AN ABSTRACT OF THE THESIS OF

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Title: _An Investigation of Factors Influencing Erythrocyte Osmotic Fragility____

Among Selected Ectothermic and Endothermic Vertebrates

Abstract approved: _David K. Saunders___

Erythrocyte Osmotic Fragility (EOF) is a quantitative measurement of erythrocyte strength and its ability to withstand varying osmotic gradients. An increased EOF is associated with decreased cell strength. I hypothesized that aquatic/semi-aquatic ectothermic vertebrates because they are likely to be exposed to varying osmotic gradients would have stronger erythrocytes than terrestrial ectothermic vertebrates that have relatively constant osmotic surroundings. The EOF for amphibians investigated supported the hypothesis, however, the EOF results for the reptiles investigated did not. Additionally, the EOF of some endotherms was investigated to compare EOF of ectotherms and endotherms. Both amphibians and reptiles showed lower EOF values than did endothermic vertebrates. The effect of temperature on EOF was also investigated at temperatures of 5, 25, and 38°C. At the higher temperatures EOF decreased which showed more osmotically resistant erythrocytes as temperature increased. This effect was found in all the ectothems and endotherms investigated. Along with EOF data presented for some mammals, birds, reptiles, and amphibians, general blood measurements were taken including packed cell volume, red blood cell counts, hemoglobin concentrations, and nucleus to cell perimeter ratios when a nucleus was present. These blood measurements were taken to provide baseline information of the

hematology of these organisms as well as to see if any of these blood properties had an effect on EOF. A significant effect was shown with mean cell volume (MCV) explaining 50% of the variation that was seen in EOF.

An Investigation of Factors Influencing Erythrocyte Osmotic Fragility Among Selected
Ectothermic and Endothermic Vertebrates

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By

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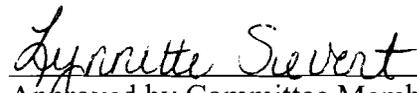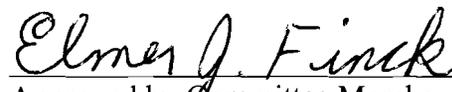
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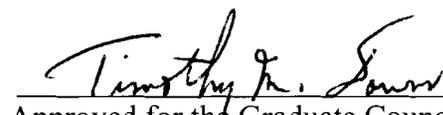
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PREFACE

My thesis was written in the style according to the instructions for submission to the Journal of Experimental Zoology.

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Introduction

Erythrocyte Osmotic Fragility (EOF) is a quantitative measurement of erythrocyte strength and its ability to withstand a variety of osmotic gradients. Basically, EOF determinations are performed by placing a blood sample in a variety of different solutions that range from isotonic to hypotonic, relative to the internal environment of the erythrocyte. Each of the solutions is evaluated for the amount of hemoglobin that is released from the lysing erythrocytes and the percent hemolysis is calculated for each of the solutions tested. From these values the erythrocyte osmotic resistance for a particular blood sample can be determined.

Those erythrocytes that lyse at relatively low osmotic gradients, or NaCl concentrations close to the osmolarity of their blood plasma, are weaker or less osmotically resistant. These weaker erythrocytes will thus show higher EOF values, which correspond to the NaCl concentration where fifty percent hemolysis occurs. Stronger erythrocytes however, will show lower EOF values because they can withstand lower NaCl concentrations, and larger osmotic gradients.

If the erythrocytes of any individual are compromised or unable to cope with the current osmotic conditions and the membranes of the erythrocytes begin to break apart there will be serious health risk to that individual. The most obvious problem involves the oxygen delivery system that will become virtually useless as an individual's erythrocytes lyse. Another problem that can occur is disruption of blood flow to various internal organs caused by particles of lysed erythrocyte membranes that remain in the blood stream.

There are several factors that have been found to have an effect on EOF. For example, Kraus et al., ('97) showed that a zinc deficiency could increase the EOF of rats and that anti-oxidant supplements such as vitamin C and vitamin E were able to counter the effects of the zinc deficiency. Martinez et al., ('88) showed that exposure to CO_2 can also increase the EOF of carp (*Cyprinus carpio*). Oyewale ('94a) has shown that pH and temperature have an effect on EOF. Bacterial infection by *Aeromonas* and *Streptococcus* in rainbow trout (*Oncorhynchus mykiss*) has been shown to increase EOF (Barham et al., '80). Finally, it has been observed that aquatic musk turtles (*Sternotherus odoratus*) show significantly stronger erythrocytes or lower EOF values compared to dogs and sprague-dawley rats (David K. Saunders. Ph.D. personal communication). These results begged the investigation of the influence of inhabiting an aquatic/semi-aquatic habitat on erythrocyte resistance to osmotic gradients.

Vertebrates such as reptiles and amphibians have representatives that are quite terrestrial as well as others that are aquatic/semi-aquatic. For example, the ornate box turtle (*Terrapene ornata ornata*) is very terrestrial compared to the western painted turtle (*Chrysemys picta bellii*) (Conant and Collins, '98). The western painted turtle is exposed to the water for long periods of time, particularly during hibernation, and therefore its erythrocytes will likely be exposed to large osmotic gradients. This effect may be dampened by the relatively water impermeable skin of these reptiles, however, water uptake likely occurs over long periods of exposure to an aquatic environment (Ultsch and Wasser, '90; Ultsch et al. '00). Additionally, comparing the giant toad (*Bufo marinus*) that is terrestrial to the bullfrog (*Rana catesbeiana*) that is much more aquatic (Conant and Collins, '98), the same analogy fits as explained with the turtles except that in this

case both individuals have skin that is highly water permeable. In fact, both the giant toad and bullfrog uptake water directly through their skin via a pelvic patch (Hillyard et al., '98).

