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By

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An Honors Thesis Submitted in Partial Fulfillment
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**Introduction**

A large advantage that all vertebrates have is their two tiered immune system. This system incorporates many different cells, tissues and soluble proteins all working together (Janeway et al., 2004). The two parts of the vertebrate immune system are the adaptive and innate sections. The adaptive is also divided into two branches. One being the cell mediated branch which is carried out by T-cells. The other is the humoral branch which involves B-cells and their production of antibodies. The innate section employs a wide range of cell types. These different types include macrophages, natural killer cells and granulocytes.

Every organism must be able to deal with the environment in which it lives and every advantage helps. The variability of that environment through the seasons poses one of the greatest challenges. The environment influences almost every aspect of an organism's existence. So it is a logical step to say that the seasons exhibit an influence on the immune system of the organism. There are several factors from the environment that contribute to either the success or failure of the organism in the struggle for life.

The changes in seasons have been shown to exhibit responses from animals in their behavior and biological patterns. It is only logical that their immune systems may also be affected. Seasonal factors have been shown to cause a broad increase in circulating leukocytes in rural Gambian infants (Collinson et al., 2008). Even the behavior of *Taricha granulosa* shows seasonal variation. They migrate between breeding ponds and upland forests. They head down to the breeding ponds in the spring and generally encountered by Elk hunters up in high elevations during the fall. The seasonal variation due to changing environment conditions has been documented in the immune systems of several other mammalian species. They are considered to be an adaptive mechanism to allow for the animals to deal with the different winter environment (Bowden et al., 2007). The adaptive value comes from being able to invest more into other biological processes such as reproduction or growth with a reduction in immune system activity as shown in certain studies (Weil et al., 2006). The characteristics of the immune system's responses are largely governed by the seasonal conditions (Leceta and Zapata, 1985). Both the immune systems of endotherms and ectotherms show variation by the seasons but both most likely have different causes (Zapata, Varas, and Torroba, 1992). There are several different environmental cues that could be the cause of these variations.

Temperature has been shown in many studies to be an important environmental cue (Bowden et al,. 2007). For ectotherms an increase in temperature means an increase in activity. Studies have also shown that with an increase in the speed and effective ness of immune responses during the spring and summer months (Wright and Cooper, 1981; Avtalion et al., 1973). In this way and other studies have also shown that temperature has direct influence on the immune systems of ectotherms (Morvan et al., 1998). When testing the primary immune response of the tortoise *Mauremys capsica* the immune system was found to variation in the responses of the summer treated subjects than in the autumn treated subjects (Leceta and Zapata, 1986). However, in this study the temperature difference was negligible so there had to be another factor influencing these variations.

Yearly variations in photoperiod have shown to affect the immune system and reproductive system of laboratory rats (Prendergast et al., 2007). In other studies the use of simulated photoperiods has resulted in affecting the timing of both seasonal physiology and behavior in Siberian hamsters (Gorman and Zucker, 1995). Data from the study done by Wen and Prendergast in 2007 showed that when subjected to short photoperiods, male hamsters showed diminished sickness behaviors. The animal's ability to detect photoperiod is very important to its survival for several reasons. Studies have shown that natural selection has favored photoperiod sensitive organisms because it allows them to predict the upcoming events of the changing seasons (Weil et al., 2006). This ability allows the organism to better allocate its resources for the upcoming stresses of the environment. At looking at the rodent model again the short photoperiod creates a phenotype for the Siberian hamster that has a reduced reproductive tract among others changes that help it to better survive the winter months (Weil et al., 2009). It is possible to link this need for energy conservation for the approaching winter with the reduction of the immune system. In so doing photoperiod is a possible defining factor. Studies have shown that biological rhythms in a certain frog *Rana temporaria*, disregard temperature variation in the environment (Zapata, Varas and Torroba, 1992).

There is another factor that has an effect upon the immune system. Research has shown evidence that certain steroid hormones are crucial to the immune system variations that coincide with the seasonal variations (Zapata et al., 1983). Other research has pointed to the rise in the levels of glucocorti-costeroids in the blood of a lizard *Chalcides ocellatus* is responsible for some part of the lymphocyte destruction that occurs in the winter months (Saad et al., 1984). The same negative result was seen in the primary antibody production in steroid-sensitive mammals (Ferreira, Moreno and Hoecker, 1973). With all of this evidence from a wide range of members of the vertebrate family it is safe to say that internal elements can also help to influence the immune system. It is these internal variations in hormones that can affect the responses of the immune system of lower vertebrates (Leceta and Zapata, 1985).

The exact timing of these variations seems to vary between species. For the red-eared slider, *Trachemys scripta* increased immune response peaked in the spring months before the peak temperatures were reached (Zimmerman et al., 2010). T-cell proliferation has also been shown to peak in the spring in reptiles (Farag and El Ridi, 1984). A study done on the tortoise *Mauremys capsia* shows that its lymphoid tissues reduces its size during the summer at the peak time of activity for this species (Leceta and Zapata, 1985). In certain frogs, *Rana temporaria*, lymphoid tissues reaches its maximum development in the summer (Zapata, Varas, and Torroba, 1992). Even the various types of leukocytes have been shown to peak in peripheral blood concentration at separate times. The highest percentage of peripheral Heterophils were found to be present in *Mauremys capsica* in the fall. This is in contrast to the lymphocyte concentrations which were found to be lowest in the fall and highest in the spring (Muñoz and De La Fuente, 2004).

