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Survival and Metabolic Responses to Freezing Temperature in the Northeast Forest Frog *Rana dybowskii*

XIANGHONG XIAO*, DONG ZHENG, CUIJUN YANG AND LONGHUI CHAI

Division of Physiology, Northeast Forestry University, Harbin 15004, China.
**Corresponding author E-mail: xiaoxh@vip.0451.com*

Abstract.- Dynamic changes in water content, crude oil, general proteins, blood sugar and hepatic glycogen during freezing temperatures in the Northeast forest frog (*Rana dybowskii* Günther, 1876) were investigated by establishing frog freeze-tolerant models. Chemical and biochemical analyses showed that a temperature drop from 4°C to -3°C resulted in (1) increase in integrative water content and decrease in in vivo moisture and dissociative water contents; (2) decrease in hepatic glycogen and crude oil and significant increase in blood sugar; (3) significant increase ($p > 0.05$) in general protein content; (4) mortality below temperatures of -1°C; (5) and increase in blood sugar and glucose levels in skeletal muscle following injection of glucose at 4°C and -2°C (hepatic glycogen levels showed similar increases in test groups injected with 650 mmol/L and 1500 mmol/L glucose-PBS, but not in groups injected with 2000 mmol/L glucose-PBS). These physiological and metabolic responses suggest that the Northeast forest frog adopts a positive freeze-tolerant strategy in which glucose serves as the primary mechanism by which damage due to freezing is prevented.

Keywords.- Freezing tolerance, moisture contents, blood sugar, crude oil, general proteins.

Introduction

The Northeast forest frog (Ranidae: Raninae: *Rana dybowskii* Günther, 1876), formerly classified as a northeastern population of *Rana chensinensis* David, 1875 (Xie et al., 1999), is found throughout eastern Asia, with records from Heilongjiang Province, Jilin Province, Liaoning Province, the northeast of the Inner Mongolia Autonomous Region, as well as the Russian Far East, eastern Mongolia, the Republic of Korea, and Tsushima island (Japan) (Fei et al., 2005). The climate in the northern province of Heilongjiang, the region from which research material was collected, is typified by intermediate and frigid-temperature zones with a continental monsoon climate. From November to March, the average temperature is usually less than 0°C, while in January, temperatures reach -15°C to -30°C. Due to climatic factors such as west-wind circumfluence, Siberian air mass, Mongolia high pressure and Baikal cyclone, winters in Heilongjiang Province are often dry and without snow.

As is typical of northern amphibians facing freezing temperatures, the Northeast forest frog hibernates to subtly adjust its physiological functions and metabolism to survive the winter. Although little physiological and biochemical investigations have been made on this species, various freeze-tolerant strategies have been examined in species with similar biochemical metabolisms, including *Rana sylvatica* LeConte, 1825, *Hyla versicolor* LeConte, 1825, *Hyla chrysoscelis* and *Rana ridibunda* Pallas, 1771 (Storey and Storey, 1986; Voituron et al., 2000). These have included studies on ecological behavior, genetic characteristics (Jiang and Zhou, 2001; Yang et al., 2001; Xia et al., 2006), classification (Jiang and Zhou, 2001; Xie et al., 1999; Yang et al., 2001), artificial breeding and reproduction (Wei et al., 2005) and biochemical composition (Xiao et al., 2005). To further explore freeze-tolerant mechanisms and cryobiology in the Amphibia, we here examine the moisture, crude oil, proteins, blood sugar and glycogen contents of the Northeast forest frog when subjected to freezing temperatures.

Materials and Methods

***Materials and freeze-tolerant models*.-** Adult male frogs weighing 20 to 22 g were collected from the Yichun area of Heilongjiang Province in September. Following one week of acclimation to room temperature (25°C), ten randomly-selected frogs were placed into separate glass boxes (40 × 40 × 40 cm) and transferred to digitally-controlled refrigerators. Temperature dropped at a rate of 1°C every 12 h and was held at 4°C for up to 60 d. One third of the water in each box was exchanged with pre-cooled fresh water (4°C) every two days.

