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Blood coagulation properties in hibernating and non-hibernating American bullfrogs (Lithobates catesbeianus)

Stephen Cain
University of Northern Iowa

BLOOD COAGULATION PROPERTIES IN HIBERNATING AND NON-HIBERNATING AMERICAN BULLFROGS (*Lithobates catesbeianus*)

An Abstract of a Thesis

Submitted

in Partial Fulfillment

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ABSTRACT

Activated partial thromboplastin times (APTT) and factor deficiency assays were performed on the plasma of twenty American bullfrogs (*Lithobates catesbeianus*) to test for potential changes and regulation of the intrinsic coagulation pathway during and after hibernation. Bullfrogs (pre-hibernating n=4, hibernating n=6, post-hibernating n=10) were housed in 10 gallon aquariums filled with RO water and placed in a temperature-controlled room. Hibernation was gradually induced by decreasing the temperature of the room from 24 C to 3 C in increments of 3 C per week. Blood (6-8 ml) was collected from individual non-hibernating frogs, hibernating frogs and post-hibernating frogs in 0.105M sodium citrate tubes and immediately centrifuged at 2500 x g for five minutes. The resulting plasma was stored at -80 C until tested for activated partial thromboplastin time. APTT times of hibernating bullfrogs were significantly longer than pre-hibernating times (p=0.000) and post-hibernating times (p=0.004). Factor deficient assays were performed for Factors VIII, IX, XI, and XII to determine their presence or absence. Factor VIII in all bullfrogs was either not present, or if present, was non-functional. Factor IX, XI, and XII were present in pre-hibernating bullfrogs, but during hibernation, these levels either decreased or were inhibited, suggesting that Factors IX, XI, and XII are regulated during hibernation. However, no difference in Factors IX, XI, and XII levels were found between hibernating and post-hibernating animals. Initial tests for a potential inhibitor failed to show that such an inhibitor existed, but additional studies are needed. Hibernating ectotherms, like hibernating endotherms, regulate levels of the

clotting factors associated with intrinsic coagulation system during hibernation to prevent clot formation during hibernation.

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has been approved as meeting the thesis requirement for the

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_____	_____
Date	Dr. David Saunders, Chair, Thesis Committee
_____	_____
Date	Dr. James Demastes, Thesis Committee Member
_____	_____
Date	Dr. David McClenahan, Thesis Committee Member
_____	_____
Date	Dr. Patrick Pease, Interim Dean, Graduate College

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Hibernation

As winter months approach, temperatures begin to drop, day length shortens, and resources become sparse. In order to survive these challenges, some animals enter a physiological state of deep torpor known as hibernation. Hibernation is a state of inactivity that is marked by a decrease in body temperature and metabolic rate (Melvin and Andrews 2009). Some endotherms and many ectotherms conserve their energy stores during hibernation by decreasing their metabolism. There are a variety of other terms used to describe the condition or degree of torpor in mammals, reptiles, amphibians and invertebrates such as: estivation, brumation, and diapause to name a few (Mayhew 1965; Geiser and Ruf 1995; Melvin and Andrews 2009). In this study, the term hibernation will be used throughout, with the understanding that hibernation strategies are varied throughout the animal kingdom. A typical hibernation season is characterized by periods of torpor that range from days to upwards of five weeks (Carey et al. 2003). Mammals that hibernate on a yearly basis undergo numerous and complex changes in morphology, physiology, and behavior in response high energy demand and lowered energy availability in the environment (Carey et al. 2003). Hibernation is a way of surviving periods of low food availability while maintaining the ability to return to a normal level of activity and often temperature when the environmental conditions become more favorable (Melvin and Andrews 2009).

Effect of Hibernation on Body Temperature in Endotherms

In order to survive long cold winter conditions, some endotherms slow down their metabolism and reset thermoregulation. By slowing down metabolism and lowering their body temperature, endotherms can conserve energy until environmental conditions become more favorable. A few endotherms can lower their body temperature to near ambient temperature or to the temperature of their hibernacula (Florant and Heller 1977; Barnes 1989; Hut et al. 2002). One of the best examples of this is the hibernating Arctic ground squirrel (*Spermophilus parryii*). Arctic ground squirrels have the ability to undergo deep and prolonged periods of supercooling with core body temperatures below the freezing point of water (0°C). They have changes in body temperature from +37°C to -3°C (Barnes 1989). European ground squirrels (*Spermophilus citellus*) have an internal temperature of 37.5°C when active and lower their internal temperature to 2.9°C during hibernation (Hut et al. 2002). Yellow-bellied marmots (*Marmota flaviventris*) lower their body temperature from 36 to 8°C as they enter hibernation. Once they reach deep hibernation at an ambient temperature of ~5°C, their body temperatures plateau around 7.5°C (Florant and Heller 1977). Alpine marmots (*Marmota marmota*) can lower their body temperature as ambient temperature falls, but they always maintain body temperatures above 3–5°C. Alpine marmots can drop their body temperature from 35°C to 9.5°C within the span of about 3 days. Once these marmots reach their threshold internal body temperature, no further decrease in body temperature is observed, even with lowered ambient temperature. Rather, metabolic rate increases to maintain the threshold body temperature (Ortmann and Heldmaier 2000). Some species of bats such as Little

brown bats (*Myotis lucifugus*) can also greatly lower their body temperature during hibernation. Little brown bats can decrease their body temperature from 30°C to 5°C on occasion (Smith et al. 1954). Typically, primates and tropical endotherms are not thought of as hibernators. However, at least one species of tropical primate from Madagascar, the Fat-tailed dwarf lemur (*Cheirogaleus medius*), is known to hibernate in tree holes for up to seven months when resources are scarce. These lemurs have active body temperatures between 34°C and 38°C with hibernating body temperatures as low as 18.3°C (Dausmann et al. 2000). Even larger endotherms such as European brown bears (*Ursus arctos*) have the ability to reduce their body temperature during a four-six month hibernation period. During this period their core temperatures can decrease from 37°C to 33°C (Hissa et al. 1994). This decrease in body temperature for the aforementioned endotherms decreases metabolic rate and greatly lowers energy demand.

Effect of Hibernation on Heart Rate of Endotherms

For some endotherms, hibernation is accompanied by a significant reduction in heart rate. Under euthermic conditions common dormice (*Glis glis*) have a heart rate of ~450 bpm, but lower their heart rate to ~10 bpm during hibernation. Anesthetised golden mantled ground squirrels (*Spermophilus lateralis*) have a change in heart rate from 372±20 to 37±9 bpm during hibernation (Milsom et al. 1993). In a study on woodchucks (*Marmota monax*) done by Lyman (1958), the heart rate of hibernating woodchucks was shown to decrease before any decrease in body temperature occurred. The heart rate of active woodchucks was usually between 80 and 95 bpm with heart rates as high as 110 bpm. As the woodchucks approached deep hibernation, their heart rate dropped to 8 to 10

bpm reaching levels as low as 3 bpm in sustained periods of deep hibernation (Lyman 1958). Accompanied by a decrease in body temperature, Grizzly Bears (*Ursus arctos horribilis)* decrease their resting heart rate from ~84 to ~19 bpm during hibernation (Nelson et al. 2008). This decrease in heart rate slows blood flow through the vessels and increases the chances of intravascular thrombosis as a result of blood stasis.

Effect of Hibernation on Body Temperature of Ectotherms

Ectotherms differ from endotherms in that they lack the ability to physiologically regulate their body temperature in contrast to the ambient temperature. Ectotherms have body temperatures that change accordingly with their surrounding temperature. This presents an array of physiological challenges for ectotherms during extended bouts of cold temperatures when their body temperatures can drop drastically. Red-sided garter snakes (*Thamnophis sirtalis parietalis*) lower their core temperature throughout their winter dormancy. Core body temperatures of female red-sided garter snakes gradually declined from 14.7°C (±0.24 °C) in mid-September to 1.1°C (±0.16 °C) in early April (Lutterschmidt et al. 2006). In a study monitoring the body temperature 38 eastern box turtles (*Terrapene carolina),* the average body temperature during hibernation was found to be 3.28°C while mean body temperature during emergence was 9.32°C (SE = 0.09) (Currylow et al. 2012). Hibernating rattlesnakes (*Crotalus viridis*) in southern British Columbia exhibited body temperatures ranging between 2°C and 7°C (Macartney et al. 1989). Some ectotherms even reach core temperatures below the freezing point of water. Another study involving eastern box turtles in southwestern Ohio found that some of the turtles experienced body temperatures of -0.3°C or below during the winter (Claussen et

al. 1991). In a previous study regarding the freeze tolerance of box turtles under lab simulated cooling, Costanzo and Claussen (1990) found that box turtles are able to withstand even colder core temperatures. Under controlled laboratory conditions, they froze 19 adult turtles to body temperatures ranging from -0.9 °C to -3.6°C with 100% survival and recovery rate (Costanzo and Claussen 1990).

Effect of Hibernation on Heart Rate of Ectotherms

Ectotherms show a higher degree of hypometabolism than endotherms. Unlike endotherms, ectotherms do not have short bouts of arousal but remain in a hypometabolic rate for the duration of hibernation. To conserve energy during periods of inactivity at low internal body temperatures, many ectotherms decrease metabolism and drop their heart rate. Some species of frogs can even stop their heart rate all together (Layne et al. 1989). Wood frogs can decrease their pre-freeze heart rate from 15 bpm to an average of 4.4 bpm at -2.4°C. Eventually, these frogs enter nonlethal cardiac arrest once they are completely frozen but heartbeat is restored soon after thawing occurs (Layne et al. 1989). Even bullfrogs have been known to experience decreased heart rates in response to decreased temperature in their hibernacula. At 4°C in air-saturated water, bullfrogs decreased their heart rates to 2–5 bpm (Lillo 1980).

Blood Coagulation

Blood coagulation is part of the host defense mechanism termed hemostasis, which helps prevent excessive blood loss from damaged tissue, blood vessels and organs. When unwanted hemostasis occurs, it is termed thrombosis. Platelet aggregation and fibrin generation are the two major bleeding defense mechanisms (Kroll 2001). The first

mechanism belongs to the cellular system. This system consists of platelets circulating in blood vessels. These platelets form a partial plug when injury occurs. Fibrin generation is based on the activation of numerous protein factors called clotting factors as well as cofactors that act in conjuction to form a blood clot. Any irregularities or imbalances in the formation of a clot could be lethal. (Kroll 2001; Kumar et al. 2007).

Fibrin generation is typically more important for hemostasis in veins and venules, while platelet aggregation is typically more important for hemostasis in arteries, arterioles, and capillaries. In both instances, coagulation occurs when the endothelium of a blood vessel is damaged. This exposes the blood to subendothelial constituents. These subendothelial constituents consist of smooth muscle cells and fibroblasts which express tissue factor (TF) as well as extracellular matrix proteins. Tissue factor is an integral cell membrane protein that initiates coagulation reactions that result in the production of fibrin. The subendothelium also contains extracellular matrix proteins which bind to both platelets and plasma von Willebrand factor (vWF). Under blood flow conditions the binding of extracellular matrix proteins results in platelet adhesion and ultimately platelet thrombus formation (Kroll 2001).

Coagulation Cascade

The coagulation cascade is a sequential series of enzymatic conversions that culminates in thrombin formation. This thrombin converts soluble fibrinogen into an insoluble fibrin clot (Kumar et al. 2007). The coagulation process is initiated mainly when tissue factor (TF) is exposed on damaged tissue or endothelium. Sometimes, when monocytes become activated they can also expose TF. There are three pathways

associated with coagulation: the intrinsic pathway, the extrinsic pathway, and the common pathway.

The intrinsic pathway is defined as blood coagulation initiated by components entirely contained within the vascular system. This pathway begins when factor XII (Hageman Factor) becomes activated on a charged surface (contact activation). Activation of factor XII is then followed sequentially by the activation of factor XI and factor IX and culminates in the activation of factor VIII (Gailani and Renne´ 2007). The extrinsic pathway begins when tissue factor binds to activated factor VII (FVIIa). FVIIa bound to tissue factor forms a complex that can activate factor IX bypassing the activation of factor XII and factor XI. The common pathway begins with factor X activation (FXa) which is where the intrinsic and extrinsic pathways converge. Factor Xa activates prothrombin to thrombin in the presence of the cofactor factor Va. The thrombin then converts fibrinogen to fibrin to complete the cascade (Gailani and Renne´ 2007).

