PHYSIOLOGICAL RESPONSE TO FREEZING IN POOL FROG *PELOPHYLAX LESSONAE* (CAMERANO, 1882)

Aliko Valbona

University of Tirana, Faculty of Natural Sciences, Department of Biology, Tirana, Albania
E-mail: valbona.aliko@fshn.edu.al

ABSTRACT

Several species of terrestrially hibernating frogs, turtles and insects have developed mechanisms, such as increased plasma glucose, anti-freeze proteins and antioxidant enzymes to resist to freezing, for survival at subzero temperatures. In the present study, the importance of glucose to cryoresistance of the anuran amphibian *Pelophyllax lessonae* has been assessed. Animals were exposed to the low temperature values (-2°C) for 10 to 18 hours and after that measurements of plasma glucose levels, liver and muscle glycogen content, haematocrit and red blood cell volume has been carried out. Blood glucose concentration were found to be increased from 33.94 ± 7.23 to 106.67 ± 18.17 mg/dl (P< 0.01) when frogs were transferred from 20°C to -2°C. Glucose accumulation in response to cold exposition in the frogs was accompanied by a decrease in liver glycogen content, which was estimated to significantly decreased (P<0.01) from 6.39 ± 0.47 to 3.265 ± 0.09 mg/100 mg tissue. These findings has been indicating that liver carbohydrate reserves were probably the primary carbon source of glucose synthesis whereas muscle carbohydrate seems unimportant (glycogen level changed from 0.74 ± 0.04 to 0.84 ± 0.05 mg/100 mg tissue). It has been found that cold exposition had no effect on the haematocrit and red blood cell volume of the frogs, suggesting that elevated glucose levels reduced erythrocyte injury. These results not only provides conclusive evidence for glucose's cryoprotective role in *Pelophyllax lessonae*, but also revealed that *pool frog* adopts a positive freeze-tolerant strategy in which glucose serves as the primary mechanism by which damage due to freezing is prevented and it is a critical determinant factor of freeze tolerance capacity in this species.

***Key words**: Pelophyllax lessonae, freeze tolerance, cryoresistance, glucose, glycogen, haematocrit, red blood cell volume.*

INTRODUCTION

Several species of amphibians are able to survive during extremely unfavorable conditions such as, subzero temperatures (Aarset, 1982; Storey & Storey, 1988, 1992; Zachariassen, 1985). Wood frogs, for example, may be frozen for 2 weeks with no cardiorespiratory activity and with up to 65% of their total body water as ice (Pinder *et al*., 1992; Storey *et al*., 1996). A cryoprotector, a low molecular weight molecule, such as glucose (in *Pseudacris crucifer* and *P.triseriata*) or glycerol (in *Hyla versicolor*), seems to play an important role in this phenomenon, warranting survival of these animals during freezing (Storey & Storey, 1984, 1985, 1986). These cryoprotectors elevate body fluid osmolarity, decreasing the extent of cell volume reduction during extracellular ice formation, preventing cell shrinkage below a critical minimum cell volume and stabilising membrane proteins (Storey *et al*., 1996). Other factors are also important to promote crioprotection, among them antifreeze proteins and an special system of antioxidant proteines (Storey *et al*., 1996).

Very little is known about the freezing tolerance/intolerance of ranidian frog *Pelophyllax lessonae*, so comprehensive explanations for the mechanisms and evolution of freeze tolerance on this species is lacking. In the presence study, for the first time in Albania, we assessed the importance of blood glucose for freezing tolerance in the pool frog, *Pelophyllax lessonae.*

MATERIAL AND METHODS

Animal source and maintenance

Experiments were performed during the active, non-aestivating period of the species from September to October. Individuals of either sex weighting 45.6±12.5 g (mean ± SEM) were obtained from a commercial supplier 2-3 weeks before experimentation. Upon arrival, the animals were kept indoors in aquaria with free access to de-chlorinated tap water at the temperature 23°C. All animals were fed with chicken liver once a week until 7 days before the experimental procedures.

Glycogen extraction and measurement

Glycogen was extracted according to the method of Sjorgen et al., (1938). Briefly, liver and muscle samples were obtained by isolating a piece of tissue weighing approximately 500 mg. 2 ml of 30% KOH was added to the samples , making sure that the tissue was completely immersed in the solution. The tubes were placed in a boiling water bath (100°C) for one hour until a homogenous solution was obtained, and were then removed from the water bath and cooled in ice. Absolute ethanol (4,5 ml) was added to precipitate the glycogen from the alkaline digest. The samples were left in ice for 10 minutes and then centrifuged at 1000 g for 10 minutes. The supernatant was carefully aspirated and the glycogen washed with distilled water and precipitated with ethanol again.

