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The effects of hibernation on the time of coagulation of the American bullfrog, Rana catesbeiana

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THE EFFECTS OF HIBERNATION ON THE TIME OF COAGULATION OF THE

AMERICAN BULLFROG, *RANA CATESBEIANA*

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Abstract

Cold conditions during winter months greatly reduce heart rate in ectothermic animals, such as bullfrogs, resulting in slow, intermittent blood flow. Slow or intermittent flow of blood often results in coagulation, yet the blood of these ectotherms fails to coagulate. In this study, the effects of the stages of hibernation from the onset of temperature decline, through the duration of constant low temperature exposure, was studied by observing the time of blood coagulation of American bullfrogs (*Rana catesbeiana*) using activated partial thromboplastin time (aPTT). It was found that the time of coagulation increased incrementally along with the decreasing temperature of the hibernation environment. These results suggest that there is a trend between a decrease in temperature and time of coagulation.

Introduction

When animals go into a state of hibernation, it results in a significant decrease in metabolism and heart rate. A consequence of this is a slow blood flow rate which puts the animal at risk for blood clotting. Research on animals that endure hibernation has mainly dealt with endotherms ("warm-blooded" animals), rather than ectotherms which are "cold-blooded" animals. The endotherms maintain a constant body temperature within the hibernation environment, whereas the ectotherms reflect the decreased temperature of the environment with their internal body temperatures. The goal of this study was to determine the effect of decreasing body temperature on the time of coagulation of blood during the duration of hibernation. By using the ectotherm, *Rana catesbeiana*, this study set a timeline of coagulation for this understudied group of ectotherms, and provides data that shows the dependency of coagulation on temperature. The purpose of this timeline is to show the role of temperature in initiating changes in coagulation and thus lead to further investigation of these changes.

Literature Review

Hibernation

When environmental conditions are less than favorable, a majority of organisms are able to enter a state of dormancy in which reduced metabolic activity is seen. A state of dormancy is a source of protection, and one example of this would be estivation; a type of dormancy exhibited by some organisms when temperatures are dry and desert-like (Boutilier et al., 1997). Another type of dormancy is hibernation, which is seen with prolonged temperature declines, and when sources of food are relatively hard to find. When these cold conditions are present, the organism will display decreased metabolism, heart rate, and body temperature (James et al., 2013). Decreased body temperatures associated with hibernation have been a subject of great physiological interest (Ultsch et al., 2004). These decreased physiological effects based on temperature can be measure by the temperature sensitivity factor, Q_{10}. When this factor is approximately 2-3 in value, this represents and implies that for every 10 degree change in temperature, the reaction rate will change by a factor of 2-3. This rate effect represents the inefficiency of metabolism and its associated enzymes as temperatures get increasingly colder within a hibernation environment (Boyer & Barnes, 1999).

Even though many characteristics of hibernation have been witnessed and recorded for ectotherms ("cold-blooded" animals), little is known about the mechanisms and evolution of this acquired trait (Costanzo et al., 2013). Some data sets have been recorded on other ectotherms, including a study by Neha Yadav (M.S. Thesis, 2011) using wood turtles, in which a decrease in temperature resulted in the time required for coagulation to increase dramatically. Similar results have been shown in the American bullfrog, *Rana catesbeiana*, with conclusions that show the presence of a cascade inhibitor (Cullinan, 2015).

Coagulation and Hemostasis

When temperature decreases during hibernation, coagulation is impaired (Cullinan, 2015), but the time and temperature of when this impairment occurs is unknown. In vertebrates, the mechanism associated with blood clotting follows the same fundamental pattern, suggesting that coagulation is conserved among all vertebrates with minor differences when comparing genomes (Jiang & Doolittle, 2003). Non-hibernating vertebrates, when tested in an environment that displays hypothermic conditions, have displayed extended clotting time in accordance with the decrease in temperature by measuring the prothrombin time (PT) and activated partial thromboplastin time (aPTT) (Martini, 2007).

Hemostasis, the process of coagulation after injury to vascular tissue, is divided into four sections: 1) primary hemostasis, 2) secondary hemostasis, 3) fibrin clot formation and stabilization, and 4) fibrinolysis. Primary hemostasis involves an injury to vascular tissue that results in a response of platelet adhesion, and thus a platelet plug will form. This plug is very unstable and must be reinforced, and the process of reinforcement is secondary hemostasis (Kamal et al., 2007). In secondary hemostasis, or the coagulation cascade, a series of reactions with proteins called coagulation factors, follow a cascade that results in the stabilization of the platelet plug into a stable fibrin clot (Gailani & Renné, 2007). This coagulation cascade is further divided into three different pathways: the extrinsic pathway, the intrinsic pathway, and the common pathway (Figure 1).