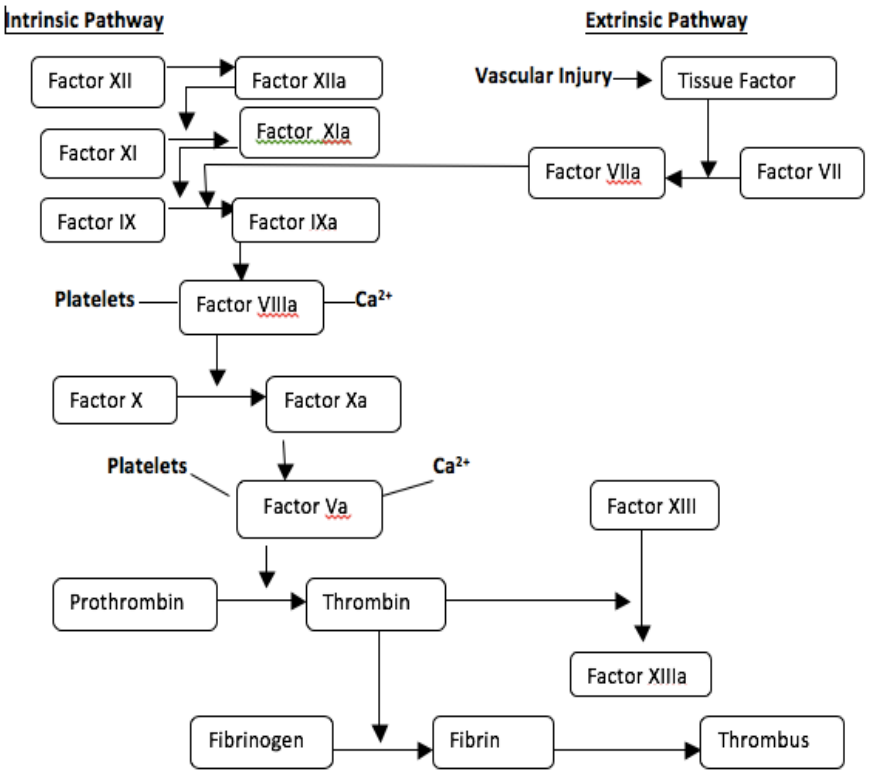

Figure 1. Coagulation Cascade.

Factors of the Coagulation Cascade

Factors involved in the coagulation cascade play a crucial role in the process of coagulation. There are a total of thirteen clotting factors which function either as a clotting factor or a co-factor in the process of clot formation (Van Beek et al. 2009). Higher than normal levels of clotting factors such as factor VIII, IX and XI are associated with an increased risk of venous thrombosis. This venous thrombosis risk is elevated as factor levels increase (Van Beek et al. 2009).

Factor VIII, which is primarily synthesized in the liver, is a glycoprotein that is involved in the middle part of the intrinsic pathway. Factor VIII circulates in the plasma as a complex with von Willebrand factor. It functions as a cofactor in clotting by accelerating the conversion of factor X to its activated form, Xa. This conversion happens in the presence of factor IXa, calcium, and phospholipid (Colman et al. 2006). However, before it can show any cofactor activity, factor VIII must undergo a minor proteolytic event caused by thrombin or another serine protease. Similar to fibrinogen, factor VIII levels will increase during an inflammatory event. Deletions and nonsense mutations of the gene for factor VIII usually result in severe hemophilia (Colman et al. 2006). When hemophilia is a result of impaired factor VIII, mutated factor VIII, or a deficiency of factor VIII, it is clinically termed hemophilia A (Lee et al. 2010). Hemophilia A results in spontaneous bleeding into joints and muscles and can cause severe bleeding when trauma occurs (Lee et al. 2010).

Factor IX, also produced in the liver, is a single-chain glycoprotein involved in the middle part of the intrinsic pathway and circulates in the plasma. Factor IX can be activated two ways. It can be activated by factor XIa or the factor VIIa/ tissue factor complex (Kaushansky et al. 2010). This activation is calcium dependent. When activated, factor IX combines with its cofactor, activated factor VII, and this complex activates factor X. Platelets are believed to be the structure responsible for the receptor/binding protein which assembles the factor IXa/VIIa complex (Lichtman et al. 2006). Activated factor X is responsible for the conversion of prothrombin to thrombin, so any deficiency in factor IX results in the bleeding disease known as Hemophilia B (Christmas Factor

Deficiency). When factor IX activity drops to less than 25 percent of normal, excessive or abnormal bleeding can occur. This risk is greatly increased after trauma. In the majority of human cases of hemophilia B, prothrombin times are normal but partial thromboplastin times are prolonged (Lichtman et al. 2006).

Factor XI, also known as a contact factor, is manufactured in the liver and circulates in the plasma as a complex with high molecular weight kininogen. Conversion of Factor XI to Factor XIa is one of the first steps in the intrinsic coagulation cascade. There are a couple of ways in which Factor XI can be converted to its activated form, Factor XIa. Factor XI can either be activated by thrombin, or by Factor XIIa. Thrombin activation can occur when circulating thrombin comes into contact with certain negatively charged materials, or thrombin can directly activate Factor XI in the presence of activated platelets. Factor XIa can then activate Factor IX and continue the coagulation cascade towards clot formation. Factor XIa also has a high affinity for binding with activated platelets. This may help localize the coagulation event.

Factor XI deficiency leads to a mild, but rare, bleeding disorder known as Hemophilia C. Factor XI deficiency is rare in humans as a whole, but is found more frequently in certain minority populations like the Ashkenazi Jews (Kroll 2001). While not as serious as Hemophilia A or B, Factor XI deficiency can lead to spontaneous bleeding events after trauma or mild surgery. Typically, surgeries that are performed on or near mucosal surfaces are the most likely to cause serious bleeding events. Individuals with Hemophilia C may also experience easy bruising, epistaxis (nosebleeds), and menorrhagia (abnormal bleeding during menstruation). Factor XI deficiency is usually

detected via screening tests that indicate a prolonged activated partial thromboplastin time or a direct test of Factor XI levels (Kroll 2001).

Factor XII is similar to Factor XI in that it is a contact factor that is manufactured in the liver and circulates in the plasma. Factor XII along with high-molecular-weight kininogen (HK) and prekallikrein (PK) are responsible for contact activation of blood coagulation. This contact activation requires protein to protein interactions as well as protein to surface interactions. These interactions convert Factor XII to Factor XIIa. Factor XIIa functions to activate Factor XI which in turn activates factor IX as the intrinsic coagulation cascade continues. Factor XII can be activated by Factor XIIa, Factor XIa, and thrombin. The activation of Factor XI by factor XIIa requires the presence of HK as well as a foreign surface (Lichtman et al. 2006).

Factor XII along with HK and PK are required for a normal activated partial thromboplastin time (aPTT), but are not required for normal hemostasis. Individuals lacking either Factor XII, HK, or PK do not seem to exhibit any bleeding tendencies other than a rare few who exhibit mild bleeding post-surgery. In humans, Factor XII is not necessary for thrombin generation. Factor XII, however, is extremely important in the inflammatory response mechanism (Lichtman et al. 2006).

Blood Clotting Inhibitors

Blood clotting involves enzymatic activity at virtually every step in the clotting cascade. Enzymes can be sensitive to temperature and pH. Changes in temperature and pH can have a significant effect on enzymatic activity and can thereby impact blood clotting times. Temperature and pH aren’t the only factors that can prolong clotting time.

There are other factors such as blood clotting inhibitors that can interfere with the activity and functionality of the clotting factors in the coagulation cascade. These inhibitors can delay or block the process of clot formation at different stages.

There are numerous inhibitors, but two of the inhibitors most specific to blood coagulation are tissue factor pathway inhibitor (TFPI) and antithrombin. Antithrombin belongs to a large family of serine protease inhibitors (SERPINS) (Kaushansky et al. 2010). Tissue factor pathway inhibitor has inhibitory sites for factor Xa and for the factor VIIa/TF complex. However, TFPI cannot inhibit the factor VIIa/TF complex unless it has also bound to factor Xa (Kaushansky et al. 2010).

Serine protease inhibitors comprise a large class consisting of a broad spectrum of protease inhibitors. Some of the SERPINS most common in clotting inhibition include: antithrombin, heparin, protein C, protein S, protein Z-related protease inhibitor, alpha-2 antitrypsin, alpha-2 macroglobulin, plasminogen activator inhibitor-1, and plasminogen activator inhibitor-2. All of these inhibitors are known to reduce the activity of the clotting factors involved in the blood coagulation cascade and thus increase the time of blood to clot. Each inhibitor mentioned above plays a different role in inhibiting the clotting process. Increase in the activity of any of these inhibitors causes prolonged clotting time. (Yadav 2011; Kaushansky et al. 2010).

Effect of Hibernation on Blood Coagulation in Endotherms

Decrease in body temperature reduces the activity of many enzymes, particularly those associated with blood coagulation in humans (Watts et al. 1998). Humans aren’t the only endothermic organisms to experience elongated clotting times from low body

temperature. Prolonged clotting times in hibernating Franklin's ground squirrels (*Spermophilus franklini)* occurred with a decrease in temperature, but were shortened when core body temperatures were increased from 7°C to 36°C. In fact, clotting times in these squirrels decreased from an average of 48 min at 7°C to an average of 2.2 min. at 36°C (Pivorun and Sinnamon 1981). Similar results of prolonged clotting times were found in dormant Little brown bats (*Myotis lucifugus*) at lower temperatures (5°C) compared to shorter times at higher temperatures (25°C) (Smith et al. 1954).

A decrease in temperature alone may not be totally responsible for increased clotting time. Another physiological effect known as acidosis also plays an important role in prolonging clotting times during hibernation. In an in vitro study performed by Dirkmann et al. (2008), hypothermia produced elongated coagulation times that were further prolonged by acidosis. Prolonged clotting times in Gray wolves (*Canis lupis*) occurred after an infusion of lactic acid (Broersma et al. 1970). In pigs (*Sus domesticus*), test groups that experienced acidosis demonstrated a 47% increase in splenic bleeding time compared to the normal control group. Pigs that experienced hypothermia showed a 57% increase in splenic bleeding time. Pigs undergoing both hypothermia and acidosis demonstrated a 72% increase in splenic bleeding time (Martini et al. 2005). Hypothermia and acidosis play important roles in increasing the clotting time in endotherms; when both conditions develop together in endotherms, it amplifies the effect on blood coagulation.

Effect of Hibernation on Blood Coagulation in Ectotherms

Many ectotherms face more extreme physiological conditions during hibernation than do endotherms. Hibernating ectotherms not only face low body temperatures during winter, but they are often subjected to extended periods of low oxygen availability. They may be trapped under ice, in anoxic mud, or in water that has been depleted of oxygen (Jackson 2002). Painted turtles (*Chrysemys picta*) often hibernate in ice covered ponds, mud, or water with little oxygen and can survive for at least 4 months at 3°C (Ultsch and Jackson 1982; Jackson 2002). When hibernating ectotherms are subjected to prolonged states of hypoxia, they rely mainly on anaerobic respiration. During prolonged anaerobic respiration lactic acid is produced, which can lead to blood and tissue acidosis. Decrease in body pH accompanied by a decrease in body temperature affects many processes in the body by altering enzyme activity. Blood coagulation is among the most important of these affected processes.

Like endotherms, ectotherms exhibit coagulation times related to body temperature. Decrease in temperature from 37°C to 15°C resulted in increased prothrombin time (PT) in Green sea turtles (*Chelonia mydas*), Kemp's ridley sea turtles (*Lepidochelys kempii*), Loggerhead sea turtles (*Caretta caretta*), Hawksbill sea turtles (*Eretmochelys imbricata*), and Leatherback sea turtles (*Dermochelys coriacea*) (Soslau et al. 2004). Two other turtle species, Painted turtles (*Chrysemys picta*) and red-eared sliders (*Trachemys scripta elegans*), demonstrated prolonged whole blood coagulation times as well as prolonged prothrombin times when acclimated to a temperature of 3°C compared to those kept at 21°C. Some of the individuals failed to even form clots at 3°C

(Barone and Jacques 1975). Likewise, prolongation of whole blood clotting time and increased prothrombin time has been observed in hibernating frogs when compared to non-hibernating Indian bullfrogs (*Hoplobatrachus tigerinus*) and other frog species (Spitzer and Spitzer 1952; Ahmad et al. 1979). The shortest clotting times were obtained from the non-hibernating frogs that were exposed to ambient temperatures between 30–37°C. Coagulation times became progressively more prolonged as the temperature decreased from 30°C to 10°C, with 10°C temperatures having the longest clotting times. The clotting times of whole blood, plasma recalcification, cephalin, and prothrombin were all significantly longer during hibernation. Thrombin time remained nearly the same for both hibernators and non-hibernators (Ahmad et al. 1979).

Hibernation in Endotherms vs Hibernation in Ectotherms

The effects of hibernation are typically more severe in ectotherms than in endotherms, because ectotherms drop their body temperature significantly during periods of low ambient temperature and for prolonged times during hibernation (Yadav 2011). Many endotherms experience a decrease in core body temperatures during hibernation, but most maintain body temperatures well above 0°C. Many hibernating ectotherms experience temperatures much closer to 0°C. Ectotherms are also more likely to experience hypoxia, because many hibernate in anoxic mud, under ice covered water or underwater where they have little access to oxygen. The drop in metabolic rates and heart rates during hibernation tend to be more severe in ectotherms relative to endotherms. A larger decrease in heart rate means ectotherms are more likely to experience stasis of the blood. When the blood is allowed to pool for long periods of time, it increases the

chances intravascular thrombosis events. Prolonged coagulation as a result of hypothermia and hypoxia may be beneficial in off-setting the increased risk of intravascular thrombosis in hibernating ectotherms (Yadav 2011).