Glycogen content was estimated with anthrone reagent. Glycogen precipitates were dissolved in 3.0 ml distilled water, transferred to a 10 ml volumetric flask and diluted to the mark with water. 4 ml antrone was carefully added to 2 ml aliquots of the final

dilution in the ice bath, and than the tubes were placed in a boiling water bath (100°C) for 10 min. After the tubes cooled down, the absorbance of the samples was measured at 620 nm on a UVICON spectrophotometer. Glucose at different concentrations (20, 30 and 50 mg/ml) was used for a calibration curve.

Plasma glucose concentration and red blood cell volume

Glucose concentration was determined quantitatively by enzymatic (glucose oxydase) method (Sigma).

To determine the cell volumes, the blood samples were examined under the light microscope with a 100x objective to which a micrometer ocular was adapted. The longest and shortest axes of red blood cells and their nuclei (50 red blood cells per animal) were measured. The cell volume was calculated according to Valeri et al., (1967) by the following formula:

$$V=0.35351426 \text{ x } D_1 \text{ x } D_2 \text{ x } (D_1 \text{ x } D_2)^{1/2}$$

were D_1 and D_2 represent the longest and shortest axis, respectively.

In the present study, we used this method to access the differences of red blood cell volume of amphibians under two different experimental conditions (20 and -2°C).

Experimental procedure

During the experiments the frogs were housed in a liter plastic chamber lined with moist paper towels. During the control period the animals were left undisturbed at 20°C for 48 hours. Control animals were decapitated (n=10), tissue (liver and leg muscle) collected for glycogen determination, and blood were collected by cardiac puncture (about 1 ml) for measurements of haematocrit and red blood cell volume. Blood was spin in a centrifuge and plasma was used for the determination of plasma glucose concentration.

Another group of frogs were transferred from 20 to -2°C by placing the animal chamber on an ice-cold bath. The same procedure described above for the control group was repeated at 6 and 10 hours after transfer to -2°C.

Calculations and statistical analysis

All values are reported as means ± SEM. The effects of freezing on blood glucose, liver glycogen, muscle glycogen and haematocrit were calculated by analysis of variance (ANOVA), and the difference between means was assessed by the Tukey-Kramer Multiple Comparisons Test. The effect of freezing on cell volume was assessed by comparing the mean values before (20°C) and after (-2°C) freezing by t-test. $P<0.05$ was considered significant.

RESULTS

All (10 of 10) of the frog specimens exposed to -2°C up to 10 hours survived. Plasma glucose concentration was significantly higher (more than 3 times) in the frogs exposed

to -2C when compared to the frogs during control conditions (20°C) (Fig.1, graph.1). Liver glycogen decreased significantly after 10 hours at -2°C, Fig.1, graph. 2. Muscle glycogen was not significantly different and cold caused no alterations in either animal species, Fig. 1, graph.3.

No significant difference in haematocrit was observed to frogs exposed to -2°C. Also, cold exposition (-2°C) caused no change in the frog's red blood cell volume, Figure 2, 3.

