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Graduate Studies

INCREASE OF EXTRACELLULAR MATRIX PROTEINS IN HIBERNATING GROUND SQUIRRELS COULD HELP MAINTAIN BONE HEALTH

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INCREASE OF EXTRACELLULAR MATRIX PROTEINS IN HIBERNATING GROUND SQUIRRELS COULD HELP MAINTAIN BONE HEALTH

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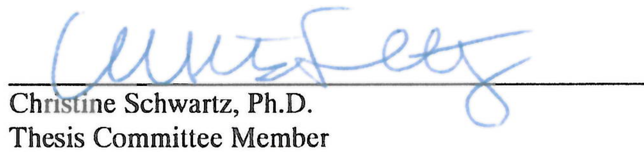

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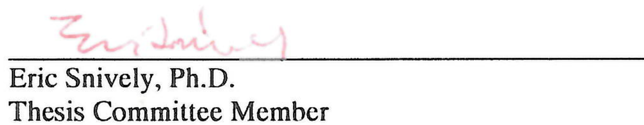

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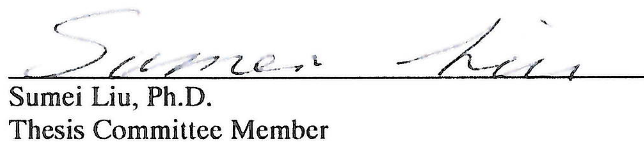

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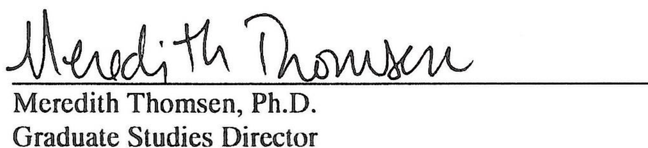

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ABSTRACT

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Whether from injury, old age, or space flight, immobility or a lack of gravitational loading has negative effects on the physiology of bone and bone marrow. Consequences include a weakening of the immune system, decreased hematopoietic cells in the bone marrow, and an overall decrease in bone density. Understanding the mechanisms behind these effects and combating their side effects is a subject of intense research. Thirteen lined ground squirrels are a common model organism for these studies because they hibernate for months and experience long periods of inactivity. Like humans under low loading and activity, the ground squirrels experience changes in the bone marrow cell make up and decreased bone density, but, unlike humans, once a 13-lined ground squirrel becomes active again, the side effects from their long rest are minute or non-existent. Transcriptomes were constructed from genes expressed in bone marrow from hibernating and non-hibernating ground squirrels. Certain extracellular matrix proteins, including COL4A2, were seen to increase drastically during hibernation and may possibly play a role in maintaining the health of the bone.

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TABLE OF CONTENTS

PAGE

LIST OF TABLES……………………….……………………………………………………vii
LIST OF FIGURES…………………………………………………………………….…..viii
INTRODUCTION 8
Bone Anatomy……………………………………………………………………………11
Compact Bone……………………………………………………………………11
Cancellous Bone………………………………………………….……………….…..12
Femur and Bone Marrow……………...………………………………………………12
Bone Turnover……..…………..…………………………………………………………13
Genes Showing Differential Expression in Bone Marrow During Hibernation.………………14
COL4A2…………………………………………………………………………….14
CHI3L1………………………………………………………………………..…16
LAMA3……………………………………………………………………………..17
ITFG3……………………………………………………………………………….18
AIM AND OBJECTIVES.. 19
MATERIALS AND METHODS.. 20
Trichrome Staining 20
Hydroxyproline Assay……………...…………………………………………………….21
Antibodies and Kits for Immunoblot and Immunohistochemistry………………..…..……..21
Immunoblot……………………………………………………………..………………22
Decalcification……………………………………………………………………………23
Immunohistochemistry…………………………………………………………………...23
RESULTS……………………………………………………………..……..………………25
Trichrome and Hydroxyproline Assay……………..………………………..………………25

Trichrome……………………………………..……………………..………..……...25

Hydroxyproline Assay………………………...……………….………………………26

Immunoblot………………………………………………………..……………….………...28

Immunohistochemistry…………………………………………………………….………..30

CHI3L1…………………………………………………………………………...…31

ITFG3……………………………………………………………………………….32

COL4A2…………………………………………………………………………….33

LAMA3……………………………………………………………………………..34

DISCUSSION…………………………...……………………………………………….…….36

CONCLUSION…………………………..………………………………………………...……40

LIST OF TABLES

TABLE PAGE

1. Cardiac Fetal Gene Expression……………………………………………………………11

2. Results from Hydroxyproline Assay……………………………………………………27

3. Percent Area of Bone Sample Stained via IHC…………………………………………35

LIST OF FIGURES

FIGURE PAGE

1. Body Temperature of 13-lined Ground Squirrels During Hibernation………………….9

2. Trichrome Stain of Femur Long Bone and Articular Cartilage………………………..25

3. Comparison of Hydroxyproline Concentrations From Femur Samples of

Hibernating and Non-Hibernating Ground Squirrels………………………………...28

4. Immunoblots for COL4A2 and LAMA3. ……………..……………………………….29

5. Immunoblots for CHI3L1 and ITFG3……………..…………………………………...30

6. Immunoblots for CHI3L1 and ITFG3……………...……………………………………31

7. Immunoblots for CHI3L1 and ITFG3. …………………………………………………32

8. IHC Using COL4A2 Antibody. ………………………………………………………..33

9. IHC Using LAMA3 Antibody……………………………………………………………34

INTRODUCTION

Homeostasis is the process of keeping the internal environment of an organism stable. A shift away from this relatively stable state could result in dysfunction or death. Mammals and other species therefore invest metabolic energy to maintain homeostasis in organs like bones and muscles. Physical activity can help maintain bone and muscle homeostasis (Russo, 2009). Examples of where homeostasis is affected by a decrease in mobility would be in individuals that experience prolonged periods of bed rest or weightlessness. Such patients commonly have drastic loss in bone density and a decrease in immune system function (Hoff et al., 2015). This can be dangerous when they resume normal activities, leading to an increased risk of injury because of increased bone fragility. Various experiments have been performed to find ways to counteract these negative side effects; however, no available methods can fully replicate mobility (McGee-Lawrence et al., 2008). Another approach to pursue is examining adaptations in animals that go through long periods of inactivity such as hibernation.

There are many animals, such as 13-lined ground squirrels (*Ictidomys tridecemlineatus*), that go through the process of hibernation during winter. Hibernation consists of two states. The first is torpor, when the animals are immobile and their heart beat and body temperature decrease to save energy (Fig. 1). The second, interbout arousal (IBA), occurs between rounds of torpor when the animals wake up and move

around for 12-18 hours before going back into torpor (Cooper et al., 2015). Similar to other mammals during torpor, ground squirrels experience bone loss that leads to a decrease in bone weight and density as well as a decrease in the number of immune cells (McGee-Lawrence et al., 2010 and Cooper et al., 2016). Some ways that ground squirrels adapt to this stress is by storing platelets as well as monocytes, lymphocytes and other immune cells in organs, and then releasing these stores upon awakening in the spring (Cooper et al., 2016). Additionally, ground squirrels lose bone density in both compact and spongy bone during hibernation, but the strength of ground squirrel bone (the femur in particular) does not change (McGee-Lawrence et al., 2011). When the ground squirrels come out of torpor for the summer, the bone is rebuilt and returns to normal density (McGee-Lawrence et al., 2011).