My study was designed to investigate two groups of ectotherms, terrestrial ectortherms and aquatic/semi-aquatic ectotherms, and compare their EOF values. Aquatic/semi-aquatic ectotherms experience very different environmental stresses than that of terrestrial ectotherms. Specifically, the aquatic/semi-aquatic ectotherms will have to cope with osmotic stresses (Schmidt-Nielsen, '90). While in their aquatic environment these ectotherms experience osmotic gradients that likely effect their erythrocytes, which causes these animals to run the risk of their erythrocytes lysing. To prevent the risk of lysing of the erythrocytes aquatic/semi-aquatic ectotherms, through natural selection pressures, potentially have evolved stronger, more osmotically resistant erythrocytes than terrestrial ectotherms. Therefore, I hypothesized that aquatic/semi-aquatic ectotherms would have stronger more osmotically resistant erythrocytes and thus lower EOF values than more terrestrial ectotherms.

Along with the investigation of EOF among aquatic and terrestrial vertebrates, a variety of blood parameters, including hemoglobin content (Hb), red blood cell counts (RBCC), packed cell volume (PCV), and erythrocyte size were determined and compared between species to look for additional hematological differences between terrestrial and aquatic/semi-aquatic ectotherms. Further, EOF values were compared between ectotherms and endotherms to investigate potential differences in the erythrocytes of these vertebrates.

Methods and Materials

Blood was collected from a variety of animals including mammals, reptiles, amphibians, and birds. The mammals included the eastern wood rat (*Neotoma floridana*), the cotton rat (*Sigmodon hispidus*), and sprague-dawley rat (*Rattus norvegicus*). There were two groups of reptiles; the terrestrial species including the red-sided garter snake (*Thamnophis sirtalis parietalis*) and the ornate box turtle, and the aquatic/semi-aquatic species the western painted turtle, and red-eared slider (*Trachemys scripta elagans*). The amphibians were separated into two groups; the more terrestrial species, the giant toad and the aquatic species, the bullfrog and neotenic tiger salamanders (*Ambystoma tigrinum*). Finally, the birds only included three individuals of domestic chicken (*Gallus gallus*) donated by Roger Ferguson.

The aforementioned animals were collected locally in Lyon County Kansas except for the tiger salamanders and the giant toad, which were ordered from Kon's Scientific (Germantown, WI). After the blood samples were collected, each locally captured animal was given a minimum of 48 hours to recover before it was released back to their place of capture. All the collecting was done under the collecting permits of Dr. Lynnette Sievert and Dr. Elmer J. Finck.

Before the blood could be collected the reptiles were anesthetized with Ketamine at a dose of 80mg/kg injected intramuscularly (Burk, '86). The neotenic salamanders were immersed in MS-222 at a concentration of 100mg/ L. For anesthetizing the mammals a minimum of 1ml of Halothane was placed into a small container with the enclosed animal. For the chickens, where venapuncture was achievable, there was no anesthetic used.

After the animals were anesthetized, blood was collected via heart puncture or other less intrusive means when possible and placed into a heparinized vacutainer. However, in most cases, heart puncture was preferred because it reduced the risk of the blood samples becoming contaminated with other fluids that could affect later measurements. Heart puncture was also preferred because it provided the best procedure for obtaining sufficiently large blood samples and it was often done without killing the animal. In most cases 1ml of blood was sufficient for EOF analysis, although to obtain a more thorough analysis that included PCV, Hb, RBCC, and blood viscosity, a sample of 3ml or more was often taken. If an insufficient blood sample would not allow for all the above tests, blood viscosity measurements were omitted as it requires more blood and was not critical for my study.

For the determination of EOF, a micro method was used as well as the method described by Harmening ('97). Using the later method, several (usually 10 to 12) NaCl solutions ranging from 0.85% NaCl to 0.0% NaCl were used. The solution sets always included a 0.0% NaCl solution (distilled water) for complete erythrocyte lysing and a 0.85% NaCl solution that should have no erythrocyte lysing, because it is at or close to the osmolarity of the plasma. The rest of the solutions contained varying concentrations of NaCl to create a gradient such that the NaCl concentration that initiated hemolysis through the NaCl concentration that resulted in complete hemolysis could be observed. Each test tube contained a total of 2.5ml of NaCl solution.

Blood samples were vortexed to ensure that they were well mixed and immediately after vortexing 50μl of blood was added to each of the solutions, then allowed to sit for 30 minutes. After 30 minutes the solutions were gently shaken for

approximately 2 minutes and then centrifuged for 5 minutes at 2000 RPM's by using a Fisher Scientific Micro 14 or Beckman Model TJ-6 centrifuge. The centrifugation pelleted out any unlysed blood cells as well as any cell membranes from lysed erythrocytes, which left the supernatant of the NaCl solution along with any hemoglobin from lysed erythrocytes. The supernatants were analyzed by using a Milton Roy Spectronic 301 Spectrophotometer set at 540nm wavelength to attain relative values of hemoglobin in the supernatant. The following equation was used to find the percent lysis at each NaCl solution used (Harmening, '97):

$$\% \text{ Hemolysis} = \frac{OD(x) - OD\ 0.85\%}{OD(o) - OD\ 0.85\%}$$

OD stands for the optical density or absorbance while (x) is the solution from which the percent hemolysis was obtained and (o) was the absorbance of the 0% NaCl solution.

