THE UNIVERSITY OF MANITOBA

Physiological Aspects of Overwintering in the Boreal Chorus Frog, *Pseudacris triseriata maculata*

by

DIANE LYNNE HUNKA

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

DEPARTMENT OF ZOOLOGY

WINNIPEG, MANITOBA

APRIL, 1974

ABSTRACT

Boreal chorus frogs, Pseudacris triseriata maculata captured in spring and fall and acclimated to cold temperatures (0°C) had lower plasma ionic concentrations than frogs acclimated to 5 or 10°C, although the overall body water content did not vary significantly over the various acclimations.

Frogs captured in the fall had a higher plasma osmolality than spring frogs, but the spring frogs had a significantly higher total body water content than the fall frogs which could explain the decrease in plasma osmolality. Increased plasma osmolality seems to be correlated with higher chloride ion levels.

Water content in isolated tissues was higher in 0°C acclimated frogs than in 10°C frogs, although a significant difference was found only in the muscle.

Chorus frogs were found to supercool to a temperature of -3.25°C in the laboratory.

The physiological changes observed were related to the animal's ability to survive the freezing temperatures of a Manitoba winter.

ACKNOWLEDGEMENTS

I wish to thank my supervisor Dr. J. W. T. Dandy and my acting supervisor Dr. K. W. Stewart for their advice and support throughout this study.

Collecting assistance by the following graduate students and friends was greatly appreciated: P. Gregory, S. Eddy, L. De March, B. De March, A. Smith, J. Mandrick, D. Hart and R. Hancox.

I also wish to thank Dr. M. Aleksiuk for the use of his refrigeration unit, Dr. R. H. Green for his patience and help in the statistical analysis, and Dr. W. J. Turnock for his critical reading of the manuscript.

I am grateful to Dr. J. G. Eales, A. Hawley and T. Narayansingh for their helpful suggestions.

This study was supported by funds provided by the National Research Council of Canada and The University of Manitoba Northern Studies Committee grants of Dr. J. W. T. Dandy.

TABLE OF CONTENTS

Table of Contents Cont'd.

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

The boreal chorus frog Pseudacris triseriata maculata has a broad distribution, ranging from Northern Ontario to Great Bear Lake in Canada, south through Colorado and Utah and intergrades with the western chorus frog in a broad band extending from the northern peninsula of Michigan to Nebraska (Conant, 1958). It is the northern-most of all the chorus frogs and is also the most common frog found in Manitoba.

These frogs are small, 2.0 to 2.8 cm (Conant, 1958) and tend to be secretive. They are semi-terrestrial and seem to share the same habitats as the wood frog, Rana sylvatica. During the breeding season (from mid-April to mid-May in Manitoba) they are found in or near temporary, shallow bodies of water. After the breeding season they tend to disperse and are found in grassy meadows and the edges of wooded areas which are adjacent to dried up ponds and marshes. By early September they are difficult to locate.

There is little information about the sites of over-wintering of the boreal chorus frog. Blanchard (1933) collecting in southern Michigan in November, 1931 found a number of amphibians including Pseudacris nigrita (=triseriata) in compact groups under litter in small depressions or cavities in the ground. Because of the time of year and the cold weather, he

assumed this was their overwintering site.

In Manitoba, poikilotherms that overwinter on land face the danger of environmental temperatures which may freeze their body fluids. Because a few specimens of Pseudacris which were collected in late fall and early spring have been located in crevices in the soil or under litter and debris, overwintering at or just beneath the soil-snow interface seems most likely. Although air temperatures may go much below 0°C during the winter months, the ground temperatures just below litter may not if the snow cover is adequate. However if the snow cover is not sufficient or if there is an excessively long period during late autumn when there is no snow and the air temperature is less than that of the soil, these animals would be subjected to temperatures which could result in freezing and subsequent death.

The exposure of poikilotherms to low temperatures may or may not result in the freezing of interstitial and intracellular body fluids. Ice formation in a variety of poikilotherms has been shown to be delayed or prevented in the following ways:

(1) Vitrification

Ice formation below zero can be prevented by very rapid cooling resulting in the formation of very tiny ice crystals or an amorphous solid (Hoar, 1966).

The survival of vinegar eels and frog spermatazoa subjected to -190°C by immersion in liquid air was found to be dependent on vitrification and devitrification (Luyet and Hartung, 1941 a,b), but apparently it is technically impossible to cool animals larger than vinegar eels with sufficient speed to ensure vitrification of their body water at very low temperatures.

(2) Supercooling

Lowe et al. (1970) define supercooling as the metastable condition in which the tissue temperature is below its freezing (melting) point without the formation of ice. As the temperature drops it passes an infinite number of supercooling points until a limit is reached and freezing ensues. But ice formation will occur rapidly if the fluid is seeded with an ice crystal or if it is agitated (Hoar, 1966).

Supercooling occurs in a number of species of invertebrates as well as in fish and reptiles. Among invertebrates the larvae of the wheat-stem sawfly Cephus cinctus will supercool between -20° and -32.7°C before freezing (Salt, 1950). The longer the period in the supercooled state, the greater the probability of freezing. But Barnes et al. (1956, 1957) found that the larvae of the European corn borer Pyrausta nubilalis collected during winter and early spring survived supercooling to -29° and sub-

sequent freezing to the same temperature. During the summer and early autumn however, their cold-hardiness decreased so that they did not tolerate being frozen after supercooling.

Scholander et al. (1957) found bottom dwelling fish off the NorthernLabrador coast had a plasma freezing point of -0.9° to -1.0°C and yet they lived in water at a temperature of -1.7°C all year round. Thus they were supercooled by -0.7°C; but they were in no danger of being seeded because ice formation did not reach the depths at which they lived.

Lowe et al. (1970) stated that reptiles are also able to escape the lethal effects of freezing by supercooling the entire body to as low as -8°C, 2 to 8°C below the freezing point of body tissues (ca. -0.6°C).

(3) Increased Solute Concentration:

An increase in the solute concentration of the body fluids of insects, fish and reptiles exposed to freezing temperatures is a well documented phenomenon.

Scholander et al. (1957) found that shallow water fish (cod and sculpin) off the coast of Labrador which were supercooled but in danger of becoming seeded, had osmoconcentrations that were twice as high as those in summer. They were almost isosmotic with sea water although they still supercooled from -0.2° to -0.4°C.

Many subarctic and a few temperate-zone marine fish living at low temperatures have elevated chloride concentration (Prosser et al., 1970), but cold temperatures do not cause an increase in ion levels in all species of fish. Goldfish acclimated to 5°C had significantly lower plasma values of Na^+, K^+ and Cl^- than in 15° and 25°C fish, suggesting that lower concentrations may be a compensation to reduced osmotic work (Prosser et al., 1970). Freshwater fish have either reduced or unaltered plasma Na^+ and Cl^- concentrations when acclimated to low temperatures (Hickman, et al. 1964).

Scholander (1957) found that in the summer, chloride (halides) accounted for approximately 80% of the osmotic pressure in both deep and shallow water Labrador fish. When the fish were transferred from warm surface water to deep cold water (-1.73°C), the total osmotic concentration increased, but the chloride rise was small and chlorides only contributed 50% of the total freezing point of the plasma.

Binyon and Twigg (1965) found very slight hemoconcentration in Natrix natrix, the grass snake during winter torpor and Zain-ul-Abedin et al. (1969) found indications of hemoconcentration in the lizard Uromastix hardwicki during the hibernating state.

The increase in body fluid solute concentration may be either active or passive. The animal may be actively making

physiological adjustments to resist freezing, or the increase in solute may be the result of dehydration i.e. environmental factors are causing the changes.

(4) Antifreeze Production

Another active adjustment some animals make in order to tolerate freezing is the production of an antifreeze such as glycerol. Glycerol is a viscous liquid which is miscible with water (White, Handler and Smith, 1968). Because of its high viscosity it has a strong tendency to supercool. Small amounts of water depress its freezing point and conversely it depresses the freezing point of water, thus facilitating supercooling.

Salt (1959, 1961) showed that glycerol accumulated in concentrations up to 5 molal in Bracon cephi, an insect parasite of the wheat-stem sawfly which tolerates extremely cold temperatures.

Glycerol has another function; it also acts as a "salt buffer", preventing strong salt concentrations from damaging cells (Lovelock, 1954; Barrington, 1968).

Recently De Vries et al. (1970) have isolated glycoproteins from the blood of Antarctic fish which can depress the freezing point of water more than would be expected on the basis of the number of particles present in solution. De Vries (1971) has proposed that the unusual behavior of these glycoproteins

can be explained by their being adsorbed onto the surface of the ice crystals thereby preventing water molecules from joining the ice lattice and effectively removing the ice crystals as nucleation sites.

If poikilotherm cannot resist freezing, the formation of ice can affect the animal in two ways:

(1) by the crystals causing a structural breakdown within the cells;

(2) by raising the electrolyte concentration in the remaining body fluids causing the denaturation of proteins and the dissolving of lipoproteins (Hoar, 1966).

Lovelock (1954) states that cells which are highly permeable to water and cells which have high surface to volume ratios lose water as freezing progresses at a rate sufficient to maintain the freezing point of their interior contents at or a little above that of their suspending fluid. By the time the medium has concentrated to saturation the electrolyte levels within the cell will be high enough to prevent freezing. But these high electrolyte concentrations may be detrimental to the cells.

Ling (1967) reviewing the effects of cooling and freezing on living cells stated that ice formation does not extend into the cells except those that are dead or injured. No specific

example was given, but he concluded that the cell membrane acts as a barrier to ice formation. Also, the increasing electrolyte concentrations may be preventing intracellular ice formation.

Maetz (1968) in a review of salt and water metabolism in lower vertebrates noted that in amphibians, cellular fluid volume is maintained at the expense of extracellular fluids during dehydration. But it has also been found that amphibians have the ability to withstand desiccation because of a remarkable tolerance of the tissues to changes in osmotic concentration. McClanahan (1964) found that two desert toads, Scaphiopus couchi and Bufo cognatus had a high tolerance to increased intracellular ionic concentration. Schmid (1965) found that terrestrial anurans had a greater tolerance to desiccation than aquatic frogs. Pseudacris nigrita (=triseriata)was found to be the most tolerant to desiccation of all the frogs and toads tested, even more so than the toads Bufo americanus and Bufo hemiophrys.

Since all evidence indicates that the boreal chorus frog would be subjected to temperatures below 0°C,they probably make some physiological adaptations that would be advantageous to their survival. The most logical adaptation would be to increase actively or passively, the osmotic concentration of the body fluids thereby lowering the freezing point. Thus the physiological basis of overwintering was investigated with reference to

the effect of varying seasonal and experimental temperatures and photoperiods on:

(1) the total body water content of the animal

(2) the freezing point depression of the body fluids, and

(3) specific ion levels in the plasma.

The ability of the animal to supercool was also examined.

MATERIALS AND METHODS

1. Experimental Animals

Preliminary experiments were performed on breeding chorus frogs collected from ditches and flooded fields in the vicinity of Winnipeg, Manitoba between April 19 and 22, 1972. Subsequent experiments that year used frogs collected in mid-August 1972 from pasturelands in the vicinity of Lake Minnedosa, Manitoba. These frogs will be referred to as "fall animals".

Experiments in the spring of 1973 were performed using breeding frogs caught on April 19, 1973 near the Seine River in Winnipeg. These animals will be referred to as "spring frogs".

No distinction was made between age and sex, although the majority of the animals were male. All frogs were kept in vented plastic boxes (10 to 20 per box) on a substrate of moist silica sand. The frogs had free access to water but were not fed before or during any experiments. Tap water (0.5 to 1.0 ppm chlorine) was used rather than dechlorinated water to check fungal infection which occurred quite frequently.

Frogs kept at low temperatures were provided with a substrate of moist soil and leaf litter.

After capture the fall frogs were kept at 10°C, 12L-12D photoperiod before final acclimation. Spring frogs were held at 8°C, 13L-11D photoperiod for four days and then dropped to 5°C

for ten more days where upon final acclimations were made.

2. Acclimations

(A) Preliminary Experiment

In subsequent experiments total body water content of the frogs held at various temperature and photoperiod regimes was to be determined. As all frogs were not fed there was the possibility that the starvation would mask the effects of the acclimations. For this reason a number of animals that were captured in the spring of 1972 were acclimated at 10°C, 12L-12D for approximately 3 months. This was the highest temperature to be used in all other experiments, so the rate of metabolism would presumably be greater at this temperature than at lower ones. Total body water percentage was determined by weight change of frogs after desiccation. These observations were made at capture and at 2 week intervals for 3 months.

(B) Fall Frogs

To see if there was any effect of temperature and photoperiod on the water content and plasma osmolality of fall frogs a series of 6 combinations of temperature and photoperiod were set up simultaneously (Table I). All acclimations were done in controlled environment chambers with temperature variations of $\pm$1.0°C. The frogs acclimated to 0°C were pre-acclimated to 5°C for 1 week before the actual 0°C acclimation was done.

Table I. A summary of the acclimations used in the various experiments.

Table I.