American Bullfrogs

American Bullfrogs, while native to North America, have a widespread global distribution. American Bullfrogs are the largest frogs in North America. They are largely aquatic, even as adults, and hibernate submerged in cold water. Hibernating *Lithobates catesbeianus* experience a decrease in body temperature as well as a drop in heart rate during winter months. During hibernation, they are able to survive water and body temperatures of around 3°C, as long as the water has a sufficient partial pressure of oxygen (PO_2) for cutaneous diffusion for O_2 extraction (Stewart et al. 2004). A study conducted by Friet (1993) suggested that adult bullfrogs overwintering beneath an ice-covered pond tended to avoid hypoxic regions found lower in the water column. Instead, the frogs more abundantly chose locations just beneath the ice where water was colder, but less hypoxic (Friet 1993). By hibernating under the water, bullfrogs are able to avoid freezing and desiccation. Warm-acclimated bullfrogs (22°C) have heart rates between 34.3 beats per minute and 38.5 beats per minute (Herman and Mata 1985). Bullfrogs acclimated to near hibernating temperature (5°C) had drastically lower heart rates between 6.6 bpm and 7.2 bpm (Herman and Mata 1985). Maintaining a low body temperature may be beneficial to hibernating ectotherms and could potentially lead to hypocoagulation or lengthened clotting times to combat intravascular clotting events. There are several other important physiological changes that occur during hibernation

that may also be responsible for the change in the blood coagulation properties of hibernating American Bullfrogs.

Bullfrogs and Wood Turtles

Previous studies at the University of Northern Iowa focused on the blood coagulation properties in hibernating vs. non-hibernating wood turtles (*Glyptemys insculpta*). These studies determined that there was a potentially unique blood clotting inhibitor present in hibernating wood turtles. This blood clotting inhibitor appeared to be a protein, but a specific protein has yet to be identified. Wood turtles are an endangered species, and as such, they are generally more difficult to obtain. Blood samples are generally small in quantity, the animals can't be sacrificed, and they must remain healthy the entire study. Therefore, the amount of plasma that can be gathered for testing is relatively limited. Bullfrogs, like wood turtles are also hibernating poikilotherms. They face similar conditions and challenges during hibernation, so direct comparisons can be made between these two organisms. Bullfrogs are more abundant and require less regulatory oversight. More blood can be taken from bullfrogs since they can be sacrificed, thus yielding more plasma. To date, there is little known about the clotting cascade and how it functions in bullfrogs.

Objectives of the Study

My study was designed to determine if hibernation alters the blood coagulation properties in American Bullfrogs (*Lithobates catesbeianus*), and if so, to determine if the response of coagulation changes during hibernation is similar between bullfrogs and wood turtles (*Glyptemys insculpta*). Another objective of the study was to better

understand the coagulation properties and processes of American Bullfrogs in general, and compare them to wood turtles. If the two organisms were similar, then we would use bullfrogs for future studies of the unknown protein inhibitor. Experiments were performed to determine if factors other than temperature effects on the enzymatic properties of clotting factors may influence blood clotting in hibernating bullfrogs. Therefore, individual clotting factors of the intrinsic pathway were tested as well as the potential role of coagulation inhibitors.

CHAPTER 2

MATERIALS AND METHODS

Set-Up

Bullfrogs were purchased from Kons Scientific in Germantown, Wisconsin. Bullfrogs (n=20) were housed in 10 gallon aquariums containing RO water placed in a temperature-controlled room at the University of Northern Iowa. Bullfrogs ranged in weight from 260 g to 436 g. The aquariums were filled with enough RO water, so that the frogs still had access to air. Day light was artificially induced using a 40 watt white fluorescent light bulb connected to a timer. The bullfrogs were exposed to 12 hours of light and 12 hours of darkness. Bullfrogs were checked daily and the water in the aquariums was changed as necessary. Bullfrogs were not fed throughout the entire study. Hibernation was gradually induced beginning on November 17, 2010. The temperature of the room, initially 24°C, was decreased approximately 3°C per week until the air temperature in the room reached 3°C. Prior to the start of reducing the temperature in the room, four animals were removed for blood collection. These animals were the pre-hibernating sample set. The remaining 16 bullfrogs were cooled to 3°C. Between the dates of February 25, 2011 and March 23, 2011, a total of six animals were periodically removed for blood collection. These bullfrogs were defined as the hibernating sample set. After removal of the hibernating group, the temperature of the cold room was increased by 3–5°C per week beginning March 21, 2011. Between April 7, 2011 and May 6, 2011, the remaining 10 individual frogs were periodically removed. These 10 bullfrogs were defined as the post-hibernating sample set.

Blood Collection

The experiments reported herein were conducted according to the protocol set forth in the "AVMA Guidelines for the Euthanasia of Animals: 2013 Edition" (American Veterinary Medical Association) (Leary et al. 2013). Blood from bullfrogs was drawn in accordance with the guidelines approved by the University of Northern Iowa Animal Care Committee (UNI Protocol Number 2010.003). Prior to drawing blood, bullfrogs were weighed and then anesthetized in a solution of 3 g/L MS222 (Tricaine Methanesulfonate) and distilled water solution with sodium bicarbonate added to the solution to bring the pH to 7.0. The frogs were bathed in this solution until they were unconscious. Blood was drawn from the bullfrogs using a 21 G x 1 ½ inch needle and sterile 10ml syringe coated with 0.105M sodium citrate. Blood samples were collected periodically from December 1, 2010 to May 6, 2011, into vacutainer tubes containing 0.105 M buffered sodium citrate solution. The amount of blood collected from the frogs ranged from 4ml to 12ml. Immediately upon removal from the bullfrogs, the blood was centrifuged at 2500 x g for five minutes to separate the plasma from the RBC's. Plasma was removed and stored in 1ml centrifuge tubes. Plasma tubes were labeled (HF1A–HF4A for pre-hibernating bullfrogs; HF5A–HF10A for hibernating bullfrogs; and XF1A–XF10A for non-hibernating bullfrogs) and stored at -80° C until the coagulation assays were performed.

Blood Coagulation Assays

Blood coagulation assays were performed to measure the blood clotting time of bullfrogs. Additional blood coagulation assays were performed to analyze the process of

blood coagulation by the intrinsic (Activated Partial Thrombin Time), extrinsic (Prothrombin Time), and the common pathways (Thrombin Time). All of the blood coagulation assays were performed using a Trinity Biotech KCΔ1 Amelung coagulation analyzer.

Coagulation Assay for Activated Partial Thromboplastin Time (APTT)

APTT assays were performed by mixing 100 µl of individual bullfrog plasma with 100 µl of Cephan 5 APTT reagent (Aniara Corporation). After mixing, the mixture was incubated at 37°C in the Trinity Biotech analyzer for 180 seconds after which 0.025M $CaCl_2$ was added. Following the addition of 0.025M $CaCl_2$, the time to clot formation was determined and this time served as the activated partial thromboplastin time. As a positive control, 100 µl of human plasma (Aniara Corporation) was used in place of bullfrog plasma, while 100µl of 0.9% saline solution, was used in place of bullfrog plasma as a negative control. This test was performed for the pre-hibernating bullfrogs (n=4), hibernating bullfrogs (n=6), and post hibernating bullfrogs (n=10). If any of the APTT assays or controls did not clot after 1,000 seconds, the assay was stopped and clotting time was recorded as “1,000 plus seconds, no clot.”

Coagulation Assay for Prothrombin Time (PT)

For PT assays, 100µl of individual bullfrog plasma was incubated at 37°C in the KCΔ1 analyzer for 60 seconds. After incubation, 100µl of Siemens Dade thromboplastin-C was added, and the time to clot formation was recorded. As a positive control, 100µl of human plasma (Aniara Corporation) was used in place of bullfrog plasma, while 100µl of a 0.9% saline solution, was used in place of bullfrog plasma as a negative control. PT

times were determined for pre- hibernating (n=4), hibernating (n=6), and post hibernating (n=10) bullfrogs. If any of the PT assays or controls ran longer than 600 seconds, the assay was stopped and clotting time was recorded as 600 plus seconds, no clot.

Coagulation Assay for Thrombin Time (TT)

For TT assays, 100µl of individual bullfrog plasma was incubated for 60 seconds, after which 100µl of Hyphen Biomed thrombin reagent was added and the time to clot formation was recorded. As a positive control, 100µl of human plasma (Aniara Corporation) was used in place of bullfrog plasma, while 100µl of a 0.9% saline solution, was used in place of bullfrog plasma as a negative control. TT assays were performed on pre-hibernating (n=4), hibernating (n=6), and post-hibernating (n=10) samples of bullfrogs. Any of the TT assays or controls that ran longer than 600 seconds were stopped and the clotting time was recorded as 600 seconds, no clot.

Factor Deficiency Assay: Determination of the Presence of Factors VIII, IX, XI, XII Using Factor Deficient Plasma

Factor deficient assays were performed to test for the presence and functionality of factors involved in the intrinsic pathway in bullfrog plasma. Plasma from individual bullfrogs was mixed with factor deficient plasma that was missing only one factor (VIII, IX, XI, or XII deficient) (Aniara Corporation) to determine if the bullfrog plasma contained sufficient amounts of that specific missing factor to recover clotting function. For factor VIII deficiency assays, 80µl of factor VIII deficient plasma (Aniara Corporation) was mixed with 20µl of bullfrog plasma and 100µl of Cephan 5 APTT solution (Hyphen Biomed). The mixture was incubated in the coagulation analyzer at

37°C for 180 seconds. Following incubation, 100µl of 0.025M $CaCl_2$ was added and the clotting time was recorded. For all positive controls, the 20µl of bullfrog plasma was substituted with 20µl of human plasma. Similarly, for all negative controls, 20µl of a 0.9% saline solution was added in place of the 20µl of bullfrog plasma. For the factor IX, XI, and XII deficiency assays, all of the above steps were the same except that in place of the 80µl of factor VIII deficient plasma, 80µl of the respective factor deficient plasma was added. The controls for these assays were the same as mentioned above. Factor VIII, IX, XI, and XII deficient assays were performed on pre-hibernating (n=4), hibernating (n=6), and post hibernating (n=10) bullfrog samples. If any of the Factor deficient assays or controls ran longer than 600 seconds, they were stopped and clotting time was recorded as 600 plus seconds, no clot.

APTT Inhibitor Assay

APTT inhibitor assays were performed by mixing human plasma with bullfrog plasma to test for the presence of blood clotting inhibitors. If any inhibitor was present in bullfrog plasma, it would inhibit the blood coagulation process of human plasma thus resulting in a prolonged clotting time. Human plasma was mixed with bullfrog plasma in different ratios: 50µl human + 50µl bullfrog; 75µl human + 25µl bullfrog; 90 µl human + 10µl bullfrog. Once the human plasma and bullfrog plasma were mixed, 100µl of Cephan 5 APTT solution was added and incubated at 37°C for 180 seconds in the coagulation analyzer. After incubation, clotting was initiated by adding 100µl of 0.025M $CaCl_2$ and the clotting time was recorded.

Protein Inhibitor Assay with Heated Bullfrog Plasma

This assay was performed to determine if the inhibitor found in bullfrog plasma was a protein. In this assay, individual hibernating and post-hibernating bullfrog plasma samples were pooled and placed in 1ml centrifuge tubes labeled heated hibernating, heated post-hibernating, room temperature hibernating, and room temperature post-hibernating respectively. The heated tubes were placed in a water filled beaker on a hot plate heated to 60°C water temperature for 1 hour to denature any proteins in the plasma. The room temperature tubes were left out at room temperature while the other tubes were being heated. Human plasma was mixed with heated or non-heated bullfrog plasma in different ratios: 50µl human + 50µl bullfrog; 75µl human + 25µl bullfrog; and 90µl human + 10µl bullfrog. For the positive controls, non-heated (room-temperature) pooled hibernating and post-hibernating bullfrog plasma was mixed with human plasma in the same ratios. For negative controls, bullfrog plasma was replaced by a 0.9% saline solution in each of the 3 different ratios. Each mixture was added to 100µl of Cephan 5 APTT solution and incubated at 37°C for 180 seconds. After incubation, clotting was initiated by adding 100µl of 0.025M $CaCl_2$ and clotting time was recorded.

Statistical Analysis

Statistical analysis was performed on APTT, APTT inhibitor, TT, factor VIII, IX, XI, and XII deficient assays. A one-way ANOVA was performed to compare the results between three time periods using IBM SPSS Version 20 statistical software. LSD post HOC tests were included for of all the aforementioned coagulation assays. PT assays and protein inhibitor assays were also performed, but were not included in the statistical

analysis. The PT assay failed to work with the frog plasma, so those results are not included. Graphs were created using GraphPad Prism 5 software.

CHAPTER 3

RESULTS

APTT Coagulation Assay

APTT coagulation time measured the time required for the plasma to clot via the intrinsic pathway. Mean APTT clotting time was longest in hibernating bullfrogs (854.6 sec), followed by post-hibernating bullfrogs (664.7 sec), and shortest in pre-hibernating bullfrogs (85.35 sec) (Figure 2). All but one of the APTT times for hibernating bullfrogs were as long as the negative control (1,000 plus sec, no clot). The post-hibernating APTT times were highly variable with some as high as 1,000 plus sec (no clot) and others closer to pre-hibernating APTT times (136 sec) (Figure 3).