The extrinsic pathway is initiated by damage to a vascular wall that releases chemicals externally to the vessel, whereas the intrinsic pathway is initiated by chemicals within the blood vessel that are released in response to damage of the tissue (Soslau et al., 2004). Both of the

pathways come together at coagulation factor X, which is the start of the common pathway

(Kamal et al., 2007). The common pathway then converts the fibrinogen of the initial platelet

plug into fibrin which makes a more stable and reliable plug. This completes secondary

hemostasis and results in completion of clot formation and stabilization, leading to the formation

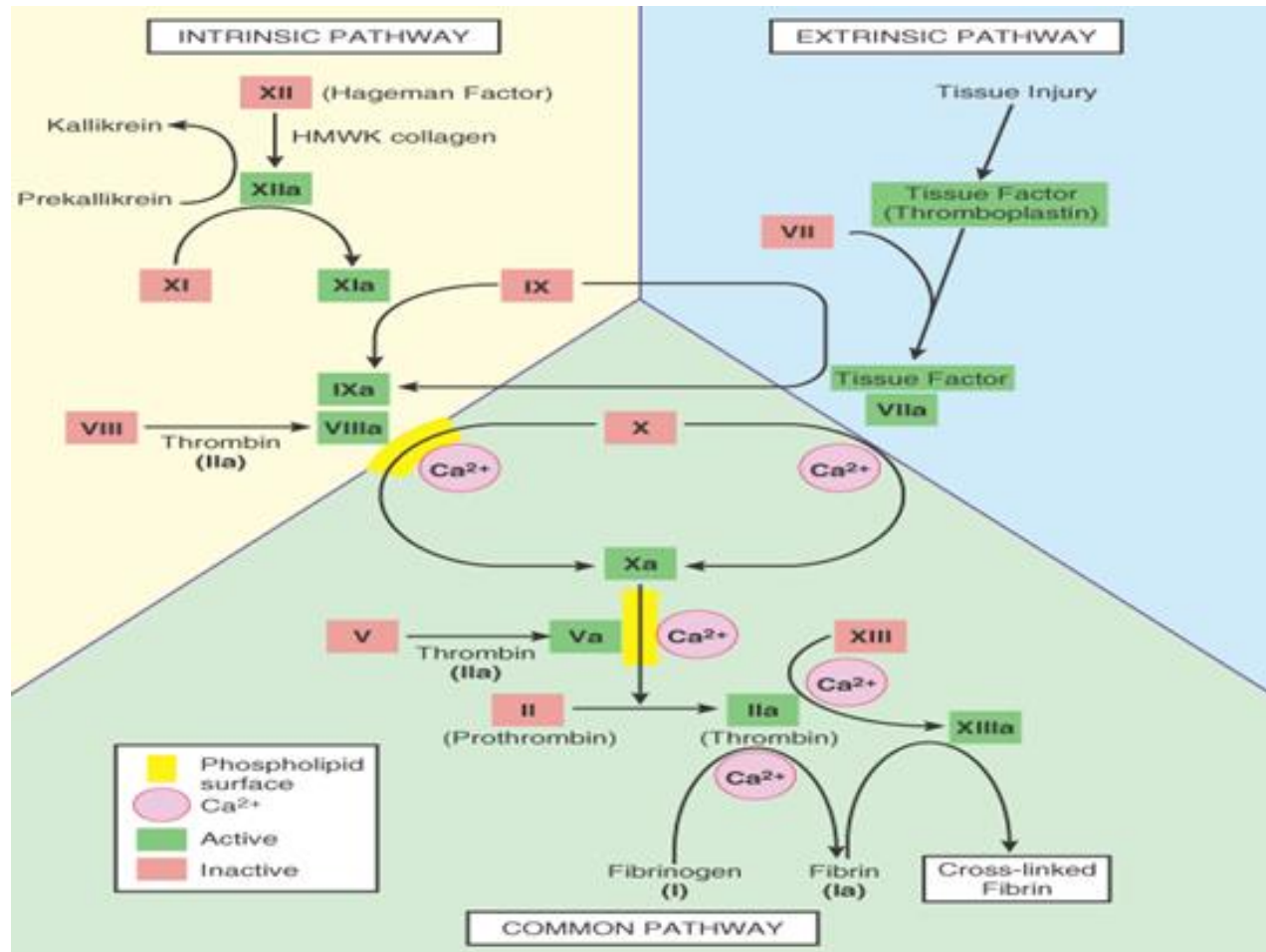of the stable plug (Soslau et al., 2004).

