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INCREASED COMPLEMENT C3 EXPRESSION IN BONE MARROW OF TORPID 13-LINED GROUND SQUIRRELS IS NOT A MECHANISM FOR INCREASED IMMUNOLOGICAL PROTECTION DURING TORPOR

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INCREASED COMPLEMENT COMPONENT C3 EXPRESSION IN THE BONE MARROW OF TORPID 13-LINED GROUND SQUIRRELS IS NOT A MECHANISM FOR INCREASED IMMUNOLOGICAL PROTECTION DURING TORPOR

By Garrett Schuh

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ABSTRACT

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Thirteen-lined ground squirrels enter torpor during winter months, characterized by decreased metabolism and decreased immune function. During torpor white blood cells almost completely disappear from circulation, and are able to return to normal levels within 2 hours of arousal. It has been recently discovered that the transcription of numerous proteins within the complement system are upregulated in the bone marrow during torpor. The complement system is part of the innate immune system and aids in killing infectious bacteria and viruses. A chemiluminescent western blot was developed to measure and compare C3 levels in the plasma of ground squirrels. Ground squirrel plasma samples were compared to determine if C3 protein levels increase during torpor. Quantitative PCR and immunohistochemistry were also used to measure complement C3 transcription and translation during torpor in tissues other than the bone marrow. The ability of the complement pathway to lyse *Escherichia. coli* cells at both summer active and torpid body temperature was assayed. There was no difference in the plasma concentration of C3 between hibernal stages. Complement C3 transcription was not increased during torpor in liver and adipose. Torpid ground squirrel serum complement was unable to lyse bacterial cells at 37 ˚C.

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INTRODUCTION

General Information

The 13-lined ground squirrel (*Ictidomys tridecemilneatus*) is a mammal that employs the seasonal adaptation of going through bouts of torpor during times of harsh weather conditions and scarce food availability. During the hibernation season ground squirrels go through some major physiological changes, including accumulation of adipose tissue, and reduction in metabolism, heart rate, and body temperature (Bouma, Carey, & Kroese, 2010). In torpor, ground squirrels also experience marked decreases in both adaptive and innate immune system function. There is an extreme reduction in adaptive immunity manifesting as reduction in the levels of circulating white blood cells. Torpor also brings about suppression of the innate immune system, especially reduction in circulating neutrophils and monocytes. It has been widely described that there is a general suppression of immunological function but some recent studies have revealed that there may be some immunologic functions that are upregulated during torpor. Transcription of many of the innate immune system proteins involved in the complement cascade, especially complement component C3, are upregulated in the bone marrow during torpor (S. T. Cooper et al., 2016). This contradicts the overall trend towards immune suppression in hibernation. The aim of this project is to investigate whether an increase in complement transcription in bone marrow leads to an increase in protein level in the blood. Additional aims are to measure if there is an increase in complement activity during torpor, and to investigate a potential role in lipid metabolism that complement

may have in torpor. Recent evidence suggests a regulatory connection between the innate immune system and fat metabolism.

Hibernation

Torpor is a seasonal adaptation that many endotherms employ to survive long periods of frigid temperatures or scarce food availability. Periods of torpor are often interrupted by periods of normothermic activity known as interbout arousals (IBA) (Bouma et al., 2010). Periods of IBA occur frequently throughout the hibernation season and usually last no more than 24 hours (Figure 1). During torpor, body temperature is maintained a few degrees Celsius above ambient temperature (Carey, Andrews, & Martin, 2003). Along with a lowered body temperature, hibernators also reduce basal metabolic rates to two to four percent of euthermic levels (Boyer & Barnes, 1999). Reduction in basal metabolic rates of hibernators also brings about a decrease in oxygen consumption to two percent of the active state. During torpor the heart rate of hibernating mammals has been observed to drop as low as four to six beats per minute (Spurrier & Dawe, 1973). Accompanying a reduced heart rate is a reduced blood flow. It is currently not known how blood can flow so slowly during torpor and not clot. Whereas all of the previously stated changes that occur during torpor have been well documented, another less well documented change that occurs during torpor is a decrease in immune system function (Bouma et al., 2010).

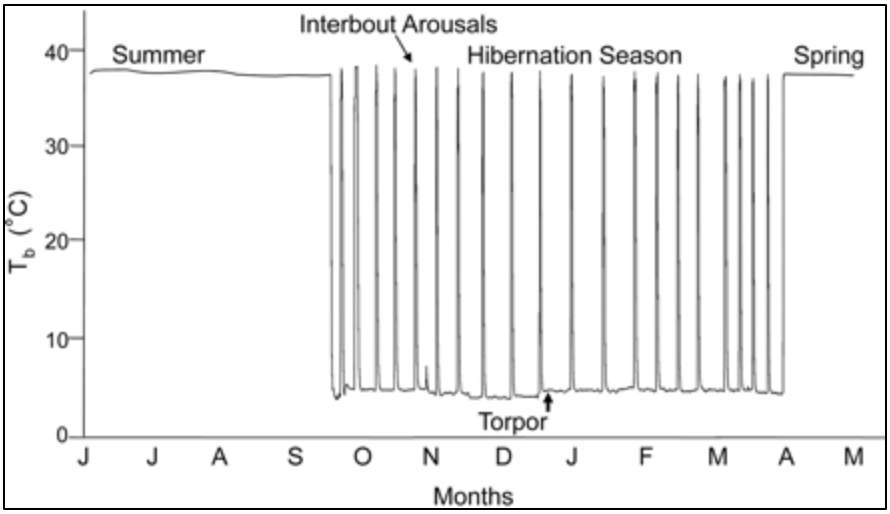

Figure 1. The hibernal cycle of 13-lined ground squirrels tracked by body temperature. Animals enter torpor in late September or early October and torpor bouts are only interrupted briefly by bouts of arousal that usually last no more than 24 hours. IBA become more frequent toward the end of torpor. Animals come out of torpor for the summer around the start of April. (Bouma et al., 2010)

Many species employ the seasonal adaptation of hibernation, and there is one species in particular that has been used as a model system to study hibernation, 13-lined ground squirrel, *(I. tridecemilneatus*). The 13-lined ground squirrel is a small rodent that enters torpor every year around October and will remain in torpor until April. This period of torpor is interrupted by multiple brief IBAs. Hibernating 13-lined ground squirrels experience a dramatic disappearance of leukocytes and platelets from the blood circulation (Bouma et al., 2010). Research suggests that platelets and many leukocytes may be sequestered in the spleen during hibernation (Yasuma, McCarron, Spatz, & Hallenbeck, 1997). Splenectomy experiments done on hibernating squirrels do not support the theory that leukocytes are being sequestered solely in the spleen (S. Cooper et al., 2014). Although there are conflicting ideas of where circulating leukocytes are

sequestered during torpor it is clear that there are dramatic changes in both adaptive and innate immune system function during torpor.

Immune System Changes during Hibernation

Adaptive Immune System Changes during Hibernation

Torpor comes with a marked reduction in adaptive immunity. The adaptive immune system is composed of the cell mediated and humoral branches. The cell mediated branch is composed of T-lymphocytes while the humoral branch creates antibodies through the action of B-lymphocytes. In torpor there is a drastic reduction in circulating T and B-lymphocytes (Bouma et al., 2010). Little is known about the mechanism by which circulating lymphocytes decrease in numbers so greatly during torpor and are able to return to 50% of summer levels during bouts of IBA. The reappearance of circulating lymphocytes during IBA is not likely due to the slower generation of new lymphocytes (Bouma et al., 2010). T-lymphocyte generation from the thymus at a rate of $1x10^5$ lymphocytes per day is much slow than the generation of neutrophils, $2x10^7$ neutrophils per day, which return to 95% of euthermic levels during arousals (Bouma et al., 2011). If new production of lymphocytes does not account for the rise in circulating lymphocytes, it has been suggested that the lymphocytes must be sequestered in a secondary lymphoid organ during torpor (Bouma et al., 2010). A decrease in T-lymphocyte numbers during torpor is at least partly attributed to a reduction in proliferation of developing lymphocytes in the thymus due to increased concentrations of released 5'-adenosine monophosphate from brown adipose tissue (Atanassov et al., 1995). With a reduction in both T and B-lymphocytes the adaptive immune response of the hibernating animal would be severely compromised in its ability

to mount both cell mediated/cytotoxic and a humoral/antibody responses. Torpor not only brings about changes in the adaptive immune system but it also brings about changes to the innate immune system.

Cellular Innate Immune System Changes during Hibernation

It has been widely observed that circulating leukocytes drop by 90% during torpor in 13-lined ground squirrels (Frerichs, Kennedy, Sokoloff, & Hallenbeck, 1994). Leukopenia includes the circulating granulocytes (neutrophils, eosinophils, and basophils) and monocytes (Szilagyi & Senturia, 1972). The leukocytes remaining in circulation during hibernation are mainly neutrophils (90%) with some lymphocytes (10%) (Bouma et al., 2010). During periods of IBA the levels of circulating neutrophils and monocytes rapidly increase to levels that are roughly equivalent to an animal during the summer months (Suomalainen & Rosokivi, 1973). In contrast lymphocytes only increase to 50% of summer levels during IBA (Suomalainen & Rosokivi, 1973).

The innate immune system is comprised of many cellular components including monocytes, neutrophils, mast cells, dendritic cells, natural killer cells. Suppression of the innate immune cells occurs in part through the induction of leukopenia. The most profoundly affected leukocytes are neutrophils and monocytes. Mature neutrophils are observed to have a larger drop during torpor (about six-fold) compared to that observed in immature neutrophils (about 1.5 fold) (Inkovaara & Suomalainen, 1973). It is possible that the reduction in mature neutrophils is due to apoptosis but it is more likely that these cells are retained in organs such as the lungs, liver, or spleen (Inkovaara & Suomalainen, 1973). During IBA, levels of neutrophils increase rapidly possibly by increased maturation of neutrophilic band cells or the release of sequestered neutrophils (Inkovaara

& Suomalainen, 1973; Yasuma et al., 1997). Recently neutrophils have been observed to be sequestered via margination (Bouma et al., 2013). Monocyte levels also drop dramatically during torpor and rise to euthermic levels upon arousal (Suomalainen & Rosokivi, 1973). Mast cells are permanent residents of tissues, and have been observed to increase in numbers in hibernating hedgehogs (HÄRmÄ & Suomalainen, 1951). Elevated mast cell numbers could be beneficial to help defend an immunocompromised hibernator against pathogens or could reflect decreased regulation by T regulatory cells during torpor. One main consequence of suppression of the innate immune system is a decreased phagocytic capacity of cells in the blood as seen by diminished number of phagocytic cells during torpor (Bouma et al., 2010; Maniero, 2002).

Protein Innate Immune System Changes during Hibernation

Innate immune system proteins include complement, toll-like receptors, and defensins. Complement activity in the blood of golden-mantled ground squirrels was reported by Maniero to be diminished during torpor and restored to euthermic levels upon arousal (Maniero, 2002). Complement activity was measured by the ability of serum to lyse antibody labelled chicken erythrocytes, through activation of the classical pathway of the complement cascade. Maniero found that euthermic animals (summer and IBA) had significantly more classical complement activity (65.33 CH_{50}/ml ± 5.17) than animals that were at any stage of hibernation (44.33 CH_{50}/ml ± 4.05) (Maniero, 2002). RNA transcripts of complement component C3 have been shown to decrease in liver samples during torpor potentially accounting for the apparent decrease in circulating complement protein (Maniero, 2002). However, RNA transcripts of C3 in bone marrow samples of 13-lined ground squirrels increase significantly during torpor, suggesting that

the bone marrow may play a role in maintaining C3 levels during torpor (S. T. Cooper et al., 2016).

Complement System

The innate immune system plays a critical role in clearing invading microbes via the recognition of pathogen-associated molecular patterns (Venkatraman Girija et al., 2013). A major component of the innate immune system is complement. The complement system comprises more than 30 serum proteins produced by cells in the liver, bone marrow, adipose, kidney, and astrocytes (James E. Marsh, 2001). Complement acts to clear infections by triggering the opsonization and lysis of invading cells (Ricklin, Hajishengallis, Yang, & Lambris, 2010). Complement also indirectly acts to clear infections by stimulating inflammatory and adaptive immune responses (Ricklin et al., 2010). In order for complement to fight an infection it first needs to be activated. Complement can be activated in three ways: the classical pathway, the alternative pathway, or the lectin pathway (Wallis, Mitchell, Schmid, Schwaeble, & Keeble, 2010) (Kindt, Goldsby, Osborne, & Kuby, 2007) (Figure 2). The three pathways differ only in how they form the enzyme C5 convertase. The lytic membrane attack complex is then formed the same way among each of the three pathways (Kindt et al., 2007).

Classical Pathway

The activation of the classical complement pathway is antibody-dependent (Gu, Jenkins, Xue, & Xu, 2012) (Figure 2). Binding of C1q to an antibody-antigen complex starts the complement cascade. Several classes of antibodies can activate the classical pathway including IgM, IgG1, IgG2, and IgG3 (Kindt et al., 2007). After C1q binds to an antibody-antigen complex, two C1r molecules bind to the C1q molecule and become

active. Active C1r molecules cleave two C1s molecules into their active state. C1r and C1s together become $C1r^2s^2$. This new complex now cleaves C2 and C4 into the split products C2a, C2b, C4a, and C4b. Split products C2a and C4b bind together to form the enzyme C3-convertase. The role of C2b after its release is not currently known but it has been hypothesized that C2b may enhance vascular permeability (Krishnan, Xu, Macon, Volanakis, & Narayana, 2009). The newly formed C3-convertase then cleaves C3 into C3a and C3b. Complement component C3b then binds to C3-convertase to create the newly formed C5-convertase. The split product of the cleavage of C3, C3a is released. Binding of C3a to the C3a receptor on mast cells causes mast cell degranulation, thus furthering inflammation by histamine release (Klos, Wende, Wareham, & Monk, 2013).

Membrane Attack Complex

All three of the pathways of complement activation result in the creation of C5-convertase. Once C5-convertase has been formed all three pathways create the membrane attack complex in exactly the same way (Figure 2). C5-convertase will work to split C5 into C5a and C5b. Split product C5a goes on to elicit a number of immunological effects to help clearance of a pathogen that include: chemotaxis of inflammatory cells, phagocytosis, cytokine and chemokine release (Manthey, Woodruff, Taylor, & Monk, 2009). While C5a elicits immune responses in different ways C5b is an essential cog in the membrane attack complex. On the surface of a microbe, complement component C5b binds to C6 creating C5b6 complex which will go on to bind to C7 and create C5b67. Newly formed C5b67 will then bind both C8 and C9 to create the fully functional membrane attack complex, which forms transmembrane channels to lyse invading microbes.

