

AN ABSTRACT OF THE THESIS OF

Nicole Marie Palenske for the Master of Science

in Biology presented on June 27, 2002

Title: Blood viscosity and hematological parameters in hibernating bullfrogs,

Rana catesbeiana

Abstract approved:

A handwritten signature in black ink that reads "David K. Saunders". The signature is written in a cursive style and is positioned over a horizontal line.

Many amphibians experience low temperature conditions associated with hibernation. Decreasing temperature results in increased viscosity, thereby potentially affecting blood flow. Therefore, amphibians might have difficulty maintaining blood flow at low temperatures. As bullfrogs hibernate underwater, the hibernation microenvironment may have low oxygen levels making it hard for them to oxygenate tissues. The purpose of this study was to evaluate hematological properties and blood viscosity in hibernating bullfrogs (*Rana catesbeiana*), a species better able to extract oxygen from its aquatic environment than some other hibernating ectothermic species. Blood was collected from bullfrogs submerged in aerated water for 20 or 50 days at 5°C, as well as 0 day bullfrogs exposed to 5°C but not submerged. A group of bullfrogs kept at 25°C served as a room temperature, nonhibernating group for comparison. No significant differences were found in hematocrit, hemoglobin, red blood cell count (RBCC), or mean cell volume (MCV) among the four groups of bullfrogs. Mean cell hemoglobin (MCH) showed a significant decrease ($P=0.001$) in the 0 and 20 day submerged bullfrogs compared to 50 day submerged bullfrogs. The values for mean cell hemoglobin concentration (MCHC) were significantly lower ($P=0.001$) in 20 day submerged bullfrogs relative to 0 and 50 day submerged bullfrogs. Apparent viscosity,

plasma viscosity, and relative viscosity (apparent viscosity/plasma viscosity) measurements were obtained and showed no significant differences among the four groups. Plasma osmolality significantly decreased ($P < 0.001$) in 5°C groups relative to the 25°C group. The results of this study suggest bullfrogs are able to extract sufficient amounts of oxygen from well-aerated water, negating the need of any hematological increases. However, because of the initial increase in hematocrit and decrease in plasma osmolality of 0 day bullfrogs, it is possible that bullfrogs in this study are trying to maintain an optimal hematocrit during hibernation.

Blood Viscosity and Hematological Parameters in
Hibernating Bullfrogs, *Rana catesbeiana*

A Thesis

Presented to

The Department of Biological Sciences

Emporia State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

By

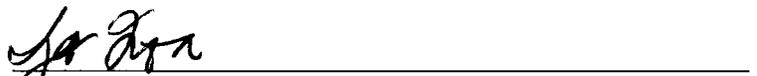
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August 2002

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Approved by Major Advisor


Approved by Committee Member


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Approved by the Department Chair


Approved by the Dean of Graduate Studies and Research

ACKNOWLEDGMENTS

I would like to thank my major advisor, Dr. David Saunders, for providing me with tremendous help and guidance throughout the entire process of completing my thesis. I appreciated his ability to listen and give support to me every step of the way. Additionally, I thank my committee members, Dr. Lynnette Sievert and Dr. Dwight Moore, for their ideas and advice. I would also like to thank Kevin Aldrich, Celestine Wanjalla, Myoung-Gwi Ryou, Sean Daly, and Sarah Swafford for their help and assistance in the lab. Thanks also to Roger Ferguson who helped with the construction of the submergence tanks and to Juanita Bartley and the biology student secretaries for always letting me borrow the master key. A special thanks to Megan Kearney, for being a wonderful friend. Her help, support, and encouragement along the way made the days bearable. I can't imagine anyone else to have been in graduate school with. This thesis would not be complete without the use of the words deleterious and ubiquitous; two words that I have become very familiar with in my numerous readings of primary literature.

My family deserves special thanks as well. They listened to me and tried to understand things the best they could. I love them all very much and I appreciate all the support they have given me. I especially thank my Mom, Sylvia Palenske, for being such a strong person who has been through so much. She put up with me and offered to do my laundry this past semester so I could devote more time to classes and my thesis. She truly is a remarkable person. Lastly, I would like to thank my Dad, Marvin Palenske. Although he is not here to see all that I have accomplished these past few years, I know that he would be very proud of me. I only wish he could be here to see this.

PREFACE

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

TABLE OF CONTENTS

	<u>Page</u>
ACKNOWLEDGMENTS	iii
PREFACE.....	iv
TABLE OF CONTENTS.....	v
LIST OF FIGURES.....	vi
INTRODUCTION.....	1
METHODS AND MATERIALS.....	5
RESULTS.....	9
DISCUSSION.....	38
LITERATURE CITED.....	46

LIST OF FIGURES

	<u>Page</u>
Figure 1. Mean hematocrit values (\pm SD) of bullfrogs at room temperature (RT), 0, 20, and 50 days of submergence at 5°C.....	11
Figure 2. Mean hemoglobin values (\pm SD) of bullfrogs at room temperature (RT), 0, 20, and 50 days of submergence at 5°C.....	13
Figure 3. Mean RBCC values (\pm SD) of bullfrogs at room temperature (RT), 0, 20, and 50 days of submergence at 5°C.....	15
Figure 4. Mean MCV values (\pm SD) of bullfrogs at room temperature (RT), 0, 20, and 50 days of submergence at 5°C.....	17
Figure 5. Mean MCH values (\pm SD) of bullfrogs at room temperature (RT), 0, 20, and 50 days of submergence at 5°C. Those groups having the same letter are not significantly different at $P = 0.001$	19
Figure 6. Mean MCHC values (\pm SD) of bullfrogs at room temperature (RT), 0, 20, and 50 days of submergence at 5°C. Those groups having the same letter are not significantly different at $P = 0.001$	21
Figure 7. Mean plasma osmolality values (\pm SD) of bullfrogs at room temperature (RT), 0, 20, and 50 days of submergence at 5°C. Those groups having the same letter are not significantly different at $P < 0.05$	23
Figure 8. Apparent viscosity at 5°C versus hematocrit at a shear rate of 150 s ⁻¹ for bullfrogs at room temperature (RT), 0, 20, and 50 days of submergence at 5°C.....	25
Figure 9. Relative viscosity at 5°C versus hematocrit at a shear rate of 150 s ⁻¹ for bullfrogs at room temperature (RT), 0, 20, and 50 days of submergence at 5°C.....	27

Figure 10. Apparent viscosity at 5°C versus shear rate at a hematocrit of 30% for bullfrogs at room temperature (RT), 0, 20, and 50 days of submergence at 5°C.....	29
Figure 11. Relative viscosity at 5°C versus shear rate at a hematocrit of 30% for bullfrogs at room temperature (RT), 0, 20, and 50 days of submergence at 5°C	31
Figure 12. Mean apparent viscosity at 5°C versus submergence time with mean hematocrit values for bullfrogs at room temperature (RT), 0, 20 and 50 days of submergence.....	33
Figure 13. Mean plasma viscosity at 5°C versus submergence time for bullfrogs at room temperature (RT), 0, 20 and 50 days of submergence.....	35
Figure 14. Mean relative viscosity at 5°C versus submergence time with mean hematocrit values for bullfrogs at room temperature (RT), 0, 20 and 50 days of submergence.....	37

INTRODUCTION

As ectotherms, bullfrogs have the ability to survive a broad range of temperatures. At low temperatures, amphibians that hibernate in aerated water can survive submerged for long periods of time (Pinder et al., '92). Field studies of hibernating bullfrogs indicate that shallow, colder, and more oxygenated waters are preferred after an initial exposure period in the deeper, warmer and more hypoxic waters (Friet, '93). Additionally, ranid frogs, *Rana lessonae*, *Rana temporaria*, and *Rana ridibunda* have been investigated to determine their preference for hibernation habitats (Sinsch, '91). Sinsch ('91) found that if *R. lessonae*, *R. temporaria*, and *R. ridibunda* were given the choice between a terrestrial and aquatic habitat, a portion of each species chose terrestrial habitats while some chose aquatic habitats. The frogs in Sinsch's study could survive the terrestrial habitats because the soil of the terrestrial sites was mostly saturated with water and provided the frogs aquatic-like conditions.