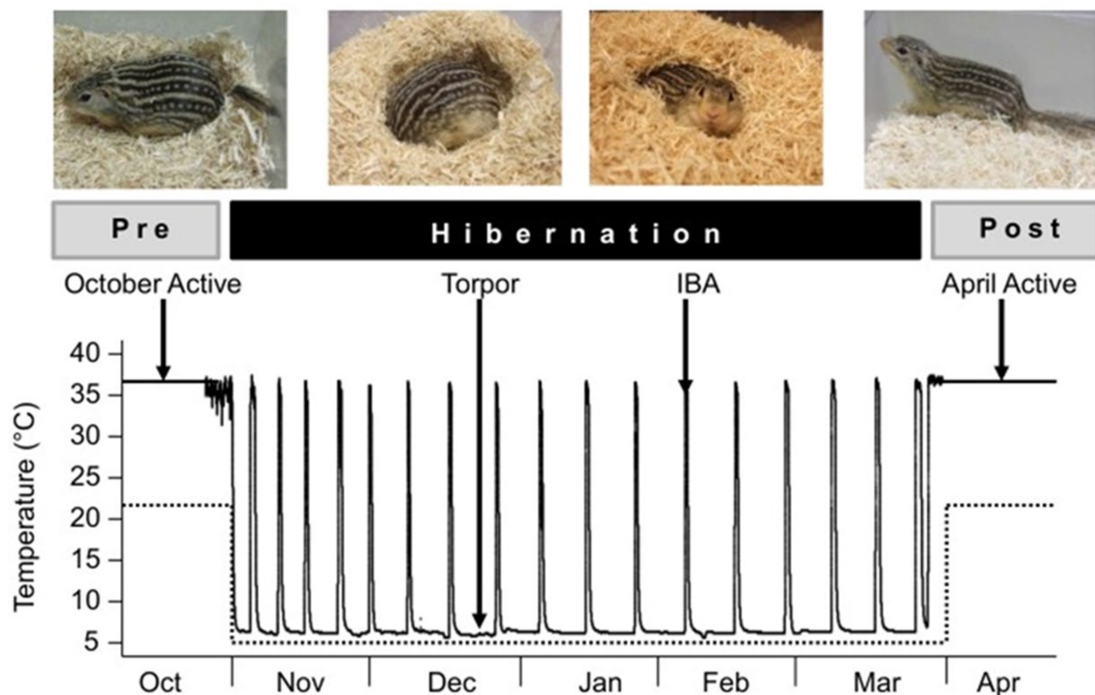

Figure 1**.** Body Temperature of 13-lined Ground Squirrels during Hibernation. 13-lined ground squirrels have a body temperature of 36˚C, and O_2 consumption of 100% and a heart rate of 300-400bpm during the summer months. This contrasts drastically with a torpor temperature of 2-10˚C, oxygen consumption of 2-3% and heart rate of 3-5bpm. About every two weeks squirrels wake up and metabolic rate and heart rate

return to the same levels seen in summer months. These periods of IBA happen continuously throughout hibernation. (Modified from Schwartz et al., 2013)

Studying the mechanisms by which ground squirrels are able to maintain bone health during immobility could have significant implications in human health. One such mechanism could be the differential expression of proteins during the hibernation period. Cooper and colleagues (Cooper 2006) extracted, sequenced, and quantified bone marrow messenger RNAs from non-hibernating (July), torpor (January) and IBA (January) ground squirrels. The transcriptome data were analyzed for differences in expression of specific genes increased during hibernation (Viguet-Carrin et al., 2006). Vermillion et al. (2015) showed increased expression of genes involved in protein synthesis and cardiac myocyte proliferation. This expression was similar to that of neonate gene expression (Table 1), indicating that 13-lined ground squirrels express certain genes at the same level as needed for the generative tissue growth in a developing fetus (Vermillion et al., 2015). The formation of new tissue at damaged sites is comparable to what is seen in the development of new, growing tissue, leading to the hypothesis that proteins that were active in fetal development and are expressed again in hibernation and are helping to maintain tissue under disuse.

Table 1**.** Cardiac Fetal Gene Expression. Vermillion et al. (2015) showed that certain cardiac genes increased during hibernation. The levels that were seen mimicked levels that are seen during neonatal development suggesting that, if these genes are translated, they could be assisting in keeping tissue healthy despite the stress of hibernation. (Adapted from Vermillion et al., 2015).

Functional Category	Cardiac Fetal Gene Program/Hypertrophy Genes	Normalized mRNA Reads			
		April	October	Torpor	IBA
Cardiac Transcription Factors	NKX2.5	973	846	1300	1108
	GATA4	536	679	864	689
	MEF2A	402	452	689	619
	NFATC1	128	161	199	179
	TBX20	332	543	577	670
	MYOCD	490	613	840	1120

Bone Anatomy

Compact Bone

Compact bone is typically found on outer surfaces of bone as in the shafts of long bone and surrounding the diploe of flat skull bones. Due to its organized structure and high density, it provides more of the skeletal support and can handle higher loading forces than spongy bone. In either endochondral or secondary ossification, as the osteoblasts lay down osteoid, it becomes trapped in its own matrix in a space called the lacuna, where the osteoblast differentiates into an osteocyte. Haversian and Volkmann canals run longitudinally and transversely through the bone respectively to allow the transduction of nutrients and signaling to the cells in the bone. The bone made by osteoblasts form circular lamellae in layers around the Haversian canal and, together with

the osteocytes in their lacunae, form an osteon. Osteocytes send out small arms or tendrils through canaliculi that allow them to communicate with each other and sense mechanical changes in pressure (Marieb, 2008).

Cancellous Bone

Cancellous (spongy) bone is found between flat bones and on the inner surfaces or ends of long bones. It forms spicules (trabeculae) that extend like bony fingers into the bone marrow. In contrast to compact bone, the osteocytes of spongy bone form an irregular pattern that results in lower density bone. Although this means spongy bone is not able to withstand as much force as compact bone, its structure provides support without adding as much density. During remodeling, spongy bone experiences the greatest change in mass due to the increased surface area exposed to active osteoblasts and osteoclasts (Marieb, 2008).

Femur and Bone Marrow

Femora are a type of long bone that consist mostly of compact bone. The shaft and epiphysis of the femur are surrounded by the dense periosteum layer. Bony spicules intervene into the bone marrow on the inner surface and are covered by the slightly thinner endosteum. Residing in the center of the bone is bone marrow. Bone marrow is a complex, spongy organ that is highly vascular and home to large numbers of adipose cells as well as hematopoietic, mesenchymal, skeletal muscle and hepatocyte stem cells.

The hematopoietic stem cells give rise to lymphoid progenitor cells (such as T-cells and B-cells) and myeloid progenitor cells (erythrocytes and other immune cells such as megakaryocytes).Mesenchymal stem cells produce cells that assist in bone formation

and maintenance,including osteoblasts, chondrocytes and adipocytes. Other stem cells are found in the bone marrow but once differentiated are transported to their respective organs via the blood stream.

Bone Turnover

Bone turnover occurs by mechanisms including hormonal or mechanical stimuli. Two major hormones involved in the maintenance of blood calcium levels, and therefore bone density, are parathyroid hormone (PTH) and calcitonin. PTH is released when blood calcium is low and leads to increased differentiation of osteoclasts via signaling from osteoblasts (Boyce et al., 2012). Osteoclasts then demineralize bone and release calcium and phosphate into circulation. PTH also plays a role in activating vitamin D which increases calcium uptake in the gastrointestinal tract, bone resorption and calcium retention in the nephron collecting duct (Ross et al., 2011). Calcitonin, a hormone released when blood calcium levels rise, inhibits osteoclast activity. Osteoblasts lay down a collagenous organic matrix that is then mineralized with calcium and potassium crystals to form new bone and remove calcium from circulation.

Mechanical stimuli can also lead to bone turnover. The thin arms of osteocytes that extend through the bone sense changes in pressure, where more pressure is experienced osteocytes signal for increased deposition of bone and thus increase bone density in that area. Conversely, when pressure is removed for extended periods of time, osteoclast activity becomes predominant and bone density is lost. Bone resorption and deposition can work in a cyclical manner to remove and repair damaged bone or to maintain blood serum calcium levels.

One or a combination of these forces account for the bone loss McGee-Lawrence et al., (2008) saw in the bone of hibernating 13-lined ground squirrels. But, how bone strength is maintained is still unknown. A possible explanation is extracellular matrix proteins aiding in support. Using a previous transcriptome study gave direction in which proteins have the largest increase during torpor and IBA, therefore, which proteins might be reinforcing the bone.