The micro method was done exactly as described above, except that with this method only 1ml of each NaCl solution was used and only 20µl of blood sample was added to each solution. This method was used due to the inability to obtain sufficiently large blood samples from some of the animals. Both, the macro and micro methods showed similar results when run together on the same animals, thereby, justifying comparisons between methods.

Graphs were made for each blood sample, which compared hemolysis and NaCl concentration. From these graphs, which were all sigmoid curves, the percent NaCl concentration at 50% hemolysis for each individual was determined. The NaCl concentrations at which 50% hemolysis occurred were then compared by using Sigma

Stat 2.0 to run a one way analysis of variance test. Differences were considered to be significant at $P \leq 0.05$.

In addition to the testing of EOF, PVCs (% volume of erythrocytes in the blood) were determined by using the microhematocrit method for each organism. Hemoglobin content (Hb) of the blood was determined by using the cyanomethemoglobin method (Sigma Chemicals, St. Louis, MO). Additionally, red blood cell counts (RBCC) were done by diluting the blood sample in a 0.9% NaCl solution and counting the red blood cells by using a hemocytometer. Mean cell volume (MCV) was calculated by dividing the PCV by the RBCC.

Results

The PCV, Hb, RBCC, and MCV are shown in Table 1. All the values given are the mean of the measurements for all individuals of that species with one standard deviation of the mean shown below each mean. The amphibians and reptiles had significantly lower values for PCV, Hb, and RBCC than did the mammals in this study. In contrast, the ectotherms had a significantly greater MCV as compared to the ectotherms investigated.

The NaCl concentration at which 50% lysing occurred is shown in Table 2 for each species investigated. Initial use of a one way analysis of variance found significant differences among groups at a P-value of <0.001. To determine specifically which species differed, a Tukeys test was performed. Those species that do not share a horizontal row or vertical column were found to be significantly different with a Tukeys test, at a P-value of <0.05. For example the sprague-dawley rat and the eastern wood rat share a vertical column and thus the NaCl concentrations at which 50% hemolysis occurred were not significantly different. The same is true for the sprague-dawley rat and the cotton rat that both share the same horizontal row. However, the cotton rat and the eastern wood rat do not share a horizontal row or vertical column and thus the NaCl concentrations at which 50% hemolysis occurred were found to be significantly different at a P-value of <0.05. The bullfrog and the neotenic tiger salamander, which have the lowest EOF, or strongest erythrocytes, share a horizontal row and both are significantly different from all the other species investigated. Note that those species that are at the top of the table have the higher EOF values and thus their erythrocytes are the weakest or

Table 1. Various blood parameters of the species investigated (means ± SD). Abbreviations as follows: PCV, packed cell volume; Hb, hemoglobin concentration; RBCC, red blood cell count; MCV, mean cell volume.

Species	PCV (%)	Hb (g/dl)	RBCC (RBC/mm^3)	MCV (μm^3)	Number of Species
Bullfrog	20.6 ± 3.6	4.57 ± 1.08	2.65 x 10^5 ± 4.98 x 10^4	785.4 ± 101.5	13
Tiger Salamander	31.6 ± 6.8	4.23 ± 1.39	1.70 x 10^5 ± 5.34 x 10^4	1881.6 ± 225.0	9
Giant Toad	26.7 ± 7.1	4.59 ± 1.72	5.53 x 10^5 ± 1.74 x 10^5	497.5 ± 71.6	8
Ornate Box Turtle	22.2 ± 8.6	6.29 ± 1.82	7.85 x 10^5 ± 4.32 x 10^4	286.9 ± 114.6	3
Western Painted Turtle	16.7 ± 5.1	5.42 ± 2.23	4.51 x 10^5 ± 1.44 x 10^5	374.6 ± 58.7	5
Red-eared Slider	25.5 ± 9.2	7.40 ± 2.02	6.61 x 10^5 ± 2.34 x 10^5	385.1 ± 29.0	3
Red-sided Garter Snake	28.3 ± 2.1	8.61 ± 0.44	8.99 x 10^5 ± 1.61 x 10^5	322.6 ± 62.4	6
Chicken	24.2 ± 3.3	7.34 ± 1.12	2.22 x 10^6 ± 4.40 x 10^5	110.9 ± 22.3	3
Cotton Rat	39.6 ± 2.9	11.51 ± 3.02	5.29 x 10^6 ± 9.68 x 10^5	76.4 ± 9.7	7
Sprague-Dawley Rat	45.7 ± 3.09	16.36 ± 2.35	8.35 x 10^6 ± 1.66 x 10^6	56.0 ± 8.5	5
Eastern Woodrat	39.7 ± 3.9	11.45 ± 1.72	7.42 x 10^6 ± 1.02 x 10^6	54.1 ± 62.4	9

Table 2. Mean % NaCl concentration, and mosM in parentheses, at which 50% lysing occurred. Those animals that do not share a horizontal row or vertical column are significantly different ($p < 0.05$).