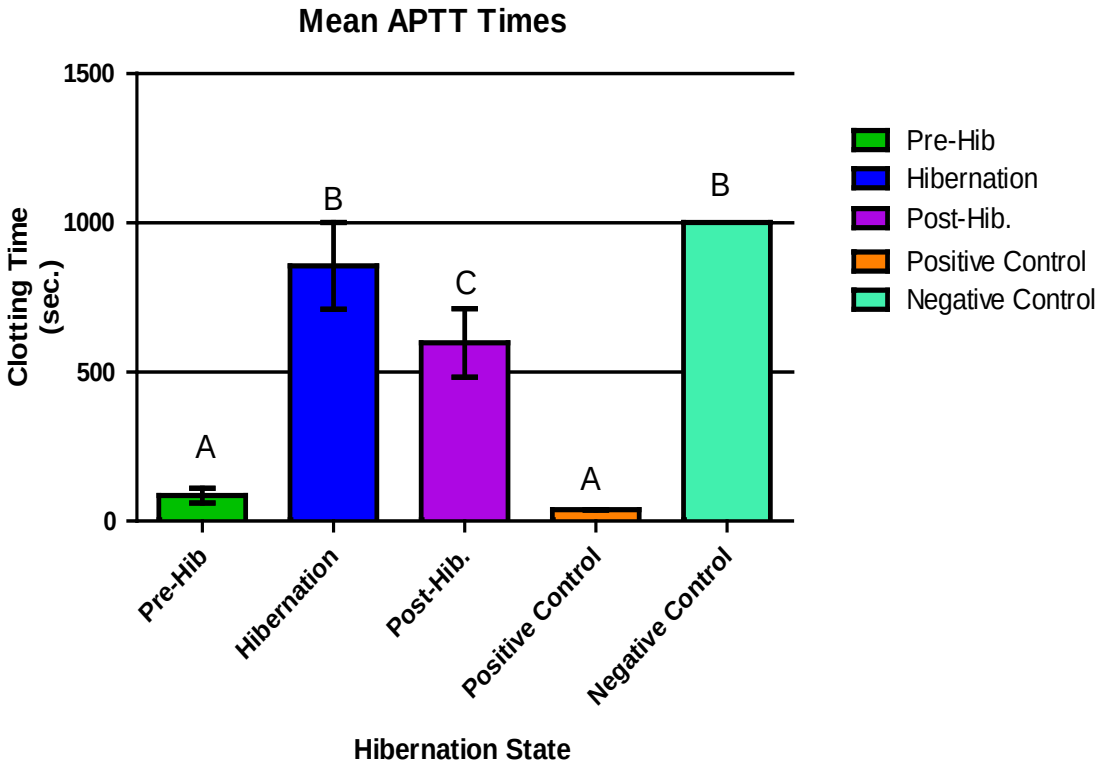

Figure 2. Mean values of bullfrog APTT clotting times in seconds ±SE for pre-hibernating frogs (n=4) hibernating frogs (n=6), post-hibernating frogs (n=10), positive controls, and negative controls. Groups with the same letter are not significantly different ($p \leq 0.05$).

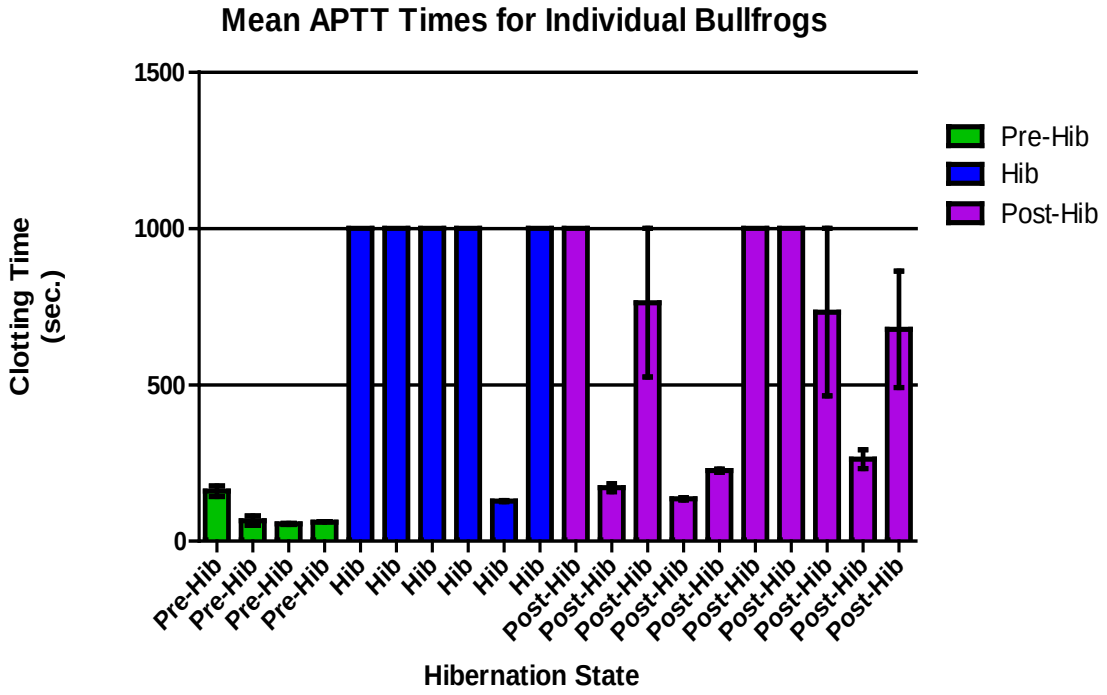

Figure 3. Mean values of bullfrog APTT clotting times in seconds ± SE for pre-hibernating frogs (n=4) hibernating frogs (n=6), post-hibernating frogs (n=10).

A one-way ANOVA was performed for the means of APTT times of bullfrogs for the three time periods (Table 1). The hibernating APTT times were significantly longer than the pre-hibernating APTT times (p=0.000). Hibernating APTT times were also significantly longer than post-hibernating APTT times (p=0.004). Post-hibernating APTT times were significantly longer than the pre-hibernating APTT times (p=0.000). Pre-hibernating APTT times were not significantly different from the positive control (human control) times (p=0.592), but post-hibernating APTT times and hibernating APTT times were both significantly different from the positive control (human control) times (p=0.000). Hibernating APTT times were not significantly different from the negative control (saline control) times (p=0.088), but both pre-hibernating and post-hibernating

APTT times were significantly different from negative control (saline control) times (p=0.000) (Table 1).

Table 1. Results of One-Way ANOVA for APTT Time.

Multiple Comparisons

Dependent Variable: Clotting Time

LSD

(I) Hibernation State	(J) Hibernation State	Mean Difference (I-J)	Std. Error	Sig.
Pre-Hib	Hibernating	-769.316917*	108.1606688	.000
	Post-Hib	-511.550250*	99.1308904	.000
	Positive Control	47.4734342	88.0801075	.592
	Negative Control	-914.650250*	96.7418432	.000
Hibernating	Pre-Hib	769.3169167*	108.1606688	.000
	Post-Hib	257.7666667*	86.5285351	.004
	Positive Control	816.7903509*	73.6095054	.000
	Negative Control	-145.3333333	83.7808938	.088
Post-Hib	Pre-Hib	511.5502500*	99.1308904	.000
	Hibernating	-257.766667*	86.5285351	.004
	Positive Control	559.0236842*	59.5530221	.000
	Negative Control	-403.100000*	71.7456711	.000
Positive Control	Pre-Hib	-47.4734342	88.0801075	.592
	Hibernating	-816.790351*	73.6095054	.000
	Post-Hib	-559.023684*	59.5530221	.000
	Negative Control	-962.123684*	55.4852524	.000
Negative Control	Pre-Hib	914.6502500*	96.7418432	.000
	Hibernating	145.3333333	83.7808938	.088
	Post-Hib	403.1000000*	71.7456711	.000
	Positive Control	962.1236842*	55.4852524	.000

LSD post-HOC test comparing mean values of pre-hibernating bullfrogs, hibernating bullfrogs, post-hibernating bullfrogs, positive controls, and negative controls. Groups are significantly different from each other if ($p \leq 0.05$).

TT Coagulation Assay

TT coagulation assays were performed to measure the functionality of the common pathway in blood coagulation. TT coagulation assays were performed for pre-hibernating, hibernating, and post hibernating bullfrogs. The mean TT clotting times for the bullfrogs were longest in the post-hibernators (130.96 sec), followed by the hibernators (93.74 sec), and pre-hibernators had the shortest mean TT time (88.17 sec) (Figure 3). The mean TT times for the pre-hibernating, hibernating and post-hibernating bullfrogs were above the positive control, but below the negative control suggesting that the assay worked in bullfrogs.

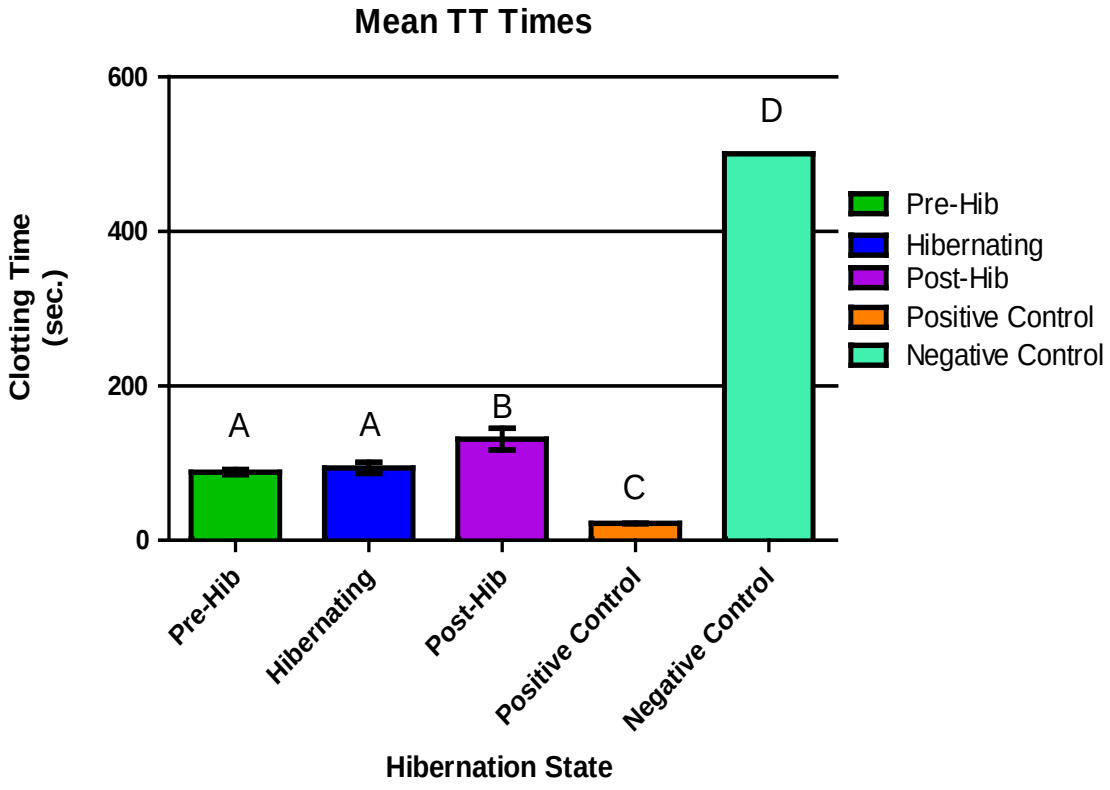

Figure 4. Mean values of bullfrog TT clotting time ± SE in seconds and mean values of human and saline control times during pre-hibernation (n=4) hibernation (n=6), and post hibernation (n=10). Groups with the same letter are not significantly different ($p \leq 0.05$).

A one-way ANOVA was performed on the means of the TT times for the three different stages of hibernation along with the controls. The results of the LSD post-HOC test showed that the mean TT times of pre-hibernating bullfrogs and hibernating bullfrogs were not significantly different ($p=0.726$). However, post-hibernating times were significantly longer than pre-hibernating times ($p=0.007$) as well as hibernating times ($p=0.009$). The mean TT times for all three stages of hibernation were each significantly different from both the positive control (human control) ($p=0.000$) and the negative control (saline control) ($p=0.000$) (Table 2).

Table 2. Results of One-Way ANOVA for mean TT times.

Multiple Comparisons

Dependent Variable: ClottingTime

LSD

(I) HibernationState	(J) HibernationState	Mean Difference (I-J)	Std. Error	Sig.
Pre-Hib	Hibernating	-5.5708333	15.6987795	.726
	Post-Hib	-42.7976190*	14.1839714	.007
	Positive Control	66.3333333*	14.5342456	.000
	Negative Control	-411.833333*	14.5342456	.000
Hibernating	Pre-Hib	5.5708333	15.6987795	.726
	Post-Hib	-37.2267857*	12.8832318	.009
	Positive Control	71.9041667*	13.2678903	.000
	Negative Control	-406.262500*	13.2678903	.000
Post-Hib	Pre-Hib	42.7976190*	14.1839714	.007
	Hibernating	37.2267857*	12.8832318	.009
	Positive Control	109.1309524*	11.4354833	.000
	Negative Control	-369.035714*	11.4354833	.000
Positive Control	Pre-Hib	-66.3333333*	14.5342456	.000
	Hibernating	-71.9041667*	13.2678903	.000
	Post-Hib	-109.130952*	11.4354833	.000
	Negative Control	-478.166667*	11.8671619	.000
Negative Control	Pre-Hib	411.8333333*	14.5342456	.000
	Hibernating	406.2625000*	13.2678903	.000
	Post-Hib	369.0357143*	11.4354833	.000
	Positive Control	478.1666667*	11.8671619	.000

LSD post-HOC test comparing mean values of pre-hibernating bullfrogs, hibernating bullfrogs, post-hibernating bullfrogs, positive controls, and negative controls. Groups are significantly different from each other if ($p \leq 0.05$).

