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Graduate Studies

# INVESTIGATION OF A POTENTIAL ROLE FOR ANGIOPOIETIN-LIKE PROTEIN 2 IN THE MIGRATION OF LEUKOCYTES DURING HIBERNATION IN THIRTEEN-LINED GROUND SQUIRRELS

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INVESTIGATION OF A POTENTIAL ROLE FOR ANGIOPOIETIN-LIKE PROTEIN 2 IN THE MIGRATION OF LEUKOCYTES DURING HIBERNATION IN THIRTEEN-LINED GROUND SQUIRRELS

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ABSTRACT

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Thirteen-lined ground squirrels enter a state of torpor during the winter months, characterized by weight loss, decreased breathing rate, and decreased immune function. Upon entrance into torpor, the squirrels' white blood cells (WBCs) almost completely disappear from the blood stream, and most levels quickly return to normal within 2 hours post arousal. It is hypothesized that these immune cells are stored in a peripheral organ or tissue during torpor; however the exact mechanism and location remain largely unknown. Angiopoietin-like protein 2 (Angptl2) is a protein primarily secreted by adipose tissue, which results in inflammation and the migration of WBCs to the adipose tissue. Angptl2 secretion is increased by conditions that are present during torpor, oxidative stress, and an increase in adipose tissue, so it was hypothesized that Angptl2 might play a role in the behavior of immune cells during torpor. A chemiluminescent western blot was used to measure and compare Angptl2 levels in plasma of non-hibernating, entering hibernation, torpid, interbout arousal and post arousal ground squirrels. Angptl2 levels were significantly higher in torpid than interbout arousal or post arousal animals. Immunohistochemical staining of adipose tissue found macrophages were approximately twice as abundant in torpid as non-hibernating tissue.

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# INTRODUCTION

## Thirteen-Lined Ground Squirrels and Hibernation

The 13-lined ground squirrel (*Ictidomys tridecemlineatus)* is native to North America, and is found in the central United States from Montana in the west to Ohio in the east. They have gradually expanded their territory northwards from Texas to as far north as central Alberta and Saskatchewan as deforestation has expanded their prairie habitat (1). These ground squirrels have an average lifespan of 7.9 years in captivity, however average lifespan is greatly reduced in the wild due to predation, especially in newborns (1). The 13-lined ground squirrel undergoes two overlapping annual cycles: a hibernal and a reproductive cycle. The 13-lined ground squirrel is one of only a few "true" hibernators, which allows it to survive the harsh northern winters. True hibernation is characterized by large reductions in metabolism, oxygen consumption, heart rate and temperature (2). Squirrels rapidly gain weight to prepare for hibernation, and maintain a state of torpor for weeks at a time throughout the winter months.

The hibernal cycle consists of two key periods: the accumulation of adipose tissue during the summer months, and hibernation, which consists of a series of multi-day bouts of torpor interspersed with brief periods of arousal (3). During these stretches of torpor, a ground squirrel's body temperature drops to just above freezing (2-6°C), as compared to a normal aroused body temperature of 37°C (2, 4). In addition, heart rate decreases from 400 to about 5 beats per minute, and breathing rate drops from 200 to 20 breaths per

minute (1, 2, 4, 5). Throughout torpor, oxygen consumption is consistently 2-3% of normal aroused levels, resulting in hypoxic conditions in the body (2, 6). Hibernation provides an energy savings of 80-85% compared with euthermic temperatures, which allows the hibernator to survive the winter without foraging for food (7). Interestingly, ground squirrels in captivity continue to go through the hibernal cycle under the constant light and temperature conditions present in an animal facility. Overall, ground squirrel regulation of their hibernal cycle remains largely unknown.

In preparation for hibernation, ground squirrels can double their body mass, and triple their fat mass. Many accumulate lipid stores equal to 20-30% of their body weight (7, 8). Ground squirrels appear to have a predetermined amount of white adipose tissue reserves that they must gain during the fall. Regulation of adipose tissue gain is more complicated than a simple increase in food intake or a change in metabolic rate. A ground squirrel with decreased daily foraging time loses weight, however their fat mass remains the same as freely-foraging squirrels. In addition, when squirrels are completely deprived of food, they accumulate fat at a faster rate once returned to normal conditions, until they have attained fat levels comparable to non-deprived controls (7). These observations suggest that sufficient levels of white adipose tissue are necessary for entrance into hibernation. Over the winter, stored lipids in white adipose tissue are slowly metabolized to fuel life during torpor and costly interbout arousals. Brown adipose tissue is also accumulated in the fall for its specific heat-producing role during hibernation. The mitochondria in brown adipose tissue are equipped with an uncoupling protein (UCP 1) with the unique ability to dissipate their proton motive source as heat, and bypass the

production of ATP (7, 9). The 13-lined ground squirrel most likely uses this pathway to create enough heat to return to euthermic (summer) body temperatures (10).

During the winter, periods of torpor can last from 6-40 days and are interspersed with interbout arousals normally lasting less than 24 hours (5, 7). During these arousals, the squirrel's body temperature and other metabolic functions rapidly return to euthermic (Figure 1).

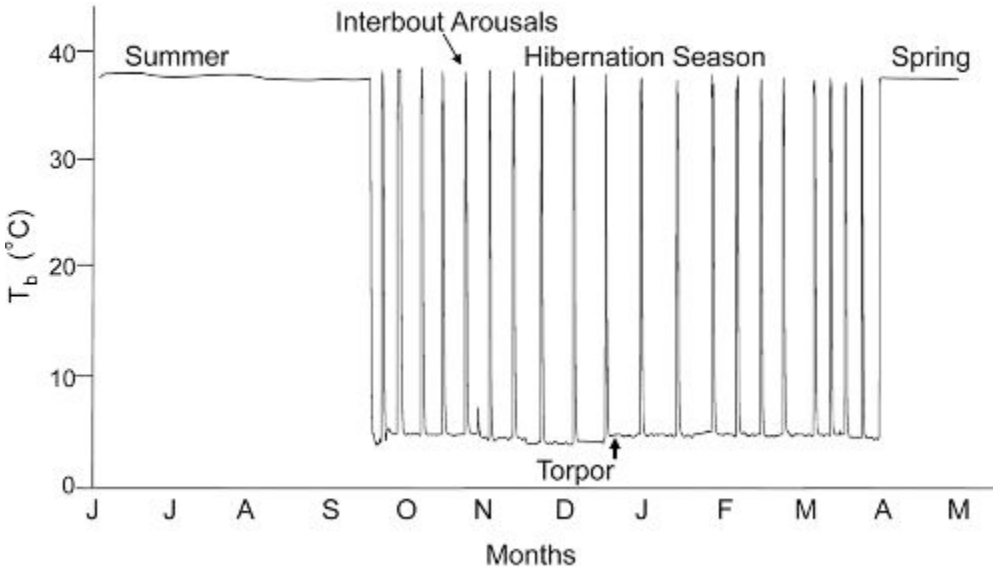


Figure 1. Body temperature trace of a 13-lined ground squirrel in captivity, housed at 4°C during hibernation (11).

The re-warming of the ground squirrel for these interbout arousals consumes 85-90% of all energy consumed during hibernation (7, 11). The reason for these costly arousals is largely unknown; however they are most likely due to a combination of several factors. Some hypothesized functions include the restoration of renal function, and normal cell growth and division, to clear waste, or to prevent muscular atrophy (7, 12). Since the squirrel's innate and acquired immune responses are repressed during

torpor, the interbout arousals could be a chance for the squirrel to briefly restore immune function. Since all immune responses to pathogens are delayed until arousal, the interbout arousals may be a way to keep the body free of infection.

## White Blood Cell Changes During Hibernation

### Total Leukocyte Changes

Entrance into torpor is accompanied by a decrease in the adaptive and innate immune responses (5, 12). One of the more notable changes that occurs during torpor is the disappearance of circulating leukocytes (11). Circulating leukocyte levels drop to 10-15% of euthermic levels after 24 hours in torpor, and remain there until the next arousal (5, 11). Torpor affects the levels of neutrophils, lymphocytes and monocytes; however small numbers of neutrophils and lymphocytes are able to remain in circulation (5). Monocytes comprise 57.7% of WBCs remaining in circulation (Cooper, personal communication). During arousal, the neutrophil and monocyte levels rapidly increase to approximately euthermic levels (1-2 hours), however lymphocytes return to only 50-60% of euthermic levels (5, 13). The decrease in leukocyte levels could be due to a combination of apoptosis, and sequestering of leukocytes in peripheral organs (5). It is also possible that leukocytes are affected by endothelial cell adhesion molecules, causing leukocytes to attach to the surface of the blood vessel endothelium. Increased expression of adhesion molecules ICAM-1, VCAM-1, and E-selectin was seen on hibernating endothelial cells in comparison to non-hibernating ground squirrel endothelial cells (13). In addition, incubation with plasma from hibernating ground squirrels increased the adherence of monocytes to rat endothelial cells (14).

**Neutrophil Changes.** Published normal neutrophil numbers in ground squirrels are 5.06 kNE/μL, measurement technique unknown (UWL unpublished normal levels were 2.83 kNE/ μL, measured with a Hemavet automated counter). Neutrophils exhibited a 94% drop in numbers in torpid individuals (0.34 kNE/μL) as compared with non-hibernators. The decrease in circulating neutrophils during torpor is mainly a decrease in mature neutrophils with segmented nuclei. During torpor, mature neutrophils decrease by about 6-fold, while immature neutrophils with band-shaped nuclei decrease by only 1.5-fold (5). Neutrophil levels were measured to have returned to slightly higher than non-hibernating levels within 2 hours post arousal (3.41 kNE/μL). The neutrophil has an average lifespan of 5.4 days in circulation in an uninfected mouse (15). Neutrophils leave the bone marrow and enter the blood stream. Upon infection; they migrate to inflamed tissues, where they will die. In one study, neutrophils were found in higher levels in the lungs, liver and spleen of torpid squirrels (5). Since neutrophils do not typically leave the tissue once they have migrated there, a specialized mechanism would be necessary for the release of neutrophils from their sequestered location.

Recent research has shown that following incidents of ischemia-reperfusion (oxygen deprivation followed by tissue damage from the return of oxygenated blood supply), neutrophils were able to reenter the blood stream, resulting in systemic inflammation and secondary organ tissue damage in humans. In incidents of reperfusion following ischemia, neutrophils were observed to undergo reverse transendothelial cell migration due to selectively reduced expression of an endothelial cell adhesion molecule, junctional adhesion molecule-C (JAM-C) (16). JAM-C has a few roles in neutrophil migration, including regulating the luminal to abluminal direction of neutrophil

migration. Decreased JAM-C expression appears to result in reverse transendothelial cell migration, from the lumen back into the blood stream. In humans and mice, these neutrophils most likely go on to cause systemic inflammation and secondary organ tissue damage (16). Conditions very similar to ischemia-reperfusion are present in hibernating squirrels during their torpor and arousal cycles. This presents the possibility that neutrophils could undergo reverse trans-endothelial cell migration during arousal, without the destructive effects seen in non-hibernators. Alternatively, neutrophils could simply be sequestered along the blood vessel endothelium throughout torpor, allowing for their quick release back into circulation.

**Lymphocyte Changes.** Published euthermic lymphocyte numbers in the 13-lined ground squirrel are 3.04 kLY/μL, measurement technique unknown (UWL unpublished normal levels were 1.56 kLY/μL, measured with a Hemavet). Lymphocytes exhibited a 41% drop in numbers in torpid individuals (0.92 kLY/μL) as compared with non-hibernators. Lymphocyte levels returned to 67% of non-hibernating levels within 2 hours post arousal (1.05 kLY/μL) (17). However, lymphocytes remained at approximately 2/3 of euthermic levels throughout the time period observed (12 days post arousal). Naïve lymphocytes have an average lifespan of weeks to months in circulation. Unlike neutrophils, lymphocytes are able to recirculate between blood, tissue and secondary lymphoid organs. The return of lymphocyte levels to only ~60% of euthermic levels upon arousal, (compared to ~95% for neutrophils) could be explained by the lymphocytes' significantly slower rates of generation by primary lymphoid organs. The normal rate of lymphocyte generation by the thymus is slower than that of neutrophils ($1x10^5$ lymphocytes per day (18) compared to $2x10^7$ neutrophils per day in mice). In addition, it

is hypothesized that lymphocyte generation by the thymus is reduced during torpor due to the release of 5′-AMP from brown adipose tissue (neutrophils and monocyte generation occurs in bone marrow, and therefore may be less affected by this product).

Previous studies have demonstrated the presence of lymphocytes in the gut and spleen during torpor (5). However, observation of the ratios of different types of lymphocytes indicates that increased lymphocytes in the gut are most likely due to the expansion of gut-derived lymphocytes rather than the influx of cells from the blood stream (5). It was previously hypothesized that lymphocytes were sequestered in the spleen during torpor, although more recent research indicates that this is unlikely. The expression of lymphocyte markers in the spleen during torpor and arousal were very similar, and splenectomies performed before and during torpor did not influence disappearance of lymphocytes during torpor, or the restoration of lymphocyte numbers upon arousal, respectively (19).

Both the spleen and gut have become improbable as possible storage sites for lymphocytes, however recent research has led to the hypothesis that lymphocytes are stored in secondary lymphoid organs during torpor (19).

**Monocyte Changes.** Published euthermic monocyte numbers in ground squirrels are 0.64 kMO/μL, measurement technique unknown (UWL unpublished levels were 0.45 kMO/μL, measured with a Hemavet) (17). Monocytes exhibited an 87% drop in numbers in torpid individuals as compared with non-hibernators (0.06 kMO/μL). Monocyte levels return to approximately non-hibernating levels within two hours post arousal (0.78 kMO/μL). Monocytes have a lifespan of about four to five days in a non-infected rat. (20) Similar to neutrophils, monocytes circulate through the blood stream until they migrate to

tissue to function as macrophages. However, monocytes/macrophages additionally migrate from tissues to lymph nodes to act as antigen presenting cells. Approximately half of ground squirrel macrophages are stored in the red pulp of the spleen. Monocytes are able to leave the spleen (usually in response to infection) and macrophages have been shown to reverse transmigrate from tissue to blood, so the potential is there for sequestered monocytes (in the case of torpor) to be released back into the blood stream (21). There is no conclusive evidence on the location of monocytes during torpor, however previous research indicates that there is increased adhesion of monocytes to the blood vessel endothelium during torpor (22).

Table 1. Current hypotheses of immune cell storage during hibernation in 13-lined ground squirrels.

| Cell type | Reduction during hibernation | Time to restoration of normal levels | Evidence for storage sites |
|---|---|---|---|
| **Neutrophil** | 94% | ≤ 2H post arousal | Lungs, liver, spleen |
| **Lymphocyte** | 41% | > 12 Days post arousal | Secondary lymphoid organs |
| **Monocyte** | 87% | ≤ 2H post arousal | Blood vessels |

Table 1 summarizes what is currently known about the location of storage during torpor for each of the observed white blood cell types. Neutrophil numbers have been observed to increase in the lungs, liver and spleen during torpor, and there is a proposed mechanism for their release back into the blood stream. The location of lymphocytes and monocytes remains largely unknown, but the potential is there for lymphocytes, monocytes or neutrophils to be stored along the blood vessel endothelium. In addition, it

is possible that each of these classes of blood cells is behaving differently upon entrance into hibernation. With limited evidence as to the behavior of leukocytes during torpor, other possibilities were explored involving the migration of leukocytes from the blood and conditions present during torpor. This exploration revealed a recently discovered protein, angiopoietin-like protein 2, which affects leukocyte migration in cases of high fat content and hypoxia (oxidative stress).

## Adipose Tissue and Blood Vessel – White Blood Cell Interaction via Angptl2 Inflammation of Adipose Tissue

Weight gain and obesity are often closely associated with chronic inflammation, although the direct cause is still unknown. Angiopoietin-like proteins (Angptls) are a class of recently discovered proteins which possess a fibrinogen-like domain, and play a role in inflammatory changes. Specifically, angiopoietin-like protein 2 (Angptl2) is secreted by adipose tissue, and is becoming well known due to its role in the emerging linkage between obesity, adipose tissue inflammation and systemic insulin resistance. Its expression is increased by obesity, or obesity-related conditions, most notably endoplasmic reticulum (ER) stress and oxidative stress (hypoxia) (23–25). Angptl2 interacts with integrin expressed on monocytes and endothelial cells to promote inflammation. Specifically, this interaction leads to attachment of leukocytes to blood vessel walls, increased vascular permeability and monocyte transendothelial migration, leading to chronic inflammation (25). Transgenic mice constitutively expressing Angptl2 in the epidermis showed local inflammation on the ears, snouts and tails (25).