Alternative Pathway

The alternative pathway for complement activation differs significantly from the classical pathway (Figure 2). One such main difference is that the alternative pathway does not require an antibody-antigen complex for activation (Segers et al., 2014). The alternative pathway is initiated after complement C3 is spontaneously cleaved and binds to cell surface elements that are foreign to the host (Kindt et al., 2007). Complement component C3 is spontaneously cleaved in serum to C3a and C3b. When C3b attaches to the surface of an invading microbe it can now attract a protein called factor B. After factor B binds to C3b, enzymatically active factor D cleaves factor B into two fragments; Bb and Ba. Fragment Bb stays connected to C3b to create C3bBb, which is analogous in function to the C3-convertase protein created during the classical pathway (Tortajada, Torreira, Montes, Llorca, & Córdoba, 2009). The smaller fragment Ba's function is not fully understood but it has been documented that Ba can interact with C3b in a specific manner (Pryzdial & Isenman, 1987). Newly formed C3-convertase can now activate unhydrolyzed C3 to produce more C3b, which will be incorporated into the C3-convertase to create C3bBb3b which is analogous to C5-convertase. From the C5-convertase the formation of the membrane attack complex is the same as the classical pathway.

Lectin Pathway

The third distinct pathway for complement activation is the lectin pathway. Lectin pathway initiation is accomplished by binding of lectins, which are proteins that recognize specific carbohydrate targets (Kindt et al., 2007). When a mannose-binding lectin (MBL) protein binds to a mannose residue on an invading microbe the complement

cascade will be initiated (Figure 2). Binding of a MBL will activate MBL-associated serine proteases 1 and 2 (MASP-1 and MASP-2). Formation of C3-convertase requires both MASP-1 and MASP-2, since MASP-1 only has the ability to cleave C2 into C2a and C2b and MASP-2 has the capability to cleave both C2 and C4 into their respective split products (Dobó et al., 2011). Once C2 and C4 have been cleaved into the split products C2a and C4b they come together to form C3-convertase. This process is similar to the formation of C3-convertase during the classical pathway of complement activation, and C3-convertase acts in the same way to cleave C3 into C3a and C3b. Much like the classical pathway C3b will then combine with C3-convertase to create C5-convertase. From the C5-convertase the formation of the membrane attack complex is the same as the classical pathway.

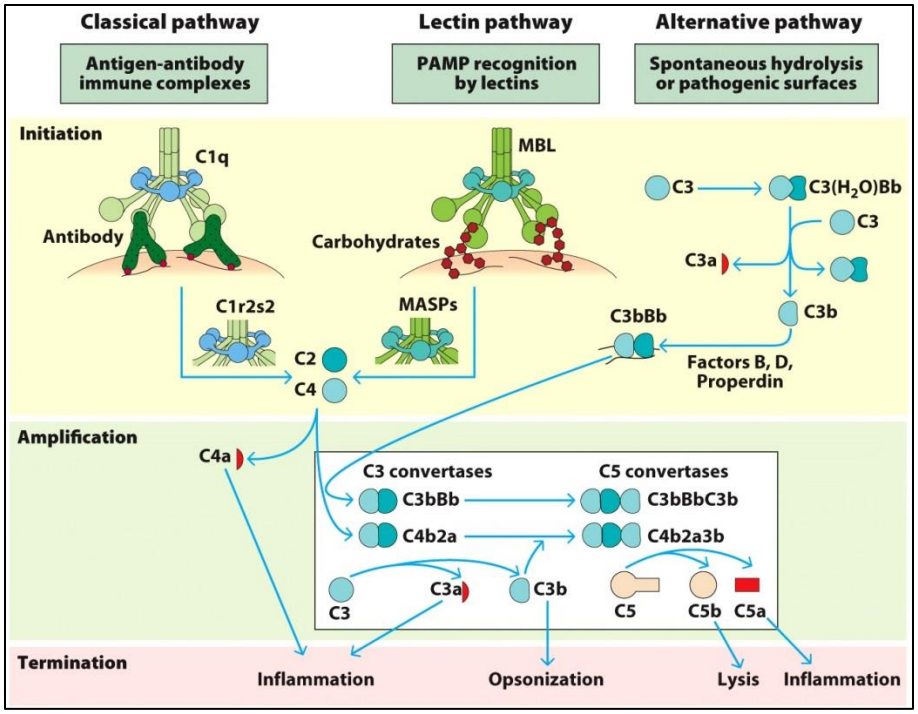

Figure 2. A summary of complement activation. Complement can be activated in one of three mechanisms that all converge at the membrane attack complex. Split products of complement also play a role in inflammation, opsonization, and lysis (Barbu, Hamad, Lind, Ekdahl, & Nilsson, 2015; Kindt et al., 2007).

Complement is often referred to as a first line of defense against an invading pathogenic microbe. This important aspect of the innate immune system could be even more important in hibernating animals than once previously thought. It has been well documented that the adaptive immune system is suppressed during torpor, potentially leaving the innate immune system and in particular complement as the primary line of defense (Bouma et al., 2010). An increase in complement components, as seen by the transcriptome data (S. T. Cooper et al., 2016), could be essential in fending off infections in immunosuppressed hibernating 13-lined ground squirrels. Another potential role for the increase in complement is that complement could potentially be involved in the leukopenia observed during torpor. Since complement split products are chemotactic for

polymorphonuclear leukocytes and monocytes the increase in complement levels may be a pivotal component to initiate leukopenia. It is clear that a further understanding of complement function during torpor will lead to a clearer understanding of the role that it plays in 13-lined ground squirrels during periods of immunosuppression during torpor.

Complement Component C3

Arguably the most important protein in the complement cascade is complement C3. Complement C3 can interact in a specific manner with up to 25 other proteins and is essential in the activation of all three pathways of complement activation (Sahu & Lambris, 2001). Human complement C3 is composed of two chains; an α-chain that is 110 kDa and a β-chain that is 75 kDa (Sahu & Lambris, 2001). Thirteen-lined ground squirrel complement C3 is of similar makeup (Sahu & Lambris, 2001). The alpha and beta chains are linked together by a single disulfide bond (Sahu & Lambris, 2001). When bound together C3α and β chains have a molecular mass of approximately 190,000 Daltons, based on the primary protein sequence. C3 is present in serum at an average concentration of 1.2 mg/ml in humans (Sahu & Lambris, 2001). The beta chain of complement C3 is capable of affecting many aspects of immune response. The attachment of C3b to acceptor molecules facilitates phagocytosis of foreign particles, elimination of self-reactive B cells, enhancement of humoral responses to antigens, and the formation of the membrane attack complex (Sahu & Lambris, 2001). Complement C3 is quite a mysterious protein with diverse functions and may be a crucial link between the innate complement system and B cell response that could help to trigger the overall acquired immune response (Sahu & Lambris, 2001).

Complement components are degraded over time and complement component C3 is no exception to this (Thurman et al.). Elucidating the pathway of complement C3 degradation will help to identify which protein subunits are identified using western blots (Figure 3). The whole protein, C3, has a molecular mass of 190 kDa, made up of two distinct domains: the alpha domain is 115 kDa and the beta domain is 75 kDa (Thurman et al.). Upon the first degradation C3 splits into C3a (9 kDa) and C3b (176 kDa). Complement component C3b then undergoes further degradation into C3f (2 kDa) and iC3b (177 kDa) (Thurman et al.). The larger iC3b consists of three distinct chains; the beta chain (75 kDa), the alpha one chain (63 kDa), and the alpha two chain (39 kDa) (Thurman et al.). The alpha one chain can then be further degraded and C3dg (39 kDa) which can be cleaved by tryptic enzymes into fragments C3d (34 kDa) and C3g (4 kDa) (Thurman et al.). After the alpha one chain of iC3b is degraded iC3b becomes C3c (136.5 kDa) and the alpha one chain is left at the new size of 22.5 kDa (Thurman et al.). Knowing this degradation pathway and the different degradation products will aid in the identification of which products are bound by any antibodies.

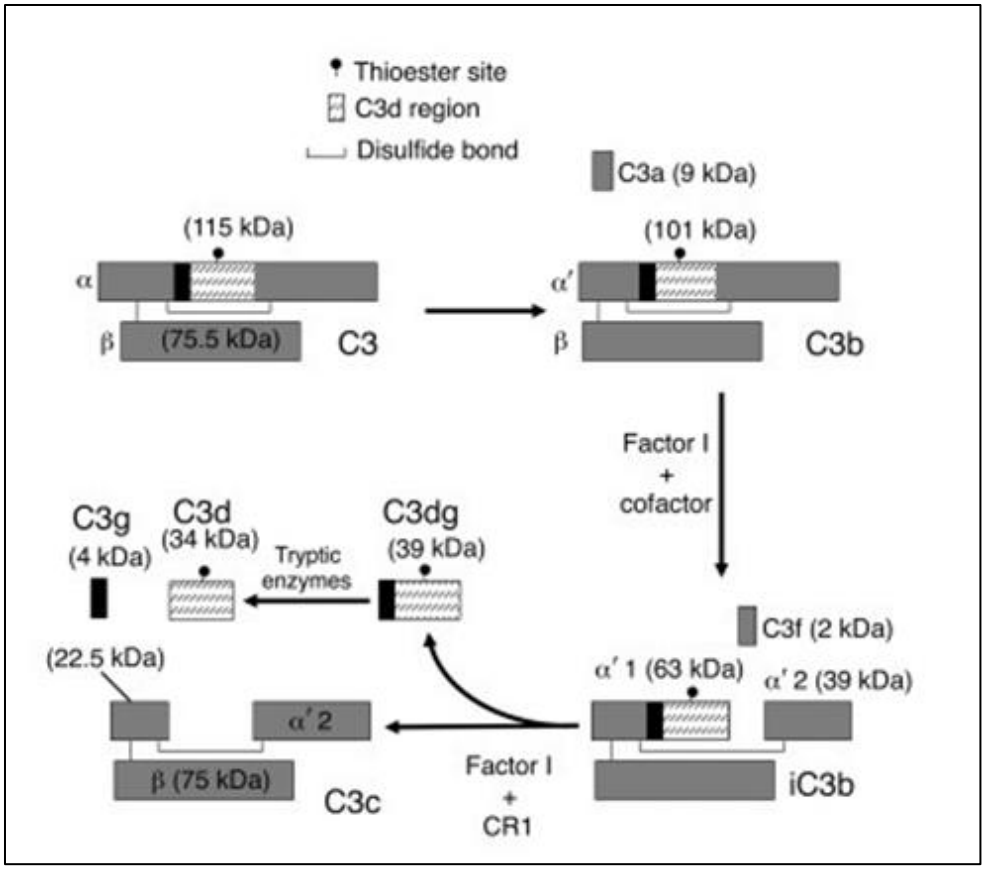

Figure 3. A summary of the degradation of complement component C3.Complement proteins are degraded over time leaving degradation products with distinct molecular masses. These different fragments of complement proteins can be detected with antibodies using a western blot (Thurman et al.).

Complement Regulatory Proteins

There are times when complement pathway activity needs to be suppressed to prevent collateral damage to host cells. To regulate the complement pathway there are numerous regulatory proteins. Complement regulatory proteins fall into two classes; fluid-phase regulatory proteins and membrane-bound regulatory proteins (Meri & Jarva, 2001). In the fluid-phase regulatory protein category is factor H. Factor H works by inhibiting the C3 convertase of the alternative pathway by competing with factor B for binding to C3 convertase (Meri & Jarva, 2001). Similar to factor H, C4b binding protein

also inhibits the C3 convertase of the classical pathway (Meri & Jarva, 2001). Clusterin is yet another fluid-phase regulatory protein. Clusterin inhibits the lytic actions of complement by binding to the terminal complement protein, C9, and preventing the insertion of the membrane attack complex into the cell membrane (Meri & Jarva, 2001).

There are also many complement regulatory proteins that are membrane bound. Among membrane-bound regulatory proteins decay accelerating factor (DAF) can be found on the membranes of peripheral blood cells, vascular endothelial cells, and numerous other types of epithelial cells. Decay accelerating factor regulates complement activity by binding to and dissociating both the alternative and classical pathway C3 and C5 convertase (Meri & Jarva, 2001). Membrane cofactor protein (CD46) is also a major player in the membrane-bound regulation of complement activation. CD46 is present on all circulating in the blood stream with the exception of erythrocytes. CD46 regulates complement function by binding to and aiding in the cleavage of C3b (Meri & Jarva, 2001). Expressed on all circulating cells, protectin (CD59) regulates complement activation by inhibiting C9 from binding to the C5b-8 complex and preventing assembly of a fully functional membrane attack complex (Meri & Jarva, 2001).

Complement Proteins and Lipid Metabolism

Besides the complement cascade's function in defending the 13-lined ground squirrel against infectious agents, such as pathogenic bacteria, viruses, and pathogenic fungi complement may also play a dual role in lipid metabolism, energy storage and insulin resistance during torpor (Harboe, Thorgersen, & Mollnes, 2011). As more research is being done on the complement system more complement proteins are being added to the list of proteins that affect lipid metabolism such as C3a, acylation

stimulating protein (ASP), properdin, adipsin, factor H, and C3aR (Gauvreau et al., 2012). Complement C3a is produced in adipocytes and acts as a potent anaphylatoxin, however when C3a is processed into ASP it serves as a positive regulator of triglyceride synthesis (Schäffler & Schölmerich, 2010). Acylation-stimulating protein acts as a co-factor to diacyl-glycerol-acyl-transferase, which is the enzyme that catalyzes the final and rate limiting step of triglyceride synthesis (Schäffler & Schölmerich, 2010). The C3a receptor, C3aR on macrophages and adipocytes, is also involved lipid metabolism (Schäffler & Schölmerich, 2010). When C3a binds to C3aR it stimulates the production of triglycerides, as illustrated by C3a null mice that are protected from diet-induced obesity (Schäffler & Schölmerich, 2010). Out of all the proteins involved in the complement cascade, complement factor properdin appears to have the most effect on lipid metabolism. Complement factor properdin is a protein that stabilizes C3 convertase (C3bBb) following alternative complement activation and can be found on adipocyte cell surface membranes (Gauvreau et al., 2012). The well-known function of properdin is to protect C3b from catalysis by complement regulator factors H and I, thus promoting the stabilization of C3 convertase (Kemper & Hourcade, 2008). Another complement protein that has duality in its function is complement factor H. Besides the role of complement factor H in ensuring that the complement cascade is only activated by potentially harmful pathogens, a role in lipid metabolism has been reported (Pangburn, 2000). Complement factor H was recently described in humans as being secreted from adipose tissue and concentration of complement factor H was found to be associated with insulin resistance, which would impair the uptake of glucose into adipose tissue (Moreno-Navarrete et al., 2010). With all of these complement proteins having dual functions in both innate

immunity and lipid metabolism in mice and humans, there is potential for the proteins, especially C3, to play a pivotal role in lipid metabolism during torpor in 13-lined ground squirrels.

Transcriptome Data

Previous liver C3 mRNA measurements by Maniero suggested that complement gene transcription is down regulated during torpor in golden-mantled ground squirrels. In contrast, in the bone marrow, many components of complement appear to be up regulated during torpor (Figure 4) (S. T. Cooper et al., 2016). Complement mRNA in the bone marrow sample is also higher during IBA than summer, but IBA levels are lower than what is seen during torpor (Figure 4). In particular C3 showed the highest increase from summer to torpor with a greater than five-fold increase (Figure 4A). Along with an increase in C3, the bone marrow transcriptome of torpid 13-lined ground squirrels showed an increase in various other components of the complement cascade upregulated less than C3 including C2, C1R, C1S, C1QA, C1QB, C1QC, and complement factor properdin (Figure 4B) (S. T. Cooper et al., 2016).

