NATURAL FREEZE TOLERANCE IN A REPTILE


SUMMARY

In temperate regions, reptiles may encounter subzero temperatures in winter and particularly during periods immediately preceding and following hibernation. Laboratory experiments, involving exposure to subzero temperatures, demonstrated that garter snakes (Thamnophis sirtalis) can survive freezing of at least 36.2% of their body water and can remain frozen for at least 48 hours without injury. In contrast to freeze tolerant insects and frogs, cryoprotective substances were not detected during freezing in garter snakes. These reptiles represent the first amniotes and the most phylogenetically advanced vertebrates for which natural freeze tolerance has been demonstrated.

KEY WORDS: Freeze tolerance, cold tolerance, supercooling, reptile, garter snake, Thamnophis sirtalis

INTRODUCTION

It has long been known that certain terrestrial insects and marine invertebrates routinely endure freezing of their extracellular body fluids under natural conditions (1). The first demonstration of freeze tolerance in any vertebrate (a frog, Rana sylvatica) occurred in one of our laboratories just over a decade ago (2). Subsequent investigations (3 - 5) have shown that four species of terrestrially hibernating frogs are physiologically well adapted to withstand extensive and repeated freezing. Although many reptiles also face seasonal cold exposure, their assumed (6) freeze susceptibility has not been examined critically. Our study provides evidence for freeze tolerance in the most phylogenetically advanced vertebrate species yet reported.
METHODS AND MATERIALS

Garter snakes (*Thamnophis sirtalis*) were collected in October, 1987 near a communal den in central Wisconsin, and maintained at 5°C in a darkened environmental chamber. Snakes were tested for freeze tolerance from 6 January to 25 February, 1988. Fourteen snakes (11 adult females; 3 juveniles of unknown sex) were cooled slowly within a well-insulated chamber submerged in an ethylene glycol-water bath maintained at -3.3°C. Core body temperatures were monitored with a 30 gauge Cu-constantan thermocouple inserted 20 mm into the hindgut. All snakes initially cooled below the melting point (ca. -0.65°C) of their tissues without freezing, but spontaneous ice nucleation usually occurred within one hour of supercooling. Body ice content was estimated using established methods (7) for the calorimetric analysis of the specific heat of wet (1.0 cal/g) and dry (0.27 cal/g) masses of frozen snakes. Mean body water content, determined in late October, was 75.0 ± 0.4% of fresh mass.

Preliminary tests revealed that estimates of ice content were reliable to ± 1.0 g ice (about ± 3% of body water content). Frozen (for 6 h) and nonfrozen snakes were assayed for cryoprotectants (glycerol, sorbitol, glucose, and trehalose) used by invertebrates and frogs (1). Blood, liver, skeletal muscle, and cardiac tissues were rapidly isolated and frozen at -70°C. Organ samples (>175 mg) were prepared (8) and analyzed (9) using HPLC. Plasma glucose concentrations were measured using a glucose kit (no. 510, Sigma Chemical Co.) and spectrophotometer.

RESULTS

The onset of ice formation was clearly marked by an exotherm and a rapid rise in body temperature from the supercooling point (SCP) to near the melting point. Snakes remained in the chamber for 6 h following the appearance of the exotherm (Fig. 1A). Frozen snakes were stiff, tightly coiled, and could be lifted from the cooling chamber without uncoiling. Thirteen of 14 snakes regained locomotory and righting abilities within a few hours at 3°C. One 12.3 g juvenile failed to recover fully although it survived, despite paralysis of the anterior one-third of its body, for 4 wk until it was euthanized. Surviving snakes were monitored closely for 3 d and appeared healthy. Two of these, subsequently exposed to summer-like conditions for 7 months, consumed fish and frogs, gained weight, basked diurnally, and shed their skins without difficulty. One previously-frozen female snake produced an apparently healthy
litter of five on July 19, 1988. Fertilization was likely dependent upon stored sperm, since this female was isolated from males after freezing.

Fig. 1. Representative cooling and freezing curves in which time zero is defined by the exotherm associated with the onset of ice formation. A. Cooling curve of a 45.8 g *Thamnophis sirtalis* frozen for 6 h and removed from the chamber prior to thermoequilibration; the projected cooling curve is shown by dots. B. The first portion of the cooling curve of a 46.2 g *T. sirtalis* frozen for 48 h. The bath temperature was raised, after nucleation, to prevent body temperature at thermoequilibration from dropping below -0.75°C.
The lower limit of freeze tolerance of invertebrates and frogs is determined primarily by the amount of internal ice formation; this value typically approximates 67% of total body water. The body ice contents of our snakes, determined by calorimetry after 6 h of freezing, ranged from 17.7 to 36.2% (mean ± SEM = 26.3 ± 2.8%; N = 6). Ice contents were significantly greater (r = -0.65; P = 0.05) at lower body temperatures. The juvenile that did not recover was substantially colder (-1.70 °C) than surviving conspecifics (Table 1), suggesting that a lethal amount of ice may have formed. One adult (42.4 g) cooled to -1.25 °C after 12 h of ice formation, but recovered fully within 24 h at 3 °C. We wished to determine whether *Thamnophis sirtalis* could survive freezing for periods longer than 6 to 12 h. Prolonged cooling under these conditions, however, would presumably have been lethal since body temperature after thermoequilibration would have approximated -2.5 °C. Higher equilibrium body temperatures were produced by raising the bath temperature after nucleation was verified by an exotherm (Fig. 1B). Three adults, frozen for 48 h in this manner, survived with body temperatures (-0.75 to -1.20 °C) similar to those of snakes tested for 6 h (Table 1). Ice content of one of these (body temperature = -1.05 °C) was 34.0% of total body water. The snakes were stiff upon removal from the chamber and their pupils appeared white; however, all recovered within 3 d at 3 °C.