Although submergence provides protection from freezing and desiccation for the non freeze-tolerant aquatic frog, other problems may occur because submerged frogs must rely entirely upon cutaneous respiration (Donohoe and Boutilier '98). Lower oxygen concentrations within bodies of water have been associated with winterkill of aquatic animals, including frogs due to their limited ability to tolerate extended periods of severe hypoxia (Greenbank, '45; Bradford, '83; Pinder et al., '92). Cutaneous gas exchange during normoxic cold submergence is sufficient to maintain metabolic requirements for periods of up to 4 months (Pinder et al., '92). Gas exchange of submerged frogs occurs through the skin and possibly the buccal cavity (Hutchison and Whitford, '66). Despite their dependence on cutaneous respiration, there is a small

amount of glycogen reserves used in liver and muscle suggesting some anaerobic metabolism (Donohoe et al., '98). Blood glucose levels appear to be higher in summer than in the winter (Wright '59).

Environmental conditions associated with hibernation, especially increasing blood viscosity, the ease at which blood flows through the body, could pose a threat to blood flow in bullfrogs. Blood viscosity is influenced by temperature, speed of blood flow, packed cell volume (hematocrit), red blood cell deformability, and plasma proteins (Chien et al., '71; Chien, '75; Fung, '81). Blood viscosity increases with decreasing temperature in a variety of vertebrate species, including *Chrysemys picta*, *Eudryptula minor*, and *Bufo woodhouseii* (Rand et al., '64; Snyder, '71; Langille and Crisp, '80; Clarke and Nicol, '93; Saunders and Patel, '98; Palenske and Saunders, in press), typically by about 3% for every 1°C decline in temperature (Merrill et al., '63; Guard and Murrish, '73). Plasma viscosity shows a similar trend of increasing 3% per 1°C decrease in temperature, similar to the change in viscosity of water (Harkness and Whittington, '70).

Some aquatic hibernators, such as musk turtles (*Sternotherus odoratus*), show significant increases in hematocrit, hemoglobin, red blood cell count (RBCC) and blood viscosity when exposed to 150 days of simulated hibernation (Saunders et al., '00). If increasing hematocrit occurs in frogs, it would increase viscosity thereby potentially influencing blood flow. Sinsch ('91) found that regardless of whether the frog species picked a terrestrial or aquatic environment in which to hibernate, the frogs showed a decrease in hematocrit and no significant difference in plasma osmolality over a 3 month period. The water content in these frogs increased only in *R. ridibunda*. In an

investigation of lymph organs of *Rana pipiens*, Cooper et al. ('92) found a decline in cell number after 90 days at 4°C. Towards the end of hibernation (day 135), cell number in the blood began to increase, and by day 30 after hibernation, blood cell levels had reached levels equivalent to or greater than prehibernation values (Cooper et al., '92).

Blood viscosity also increases with a decrease in the rate of blood flow (Wells et al., '62). At low temperatures, a low heart rate should lead to slower blood flow, resulting in increased blood viscosity. Herbert and Jackson ('85) found decreases in heart rate in *Chrysemys picta bellii* during anoxic submergence. Blood viscosity also increases with a decrease in red blood cell deformability. The deformability of red blood cells decreases with a decrease in temperature (Koutsouris et al., '85). Bullfrogs have nucleated red blood cells that are less deformable than non-nucleated red blood cells found in mammals (Chein, '75; Gaetgens et al., '81).

The increase in water uptake associated with submerged hibernating frogs would likely have an effect in decreasing plasma osmolality because of dilution of the solutes in the plasma. Amphibian skin plays an important role in osmoregulation, due to its high water permeability and active uptake of electrolytes (Krogh, '37). Seasonal changes of *R. pipiens* have a significant effect on the osmotic water permeability of the skin (Parsons et al., '78). Both field and laboratory observations show that when warm-adapted adult anurans are exposed to low temperatures, they rapidly gain weight and increase water content (Jorgensen et al., '78; Bradford, '84). Schmid ('82) found that ranid frogs should be able to survive temperatures as low as -0.3°C because of osmotic effects that occur during hibernation. With the absence of food, the increase in weight of the cold frogs represents a net uptake of the water in which they were kept (Miller et al., '68).

Experiments with ranid frogs (*R. esculenta* and *R. catesbeiana*) and toads (*Bufo bufo*) submerged in 2-5°C water in laboratory conditions showed a rapid increase in mass due to water uptake, and stabilization of body mass after several days in water, which continued over weeks to months (Jorgensen, '50; Schmidt-Nielsen and Forster, '54; Miller et al., '68; Jorgensen et al., '78). The effect of cold temperature and water uptake with dilution of plasma salts has been found in *R. pipiens* (Miller et al., '68; Parsons and Lau, '76). When *R. temporaria* were transferred to cold temperatures, plasma osmolality decreased (Parsons and van Rossum, '61). Dilution of the blood appears to cause hematocrit levels to decrease (Bradford, '84).

This study was undertaken to determine if, or how, bullfrogs adapt to hibernating conditions. The results of this study will be beneficial in determining ideal hibernation sites for bullfrogs. In comparing warm room adapted frogs with those exposed to 5°C for up 50 days, I predicted bullfrogs would not show changes in hematocrit, hemoglobin, RBCC, and blood viscosity. I also expected to see a decrease in plasma osmolality the longer the bullfrogs were submerged because of water uptake occurring through their skin.

METHODS AND MATERIALS

Thirty-three bullfrogs were obtained from Kons Scientific (Germantown, WI). The bullfrogs were kept in glass aquaria (20 cm x 10 cm x 12 cm) with shallow water, which allowed air breathing. The bullfrogs were kept on a 12:12 photoperiod centered at $25 \pm 1^\circ\text{C}$ at the Emporia State University animal care facilities. The bullfrogs were provided with basking lamps and were fed crickets daily for the first week. After one week, 27 bullfrogs were moved to an environmental chamber, where the temperature was 18°C , and the remaining bullfrogs were kept at 25°C and represented nonhibernating bullfrogs. The temperature in the environmental chamber was decreased 1°C per day to a final temperature of 5°C . Bullfrogs were acclimated to this temperature for one week. The aquaria were filled with water to a level 4 cm from the top and the bullfrogs were submerged. A piece of plastic grating was placed into each aquarium, below the surface of the water, to prevent the bullfrogs from gaining access to room air. Aerators were placed above the grating to maintain constant aeration of the water.

Nine bullfrogs were removed at the end of 20 days and eight bullfrogs removed at the end of 50 days of submergence. Data were also collected at 0 days hibernation on ten cold-acclimated, nonsubmerged bullfrogs, and on a room temperature group of six bullfrogs not exposed to 5°C .

Before blood samples were taken, each bullfrog was properly anesthetized. Approximately 1 ml of halothane was used for anesthetization and each bullfrog was then pithed before blood samples were taken. Blood was taken by heart puncture, using a heparinized syringe. Approximately 5 ml of blood were collected from each frog and placed into vacutainers containing heparin. All procedures were performed with the

approval of the Emporia State University Animal Care and Use Committee. Vacutainers were vortexed to ensure mixing of blood. Hematocrit was determined for each frog using the microhematocrit method. For viscosity studies, blood from each animal was placed into three Eppendorf tubes. The tubes were centrifuged at 1,200g for 5 minutes. Plasma from the first tube was added to the second tube to create packed cell volumes (PCV) above and below that of the original sample. The PCV of the third tube remained unaltered, representing the original blood sample.