**Goal of this Study**

The goal of this study is to focus in on the percentages of leukocytes found in the peripheral blood of the newt *Taricha granulosa*. The specific affects of temperature, photoperiod, and hormonal control will not be looked at in such detail. The specific type of leukocyte will not be of importance either. The purpose of this study is to determine if there is a cycle present in the leukocyte concentrations in the peripheral blood of *Taricha granulosa* and more importantly if this cycle corresponds to the change in the seasons. The other goal is to determine when, if there are varying levels of leukocyte percentages, the peak takes place. Will it be in the spring time like for the red-eared slider (Zimmerman et al., 2010)? According to the evidence provided from previous research a cycle is an almost a certainty ((Leceta and Zapata, 1985). Also with the evidence that shows ectotherm's increased physical and immune system activity coincide with warmer temperatures (Wright and Cooper, 1981; Avtalion et al., 1973). The *Taricha granulosa*, will exhibit a low level of peripheral leukocytes in the winter with increases through the spring. The highest percentages will be recorded in the summer months when the temperatures are at their highest. The peripheral leukocyte levels will decrease through the fall before reaching a value close to that of the previous winter.

**Methods and Materials**

*Animal* **-** The main animal used in the study was the rough skinned newt, *Trachia granulosa*. These animals were a likely choice for this study due to their abundant nature in the region. Another key factor is the few numbers of natural predators that exist for *Trachia granulosa*. The main reason for this is their potent tetrodotoxin which deters most predators except for local Garter snakes. The samples were taken from a local farmer's pond with permission every month. Upon capture the time and sex of the animal was noted. Then specimen was then sedated by a slight knock to the head and a blood smear was taken. This was accomplished by using a razor to remove the tip of the tail and then drawing the blood onto three prepared slides per specimen. Some months several specimens were able to be found while other months certain factors prevented the capture of any.

*Capture Technique* - The most important part of my equipment in the field was my fine mesh net. During the colder months when the vegetation along the shore was highly reduced the newts were easily spotted in the shallows. They spent most of the time in the shallows during the winter months and this allowed for walking the bank to be a very effective technique. However, as the temperature began to increase the vegetation began to grow and the newts became harder to spot. Also with the seasons shifting towards summer the pond became full of a large population of small green frogs. These frogs spooked easily and prevented walking the bank from being successful. In order to prevent spooking the frogs, the farmer's canoe was used. When on the water you have to wait for the newt to rise to the surface or catch one floating right beneath the surface. This was more easily said than done. In the colder months of fall prey capture became more difficult because the newts were scarce or the pond had frozen over. The reason for this may have been the seasonal activity of the newts themselves. They tend to migrate to breading ponds in the spring time. Then they turn and leave and head towards upland forests to grown until sexual maturity. It is not until this point that they return to the ponds. This behavior may have been to blame for my difficulty in collecting samples in the late fall and early winter months.

*Blood Samples* - The blood smears were taken from the field and labeled A, B and C for each specimen. The air dried blood smears were brought into the laboratory and dipped into absolute methanol. They were then allowed to air dry. After they had been thoroughly dried they were placed into undiluted Giemsa stain for approximately 5 minutes. After the time was up the slides were removed and the excess stain was wicked away by placing the end of the slide against the absorbent pad. These treated slides were then placed within distilled water for approximately 5 minutes. This allowed for the rest of the excess stain to be removed from the slide. The slides were then removed and placed out to air dry for several minutes. When they had been sufficiently dried they were stored for further analysis.

*Lab Techniques* - The treated slides were then examined under a common light microscope. Each slide had three sectors selected at random that were counted. The sectors were viewed under 10X and when there were over 300 cells present the power was increased to 40X magnification. The amount of red blood cells and white cells were counted for each sector. The percentages were then tallied for each sector. With three sectors per slide and three slides per specimen it allowed 9 data points for each specimen. The overall white blood cell percentage was calculated by taking the average of all sectors for all three slides.

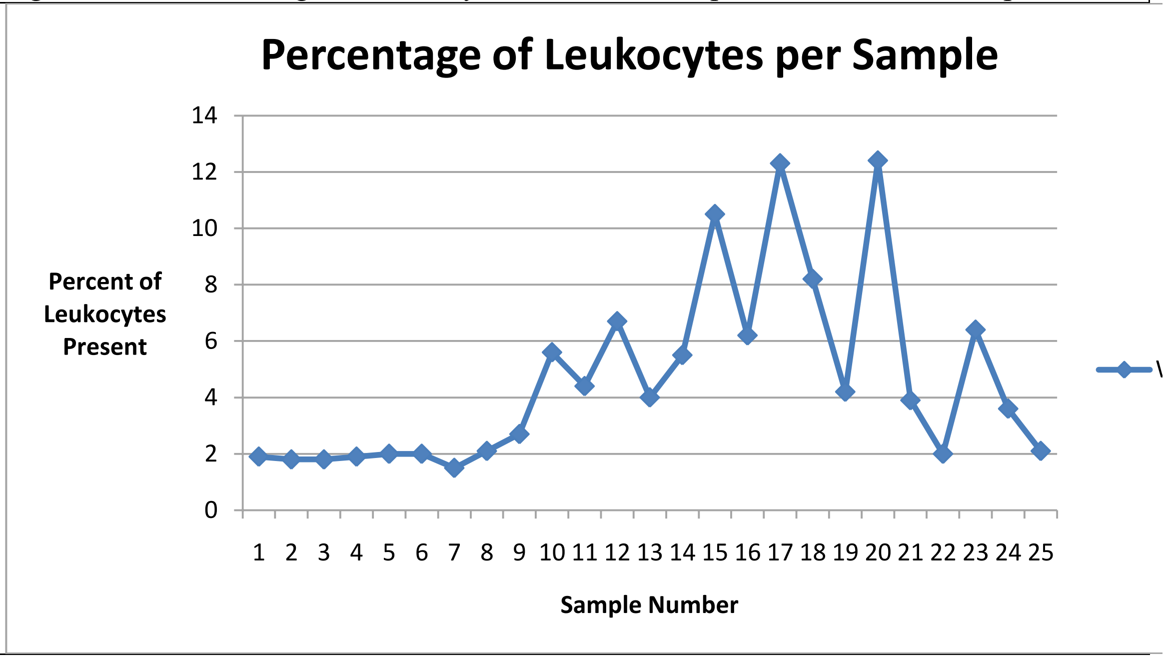
*Data Analysis* - The data for each specimen was recorded and the totals were added up for both the White blood cells and the red blood cells. The final Leukocyte percentage was derived from these total values of each specimen. These where then organized by sex and month caught. Also the data was organized into three month seasons. December through February was designated the winter season. The spring season was made to be the months of March through May. The summer season included June through August. Finally the remaining months of September through November were labeled the fall season. With this study stretching from January 2009 to February