***Freeze temperature impacting survival ratios*.-** To investigate survival ratios at different temperatures, six test groups (held at 2°C, 1°C, 0°C, -1°C, -2°C, and -3°C,

respectively) and one control group (25°C) were established with ten frogs per group. For each test group, water temperature was lowered from 4°C to the target temperatures specified above at a rate of 2°C per day. Frogs in those test groups with target temperatures less than -1°C were placed on water-soaked sponges. Survivorship was checked once daily and PT100 thermo-sensors were used to monitor temperatures. Following 10 d of freezing stress, each test group was returned to room temperature (25°C) for 24 hrs and survivorship examined, followed by euthanasia and biochemical analysis. Each test was repeated three times.

Measurement of moisture content, crude oil, general proteins, blood sugar and glycogen.- The drying method outlined by Han et al. (2005) was used to measure dissociative and integrative water content: three 10 g samples from each frog (recorded as W_w) were incubated at 70°C for 6 or 7 hrs until the semi-dry samples reached a constant weight (recorded as W_{70}). ; the semi-dried samples were incubated at 105°C for 5 or 6hrs until the samples again reached a constant weight (recorded as W_{105}). Calculated dissociative and integrative water percentages were calculated as follows:

$$\text{Dissociative water (\%)} = 100 - \frac{W_{70}}{W_w} \times 100$$

$$\text{Integrative water (\%)} = \frac{W_{70} - W_{105}}{W_w} \times 100$$

The Soxhlet extraction method was used for measuring crude oil (Wei et al., 2004): for each group, 10 dry, 1 g samples (recorded as W_{fat}) were placed into the Soxhlet extraction flask and degreased with low-melt-point aether/petroleum; samples were packaged and incubated at 105°C for 1hr until the weight again became constant (recorded as W'_{fat}). Crude oil percentage was calculated using the following equation:

$$\text{Crude oil (\%)} = \frac{W_{fat} - W'_{fat}}{W_{fat}} \times 100$$

General protein content was determined by the Kjeldahl nitrogen determination method: for each group, semi-dried 0.5 g samples were transferred into a digestion tube; 2.5 g Na_2SO_4, 0.13 g $CuSO_4$, and 10 ml H_2SO_4 were added and digested at 400°C for 3 h until the solution color changed to pea green; the tubes were then placed on the Kjeldahl nitrogen determination apparatus for distillation; 15 ml 1% H_3BO_3 were added to the Erlenmeyer flask and connected to the condensator exit; 20 ml saturated NaOH was added to the reaction and methyl red/bromocresol green indicator was added until the solution turned grey; the solution was finally titrated with HCl standard buffer until the solution color changed from grey to blue. General proteins percentages were calculated with following equation:

$$\text{General proteins (\%)} = \frac{(V_s - V_o)}{W} \times 0.01 \times 0.014 \times 6.25 \times 100$$

V_S is the volume of HCl standard buffer added to adjust the distilled sample solution, V_0 is the volume of HCl standard buffer needed to adjust the control solution, and W is the dry weight of sample.

The ortho-toluidine o-toloidine colorimetry method and the anthrone colorimetry method were used for measuring blood sugar and glycogen, respectively (He et al., 2004): after 24 h of freezing-temperature stress, blood sugar was measured from heart tissue (immediately treated with heparin from phlebotomized specimens within each group); 1 g of liver tissue was used to measure glycogen; tissue was homogenated and centrifuged, the supernatant was collected and added to an equal volume of ethanol; the solution was centrifuged, and 100 ml of distilled water was added to the precipitated glycogen, which was then measured.

Exogenous glucose intervention assay.- The method described by Costanzo et al. (1991) was used to detect the effects of exogenous glucose on glucose metabolism in frog liver and muscle tissue, and to demonstrate whether glucose was involved in any freeze-tolerance mechanisms. For each target temperature (4°C or -2°C), ten frogs in three test groups and one control group (25°C) were examined. For the control group, approximately 2.3–2.5 ml 115 mmol/L PBS was injected into the dorsal lymph bursa, comprising approximately 6.7 % of total volume in the bursa. (For the three test groups, different concentrations (650, 1500, 2000 mmol/L, respectively) of glucose-PBS (pH 7.4) were injected to again comprise approximately 6.7% of total bursa volume. All frogs were euthanized following 72 hrs of freezing temperatures. One gram of blood, liver and muscle were immediately collected to analyze glucose contents by the same methods described above.

