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# Anti-proliferative effect of hibernating American bullfrog, *Lithobates catesbeianus*, blood plasma on the THP-1 cells

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ANTI-PROLIFERATIVE EFFECT OF HIBERNATING AMERICAN BULLFROG,  
*LITHOBATES CATESBEIANUS*, BLOOD PLASMA ON THP-1 CELLS

An Abstract of a Thesis  
Submitted  
in Partial Fulfillment  
of the Requirements of the Degree  
Master of Science

Sean L Robbins  
University of Northern Iowa  
July 2017

## ABSTRACT

Hibernation is a remarkable process that allows an organism to go dormant for long periods of time during the winter, when food is scarce and living conditions can be lethal. Much is unknown about the physiology behind hibernation, and most research comes from mammalian models. For example, it is known that during hibernation many of the cells in the body are impeded from going through mitosis but the mechanism that caused this was unknown. It was recently discovered that 13-lined ground squirrels (*Ictidomys tridecemlineatus*) and woodchucks (*Marmota monax*) have a protein, alpha-2-macroglobulin, in their blood plasma, which has significant anti-proliferative effects on spleen cells (Sieckman et al., 2014).

The goal of my study was to determine if an ectothermic organism, the American bullfrog, *Lithobates catesbeianus*, also actively suppresses mitosis while hibernating and if so, determine if it is caused by a component in their blood plasma. Cellular assays using Human THP-1 cells were conducted by incubating cells in different treatments of plasma collected from active and hibernating bullfrogs. After incubation, the cells were either counted to determine if the rate of cell growth had been inhibited or stained with a dye that attaches to DNA and run through a flow cytometer. Results of the cell count assays were inconclusive regarding how the rate of growth had been affected by hibernating plasma treatment. Flow cytometry assays found that the majority of cells in the hibernating plasma treatment were in

the M phase of the cell cycle, instead of the  $G_0$  phase. This suggests that the majority of cells had replicated their DNA, but mitosis was halted before the cell divided. This may be because there is substantial cell death throughout hibernation and by stopping mitosis just after DNA replication, the cells are better prepared for proliferation when the animal is exiting hibernation.

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*LITHOBATES CATESBEIANUS*, BLOOD PLASMA ON THP-1 CELLS

A Thesis submitted  
in Partial Fulfillment  
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July 2017

This Study By: Sean L Robbins

Entitled: Anti-proliferative Effects of Hibernating American Bullfrog, *Lithobates catesbeianus*, Blood Plasma on THP-1 Cells

has been approved as meeting the thesis requirement for the

Degree of Master of Science

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Dr. Kavita Dhanwada, Dean, Graduate College

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To all, thank you for believing in me.



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## CHAPTER 1

### INTRODUCTION AND LITERATURE REVIEW

#### Hibernation

Many groups of organisms can enter into a state of dormancy, or torpor, whether to live throughout a period of time when food is sparse or conditions are unfavorable. In this dormancy the organism goes through a significant metabolic suppression. The heart and respiration rate dramatically decreases. The digestive tract nearly shuts down and the core body temperature drops down to ambient temperatures. Some organisms such as reptiles, amphibians, mollusks, fish, arthropod and mammals, which live in very hot and dry climates, can go into a state of torpor known as aestivation. In general these organisms go into aestivation during the period of the year when precipitation is very low and therefore there is a risk of desiccation (Boutilier et al., 1997). Aestivation is also mostly characterized as occurring in shorter cycles and the organism is easily able to come out of aestivation fairly quickly. The African lungfish, for example, will burrow under the mud of pools of water before that pool dries up and go into aestivation until adequate precipitation occurs.

In contrast, hibernation is when an organism enters into a state of long term torpor in order to essentially skip over those months in which there is little food present. As a result the organism has to contend with different environmental pressures, such as preventing ice from forming in their bodies and having sufficient

energy reserves to service throughout the winter. Organisms such as amphibians, fish, arthropods, a few birds, and mammals have adapted to use hibernation.

### Mammalian Hibernation

Most of what we know about the physiology of hibernation is drawn from experiments using mammalian models, most commonly the thirteen-lined ground squirrel (*Ictidomys tridecemlineatus*). Hibernation in mammals is thought to be triggered by a change in ambient temperature and photoperiod length. This causes a behavioral response to over feed so that they gain fat, which will then be burned for energy throughout hibernation. They may also start to collect and store excess food in their burrows. Mammals are endothermic and homoeothermic organisms. As such they produce and maintain their own body heat. However, prior to hibernation their bodies go through extensive physiological changes so that they essentially become ectothermic and their core body temperatures will then mimic the ambient temperature of their burrows. Their heart rate will drastically slow down to the point that their blood will become viscous. The average heart rate of a thirteen-lined ground squirrel during hibernation is three to five beats per minute (Cooper et al, 2012). While in hibernation blood platelet counts in these animals decrease by 90% and their clotting factors decline by 70% to prevent the blood from clotting (Cooper et al, 2012). Metabolic rate is also reduced to below 5% of the squirrel's normal metabolic rate in order to preserve as much energy as possible (Wu & Storey, 2012). Mammals also do not constantly stay in a state of deep torpor throughout

hibernation. They will periodically become aroused out of torpor, raising their core body temperature, then lower their body temperature again, entering deep torpor, multiple times throughout hibernations (Carey et al., 2003). Why mammals periodically exit torpor during hibernation is poorly understood but it is speculated that doing so is important in order to restore metabolic homeostasis in the spring (Ratigan and McKay, 2016).

### Amphibian Hibernation

Amphibian hibernation differs from mammalian hibernation in a few ways. Amphibians, unlike mammals, are ectothermic and poikilothermic. This means that they do not need a specific mechanism to drop their core body temperature because their core body temperature is already the same as the surrounding temperature. Also, unlike mammals, amphibians rely on breathing subcutaneously and therefore need moisture in order for that to occur.

In frogs there are two general methods of hibernation: terrestrial hibernation and, the more commonly used, aquatic hibernation. For aquatic hibernation, individual frogs seek out a pool of water, and once there, the frog will swim to the bottom of the water and hibernate on top of the mud where it can breathe subcutaneously (Stewart et al. 2004). At the onset of hibernation, several biochemical and physiological changes take place. The bone marrow no longer generates new blood cells and instead becomes a site for fat storage, acting as an energy reserve during hibernation (Cooper et al. 1992). The antioxidant defense

system of the frog shuts down, as there is a lack of antioxidant enzymes, such as superoxide dismutase and catalase, present in the heart and muscle tissue during hibernation (Feidantsis et al., 2013). The immune system of the frog also almost completely shuts down (Cooper et al. 1992). The heart rate significantly decreases and respiration is slowed down as the frog relies solely on subcutaneous respiration. On the other hand, brain activity is fully active during hibernation. It has been found that there is an increase in the proliferation of brain cells during hibernation. This is to combat massive cell death due to cold temperatures and to keep the brain active so that baseline functions, needed to maintain life, can still be carried out (Cerri et al., 2009).