2010 the first winter season only contains the data for the first two months of January and February of 2009.

**Results**

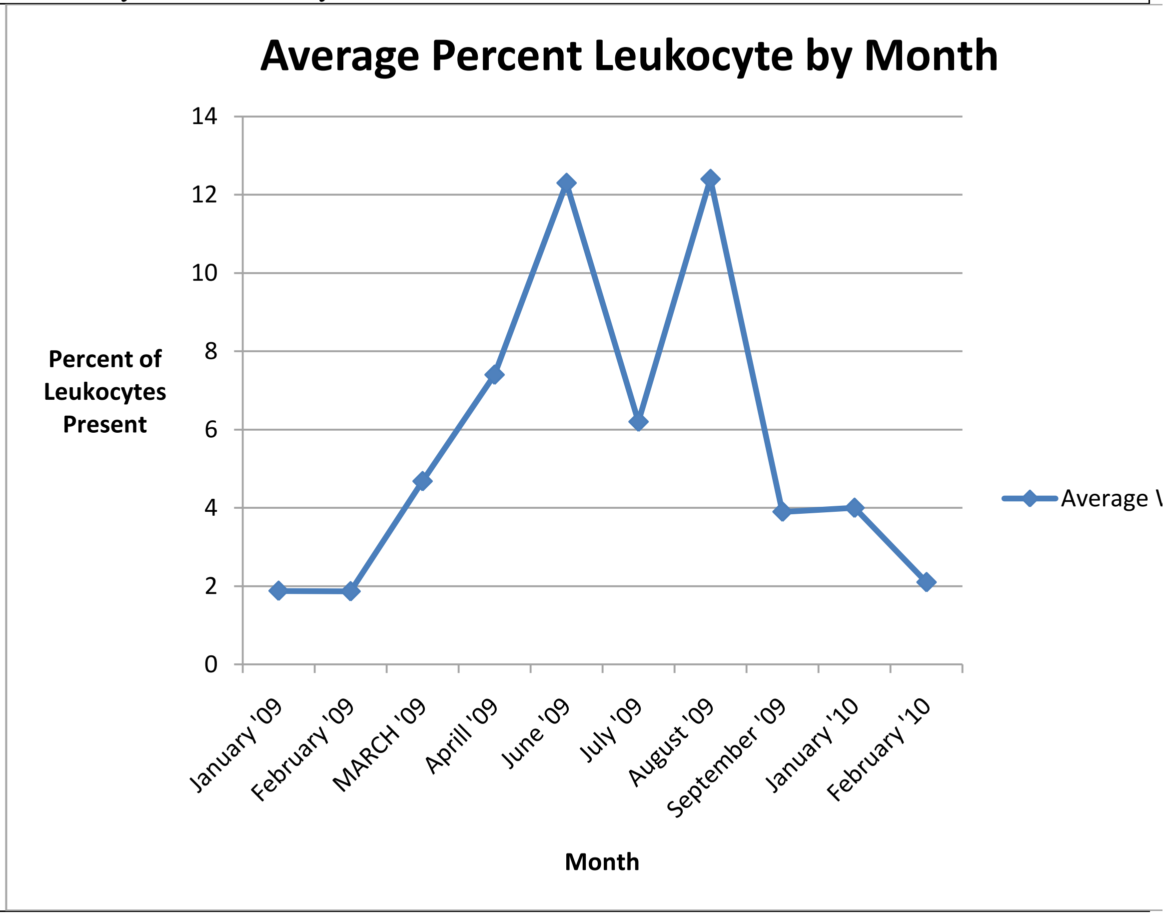
There were a total of 28 samples caught between January of 2009 and February of 2010. Of these 28 samples only 25 of them produced blood smears that could be analyzed. The results showed a fairly stable level of leukocyte percentage in the peripheral blood of approximately 2% until sample 10 (Figure 1). Sample 10 was caught in March of 2009 and the subsequent specimens all showed increases leukocyte percentage until sample 21 which was caught in September of 2009. This specimen recorded a Leukocyte percentage of 3.9% which was a decrease from those previously caught. The final four specimens, samples 22-25 were caught in January and February of 2010. There was a substantial amount of variation between the three caught in January. Specimens 22, 23 and 25 had leukocyte percentages of 2, 6.4, and 3.6% respectively while the final specimen 25, had a percentage of 2.1 (Figure 1).

Figure 1: The Percentage of Leukocytes within the Peripheral Blood of each Specimen.