Experiment Title	Sample Size (n)	Preacclimation Temperature C°	Preacclimation Photoperiod	Preacclimation Time (days)	Acclimation Temperature C°	Acclimation Photoperiod	Acclimation Time (days)	Sampling period (days)
1. Starvation	5-10 animals /sample period	-	-	-	10	12L-12D	0 - 90	Animals were sampled in the field and subsequently at 2 week intervals for 3 months
2. Fall	10	-	-	-	10	12L-12D	16	
	10	-	-	-	10	0L-24D	16	All animals were sampled at the end of the acclimation period
	10	-	-	-	5	12L-12D	16	
	10	-	-	-	5	0L-24D	16	
	10	5	12L-12D	7	0	12L-12D	16	
	10	5	12L-12D	7	0	0L-24D	16	
3. Spring	10	5	13L-11D	10	10	12L-12D	17	All animals were sampled at the end of the acclimation period.
	10	5	13L-11D	10	0	8L-16D	17	

(C) Spring Frogs

Again, temperatures and photoperiod acclimations were simultaneously set up. But this time more natural conditions were simulated instead of extremes. Also the number of acclimations were restricted due to lack of facilities (Table I).

3. Sampling Procedure

When acclimations were completed the frogs were individually surface dried and weighed in plastic bags. Before weighing gentle pressure was applied to the lower abdomen to eliminate any urine from the bladder.

After weighing, blood samples were obtained by cutting the femoral vein and artery in the thigh and the blood was collected in heparinized microhematocrit capillary tubes which were immediately sealed and kept cool. The blood samples were centrifuged in an International IEC micro-capillary centrifuge at 1500 X G for 3 minutes and the plasma was collected again in heparinized capillary tubes. Each plasma sample (5 to 10 u l) was moved to the centre of the capillary tube to allow for air spaces on both sides and the tubes were sealed with microhematocrit tube closures. The capillary tubes were put in capped plastic test tubes and were immediately frozen for analysis at a

later date.

After the blood samples were drawn the frogs were then killed and put in a drying oven at approximately 105°C and were dried until constant weights were obtained (24 hours). They were then put in a box containing calcium chloride and weighed to 0.1 mg.

In the case of the "spring frogs" after total wet weights were determined, heart, liver, forelegs and hind legs were removed and placed in pre-weighed glass vials with stoppers. The remaining carcass was wrapped in pre-weighed aluminium foil on which the dissections were done. All tissues and the carcasses were weighed to determine the wet weights. The vial stoppers were then removed and the pieces of foil on which the carcasses lay were unwrapped; the vials and foil containing the tissue and remaining carcasses were put in the oven at 100°C and dried until constant weights were determined (24 hours). All weighing was done as quickly as possible to minimize water evaporation from wet tissues and absorption of moisture by dry tissues.

4. Osmolality Determinations

The ionic concentrations of the plasma samples were determined by the comparative melting point method (Gross, 1954; Schmid, 1965).

Frozen capillary tubes containing standards of 0.22, 0.20,

0.18, 0.16, 0.14 and 0.12 molal NaCl as well as 2 frozen plasma samples were set on a plexiglass rack and the rack was submerged in a 25% alcohol bath that had previously been chilled to -5.0°C with dry ice. The volumes of standards/tube equalled the volumes of the samples.

The alcohol bath (Figure 1) agitated by an electric stirer was warmed up at the rate of 1°C per 20 to 25 minutes.

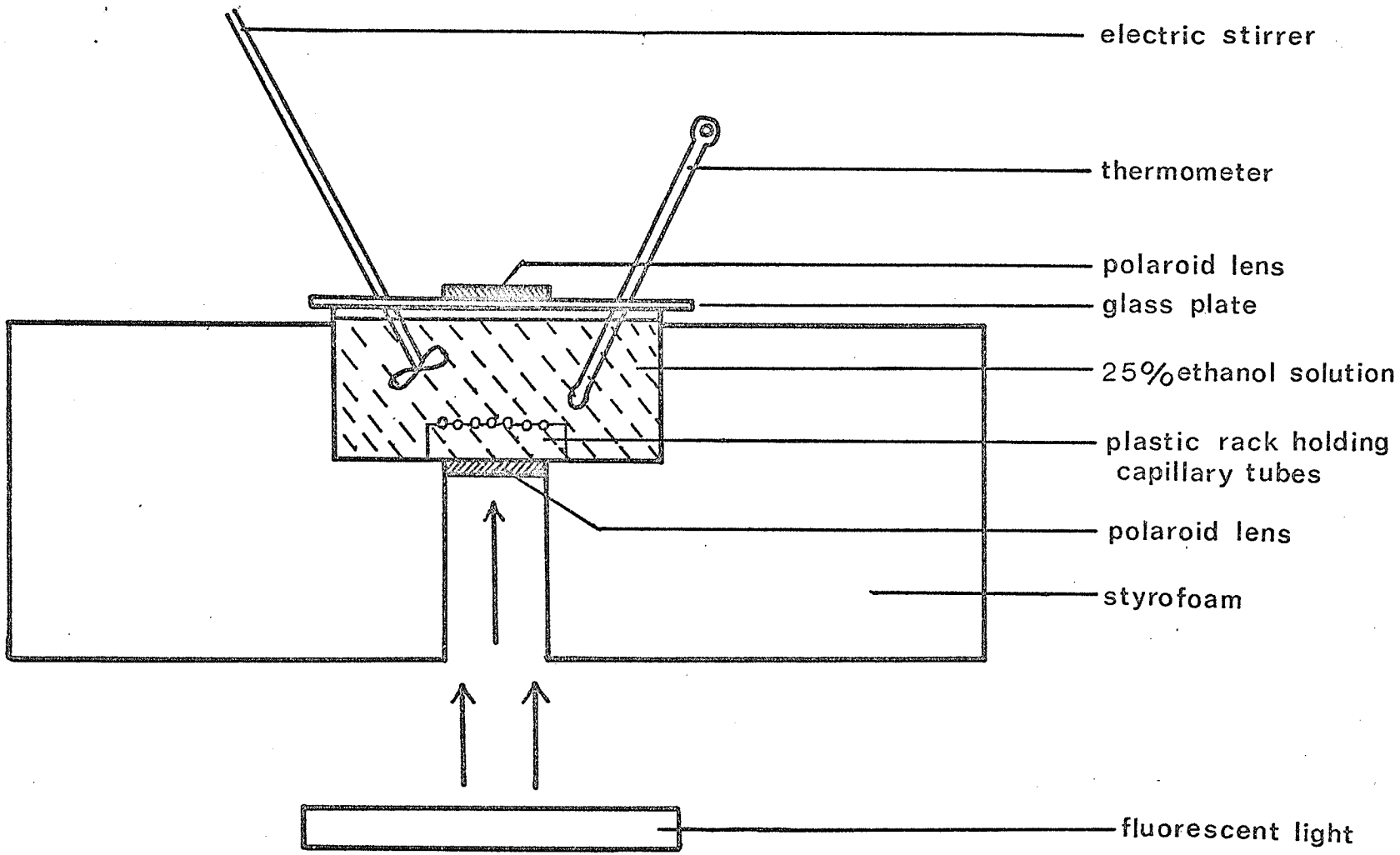
When the frozen samples were observed using transmitted light and crossed polarizing filters, they fluoresced white against a dark blue background. As the samples melted the fluorescence decreased, thus making it easy to determine when the samples melted. The melting point was taken at the time at which approximately 90% of the sample had melted.

As the bath warmed up, the standards melted in order of concentration. The osmolalities of the unknowns were determined by interpolation of the graph of osmolalities of standards vs. melting times. The graph should theoretically, be a straight line, so a linear regression analysis was performed to obtain the best straight line from the standard results. All plasma osmolalities were then expressed in terms of NaCl molality.

5. Ion Determinations

Chloride determinations were done using a Type C101 titration electrolyte for a Radiometer titrator Type CMT20.

Figure 1. Apparatus used for freezing point determinations. By using crossed polaroid lenses and transmitted light, the frozen standard salt solutions and plasma samples glowed against a dark blue background making it easy to observe melting times. This diagram is approximately 1/3 the actual size.


electric stirrer
thermometer
polaroid lens
glass plate
25% ethanol solution
plastic rack holding capillary tubes
polaroid lens
styrofoam
fluorescent light

Sodium and potassium determinations were made using an Instrumentation Laboratory IL 143 Digital flame photometer.

Appropriate dilutions were made for both machines. In some instances it was necessary to pool samples to obtain enough plasma for the proper dilutions.

There was the possibility that the capillary tubes in which the plasma samples were stored had been treated with sodium heparin by the manufacturer. Sodium and potassium determinations were done on blank tubes and the amount of sodium was found to be negligible ($Na+ \bar{x} = 1.96 \pm 0.07$ mequiv/1; $K+ \bar{x} = 0.1 \pm 0.02$ mequiv/1).

6. Supercooling Method

Supercooling and freezing points were done on live animals by inserting a YSI 402 probe in the esophagus of the animal. The frog and probe were then taped to a cardboard disc and put into a 2 1/2 x 3 1/2 inch plastic jar which was then stoppered. A YSI Model 42SC telethermometer and YSI Model 81 dual channel recorder were used to record body temperatures. The jar was then submerged in a Neslab Bath Cooler PBC-2 containing ethylene glycol at the temperature at which the animal had been acclimated (0° or 5°C). As these temperatures were quite low, oxygen consumption would also be low, so no provision was made for supplying air to the animals.

In the bath, the body temperature of the animal was allowed to stabilize for half an hour, and then the bath temperature was decreased 2°C per half hour until first the supercooling limit and then the freezing point of the animal was reached.

When the frog was removed from the container it was placed on its back on a wet paper towel. If it showed signs of life i.e. the heart was still beating, the time it took the animal to right itself was recorded.

RESULTS

1. Starvation Experiment

During a 3 month period there was no effect of starvation on the water content of the frogs (Figure 2). A one-way analysis of variance showed there was no significant change in the water contents ($P > .05$) during this time (see Appendix A for statistical analysis), although there was an upward trend after approximately one month of starvation.

Since all further experiments were carried out on animals that were held for less than 3 months, starvation would not effect their water contents.

2. Fall Frog Acclimations

Six acclimations were set up simultaneously for 16 days (10 frogs/treatment) to test the effect of temperatures and photoperiod on the percentage body water, plasma osmolality and plasma chloride levels.

A. Water Content

There were no significant trends in the results (Table II). A two-way analysis of variance showed that acclimation temperature alone and photoperiod alone do not have an effect on the water contents of the frogs, but there is a significant interaction effect at $P < .05$. Duncan's New Multiple Range Test showed that the water content of the 10°C 12L-12D frogs was significantly higher than the water content of the 5°C 0L-24D frogs. See Appendix B (III) for the statistical analysis.

Figure 2. The effects of starvation on the percent body water of the chorus frog, Pseudacris triserata maculata. The numbers in brackets indicate the sample size (n) and the vertical bars represent the 95% confidence intervals.

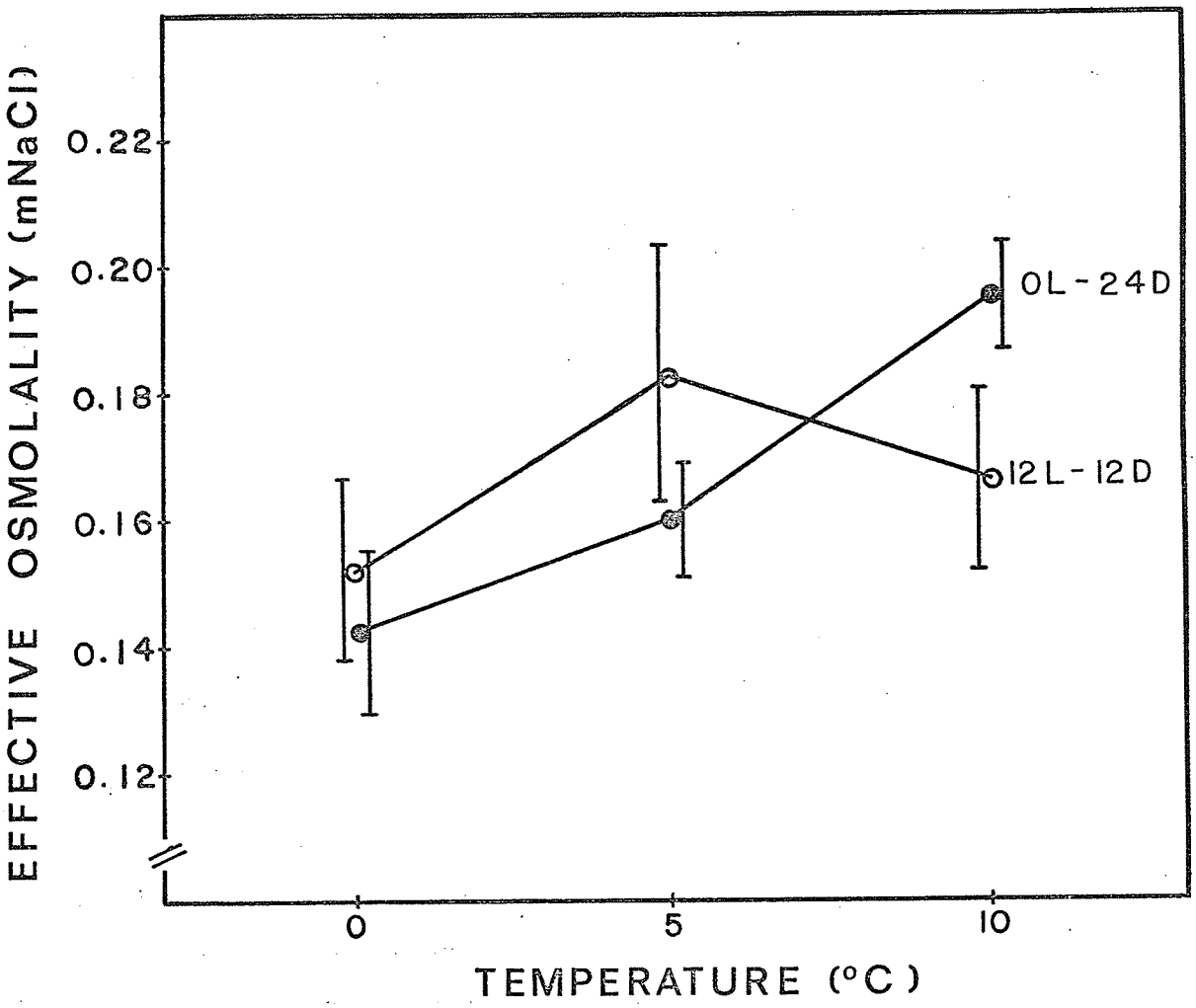

EFFECTIVE OSMOLALITY (mNaCl)
0.22
0.20
0.18
0.16
0.14
0.12
0
5
10
TEMPERATURE (°C)
0L-24D
12L-12D

Table II. The effect of acclimation temperature and photoperiod on the total water content of chorus frogs.