Factor VIII Deficiency Assay

Factor VIII deficient human plasma was combined with bullfrog plasma and analyzed for the functionality of Factor VIII. Since the human plasma lacked Factor VIII, this assay was used specifically to determine if the bullfrog plasma had sufficient Factor VIII present to overcome this deficiency. The results showed that the Factor VIII deficiency clotting times were most prolonged in pre-hibernators (104.9 sec), closely followed by hibernators (104.6 sec), and post hibernators (100.64 sec). Pre-hibernating and hibernating times were above the negative control (102.49 sec), while post-hibernating times were just shorter than the negative control (Figure 4). This suggests that Factor VIII is either non-functional in the bullfrogs or is at levels too low to cause clotting in Factor VIII deficient plasma.

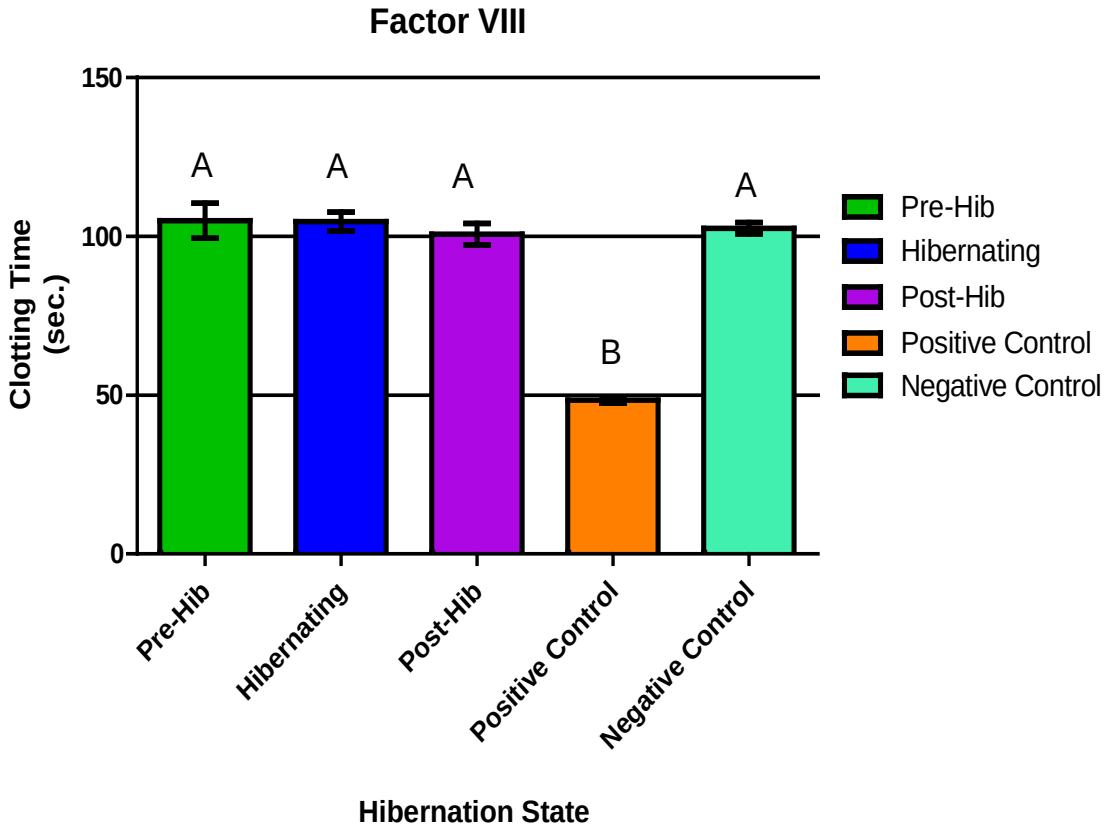

Figure 5. Mean values of bullfrog Factor VIII deficient plasma clotting times in seconds ± SE for pre-hibernating (n=4), hibernating (n=6), and post-hibernating bullfrogs (n=10). Groups with the same letter are not significantly different as determined by a one way ANOVA.

A one way ANOVA was performed on the mean values of the Factor VIII deficient plasma times for the three different stages of hibernation. The ANOVA showed no significant difference between the three main groups. Pre-hibernating coagulation times were not significantly longer than hibernating times or post-hibernating times ($p=0.952$ and $p=0.356$, respectively). Hibernating clotting times were also not significantly longer than post-hibernating times ($p=0.327$). The positive control was significantly different than all other groups with the same significance for each group

(p=0.000). The negative control, however, was not significantly different than pre-hibernating times (p=0.606), hibernating times (p=0.607), or post-hibernating times (p=0.604) (Table 3).

Table 3. Results of One-Way ANOVA for Factor VIII Deficiency Means

Multiple Comparisons

Dependent Variable: Clotting Time

LSD

(I) Hibernation State	(J) Hibernation State	Mean Difference (I-J)	Std. Error	Sig.
Pre-Hib	Hibernating	.2999917	4.9741654	.952
	Post-Hib	4.2633247	4.5588979	.356
	Positive Control	56.4999917*	4.6306960	.000
	Negative Control	2.4111028	4.6306960	.606
Hibernating	Pre-Hib	-.2999917	4.9741654	.952
	Post-Hib	3.9633330	3.9793324	.327
	Positive Control	56.2000000*	4.0613891	.000
	Negative Control	2.1111111	4.0613891	.607
Post-Hib	Pre-Hib	-4.2633247	4.5588979	.356
	Hibernating	-3.9633330	3.9793324	.327
	Positive Control	52.2366670*	3.5406369	.000
	Negative Control	-1.8522219	3.5406369	.604
Positive Control	Pre-Hib	-56.4999917*	4.6306960	.000
	Hibernating	-56.2000000*	4.0613891	.000
	Post-Hib	-52.2366670*	3.5406369	.000
	Negative Control	-54.0888889*	3.6326168	.000
Negative Control	Pre-Hib	-2.4111028	4.6306960	.606
	Hibernating	-2.1111111	4.0613891	.607
	Post-Hib	1.8522219	3.5406369	.604
	Positive Control	54.0888889*	3.6326168	.000

Table 3. LSD post-HOC test comparing mean values of pre-hibernating bullfrogs, hibernating bullfrogs, post-hibernating bullfrogs, positive controls, and negative controls. Groups are significantly different from each other if ($p \leq 0.05$).

Factor IX Deficiency Assay

Factor IX deficient human plasma was combined with bullfrog plasma and analyzed for the functionality of Factor IX. Since the human plasma lacked Factor IX, this assay was used specifically to determine if the bullfrog plasma had sufficient Factor IX present to overcome this deficiency. The results showed that Factor IX clotting times were longest in post-hibernating bullfrogs (171.94 sec), followed by hibernators (159.25 sec), and pre-hibernators (139.87 sec). All three stages of hibernation were above the positive control (50.65 sec) but under the negative control (179.68 sec) (Figure 5). This suggests that Factor IX is likely present in the blood stream in some quantity before the onset of hibernation. Then, the level of Factor IX in the blood is either decreased or Factor IX is inhibited during hibernation and post-hibernation or both.

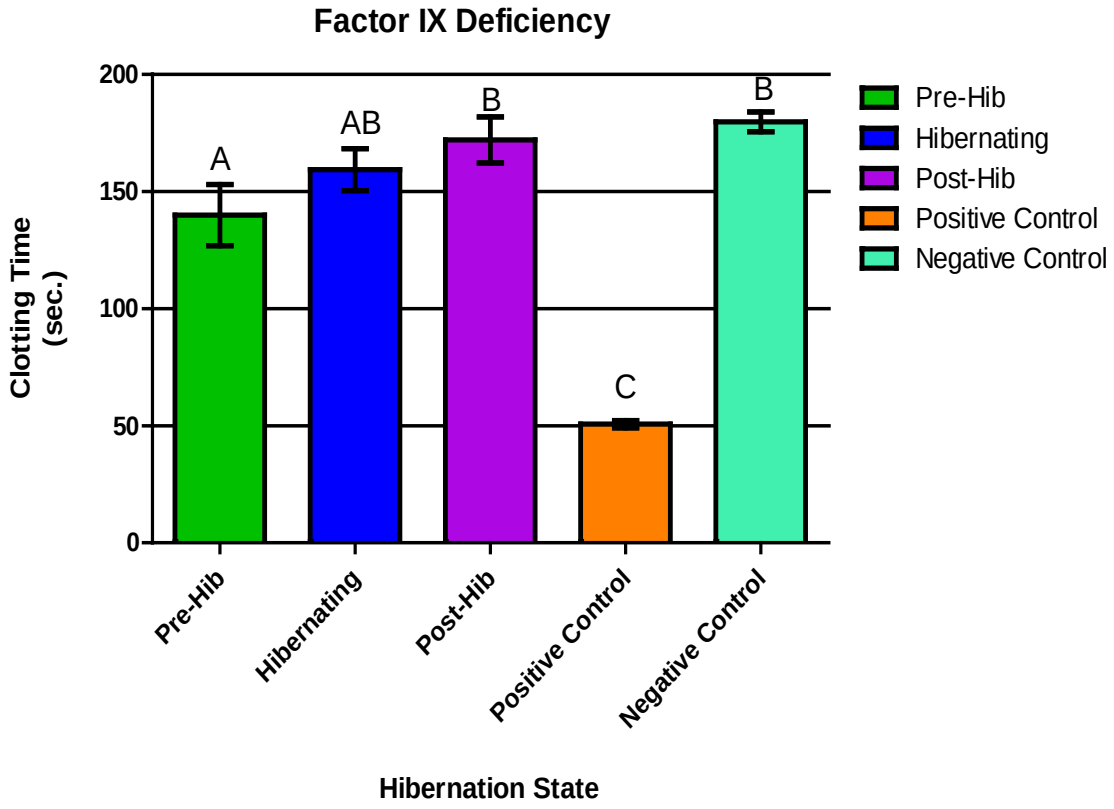

Figure 6. Mean values of bullfrog Factor IX deficient plasma clotting times in seconds ±SE for pre-hibernating bullfrogs (n=4), hibernating bullfrogs (n=6), post-hibernating bullfrogs (n=10), positive controls, and negative controls. Groups with the same letter are not significantly different as determined by a one way ANOVA.

A one way ANOVA was performed on the mean values of the Factor IX deficient plasma times for the three different stages of hibernation. The ANOVA showed that pre-hibernating times were not significantly different from hibernating times ($p=0.226$), but they were significantly shorter than post-hibernating times ($p=0.035$). Hibernating times were not significantly different from post-hibernating times ($p=0.320$). However, pre-hibernating times, hibernating times, and post hibernating times were all significantly different from the positive control ($p=0.000$ for all groups). Pre-hibernating times were

also significantly different from the negative control ($p=0.029$) while hibernators and post-hibernators were not significantly different from the negative control ($p=0.203$ and $p=0.594$ respectively) (Table 4).

Table 4. Results of One-Way ANOVA for Factor IX Deficiency Means.

Multiple Comparisons

Dependent Variable: Clotting Time

LSD

(I) Hibernation State	(J) Hibernation State	Mean Difference (I-J)	Std. Error	Sig.
Pre-Hib	Hibernating	-19.3833250	15.5969748	.226
	Post-Hib	-32.0733250*	14.2948635	.035
	Positive Control	89.2166750*	17.0856299	.000
	Negative Control	-39.8083250*	17.0856299	.029
Hibernating	Pre-Hib	19.3833250	15.5969748	.226
	Post-Hib	-12.6900000	12.4775799	.320
	Positive Control	108.6000000*	15.5969748	.000
	Negative Control	-20.4250000	15.5969748	.203
Post-Hib	Pre-Hib	32.0733250*	14.2948635	.035
	Hibernating	12.6900000	12.4775799	.320
	Positive Control	121.2900000*	14.2948635	.000
	Negative Control	-7.7350000	14.2948635	.594
Positive Control	Pre-Hib	-89.2166750*	17.0856299	.000
	Hibernating	-108.600000*	15.5969748	.000
	Post-Hib	-121.290000*	14.2948635	.000
	Negative Control	-129.025000*	17.0856299	.000
Negative Control	Pre-Hib	39.8083250*	17.0856299	.029
	Hibernating	20.4250000	15.5969748	.203
	Post-Hib	7.7350000	14.2948635	.594
	Positive Control	129.0250000*	17.0856299	.000

LSD post-HOC test comparing mean values of pre-hibernating bullfrogs, hibernating bullfrogs, post-hibernating bullfrogs, positive controls, and negative controls. Groups are significantly different from each other if ($p \leq 0.05$).

Factor XI Deficiency Assay

Factor XI deficient human plasma was combined with bullfrog plasma and analyzed for the functionality of Factor XI. Since the human plasma lacked Factor XI, this assay was used specifically to determine if the bullfrog plasma had sufficient Factor XI present to overcome this deficiency. The results showed that Factor XI clotting times were most prolonged in hibernating bullfrogs (148.01 sec), followed by post hibernators (139.34 sec), and pre-hibernators (118.81 sec) (Figure 6). All bullfrogs had coagulation times that were longer than the positive control, but shorter than the negative control. This suggests that Factor XI is likely present in the blood stream in some quantity before the onset of hibernation. Then, the levels of Factor XI in the blood are either decreased or Factor XI is inhibited during hibernation and post-hibernation, or both.

