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Freeze Tolerance and Cryoprotectant Synthesis of the Pacific Tree Frog *Hyla regilla*

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Freeze tolerance and cryoprotectant synthesis was examined in *Hyla regilla*, collected from Northern California in the spring and fall. Specimens frozen at 2 C for six and 12 hours had a survival rate of 10% and 80%, respectively, in both seasons. This is the first report of freeze tolerance for *H. regilla*. Freezing caused a fivefold increase in plasma glucose levels in the spring and a 14-fold increase in the fall. Ice formation induced a rise in liver glucose and glycerol production in both seasons with concentrations of liver glucose being greater in the fall than in the spring. The increase in glucose was accompanied by a significant decline in liver glycogen. Seasonal differences in muscle glycogen levels in response to freezing were not shown, suggesting that the liver is the organ responsible for cryoprotectant synthesis. The rise in plasma glucose, along with increased levels of liver glucose and glycerol in response to freezing, suggests that these compounds are being used as cryoprotectants, with glucose being the primary component.

TERRESTRIAL exotherms inhabiting temperate regions where subzero temperatures may be encountered are faced with the possibility of freezing. As an adaptation for winter survival, many insect species and a select group of amphibians and reptiles are able to tolerate the freezing of their extracellular body fluids (Zachariassen, 1985; Storey and Storey, 1988). Freeze tolerance in anurans has been demonstrated in five species: *Rana sylvatica, Hyla crucifer, Hyla versicolor* (Schmid, 1982), *Pseudacris triseriata* (Storey and Storey, 1986), and *Hyla chrysoscelis* (Costanzo et. al., 1992). These terrestrially hibernating frogs can revive after days or weeks with as much as 65% of their body water as ice (Layne and Lee, 1987; Storey and Storey, 1988).

The Pacific Tree Frog, *Hyla regilla*, has a range that extends from British Columbia to Baja California and from Montana, Idaho, and Nevada west to the Pacific coast. The species can be found from sea level to elevations of more than 10,000 feet (National Audubon Society, 1979). For classification purposes, it has been suggested, based on phylogenetic relationships, that *Hyla regilla* be transferred to the genus *Pseudacris* (Hedges, 1986). This study examines the freeze tolerance of *H. regilla* collected in the Sierra Nevada Mountains of Northern California (elev. 6000 ft; lat. 40.0 N). During the winter, this area is exposed to subzero temperatures and receives a substantial amount of snow, thus providing conditions that could favor the development of freeze tolerance. Anuran species that utilize freeze tolerance as an overwintering strategy inhabit shallow, terrestrial hibernacula (Storey and Storey, 1986, 1988; Costanzo et al., 1992; Swanson et al., 1996). To our knowledge, there is no documentation concerning the hibernacula characteristics used by this species for overwintering. Such information would be important to show that they are indeed exposed to subfreezing environmental temperatures.

MATERIALS AND METHODS

All chemicals were purchased from Sigma Chemical Co., St. Louis, Missouri. Male Pacific Tree Frogs, *H. regilla* (4.48 ± 0.10 g) were collected at an elevation of 6000 feet, near streams and breeding ponds above Butte Meadows, Northern California. Two collections were made in the spring of 1996. The first was on the initial nights that the animals were active (calling) after their spring emergence (15 May). These frogs (n = 55) were used for the spring experiments. The second collection was made 15 June. These animals (n = 60) were kept through the summer for testing in the fall. They were placed in terrariums, held at room temperature, and fed a diet of crickets and mealworms ad libitum.

The presence of food in the gut of freeze-tolerant animals is believed to cause uncontrolled ice nucleation and thus reduce their survivorship during freezing (Storey and Storey, 1987). To allow time for gut content evacuation for the spring and fall frogs and to simulate light and temperature conditions leading up to hibernation in the fall, the preparation of spring frogs prior to freezing was different than for the fall samples.

On 16 May, for the spring frogs, and on 22 October, for the fall frogs, specimens were

weighed and placed into individual plastic containers with damp sphagnum moss. Both the spring and fall groups were fasted and maintained at 7 C but were exposed to two different photoregimes. Prior to testing, spring specimens were held without light for two weeks, and fall specimens were held under a photoregime (fluorescent light) adjusted to natural photoperiod for one month.