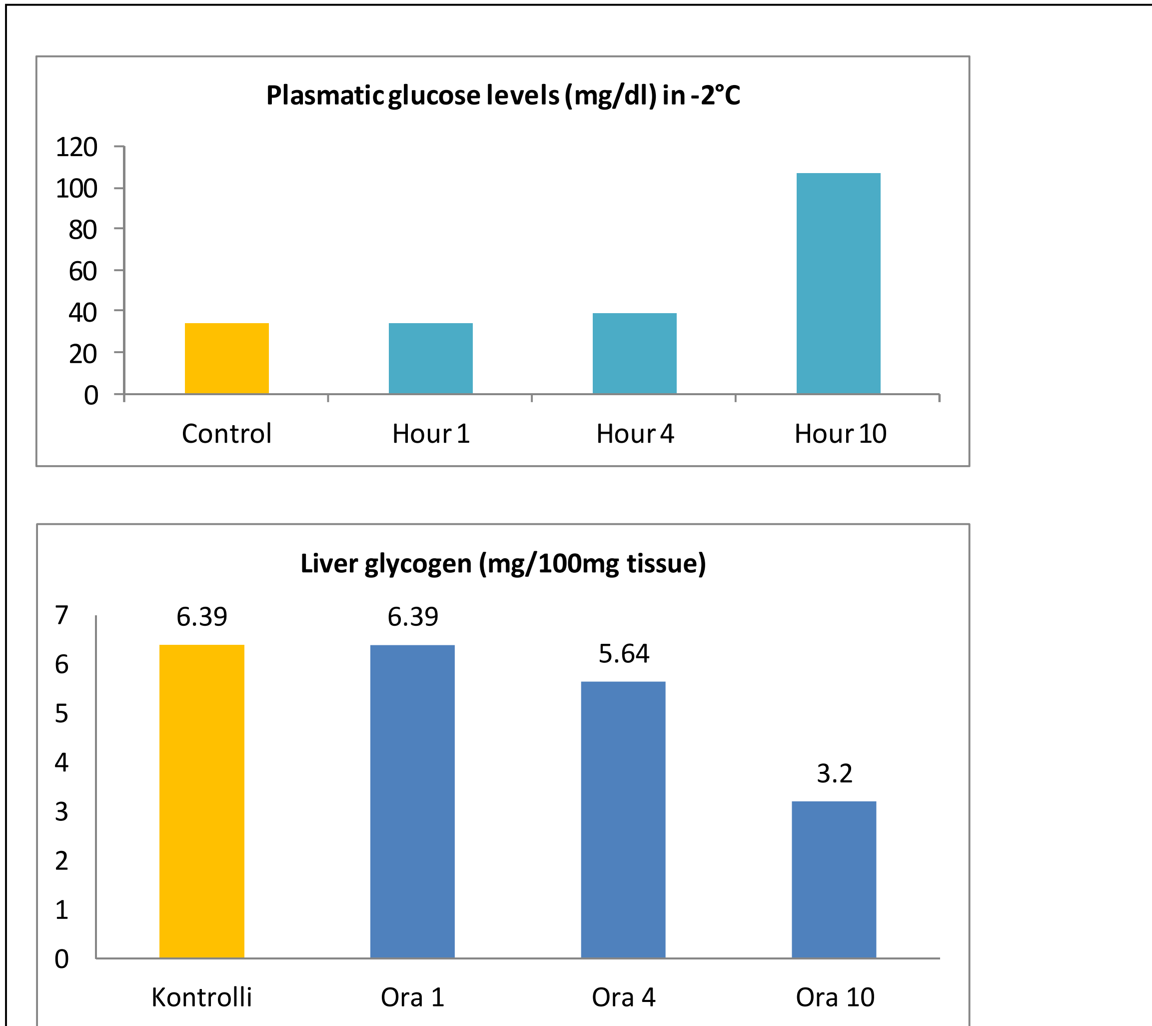

Figure1. Effect of freezing on blood glucose (graph.1), liver glycogen (graph.2), and muscle glycogen

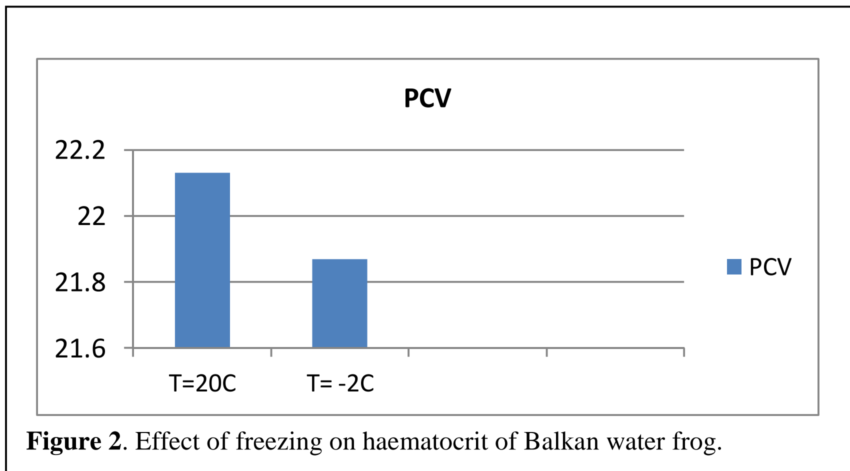

Figure 2. Effect of freezing on haematocrit of Balkan water frog.