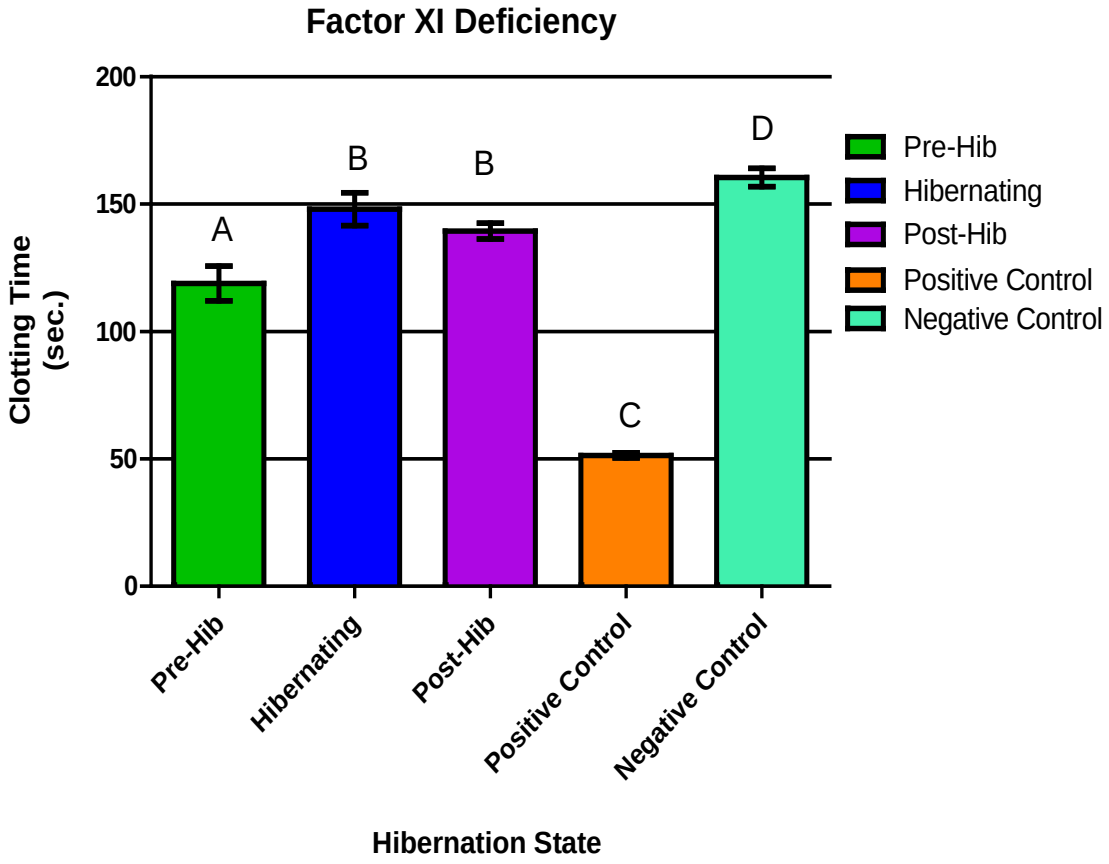

Figure 7. Mean values of bullfrog Factor XI deficient plasma clotting times in seconds ±SE for pre-hibernating (n=4), hibernating (n=6), and post-hibernating bullfrogs (n=10). Groups with the same letter are not significantly different as determined by a one way ANOVA.

A one way ANOVA was performed on the mean values of the Factor XI deficient plasma times for the three different stages of hibernation. The ANOVA showed that pre-hibernating coagulation times were significantly shorter than hibernating times and post-hibernating times ($p=0.000$ and $p=0.002$, respectively). Hibernating clotting times were not significantly different than post-hibernating clotting times ($p=0.122$). The positive controls were significantly different than all other groups with the same significance for

each group (p=0.000). The negative control was also significantly different than all other groups. The significance value was the same for all groups except for hibernators (p=0.038). All other groups had a significance value of (p=0.000) (Table 5).

Table 5. Results of One-Way ANOVA for Factor XI Deficiency Means.

Multiple Comparisons

Dependent Variable: Clotting Time Factor XI
LSD

(I) Hibernation State	(J) Hibernation State	Mean Difference (I-J)	Std. Error	Sig.
Pre-Hib	Hibernating	-29.1930556*	6.8034977	.000
	Post-Hib	-20.5274967*	6.2355086	.002
	Positive Control	67.4750000*	6.4543646	.000
	Negative Control	-41.5625000*	6.4543646	.000
Hibernating	Pre-Hib	29.1930556*	6.8034977	.000
	Post-Hib	8.6655589	5.4427982	.122
	Positive Control	96.6680556*	5.6922146	.000
	Negative Control	-12.3694444*	5.6922146	.038
Post-Hib	Pre-Hib	20.5274967*	6.2355086	.002
	Hibernating	-8.6655589	5.4427982	.122
	Positive Control	88.0024967*	4.9995294	.000
	Negative Control	-21.0350033*	4.9995294	.000
Positive Control	Pre-Hib	-67.4750000*	6.4543646	.000
	Hibernating	-96.6680556*	5.6922146	.000
	Post-Hib	-88.0024967*	4.9995294	.000
	Negative Control	-109.037500*	5.2699667	.000
Negative Control	Pre-Hib	41.5625000*	6.4543646	.000
	Hibernating	12.3694444*	5.6922146	.038
	Post-Hib	21.0350033*	4.9995294	.000
	Positive Control	109.0375000*	5.2699667	.000

LSD post-HOC test comparing mean values of pre-hibernating bullfrogs, hibernating bullfrogs, post-hibernating bullfrogs, positive controls, and negative controls. Groups are significantly different from each other if ($p \leq 0.05$).

Factor XII Deficiency

Factor XII deficient human plasma was combined with bullfrog plasma and analyzed for the functionality of Factor XII. Since the human plasma lacked Factor XII, this assay was used specifically to determine if the bullfrog plasma had sufficient Factor XII present to overcome this deficiency. The results showed that Factor XII clotting times were most prolonged in hibernators (176.70 sec), closely followed by post-hibernators (174.98 sec), and shortest in pre-hibernators (125.39 sec). All three states of hibernation had coagulation times that were longer than the positive control (60.65 sec), but shorter than the negative control (300 sec) (Figure 7). This suggests that Factor XII is likely present in the blood stream in some quantity before the onset of hibernation. Then, the levels of Factor XII in the blood are either decreased or Factor XII is inhibited during hibernation and post-hibernation or both.

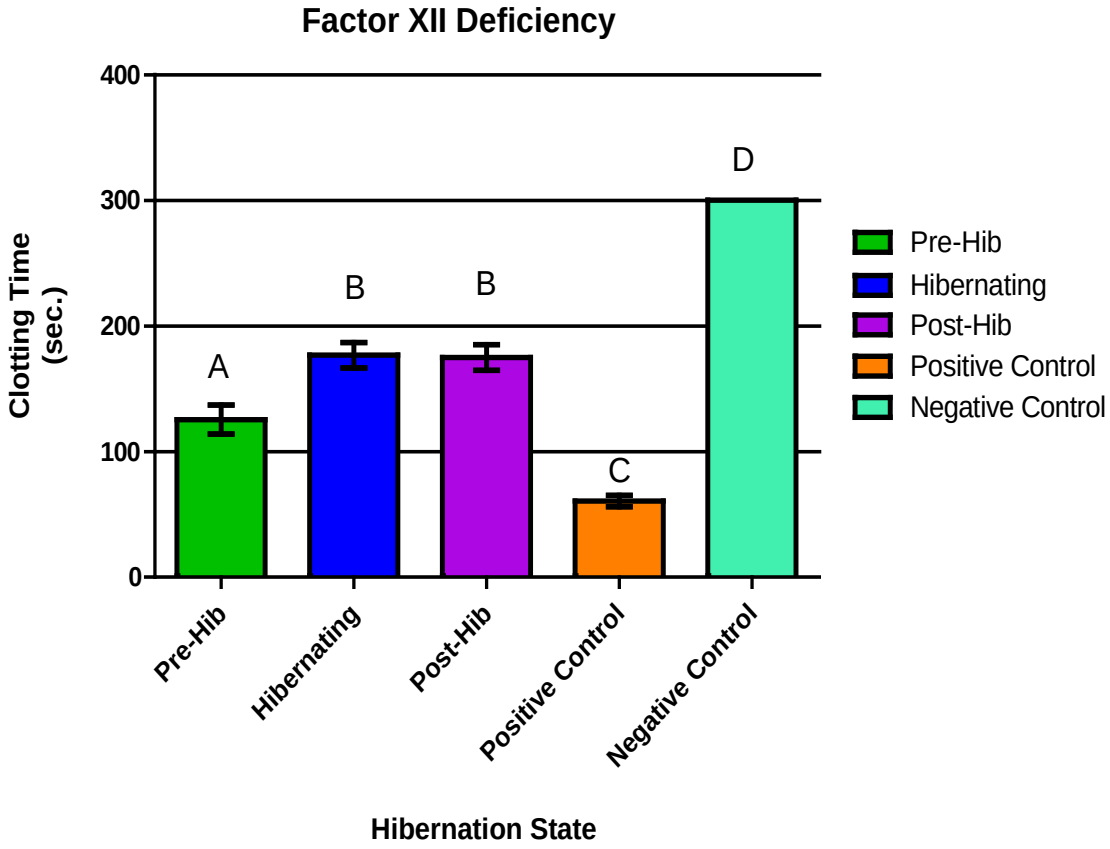

Figure 8. Mean values of Factor XII deficient plasma clotting times in seconds ± SE for pre-hibernating bullfrogs (n=4), hibernating bullfrogs (n=6), post-hibernating bullfrogs (n=10), positive controls, and negative controls. Groups with the same letter are not significantly different as determined by a one way ANOVA.

A one-way ANOVA was performed on the mean values of the Factor XII deficient plasma times for the three different stages of hibernation. The ANOVA showed that pre-hibernating clotting times were significantly shorter than hibernating times ($p=0.002$) as well as post hibernating times ($p=0.001$). Hibernating clotting times were not significantly longer than post-hibernating times ($p=0.888$). The positive controls were significantly shorter than all other groups with the same significance value for all groups

(p=0.000). The negative controls were significantly longer than all other groups with the same significance value for all groups (p=0.000) (Table 6).

Table 6. Results of One-Way ANOVA for Factor XII Deficiency Means.

Multiple Comparisons

Dependent Variable: Clotting Time Factor XII

LSD

(I) Hibernation State	(J) Hibernation State	Mean Difference (I-J)	Std. Error	Sig.
Pre-Hib	Hibernating	-51.3083250*	15.1173660	.002
	Post-Hib	-49.5883250*	13.8552948	.001
	Positive Control	64.7416750*	15.1173660	.000
	Negative Control	-503.208325*	15.1173660	.000
Hibernating	Pre-Hib	51.3083250*	15.1173660	.002
	Post-Hib	1.7200000	12.0938928	.888
	Positive Control	116.0500000*	13.5213832	.000
	Negative Control	-451.900000*	13.5213832	.000
Post-Hib	Pre-Hib	49.5883250*	13.8552948	.001
	Hibernating	-1.7200000	12.0938928	.888
	Positive Control	114.3300000*	12.0938928	.000
	Negative Control	-453.620000*	12.0938928	.000
Positive Control	Pre-Hib	-64.7416750*	15.1173660	.000
	Hibernating	-116.050000*	13.5213832	.000
	Post-Hib	-114.330000*	12.0938928	.000
	Negative Control	-567.950000*	13.5213832	.000
Negative Control	Pre-Hib	503.2083250*	15.1173660	.000
	Hibernating	451.9000000*	13.5213832	.000
	Post-Hib	453.6200000*	12.0938928	.000
	Positive Control	567.9500000*	13.5213832	.000

LSD post-HOC test comparing mean values of pre-hibernating bullfrogs, hibernating bullfrogs, post-hibernating bullfrogs, positive controls, and negative controls. Groups are significantly different from each other if ($p \leq 0.05$).

APTT Inhibitor Assay

APTT inhibitor time measured the time required for the plasma to clot when bullfrog plasma was mixed with human plasma. This assay was used to determine if bullfrog plasma had an inhibitor present that would lengthen the normal clotting time of human plasma. For the 50µl/50µl mixture, clotting times were longest in post-hibernators (83.64 sec), followed by pre-hibernators (71.39 sec), and shortest in hibernators (70.63 sec). All three stages of hibernation had clotting times that exceeded the control (53.86 sec). For the 25µl/75µl mixture, clotting times were longest in post-hibernators (51.19 sec), closely followed by pre-hibernators (46.1 sec), and hibernators (46.06 sec). All three stages of hibernation had clotting times that exceeded the control (41.89 sec) For the 10µl/90µl mixture, clotting times were most prolonged in post-hibernators (44.28 sec), followed by hibernators (41.64 sec), and pre-hibernators (40.94 sec). Once again, all three stages of hibernation had clotting times that were greater than the control (39.54 sec) (Figure 8).