Figure 1 - Secondary Hemostasis: Extrinsic, Intrinsic, and Common Pathways (Reed, 2012)

By researching the time of coagulation and its relationship to environmental temperature,

this study established a timeline of changes in coagulation through hibernation and provided a

structure for further research into the complex cascade of coagulation and homeostasis.

Methodology

Twenty-one American bullfrogs (*Rana catesbeiana*) were placed in an environmental chamber at room temperature (24°C) and over the time of this experiment, the environmental temperature was decreased to the hibernation environmental temperature of 4˚C. Plasma was then extracted at different temperature increments following the protocol previously described (Cullinan, 2015). This process, with modifications, provided plasma samples to be used with the KC1Δ Amelung coagulation analyzer that measured activated partial thromboplastin time (aPTT) of blood coagulation.

The procedure described by Cullinan (2015) was done with the following modifications: The grouping of the frogs was done in pairs. Two frogs were tested at each stage within the hibernation sequence, starting first with two pairs of room temperature frogs, and then followed by another pair of frogs that had their plasma extracted approximately every four weeks (over a 4-month period) as temperature decreased to represent the actual timeline of hibernation. The temperature in the environmental chamber was lowered by approximately 4°C every four weeks until at constant temperature of 4°C was reached. This process is shown in Figure 2.

The time of coagulation was recorded and averaged for each frog to obtain a R_1 value for the Q_{10} equation: $Q_{10} = \left[\frac{R_2}{R_1}\right]^{\frac{10}{T_2 - T_1}}$ (R_2 = clotting time at room temperature, R_1 = clotting time at hibernation temperature, T_2 = room temperature, T_1 = hibernation temperature).
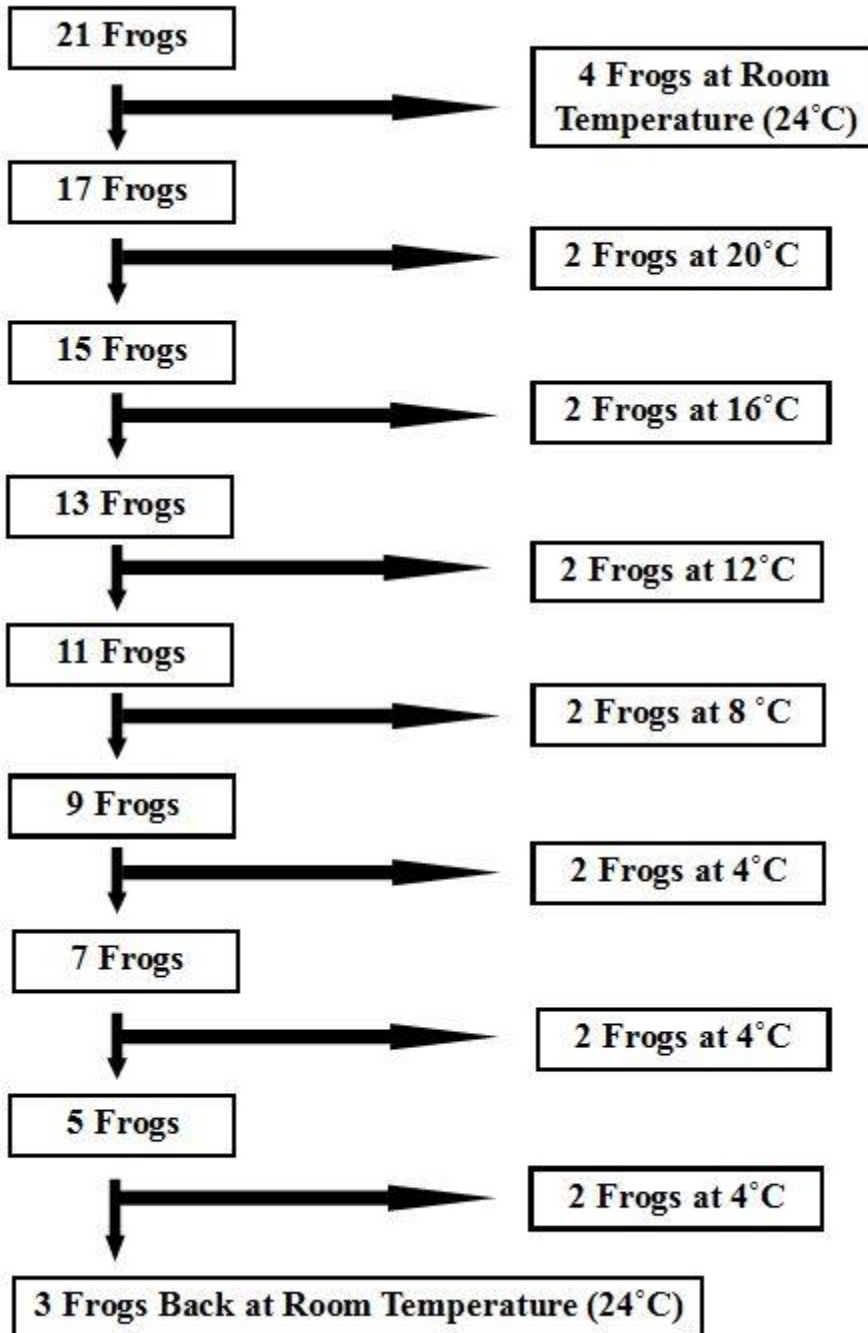
Figure 2 – Methodology of Plasma Extraction with Temperature Change