#### Anti-proliferation During Hibernation

It is known that during hibernation, most cellular activity is suppressed throughout the body. It costs energy and resources in order for cells to go through mitosis. Other studies have found that the synthesis of DNA, RNA, and proteins in hibernating tissues is significantly decreased during hibernation (Kolaeva et al. 1980). However, the mechanism, which prevents the cells from going through mitosis, is poorly understood. There could be multiple factors at play causing anti-proliferation, one of which is that enzymatic activity is greatly reduced with declining temperature. Biological processes have an approximate  $Q_{10}$ , or temperature sensitivity factor, of 2-3, such that the rate of an enzymatic reaction is changed by a factor of 2-3 for every 10-degree change in temperature (Boyer and

Barnes, 1999). Therefore, it could be that the temperature is too low for enzymatic reactions to take place.

Another possibility is that during hibernation there may be a down regulation of proteins called growth factors. In order for cells to start mitosis, there must be a certain concentration of growth factors present around the cell, which will create an extracellular signal to start the process of mitoses in the cell. Once this signal starts, it activates cyclin-CDK complexes, which regulate the progression of cell proliferation. One study measured the level of concentration of these proteins in the liver cells of hibernating thirteen-lined ground squirrel, *Ictidomys tridecemlineatus*, in order to analyze the stasis of the cell cycle during hibernation. They found that during hibernation the cells have a reduction of cyclin D and E, which are the cyclins responsible for getting the cells out of G<sub>0</sub> or interphase (Wu & Storey, 2012). This suggests that there is a degree of under regulation of growth factors at play during hibernation.

Another mechanism for the suppression of mitosis during hibernation could be that a protein is produced which causes an inhibitory response to cell proliferation. It has been shown that the rate of proliferation of tumor cells that have been transplanted into hibernating hamsters was dramatically decreased (Lyman and Fawcett, 1954). Also blood plasma taken from hibernating ground squirrels has been shown to induce hibernation when transferred to summer active ground squirrels (Dawe and Spurrier, 1969). These studies indicated that there was



something in the plasma causing a suppression of mitosis. The substance was identified much later as being a protein (Siechmann et al., 2014). Cultured mouse spleen cells, incubated in different concentrations of plasma from hibernating thirteen-lined ground squirrels found that the higher the concentration of plasma incubated with the cells the lower the uptake of the radioactive thymidine in the cells, indicating that the rate of mouse spleen cells division was significantly slowed. A western blot test analysis found that it was a protein responsible in the plasma and that this protein was in the alpha-2-macroglobulin family (Sieckmann et al., 2014). This gives evidence to the presence of an inhibitory protein that is causing mitotic suppression.

For ectothermic organisms there are no known studies that have been performed to determine the mechanism causing anti-proliferation in their cells during hibernation. The goal of this study is to determine if an ectothermic vertebrate, such as the American bullfrog, *Lithobates catesbeianus*, also actively suppresses mitosis while hibernating, and if so, determine if it is caused by a component in their blood plasma. This was done by conducting cellular assays in which a human cell line, THP-1 cells, were incubated in the blood plasma collected from bullfrogs that were at different durations of hibernation and bullfrogs that remained active, at room temperature, when plasma was extracted. A cell count was performed at the end of the incubation period to measure the rate of proliferation within the cell population. Assays were also conducted to determine how the

hibernating plasma affected the cell cycle in comparison to plasma collected from active bullfrogs. This was done by staining the cells with a fluorescent dye that attaches to DNA and using a flow cytometry to determine the percentage of cells in the various mitotic stages.

## CHAPTER 2

### MATERIALS AND METHODS

#### Bullfrogs

All bullfrogs used throughout this study were treated as humanely as possible, following protocol designed to minimize pain or discomfort to the animals. Twenty-one of American bullfrogs (*Lithobates catesbeianus*) were purchased from Kons Direct (Germantown, WI 53022). The Bullfrogs were delivered on November 12<sup>th</sup> 2015. Once the frogs were delivered they were separated and placed into four, twenty-gallon transparent plastic storage containers with ventilated lids and placed in a temperature controlled room held at 28°C. Each container was filled with approximately four centimeters of tap water. The water used to fill the containers once the temperature was lowered in the cold room was stored in the same cold room as the hibernating bullfrogs to ensure that the water was at the same temperature as that of the surrounding environment, thus reducing potential stress. The water in each of the containers was changed once per week.

Four of the bullfrogs were randomly selected and placed in a container that was kept separate and at room temperature in the laboratory. These bullfrogs were classified as the active non-hibernating bullfrogs. The remaining 17 bullfrogs were randomly assigned and placed into one of three containers, with five individuals in one container and six individuals in each of the other two containers. These

containers were then kept in a temperature controlled cold room. The individuals kept in this manner were classified as the hibernating bullfrogs.

Starting on November 19<sup>th</sup>, 2015 the temperature of the cold room was lowered to 24° C. The temperature was then lowered by 4°C every seven days until the temperature of the cold room reached 4°C on December 17<sup>th</sup> 2015. The temperature was maintained at this temperature until March 31<sup>st</sup>, 2016, when it was raised to 9°C. The temperature was then raised to 15°C on April 7<sup>th</sup>, 2016. No bullfrogs were fed during hibernation.

#### Drawing Blood Plasma

The experiments reported herein were conducted according to the protocol set for in the “AVMA Guidelines for the Euthanasia of Animals: 2013 Edition,” American Veterinary Medical Association, 2013. The four bullfrogs kept at room temperature in the lab, here after referred to as active bullfrogs, were kept in the laboratory for three weeks. Blood was drawn from two individuals on December 1<sup>st</sup>, 2015. The individuals were anesthetized using a solution of tricaine mesylate, also known as MS-222, purchased from Syndel USA (Ferndale, WA 98248) with 2.5 grams of MS-222 per liter of water. MS-222 is acidic with an approximate pH of 5, thus sodium bicarbonate was added to the solution to raise the pH to approximately 7-7.5. The bullfrogs were submerged in the solution until they were immobile, which occurred approximately 8 to 12 minutes after submersion in active bullfrogs. Once anesthetized, the individual was placed on their dorsal side and blood was

drawn through a cardiac puncture. To expose the heart, a vertical incision was made on the ventral side, just to the right of the sternum. A sterile syringe, flushed with 0.105M (3.5%) sodium citrate was used to draw blood from the ventricle. The sodium citrate was used to prevent coagulation of blood entering the syringe. The volume of the drawn blood was initially placed into tubes containing 0.105 M sodium citrate. Blood from these tubes was separated into centrifuge tubes, with approximately 1.5 ml of blood per centrifuge tube. The blood in each of the centrifuge tubes was centrifuged for five minutes at 2500g to separate the plasma from the blood cells. The plasma was then pipetted into a separate centrifuge tubes, with approximately 1ml of plasma per tube. Each plasma sample was then stored at -80°C. This same procedure was used for each individual bullfrog in this study. The blood from the other active bullfrogs was drawn within nine days of that for the first active bullfrog.