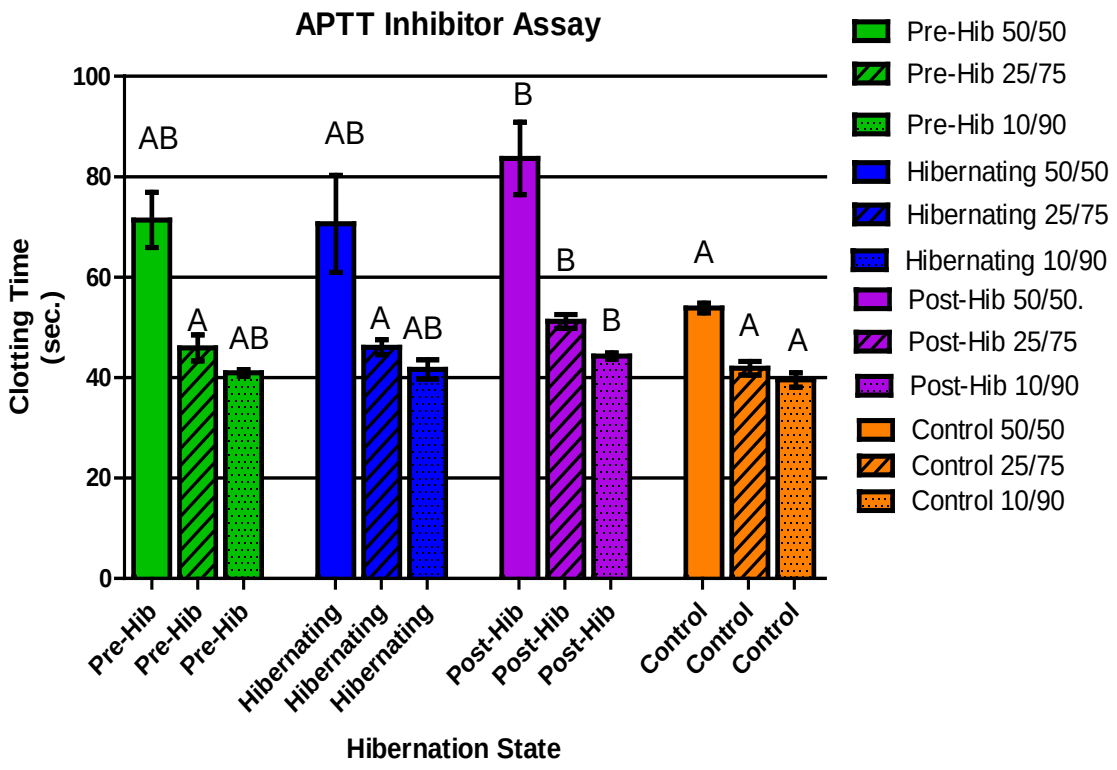

Figure 9. Mean values of APTT inhibitor clotting times in seconds ± SE for pre-hibernating bullfrogs (n=4), hibernating bullfrogs (n=6), post-hibernating bullfrogs (n=10), and saline controls (in dilutions of 50µl/50µl, 25µl/75µl, and 10µl/90µl). Similar dilutions with the same letter are not significantly different as determined by a one-way ANOVA.

A one way ANOVA was performed on each of the dilution groups (50/50, 25/75, 10/90) comparing the mean coagulation values of the three different stages of hibernation. The ANOVA for the 50µl/50µl dilution showed that pre-hibernating clotting times were not significantly different from hibernating times (p=0.941), post-hibernating times (p=0.180), or the saline controls (p=0.072). Likewise, hibernating times were not significantly different from post-hibernating times (p=0.174) or the saline controls (p=0.097). Post-hibernating times were significantly longer than the saline controls (p=0.002) (Table 7).

Table 7. Results of One-Way ANOVA for APTT Inhibitor Assay for 50µl/50µl Dilution.

Multiple Comparisons

Dependent Variable: Clotting Time 50/50 ratio

LSD

(I) Hibernation State	(J) Hibernation State	Mean Difference (I-J)	Std. Error	Sig.
Pre-Hib	Hibernating	.7523810	10.0432214	.941
	Post-Hib	-12.2542857	8.8961367	.180
	Saline Control	17.5232143	9.3428123	.072
Hibernating	Pre-Hib	-.7523810	10.0432214	.941
	Post-Hib	-13.0066667	9.3220274	.174
	Saline Control	16.7708333	9.7492096	.097
Post-Hib	Pre-Hib	12.2542857	8.8961367	.180
	Hibernating	13.0066667	9.3220274	.174
	Saline Control	29.7775000*	8.5628289	.002
Saline Control	Pre-Hib	-17.5232143	9.3428123	.072
	Hibernating	-16.7708333	9.7492096	.097
	Post-Hib	-29.7775000*	8.5628289	.002

LSD post-HOC test comparing mean values of pre-hibernating bullfrogs, hibernating bullfrogs, post-hibernating bullfrogs, and positive controls for the 50µl/50µl dilution of the APTT inhibitor assay. Groups are significantly different from each other if ($p \leq 0.05$).

The ANOVA for the 25µl/75µl dilution showed that post-hibernating times were significantly different than pre-hibernating times (p=0.038), hibernating times (p=0.024), and saline control times (p=0.000). Pre-hibernating times were not significantly different from hibernating times (p=0.950) or the saline controls (p=0.135). Hibernating times were also not significantly different from the saline controls (p=0.089) (Table8).

Table 8. Results of One-Way ANOVA for APTT Inhibitor Assay 25µl/75µl Dilution.

Multiple Comparisons

Dependent Variable: Clotting Time

LSD

(I) Hernation state	(J) Hernation state	Mean Difference (I-J)	Std. Error	Sig.
Pre-Hib	Hibernating	-.1555556	2.4740314	.950
	Post-Hib	-5.2900000*	2.4240458	.038
	Saline Control	4.0142857	2.6115809	.135
Hibernating	Pre-Hib	.1555556	2.4740314	.950
	Post-Hib	-5.1344444*	2.1568106	.024
	Saline Control	4.1698413	2.3656265	.089
Post-Hib	Pre-Hib	5.2900000*	2.4240458	.038
	Hibernating	5.1344444*	2.1568106	.024
	Saline Control	9.3042857*	2.3132997	.000
Saline Control	Pre-Hib	-4.0142857	2.6115809	.135
	Hibernating	-4.1698413	2.3656265	.089
	Post-Hib	-9.3042857*	2.3132997	.000

LSD post-HOC test comparing mean values of pre-hibernating bullfrogs, hibernating bullfrogs, post-hibernating bullfrogs, and positive controls for the 25µl/75µl dilution of the APTT inhibitor assay. Groups are significantly different from each other if ($p \leq 0.05$).

The ANOVA for the 10µl/90µl dilution showed that pre-hibernating times were not significantly different than hibernating times (p=0.747), post-hibernating times (p=0.150), or saline control times (p=0.530). Similarly, hibernating times were not significantly different than post-hibernating times (p=0.150) or saline control times (p=0.272). Post-hibernating times were significantly different than saline control times (p=0.015) (Table 9).

Table 9. Results of One-Way ANOVA for APTT Inhibitor Assay 10µl/90µl Dilution.

Multiple Comparisons

Dependent Variable: Cliotting Time

LSD

(I) Hibernation State	(J) Hibernation State	Mean Difference (I-J)	Std. Error	Sig.
Pre-Hib	Hibernating	-.7044444	2.1604243	.747
	Post-Hib	-3.3400000	2.1214945	.127
	Saline Control	1.4025000	2.2081215	.530
Hibernating	Pre-Hib	.7044444	2.1604243	.747
	Post-Hib	-2.6355556	1.7796592	.150
	Saline Control	2.1069444	1.8820862	.272
Post-Hib	Pre-Hib	3.3400000	2.1214945	.127
	Hibernating	2.6355556	1.7796592	.150
	Saline Control	4.7425000*	1.8372681	.015
Saline Control	Pre-Hib	-1.4025000	2.2081215	.530
	Hibernating	-2.1069444	1.8820862	.272
	Post-Hib	-4.7425000*	1.8372681	.015

LSD post-HOC test comparing mean values of pre-hibernating bullfrogs, hibernating bullfrogs, post-hibernating bullfrogs, and positive controls for the 10µl/90µl dilution of the APTT inhibitor assay. Groups are significantly different from each other if ($p \leq 0.05$).

Protein Inhibitor Assay

Protein inhibitor time measured the time required for the plasma to clot when bullfrog plasma was mixed with human plasma after the bullfrog plasma had been heated. The protein inhibitor assay was performed to determine if a protein inhibitor was a potential cause of lengthened clotting time in post-hibernating bullfrogs. The results of the assay showed that mean clotting time was longest in post-hibernating heated plasma (64.55 sec), followed by post-hibernating room temperature times (57.05 sec).

Hibernating heated plasma had longer mean clotting times (56.55 sec) than hibernating room temperature plasma (48.4 sec). The mean saline control times were identical to those of the hibernating room temperature plasma samples (48.4 sec) (Figure 9). The plasma was heated to an extent that would denature the proteins in it. If a protein inhibitor is present in bullfrog plasma, the clotting time would be expected to be shorter in heated plasma samples versus room temperature plasma samples. However, the opposite result was observed. Heated plasma samples had lengthened clotting times when compared to room temperature samples for both pooled hibernating plasma and pooled post-hibernating plasma. This indicates that if a blood coagulation inhibitor is present in post-hibernating bullfrogs, it is probably not a protein. Statistical analysis was not performed for this assay due to lack of sufficient replicates.

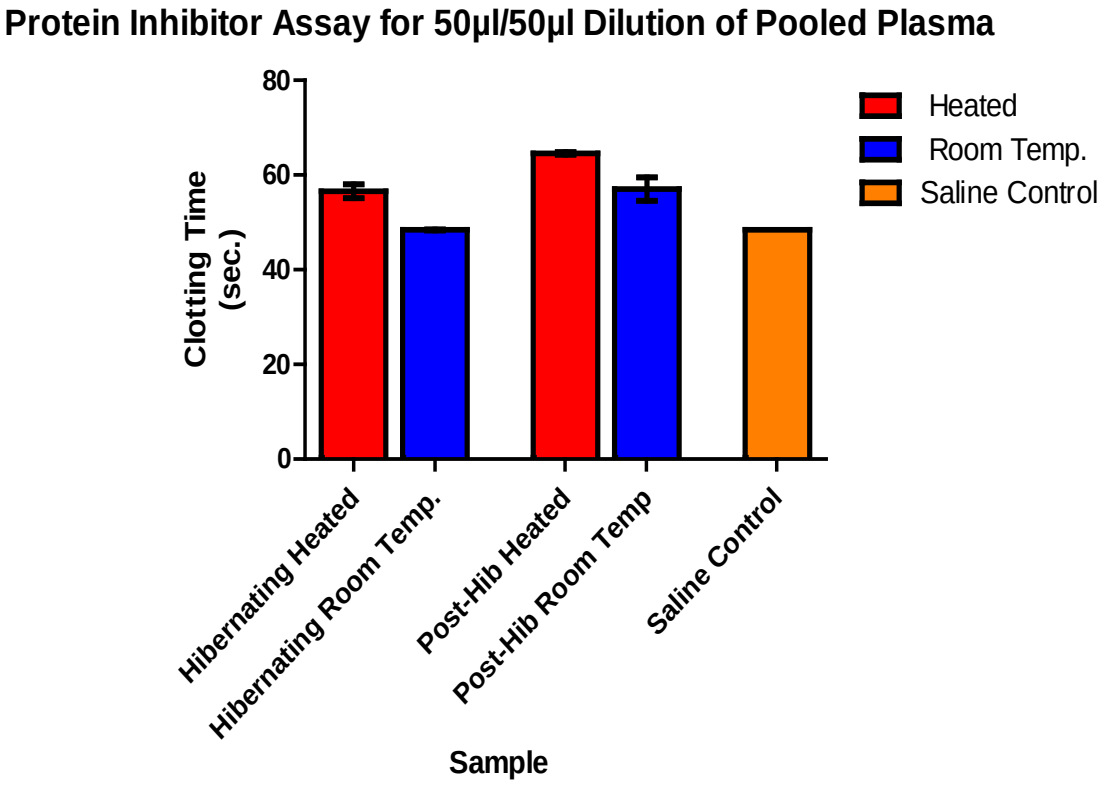

Figure 10. Mean values of Protein Inhibitor clotting times in seconds ±SE for heated and room temperature pooled hibernating bullfrog plasma, pooled post-hibernating bullfrog plasma, and saline controls in a 50µl/50µl dilution.

CHAPTER 4

DISCUSSION

Hibernation has been known to cause a variety of physiological changes in many ectotherms and endotherms, including the lengthening of blood coagulation times (Biörgk et al. 1962; Ahmad et al. 1979; Yadav 2011). Factors that lead to prolonged clotting time during hibernation include: change in core body temperature, blood pH, oxygen availability, and metabolic rate, among others (Biörgk et al. 1962; Barone and Jacques 1975; Ahmad et al. 1979; Pivorun and Sinnamon, 1981; Soslau et al. 2004; Dirkmann et al. 2008; Yadav 2011). Body temperatures of frogs can vary greatly depending on hibernacula temperature, but they are able to acclimate and survive in temperatures as low as 3°C (Stewart et al. 2004). A decrease in internal temperature will lead to increased coagulation times by lowering the efficiency of clotting enzymes. In fact, humans were also found to have prolonged APTT times when their body temperatures decreased (Dirkmann et al. 2008). This suggests that a decrease in internal body temperature significantly impacts the process of blood coagulation.