Acclimation Temperature (°C)	Photoperiod	n	Average Body Weight (g.)	Percentage Body Water (%) $\bar{x}$ ± S.E.
0	0L-24D	10	0.5015	80.6183 0.5584
	12L-12D	10	0.4864	79.0026 0.6432
5	0L-24D	10	0.4313	78.3105 0.7884
	12L-12D	10	0.4664	79.7940 0.7695
10	0L-24D	10	0.5039	78.8942 0.5819
	12L-12D	10	0.3691	80.6480 0.3260

B. Osmolality

There was a trend of decreasing plasma osmolality with decreasing temperature (Figure 3). A two-way analysis of variance (for unequal sample size) showed a significant effect of temperature, and an interaction of temperature and photoperiod at $P<.01$ (Appendix B (Ib, IV, V). The least significant difference method was used to determine which temperature and photoperiod acclimation pairs differed significantly. At the 0L-24D photoperiod, the 10°C mean plasma osmolality was significantly ($P<.05$) higher than the 0°C and 5°C values. At the 12L-12D photoperiod, the 5°C mean plasma osmolality was significantly ($P<.05$) higher than the 0°C value. See Appendix B (V) for the comparison of all the means.

C. Chloride Determinations

The results are shown in Table III and Figure 4. There was a high positive correlation between the chloride levels in the plasma and osmolality of the plasma; the correlation coefficient, r is equal to 0.8225 and it is significant at the $P<.05$ level.

3. Spring Frog Acclimations

Two groups were simultaneously acclimated to 0°C 8L-16D and 10°C 12L-12D for 17 days. Total body water contents, plasma osmolality and plasma Na+ and K+ levels were determined (Table IV). The mean osmolality of the 0°C 8L-16D acclimated frogs was significantly lower than the osmolality of the 10°C 12L-12D frogs ($P<.05$). Total body water contents, Na+ and K+ levels in the plasma were not found to vary significantly between the two treatments.

Figure 3. Chorus frog plasma osmolalities at various temperature and photoperiod acclimations. Bars represent 95% confidence intervals.

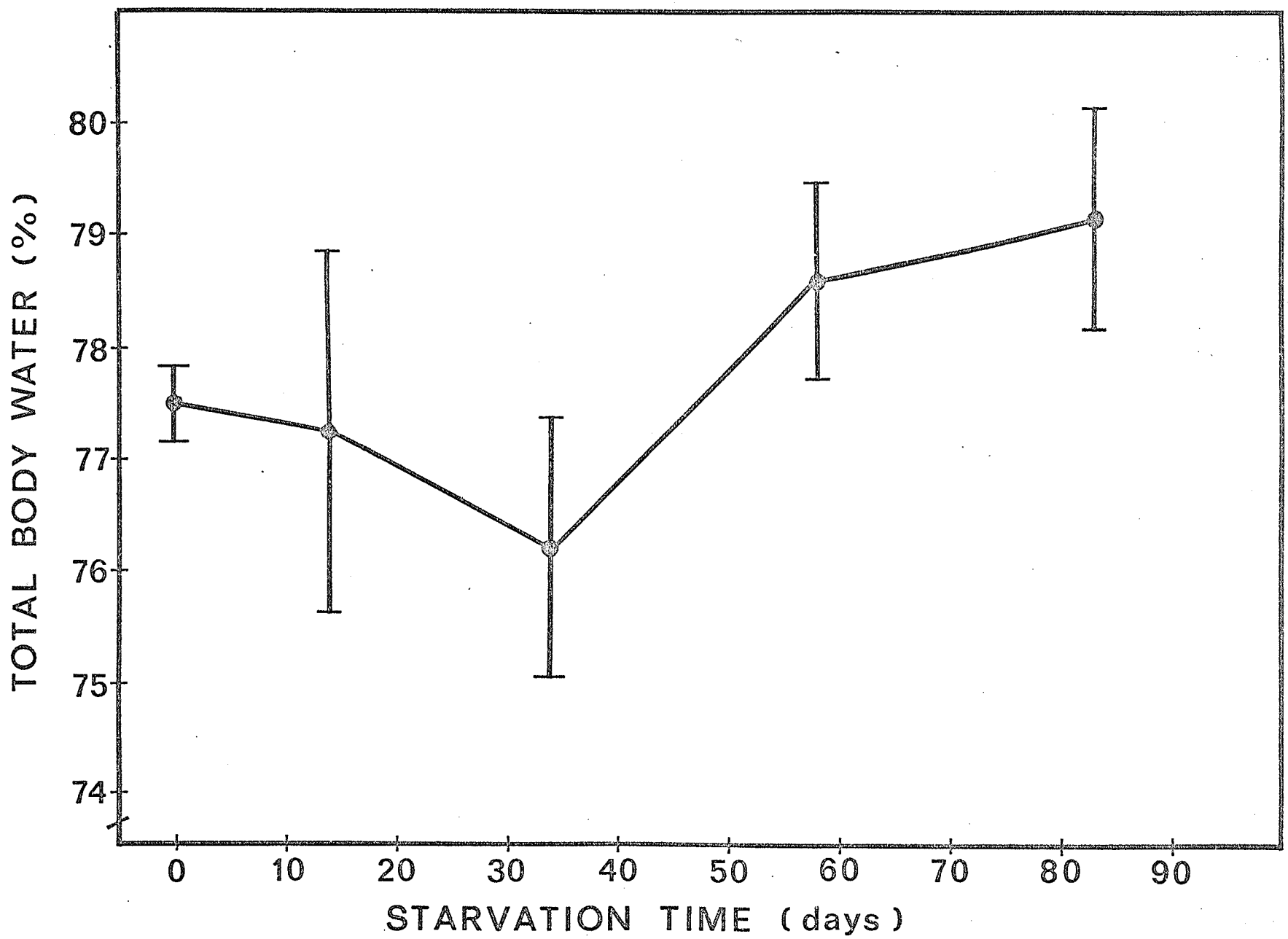

TOTAL BODY WATER (%)
80
79
78
77
76
75
74
0
10
20
30
40
50
60
70
80
90
STARVATION TIME (days)

Table III: Plasma osmolalities and chloride ion levels at various fall acclimations.

Acclimation Temperature (°C)	Photoperiod	(n)	Osmolality m NaCl $\bar{x} \pm$ SE	Cl⁻Level m equiv/l $\bar{x} \pm$ SE
0°	12L-12D	10	0.152 ± 0.006	73.50 ± 9.00
0°	0L-24D	7	0.142 ± 0.005	81.25 ± 5.04
5°	12L-12D	8	0.183 ± 0.009	111.00 ± 7.55
5°	0L-24D	8	0.148 ± 0.006	102.30 ± 0.88
10°	12L-12D	8	0.166 ± 0.006	96.00 ± 4.58
10°	0L-24D	8	0.196 ± 0.004	106.00 ± 2.34

Figure 4. The relationship between plasma chloride ion levels and plasma osmolality in the chorus frog. The correlation is significant r (4,.05) = 0.8110.

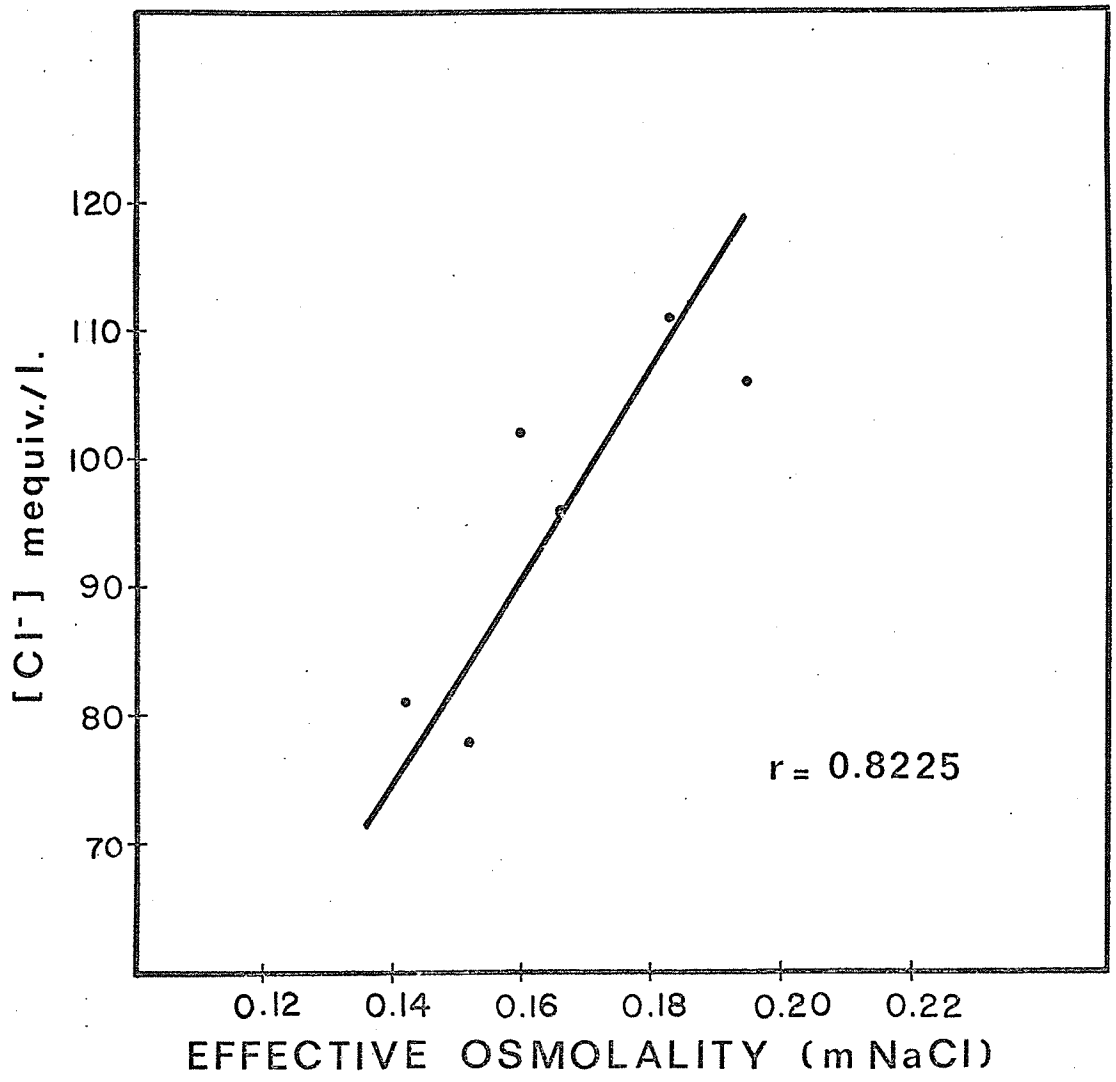

120
110
100
90
80
70
[Cl⁻] mequiv./l.
r = 0.8225
0.12
0.14
0.16
0.18
0.20
0.22
EFFECTIVE OSMOLALITY (m NaCl)

Table IV. Acclimation effects on water content, plasma osmolality, plasma Na+ levels, and plasma K+ levels in spring frogs. Tests for homogeneity of variance are found in Appendix C.

	Acclimations					
	0°C 8L-16D		10°C 12L-12D		t	Signif-icance
	$\bar{x} \pm$ SE	n	$\bar{x} \pm$ SE	n	value	
% Total Body Water	78.25 ± 0.575	9	77.97 ± 0.47	10	0.371	N.S.
Plasma Osmolality (m NaCl)	0.122 ± 0.003	9	0.147 ± 0.006	9	3.059	$P<.05$ Sign.
Plasma Na+ Levels (m equiv/1)	107.0 ± 2.601	6	109.3 ± 2.516	9	0.637	N.S.
Plasma K+ Levels (m equiv/1)	3.86 ± 0.503	6	3.53 ± 0.385	9	0.522	N.S.

4. Tissue Water Content

The percentage water contents were determined in tissues of spring frogs simultaneously acclimated for 17 days to 10°C 12L-12D and 0°C 8L-16D. This was done to see if there were any shifts of water into or out of specific tissues. The tissues used were: (1) muscle of the hind and forelegs; (2) liver; (3) heart; and (4) the remaining carcass.

The tissue water contents were consistently higher in frogs at 0°C (Figure 5). Both the hind and forelegs were significantly higher ($P < .05$) in the 0°C frogs than in the 10°C frogs, but no significant differences between 0°C and 10°C frogs were found in the liver, heart, remaining carcass or total body water. There was an upward trend in the water contents of all tissues with decreasing temperature.

