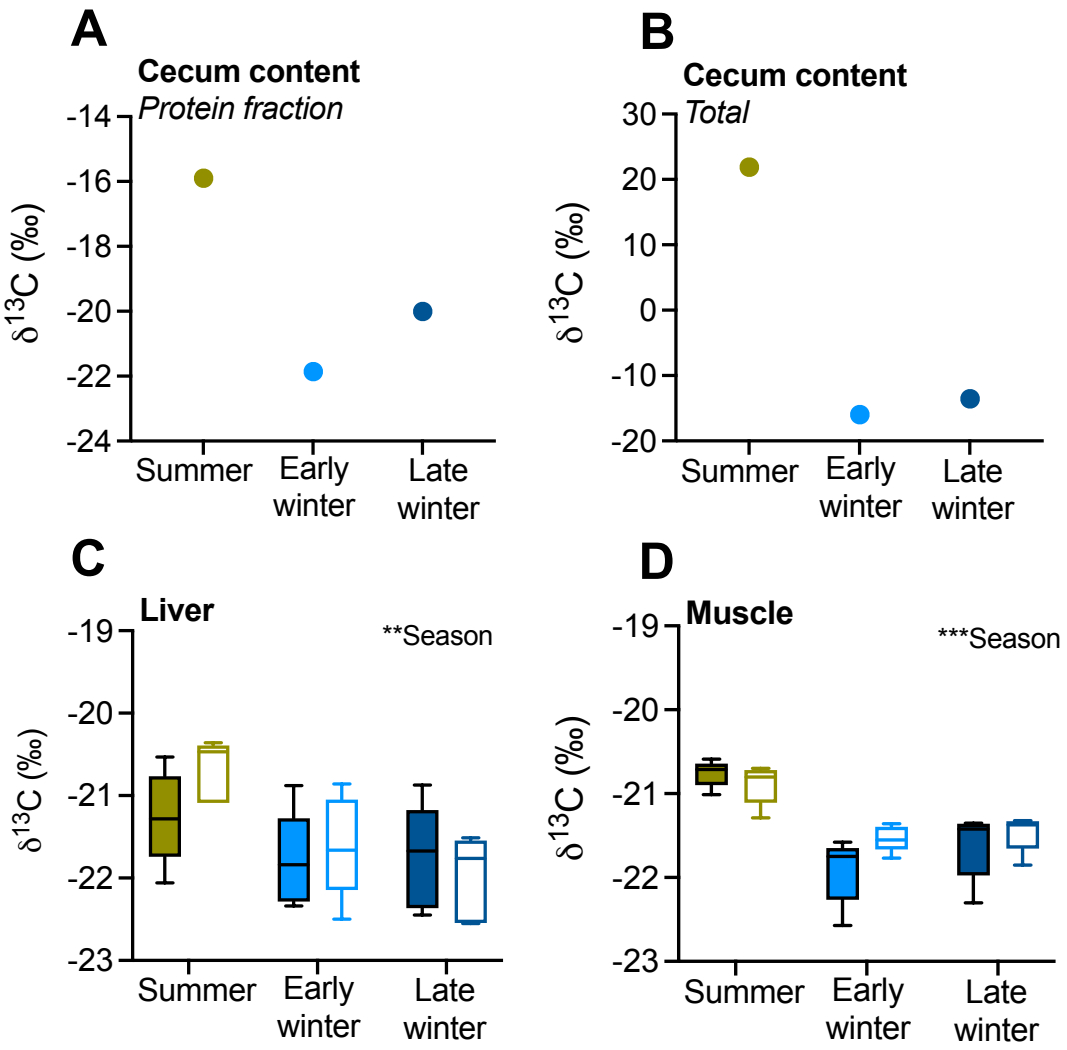


Figure S2. Isotope ratio mass spectrometry-based measurements of ^{13}C-urea carbon incorporation into the protein fraction (A) and total milieu (B) of cecum content, as well as protein fractions of (C) liver and (D) quadriceps muscle. Solid bars represent samples from microbiota-intact squirrels, white bars from microbiota-depleted squirrels. Cecum content panels (A, B) lack microbiota-depleted data because ABX-treatment caused 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, B; n=1, pooled samples).