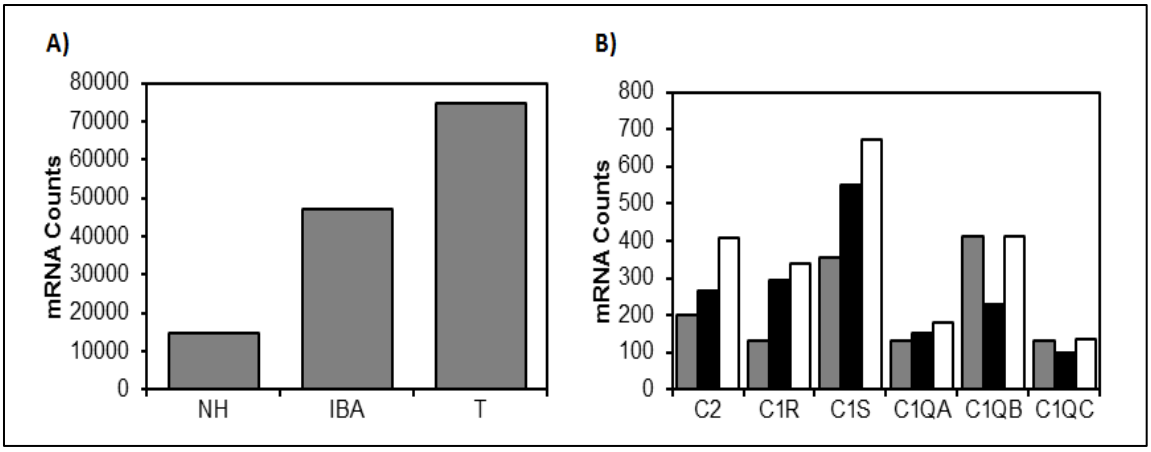

Figure 4. A) RNA transcription in bone marrow of 13-lined ground squirrels of complement component C3 was shown to jump from a summer (NH) level of 14,677 mRNA counts to 74,655 mRNA counts during torpor (T). This change is a 5-fold change. C3 counts were also significantly elevated during interbout arousal (IBA) at a level of 47,020. B) Transcriptome data from the bone marrow of 13-lined ground squirrels for proteins that are directly involved in the activation of the complement cascade. Messenger RNA counts were taken during the summer active period (gray), IBA (black), and during bouts of torpor (white).

Many of the complement pathway regulatory proteins, both fluid-phase and membrane-bound proteins, are differentially transcribed throughout the hibernal cycle (Figure 5). Of the fluid-phase regulatory proteins factor H and clusterin are upregulated during torpor (3-fold and 2.1-fold increase respectively) while C4b binding protein has a relatively constant expression. Conversely, expression of the membrane-bound regulatory protein CD46 is down regulated during torpor (1.9-fold decrease) while decay accelerating factor expression is up regulated during torpor and CD59 expression is relatively constant throughout the hibernal cycle.

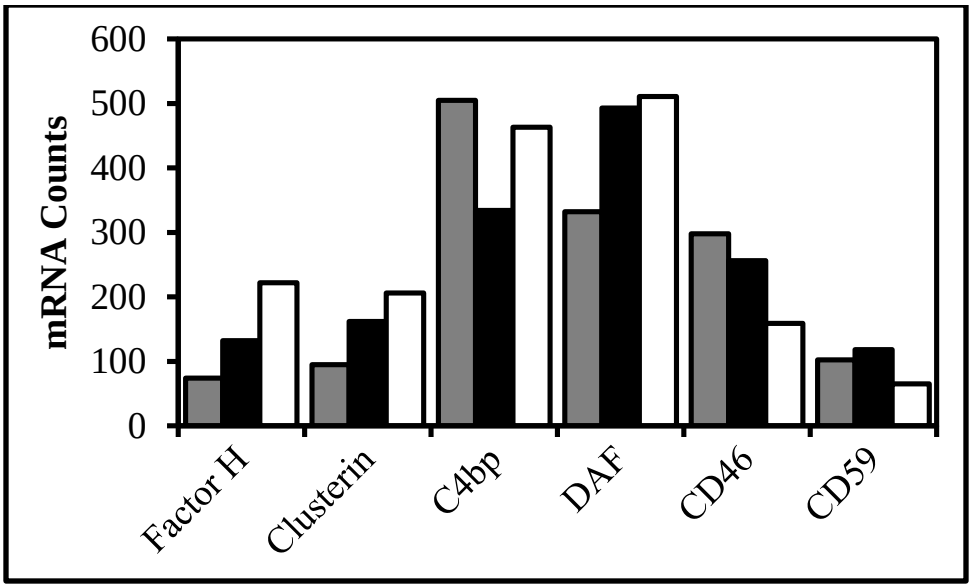

Figure 5. Transcriptome data from the bone marrow of 13-lined ground squirrels for complement pathway regulatory proteins, both fluid-phase and membrane-bound. Messenger RNA counts were taken during the summer active period (gray), IBA (black), and during bouts of torpor (white).

While numerous components of the complement cascade appear to be transcriptionally upregulated in bone marrow during torpor, there appears to be an up regulation of proteins indirectly related to complement including complement factor H-related 2 (CFHR2), C1q and tumor necrosis factor related protein 1 (C1QTNF1), complement component 5a receptor 1 (C5AR1), and complement regulatory protein (CD59) (Figure 6A) (S. T. Cooper et al., 2016). Plasma protein CFHR2 forms homodimers and binds to fragments of C3 to inhibit the formation of C3 convertase in the alternative pathway for complement activation (Eberhardt et al., 2013). It is curious that CFHR2 was seen to be upregulated along with components of the complement cascade given CFHR2's role in inhibiting complement activation. C1q and tumor necrosis factor related protein 1 is an adipokine that serves many functions in many different tissue types (Li et al., 2014). Functions of C1QTNF1 include: energy metabolism, inflammation, host

defense, apoptosis, cell differentiation, and organogenesis (Li et al., 2014). Complement component 5a receptor, a G-protein receptor, functions by binding the split product of complement activation C5a. Binding of C5a to C5AR1 induces inflammation (Rabiet, Huet, & Boulay, 2008). Up regulation of C5AR1 during torpor may help a hibernating 13-lined ground squirrel to induce inflammation more readily and thus provide greater protection from infections. Complement regulatory protein CD59 was the only complement associated protein to be seen to be down regulated during torpor (S. T. Cooper et al., 2016). CD59 is a regulatory protein which inhibits cell lysis by preventing the formation of the membrane attack complex (Tone et al., 1999). Less CD59 expression on host cells could make logical sense as it would allow complement to provide greater protection against infection. Complement factor properdin was also seen to be increased during torpor (Figure 6B). Since complement factor properdin has functions in lipid metabolism and stabilizing C3 convertase and increase in levels could help the animal metabolize the lipids it needs to survive times of scarce food availability while simultaneously stabilizing C3 convertase to more effectively ward off foreign agents (Gauvreau et al., 2012).

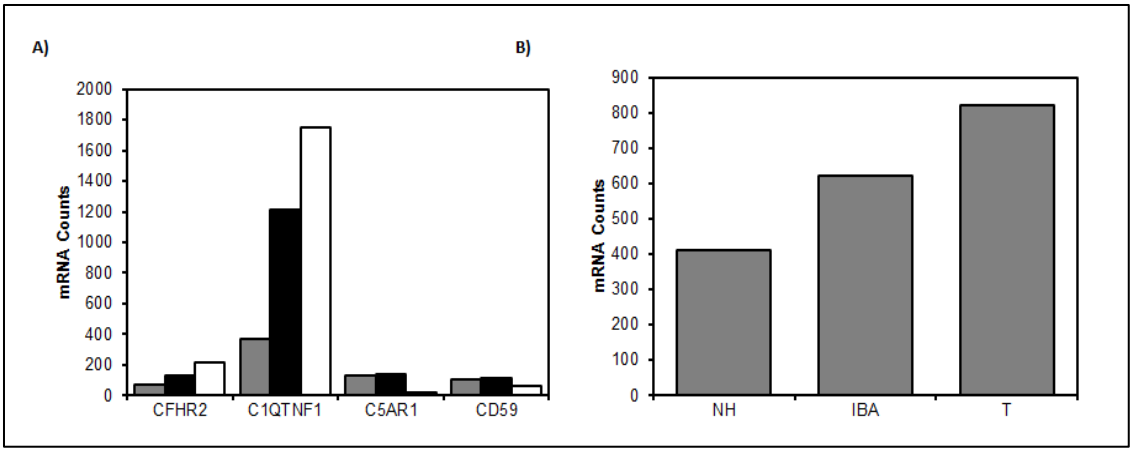

Figure 6. A) Transcriptome data collected from bone marrow samples of 13-lined ground squirrels measured proteins indirectly associated with the activation of the complement cascade. Transcriptome counts were measured during summer active periods (gray), IBA (black), and torpor (white). B) Complement factor properdin was shown to be elevated during torpor (T), 822 mRNA counts compared to levels during interbout arousal (IBA), 623 mRNA counts, and summer (NH), 413 mRNA counts.

Measuring Complement Concentration and Function

The concentration of soluble proteins, such as complement C3, can be assessed in a number of different ways but the most commonly used are an enzyme linked immunosorbent assay (ELISA), a semi-quantitative western blot, or an immunoprecipitation assay (Kirschfink & Mollnes, 2003). A capture ELISA would be a better way to get a more accurate picture of the protein concentration of C3 but it would requires two monoclonal antibodies against the targeted protein. A semi-quantitative western blot would only give a rough estimate as to what the targeted proteins concentration would be, but is more readily usable as it only would require one monoclonal antibody against the targeted protein. An immunoprecipitation assay such as a radial immunodiffusion assay could also be used to determine protein concentration and again would require one monoclonal antibody against the targeted protein.

While there are numerous ways in which the concentration of complement C3 could be elucidated there are a limited number of assays in which to assess the functional capabilities of complement C3. The most widely encountered assay to measure complement function is the CH50 assay. The CH50 is used to assess classical pathway activation and is based on the use of antibody coated sheep erythrocytes which are lysed by the complement cascade (Mollnes et al., 2007). Results for the CH50 assay are reported as the reciprocal dilution of serum required to achieve 50% lysis of a known amount of erythrocytes. To assess the function of the alternative pathway a similar assay exists, the AH50 assay. The AH50 assay is done using rabbit erythrocytes which activate the alternative complement pathway when the serum is diluted in a buffer containing Mg^{2+} ions while Ca^{2+} ions are chelated with ethylene glycol tetraacetic acid (EGTA) to block the activation of the classical and lectin pathways (Mollnes et al., 2007). More modern techniques which can quantify complement derived split products are used to show that the complement system has been activated. These newer techniques take advantage of the advances in monoclonal antibody production and use monoclonal antibodies that have specific binding sites on complement component activation products (Mollnes et al., 2007). These assays will then only detect complement proteins after the complement system has been activated.

RATIONALE AND MAIN GOALS

Rationale

Increased transcription of complement genes, especially C3, in the bone marrow of torpid 13-lined ground squirrels suggests that complement has an important role in adaptation to hibernating conditions in this species. This assumes that RNA transcripts of C3 are being translated into protein. Two main roles reported for complement are as an innate immune system effector and as a recently discovered facilitator of lipid metabolism (Barbu et al., 2015; Kindt et al., 2007). The increase in complement transcription in torpid 13-lined ground squirrels is intriguing given that the majority of studies have shown suppression in immune system function during hibernation, while complement C3 transcript levels, at least in the bone marrow, suggest a potential increase in complement function during hibernation.

The main goals of this study were; (1) To determine if there is also an increase in transcription of complement component C3 in liver and adipose tissue during torpor. (2) To determine if an increase in complement C3 transcription in the bone marrow of torpid 13-lined ground squirrels is reflected by increased C3 protein in the blood of torpid squirrels. (3) To determine if C3 protein levels in the blood of non-hibernating and torpid ground squirrels correlate with the degree of complement-mediated killing of bacteria. (4) Assay 13-lined ground squirrel plasma to determine if it is a possibility that the classical complement pathway is activated or whether just the alternative and lectin pathways are

activated. (5) Look for increased presence of C3 protein localization in liver, adipose tissue and bone marrow of hibernating 13-lined ground squirrels.

Strategy

To meet these goals (1) quantitative polymerase chain reaction was used to determine the level of transcription of complement component C3 relative to a reference gene (GAPDH) in liver tissue. (2) C3 protein in the blood was detected using a polyclonal antibody confirmed by Western blot to specifically bind to 13-lined ground squirrel C3. C3 protein levels were measured in the blood of torpid, IBA, and non-hibernating squirrels using a quantitative western blot. (3) The ability of serum complement to lyse *E. coli* bacterial cells at physiological temperature in torpid, IBA, and non-hibernating squirrels was measured. Since torpid 13-lined ground squirrel body temperature is 4˚C serum from torpid ground squirrels and summer was tested at this temperature. (4) Flow cytometry was used to assay 13-lined ground squirrel plasma for the presence of antibodies specific for *E. coli* cells. (5) Adipose tissue was stained with the 13-lined ground squirrel C3 specific antibody to look for C3 protein in liver, adipose tissue and bone marrow during hibernation. All of this data together will broaden the picture of complement function in hibernating 13-lined ground squirrels.

MATERIALS AND METHODS

Thirteen-Lined Ground Squirrel Sample Collection

A colony of 13-lined ground squirrels has been established at the University of Wisconsin at La Crosse. The colony includes squirrels that were caught in the wild and squirrels that were bred at the facility. Squirrels have been sacrificed throughout the hibernal cycle; non-hibernators (spring and summer), entering hibernation, torpid animals, during IBAs, and post-arousal animals (2 hours to 1 week post arousal). Squirrels were weighed before being sacrificed. Non-hibernators were euthanized via carbon dioxide suffocation followed by exsanguination, while torpid squirrels were euthanized via cervical dislocation followed by exsanguination. Samples of subcutaneous adipose tissue from the abdomen were taken and placed on dry ice. Tissue sections were placed into a cyro mold and immersed in frozen tissue embedding medium (Fisher Chemical, Fair Lawn, NJ) before being flash frozen in 2-methylbutane immersed in liquid nitrogen. Flash frozen tissues were then placed at -80˚C for long term storage. Serum and plasma samples were collected from each of the 40 squirrels sacrificed (10 entering hibernation, 10 torpid, 10 IBA, 10 post arousal, and 10 non-hibernators) and stored at -20˚C. Plasma was anti-coagulated with acid-citrate-dextrose (ACD).