5. Comparison of Spring and Fall Frogs

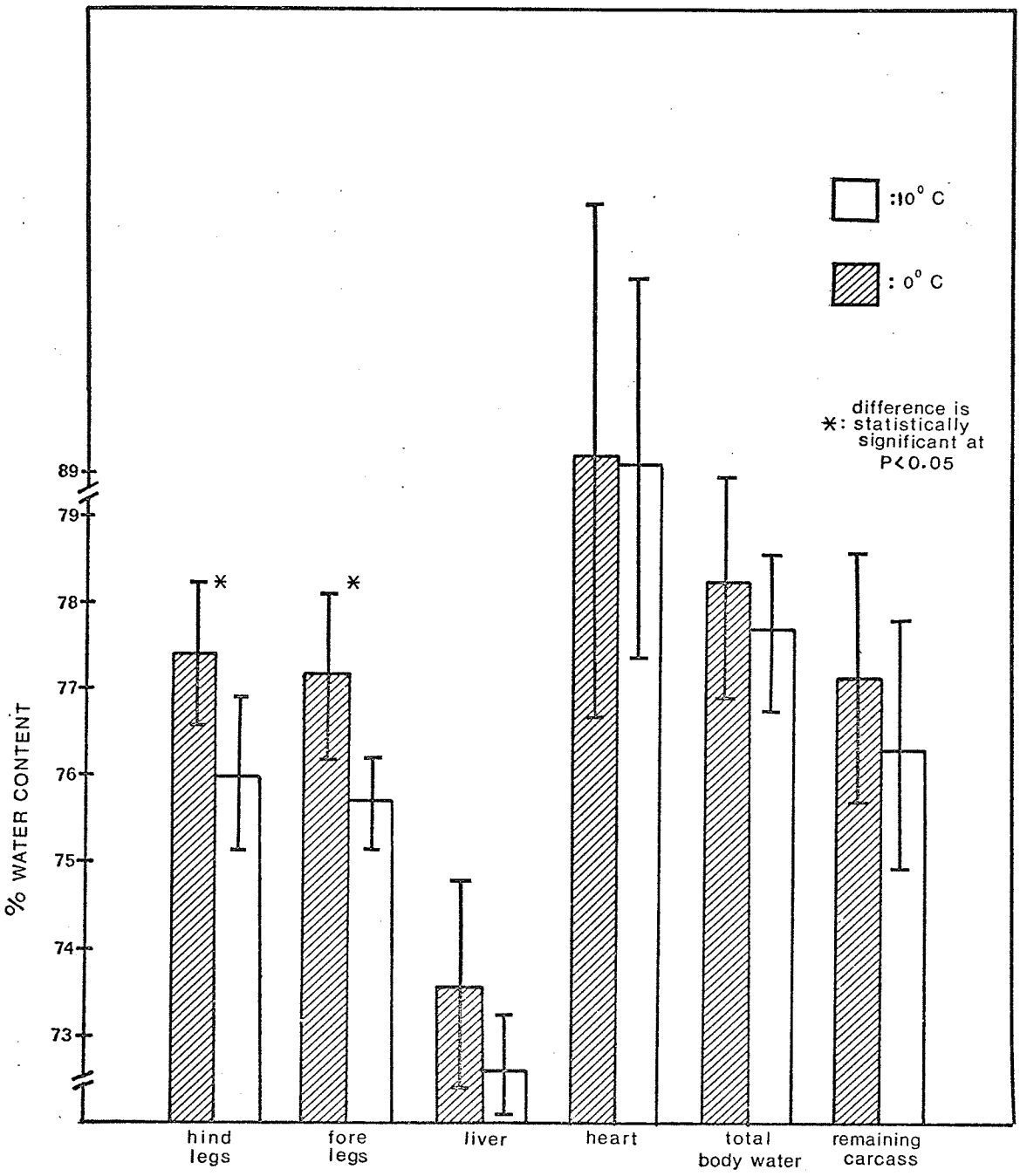
Frogs had been simultaneously acclimated to 0°C and 10°C in both fall and spring. To see if there were any seasonal variations in total water content and plasma osmolality a comparison was made between the two groups (i.e. spring and fall). See Appendix E for homogeneity of variance.

A. Relationship of Size to the Various Parameters Measured

(i) Size vs. Total Body Water Content

In all experiments the smaller frogs tended to have a

Figure 5. Percent total water contents of various tissues of chorus frogs acclimated to 0°C 8L-16D and 10°C 12L-12D. The vertical bars represent 95% confidence intervals. Tests for homogeneity of variance are found in Appendix D.


:10° C
: 0° C
difference is
*: statistically
significant at
P<0.05
% WATER CONTENT
89
79
78
77
76
75
74
73
*
*
hind
legs
fore
legs
liver
heart
total
body water
remaining
carcass

higher percentage body water than the larger animals.

In comparing the water contents of the spring and fall frogs, the difference in size ranges must be taken into account (mean weight of fall frogs = 0.4598 $\pm$ 0.0167 g; mean weight of spring frogs = 0.9572 $\pm$ 0.0398 g).

Initially a plot of wet weight of the fall animal vs. the percent body water was made and there was an obvious negative correlation (Figure 6). But since these are not independent variables it was decided to plot dry weight vs. water weight per animal.

The model assumed was $W = a\ D^{b}$, where W = water content and D = dry weight. The value of "b" would equal 1 if the water content is in constant proportion with the dry weight, but if the proportion was significantly higher in smaller frogs then "b" would be less than 1. If the relationship between proportion of water and size of frog was similar in spring and fall frogs, then the value of "b" should not differ significantly between the two groups. If this relationship was similar for spring and fall frogs, but for any given size of frog the water content was higher for one group than the other, then the value of "a" would differ between the two groups.

The model can be transformed to a linear additive model by taking logarithms of both sides: $\log W = \log a + b \log D$. The

Figure 6. The correlation between the wet weight and the percent body water in the chorus frog. The correlation coefficient is-0.6087 and it is significant at $P < .05$.

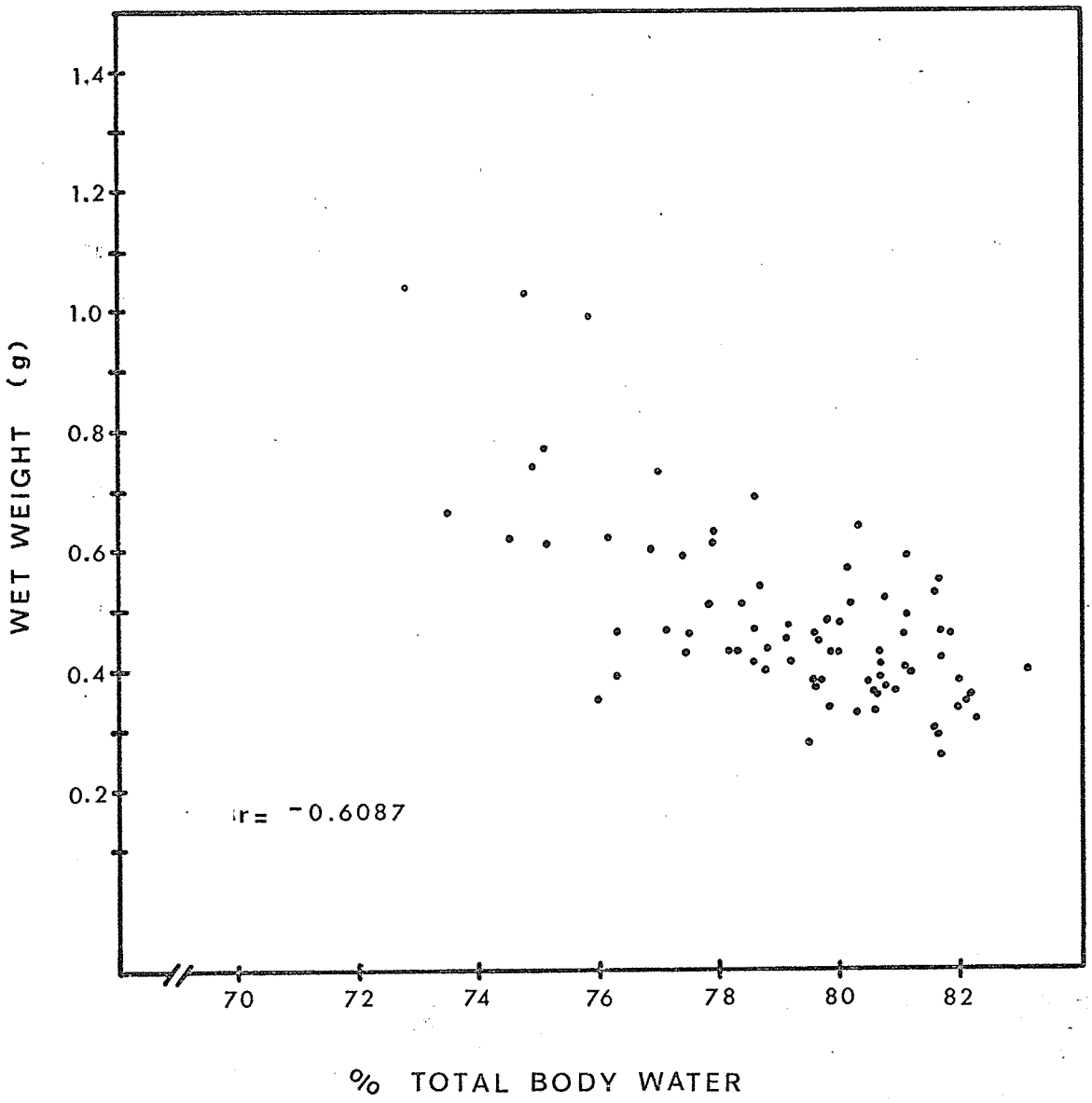
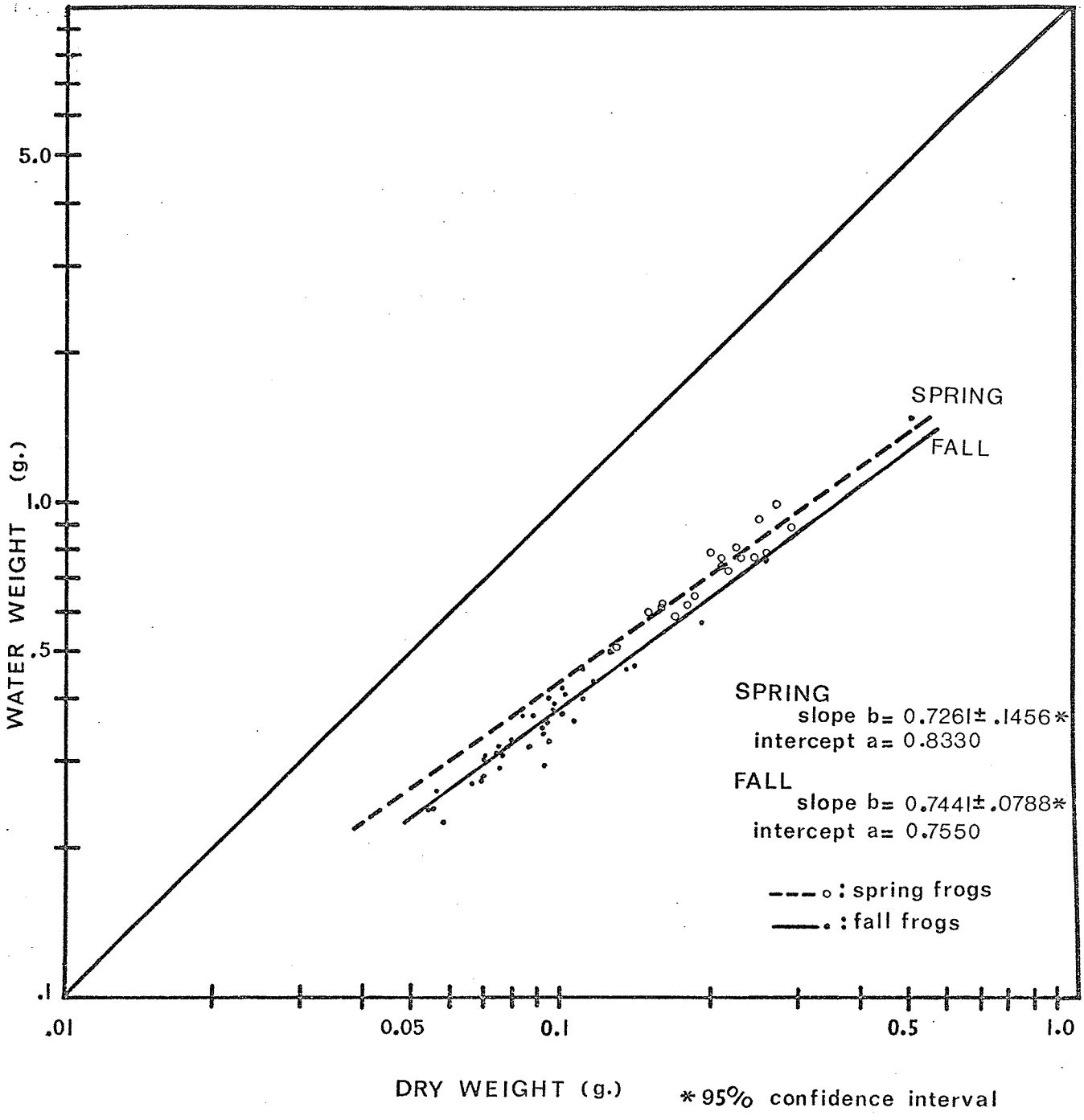

1.4
1.2
1.0
0.8
0.6
0.4
0.2
WET WEIGHT (g)
r= -0.6087
70
72
74
76
78
80
82
% TOTAL BODY WATER

Figure 7. The relationship between size and water content in chorus frogs. Each point represents one frog.


