ABSTRACT

UREA HYDROLYSIS BY GUT BACTERIA: FIRST EVIDENCE FOR UREA-NITROGEN RECYCLING IN AMPHIBIA

by James Michael Wiebler

Enteric bacteria contribute to nitrogen balance in diverse vertebrates because they produce urease, the enzyme needed to liberate nitrogen from urea. Although this system of urea-nitrogen recycling is as yet unknown in Amphibia, this study of the wood frog (*Rana sylvatica*), a terrestrial hibernator that is strongly hyperuremic during winter, documented robust urease activity in bacteria inhabiting the hindgut. Despite a ~33% reduction in the number of bacteria, ureolytic capacity in hibernating winter frogs was superior to that of active summer frogs, and was further enhanced by experimentally augmenting urea within the host. Bacterial inventories constructed using 16S rRNA sequencing revealed that the assemblages hosted by hibernating and active frogs were equally diverse but markedly differed in community membership and structure. Approximately 38% of the 96 observed bacterial genera were exclusive to one or the other group. Although ~60% of these genera possess urease-encoding genes and/or have member taxa that reportedly hydrolyze urea, hibernating frogs hosted a greater relative abundance and richer diversity of ureolytic organisms, including, notably, species of *Pseudomonas* and *Arthrobacter*. Amphibians, in whom urea accrual has a major osmoregulatory function, likely profit substantially by repurposing the nitrogen liberated from the bacterial hydrolysis of urea.

UREA HYDROLYSIS BY GUT BACTERIA: FIRST EVIDENCE FOR UREA-NITROGEN RECYCLING IN AMPHIBIA

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Introduction

Osmoregulators adapt to stresses of dehydration or saline exposure by accumulating one or more small organic osmolytes, or "compatible solutes." Despite its potentially destabilizing effects on macromolecular structure and function, urea is an important balancing osmolyte in some ectotherms (Yancey, 2005). Amphibians respond to osmotic challenge by accruing urea (up to 0.3 mol l^{-1} in some species) by ceasing urination, reabsorbing urea from the renal tubules and bladder fluid, reducing renal filtration rate, and, in some cases, upregulating hepatic ureagenesis (Shoemaker et al., 1992). Hyperuremia, which they readily tolerate, preserves the water potential gradient conducive to retaining body water whilst also limiting the injurious rise in ionic concentration. Given its crucial role in the water economy of amphibians—and ultimately in their survival of environmental extremes and exploitation of severe habitats—it is surprising that the ultimate fate of accrued urea is as yet unknown.

Amphibians would benefit from recouping the nitrogen in surplus urea, although, as with other vertebrates, they lack urease, the enzyme needed to hydrolyze the compound. Nevertheless, some mammals, birds, fishes, and reptiles recycle urea's nitrogen through a symbiotic relationship with certain gut bacteria that produce urease (Singer, 2003; Stewart and Smith, 2005). In this system, urea's hydrolysis to carbon dioxide and ammonia occurs within the rumen of foregut fermenters, the midgut of some non-ruminants, or the hindgut (caecum and/or proximal colon) of monogastric herbivores, omnivores, and carnivores. Changes in the composition of gut bacteria and their nutritional contribution to the host—triggered by changes in the host's diet, metabolic state, and/or gut morphology (Carey and Assadi-Porter, 2017)—and/or altering the expression of urease in bacteria that sense change in the availability of urea (Mobley and Hausinger, 1989), possibly influences the urea-nitrogen recycling system.

Postulating that urea-nitrogen recycling would be especially beneficial in amphibians that accumulate urea during periods of activity and dormancy, I focused my research on the wood frog (*Rana sylvatica*), a terrestrial hibernator in which urea is not only an osmoprotectant, but also is a cryoprotectant (Costanzo and Lee, 2005) and metabolic depressant (Muir et al., 2007). In the weeks preceding hibernation, urea accrues to high levels (reaching 0.25 mol l^{-1} in the blood of some individuals) that persist until late winter (Costanzo et al., 2015). Conceivably,

recovery of the constitutive nitrogen and its incorporation into biosynthetic molecules could help restore body condition at a time when its loss from the body could not soon be remedied by feeding.

In the present study, I sought evidence for urea-nitrogen recycling in Amphibia by determining capacity for urea hydrolysis by bacteria in the gut of hibernating *R. sylvatica*. I compared the gross morphology of guts from hibernating and active frogs because downregulation of the gastrointestinal tract in response to aphagia and seasonal dormancy (Secor, 2005; Secor and Lignot, 2010) potentially influences the microbiome, and hence ureolytic capacity. Finally, I inventoried and compared the enteric bacterial communities in hibernating and active frogs in order to test the supposition that the former host a greater abundance of ureolytic taxa.

Methods

Animals, acclimatization, and sampling

I collected male wood frogs (*R. sylvatica*) in late winter from breeding ponds in southern Ohio. Frogs were housed individually in plastic tubs containing damp moss and kept at 4°C in darkness. Some of these "late winter" frogs were used ~4 weeks later in a urea-loading experiment (see below), whereas others were kept until April and then released in an outdoor enclosure. Situated in a mature, deciduous woodlot, this 48-m^2 outdoor pen provided herbaceous cover, cool, moist conditions, and a small pool of water (Costanzo and Lee, 2005). Vitamin-fortified crickets (*Gryllodes sigillatus*; Ghann's Cricket Farm, Augusta, GA) were stocked thrice weekly, although the frogs' diet was enriched with various arthropods drawn to a UV-A light. In June, some individuals (hereafter, "active" frogs) were collected, returned to the laboratory, and immediately sampled for use in experiments that have a seasonal comparison. Additional frogs were gathered in November, after feeding had ceased, and placed in simulated hibernation (4°C, darkness) as described above; these "hibernating" frogs were sampled two months later and used in experiments with a seasonal comparison, as well as for characterization of morphometrics, bacterial load, and ureolytic capacity of frog gut.

Experiment 1: Morphometrics, bacterial load, and ureolytic capacity of frog gut

Frogs were dissected and samples were collected in a refrigerated room (4°C) using aseptic technique and filter-sterilized reagents. Hibernating frogs (*N*=10) were purged of bladder fluid, weighed, euthanized by double-pithing, measured to determine snout–ischium length, and dissected. Blood was drawn from an incision in the aortic trunk into heparinized capillary tubes and centrifuged (2,000 *g*, ~5 min); the resultant plasma was frozen in liquid nitrogen, stored at -80°C, and ultimately assayed for urea (B7551-120, Pointe Scientific, Canton, MI). The foregut, midgut, and hindgut were ligated at each end with suture silk, removed from the coelom, rinsed externally with phosphate-buffered saline (PBS), and opened longitudinally. Bacteria within each gut segment were separately collected by gently scraping the mucosa with a spatula, along with any luminal matter, into 700 µl sodium phosphate buffer (10 mmol l^{-1}; pH 7.0), centrifuging this suspension (400 *g*, 5 min) to pellet coarse debris, and isolating the supernatant. The debris pellet was washed in 700 µl fresh buffer, centrifuged (400 *g*, 5 min), and the resultant supernatant was combined with that reserved from the first centrifugation. Bacteria were coalesced by centrifugation (14,000 *g*, 20 min) and resuspended in 800 µl fresh buffer. Cells in a 20-µl aliquot of this suspension were fixed with 1% glutaraldehyde (Kepner and Pratt, 1994) for enumeration (see below), whereas the remainder was centrifuged (14,000 *g*, 20 min) and the resultant bacterial pellet was stored at –80°C for 4-6 weeks before I assayed urease activity (see below). Finally, I measured the resting length and, after drying at 65°C, the mass of each gut segment. Unused lysate prepared from hindgut bacteria from five of these frogs was used in an effort to validate that the ammonia produced in urease assay resulted from enzymatic hydrolysis of urea. These lysates were removed from frozen storage (–80°C), thawed, and divided; portions were assayed without further treatment, after preincubation with the urease inhibitor, acetohydroxamic acid (15 mmol l^{-1}, 30 min), or after heating (95°C, 10 min).