Viscosity was measured using a Wells-Brookfield cone/plate viscometer (Model DV-II+, Brookfield Engineering Lab, Stoughton, MA, USA), with a CP-40 cone using a sample of 0.50 ml blood. The viscometer was calibrated using distilled water before the collection of blood viscosity data (using values stated in the Handbook of Chemistry and Physics at 5°C). Blood viscosity was determined for each blood sample at 5°C. The temperature of the sample cup was kept at 5°C with an external water bath. Blood viscosity measurements were made at 8 shear rates (15, 18.8, 30, 37.5, 75, 150, 375, and 750 s⁻¹). Due to the limited range of the viscometer at 5°C, I was unable to determine the viscosity for many of the samples at shear rates above 150 s⁻¹.

Once viscosity values were determined for the blood in each of the three Eppendorf tubes, for each animal, a linear regression was calculated for each shear rate on plots of log apparent viscosity versus PCV. This process was performed for the blood of each frog. From the regression equation of each frog, apparent (whole) blood viscosity values were predicted for a range of PCV (from 10 to 70%) for each animal. The apparent blood viscosity values of all frogs were then compared at a constant PCV.

Eppendorf tubes containing 1 ml of blood were placed into a Micro 14 microcentrifuge (Fisher Scientific, Denver, CO) and centrifuged at 5,000 rpm for 4 min. Plasma was removed and stored in a separate Eppendorf tube. Plasma viscosity was determined for each frog at a shear rate of 375 s^{-1} at 5°C . Relative viscosity values for each hematocrit (10-70%) were determined for each frog by dividing apparent blood viscosity by the plasma viscosity.

Hemoglobin (Hb) concentration was measured using the cyanomethemoglobin method (Sigma Chemicals, St. Louis, MO). Red blood cell counts (RBCC) were determined after diluting the blood in a standard red blood cell pipette (1:100) with a 0.9% NaCl solution. The diluted blood was placed on a hemocytometer and cells were counted using the method for fishes (Hesser, '60). Mean cell hemoglobin (MCH), mean cell volume (MCV), and mean cell hemoglobin concentration (MCHC) were calculated using the following equations:

$$\text{MCH} = (\text{Hb}/\text{RBCC}) \times 10$$

$$\text{MCV} = (\text{PCV}/\text{RBCC}) \times 100$$

$$\text{MCHC} = (\text{Hb}/\text{PCV}) \times 100$$

Plasma osmolality values were determined using a Vapro® vapor pressure osmometer (Model 5520, Wescor Inc., Logan UT). The osmometer was calibrated before measurements were taken, using standard solutions of 100, 290, and 1000 mOsm. Plasma osmolality values were taken for each group of frogs. Five trials were run for all frogs in each group and the means of each group were calculated.

Statistical analyses were performed using SigmaStat Software. A one-way analysis of variance (ANOVA) was used to determine if changes occurred in

hematological parameters and plasma osmolality among all groups. A multiple comparison was also performed among the four groups using Tukey's method. For those variables that failed a normality test (hematocrit and plasma viscosity,), a one-way ANOVA on ranks (Kruskal-Wallis) was used. The 95% level of significance was used throughout.

RESULTS

No significant differences among the groups were found in hematocrit ($H=2.520$, $d.f.=3$, $P=0.472$), hemoglobin ($F=1.843$, $d.f.=3$, $P=0.161$), RBCC ($F=1.882$, $d.f.=3$, $P=0.155$), and MCV ($F=1.493$, $d.f.=3$, $P=0.238$) (Figures 1-4). The MCH values showed a significant difference ($F=7.138$, $d.f.=3$, $P=0.001$) when comparing the 50 day submerged bullfrogs with the 0 and 20 day submerged groups (Figure 5). The values for MCHC were significantly different ($F=6.691$, $d.f.=3$, $P=0.001$) when comparing the 20 day submerged bullfrogs with both the 0 and 50 day submerged groups (Figure 6).

A significant difference ($F=7.981$, $d.f.=3$, $P<0.001$) was found in plasma osmolality, when comparing the room temperature (25°C) bullfrogs with the 0, 20 and 50 day submerged groups at 5°C (Figure 7).

No significant differences were found in apparent viscosity ($F=0.235$, $d.f.=3$, $P=0.871$) or relative viscosity ($F=0.598$, $d.f.=3$, $P=0.622$) when compared at both constant hematocrit and constant shear rate (Fig. 8-11). No significant differences were found when comparing submergence time with apparent viscosity ($F=1.136$, $d.f.=3$, $P=0.351$), plasma viscosity ($H=3.180$, $d.f.=3$, $P=0.365$), and relative viscosity ($F=0.429$, $d.f.=3$, $P=0.734$) between the four groups of bullfrogs (Fig. 12-14) at each group's mean hematocrit.

Figure 1. Mean hematocrit values (\pm SD) of bullfrogs at room temperature (RT), 0, 20, and 50 days of submergence at 5°C.

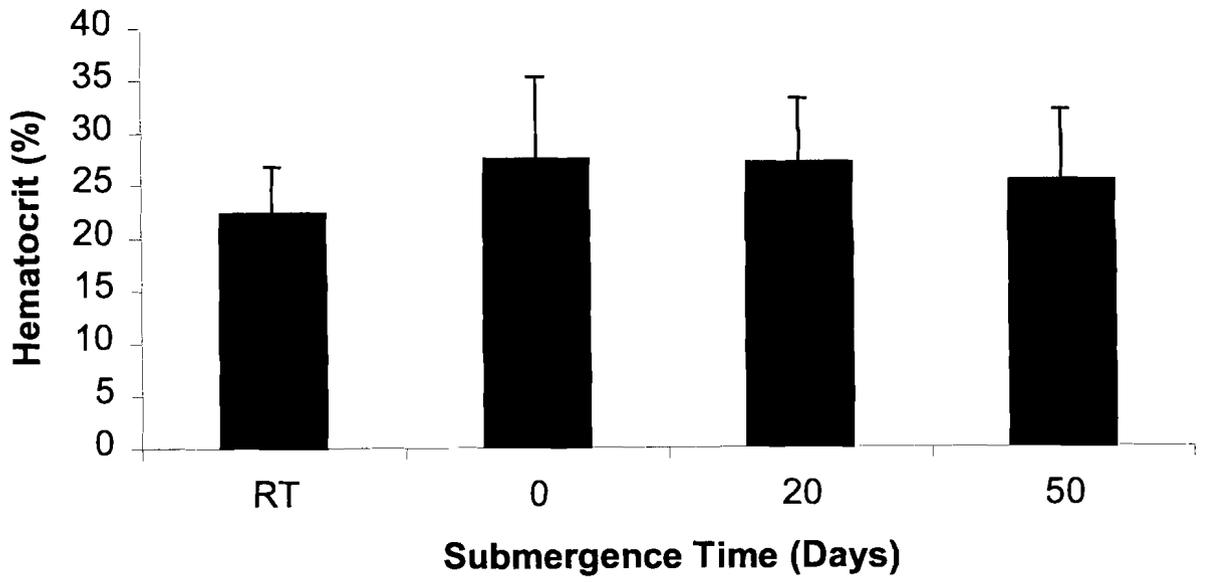


Figure 2. Mean hemoglobin values (\pm SD) of bullfrogs at room temperature (RT), 0, 20, and 50 days of submergence at 5°C.