5.0
1.0
.5
.1
WATER WEIGHT (g.)
.01
0.05
0.1
0.5
1.0
DRY WEIGHT (g.)
SPRING
FALL
SPRING
slope b= 0.7261± .1456*
intercept a= 0.8330
FALL
slope b= 0.7441± .0788*
intercept a= 0.7550
---o : spring frogs
——• : fall frogs
*95% confidence interval

first test, for similar values of "b" between the two groups, amounts to a test for similarity of slopes in the regression of log W on log D. The slopes were not significantly different from each other ($P > .05$); but were significantly less than b = 1 ($P < .05$), implying that the relationship between proportion of water and size of frog is similar for spring and fall frog. To test whether the value of "a" differs between the two groups, the appropriate statistical test is for difference in the intercepts between the two regressions. The intercepts are significantly different ($P < .01$), implying that water content is greater for a spring frog than for a fall frog of the same size.

The two seasonal groups were compared after correcting for size differences. For each group the logarithm of water content of each frog was corrected to the value appropriate to a frog of "standard dry weight", i.e. mean dry weight = 0.1177g. (See Appendix G for calculations).

(ii) Size vs. Osmolality

There was no significant correlation between the dry weight of the frogs and the osmolality.

B. Water Content Comparison Between Spring and Fall Frogs

Although the average actual water content values at each

acclimation temperature were significantly lower ($P<.05$) in spring than in fall frogs, once the water content values were corrected for size differences, the water content was significantly higher in the spring frogs. See Figure 8a.

C. Plasma Osmolality Comparison Between Spring and Fall Frogs

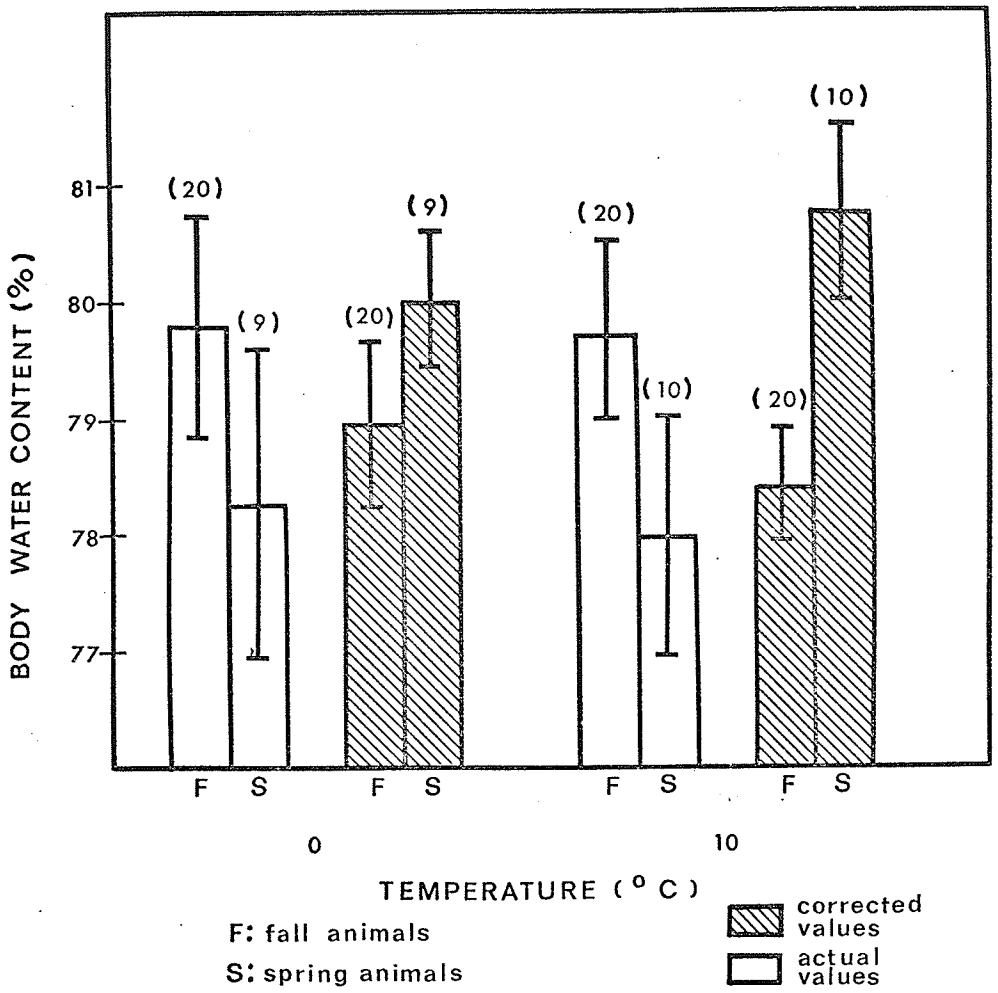
The mean plasma osmolality of the fall frogs at 10°C was 0.1810 m NaCl and that of the fall frogs at 0°C was 0.1482 m NaCl. The difference was statistically significant at the $P<.05$ level. The mean osmolality of spring frogs at 10°C was 0.1467 m NaCl which was significantly higher ($P<.05$) than the osmolality of the 0°C frogs which was 0.1223 m NaCl. In both spring and fall animals the osmolality was higher at 10°C than at 0°C. (Figure 8b). But the seasonal effect on osmolality can be seen in both acclimation temperatures (10°C and 0°C). At both acclimations the osmolality in the fall is significantly higher than in the spring ($P<.05$).

6. Supercooling

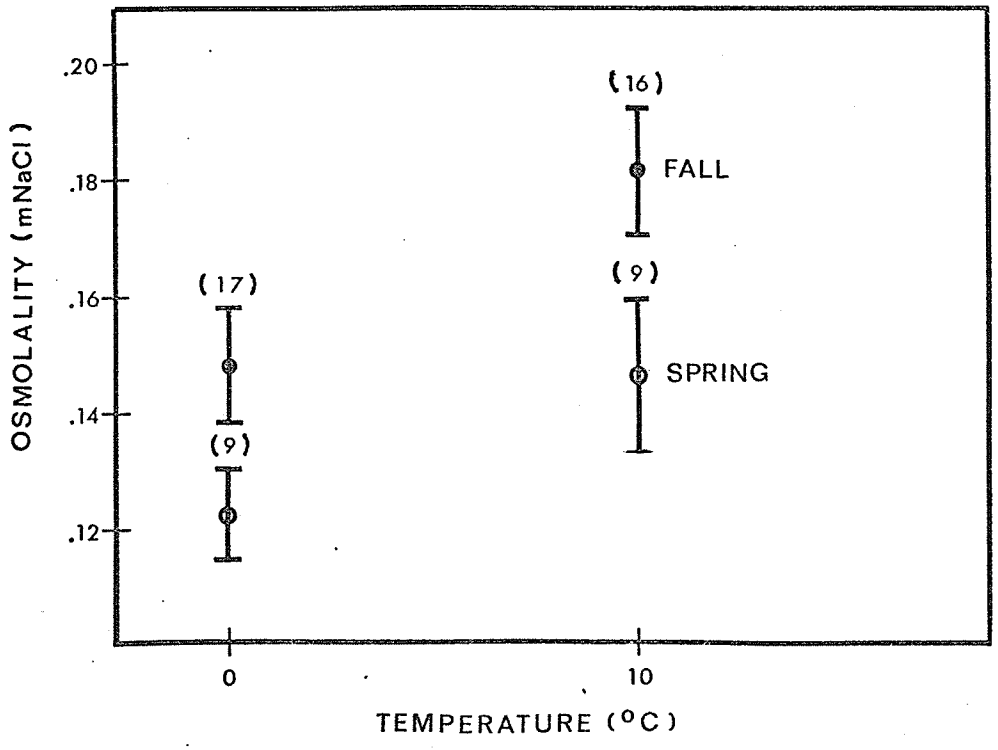
This experiment was performed to see if the chorus frog actually did have the ability to supercool and if so, what the limits were. Spring frogs that had been acclimated to 0°C and 5°C OL-24D photoperiod for at least two weeks were used.

Figure 8a. Actual and corrected percent total body water values in spring and fall chorus frogs, held at 0° and 10°C acclimations. Vertical bars represent 95% confidence intervals and the bracketed numbers, the sample sizes (n).

Figure 8b. Comparison of plasma osmolalities of spring and fall chorus frogs held at 0° and 10°C acclimations. Vertical bars represent 95% confidence intervals and the bracketed numbers, the sample sizes (n).


BODY WATER CONTENT (%)
81
80
79
78
77
(20)
(9)
(20)
(9)
(20)
(10)
(20)
(10)
F S F S F S F S
0
10
TEMPERATURE (°C)
F: fall animals
S: spring animals
corrected values
actual values

A


OSMOLALITY (mNaCl)
.20
.18
.16
.14
.12
(16)
FALL
(9)
SPRING
(17)
(9)
0
10
TEMPERATURE (°C)

B

A standard cooling curve was recorded for all animals with variations observed in the supercooling limits and freezing points. Figure 9 gives a typical recording pattern. The animals supercool to a point where the body temperature will rise and freezing occurs. The supercooling limit is easily recognizable on the recording because the body temperature will drop with the lowering of the external temperature until it reaches a point where supercooling ends and freezing ensues. At this point the body temperature rises to near 0°C where it remains until the animal is completely frozen. The temperature again declines with the external temperature.

The mean supercooling limit for the 5°C acclimated frogs was -3.1°C and for the 0°C frogs it was -2.9°C. There is not a significant difference at $P > .05$ level. (Table V).

It was observed that even if the frog had reached its supercooling limit and was at its freezing point, the animal would survive if it did not freeze solid. As long as the internal organs (specifically noted was the heart) were not frozen, the animal would revive with no apparent harmful effects.

As an animal froze it was observed that the eyes would become opaque and ice crystals could be felt in the appendages and abdomen. Once removed from the chamber and left at room temperature, the frogs would revive fully within 1 hour. By the

Figure 9. An example of a recording made when a chorus frog was supercooled. In this case the supercooling limit was approximately -3.0°C and the freezing point was -0.5°C.

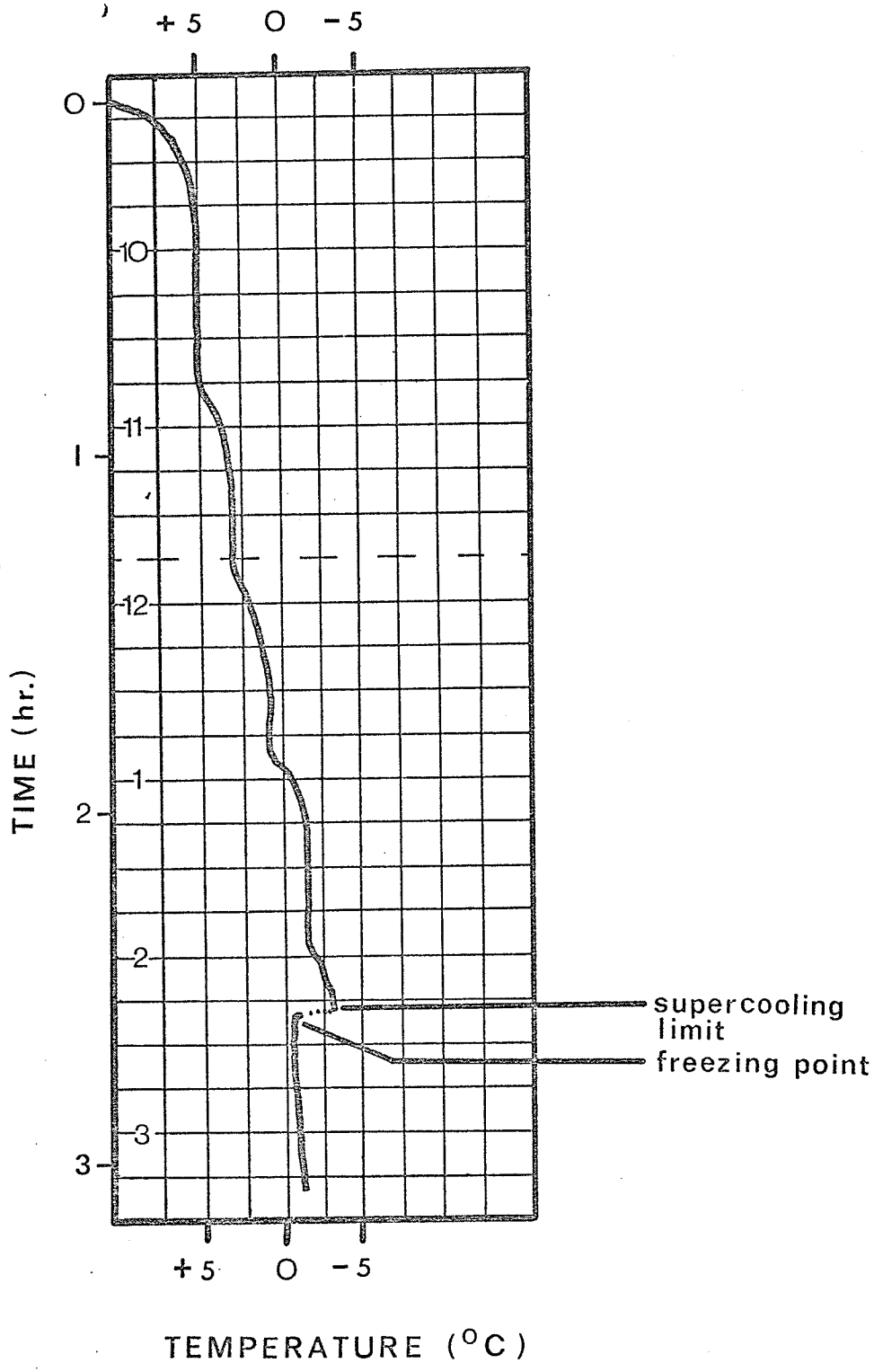

+5
0
-5
0
10
11
1
12
1
2
2
3
3
+5
0
-5
TIME (hr.)
TEMPERATURE (°C)
supercooling limit
freezing point

Table V. Supercooling limits and freezing points in the chorus frog and garter snake.