Genes Showing Differential Expression in Bone Marrow During Hibernation

From the ground squirrel bone marrow transcriptome, genes that showed 1.5 fold or greater differential expression between non-hibernators and hibernators were analyzed. Additional genes were selected that showed more expression in bone marrow than other tissues, or have a function in the stability of the bone. Using these criteria COL4A2, CHI3L1, LAMA3 and ITFG3 were chosen for further study because they showed 4.9, 13.8, 3.3 and 1.8 fold increases respectively during hibernation (Cooper et al.,2016).

COL4A2

COL4A2 is a gene that codes for one of the three subunits found in type IV collagen which makes up basement membranes. Basement membranes are key in the development and maintenance of tissues, allowing for the adhesion and binding of growth factors as well as simultaneously supplying strength and flexibility at tissue and organ levels (Malara et al., 2013). Collagens are abundant in extracellular matrices and contain three subunits that intertwine in triple helix formation to create a total of 28 different collagen types (Frantz et al., 2010). For example, type IV collagen consists of

two α1 subunits and one α2 subunit (Lodish et al., 2000). Disruption of the triple helix at varying locations along the protein allows type IV collagen to form into a flexible meshwork, which is found solely in basement membranes (Lodish et al., 2000, Gelse et al., 2003). Further modifications after assembly of the triple helix can consist of hydroxylation of proline residues (Gelse et al.,2003). These hydroxyproline residues have a unique presence in collagen and can be used to quantify total collagen levels in tissue samples.

Type IV collagen is a basement membrane protein that can be found in all tissue types but is critical in the vasculature as mutations in COL4A2 lead to vascular diseases (Loscertales et al., 2016 and Gunda et al., 2014). Type IV collagen is expressed by megakaryocytes (MKs), platelet progenitor cells found in bone marrow (Malara et al., 2013). A decrease of platelet levels in the blood system is one of the known stimulants for increased expression of type IV collagen by MKs, which in turn acts in a negative feedback loop to support proplatelet formation (Malara et al., 2013). Low levels of platelets during hibernation could be triggering the increased gene expression in the transcriptome (Cooper 2012, Cooper 2016). Additionally, in studies where MKs were exposed to individual extracellular matrix ECM proteins, exposure to type IV collagen increased the number of MKs, which would create more collagen and platelets (Malara et al., 2013).

CHI3L1

CHI3L1 is a gene that codes for a chitinase-like protein. Chitinase proteins are typically found in organisms (e.g. fungi) that need to break down chitin (Renkema et al., 1997), however, several homologs have been found in humans including gp-39 (YDL-40). The exact role of this protein in humans is not fully known. While a 50kDa paralog has strong chitin binding ability, gp-39 only binds weakly and lacks any enzymatic activity (Renkema et al., 1997). Gp-39 is secreted primarily from macrophages but is also expressed in other cells such as fibroblast-like synovial cells, hepatic stellate cells, arthritic or injured chondrocytes and vascular smooth muscle cells. All of these cells show increases in the protein expression level in cases of ECM remodeling and inflammation (Johansen 2006). It has been hypothesized that gp-39 exerts growth factor properties on multiple cell types and protects cells from apoptosis (Johansen 2006). Gp-39 also has heparin/heparin sulfate binding properties that links it to functioning in changes of the ECM (Johansen 2006).

Two different isoforms of gp-39 exist, a 50kDa form and a more common 39kDa form which is produced by macrophages osteoblasts, and chondrocytes The HC gp-39 is found in synovial fluid of rheumatoid arthritis patients and patients with breast cancer. It has also been used as a marker for maturation of monocytes to macrophages. It could act as an opsonin, a type of antibody that tags foreign organisms for phagocytosis.

LAMA3

LAMA3 is a gene coding for the α3 subunit of laminin. Laminins are heterotrimers composed of different α-, β-, and γ-subunits that combine in varying combinations to form 15 different cross-like structures (Klees et al., 2005). Laminins are found in the extracellular matrix (ECM), primarily basement membranes, and have a role in cell attachment (Paulsson, 1992). Laminins also influence epithelial and mesodermal tissues cellular differentiation, proliferation and motility (Klees et al., 2005 and Paulsson, 1992). The α3 subunit is found in laminin 5, 6 and 7 (Klees et al., 2005), making all of these proteins possible candidates that may increase during hibernation.

Laminin 5 (Ln-5) is comprised of α3, β3, and γ2 subunits. Although typically found in only endo- and ectoderm, expression has been seen in vascular smooth muscle and it is thought to also play a role in the development of the skeletal system (Klees et al.,2005). Ln-5 also acts in cell adhesion and motility via integrins such as α3β1 (Ryan et al., 1999). Increased interactions of Ln-5 and integrin α3β1 are seen at sites of injury and promote cell migration to aid in healing (Lampe et al., 1998). Positive staining has also been seen in the periosteum of rat ribs (Klees et al., 2005).

Laminin 6 (Ln-6) is another laminin containing the alpha 3 subunit (α3β1γ1), and when coupled with Ln-5, the long arm of Ln-6 interacts with integrin and amplifies the effects of Ln-5 on cell spreading (Hirosaki et al., 2002). When not in complex with Ln-5, Ln-6 is more commonly found free and does not promote cell spreading (Hirosaki et al., 2002). In the lungs it acts with perlecan (a basement membrane heparin sulfate proteoglycan) as a stretch receptor to prevent injury (Jones et al., 2005).

Laminin 7 (Ln-7) is typically found associated Ln-5 and Ln-6 in the basement membranes of blood vessels and nerves (Champliaud et al., 1996). Ln-6/7 act as intermediates to assist Ln-5, bound to epithelial cells, in binding to the underlying hemidesmosome (Champliaud et al., 1996).

ITFG3

Integrin alpha FG GAP containing repeat 3 (ITFG3) is a protein that contains repeats of the amino acids phenylalanine-glycyl (FG) and glycyl-alanyl-prolyl (GAP) (GAP; Springer, 1996). This sequence can repeat up to 7 times and is located at the N-terminus forming a ligand binding, extracellular domain of integrin called the beta propeller. The 8 different β propeller subunits can complex with 18 α propeller subunits to form a plethora of complexes (Springer, 1996). An increase of the FG GAP containing repeat 3 mRNA has been seen during osteoclast differentiation (Itou et al., 2014). This protein is also thought to be an intracellular membrane protein localized to the mitochondria and nucleoplasm (FG-GAP repeat (IPR013517), 2018).

AIM AND OBJECTIVES

The aim of this research is to determine if the increase of COL4A2, CHI3L1, LAMA3 and ITFG3 mRNAs in hibernating 13-lined ground squirrels is also observed in their corresponding proteins.

Objectives

1. Use a trichrome and a hydroxyproline assay to look for differences in total collagen proteins in the femur of summer and hibernating ground squirrels.

2. Use immunohistochemistry to determine the amount and location of Type IV collagen, laminin α3, chitinase-like protein, and integrin FG-GAP repeat-3 in the femurof 13-lined ground squirrels between summer and hibernation.

MATERIALS AND METHODS

Trichrome Staining

Five µm thick sections were made from decalcified, paraffin-embedded femur obtained from ground squirrels collected at four time points: July non-hibernator, October entrance into hibernation, January hibernator, and one week post arousal in March. Sections were deparaffinized in a 60˚C oven until the paraffin had melted then rehydrated through 100% alcohol, 95% alcohol and 70% alcohol. Slides were then rinsed in DI water and re-fixed in Bouin's solution (75 ml Picric acid (saturated), 25ml 40% formaldehyde, 5ml glacial acetic acid) at 56˚C for 1hr. The slides were then rinsed under running tap water for 5min and incubated in Weigert's iron hematoxylin (1 part Stock Solution A: 1 g Hematoxylin, 100ml 95% Alcohol. 1 part Stock Solution B: 4ml 29% Ferric chloride in water, 95ml Distilled water, 1ml concentrated hydrochloric acid) for 10min, rinsed, then incubated in phosphomolybdic-phosphotungstic acid solution (25 ml 5% Phosphomolybdic acid, 25ml 5% Phosphotungstic acid) for 10min. Sections were directly transferred into aniline blue for 5min, rinsed in water and then placed in 1% acetic acid solution for 2 min. After a final wash slides were dehydrated through 95% alcohol, 100% alcohol and toluene.