0.456 (156) **Eastern Woodrat**			
0.443 (152) **Sprague Dawley Rat**	**0.391 (134)** **Cotton Rat**		
	0.332 (110) **Chicken**	**0.278 (95)** **R.S.G. Snake**	
		0.234 (80) **R. E. Slider**	**0.189 (65)** **0. 216 (74)** **0.227 (78)** **Giant Toad O. Box Turtle W. P. Turtle**
0.119 (41) **Bullfrog**	| **0.145 (50)** **Neotenic Tiger Salamander**		

the least osmotically resistant. Those species that are lower in the table have lower EOF values and their erythrocytes are stronger or more osmotically resistant.

A linear regression model compared the effect of erythrocyte volume on EOF (Figure 1). The relationship was significant at a $P<0.05$. The regression model shows that as erythrocyte sizes increase, a resulting decrease occurs in the EOF value ($R = 0.707$ and $R^2 = 0.500$). Thus, the larger erythrocytes tend to be the stronger or more osmotically resistant. After further investigation of the effect of erythrocyte size on EOF it seemed that a cubic polynomial regression model might better explain the data (Figure 2). The cubic polynomial model makes more sense because volume is also a cubic function. Figure 3 shows the same cubic function without the tiger salamander erythrocytes to show how well remaining data fit this model.

Figure 1. Linear regression of mean cell volume (MCV) and % NaCl concentration at which 50% hemolysis occurred. Species labeled as follows: a, neotenic tiger salamander; b, bullfrog, c, giant toad; d, ornate box turtle; e, western painted turtle; f, red-eared slider; g, red-sided garter snake; h, chicken; I, cotton rat; j, Spague-Dawley rat; k, eastern woodrat.
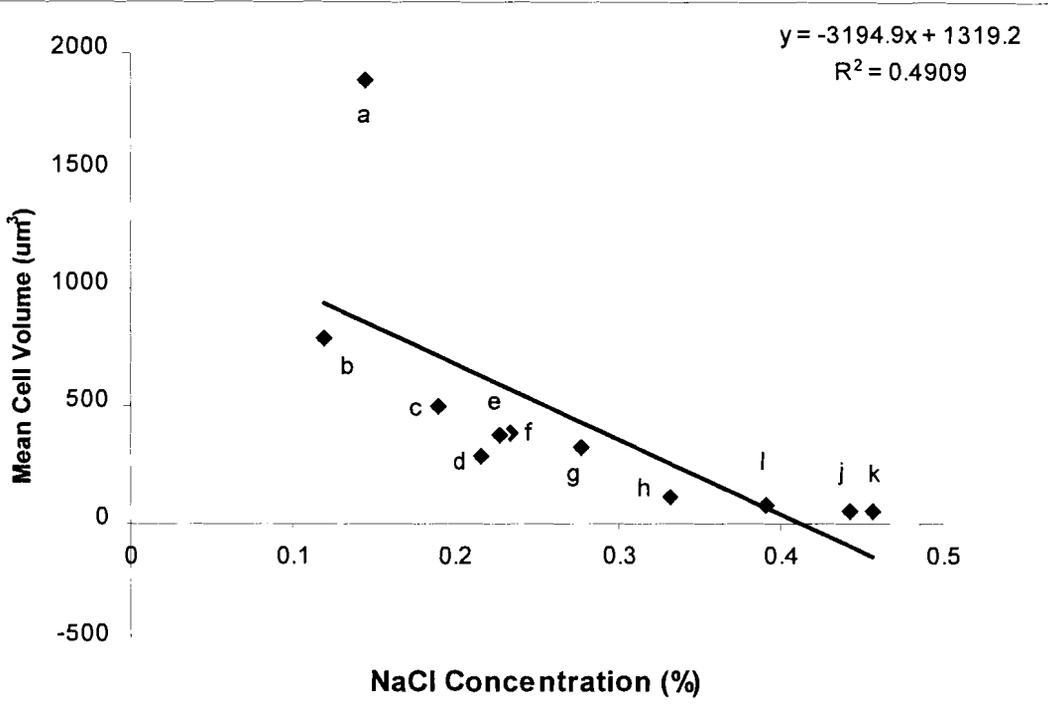
Figure 2. Cubic regression of mean cell volume (MCV) and % NaCl concentration at which 50% hemolysis occurs. Species labeled as follows: a, neotenic tiger salamander; b, bullfrog, c, giant toad; d, ornate box turtle; e, western painted turtle; f, red-eared slider; g, red-sided garter snake; h, chicken; I, cotton rat; j, Spague-Dawley rat; k, eastern woodrat.