**What is Angiopoietin-Like Protein 2?**

Angiopoietin-like proteins (Angptls) are a class of proteins that are structurally similar to angiopoietins. There are a total of seven angiopoietin-like proteins, which have a variety of different functions associated with them, including the regulation of lipid, glucose and energy metabolism, and the induction of inflammation and angiogenesis (26, 27). Angptls have a coiled-coil domain at the N-terminus and a fibrinogen-like domain at the C-terminus, which is also seen in angiopoietin (27). At the N-terminus, Angptls also exhibit a hydrophobic region that is typical of a signal sequence for protein secretion (28). Angptl2 has a molecular mass of 64 kDa, however deglycosylation reduces its apparent molecular mass to 57 kDa, the mass predicted by the DNA sequence. This indicates that Angptl2 is a glycoprotein, which allows for efficient secretion (28).

Angptl2 mRNA is expressed in the skeletal muscle, heart, intestine, stomach, adipose tissue and uterus, but it was found to be largely secreted by adipose tissue. Its expression was also found to be up-regulated in other organs in association with cancer, tumor growth, or inflammation-based autoimmune diseases (24). Angptl2 levels in serum and adipose tissue are up-regulated in obesity, which results in local inflammation, abundant leukocyte attachment to vessel walls and increased blood vessel permeability. Angptl2 levels are up-regulated by hypoxia and ER stress, however the number of blood vessels appears to remain stable with the up-regulation of Angptl2, suggesting that Angptl2 promotes vascular inflammation, but not angiogenesis *in vivo* (27). This is contradictory to other research which has found that Angptl2 promotes angiogenesis in avascular tissue, such as the cornea, indicating that Angptl2 may have a different effect on endothelial cells than on other tissues (27).

**Angptl2 Interacts with Integrin on Endothelial Cells**

Angptl2 binds to integrin on endothelial cells in a dose dependent manner (25). Data indicate that Angptl2 binds specifically to integrin α5β1 on the endothelium, which promotes the migration of endothelial cells during angiogenesis. Through integrin α5β1, Angptl2 activates Rac1 in endothelial cells, which leads to protrusion of the lamellipodia, membrane ruffling and subsequent cell migration (23, 25, 29). Angptl2 activation of integrin α5β1 also initiates NF-κB translocation to the nucleus of the endothelial cell (23, 25). This increases levels of NF-κB-dependent inflammatory gene expression in the endothelial cell, specifically leading to the synthesis of pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, and IL-8 (24, 25, 30). Mice engineered to over express Angptl2 showed increased levels of inflammatory cytokines Il-6, TNF-α and IL-1β as compared with wild-type mice. In these same mice, there were increased numbers of adherent leukocytes in blood vessels within the adipose tissue. This observation was confirmed by RT-PCR analysis, which showed an increase in both general (CD68) and inflammatory (CCR2) macrophage markers in the adipose tissue (25). Along with triggering cytokine secretion, NF-κB also increases endothelial cell expression of the adhesion molecules E-selectin, VCAM-1, and ICAM-1, which promote leukocyte adhesion and transmigration (30).

**Angptl2 Interacts with Integrin on Immune Cells**

Angptl2 increases the migration of monocytes/macrophages in two distinct ways. Endothelial cell activation by Angptl2 leads to increased expression of adhesion molecules, as previously stated. Initial contact between the leukocyte and the activated endothelium of vessel walls results in the leukocyte rolling along the endothelium. The

cell adhesion molecules on the endothelial cell activate integrins on the leukocyte to a high-affinity conformation, resulting in firm adhesion to the endothelium (29). Leukocytes then become polarized and can travel along the endothelium until they find a junction between endothelial cells, and can migrate into the tissue beneath (25). In addition, Angptl2 can directly interact with integrin α4 or β2 to induce the transmigration of circulating monocytes/macrophages (24, 25). The increase in migration of human monocytes in response to Angptl2 occurs in a dose dependent manner; however the exact mechanism remains unknown.

**Accumulation of Macrophages in Adipose Tissue**

Obesity is associated with chronic inflammation in the adipose tissue accompanied by macrophage infiltration. There is a positive correlation between adipose deposits, Angptl2 levels, and macrophage infiltration into the adipose tissue. Macrophages are numerous in the adipose tissue of obese mice, but are only rarely found in the adipose tissue of normal mice (31). In addition, Angptl2 interaction with integrin α5β1 on leukocytes activates NF-κB, resulting in the production of pro-inflammatory cytokines and further increasing macrophage infiltration (25).

Adipose tissue appears to be an active site of immune trafficking during the onset of diet-induced obesity, involving both B and T lymphocytes as well as natural killer cells and macrophages (32). To confirm that the migration of macrophages in obesity is organ-specific, no significant difference in macrophage levels is seen in the muscles and liver or obese and non-obese mice (33). Most research on immune system changes in obesity has focused on macrophages, however in addition to the accumulation of macrophages in the adipose tissue of obese mice, recent research has discovered that

there is an increase in natural killer cells and T-lymphocytes as well (32, 33). B cells were found to accumulate in the adipose tissue very early in a high fat diet, before there is a significant change in fat mass. But further research has not been performed specifically to look at this occurrence (32). In a recent study, mice were genetically engineered to not produce T or B-lymphocytes. In the absence of these adaptive immune cells, there was a significant increase in the accumulation of the innate immune cells (Natural killer and macrophages) in the adipose tissue as compared to wild type obese mice (32). This finding indicates that the accumulation of T or B-lymphocytes in the adipose tissue is not the trigger for the recruitment of macrophages.

**What Suggests that Angptl2 May Play a Role in Leukocyte Changes During Hibernation?**

Angptl2 secretion in obesity results in effects very similar to those seen during hibernation. During torpor, there is a significant migration of white blood cells (both innate and acquired) out of the blood stream to a location or locations that remain unclear. Angptl2 secretion is induced by ER stress in the body, a result of adipose tissue expansion. ER stress is significantly increased in adipocytes in obese mice compared to non-obese mice (25).

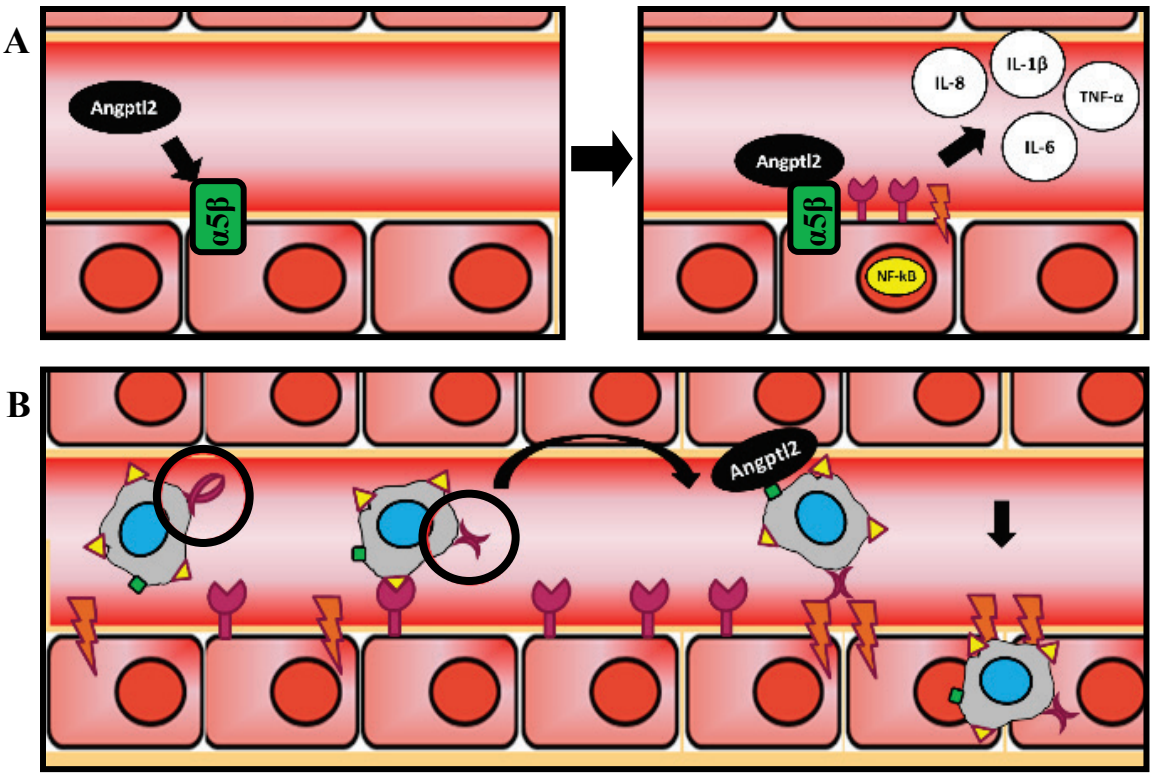


Figure 2. Angptl2 interacts with monocytes and endothelial cells to induce white blood cell migration. (A) Angptl2 interacts with integrin α5β1 on the surface of endothelial cells to increase production of proinflammatory cytokines and increase endothelial cell expression of adhesion molecules. (B) Circulating monocytes/macrophages interact with adhesion molecules on activated endothelial cells to induce rolling adhesion and transmigration. Angptl2 further interacts with monocytes to increase migration.

Since ground squirrels can triple their fat mass in preparation for hibernation, it can be assumed that they would also have significantly increased ER stress due to adipose tissue expansion. This would lead to increased levels of Angpt2.

Angptl2 secretion is induced by hypoxia, or oxidative stress. During torpor, oxygen consumption is held steady at 2-3% of euthermic rates, however since metabolic function is also greatly reduced during hibernation, it is a matter of debate when, if at all, ground squirrels are hypoxic during torpor. Although ground squirrels may not be truly

hypoxic during torpor, there is evidence to suggest that they do experience some degree of oxidative stress, which could induce Angptl2 secretion.

## Oxidative Stress Levels During Torpor

It is hypothesized that hibernators experience ischemic events (hypoxia) due to variable rates of tissue perfusion during entrance into, and arousal from torpor (14). This would be a result of a decrease in heart rate and cardiac output prior to the significant reduction in temperature, and thus the significant reduction in metabolism. This would result in hypoxic conditions in tissues that are still undergoing biochemical reactions at euthermic rates, while heart rate and oxygen supply have already been significantly reduced. In addition, the rapid increase in body temperature upon arousal increases the risk of regional ischemia-reperfusion injury in areas where blood flow is not fully restored prior to the elevated body temperature (14).

There are also indicators that some tissues are vulnerable to oxidative stress during torpor. For example, the intestines shift to a more oxidized state during torpor, which suggests that a change in redox homeostasis may be an adaptation for mammalian hibernation (Figure 2). In addition, ground squirrels fed diets high in α-tocopherol, an important reactive oxygen species scavenger, are less likely to enter torpor, and less likely to survive torpor. This supports the concept that a minimum amount of oxidative stress may be required for normal hibernation (14).

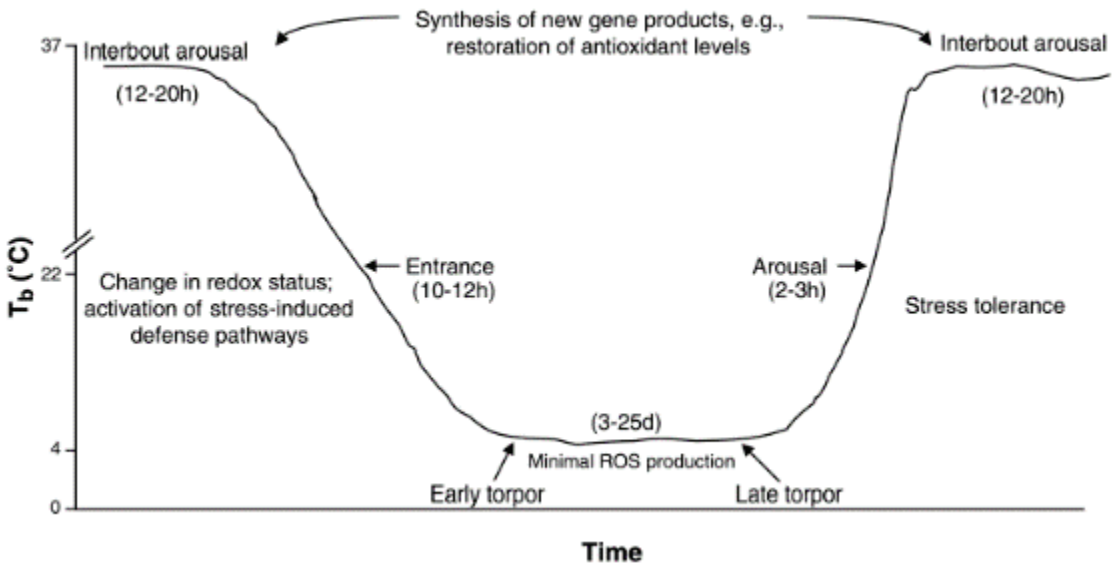


Figure 3. Model of stress induction and tolerance during torpor-arousal cycles in hibernators, based on intestinal mucosa (14).

Although oxidative stress can be difficult to quantify, a study was performed observing levels of ubiquitin-conjugated proteins in hibernating squirrels. Ubiquitin is a highly conserved polypeptide which is conjugated to cellular proteins to tag them for degradation. An increase in proteins conjugated to ubiquitin in tissues has been previously used as an indicator for stress-induced protein damage. In this study, the gut and liver of 13-lined and golden-mantled ground squirrels were tested for protein-ubiquitin conjugate concentrations. In both tissues measured, ubiquitin conjugate concentrations increased during entrance into torpor and were elevated 2-3 fold by late torpor as compared with levels in active squirrels (34).

**Angptl2 Secretion Could be Increased During Hibernation**

A molecular approach can also be taken to examine the likelihood that Angptl2 is secreted during torpor. C-jun is an early-intermediate hibernation gene product which can

interact with the promoter of Angptl2 to induce Angptl2 expression (35). Since c-Jun is expressed early in hibernation, it has the potential to increase the secretion of Angptl2 upon entrance into torpor (36).

Transcription of mRNA is depressed during hibernation to rates as low as 11% of euthermic rates, with the exception of a select few necessary genes (14). This slowing of transcription appears to be simply a result of the decrease in body temperature, and not a specific mechanism unique to hibernators. The transcriptomes of several organs in hibernating 13-lined ground squirrels over a yearly hibernal cycle have been measured and analyzed (36). Angptl2 mRNA levels in white adipose tissue were higher in torpor than in the spring or summer (n=36) however the overall significance of this transcriptome concerning Angptl2 levels is uncertain (36). Even the slight increase in Angptl2 mRNA levels seen during torpor could be considered significant considering that transcription is overall significantly decreased during hibernation. In addition, gene regulation can be controlled at many levels, including mRNA transcription, processing and stability, as well as protein translocation, processing, stability and post-translational modifications. Indeed, it has been hypothesized that genes necessary during cycles of torpor and arousal may be regulated less by amounts of gene products, and more by rapidly reversible molecular switches (such as phosphorylation, or glycosylation) to control protein activity (14). Since the hibernating ground squirrel arouses periodically from torpor, the ability to function at euthermic temperatures must be constantly maintained. The transcriptomes were also observed for increased transcription of genes expressed in association with Angptl2 secretion. VCAM-1 mRNA levels in white adipose tissue were higher in torpor (66) than in the spring or summer (11 and 16, respectively)

(n=36). There was also a slight increase in mRNA levels of general macrophage marker CD68 in during torpor (17) as compared with spring or summer (12 and 8, respectively). A significant difference was not seen in mRNA levels of IL-6, IL-1β, adhesion molecules E-selectin or ICAM-1 or in inflammatory macrophage marker CCR2.

