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EFFECTS OF HIBERNATION ON GASTROINTESTINAL TRANSIT AND NEUROCHEMICAL CODING IN THE ENTERIC NERVOUS SYSTEM OF THIRTEEN-LINED GROUND SQUIRREL (*Ictidomys tridecemlineatus*) STOMACH

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EFFECTS OF HIBERNATION ON GASTROINTESTINAL TRANSIT AND NEUROCHEMICAL CODING IN THE ENTERIC NERVOUS SYSTEM OF THIRTEEN-LINED GROUND SQUIRREL (*Ictidomys tridecemlineatus*) STOMACH

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ABSTRACT

Brandenburg, C.J. Effects of hibernation on gastrointestinal transit and neurochemical coding in the enteric nervous system of thirteen-lined ground squirrel (*Ictidomys tridecemlineatus*) stomach. MS in Biology – Cellular and Molecular, May 2014, 25pp. (S. Liu)

Hibernating animals experience dramatic changes in the structure and function of their digestive system, along with other organ systems. Gut motility mixes food with digestive enzymes and propels it from the stomach to the more distal regions of the digestive tract for further processing. During the inter-digestive state, gut motility helps to clean out the indigestible matter and prevent bacteria overgrowth in the intestine. Despite its importance, changes in gut motility during hibernation have received minimal attention. The purpose of this study was to investigate the effects of hibernation on gut motility and its neuronal regulation. Thirteen-lined ground squirrels in summer active, winter torpor, and interbout arousal states were used in the study. Gastrointestinal transit was measured using a charcoal meal method. Immunofluorescence was used to measure the expression of neurotransmitter synthetic enzymes in the enteric nervous system of the stomach. Gastrointestinal transit was significantly slowed during torpor, but returned to normal level during interbout arousal. Choline acetyltransferase is an enzyme used in the synthesis of acetylcholine, a major excitatory neurotransmitter in the enteric nervous system. Nitric oxide synthase is an enzyme used in the synthesis of nitric oxide, a major inhibitory neurotransmitter in the enteric nervous system. There was no significant change in the number of neurons expressing choline acetyltransferase or nitric oxide synthase in the myenteric plexus of the stomach among arousal states. Further studies are needed to explore the changes of other neurotransmitters in the enteric nervous system during hibernation. Understanding the mechanisms responsible for the changes in gut motility during hibernation may provide insights into the basis of gastrointestinal motility disorders in humans.

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INTRODUCTION

The ability to hibernate is a dramatic example of phenotypic plasticity displayed by animals. Mammalian hibernators undergo complex morphological, physiological and behavioral changes in response to periods of high-energy demand coupled with seasonal reduced energy availability in the environment (1). For a small mammal such as the thirteen-lined ground squirrel (*Ictidomys tridecemlineatus*), a typical hibernation season includes extended bouts of torpor separated by periods of interbout arousal (Fig. 1). During torpor, core body temperature is maintained at 4–6°C, and relative to arousal, oxygen consumption is reduced to 2-3%, basal metabolic rate is reduced to 2-4%, heart rate drops from 200-400 beats/min to 3-10 beats/min, and respiration rate drops from 100-200 breaths/min to 4-6 breaths/min (2). During the periodic interbout arousals, core body temperature returns to near normal range and is maintained for 12-24 hours before reentry into torpor (1). The basal metabolic rate, heart rate, respiration, and other physiological parameters are also restored rapidly to near normal levels during interbout arousals (2). The periodicity of arousal episodes constitutes one of the most remarkable features of the hibernation cycle, although the trigger and the functional significance of interbout arousals are still not clear.

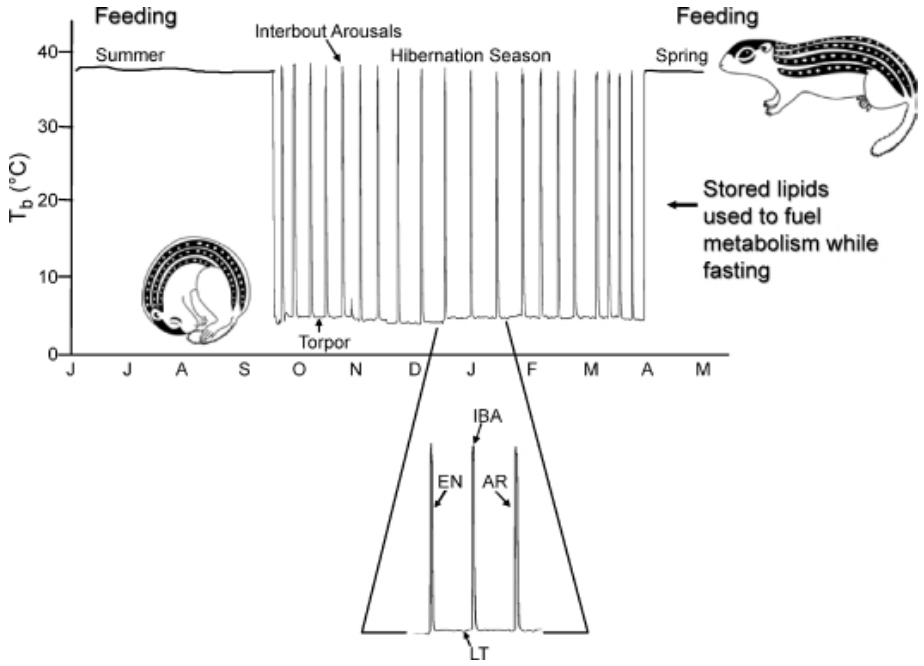

Figure 1. Annual torpor and arousal cycles during hibernation in 13-lined ground squirrels. Body temperature plotted against month of the year in the annual cycle. EN, entering torpor; LT, late torpor; AR, arousing from torpor; IBA, interbout arousal (6) Reprinted with permission from author.