Quantitative Polymerase Chain Reaction

The absolutely RNA miniprep kit (Agilent Technologies, Santa Clara, CA) protocol was followed to isolate RNA from 13-lined ground squirrel liver samples. Livers from 5 non-hibernating, 6 IBA, and 6 hibernating 13-lined ground squirrels were analyzed. After RNA isolation was complete the concentration of RNA was assayed on a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Rockford, IL). The affinity script qPCR cDNA synthesis kit (Lamda Biotech, St. Louis, MO) protocol was followed to synthesize cDNA from the previously isolated RNA samples using a consistent amount of RNA in each reaction. Complementary DNA synthesis reactions were set up so that each reaction had an equal concentration of cDNA. The thermocycler was set at 25˚C for 5 minutes to allow primer annealing, followed by 42˚C for 15 minutes to allow cDNA synthesis, and 95˚C for 5 minutes to terminate the cDNA synthesis reaction. All cDNA samples were stored at -20˚C until qPCR was performed.

Real time qPCR reactions were set up using EvaGreen qPCR Mastermix (MidSci, Valley Park, MO). One reaction was set up with primers for complement C3 and a control was set up with primers for glyceraldehyde phosphate dehydrogenase (GAPDH). Glyceraldehyde phosphate dehydrogenase was chosen for the reference gene based on observations that GAPDH has stable expression in liver tissue throughout the hibernal cycle (Otis, Ackermann, Denning, & Carey, 2010). Commercially synthesized forward and reverse primers (Integrated DNA Technologies, Coralville, IA) were added at a concentration of 100 nM. Each reaction then received cDNA that was previously synthesized from isolated 13-lined ground squirrel RNA, along with nuclease free water and a 2X master mix to bring the total reaction volume to 20 µl. The thermocycler was

set for 3 minutes at 95˚C followed by 50 cycles of 95˚C for 10 seconds, 55˚C for 30 seconds, and 72˚C for 30 seconds and then ended by heating the reactions up to 95˚C. Relative gene expression of complement component C3 compared to the reference gene GAPDH was calculated (equation 1). Each 13-lined ground squirrel liver sample was run four times.

$$(1) \quad \text{Relative Gene Expression} = \frac{2^{CP-GAPDH}}{2^{CP-C3}}$$

RNA Isolation from Adipose Tissue

The isolation of RNA from the adipose tissue of 13-lined ground squirrels was done using a protocol adapted from one used to isolate RNA from bone marrow. Flash-frozen adipose tissue (50-100 mg) was placed into TRI reagent (Thermo Scientific, Rockford, IL). The adipose tissue was incubated for 5 minutes in TRI reagent before 100 µl of 1-bromo-3-chloropropane per 1 ml of TRI reagent was added, mixed and incubated for 15 minutes. After incubation the tube was centrifuged for 15 minutes at 1400 rpm. The aqueous phase was transferred into a new tube containing 600 µl of buffer RLT from a Qiagen RNeasy Mini kit (Qiagen, Germantown, MD). The lysate was then centrifuged at max rpm for 3 minutes before the supernatant was removed and transferred into a new microcentrifuge tube. Next 500 µl of 70% ethanol was added to the supernatant and mixed well before 700 µl of this mixture was added to a RB column from a Total RNA Mini kit (IBI Scientific, Peosta, IA). The Total RNA Mini kit protocol was then followed beginning with step three (wash) and concluding with the end of step four (RNA elution). Complementary strand DNA synthesis and qPCR were then performed as previously

described. Relative gene expression was normalized to the stably expressed housekeeping gene GAPDH (Otis et al., 2010).

Chemiluminescent Western Blot to Detect Ground Squirrel C3

Confirmation of an Antibody Specific for Ground Squirrel C3

A commercial polyclonal goat anti-mouse complement component C3 antibody (MP Biomedicals, Solon, OH) was tested for specificity to 13-lined ground squirrel complement C3. The polyclonal goat anti-mouse complement C3 antibody had reactivity with mouse and since mouse and ground squirrel are phylogenetically close, there was a reasonable probability for the antibody to also be reactive to ground squirrel complement C3. Ground squirrel plasma and mouse plasma were electrophoresed on a 10% sodium dodecyl sulfate (SDS) polyacrylamide gel and transferred to a polyvinylidene fluoride (PVDF) membrane and probed with the goat anti-mouse complement C3 polyclonal antibody. Mouse plasma was included as a positive control as the antibody was known to have reactivity with mouse complement C3. Goat gamma globulin isotype control (Thermo Scientific, Rockford, IL) was used as a negative control.

Ground squirrel plasma was serially diluted (50, 40, 30, 20, 10 and 5 µl/ml) in sample buffer containing β-mercaptoethanol and mouse plasma was diluted 1/100 in the same sample buffer. Samples were heated for five minutes at 95˚C to denature the proteins. Ten microliters of each sample (ground squirrel and mouse) were loaded onto the Mini-PROTEAN TGX Precast Gel (Bio-Rad, Hercules, CA) along with a SuperSignal Molecular Weight Protein Ladder (Thermo Scientific, Rockford, IL). The SDS PAGE gel was run at 250 V for 28 minutes. The gel and PVDF membrane were equilibrated in a semi-dry transfer buffer for 20 minutes. Proteins were transferred onto

the PVDF membrane at 15V for 15 minutes using a semi-dry blotting apparatus. The membrane was blocked overnight with 1% bovine serum albumin (BSA)/0.05% Tween 20/ PBS solution. The ground squirrel and mouse plasma lanes were incubated with the polyclonal goat anti-mouse complement C3 antibody (MP Biomedicals, Solon, OH) (1/5,000 in 0.05% Tween/PBS, PBST) for ground squirrel lanes and 1/20,000 in 0.05% PBST for mouse lanes) for 75 minutes at room temperature with agitation. The membrane was then washed three times for 5 minutes with PBST with agitation. The lanes were then incubated with a biotinylated rabbit anti-goat IgG antibody (Novex, Frederick, MD) (1/40,000 in PBST) for 65 minutes at room temperature with agitation. The membrane was washed again as previously described and incubated with streptavidin conjugated to the enzyme horseradish peroxidase (BD Biosciences, San Jose, CA) (1/140,000 in PBST) for 65 minutes at room temperature with agitation. The membrane was washed again as previously described and incubated with ECL plus western blotting substrate (Thermo Scientific, Rockford, IL) for 5 minutes at room temperature in the dark. After incubation, the substrate was removed and the membrane was placed in a plastic sheet protector. The protected membrane was then placed into a film cassette where film was exposed to the membrane for 6-10 seconds in a dark room and subsequently developed in a film processor.

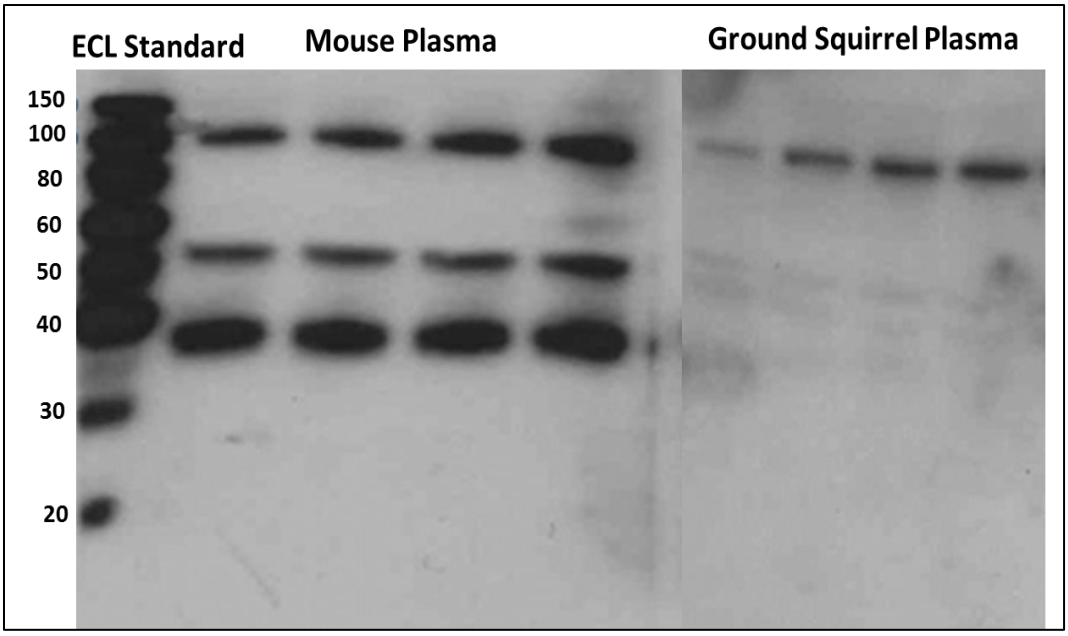

Figure 7. Western blot investigation into the specificity of a commercial goat anti-mouse complement C3 antibody (MP Biomedicals, Solon, OH) for ground squirrel complement C3. Mouse plasma (1/100 dilution) and ground squirrel plasma (5-50 µl/ml (left to right) dilutions) were run on a 10% Mini-PROTEAN TGX Precast Gel (Bio-Rad, Hercules, CA) and probed with either goat anti-mouse C3 or goat gamma globulin isotype control (negative control). Prominent bands were seen at about 115 kDa (mouse and ground squirrel), 55 kDa (mouse), and 40 kDa (mouse).

In both the ground squirrel and mouse plasma samples there was a prominent band visible at about 115 kDa (Figure 3). This band was believed to be complement C3b alpha domain. In the mouse samples there were two additional prominent bands (55 kDa and 40 kDa) that are believed to be the C3b beta domain (55 kDa) and a degradation fragment (40 kDa). The ground squirrel 55 kDa C3b beta domain was less reactive with the goat anti-mouse C3 antibody, appearing as a faint band.

Semi-Quantitative Western Blot

Linear Range

A semi-quantitative western blot was chosen to compare complement component C3 protein concentration in the plasma across the hibernal cycle because it only required the confirmation of one antibody specific for ground squirrel C3. Ground squirrel plasma was serially diluted (50, 40, 30, 20, 10, and 5 µl/ml) to determine which dilution would produce a chemiluminescent signal that falls within a linear range for quantitative western blot analysis. The western blot was carried out as previously described. Once the film was developed it was scanned into the computer and imported into the western blot analysis software Image Studio Lite version 5.2 (LI-COR Biotechnology, Lincoln, NE). A box of equal size was drawn around the 115 kDa band in each lane. The median signal from a strip 3 pixels-wide on the top and bottom of the analyzed box was used for background subtraction (equation 2).

$$(2) \qquad \textit{Corrected Signal} = \textit{Signal} - (\textit{Median background signal} \times \textit{Area}) \tag{2}$$

The chemiluminescent signal from each 115 kDa C3 band was plotted versus the concentration of ground squirrel plasma. The exponential curve was used to determine a linear range to ensure that samples below the linear range or in the plateau range would be excluded from analysis. The linear range was determined to be samples that gave between 20,000 and 85,000 chemiluminescent signals (Figure 8).

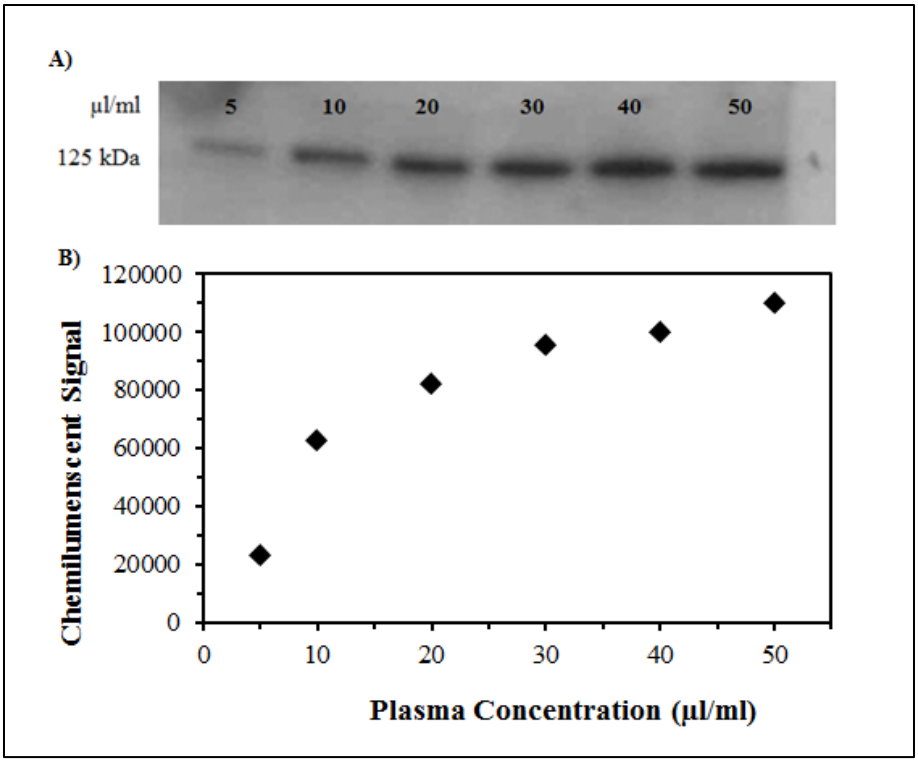

Figure 8. Complement component C3 linear range plot. **A)** Ground squirrel plasma serially diluted (5, 10, 20, 30, 40 and 50 µl/ml) was run on a 10 % SDS PAGE gel and transferred to PVDF membrane. The membranes were developed for C3 and the chemiluminescent signal of the 115 kDa band was analyzed and plotted against serum concentration. **B)** The plot was used to determine a linear range for analysis of C3 protein concentration in ground squirrel plasma.

Sample Analysis

Thirty ground squirrel plasma samples from time points across the hibernal cycle were analyzed by semi-quantitative western blot. Ten non-hibernating, 10 IBA, and 10 hibernating ground squirrel plasma samples were analyzed. A pooled ground squirrel plasma standard consisting of a combination of 30 different ground squirrel plasma samples was run on all gels to serve as a normalization control among gels. Three non-hibernating, IBA, and torpid ground squirrel plasma samples were run per SDS gel along with the pooled ground squirrel plasma standard and an enhanced chemiluminescent molecular weight standard as previously described. The Mini-PROTEAN TGX Precast

Gels had a trihalocompound incorporated that when exposed to UV light activated a reaction between tryptophan residues on the sample proteins and trihalocompound, resulting in fluorescence (Gilda & Gomes, 2013). A picture of the protein bands on the gel was taken using a gel doc system and a 120 second exposure to UV light. The proteins were then transferred to a PVDF membrane as previously described. The membrane was blocked overnight at 4˚C with 1% BSA/PBST and incubated with goat anti-mouse C3 (diluted 1/5000 in 1% BSA/PBST) at room temperature on a rocker for 75 minutes. The membrane was washed as previously described and incubated with a biotinylated rabbit anti-goat IgG (diluted 1/40,000 in 1% BSA/PBST solution) at room temperature on a rocker for 65 minutes. The membrane was washed as previously described and incubated with streptavidin-HRP (diluted 1/140,000 in 1% BSA/PBST) at room temperature on a rocker for 65 minutes. The membrane was then developed as previously described.

