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THE EFFECTS OF HIBERNATION ON THE HEMOSTATIC PROPERTIES OF THE AMERICAN BULLFROG, *RANA CATESBEIANA*

A Thesis Submitted

in Partial Fulfillment

of the Requirements for the Designation

University Honors

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May 2015

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Entitled: The Effects of Hibernation of the Hemostatic Properties of the American Bullfrog, *Rana catesbeiana*

has been approved as meeting the thesis or project requirement for the Designation University Honors.

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Abstract

It is known that hibernating animals display a drastic reduction in metabolic rate, accompanied by reduced heart rate and rate of blood flow through vessels. Sluggish blood flow increases the risk for developing clots, yet it has been observed that hibernators conclusively display elongated clotting times. This observed impairment may be caused by the decrease in body temperature, due to a negative impact on the functioning of the enzymes involved in coagulation. Some hibernating endotherms, or "warm-blooded" animals, employ biological mechanisms as preventative means to prevent clotting during hibernation, such as the down-regulation of certain clotting factors or the production of an inhibitor. In this study, the hemostatic properties of ectotherms, or "cold-blooded" animals, were studied by comparing the clotting times of non-hibernating and hibernating American bullfrogs (*Rana catesbeiana)* using activated partial thromboplastin time (aPTT). It was found that blood coagulation in ectotherms is less impacted by the change in temperature than in endotherms, and that the difference in clotting time between non-hibernating and hibernating frogs could not be explained by temperature alone. Bullfrogs appear to down-regulate levels of coagulation factors XI and XII, as well as produce a non-protein coagulation inhibitor. These results suggest that hibernating ectotherms such as frogs employ similar physiological mechanisms to prevent clotting during hibernation as do endotherms.

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Introduction

During hibernation, animals display a significant reduction in metabolic rate and heart rate, which consequently slows the rate of blood flow through vessels. Sluggish circulation puts hibernators at higher risk for developing unwanted blood clots. However, it has been shown that animals in hibernation conclusively display elongated coagulation times compared to those in a non-hibernating state, likely as a preventative measure to protect against increased clotting risk. Most research into the effects of hibernation on coagulation has been focused on endotherms, “warm-blooded” animals that maintain a constant internal temperature (mammals and birds), rather than ectotherms, “cold-blooded” animals whose internal temperature is primarily dependent on the temperature of their environment (amphibians, reptiles, fish, insects, and arachnids). The goal of this study is to determine whether ectothermic mechanisms for preventing clotting during hibernation are similar to those which have been found in endotherms. Using the American bullfrog, *Rana catesbeiana,* as a model, I examined the role of temperature in causing elongated clotting and identified physiological changes in the hemostatic system during hibernation. This study seeks to provide a greater understanding of how hibernation affects hemostasis in this understudied groups of animals.

Literature Review

Hemostasis

Hemostasis refers to the process of stopping blood flow following vascular damage by the formation of a clot. This system must be highly regulated in order to maintain a constant balance between excessive clotting and insufficient clotting, as both can be deadly. Too much clotting causes the formation of thrombi, which can occlude vessels or break off and travel to the heart, brain, or lungs. Too little clotting causes hemorrhaging, the uncontrolled loss of blood. The process of hemostasis is characterized by four components: primary hemostasis, secondary hemostasis, fibrin clot formation and stabilization, and fibrinolysis.

Primary hemostasis is the formation of a platelet plug. It is stimulated by an injury to the epithelial lining on the inside of a blood vessel that exposes the underlying collagen [1]. Adhesion, the first step in this process, occurs when exposed collagen triggers the release of chemicals that induce vasoconstriction [2,3], the narrowing of blood vessels, to reduce blood flow to the area, and cause circulating platelets to adhere to the damaged epithelium. This is followed by aggregation, in which these platelets initiate a positive feedback loop by releasing chemicals that attract additional platelets, ultimately leading to the formation of a platelet plug [1,3,4]. The platelet plug is an unstable and temporary means to stem blood loss, and must be reinforced by a stable, permanent clot produced by secondary hemostasis.

Secondary hemostasis, also known as the coagulation cascade, involves a series of enzymatic reactions which produce amplified amounts of product at each step. The

proteins in the blood that are involved in the coagulation cascade are called coagulation factors. These factors are produced by the liver [2] and circulate in the blood as zymogens, inactive enzyme precursors that require a biochemical change to become active. Each step in the cascade activates a zymogen, which in turn can activate an increasing amount of the next zymogen in the process [5]. This amplification ultimately results in the formation of a stable fibrin clot. It should also be noted that platelets play an important role in secondary hemostasis, as they provide the surface on which these zymogen reactions occur [1,6].

Traditionally, the coagulation cascade has been divided into three pathways: the extrinsic pathway, the intrinsic pathway, and the common pathway. The extrinsic pathway is initiated by chemicals external to the blood vessel that are released from damaged tissues. The intrinsic pathway, however, initiates coagulation when damage is within the blood vessel, and all necessary components are present in the circulating blood [2]. The extrinsic pathway does not act exclusive to the intrinsic pathway. Recent research indicates that the extrinsic pathway is involved in clot initiation, while the intrinsic pathway is responsible for clot growth [4,5,7]. However, the intrinsic pathway may act alone when the endothelial lining is damaged but the blood vessel wall has not been broken. Both pathways converge at the activation of coagulation factor X, the point at which the common pathway begins (Fig. 1).

