HIBERNATION

Nitrogen recycling via gut symbionts increases in ground squirrels over the hibernation season

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Hibernation is a mammalian strategy that uses metabolic plasticity to reduce energy demands and enable long-term fasting. Fasting mitigates winter food scarcity but eliminates dietary nitrogen, jeopardizing body protein balance. Here, we reveal gut microbiome–mediated urea nitrogen recycling in hibernating thirteen-lined ground squirrels (*Ictidomys tridecemlineatus*). Ureolytic gut microbes incorporate urea nitrogen into metabolites that are absorbed by the host, with the nitrogen reincorporated into the squirrel's protein pool. Urea nitrogen recycling is greatest after prolonged fasting in late winter, when urea transporter abundance in gut tissue and urease gene abundance in the microbiome are highest. These results reveal a functional role for the gut microbiome during hibernation and suggest mechanisms by which urea nitrogen recycling may contribute to protein balance in other monogastric animals.

Hibernation is an adaptation to seasonal food scarcity. The hallmark of hibernation is torpor, a metabolic state that reduces rates of fuel use by up to 99% relative to active season rates. Torpor enables seasonal hibernators such as the thirteen-lined ground squirrel (*Ictidomys tridecemlineatus*) to fast for the ~6-month hibernation season, solving the problem of winter food scarcity; however, fasting deprives the squirrel of dietary nitrogen, thus jeopardizing protein balance.

Despite dietary nitrogen deficiency and prolonged inactivity, hibernators lose little muscle mass and function during winter (*1*). Moreover, late in hibernation, squirrels elevate muscle protein synthesis rates to active season levels (*2*). It is unknown how hibernators preserve tissue protein during hibernation, but one hypothesis is that they harness the ureolytic capacities of their gut microbes to recycle urea nitrogen back into their protein pools (*3*). This process, termed urea nitrogen salvage, is present in ruminants and at least some nonruminant animals (*4*), but there is minimal evidence of its use by mammalian hibernators (*5*).

We hypothesized that squirrels use this mechanism to recoup urea nitrogen to facilitate tissue protein synthesis during, and particularly late in, hibernation (Fig. 1A). We tested this hypothesis with three seasonal squirrel groups: summer (active), early winter (1 month of hibernation and fasting), and late winter (3 to 4 months of hibernation and fasting) squirrels. Early and late winter squirrels were studied during induced interbout arousals at euthermic metabolic rates and body temperatures. Each seasonal group contained squirrels with intact and antibiotic-depleted gut microbiomes. For each group, we administered two intraperitoneal injections of ^{13}C,^{15}N-urea (~7 days apart depending on season, with unlabeled urea used as the control; fig. S1 and table S2). We then examined the critical steps of urea nitrogen salvage (Fig. 1).

This process begins with hepatic urea synthesis and transport into the blood. Urea that is not excreted by the kidneys can be transported into the gut lumen through epithelial urea transporters (UT-Bs) (*6*) where, in the presence of ureolytic microbes, it is hydrolyzed into ammonia and CO_2. Plasma urea concentrations in early and late winter squirrels were lower than those in summer squirrels (Fig. 2A), as observed previously (*7*) (fig. S2). However, UT-B abundance in the ceca of squirrels untreated with urea was about three times as high in late winter squirrels relative to summer squirrels (Fig. 2B), suggesting that lower plasma urea concentrations in winter may be partially offset through enhanced capacity for urea transport into the gut. Although this must be verified by future UT-B inhibition experiments, the observations that microbiome depletion increases UT-B expression (Fig. 2B), lowers plasma urea (Fig. 2A), and increases luminal urea concentrations (fig. S3) in summer squirrels support a role for UT-B in urea nitrogen salvage during the hibernation season. The mechanism underlying greater UT-B abundance in late winter squirrels and microbiome-depleted summer squirrels may involve luminal ammonia, which inhibits UT-B expression in ruminants (*8*). Commensurate with this, luminal ammonia levels were lower during hibernation in late winter squirrels than in summer squirrels (*9*) (fig. S4A) and in microbiome-depleted relative to microbiome-intact squirrels (Fig. 4A).

Next, we measured microbial ureolytic activity in vivo using stable isotope breath analysis where, because vertebrates lack urease, elevated $^{13}CO_2$:$^{12}CO_2$ ($\delta^{13}C$) after injection of ^{13}C ,^{15}N-urea indicates microbial ureolysis. Breath $\delta^{13}C$ increased after ^{13}C,^{15}N-urea injection (Fig. 3, A to C) in microbiome-intact—but not microbiome-depleted—squirrels (Fig. 3D), thus confirming microbial ureolytic activity. Ureolysis was greatest in summer squirrels (Fig. 3D and fig. S4B), consistent with greater bacterial abundance in summer versus hibernating squirrels (fig. S5). Nevertheless, microbial ureolysis continued throughout hibernation.

The metagenomes of hibernating squirrels trended toward higher percentages of urease genes than those of summer squirrels (Fig. 3E) across the seven urease-related genes (Fig. 3F). This suggests that during hibernation, a higher percentage of microbes have the potential to hydrolyze urea. Indeed, *Alistipes*—the bacterial genus with the greatest detectable genomic representation of urease genes in early and late winter squirrels (Fig. 3G)—is predominant during hibernation, with a six-fold population increase between the summer and late winter groups (*9*).

To benefit the host, microbial ureolytic activity would need to provide nitrogenous compounds such as amino acids and/or ammonia. Using two-dimensional ^{1}H-^{15}N NMR spectroscopy, we found that more ^{15}N was incorporated into the cecal content and liver metabolomes of microbiome-intact compared with microbiome-depleted squirrels (Fig. 4, A and B), a trend that—with a few exceptions—also held for specific metabolites such as ammonia, glutamine, and alanine (Fig. 4, A and B). ^{15}N-metabolite levels also varied seasonally. In cecum contents, early and late winter metabolite levels were generally lower than summer levels, whereas in the liver, early and late winter metabolite levels were generally higher than summer levels and were typically highest in the late winter group (Fig. 4, A and B). For muscle, metabolite ^{15}N incorporation was generally unaffected by the presence of a microbiome (Fig. 4C), which could be due to the timing of our ^{13}C,^{15}N-urea dosing and tissue sampling protocols. When tissues were sampled, the ^{15}N-amino acids from the initial ^{13}C,^{15}N-urea dose may have already been incorporated into muscle protein, thus explaining the microbiome-dependent ^{15}N-protein

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