The chemiluminescent signal for each band was quantified using Image Studio Lite software. A box of equal size was drawn around the 115 kDa C3 band in each lane. The median signal from a strip 3 pixels-wide on the top and bottom of the analyzed box was used for background subtraction (equation 2). The chemiluminescent signal of each 115 kDa C3 band on each gel was then normalized to the chemiluminescent signal of the pooled ground squirrel sample with the highest chemiluminescent signal using equation 3.

$$(3) \qquad \textit{Normalized Band Intensity} = \frac{Complement\ Band\ Intensity}{\left(\frac{Pooled\ \ Plasma\ Intensity}{Highest\ Pooled\ Plasma\ Intensity}\right)}$$

Total ground squirrel plasma protein was calculated based on the intensity of all the bands from the stain-free SDS gel using Image Studio Lite software. A box of equal size was drawn around entire lanes. The median signal from a strip 3 pixels-wide on the top and bottom of the analyzed box was used for background subtraction (equation 2). The total protein signal on each gel was then normalized to the total protein signal of the pooled ground squirrel sample with the highest intensity (equation 3). The relative concentration of complement C3 was then calculated to a percentage of total protein using equation 4. Relative protein concentration was calculated for the 10 non-hibernating, 10 IBA, and 10 hibernating ground squirrel plasma samples.

$$(4) \qquad \% \ Total \ Protein = \frac{Complement \ C3 \ Intensity}{Total \ Protein \ Intensity} \ X \ 100\%$$

Bacterial Lysis Functional Assay at 37˚C

Escherichia coli NU2-56B, a strain isolated from a human urinary tract infection and kept in stock culture at the University of Wisconsin-La Crosse, was brought out of the -80˚C freezer and streaked onto a tryptic soy agar (TSA) plate. The TSA plate of *E. coli* NU2-56B was placed at 35˚C for 24 hours, and then it was sub-cultured into a tryptic soy broth (TSB). The TSB culture was placed at 35˚C for 24 hours and then sub-cultured into another TSB which was incubated for 24 hours at 35˚C. The twice sub-cultured *E. coli* NU2-56B was diluted 1:100 with phosphate buffered saline (PBS) and absorbance was read at 600 nm to ensure it was between 0.02 and 0.03. The spectrophotometer was blanked with PBS. Once the bacterial dilution was in the appropriate absorbance range, 25 µl of *E. coli* was mixed with 25 µl of ground squirrel serum that had been stored at -

20˚C, or 25 µl of PBS and incubated in a 37˚C water bath for 90 minutes. After incubation 50 µl of *E. coli*/ground squirrel serum mix was added to 450 µl of PBS to make a 10^{-1} dilution. The 10^{-1} dilution was then serially diluted in PBS out to 10^{-4}. Fifty microliters of *E. coli*/PBS was also diluted in 450 µl of PBS to make a 10^{-1} dilution and that was serially diluted out to 10^{-7}. Serially diluted *E. coli*/ground squirrel serum (100 µl) and *E. coli*/PBS mixes (100 µl) were spread onto TSA plates with a spreading triangle. The 10^{-1} through the 10^{-4} dilutions were plated for the *E. coli*/ground squirrel serum mix while the 10^{-4} through the 10^{-7} dilutions were plated for the *E. coli*/PBS mix. All of the TSA plates were placed in a 35˚C incubator for 24 hours. Plates were removed from the incubator after 24 hours and a countable plate, with between 30 and 300 colonies, was selected. The colony forming units (CFU) per milliliter were calculated for the *E. coli*/ground squirrel serum and *E. coli*/PBS mixes. For each individual ground squirrel serum sample from non-hibernating and IBA animals the assay was run in triplicate and averaged. Twelve non-hibernating, 8 IBA, and 8 hibernating ground squirrel serum samples were assayed.

Bacterial Lysis Functional Assay at 4˚C

Non-hibernating 13-lined ground squirrel serum samples were tested for the capacity of complement to lyse bacterial cells at 37˚C because that is the normal physiological temperature of the squirrel during the active summer months. To assess the ability of complement to adequately protect 13-lined ground squirrels during hibernation it is imperative that complement's capacity to lyse bacterial cells at torpid body temperatures be tested. Normal body temperature for torpid 13-lined ground squirrels is slightly above the ambient temperature of the hibernaculum, so 4˚C was chosen for the

assay. Serum samples from non-hibernating and torpid 13-lined ground squirrels, was mixed with an equal volume of diluted *E. coli* NU2-56B and then placed at 4˚C for 12 hours before being serially diluted and plated onto TSA plates as previously described in the section: bacterial lysis functional assay. After the TSA plates had incubated for 24 hours the CFU/ml was calculated to determine how effective complement was at lysing bacterial cells at torpid temperatures. Eight hibernating and 7 non-hibernating ground squirrel serum samples were assayed. Serum samples from IBA 13-lined ground squirrels were not analyzed due to lack of sample availability.

Flow Cytometry for Detection of Ground Squirrel Anti-*E. coli* Antibodies

Escherichia coli O157:H7 was brought out of a -80˚C freezer and plated onto a TSA plate. *E. coli* cells were suspended in sterile PBS and matched to a 0.5 McFarland standard or 1 x 10^6 cells/ml. The cells were pelleted by centrifugation for 5 minutes at 24.1 g. Once the *E. coli* cells had been pelleted the PBS was removed without disturbing the *E. coli* pellet. The pellet was then re-suspended in one of three ways: (1) for the positive control the pellet was re-suspended in a mouse anti-*E. coli* O157 monoclonal antibody (Thermo Scientific, Rockford, IL) diluted 1:10 in sterile PBS. (2) For the negative control the pellet was re-suspended in a mouse monoclonal anti-human CD3 antibody supernatant (undiluted). (3) The pellet was re-suspended in the plasma of 13-lined ground squirrels diluted 1:10 in sterile PBS. The primary antibody was incubated for 30 minutes at room temperature. After incubation the *E. coli* cells were washed with the addition of 250 µl of PBS, pelleted in a centrifuge and re-suspended in 250 µl of PBS. The wash step was repeated three times before the pellet was suspended in secondary antibody, FITC sheep $(Fab)^2$ anti-mouse Ig heavy and light chain (Sigma Aldrich, St.

Louis, MO) diluted 1:50 in sterile PBS. The secondary antibody was incubated for 30 minutes at room temperature in the dark. The *E.coli* cells were then washed in the same way as previously described. After the wash the cells were re-suspended in 400 µl of PBS. The cells were then passed through a flow cytometer until 10,000 events had been recorded. Plasma samples from 10 non-hibernating, 10 IBA, and 10 hibernating animals were analyzed via this method.

Immunohistochemistry for C3 Detection

Liver tissue samples from non-hibernating, torpid, and IBA ground squirrels were preserved in embedding media at -80˚C. Tissue sections were cut to a thickness of 5 µm at -30˚C in a cryotome and transferred to poly L-Lysine coated slides and allowed to dry for two hours at room temperature.

Subcutaneous abdominal adipose tissue and bone marrow from femurs were embedded in paraffin. Paraffin embedded tissues were cut to a thickness of 4 µm in a microtome and transferred to poly L-Lysine coated slides and allowed to dry for two hours. Tissue sections were deparaffinized at 60˚C for 1 hour. Tissue sections then went through heat induced epitope retrieval. Tissue sections were soaked 3 times in toluene for 3 minutes each before being soaked in progressively more dilute ethanol (100%, 95%, 80%, and 75%) for 3 minutes each. Tissue sections were soaked in DI water for 5 minutes before being incubated in a pH 9.0 buffer heated to 90˚C for 21 minutes.

Liver, adipose, and bone marrow tissue sections were fixed in cold (4˚C) acetone for 5 minutes and circled with a wax pen. To inhibit endogenous peroxidase activity tissue sections were incubated with 50-100 µl of a 0.3% H_2O_2, 0.1% NaN_3 mixture for 30 minutes in a humidity chamber. Tissue sections were then rinsed for 5 minutes in PBS

with agitation and then blocked with 10% goat serum/PBS for 30 minutes at room temperature in a humidity chamber. Serum was not washed off but excess serum was allowed to drain off and remaining serum was blotted around the edges with a kimwipe. Tissue sections were incubated with a polyclonal goat anti-mouse complement component C3 antibody (MP Biomedicals, Solon, OH) that had previously been shown to be reactive with ground squirrel complement C3 or mouse anti-human CD20 (undiluted hybridoma supernatant, negative control) overnight at 4˚C. Excess antibody was removed and tissue sections were washed three times for 5 minutes in PBS with agitation before incubation with a biotinylated rabbit anti-goat IgG antibody (Thermo Scientific, Rockford, IL) diluted 1:1000 in 1% ground squirrel serum/PBS to help block tissue IgG cross reactivity with the anti-Ig secondary antibody. Secondary antibody was incubated at room temperature in a humidity chamber for 30 minutes then slides were washed for 5 minutes in PBS with agitation. Tissue sections were incubated for 30 minutes at room temperature in a humidity chamber with streptavidin conjugated to the enzyme horseradish peroxidase (Thermo Scientific, Rockford, IL) diluted 1:400 in PBS. Excess enzyme conjugate was drained and slides were washed for 5 minutes in PBS with agitation. Freshly prepared aminoethylcarbazole (AEC) working solution was applied to the tissue sections for 10 minutes. The AEC reaction was stopped with DI H_2O before counterstaining the tissue sections in Mayer's hematoxylin for 1 minute. To fade the Mayers hematoxylin counterstain, tissue sections were dipped in Scott's solution for 15 seconds. Slides were mounted in aqueous medium and covered with a coverslip to make permanent slides and then analyzed under 100X and 200X magnification on a compound light microscope for complement C3.

RESULTS

Relative Gene Expression of Complement Component C3 in the Liver throughout the Hibernal Cycle

The transcription level of complement component C3 in liver of 13-lined ground squirrels relative to the reference gene GAPDH was determined by qPCR. There was an increase in the relative gene expression of C3 in torpid ground squirrels (16.43 ± 9.73) as compared to ground squirrels in IBA (9.76 ± 4.92) and summer/non-hibernating ground squirrels (9.62 ± 4.97) (Figure 9). The observed increase in relative transcription level of C3 between torpid ground squirrels and non-hibernating ground squirrels was not statistically significant as analyzed by T-test (p-value = 0.174).

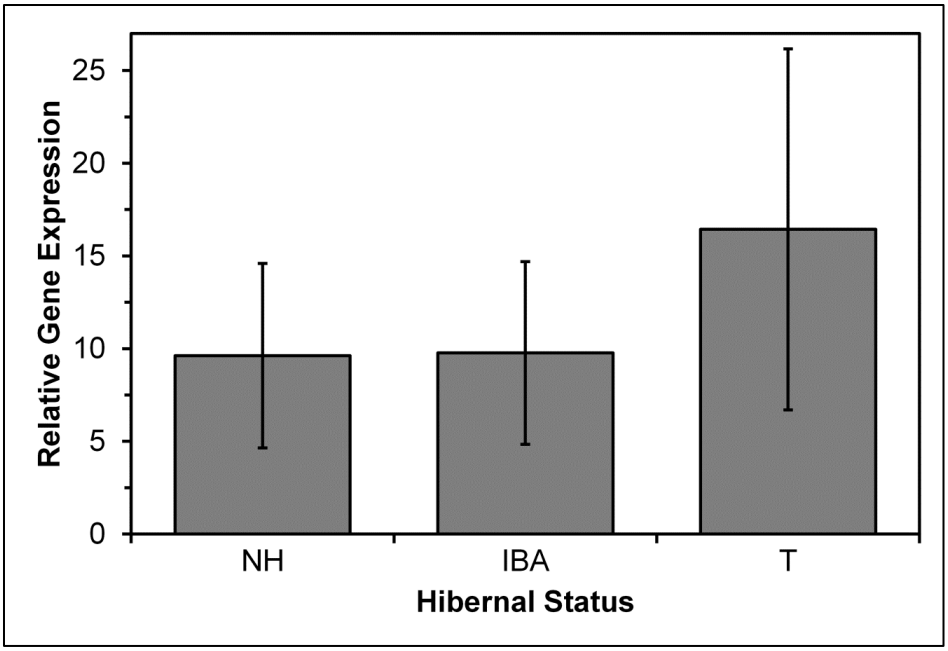

Figure 9. Expression of complement component C3 was measured relative to the housekeeping gene GAPDH at different stages of the hibernal cycle, non-hibernator (NH) (n=5), inter-bout arousal (IBA) (n=6), and torpor (T) (n=6) using qPCR.

Relative Gene Expression of Complement Component C3 in Adipose Tissue throughout the Hibernal Cycle

The transcription level of complement component C3 in adipose tissue of 13-lined ground squirrels relative to the reference gene GAPDH was measured by qPCR. Complement component C3 transcription in torpid 13-lined ground squirrels (0.713 ± 0.515) was slightly lower than that of non-hibernating 13-lined ground squirrels (1.354 ± 0.713) (Figure 10). The relative C3 transcription level of animals in IBA was not measured due to the lack of availability of samples. The difference in C3 transcription level between non-hibernating and torpid 13-lined ground squirrels was not significant as analyzed by a T-test (p-value = 0.222).

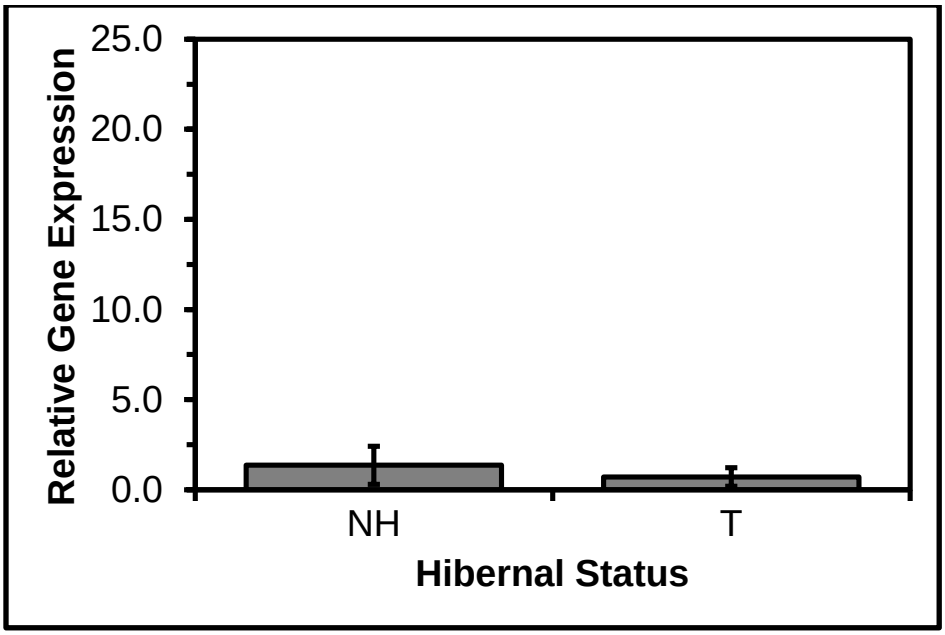

Figure 10. Relative gene expression of complement component C3 in adipose tissue was measured relative to the reference gene GAPDH. Expression was measured in non-hibernating (NH) (n=6) and hibernating (T) (n=6) 13-lined ground squirrels.