Blood was drawn from two hibernating individuals on the same day. The first blood to be drawn from hibernating bullfrogs was drawn on December 8<sup>th</sup>, 2015, five days after the temperature of the cold room was lowered to 12°C. The same procedure that was used on the active Bullfrog specimens was repeated on all of the hibernating bullfrogs. Blood was drawn from hibernating frogs on the proceeding dates: on December 18<sup>th</sup>, after the temperature of the cold room was lowered to 8°C, January 21<sup>st</sup>, 2016, at 4°C, February 22<sup>nd</sup> at 4°C, and March 23<sup>rd</sup> at 4°C. The temperature was gradually increased to 15°C. Once this temperature was reached,

blood was drawn from two individuals on April 14<sup>th</sup>, two individuals on May 12<sup>th</sup>, and three individuals on June 3<sup>rd</sup>.

### Cell Colony

THP-1 cells were used to conduct cellular assays throughout the entirety of my study. THP-1 cells are monocytic cells that were acquired from a one-year-old patient with acute monocytic leukemia. Cells are spherical in shape and non-adhesive. The cells were kept in a cell media which was comprised of the Roswell Park Memorial Institute (RPMI) medium, 10% Fetal Bovine Serum (FBS), and .35  $\mu$ l 2-Mercaptoethanol. Cells were kept in an incubator set at 37°C and 5% CO<sub>2</sub>. The colony was kept at 10 ml in a cell culture flask. Once per week, half of the cell suspension was removed and 5ml of fresh media was added back into the flask.

### Cell Count Assays

Cell count assays were conducted by incubating the THP-1 cells in different treatments of bullfrog plasma in order to determine if there was a difference in rate of cell proliferation between cells incubated in the active bullfrog's plasma and the hibernating bullfrog's plasma. The cells were washed free of any storage media before use in the cell assays by drawing up 1 ml of cell suspension from the cell colony and placing it in a sterile 5 ml Falcon tube. This tube was then centrifuged at 10,000 xg for five minutes. The liquid in the Falcon tube was poured off carefully as to not to dislodge the clump of cells at the bottom from the tube. 1ml of phosphate-buffered saline (PBS) was then added to the tube, followed by mixing with a

vortexer for ten seconds. The Falcon tube was again centrifuged at 10,000 xg for five minutes and the top liquid was poured out. Another 1 ml of PBS was added to the tube and the contents were agitated again using a vortexer.

To conduct the cell count assays, the initial cell count had to be determined for each separate assay. 10 $\mu$ l of the washed cell suspension was placed into each of the two chambers of a hemocytometer. The hemocytometer was then placed under a compound light microscope. Using the low power objective (4x), cells in each of the nine largest squares were counted in each of the two chambers using an electronic counter. The number of cells obtain from the count was divided by 18 and then multiplied by 10,000 to determine the approximate number of cells total in a 1ml cell suspension. The estimated number of cells had to be within the range of 300,000-500,000 in order to be used in the cellular assay.

Sterile 96 well plates with lids were used for all cellular assays. Before adding the cell suspension, wells were filled with 100  $\mu$ l of treatment solution. Treatment solutions varied from assay to assay but always consisted of a control group, an active bullfrog plasma group, and several hibernating bullfrog plasma groups. The control group consisted of the same FBS media used to grow the cell colony. The active bullfrog plasma group consisted of blood plasma that was drawn from a bullfrog that was kept at room temperature in the lab. The hibernating bullfrog plasma groups consisted of blood plasma that was drawn from the individuals that were in hibernation while being kept in the cold room.

Once the initial cell count was determined and the plasmas were added to the wells, 10  $\mu$ l of the cell suspension was pipetted into each of the respective wells. The well plate was then placed in an incubator set at 37°C and at 5% CO<sub>2</sub> for 72 hours. At the end of the incubation period the plate was taken out and a sample from each well was pipetted into a hemocytometer to be counted. Every cell within the nine square grid was counted in both of the chambers. The number of cells in 1ml was determined by dividing the total count by 18 and multiplying by 10,000 and then by 10 (for the factor of dilution). This number represents the approximate number of cells that were in 1 ml of plasma in that well at the end of the 72 hour incubation.

#### Flow Cytometry Assays

To determine if the cell cycle was being effected differently between THP-1 cells incubated in active bullfrog plasma and THP-1 cells incubated in hibernating bullfrog plasma, the cells were incubated with a fluorescent dye that attaches to DNA. An Applied Biosystems Attune Acoustic Flow Cytometer (Life Technologies) was used to measure the proportionality of the cells that were in the different stages of the cell cycle. The flow cytometer measured the intensity of the dye bound to the cell's DNA, which then indicates if the DNA has been duplicated. For these assays the same procedure previously stated for washing the cells was used with the exception that 1.5 ml of cell suspension from the colony was used instead of 1 ml of suspension. The same general treatment groups were also used for these assays as well. Sterile 96 well plates with lids were used for these assays. 100  $\mu$ l of either FBS



treatment, plasma from active frogs, or plasma from hibernating frogs were added to each of the wells being used in the experiment. A sample was drawn from the cell suspension after the washing process to determine number of cells in 1 ml using a hemocytometer. For the flow cytometer assays, the initial cell count had to be at, or over, one million cells in order for it to be used in the assay. Once the initial cell count was determined, 10 $\mu$ l of the washed cells were pipetted into each of the treatment well. The well plate would then be incubated for 72 hours in an incubator at 37°C and 5% CO<sub>2</sub>.

Following incubation, the contents of two of the wells, within the same treatment group, were combined into one loose-capped test tube and 850 $\mu$ l of PBS was added to bring the total volume of liquid to 1 ml. 2  $\mu$ l of Vybrant DyeCycle Green stain (Fisher Scientific) was then added to each of the test tubes and each test tube was mixed well using a vortexer. The test tubes were placed in the incubator for 30 minutes to allow the dye to stain the DNA.

The blue light laser of the flow cytometer was used to measure the amount of dye in each sample. A 1 ml sample of the original cell colony was also stained with the Vibrant DyeCycle Green stain and incubated with the treatment test tubes. This sample was the first to be drawn through the flow cytometer in order to adjust the appropriate parameters in the software so that it would measure cells that were within the same size range as the THP-1 cells. This was done by adjusting the forward scatter (FSC), side scatter (SSC), and blue light frequency (BL1) until the

cell population could be seen on a dot diagram. For all of the separate flow cytometer assays, the machine was set to analyze a maximum of 10,000 events, objects that fit in the pre-established parameter of size, or 750  $\mu$ l of sample liquid. After calibration was completed, each sample was agitated with a vortexer and then run through the flow cytometer. A histogram comparing the event count and BL1 was set up for each sample and three gates were established on histograms. The first gate marked the estimate frequency range, which would represent the cells in the  $G^0$  or  $G^1$  phase. The second gate was to frame the cells in S phase and the last gate framed the cells in  $G^2$  or M phase.