Statistical analysis.- SPSS (ver.15.0) software was used for statistic analysis. Confidence intervals were set to 0.05. Data were presented as means±standard error (SE) and analyzed using a one-way ANOVA; these results were subsequently analyzed using the Tukey test.

Results

Freezing temperatures and their impact on survival ratios.- During the temperature decrease from 4°C to -1°C, ice crystals became visible on the skin of the frogs. After ten days, mortality was observed only below -1°C (see Fig. 1).

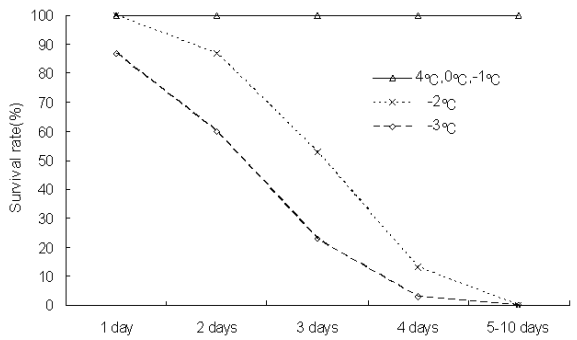

Figure 1. Impact of freeze temperatures and their duration on survival ratios of *Rana dybowskii*. Y-axis is survival ratios in percentage, X-axis is duration. The continuous line with empty triangles represents data from the test groups at 4°C, 0°C and -1°C. The dotted line with crosses is for the test groups at -2°C. The dashed line with empty diamonds is for the test groups at -3°C.

Changes of moisture contents* in vivo*, crude oils, general proteins and glycogen.- Following the temperature decrease from 4°C to -3°C, moisture contents *in vivo* and dissociative water contents decreased gradually, whereas integrative water was found to increase; furthermore, blood sugar was found to increase while hepatic glycogen and crude oil decreased significantly. During freezing-temperature stress, general proteins were found to decrease slightly with decreases in temperature ($p > 0.05$) (see Table 1).

Changes in blood sugar and glycogen levels with addition of exogenous glucose under freezing-temperatures.- At 4°C and -2°C, blood sugar and glucose concentrations in skeletal muscle were always found to increase with the addition of exogenous glucose. Hepatic glycogen also increased with increasing concentrations of exogenous glucose in the test groups injected with 650 mmol/L and 1,500 mmol/L glucose-PBS, however, it was found to decrease in the test groups injected with 2,000 mmol/L, particularly at -2°C ($p < 0.01$) (see Table 2–3).

Discussion

Since the 1980s, investigators have studied the freezing-tolerance mechanisms of amphibians, and have found that species such as *Rana sylvatica* can precisely regulate their metabolic levels in order to tolerate extracellular ice crystallization (Storey and Storey, 1988), which plays as key role in survival and evolution. Investigations on *Rana sylvatica*, *Pseudacris triseriata* and *Rana ridibunda* (Churchill and Storey, 1995; Costanzo et al., 1991; Edwards et al., 2004; Layne and Jones, 2001; Storey and Storey, 1985) illustrate that in at least some amphibians, endogenous glucose is used as a protectant during hibernation.

In the present study, it was found that in *Rana dybowskii,* freezing temperatures are associated with dehydration, an increase in blood sugar and a decrease in hepatic glycogen; temperatures below -1°C are also associated with increased mortality. Some investigations have proposed that endogenous water redistribution aids in the tolerance of freezing temperatures by changing dissociative water into integrative water, condensing extra-cellular solutes and promoting intracellular water trafficking out of cells. This prevents intracellular icing, lowers the freezing point of the body, induces antifreeze

Table 1. Effects of lowering temperature on percentage of water, crude oil, general proteins and sugar in *Rana dybowskii.*