Relative Plasma Complement C3 Protein Concentration

Total plasma protein concentration was determined for ground squirrels at time points throughout the hibernal cycle. Relative protein intensity was determined by total band intensity of plasma samples run on Mini-PROTEAN TGX Precast Gel (Bio-Rad, Hercules, CA). There was a higher relative protein intensity in IBA ground squirrels (218,041.64 ± 94,073.39) than was present in non-hibernating (216,060.23 ± 73,197.03) or torpid ground squirrels (202,423.53 ± 82,776.01) (Figure 11). The statistical significance of the decrease in total protein concentration from non-hibernating and IBA ground squirrels to torpid ground squirrels was evaluated with an unequal variance T-test. It was found that this decrease was not statistically significant (p-value = 0.70 and 0.69 respectively).

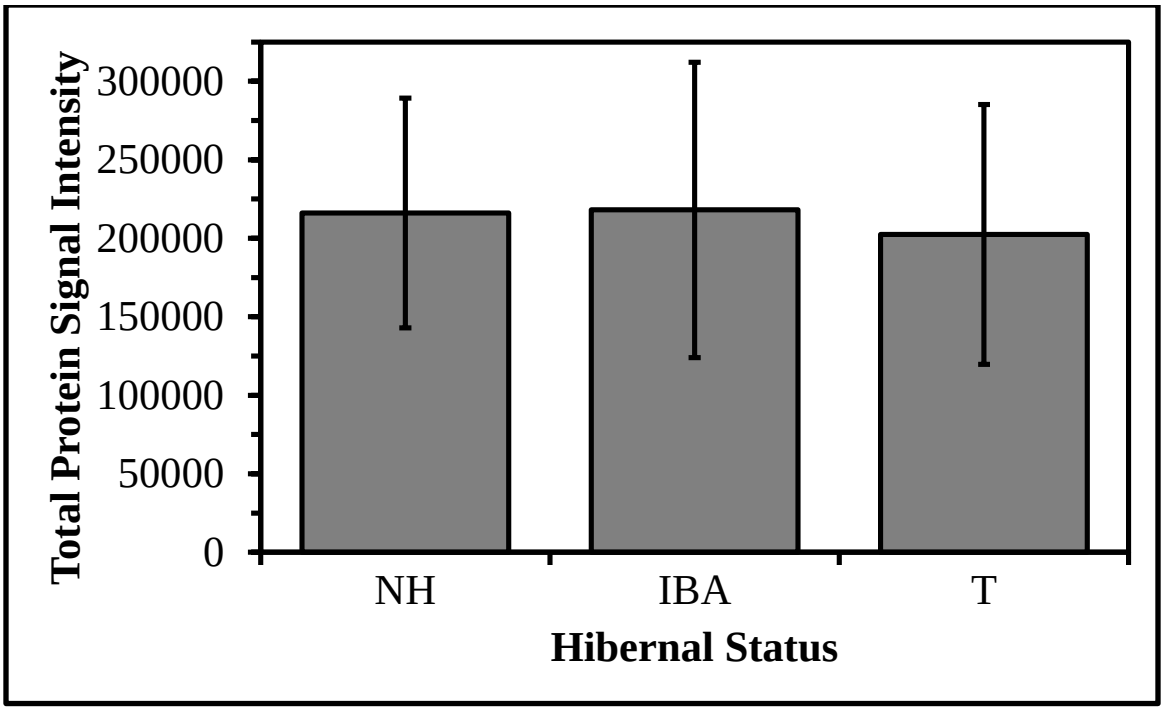

Figure 11. Relative total plasma protein intensity was determined for non-hibernating (NH) (n=10), interbout arousal (IBA) (n=10), and hibernating (H) (n=10) ground squirrels. Total protein concentration is expressed as the signal intensity of all protein bands produced when stain-free gels with ground squirrel plasma run on them were analyzed with Studio Image Lite.

The complement component C3 relative protein concentration of 30 ground squirrel plasma samples was determined by semi-quantitative western blot. Ground squirrel plasma samples were normalized by expressing relative C3 concentration as a percentage of total protein. Percent C3 was at the lowest level in torpid ground squirrels (33.58% ± 13.58%), and C3 was at a slightly higher percent in non-hibernating (35.15% ± 14.21%) and IBA ground squirrels (34.19% ± 17.23%) (Figure 12). The statistical significance of the decrease in C3 concentration during torpor was evaluated using an unequal variance T-test and it was found that there is not a statistically significant

decrease in C3 concentration from non-hibernating to torpid ground squirrels (p-value = 0.80).

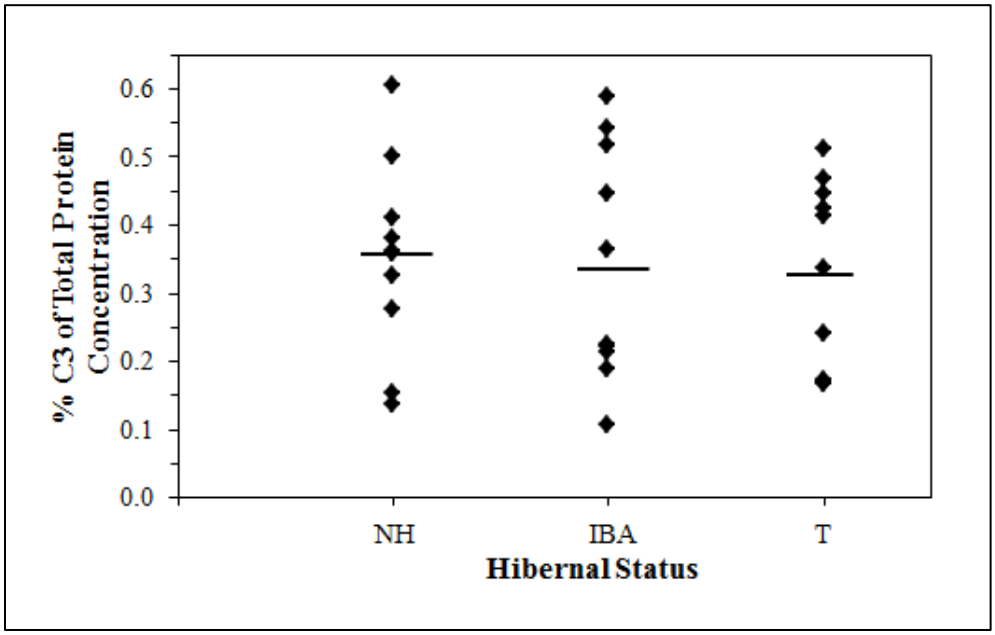

Figure 12. Relative percent complement C3 normalized to plasma protein in ground squirrels in different stages of hibernation. Plasma complement C3 concentrations were determined by semi-quantitative western blot and normalized for total plasma protein concentration to be expressed as a percentage of total plasma protein. The percent C3 of total plasma protein was plotted for each of the three measured time points in the hibernal cycle (n=10 for each stage). The horizontal bar represents the mean relative percent of C3 for that time point.

Complement Bacterial Lysis Ability at 37 ˚C

Ground squirrel serum was assayed for the ability of complement to lyse a complement-sensitive strain of *E. coli* at body temperature for an active non-hibernating ground squirrel (37 ˚C). A mixture of *E. coli* and PBS was used to set a baseline CFU/ml level ($1.804 \times 10^7 \pm 9.08 \times 10^6$) (Figure. 13). Serum from non-hibernating ground squirrels was able to lower the CFU/ml to $6.44 \times 10^5 \pm 7.53 \times 10^5$, equivalent to a 28.1 fold decrease in *E. coli* when incubated with non-hibernating ground squirrel serum. Serum from IBA

and torpid ground squirrels was not able to lower the CFU/ml and actually resulted in an increase in CFU/ml ($2.90 \times 10^7 \pm 1.67 \times 10^7$ and $3.15 \times 10^7 \pm 8.12 \times 10^7$ respectively), equivalent to a 1.8 fold increase in *E. coli* when incubated with torpid ground squirrel serum and a 1.6 fold increase in *E. coli* when incubated with serum from an IBA ground squirrel. Statistical differences between groups were evaluated using a T-test assuming equal variance. Non-hibernating ground squirrel serum significantly lowered the CFU/ml as compared to the PBS baseline (p-value = 7.62×10^{-9}), while the CFU/ml was significantly greater for both IBA and torpid ground squirrel serum as compared to the PBS baseline (p-value = 0.004 and 0.0001 respectively). Non-hibernating ground squirrel serum was able to significantly lower CFU/ml as compared to IBA and torpid ground squirrel serum (p-value = 6.13×10^{-6} and 5.05×10^{-11} respectively).

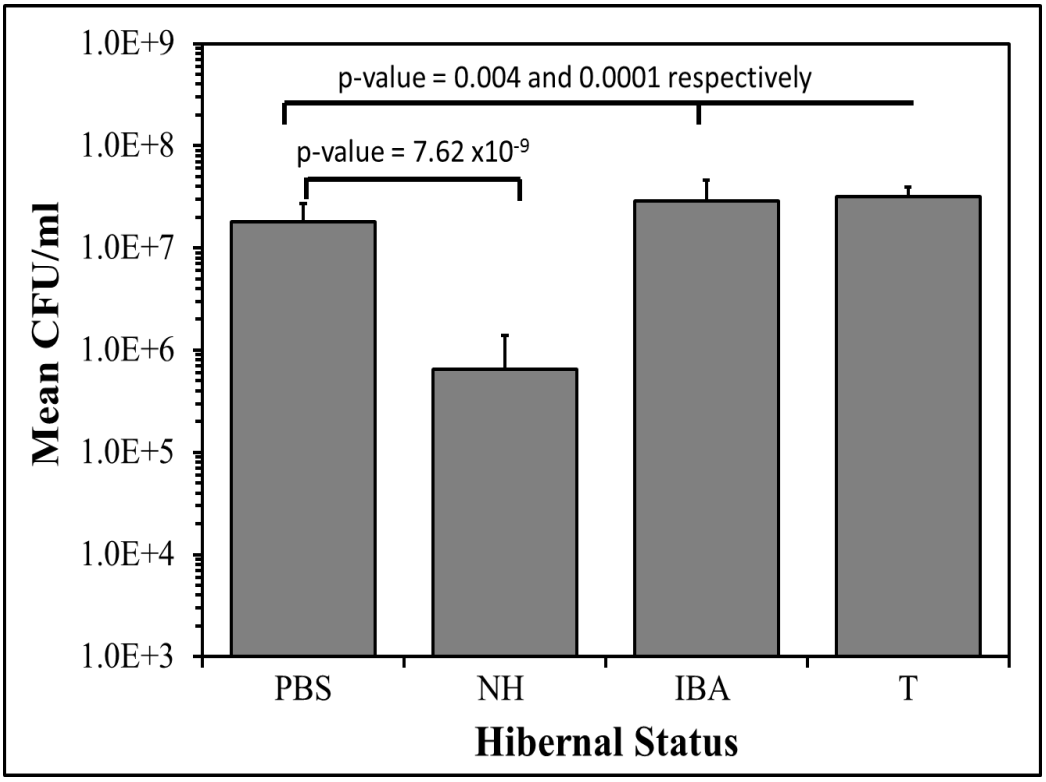

Figure 13. The ability of complement in the serum of ground squirrels from various time points in the hibernal cycle (non-hibernating (NH) (n=12), interbout arousal (IBA) (n=8), and hibernating (H) (n=8)) to kill complement sensitive *E. coli* cells was assayed. The assay was conducted at normal body temperature of a non-hibernating ground squirrel (37 ˚C). This was measured by calculating CFU/ml after incubation with ground squirrel serum. Statistical significance was measured using a T-test assuming equal variance. Solid black bars indicate statistical significance between those two groups.

Complement Lysis of Bacteria at 4 ˚C

Ground squirrel serum was assayed for the ability of complement to lyse a complement-sensitive strain of *E. coli* at body temperature for a torpid ground squirrel (4 ˚C). A mixture of *E. coli* and PBS was used to set a baseline CFU/ml level ($4.825 \times 10^6 \pm 8.62 \times 10^5$) (Figure. 14). Ground squirrel serum from non-hibernating animals was able to slightly reduce the CFU/ml as compared to the baseline ($3.51 \times 10^6 \pm 1.12 \times 10^6$), equivalent to a 1.4 fold decrease in *E. coli* cells when incubated with non-hibernating

ground squirrel serum. Torpid ground squirrel serum was also able to slightly reduce the CFU/ml as compared to the baseline ($3.53 \times 10^6 \pm 4.65 \times 10^5$). There was a 1.4 fold decrease in *E. coli* cells when incubated with torpid ground squirrel serum. Statistical differences between groups were evaluated using a T-test assuming equal variance. The small reduction in *E.coli* CFU/ml obtained with non-hibernating and torpid ground squirrel serum was statistically significant as compared to the baseline (p-value = 0.037 and 0.0031 respectively). There was no statistically significant difference between the amount of reduction in the CFU/ml from non-hibernating and torpid ground squirrel (p-value = 0.48).

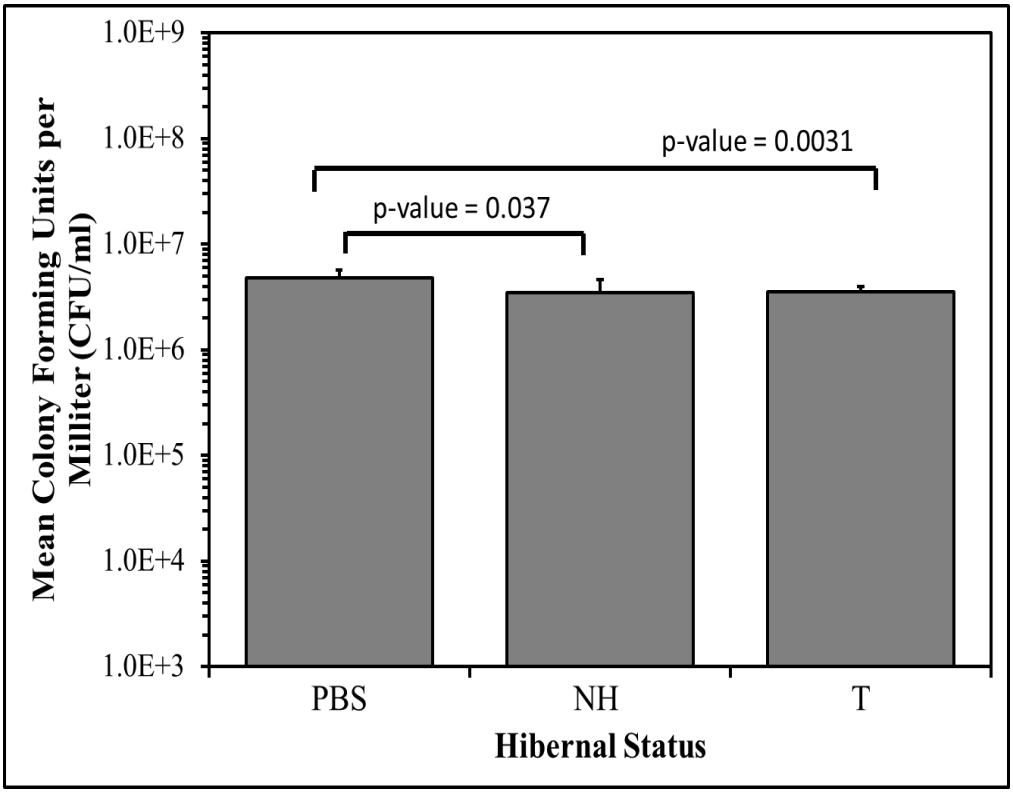

Figure 14. The ability of complement in the serum of ground squirrels from various time points in the hibernal cycle (non-hibernating (NH) (n=7) and hibernating (H) (n=8)) to kill complement sensitive *E. coli* cells was assayed. The assay was conducted at normal body temperature of a torpid ground squirrel (4 ˚C). This was measured by calculating CFU/ml after incubation with ground squirrel serum. Statistical significance was measured using a T-test assuming equal variance. Solid black bars indicate statistical significance between those two groups.