#### Statistical Analysis

Data collected was analyzed using the statistical software R Studio. Data collected from the cell count assays was normalized using log transformation and an ANOVA test was used to compare the differences between the active bullfrog plasma group and the hibernating bullfrog plasma group. Data from the flow cytometer assays was normalized using the arcsine-square root transformation on the proportion of cells in the different gates. The data was then grouped together based on the temperature of the bullfrog when the plasma was collected. The groups were as followed: FBS, room temperature (RT), hibernation, and post-hibernation. The RT group consisted of data collected from cells incubated in active bullfrog plasma. The hibernation group consisted of data collected from bullfrogs that were at 4°C when plasma was collected and were at various durations of time in hibernation. The

post-hibernation group consisted of data from bullfrogs that were at 15°C when plasma was collected. An ANOVA test was run on the transformed data to determine if any significant differences existed between the active and hibernating bullfrog plasma treatment groups in each of the gates set up on the histograms.

## CHAPTER 3

### RESULTS

For this study, I conducted experiments to try to discover evidence that an ectothermic organism does regulate cellular division during hibernation and that this regulation was not directly dependent on temperature. To accomplish this goal, cellular assays were carried out using a human cell line (THP-1 cells) that was incubated with different treatments of bullfrog plasma. The data collected from cell count assays had too much variance within all experimental groups, including the positive control, to ascertain any statistical significance or trend.

Results from the flow cytometer assays showed a trend that the majority of cells grown in hibernating bullfrog plasma treatments were in the  $G_2/M$  phase of the cycle. Inversely, the cells grown in the plasma extracted from the active bullfrogs show a normal distribution on the histogram, and more of the cells were in the  $G_0/G_1$  phase. Figure 1 is an example of a histograms produced from the flow cytometry data collected from active bullfrog plasma. Table 1 represents the statistical readings gained from that histogram in the form of the proportionality of the cells residing under each peak. The first peak on the left-hand side, which is in the gate labeled R2, represents the proportion of cells in the  $G_0/G_1$  phase and the taller peak on the right-hand side, which is in the gate labeled R4, represent the cells in  $G_2/M$  phase. In this example 41.89% of the total cells fall within the R2 gate and are in the  $G_0/G_1$  phase, while 28.15% of the total cells are in the R4 gate and thus in

the G<sub>2</sub>/M phase. Figure 2, shows a histogram produced from the data collected from cells incubated in hibernating blood plasma. Table 2 shows the proportionality of cells within each of the gates in figure 2, indicating that 51.16% of the total cells fall within gate G4 and thus are in the G<sub>2</sub>/M phase and only 11.55% of the cells are in the G<sub>0</sub>/G<sub>1</sub> phase. This pattern is consistent for flow cytometry data collected from cells incubated in hibernating bullfrog plasma.

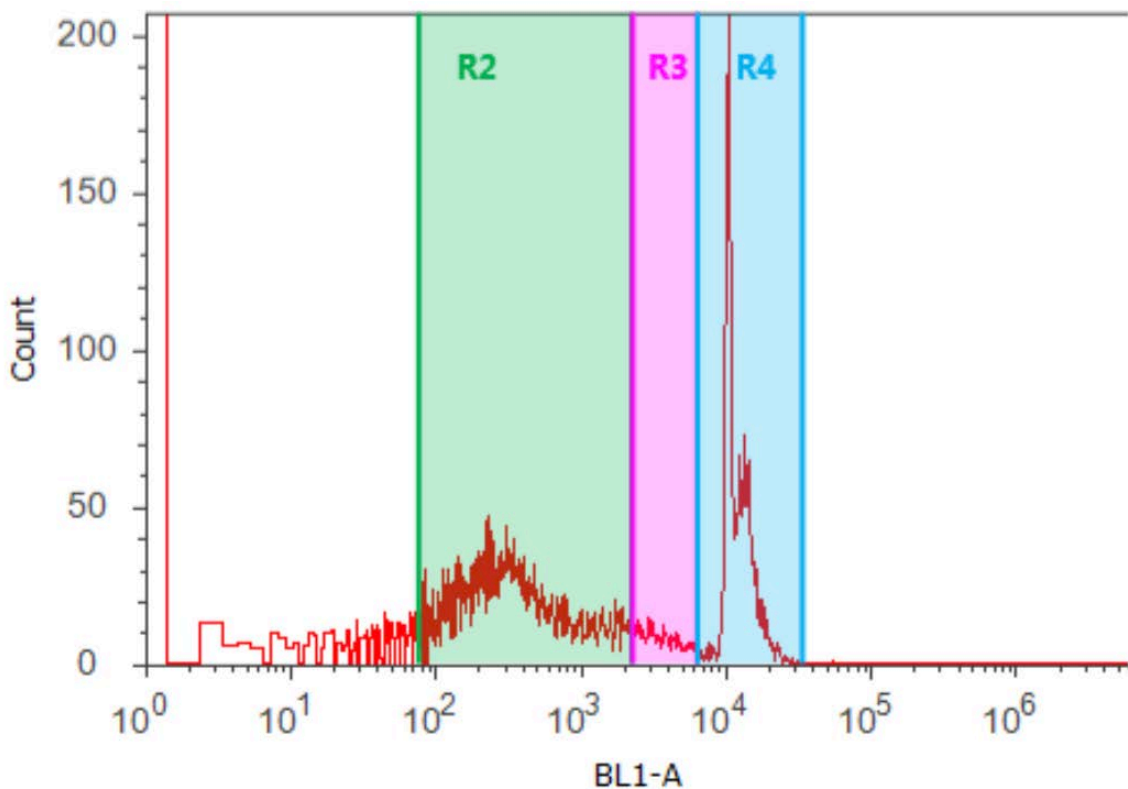


Figure 1. A histogram showing the results from the flow cytometry analysis carried out on THP-1 cells that were incubated in plasma extracted from an active bullfrog. The R2 region represents the cells in the G<sub>0</sub>/G<sub>1</sub> phase, The R3 gate represents the cells in the S phase, and the R4 gate represents the cells in the G<sub>2</sub>/M phase.

Table 1. The number of cells within each gate in the histogram of figure 1 and their proportion to the total number of cells. The phase of the cell cycle that each gate represents is noted in the parenthesis.