Several aspects of hibernation could be considered stressful to the gut, including extended fasting, prolonged hypothermia, rapid changes in metabolism, and redistribution of blood flow. In response to long-term absence of luminal nutrition during the winter fast, gradual atrophy of the intestinal mucosa occurs, marked by reduction in villus height and crypt depth. However, the overall structure of the intestinal epithelium is well preserved with enterocyte microvillus height unchanged and microvillus density slightly increased. Activities of digestive enzymes are only slightly reduced if at all (3). Interestingly, the absorption of nutrients, when normalized to mucosal surface area, was actually greater in winter torpor than in spring or summer squirrels, but not significantly different from fall squirrels (4). Also, an enhanced secretory capacity has been observed in the jejunum of fasted squirrels (5).

Gut motility functions to mix food with digestive enzymes and propel food through the digestive tract during the fed state. In the periods between digestion, gut motility helps to clean out the indigestible matter and prevent bacterial overgrowth in the small intestine. Movement in the gut is generated by the smooth muscle in the wall of the gut and controlled by the enteric nervous system (ENS) (Fig. 2) in concert with inputs from the central nervous system (CNS).

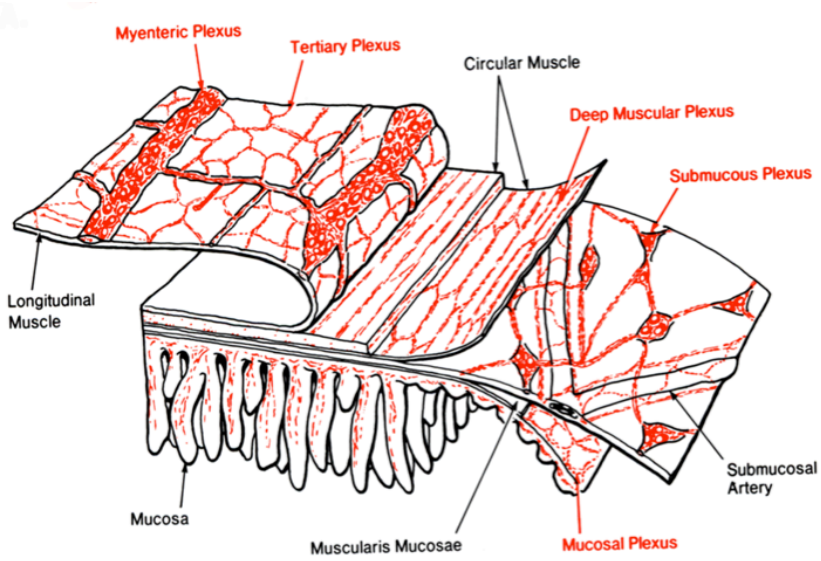

Figure 2. The intestinal wall. The enteric nervous system is located within the intestinal wall and consists of two neuronal plexuses. The submucosal plexus is located underneath the mucosa and regulates secretion and local blood flow. The myenteric plexus is located in between the circular and longitudinal muscle layers and regulates motility (7). Reprinted with permission from author.

Different motility patterns are observed in the stomach and small and large intestines during the fed and fasted states. After a meal, peristaltic waves move from the fundus of the stomach toward the pylorus. As the contractions approach the pylorus, they become much more powerful (8). The vigorous peristalsis mixes food with gastric secretion and breaks up food into small particles that could be emptied into the small

intestine. Normally, each peristaltic wave reaching the pyloric region only empties a small amount of food to the small intestine; the rest is propelled back into the stomach, where it is mixed further. This back and forth grinding action effectively breaks up solid food in the gastric contents. Once food is emptied into the small intestine, segmentation will occur. Segmentation is characterized by alternating contraction and relaxation of the circular muscles in a segment of the small intestine. The rhythmic contraction and relaxation help to mix food with digestive enzymes. Segmentation also moves intestinal contents slowly and steadily toward the large intestine at a rate that allows digestion and absorption to complete. Once most of the nutrients have been absorbed and segmenting movement wanes, the gut enters the fasted state. During this period of time, strong peristaltic waves are initiated in the pylorus or the proximal duodenum every 90-120 minutes and sweep slowly along the intestine, moving 50-70 cm before dying out. Each successive wave is initiated a bit more distally, and propagates further toward the large intestine. The type of motility pattern seen during the fasted state is called the migrating motor complex. It helps to sweep the remnants of food toward the anus and prevent the overgrowth of bacteria in the intestinal lumen. Upon re-feeding, the migrating motor complex disappears and segmentation takes over in the small intestine (9).