Detection of Anti-*E. coli* Antibodies in Plasma

Flow cytometry was used to assay for the presence of anti-*E. coli* antibodies in plasma samples of 13-lined ground squirrels in different stages of the hibernal cycle (n=10 in each stage). The negative control had 84.9% of the events with fluorescence to the left of the designated divide and 15.1% of events had of fluorescence to the right of the designated divide (Figure. 15A). The divide between higher and lower amounts of fluorescence is delineated by a vertical line positioned at 10^3 on the FL1-A axis. The

positive control was the inverse of the negative control having only 9.5% of events with little fluorescence and 90.5% of events with higher levels of fluorescence (Figure. 15B). A ground squirrel with no antibody titer was approximated to be any plasma sample that had a percentage of events that had higher levels of fluorescence that was lower or equal that of the negative control (15.1%) (Figure. 15C). A ground squirrel that had an antibody titer against *E. coli* was any plasma sample that had a higher percentage of events with higher levels of fluorescence than the negative control (Figure. 15D and E). Using this approximation, of the 30 plasma samples assayed five ground squirrels (16.6%) had anti-*E. coli* antibodies present. Of the five ground squirrels that tested positive two were from non-hibernating animals, two were from IBA animals, and one was from a hibernating animal.

Ground squirrel microbiome data shows *E. coli* was not present in detectable level but microbiome results showed that ground squirrels have some *Lactobacillus* species and thus these bacteria might show what a strong antibody-positive would look like for this assay. *Lactobacillus helveticus* was chosen to test this. When *L. helveticus* was reacted with ground squirrel plasma it showed no positive result. The negative control showed that 0.1% of events had higher fluorescence than the previously specified threshold. When *L. helveticus* was incubated with ground squirrel serum diluted 1/10 in PBS 0.7% of events had higher fluorescence than the threshold.

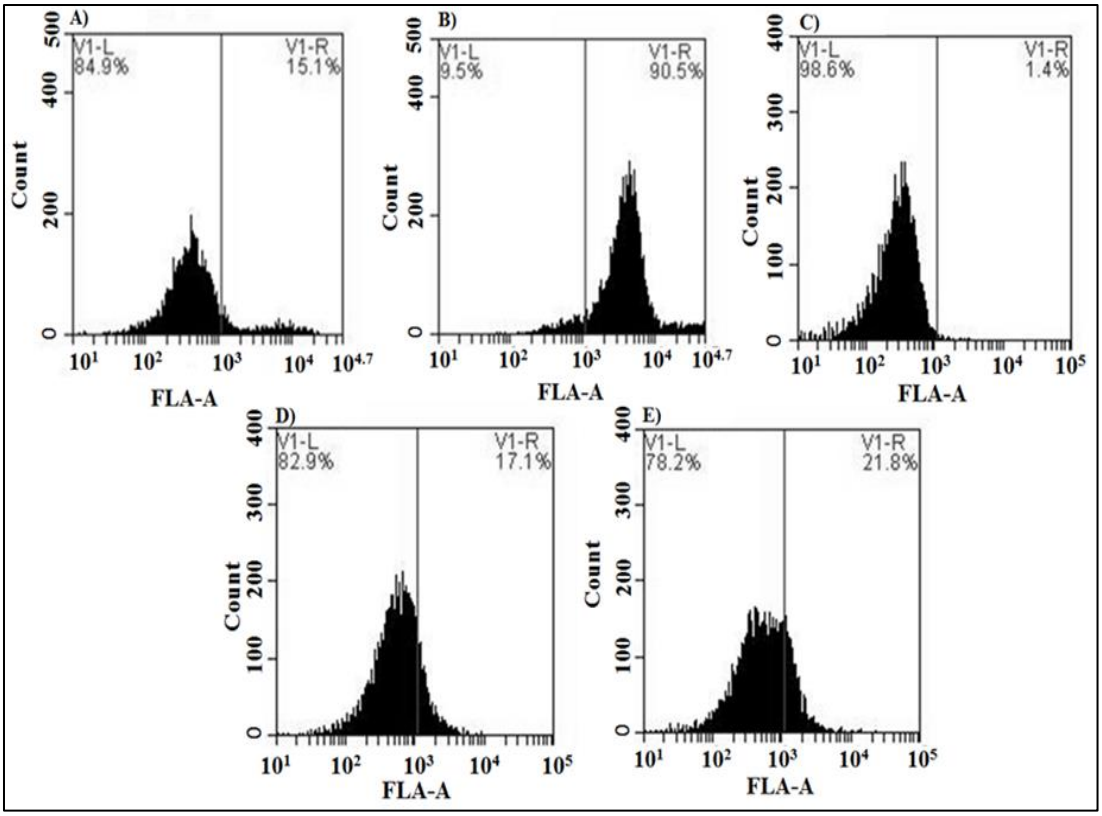

Figure 15. Flow cytometry was used analyze 13-lined ground squirrel plasma for the presence of anti-*E. coli* antibodies. Histograms were created plotting the cell counts against the relative amount of fluorescence. A) Negative control. B) Positive control. C) Shows a plasma sample from a ground squirrel with little to no antibody titer against *E. coli.* D) Shows a plasma sample from a ground squirrel with low levels of antibody titer against *E. coli.* E) Shows a plasma sample from a ground squirrel with higher antibody titer against *E. coli*.

Liver Tissue Staining

Liver tissue sections from non-hibernating, torpid, and IBA ground squirrels were stained with goat anti-mouse C3 antibody and observed under 100X and 200X magnification (Figure 16). The presence of C3 was determined by HRP on the secondary antibody producing red AEC stain. AEC stained C3 was present in blood vessels in non-hibernating, torpid and IBA animals, however the intensity of stain was much stronger in

non-hibernating animals. There was very little AEC-staining in any parts of the tissue section other than the areas around the blood vessels.

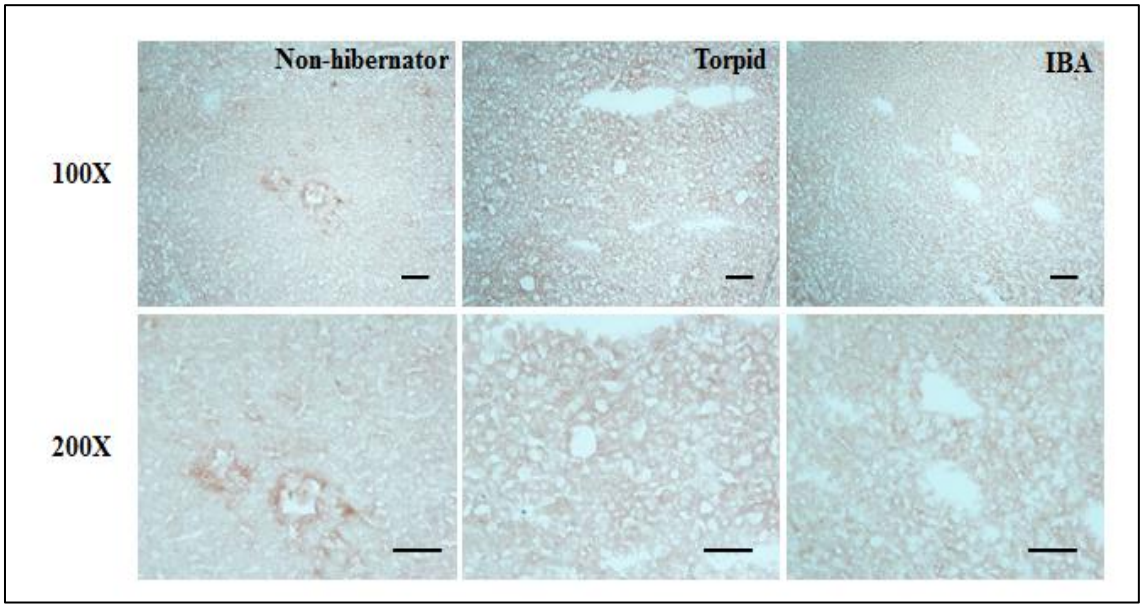

Figure 16. Ground squirrel liver tissue stained with mouse anti-human CD20 antibody (Negative control) (Not shown). Non-hibernating, torpid, and IBA tissue sections were stained with goat anti-mouse C3 antibody. Slides were counterstained with hematoxylin and Scott's solution, and viewed at 100X and 200X magnification. Scale bars represent 100µm.

Adipose Tissue Staining

Adipose tissue sections from non-hibernating, torpid, and IBA ground squirrels were stained with goat anti-mouse C3 antibody and red AEC substrate and observed under 100X and 200X magnification (Figure 17). There was very little staining in any of the adipose sections. When present, staining was seen sporadically throughout the tissue. In general there seemed to be slightly more staining in non-hibernating ground squirrel adipose tissue than was present in torpid or IBA ground squirrel adipose tissue.

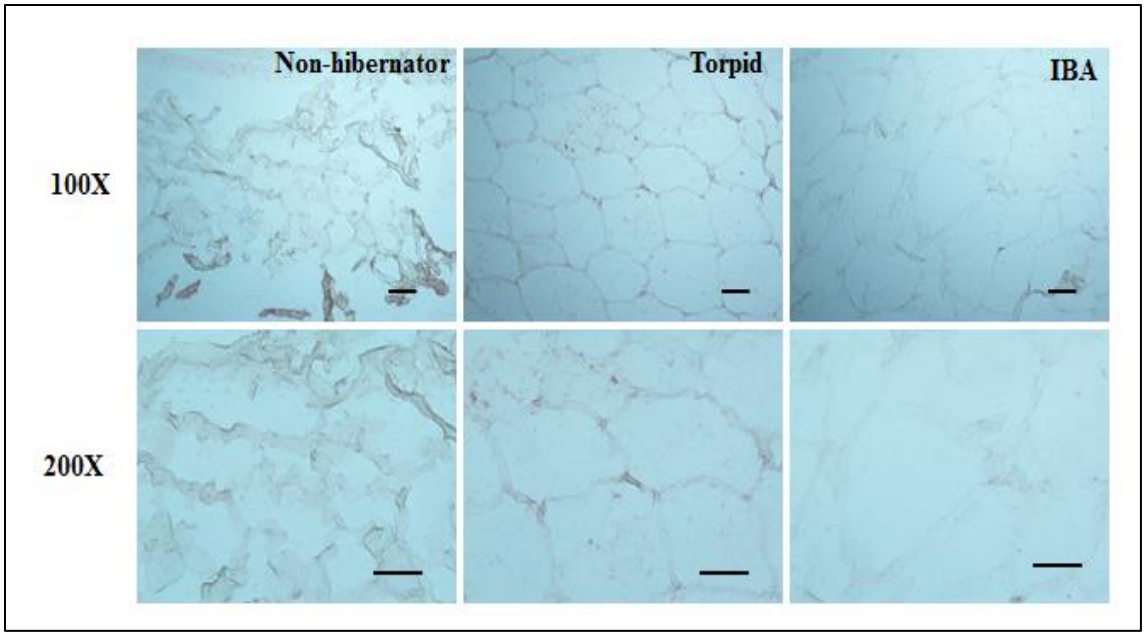

Figure 17. Ground squirrel adipose tissue stained with mouse anti-human CD20 antibody (Negative control) (Not shown). Non-hibernating, torpid, and IBA tissue sections were stained with goat anti-mouse C3 antibody. Slides were counterstained with hematoxylin and Scott's solution, and viewed at 100X and 200X magnification. Scale bar represents 100 µm.

Bone Marrow Tissue Staining

Bone marrow tissue sections from the femur of non-hibernating, torpid, and IBA ground squirrels were stained with goat anti-mouse C3 antibody and red AEC substrate and observed under 100X and 200X magnification (Figure 18). C3 was diffusely present only where the non-adipocyte components of the bone marrow were present. Staining was not present in the adipocytes that are present within the bone marrow. The red staining was more intense in torpid ground squirrel bone marrow than it was in either non-hibernating or IBA bone marrow tissue.

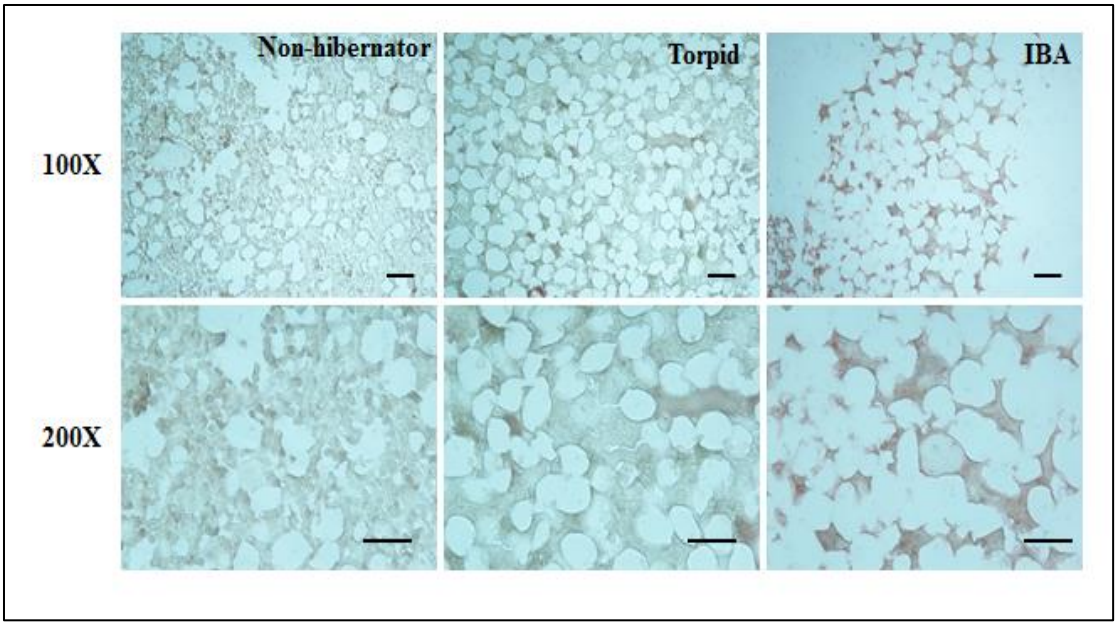

Figure 18. Ground squirrel bone marrow tissue stained with mouse anti-human CD20 antibody (Negative control) (Not shown). Non-hibernating, torpid, and IBA tissue sections were stained with goat anti-mouse C3 antibody. Slides were counterstained with hematoxylin and Scott's solution, and viewed at 100X and 200X magnification. Scale bar represents 100 µm.