Name	Event Count	% Total
<b>All Events</b>	10,000	100%
<b>Gate R1</b>	8,717	87.17%
<b>Gate R2 (G<sub>0</sub>/G<sub>1</sub>)</b>	4,189	41.89%
<b>Gate R3 (S)</b>	540	5.4%
<b>Gate R4 (G<sub>2</sub>/M)</b>	2,815	28.15%

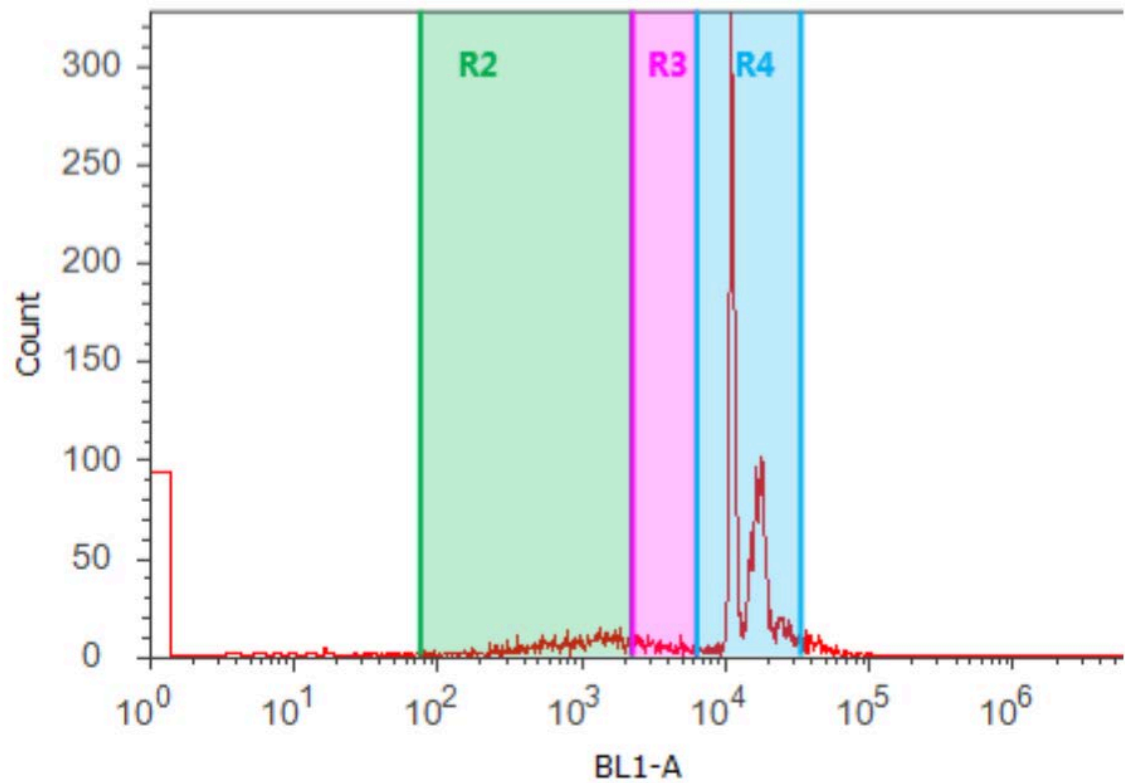


Figure 2. A histogram showing the results from the flow cytometry analysis carried out on THP-1 cells that were incubated in plasma extracted from a hibernating bullfrog. The R2 region represents the cells in the  $G_0/G_1$  phase, The R3 gate represents the cells in the S phase, and the R4 gate represents the cells in the  $G_2/M$  phase.

Table 2. The number of cells within each gate in the histogram of figure 2 and their proportion to the total number of cells. The phase of the cell cycle that each gate represents is noted in the proceeding parentheses.

Name	Event Count	% Total
<b>All Events</b>	8,145	100%
<b>Gate R1</b>	5,818	71.43%
<b>Gate R2 (G<sub>0</sub>/G<sub>1</sub>)</b>	941	11.553%
<b>Gate R3 (S)</b>	340	5.844%
<b>Gate R4 (G<sub>2</sub>/M)</b>	4,167	51.160%

The average percentage of cells in each gate for each individual bullfrog in the study was calculated and plotted over time (figure 3 and 4), which illustrated the trend that the longer the duration of hibernation, the greater the proportion of the cells in the G<sub>2</sub>/M phase. Figure 3 illustrates the data derived for each individual from all of the flow cytometer readings within the R2 gate, which represented the cells in G<sub>0</sub>/G<sub>1</sub> phase. Figure 4 illustrates all of the data for each individual derived from the flow cytometer readings within gate R4, representing the cells in the G<sub>2</sub>/M phase.



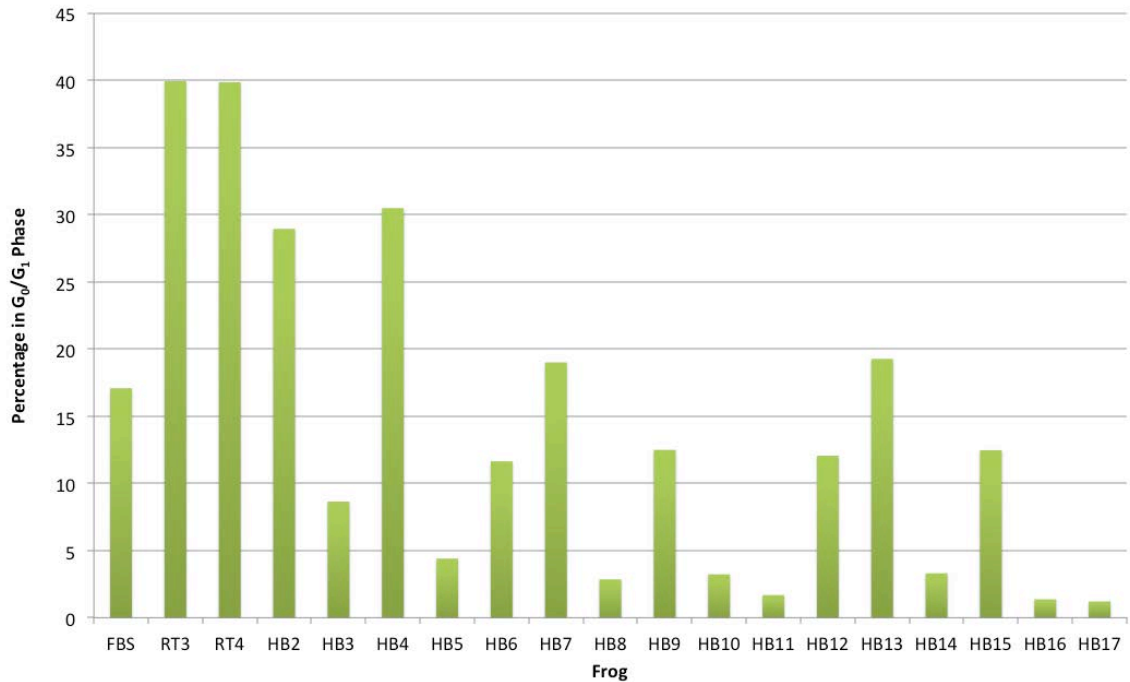


Figure 3. Bar graph showing the mean percentage of cells found in gate R2 per individual bullfrog. This indicates the mean percentage of cells that were in the G<sub>0</sub>/G<sub>1</sub> phase of the cell cycle for each individual bullfrog. FBS stands for the fetal bovine serum group, which served as a positive control group. RT stands for the room temperature frogs that remained active. HB2 was a hibernating bullfrog whose plasma was collected after the temperature of the cold room reached 12°C. Individuals HB3-HB10 were hibernating bullfrogs that were at 4°C when plasma was collected from them. Individuals HB11-HB17 were hibernating bullfrogs that were at 15°C when plasma was collected and was considered the post-hibernating group.