Additional hibernating frogs were used to investigate the thermal sensitivity of urease activity in hindgut bacterial lysates. I assayed activity at 20, 5, or 0°C, extending incubation time (up to 5 h) to accommodate the reduced catalytic activity occurring at low temperature. Combining lysates from three individuals was necessary to provide sufficient material to assay at each temperature; results are reported for three separate pools. For context, I performed identical tests on lysates prepared from bacteria harvested from the caeca of euthanized laboratory mice (*N*=3 lysate pools, each prepared from three individuals).

Experiments 2 and 3: Ureolytic capacity influenced by host's urea level and season

In experiment two, I tested the hypothesis that ureolytic capacity of hindgut bacteria is enhanced in hyperuremic frogs by experimentally manipulating urea levels in late-winter frogs, which are cold hardy but maintain low levels of urea (Costanzo and Lee, 2005). Following Muir et al. (2007), I injected a volume (~3% of standard body mass) of PBS or PBS containing 1.5 M urea into the dorsal lymph pad. Frogs (N=7, each group) were held in darkness at 4°C for 10 d. Frogs were then sampled as in experiment one, except only hindgut (not foregut or midgut) was removed, and lysates, prepared from hindgut bacteria as above, were assayed for urease activity and protein (see below). Control (N=7) and urea-loaded (N=6) frogs were matched for standard body mass (14.6±0.6 *versus* 13.7±0.7 g; P=0.35) and snout-ischium length (52.6±1.2 *versus* 53.4±0.7 mm; P=0.56).

I further tested this hypothesis in experiment three, where I compared ureolytic capacity of hindgut bacteria of hyperuremic, hibernating frogs (same individuals as described in experiment one) with that of normouremic, active frogs, which were sampled identically to the hibernating group. In addition, I compared the length, dry mass, and bacterial load of gut segments between these groups. Hibernating and active frogs were indistinguishable with respect to standard body mass (15.7±0.5 *versus* 14.8±0.5, respectively; P=0.79) and snout-ischium length (53.6±0.9 *versus* 52.7±0.6 mm, respectively; P=0.43).

Experiment 4: Bacterial inventories from hibernating and active frogs

I investigated whether seasonal variation in ureolytic capacity in the frog hindgut is associated with differences in the bacterial community by comparing inventories obtained for hibernating (N=5) and active (N=8) frogs. These individuals were not used in any other experiment. Frogs were sampled and hindgut contents were collected as described in experiment one, except that gut contents were suspended in 200 µl PBS and frozen at –20°C. Total DNA was extracted using the QIAamp PowerFecal DNA Kit (12830, MO BIO Laboratories, Carlsbad, CA) following the kit's instructions, except that I repeated the elution to increase the DNA yield. Quantity and quality of the isolated DNA were determined using a NanoDrop 2000 spectrophotometer. Samples were shipped under dry ice to LC Sciences, LLC (Houston, TX) for

amplification of the V3-V4 region of the 16S rRNA gene and sequencing using the Illumina MiSeq platform (Caporaso et al., 2012).

I used QIIME (Caporaso et al., 2010) to analyze the sequences. After implementing standard quality control measures, sequences were grouped into operational taxonomic units (OTUs) using the open reference method against the Greengenes core set (DeSantis et al., 2006). Sequences were grouped with UCLUST (Edgar, 2010) using a minimum sequence identity of 99%. I aligned the most abundant sequences within each OTU against the Greengenes core set (DeSantis et al., 2006), removed the hypervariable regions, and classified the OTUs using UCLUST (Edgar, 2010). Phylogenetic trees of representative sequences were constructed with FastTree (Price et al., 2009).

Enumeration of enteric bacteria

To estimate the bacterial load within the gut, I stained glutaraldehyde-fixed samples for 40 min with 10 μg ml^{-1} DAPI (Yu et al., 1995) and counted bacteria in a Bright-line Petroff-Hausser chamber viewed at 1000× (Pryor, 2008). Stained samples were counted in triplicate, where the chamber was re-loaded for each replicate. The average of three replicates was taken to represent each sample.

Urease activity

Bacterial pellets were thawed on ice, mixed with cold sodium phosphate buffer, and processed for 4 min at 4°C using 0.1 mm glass beads and a bead mill (BBY24M; Next Advance, Averill Park, NY). I centrifuged the lysate solution (14,000 *g*, 5 min) and assayed the clear supernatant (~250 μl) for urease activity using a kit (MAK120, Sigma Aldrich, MO) that quantifies ammonia produced from urea hydrolysis. Except as otherwise noted, lysate was incubated with substrate (urea) at 20°C for 1 h (hindgut), 5-12 h (midgut), or 30 h (foregut). Urease quantity is reported in mU, where one unit (U) is the amount of enzyme hydrolyzing 1.0 μmol urea min^{-1}. I used protein concentration of the lysate, a sensitive and reliable proxy for bacterial density (Nittayajarn and Baker, 1989), to normalize urease activity; protein was measured using the NanoDrop 2000 protocol for the Coomassie Plus (Bradford) protein assay (23236, Pierce, Rockford, IL) with bovine serum albumin as a standard.

Statistical inferences

Summary statistics for morphological and physiological variables are presented as mean ± SEM. Data from different groups were compared using a Student's *t*-test or Analysis of Variance (ANOVA), followed by Student-Newman-Keuls procedure. Two-factor ANOVA was used to compare morphometric variables of gut segments between hibernating and active frogs, with pairs of means distinguished using Bonferroni tests. Data sets failing to meet assumptions of normality and homoscedasticity were transformed or, if necessary, analyzed using a nonparametric equivalent (Mann-Whitney *U*-test or Kruskal-Wallis/Dunn's test). Significance was judged at $P<0.05$.