In summary, ANGPTL2 expression in mice results in leukocyte attachment to blood vessel walls and the migration of leukocytes from the blood stream into adipose tissue. ANGPTL2 secretion is increased by conditions that are present during hibernation: endoplasmic reticulum stress resulting from increased stores of adipose tissue, and acute hypoxia from a lowered breathing rate.

## RESEARCH GOAL AND OBJECTIVES

The main goal of this research was to determine if Angptl2 could play a role in the migration of WBC out of the blood stream during hibernation in 13-lined ground squirrels. Angptl2 levels in serum and adipose tissue of mice and humans are up-regulated by fat accumulation (ER stress) and oxidative stress, both conditions which are unavoidably present during hibernation. Increased expression of Angptl2 results in local inflammation, abundant leukocyte attachment to vessel walls and increased blood vessel permeability, a phenomenon very similar to the loss of leukocytes observed during the entrance into hibernation. Based on this previous knowledge, it was hypothesized that:

1. Angptl2 levels would be highest in torpid squirrels.
2. Macrophages would be most abundant in torpid adipose tissue

The following objectives were addressed:

1. A method to detect 13-lined ground squirrel Angptl2 was established
2. Angptl2 levels in plasma of non-hibernating, entering hibernation, torpid, interbout arousal, and post arousal 13-lined ground squirrels were measured and compared.
3. Angptl2 levels in plasma were correlated with fat levels throughout the hibernal cycle.

4. Adipose tissue of non-hibernating and torpid ground squirrels were stained and examined for the comparative presence of WBC in tissue and on blood vessel walls.

## METHODS AND MATERIALS

### Thirteen-Lined Ground Squirrels

A thirteen-lined ground squirrel colony has been established at UW-La Crosse. The colony is comprised of a combination of locally wild-caught squirrels and squirrels bred at the facility. Squirrels have been sacrificed at different periods throughout the hibernal cycle; non-hibernating (both spring and summer) entering hibernation, torpid, during interbout arousals and post arousal (2 hours to 1 week). The squirrels were first weighed, and torpid animals were euthanized via cervical dislocation and exsanguination while non-hibernators were euthanized via suffocation with carbon dioxide, followed by exsanguination. Samples of the subcutaneous abdominal adipose tissue were removed and placed on dry ice. The tissue sections were placed in a cryo mold, covered in O.C.T. compound (Tissue-Tek, Torrance, CA) and rapidly frozen in 2-methylbutane immersed in liquid nitrogen, then placed in long-term storage at -80 °C. Fifty-five plasma samples (eight entering hibernation, ten torpid, nine interbout arousal, ten post arousal (2 hours to 1 week) and eighteen non-hibernating samples (spring and summer)) were frozen at -20 °C at a 1:2 dilution in 25% glycerol.

### Chemiluminescent Western Blot to Detect Ground Squirrel Angptl2

#### Rabbit Anti-Mouse Angptl2 (Polyclonal)

A commercial polyclonal rabbit anti-mouse Angptl2 antibody (Biorbyt, San Francisco, CA) was tested for specificity to ground squirrel Angptl2. The rabbit anti-

mouse Angptl2 had known reactivity with human, mouse, rat, rabbit, pig, horse, chicken, cow and dog Angptl2, so there was a reasonable probability that it would be reactive with ground squirrel Angptl2. Ground squirrel plasma and human serum were electrophoresed on an SDS PAGE gel, transferred to PVDF membrane and probed with the rabbit anti-mouse Angptl2 antibody. Human serum was included as a positive control, since the antibody had known reactivity with human Angptl2. Polyclonal rabbit IgG was used as a negative control.

A 0.75 mm SDS page gel was poured with a 10% acrylamide running gel under a 4% stacking gel. Ground squirrel plasma (1/20 in 2x Laemmli sample buffer) and human serum (diluted 1/80 in 2x sample buffer) were heated at 95 °C for five minutes to denature the proteins. Ten microliters of each sample were loaded on to the gel along with chemiluminescent molecular weight standards, and the gel was run at 150 V. Proteins were blotted on to a PVDF membrane at 15 mAmps for 15 minutes using a semi-dry blotting apparatus. The membrane was blocked overnight in a 1% BSA/0.05% Tween 20/PBS solution. The ground squirrel plasma and human serum lanes were incubated with the rabbit anti-Angptl2 antibody (diluted 1/3,000 in 0.05% Tween/PBS (PBST)) for 2 hours at room temperature. The membrane was rinsed three times for 5 minutes in PBST with agitation. The lanes were then incubated with biotinylated goat anti-rabbit IgG (1/5,000 in PBST) for one hour at room temperature. All lanes were washed as described previously, and incubated in streptavidin-horseradish peroxidase (HRP) (diluted 1/140,000 in PBST) for an hour at room temperature. Lanes were washed five times for 5 minutes in PBST with agitation, then soaked in a freshly mixed 1:1 chemiluminesence working solution (Thermo Scientific, Rockford, IL) for three minutes,

drained and placed in plastic wrap. The membrane was exposed to film for 20 seconds, and the film developed. A band of approximately 57 kDa was expected for reactivity with Angptl2 (Figure 4).

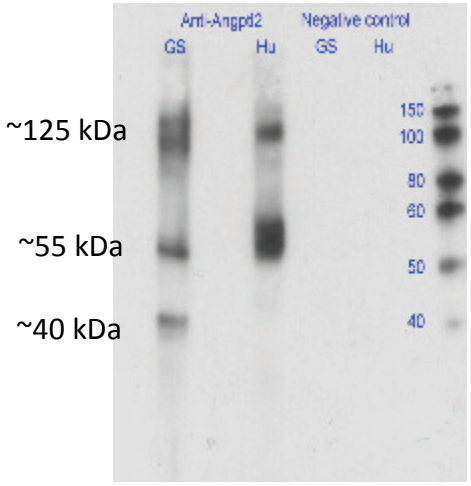


Figure 4. Western blot investigating the specificity of the commercial rabbit anti-mouse Angptl2 antibody (biorbyt) for ground squirrel Angptl2. Ground squirrel plasma and human serum (1/20 dilutions) were run on a 10% SDS PAGE gel and probed with either rabbit anti-mouse Angptl2, or polyclonal Rabbit IgG (negative control). Prominent bands were seen at about 55 kDa and 125 kDa (GS and human) and around 40 kDa (GS only).

Prominent bands were seen on the gel at about 125 and 55 kDa (GS and human samples) and 40 kDa (GS plasma only). While the 55 kDa band seemed a likely candidate for Angptl2, the presence of multiple prominent bands indicated that the antibody was reacting with other proteins. Since this rabbit anti-Angptl2 antibody could be reacting with plasma proteins other than Angptl2, it could not be used as a capture antibody in an ELISA. This antibody could still be useful as the detecting antibody in a capture ELISA utilizing a very specific capture antibody.

**Rat Anti-Mouse Angptl2 (Monoclonal)**

The specificity of a monoclonal rat anti-mouse Angptl2 antibody (R&D systems, Minneapolis, MN) for ground squirrel Angptl2 was confirmed with a chemiluminescent western blot. The rat anti-mouse Angptl2 had known reactivity with human and mouse Angptl2, and minimal cross reactivity with angiopoietins and other angiopoietin-like proteins. Mouse serum (a positive serum control) recombinant mouse Angptl2 (R&D systems, Minneapolis, MN) and ground squirrel plasma were electrophoresed, transferred to PVDF and probed with rat anti-mouse Angptl2 or purified rat IgG (negative control, R&D systems, Minneapolis, MN), the biotinylated sheep anti-rat IgG, streptavidin-HRP

Ground squirrel plasma and mouse serum (both diluted 1/20 in 2x sample buffer) and recombinant Angptl2 (1/60 in sample buffer, 50 ng per well) were run on a 10% SDS PAGE gel as previously described. The lanes were blocked the same as above, then incubated in rat anti-mouse Angptl2 (1/500 dilution in PBST) or rat IgG (1/1000) for 2 hours, washed as previously described, and then incubated in the biotinylated sheep anti-rat IgG (1/2,000 in PBST) for 1.5 hours. The membrane was washed, and then incubated in streptavidin- HRP (diluted 1/150,000 in PBST) for an hour. The membrane was washed and developed as previously described.

Two separate ground squirrel plasma samples produced strong bands around 55 kDa (Figure 5), indicating reactivity of the rat anti-mouse Angptl2 antibody with ground squirrel Angptl2. In addition, the prominent ground squirrel plasma band was the same size as the band produced by detection of recombinant Angptl2.

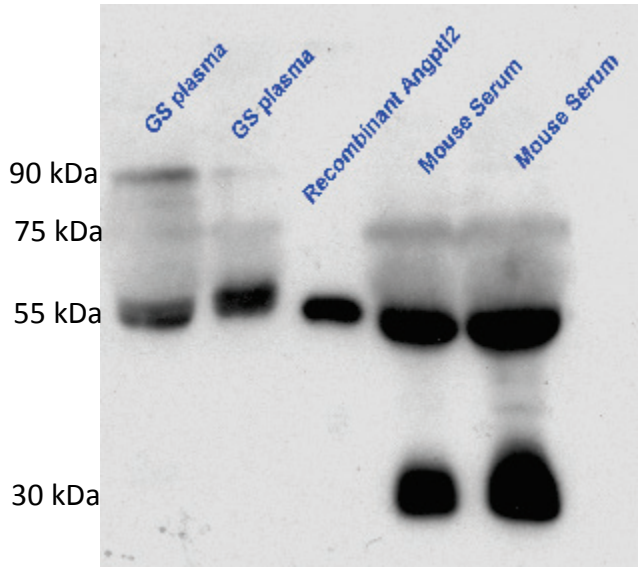


Figure 5. Western blot investigating the specificity of the commercial rat anti-mouse Angptl2 antibody (R&D Systems) for ground squirrel Angptl2.Ground squirrel plasma and mouse serum were run on a 10% SDS PAGE gel alongside recombinant mouse Angptl2. The blot was developed for with rat anti-mouse Angptl2. Prominent bands are seen at about 57 kDa in all samples, and around 30 kDa in the mouse serum only.

In both the ground squirrel and mouse samples, there was a fainter smear running from the most prominent band (55 kDa) to about 70 kDa (mouse samples) or 95 kDa (GS samples). Within these smears, prominent bands can be observed at 70 kDa (GS and mouse samples) and 95 kDa (GS samples only). It was hypothesized that these fainter bands correspond to glycosylated versions of Angptl2, resulting in a protein with a higher molecular weight. Human Angptl2 has a documented N-glycosylation site at amino acid position 164, and another potential N-glycosylation site at position 192 (37). In addition, human Angptl2 has a documented O-linked glycosylation site at amino acid position 229 (38). The two upper bands could correspond with Angptl2 with one, two or potentially three glycosyl groups added. These upper bands appeared with some variability across

ground squirrel plasma samples, which could be due to differences in storage procedures or the ground squirrels' stage in the hibernal cycle.

There was also a notable lower band seen on mouse serum samples (Figure 5), as well as in some ground squirrel plasma samples (Figure 6). A smaller Angptl2 fragment has been previously documented at approximately 36 kDa on SDS page gels run with flagged Angptl2 (39). The lower band seen in Figure 4 is a similar size (subtracting the mass of the flag, which appears to add a mass of 5-10 kDa to Angptl2). A significant difference in protease activity between serum and plasma that would account for this extra band has not been documented.

**PNGase F Digestion**

To determine if the additional bands seen above the prominent band were due to different degrees of glycosylation, a digestion was performed to cleave glycosyl groups from proteins. Plasma proteins were digested using the deglycosylation enzyme PNGase F, and compared against normal ground squirrel plasma and a mock digestion (negative control).

Ground squirrel plasma was diluted 1/12 and 1/24 (approximately 100 and 50 µg plasma protein per digestion, respectively) in sterile water. One microliter of 5% SDS and 1 µL of 1M dithiothreitol (DTT) were added to a 12 µL aliquot of each plasma dilution (1/24 and 1/12). The samples were denatured by heating at 95 °C for 5 min, then allowed to cool at room temperature for 5 min. Two microliters of 0.5M sodium phosphate buffer (pH 7.5) and 2 µL of 10% Triton X-100 were added to each sample followed by 2 µL of PNGase F (Promega, Madison, WI). The samples were incubated at 37 °C for 2 hours. As a negative control, a 12 µL aliquot of the 1/24 plasma dilution was

taken through all the digestion steps described above, with the exception that 2 µL of sterile water was added in the place of PNGase F. The three digested samples were diluted 1:1 in sample buffer, and 20 µL of each sample was loaded on a gel along with 10 µL of undigested ground squirrel plasma (diluted 1/40 in sample buffer). The gel was run as described previously. The membrane was blocked overnight in 1% BSA/PBST at 5 °C. The membrane was probed for Angptl2 as described above with the exceptions that the rat anti-Angptl2 antibody was at a dilution of 1/1000 in PBST and the streptavidin- HRP was at a dilution of 1/120,000 in PBST.

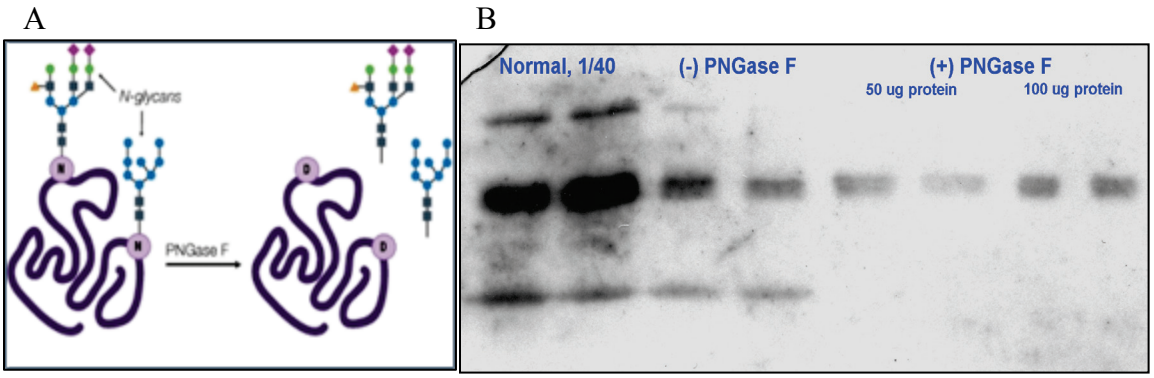


Figure 6. PNGase digestion of ground squirrel plasma to determine glycosylation of Angptl2 (a) The enzyme PNGase F cleaves glycans off of glycosylated proteins. (b) Ground squirrel plasma (50 µg and 100 µg total plasma protein) was digested with PNGase F for 2 hours at 37 °C, and run with normal plasma and plasma which underwent a mock digestion on an SDS PAGE gel. The membrane was reacted with rat anti-mouse Angptl2.

The upper and lower bands present in the normal sample became fainter after the mock digestion (negative control), but were missing completely from both of the digested samples (Figure 5). This indicated that the upper bands present in undigested samples were from ground squirrel Angptl2 in different states of glycosylation. The lower bands

were most likely a result of glycosylated fragments of Angptl2. Once the glycosylation was removed, these proteins were too small to be resolved on this gel. Overall, it was concluded that the rat anti-Angptl2 is specific for GS Angptl2.

**Capture ELISA to Detect and Quantify Angptl2**

A capture ELISA was designed to quantify the amount of Angptl2 in ground squirrel plasma samples. The rat anti-mouse Angptl2 antibody was used as the capture antibody, since it was determined to be specific for GS Angptl2. Since this antibody was monoclonal, a different antibody was needed as the detecting antibody in the capture ELISA. The Biorbyt polyclonal rabbit anti-Angptl2 was tested for use as a detecting antibody.

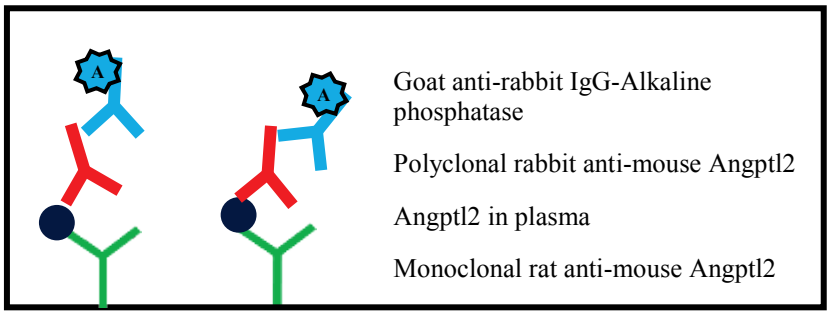


Figure 7. Components of the capture ELISA for the detection of Angptl2 in ground squirrel plasma.