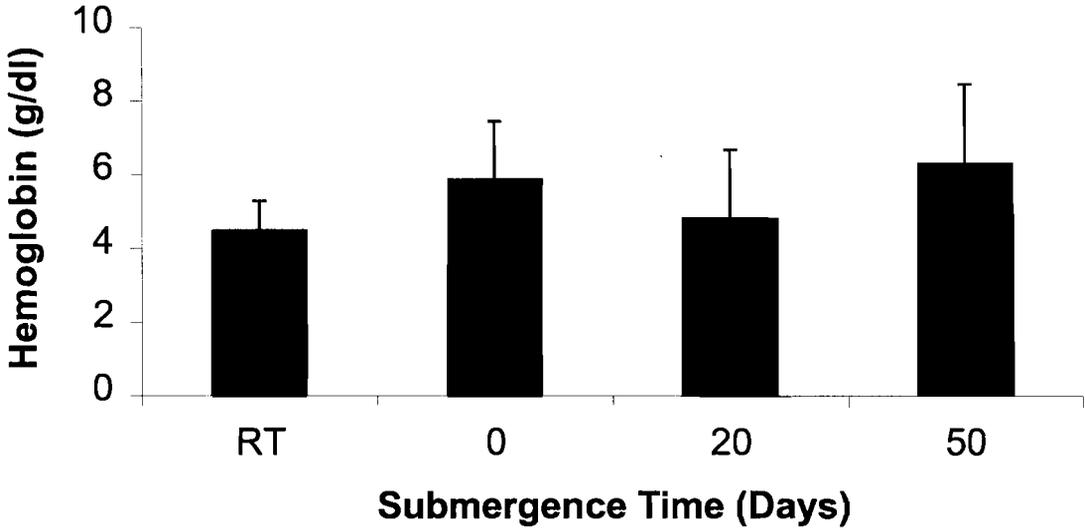


Figure 3. Mean RBCC values (\pm SD) of bullfrogs at room temperature (RT), 0, 20, and 50 days of submergence at 5°C.

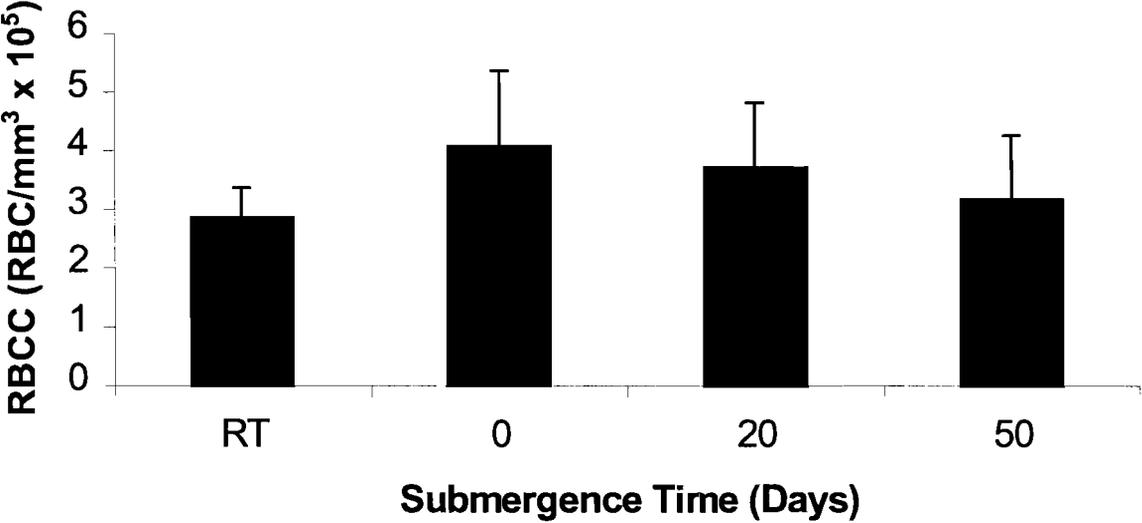


Figure 4. Mean MCV values (\pm SD) of bullfrogs at room temperature (RT), 0, 20, and 50 days of submergence at 5°C.

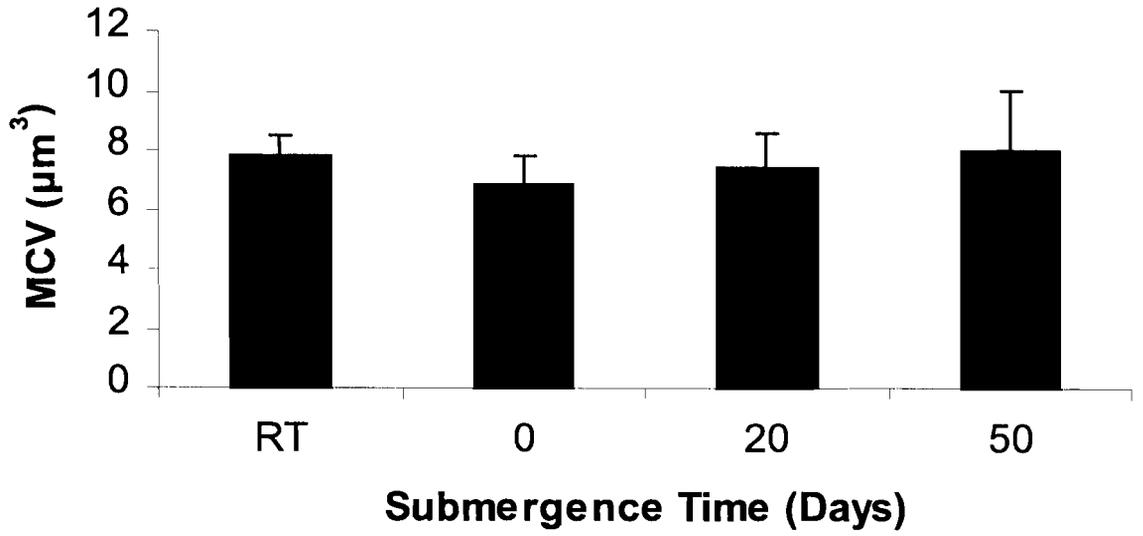


Figure 5. Mean MCH values (\pm SD) of bullfrogs at room temperature (RT), 0, 20, and 50 days of submergence at 5°C. Those groups having the same letter are not significantly different at $P = 0.001$.

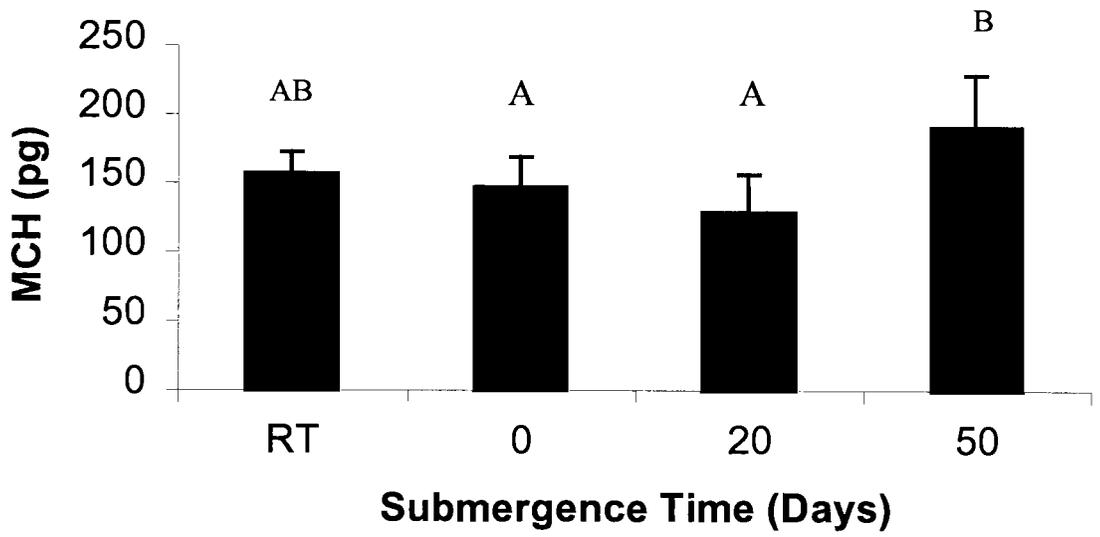


Figure 6. Mean MCHC values (\pm SD) of bullfrogs at room temperature (RT), 0, 20, and 50 days of submergence at 5°C. Those groups having the same letter are not significantly different at $P = 0.001$.