Results

Data from aPTT testing established a trend between a decrease in temperature within the hibernation environment and increased time of coagulation due to the slope of 3.1599 and R_2 value of 0.267 which displays an increasing and consistent trend through the duration of changes in temperature. (See Figure 3).
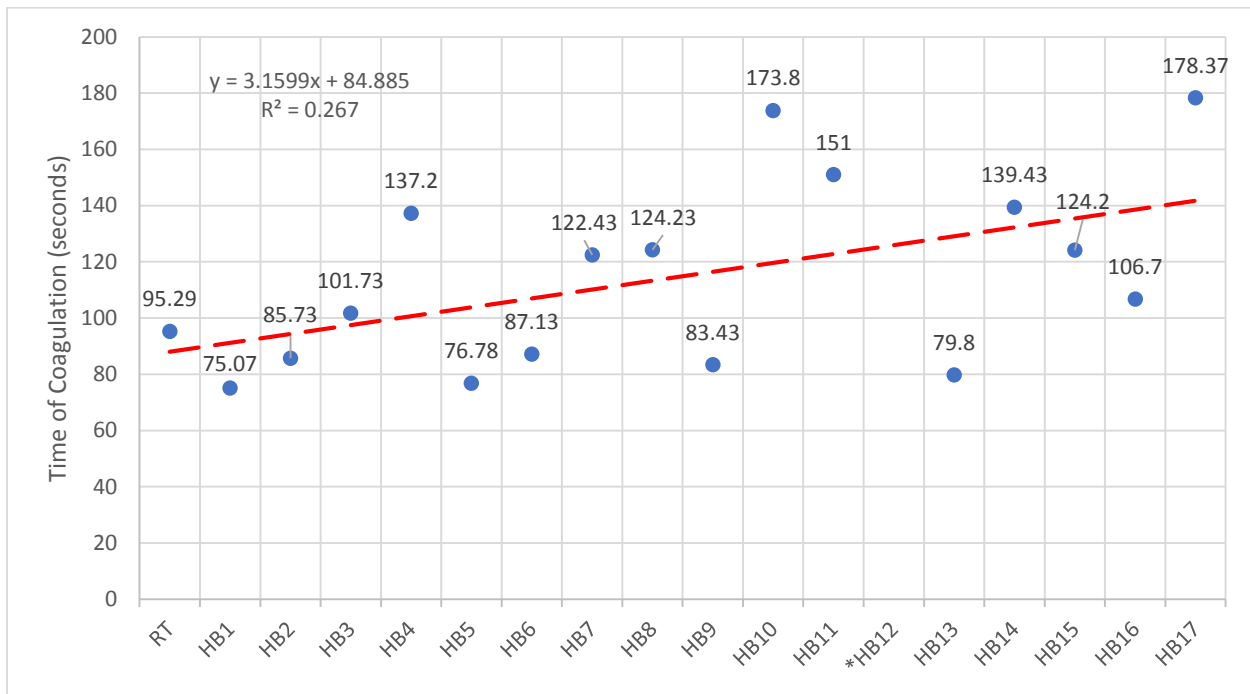

Figure 3 – Averaged Time in Seconds of Coagulation of *Rana catesbeiana* Within a Hibernation Environment

HB12 had a coagulation time over 300 seconds, and thus was not included in the graph as a data point

RT = room temperature, HB1 and HB2 = 20°C, HB3 and HB4 = 16°C, HB5 and HB6 = 12°C, HB7 and HB8 = 8°C, HB9 and HB10 = 4°C, HB11 and HB12 = 4°C (for 3 additional weeks), HB13 and HB14 = 4°C (for 6 additional weeks), HB15, HB16, and HB17 = 24°C.