Table 1. Mass and thermal data for garter snakes frozen under laboratory conditions, during winter, for 6 h. All adults and two of three juveniles survived. Mean values are shown ± SEM. Abbreviations: SCP = supercooling point; Tb = body temperature after 6 h of freezing.

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Mass (g)</th>
<th>Cooling Rate (degrees C/h)</th>
<th>SCP (C)</th>
<th>Tb (C)</th>
</tr>
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<tr>
<td>Adults</td>
<td>11</td>
<td>52.1 ± 1.7</td>
<td>-3.7 ± 0.3</td>
<td>-1.35 ± 0.16</td>
<td>-0.18 ± 0.03</td>
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<tr>
<td>Juveniles</td>
<td>3</td>
<td>14.4 ± 3.0</td>
<td>-7.2 ± 0.3</td>
<td>-1.72 ± 0.62</td>
<td>-1.35 ± 0.18</td>
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<tr>
<td>Combined</td>
<td>14</td>
<td>45.7 ± 4.9</td>
<td>-4.4 ± 0.4</td>
<td>-1.43 ± 0.17</td>
<td>-0.93 ± 0.03</td>
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DISCUSSION

Our data suggest that environmental insulation is critical to freezing survival under laboratory and, presumably, natural conditions. Recent studies with frogs have shown that added insulation slows cooling and thereby retards the rate of ice formation. One adult (59.6 g) *Thamnophis sirtalis* failed to recover.
from an 18 h freeze exposure inside a poorly insulated cooling chamber. It
cooled more quickly (-6.9 °C/h) than did snakes in the heavily-insulated
chamber, reaching -0.80 °C in only 2.5 h, and, finally, -2.30 °C at
thermoequilibrium. Although its heart pulse could be felt, the snake showed no
righting response and died 3 d later. Juveniles cooled faster and had lower body
temperatures after 6 h of freezing than did larger adults tested within the same
chamber (Table 1). This suggests that small snakes may be more susceptible to
freeze injury and death under comparable field conditions. In cold climates,
active garter snakes select protected overnight refugia (12) which may retard
cooling.

Differences are apparent between *T. sirtalis* and the freeze tolerant
frogs. These anurans tolerate prolonged freezing at lower temperatures (-6 to
-8 °C) than do *T. sirtalis* (1). A threshold survival temperature has yet to be
determined for *T. sirtalis*, but our data suggest that it is probably near -1.7 °C.
Also, plasma concentration of glucose, a cryoprotectant reported in frogs, can
increase one hundred-fold within a few h of freezing (1). In contrast, there
was no indication of cryoprotectant accumulation (glucose or any other) in
garter snakes (Table 2). The propensity for freeze tolerance in *T. sirtalis*, in
the apparent absence of cryoprotectants, is not unique; a similar situation has
been demonstrated in marine invertebrates (1).

Table 2. Cryoprotectant (glucose) concentrations in garter snakes frozen
for 6 h during mid-winter and wood frogs (*Rana sylvatica*) for
12 h during early spring relative to cold, but nonfrozen (control)
animals. Mean (± SEM) concentrations are in umol/g wet liver
mass and umol/ml plasma. Wood frog data are from Storey and

<table>
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<th>Glucose Source:</th>
<th>Garter Snakes:</th>
<th>Wood Frogs:</th>
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<tr>
<td></td>
<td>Control Frozen</td>
<td>Control Frozen</td>
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<tr>
<td>Liver</td>
<td>26.4 ± 1.4</td>
<td>8.1 ± 2.5</td>
</tr>
<tr>
<td>Plasma</td>
<td>3.2 ± 1.7</td>
<td>6.8 ± 3.5</td>
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<tr>
<td>N</td>
<td>2</td>
<td>3</td>
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Survival of reptiles exposed to sub-freezing temperatures in nature
typically has been attributed to supercooling (6, 12, 13). Erroneously, it is often
assumed that reptiles are protected indefinitely from internal freezing at
temperatures above their reported SCP. An individual's SCP depends upon
cooling rate, body size, and hydration state. Also, the likelihood of ice nucleation increases over time and with decreasing body temperature (1). In this study, garter snakes remained supercooled for only 0.77 ± 0.21 h, and SCP's were quite high (Table 1). Therefore, unlike the situation with many terrestrial insects, it is doubtful that supercooling represents a major strategy of freeze avoidance in these snakes. Behavioral avoidance of subzero temperatures is doubtless important among reptiles, but we suspect that freeze tolerance, as an additional strategy, is not limited to T. sirtalis.

Successful methods have not yet been developed for the cryopreservation of mammalian organs (14). The garter snake represents the most highly evolved organism known to be capable of natural freeze tolerance. We anticipate that these common amniotes may serve as useful models for the advancement of cryobiological research.

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REFERENCES