Animal	Acclimation Temperature °C	n	Supercooling Limit °C $\bar{x} \pm$ SE	Freezing Point °C $\bar{x} \pm$ SE
Pseudacris	0°	3	-2.96 ± 0.13	-0.88 ± 0.12
Pseudacris	5°	4	-3.12 ± 0.12	-0.70 ± 0.10
Thamnophis	1°	2	-2.35 ± 0.25	-0.62 ± 0.12

following day, the eyes cleared and vision seemed normal. All revived, supercooled frogs were kept for over one month.

Frogs which were supercooled, frozen and kept in the bath until their internal temperatures started to drop to that of the air inside the chamber, would freeze solid and not revive.

To see if other poikilotherms of Manitoba also had this ability to supercool, the garter snake Thamnophis sirtalis parietalis was also tested. Animals used were those that had just come out of hibernation or were still in hibernation. The supercooling limit was found to be -2.0°C.

So it appears that the boreal chorus frog does have the ability to supercool, apparently to a slightly greater degree than the garter snake under laboratory conditions.

DISCUSSION

1. Temperature and photoperiod effects on fall and spring frogs.

Among frogs caught in the fall and acclimated to 0°, 5° and 10°C with a 12L-12D or 0L-24D photoperiod, the plasma osmolality decreased with decreasing temperatures. There was an exception to this in the case of the 10°C, 12L-12D acclimated frogs, where the osmolality dropped from that at 5°C. This reduction in osmolality is not in agreement with the results in the 0L-24D acclimations or with subsequent experiments (see spring experiment results - figure 8b). It is believed that the plasma osmolality of this group of frogs may not be indicative of the values in the normal frogs as these animals were smaller than all others sampled and did not appear healthy (possible fungal infection).

In more aquatic frogs, Rana clamitans and R. pipiens, a decrease in plasma osmolality is common at low temperatures (Schmidt-Nielsen and Forester, 1954; Miller et al., 1968). The aquatic green frog R. clamitans becomes hydrated at 5°C and the plasma osmolality correspondingly decreases. When these frogs are warmed up the excess water is excreted. This phenomenon occurs in the frog's natural habitat in fall and winter as well. The dilution of electrolytes in cold treated R. pipiens has also been accounted for by hydration. Since these frogs are at a low

temperature, metabolism would be reduced and therefore osmoregulation might not be as efficient.

Thus in the case of the chorus frog, this decrease in plasma osmolality at low temperatures could be a result of:

(1) an overall hydration of the frog;

(2) movement of water out of the tissues and into the plasma;

(3) movement of ions out of the plasma and into the tissues.

Both the movement of water out of and the movement of ions into the tissues would increase tissue freezing resistance.

In reference to the overall hydration of the frog, the percent total body water values at the various acclimations did not change significantly ($P < .05$). But the hydration necessary to decrease the plasma osmolality may be so slight that it would not be detectable due to the lack of sensitivity in the procedure used.

To test this idea, the fall animal data was examined. The mean weight of these animals was 0.4788 ± 0.0179 g. Plasma volume is approximately 7% of the body weight (Gordon, 1972), thus the amount of plasma in a frog this size should be approximately 0.0335 g. The mean osmolality of the 10°C fall frogs was 0.1956 m NaCl and for the 0°C frogs it was 0.1425 m NaCl. It was

calculated that 0.0125 g of water must be added to 0.0335 g. of plasma to decrease the osmolality from 0.1956 to 0.1425 m NaCl. This addition of water should result in a 2.6% increase in percent body water of the animals at 0°C. Comparing the mean percent body water values at the two acclimation temperatures the difference is 1.724% which is less than the expected change. If the 95% confidence intervals are observed, rather than the means, the maximum possible change is 2.538% which is slightly larger than the theoretical expected change. It is possible that this small hydration does account for the dilution of the plasma.

In the spring animals the 0°C 8L-16D acclimated frogs had a plasma osmolality which was significantly lower than that of the 10°C 12L-12D acclimated frogs. Again the water contents did not vary significantly between the two temperature acclimations. The theoretical change in water content was again calculated and found to be 1.37%. The 95% confidence intervals of the two mean water contents (0° and 10°C) allows a maximum possible difference of 1.057%. This value is very close to the theoretical percent change expected.

It is possible that slight hydration is occurring, but if it is not then the plasma must be losing ions to the tissues or else water is being shifted out of the tissues and into the

plasma. Hydration would allow the animal to tolerate greater desiccation which a terrestrial overwintering frog might be subjected to during the winter. However, hydration would not be advantageous to the animal if it were subjected to temperatures at or below 0°C because it would be diluting body fluids and therefore the animal would be less resistant to freezing.

2. Tissue water contents in spring animals

Because plasma osmolality changed with temperature and there was no significant change in total body water, partitioning of water within the frog seemed likely. The water content was found to significantly increase ($P < .05$) at low temperatures in the muscle and although the differences were not significant in the other tissues, there was a trend towards increasing water content at low temperatures.

Stangenberg (1955) reported a higher muscle water content in cold-adapted Rana esculenta, but Rieck (1960) and Bishop and Gordon (1967) reported that Rana pipiens muscle water content was constant during cold acclimation. So it appears that adaptation to cold in terms of water content can vary even among frogs which overwinter subaquatically.

In Pseudacris there is an increase in the water content of the tissues as well as a dilution of the plasma at low temperatures. Even though the total body water content did not

increase significantly at the low temperature, this tissue water content data tends to support the idea of overall hydration. If in fact, the total body water values do not change at low temperatures possibly the ions in the plasma are being shunted into the tissues as an adaptation to cold i.e. resistance to freezing. But the mechanism may be imperfect and water may follow into the tissues by osmotic pressure. The result of this would be:

(1) no change in total body water content;

(2) an increase in tissue ion concentration and water content;

(3) a decrease in plasma osmolality;

(4) a decrease in plasma volume.

Three of these effects were observed i.e. (1) no significant change in total body water content (assuming the method of water determination was sensitive enough), (2) an increase in tissue water content, (3) a decrease in plasma osmolality. Tissue osmolality (intracellular) and plasma volumes were not measured because of limited facilities and experimental animals. An attempt was made to record hematocrit values in blood samples but it was found that lymph was sometimes taken up with the blood samples causing the hematocrit values to fluctuate .

Increasing the tissue osmolality would be advantageous as an antifreeze mechanism. But it must be noted that these

frogs in addition would lose water to the freezing environment causing the ionic concentration in the tissues, particularly the muscle to increase. Thus these animals must be able to tolerate high intracellular salt concentrations.

3. Ion levels in the plasma

In the experiments on fall frogs, chloride ion levels were determined in the plasma samples. There was a high correlation between Cl^- levels and plasma osmolality (r=0.8225). Hibernating green frogs (genus and species not named) taken from the ponds during winter and brought into a warm laboratory were found to give off large quantities of urine rich in chloride ions (De Haan, 1972). This supports the idea that changes in plasma osmolality may be due to fluctuations in chloride ion levels.

In the experiments on spring frogs, Cl^- levels were not determined, instead Na+ and K+ readings were done. Both Na+ and K+ levels did not change significantly between the 0°C and 10°C acclimations, although the plasma osmolalities did.

Moore (1964) gives the Cl^- levels in frog plasma as ranging between 64 and 96 m moles/liter (genus: Rana, but no species named). Gordon (1972) gives Cl^- values in Rana esculenta of 75 m moles/1 and Na+ levels of 105 m moles/1. In Rana temporaria there was 74 m moles Cl^-/1, 104 m moles Na+/1, and 2.2 m moles K+/1.

Chorus frogs collected in the fall had much higher Cl^- levels than those reported in the literature. This again supports the idea that a chloride ion level increase causes a change in the plasma osmolality.

4. Seasonal variation in water content and plasma osmolality.

When size difference was corrected for, it was found that the spring frogs actually had a significantly higher water content than the fall frogs. Therefore the low plasma osmolality in the spring frogs may be due to the increase in overall water content of the animal. This increase could be explained by the fact that although these frogs are mainly terrestrial, those caught in the spring were in breeding pools. Therefore their osmoregulatory mechanism may not be as efficient as aquatic frogs in dealing with excess water intake. Also, in the spring, water temperatures were quite low and as discussed before the frogs might not have been capable of effective osmoregulation. This has been found in the aquatic bullfrog, Rana esculenta which increases body water when transferred from room temperature to low temperatures of 2 to 3°C (Jørgensen, 1949), so it may be occuring in Pseudacris as well.

In the winter when the surrounding free water freezes, these frogs are subject to desiccation. Therefore, prior to emergence in the spring they are probably at their tolerance limit for desiccation. On emergence, due to the availability of

water i.e. spring run-off, they dehydrate rapidly.

From the results, the fall frogs seem to be dehydrated when compared to spring frogs.

Schmid (1965) determined the osmolarities of plasma samples from a number of anuran species (osmolarity and osmolality are considered equivalent at the level of biological fluids (Gordon, 1972). The values ranged from 0.153 M NaCl in the aquatic mink frog, Rana septentrionalis to 0.1710 M NaCl in the terrestrial common toad, Bufo americanus and 0.1625 M NaCl in the semi-terrestrial wood frog, R. sylvatica. From the above values there seems to be a trend of increasing plasma osmolality in the more terrestrial species of anurans. Since Pseudacris is semi-terrestrial, one would expect a plasma osmolality somewhere between the aquatic and terrestrial values. But the experimental value obtained in fall frogs is higher even than that of the terrestrial toad B. americanus, suggesting hemoconcentration in Pseudacris.

Spring animals had plasma osmolality values that were even lower than the values found for the aquatic mink frog, R. septentrionalis, suggesting hydration of the chorus frogs in the spring.

In the gartersnake Thamnophis sirtalis parietalis, the water content decreases in the fall (Aleksiuk and Stewart, 1971). It was stated that this could be a mechanism of resistance to freezing i.e. hemoconcentration. Farrell (1971) found that the tree frog Hyla crucifer had a reduced water content at 7°C as compared to 20°C. Their tolerance to desiccation was also greater at 7°C.

Poikilotherms lose water to varying degrees when exposed to cold or they will resist water loss completely. In the two turtles Terrapene cardina carolina and Pseudemys scripta elegans, hemoconcentration occurs at low temperatures (Hutton and Goodnight, 1957). In the grass snake, Natrix natrix there is a very slight degree of hemoconcentration (Binyon and Twigg, 1965) and in the turtle Chrysemys picta, hemodilution occurs in cold torpor (Musacchia and Sievers, 1956).

Pseudacris does have an increased plasma osmolality in the fall, suggesting that it does hemoconcentrate and that this change is not induced by temperature alone, but is a seasonal adjustment.

5. Ability to supercool .

Chorus frogs were found to supercool by approximately 3°C below the freezing point of their body fluids.

Lowe et al. (1970) state that the supercooling limit is directly proportional to the freezing point. Since it was found

that the plasma osmolality decreased at low temperatures during short term acclimations it would be expected that the freezing point and associated supercooling limit would not be as great at 0°C as at 5°C. Also the fall animals with a much higher osmolality would be expected to supercool to an even lower temperature.

To see if other poikilotherms common to Manitoba are capable of supercooling, the red-sided garter snake, Thamnophis sirtalis parietalis was tested. They supercooled to -2.0°C. Pseudacris, therefore, can supercool to a slightly lower level than the garter snake.

Bailey (1949) found that Thamnophis radix could withstand a minimum temperature of -2.0°C. He attributed this tolerance to (1) gradual physiological preconditioning to low temperatures (2) the low temperature of crystallization of protoplasm as compared to water and (3) supercooling of the body tissues. Supercooling limits in T. sirtalis parietalis seem to agree with results obtained by Bailey (1949).

Pseudacris may supercool to a greater degree in nature than that observed in the laboratory since the rate of cooling is slower in nature. The rate used here was 2°C/0.5 hr. Preliminary experiments were done with faster rates and the results were the same. Possibly with slower cooling rates, more

in line with natural situations, the supercooling limit would have decreased.

Kirton et al. (1973) tagged wood frogs R. sylvatica in Alaska and followed them to their overwintering sites. He monitored the temperatures at these sites and found three frogs survived temperatures of -5°, -7° and -9°C.

Vincent (1971) monitored ground temperatures at a depth of 21 cm. and 56 cm. in a natural snake den and found the temperatures never went to below -3.0°C and in an artificial limestone den the lowest temperature was approximately -5.0°C at 0.3 m below the surface.

The most likely overwintering sites for Pseudacris in Manitoba are under litter or in soil crevices, usually in woodlands. The protection of even a small amount of overlying litter or soil, and the tendency for a deep layer of snow to accumulate in such areas probably provides sufficient insulation to prevent ambient temperatures from dropping below -5.0°C. Although this value is lower than the supercooling point of chorus frogs in the laboratory, they may well supercool to a lower temperature in nature. Also, ambient temperatures of -5.0°C at or near the soil interface are not a regular feature of soil temperature profiles in Manitoba, nor do they persist for a great length of time. Temperatures between -1°C and -3°C at the soil-snow

interface are more usual over the winter as a whole, and these temperatures are within the observed supercooling range determined in the laboratory for Pseudacris.