By grouping and averaging the time of coagulation of frogs with the same environmental temperature in which they were extracted, the same trend between a decrease in temperature within the hibernation environment and increased time of coagulation (slope of 6.0312 and R_2 value of 0.4617) is seen in Figure 4.
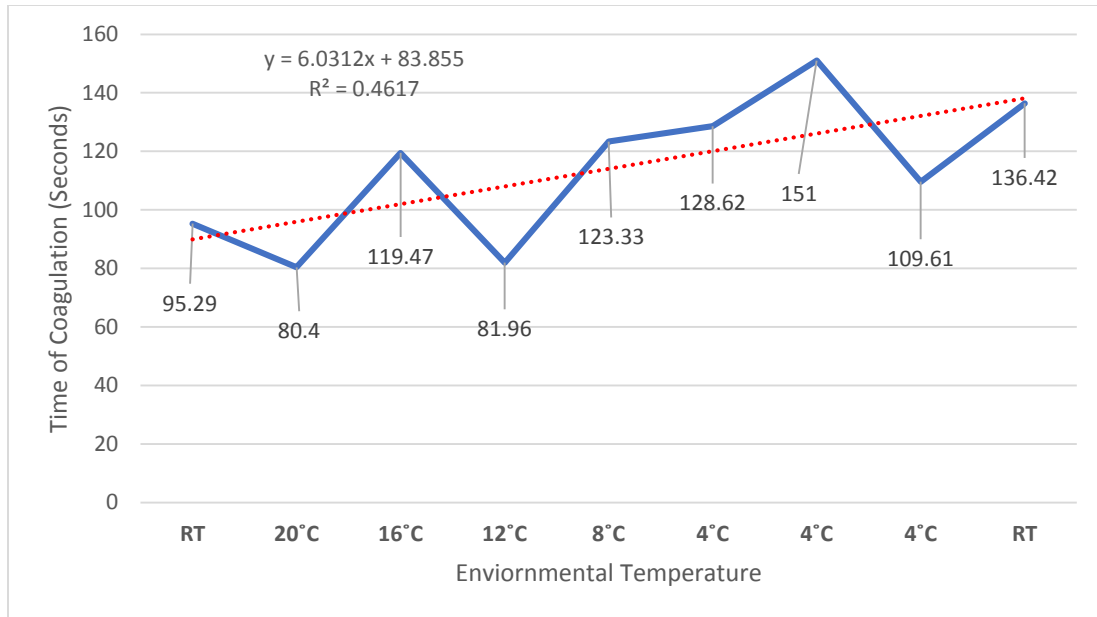

Figure 4 – Effects of Environmental Temperature on Time of Coagulation

Times of coagulation per 4 ˚C increments are seen in Figure 5: RT (95.29 ± 18.68), 20˚C (80.4 ± 8.73), 16 ˚C (119.47 ± 21.77), 12 ˚C (81.96 ± 8.19), 8 ˚C (123.33 ± 11.73), 4˚C (128.62 ± 46.71), 4˚C for 3 weeks (151.0 ± 22.13), 4˚C for 6 weeks (109.62 ± 36.54), and 24˚C (136.42 ± 34.63).