Hydroxyproline Assay

A Hydroxyproline Assay Kit (STA-675, Cell Biolabs, Inc., San Diego, CA) was used to quantify levels of collagen. Femur from non-hibernators, entrance into hibernation, IBA, torpor and post arousal specimens were ground with a mortar and pestle then a tissue homogenizer in 100µl DI water/10mg sample. 100µl of homogenate was transferred to 2ml Nalgene vials (5000-0020, Thermo Fisher Scientific, Waltham, MA) and mixed with 100µl 12N HCl under a fume hood. Samples were hydrolyzed for 3hrs at 120˚C then vortexed with 5mg of activated charcoal after a brief cooling period. The mixtures were then centrifuged at 10,000 g for 5min and supernatant was isolated and stored at 4˚C. Acid hydrolyzed samples (10µl) were pipetted into a flat bottom microplate (96 well) and evaporated to dryness at 70˚C for 45 min. Standards were prepared according to the kit protocol and 10µl of each concentration were added to separate wells. Chloramine T (100µl: 6µl chloramine T/94µl assay buffer) was added to each well and incubated for 30 min at room temperature followed by Ehrlich's Reagent (100µl: 50µl 2X Ehrlich's Concentrate/50µl isopropanol/perchloric acid) incubated at 60˚C for 90min and optical density (OD) was determined at 550nm using a Vmax plate reader.

Antibodies and Kits for Immunoblot and Immunohistochemistry

Primary antibodies were diluted at 1:1000 for immunoblot and 1:50 for immunohistochemistry (IHC) in dilution buffer (Dako EnVision Kit). The antibodies used for each antigen were as follows: CO4A2 (PA5-27708, Thermo Fisher, Rockford, IL), LAMA3 (TA314144, OriGene, Rockville, MD), ITFG3 (PA5-31403, Thermo Fisher,

Rockford, IL) and CHI3L1 (H00001116-D01P, Abnova, Taiwan). The secondary antibody, Pierce Goat Anti-rabbit IgG Secondary Antibody, HRP (SA1-9510, Thermo Scientific, Rockford, IL), was diluted to 1:500. To visualize the antibodies a Dako EnVision+ System-HRP (DAB) was used (Dako North America, Inc., Carpinteria, CA).

Immunoblot

ProteinAtlas.com was used to identify tissues that have been shown to express the proteins of interest in order to prepare standards for immunoblotting. The antibodies were tested on samples as follows- CHI3L1: liver, brain bone; LAMA3: heart, liver, lung, brain; ITFG3: heart, brain, lung; COL4A2: bone, heart, lung, liver, brain. Lysates were created from tissue samples taken from 13-lined ground squirrels and then frozen at -80˚C. Approximately 5mg of each tissue was homogenized with 500µl 1% NP-40 and 1:100 protease inhibitor. Homogenate was gently rotated at 4˚C for two hours, centrifuged at 12,000rpm for 10min, and the supernatant was isolated. Protein concentrations in lysates were measured using a NanoDrop Lite spectrophotometer (Thermo Scientific, Rockford, IL).

Samples were diluted to 10mg/ml with DI water and 10µl were mixed with an equal amount of 2x loading buffer and 1:20 volume of β mercaptoethanol. Samples were heated at 100˚C for 5 min then 20µl of each were loaded into a 12% acrylamide gel. A ladder (5µl) was loaded in lanes 3 and 7. The gel was run in 1X SDS PAGE buffer at 100V for 5min then 200V for 45min.

PVDF membrane was soaked in methanol for 1min to hydrate. Filter paper pads, gel and PVDF membrane were then soaked in transfer buffer (25 mM Tris-HCl pH 7.6,

192 mM glycine, 20% methanol) for 1min. A dry transfer was run at 320mA for 1hr. When the transfer was complete, the membrane was blocked with 1% dried milk in TBST (20mM Tris pH 7.5, 150 mM NaCl, 0.1% Tween 20) for 1hr. The membrane was then cut into strips and incubated in 1:1000 dilutions of 1˚ antibody for COL4A2, ITFG3, LAMA3 and CHI3L1 at 4˚C overnight. The next morning membranes were washed 3 times in 1X TBST for 5min each then incubated at room temperature in 2˚ antibody 1:10,000 dilution of DyLight 649 Conjugated Affinity Purified Anti-rabbit IgG (H&L) (Goat) (611-143-002, Rockland, Pottstown, PA) for 1hr. The three washes were repeated and bands on the membrane were visualized using Typhoon Fla 9500 (GE health care life sciences, Pittsburg, PA, USA) at 500, 600 and 700V.

Decalcification

Femur isolated from summer, torpor and IBA animals were stored in 10% formalin. They were rinsed with DI water then incubated in DeltaCal for 2hrs or until all parts of the bone were flexible. Tissues were then fixed and embedded in paraffin. These samples were used for IHC and trichrome staining.

Immunohistochemistry

Experiments were performed in triplicate using slides prepared with 4µm thick sections from paraffin embedded femur from summer, torpor and IBA animals. Slides were incubated at 60˚C for 1hr or until paraffin was melted off the slides. Samples were then deparaffinized in toluene (3x), 100% ethanol (3x), 95% ethanol (1x) and DI water (1x) for 2min in each. Heat induced epitope retrieval was then performed, using 1X Dako pH9 buffer heated in a water bath to 90˚C, for 21min. Buffer and slides were allowed to

cool at room temperature for 21min then a PAP pen was used to form barriers around tissue. Slides were then rinsed in 1X TBST for 5min with gentle rotation. Four drops of peroxidase block were applied to each slide and incubated in a humidifying chamber on a rocker for 5min. Slides were rinsed twice, each time in fresh 1X TBST buffer for 5min each followed by a 30min incubation in Triton 100X detergent. After being rinsed again for 5min slides were incubated with 1:50 dilutions of the 1˚ antibodies for 1.5hrs. A 30min incubation with 4 drops of 1:500 dilution of Pierce Goat Anti-rabbit IgG Secondary Antibody, HRP (SA1-9510, Thermo Scientific, Rockford, IL) was performed followed by two 5min rinses in wash buffer. DAB+chromogen was applied and incubated for 5min and then tissues were counterstained in hematoxylin and dehydrated back to toluene.

Image-J_win64 software was used to calculated the average percent of staining area for certain samples.

RESULTS

Trichrome and Hydroxyproline Assay

Trichrome

Femur stained with trichrome stain did not show an increase in the intensity of blue staining, indicating no dramatic increase in the overall collagen levels (Fig. 2). There was, however, an observable increase in eosin staining in the articular cartilage of the femur indicating an increase of extracellular matrix proteins (Fig.2) (Cooper et al., 2016).

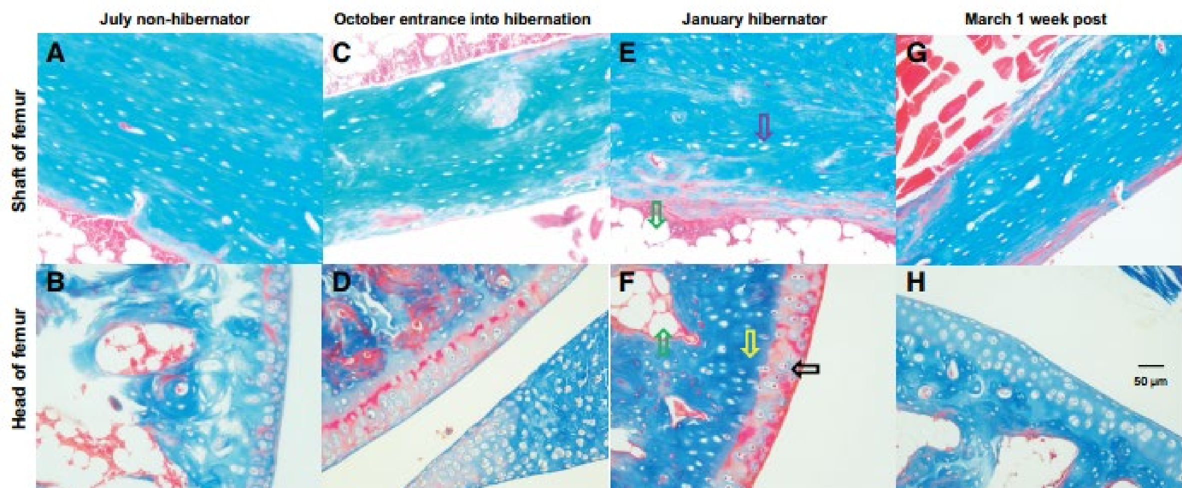

Figure 2. Trichrome Stain of Femur Long Bone and Articular Cartilage. The long bones of femurs were stained via trichrome and exemplary slides were chosen for each group and observed under 200X magnification. A) Non-hibernator C) Entrance into hibernation E) Hibernators G) PA. Collagen stained blue, adipose and empty spaces white, and extracellular matrix and muscle bright pink. No significant difference in staining was seen between the different samples. Arrows indicate; green: adipocyte, purple: osteocyte, yellow: collagen, and black: chondrocyte. (Cooper et al., 2016)