Mouse serum was used as a positive Angptl2 control, since it had known reactivity with both antibodies. Two negative controls were employed: (1) wells with only the monoclonal rat anti-Angptl2 antibody, blocking agent, polyclonal rabbit anti-Angptl2, goat anti-rabbit IgG-Alkaline phosphatase and substrate and (2) wells with the monoclonal rat anti-Angptl2 antibody, blocking agent, bovine serum albumin, polyclonal

rabbit anti-Angptl2, goat anti-rabbit IgG-Alkaline phosphatase and substrate (secondary antibody control).

An optimization was performed where both the capture (rat anti-mouse Angptl2) and detecting (rabbit anti-mouse Angptl2) antibodies were titrated. Wells of a 96-well plate were incubated with 50 µl of the rat anti-mouse Angptl2 (2-fold diluted from 1/1000 to 1/128,000 in TBS) for 24 hours at 4 °C (Figure 7). The plate was washed three times with a TBS/0.05% Tween 20 solution (TBST). Fifty microliters of blocking agent (1% milk in TBST) was added and incubated for 30 minutes at room temperature. After washing with TBST, 50 µL of ground squirrel plasma (diluted 1/10 in TBS) was added to wells and incubated for one hour at room temperature. After washing, the polyclonal rabbit anti-mouse Angptl2 (2-fold diluted from 1/1000 to 1/128,000 in TBS) was added to appropriate wells and incubated for one hour at room temperature. The plate was washed, and 50 µl of goat anti-rabbit IgG-AP (1/1000 dilution in TBS, Sigma-Aldrich) was added to all wells and allowed to incubate for 30 minutes at room temperature. The plate was washed, and 100 µL of Alkaline phosphatase yellow (pNPP) liquid substrate solution for ELISA (Sigma-Aldrich, St. Louis, MO) was added to all wells. One hundred microliters of 3N NaOH stop solution was added to all wells after 15 minutes and the absorbance was read at 405 nm. Average optical density was 0.20 (range 0.286-0.151) with no significant change in absorbance with an increase of either antibody. Ground squirrel plasma was then titrated against the capture antibody. Ground squirrel plasma (1/5 to 1/200) and the capture antibody (1/100 to 1/1000) were diluted 2 fold (detecting antibody was at a 1/5000 dilution). An increase in absorbance was seen in the 1/5 and 1/10 plasma dilutions as compared with the 1/20 dilution, but again there was no change

in absorbance with an increase in capture antibody at these plasma dilutions. A small reaction with the BSA negative control (0.08 Abs) was observed (compared with a 0.03 blank), and there was no reaction with the recombinant mouse Angptl2 positive control. Given that the detecting antibody (rabbit anti-Angptl2) was polyclonal, it was unlikely that the antibody was indeed specific for mouse Angptl2, but not reactive with recombinant mouse Angptl2. Due to the time and expense required to ensure an antibody is specific for ground squirrel Angptl2, an alternate method to detect ground squirrel Angptl2 was chosen that would require only one ground squirrel Angptl2 specific antibody.

## Quantitative Western Blot to Determine Relative Angptl2 Levels in Ground Squirrel Plasma

### Standard Curve

A semi-quantitative western blot was chosen to compare Angptl2 levels in plasma samples because it only required one ground squirrel Angptl2-specific antibody. Recombinant mouse Angptl2 of known concentration was serially diluted in sample buffer to generate a standard curve, which was then used to calculate the Angptl2 concentration of a pooled plasma standard. The pooled ground squirrel plasma standard was a combination of three different squirrel plasma samples, and was run in duplicate along with the standard curve. An aliquot of the pooled sample was digested and run along with each sample gel to normalize for differences across gels.

Angptl2 concentration standards (1200, 1100, 1000, 800 and 700 ng Angptl2/mL) and the ground squirrel pooled plasma standard (diluted 1/20 in 2X sample buffer (final dilution of 1/40)) were run on an SDS PAGE gel and transferred to a PVDF membrane as

previously described. The membrane was blocked overnight in a 1% BSA/PBST solution and incubated with the rat anti-Angptl2 (diluted 1/1000 in PBST) for 2 hours at room temperature. The membranes were washed in PBST as previously described, and incubated in biotinylated sheep anti-rat IgG (1/2,000 in PBST) for one hour at room temperature. All lanes were washed as described previously, and incubated in streptavidin-HRP (diluted 1/120,000 in PBST) for an hour at room temperature. The membrane was then washed and developed as previously described.

The chemiluminescent signal of each band was quantified using the program Image Studio Lite. A box of equal size was drawn around the prominent band in each lane. The median signal from a strip 3 pixels-wide on the left and right hand sides of the analyzed box was used for background subtraction (equation 1).

$$(1) \qquad Corrected\ signal = Signal - (Median\ background\ signal \times Area)$$

The chemiluminescent signal from each Angptl2 standard band was plotted versus Angptl2 concentration and fit to an exponential curve. Although many standard curves are linear, chemiluminescent standard curves have been shown produce standard curves ranging from linear to exponential, depending on the primary antibody (40). The exponential curve was used to calculate the concentration of the pooled ground squirrel plasma standard, 39.44 µg/mL, with a coefficient of variation (CV) of 4.07%.

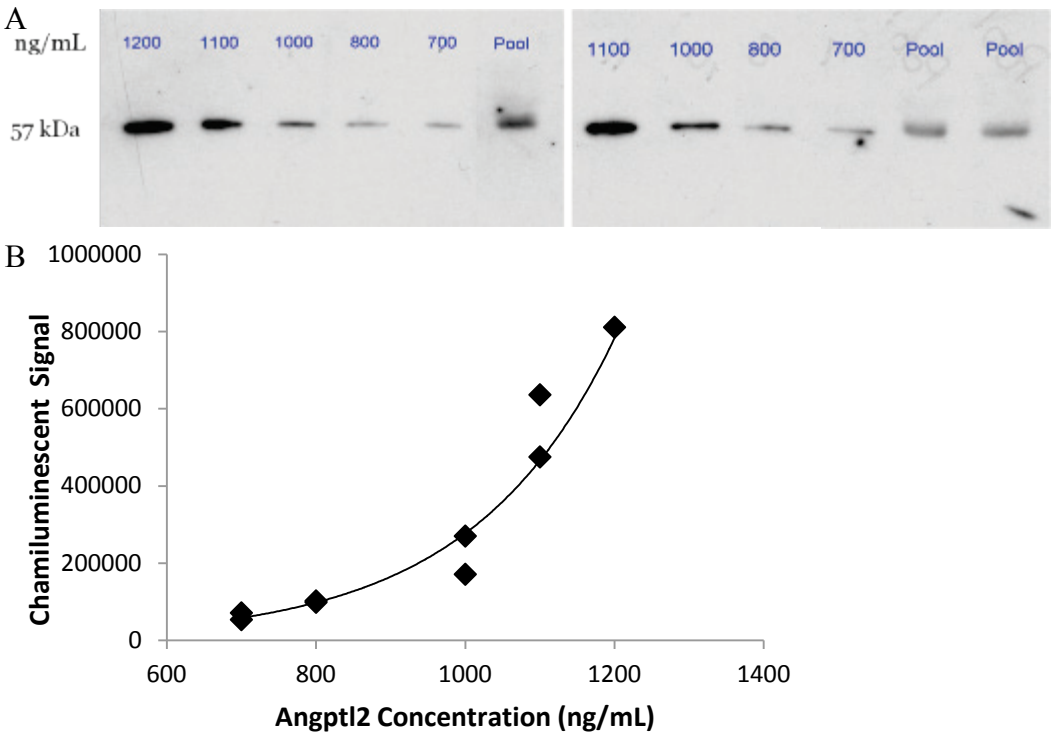


Figure 8. Angptl2 standard curve measured by chemiluminescent western blot (a) Recombinant Angptl2 standards (1200, 1100, 1000, 800 and 700 ng Angptl2/mL) along with the pooled plasma standard (1/40 final dilution) were run on duplicate SDS PAGE gels. The membranes were developed for Angptl2, and the chemiluminescent signal of the bands from both gels was analyzed and plotted against Angptl2 concentration. (b) An exponential curve was fit to the data, $y = 1543.6e^{0.0052x}$, $R^2 = 0.9521$.

**Sample Analysis: Angptl2**

Fifty-five ground squirrel plasma samples from various time points in the hibernal cycle were analyzed by semi-quantitative western blot. The pooled plasma sample was run on all sample gels to serve as a normalization control between gels, and as a concentration standard to allow for the approximate quantification of samples. To enhance the quantitative nature of this western blot, all plasma samples were normalized for total protein concentration and a loading control protein was added to the sample buffer and detected to check for differences in loading volumes. A loading volume

control protein needed to be chosen that would not change with hibernation, and was detectable in ground squirrel plasma. Beta-actin had been previously used as an internal control in a proteomics study looking at liver tissue of hibernating ground squirrels. However, beta-actin was not present in detectable levels in ground squirrel plasma and therefore was not be useful as an internal loading control. Albumin and transferrin are common internal controls for quantitative analysis of plasma samples. Albumin has been shown to fluctuate during hibernation, and the effect of hibernation on transferrin levels remains unknown, therefore neither of these options would provide a reliable internal loading control. In the absence of a suitable internal control, Beta-casein was chosen as an external loading control because it is a foreign protein that is not typically found in plasma. Casein was added to the pool of Laemmli sample buffer (6 ng/µL) and then detected using an anti-beta casein antibody.

Ground squirrel plasma samples and the pooled standard were diluted 1/20 in 2x Laemmli sample buffer (final 1/40 plasma dilution, 6 ng/µL of casein) and run on an SDS PAGE gel as previously described. The membrane was developed for Angptl2 using the same protocol as for the standard curve. The chemiluminescent signal of each sample was analyzed using Image Studio Lite. Total band analysis was used for all further analysis because it included all glycosylated forms of Angptl2 in the quantification (figure 9).

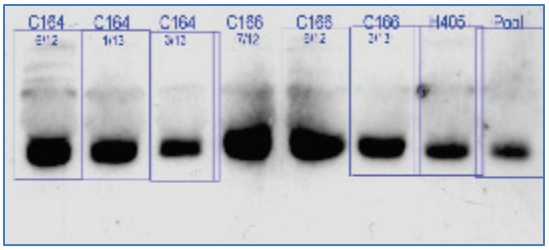


Figure 9. Detection of Angptl2 in ground squirrel plasma samples Ground squirrel plasma samples (final 1/40 dilution) were run on a 10% SDS PAGE gel and detected for Angptl2. The chemiluminescent signal of each sample lane was quantified using Image Studio Lite. The chemiluminescent signal of all sample bands was measured.

Approximate Angptl2 concentration of ground squirrel plasma samples was calculated based on the signal of sample to signal of pool ratio (equation 2)

$$(2) \qquad Angptl2\ Conc. = \frac{Signal\ of\ Plasma\ Sample}{Signal\ of\ Pool} \times Angptl2\ Conc.\ of\ Pool$$

**Sample Analysis: Beta-Casein**

After detection of Angptl2, the membranes were washed overnight in PBST at 4 °C and probed for beta-casein. The membranes were probed with polyclonal rabbit anti-beta casein (1/10,000 in PBST) (Bioss, Woburn, MA) for 1 hour at room temperature. The membranes were washed in PBST as described previously, and then incubated with biotinylated goat anti-rabbit IgG (1/40,000) for 1 hour at room temperature. The blot was washed as described previously and then incubated with a 1/120,000 dilution of streptavidin-HRP. The membranes were washed and developed as described previously. Bands at approximately 25 kDa were expected for beta-casein. The beta-casein blot was visually analyzed to look for consistency in loading volumes, indicated by consistent

beta-casein bands (Figure 10). Plasma samples were repeated when the beta-casein band's intensity did not match that of the pooled standard (figure 10, a).

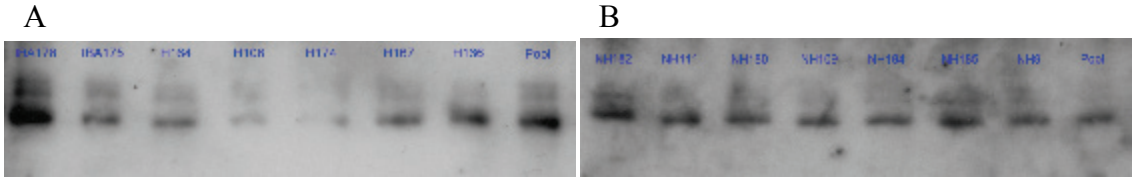


Figure 10. Detection of the beta-casein loading control in ground squirrel plasma samples. Ground squirrel plasma was diluted in sample buffer containing casein (final concentration of 6 ng/µL) and run on an SDS PAGE gel. The membrane was probed for Angptl2, then washed and probed for beta-casein. The beta-casein bands produced were visually analyzed for relative sample volumes loaded. (a) Variable Beta-casein bands, indicating variable loading volumes (b) Consistent beta-casein bands

**Total Protein Analysis**

To account for differences in sample collection procedures, as well as the total protein differences that may be present between squirrels, the total protein concentration of all plasma samples was measured using a bicinchoninic acid (BCA) protein assay. Bovine serum albumin (BSA) protein standards ranging from 25 µg/mL to 2000 µg/mL were run with each plate of samples to create a standard curve. Plasma samples were diluted to a final dilution of 1/150 in PBS. Twenty-five microliters of BSA protein standard or the diluted sample were placed in triplicate into wells of 96 well microtiter plates. The BCA working reagent was prepared according to manufacturer specifications (Thermo Scientific, Waltham, MA) and 200 µL was added to each well. The plates were incubated for 30 min at 37 °C, and the absorbance was read at 562 nm. The absorbencies of the protein standards were plotted and used to calculate the protein concentrations of

the plasma samples. Normalization of plasma samples for total plasma concentration was performed by expressing sample angptl2 levels as a percent of total plasma protein (Equation 3)

$$(3) \qquad \%Angptl2\ of\ Total\ Plasma\ Protein\ = \frac{Sample\ Angptl2\ Conc.}{Total\ Plasma\ Protein\ Conc.} \times 100$$

Significant differences in % Angptl2 as a percentage of total plasma protein among ground squirrels at different points in the hibernal cycle were statistically analyzed with the unequal variance student t-test.

**Correlation of % Angptl2 with Fat Levels**

The Angptl2 levels measured were tested for correlation with ground squirrel fat levels. The pre-harvest weights of all sampled ground squirrels were recorded before blood samples and organs were removed. The plasma Angptl2 levels of each squirrel were measured by chemiluminescent western blot, and the Angptl2 levels were plotted against the squirrels' weight (as an approximation of its adipose levels). This graph was analyzed to look for a correlation between adiposity and Angptl2 levels. Correlations were looked for both among squirrels in the same (hibernating vs. hibernating) and at different points in the hibernal cycle (hibernating vs. non-hibernating).

