Natural Freeze Tolerance in the Terrestrial Turtle, *Terrapene carolina*

JON P. COSTANZO AND DENNIS L. CLAUSSEN

Department of Zoology, Miami University, Oxford, Ohio 45056

**ABSTRACT** To investigate freeze tolerance in box turtles (*Terrapene carolina*), we froze 19 adults to body temperatures as low as −3.6°C under controlled laboratory conditions. Our data reveal that box turtles tolerate the freezing of at least 58% of their body water and can remain frozen, without injury, for at least 73 hr. Supercooling occurred in 63% of the turtles but only lasted from 0.1 to 2.0 hr. Supercooling points (X ± SEM = −1.1 ± 0.3°C) were high, and thus supercooling is not an effective strategy for freeze avoidance in *T. carolina*. Body ice contents (7–58% of total body water) of turtles frozen for 0.7–50 hr, determined calorimetrically, were inversely related to core body temperature. Significant thermal gradients occurred within the turtles' bodies during prefreeze cooling, freezing, and thawing. Since *T. carolina* hibernates in shallow terrestrial burrows, where exposure to subzero temperatures is likely, freeze tolerance represents a remarkable adaptation that probably enhances winter survival. These turtles are the largest (up to 0.5 kg) animals, by nearly an order of magnitude, for which natural freeze tolerance has been demonstrated.

Natural freeze tolerance, the ability of an animal to endure a significant portion of its body water as ice, is a remarkable adaptation of some temperate zone animals. Although freeze tolerance has long been known in many invertebrates of terrestrial and intertidal habitats, only recently have vertebrates been examined in this regard.

At least four species of terrestrially hibernating anurans tolerate extensive and repeated freezing of their extracellular fluids (see review by Storey and Storey, '88). Recent investigations on reptiles demonstrated that eastern garter snakes (*Thamnophis s. sirtalis*) and hatching painted turtles (*Chrysemys picta*), species that encounter subzero temperatures in nature, also tolerate substantial body freezing (Costanzo et al., '88; Storey et al., '88). These studies, in addition to increasing significantly our knowledge of the thermal biology of amphibians and reptiles, have provided useful models for the study of vertebrate cryopreservation.

Body freezing is, in fact, probably tolerated by several reptiles, especially those that inhabit high latitudes (or altitudes) and hibernate terrestrially or in shallow water. We here provide conclusive evidence for freeze tolerance in the box turtle, *Terrapene carolina*. This species is widely distributed over the central and eastern United States, where it inhabits open woodlands, bottomlands, and meadows (Carpenter, '57; Schwartz and Schwartz, '74; Stickel, '78). Box turtles hibernate for 6 months or more within burrows excavated in loose soil or organic debris (Dolbeer, '71; Russo, '72). We hypothesized that box turtles would be freeze tolerant because their hibernacula are often shallow (Carpenter, '57; Dolbeer, '71) and because turtles are active and are potentially exposed to severe weather in early spring and late fall (Cahn, '33; Neill, '48; Carpenter, '57).

**MATERIALS AND METHODS**

Adult eastern box turtles (*Terrapene c. carolina*) were collected from March 3 to July 5, 1989, from Dearborn, Ohio, and Switzerland Counties, southwestern Indiana, and from Butler, Pike, Preble, and Scioto Counties, southern Ohio. Turtles were acclimated to 5°C in a darkened room, for 10–41 days (X ± SEM = 20 ± 2 days) before testing. Water was always available, but food was withheld.

Testing for freeze tolerance, conducted from March 16 to July 17, largely followed procedures described by Costanzo (‘88). Turtles (mean mass

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RESULTS

Following freezing, all 19 turtles behaved normally, indicating that they had recovered fully within 3 days of testing.\(^1\) The turtles cooled to a core body temperature (\(X \pm SEM\)) of -0.83°C ± 0.19°C before freezing commenced. Supercooling occurred in 63% of the turtles, lasting from 0.1 to 2.0 hr (\(X \pm SEM = 0.5 \pm 0.2\) hr). Mean ± SEM supercooling point (SCP) for these individuals was -1.12°C ± 0.28°C. Body temperature (\(X \pm SEM\)) immediately following the onset of freezing, a close approximation of the melting point of body fluids under our experimental conditions, was -0.38°C ± 0.02°C.

For the eight turtles allowed to thaw within the cooling chamber, body temperatures, at the termination of the freezing phase, ranged from -0.90°C to -3.60°C (\(X \pm SEM = -1.68°C \pm 0.21°C; N = 8\)). Thawing time, arbitrarily determined as the time elapsed before the turtle warmed to -0.2°C, ranged from 16.5 to 35.0 hr (\(X \pm SEM = 21.4 \pm 2.2\) hr). Correlation analysis showed that thawing time was directly related to body mass (\(r = 0.65; P = 0.039\)).

Analysis of the thermal gradients within the turtles’ bodies during prefreeze cooling, freezing, and thawing (Table 1) showed that, relative to core temperature, external body surfaces were colder during prefreeze cooling and freezing but warmer during thawing. High core-to-surface thermal differentials (up to 1.10°C) were recorded just after ice nucleation, but these were reduced significantly by 48 hr of freezing. Differentials were highest (up to 2.70°C) during thawing.