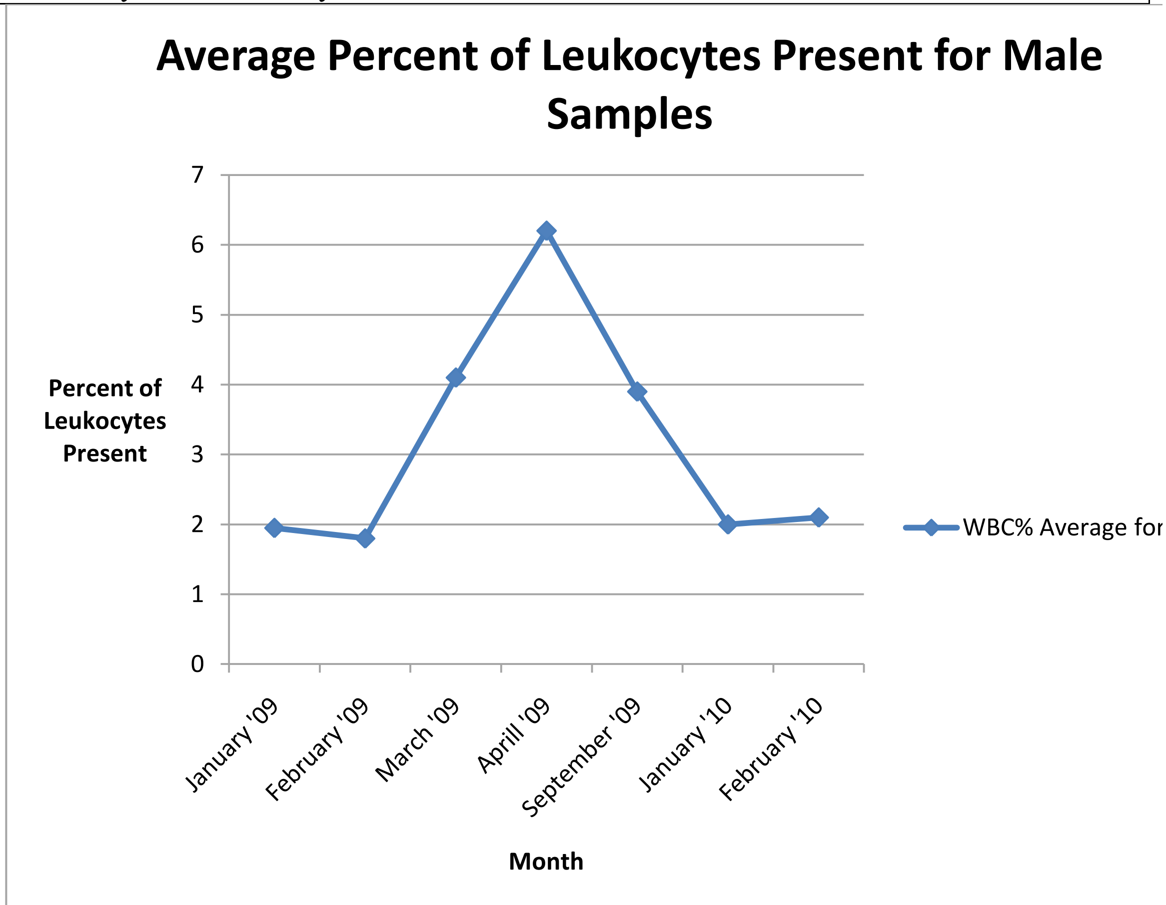

The data was then organized by month by averaging the leukocyte percentages of all specimens caught in that month. The results showed again a rise starting when the leukocyte percentages jumps from below 2% in February of 2009 to 4.68% in March of that year (Figure 2). The percentages climbed with each month until they hit a high point in June of 2009 with a value of 12.3%. This was followed by a drop in July of 2009 when the leukocyte percentages were tallied at 8.2%. Then in August the percentages again increased but this time even higher to 12.4%. Then in September of 2009 the leukocyte percentage fell to 3.9%. In January and February of 2010 the leukocyte percentages were recorded at 4 and 2.1% respectively.\

Figure 2: The Percentage of Leukocytes Present in the Peripheral Blood for the Months of January 2009-February 2010.