Lack of oxygen or low oxygen availability leads to an increase in anaerobic respiration and thus the production of lactic acid. As blood pH becomes more acidic, the activity of coagulation factor complexes becomes highly reduced, prolonging clotting times. In humans, a blood pH of 7.2 will reduce the activity of FXa/Va complex by as much as 50%, and as much as 90% at a pH of 6.8 (Hess et al. 2008). Along with prolonging clotting times, acidemia reduces clot strength and leads to degradation of fibrinogen (Hess et al. 2008). Blood pH and oxygen availability are both heavily tied to

hibernacula conditions. While frogs can tolerate short periods of anoxia, they likely cannot survive anoxic conditions for longer than 3-7 days (Stewart et al. 2004). To combat this, bullfrogs are not completely torpid during winter months. They can move to areas of shallow ponds and lakes where oxygen is more readily available (Stinner et al. 1994). European common frogs (*Rana temporaria*) may, in fact, select microhabitats under frozen bodies of water to maximize their energy savings and possibly reduce the effects of hypoxia (Tattersall and Boutilier 1997). Thus, it is not clear if hibernating frogs do become hypoxic and acidotic, but doing so would lead to prolonged clotting times.

In contrast, a decrease in heart rate allows blood to pool for longer periods of time leading to an increased probability of clot formation. During hibernation, heart rate in bullfrogs is slowed to nearly 1/6 of its normal rate (Herman and Mata 1985). It is evident that in hibernating animals studied thus far, the impact of cold temperatures in prolonging clotting time is not sufficient to offset the effects of slow blood flow on stimulating clotting, due to the reduction in clotting factors associated with hibernation in these animals.

Prolonged APTT time has been found in many hibernating endotherms such as Ground squirrels (*Citellus tridecemlineatus*) (Lechler and Penick 1963), Little brown bats (*Myotis lucifugus*) (Smith et al. 1954), as well as in hibernating ectotherms like the Indian bullfrog (*Rana tigrina*) (Ahmad et al. 1979), sea turtles (*Chelonia mydas, Lepidochelys kempii, Eretmochelys imbricata, Caretta caretta, Dermochelys coriacea*) (Soslau et al. 2004), and Wood turtles (*Glyptemys insculpta*)(Yadav 2011). Previous studies have shown changes in the levels of clotting factors during hibernation. Studies performed on

sea turtles found decreased levels of Factor XI and Factor XII during prolonged hypoxic diving, while Factor VIII and Factor IX were found to be present and functioning (Soslau et al. 2004). In ground squirrels, Factor VIII and Factor IX showed a significant decrease in activity during hibernation as compared to non-hibernating ground squirrels (Lechler and Penick 1963). In Wood turtles, Factor VIII, Factor IX, and Factor XII have decreased levels of activity or no activity at all in hibernating Wood turtle samples as compared to non-hibernating, pre-hibernating, and post-hibernating Wood turtles. This suggests that the levels of circulating clotting factors are somehow regulated depending on the state of hibernation (Yadav 2011). This would likely allow Wood turtles to form clots for injury survival during active phases when they might be more prone to injury, while reducing clotting during periods of cold torpor, low blood flow, and slower heart rate.

In my study, I also found prolonged APTT times during hibernation. In fact, the APTT times were so prolonged that the bullfrogs did not seem to exhibit functional APTT times at all during hibernation and exhibited minimal functionality post-hibernation. These prolonged APTT times could largely be explained by the decreased activity of the circulating clotting factors. Again, the results of the Factor VIII deficient assay suggest that Factor VIII is not present or it is not active in pre-hibernating bullfrogs, hibernating bullfrogs, or non-hibernating bullfrogs. For Factor IX, Factor XI, and Factor XII the level of activity decreases during periods of hibernation and post-hibernation when compared to pre-hibernating levels (Figure 10).

Bullfrogs also appear to regulate the APTT clotting mechanism during hibernation as APTT clotting times increased significantly in frogs in the hibernating and

post-hibernating periods. Further, clotting times in the post-hibernating frogs were significantly decreased relative to frogs in hibernation, but still significantly longer than those frogs in the pre-hibernating group, suggesting a potential imminent return to pre-hibernating APTT clotting times upon exiting hibernation. My findings on clotting times for bullfrogs at "warm" temperatures were similar to previous studies (Dent and Schuellein 1950). These changes in APTT clotting times also align with changes in two of the specific clotting factors associated with the intrinsic clotting pathway that is measured through the use of APTT (Figure 10). Factor deficient assays involving factors XI and XII found significantly longer clotting times for hibernating and post-hibernating animals relative to pre-hibernating animals suggesting a decrease in these two factors during the hibernation and post-hibernation periods. Interestingly, neither factor was significantly lowered during post-hibernation relative to the hibernating group, even though APTT times were significantly shorter in the post-hibernation group compared to the hibernation group.

In contrast, factor VIII did not appear to be present in hibernating bullfrogs, suggesting that Factor VIII is not an essential clotting factor in American Bullfrogs. This is in stark contrast with the human clotting cascade in which the lack or impairment of factor VIII results in severe hemophilia (Lee et al. 2010). Additionally, it did not appear that bullfrogs regulated factor IX during hibernation, as no significant difference was found between clotting times of pre-hibernating and hibernating frog plasma mixed with factor IX deficient plasma. Interestingly, data from post-hibernating frogs indicated a significant increase in clotting times relative to pre-hibernating frogs when tested with

factor IX deficient plasma. Looking at the combined effect of longer clotting times associated with decreases in concentration of activity of factors IX, XI, and XII during post-hibernation, one would have expected that the APTT times of post-hibernating frogs would have been equal to or longer than the APTT times of hibernating frogs. Such was not the case, as post-hibernating frogs had shorter APTT times relative to hibernating animals.

Another confounding issue with post-hibernating frogs and their return to a faster coagulation time is the potential presence of an inhibitor to coagulation during this time. Plasma from post-hibernating frogs took significantly longer to clot when a concentration of 25 µl post-hibernating frog plasma was mixed with human plasma, relative to plasma mixed in similar concentrations for pre-hibernating and hibernating frogs. Inferring from these data alone, it would suggest that APTT clotting times should be even further prolonged in post-hibernating frogs relative to hibernating animals, the opposite of what was shown for measured APTT times. Other factors may be involved in the regulation of the APTT mechanism in frogs of which I am not aware and did not measure.

Large variations in APTT times were found in the post-hibernating frogs (Figure 3), with some frogs having coagulation times that were as prolonged as hibernating animals, with other frogs having APTT times more resembling those of pre-hibernating frogs. Further, there did not appear to be any effect of time in post-hibernation on APTT times. No pattern emerges, such as decreasing APTT time with the amount of time in post-hibernation. Such a pattern might have been predicted should the production of

clotting factors, or the functionality of clotting factors returned as a result of prolonged exposure to non-hibernating temperatures.

As bullfrogs begin to come out of hibernation, they may start to produce some of these clotting factors again causing APTT times to shorten as seen in Figure 1 and Figure 2. As the bullfrogs slowly come out of hibernation, production of these clotting factors may be delayed depending on the availability of essential nutrients such as Vitamin K. Vitamin K is essential in several key vitamin K-dependent proteins including prothrombin, Factor VII, Factor IX, and Factor X. Without Vitamin K, these proteins are not biologically active (Dowd et al. 1995; Shearer 1995). Bullfrogs in my study were not fed as they were coming out of hibernation so it is likely that their Vitamin K levels and levels of other nutrients had been depleted. This might explain why clotting times of post-hibernating Factor IX and Factor XII didn't return to pre-hibernating times for all frogs in the study.

It is also possible that bullfrogs may reduce their levels of platelets or promote a platelet shape change as they begin to enter hibernation, thereby prolonging coagulation times. Platelets circulate and are membrane bound when non-activated. When endothelium damage or vascular injury occurs, platelets come into contact with extracellular matrix (ECM) constituents and proteins such as vWF that are not exposed under normal conditions. When platelets encounter these proteins, they undergo three reactions. The first is platelet adhesion to ECM and shape change, followed by the secretion of α-Granules and δ granules that include calcium and ADP. The last reaction is aggregation of platelets. As the platelets clump together at the site of injury they form the

primary hemostatic plug (Kumar et al. 2007). A decrease in the amount of platelets during hibernation would decrease the chances of plug formation or slow the process greatly. Likewise, any platelet shape change prior to contact with ECM proteins would block the binding sites of the ECM constituents and help to prevent platelet aggregation. However, changes in platelet morphology or numbers cannot explain the incongruities associated with APTT times and clotting factor assays, as all clotting times were performed on plasma free of platelets.

Thrombin Times (TT) of pre-hibernating and hibernating bullfrogs were not significantly different from each other. However, post-hibernating TT times were significantly prolonged when compared to pre-hibernating and hibernating bullfrogs. This is similar to my findings for APTT times. Measurements were not made for those factors involved in TT clotting times, thus it is not known if bullfrogs also regulate these factors during hibernation and post-hibernation. Potential mechanisms for the elongation of TT time in post-hibernating frogs may be the result of insufficient concentrations, or inactivation of factors VII and/or X, as well as insufficient concentrations or inactivation of prothrombin, thrombin, and/or fibrinogen. This result may contradict the belief that there is no significant decrease in the plasma fibrinogen content in frogs during hibernation (Ahmad et al. 1979).

It appears that the decreased activity of multiple clotting factors (Factors IX, XI, and XII) significantly lengthens APTT time in hibernating bullfrogs (Figure 11). When bullfrogs begin to enter hibernation, they may decrease the level of these factors by ceasing the production of Factors IX, XI, and XII and/or they release some inhibitors to

interfere with the activity of these factors. My results suggest that if an inhibitor is released, that inhibitor is not a protein and is only present during the post-hibernation time period. These results differ from that of wood turtles (Yadav 2011). Finally, bullfrogs, which are ectotherms, show similar regulation of clotting factors during hibernation as do hibernating endotherms thus far studied. Interestingly, I was unable to show the presence and/or activity for Factor VIII in the intrinsic coagulation pathway of any of the bullfrogs in my study. This contrasts starkly with the importance of Factor VIII in coagulation of most mammalian species. This also differs from the intrinsic pathway in various Sea turtle species (Soslau et al. 2004) and Wood turtles (Yadav 2011) in which Factor XII is not regulated. Thus, the use of bullfrogs as a substitute for Wood turtles in the study of a potential inhibitor in the intrinsic coagulation pathway seems unwarranted.

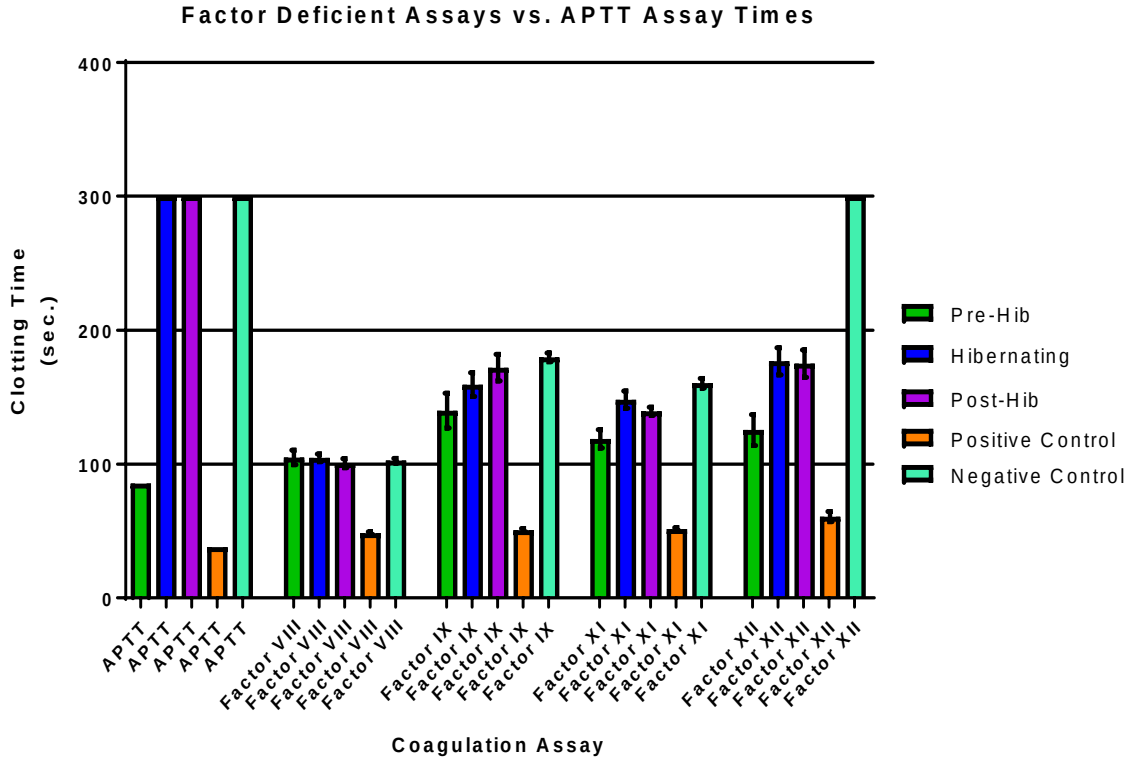

Figure 11. Mean values of Factor VIII, Factor IX, Factor XI, Factor XII, and APTT clotting times in seconds ± SE for pre-hibernating bullfrogs (n=4), hibernating bullfrogs (n=6), post hibernating bullfrogs (n=10), positive controls, and negative controls. Actual mean time of Hibernating APTT times.

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