I compared the number of 16S rRNA gene sequences in each group using Student's *t*-tests. Metrics of alpha diversity (Shannon Index, evenness, observed OTUs, and Faith's Phylogenetic Diversity) were also compared between groups using Student's *t*-tests; here, I calculated the mean of 20 iterations of a random sub-sampling of 17,800 sequences for each sample. Beta-diversity metrics of community membership and community structure were calculated from unweighted and weighted Unifrac distances, respectively, using 17,800 sequences per sample, and compared using adonis, a permutational MANOVA (Anderson, 2001). I used principal coordinates analysis (PCoA) to visually compare these results. Relative abundances of bacterial phyla and genera underwent a variance-stabilizing transformation of $\arcsin(\text{abundance}^{0.5})$ (Kumar et al., 2012; Shchipkova et al., 2010) and were compared using the Response Screening function in JMP 12.0, which performs multiple *t*-tests. *P*-values were adjusted using the False Discovery Rate correction for multiple comparisons (Benjamini and Hochberg, 1995); significance was accepted at $P<0.05$.

Results

Experiment 1: Morphometrics, bacterial load, and ureolytic capacity of frog gut

The gastrointestinal tracts of hibernating frogs contained small amounts of mucus and presumably autochthonous matter but were largely devoid of recognizable ingesta. Mass and length of the foregut, midgut, and hindgut varied markedly, the latter being the tract's smallest segment (Table 1). Despite its diminutive size, the hindgut harbored 2.4-fold more bacteria than the midgut and 40-fold more bacteria than the foregut. Accordingly, abundant urease (nearly 5

mU) occurred only in this segment. Indeed, despite the exquisite sensitivity of the assay (lower limit of detection, 0.0005 mU ml^{-1}), I did not detect urease activity in the foreguts of two frogs and the midguts of three frogs.

Validation tests, using residual lysates prepared from the hindgut bacteria of hibernating frogs, suggested that the ammonia accrued in urease assays was produced by an enzymatic process. Relative to results for freshly prepared lysates, urease activity in frozen/thawed samples was reduced by 24% (range: 10-37%; P=0.027). Pre-treating the lysate with acetohydroxamic acid, an inhibitor of urease activity, before assay reduced urease activity by 97% (range: 95-98%; P=0.003), whereas heating the lysate before assay reduced urease activity by 99% (range: 98-100%; P=0.003).

Urease activity in lysates prepared from hindgut bacteria was strongly dependent (repeated measures ANOVA; P=0.0002) on assay incubation temperature, with the activity measured at 0°C (37.2±6.9 mU mg^{-1} lysate protein) being only one-third of that measured at 20°C (106.9±11.2 mU mg^{-1} lysate protein). The overall temperature coefficient (Q_{10}) was 1.69 (Fig. 1). The Q_{10} of urease activity in lysates prepared from mouse caecal bacteria was similar, although the urease activity measured at 20°C (71.7±6.1 mU mg^{-1} lysate protein) was ~33% below that of samples from frogs (P=0.046; Fig. 1).

Experiments 2 and 3: Ureolytic capacity influenced by host's urea level and season

Frogs administered urea solution had urea levels 5-fold higher than controls (42.2±4.5 *versus* 8.4±1.1 mmol l^{-1}; P<0.0001). Urea augmentation failed to raise the number of hindgut bacteria, as the complements in urea-loaded frogs (1.0±0.2 $\times 10^8$) and controls (1.0±0.1 $\times 10^8$) were indistinguishable (P=0.357). However, results suggested that hyperuremia enhanced ureolytic capacity, as the quantity of urease in hindgut was nominally (albeit not significantly; P=0.126) greater in urea-loaded frogs (1.7±2.0 *versus* 0.6±0.2 mU), and urease activity in bacterial lysates was 2.7-fold higher in urea-loaded frogs as compared to controls (215.7±68.3 *versus* 79.9±29.2 mU mg^{-1} lysate protein; P=0.037).

The austere gut of hibernating frogs contrasted with that of active frogs in which the foregut usually contained insect parts, the epithelium of the midgut supported well-formed villi, and the hindgut contained feces. Gastrointestinal tracts of hibernators weighed 61% less (P<0.0001) and were 25% shorter (P=0.005) and half as dense (i.e., mass per unit length; P<0.0001) as those of active frogs (Fig. 2). However, activity state × gut segment interaction for both mass (P=0.004)

and density (P=0.0001) indicated that such disparities were not uniform among the tract's components. Indeed, intergroup variation in mass and density of hindgut was relatively small relative to that of foregut and midgut, and, furthermore, hindgut length was indistinguishable between hibernating and active frogs (Fig. 3).

Expectedly, plasma urea concentration was markedly higher (P<0.0001) in hibernating frogs as compared to active frogs (23.5±2.9 *versus* 3.7±0.5 mmol l^{-1}). The hindgut of hibernators harbored 33% fewer bacteria (P=0.020) but nevertheless held twice the quantity of urease (P=0.024); moreover, urease activity was 2.8-fold higher (P<0.0001) in hibernators than in active frogs (Fig. 4).

Experiment 4: Bacterial inventories from hibernating and active frogs

I obtained 26,679±1,692 sequences per sample, finding no difference (P=0.929) in the number of sequences between hibernating (N=5) and active (N=8) frogs. These groups did not differ (t-tests: P>0.261, all cases) in metrics of alpha diversity: Shannon Index (hibernating: 6.42±0.26 *versus* active: 6.56±0.29), observed OTUs (1,370±101 *versus* 1,180±124), evenness (0.62±0.02 *versus* 0.64±0.02), and Faith's Phylogenetic Diversity (43.89±3.98 *versus* 43.35±3.22). Conversely, they differed markedly in bacterial community membership (adonis: R^2 = 0.166; P=0.001; Fig. 5a) and structure (adonis: R^2 = 0.168; P=0.028; Fig. 5b). Clustering suggested that inter-individual variation among hibernating frogs was relatively high with respect to community membership, but (with the exception of one individual) extremely low with respect to community structure. Of the 9,056 observed bacterial OTUs, 2,042 (22.5%) occurred in both groups, whereas 3,024 (33.4%) were exclusive to hibernating frogs and 3,990 (44.1%) were exclusive to active frogs.

Sequence analysis identified 15 bacterial phyla of which three (Acidobacteria, Deferribacteres, WPS-2) were exclusive to hibernating frogs and one (Fusobacteria) exclusive to active frogs. I identified 96 genera, twenty of which were exclusive to hibernating frogs and sixteen of which were exclusive to active frogs; thus, ~38% of all genera occurred only in one or the other group.

Relative abundance data for all observed taxa are presented in Table 2. Group differences were found for two phyla, Actinobacteria (FDR-corrected P=0.004) and Acidobacteria (FDR-corrected P=0.004), which were more abundant in hibernating frogs. Five phyla accounted for ~95% of all bacteria identified from the frog hindgut (Table 3). Bacteroidetes composed >56%

of the bacteria from both hibernating and active frogs, and Proteobacteria and Firmicutes jointly accounted for much of the remainder (24%, hibernating; 40%, active). Actinobacteria composed ~5% of bacteria from hibernating frogs, but was poorly represented in active frogs.

Fourteen of the 96 genera observed differed in relative abundance (Table 2), six being more abundant (FDR-corrected $P<0.029$, all cases) in hibernating frogs and eight being more abundant (FDR-corrected $P<0.049$, all cases) in active frogs. Eleven genera were particularly well-represented (i.e., relative abundance ≥1%) overall, including *Bacteroides*, which composed 40-50% of all bacteria (Table 3). *Desulfovibrio*, *Parabacteroides*, and *Oscillospira* collectively accounted for ~20% of bacteria from active frogs, but only 7% of bacteria from hibernating frogs. Approximately 9% of bacteria from hibernating frogs belonged to three genera (*Pseudomonas*, *Anaerovorax*, and *Arthrobacter)* that were poorly represented in active frogs.