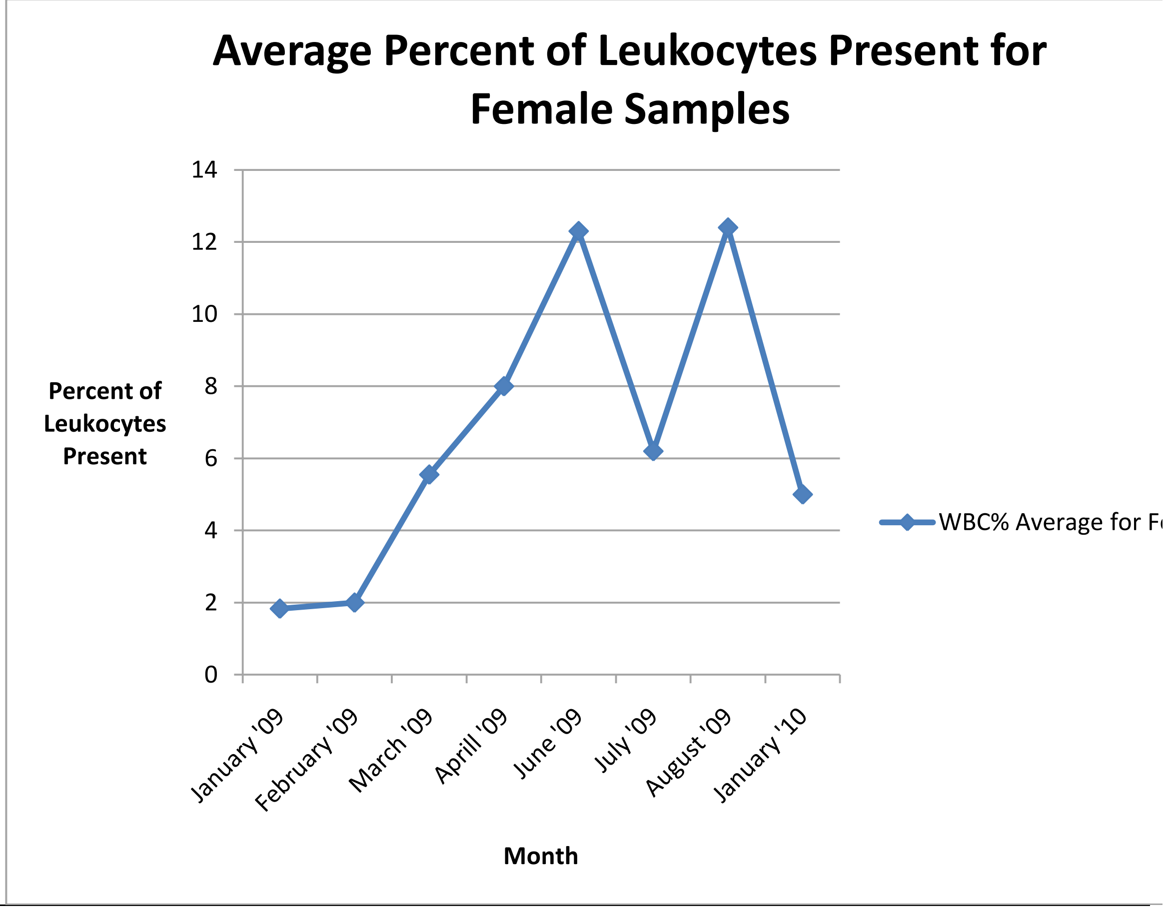

The data was then divided between the male and female results. First the male leukocyte percentages were analyzed on a monthly basis for the time of January 2009 to February 2010. There were two significant holes in the data for male specimens due to lack of males caught during the months of May 2009-August 2009 and October 2009-December 2009. The resulting data shows an increase in the leukocyte percentage starting in March of 2009 and a decline starting in September of 2009. In 2009 the male data showed percentages of 1.95% and 1.8% for the months of January and February respectively. In 2010 the data for January was a leukocyte percentage of 2% and of 2.1% for February.

Figure 3: The Average Percent of Leukocytes Present for Male Samples for the Months of January 2009-February 2010.



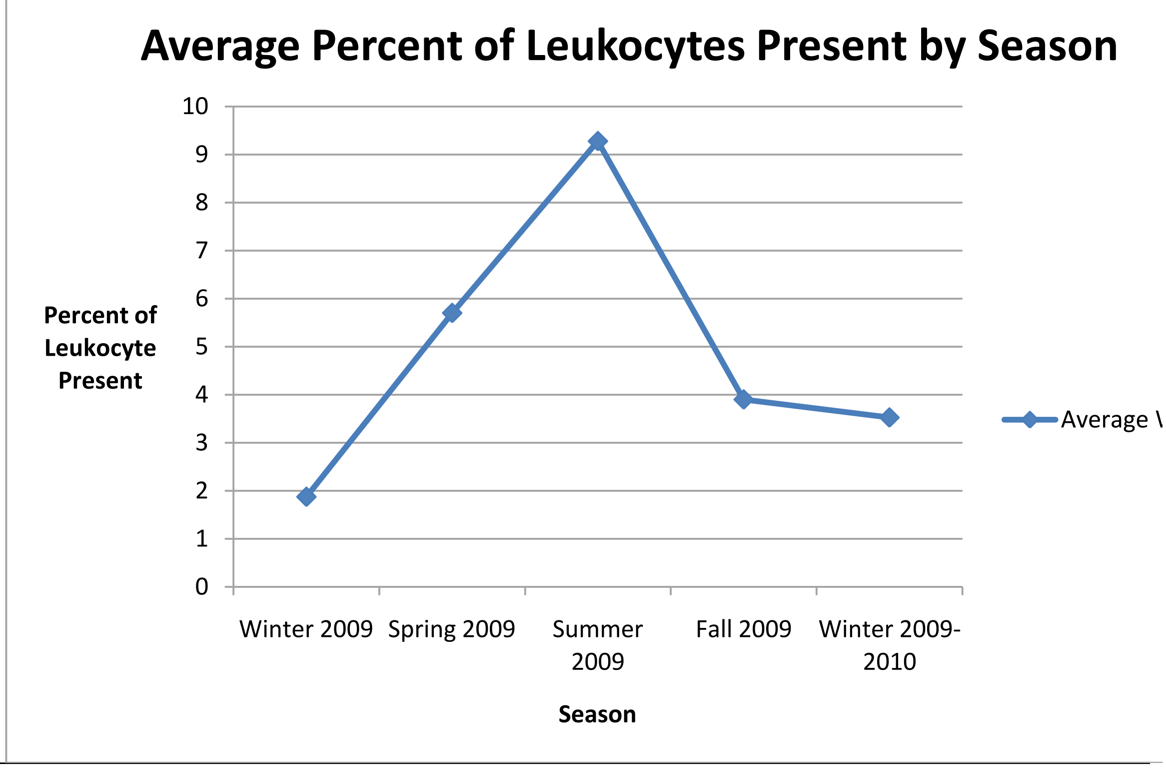

The data for female samples shows an increase in the leukocyte percentage starting in March of 2009 when it increased from 2% in February to 5.55% (Figure 4). The percentage continued to increase until it hit a high point in June of 2009 when the leukocyte percentage was recorded as 12.3%. Then in July the percentage dipped to 6.2%. Then there was another sharp increase with the leukocyte percentage of August being 12.4%. The female data then showed a decrease to the leukocyte percentage of 5% in the month of January in 2010.