Hydroxyproline Assay

A hydroxyproline assay was performed to measure the concentration of hydroxyproline in femur samples, this can be used to indicate total collagen levels. An initial trial was performed to affirm the efficacy of the assay and then following trials to increase the sample size. All samples were assayed in triplicate except for the final one, which was in duplicate. This trial included NH218-NH222 and a repeat of HIB74, HIB75 and HIB78.

Five samples were excluded from the final t-test for the following reasons: Residual shards of bone were pipetted into the plate wells for sample NH218, increasing the concentration compared to other samples. After the final 60˚C incubation those wells were more black than pink giving a falsely high concentration (Table 2), because of this NH218 was removed from the final t-Test. The results for the repeat HIB samples were much lower than originally seen and so not included when calculating t-test values since protein degradation had most likely occurred between the first and final trials (data not included). During the run including the 120˚C incubation of I213, I217 and IBA130304 with HCl, the vial caps of some of the samples were not tight enough, allowing sample to boil off. This could account for the low concentration of I217 (see Table 2).

A t-test was performed to compare the concentration of hydroxyproline from all of the non-hibernators (M=1606.619, SD=637.325), IBA (M=2513.238, SD=1475.320) and torpor animals (M=2716.946, SD=1677.823). The P-values were as follows; NH:IBA= 0.167, NH:TOR=0.128 and TOR:IBA=0.822 (Table 2). Although the t-Test shows a higher correlation between torpor and IBA, the variance between torpor/IBA and

non-hibernators is not significant, indicating that there is no significant increase of collagen during hibernation. A conditional error probabilities test was performed on these p-values which had the following results: NH:IBA= 44.8%, NH:TOR=41.7% and TOR:IBA=30.5%.

Table 2. Results from Hydroxyproline Assay. Comparing the concentrations of hydroxyproline (an indicator of overall collagen concentrations) using a t-test. Sample sizes of 8, 7 and 7 for non-hibernators, torpor and IBA respectively were tested with a hydroxyproline assay. P-value results (seen in column 4) were as follows: NH:IBA=0.167, NH:TOR=0.128 and TOR:IBA=0.822.

Sample Concentration (µM)						**P-Values**	
NH		**Torpor**		**IBA**			
NH100	1137.762	**I213**	4411.571	**Hib214**	703.952	**NH:IBA**	0.167
NH110	1398.714	**IBA130304**	671.5714	**Hib215**	750.143	**NH:TOR**	0.128
NH99	995.857	**I217***	185.381	**Hib15wcol**	2267.762	**TOR:IBA**	0.822
NH218*	3859.333	**IBA130301**	2150.619	**HIB78**	2802.048		
NH219	2748.222	**IBA209**	1042.048	**HIB74**	3101.571		
NH220	1321.000	**IBA206**	3323.000	**HIB75**	4073.952		
NH221	2230.444	**IBA208**	3480.619	**H108**	5319.190		
NH222	1254.333						

Grouping the data from the hibernators and comparing these to that of the non-hibernators showed a statistically significant difference between the two with a P-value of 0.0259 (Fig. 3).

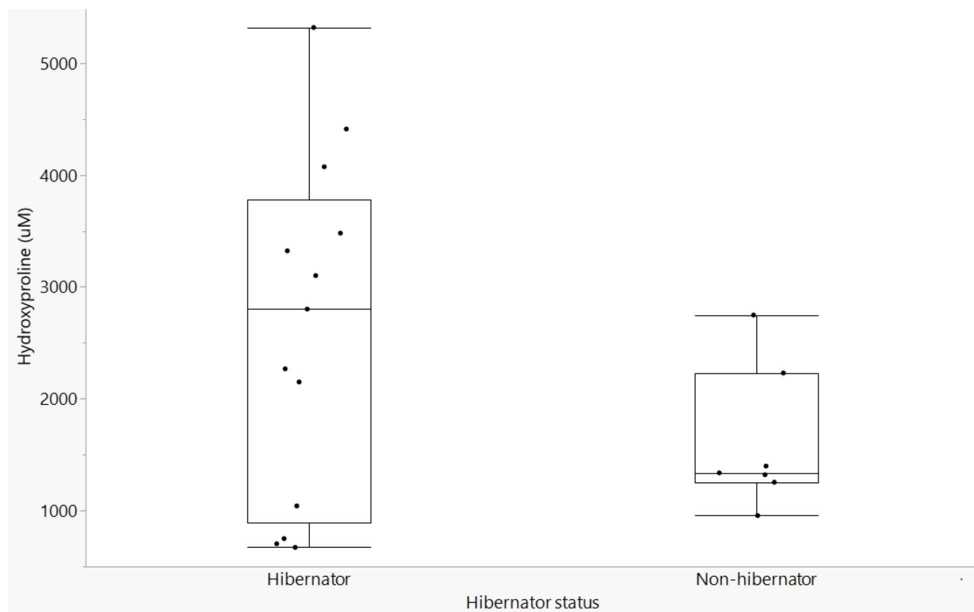

Figure 3. Comparison of Hydroxyproline Concentrations From Femur Samples of Hibernating and Non-Hibernating Ground Squirrels. A box plot comparing the concentrations of hydroxyproline between hibernators and non-hibernators. When grouping IBA and torpor animals, and comparing to the concentrations of non-hibernators, a statistically significant difference was seen with a P-value of 0.0259. These data support a statistically significant increase of hydroxyproline (and thus collagen) during hibernation.

Immunoblot

The type IV collagen alpha 2 chain was predicted to be about 167kDa. Staining with anti-col4a2 antibodies showed clear bands at 55kDa from the brain sample and a fainter band from the lung sample (Fig. 4). Additional faint bands could be seen in the brain sample at 34, 30, 26 and 17kDa which, along with the 55kDa band add up to 162kDa, close to the expected 167kDa (Fig. 4). Additional faint bands were seen in the lung samples but nothing that is definitive. Samples of brain, kidney and lung were used as positive controls to blot for laminin, and the kidney lane had multiple bands but two dark bands at 72kDa and ~30kDa (Fig. 4). Faint bands from the lung sample showed staining at 72, 55, 30 and 17kDa. These bands can be seen in the kidney lane as well and

together add up to 174kDa. Based on GeneCards human gene database, the human laminin alpha 3 subunit should be 170kDa.