I attempted to determine which among the observed genera had at least one member that potentially can hydrolyze urea by querying the Kyoto Encyclopedia of Genes and Genomes (KEGG), an online resource that contains gene catalogs from sequenced organisms, for entries having urease-encoding genes using the KEGG orthology (KO) identifiers K01427, K01428, K01429, K01430, and K14048. In 17 cases for which the gene was not reported, I qualified the particular genus as ureolytic by obtaining published evidence of urea hydrolysis in a member taxon. Ureolytic potential was determined for 56 (58.3%) of the observed genera (Table 2), more of which were hosted by hibernating frogs (27.0±3.1 *versus* 19.5±2.3; $P=0.036$). Relative abundance differed between the groups for seven of the ureolytic genera, which overall were better represented in hibernating frogs (Fig. 6).

Discussion

Ubiquitous in nature, urease-producing bacteria benefit ecosystems by making nitrogen from urea available to life (Mobley and Hausinger, 1989), and many are enteric symbionts that contribute to nitrogen balance in their host (Singer, 2003). This study demonstrating ureolytic activity in bacteria hosted in the frog gut provides the first evidence that amphibians potentially can salvage nitrogen from the urea they produce.

Urea hydrolysis mainly occurs in the foregut of ruminants or the hindgut and/or caecum of non-ruminants (Stewart and Smith, 2005), but potentially occurs in other alimentary organs,

which host distinct bacterial communities (Mashoof et al., 2013; Zhang et al., 2017). In the domestic goat, for example, urease activity is expressed not only in the rumen but also in the small intestine, caecum, and colon (Šimůnek et al., 1995). Limited urea hydrolysis occurs in the stomach and/or small intestine of rats (Kim et al., 1998; Takebe and Kobashi, 1988), rabbits (Marounek et al., 1995), and grouse (Vecherskii et al., 2015). In hibernating frogs, bacterial urease was scanty or absent from the foregut and midgut but highly abundant in the hindgut, suggesting the latter is the primary site of urea hydrolysis in amphibians as it is in non-ruminant mammals, birds, fish, and probably reptiles (Singer, 2003). Nevertheless, the possibility that urea hydrolysis occurs elsewhere cannot be excluded. For example, ureolytic bacteria reportedly occur in parenteral organs of healthy sharks (Grimes et al., 1985), which suggests that urea hydrolysis may take place outside of the gastrointestinal tract in some species.

Ureolytic capacity in lysates of bacteria sampled from the hindgut of hibernating frogs was robust. Measured at 20°C, urease activity in these samples was ~1.5-fold higher than that determined for bacteria sampled from mouse caecum, and 6-11-fold higher than activities measured at 37°C for bacteria sampled from bovine rumen (Jin et al., 2017). Amphibians can accumulate substantial quantities of urea for osmoprotection (Shoemaker et al., 1992) and, therefore, might possess a greater ureolytic capacity than mammals, which do not normally accrue this substrate. The relatively low temperature coefficient, 1.69, for urease activity in these samples is comparable to, if not slightly lower than, that previously reported (Larson and Kallio, 1954; Magaña-Plaza et al., 1971) and likely facilitates urea hydrolysis in frogs even at winter temperatures.

The gastrointestinal tract of amphibians, like that of other vertebrates, undergoes profound transformations in structure and function as animals adapt to altered physiological and nutritional states in dormancy (Secor, 2005; Secor and Lignot, 2010). Accordingly, the gastrointestinal tract of hibernating frogs was reduced in mass (25%) and length (61%), but also in density (~50%), perhaps owing to degeneration of the mucosa and musculature, particularly of the midgut. Remodeling responses vary among alimentary organs. For example, the hindgut is little altered, whereas the size of the foregut and/or midgut is reduced by 50-85% in estivating frogs (Cramp and Franklin, 2003) and salamanders (Smith and Secor, 2017), and in hibernating toads (Naya et al., 2009). Results for hibernating frogs were comparable. Selectively maintaining the hindgut's

morphology in dormancy presumably benefits the host by providing symbiotic bacteria with diverse colonization niches (Donaldson et al., 2016).

Dormant animals commonly host reduced populations of enteric bacteria, as those of the hindgut fall by 80-90% during underwater hibernation in the frogs *R. pipiens* and *R. catesbeiana* (Banas et al., 1988; Carr et al., 1976; Gossling et al., 1982a; Gossling et al., 1982b). By contrast, the reduction in *R. sylvatica*, a terrestrial hibernator, was only ~33%. Despite this drop, hibernating frogs harbored twice the bacterial urease and nearly triple the urease activity as active frogs. Greater abundance of enzyme in these hyperuremic frogs potentially derived from the inductive effect of high urea on urease expression (Mobley et al., 1995) and remodeling of the bacterial community (Wong et al., 2014; Zhou et al., 2017). Indeed, relative to active frogs, hibernating frogs hosted a higher relative abundance and richer diversity of ureolytic organisms, including, notably, species of *Pseudomonas* and *Arthrobacter*. The putative positive effect of increasing substrate availability on ureolytic capacity is underscored by the finding that experimentally augmenting the host's urea levels markedly increased urease activity in hindgut bacteria.

Few comprehensive analyses of the enteric bacterial community in amphibians are available. The principal phyla hosted by *R. sylvatica*, Bacteroidetes, Firmicutes, Proteobacteria (and, to a lesser extent, Actinobacteria and Verrucomicrobia) were the same as those found in other anurans (Chang et al., 2016; Kohl et al., 2013; Mashoof et al., 2013; Weng et al., 2016). *Bacteroides* (Bacteroidetes), which is well represented in other ranids (Banas et al., 1988; Gossling et al., 1982a; Gossling et al., 1982b; Kohl et al., 2013), composed >40% of hindgut bacteria in *R. sylvatica*. Complexity of the bacterial community often is reduced in hibernating mammals (e.g., (Carey et al., 2013; Dill−McFarland et al., 2014; Sommer et al., 2016; Stevenson et al., 2014) and frogs (Gossling et al., 1982a; Gossling et al., 1982b; Van der Waaij et al., 1974; Weng et al., 2016). I observed no change in alpha diversity metrics but found marked alterations in community membership and structure of the bacteria hosted by hibernating *R. sylvatica*. Indeed, the bacterial assemblage of hibernators uniquely included several phyla (Acidobacteria, Deferribacteres, and WPS-2) and 21% of the observed genera. *Pseudomonas*, *Anaerovorax*, and *Arthrobacter* predominated in hibernating frogs but were rare in active frogs. *Pseudomonas* (Proteobacteria), a well-known psychrophile, is highly abundant in cold-acclimated *R. sylvatica* and other ranids but otherwise is uncommon (Banas et al., 1988; Carr et al., 1976; Gossling et

al., 1982a; Lee et al., 1995). Extensive remodeling of the microflora in hibernating animals seems essential to maintain the host-bacteria symbiosis under altered biotic and abiotic conditions within the gut (Carey et al., 2013; Stevenson et al., 2014).