CONCLUSIONS

1. Plasma osmolality decreased at low temperature acclimations in both fall and spring frogs. The biological significance for this is not obvious.

2. Fall animals had a much higher plasma osmolality and a lower total body water content than spring animals. This hemoconcentration may be an active freezing resistance mechanism, but it is not extensive enough to allow the animals to tolerate very low temperatures without freezing.

3. Temperature affected the total water content, plasma osmolality and chloride ion levels of chorus frogs, whereas photoperiod did not. This shows that temperature plays a more important role in triggering overwintering physiological adaptations in Pseudacris.

4. The boreal chorus frog can supercool to approximately -3.0°C. Selection of appropriate overwintering sites and the ability to supercool probably allows the chorus frog to survive without additional freezing-resistance mechanisms. In fact, freezing may not be as serious a problem to these frogs as dehydration would be.

BIBLIOGRAPHY

Aleksiuk, M. and K. W. Stewart 1971. Seasonal changes in the body composition of the garter snake (Thamnophis sirtalis parietalis) at Northern latitudes. Ecology 52 (3), pp. 485-490.

Bailey, R. M. 1949. Temperature toleration of garter snakes in hibernation. Ecology 30, pp. 238-242.

Barnes D. and A. C. Hodson 1956. Low temperature tolerance of the European corn borer in relation to winter survival in Minnesota. J. Econ. Ent. 49, pp. 19-24. Cited in: Smith, A. U. 1958.

Barnes D. and A. C. Hodson 1957. Low temperature tolerance of the European corn borer in relation to winter survival in Minnesota. Rev. appl. Ent. 45A, pp. 93-94. Cited in: Smith A. U. 1958.

Barrington, E. J. W. 1968. The Chemical Basis of Physiological Regulation. Scott, Foreman and Co. 274 pp.

Binyon, E. J. and G. I. Twigg 1965. Seasonal temperature concentration changes in the blood and thyroid of the grass snake, Natrix natrix. Nature 207,(4998) pp. 779-780.

Bishop, L. G. and M. S. Gordon 1967. Thermal adaptation of metabolism in Anuran amphibians. In: A Symposium on Molecular Mechanisms of Temperature Adaptation. 27-29 December, 1965. Berkeley, California. American Association of Advanced Science Publ., pp. 263-280.

Blanchard, F. N. 1933. Late autumn collection and hibernating situations of the salamander Hemidactylum scutatum (Schlegel) in Southern Michigan. Copeia No. 4, p. 216.

Conant, R. 1958. A field Guide to Reptiles and Amphibians. Houghton Mifflin Company, Boston. 366 pp.

De Haan, J. 1927. Variations in the blood and tissue fluids in summer and winter frogs. Biol. Generalis 3 (1/2), pp. 1-14.

De Vries, A. L. 1971. Glycoproteins as biological antifreeze agents. Science 172, pp. 1152-1155.

De Vries, A. L., S. K. Komatsu and R. E. Fenney 1970. Chemical and physical properties of freezing point depressing glycoproteins from Antarctic fishes. J. Biol. Chem. 245 (11): pp. 2901-2908.

Farrell, M. P. 1971. Effect of temperature and photoperiod acclimation on the water economy of Hyla crucifer. Herpetologica 27, pp. 41-48.

Gordon, M. S. 1972. Animal Physiology: Principles and Adaptations. The MacMillan Company, New York. Second Edition. 592 pp.

Gross, W. J. 1954. Osmotic responses in the sipunculid, Dendrostomum zostericolum. J. Exp. Biol. 31pp. 402-423.

Hickman, C. P. Jr., R. A. McNabb, J. S. Nelson, E. D. Van Breeman, and D. Comfort. 1964. Effects of cold acclimation on electrolyte distribution in rainbow trout (Salmo gairdnerii). Cdn. J. Zool. 42, pp. 577-597.

Hoar, W. S. 1966. General and Comparative Physiology. Prentice-Hall, Inc. Englewood Cliffs, New Jersey. 815 pp.

Hutton, K. E.&Goodnight, C. J. 1957. Variations in the blood chemistry of turtles under active and hibernating conditons. Physiol. Zool. 30, pp. 198-207.

Jørgensen, C.B. 1950. Osmotic regulation in the frog. R. esculenta (L) at low temperatures. Acta Physiol. Scand. 20:46, pp. 46-55.

Kirton, M. P. 1973. Fall movements and hibernation in Rana sylvatica from interior Alaska. Paper presented at the American Society of Ichthyologists and Herpetologists, 53rd Annual Meeting June 24-30, 1973. San Jose, Costa Rica.

Ling, G. N. 1967. Effects of temperatures on the state of water in the living cell. In: Thermobiology 1967. Edited by A. H. Rose. Academic Press, London and New York, pp. 18-24.

Lovelock, J. E. 1954. Biophysical aspects of the freezing and thawing of living cells. Proceedings of the Royal Society of Medicine. 47 (1), pp. 60-62.

Lowe, C. H., P. J. Lardner and E. A. Halpern 1971. Supercooling in reptiles and other vertebrates. Comp. Biochem, Physiol. 39A, pp. 125-135.

Luyet B. J. and M. C. Hartung 1941a. Survival of Anguilla aceti after solidification in liquid air. Biodynamica 3, p. 353-362.

Luyet, B. J. and M. C. Hartung 1941b. Factors in revival of Anguilla aceti after its solidification in liquid air. Am. J. Physiol. 133, p. 368.

Maetz, J. 1968. Salt and Water Metabolism. In: Perspectives in Endocrinology. E. J. W. Barrington and C. Barker Jorgensen 1968. Academic Press, London and New York. pp. 47-162.

McClanahan, L. 1964. Osmotic tolerance of the muscles of two desert inhabiting toads, Bufo cognatus and Scaphiopus couchi. Comp. Biochem. Physiol. 12, pp. 501-508.

Miller, D. A. M.,I Standish and A. Thurman Jr. 1968. Effects of temperature on water and electrolyte balance in the frog. Physiol. Zool. 41 (4), pp. 500-506.

Moore, J. A. 1964. Physiology of the Amphibia. Academic Press: New York and London. 654 pp.

Musacchia, X. L. and Sievers, M. L. 1956. Effects of induced cold torpor on blood of Chrysemys picta. Am. J. Physiol. 187 (1), pp. 99-102.

Prosser C. L., W. MacKay and K. Kato 1970. Osmotic and ionic concentrations in some Alaskan fish and goldfish from different temperatures. Physiol. Zool. 43 (2), pp. 81-89.

Rieck, A. F., J. A. Belli and M. E. Blaskovics 1960. Oxygen consumption of whole animal and tissues in temperature acclimated amphibians. Proc. Soc. Exptl. Biol. Med. 103, pp. 436-439.

Salt, R. W. 1950. Time as a factor in the freezing of under-cooled insects. Canada J. Res. D, 28, pp. 285-91. Cited in: Smith, A. U., 1958.

Salt, R. W. 1959. Role of glycerol in the cold-hardening of Bracon cephi (Gahan). Cdn. J. Zool. 37, pp. 59-69.

Salt, R. W. 1961. Resistance of poikilothermic animals to cold. British Medical Bulletin 17, pp. 5-8.

Schmid, W. D. 1965. Some aspects of the water economies of nine species of amphibians. Ecology 46, pp. 261-269.

Schmidt-Nielsen, B. and R. P. Forester 1954. The effect of dehydration and low temperature on renal function in the bull frog. J. Cell. Comp. Physiol. 44, p. 233.

Scholander, P. F., L. Van Dam, J. W. Kanwisher, H. T. Hammel and M. S. Gordon 1957. Supercooling and osmoregulation in Arctic fish. J. Cell and Comp. Physiol. 49, pp. 5-24.

Smith, A. U. 1958. The resistance of animals to cooling and freezing. Camb. Biol. Rev. 33, pp. 197-253.

Stangenberg, G. 1955. Der Temperatureinfluss auf Lebensprozesse und Der Cytochrom C Gehalt Bein Wasserfrosch. Arch. ges. Physiol. 260, pp. 320-332. Cited in: Bishop, L. G. and M. S. Gordon, 1967.

Vincent, T. K. 1971 (Thesis) Resistance to cold stress in the red-sided garter snake. Thamnophis sirtalis parietalis. 54 pp. The University of Manitoba, Winnipeg, Manitoba.

White, A., P. Handler and E. Smith 1968. Principles of Bio-Chemistry. Fourth Edition. McGraw-Hill Book Co. 1187 pp.

Zain-ul-abedin, M. Zeb-un-nisa Behleem and M. Ataur Raham 1969. Blood electrolytes of a lizard. Pak. J. Biochem. 2 (2), pp. 47-49.

Appendix A.

Starvation Experiment

I. Bartlett's Test

Treatment	d.f.	Variance s^2
1	4	0.5951
2	6	19.0649
3	8	10.9651
4	9	9.2575
5	4	5.3599

M/C = 9.1280

X^2 (.05,4) = 9.49 (4 d.f.)

$\therefore$ Variances are homogeneous at $P < .05$ level.

II. One-way Analysis of Variance

Source of Variation	d.f.	SS	MS
Treatment	4	53.6968	13.4242
Error	31	309.2490	9.9757
Total	35	362.9458	

F = 1.3456

$F_{.05(4,31)} = 2.68$

$\therefore$ Means are not significantly different

Appendix B

Fall Frog Experiments

I. Bartlett's Tests

a. Water Content

Treatment	d.f.	Variance s^2
1	9	4.1192
2	9	3.1127
3	9	5.9004
4	9	6.2011
5	9	1.0628
6	9	3.3504

M/C = 7.1917 (5 d.f.)

X^2 (.05,5) = 11.07

∴ Variances are homogeneous at $P < .05$ level.

b. Plasma Osmolality

Treatment	d.f.	Variance s^2
1	9	0.0004
2	6	0.0002
3	7	0.0006
4	7	0.0001
5	7	0.0003
6	7	0.0001

M/C = 8.4493 (5 d.f.)

X^2 (.05,5) = 11.07

∴ Variances are homogeneous at $P < .05$ level.

c. Chloride Levels

Treatment	d.f.	Variance s^2
1	1	162.0
2	3	101.583
3	2	171.0
4	2	2.333
5	2	63.0
6	3	22.0

M/C = 6.6664 (5 d.f.)

X^2 (.05,5) = 11.07

$\therefore$ Variances are homogeneous at $P < .05$ level.

II. Two-way Analysis of Variance - Effect of Temperature and Photoperiod on Total Water Contents of Fall Frogs

Anova Table

Source	d.f.	SS	MS	F	Significance
*T	2	7.289	3.645	0.9216	N.S.
P	1	4.400	4.400	1.1125	N.S.
TP	2	35.160	17.580	4.4450	SIGN
Within	54	213.550	3.955		
Total	59				

F.05(2,54) = 3.17
F.05(1,54) = 4.02
F.05(2,54) = 3.17

*T = Temperature
P = Photoperiod

Acclimation temperature and photoperiod individually do not have an effect on the water content of the frogs. There is a significant interaction at $P < .05$ level.

III. Duncan's New Multiple Range Test on Pairs of Means of Water Contents in Fall Frogs.

	A	B	C	D	E	F
Acclimations:	0°C OL-24D	5°C OL-24D	10°C OL-24D	0°C 12L-12D	5°C 12L-12D	10°C 12L-12D
$\bar{X}$ (%)	80.62	78.31	78.89	79.0	79.79	80.65

$S_{\bar{x}} = \sqrt{\text{error M.S.}/r}$

$= 0.629$

d.f. = 54

Values of P:	2	3	4	5	6
SSR	2.846	2.996	3.086	3.156	3.206
LSR	1.790	1.884	1.941	1.985	2.016

Pairs		Difference			Significance
F-B	=	2.34	>	2.016	Significant
F-C	=	1.76	<	1.985	NS
F-D	=	1.65	<	1.941	NS
F-E	=	0.86	<	1.884	NS
F-A	=	0.03	<	1.790	NS
A-B	=	2.31	>	1.985	Significant
A-C	=	1.73	<	1.941	NS
A-D	=	1.62	<	1.884	NS
A-E	=	0.83	<	1.790	NS
E-B	=	1.48	<	1.941	NS
E-C	=	0.90	<	1.884	NS
E-D	=	0.79	<	1.790	NS
D-B	=	0.69	<	1.884	NS
D-C	=	0.11	<	1.790	NS
C-B	=	0.58	<	1.790	NS

IV. The Effect of Temperature and Photoperiod on the Plasma Osmolalities of Acclimated Frogs. Two-Way Analysis of Variance

Preliminary ANOVA

SOURCE	d.f.	S.S.	M.S.	F
Among Cells	5	0.0160	0.0032	8.9385*
P ignoring T	1		.000032	
T ignoring P	2		.009100	
Within Cells	43	0.0154	0.000358	

* Significant at $P < .01$ level

$F_{.01}(5,43) = 3.475$

Simultaneous Equations

Assume $YijK = u + \alpha i + Bj + EijK$

$\therefore$

$m \quad 8.1190 = 49m + 23P_1 + 26p_2 + 17t_1 + 16t_2 + 16t_3$

$p_1 \quad 3.8010 = 23m + 23p_1 + 0p_2 + 7t_1 + 8t_2 + 8t_3$

$p_2 \quad 4.3180 = 26m + 0p_1 + 26p_2 + 10t_1 + 8t_2 + 8t_3$

$t_1 \quad 2.5195 = 17m + 7p_1 + 10p_2 + 17t_1 + 0t_2 + 0t_3$

$t_2 \quad 2.7035 = 16m + 8p_1 + 8p_2 + 0t_1 + 16t_2 + 0t_3$

$t_3 \quad 2.8960 = 16m + 8p_1 + 8p_2 + 0t_1 + 0t_2 + 16t_3$

Solutions: $m = 0.0493$, $p_1 = -0.1510$, $p_2 = -0.1480$

$t_1 = 0.2482$, $t_2 = 0.2692$, $t_3 = 0.2813$

$R(t, p) = mY... + \sum_i t_i Y_i + \sum_j piYj - CT$

$= 0.0096$

Final ANOVA

SOURCE	d.f.	S.S.	M.S.	F
Photoperiod	1	0.0001134	0.0001134	0.3167
Temperature	2	0.0094002	0.0047	19.70949*
Interaction	2	0.0064	0.0032	8.93850*
Within	43		0.000358	

$F.01(2,43) = 5.135$
$F.01(1,43) = 7.24$

* Temperature and Interaction are Significant at $P < .01$ Level.