Figure 5 – Averaged Times of Coagulation by Hibernation Temperature
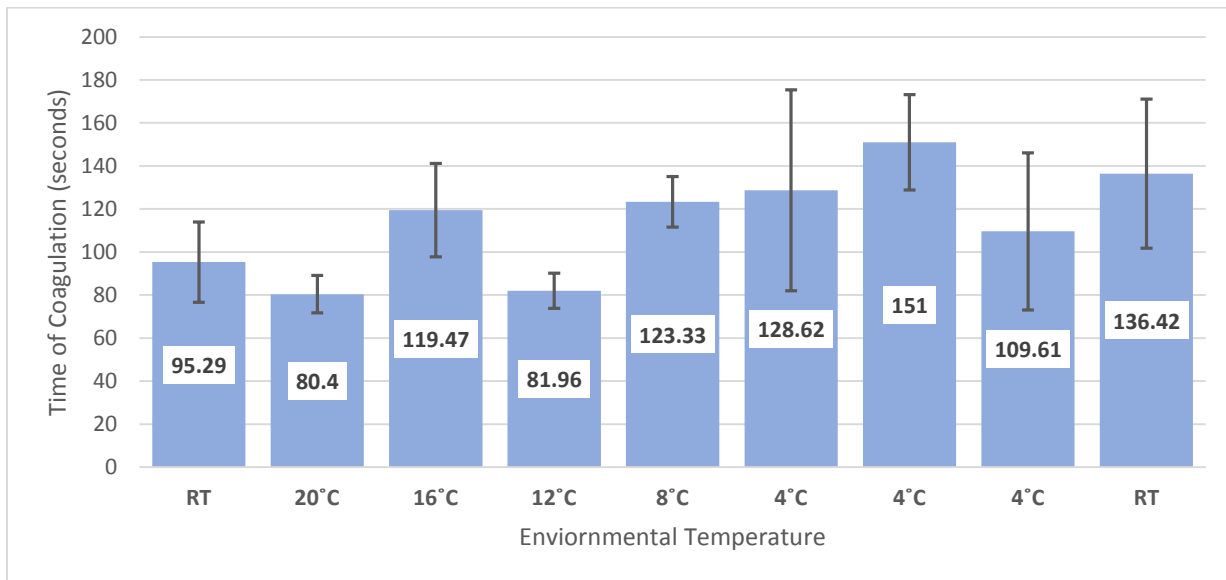
By calculating the percent change from room temperature (seen in Figure 6), it provided information that supported the trend seen in Figures 3-5, and showed that the changes between each 4˚C increments are not consistent with one another. Changes are also seen at the durational 4˚C (for the duration of 6 weeks).
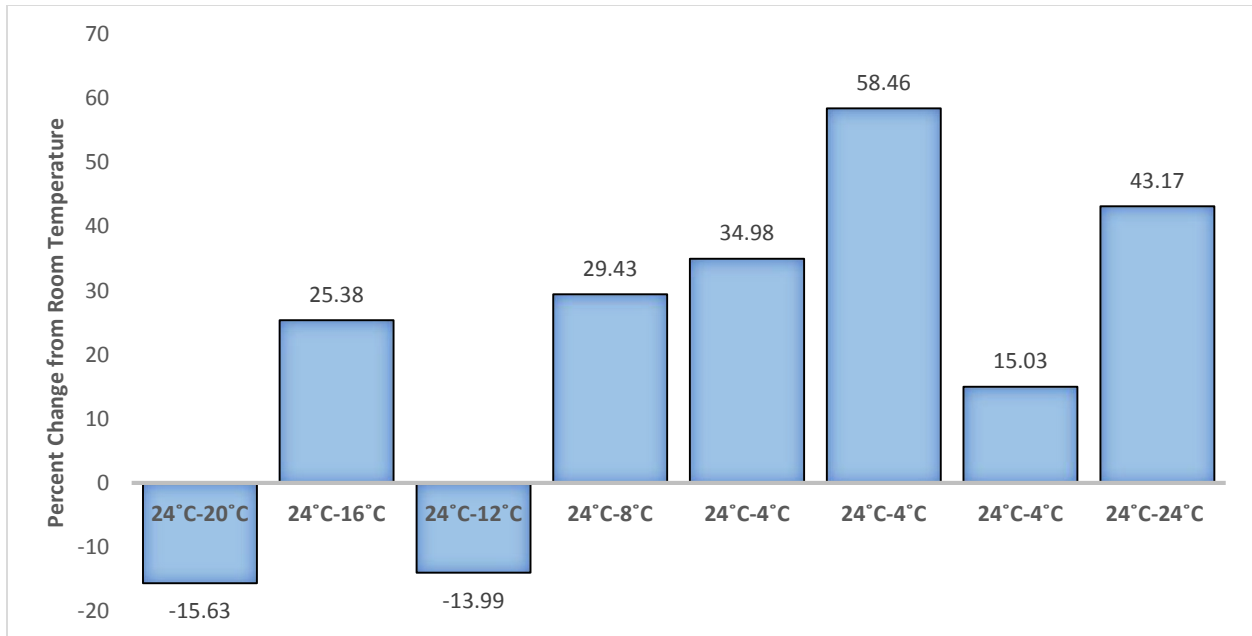
Figure 6 – Percent Change from Room Temperature

Figure 7 shows the calculated Q_{10} values of each decrease of 4˚C and the Q_{10} of 24˚C to 4 ˚C. This data shows that the same decrease of 4˚C between temperatures does not follow consistent increments and is not equal to the overall Q_{10} (0.794). This indicates that perhaps more than one factor is affecting the time of coagulation as the temperature is decreased over time.