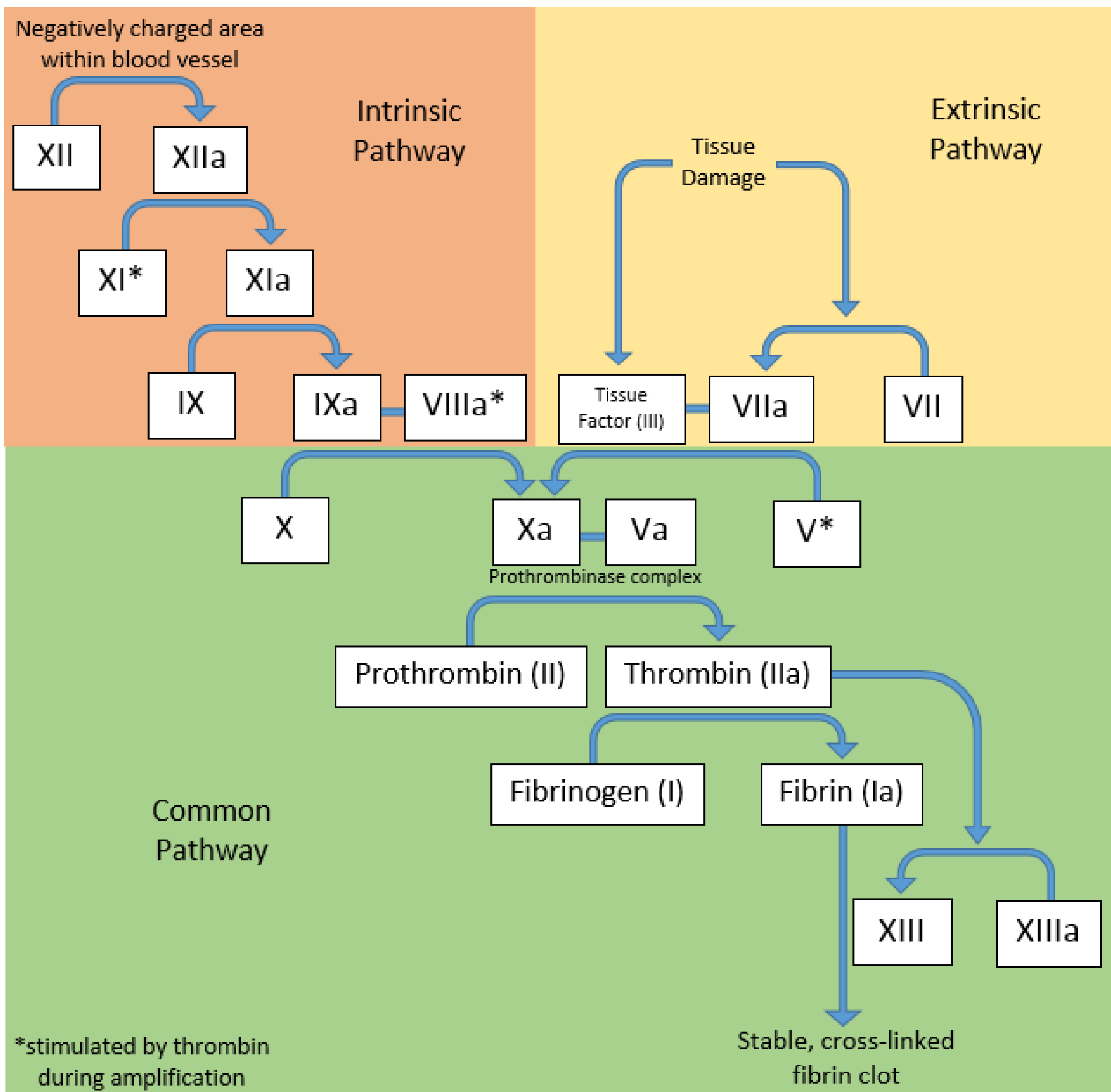

Figure 1 - Secondary Hemostasis and Fibrin Clot Formation & Stabilization

Due to the complex nature of the coagulation cascade and its pathways, it is helpful to think of secondary hemostasis as three phases: initiation, amplification, and propagation. Initiation begins via the extrinsic pathway when damaged tissues release a protein called tissue factor (TF), also known as coagulation factor III [4,6,7]. Tissue factor is unique in that it is always present in its activated form and it is thought to be essential to life [5,8]. Tissue damage also causes the activation of another coagulation factor in the blood, factor VII, which combines with TF to form the TF-VIIa complex. The “a”

notation indicates an activated enzyme. One function of this complex is to activate factor X to factor Xa and begin the common pathway (Fig. 1). Under normal physiological circumstances, the extrinsic pathway is the primary means for the initiation of coagulation [2,8].

Coagulation may also be initiated by the intrinsic pathway, and is triggered by the appearance of negatively charged areas within the vessel when the epithelial lining is damaged. Contact with these areas results in the activation of coagulation factor XII to factor XIIa [2,5,7]. The cascade proceeds as follows: factor XIIa activates factor XI, factor XIa activates factor IX, and factor IXa complexes with factor VIIIa. The factor IXa-factor VIIIa complex catalyzes the conversion of factor X to factor Xa, the first enzyme in the common pathway [1] (Fig. 1).

In addition to beginning the common pathway, the activation of factor Xa is the first step in the propagation phase. In this phase, factor Xa joins with factor Va to form the prothrombinase complex. The role of the prothrombinase complex is to catalyze the conversion of prothrombin (factor II) to thrombin (factor IIa) [5]. The production of thrombin is the key event in the coagulation cascade [9]; once it has been formed, the amplification phase can begin. This phase is characterized by the positive feedback on the system initiated by thrombin. Thrombin is capable of activating zymogens earlier in the intrinsic and common pathways. These include factor XI, factor VIIIa, and factor V, as well as the activation of additional platelets [6]. The combined role of the propagation and amplification phases is to produce sufficient thrombin to generate widespread clot formation [6].