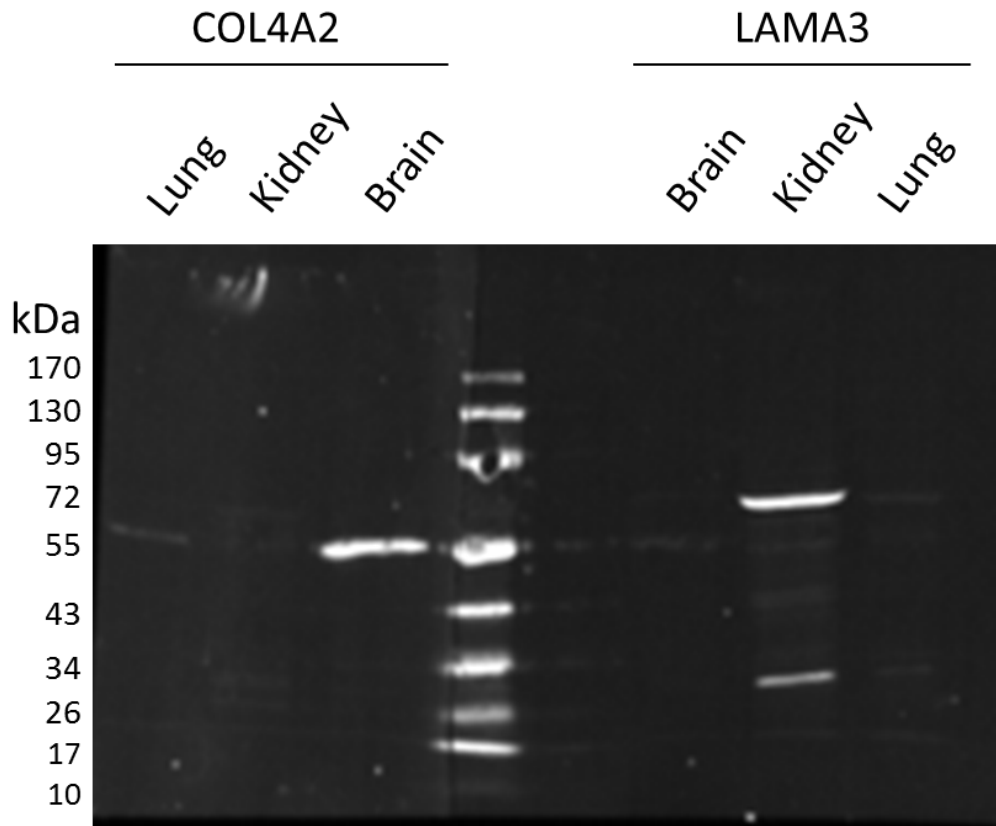

Figure 4. Immunoblots for COL4A2 and LAMA3. Immunoblots were performed on tissue samples from 13-lined ground squirrels. Membranes stained with anti-COL4A2 gave a band at 55kDa in the lanes containing samples of brain and lung. The lanes stained with anti-LAMA3 showed a bright band from the kidney sample at 72kDa and a degradation band at ~30kDa. Faint bands from the lung sample showed staining at 72, 55, 30 and 17kDa. These bands can be seen in the lane containing kidney as well and together add up to 174kDa.

Chitinase-like protein stained at 50kDa indicating the presence and successful binding of the antibodies to the 50kDa isoform (Fig. 5, Renkema et al., 1997). ITFG3 had a faint band from the lung samples at 72kDa (Fig. 5). Although the bands are faint they

were seen over repeated trials. Uniprot indicates a human isoform at ~60kDa. The discrepancy could be due to the different species and posttranslational modifications.

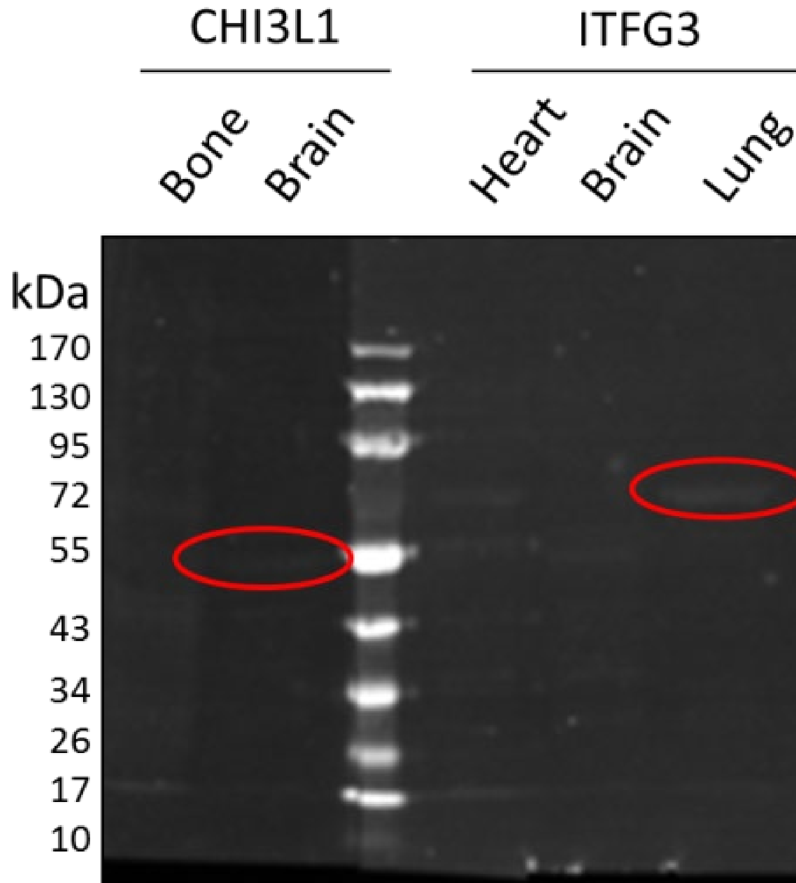

Figure 5. Immunoblots for CHI3L1 and ITFG3. Immunoblots blots performed on prepared samples of the ground squirrel organs. Membranes were stained with rabbit anti-human CHI3L1 and ITFG3 antibodies. When stained with CHI3L1, brain samples showed bands at 50kDa. Lung samples stained with ITFG3 antibodies showed bands at 72kDa over repeated trials.

Immunohistochemistry

If the mRNA quantified in the transcriptome correlates with protein expression, an increase in the density or area of tissue stained by immunoblot would be expected during hibernation. Based on the localization of the staining for each antibody in the

bone, a prediction of the cells that are expressing each protein can be made and thus identify the role that each protein may be playing during hibernation. Full sections of ground squirrel femur collected from non-hibernating, torpor and IBA squirrels stained with antibodies gave the following results.

CHI3L1

No staining was seen in the shaft of the femur but positive results in the bone marrow and articular cartilage show darker staining in the hibernating samples (Fig. 6). In the bone marrow, staining was localized near adipose cells and although the cells were enlarged the staining is still present in intervening spaces (Fig. 6 A-C). In the articular cartilage chitinase-like protein was found in the lacunae of the chondrocytes and specifically of the active cells (Fig. 6 F).

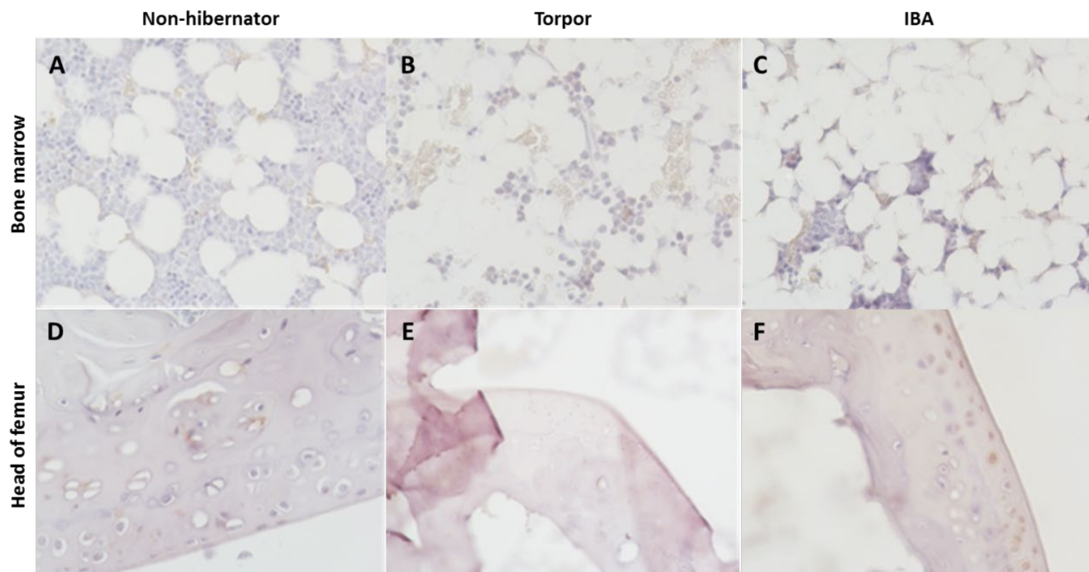

Figure 6. IHC Using CHI3L1 Antibody. Femora were stained via IHC with rabbit anti-human CHI3L1 antibodies and exemplary slides were chosen for each group and observed under 200X magnification. A,D) Non-hibernator B,E) Torpor C,F) IBA. The chitinase like protein stained brown while other tissues were

white or a purple-brown. Intensity of staining increased across all samples. In the bone marrow, staining was observed near adipose cell. In articular cartilage staining appeared in the chondrocyte lacunae.