**Detection of Macrophages in Ground Squirrel Adipose Tissue**

Adipose tissue from hibernating and non-hibernating ground squirrels was preserved in cryo-embedding media at -80 °C. The adipose tissue sections were cut to a thickness of 10 μm in a cryotome at -30 °C and transferred to poly L-Lysine coated slides and allowed to dry for 2 hours. The tissue sections were fixed in acetone for 5 minutes

and circled with a wax pen, then incubated for 15 minutes with 0.3% $H_2O_2$ , 0.1% $NaN_3$ to reduce endogenous peroxidase activity. The tissues were washed for 5 minutes in PBS with gentle agitation and then sections were blocked for 1 hour in 10% rabbit serum/PBS at room temperature. Excess block was removed, and the sections were incubated in mouse anti-ground squirrel macrophage antibody (undiluted 9G8.B4 hybridoma supernatant generated by Bayan Shaheen at UWL) or mouse anti-human CD20 (undiluted hybridoma supernatant, negative control) for 20 min. The tissues were washed three times for five minutes in PBS with gentle agitation, then incubated for 20 min in biotinylated goat anti-mouse (diluted 1/500 in 1% BSA/PBS). The slides were rinsed three times in PBS and then incubated in streptavidin-HRP (1/1,000 in PBS) for 20 min. The slides were washed three times in PBS, and developed in fresh AEC working solution for 10 minutes. The slides were rinsed in distilled water and then counterstained with hematoxylin for 40 seconds followed by Scott's solution for 15 seconds. The slides were rinsed with water and preserved in mounting media, sealed under a glass coverslip. The slides were observed under a light microscope at 100x and 200x total magnification for red color in the spaces between the adipocytes, indicating the presence of macrophages. The stained hibernating and non-hibernating tissues were compared to the negative controls and to each other to look for relative macrophage numbers. A blinded panel was designed to provide an unbiased comparison of the hibernating and non-hibernating adipose sections. Four macrophage-stained adipose tissue sections from both hibernating and non-hibernating ground squirrels were compared alongside two sections of hibernating and non-hibernating anti-human CD-20 stained negative control sections. The total number of macrophages per field of view was recorded for a total of ten fields

of view for both hibernating and non-hibernating anti-macrophage stained tissues, and five fields of view were observed for hibernating and non-hibernating tissue sections stained with anti-human CD20 as a negative control. Fields of view were chosen where tissue was completely intact, and from a range of number of blood vessels present.

# RESULTS

## Comparison of Angptl2 Levels in Plasma of Ground Squirrels throughout the Hibernal Cycle

The Angptl2 concentration of 55 ground squirrel plasma samples was determined by semi-quantitative western blot. Seven of the 55 ground squirrel plasma samples were analyzed in duplicate to assess the reproducibility of the assay. The average coefficient of variation was 3.69%, with a range from 0.25 to 10.15%, indicating this assay has reproducibility within an acceptable range. Ground squirrel plasma samples were normalized for differences in total plasma protein concentration by expressing Angptl2 as a percent of total plasma protein. Percent Angptl2 increased (though not significantly) in torpid animals compared with entering hibernation and non-hibernating animals (Figure 11). Angptl2 percentages dropped significantly in interbout arousal ($p = 0.0132$) and post arousal ($p = 0.0156$) samples from peak torpor levels.

Table 2. Semi-quantitative western blot analysis of seven 13-lined ground squirrel plasma samples performed in duplicate.

| Sample | Image | Signal | Angptl2 Concentration (µg/mL) | Average Concentration (µg/mL) | Standard Deviation | %CV |
|---|---|---|---|---|---|---|
| C160 (3/13) | F | 2420000 | 41.52 | 41.07 | 0.64 | 1.55 |
| C160 (3/13) | N | 1640000 | 40.62 | | | |
| H57 | H | 1600000 | 34.89 | 34.95 | 0.09 | 0.25 |
| H57 | C | 1500000 | 35.01 | | | |
| IBA175 | G | 240000 | 34.40 | 35.94 | 2.18 | 6.07 |
| IBA175 | L | 172000 | 37.49 | | | |
| NH115 | A | 2650000 | 42.06 | 41.34 | 1.02 | 2.46 |
| NH115 | C | 3110000 | 40.62 | | | |
| PA134 | A | 614000 | 30.81 | 33.19 | 3.37 | 10.15 |
| PA134 | H | 1750000 | 35.58 | | | |
| PA135 | A | 973000 | 34.35 | 34.47 | 0.16 | 0.47 |
| PA135 | F | 748000 | 34.58 | | | |
| PA138 | A | 1870000 | 39.38 | 40.78 | 1.99 | 4.87 |
| PA138 | F | 2010000 | 42.19 | | | |

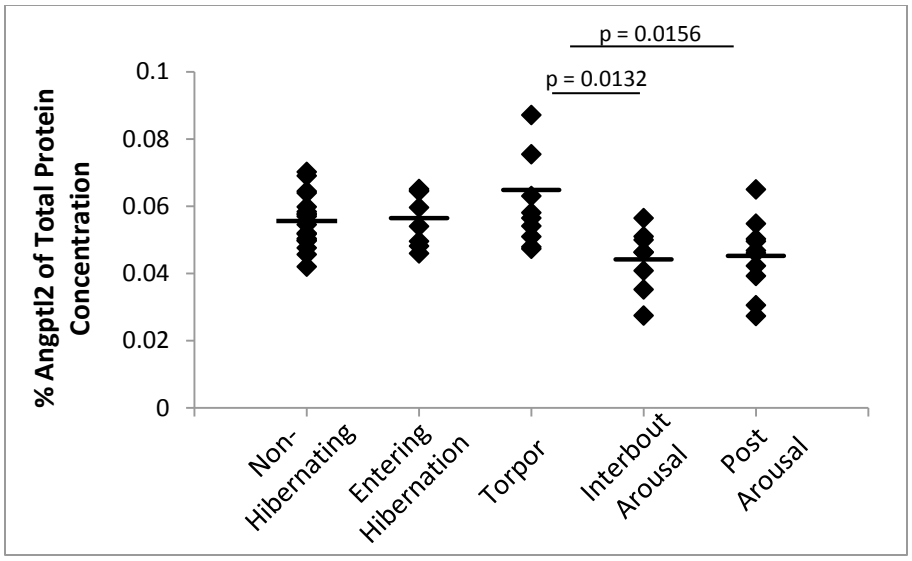


Figure 11. Average % Angptl2 in ground squirrels in different stages of hibernation. Plasma Angptl2 concentrations were determined by semi-quantitative western blot, and normalized for total plasma protein concentration to be expressed as a % of total plasma protein. (a) The %Angptl2 of total plasma protein was plotted for each of the five measured time points in the hibernal cycle. The bar represents the average %Angptl2 for that time point. P values were calculated using the unequal variance T Test.

Plasma samples from five individual ground squirrels were collected at multiple time points through one hibernal cycle (non-hibernating in June and July, entering hibernation, torpid, and interbout arousal). For each ground squirrel, percent Angptl2 was plotted against the month of sample collection for each ground squirrel to observe trends in Angptl2 levels over the year (figure 13). For each individual ground squirrel, percent Angptl2 and weight were both plotted over a hibernal cycle. While in most animals Angptl2 was at its highest plasma concentration during hibernation, the sample size for this analysis was too small to determine statistical significance.

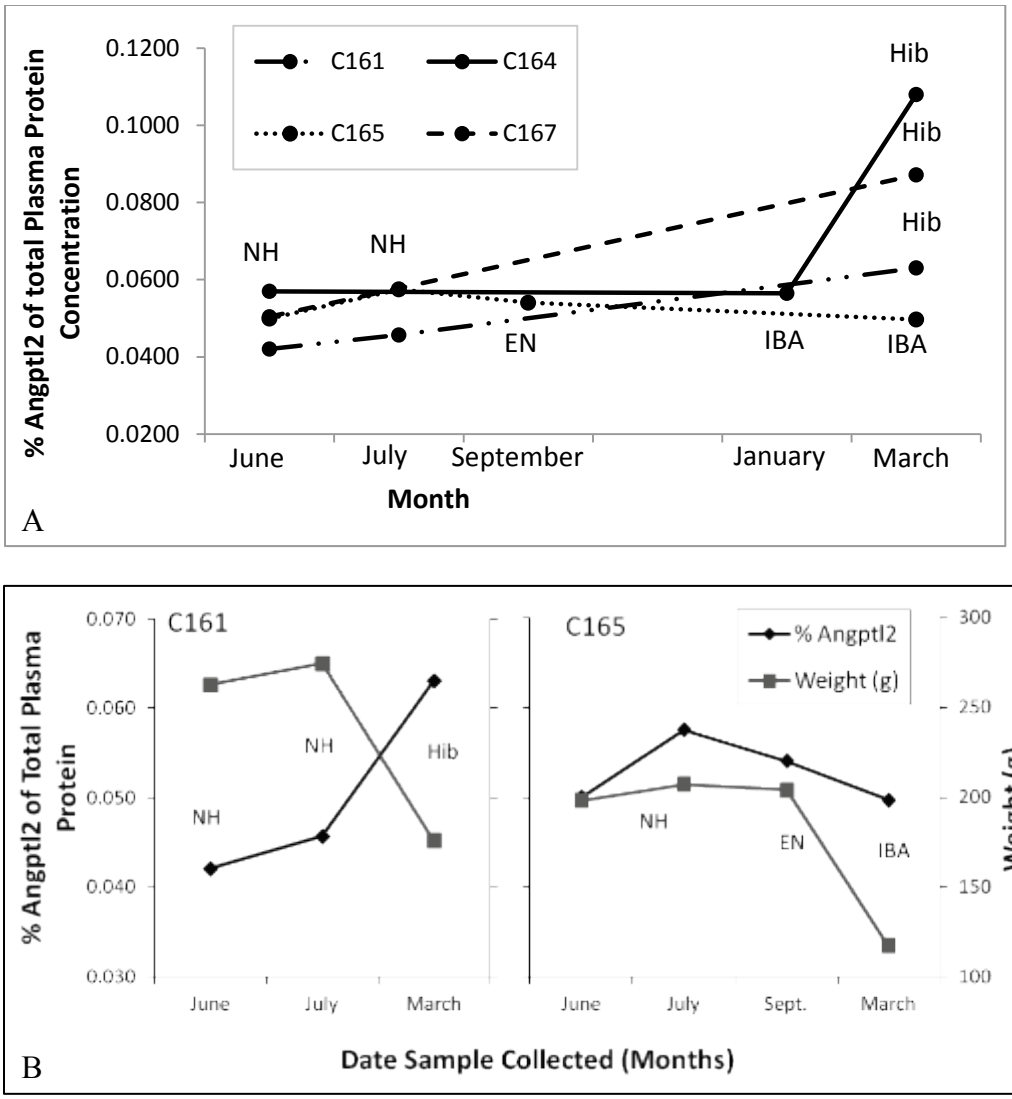


Figure 12. Percent Angptl2 in four ground squirrels measured at multiple time points over one year. (a) Plasma Angptl2 levels in four squirrels were measured at time points through one hibernal cycle. (b) Percent Angptl2 and weight were plotted against the month of sample collection in two ground squirrels (C161 and C165). Note; no hibernating plasma was available for C165.

**Correlation of Angptl2 Levels in Plasma with Fat Levels**

**throughout the Hibernal Cycle**

Percent Angptl2 was plotted versus ground squirrels' pre-harvest weights (in grams) as an approximation of their fat levels (Figure 12). When samples from all time points were plotted, there was an overall slight negative trend in the relationship between plasma Angptl2 and fat. Higher Angptl2 levels were slightly correlated with lower mass. This was contrary to the positive correlation described in humans between log (serum Angptl2) and body mass index (25).

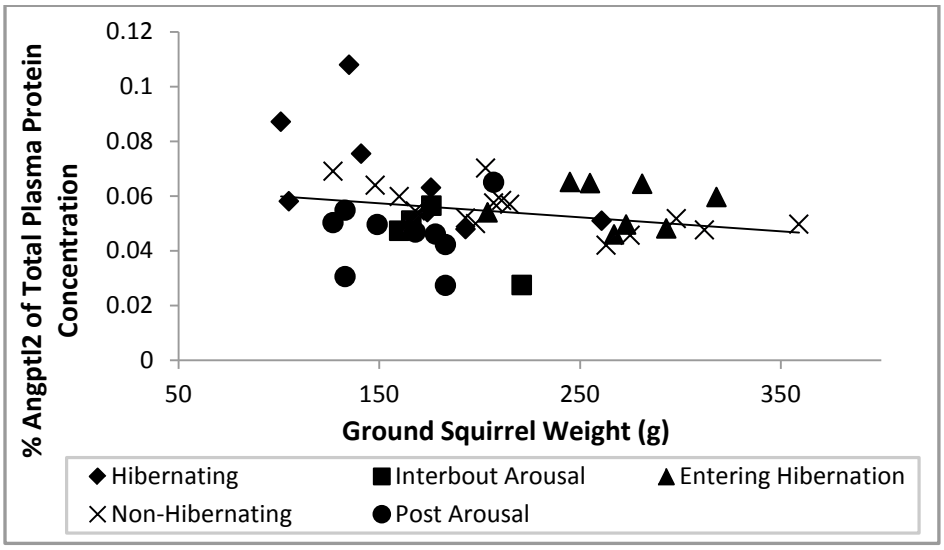


Figure 13. Percent Angptl2 plotted against weight as an approximation of fat levels. Angptl2 levels in 55 ground squirrels were measured by semi-quantitative western blot and expressed as a % of total plasma protein. Percent Angptl2 was plotted against pre-harvest weight, as an approximation of fat levels. All data points from the entire hibernal cycle were used to generate the trend line shown.

## Examination of Adipose Tissue for the Relative Presence of Macrophages in Torpid and Non-Hibernating Squirrels

Adipose tissue sections from one torpid and one non-hibernating squirrel were stained with mouse-anti-ground squirrel macrophage antibody, and observed under 100x and 200x total magnification (Figure 14-15). Macrophages in the tissue sections were marked by a red stain. Both sections were highly variable in the number of macrophages seen per field of view, however there did appear to be more macrophages in torpid than non-hibernating ground squirrel adipose tissue. Red stained macrophages or monocytes were present in blood vessels in both torpid and non-hibernating sections, however animal in torpor appeared to have larger, and more numerous red stained cells in vessels. In both hibernating and non-hibernating sections, the considerable variation in number of macrophages per field of view may have related to macrophages being more abundant in sections of adipose with a greater number of blood vessels. Macrophage staining was more difficult to differentiate from stained adipose cells at 100x magnification, but the red staining was much more apparent at 200x magnification (Figure 14-15).

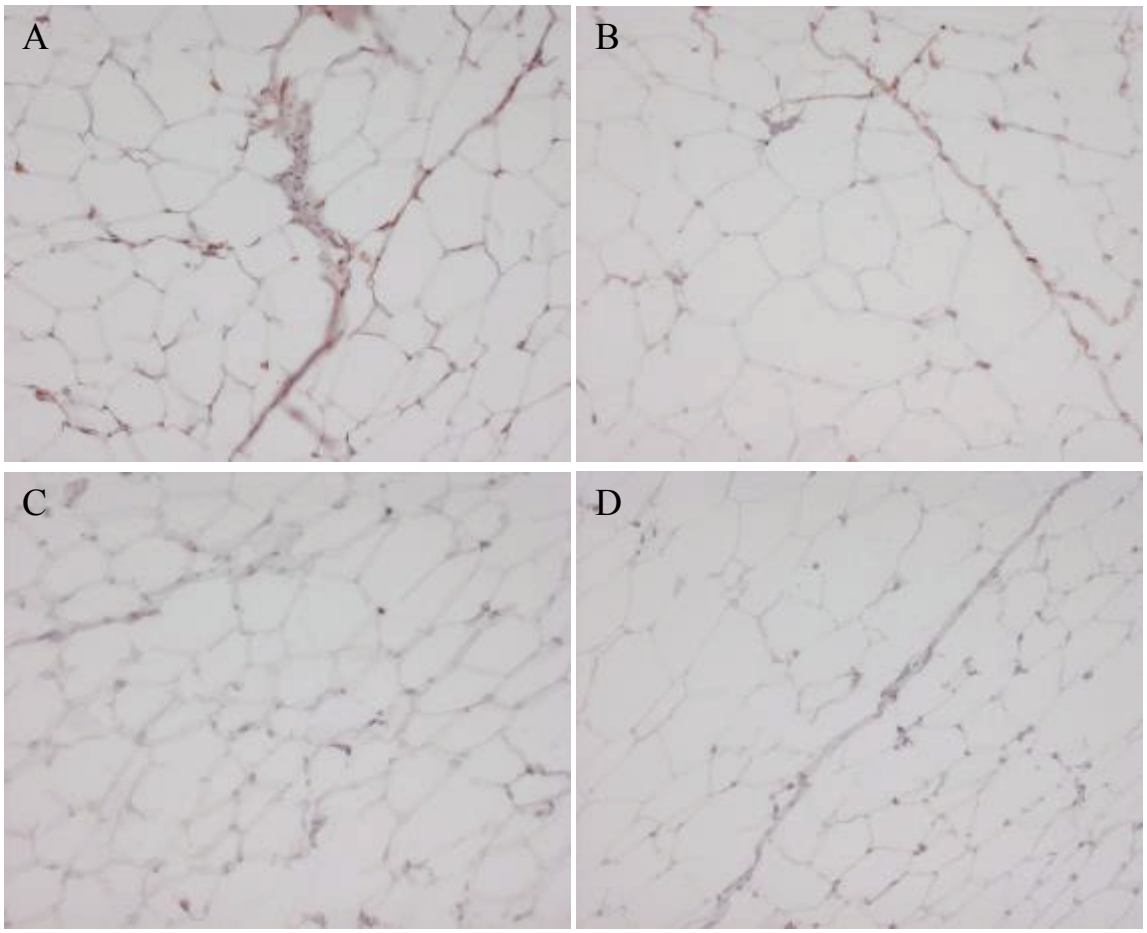


Figure 14. Torpid ground squirrel adipose tissue stained with mouse anti-ground squirrel macrophage antibody (A) or mouse anti-human CD20 (negative control) (C). Non-hibernating ground squirrel adipose tissue stained with mouse anti-ground squirrel macrophage antibody (B) or mouse anti-human CD20 (negative control) (D). Slides were counterstained with hematoxylin and Scott's solution, and viewed at 100x magnification.