DISCUSSION

The overall goal of this study was to further investigate the 5-fold increase in transcription of complement component C3 in the bone marrow of 13-lined ground squirrels during torpor. Interest in this increased transcription was sparked because it contradicts most previous reports of a general suppression of the immune system during hibernation. Four supporting hypotheses were made: (1) That transcription of C3 would be increased in other tissues, (2) That there would be a higher plasma concentration of C3 during torpor, (3) Increased levels of C3 would lead to an increased ability for torpid ground squirrels to lyse bacteria, and (4) That increased protein levels during torpor would be seen not only in the bone marrow but also in the liver and adipose tissue.

Comparison of C3 Expression Levels in Liver and Adipose Tissue throughout the Hibernal Cycle

Quantitative PCR was used to assay the transcription level of C3 in both liver and adipose tissue of ground squirrels throughout different stages of the hibernal cycle. The liver was chosen because it is the primary producer of plasma proteins, so if C3 expression is higher during torpor there would be a reasonable belief that there could be a higher plasma concentration of C3 during torpor. C3 expression relative to the housekeeping gene GAPDH was highest in torpid ground squirrels, with non-hibernating and IBA ground squirrels having similar expression levels. The increase in C3 transcription during torpor in the liver was nowhere near as dramatic as with what was

seen in the bone marrow. Due to high variation among individual ground squirrels this increase was not statistically significant.

Due to the recent discoveries linking C3 with adipose tissue breakdown (Harboe et al., 2011), C3 transcription in adipose tissue throughout the hibernal cycle was also assayed to see if there was a potential role for C3 in aiding in lipid metabolism during torpor. Adipose tissue was also selected to determine if the increase in transcription of C3 in the bone marrow during torpor is the effect of the invasion of bone marrow tissue with adipocytes during torpor. The transcription level of C3 was seen to be higher in non-hibernating ground squirrels than it is in torpid ground squirrels. Again high variation in expression levels between individual ground squirrels caused these results to not be statistically significant. The relative transcription level of C3 in the adipose was much lower than transcription levels in the liver which was to be expected since the liver is one of the primary sites for C3 production. The relative C3 transcription level in both the liver and adipose tissue were in agreement with previous transcriptome work done on these tissues (Scott Cooper personal communication).

Development of a Method to Detect and Quantify Ground Squirrel C3

A semi-quantitative western blot utilizing a commercial polyclonal goat anti-mouse C3 antibody was developed to measure the relative plasma concentration of C3 in ground squirrels. Specificity of the goat anti-mouse C3 antibody for ground squirrel C3 was confirmed with a chemiluminescent western blot, where a prominent band at 115 kDa was present along with faint bands at 55 kDa and 40 kDa. The presence of multiple distinct bands is most likely due to the polyclonal nature of the commercial antibody. The three bands present on ground squirrel samples exactly matched the three prominent

bands that were present when the antibody was reacted against mouse plasma. These results indicated that the goat anti-mouse C3 antibody was specific for ground squirrel C3.

The goat anti-mouse C3 antibody was used in a semi-quantitative western blot assay to detect and compare the plasma concentration of C3 in 30 plasma samples from ground squirrels at varying time points throughout the hibernal cycle. Plasma concentration was plotted against its given chemiluminescent signal to identify a linear range. It was imperative to be working within the linear range to ensure that the concentration of C3 could be accurately measured. A plasma sample that consisted of an aliquot of each tested ground squirrel plasma sample was used as a control to help normalize for any variation between gels and for any variation in plasma protein concentration between ground squirrels. A picture of each gel was taken so that an image of total protein level could be obtained and then plasma concentration of C3 could be calculated based on a percentage of total protein. This western blot assay was only semi-quantitative, so plasma C3 concentrations produced are more useful for comparing relative variation in plasma C3 concentration in the different stages of the hibernal cycle

Comparison of C3 Plasma Concentration throughout the Hibernal Cycle

Relative plasma concentration of C3 in 30 plasma samples was measured by semi-quantitative western blot and compared to a pooled plasma sample to determine the relative C3 concentration as a percentage of total protein. Total protein levels varied only slightly between non-hibernating, IBA, and torpid ground squirrels with torpid ground squirrels having slightly less total protein although this was not statistically significant. High variation among squirrels could have been a result of varying qualities of the

pictures taken or different ground squirrels really have a high degree of variation in total plasma protein concentration or variation in the ratio of anticoagulant to plasma when the blood was collected. Overall this is a good representation of the relative average total plasma protein levels of ground squirrels in different stages of the hibernal cycle. Similarly to total protein levels, plasma C3 concentrations did not vary much between ground squirrels in different stages of the hibernal cycle. Plasma C3 concentration was at its lowest in torpid ground squirrels but this was not a statistically significant difference from ground squirrels who were non-hibernating or in IBA. Again there was a high degree of variation among the small number of individual animals tested which made it difficult to reveal statistically significant changes in plasma C3 concentration. The large variation among individual ground squirrels could have been due to unaccounted for variables such as genetic background or an ongoing infection resulting in levels of C3 present in the plasma. These results indicate that if the transcripts in the bone marrow are being translated into protein that protein is not significantly affecting concentrations in the plasma but instead could be sequestered inside cells of another tissue or staying within the bone marrow.

Development of a Method to Quantify the Ability of Complement to Lyse Bacterial Cells

A functional assay was designed to measure the ability of complement to lyse bacterial cells at an active non-hibernating body temperature (37 ˚C) and also at the body temperature of a torpid ground squirrel (4 ˚C). A mixture of ground squirrel serum and *E. coli* were incubated for two hours at 37 ˚C to simulate the physiological conditions of a non-hibernating ground squirrel. A mixture of ground squirrel serum and *E. coli* was also

incubated for 12 hours at 4 ˚C to simulate the physiological conditions of a torpid ground squirrel. An effect of temperature on complement function was tested by running the assay at both non-hibernating and torpid ground squirrel body conditions. It was essential that the strain of bacteria used was sensitive to the lytic actions of the complement pathway. Through the work of the University of Wisconsin La Crosse Microbiology Department the bacterial strain used, *E. coli* NU2-56B, has been previously documented to be sensitive to complement (Bernadette Taylor, personal communication).

Numerous assays are available to measure the activity level of complement, such as the CH50 assay to measure the activity of the classical pathway or the AH50 assay to measure the activity of the alternative pathway (Mollnes et al., 2007). Both the CH50 assay and the AH50 assay utilize rabbit erythrocytes to activate the complement pathway and lyse the erythrocytes. The argument can be made that these assays are measuring complement activity in a non-natural environment. By measuring the ability of complement to lyse and kill bacterial cells it allows a more accurate natural portrayal of true complement activity as it relates to immune system function.

Comparison of the Ability of Complement to Lyse Bacterial Cells at 37 ˚ and 4 ˚C

The ability of complement to kill *E. coli* cells was measured at both 37 ˚C and 4 ˚C. At 37 ˚C non-hibernating ground squirrel serum was able to significantly lower the CFU/ml of the mixture as compared to the control (p-value = 7.62×10^{-9}). Non-hibernating ground squirrel serum also was shown to lower the CFU/ml significantly more than both IBA and torpid ground squirrel sera (p-value = 6.13×10^{-6} and 5.05×10^{-11} respectively). Non-hibernating ground squirrel complement was also more active than complement from a torpid ground squirrel. These results give a clear indication that

complement is much more active in non-hibernating ground squirrels at 37 ˚C. Even though the complement of IBA and torpid ground squirrels was not able to lower the CFU/ml as compared to the control doesn't mean that complement was not active. Since the *E. coli* used in this assay was viable and able to replicate if the complement was not active that would have meant that the CFU/ml of both the IBA and torpid ground squirrel serum would have been higher than that of the control. To control for this a separate control with heat inactivated serum could have been run alongside serum that was not heat inactivated. The heat inactivated serum would give a control give a baseline for how much replication would occur during the 37 ˚C incubation. Since *E. coli* cells can double once every 30 minutes when grown at 37 ˚C complement activity was still able to hold *E. coli* numbers in check (Dill, Ghosh, & Schmit, 2011). Higher complement activity from non-hibernating ground squirrels as compared to torpid ground squirrels at 37 ˚C is in agreement with previous complement work with the exception of high levels of complement activity during IBA (Maniero, 2002).

At 4 ˚C both non-hibernating and torpid ground squirrel serum was able to slightly lower the CFU/ml as compared to the control (p-value = 0.037 and 0.0031 respectively). Torpid ground squirrel complement was able to lower the CFU/ml slightly more than that of non-hibernating ground squirrel serum. With slight bacterial killing occurring at 4 ˚C it shows that the complement pathway can function at low temperatures associated with physiological conditions of a torpid ground squirrel. At 4 ˚C *E. coli*'s ability to divide is greatly reduced (Dill et al., 2011), the control is most likely an accurate representation of how many cells were present at the beginning of the assay. This assay then gave an accurate picture as to the level of complement activity without

having to deal with the variable of how much were the bacterial cells dividing during the incubation period. Complement may be helping to protect torpid ground squirrels from bacterial sepsis.

The lack of complement killing activity in torpid ground squirrel serum at 37 ˚C is a perplexing finding. IBA animals were not included at 4 ˚C due to the lack of sample availability. If serum from IBA ground squirrels was available it would be speculated that the results would have been similar to what was seen with the serum from torpid ground squirrels. It is worth considering that the levels of the other complement pathway genes may be down regulated during torpor not allowing the pathway to be completed. When looking at the bone marrow transcriptome a down regulation in other complement pathway proteins during torpor is not seen (S. T. Cooper et al., 2016). Plasma protein concentration of other complement pathway proteins should be analyzed to more accurately answer this question. The complement pathway is regulated by numerous protein both fluid phase regulators and membrane bound regulators (Meri & Jarva, 2001). There is the possibility that complement pathway regulatory proteins are upregulated during torpor accounting for the decreased complement activity. Of the fluid-phase regulatory proteins detected in the bone marrow transcriptome it was seen that factor H and clusterin were both upregulated during torpor while C4b binding protein remains constant throughout the hibernal cycle (S. T. Cooper et al., 2016). Interestingly membrane bound regulatory proteins CD46 and CD59 are down regulated in the bone marrow during torpor while decay accelerating factor was seen to be upregulated (S. T. Cooper et al., 2016). The upregulation of fluid-phase regulatory proteins during torpor in the bone marrow could be an explanation for the lack of complement activity from torpid

ground squirrels at 37 ˚C, but requires additional analysis. This upregulation of complement regulatory proteins during torpor could help protect the host from complement-mediated damage.

Electrolyte levels throughout the hibernal cycle also needs to taken into account. Without proper electrolyte balance the complement proteins would not be able to function properly. There are conflicting reports of electrolyte levels in the literature with some studies describing an increase in certain electrolytes like magnesium during torpor (Pengelley & Chaffee, 1966; Riedesel, 1957). Other studies have described a decrease in other electrolytes such as sodium and potassium during torpor (Pengelley & Kelly, 1967). More studies have described no change to sodium levels while magnesium levels were increased in torpid animals and potassium was highest in aroused animals (Soivio & Kristoffersson, 1974). There is an agreement among the literature that magnesium levels are increased during torpor and potassium levels highest in active animals. Reports have placed calcium levels are the highest during torpid ground squirrels (Soivio & Kristoffersson, 1974). It is unclear how the changing electrolyte levels potentially impact complement function and further studies are needed to elucidate any effects.

Post translational modification of complement proteins during torpor could also be responsible for the decrease in complement activity in torpid animals at 37 ˚C. Further protein analysis would be required to test for any post translational modification. Future studies could include 2D gel electrophoresis or mass spectroscopy to compare proteins from summer and torpid serum for post translational modification.

Teasing Apart which Complement Pathway was activated

The next logical question after assaying complement activity was to know which pathway the complement system was being activated by. Flow cytometry was used to assay if ground squirrels had anti-*E. coli* antibodies present. If no anti-*E. coli* antibodies were present that would eliminate the possibility of the classical pathway being activated and leave complement to be activated via the alternative or lectin pathway. Only five ground squirrels (16.6 %) had anti-*E. coli* antibodies present. With this number being fairly low it is more likely that complement was activated via the alternative or lectin pathway but the possibility of the classical pathway being activated cannot be ruled out. To get a more accurate idea of which pathway or pathways were activated further studies involving developing ELISA's to detect spilt products for each pathway would need to be developed for use against ground squirrel complement but that was beyond the scope of this study.

Comparison of C3 Protein Levels in Liver, Adipose, and Bone Marrow Tissue throughout the Hibernal Cycle using Immunohistochemistry

Non-hibernating, IBA, and torpid ground squirrel liver, adipose, and bone marrow tissue were stained with goat anti-mouse C3 antibody and observed under 100X and 200X magnification for the relative abundance and location of C3. Liver C3 staining was concentrated around blood vessels and was more intense in non-hibernating ground squirrels. The intensity of staining backed up the qPCR data that was collected for the liver. Adipose tissue showed lower levels of staining than in the liver and the staining was not concentrated in any particular region of the tissue. Staining did appear to be slightly more intense in non-hibernating ground squirrel adipose tissue. Low staining

intensity in the adipose tissue also agreed with the qPCR data for adipose tissue. Torpid ground squirrel bone marrow stained much more intensely than non-hibernating or IBA ground squirrel bone marrow. Intense torpid ground squirrel bone marrow staining agreed with the transcriptome data. Bone marrow staining revealed that the high level of transcripts of C3 being expressed during torpor do appear to be being translated into protein and that C3 is staying within the bone marrow.

Why is C3 so highly Expressed in Bone Marrow of Torpid Ground Squirrels?

This project failed to uncover the real reason why C3 is being expressed as highly as it is in the bone marrow of torpid ground squirrels. Armed now with the knowledge that C3 protein does not increase in the plasma, it could be staying in the bone marrow. Thus, it is logical to hypothesize that C3 is playing an essential and potentially novel role within the bone marrow during torpor. Bone marrow becomes enriched with adipocytes during periods of hibernation, potentially displacing other cell types normally present within the bone marrow (S. T. Cooper et al., 2016). It is possible that bone marrow derived C3 is functioning in a different way than liver derived C3 and it has been hypothesized that C3 may be functioning in lipid storage within the bone marrow (Barbu et al., 2015). While lipid storage is a possible role for bone marrow derived C3, another is that C3 is helping the bone marrow regenerate after the animal comes out of torpor (Janowska-Wieczorek, Marquez-Curtis, Shirvaikar, & Ratajczak, 2012). Recent studies have shown that C3 -/- knockout mice who underwent irradiation displayed a slowed response and a 5-7 day delay in bone marrow regeneration (Ratajczak et al., 2004). Activated products of C3 may be directly involved in promoting the regeneration of the bone marrow after periods of hibernation (Ratajczak et al., 2004). This novel function of

C3 in mice may be playing the same role in hibernating ground squirrels which would explain why the expression of C3 is so high in the bone marrow during torpor.

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