ITFG3

Integrin was located near adipose cells of the bone marrow and chondrocytes of the articular cartilage (Fig. 7). Contrary to what was expected, the intensity of staining seemed to decrease as the animals were in hibernation. Darker staining showed the majority of protein to be found in the active chondrocytes (Fig. 7 D).

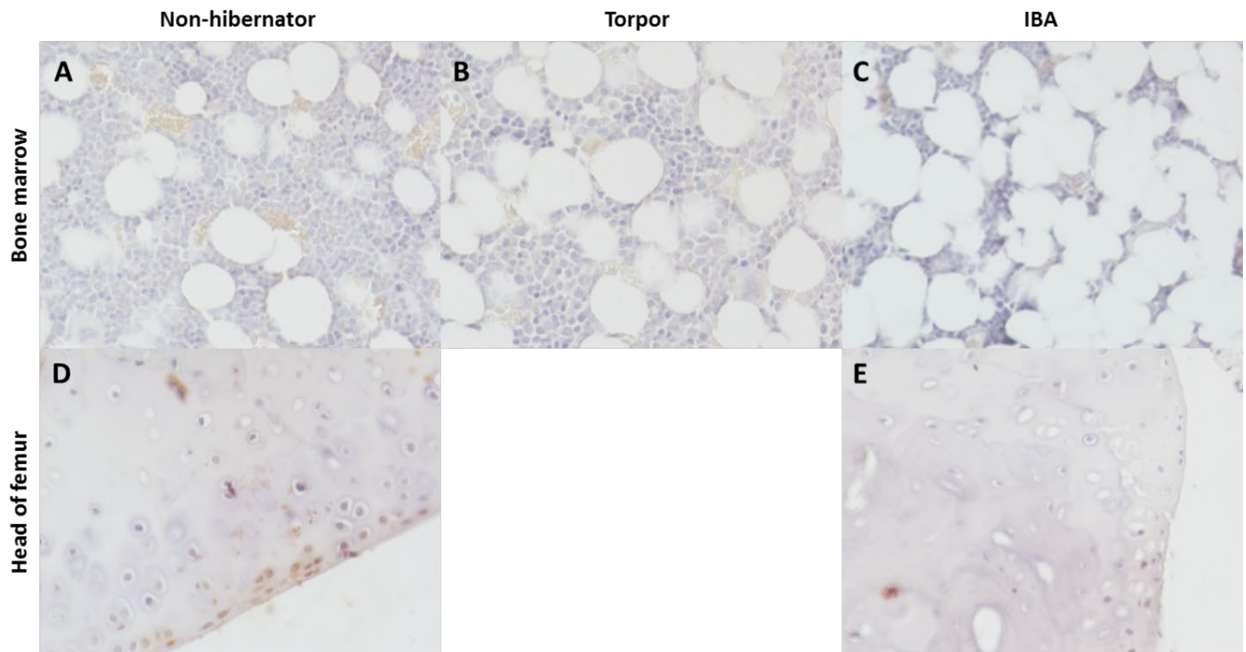

Figure 7. IHC Using ITFG3 Antibody. Staining of femora with rabbit anti-human ITFG3 antibody showed localization of protein (brown) near adipose cells (white) in the bone marrow and in the lacunae of chondrocytes of articular cartilage. Staining appeared more intense in the non-hibernating samples, particularly in the more active chondrocytes. No good images could be obtained from the articular cartilage of torpid animals.

COL4A2

Type IV collagen was located near adipose cells in the shaft of bone marrow. The samples obtained from torpor animals had significantly more staining present although the IBA samples did not appear to have the same concentrations as the torpor samples based on the IHC (Fig. 8 A-C). In one of the samples staining was more intense at the end proximal to the hip and decreased in the bone marrow closer to the knee. Dark staining was also seen in the lacunae of chondrocytes from torpor samples and little to none in the non-hibernator samples (Fig. 8 D-E)

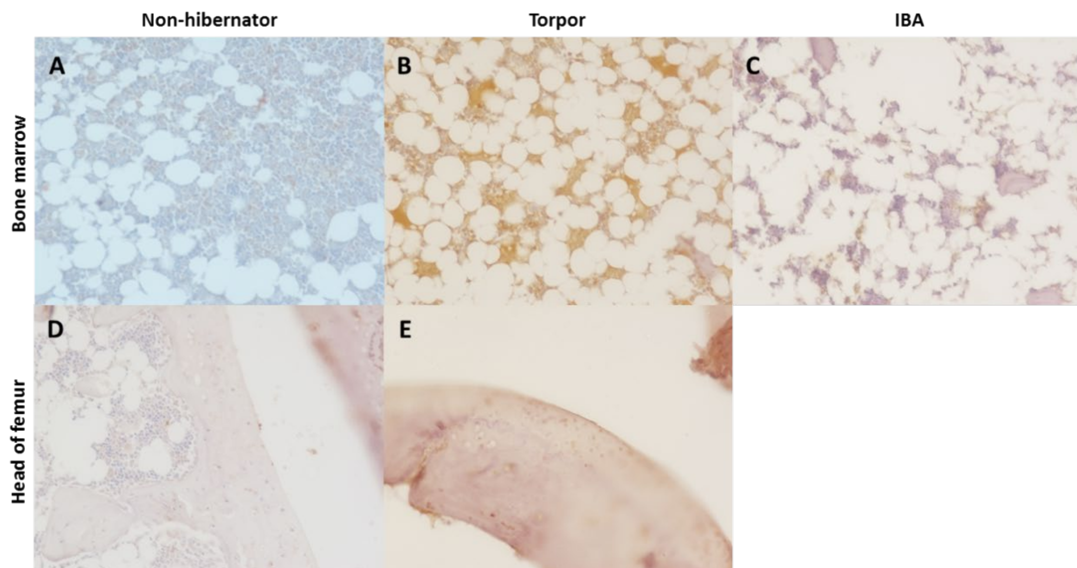

Figure 8. IHC Using COL4A2 Antibody. Rabbit anti-human COL4A2 antibodies were used to stain femur sections and exemplary slides were chosen for each group and observed under 100X magnification. A,D) Non-hibernator B,E) Torpor C) IBA. The collagen protein stained brown while other tissues were white or a purple-brown and adipose was white. Torpor samples had the darkest staining followed by IBA then non-hibernators. In the bone marrow, staining was observed near adipose cell. Very little to no staining was seen in the articular cartilage of non-hibernators whereas coloration was present in the lacunae of active chondrocytes.

LAMA3

Slight staining for the laminin α3 subunit was scattered through the bone marrow of non-hibernator samples and grew much darker in torpor samples (Fig. 9 A-B). IHC of IBA animals had staining more similar to non-hibernators that torpor (Fig. 9 C). Staining in the articular cartilage showed a unique trend where laminin was located not only in the lacunae of chondrocytes but along the tidemark (the margin between calcified and non-calcified cartilage) of all samples as well (Fig. 9 D-F). Staining was darker in both torpor and IBA animals when compared to non-hibernators.