Despite the importance of gut motility in nutrient digestion and absorption, it is not known how gut motility is modified during the hibernation season when there is a low core body temperature and prolonged fasting experienced by the hibernating squirrels. The intestinal lumen of the hibernating squirrels contains mucus, food debris and microorganisms. It is not known whether these materials accumulate statically or are in transit through the gut during winter torpor. In addition, fecal matter was observed in

cages hosting winter torpor squirrels (unpublished observation). It is unknown whether this fecal matter was passed during torpor or interbout arousal. This study used the 13-lined ground squirrel as an animal model to compare the rates of gastrointestinal transit during summer active, winter torpor and interbout arousal periods. Gastrointestinal motility is controlled moment to moment by the ENS within the wall of the gut. Thus, we investigated changes in the expression of enzymes for the synthesis of specific neurotransmitters in the ENS during hibernation. Understanding the mechanisms underlying the changes in gut motility during hibernation may lead to new targets for drug development to treat gastrointestinal motility disorders.

MATERIALS AND METHODS

Animals

All experiments using 13-lined ground squirrels were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Wisconsin-La Crosse. The ground squirrels were either captured from a local golf course or bred in captivity. Five summer active, five winter torpor, and five interbout arousal ground squirrels were used in the study, including both male and female squirrels. The group size is similar to related studies in the literature (10). The same squirrels were used for all experimental objectives.

During the spring and summer, squirrels were housed individually in rat caging on a Wisconsin photoperiod to emulate environmental conditions that trigger hibernation. They were provided with water, rat chow and cat food *ad libitum* and supplemented with sunflower seeds to provide n-6 polyunsaturated fatty acids. In the fall, when body temperatures dropped to 25°C (ambient), the squirrels were placed in a hibernation cage with bedding and moved into a 4°C hibernaculum. Food and water were removed once squirrels began regular torpor bouts. Active squirrels were studied in the summer. Hibernating squirrels were studied at least 4 weeks after entering hibernation and in torpor. Squirrels in natural interbout arousal were also studied.

Effects of Hibernation on Gastrointestinal Transit

Gastrointestinal motility is usually measure by gastrointestinal transit time, the time it takes for food to leave the stomach and travel through the small intestine. Several techniques have been used to measure transit times in humans and animals. We used the charcoal meal method (11) in the current study because it is easy to operate in rodents. Summer active squirrels were fasted for 24 hours with free access to water before the experiment, while the winter torpor and interbout arousal squirrels were naturally fasted. Summer active and interbout arousal squirrels were anesthetized slightly with isoflurane and gavaged with a single dose of 1.5 ml suspension of charcoal meal (20% charcoal in 5% gum arabic). Winter torpor squirrels were not anesthetized before gavage. Summer active and interbout arousal squirrels were euthanized 30 min post gavage via CO_2 inhalation. Winter torpor squirrels were euthanized via cervical dislocation while remaining in torpor. Death was assured in all groups by cutting the diaphragm to stop breathing. The abdomen was opened and the small intestine removed from the pyloric junction to the cecal end. The total length of the small intestine and the distance traveled by the charcoal meal were measured. Gastrointestinal transit (%) was expressed as the following formula: Gastrointestinal transit (%) = (the distance traveled by the charcoal/the total length of the small intestine) X 100%. The one-way analysis of variance (ANOVA) was used to determine whether there were any significant differences in the gastrointestinal transit rate between summer active, winter torpor, and interbout arousal squirrels.

Effects of Hibernation on Neurons in the Myenteric Plexus of the Stomach

Gut motility is under the control of the ENS, which is located within the wall of the gut. In order to investigate whether changes in gut motility during hibernation are due to changes in the ENS, the total number of neurons, the number of neurons expressing choline acetyltransferase (ChAT), and the number of neurons expressing nitric oxide synthase (NOS), were observed using immunofluoresence staining. ChAT is an enzyme used in the synthesis of acetylcholine, a major excitatory neurotransmitter in the ENS, and NOS is an enzyme used in the synthesis of nitric oxide, a major inhibitory neurotransmitter in the ENS.

The stomach of each animal was harvested and the tissue was stretched taut and fixed in Zamboni's fixative (4% formaldehyde and 15% saturated picric acid in 0.1M phosphate buffer; pH 7.4) over night at 4°C. The myenteric plexus was separated by dissection. The myenteric plexus preparations were incubated in phosphate buffered saline (PBS) containing 10% normal donkey serum and 0.3% Triton X-100 for one hour to block the non-specific binding and to permeabilize the membrane. The preparations were then incubated with the primary antibody for human neuronal protein HuC/D (a neuronal marker, mouse, 1:50, Molecular Probe, cat# A-21275) overnight at 4°C. After being washed, the tissues were incubated in fluorescein isothiocyanate (FITC)-labeled donkey anti-mouse immunoglobulin G (IgG) (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) at room temperature for 1 h. The tissues were washed in PBS and cover slipped with VECTASHIELD mounting medium (Vector Labs). Fluorescence labeling was examined under a Nikon Eclipse 80i fluorescence microscope to ensure the quality of staining. The tissues were then washed in PBS and subsequently incubated

with the primary antibody against ChAT (goat, 1:100, Millipore, cat#144P), or the primary antibody against NOS (sheep, 1:500, Millipore, cat#AB1529). The tissues were then washed in PBS and incubated with cyanine 3(Cy3)-labeled donkey anti-goat IgG for ChAT (Jackson ImmunoResearch Laboratories), or Cy3-labeled donkey anti-sheep IgG for NOS (Jackson ImmunoResearch Laboratories) at room temperature for 1 h. After a thorough rinse, the tissues were cover slipped with VECTASHIELD mounting medium and examined under the fluorescence microscope. Neurons in at least 30 ganglia in the myenteric plexus were counted for each preparation.