The general freezing protocol was as follows: Frogs were moved from the 7 C cold room to an incubator set at 1 C. The temperature was lowered by 1 C every 12 h until −2 C was reached. Physical examination was used to determine when frogs were frozen. Examination consisted of a visual inspection and lightly squeezing the frog's midsection. Frozen frogs were observed to have opaque eyes, stiff limbs, and solid abdomens. Pulmonary breathing and cardiac activities were absent. Previous experiments used an observed exotherm as a beginning time for freezing (Costanzo and Lee, 1993; Layne and Lee, 1989; Storey, 1987b). To be judged frozen by physical examination resulted in specimens being frozen longer before starting the time of freeze duration. Control frogs were handled similar to the experimental specimens in their respective group, except they were kept in the dark and sampled directly from the 7 C cold room.

Determination of freezing survival was as follows: freezing regime was as described earlier in the general freezing protocol. Once frozen, frogs were removed at intervals of 6, 12, and 24 h (n = 5, 10, and 5, respectively) and thawed at 7 C. Recovery was based upon the frog's ability to show regular breathing, normal posture, and normal locomotor capabilities (jumping) within 48 h.

Analysis of plasma was as follows: following the general freezing protocol, frogs (n = 10) were frozen at −2 C for 12 h and then thawed at 7 C. Once the specimens regained normal locomotor and respiratory movements (usually within four to eight hours), they were double pithed and dissected. A midsagittal incision in the ventral body wall was made to expose the heart. The aorta and ventricle were snipped and a blood sample taken using a heparinized capillary tube. Blood plasma was separated using a microcapillary centrifuge. The plasma was immediately assayed for glucose and glycerol. Glucose concentrations were determined spectrophotometrically (Sigma glucose procedure 115-A). Glycerol concentrations were measured using a Sigma triglyceride kit. All spectrophotometric data were collected using a Perkin Elmer model LAMBDA2 UV-Vis spectrophotometer.

Analysis of tissue was as follows: tissue samples were removed from specimens frozen at −2 C for 48 h (n = 10) and then quickly thawed in water (25 C). Tissue samples of the liver (trimmed of gallbladder) and both gastrocnemius muscles were excised and weighed. To quick freeze tissues, samples were enveloped by wax paper and pressed between two blocks of dry ice for approximately one minute. The sample was labeled, put into a plastic bag, and placed into an ice chest filled with dry ice. Frozen samples were stored at −70 C until analysis. Great care was taken to ensure the tissues were processed as rapidly as possible after thawing.

Glycogen concentrations were determined as described by Keppler and Decker (1974). Preparation of tissue samples for metabolite assays was similar to methods used by Storey and Storey (1984). Frozen tissues were homogenized (1:5w/v) in ice-cold perchloric acid. Potassium bicarbonate (2 M) was then added to neutralize the homogenate. An aliquot (200 μl) of homogenate was placed into a 25 ml Erlenmeyer flask and kept in an ice bath for later use in glycogen hydrolysis. The remaining homogenate was centrifuged for 15 min. A portion (20 μl) of the supernatant was used for glucose determination.

Glycogen hydrolysis was accomplished by incubating the 200 μl homogenate with 2.0 ml amyloglucosidase solution in a stoppered 25 ml Erlenmeyer flask with shaking at 40 C for six hours. The mixture was then neutralized by the addition of 2 M potassium bicarbonate. The sample was centrifuged and a 20 μl portion of the supernatant was removed for the determination of glucose using the Sigma glucose procedure 115-A.

Determination of supercooling point was as follows: whole animal supercooling point was determined by securing a YSI thermister to the frog's abdomen with tape (n = 5). The specimen and thermister were then secured (using masking tape) onto a broad sheet of cardboard and damp sphagnum moss was positioned around the animal. The frog was placed in an incubator at −3 C, and body surface temperature was monitored using a digital multimeter (Hewlett Packard, 3476A). Ice formation was detected as a sudden rise in body surface temperature. The freezing point was recorded as the rebound temperature, which was the value to which the body temperature rose immediately after ice nucleation. The freezing procedure was similar to that used by Storey (1987b).