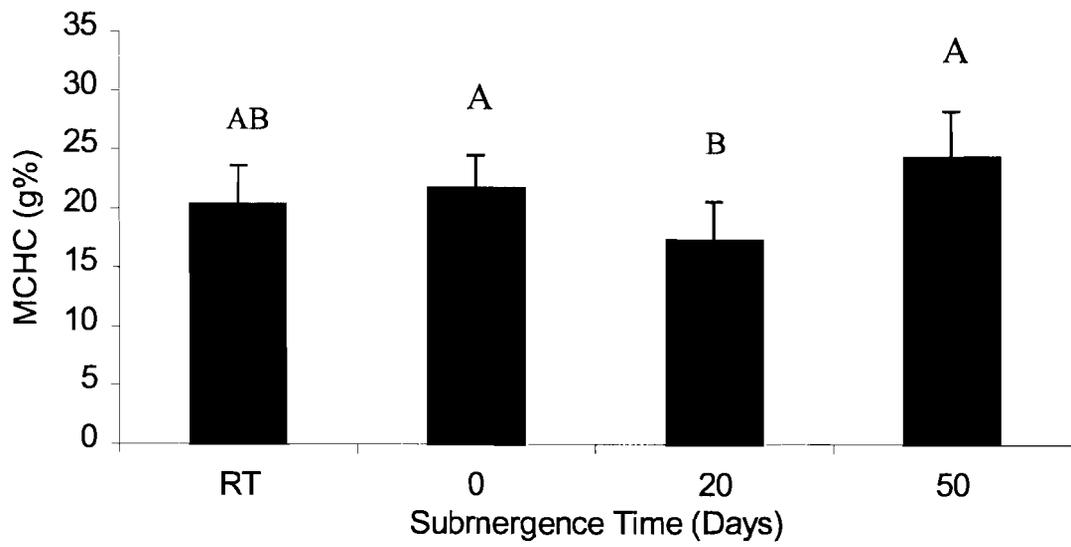


Figure 7. Mean plasma osmolality values (\pm SD) of bullfrogs at room temperature (RT), 0, 20, and 50 days of submergence at 5°C. Those groups having the same letter are not significantly different at $P < 0.05$.

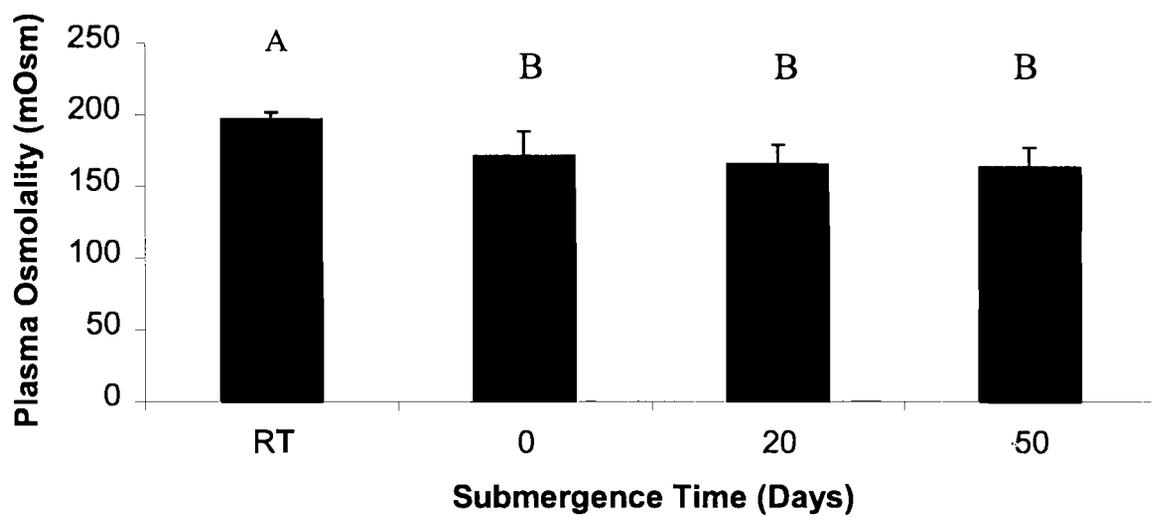


Figure 8. Apparent viscosity at 5°C versus hematocrit at a shear rate of 150 s⁻¹ for bullfrogs at room temperature (RT), 0, 20, and 50 days of submergence at 5°C.

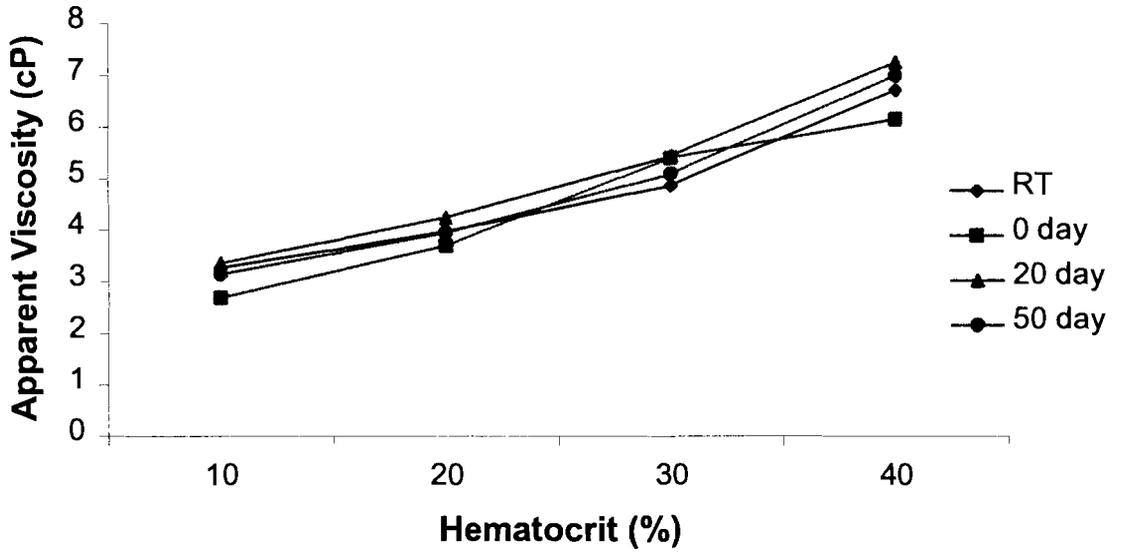


Figure 9. Relative viscosity at 5°C versus hematocrit at a shear rate of 150 s⁻¹ for bullfrogs at room temperature (RT), 0, 20, and 50 days of submergence at 5°C.

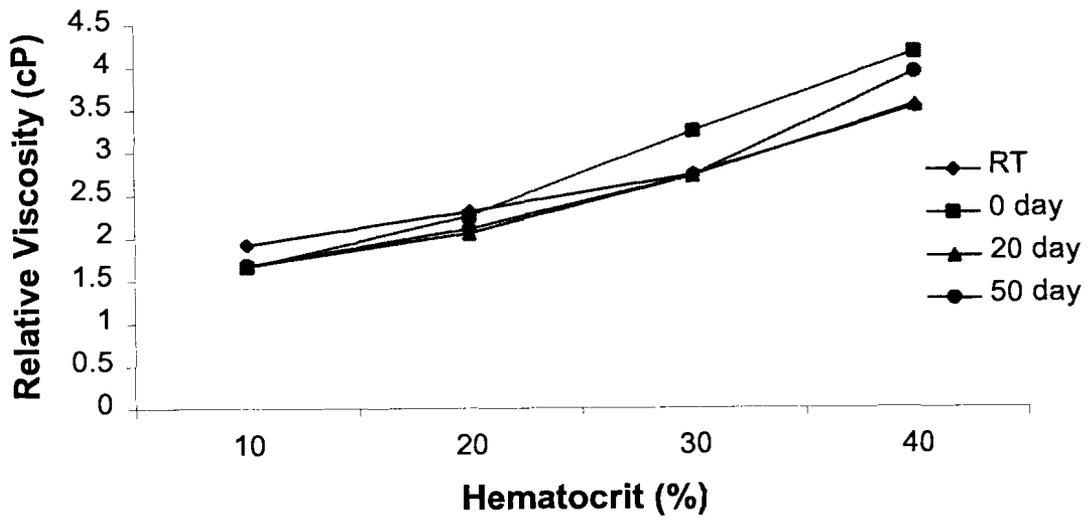


Figure 10. Apparent viscosity at 5°C versus shear rate at a hematocrit of 30% for bullfrogs at room temperature (RT), 0, 20, and 50 days of submergence at 5°C.