Urea-nitrogen recycling presently is unknown in Amphibia despite the universal role of urea accrual in their osmoregulation (Shoemaker et al., 1992). The hindgut of *R. sylvatica*, a species that remains hyperuremic throughout the winter, harbors a rich diversity of urease-containing bacteria capable of hydrolyzing the host's urea. Nitrogen liberated by ureolytic bacteria presumably is incorporated into the biosynthetic compounds needed to restore body condition following hibernation; however, this system potentially is of general importance to nitrogen balance in all amphibians.

Tables

Table 1.

Morphometrics, number of bacteria, and urease content of gut segments in hibernating frogs

	Foregut	Midgut	Hindgut	*P*
Mass (mg)	24.3 ± 1.3^{a}	15.4 ± 0.8^{b}	7.4 ± 0.4^{c}	<0.0001
Length (mm)	21.3 ± 1.3^{a}	46.2 ± 3.6^{b}	12.5 ± 1.5^{c}	<0.0001
Bacteria ($\times10^{8}$)	0.03 ± 0.01^{a}	0.5 ± 0.3^{b}	1.2 ± 0.2^{c}	0.0003
Urease (mU)	0.007 ± 0.004^{a}	0.01 ± 0.01^{a}	4.87 ± 0.95^{b}	<0.0001
N	9-10	9-10	9-10	

Within each row, means (± SEM) denoted by different letters were statistically distinguishable ($P<0.05$)

Table 2.

Relative abundances (%) of bacteria sampled from the hindgut of hibernating and active frogs

	Hibernating	Active	FDR-corrected *P*
Phyla			
Acidobacteria	0.09±0.08	N. D.	**0.004**
Actinobacteria	4.50±1.93	0.22±0.08	**0.004**
Bacteroidetes	63.16±12.61	56.16±7.44	0.700
Chloroflexi	0.004±0.003	0.001±0.001	0.601
Deferribacteres	0.002±0.001	N. D.	0.510
Firmicutes	10.29±1.90	22.77±3.93	0.056
Fusobacteria	N. D.	0.94±0.84	0.282
Gemmatimonadetes	0.003±0.002	0.001±0.001	0.680
Planctomycetes	0.006±0.004	0.004±0.002	0.680
Proteobacteria	14.05±10.42	16.89±5.88	0.723
Spirochaetes	0.05±0.04	0.09±0.05	0.776
TM7	0.002±0.001	0.01±0.01	0.680
Tenericutes	0.12±0.12	0.05±0.01	0.680
Verrucomicrobia	1.27±0.75	0.08±0.07	0.087
WPS-2	0.03±0.02	N. D.	0.087
Genera			
Acetobacteraceae			
Acidisoma	0.02±0.02	N. D.	0.252
Acidocella	0.005±0.004	N. D.	0.374
Acidaminococcaceae			
Phascolarctobacterium	0.07±0.07	0.04±0.03	0.950
Acidobacteriaceae			

Candidatus Koribacter	0.003±0.002	N. D.	0.374
Terriglobus	0.004±0.004	N. D.	0.374
Actinoplanaceae			
Actinoplanes	0.009±0.008	N. D.	0.374
Bacillaceae			
Bacillus	0.004±0.003	0.05±0.03	0.207
Bacteroidaceae			
Anaerorhabdus	0.01±0.01	0.003±0.002	0.973
Bacteroides	51.56±11.58	40.48±7.57	0.544
Brachyspiraceae			
Brachyspira	0.05±0.04	0.09±0.05	0.837
Bradyrhizobiaceae			
Bosea	0.03±0.01	N. D.	**0.0003**
Brucellaceae			
Pseudochrobactrum	0.001±0.001	N. D.	0.200
Burkholderiaceae			
Burkholderia	0.42±0.39	N. D.	0.162
Cellulomonadaceae			
Cellulomonas	0.001±0.001	0.007±0.004	0.374
Oerskovia	N. D.	0.001±0.001	0.374
Christensenellaceae			
Christensenella	0.003±0.002	0.05±0.03	0.260
Chthoniobacteraceae			
DA101	0.005±0.003	0.003±0.001	0.950
Clostridiaceae			
Anaerotruncus	0.08±0.03	0.18±0.04	0.129
Dorea	0.05±0.02	0.36±0.10	**0.012**
Coxiellaceae			
Rickettsiella	0.006±0.006	0.005±0.003	0.973
Cyclobacteriaceae			
Algoriphagus	N. D.	0.001±0.001	0.374

Deferribacteraceae			
Mucispirillum	0.002±0.001	N. D.	0.374
Dehalobacteriaceae			
Dehalobacterium	0.01±0.01	0.006±0.002	0.762
Desulfovibrionaceae			
Bilophila	0.28±0.16	2.13±0.71	**0.005**
Desulfovibrio	1.36±0.45	9.16±5.35	0.161
Enterobacteriaceae			
Citrobacter	0.03±0.03	1.02±0.73	0.181
Enterobacter	0.003±0.003	0.007±0.004	0.544
Erwinia	0.03±0.03	0.007±0.005	0.837
Morganella	N. D.	0.01±0.01	0.374
Plesiomonas	0.30±0.18	0.06±0.04	0.204
Proteus	N. D.	0.04±0.03	0.374
Providencia	N. D.	0.06±0.05	0.374
Serratia	0.15±0.09	0.20±0.07	0.553
Erysipelotrichidae			
Coprobacillus	0.09±0.04	0.05±0.02	0.311
Holdemania	0.02±0.02	0.03±0.02	0.785
Eubacteriaceae			
Anaerofustis	N. D.	0.002±0.001	**0.044**
Anaerovorax	1.15±0.24	0.16±0.06	**<0.00001**
Pseudoramibacter Eubacterium	0.09±0.01	0.15±0.04	0.479
Flavobacteriaceae			
Flavobacterium	0.05±0.03	0.004±0.004	0.063
Gillisia	N. D.	0.003±0.001	**0.045**
Fusobacteriaceae			
Fusobacterium	N. D.	0.94±0.84	0.260
Geodermatophilaceae			
Modestobacter	0.002±0.001	N. D.	0.200
Gordoniaceae			