Data and statistics analysis was as follows: mean values ± SEM between unfrozen and frozen samples for blood plasma, tissue metabo-

TABLE 1. SURVIVORSHIP OF *Hyla regilla* TO FREEZING. Specimens were frozen at −2 C in the spring and fall (1996) and thawed at 7 C. * = survivors/n.

Sample	Time frozen (hours)		
	6	12	24
Spring	5/5*	8/10	0/5
Fall	5/5	8/10	1/5

lites, and supercooling/freezing point were compared with Student's *t*-test. $P \leq 0.05$ was required to assign significance.

RESULTS

Once frozen, as determined by physical examination, individual frogs were removed from the −2 C incubator at 6, 12, or 24 h postfreezing. They exhibited stiff limbs and solid abdomens, indicating that a large amount of ice had formed within their body. The eyes were opaque in many of the specimens frozen for 12 or more hours. Respiratory and cardiac activity was not observed in any of the animals. Both the spring and fall samples had similar survival rates (Table 1). All frogs (5/5) survived 6 h of freezing and 8/10 survived 12 h of being frozen. None (0/5) of the spring specimens tolerated being frozen for 24 h, whereas 1/5 of the fall sample survived. Frogs that survived freezing regained normal locomotor and respiratory movements within 2–10 h of being thawed at 7 C. The opaque discoloration of the eyes cleared to the normal black pigmentation within a few hours, and vision did not seem to be impaired.

Freezing induced a substantial increase in plasma glucose levels of frogs tested in both seasons. Plasma glucose significantly increased by nearly fivefold in the spring and 14-fold in the fall in response to freezing ($P \leq 0.05$). Glycerol content was below the reliable detection limits (1 μmol/ml) in the control and frozen frogs of both seasons.

Liver glycerol, glucose, and glycogen concentrations of control and frozen frogs from the spring and fall are shown in Figure 1. There was a substantial rise in glycerol and glucose levels in response to freezing. Glycerol concentrations significantly increased by nearly three times in the spring and by two times in the fall ($P \leq 0.05$). Glucose levels were also significantly higher, with a 5.5-fold increase in the spring and an 11-fold increase in the fall.

Liver glycogen content was reduced in specimens frozen in both the spring and fall (Fig. 1).

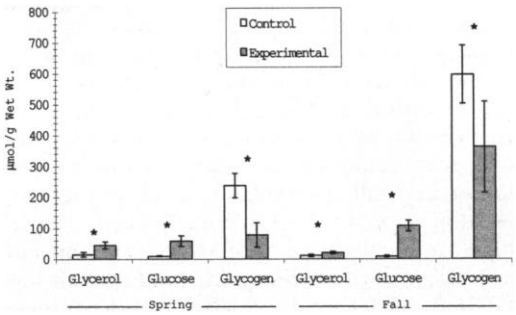

Fig. 1. Effect of freezing exposure on metabolite levels in liver of *Hyla regilla* Examined in the spring and fall of 1996. Values are means ± SEM. Units expressed in μmol/g wet weight with glycogen expressed in glucose units. * Significantly different from corresponding control value by the Student's *t*-test, $P \leq 0.05$. Error bars represent a 95% confidence interval.

Control values for the spring (237.87 μmol/gww) and fall (596.97 μmol/gww) were significantly reduced to 78.48 μmol/gww and 362.39 μmol/gww, respectively ($P \leq 0.05$).

Muscle glycerol, glucose, and glycogen concentrations of control and frozen frogs from the spring and fall are shown in Figure 2. After freezing, spring values of muscle glucose and glycogen concentrations were statistically indistinguishable from control specimens. However, glycerol levels significantly increased (twofold) in concentration due to freezing ($P \leq 0.05$). In contrast, only glucose values in the muscle samples of the fall frogs were significantly higher (about 1.5 times, $P \leq 0.05$). Muscle glycerol and glycogen of fall frogs did not change in response to freezing.