$$y = -8195.1x^3 + 24130x^2 - 15116x + 2736.2$$
$$R^2 = 0.6154$$
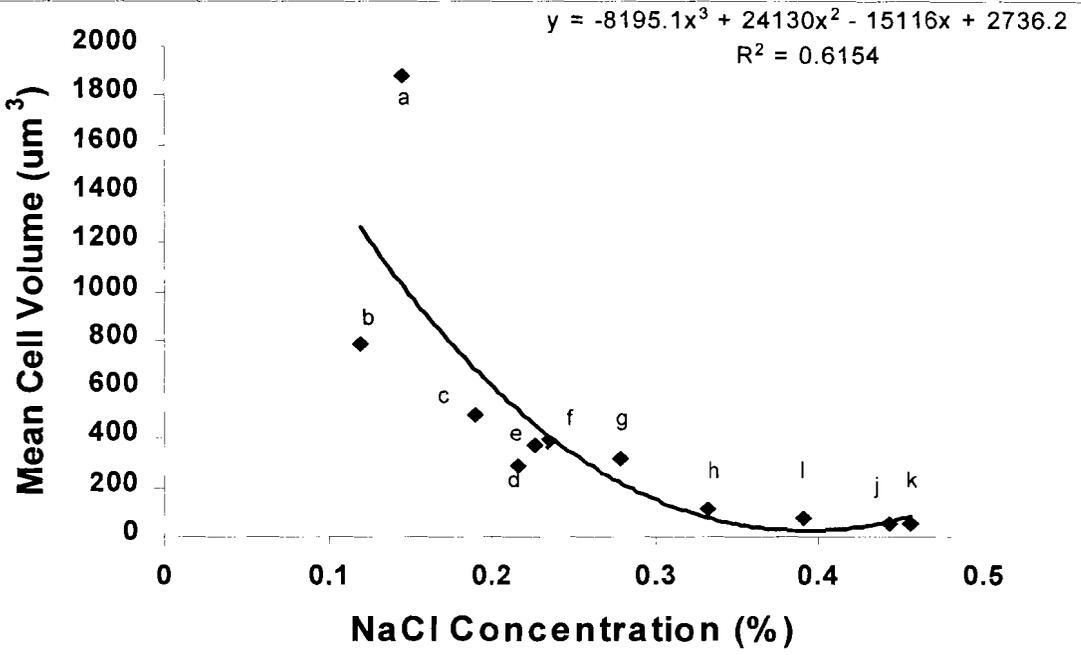
Figure 3. Cubic regression of mean cell volume (MCV) and % NaCl concentration at which 50% hemolysis occurred without the tiger salamander. Species labeled as follows: a, neotenic tiger salamander; b, bullfrog, c, giant toad; d, ornate box turtle; e, western painted turtle; f, red-eared slider; g, red-sided garter snake; h, chicken; I, cotton rat; j, Spague-Dawley rat; k, eastern woodrat.

$y = -9284.4x^3 + 14669x^2 - 8032.5x + 1540.7$
$R^2 = 0.9549$
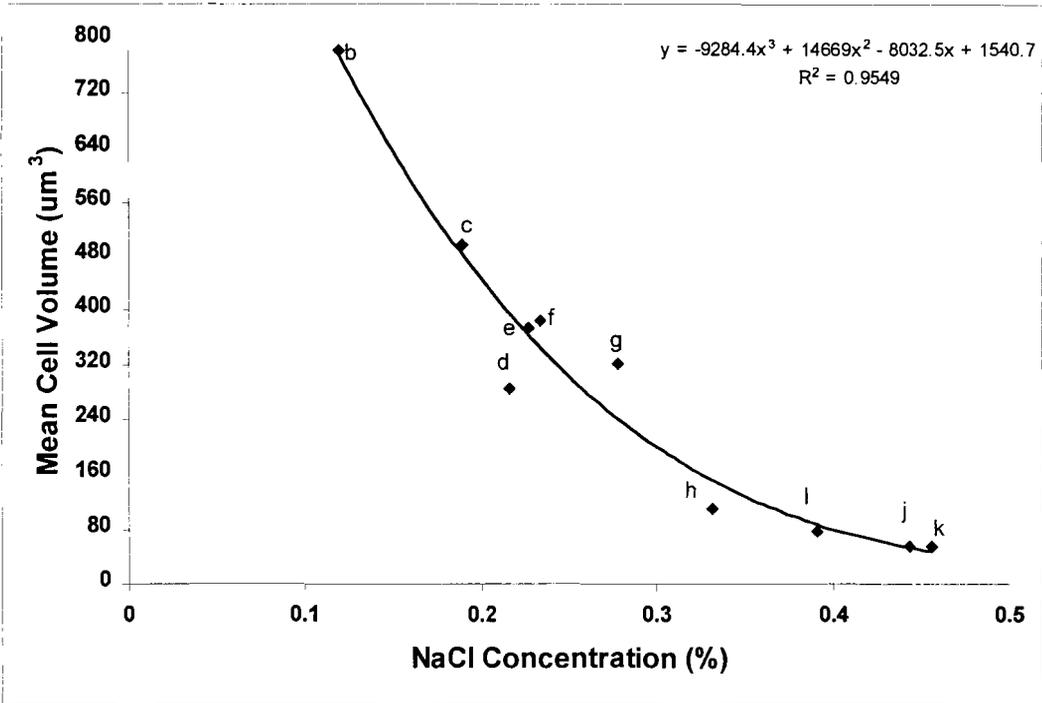
Discussion

The erythrocytes of reptiles (Oyewale, '94a,b) and amphibians (Lake et al., '77; Costanzo and Lee, '91) are more osmotically resistant than those of mammals (Perk et al., '64). The purpose of my study was to investigate if such differences were the result of the aquatic nature of many amphibians and reptiles. As such I hypothesized that differences in EOF would be seen when comparing aquatic ectotherms to terrestrial ectotherms. This hypothesis was only partially supported. The more aquatic amphibians, the bullfrog and neotenic tiger salamander, had more osmotically resistant erythrocytes compared to the more terrestrial amphibian, the giant toad. This was not the case with the reptilian species investigated. The two semi-aquatic reptiles, the red-eared slider and western painted turtle, did not show a significant decrease in EOF, compared to the much more terrestrial ornate box turtle. These results raise the question of why the amphibians seem to fit the hypothesis and the reptiles investigated do not.