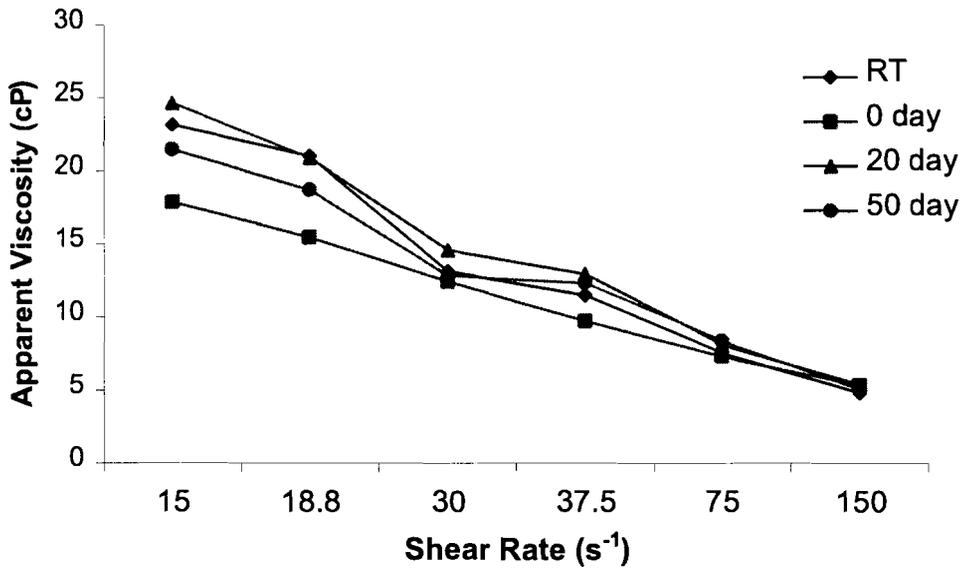


Figure 11. Relative viscosity at 5°C versus shear rate at a hematocrit of 30% for bullfrogs at room temperature (RT), 0, 20, and 50 days of submergence at 5°C.

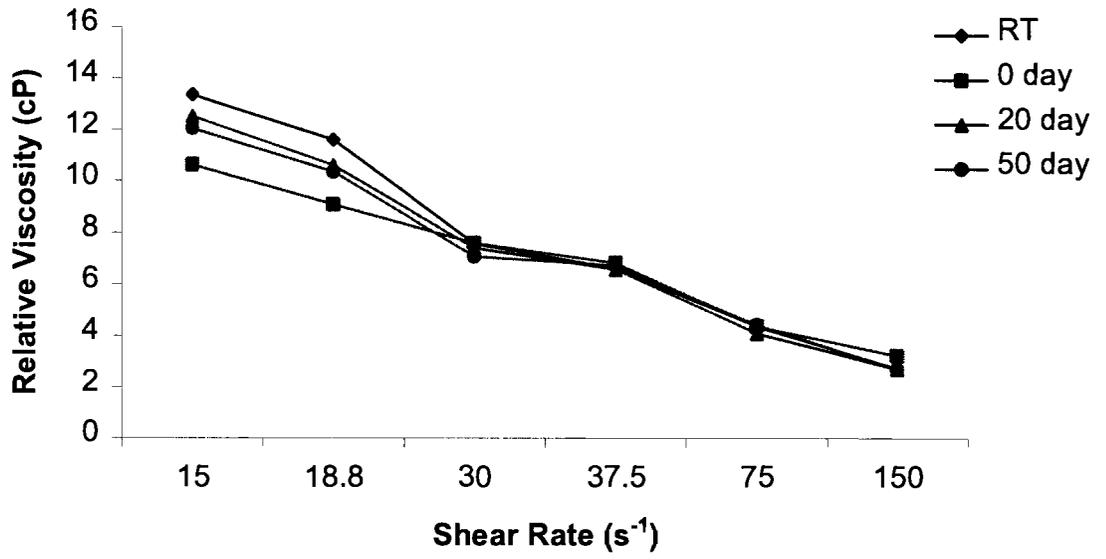


Figure 12. Mean apparent viscosity at 5°C versus submergence time with mean hematocrit values for bullfrogs at room temperature (RT), 0, 20 and 50 days of submergence. (Hematocrit values: RT=22.33%, 0 day=27.4%, 20 day=27.0%, 50 day=25.31%).

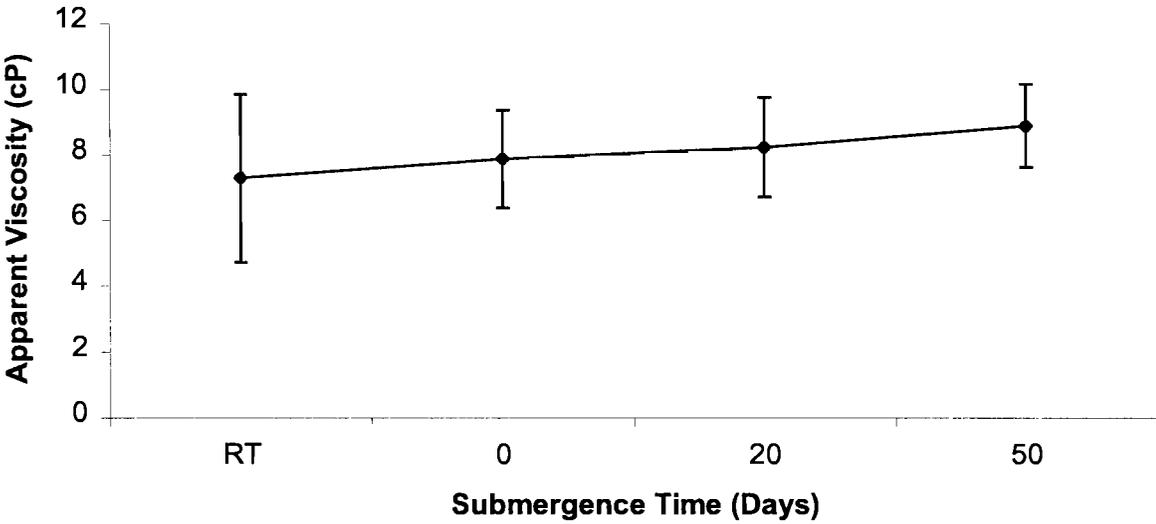


Figure 13. Mean plasma viscosity at 5°C versus submergence time for bullfrogs at room temperature (RT), 0, 20 and 50 days of submergence.