V. Comparing Plasma Osmolities (Cont'd).

Multiple Range Test - Least Significant Difference.

$$\bar{x} - \bar{x}j \rangle t\ (\alpha,\gamma)\ S \sqrt{\frac{1}{ni} + \frac{1}{nj}}$$

t (.05,43) = 2.017

s = 0.01892

	0°C OL-24D A	5°C OL-24D B	10°C OL-24D C	0°C 12L-12D D	5°C 12L-12D E	10°C 12L-12D F
$\bar{x}$	0.1425	0.1548	0.1956	0.1522	0.1831	0.1663

Comparisons

	$\bar{x}_i - \bar{x}_j$	$t\ (\alpha,\gamma)\ s\sqrt{\frac{1}{n_i} + \frac{1}{n_j}}$	Significance
C-A =	0.0531	0.0212	Sign.
C-D =	0.0434	0.0192	Sign.
C-B =	0.0408	0.0204	Sign.
C-F =	0.0293	0.0204	Sign.
C-E =	0.0125	0.0204	N.S.
E-A =	0.0406	0.0212	Sign.
E-D =	0.0309	0.0192	Sign.
E-B =	0.0283	0.0204	Sign.
E-F =	0.0168	0.0204	N.S.
F-A =	0.0238	0.0212	Sign.
F-D =	0.0141	0.0192	N.S.
F-B =	0.0115	0.0204	N.S.
B-A =	0.0123	0.0212	N.S.
B-D =	0.0026	0.0192	N.S.
D-A =	0.0097	0.0212	N.S.

Appendix C.

Spring Frog Experiments

I. Testing for Homogeneity of Variances (2-tailed F test)

	Treatment	d.f.	s^2
a) Water Content	1	9	2.2473
	2	8	2.9732

F = 1.323

$F_{.05\ (8,9)} = 4.10$

Variances are Homogeneous

b) Osmolality	1	8	0.0003
	2	8	0.0001

F = 3.0

$F_{.05\ (8,8)} = 4.43$

Variances are Homogeneous

c) Na^+ Levels	1	8	56.9652
	2	5	40.6000

F = 1.4031

$F_{.05\ (8,\ 5)} = 6.76$

Variances are Homogeneous

d) K+ Levels	1	8	1.3375
	2	5	1.5164

F = 1.1338

$F = {}_{.05\ (5,\ 8)} = 4.65$

Variances are Homogeneous

Appendix D.

Tissue Water Contents

I. Testing for Homogeneity of Variances

	Treatment	d.f.	Variance s^2
a) Hind Legs	1	9	1.3374
	2	9	1.5266
F = 1.1414			
$F_{.05\ (8,8)} = 4.43$			
Variances are Homogeneous			
b) Forelegs	1	9	1.9449
	2	8	0.6675
F = 2.9137			
$F_{.05\ (9,8)} = 4.30$			
Variances are Homogeneous			
c) Heart	1	9	27.8653
	2	9	10.3032
F = 2.7045			
$F_{.05\ (9,9)} = 3.96$			
Variances are Homogeneous			
d) Liver	1	9	3.1658
	2	9	0.6999
F = 4.5232			
$F_{.05\ (9,9)} = 3.96$			
Variances are not Homogeneous			
e) Total Body Water	1	8	2.9713
	2	9	2.2473
F = 1.322			
$F_{.05\ (8,9)} = 4.10$			
Variances are Homogeneous			

Appendix E.

Comparison of Spring and Fall Frogs

i. Testing for Homogeneity of Variances (2-Tailed F Test)

	Treatment	d.f.	Variance s^2
a) Total Body Water			
Body Water at 10°C	1 (spring)	9	0.5811
	2 (fall)	19	1.3446
F = 2.3138			
$F_{.05\ (19,9)} = 2.77$			
Variances are Homogeneous			
Body Water at 0°C	1 (spring)	8	1.0659
	2 (fall)	19	1.3501
F = 1.2666			
$F_{.05\ (19,8)} = 2.91$			
Variances are Homogeneous			
b) Osmolality			
10°C	1 (spring)	8	0.0003
	2 (fall)	15	0.0004
F = 1.33			
$F_{.05\ (15,\ 8)} = 4.10$			
Variances are Homogeneous			
0°C	1 (spring)	8	0.0001
	2 (fall)	16	0.0004
F = 4.0			
$F_{.05\ (16,8)} = 4.10$			
Variances are Homogeneous			

Appendix F.

Covariance Analysis. To test if size has an effect on the water contents of chorus frogs.
N.B. Analysis done on natural log values.

Group	Means x	Means y	S.D. x	S.D. y	Regression a	Regression b	Correlation Coefficient	t value	d.f.
Fall	-2.40	-1.03	0.317	0.247	0.755	0.7411	0.951	19.0	38
Spring	-1.57	-0.31	0.226	0.176	0.833	0.7261	0.931	10.5	17
Combined	-2.14	-0.80	0.486	0.406	0.955	0.8194	0.981	38.69	57

		Sum of Products			Y Adjusted for X		
Source of Variance	DF	XSS	XYSP	YSS	Red. YSS Due to Regression	Deviations From Regression DF	SS	Mean Square
Between Groups	1	8.86	7.65	6.60		1	0.05	0.049
Within Groups	57	4.83	3.57	2.94	2.63	56	0.30	0.005
Total	58	13.69	11.22	9.54	9.19	57	0.35	0.006

Significance of differences between adjusted group Y means. F = 9.126 with 1 and 56 degree of freedom.
**P .01 ∴ intercepts are significantly different.

Testing of Covariance Assumptions.
Homogeneity of within group variances.
Bartlett's chi-square = 0.522 with 1 degree of freedom.

∴ is non significant

∴ variances are homogeneous.

Homogeneity of within group regression coefficients.
F = 0.023 with 1 and 55 d.f.

∴ not significant at P$>$.01.

Pooled regression coefficient = 0.738 n.s. (P$>$.05) i.e. same regression coefficient for spring and fall animals.

Appendix G.

Correcting Spring and Fall Water Contents of Frogs for Size Variation.

$\log_e [\bar{x}$ dry weight (for all animals $] = -2.14$

$\bar{x}$ dry weight $= 0.1177$g.

Spring standard water weight at $\bar{x}$ weight:

WW = water weight

DW = dry weight

$\log WW = \log a + b \log DW$

$= .833 + .7261 (-2.14)$

$= -0.7208$

$\therefore WW = 0.4863$ g.

Fall standard water weight at $\bar{x}$ dry weight:

$\log WW = \log a + b \log DW$

$= .7550 + .7441 (-2.14)$

$= -0.8374$

$\therefore WW = 0.4328$ g.

Appendix G.

Actual, Theoretical and Corrected Water Weights

A. Spring

	log (Theoretical water weight)	log (Actual waterweight)	log deviation	standard WW ± Dev.	anti-log	corrected % water
10°C Acclimation						
1.	-0.1535	-0.1475	+0.0060	-0.7148	+0.4893	80.61
2.	-0.2430	-0.2015	+0.0415	-0.6793	0.5070	81.16
3.	-0.1068	-0.0077	+0.0991	-0.6217	0.5370	82.02
4.	-0.1750	-0.0668	+0.1082	-0.6126	0.5419	82.15
5.	-0.0620	-0.1028	-0.0408	-0.7616	0.4669	79.87
6.	-0.2771	-0.3139	-0.0368	-0.7576	0.4688	79.93
7.	-0.3359	-0.2195	+0.1164	-0.6044	0.5464	82.28
8.	-0.4085	-0.4695	-0.0610	-0.7818	0.4576	79.54
9.	-0.1818	-0.2472	-0.0654	-0.7862	0.4556	79.47
10.	-0.2940	-0.3008	-0.0068	-0.7276	0.4831	80.41
0°C Acclimation						
11.	-0.5340	-0.5107	+0.0233	+0.6975	0.4978	80.88
12.	-0.2923	-0.2421	+0.0502	-0.7710	0.4625	79.71
13.	-0.4519	-0.5336	-0.0817	-0.8025	0.4482	79.20
14.	-0.5022	-0.4671	+0.0351	-0.6857	0.5037	81.06
15.	-0.4744	-0.4763	-0.0019	-0.7227	0.4854	80.48
16.	-0.3911	-0.4219	-0.0308	-0.7516	0.4716	80.03
18.	-0.1326	-0.2421	-0.1095	-0.8303	0.4359	78.74
19.	-0.2081	-0.2588	-0.0507	-0.7715	0.4623	79.71
20.	-0.6602	-0.6593	+0.0009	-0.7199	0.4868	80.53

Appendix G.

B. Fall

log (Theoretical water weight	log (Actual water weight)	log deviations	standard WW ± Dev.	antilogs	corrected % water
0°C Acclimation					
1. -0.2456	-0.2577	-0.0121	-0.8495	0.4276	78.41
2. -1.1704	-1.2259	-0.0555	-0.8929	0.4095	77.67
3. -1.2222	-1.2903	-0.0681	-0.9055	0.4043	77.45
4. -0.8813	-0.9113	-0.0300	-0.8674	0.4200	78.11
5. -1.0619	-0.9859	+0.0760	-0.7614	0.4670	79.87
6. -0.9981	-1.0035	-0.0054	-0.8428	0.4305	78.53
7. -1.1773	-1.1351	+0.0422	-0.7952	0.4515	79.32
8. -1.0671	-1.1114	-0.0443	-0.8817	0.4141	77.87
9. -1.0148	-1.2087	-0.1939	-1.0313	0.3565	75.18
10. -0.9872	-0.9547	+0.0325	-0.8049	0.4471	79.16
11. -0.9515	-0.8687	+0.0828	-0.7546	0.4710	80.01
12. -0.7834	-0.6607	+0.1227	-0.7147	0.4893	80.61
13. -0.8460	-0.8456	+0.0004	-0.8370	0.4330	78.63
14. -0.7079	-0.7664	-0.0585	-0.8959	0.4082	77.62
15. -1.2059	-1.1979	+0.0080	-0.8294	0.5030	78.75
16. -0.9561	-0.8059	+0.1502	-0.6872	0.5030	81.04
17. -0.9703	-0.8276	+0.1427	-0.6947	0.4992	80.92
18. -1.2442	-1.0933	+0.1509	-0.6865	0.5033	81.05
19. -1.0943	-0.9787	+0.1156	-0.7218	0.4859	80.50
20. -1.1754	-1.1751	+0.0003	-0.8371	0.4330	78.63

Appendix G.

B. Fall (cont'd)

log (Theoretical water weight)	log (Actual water weight)	log deviations	standard WW ± Dev.	anti-logs	corrected % water
10°C Acclimation					
51. -0.8654	-0.7813	+0.0841	-0.7533	0.4708	80.00
52. -1.1337	-1.1050	+0.0287	-0.8087	0.4454	79.10
53. -1.4005	-1.4057	-0.0052	-0.8426	0.4306	78.53
54. -1.2698	-1.3112	-0.0414	-0.8788	0.4153	77.92
55. -1.0203	-1.0697	-0.0494	-0.8868	0.4120	77.78
56. -1.1724	-1.1603	+0.0121	-0.8253	0.4381	78.82
57. -1.4059	-1.4114	-0.0055	-0.8429	0.4305	78.53
58. -1.2290	-1.2438	-0.0148	-0.8522	0.4265	78.37
59. -1.3832	-1.3360	+0.0472	-0.7902	0.4538	79.41
60. -1.3611	-1.4876	-0.1265	-0.9630	0.3817	76.43
71. -0.4757	-0.5490	-0.0733	-0.9107	0.4022	77.36
72. -0.7356	-0.7713	-0.0357	-0.8731	0.5354	78.02
73. -0.9465	-0.8869	+0.0596	-0.7778	0.4594	79.60
74. -1.2312	-1.2420	-0.0108	-0.8482	0.4282	78.44
75. -0.9227	-1.0178	-0.0951	-0.9325	0.3936	76.98
76. -1.0044	-0.9086	+0.0958	-0.7416	0.4764	80.19
77. -0.9613	-0.9729	-0.0116	-0.8490	0.4278	78.42
78. -0.9864	-0.9610	+0.0254	-0.8120	0.4440	79.05
79. -1.0020	-1.0838	-0.0818	-0.9192	0.3988	77.21
80. -1.0293	-1.0314	-0.0021	-0.8395	0.4319	78.58