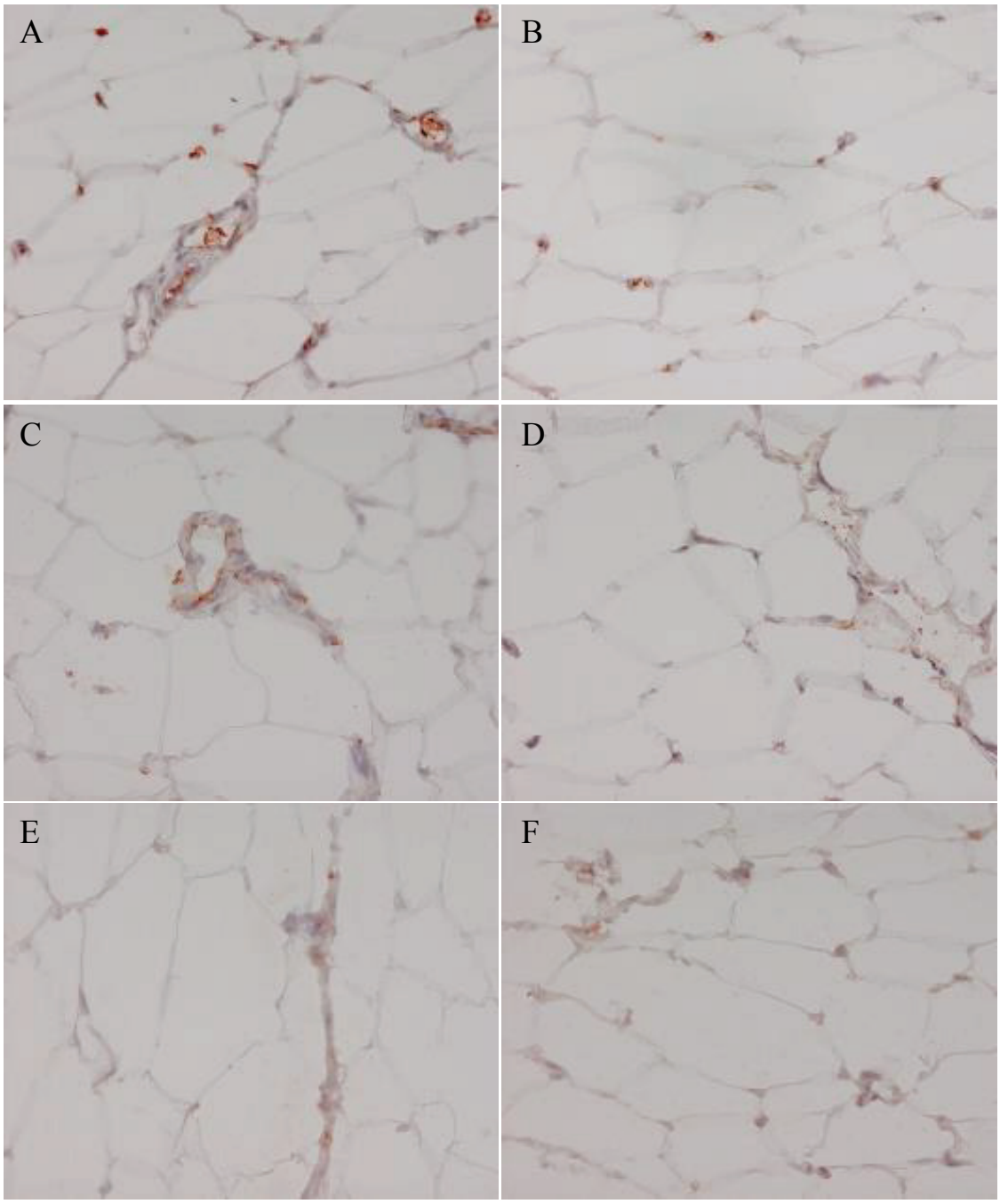


Figure 15. Torpid (A-C) or non-hibernating (D-F) ground squirrel adipose tissue was stained with mouse anti-ground squirrel macrophage antibody and counterstained with Hematoxylin and Scott's solution. 200x magnification. Blood vessels appear as a thicker purple line with more numerous red stained cells.

Due to the variability in number of macrophages per field of view seen within stained tissue sections, a blind panel was designed to achieve an unbiased comparison of macrophages in torpid and non-hibernating tissue sections. A total of ten fields of view were observed across the four anti-macrophage stained tissue sections for both torpid and non-hibernating squirrels, and a total of five fields of view were observed for torpid and non-hibernating negative control sections. Twice as many macrophages were observed in the torpid ground squirrel adipose tissue sections as in the non-hibernating sections (Table 3).

Table 3. Macrophages per field of view in torpid and non-hibernating ground squirrel adipose tissue sections.

| | Hibernating | | Non-Hibernating | |
|---|---|---|---|---|
| | Anti-ground squirrel macrophage[a] | Negative control[b] | Anti-ground squirrel macrophage[a] | Negative control[b] |
| Total cells counted | 172 | 2.0 | 83 | 14 |
| Average macrophages per field of view | 17.2 | 0.4 | 8.3 | 2.8 |
| Range | 0-45 | 0-1 | 3-22 | 2-4 |

[a] A total of ten fields of view were observed
[b] A total of five fields of view were observed

# DISCUSSION

The overall goal of this study was to determine if Angptl2 could play a role in the migration of monocytes/macrophages out of the blood stream during hibernation in 13-lined ground squirrels. Interest in such a role for Angptl2 was produced by previous studies in mice and humans that showed that leukocyte migration is increased during obesity and obesity-related conditions. Two supporting hypotheses were made: (1) That Angptl2 levels would be higher in torpid squirrels than in non-hibernating squirrels, and (2) Macrophages would be more abundant in torpid than non-hibernating ground squirrel adipose tissue.

## Development of a Method to Detect and Quantify Ground Squirrel Angptl2

A semi-quantitative western blot utilizing a commercial monoclonal rat anti-mouse Angptl2 antibody was developed to measure Angptl2 levels in ground squirrel plasma. The rat anti-mouse Angptl2 antibody specificity for ground squirrel Angptl2 was confirmed with a chemiluminescent western blot, in which a prominent band at 57 kDa appeared as expected, along with a faint upper and lower band. A PNGase digestion was performed to determine if the additional bands were due to Angptl2 in different states of glycosylation. While both mock and PNGase digestion reduced the intensity of all bands compared with the undigested control (likely a result of protein degradation during the two hour incubation at 42 °C), the digestion produced a single prominent band at 57 kDa,

indicating that additional bands on the western blot were due to glycosylated forms of Angptl2, and that the rat anti-mouse Angptl2 was specific for ground squirrel Angptl2.

The rat anti-Angptl2 antibody was used in a semi-quantitative western blot assay to detect and compare Angptl2 levels in 55 plasma samples from ground squirrels at different time points in the hibernal cycle. A standard curve was created with commercial recombinant mouse Angptl2, and used to calculate the Angptl2 concentration of each sample. Two controls were utilized to increase the quantitative-nature of the assay, a beta-casein loading control, and a total protein assay to normalize for variation in plasma protein concentration between ground squirrels. The average Angptl2 concentration of all plasma samples was 40.0 μg/mL, which is 10,000-fold higher than the previously reported serum Angptl2 concentration in humans (1.36-4.98 ng/mL) (25). All plasma Angptl2 concentrations were based upon the concentration of the recombinant mouse Angptl2. Inaccuracy in the reported concentration of in the commercial recombinant protein could extrapolate into a large error in plasma Angptl2 concentration. In addition, a western blot can only be made semi-quantitative, so Angptl2 concentrations produced from this assay are more useful for comparing relative plasma Angptl2 levels. To assess the reproducibility of the assay, seven of the 55 plasma samples were run in duplicate on separate gels. The average coefficient of variation for concentration of Angptl2 in the seven samples was 3.69%, with a range from 0.25 to 10.15%. Both the average and range are within what is typically considered acceptable for an assay. It is also possible that 13-lined ground squirrels naturally produce higher concentrations of Angptl2 in their plasma than humans or mice.

## Comparison of Angptl2 Levels in Plasma of Ground Squirrels throughout the Hibernal Cycle

Plasma Angptl2 levels in 55 plasma samples were measured by semi-quantitative western blot and compared against a pooled standard to determine an approximate Angptl2 concentration. Angptl2 levels were significantly higher in torpid ground squirrels than in interbout arousal ($p = 0.0132$) and post arousal ($p = 0.0156$) animals, mean differences of 0.0200% Angptl2 and 0.0196% Angptl2, respectively. There was also a small increase in Angptl2 levels in torpid animals as compared with entering hibernation or non-hibernating squirrels. Angptl2 levels in four ground squirrels were measured at multiple time points over a hibernal cycle. In each torpid squirrel, Angptl2 levels increased, while a decrease was seen in the interbout arousal squirrels. Angptl2 levels were higher in torpid and entering-hibernation squirrels compared with non-hibernators, however this was not statistically significant. Weight may have interfered with this trend, as will be discussed below. Higher plasma Angptl2 levels were seen in torpor than interbout arousal or post arousal ground squirrels, which supports our hypothesis that a role for Angptl2 in macrophage migration would be associated with higher Angptl2 levels during torpor.

## Correlation of Angptl2 Levels in Plasma with Fat Levels throughout the Hibernal Cycle

Plasma Angptl2 levels in 55 ground squirrels from multiple time points in the hibernal cycle were measured by chemiluminescent western blot, and compared against their pre-harvest weight as an approximation of fat levels. A positive correlation between weight and Angptl2 levels was predicted based on a previously published plot of human

serum Angptl2 vs. Body mass index (BMI). In this study, serum Angptl2 concentration in humans was quantified by capture ELISA, and plotted against BMI, resulting in the positive correlation previously described (n = 98) (25). This was contradictory to what was observed when %Angptl2 of total plasma protein in ground squirrel samples was plotted versus the ground squirrel's pre-harvest weight, an overall negative trend was observed.

There were some differences between these two experimental studies that may have contributed to the difference seen in results. The previous study measured serum instead of plasma levels, and compared it to BMI rather than weight, which alone could make the plots non-comparable. In addition, the human study did not utilize a control for normal variation in total protein concentration between serum samples. It is also possible that hibernation may interfere with this expected trend in comparison with a non-hibernating species (humans). A different trend might have been observed when plotting samples taken from many squirrels of different weights from the same time point in the hibernal cycle. However, considering that 98 subjects were used in the human study, a large number of ground squirrel plasma samples from each time point would need to be analyzed, which put that experiment outside of the scope of this project.

Since the relationship between Angptl2 levels and weight remains uncertain, the best comparison of Angptl2 levels would be between groups of ground squirrels with similar weights (Figure 16). For this reason, the comparison between torpor and interbout arousal or post arousal ground squirrels may be the most significant. For all three of these groups, both average weight, and the time of year that the sample was collected are all

approximately equal. The Angptl2 levels in the torpid group were significantly higher than that of either the interbout arousal or post arousal animals.

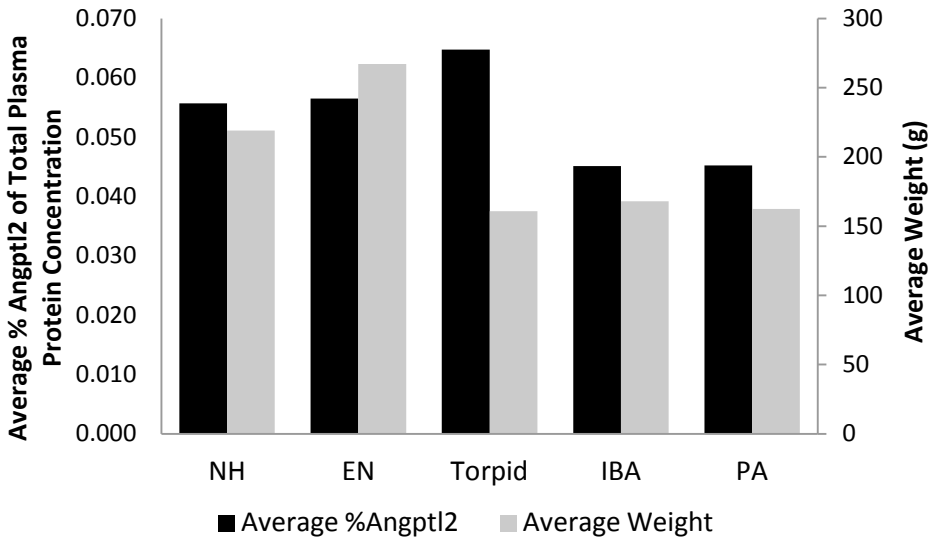


Figure 16. Average % Angptl2, as determined by semi-quantitative western blot, and average weight were plotted for each time point in the hibernal cycle. (Angptl2 – NH n=18; EN n=8; Hib n=10; IB, n=9; PA n=10. Weight – NH n=16; EN n=8; Hib n=8; IBA n=5; PA n=9).

To reduce variation in Angptl2 levels due to natural differences between squirrels, five ground squirrels were sampled at multiple time points over a yearly hibernal cycle. Their plasma Angptl2 levels were measured by chemiluminescent western blot, and plotted, along with weight, versus month of sample collection. All of the torpid ground squirrels exhibited some degree of increase in Angptl2 levels in late hibernation, as well as a decrease in weight over the same time period, as compared to summer non-hibernating levels. The average % increase in Angptl2 and decrease in weight as

compared with summer non-hibernating levels was plotted for both late hibernating and interbout arousal ground squirrels (Figure 17).

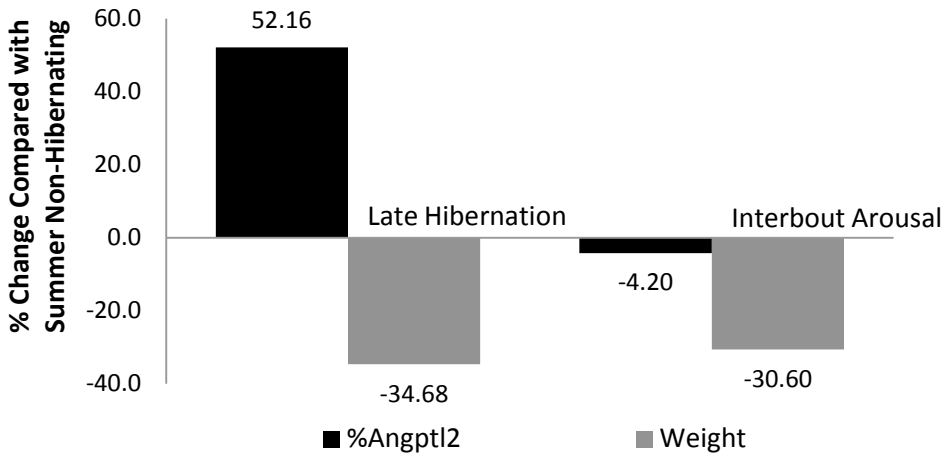


Figure 17. Plasma Angptl2 levels in five squirrels was measured at time points through the hibernal cycle. %Change in weight and %Angptl2 in late hibernating (n=5) and interbout arousal (n=2) as compared with their summer non-hibernating levels. Summer non-hibernating levels were the average of June and July collections, if available.