Figure 4: The Average Percent of Leukocytes Present in the Female Samples for the months of January 2009-February 2010.



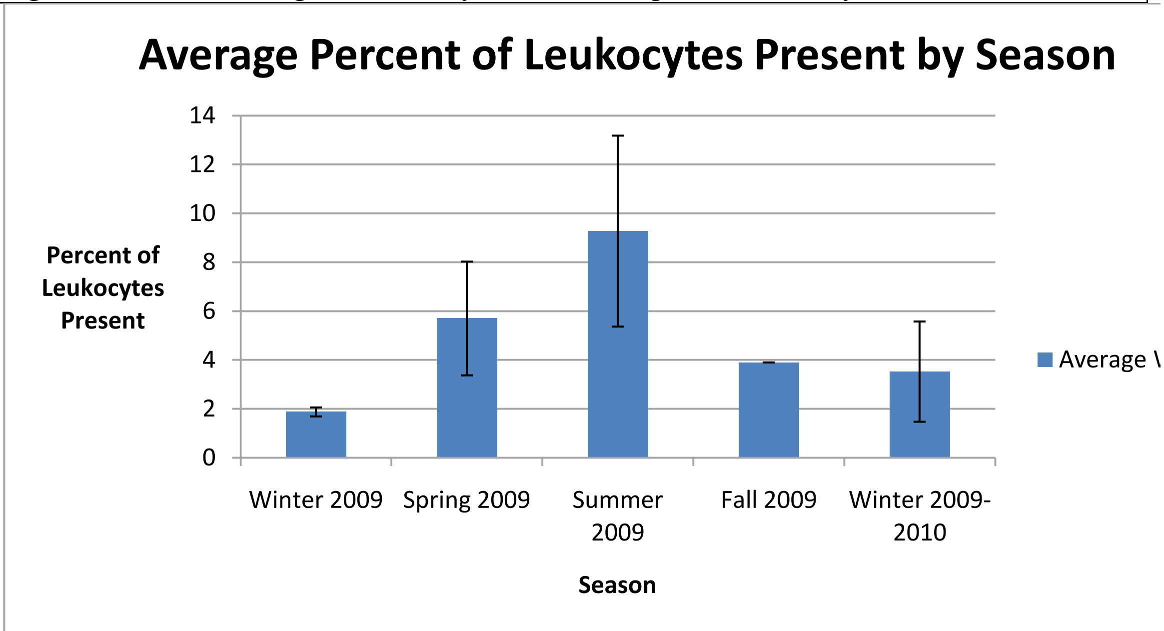

The data was then organized into the three month seasons of winter, spring, summer, and fall in attempts to provide another way to look at the results. The data showed an increase from the leukocyte percentage of 1.875% in the winter of 2009 to the spring of 2009 when the percentage was shown to be 5.7% (Figure 5). The leukocyte percentage peaked in the summer of 2009 at 9.275%. Then the percentages decreased to 3.9% and 3.525% in the Fall of 2009 and the Winter of 2009-2010 respectively.

Figure 5: The Average Percent of Leukocytes Present in the Peripheral blood by Season.