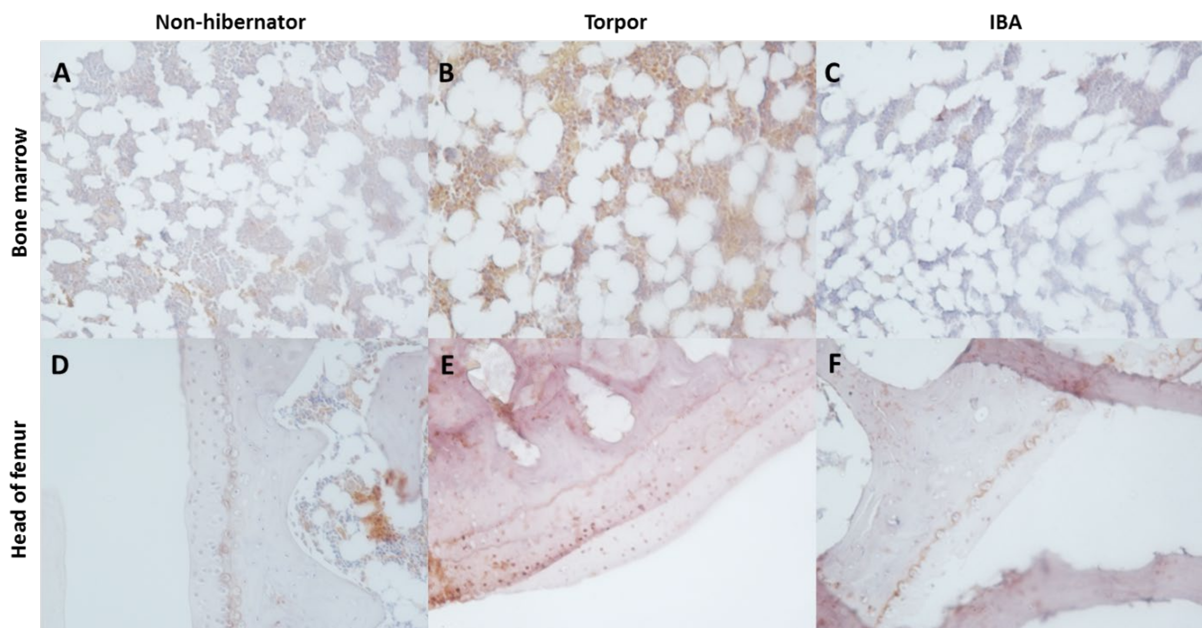

Figure 9**.** IHC Using LAMA3 Antibody. Femur sections were stained via IHC with rabbit anti-human LAMA3 antibodies and exemplary slides were chosen for each group and observed under 100X magnification. A,D) Non-hibernator B,E) Torpor C,F) IBA. Intensity of staining was highest in torpor samples. In the bone marrow, staining was observed near adipose cell. In articular cartilage staining appeared in the lacunae of active chondrocytes as well as along the tidemark.

ImageJ-win64 was used to calculate the average area that was stained on samples and the averages from each season calculated (Table 3). T-tests were performed but showed no statistically significant difference between seasons for any of the proteins.

Table 3. Percent Area of Bone Sample Stained via IHC. Average percent area stained from each season for proteins COL4A2, LAMA3, ITFG3 and CHI3L1 were not significantly different between the seasons.

	Antibody			
Season	COL4A2	LAMA3	ITFG3	CHI3L1
Hib	10.5	15.5	18.3	11.8
IBA	10.5	6.0	5.0	5.7
NH	5.0	8.3	3.4	3.4

DISCUSSION

It has been seen during hibernation of 13-lined ground squirrels that their bone strength does not decrease even though bone cell numbers decline and microstructural mass is lost (McGee-Lawrence et al., 2011). One possible explanation for this observation is an increase in extracellular matrix proteins, such as collagen, to strengthen the bone. This is supported by bone marrow transcriptome data showing increases in the expression of multiple extracellular matrix genes (Cooper et al., 2016). Due to the high amount of collagen known to be in bone, along with additional parameters, a collagen coding gene COL4A2 was selected for further study. A trichrome stain did not indicate a difference in the total amount of collagen in the femur shaft, although further study with a hydroxyproline assay indicated a slight, but debatably significant, increase in collagen levels during hibernation. And, after performing conditional error probabilities on the p-values, we saw that there is nearly a 43% chance that the p-values comparing non-hibernators to torpor and IBA samples are due to chance alone and there is zero difference between the collagen levels across seasons. IHC further confirmed the lack of type IV collagen in the shaft of the femur but staining increased around adipocytes of the bone marrow and chondrocytes of the articular cartilage in IBA and torpor samples. While total collagen does not increase significantly, there are many subtypes of collagen and other ECM proteins that could increase and have a significant contribution to bone integrity. Making up 90% of the bone matrix (Pietzak and Mucksavage, 2016) and being the primary type of collagen synthesized by osteoblasts (Shaw and Högler, 2012), type I collagen would appear to be a more probable agent for bone strength than type IV.

Obvious changes in the extracellular matrix of the articular cartilage through the seasons can be seen via trichrome stain by the decrease of collagen (blue) and a seeming increase of proteoglycans/ECM (pink); whereas an increase of type IV collagen in the articular cartilage was seen by immunohistochemistry. These contradictory results would appear to be a mystery. It couldn't be osteoarthritis, where collagen levels increase and proteoglycans decrease (Maldonado and Nam, 2013) which is the opposite of our trichrome resultsOther inflammatory diseases would predict a decrease in type IV collagen (Foldager et al., 2016) which is the opposite of our IHC results. A possible explanation for the increased eosin staining is an increase in production of ECM protein at the head of the femur could protect ground squirrel joints during immobility. While most research has focused on the impact of immobility on loss of bone strength, the ends of bones and joints can also be affected by immobility (Liphardt et al., 2015). Further studies into the role of proteoglycans in the articular cartilage of hibernating ground squirrels, and looking into changes in type I and II collagen, would be an interesting path to pursue.

When the trichrome didn't explain how the bone was maintaining strength, we looked to genes that had increased in the transcriptome data. Transcription of genes for laminin, chitinase-like protein and ITFG3 increased during torpor and are all extracellular matrix proteins that could stabilize bone. Immunohistochemistry results indicated that amounts of all the proteins except for ITFG3 increased during hibernation. ITFG3 responded in the opposite manner and staining decreased during hibernation, For all of the proteins, staining was found primarily in the bone marrow near adipocytes and in the

articular cartilage in the lacunae of chondrocytes. The specific functions these proteins contribute to bone would require further investigation.

Chitinase 3 like 1 has been seen in many other mammals to play roles including inducing primary and secondary adaptive immune responses, controlling and repairing oxidative tissue damage, and regulation of apoptosis (Lee et al., 2011). This information, coupled with studies that show it is produced by damaged chondrocytes and helps with the repair of the ECM, indicates that first: the tissue is damaged during hibernation but, second: the chitinase like protein is supporting tissue health (Johanson et al., 2006).

Integrin, a protein bound to cells, appeared to decrease in the IHC results. Due to other cells getting crowded out by adipose it could be that subsequently the integrin bound to those cells was lost too. Laminin is frequently found co-localized with type IV collagen. In damaged cartilage, laminin expression patterns mimic that of type IV collagen, though the changes are not as drastic (Foldager et al., 2016). Its primary role, to support collagen, would explain why this correlation is seen.

To see if there was a statistical change in the amount of staining from non-hibernating to hibernating samples, we used Image-J to calculate the percent area stained of a sample. This showed that there was no significant increase or decrease of area stained from non-hibernating to hibernating samples. A larger sample size may show a significant difference. Additionally, data was poor due to only having one sample for some of the seasons, making P-values for every comparison unattainable.

To see if expression of these genes is similar in pattern to what Vermillion et al., 2016 saw, the 4 genes investigated in this study would need to be measured in fetal or

neonate ground squirrels. Exploring whether gene levels in hibernating squirrels are similar to expression in neonates provides yet another intriguing exploratory outlet.

All of the proteins investigated play roles in microstructural integrity and homeostasis, but whether these roles are significant enough to affect the total strength of bone is still unknown.

CONCLUSION

Investigating the location and amount of type IV collagen α2 as well as laminin α3, chitinase-like protein and ITFG3 showed localization, not in the shaft of the long bone as expected, but in the bone marrow near adipocytes and in the lacunae of chondrocytes in the AC. All of the proteins increased during hibernation except for ITFG3 which appeared to decrease. Further studies would need to be done to know whether or not these are proteins that are maintaining the integrity of the bone during immobility.

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