This experiment had a small sample size (n=5), which greatly increases the error in these comparisons. A similar comparison was made utilizing all 55 ground squirrel samples. Plasma samples were separated by sample collection season and hibernal stage, then average %Angptl2 and weight for each group were plotted over a yearly hibernal cycle (Figure 18). The trends seen in this plot are very similar to those seen when observing the five individual ground squirrels. There was an increase in %Angptl2 as squirrels entered hibernation, and an even greater increase as squirrels entered late hibernation (Spring). There was a decrease in %Angptl2 in interbout arousal and post arousal squirrels as compared with non-hibernating animals. Although these data sets (individual squirrel and

collective squirrel analysis) agree with each other, neither one has enough data points in each group for the data to be considered statistically significant. The trends from this preliminary data support the hypothesis that Angptl2 levels are higher in torpid than non-hibernating ground squirrels.

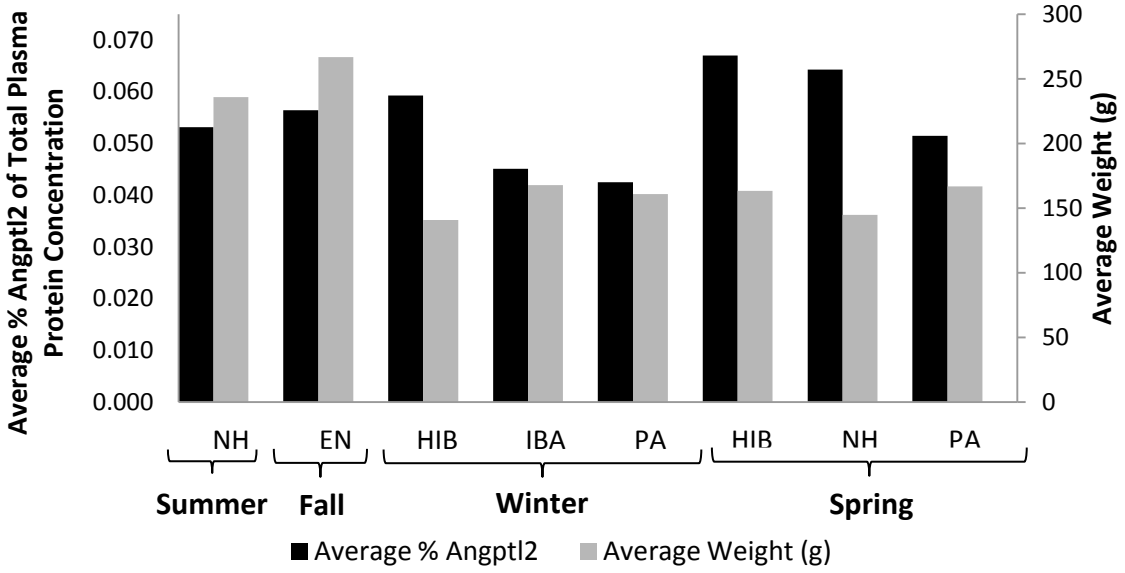


Figure 18. Average % Angptl2 and average weight (n=55) were for grouped by season of sample collection and hibernal stage.

**Examination of Adipose Tissue for the Relative Presence of Macrophages in Hibernating and Non-Hibernating Squirrels**

Torpid and non-hibernating ground squirrel adipose tissue sections were stained with mouse anti-ground squirrel macrophage antibody, and observed under 100x and 200x total magnification for the relative presence of macrophages. An increase in the number of macrophages per field of view did appear to correspond with the number of blood vessels in that area, which contributed to variation between fields of view. Both

torpid and non-hibernating ground squirrel adipose sections were highly variable in the number of macrophages seen per field of view, however when an unbiased comparison of macrophage numbers between torpid and non-hibernating grounds squirrel adipose tissue was performed, there were about twice as many macrophages in torpid than non-hibernating sections. A greater number of macrophages observed in torpid than non-hibernating adipose tissue agreed with the hypothesis that increased Angptl2 levels during torpor would result in the migration of macrophages to the adipose tissue. Additionally, many macrophages were observed inside of the blood vessels of torpid ground squirrels, which supported the hypothesis that leukocytes could be sticking to the inside of blood vessels during torpor. This data is only preliminary, given the small sample size, and the difficulty of discriminating individual macrophages in tissue sections. Tissue staining should be performed with additional torpid and non-hibernating samples, as well as interbout arousal and post arousal animals, since that was where a significant difference was seen in Angptl2 levels. Angptl2 also has high transcription levels in the heart and lungs, so those organs should also be examined for an increase in macrophages during hibernation (25).

Angptl2 levels were significantly higher in torpid ground squirrels than in interbout arousal or post arousal animals, with no significant change in average weight across those three groups. There was also a small increase in Angptl2 levels in torpid squirrels compared with entering hibernation or non-hibernating squirrels. When four squirrels were sampled at multiple points throughout the hibernal cycle, Angptl2 levels increased in late hibernation, while weight decreased. Overall, Angptl2 levels were

higher in torpid animals. This supports the first hypothesis: Angptl2 could play a role in the migration of macrophages during torpor.

Approximately twice as many macrophages were observed in torpid than non-hibernating ground squirrels, which supported the hypothesis that macrophages would be more abundant in torpid adipose tissue. Due to time limitations, immunohistochemistry was only performed on adipose tissue from one ground squirrel from each hibernal group. Additional experimentation should be performed on a larger number of ground squirrels, along with adipose tissue from interbout arousal and post arousal squirrels, which would give more insight into relative macrophage numbers throughout the hibernal cycle. Other organs, such as the heart and lungs, could also be stained to look at the relative presence of macrophages in torpid and non-hibernating tissues.

Contrary to what was expected, when %Angptl2 was plotted versus the ground squirrel's pre-harvest weight, there was an overall slight negative trend. It was hypothesized that a positive correlation between fat mass and Angptl2 level would be seen, similar to the plot of log(serum Angptl2) vs. body mass index) previously published (25). Torpor with associated weight loss as fat is used as fuel for metabolism may have an effect on this correlation. Additional experimentation to expand the number of ground squirrels sampled in each stage of hibernation would allow the %Angptl2 versus weight to be plotted for each stage of hibernation. In the future, the squirrel's length could be measured as well as weight, so BMI could be used as a more accurate approximation of fat levels. It is also possible that Angptl2 is regulated differently in ground squirrels compared to non-hibernating animals due to its potential role in hibernation.

The evidence presented above supports the overall hypothesis, that Angptl2 could play a role in the migration of macrophages during the hibernation of 13-line ground squirrels. However, to definitively determine if Angptl2 is necessary for the migration of macrophages during hibernation, an experiment would need to be designed to block Angptl2 levels and observe the effect on the hibernating immune system. Two options are available for this: (1) an Angptl2 knock out ground squirrel could be bred or (2) Normal ground squirrels could be dosed with an anti-Angptl2 antibody, so all of the Angptl2 in their system would be rendered useless. The creation of a knock out ground squirrel would be very time consuming and expensive, given that there are not easily accessible inbred lines of laboratory ground squirrels available. In addition, the genetic manipulation performed to create a knock out might change other aspects of the ground squirrels physiology, resulting in skewed results. The second option, blocking Angptl2 in the body with an antibody, is a more inexpensive experiment, and also has the benefit of being usable with a wide range of squirrels (wild caught or lab bred). However, with this method, additional experimentation would be necessary to confirm that Angptl2 was indeed blocked and non-functional, the time period that the blocking would last, as well as the potential effects of hibernation on this type of blocking process.

This study will contribute to current information about the sequestration of white blood cells during hibernation in 13-lined ground squirrels. Scientists hope that information about hibernation will someday be helpful in treating humans who have undergone ischemic events, such as a stroke or heart attack, by offering insights into migratory control of leukocytes that participate in inflammation.

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# APPENDIX A

# 13-LINED GROUND SQUIRREL SAMPLE LOG

| Sample | Date Sacrificed | Weight (g) | Stage in Hibernal Cycle |
|---|---|---|---|
| C165 (9/12) | 9/28/2012 | 204 | Entering Hibernation |
| EN186 | 9/20/2013 | 318 | Entering Hibernation |
| EN189 | 9/20/2013 | 273 | Entering Hibernation |
| EN190 | 9/20/2013 | 245 | Entering Hibernation |
| EN191 | 9/20/2013 | 281 | Entering Hibernation |
| EN197 | 10/14/2013 | 255 | Entering Hibernation |
| EN199 | 10/14/2013 | 267 | Entering Hibernation |
| EN201 | 10/14/2013 | 293 | Entering Hibernation |
| C160 (3/13) | 3/8/2013 | 260.8 | Hibernating |
| C161 (3/13) | 3/8/2013 | 175.8 | Hibernating |
| C164 (3/13) | 3/13/2013 | 135 | Hibernating |
| C167 (3/13) | 3/13/2013 | 101 | Hibernating |
| C168 (3/13) | 3/8/2013 | 105 | Hibernating |
| H108 | 3/14/2011 | 193 | Hibernating |
| H174 | 1/11/2013 | NA | Hibernating |
| H405 | 3/14/2011 | 174 | Hibernating |
| H57 | 2/4/2010 | 141 | Hibernating |
| H6 | 12/27/2006 | NA | Hibernating |

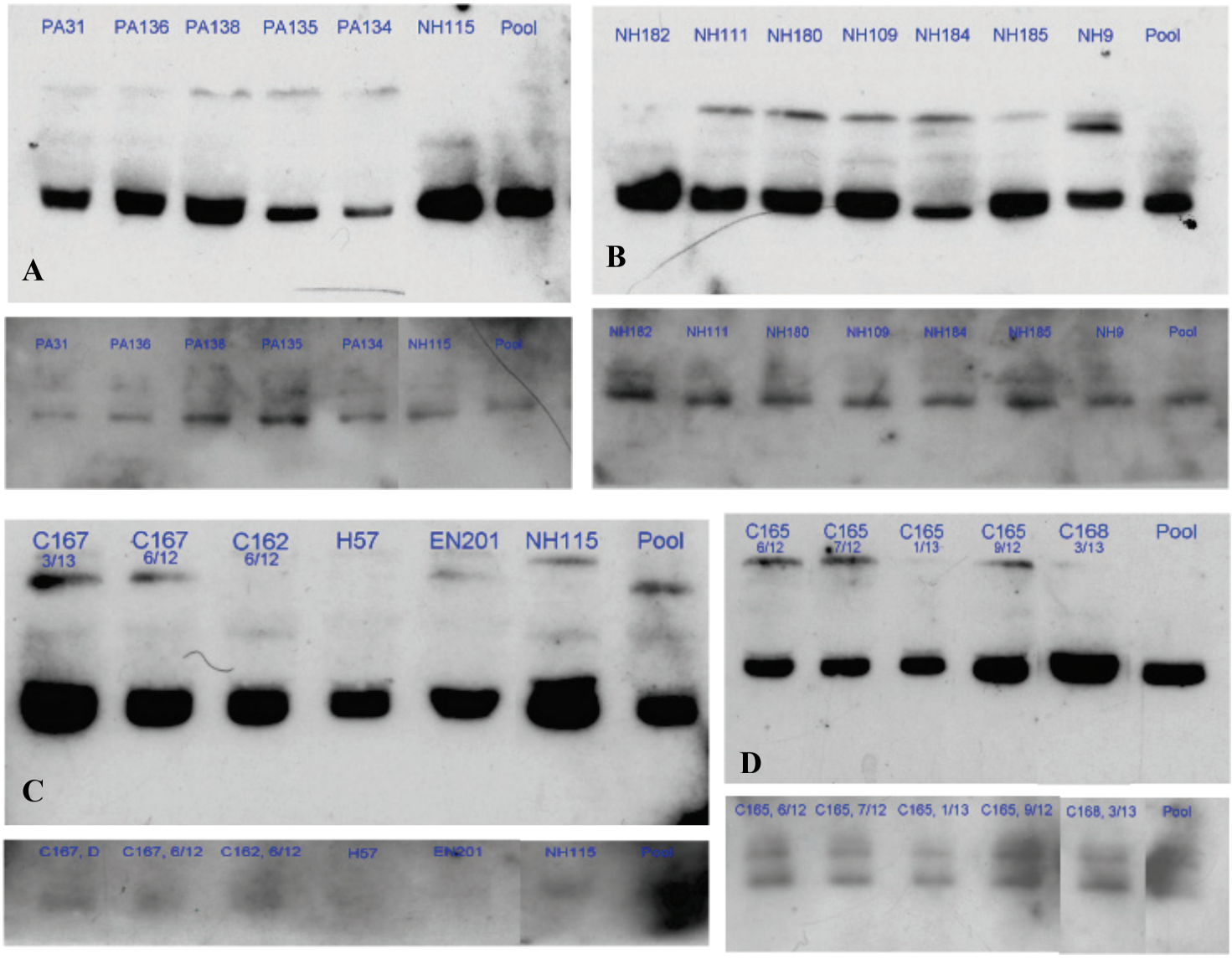
[a] Wild Caught, put down same Day
[b] 1 month PA

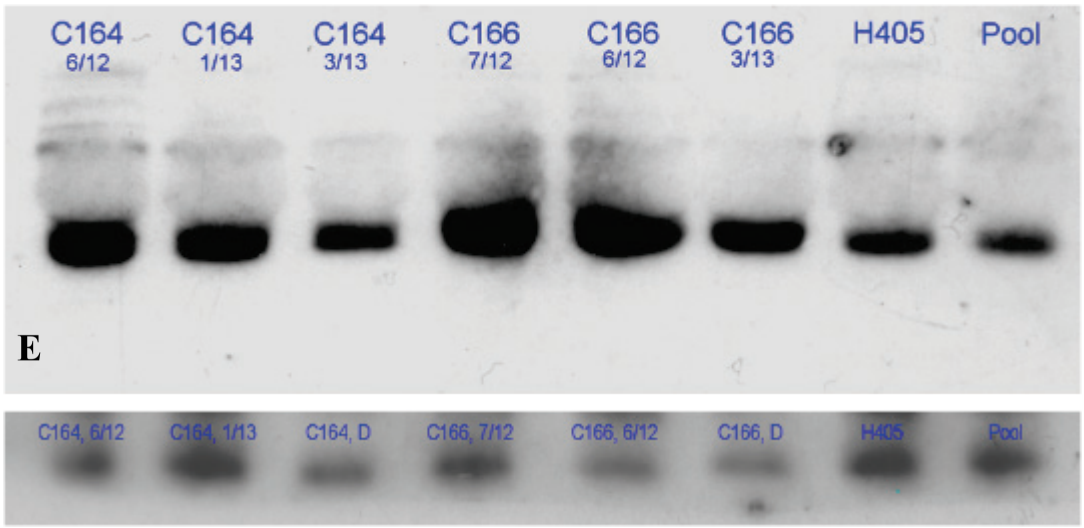
| Sample | Date Sacrificed | Weight (g) | Stage in Hibernal Cycle |
|---|---|---|---|
| C161 (6/12) | 6/26/2012 | 263 | Non-Hibernating |
| C161 (7/12) | 7/31/2012 | 275 | Non-Hibernating |
| C162 (6/12) | 6/26/2012 | 211 | Non-Hibernating |
| C164 (6/12) | 6/26/2012 | 215 | Non-Hibernating |
| C165 (6/12) | 6/26/2012 | 198 | Non-Hibernating |
| C165 (7/12) | 7/31/2012 | 207 | Non-Hibernating |
| C167 (6/12) | 6/26/2012 | 167 | Non-Hibernating |
| NH67 | | | Non-Hibernating |
| NH109[b] | 4/11/2011 | 127 | Non-Hibernating |
| NH111[a] | 5/10/2011 | 160 | Non-Hibernating |
| NH115[a] | 5/11/2011 | 148 | Non-Hibernating |
| NH180 | 7/1/2013 | 203 | Non-Hibernating |
| NH181 | 7/1/2013 | 168 | Non-Hibernating |
| NH182 | 7/1/2013 | 312 | Non-Hibernating |
| NH183 | 7/3/2013 | 193 | Non-Hibernating |
| NH184 | 7/3/2013 | 298 | Non-Hibernating |
| NH185 | 7/3/2013 | NA | Non-Hibernating |
| NH9 | 3/14/2011 | NA | Non-Hibernating |
| PA134 | 2/16/2012 | 183 | Post Arousal (2 hours) |
| PA135 | 2/16/2012 | 149 | Post Arousal (2 hours) |
| PA136 | 2/16/2012 | 168 | Post Arousal (2 hours) |
| PA138 | 2/16/2012 | 133 | Post Arousal (2 hours) |
| PA144 | 2/22/2012 | 178 | Post Arousal (1 week) |
| PA145 | 2/22/2012 | 183 | Post Arousal (1 week) |
| PA146 | 2/22/2012 | 133 | Post Arousal (1 week) |
| PA30 | 4/11/2011 | 127 | Post Arousal (2 hours) |
| PA31 | 3/11/2011 | NA | Post Arousal (2 hours) |
| PA33 | 3/11/2011 | 207 | Post Arousal (2 hours) |