Figure 7 – Q_{10} Values

Discussion

When looking at the cascade of blood physiology, a step-wise process is seen and can be used as an explanation when analyzing this data. 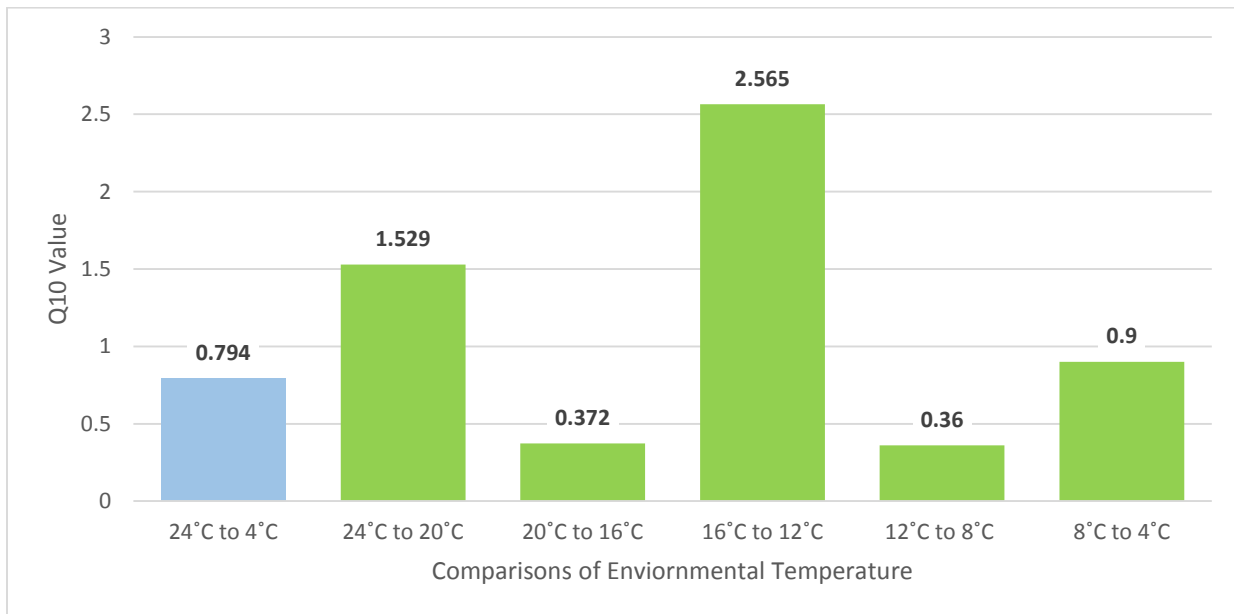As the temperature decreased 4°C, we see that the clotting times increased in a steady fashion. Since temperature affects the system as a whole and leads to a steady change of coagulation physiology, finding out exactly what causes this change is the next step for further research. Previous research and conclusions by Cullinan (2015), state that the time of coagulation of plasma with decreasing temperature have shown a decrease in factor XI and XII of the coagulation cascade within the same species, *Rana catesbeiana*, as the temperature decreases. Another conclusion made by Cullinan (2015) is that there is an inhibitor that is produced as the temperature decreases.

Using the data that was collected with this experiment, the conclusion of decreased factors within the blood and an increase of inhibitor as the temperature decreases is supported. The trend of an increase of coagulation times throughout the whole duration of decreasing temperature suggests that more than one variable is affecting the coagulation of the frogs. If one or multiple clotting factors were decreased and further depleted, a plateau would have been exhibited in this experiment's data. The effect of the decrease of clotting factors and the presence of an inhibitor independently cannot be explained from this data, but further investigations can be used to derive additional explanations.

The Q_{10} calculations that were made for each decrease of 4°C and the full range from 24 °C to 4°C are not consistent with one another. This shows that the constant incremental decrease of temperature and its impact on the enzymatic components of the coagulation cascade is not the only variable that is causing the change in time of coagulation. These values support the idea that

there is/are other variables changing the rate of coagulation as these temperature decreases take place.