The generation of thrombin triggers the third major event in coagulation, fibrin clot formation and stabilization. The role of thrombin is to convert fibrinogen (factor I), a soluble blood protein, to fibrin (factor Ia), which forms polymers and becomes insoluble [4]. Fibrin makes up the molecular scaffolding of a clot. Factor XIII, activated by thrombin to factor XIIIa, cross-links the long fibrin strands over top of the unstable platelet plug produced by primary hemostasis, stabilizing it [3] (Fig. 1).

Once a clot is no longer needed, fibrinolysis takes place to return normal blood flow to the area. To begin this process, endothelial cells on the inside of the blood vessel release a protein called tissue plasminogen activator (tPA). The role of tPA is to catalyze the conversion of plasminogen to plasmin, the major enzyme responsible for fibrin clot degradation [4]. To constantly maintain hemostasis, organisms must employ a complex combination of primary hemostasis, secondary hemostasis, and fibrinolysis.

Hibernation

Many organisms are capable of entering a state of dormancy, also called torpor, when environmental conditions are less than optimal. This state of dormancy is characterized by a marked reduction in metabolic activity. Aestivation is one type of torpor used by some organisms, and takes place during the summer months when conditions are hot and dry. Aestivation protects the organism from damage caused by high temperatures and from the risk of desiccation [10] and is common in mollusks, arthropods, fish, amphibians, and reptiles. Some small desert mammals have also been shown to use this adaptation [11].

On the other end of the spectrum, hibernation is an adaptation for winter months, when temperatures are cold and food can be scarce. This dormancy period is characterized by a significant reduction in heart rate, breathing rate, body temperature and metabolism. Preparation for hibernation is marked by an increase in stores of fat and glycogen that are depleted over the course of the winter [12,13]. Hibernation has also been shown to inhibit the immune system [14,15]. A large portion of the current understanding about hibernation is based on studies of mammalian hibernation, but it is also observed in reptiles, amphibians, insects, and birds. Preparation for, and maintenance of, hibernation requires a complex series of biochemical changes that are still not completely understood.

The overall reduction in body temperature may be expected to play a significant role in hibernation physiology [16,17], as the Q_{10}, or temperature sensitivity factor, for biological processes is approximately 2-3 [12]. This means that for every 10 degree change in temperature, the rate of reaction changes by a factor of 2-3. Therefore, as hibernating animals get increasingly colder, various metabolic enzymes become increasingly inefficient.

In addition, hibernation is accompanied by a strong suppression of transcription and translation [12,16]. Transcription is the process of making a copy of a sequence in the genome, and translation is the process of turning that copy into a new protein molecule. Reducing these processes during hibernation is beneficial, as they are energetically expensive to carry out. However, not all proteins are down-regulated, some are expressed in higher amounts during hibernation. For example, the enzyme

pancreatic lipase is responsible for breaking down fats into free fatty acids that can be used for energy, which is necessary when the organism is relying on fat stores for its survival [12]. This study examines how these two key aspects of hibernation, decreased temperature and changes in protein expression, impact the hemostatic system of the American bullfrog.

Hibernation & Hemostasis

The metabolic depression seen during hibernation affects the cardio-circulatory system by reducing heart rate and subsequently reducing the rate of blood flow through vessels [18]. Decreased temperature also contributes to reduced blood flow by increasing blood viscosity [19]. Sluggish circulation leads to an increased risk of forming an unwanted intravascular clot. It was observed as early as the 18th century that animals in hibernation have extended clotting times, and this is probably an adaptive mechanism used to compensate for the increased risk of forming clots [20].

The reduction in temperature itself would predict an impairment in coagulation, as the clotting factors, which are enzymes, would be expected to work less efficiently at increasingly colder temperatures. Recall that the Q_{10} (temperature sensitivity factor) for most biological processes is 2-3, meaning that they work faster as temperature increases and slower as temperature decreases. Indeed, non-hibernating animals exposed to hypothermic conditions have demonstrated extended clotting times, as measured by both prothrombin time (PT) and activated partial thromboplastin time (aPTT) [21]. These laboratory tests are used to measure the integrity of the extrinsic (PT) and intrinsic (aPTT) pathways. Hypothermia appears to cause a delay in the initiation of

thrombin, but it has no effect on the propagation phase and therefore the ultimate strength of the clot [21,22]. Hypothermic conditions have also been shown to cause platelet dysfunction by decreasing both platelet count and aggregability [19,22,23].

In addition to the impact of temperature, blood coagulability can be reduced when organisms alter the expression of certain types of proteins. One strategy is to down-regulate the production of certain coagulation factors. Hibernating hamsters and ground squirrels both display a reduction in the amount factor V from the common pathway, as well as a marked reduction in the amount of prothrombin (factor II) present in their blood [24,25,26,27]. Hamsters have also been shown to suppress production of factor VII of the extrinsic pathway [24].

Another strategy is to up-regulate the production of a clotting inhibitor. Ground squirrels have been shown to upregulate the production of the plasma protein α_2-macroglobulin. α_2-Macroglobulin is a broad spectrum inhibitor of enzymes, known as proteases, that break apart bonds in proteins [20]. It has been shown to be a particularly potent inhibitor of factor Xa, the enzyme which begins the common pathway [20]. Hedgehogs display a rise in the number of mast cells, also called heparinocytes, which produce the endogenous anticoagulant heparin [28]. Heparin increases the activity of the regulatory protein antithrombin. As its name suggests, the role of antithrombin is to inhibit thrombin, preventing clotting by preventing the formation of fibrin strands. In cats [29], bats, turtles [30], and dogs [23] there appears to be an increase in heparin or another heparin-like substance, but no increase in mast cells. This study seeks to determine whether ectothermic animals employ similar mechanisms for preventing

coagulation during hibernation as those observed in endotherms, and to compare the impact of temperature on clotting in these two groups of animals.