Data Analysis

Data are expressed as means ± SEM with *n* values representing the numbers of animals in the group. One-way analysis of variance followed by Tukey's honestly significant difference (HSD) test was used to determine statistical significance. $P < 0.05$. was considered statistically significant.

RESULTS

Effects of Hibernation on Gastrointestinal Transit

Gastrointestinal motility was measured by gastrointestinal transit of a charcoal meal in 30 min according to the method by Mittelstadt et al. (11). Gastrointestinal transit, expressed as percent total length of the small intestine traversed by the charcoal meal, was significantly slowed in the winter torpor group (1.1% ± 2.9; n=5; $P<0.001$) versus the summer active group (89.8% ± 7.1; n=5). In fact, there was no gastric emptying in 4 out of 5 of the winter torpor squirrels 30 min after the charcoal meal. Only one squirrel in torpor had gastric emptying with gastrointestinal transit only 5.7%. Gastrointestinal transit in the interbout arousal group (95.9% ± 2.5; n=5; $P>0.05$) returned to the typical levels of the summer active group (Fig. 3).

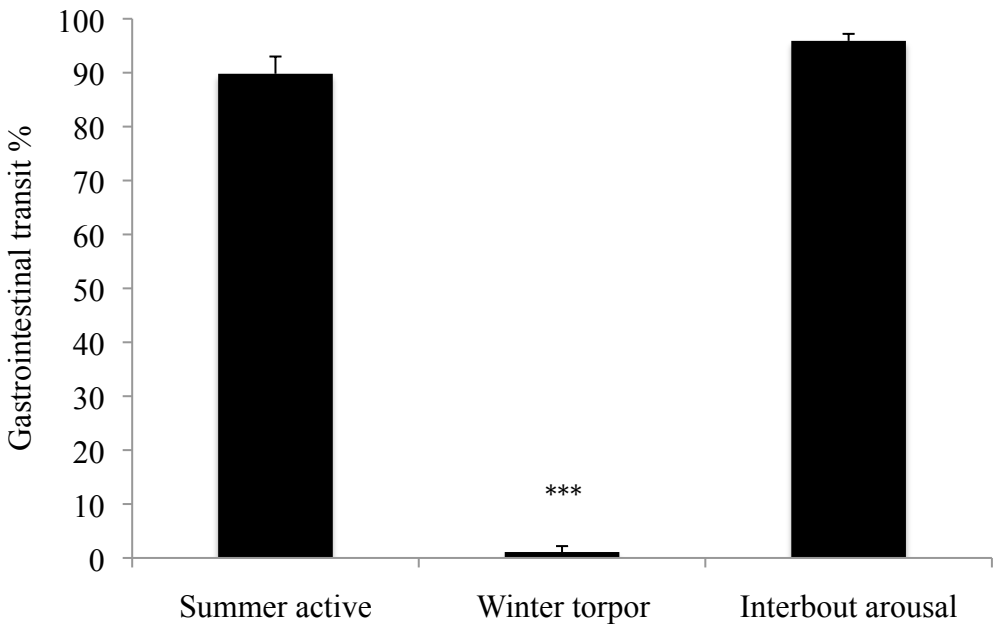

Figure 3. Gastrointestinal transit in summer active, winter torpor and interbout arousal ground squirrels. Data are expressed as means ± SEM, ***P<0.001 compared to the summer active group; n=5/group

Effects of Hibernation on Neurons in the Myenteric Plexus of the Stomach

The myenteric plexus of the ENS regulates gastrointestinal movement by controlling the activity of smooth muscle in the wall of the gut. The smooth muscle in the gut wall is innervated by both excitatory and inhibitory motor neurons in the ENS. In order to understand whether changes in gastrointestinal transit during hibernation were due to alterations in the ENS, the total number of neurons (labeled by the pan neuronal marker human neuronal protein HuC/D), the neuron populations expressing ChAT (a marker for cholinergic excitatory neurons), and the neuron populations expressing NOS (a marker for nitrergic inhibitory neurons) in the myenteric plexus of the gastric corpus and antrum were compared between animal groups. There was no change in number of cell bodies containing the neuronal marker Hu C/D in the investigated regions of the gut

in the winter torpor squirrels compared with summer active and interbout arousal squirrels (Fig. 4,7). No statistically significant differences were found in neuron populations expressing ChAT (Fig. 5,7) or NOS (Fig. 6,7) among summer active, winter torpor and interbout arousal squirrels.