Future Investigations

      This study can be used in many ways in future research in reference to the mechanism of changing the blood physiology. This mechanism could be due to the production of an inhibitor of the coagulation cascade that is developed as a result of the lowering in temperature, and/or the effects of exposure to the lowered temperature over time. In order to determine if there is a decrease in clotting factors produced, an experiment where factor-deficient plasma is mixed with hibernating frog plasma and then the clotting times can be recorded using this study's methodology and analyzed looking for patterns and differences between the normal plasma and factor-deficient plasma.

      Also, in reference to exposure to the lowered temperature, a very similar experiment can be done like this study, but to start with 36 frogs, leaving two frogs at each change in temperature change for the duration of the experiment. This would include a total of four frogs at each increment as used in this experiment (four frogs x nine increments = 36 frogs). Analyzing this data for the time of coagulation of the frogs as they remain at a lower constant temperature below room temperature, and comparing it to that of the frogs that endure to full range of hibernating temperatures (20˚C - 4˚C) within the experiment. The trends exhibited by this data would show if exposure duration to lowered temperature has an effect on the coagulation cascade of the frog.

      Another future investigation that could take place following this experiment would be to determine if the rate of clotting factors decrease at a constant rate with decreasing temperature.

By following the same procedure of pulling out the frogs at each 4°C drop of temperature, and then determining the amount of each clotting factor present, the procedure would allow for a further conclusion on the rate of decrease of clotting factors.

For this study and future studies, the use of human cephen with that of the frog plasma can be another variable to investigate. The use of frog cephen could be an approach to seeing if this change of material would change the result of this study and whether composition of cephen from different species is conserved. The process of coagulation also was incubated at 37 °C, which if the frogs are put into this temperature, would not be able to survive. A test where the process of coagulating the blood is done at different temperatures would also provide data on whether the temperature of the coagulation process would change the time of coagulation.

Conclusion

The goal of this study was to determine if there was a pattern between the time of coagulation and the decreased temperature of the hibernation environment, and results indicate that there is a correlation that does exist between these two factors. Even though the results of this experiment can only provide a timeline of coagulation and present a correlation between the decreased temperature and increased coagulation time, it lays the groundwork for future experiments.

This study faces some limitations including the use of human cephen in coagulation assays of the frog's blood. Also, using *Rana catesbeiana* only represents a small group within the larger ectotherm community, and thus these results cannot represent the whole community of ectotherms. Further research is needed to make more conclusions about these ectotherms, but this study provides a timeline of coagulation that was not previously present, and lays the groundwork for an

insight into the specific physiological timeline of the coagulation of ectotherms during cold temperature exposure. With little research done in this area, this study provides data for which many experiments can be done to further the fields of blood coagulation and more specifically, the impact of temperature and hibernation on this process.

Acknowledgements

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Appendix A

Table 1

Times and Calculations of Coagulation

Temperature	Identity	Time of Coagulation				Average	AVG per Temp.	STD
24˚C	RT1	105.7	113.6			105.7	92.783	18.675
	RT3	71.7	70.6			71.7		
	RT4	108.3	93.6	113.3		100.95		
20˚C	HB1	83.5	66.4	75.3		74.95	80.825	8.728
	HB2	81.3	92.1	83.8		86.7		
16˚C	HB3	88.2	97.9	111.9	108.9	99.333	116.533	21.768
	HB4	130.3	147.4	123.5	147.6	133.733		
12˚C	HB5	75.5	79.2	74.0	78.4	76.233	82.367	8.186
	HB6	78.2	88.4	98.9	83.0	88.50		
8˚C	HB7	105.2	126.2	125.1	133.2	118.833	123.450	11.728
	HB8	137.1	134.1	113.0	112.7	128.067		
4˚C	HB9	83.7	83.9	80.2	85.9	83.425	127.75	46.709
	HB10	172.9	174.7			173.80		
4˚C (3 Weeks)	HB11	133.9	176.0	143.1		151.0	151.0	22.134
	HB12	*Over 300s, thus excluded from calculations						
4˚C (6 Weeks)	HB13	99.5	72.1	67.8		79.80	109.617	36.542
	HB14	125.2	161.5	131.6		139.43		
24˚C	HB15	137.5	121.4	113.7		124.20	136.422	34.630
	HB16	102.6	108.0	109.5		106.70		
	HB17	158.9	175.3	200.9		178.37		