There are several possible explanations for the conflicting results. One reason is that the erythrocytes of the reptiles while in their aquatic environment are not experiencing large variation in osmotic gradients. Ultsch et al., ('98) suggested that turtles that use integumentary gas exchange will in turn enhance osmotic uptake of water. Therefore, a turtle such as the red-eared slider that does not depend greatly on integumentary gas exchange during short dives (Ultsch and Wasser, '90) might have skin that is relatively impermeable to water. However, with prolonged submergence during hibernation these turtles do have significant water uptake (Ultsch, '89).

Amphibians have skin that tends to be more water permeable than that of the reptiles. Most amphibians acquire water by direct absorption through the skin rather than

by drinking (Hillyard et al., '98), therefore, the erythrocytes of amphibians in an aquatic environment will probably experience large osmotic gradients. Those amphibians that are often exposed to aquatic environments should experience natural selection pressures to develop stronger more osmotically resistant erythrocytes. As such, this might explain why the amphibians do fit the hypothesis that the more aquatic species will have the more osmotically resistant erythrocytes.

Additionally, reptiles may not be showing any effect of an aquatic environment on erythrocyte strength as they might have developed relatively strong erythrocytes to cope with environmental influences other than osmotic stresses. For example, reptiles may have developed strong erythrocytes to cope with the effect of temperature variations that their erythrocytes will invariably experience. A decrease in temperature may increase EOF values, reducing erythrocyte osmotic resistance in some species (Oyewale, '94a). As such, possessing erythrocytes that are highly resistant to osmotic fluxes may be beneficial to ectotherms during cold temperature exposure. Table 2 shows most ectotherms in my study possessed erythrocytes that are highly osmotically resistant relative to the endotherms investigated.

The variation in erythrocyte size in animals is extensive (Chien et al., '71). The reason for this tremendous size variation is somewhat unclear. Snyder and Sheafor ('99) suggested that observed differences in erythrocyte size were due to the variation in vessel size. Erythrocytes must be able to deform as they pass through increasingly smaller vessels. The deformation of these erythrocytes aids in the release of oxygen to the surrounding tissues. Therefore if the vessels are large, the erythrocytes also will tend to be larger to aid in the release of oxygen.

In my study, organisms that had larger erythrocytes tended to be those that also had the more osmotically resistant erythrocytes. A significant relationship between erythrocyte size and EOF was found (Figure 1). Fifty percent of the variation in EOF can be explained by changes in erythrocyte size with the linear regression. However, with the cubic regression 61.5 % of the variation in is explainable by changes in erythrocyte size (Figure 2). More specifically, the larger the erythrocyte the more osmotically resistant it tended to be. Olowookorun and Makinde, ('98) who investigated EOF of ostrich (*Struthia camelus*) and domestic chicken (*Gallus gallus*) found similar results. The reason for this size effect is likely due to the membrane of the erythrocytes being like a plastic bag, the erythrocyte is flexible but not very elastic (Harmening, '97). As pressure increases in the erythrocyte from water influx, the erythrocyte starts to change from a disk shape to a spherical shape. As this change occurs, the pressure within the erythrocyte will begin to decrease. With the change in shape of a larger erythrocyte the decrease in internal pressure will be larger thus allowing the erythrocyte to better withstand a larger osmotic gradient. Therefore, erythrocyte size could be an evolutionary adaptation for coping with osmotic pressures as well as a response to arteriole size.

Although erythrocyte size has a significant effect and can explain 61.5% of the variation in EOF, 39.5% of the variation must be explained by other factors. One such factor is the membrane composition. Norman and Dewey ('85) suggested that EOF might be correlated with relative quantities of membrane proteins such as spectrin. With more spectrin the membrane tends to be more flexible, thus affecting EOF (Baumann and Sowers, '96). Another factor affecting EOF could be the differences in erythrocyte life span between ectotherms and endotherms. For example, reptilian erythrocytes seem to

have a much longer life span, compared to mammalian erythrocytes, with a life of reptilian red blood cells lasting about 600-800 days (Sypek and Borysenko, '88). The long life span of the reptilian red blood cell might be due to their low metabolism (Sypek and Borysenko, '88). However, it might be that ectothemic red blood cells have different membrane compositions making them stronger and more durable, thus, allowing them to last longer, in addition to making them more osmotically resistant.

My original hypothesis of more aquatic ectotherms having more osmotically resistant erythrocytes was supported only with the amphibians tested. Yet even the terrestrial amphibians investigated in my study, the giant toad, possessed erythrocytes that were much more osmotically resistant relative to the endotherms examined. This coupled with the lack of a significant effect of aquatic environment on reptilian erythrocyte osmotic resistance suggested that exposure to an aquatic environment might not play a major role in the development of erythrocyte osmotic resistance in ectotherms. Because the ectothermic vertebrates investigated tended to have stronger erythrocytes than those of the endothermic vertebrates in my study it is possible that temperature range exposure or erythrocyte life span may be an important determinant of EOF, with longer erythrocyte life span and exposure to low temperatures correlating with increased osmotic resistance. Finally the relationship of erythrocyte size showed that those organisms with large erythrocytes tend to have more osmotically resistant erythrocytes.