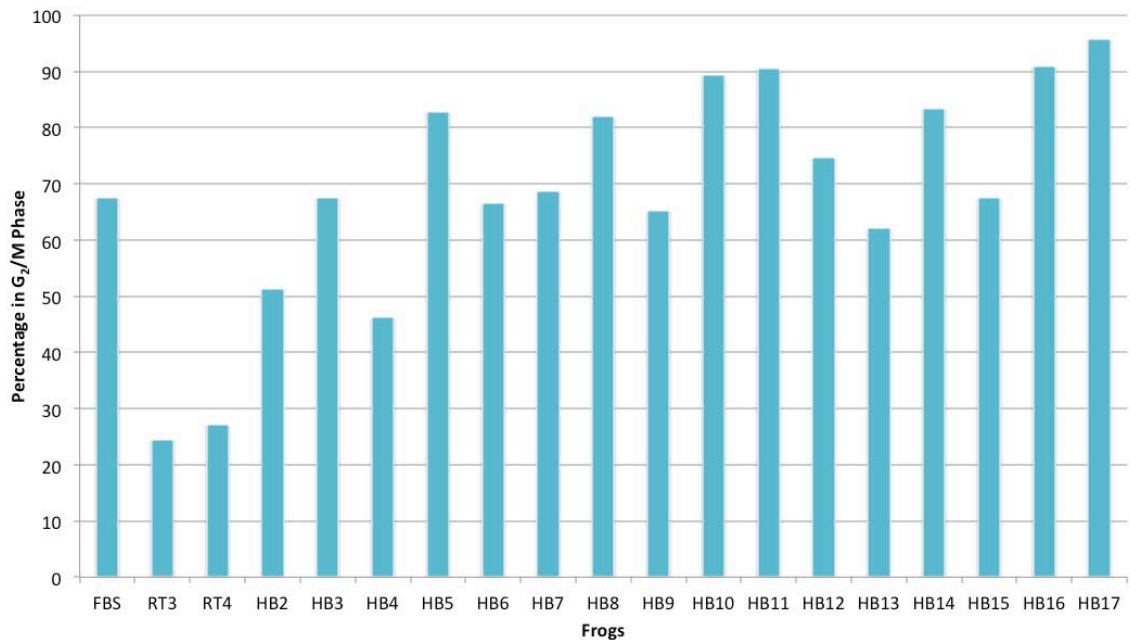


Figure 4. Bar graph showing the mean percentage of cells found in gate R4 per individual bullfrog. This indicates the mean percentage of cells that were in the G<sub>2</sub>/M phase of the cell cycle for each individual bullfrog. FBS stands for the fetal bovine serum group, which served as a positive control group. RT stands for the room temperature frogs that remained active. HB2 was a hibernating bullfrog whose plasma was collected after the temperature of the cold room reached 12°C. Individuals HB3-HB10 were hibernating bullfrogs that were at 4°C when plasma was collected from them. Individuals HB11-HB17 were hibernating bullfrogs that were at 15°C when plasma was collected and was considered the post-hibernating group.

An ANOVA was conducted on the data collected from the flow cytometry assays. Bullfrogs were separated into groups based on the temperature exposure of bullfrog when plasma was extracted. The groups consisted of the FBS control group, the room temperature or RT group, the hibernation group, and post-hibernation group. Figure 5, 6, and 7 shows the means for each of the groups within their respective gates on the flow cytometer readings. The ANOVA test showed data within each of the three gates were significant with a p value for the R2 gate being  $2.038 \times 10^{-12}$ , the R3 gate being 0.0004069, and the R4 gate being  $2.2 \times 10^{-16}$ .

A Tukey HSD test was used to determine the level of significance between each of the groups, the results of which are illustrated in Figures 5, 6, and 7. In the R2 gate data, cells within the hibernating group and the post-hibernating group are significantly different from the room temperature group. Likewise, Tukey tests showed that both of the hibernation groups are significantly different from the room temperature group.

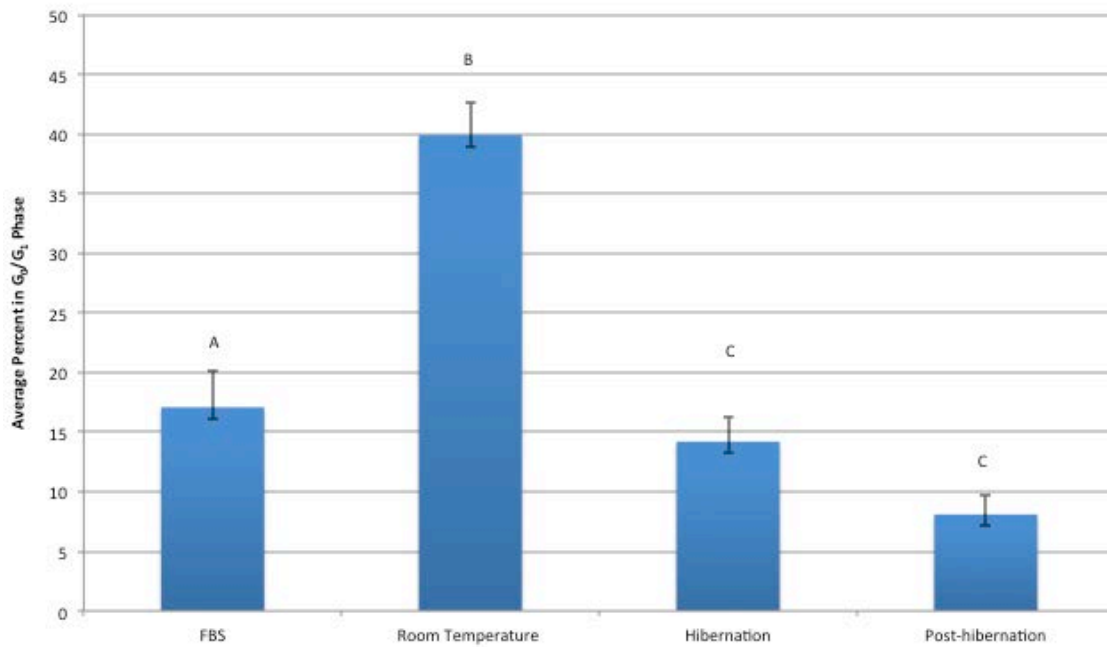


Figure 5. Bars showing the average of the percent of cells within the R2 gate, which indicates cells were in the G<sub>0</sub>/G<sub>1</sub> phase, and the standard error within each group. Room Temperature represents the group treated with active bullfrog plasma. Statistical significance recognized with a p value of less than 0.05. Means with same letter are not significantly different.