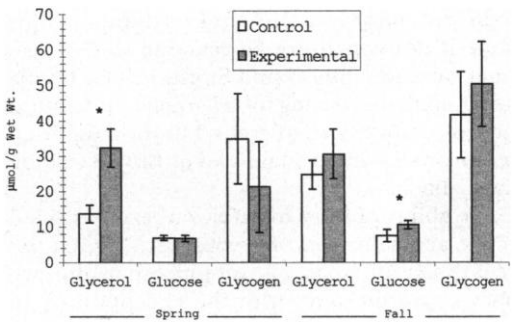

Fig. 2. Effect of freezing exposure on the metabolite levels in muscle of *Hyla regilla* examined in the spring and fall of 1996. Values are means ± SEM. Units expressed in μmol/g wet weight with glycogen expressed in glucose units. Significantly different from corresponding control value by the Student's *t*-test, $P \leq 0.05$. Error bars represent a 95% confidence interval.

Individual whole animal supercooling and freezing points were measured in spring (n = 5) and fall (n = 5) specimens. Freezing point was recorded as the rebound temperature, which is the value to which the body temperature rose immediately after ice nucleation. Spring and fall specimens had an average supercooling point of −1.5 ± 0.37 C and −1.1 ± 0.6 C, respectively (x ± SEM). Spring and fall animals' mean freezing point temperature was −0.64 ± 0.04 C and −0.70 ± 0.16 C, respectively. There was no significant difference ($P \leq 0.05$) in the supercooling and freezing point between these samples. The duration of each exotherm varied between specimens, with the shortest lasting four hours and the longest lasting more than eight hours.

Discussion

Freeze tolerance is an adaptation that allows an organism to survive extensive ice formation within the body fluids at temperatures normally encountered during winter hibernation. All five species of tree frogs known to be freeze tolerant are located in northern temperate regions of the United States and Canada. It is likely that other terrestrially hibernating anurans exposed to similar ecological conditions will also have developed this unusual method of surviving subzero temperatures.

In the present study, *H. regilla* (collected at an elevation of 6000 feet in Northern California) survived the internal freezing of its body fluids. These data represent the first report of freeze tolerance for this species. *Hyla regilla* was found to be just as freeze tolerant in the early spring as in the fall; it tolerated being frozen at −2 C for at least 12 h. Although one fall specimen did survive a 24-h freeze duration, this alone is not conclusive evidence to show a seasonal variation. Storey and Storey (1988) briefly mentioned the testing of *H. regilla* and found they were not freeze tolerant, but the frogs used were from the lower mainland of British Columbia, Canada.

The ability of frogs to survive freezing is aided by the accumulation of cryoprotectants. Of the freeze-tolerant frogs, all use glucose as the primary cryoprotectant, with the exception of *H. versicolor*, which uses glucose and glycerol. Freeze tolerant anurans derive their cryoprotectants primarily from liver glycogen stores, which increase prior to hibernation (Storey and Storey, 1992). Seasonal decline in liver glycogen coincides with a general reduction of cryoprotectant production upon freezing, with spring quantities being lower than fall quantities (Storey and Storey, 1987). Current data suggest a similar situation for *H. regilla* in both the type of cryoprotectants synthesized and the seasonal variation in their production. There was a definite increase in plasma glucose and tissue glucose/glycerol levels in response to freezing. Plasma glucose increased by nearly fivefold in the spring (1.37–6.60 μmol/ml) and 14-fold in the fall (1.23–17.37 μmol/ml), as compared to unfrozen frogs. Plasma glycerol levels in unfrozen and frozen frogs were below reliable detection in both seasons. The significant rise of plasma glucose and the undetectable levels of plasma glycerol in response to freezing suggests glucose is the primary cryoprotectant being utilized.

The rise in plasma glucose levels of *H. regilla* is similar to the freeze-tolerant frogs *H. chrysocelis* and *R. sylvatica*, which produced 24.9 ± 3.3 μmol/ml (winter tested) and 14.6 ± 0.4 μmol/ml (spring tested), respectively, when under a similar freeze stress (Costanzo et al., 1992; Layne and Lee, 1987). Both of these frog species survived extended exposure to freezing (24 h or more) using similar amounts of cryoprotectants. The method of freezing used in these previous studies was different than that used in the current study. Their frogs were held in individual plastic centrifuge tubes that were placed into a metal canister submerged in a refrigerated bath. Because *H. regilla* had comparable cryoprotectant levels, but lacked the corresponding survival rates after prolonged freezing, further research is needed to determine whether the development of a different freezing regime than the one used in this study could enhance survival.