Materials & Methods

Twenty-two American Bullfrogs, *Rana catesbeiana*, were obtained from Kons Scientific (Germantown, WI). The average weight of each frog was 220.2 grams. Five frogs were randomly selected to serve as a control group and kept at room temperature in a plastic tub with a ventilated lid filled with approximately 2-3 inches of tap water. The remaining seventeen frogs were separated into three groups, A, B, and C, to be kept in hibernation for increasing lengths of time. They were kept in three additional tubs and placed in an environmental chamber on September 30th, 2014. To best mimic the natural process of hibernation, the temperature of the chamber was decreased steadily at a rate of three degrees every three days until it reached 3°C (on October 23rd, 2014). No group of frogs was fed during this time.

Blood was drawn from the five room temperature frogs on October 23rd, 2014. An anesthetic solution was prepared using tricaine mesylate, more commonly known as MS-222, in a ratio of 2.5g MS-222 per liter of water. The resulting solution was mildly acidic (pH ~ 5), and was buffered using sodium bicarbonate to a neutral pH of approximately 7-7.5. Individually, frogs were placed into the anesthetic solution until rendered immobile, typically about 2-3 minutes, and then weighed. To expose the heart, a vertical incision was made on the ventral side of the frog, just to the right of the sternum. Using a syringe flushed with 0.105M (3.2%) sodium citrate (to prevent

coagulation upon withdrawal), blood was drawn by cardiac puncture (Fig. 2). The blood was placed into centrifuge tubes and centrifuged for 5 minutes at 2500g to separate the plasma from the blood cells. The plasma, containing the coagulation factors, was then pipetted into new centrifuge tubes in 0.5mL amounts and stored at -80°F.

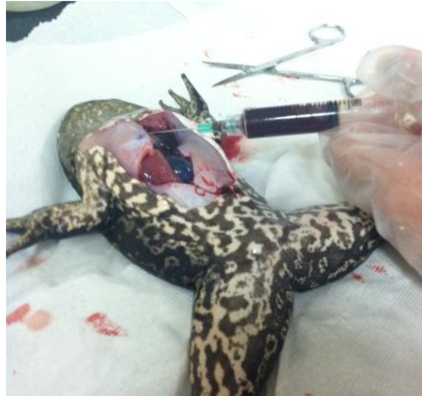

Figure 2 - Drawing blood from *Rana catesbeiana* by cardiac puncture

Blood was drawn from the first group of hibernating frogs (A) on October 30th, 2014, one week after hibernation temperature was reached. Plasma was obtained from 5 frogs. Blood was drawn from the second group of hibernating frogs (B) on November 13th, 2014, three weeks after hibernation temperature was reached. Plasma was obtained from 5 frogs. Finally, blood was drawn from the last group of hibernating frogs (C) on December 4th, 2014, six weeks after hibernation temperature was reached. Plasma was obtained from the remaining 7 frogs.

Laboratory tests were performed throughout the spring 2015 semester. Evaluation of the integrity of the intrinsic pathway was performed by measuring activated partial thromboplastin time (aPTT), using the KC1Δ Amelung coagulation analyzer.

Effect of Temperature on Coagulation

To investigate the effects of temperature on coagulation, three groups were compared: room temperature frogs, hibernating frogs, and an endotherm (sheep). To better understand the impact of temperature on coagulation, the aPTT tests were performed at two different temperatures: typical endothermic body temperature (37°C) and hibernation temperature (3°C). To test at 37°C, the sample cuvette was allowed to incubate for 3 minutes following the addition of 100 microliters of plasma and 100 microliters of Cephen 5 reagent. Measurement of coagulation time began following the 3 minute incubation period and the addition of 100 microliters of calcium chloride reagent. To test at 3°C, the plasma samples, Cephen 5 reagent and calcium chloride reagent were all placed in an ice bath to keep them cold. The plasma sample and Cephen 5 reagent were added to the sample cuvette, and this mixture was kept in an ice bath for at least two minutes to ensure that it reached approximately 3°C. An infrared thermometer was used to measure the temperature of the sample. When the sample reached 3°C, it was placed into the analyzer and calcium chloride was added immediately to begin the coagulation time measurement. Once the sample had clotted, the temperature was measured again. These two temperature values were averaged to find the value for T_1 in the equation for Q_{10}, which was calculated using the formula $Q_{10} = \left[\frac{R_2}{R_1}\right]^{\frac{10}{T_2 - T_1}}$. A one-way ANOVA was performed to determine potential statistical significance within the three groups for coagulation time at 3°C and 37°C and between the groups for Q_{10} value.

Changes in Coagulation Factor Levels

Despite a drastic reduction in the rate of blood flow, frogs in hibernation still experience micro-tears in their vasculature, which would initiate coagulation via the intrinsic pathway. The aPTT test can provide information about the presence of factors VIII, IX, XI and XII in the hibernating frogs compared to the room temperature frogs. To determine whether some coagulation factors are down-regulated during hibernation, rescue assays were performed using factor-deficient human plasma. Twenty microliters of frog plasma was mixed with 80 microliters of reconstituted human plasma that is deficient in one factor of the intrinsic pathway. Then aPTT was used to assess the clotting time. If this combination clotted faster than the factor-deficient plasma by itself, then it has been "rescued" and the frog plasma sample can be concluded to contain that clotting factor in sufficient amounts. Failure to rescue the factor-deficient plasma indicates a lack of that factor. This method was used to determine the relative levels of factors VIII, IX, XI and XII in the room temperature and hibernating frogs. A one-way ANOVA was again used to determine statistical significance between the three groups for each coagulation factor.