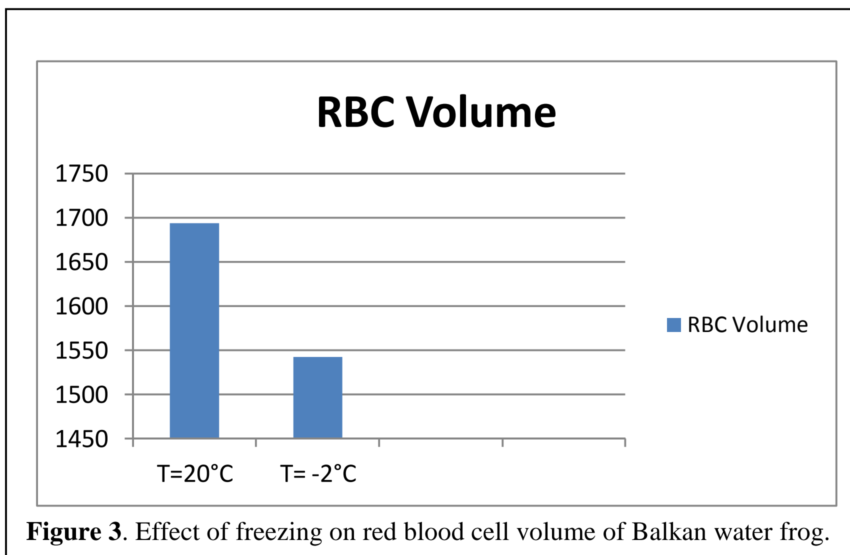

Figure 3. Effect of freezing on red blood cell volume of Balkan water frog.

DISCUSSION

In the present study, we assessed the effects of cold exposure with respect to plasma glucose, liver glycogen, haematocrit and blood cell volume in Balkan water frog. The

frog data is consistent with previous reports (Storey *et al*., 1996). Our study provides evidence that increased plasma glucose is needed to prevent cell shrinkage.

Exposure to cold (-2°C) was not lethal for frog specimens, indicating that this species is freeze-tolerant. We observed a significant increase in blood glucose concentration when animals were exposed to -2°C. Both basal glucose levels and the cold-induced hyperglycemia were significantly higher in the frog specimens exposed to cold (Fig. 1, graph.1). It is known that the accumulation of high concentrations of cryoprotector (glucose) is essential for freezing tolerance (Canty *et al*., 1986; Costanzo & Lee, 1991). Besides glucose, other factors may also be involved, at least in frogs, such as antifreeze proteins (Davies & Hew, 1990), increased membrane glucose transporters (King *et al*., 1993, 1995) and an increased activity of antioxidant enzymes (Storey, 1996).

We observed a significant decrease in liver glycogen content in frogs (Fig. 1.graph.2). As to muscle glycogen, no significant change was observed, Fig.1, graph.3. Synthesis of blood glucose is based on the catabolism of liver glycogen (Pinder et al., 1992; Storey & Storey, 1992) that seems to be well correlated with our data. Recently it has been shown that glycogen phosphorylase activity is higher in freeze-tolerant frogs than in freeze-intolerant species (Swanson et al., 1996). In agreement with the above mentioned study, we observed a pronounced decrease in liver glycogen content in frogs (6.39 ± 0.47 to 3.265 ± 0.09 mg/100 mg tissue at -2°C). Furthermore, the high basal liver glycogen content in frogs could be related with the ability of frogs to obtain more energy from non aerobic metabolism during intense activity.

Accumulation of glucose in frog blood, as a colligative cryoprotectant, functions primarily to prevent cell shrinkage below a critical minimum cell volume within the range of naturally encountered freezing temperatures, avoiding osmotic shock (Storey & Storey, 1992). This cell protection mechanism, i.e., hyperglycemia, might be related to the differences observed in the frog haematocrit (Fig.2).

Perhaps, haematocrit did not change because red blood cell glucose transporter (GLUT 1) may be more effective during freezing than the transporters of another cell type.

In conclusions, the present study shows that Balkan water frog has a great increase in plasma glucose during exposure to cold temperatures. This physiological aspect indicates that the freezing tolerance of this specimen is related with a pre-existing mechanism which has been enabled frog surviving to water stress during evolution, and had determined the wide ecological distribution of this frog.

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