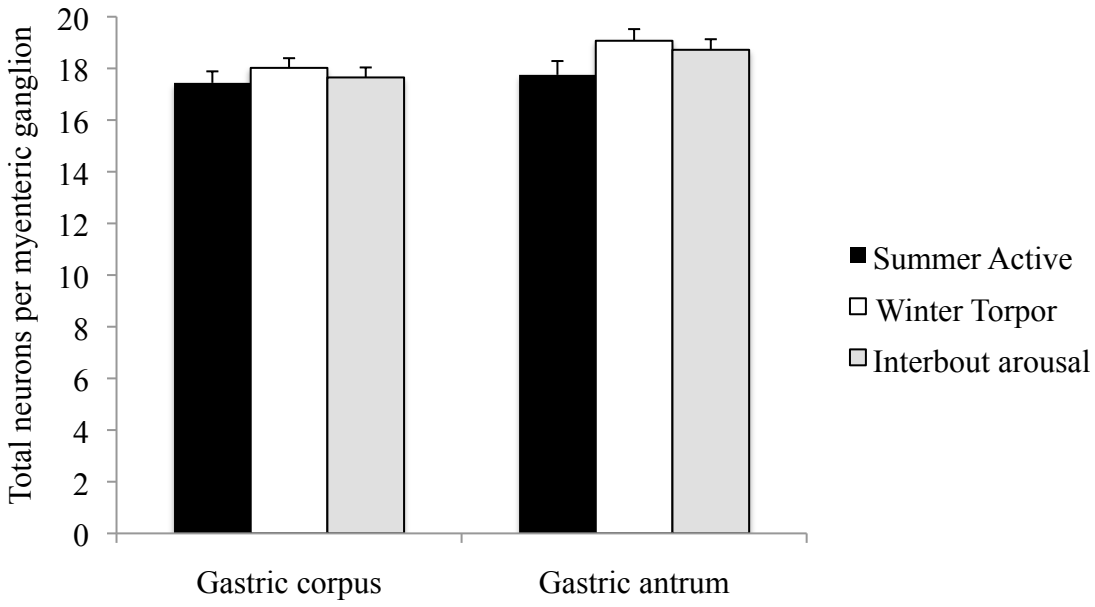

Figure 4. Total neuron populations in the myenteric plexus of the gastric corpus and antrum of summer active, winter torpor and interbout arousal squirrels. Myenteric neurons were labeled with an antibody against the pan neuronal marker, human neuronal protein HuC/D. No statistically significant differences were found among groups (n=5/group). Data are expressed as means ± SEM.

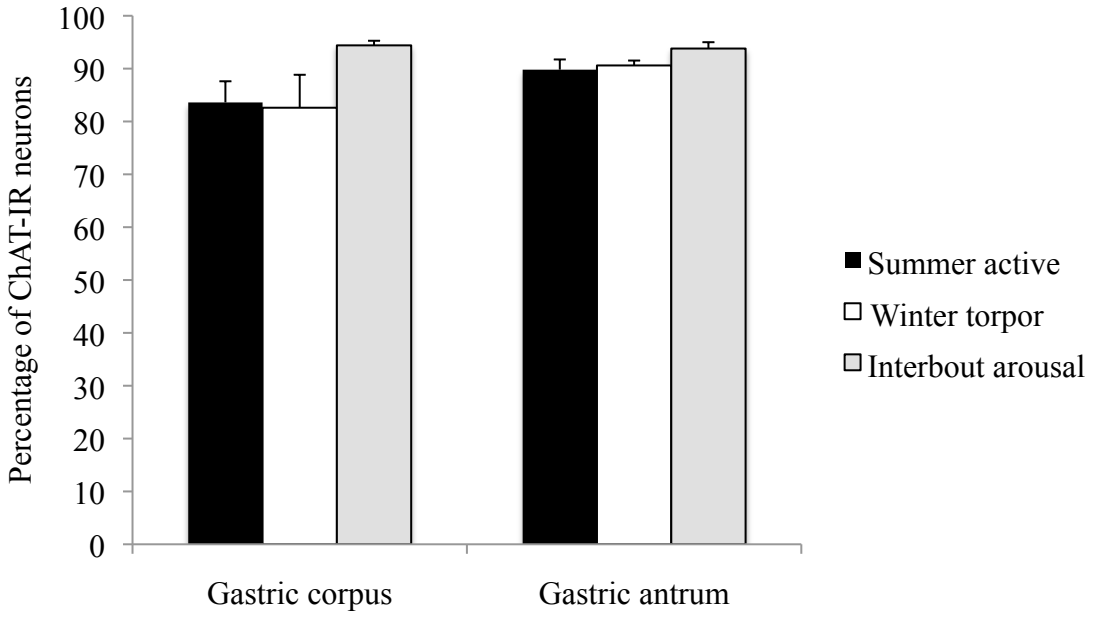

Figure 5. Percentage of ChAT-immunoreactive (IR) neurons in the myenteric plexus of the gastric corpus and antrum in summer active, winter torpor and interbout arousal squirrels (n=5/group). No statistically significant differences were found among groups.