Chapter 2

Introduction

Ectothermic vertebrates experience a wide range of body temperatures, both daily and seasonally (Pough et al., '98; Brattstrom, '65), while most non-hibernating endothermic vertebrates rarely experience such fluctuations in body temperature. These temperature fluctuations likely cause physiological stress on the erythrocytes of these animals. Cossins and Lee ('85) suggested that at lower temperatures, passive permeability of the skin might become enhanced, leading to erythrocyte exposure to increased osmotic gradients. If freeze-tolerant frogs are considered, the risk of damage to erythrocytes seems high. However, these animals are obviously able to survive despite the exposure to extreme temperature variation (Costanzo and Lee, '91). Some studies have shown that ectotherms often have more osmotically resitstant erythrocytes than endotherms (Lake et al., '77; Costanzo and Lee, '91;).

Martinez et al., ('88) found that erythrocytes of one ectotherm, the carp (*Cyprinus carpio*) experienced no significant change in EOF (increased erythrocyte osmotic resistance) at temperatures of 5 °C, 11 °C, and 20 °C. Thus ectotherms might have more osmotically resistant erythrocytes to deal with wide variations in body temperature. However, Oyewale ('94a) found that the erythrocytes of the African Toad (*Bufo regularis*) showed a significant decrease in erythrocyte osmotic fragility or EOF with an increase in temperature.

The following comparative investigation was performed to determine if there was a significant effect of temperature on the EOF of ectotherm and endotherm erythrocytes. Specifically, differences in EOF within and among some ectothemic species and some

endothermic species were investigated. I hypothesized that the erythrocytes of

ectothermic species would be less affected by changes in temperature than endothermic

erythrocytes since ectotherms are more likely to have developed physiological responses

to deal with body temperature changes that are associated with their environment.

Methods and Materials

Blood was collected from a variety of mammals and amphibians. The mammals included the eastern wood rat (*Neotoma floridana*), the cotton rat (*Sigmodon hispidus*), and sprague-dawley rat (*Rattus norvegicus*). The amphibians included the more terrestrial species, the giant toad (*Bufo marinus*) and the more aquatic species, the bullfrog (*Rana catesbeiana*). All animals were collected locally from Lyon County Kansas except for the giant toad, which was ordered from Kon's Scientific (Germantown, WI). All the collecting was done under the collecting permits of Dr. Lynette Sievert and Dr. Elmer J. Finck.

Before any blood samples were taken each animal was properly anesthetized. For anesthetizing the cotton rat and sprague-dawley rat a minimum of 1ml of Halothane was placed into a small container with the enclosed animal. An intramuscular injection of Ketamine of 80mg/kg was used to anesthetize the eastern wood rat. The bullfrog and giant toad were cooled in a refrigerated room and pithed before blood samples were taken.

After the animals were anesthetized, blood was collected via heart puncture and placed into a heparinized vacutainer. Blood was collected from the endothermic animals at their normal body temperatures (approximately 38^0C) while blood from the ectotherms were collected while the animals were at room temperature (approximately 25^0C). Heart puncture was used because it reduces the risk of the blood samples becoming contaminated with other fluids that could affect later measurements. Heart puncture also provides the best procedure for obtaining a sufficiently large blood sample. In most cases 1ml of blood was sufficient for EOF analysis, although to obtain a more thorough

analysis that included packed cell volume, hemoglobin content, and red blood cell counts, a sample of 3ml or more was often taken.

For the determination of EOF, the method described by Harmening ('97) was modified by decreasing the amount of solution from 2.5ml to 1ml and the amount of blood sample from 50μl to 20μl. Using this method, several (usually 10 to 12) NaCl solutions ranging from 0.85% NaCl concentration to 0.0% NaCl (distilled water) were used. Three sets of solutions were made for each animal investigated, with each solution set incubated at 5 °C, 25 °C, and 38 °C for at least 30 minutes prior to the addition of any blood sample. These temperatures were chosen to simulate a temperature range from a low of 5 °C that might be experienced by some of the ectotherms to a high temperature of 38 °C, the approximate normal body temperature for the endotherms investigated. Each solution set always included a 0.0% NaCl solution (distilled water) for complete erythrocyte lysing and a 0.85% NaCl solution that should cause no erythrocyte lysing, because it is at or close to the osmolarity of the animal's plasma. The rest of the solutions contained varying concentrations of NaCl to create a gradient such that the NaCl concentration that initiated hemolysis through the NaCl concentration that resulted in complete hemolysis could be observed. Each test tube contained a total of 1ml of NaCl solution.