Body ice contents of eight turtles, frozen for 18–50 hr, ranged from 33% to 58% (\(X \pm SEM = 45\% \pm 3\%\) of total body water (Fig. 1). Values for turtles frozen for 0.7, 2.5, and 15.0 hr were 7%, 8%, and 26%, respectively. More ice formed in turtles with lower body temperatures (Fig. 1). Calorimetric analysis of a cold, but unfrozen, turtle predicted that 3.3 g of ice was present; this is within the error reported for this methodology by Costanzo et al. (’88).

DISCUSSION

Numerous anecdotal reports (e.g., Cahn, ’33; Neill, ’48; Schwartz and Schwartz, ’74) imply that box turtles die when body freezing occurs (but, see Legler, ’60). However, no previous study has tested this notion under controlled experimental conditions. Since these turtles occupy terrestrial hibernacula, which, across their range, are often

\(^1\)All turtles met our criteria for survival; however, four individuals later died 17, 10, 22, and 24 days after testing. The period of cold acclimation and food denial for these turtles was significantly (Student’s t test, \(P = 0.02\)) longer than for survivors and may have contributed to their demise.
shallow (5–12 cm, Carpenter, '57; 2–10 cm, Dolbeer, '71; 0–5 cm, Gatten, '87), and, because in nature they may survive 80 winters or more (Stickel, '78), this species must be well-adapted to withstand the subzero temperatures that they inevitably encounter.

Our data clearly demonstrate that box turtles tolerate a substantial and prolonged freezing of their body fluids. The degree of freeze tolerance in this species is comparable to that of Chrysemys picta hatchlings, which survive the freezing of 52–53% of their extracellular fluid, for 24 hr, at an ambient temperature of −4°C (Storey et al., '88). Although we did not determine the ice contents of turtles frozen to temperatures lower than −3.1°C, extrapolation of the data in Figure 1 suggests that ice contents of T. carolina, if frozen to −4°C, would be similar.

Box turtles apparently tolerate more body ice than garter snakes. The relationship between body temperature and ice content in Thamnophis sirtalis (Costanzo et al., '88) is strikingly similar to that in T. carolina; however, these snakes die at higher (ca. −1.7°C) subzero temperatures. Garter snakes typically hibernate below frostline (Costanzo, '86) and probably encounter freezing temperatures only intermittently while abroad during late fall and early spring (Costanzo, '88). In contrast, box turtles occupy relatively shallow hibernacula where, like painted turtle hatchlings (Breitenbach et al., '84; Storey et al., '88), they may experience prolonged subzero temperatures (Russo, '72; Adams et al., '89).

Supercooling, a strategy of freeze-avoidance employed by many invertebrates, is not an effective alternative to freezing in T. carolina since they remained unfrozen for very short periods when cooled below the melting point of their body fluids. Without the aid of antifreeze compounds,
large ectotherms, such as *T. carolina*, are incapable of long supercooling to low temperatures because their fluid volumes are too massive (see discussion by Costanzo, '88). Moreover, *T. carolina* hibernates in moist or wet substrates (Carpenter, '57; Reagan, '74) where trans integumentary ice nucleation likely inhibits supercooling (e.g., Layne et al., '90). Lowe et al. ('71) reported a SCP of −6.7°C for *T. carolina*, but this result is suspect because of their extremely high cooling rate.

Our data show substantial thermal gradients within box turtles during prefreeze cooling, freezing, and thawing. Measuring the surface temperature of freezing animals, especially with uninsulated probes (e.g., Layne and Lee, '87; Storey et al., '88), very likely misrepresents core body temperatures. Although these gradients can be reduced by slow cooling and, fortunately, are negligible near equilibrium ice content, investigators should monitor core temperatures whenever possible.

In nature, box turtles hibernate under leaf litter and organic debris, which provides some protection against freezing (Reagan, '74; Schwartz and Schwartz, '74). Should body freezing occur, this insulation, by retaining the heat of fusion, would reduce the rate of ice formation. Thawing rates would also be moderated. In this regard environmental insulation is critical to the survival of frozen reptiles (Costanzo et al., '88).

Further study is needed to determine whether cryoprotectants, such as glucose and glycerol, are utilized by box turtles during freezing. In freezing frogs these substances function colligatively to reduce cellular dehydration, but they may also serve in other capacities (e.g., membrane stabilization; Storey and Storey, '88). Freeze tolerance is not necessarily dependent upon these cryoprotectants, however; painted turtle hatchlings and garter snakes accumulate little or no carbohydrate during freezing (Storey et al., '88; Costanzo et al., '88). Interestingly, a significant increase in osmotically active plasma constituents (glucose, uric acid, inorganic ions) has been reported for hibernating *T. carolina* (Hutton and Goodnight, '57).

Freeze tolerance depends, in part, on glycolytic energy metabolism during ischemia (Storey et al., '88). The high anaerobic capacity of *T. carolina* (Gatten, '87) may indeed promote freez e tolerance, but this notion requires experimental validation. The box turtle, the largest (up to 0.5 kg) animal by nearly an order of magnitude for which freeze tolerance has been demonstrated, is an ideal model for the study of vertebrate cryobiology.

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**LITERATURE CITED**


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