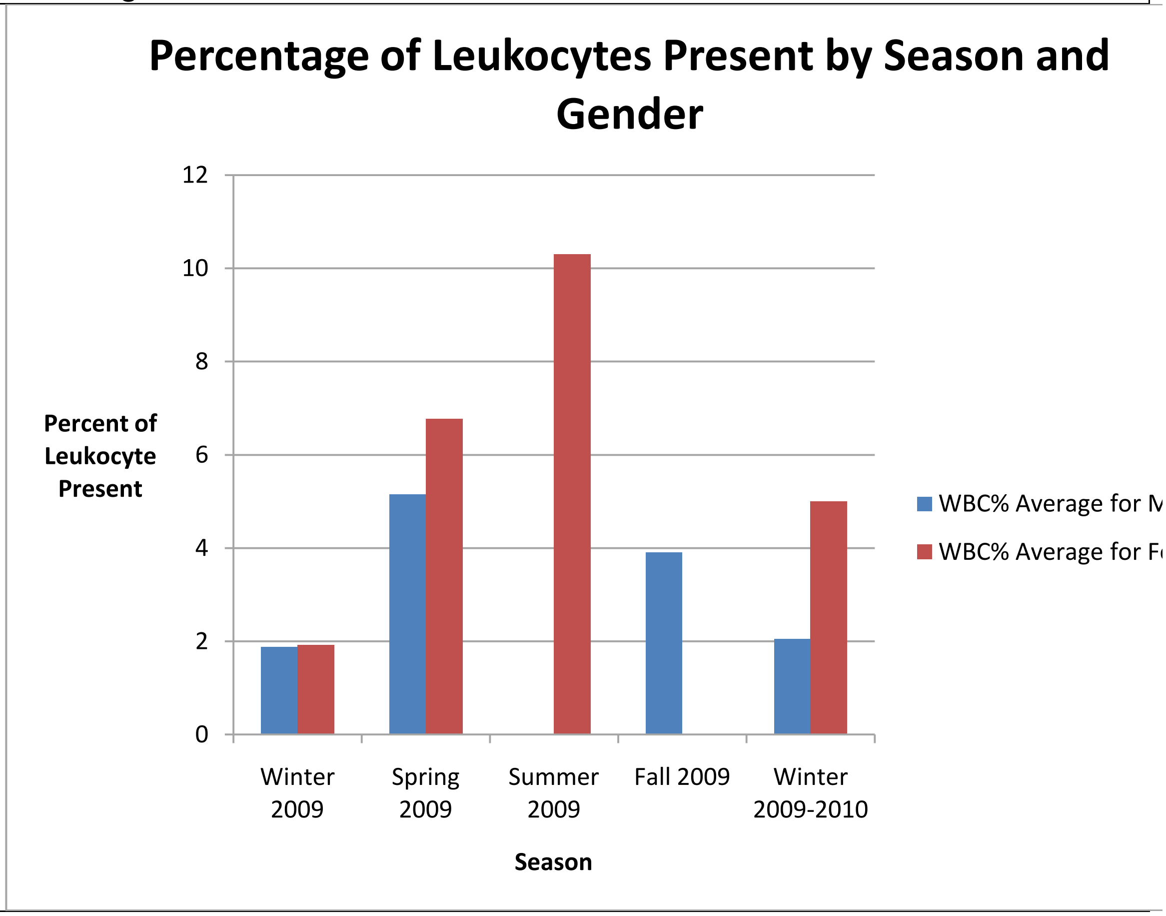

The data was then taken and the standard deviation was found for each season and the summer of 2009 had the greatest with a deviation of 3.908 (Figure 6). The Standard deviation for the winter of 2009 was the lowest at 0.18. The deviation was unable to be recorded as a zero for the fall of 2009 due the fact that there was only one specimen for that season. For both the Spring of 2009 and the Winter of 2009-2010 registered standard deviation values of over 2. They were 2.32 and 2.05 respectively.

Figure 6: The Percentage of Leukocytes in the Peripheral Blood by Season with Error.




The final results are from a comparison of each gender's leukocyte percentage according to the seasons (Figure 7). For the winter of 2009 the male average leukocyte percentage was 1.875% and the female average was 1.916%. In the spring of 2009 the male average was shown to be 5.15% and the female average was shown to be 6.775%. In the summer of 2009 there were no male specimens captured so there is no average leukocyte percentage. However, the female average was shown to be 10.3%. In the fall of 2009 the male average was recorded as 3.9% while there were no female specimens caught. Finally in the winter of 2010 the male leukocyte percentage was 2.05% and the female percentage was found to be 5%.\

Figure 7: The Percent of Leukocytes Present in the Peripheral Blood for both Genders According to the Seasons.




**Discussion**

The results of this study showed that the percentage of leukocytes in the peripheral blood for *Taricha granulosa,* increased in the spring and peaked in the summer. This is similar to the findings in studies done on *Mauremys capsica* (Leceta and Zapata, 1985). However, difference comes in the peak of activity. For the tortoise the immune activity peaked in the spring months while the Newt's peaked in the summer. There are several possible reasons for this difference in timing. It could just be a case of species-specific differences that are responsible for the variation in the immune system response (Zapata, Varas, and Torrob, 1992).

The reason for these variations may be due to a difference in annual mating timing or variations in amount of energy necessary for reproduction. Research into the influence of immune response activation on reproductive success has shown a decrease in fitness when accompanied by a strong immune response (Martin et al., 2008). The pros and cons of the investment of energy into an immune response or other expensive biological processes have been well studied (Sheldon and Verhulst, 1996; Norris and Evans, 2000). This effect has been shown in the reduction of rodent's reproductive tracts (Weil et al., 2006). However, this cannot be determined from the data in our study but it provides a plausible explanation behind the trend.

Another reason that could be behind the trend is the variation in photoperiod. Several studies have shown that the photoperiod acts as an important environmental cue for several species (Zapata, Varas, and Torroba, 1992; Wen and Pendergast, 2007; Paul, Zucker, Schwartz, 2008; Weil et al., 2009). However, the opposite has also been shown. Studies conducted with seasonal breeding rodents showed a small portion of the population actually was non-responsive to photoperiod (Prendergast et al., 2001). This data was found in a mammalian model and may or may not apply to *Taricha granulosa.* Most likely the photoperiod has major effects upon the behavior and physiology of the Newts but that is for another study.

Temperature is the other environmental factor that varies with the seasons and may be responsible for the trend within the peripheral leukocyte percentages found. The immune system of ectotherms has been shown to directly influenced by temperature (Le Morvan et al., 1998). Many immune system responses have been shown to be suppressed at low temperatures in some amphibians (Macela and Romanovsky, 1970). However, immune responses have also been shown to be temperature independent in certain teleosts (Wright and Cooper, 1981). More importantly certain tropical reptiles have shown a decrease in circulating lymphocytes during the colder temperatures of the winter months (Hussein et al., 1979). Since the *Taricha granulosa* is a temperate reptile the more relevant data comes from the study done in 1970 that showed a reduction in circulating lymphocytes in hibernating temperate frogs and reptiles (Duguy, 1970). This data is a highly probable candidate to explain the trend that is shown in this study. However, there are still very different aspects of the behavior between these organisms. The most important of these being that the *Taricha granulosa* do not hibernate and remain active during the winter months.