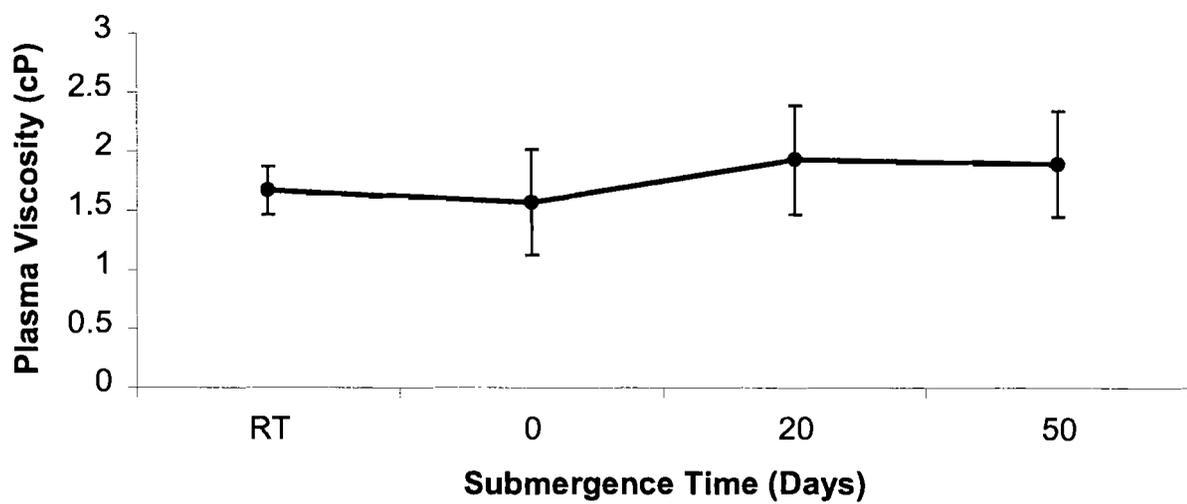
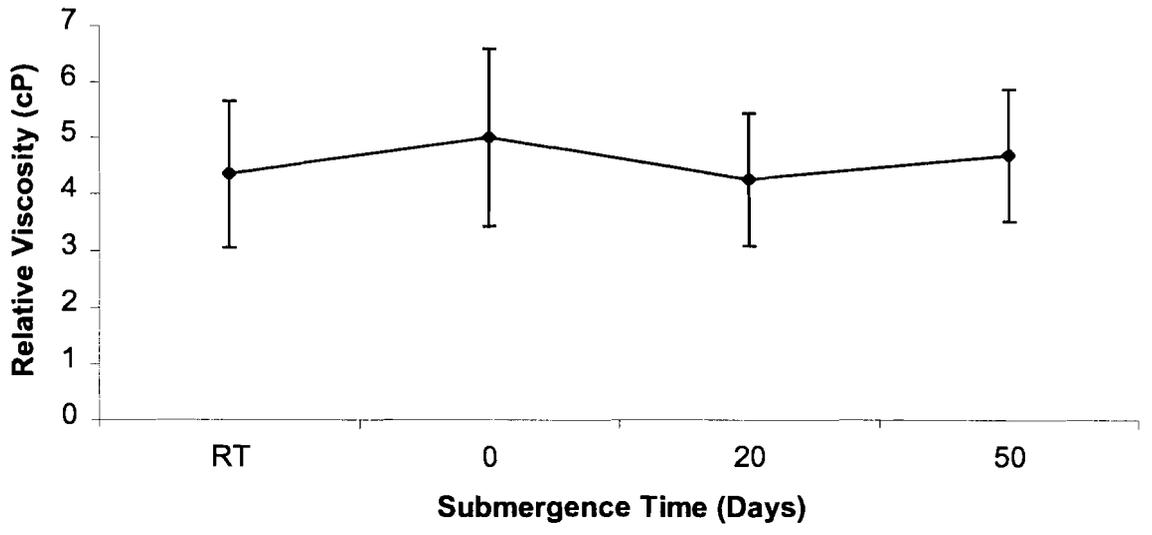


Figure 14. Mean relative viscosity at 5°C versus submergence time with mean hematocrit values for bullfrogs at room temperature (RT), 0, 20 and 50 days of submergence. (Hematocrit: RT=22.33%, 0 day=27.4%, 20 day=27.0%, 50 day=25.31%).



DISCUSSION

No significant differences were found in hematocrit or RBCC in the bullfrogs of this study. Under normoxic conditions, the normal hematocrit of adult bullfrogs ranges from 22.2-27.0% (Tazawa et al., '79; Pinder and Burggren, '83). The nonsubmerged bullfrogs in this study fell within this range, with an average hematocrit of 22.33%. Bullfrogs kept at temperatures of 5 and 20°C in aquaria containing water for a period up to two months, had average hematocrits of 24.4 and 28.1%, respectively (Weathers, '76). The average hematocrit for bullfrogs in this study was 26.6% at 5°C and 22.33% at 25°C. The bullfrogs in my study showed an initial increase in hematocrit and RBCC after being exposed to the cold conditions. Then, after 20 and 50 days submergence, both hematocrit and RBCC began to decrease towards the values of the room temperature bullfrogs. Cline and Waldman ('62) also observed an initial increase in hematocrit and then a decrease in hematocrit after 11 days at 4°C in *R. pipiens*. However, the experimental setup used by Cline and Waldman is unclear. In my study, bullfrogs had been acclimated at 18°C for seven days and the temperature decreased the following 13 days until 5°C was reached. Then the bullfrogs were acclimated to 5°C for seven days before the 0 day group was removed. It seems as though the initial exposure to the cold conditions causes an increase in hematocrit, which may indicate an increase in red blood cell production.

Bradford ('84) however, found hematocrit to decrease during overwintering in *R. mucosa* provided with adequate oxygen, largely due to hemodilution. Frogs in Bradford's study were placed in dechlorinated tap water and were not forcibly submerged, as in my study. This decline in hematocrit was found initially over the first month of exposure to 4°C, and then seemed to increase slightly (Bradford, '84). Bradford

('84) suggested that the initial decline in hematocrit was due to an increased water content in the extracellular space resulting in hemodilution. A similar decrease in hematocrit occurred in *R. pipiens* that were collected seasonally and placed in pools containing constantly aerated well water (Harris, '72). Other ranid frogs, (*R. temporaria* and *R. lessonae*) having access to water and soil, have also shown a continual decrease in hematocrit with exposure to 4°C for 3 months (Sinsch, '91).

Bradford ('84) found a 30% decrease in hematocrit after thirty days of exposure to 4°C, coupled with a significant decrease in peritoneal osmolality values. The 0 day group of bullfrogs in my study was exposed to cold conditions for 27 days before blood samples were taken. In this group of bullfrogs, I saw an increase in hematocrit of 23% and a decrease in plasma osmolality of 15% in the 0 day bullfrogs that were exposed to 5°C but not submerged relative to the room temperature nonhibernating group. Assuming red blood cell production equaled red blood cell removal, the decrease in osmolality should result in a decrease in hematocrit due to water being taken up by the 0 day group, but a significant decrease in hematocrit relative to the room temperature group was not found. The initial increase in both hematocrit and RBCC in the face of decreased osmolality suggests that bullfrogs are increasing their rate of red blood cell production with the initial cold exposure.

As the length of submergence time increased from 0 to 20 days of submergence, hematocrit decreased by 1.6% and plasma osmolality decreased by 3.5%. Additionally, as the length of submergence time increased from 20 to 50 days, hematocrit decreased by 6.7% and plasma osmolality decreased by about 1.1%. This seems to suggest that no further hemodilution took place with prolonged submergence. If no further hemodilution

occured and RBCC and hematocrit remain constant, as in this study, then likely the production of red blood cells once again equaled red blood cell removal. The bullfrogs in this study appear to maintain a stable hematocrit despite being exposed to cold temperatures and being submerged underwater.

Bullfrogs in this study showed a significant decrease in plasma osmolality relative to room temperature bullfrogs after initial exposure to cold temperatures. Little change occurred in plasma osmolality after being submerged for 20 and 50 days relative to 0 day bullfrogs. There may be a decrease in osmolality of body fluids in frogs during hibernation because of water uptake (Penney, '87; Jorgensen et al., '50) and ion loss (Penney, '87). Bradford ('84) found peritoneal fluid osmolality values to decrease during the first month of cold exposure by 9.6% relative to the control group at 17°C (Bradford, '84). The initial decrease in plasma osmolality in my study was likely because of water uptake, but may have been due to ion loss; however ion concentrations were not measured in the plasma. Miller et al. ('68) compared cold and warm, unfed *R. pipiens* and found that cold frogs weighed 5.7% more than initially and 12% more than the warm adapted frogs. It was calculated that the 5.7% increase in weight of the cold frogs represented a 6.9% increase in water (Miller et al., '68). The frogs in Miller's study were not submerged but were maintained in demineralized water.