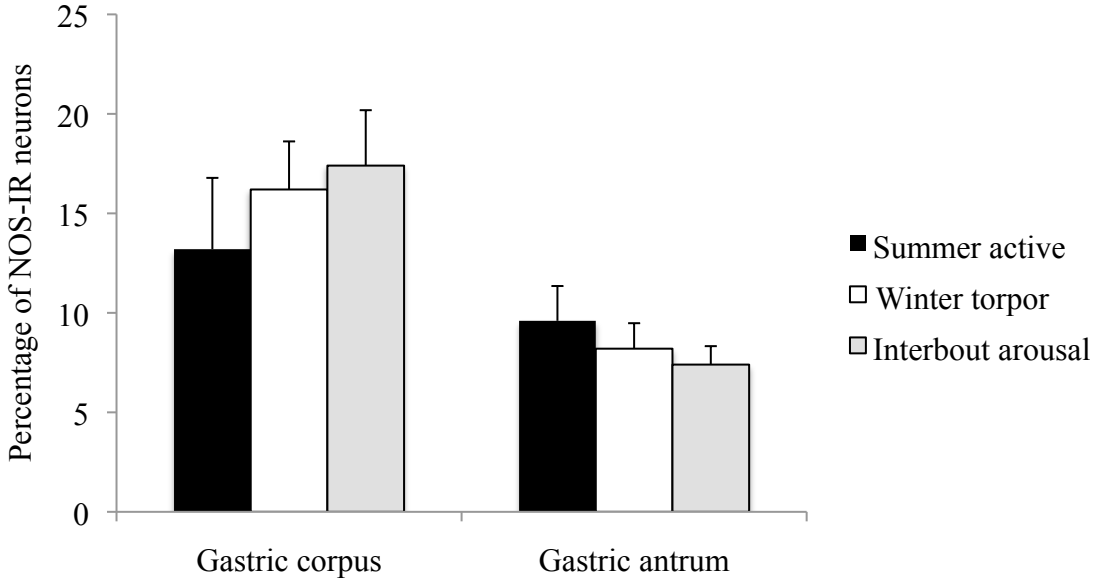

Figure 6. Percentage of NOS-IR neurons in the myenteric plexus of the gastric corpus and antrum in summer active, winter torpor and interbout arousal squirrels (n=5/group). No statistically significant differences were found among groups. Data are expressed as means ± SEM.

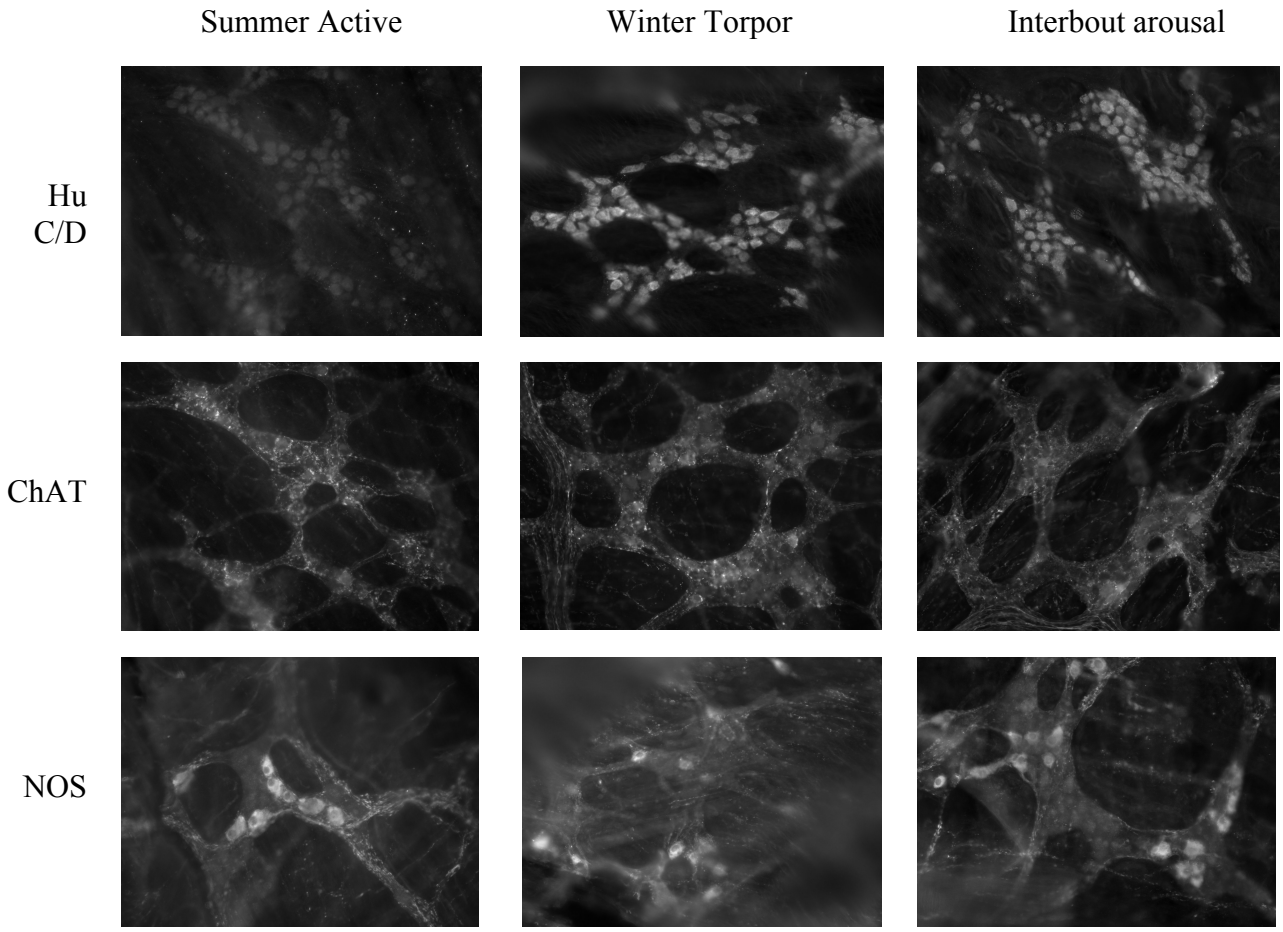

Figure 7. Immunofluoresence staining for the neuronal marker Hu C/D, choline acetyltransferase (ChAT), and nitric oxide synthase (NOS) in the myenteric plexus of the gastric corpus. No significant differences in neuron number per ganglia were observed among groups.