After the NaCl solution set was made the blood sample was vortexed to ensure that it was well mixed, immediately after vortexing 20μl of blood was added to each of the solutions for all three solution sets. The solutions were then placed back into the appropriate water bath set at 5 °C, 25 °C, or 38 °C and allowed to sit for 30 minutes. After 30 minutes the solutions were gently shaken and centrifuged for 5 minutes at 2000

RPM's with a Fisher Scientific Micro 14 or Beckman Model TJ-6 centrifuge. The centrifugation pelleted out any unlysed blood cells as well as any cell membranes from lysed erythrocytes, which leaves the supernatant of the NaCl solution along with any hemoglobin from lysed erythrocytes. The supernatants were analyzed with a Milton Roy Spectronic 301 Spectrophotometer set at 540nm wavelength, to attain relative values of hemoglobin in the supernatant. The following equation was used to find the percent lysis at each NaCl solution used (Harmening, '97):

$$\% \text{ Hemolysis} = \frac{OD(x) - OD\ 0.85\%}{OD(o) - OD\ 0.85\%}$$

OD stands for the optical density or absorbance while (x) is the solution from which the percent hemolysis was obtained and (o) is the absorbance of the 0% NaCl solution.

Graphs were made for each blood sample by comparing hemolysis and NaCl concentration. From these graphs, which were all sigmoid curves, the percent NaCl concentration at 50% hemolysis for each individual was determined. The NaCl concentrations at which 50% hemolysis occurred were then compared at all three temperatures with Sigma Stat 2.0 to run a two way analysis of variance test on the three different temperatures for all animals investigated. Differences were considered to be significant at $P \leq 0.05$.

Results

There was a significant effect of species on EOF (P < 0.001 DF= 5). The effect of

temperature on EOF was also shown to be significant (P < 0.001 DF= 5). Erythrocytes in

the 5 °C solutions showed the least osmotic resistance and the erythrocytes placed in the

solutions at 25 °C showed more osmotically resistant erythrocytes with those in the

solutions at 38 °C showing the most osmotic resistance (Table 3). The same trend was

observed with all species investigated and no interaction of species and the temperature

effect was shown. Thus, different species did not show a significant difference in the

occurrence or magnitude of the effect of temperature on EOF (P = 0.983 DF= 5). The

percent decrease in EOF from 5 °C to 25 °C was 10.0% for the bullfrog, 8.5% for the

giant toad, 4.3% for the cotton rat, 6.4% for the sprague-dawley rat, and 6.1% for the

eastern wood rat. The percent decrease found from 25 °C to 38 °C was 6.8% for the

bullfrog, 1.0% for the giant toad, 1.5% for the cotton rat, 2.9% for the sprague-dawley

rat, and 1.9% for the eastern wood rat.

Table 3. Mean EOF value (%NaCl concentration at 50% hemolysis) for each species studied at 5 °C, 25 °C, and 38 °C.

Species	5 °C	25 °C	38 °C	
Bullfrog	0.130	0.117	0.109	
Giant Toad	0.212	0.194	0.192	
Cotton Rat	0.420	0.402	0.396	
Sprague-Dawley Rat	0.471	0.441	0.428	
Eastern Wood Rat	0.492	0.462	0.453	

Discussion

The results suggested that temperature does have an effect on EOF. Specifically, there appears to be an inverse relationship between temperature and EOF and thus the erythrocytes are more osmotically resistant at higher temperatures. The opposite is also true that as temperature is decreased the EOF values increase. Temperature is known to affect properties such as enzyme activities, membrane pumps, as well as membrane flexibility and thus these results are not surprising (Lodish et al., '95).

Perhaps the most interesting thing about my results is that there was no significant interaction between the species and the effect of temperature on EOF. In fact, regardless of the species investigated, whether endothermic or ectothermic, all showed the same effect of temperature on EOF. This suggests that the erythrocytes of the ectotherms and endotherms used in the study do not possess physiological mechanisms to maintain osmotic resistance relative to temperature fluctuations. However, it is possible that ectotherms kept at a specific ambient temperature may have adaptations in their erythrocytes, making them more resistant to osmotic lysing at that ambient temperature. For example, if adaptation in the erythrocytes were to occur, ectotherms kept at 5^0C should have erythrocytes that would be more osmotically resistant than the erythrocytes of the same animal kept at 25^0C when EOF is compared at 5^0C. Yet, the data collected from this study would suggest that such adaptation is likely unnecessary as the erythrocytes of ectotherms already possess relatively high osmotic resistance.

A possible explanation for the lack of a maintained erythrocyte osmotic resistance of ectotherms to temperature fluctuation is that there is no apparent need for such mechanisms. As shown in Table 3, the ectotherms have drastically lower EOF values

than the endotherms. The erythrocytes of ectotherms may be sufficiently strong such that the slight increase in EOF at low temperatures is not large enough to cause damage to their erythrocytes at the normal osmotic gradients they experienced in nature. As such, the erythrocytes of ectotherms, due to their strong osmotic resistance, would require no additional mechanisms of protection from lysing under low temperature exposure. These findings are simular to those of Oyewale ('94a) for the African toad.

The high osmotic resistance of amphibian erythrocytes may benefit these animals during cold exposure like that experienced during hibernation. Many of these amphibians such as the spring peeper (*Pseudacris crucifer*), western chorus frog (*Pseudacris triseriata*), and gray treefrog (*Hyla versicolor*) will actually experience temperatures below 0 °C during hibernation (Pough et al, '98). Possibly similar results would be seen comparing hibernating and non-hibernating endotherms. Hibernating animals might use physiological mechanisms to increase erythrocyte osmotic resistance.

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An Investigation of Factors Influencing Erythrocyte Osmotic Fragility Among Selected

Ectothermic and Endothermic Vertebrates
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