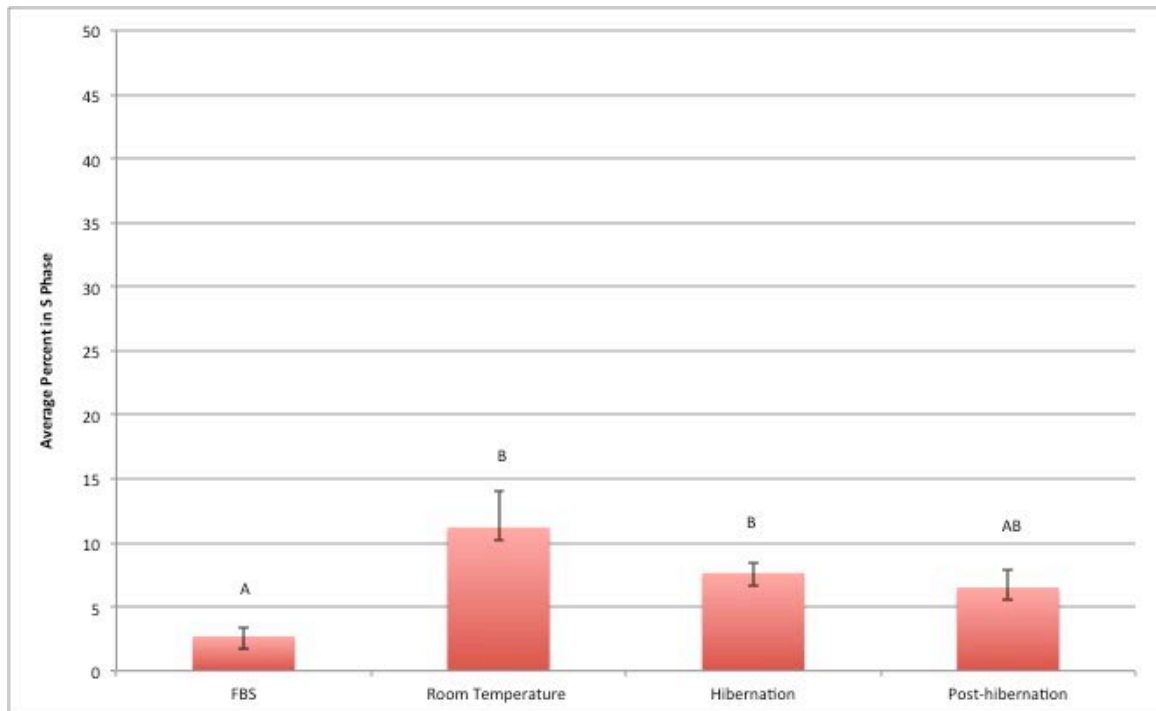


Figure 6. Bars showing the average of the percent of cells within the R3 gate, which indicates cells were in the S phase, and the standard error within each group. Room Temperature represents the group treated with active bullfrog plasma. Statistical significance recognized with a p value of less than 0.05. Means with the same letter are not significantly different.

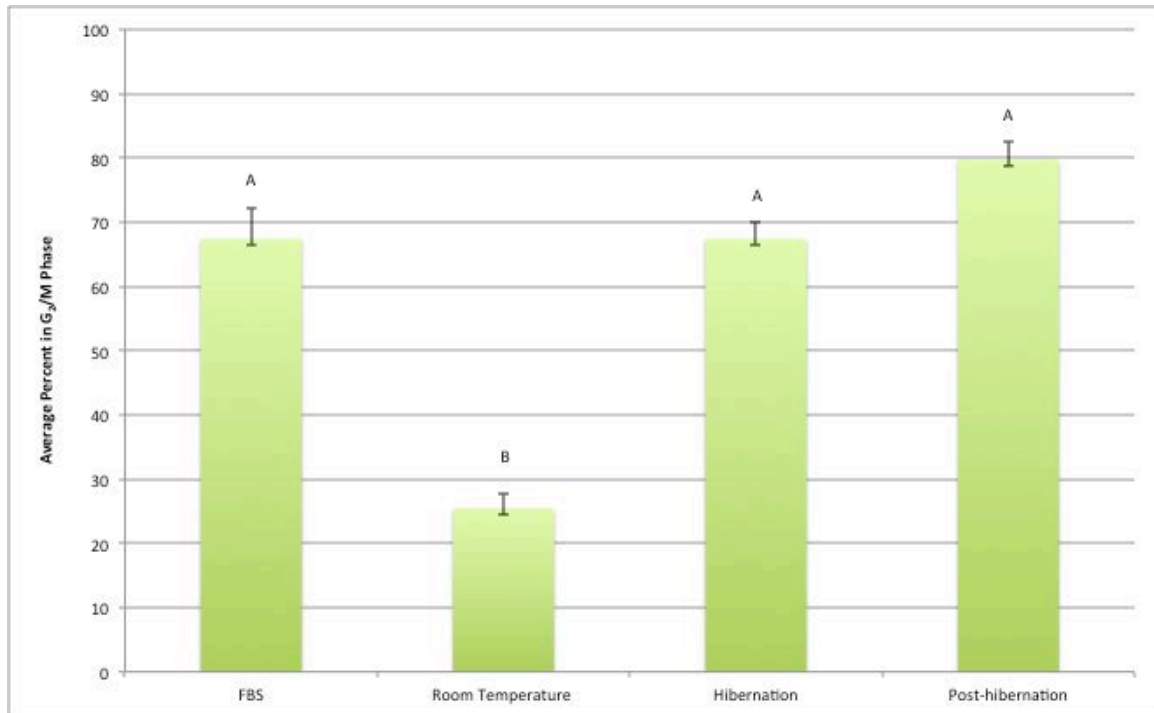


Figure 7. Bars showing the average of the percent of cells with in the R4 gate, which indicate that they were in the G<sub>2</sub>/M phase, and the standard error within each group. Room Temperature represents the group treated with active bullfrog plasma. Statistical significance recognized with a p value of less then 0.05. Means with the same letter are not significantly different.

## CHAPTER 4

### DISCUSSION

Hibernation is the ability for some organisms in colder regions to go dormant in order to wait out the cold season. The organism undergoes complex physiological changes in order to enter and exit hibernation. During hibernation, the rate of most of the vital systems in the body is dramatically decreased so that the animal can conserve as much energy as possible (Geiser, 2013). One aspect of this is halting or significantly slowing down the process of cellular proliferation (Sieckmann et al., 2014). If the cells are proliferating, it costs the organism energy and resources, which it is trying to conserve in order to survive throughout the cold season.

There are several mechanisms that could be causing this suppression of mitosis in the organism. One of these factors is that enzymatic activity drastically slows down by a factor of 2 to 3 for every 10-degree decrease in temperature (Boyer and Barnes, 1999). This means that decreasing the body's core temperature to that of their surroundings during winter may cause enzymatic activity to decrease to a degree where the cells are no longer capable of going through mitosis. This prediction, however, is not supported by the background literature. Several studies have measured the metabolic rate of hibernating mammalian models and have concluded that the degree of metabolic suppression could not be explained by the decrease in temperature alone and that a physiological inhibition must be occurring (Geiser, 2004).