Hemoglobin does not follow the same patterns of RBCC and hematocrit. Although none of the changes are significant, the blood of the 50 day submerged bullfrogs show an increase in hemoglobin; whereas RBCC and hematocrit continue to slightly decrease in the bullfrogs submerged for 50 days. In this study, the normal hemoglobin levels were 4.48 g/dl in the group of frogs not submerged. Normal

hemoglobin levels range from 5.7-6.6 g/dl in adult bullfrogs where hemoglobin was measured using the cyanomethemoglobin method (Lenfant and Johansen, '67; Hazard and Hutchison, '78). It is likely that no significant differences were observed in hemoglobin concentration because the bullfrogs were able to extract sufficient amounts of oxygen from the water. Boutilier et al. ('86) found that bullfrogs will direct their blood to the site of gas exchange where the most oxygen is available. Bullfrogs exposed to aquatic hypoxia showed a reduction in the proportion of pulmocutaneous blood flowing to the skin and increased blood flow to the lungs as compared to bullfrogs in normoxic conditions (Boutilier et al., '86). This reduction in cutaneous blood flow could be a way of conserving body oxygen stores (Shelton, '70). Conversely, if bullfrogs can shift blood flow from the lungs to the skin in well-aerated water, it may help improve blood oxygenation during prolonged exposure to underwater hibernation (Boutilier et al., '86) limiting the need for hematological acclimation.

Of the animals in this study, the initial exposure of bullfrogs to the cold temperature resulted in the lowest MCV of all bullfrog groups with a value of $681 \mu\text{m}^3$. There was no significant increase in MCV at day 0 of submergence, despite a decrease in plasma osmolality values. This seems to indicate that no cell swelling is occurring with initial exposure of the bullfrogs to cold temperatures. I saw a significant increase in MCH in the 50 day submergence group relative to the 0 and 20 day submergence group of bullfrogs. Hemoglobin values were highest in the 50 day group of submerged bullfrogs and RBCC in the same group of bullfrogs was near its lowest. A significant decrease was also seen in MCHC, comparing the 20 day submergence group with the 0 and 50 day submergence groups. The significant difference between these groups could

again be an artifact. In the 20 day group of submerged bullfrogs, there are low values for hemoglobin and high values for hematocrit, whereas the 0 day bullfrogs had higher hemoglobin and hematocrit values, and the 50 days bullfrogs had high hemoglobin and low hematocrit values as compared to the 20 day bullfrogs. These artifacts are likely the result of a Type I error.

Bullfrogs in this study exposed to room temperature, 0, 20, and 50 days submergence did not show significant differences in apparent viscosity, plasma viscosity, or relative viscosity when all groups were compared at a constant temperature of 5°C with a constant shear rate and constant hematocrit. Apparent viscosity did increase slightly the longer the bullfrogs were exposed to the cold temperature, but not significantly (Fig. 12). Apparent viscosity also increased with increasing hematocrit (Fig. 8). This is supported by findings by Chein, '75 and Fung, '81 where increased viscosity is associated with increased hematocrit. Viscosity increases as the velocity of flow decreases because viscosity is inversely related to the velocity of blood flow (Wells et al., '62; Chein et al., '71). Blood viscosity is influenced by shear rate, the ratio of the velocity of flow to the distance between layers within a flowing fluid stream (Wells et al., '62). At colder temperatures, there is slower blood flow resulting from decreased heart rate and thus increased blood viscosity. Jones ('68) found a decrease in heart rate in *R. pipiens* and *R. temporaria* at 4-5°C that were placed in water but not denied access to the surface of the water. Although the heart rate was not measured in the bullfrogs in my study, I did notice that as I was collecting blood by heart puncture, it took several minutes to collect blood because the heart was beating very slowly. In this study, the blood viscosity of frogs exposed to colder temperatures did not show any significant differences

from those kept at room temperature, when compared at 5°C. This suggests that even though some bullfrogs were exposed to cold conditions, there was no acclimation of blood properties affecting viscosity in these bullfrogs as compared to warm temperature bullfrogs. This is consistent with a previous study showing no acclimation in blood properties at low temperatures in amphibians and mammals (Palenske and Saunders, in press). The apparent viscosity showed an increasing non-significant trend the longer the bullfrogs were submerged relative to the warm temperature group.

No significant differences in plasma viscosity occurred among the four groups of bullfrogs. The plasma viscosity initially decreased in the bullfrogs exposed to the cold temperature and viscosity then increased to levels slightly higher than that of the warm group of bullfrogs. The viscosity of the plasma was not significantly affected by the possible dilution thought to cause the decrease observed in plasma osmolality.

Since the entire blood sample (apparent viscosity) and the plasma viscosity of the bullfrogs did not show a difference between the hibernating and nonhibernating groups of bullfrogs, the role of red blood cells was further investigated. Relative viscosity, which indicates the amount of viscosity due to the red blood cells alone, also showed no significant differences among the groups of bullfrogs. Because no change was seen in plasma viscosity or relative viscosity, it suggests that the plasma and red blood cells of bullfrogs do not undergo acclimation for blood flow at lower temperatures.

The results of this study suggest that bullfrogs acclimate to oxygenated hibernating conditions by extracting sufficient amounts of oxygen from the surrounding water, negating the need for hematological acclimation. The decrease in plasma osmolality suggests water uptake into the blood and a possible dilution of hematocrit and

RBCC. Because of the initial increase in hematocrit and decrease in plasma osmolality of 0 day bullfrogs, it is possible that the bullfrogs in this study are trying to maintain an optimal hematocrit, defined as the hematocrit which provides the greatest oxygen transport (Weathers, '76). A specific hematocrit can influence oxygen carrying capacity and blood viscosity (Birchard, '97). Poiseuille's model for laminar blood flow ($Q = \Delta P \pi r^4 / 8 \eta l$) where Q =fluid flow; ΔP =change in pressure; r =radius; η =viscosity of the fluid; and l =length of the tube, shows the effect of viscosity on blood flow. High oxygen carrying capacity is found with high hematocrit values, but can result in high blood viscosity. Low hematocrit values result in decreased blood viscosity and thus decreases the oxygen carrying capacity of the blood. Thus, the optimal hematocrit theory suggests that oxygen transport is maximized at a particular hematocrit because of the relationship between oxygen carrying capacity and blood viscosity as compared to hematocrit. The lack of a significant difference in hematocrit with prolonged submergence is likely due to an equilibration between the internal fluid compartments of the frog and its surrounding environment. Thus, there is no longer hemodilution coupled with static erythropoiesis.

Although initially it appears that bullfrogs do not acclimate hematologically or rheologically to hibernation, they may in fact do. Bullfrogs exposed to cold but not submerged, show an initial increase in hematocrit by 23% and an initial significant decrease in plasma osmolality by 15%, suggesting an increase in erythropoietic activity. In the submergence groups that follow, it seems no more dilution of the blood is taking place, because no further significant changes are observed in plasma osmolality. Additionally, no further increase in erythropoiesis appears to be occurring, because the hematocrit and RBCC remain fairly constant when compared to the 0 day group of

bullfrogs. These two factors, coupled with the fact that no significant changes were observed in blood viscosity, indicate that the bullfrogs in this study may be trying to maintain an optimal hematocrit. The maintenance of an optimal hematocrit could be desired in these bullfrogs as a way to maximize oxygen transport to the tissues. This could be beneficial to the bullfrogs as they may be limited to the amount of oxygen that they can extract from the water during prolonged hibernation.

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July 22, 2002
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Blood Viscosity and Hematological Parameters
in Hibernating Bullfrogs, *Rana catesbeiana*
Title of Thesis

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July 22, 2002
Date Received

01/2002