DISCUSSION

This study is the first to report a seasonal change in gastrointestinal transit in a hibernating mammal. Our results indicated that gastrointestinal transit in torpid 13-lined ground squirrels was significantly reduced. In four out of five of the torpid squirrels, there was no gastric emptying 30 min after the charcoal meal. In one torpid squirrel that did show gastric emptying, the charcoal meal passed through the small intestine at about 6% of the rate at which it traversed the intestines of summer-active animals. Our observation is in agreement with a previous report in hibernating frogs (*Rana pipiens*), which demonstrated that passage of material through the gut of hibernating frogs slowed down significantly compared to active frogs (12).

Two factors may contribute to the reduced gastrointestinal motility during winter torpor: low body temperature and prolonged fasting. We found that gastrointestinal transit times were rapidly restored to summer active levels in interbout arousal squirrels, suggesting that prolonged fasting may not be the cause of reduced gastrointestinal motility since squirrels in interbout arousal do not have food intake. *In vitro* studies using isolated intestinal segments from hibernating big brown bats and laboratory mice (non-hibernator) showed that as the gut temperature declined, the frequency of contraction decreased significantly, with contraction ceasing at 15°C for mice and 7°C

for bats (13). Although the critical temperature for the ground squirrel's gut to stop contracting is unknown, duodenal segments from hypothermic or normothermic squirrels maintained at 4°C *in vitro* did not show any spontaneous motility (14). The 13-lined ground squirrels in the present study hibernated in a 6-7°C hibernaculum and their anal temperatures were 9.8 ± 0.7°C (n=5). Reduced core body temperature may play a major role in the decrease of gastrointestinal transit times in winter torpor ground squirrels.

The mechanism underlying the temperature effect on gastrointestinal motility remains unclear. It is possible that reduced temperatures have an effect on the pacemaker cells of the gut, the interstitial cells of Cajal that are responsible for generating spontaneous contraction of gastrointestinal smooth muscle. Since the heart rate is slowed in hibernators as well, it would be interesting to compare activities in the cardiac pacemaker to the gut pacemaker. Unfortunately, information on this in the literature is lacking. It is known that between 10 and 15°C, the hearts of non-hibernating mammals become arrhythmic and/or fibrillate and no longer function. This is not true of hibernating mammals, whose hearts continue to function at temperatures approaching 0°C, regardless of the season. Literature suggests that entrance into hibernation shows an initial large increase in parasympathetic tone that reduces heart rate by as much as 50% before body temperature begins to fall substantially. The parasympathetic tone is slowly replaced by the Q_{10} effects of temperature (15). This relationship should be further explored in the gut.

The ENS acts as the "brain-in-the-gut" and is essential for normal smooth muscle activity in the gut. Neurons in the ENS form neuronal circuits that are designed to control gut motility independent of input from the brain and the autonomic nervous

system. To further investigate the mechanisms that may contribute to the clear slowing of gastrointestinal motility observed in winter torpor squirrels, total number of neurons in the myenteric plexus, the number of neurons expressing a major excitatory neurotransmitter, and the number of neurons expressing a major inhibitory neurotransmitter were compared across animal groups. ChAT is an enzyme used in the synthesis of acetylcholine, a major excitatory neurotransmitter in the ENS. ChAT is used as a marker for cholinergic excitatory neurons. NOS is an enzyme used in the synthesis of nitric oxide, a major inhibitory neurotransmitter in the ENS. NOS is used as a marker for nitergic inhibitory neurons. Due to fundamental differences in the contractile activity of the gastric corpus and antrum, these two regions were compared across animal groups. No statistical differences were found for the ChAT-IR and NOS-IR neurons in the myenteric plexus across animal groups. Several factors may contribute to the negative results. First, the methodology we used to count the neurons may not provide an accurate measure if there are small-scale differences. Second, bias in the counting is inherent due to the quality of the fluorescence staining and ambiguous lines between neurons. Further analysis measuring specific levels of neurotransmitter expression through methods such as ELISA are prudent.