Williamsia	0.001±0.001	0.001±0.001	0.979
Hyphomicrobiaceae			
Devosia	0.04±0.02	N. D.	**0.029**
Hyphomicrobium	N. D.	0.001±0.001	0.374
Pedomicrobium	0.001±0.001	0.0009±0.0009	0.519
Rhodoplanes	0.03±0.01	0.004±0.002	0.192
Lachnospiraceae			
Anaerostipes	N. D.	0.004±0.003	0.309
Coprococcus	0.11±0.06	0.62±0.20	**0.016**
Roseburia	N. D.	0.07±0.07	0.124
Methylobacteriaceae			
Methylobacterium	0.0007±0.0007	0.003±0.001	0.314
Microbacteriaceae			
Agromyces	0.001±0.001	0.003±0.002	0.693
Clavibacter	0.03±0.01	0.002±0.001	**<0.00001**
Mycetocola	0.001±0.001	N. D.	0.200
Salinibacterium	0.08±0.06	N. D.	0.242
Micrococcaceae			
Arthrobacter	3.31±1.64	N. D.	**0.018**
Renibacterium	0.06±0.05	N. D.	0.162
Moraxellaceae			
Acinetobacter	0.0007±0.0007	0.03±0.02	0.519
Mycobacteriaceae			
Mycobacterium	0.04±0.02	0.005±0.002	0.260
Nocardiaceae			
Nocardia	0.002±0.001	0.0005±0.0005	0.374
Rhodococcus	0.007±0.002	0.003±0.002	0.347
Nocardioidaceae			
Nocardioides	0.004±0.004	0.0005±0.0005	0.551
Odoribacteraceae			
Butyricimonas	0.05±0.03	0.03±0.02	0.704

Oxalobacteraceae			
Janthinobacterium	0.04±0.03	N. D.	0.374
Paenibacillaceae			
Paenibacillus	0.005±0.002	0.004±0.002	0.676
Peptococcaceae			
Desulfotomaculum	0.02±0.02	N. D.	0.374
Peptostreptococcaceae			
Clostridium	0.008±0.004	1.06±0.58	0.065
Planococcaceae			
Paenisporosarcina	N. D.	0.001±0.001	0.374
Porphyromonadaceae			
Dysgonomonas	0.002±0.002	0.13±0.06	**0.049**
Parabacteroides	4.49±1.71	8.84±1.83	0.192
Odoribacter	0.91±0.38	0.83±0.19	0.937
Pseudomonadaceae			
PSB-M-3	0.005±0.005	0.001±0.001	0.950
Pseudomonas	4.63±4.10	0.005±0.004	**0.029**
Pseudonocardiaceae			
Actinomycetospora	0.005±0.003	0.0005±0.0005	0.168
Pseudonocardia	0.002±0.002	0.0005±0.0005	0.837
Rhizobiaceae			
Agrobacterium	0.006±0.003	0.0003±0.0003	0.135
Kaistia	0.002±0.001	0.0005±0.0005	0.374
Rhizobium	0.12±0.06	0.002±0.001	0.052
Rhodanobacteraceae			
Luteibacter	0.07±0.06	N. D.	0.299
Rhodocyclaceae			
Uliginosibacterium	N. D.	0.004±0.003	0.252
Rikenellaceae			
AF12	0.12±0.06	0.25±0.09	0.605
Alistipes	0.001±0.001	0.0007±0.0007	0.837

PW3	0.0006±0.0006	0.52±0.42	0.065
Rikenella	N. D.	0.004±0.002	**0.049**
Ruminococcaceae			
Oscillospira	1.35±0.36	2.04±0.31	0.374
Ruminococcus	0.24±0.03	0.76±0.13	**0.0002**
Selenomonadaceae			
Propionispira	0.003±0.003	N. D.	0.374
Solibacteraceae			
Candidatus Solibacter	0.002±0.001	N. D.	0.204
Sphingobacteriaceae			
Pedobacter	0.10±0.06	0.002±0.002	0.160
Novosphingobium	0.0009±0.0009	0.003±0.003	0.837
Sphingomonas	0.04±0.03	0.002±0.002	0.416
Streptococcaceae			
Lactococcus	N. D.	0.006±0.005	0.374
Streptomyces	0.002±0.002	0.002±0.001	0.605
Verrucomicrobiaceae			
Akkermansia	1.26±0.74	0.07±0.07	0.095
Xanthobacteraceae			
Labrys	0.003±0.002	0.001±0.001	0.495
Xanthomonadaceae			
Lysobacter	N. D.	0.003±0.002	0.290

Mean ± SEM; *N*=5 (hibernating frogs) or *N*=8 (active frogs)

Genera in bold typeface are potentially ureolytic; see text for details.

N. D., not detected

Table 3.

Relative abundances (%) of the most abundant taxa of bacteria sampled from hindgut of hibernating and active frogs

Hibernating		Active	
Phyla			
Bacteroidetes	63.16±12.61	Bacteroidetes	56.16±7.44
Proteobacteria	14.05±10.42	Firmicutes	22.77±3.93
Firmicutes	10.29±1.90	Proteobacteria	16.89±5.88
Actinobacteria	**4.50±1.93****	Actinobacteria	0.22±0.08
Verrucomicrobia	1.27±0.75	Verrucomicrobia	0.08±0.07
Genera			
Bacteroides	51.56±11.58	*Bacteroides*	40.48±7.57
Pseudomonas	**4.63±4.10***	*Desulfovibrio*	9.16±5.35
Parabacteroides	4.49±1.71	*Parabacteroides*	8.84±1.83
Arthrobacter	**3.31±1.64***	*Bilophila*	**2.13±0.71****
Desulfovibrio	1.36±0.45	*Oscillospira*	2.04±0.31
Oscillospira	1.35±0.36	*Clostridium*	1.06±0.58
Akkermansia	1.26±0.74	*Citrobacter*	1.02±0.73
Anaerovorax	**1.15±0.24****		

Mean ± SEM; N=5 (hibernating frogs) or N=8 (active frogs)

Bold typeface signifies that the mean was greater (*P<0.05; **P<0.01) than that for the other group

Figures

Figure 1.

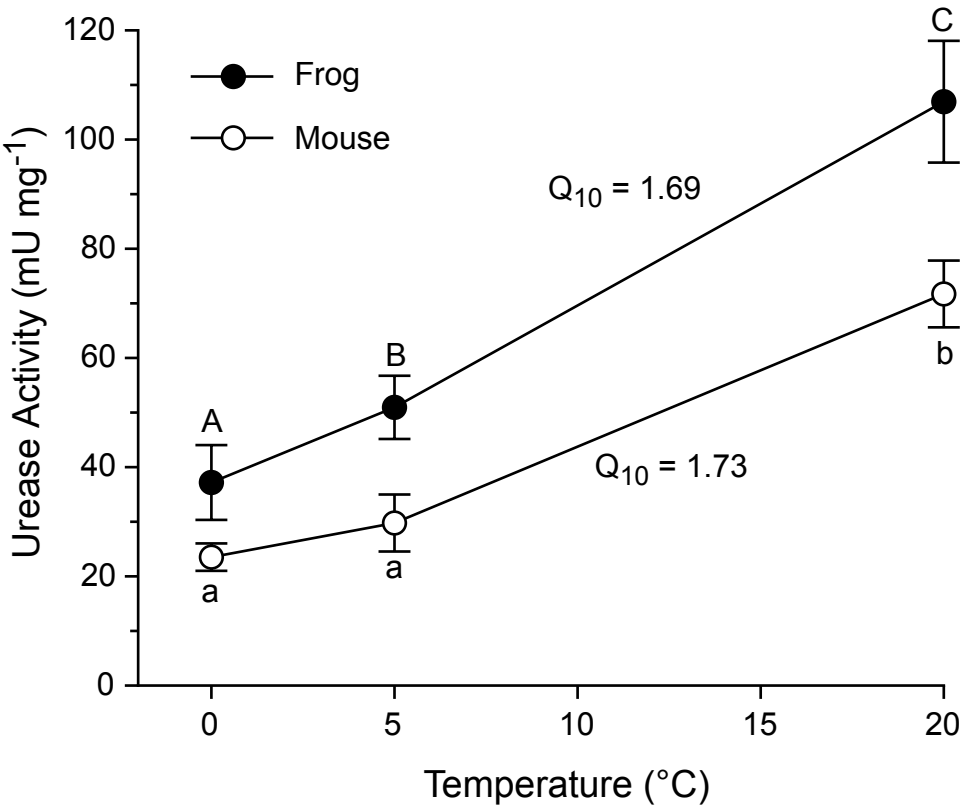


Figure 2.