All of the previous mentioned seasonal variations may be the cause outside cause behind the trend we see. However, the internal biological cause maybe the fluctuating levels of corticoids. The steroid level has been linked to changes in number of leukocytes (Cupps and Fauci, 1982). Strong immune responses and fully developed lymphoid organs are present with low corticosteroid levels in *Chalcides ocellatus* during the summer months (Zapata, Varas, Torroba, 1992). These fluctuating hormone levels are most likely due to the environmental variations of the seasons. It is then most likely these fluctuating levels that influence that change in immune response.

The differences in immune responses may also be affected by the sex or age of the organism (Zimmereman et al., 2010). That was the whole reason for the analysis carried out in this study (Figure 7). In our results a trend of variation between the sexes was shown however, the samples sizes were too small to enable the claim of a cycle.

Future research is needed in several areas. There has been much done on the effects of seasonal variation on mammalian, fish, and tropical reptilian immune systems. For a complete understanding of the functioning of the vertebrate immune system more research needs to be done with species like *Taricha granulosa*, which remain active even in the colder winters of more northern environments. With the evidence towards the trend of peaking peripheral blood leukocyte percentages in the summer presented by this study the causing factors can now be looked at in more detail. Research geared toward whether temperature, photoperiod, or hormone levels can all be used to help explain the trend. Most importantly a more comprehensive study of the *Taricha granulosa* is needed in order to establish whether the trend found in this study is a statistically significant cycle.

Another need for research is to see if the trend is part of an annual cycle or not. This would require a multi-year study. The main improvement needed on this study is an expansion of sample size which would allow for an increase in data and a likelihood of establishing statistical evidence of an annual cycle.

Further study can also be directed towards the differences between the sexes when it comes to the possibility of the females expressing higher leukocyte percentages. The trend could be further explored and confirmed. Research could also be directed towards establishing a correlation between the reproductive investment of the female and the seemingly high level of immune system activity. Both of these future studies should again be carried out with a larger sample size as well as maybe multiple populations. This way the possibility of a species wide trend could be established.

The conclusion of this study shows that there is evidence of a trend in the leukocyte percentage in the peripheral blood. A statistical signifigince cannot be shown for either the trend in percentages or the differences between the sexes. It goes from low levels in the winter months to rising through the spring and peaking in the summer. It returns to lower levels by decreasing during the fall and bottoming out in the winter. The female samples seem to show a trend of having higher leukocyte percentages within their peripheral blood as well.

**Appendix**

Table 1: The month the specimen was captured and the recorded leukocyte percentage.

| Sample # | Month | WBC% |
|---|---|---|
| 1 | January | 1.9 |
| 2 | January | 1.8 |
| 3 | January | 1.8 |
| 4 | January | 1.9 |
| 5 | January | 2 |
| 6 | February | 2 |
| 7 | February | 1.5 |
| 8 | February | 2.1 |
| 9 | March | 2.7 |
| 10 | March | 5.6 |
| 11 | March | 4.4 |
| 12 | March | 6.7 |
| 13 | March | 4 |
| 14 | April | 5.5 |
| 15 | April | 10.5 |
| 16 | April | 6.2 |
| 17 | June | 12.3 |
| 18 | June | n/a |
| 19 | July | 8.2 |
| 20 | July | 4.2 |
| 21 | August | 12.4 |
| 22 | Septembe | 3.9 |
| 23 | October | n/a |
| 24 | October | n/a |
| 25 | January | 2 |
| 26 | January | 6.4 |
| 27 | January | 3.6 |
| 28 | February | 2.1 |

Table 2: The average leukocyte percentage per month in the peripheral blood.

| Month | Average WBC% |
|---|---|
| January '09 | 1.88 |
| February '09 | 1.866666667 |
| MARCH '09 | 4.68 |
| Aprill '09 | 7.4 |
| May '09 | n/a |
| June '09 | 12.3 |
| July '09 | 6.2 |
| August '09 | 12.4 |
| September '09 | 3.9 |
| October '09 | n/a |
| November '09 | n/a |
| December '09 | n/a |
| January '10 | 4 |
| February '10 | 2.1 |

| Table 3: Average leukocyte percentage according to season with standard deviation | | |
|---|---|---|
| Season | Average WBC% | Standard Deviation |
| Winter 2009 | 1.875 | 0.183225076 |
| Spring 2009 | 5.7 | 2.327475641 |
| Summer 2009 | 9.275 | 3.908431058 |
| Fall 2009 | 3.9 | 0 |
| Winter 2009-2010 | 3.525 | 2.051625372 |

| Table 4: The percent of leukocytes in the peripheral blood according to month and sex. | | |
|---|---|---|
| Month | WBC% Average for Male | WBC% Average for Female |
| January '09 | 1.95 | 1.833333333 |
| February '09 | 1.8 | 2 |
| March '09 | 4.1 | 5.55 |
| Aprill '09 | 6.2 | 8 |
| May '09 | n/a | n/a |
| June '09 | n/a | 12.3 |
| July '09 | n/a | 6.2 |
| August '09 | n/a | 12.4 |
| September '09 | 3.9 | n/a |
| October '09 | n/a | n/a |
| November '09 | n/a | n/a |
| December '09 | n/a | n/a |
| January '10 | 2 | 5 |
| February '10 | 2.1 | n/a |

| Table 5: The percent of leukocytes in the peripheral blood according to season and sex. | | |
|---|---|---|
| Season | WBC% Average for Male | WBC% Average for Female |
| Winter 2009 | 1.875 | 1.916666667 |
| Spring 2009 | 5.15 | 6.775 |
| Summer 2009 | 0 | 10.3 |
| Fall 2009 | 3.9 | 0 |
| Winter 2009-2010 | 2.05 | 5 |

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