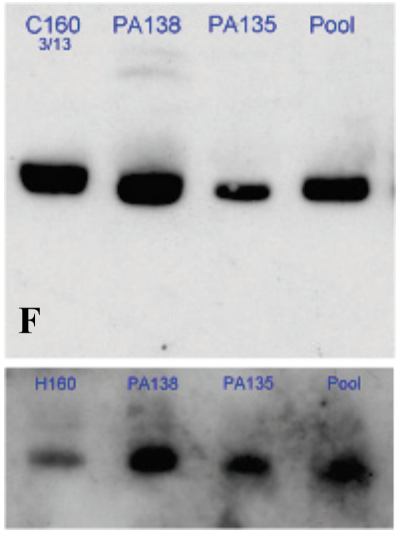
[a]Wild caught, put down same day
[b] 1 month PA

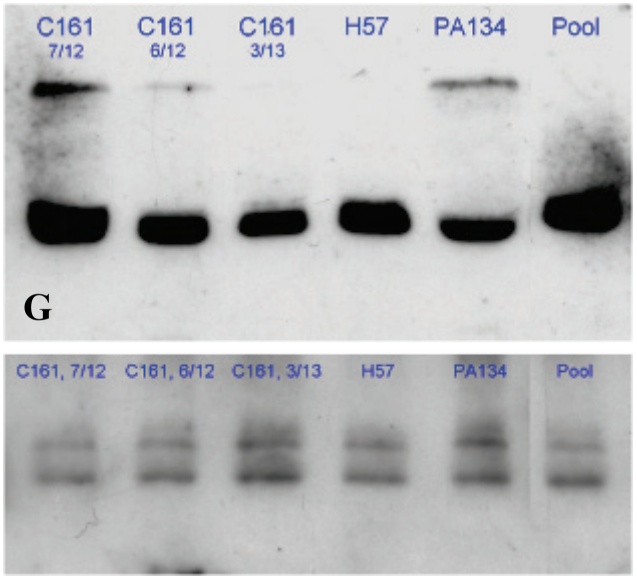
# APPENDIX B

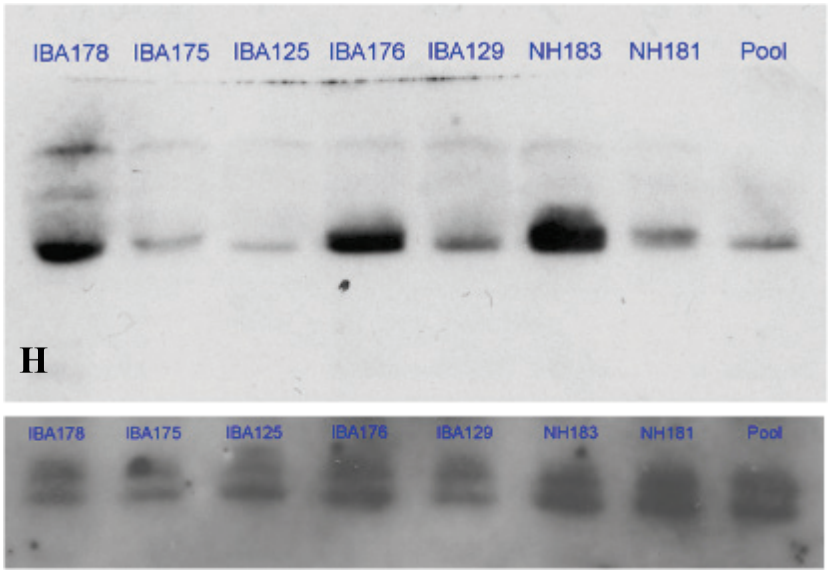
# ANGPTL2 (TOP) AND BETA-CASEIN (BOTTOM) CHEMILUMINESCENT WESTERN BLOT IMAGES FOR ALL PLASMA SAMPLES

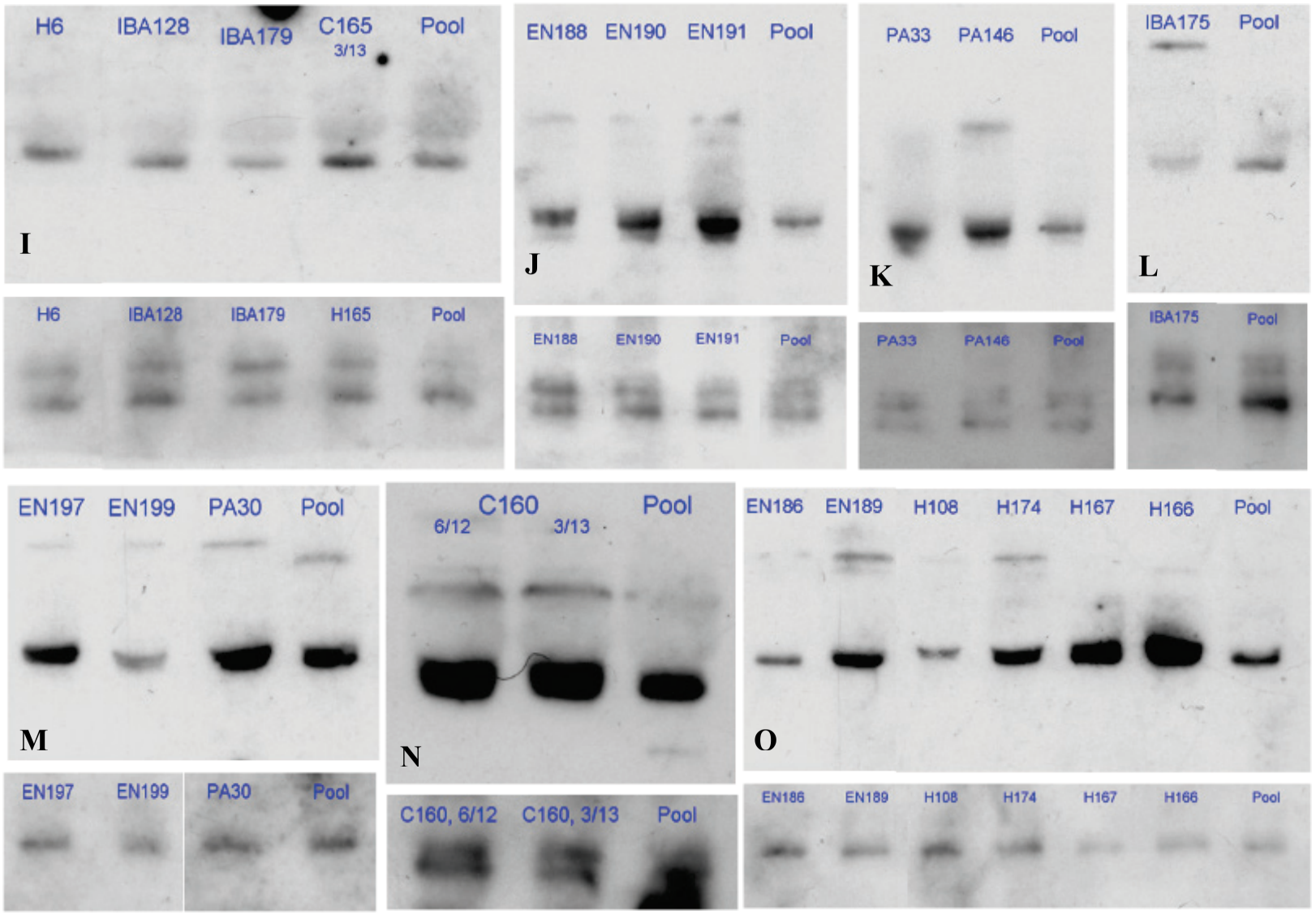

PA31 PA136 PA138 PA135 PA134 NH115 Pool
A
NH182 NH111 NH180 NH109 NH184 NH185 NH9 Pool
B
PA31 PA136 PA138 PA135 PA134 NH115 Pool
NH182 NH111 NH180 NH109 NH184 NH185 NH9 Pool
C167 3/13
C167 6/12
C162 6/12
H57
EN201
NH115
Pool
C
C165 6/12
C165 7/12
C165 1/13
C165 9/12
C168 3/13
Pool
D
C167, D
C167, 6/12
C162, 6/12
H57
EN201
NH115
Pool
C165, 6/12
C165, 7/12
C165, 1/13
C165, 9/12
C168, 3/13
Pool


C164 6/12
C164 1/13
C164 3/13
C166 7/12
C166 6/12
C166 3/13
H405
Pool
E
C164, 6/12
C164, 1/13
C164, D
C166, 7/12
C166, 6/12
C166, D
H405
Pool


C160 3/13
PA138
PA135
Pool
F
H160
PA138
PA135
Pool


C161 7/12
C161 6/12
C161 3/13
H57
PA134
Pool
G
C161, 7/12
C161, 6/12
C161, 3/13
H57
PA134
Pool


IBA178
IBA175
IBA125
IBA176
IBA129
NH183
NH181
Pool
H
IBA178
IBA175
IBA125
IBA176
IBA129
NH183
NH181
Pool


H6
IBA128
IBA179
C165
3/13
Pool
I
H6
IBA128
IBA179
H165
Pool
EN188
EN190
EN191
Pool
J
EN188
EN190
EN191
Pool
PA33
PA146
Pool
K
PA33
PA146
Pool
IBA175
Pool
L
IBA175
Pool
EN197
EN199
PA30
Pool
M
EN197
EN199
PA30
Pool
C160
6/12
3/13
Pool
N
C160, 6/12
C160, 3/13
Pool
EN186
EN189
H108
H174
H167
H166
Pool
O
EN186
EN189
H108
H174
H167
H166
Pool

# APPENDIX C

# BRADFORD ASSAY AND WESTERN BLOT ANALYSIS DATA OF ALL PLASMA SAMPLES

**Hibernating Samples**

| Sample | Image | Total Protein Conc. (mg/ml) | Signal | Angptl2 Conc. (ug/mL) | % Angptl2 of Total Protein Conc. |
|---|---|---|---|---|---|
| C160 (3/13) | Average | 80.56 | | 41.07 | 0.0510 |
| C161 (3/13) | H | 53.26 | 1350000 | 33.58 | 0.0631 |
| C164 (3/13) | E | 37.69 | 1130000 | 40.69 | 0.1080 |
| C167 (3/13) | C | 49.13 | 4140000 | 42.82 | 0.0872 |
| C168 (3/13) | D | 71.73 | 1960000 | 41.68 | 0.0581 |
| H108 | O | 69.40 | 294000 | 33.26 | 0.0479 |
| H174 | O | 77.44 | 1150000 | 43.75 | 0.0565 |
| H405 | E | 78.22 | 1400000 | 42.34 | 0.0541 |
| H57 | Average | 46.30 | | 34.95 | 0.0755 |
| H6 | I | 76.28 | 355000 | 36.13 | 0.0474 |

**Entering Hibernation Samples**

| Sample | Image | Total Protein Conc. (mg/ml) | Signal | Angptl2 Conc. (ug/mL) | % Angptl2 of Total Protein Conc. |
|---|---|---|---|---|---|
| EN186 | O | 57.71 | 342000 | 34.42 | 0.0596 |
| EN189 | O | 89.64 | 1260000 | 44.46 | 0.0496 |
| EN190 | J | 70.98 | 856000 | 46.24 | 0.0651 |
| EN191 | J | 77.05 | 1340000 | 49.69 | 0.0645 |
| EN197 | M | 58.82 | 867000 | 38.07 | 0.0647 |
| EN199 | M | 66.22 | 322000 | 30.46 | 0.0460 |
| EN201 | C | 76.95 | 1960000 | 37.07 | 0.0482 |
| C165 (9/12) | C | 77.69 | 2040000 | 41.99 | 0.0540 |

**Non Hibernating Samples**

| Sample | Image | Total Protein Conc. (mg/ml) | Signal | Angptl2 Conc. (ug/mL) | % Angptl2 of Total Protein Conc. |
|---|---|---|---|---|---|
| C160 (6/12) | N | 86.02 | 2850000 | 42.78 | 0.0497 |
| C161 (6/12) | H | 82.40 | 1550000 | 34.64 | 0.0420 |
| C161 (7/12) | H | 88.10 | 3210000 | 40.24 | 0.0457 |
| C162 (6/12) | C | 63.61 | 1980000 | 37.15 | 0.0584 |
| C164 (6/12) | E | 82.99 | 2670000 | 47.31 | 0.0570 |
| C165 (6/12) | D | 80.24 | 1590000 | 40.07 | 0.0499 |
| C165 (7/12) | D | 69.42 | 1560000 | 39.92 | 0.0575 |
| C167 (6/12) | C | 76.34 | 2350000 | 38.47 | 0.0504 |
| NH109 | B | 59.49 | 2350000 | 41.10 | 0.0691 |
| NH111 | B | 65.61 | 1850000 | 39.25 | 0.0598 |
| NH115 | Average | 64.63 | | 41.34 | 0.0640 |
| NH180 | B | 58.14 | 2270000 | 40.83 | 0.0702 |
| NH181 | G | 72.31 | 463000 | 39.46 | 0.0546 |
| NH182 | B | 86.13 | 2330000 | 41.03 | 0.0476 |
| NH183 | G | 95.97 | 1800000 | 49.90 | 0.0520 |
| NH184 | B | 75.60 | 1820000 | 39.13 | 0.0518 |
| NH185 | B | 68.88 | 1990000 | 39.82 | 0.0578 |
| NH9 | B | 60.69 | 1820000 | 39.13 | 0.0645 |

**Interbout Arousal Samples**

| Sample | Image | Total Protein Conc. (mg/ml) | Signal | Angptl2 Conc. (ug/mL) | % Angptl2 of Total Protein Conc. |
|---|---|---|---|---|---|
| IBA125 | G | 119.01 | 193000 | 32.72 | 0.0275 |
| IBA128 | I | 74.18 | 281000 | 34.33 | 0.0463 |
| IBA129 | G | 83.71 | 707000 | 42.71 | 0.0510 |
| IBA175 | Average | 101.93 | | 35.94 | 0.0353 |
| IBA176 | G | 103.02 | 1370000 | 47.80 | 0.0464 |
| IBA178 | G | 102.21 | 2110000 | 51.12 | 0.0500 |
| IBA179 | I | 81.40 | 244000 | 33.24 | 0.0408 |
| C164, 1/13 | E | 80.61 | 2120000 | 45.53 | 0.0565 |
| C165, 3/13 | I | 77.69 | 490000 | 38.61 | 0.0497 |

**Post Arousal Samples**

| Sample | Image | Total Protein Conc. (mg/ml) | Signal | Angptl2 Conc. (ug/mL) | % Angptl2 of Total Protein Conc. |
|---|---|---|---|---|---|
| PA134 | Average | 121.31 | | 33.19 | 0.0274 |
| PA135 | Average | 69.48 | | 34.47 | 0.0496 |
| PA136 | A | 81.42 | 1590000 | 38.13 | 0.0468 |
| PA138 | Average | 133.38 | | 40.78 | 0.0306 |
| PA144 | H | 85.75 | 2930000 | 39.54 | 0.0461 |
| PA145 | H | 88.87 | 2270000 | 37.58 | 0.0423 |
| PA146 | K | 85.28 | 1010000 | 46.76 | 0.0548 |
| PA30 | M | 80.30 | 1170000 | 40.38 | 0.0503 |
| PA31 | A | 92.98 | 1290000 | 36.52 | 0.0393 |
| PA33 | K | 70.46 | 893000 | 45.82 | 0.0650 |