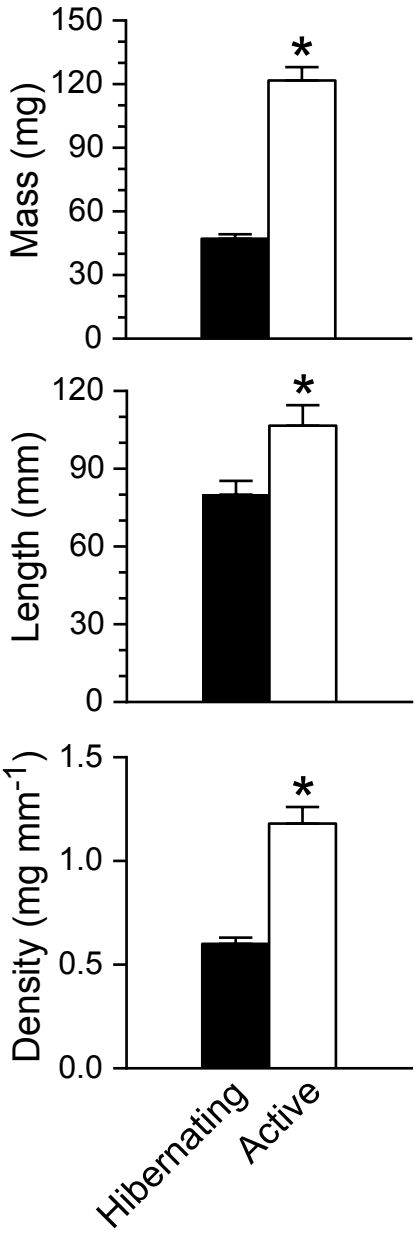

Mass (mg)
150
120
90
60
30
0
*
Length (mm)
120
90
60
30
0
*
Density (mg mm-1)
1.5
1.0
0.5
0.0
*
Hibernating
Active

Figure 3.

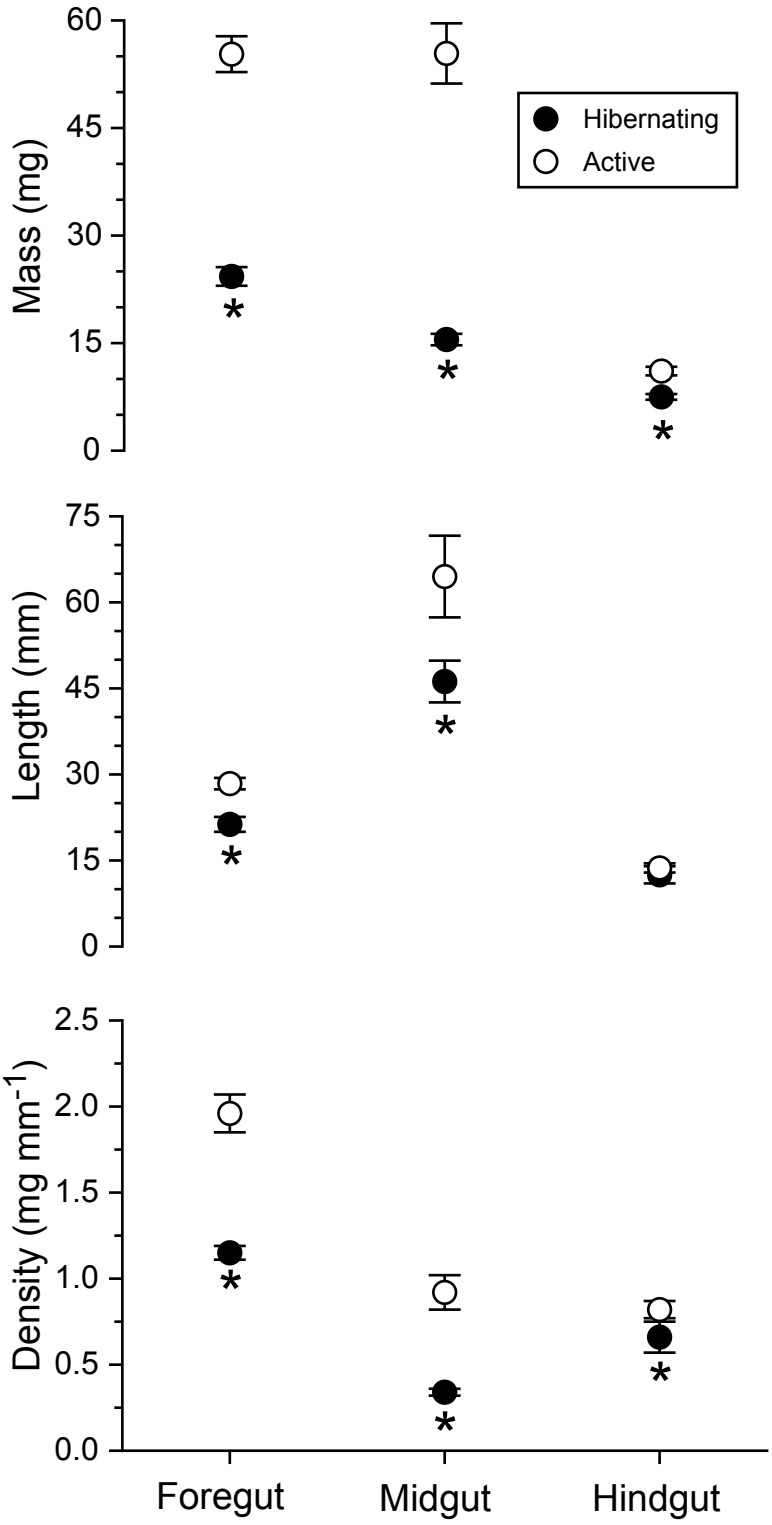

Mass (mg)
Length (mm)
Density (mg mm-1)
Hibernating
Active
Foregut
Midgut
Hindgut

Figure 4.