It is possible that the acclimatization protocol used in this study to induce an overwintering, freeze-tolerant state, was not adequate. The difficulty in finding samples during the early autumn months made it necessary to catch frogs in the spring and keep them over the summer for use in the fall. Laboratory conditioning to induce freeze tolerance has been successful in previous studies using *H. versicolor* collected in the late summer (Storey and Storey, 1985) and in the early summer (Layne and Lee, 1989). Although photoperiod and temperature were reduced in a way that was believed to simulate conditions prior to hibernation, it is probable that certain environmental cues were not present that are necessary to stimulate proper physiological adjustments for hibernation. Future investigations comparing different acclimatization programs would be beneficial in determining the best protocol for full overwinter conditioning of *H. regilla*.

Freeze-tolerant frogs do not accumulate their cryoprotectants before freezing. It is only during the initial minutes and hours of ice formation that cryoprotectants from the liver are rapidly distributed via the blood to other organs of the body (Storey, 1987a,b). To determine whether *H. regilla* was synthesizing its cryoprotectants from the liver as in other freeze-tolerant frogs, metabolite levels of the liver and skeletal muscle were analyzed. Seasonal variations in liver glycogen stores of nonfrozen animals were evident with the spring animals having substantially less glycogen than the fall specimens. Freezing stimulated a significant elevation in liver glucose and glycerol production in both seasons, with the rise in glucose closely relating to that of blood levels. This was accompanied by a significant decline in liver glycogen. The substantially higher amounts of glucose produced in the fall relative to the spring likely reflect the diminished glycogen stores in the liver of spring frogs. Glycogen is a metabolic fuel used during hibernation, and its consumption during the winter would result in a decrease in availability for cryoprotectant synthesis in the spring. Analysis of metabolite levels in skeletal muscle (gastrocnemius) showed little difference in glycogen, glucose, and glycerol amounts between control and frozen animals. This suggests that the liver is the major location of cryoprotectant synthesis via glycogenolysis as would be anticipated.

Ice formation of many freeze-tolerant animals generally begins just below the freezing point of their body fluids. *Hyla regilla* tested in this study had supercooling points between −1 and −1.5 C and a freezing point of around −0.6 C, with no statistical evidence of seasonal variation. The supercooling and freezing points of *H. regilla* was similar to other freeze-tolerant anurans which typically supercool from −1.2 to −3.0 C (Storey and Storey, 1986, 1987). This limited ability to supercool ensures that the rate of ice formation will be slow, allowing time for cell volume regulation, cryoprotectant synthesis, and distribution and gradual transition into ischemia (Storey and Storey, 1992). It should be noted that the frogs were placed in damp sphagnum moss during the supercooling and freezing point determination as well as all other freeze experiments. This was done to simulate the physical characteristics that might be found within the hibernaculum. Previous research found that freezing occurred at higher subzero temperatures on a wet substrate than on a dry substrate indicating that anurans were susceptible to inoculative freezing (Swanson et al., 1996). It is possible that the ice nucleation of *H. regilla* was seeded by contact with ice forming in the moss as opposed to spontaneous nucleation within the body fluids. Future tests should include supercooling and freezing point experiments on dry substrates.

Hyla regilla used in this study is presumed to hibernate (as do the five other species of freeze-tolerant frogs) just beneath the forest ground cover. With a deep snow pack, it is unlikely that *H. regilla* will encounter subzero temperatures, but if they do, they can survive being frozen for at least 12 h. Based on current data, it appears this species can tolerate limited durations of freezing that may accompany subzero temperature fluctuations within its hibernaculum. At present, we do not have any information detailing the microenvironment of *H. regilla*'s hibernaculum. We can only speculate that this limited freeze tolerant adaptation would allow these frogs to perhaps, survive cycles of internal ice formation, such as freezing at night and thawing during the day.

The present study represents an initial report of the freeze tolerance abilities of *H. regilla* collected in the Sierra Nevada Mountains of Northern California. Additional studies should focus on the maximum ice content that *H. regilla* can survive, glycogen levels in other tissues, and the effect of ice crystallization on initiation of cryoprotectant synthesis. Because overwintering conditions experienced by these frogs will likely vary at different elevations and latitudes, it would be interesting to study the freezing survival and metabolic changes in specimens sampled at lower elevations and more northerly latitudes.

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