Presence of a Coagulation Inhibitor

To determine the possibility of a coagulation inhibitor produced by hibernating frogs, aPTT was performed using 50 microliters room temperature plasma combined with 50 microliters hibernating plasma. If the addition of the hibernating plasma elongated the clotting time of the room temperature plasma, it would suggest the presence of a coagulation inhibitor in the hibernating plasma. The inhibitor could be a

protein, such as the α_2-macroglobulin protein that is upregulated in ground squirrels during hibernation, or it could be another type of molecule such as a polysaccharide (carbohydrate) like heparin. To test this question, samples of hibernating plasma were placed in a water bath at 80°C for approximately one hour. If the inhibitor is a protein, the heat from the water bath would have resulted in denaturation, rendering the protein nonfunctional. If addition of the heated hibernating plasma to room temperature plasma did not elongate the coagulation time, it could be concluded that the inhibitor is a protein. One-way ANOVA was used to identify any statistical significance between the coagulation time of the room temperature plasma by itself and when it was mixed with hibernating and denatured hibernating plasma.

Results

Results from aPTT testing indicated that there was no significant difference ($p=0.22$) between the coagulation times of Group A, which had been at hibernation temperature for one week (61.64 ± 13.06 sec), Group B, which had been at hibernation temperature for three weeks (56.74 ± 11.09 sec), and Group C, which had been at hibernation temperature for the longest, at six weeks (56.11 ± 8.07 sec) [Fig. 3]. Because no significant difference occurred between these three groups, the data from all three were pooled for subsequent comparisons and analysis.

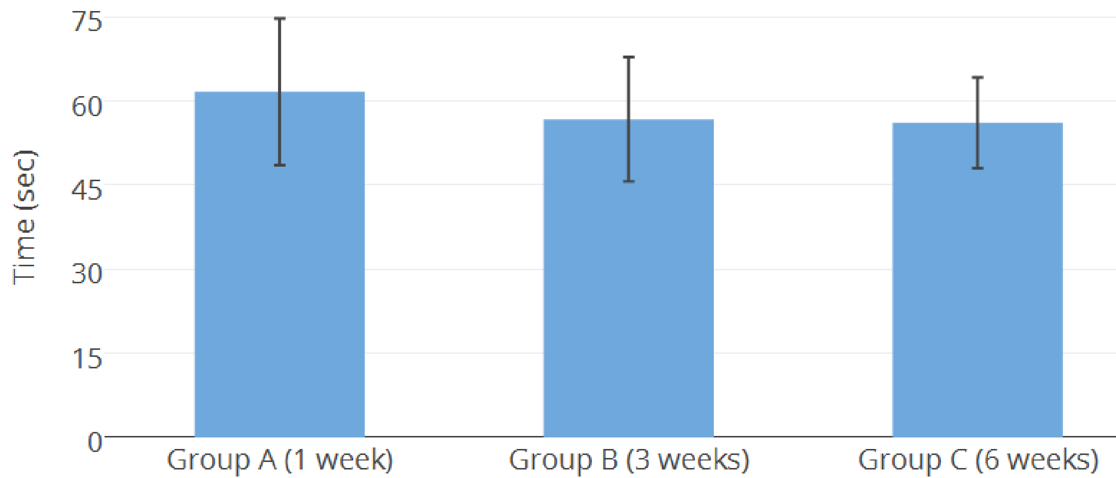

Figure 3 - Coagulation Times of Hibernating Groups A-C

At both 3°C and 37°C, the hibernating frogs displayed elongated coagulation times (98.33 ± 31.34 sec at 3°C, 59.58 ± 11.48 sec at 37°C) when compared to the room temperature frogs (67.66 ± 20.85 sec at 3°C, 48.64 ± 11.23 sec at 37°C) [Fig. 4]. The difference between these groups was significant at both temperatures.

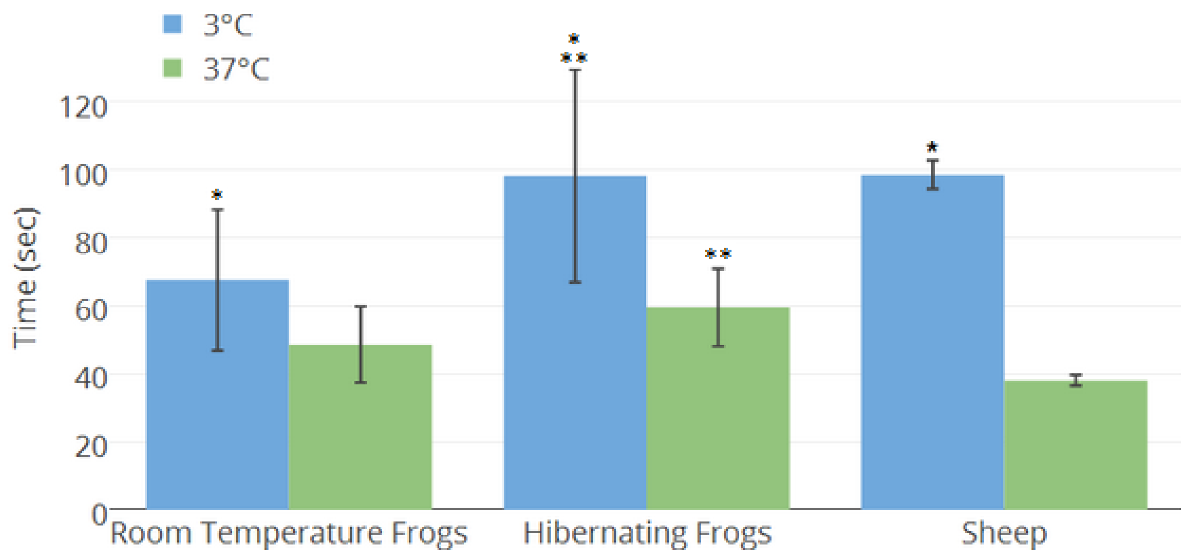

Figure 4 - Coagulation Times at 3°C and 37°C
(* indicates significant difference between 3°C and 37°C within sample group [p≤0.05])
(**indicates significant difference from room temperature frogs [p≤0.05])

Effect of temperature on Coagulation

The sheep plasma clotted faster than both frog groups at 37°C (38.10 ± 1.63 sec) and was greatly elongated at 3°C (98.62 ± 4.18 sec) [Fig. 4]. Calculation of Q_{10} values for these three groups showed that the coagulation time of sheep plasma was most impacted by the change in temperature (Q_{10}= 0.651). The difference in Q_{10} between the room temperature frogs (Q_{10}= 0.902) and hibernating frogs (Q_{10}= 0.830) [Fig. 5] was not found to be statistically significant (p= 0.15).