Another mechanism by which the cells could be impeded from going through mitosis is the down regulation of growth factors in the body. In order for mitosis to be initiated, an extracellular signal must be received on the cell membrane in the form of growth factors, which are proteins secreted by the endocrine system. During hibernation, many systems are stopped or significantly slowed (Sieckmann et al., 2014). By analyzing the concentration of cyclins and CDKs in the liver cells of hibernating thirteen-lined ground squirrels, scientists have found that there is far less cyclin D in the cells during hibernation (Wu & Storey, 2012). Cyclin D is the first cyclin produced when the cell starts the process of proliferation. This could mean that there is a lack of growth factor present to signal the cell to initiate mitosis.

Another possibility is that there is a protein that is actively inhibiting mitosis in the cells. In the plasma of hibernating thirteen-lined ground squirrels and woodchucks, there exists a protein, alpha-2-macroglobulin, which inhibits mouse spleen cells from going through mitosis (Sieckmann et al., 2014). Almost all previous studies were conducted on mammalian models and very little is known about the mechanism, which causes anti-proliferation in hibernating ectothermic organisms.

The goal of this study was to determine if there was a mitotic inhibitor present in the blood plasma of the hibernating American bullfrog, which is an ectothermic organism, and if so, how this inhibitor affects the cell cycle. I hypothesized that there would be anti-proliferative effect on the THP-1 cells when they are incubated in plasma from hibernating bullfrogs. This correlated with the



hypothesis that there is a protein in the plasma that actively inhibits mitosis, similar to the alpha-2-macroglobulin found in thirteen-lined ground squirrels. The results collected from the cell count assays were inconclusive due to extreme variance within the data and a non-normal distribution, possibly due to the fact that a human cell line was used with the bullfrog plasma.

The data collected from the flow cytometry assays showed that there was a clear difference between cells that had been incubated in active bullfrog plasma and cells that were incubated in hibernating bullfrog plasma. The cells that were incubated in the active bullfrog plasma (room temperature group shown in figures 5, 6, and 7) were predominately in the  $G_0$  or  $G_1$  phases of the cell cycle. The  $G_0$  phase is the stage of the cell cycle in which the cell spends the majority of its time and is when the cell is in a resting state. This is as expected for this group because the cells were incubated in plasma from adult bullfrogs and, therefore, the plasma would have less growth factors leading to a majority of the cells in their resting state.

The cells incubated in the plasma taken from hibernating bullfrogs showed that a vast majority of the cells were in the  $G_2$  or M phase of the cell cycle, shown in figure 7. At these phases of the cell cycle the DNA has been duplicated resulting in greater binding to the dye and a higher intensity of light is emitted when passing through the flow cytometer. As a result, it is speculated that the majority of the cells incubated in the hibernating plasma have gone through the  $G_1$  and S phase and are being stopped in the  $G_2$  or M phase, after the DNA has been duplicated. It is the

cyclin B/ Cdk 1 complex that regulates the division of the cell once it is in the M phase (Wu and Strorey, 2012). Therefore, the substance in the hibernating plasma causing the majority of cells to be in the M phase could be playing a roll in the negative regulation of the cell cycle and inhibiting this complex from activating.

Stopping at this point in the cell cycle could be a mechanism for the bullfrog to more quickly recover from cellular death during hibernation. It is well known that many cells die during the process of hibernation due to the stresses of cold and being in a reduced metabolic state (Cerri et al., 2009). Therefore, by having the cells already duplicate their DNA and then stop before going through the M phase, the cells can divide quickly while the frog is exiting hibernation reducing the time needed to replace damaged cells. Further experimentation is needed in order to determine whether or not this substance is in fact a protein, similar to the inhibiting protein found in the thirteen-lined ground squirrel. The data of this study does not coincide with a previous study that used a flow cytometer assays as well, in which researchers found that the majority of the cells in hibernating mammal organs were in the G<sub>1</sub> phase of the cell cycle (Kolaeva et al., 1980). The reason that my data differed from theirs could be because in that study they used a mammalian model. This could indicate that there may be a difference in the mechanism of mitotic suppression between amphibian and mammals.

Other studies looking at the mitotic index of several immortal mammalian cell lines have found similar results to this study. As the temperature lowers on

mammalian cell cultures, which proliferate optimally at 37°C, the rate of proliferation hits a threshold where the cell progresses through G<sub>2</sub> but is stopped before entry into M phase (Rieder and Cole, 2002). This suggests that there exists a temperature dependent mechanism that acts as a checkpoint just after G<sub>2</sub>, which prevents the cell from entering M phase if the temperature is too cold. The majority of the THP-1 cells incubated in plasma extracted from hibernating bullfrogs in this study also progressed through the cell cycle until they were stopped in the G<sub>2</sub> phase. However, all of the cells used in this study were incubated at 37°C. Other studies have found that when mammalian immortal cell lines, such as HeLa and C3-H.Ep-2 cells, are incubated at 4°C for one hour and then brought back up to 37°C their mitotic index drops for about 17 hours, after which the mitotic activity increases dramatically for 2 hours with 60-80% of the cells entering into mitosis (Newton and Wildly, 1959, Rapp, 1960, Manabe, 1969). This gives merit to the above speculation that the cells are being inhibited from entering M phase as a mechanism to more quickly duplicate cells while exiting hibernation.

All experiments were carried out in an incubator set at 37°C, and the corresponding differences in mitotic suppression cannot then be explained by incubation in differing temperatures. The information gathered from this study also leads me to believe that this occurrence is not caused by a down regulation of growth factors because the majority of the cells incubated in hibernating plasma were in the M phase of the cell cycle. If the suppression of mitotic activity was

caused a down regulation of growth factors one would expect that the majority of the cells would be in the  $G_0$  phase because without a concentration of growth factors the cells would not have been triggered to duplicate their DNA. Therefore, it is believed that there is an inhibitory agent present in the hibernating plasma, which is causing an anti-proliferative effect on the cells during hibernation. Cells incubated in the active bullfrog plasma had normal growth with the expected majority of the cells being in the  $G_0$  phase of the cell cycle. On the other hand, the cells incubated in hibernating plasma showed that the majority of cells had duplicated their DNA and were in either the  $G_2$  or M phase. This means that there is most likely a substance in the hibernating plasma, which is not in the active plasma causing a different effect on the cell cycle in these cells. The findings of this study does support my hypotheses, that this regulation of mitosis would be caused by a protein, or other substance, in the blood plasma of hibernating bullfrogs, which is actively causing an inhibition of proliferation.

Conducting research to better understand this mechanism that hibernating organisms use to actively suppressing cellular proliferation is important and has significant applications in a wide array of fields. Knowledge gained about the biochemical response to the body when hibernation is initiated could lead to great medical advancements in organ donation and transplantation. By isolating the substance that causes the mitotic inhibition of cells and decreases the metabolic rate one could drastically extend the viability of a donated organ before the tissue

becomes too damaged, thus increasing transplant success rates. Discovery of this substance that is causing mitotic inhibition could also lead to safer cancer treatment. The cancerous tumor could be inoculated with this substance, causing the rate of proliferation in the cancer cells to dramatically slow down while other treatments such as radiation and chemotherapy have more time to work. Further possible application for this research could also reach to placing humans into suspended animation for deep space travel.

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