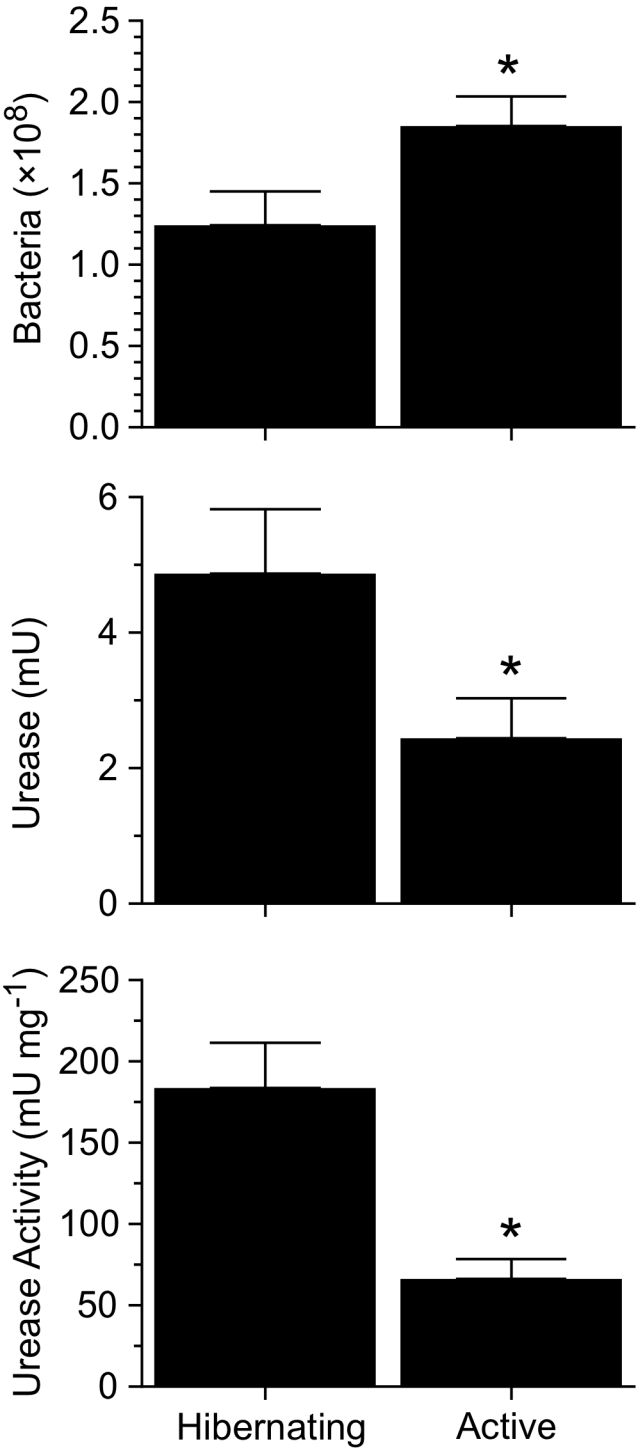

Bacteria (×10^8)
2.5
2.0
1.5
1.0
0.5
0.0
*
Urease (mU)
6
4
2
0
*
Urease Activity (mU mg^-1)
250
200
150
100
50
0
*
Hibernating
Active

Figure 5.

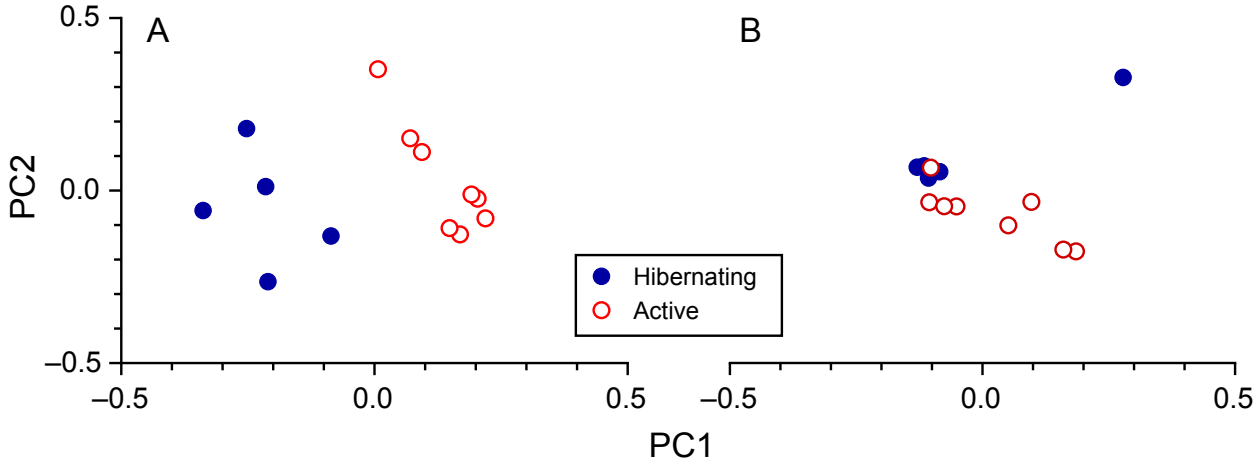
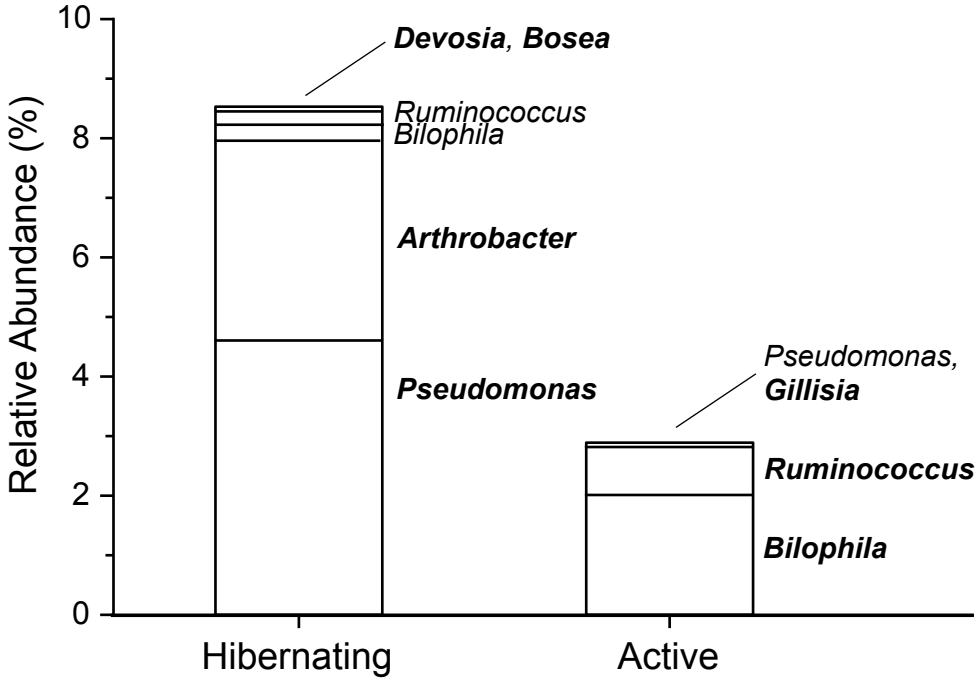


Figure 6.


Relative Abundance (%)
10
8
6
4
2
0
Devosia, Bosea
Ruminococcus
Bilophila
Arthrobacter
Pseudomonas
Pseudomonas,
Gillisia
Ruminococcus
Bilophila
Hibernating
Active

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