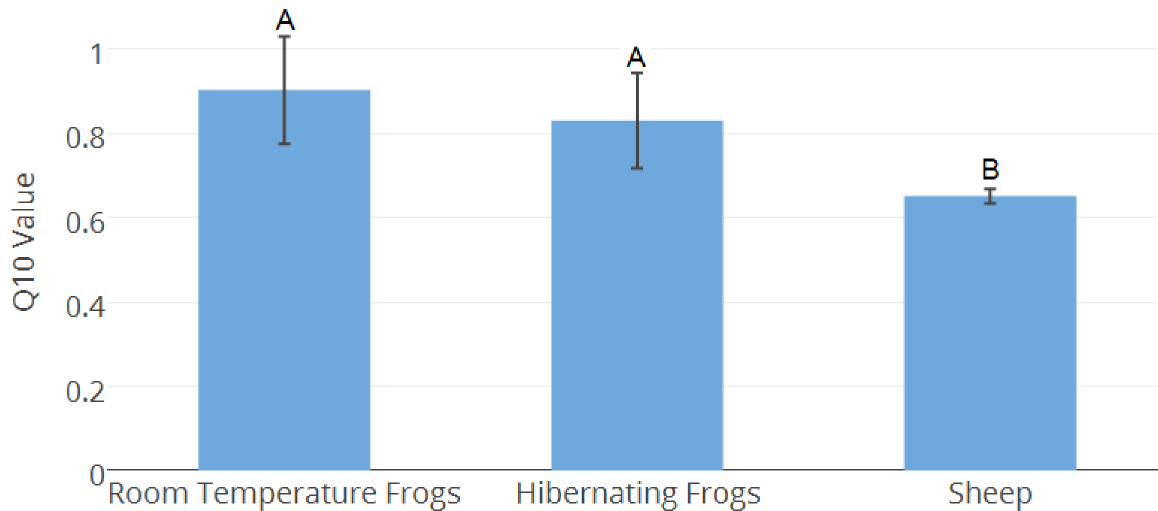

Figure 5 - Q_{10} Values
(Groups with the same letter indicate no significant difference [p>0.05])

Changes in Coagulation Factor Levels

Results from rescue assays indicated that coagulation factor VIII did not appear to be present in either room temperature frogs (81.37 ± 5.22 sec) or hibernating frogs (87.10 ± 4.17 sec), as both groups had longer coagulation times than the factor-deficient plasma by itself (76.77 ± 3.66 sec). Similarly, coagulation factor IX also did not appear to be present in either room temperature frogs (124.91 ± 7.66 sec) or hibernating frogs

(125.82 ± 7.75 sec), as both groups had longer coagulation times than the factor-deficient plasma by itself (110.0 ± 4.0 sec) [Fig. 6].

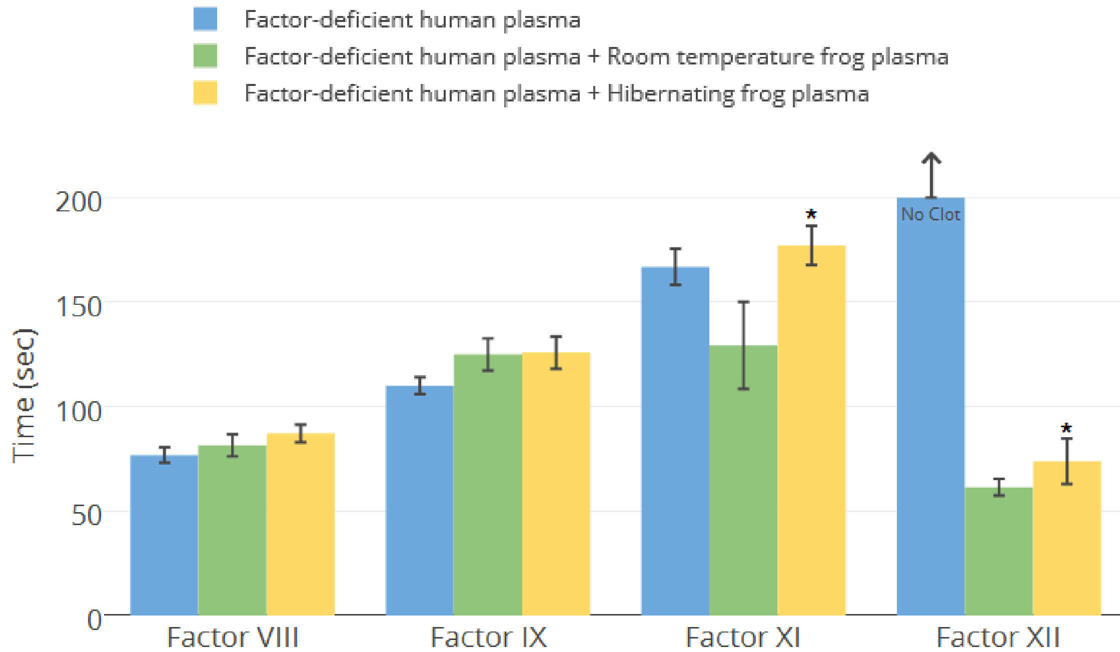

Figure 6 - Relative Coagulation Factor Levels
(* indicates significant difference from room temperature [p≤0.05])

Coagulation factor XI was found to be present in sufficient amounts in the room temperature frogs (129.28 ± 20.89 sec) so as to rescue the factor-deficient plasma (166.84 ± 8.63 sec), but was not found in sufficiently high levels in the hibernating frogs (177.08 ± 9.30 sec) for rescue [Fig. 6]. These results indicate that coagulation factor XI is down-regulated in hibernating frogs, leading to a prolonged clotting time.