	Dissociative water (%)	Integrative water (%)	Moist contents (%)	Crude oil (%)	General proteins (%)	Blood sugar (mg%)	Hepatic glycogen (%)
Control groups at 25°C	81.25±0.70d	1.44±0.11a	82.68±0.73c	4.64±0.06b	70.49±1.71b	152.38±6.57a	5.60±0.02e
Test groups at 4°C	79.69±0.47cd	1.79±0.06ab	81.49±0.50c	4.63±0.03b	64.90±0.61ab	184.97± 13.18a	4.68±0.08d
Test groups at 0°C	77.67±1.44bcd	1.90±0.13ab	79.58±1.57bc	4.55±0.03ab	64.51±0.66a	196.98± 15.08a	4.12±0.05c
Test groups at -1°C	76.24±2.07bc	2.07±0.14b	78.32±2.21bc	4.49±0.03ab	63.33±1.50a	203.78± 1.84a	3.65±0.03b
Test groups at -2°C	73.33±1.24ab	2.18±0.02b	75.52±1.23ab	4.41±0.05a	63.19±1.18a	266.98± 23.08b	3.21±0.02a
Test groups at -3°C	70.40±0.94a	2.26±0.04b	72.66±0.91a	4.41±0.09a	63.09±1.90a	305.26± 5.88b	3.10±0.01a

*Different letters in the same column represent significant differences ($p < 0.05$).

Table 2. Effect of exogenous glucose on blood sugar and liver glycogen in *Rana dybowskii* at 4°C.

	Blood sugar (mg %)	Hepatic glycogen (%)	Skeletal-muscle glucose (%)
Control groups	124.20±0.69a	5.15±0.45a	0.10±0.01a
Test groups injected with 650 mmol/L	475.50±1.27b	28.68±0.55b	2.03±0.07b
Test groups injected with 1,500 mmol/L	539.07±1.24c	47.65±2.16d	2.57±0.06c
Test groups injected with 2,000 mmol/L	945.07±1.65d	37.94±0.89c	4.59±0.13d

*Different letters in the same column represent significant differences ($p < 0.05$).

Table 3. Effect of exogenous glucose on blood sugar and liver glycogen in *Rana dybowskii* at -2°C.

	Blood sugar (mg %)	Hepatic glycogen (%)	Skeletal-muscle glucose (%)
Control groups	131.18±0.17a	4.19±0.59a	1.63±0.03a
Test groups injected with 650 mmol/L	651.69±0.28b	18.43±1.13b	2.54±0.07b
Test groups injected with 1,500 mmol/L	715.67±2.16c	33.92±1.66c	5.66±0.02c
Test groups injected with 2,000 mmol/L	1,012.58±3.90d	20.99±1.67b	7.33±0.24d

*Different letters in the same column represent significant differences ($p < 0.05$).

synthesis, and prevents damage to critical organs from intracellular ice crystallization (Churchill and Storey, 1995; Hermes-Lima and Storey, 1996; Horton, 1996). Below a certain temperature, however, small ice crystals enlarge to a point that causes damage to cell membranes and cellular substructure, thereby causing death. Stability of the cell membrane would also be compromised, resulting in plasma-membrane fusion, phase transformation from liquid crystal to gels, membrane lipids deficiencies, phospholipid separation, etc., and would destabilize membrane structure (Tong and Nie, 1996). In amphibians glucose is known to function as antifreeze, lowering the freezing-point of the body, maintaining cell membranes and stablizing internal environments (Costanzo and Lee, 1994; Katz, 1989; King *et al*., 1995). In situations involving dehydration, such as those observed here, hydrogen bond formation between glucose hydroxyls and the heads of membrane phospholipids have been found to stabilize lipid bilayers at the liquid crystalline state, preventing membrane fusion, phase change, side phase separation, cell leakage and membrane protein displacement (Tong and Nie, 1996). Simultaneously, freezing temperatures stimulate the catabolism of hepatic glycogen and crude oils, increasing blood sugar concentrations, initiating the glucose antifreeze system, stabilize cell membranes, and enhancing physiological cold-tolerance. These factors combined provide a physiological and biochemical strategy that the Northeast forest frog uses to survive freezing annual temperatures.

In the present study, it has been illustrated that the injection of exogenous glucose into the lymph bursa results in increased levels of blood sugar and skeletal muscle glucose levels, increasing as higher concentrations of glucose solution are injected, particularly in the test groups kept at -2°C. In comparison, hepatic glycogen levels also showed an increase with higher concentration of the injected glucose solution, although the test groups injected with 2000 mmol/L glucose-PBS showed a significant decrease. A possible explanation for this unexpected result is that extremely high concentrations of glucose induce diabetes-like symptoms, causing glycol-metabolism disorders and decreased glycogen synthesis (Wang and Zhuang, 2001).

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