Previous studies show clear changes in gut structure (5) and function (3, 4) during hibernation, including enhanced ion secretion and absorptive capacity of nutrients in ground squirrels. Since these functions are controlled by the ENS moment to moment, studies on alterations in the neurochemical composition of the enteric neurons are of particular importance. Reports on the effects of hibernation on the myenteric plexus of the golden hamster small and large intestine show changes in the number of myenteric

neurons expressing several different types of excitatory and inhibitory neurotransmitters. There was a significant increase during hibernation in the number of neurons immunoreactive to vasoactive intestinal polypeptide, substance P and calcitonin gene-related peptide. Neuronal cell bodies positive for tyrosine hydroxylase, were almost absent in the control animals, but were prominent in the hibernating animals. There was also a significant decrease in the number of neurons immunoreactive to 5-hydroxytryptamine and no significant changes in the numbers of neurons immunoreactive to NOS (16). ChAT was not measured in the hamster study; however, the results from the present study support their findings for total neurons and NOS. Even though no changes in neurotransmitters were observed, the myriad of other neurotransmitters and hormones that contribute to motility and have been implicated by other studies should be explored. Gastrointestinal motility is directly controlled by excitatory and inhibitory neurotransmitters; therefore, further investigation of the roles of specific neurotransmitters in hibernating ground squirrels is warranted.

Work building on the preliminary results of this study should provide insight into gastrointestinal disorders characterized by weakened, spastic or failed propulsion of food through the digestive system in humans. Along with this, further studies on the thickness of the gut wall would be helpful. Smooth muscle cells in the gut wall are responsible for generating gastrointestinal motility. However, there are currently no reports on whether the smooth muscle layer in the gut wall atrophies during hibernation. Elucidating the underlying mechanisms that cause the reduction in gastrointestinal transit during torpor in thirteen-lined ground squirrels may provide insight into potential treatments for gastrointestinal hypomotility in humans.

LITERATURE CITED

1. Carey, H.V., Andrews, M.T., & Martin, S.L. (2003). Mammalian hibernation: cellular and molecular responses to depressed metabolism and low temperature. *Physiology Review, 83,* 1153–1181.

2. Hampton, M., Bethany, L., Nelson, T., & Andrews, M.T. (2010). Circulation and metabolic rates in a natural hibernator: an integrative physiological model. Am J *Physiol Regul Integr Comp Physiol, 299,* R1478–R1488.

3. Carey, H.V., Mangino, M.J., & Southard, J.H. (2001). Changes in gut function during hibernation: implications for bowel transplantation and surgery. *Gut, 49,* 459-461.

4. Carey, H.V. (1990). Seasonal changes in mucosal structure and function in ground squirrel intestine. *Am J Physiol Regul Integr Comp Physiol, 259,* 385-392.

5. Carey, H.V. (1992). Effects of fasting and hibernation on ion secretion in ground squirrel intestine. *Am J Physiol Regul Integr Comp Physiol, 263,* 1203-1208.

6. Nelson, C.J., Otis, J.P. & Carey, H.V. (2009). A role for nuclear receptors in mammalian hibernation. *The Journal of Physiology, 587,* 1863-1870.

7. Furness, J.B. & Costa, M. (1980). Types of nerves in the enteric nervous system. *Neuroscience, 5,* 1-20.

8. Kunze, W.A.A. & Furness, J. B. (1999). The enteric nervous system and regulation of intestinal motility. *Annual Review of Physiology, 61,* 117-142.

9. Olsson, C. & Holmgren, S. (2001). The control of gut motility. Comparative *Biochemistry and Physiology, 128,* 479-501.

10. Martinez, V., Wang, L., Rivier, J., Grigoriadis, D., & Taché, Y. (2004). Central CRF, urocortins and stress increase colonic transit via CRF1 receptors while activation of CRF2 receptors delays gastric transit in mice. *Journal of Physiology. 556,* 221-34.

11. Mittelstadt, S.W., Hemenway, C.L., & Spruell, R.D. (2005). Effects of fasting on evaluation of gastrointestinal transit with charcoal meal. *Journal of Pharmacological and Toxicological Methods, 52,* 154-158.

12. Gossling, J., Loesche, W.J, Ottoni, L.D., & Nace, G.W. (1980). Passage of material through the gut of hibernating Rana pipiens (Amphibia, Anura, Ranidae). *Journal of Herpetology, 14,* 407-409.

13. Studier, E.H., Behrend, T.A., & Freed A.L. (1976). Effects of temperature on intrinsic intestinal motility in a hibernator. *Journal of Thermal Biology, 1,* 149-151.

14. Weekley, B. & Harlow, H.J. (1986). The influence of cholecystokinin octapeptide and angiotensin II on *in Vitro* duodenal motility in hibernating and aroused 13-lined ground squirrels. *Cryobiology, 23,* 447-452.

15. Milsom, W.K., Zimmer, M.B., & Harris, M.B. (1999). Regulation of cardiac rhythm in hibernating mammls. *Comparative Biochemistry and Physiology, 124,* 383-391.

16. Toole, L., Belai, A., Shochina, M., & Burnstock, G. (1999). The effects of hibernation on the myenteric plexus of the golden hamster small and large intestine. *Cell Tissue Research, 296,* 479-487.

17. Hume, L.D., Beiglbock, C., Ruf, T., Frey-Roos, F., Bruns, U., & Arnold, W. (1990). Seasonal changes in morphology and function of the gastrointestinal tract of free-living alpine marmots. *Journal of Comparative Physiology, 172,* 197-207.

18. Vanden, B.P., Coulie, B., Tack, J., Mawe, G.M., Schemann, M., & Janssens, J. (1999). Neurochemical coding of myenteric neurons in the guinea-pig antrum. *Cell Tissue Research, 297,* 81-90.