The factor-deficient human plasma lacking coagulation factor XII did not clot at all during aPTT testing, but was rescued by both the room temperature frog plasma (61.38 ± 4.01 sec) and the hibernating frog plasma (73.81 ± 10.92 sec) [Fig. 6]. The difference between these two groups was shown to be statistically significant

(p= 0.0022), indicating that coagulation factor XII is also down-regulated during hibernation.

Presence of Coagulation Inhibitor

Results showed that when hibernating plasma was added to room temperature plasma, its clotting time was significantly elongated (from 48.64 ± 11.23 sec to 84.86 ± 16.18 sec), which suggests that the hibernating plasma does, in fact, contain some kind of coagulation inhibitor. When the hibernating plasma was denatured in a hot water bath before being combined with the room temperature plasma, the coagulation time lengthened again (118.66 ± 23.45 sec) [Fig. 7], indicating that the inhibitor may not be a protein.

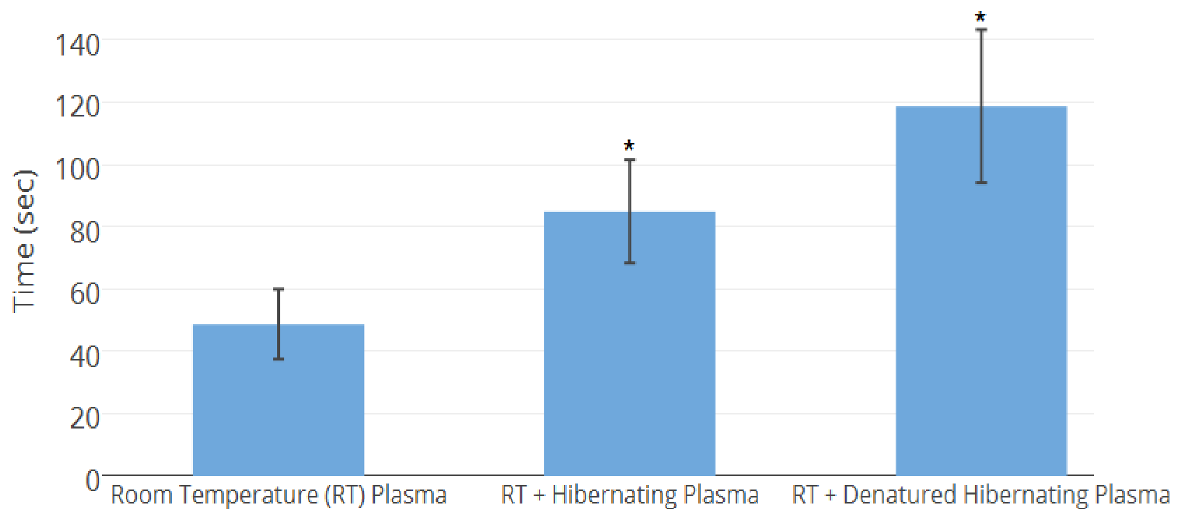

Figure 5 - Quantification of Coagulation Inhibitor
(* indicates significant difference from room temperature [p≤0.05])

Discussion

Given that the physiological changes that accompany a process like hibernation are typically gradual, a stepwise progression of changes in the hemostatic properties of hibernating frogs was predicted. For example, that the frogs that were exposed to the cold temperature for the longest amount of time would be predicted to display the longest clotting time. However, this was not the case. While each group of hibernating frogs displayed elongated clotting times when compared to room temperature frogs, there was no significant difference in coagulation time between the frogs that were at hibernation temperature (3°C) for one week, three weeks, and six weeks. It seems that hibernating frogs need to make the necessary changes to their hemostatic system prior to reaching 3°C, indicating that the risk for coagulation at that temperature is the same regardless of the length of exposure. It would be beneficial to conduct future studies on the timing of hemostatic changes that focus on the span of time during which hibernation is being induced (period of time during which frogs go from 25°C to 3°C). This is likely the time in which the majority of physiological changes occur to prepare the animal for entering an extended period of dormancy.

Effect of Temperature on Coagulation

One factor that is known to elongate clotting in hibernating endotherms is decreased temperature because the enzymes of the coagulation cascade cannot work as efficiently at lower temperatures. However, given that endothermic animals experience a very narrow range of internal temperatures, we would expect that

hemostasis in this group would be much more impacted by the change in temperature than would be the case for ectotherms, who naturally experience a much greater range of internal temperatures. Indeed, at 37°C, the sheep plasma clotted quickly and efficiently, because this is typical endothermic body temperature, but at 3°C, a temperature that a sheep would never actually reach in nature, the clotting time increased dramatically. While both frog groups showed elongated clotting at 3°C compared to 37°C, the difference was less extreme than in sheep. This difference in the ability of enzymes, in this case the coagulation factors of the intrinsic pathway, to function at these two temperatures can be illustrated by the temperature coefficient, or Q_{10} value. A Q_{10} value equal to one would indicate no change in enzyme rate as temperature increases, whereas a Q_{10} value equal to two would mean that for a 10 degree increase in temperature, the rate of the enzymatic reaction would increase twofold, and so on.

In this study, it was found that the Q_{10} values for all groups were less than one, which would indicate that as temperature increased the rate of the enzymatic reaction decreased. This is somewhat counterintuitive because of the nature of the enzymatic reactions involved in coagulation. When the temperature is increased, the coagulation factors behave as enzymes typically do and perform their function more quickly (meaning the enzymatic rate increases), which would typically give a Q_{10} value greater than one. However, because the clotting enzymes are working faster, this subsequently causes coagulation to occur more rapidly and the overall rate of the reaction that can be measured actually decreases. This gives a Q_{10} value that is less than one. Due to this

counterintuitive relationship, the Q_{10} value can be thought of as the factor by which the enzyme function changes as the temperature decreases, rather than increases.

If the hypothesis that endotherms are more impacted by temperature than ectotherms is correct, we would expect that the sheep plasma would display a Q_{10} value further away from one in either direction. Indeed, sheep displayed a Q_{10} value of 0.651, while room temperature and hibernating frogs displayed values of 0.902 and 0.830 respectively. In essence, when the temperature dropped 10 degrees, the sheep's enzymes performed at only about 65% of the speed that they did at the higher temperature, whereas the frogs' enzymes performed at 90% and 83% of their original speed. Therefore, it can be concluded that changing temperature does, in fact, impact coagulation in ectotherms, but to a lesser degree than it does in endotherms. Additionally, the difference in Q_{10} values between room temperature and hibernating frogs was not found to be significant, which indicates that the difference in coagulation times between these groups cannot be solely attributed to the change in temperature. Hibernating frogs must employ other of biological mechanisms to account for their elongated clotting times.

Changes in Coagulation Factor Levels

Research has shown that some hibernating endotherms alter the levels of coagulation factors in their blood, effectively reducing their ability to initiate clotting. This study indicates that hibernating frogs employ the same technique. The factors that were examined were those of the intrinsic pathway (VIII, IX, XI and XII) as this is the "internal" pathway that would typically initiate clotting during hibernation. It is

suspected that both factor VIII and factor IX are absent in both room temperature and hibernating frogs, because neither sample rescued the factor-deficient human plasma and enabled it to clot. The reason that frogs appear to lack these two factors completely is not entirely known. The coagulation cascade may not be a system that has been completely conserved throughout vertebrate evolution. Perhaps factors VIII and IX evolved later, with the development of mammals. Alternatively, perhaps frogs do have factors that perform the same role in coagulation, but they are sufficiently different from those of humans such that they would not be able to rescue human plasma. Future studies are required to address this question.

Results do indicate that hibernating frogs alter the levels of the other two intrinsic coagulation factors, factor XI and factor XII, somewhat like an induced hemophilia. It appears as though the production of factor XI may be completely shut off in hibernators, given that the hibernating plasma was unable to rescue the factor-deficient plasma. On the other hand, human plasma deficient in factor XII was rescued by both room temperature and hibernating frog plasma, indicating that this factor may still be produced in hibernators but to a lesser degree. By down-regulating the production of these factors during hibernation, the initiation of hemostasis takes longer and the frogs can preemptively protect themselves against the increased risk of clotting due to sluggish blood flow.

Presence of a Coagulation Inhibitor

Certain endotherms have also been shown to produce an inhibitor as a means to prevent coagulation during hibernation, such as the protein α_2-Macroglobulin or the

polysaccharide heparin. It is reasonable to expect that ectotherms may employ a similar strategy during hibernation. Given that the addition of hibernating plasma to a sample of room temperature plasma increased the coagulation time, it appears as though the plasma of hibernating frogs does contain some kind of inhibitor. Results from the rescue assays provide additional evidence for this hypothesis. If there was no inhibitor present, it would be expected that the clotting time of the factor-deficient human plasma would remain unchanged following the addition of a frog plasma sample if that sample did not contain the missing factor. However, for factors VIII, IX, and XI, in which the hibernating plasma did not rescue the factor-deficient plasma, the clotting time is actually slightly longer than the factor-deficient plasma alone. This elongation is likely due to the presence of an inhibitor.

Although the macromolecular class of this inhibitor remains unknown, results from this study indicate that it does not appear to be a protein. If it were a protein, one would expect that heating the hibernating plasma sample would cause denaturation and destroy its inhibitory ability, thereby causing the clotting time to decrease when compared to unheated hibernating plasma. Results showed that the addition of denatured hibernating plasma actually increased the clotting time further. This observed elongation may be due to the fact that the water bath would denature the coagulation enzymes as well, and the sample became sufficiently diluted that the remaining functional enzymes from the room temperature plasma were less efficient at clotting the sample. If this is true, it could be possible that the inhibitor is actually a protein, but that the loss of its anti-coagulatory function is masked by the loss of the

coagulatory function of the clotting factors. It would be beneficial to conduct future studies using varying ratios of room temperature and hibernating plasma to better understand what role dilution may play in these results.

Conclusion

The purpose of this study was to determine whether ectothermic regulation of hemostasis during hibernation is similar to that of endotherms, and results indicate that these groups do in fact use similar physiological mechanisms. The American bullfrog displays a down-regulation of certain coagulation factors during hibernation as well as the production of a coagulation inhibitor, both of which are strategies employed by hibernating endotherms. Ectotherms, however, are less impacted by the reduction in temperature accompanying hibernation than are endotherms, and the change in temperature could not account for the difference in clotting times between hibernating and non-hibernating frogs, creating the need for such physiological mechanisms to prolong coagulation.

This study does face some limitations, such as the limited sample of frogs from which plasma was drawn and the fact that the rescue assays were performed using factor-deficient human plasma, which may not be entirely comparable to frog plasma. Additionally, amphibians such as the American bullfrog represent only one class of animals in the large and diverse group of ectotherms, and therefore cannot be assumed to represent all ectothermic animals as a whole. Additional studies are needed to better understand the progression and timing of these hemostatic changes when hibernation is

being induced, and to determine the identity of the coagulation inhibitor. Despite these limitations and the need for future research, this study provides valuable insight into how hibernation affects hemostasis in ectotherms, a group for which little was previously known.

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