PERGAMON

Journal of Thermal Biology 24 (1999) 161–166

Journal of
THERMAL BIOLOGY

Behavioral responses of hatchling painted turtles (*Chrysemys picta*) and snapping turtles (*Chelydra serpentina*) at subzero temperatures

Jon P. Costanzo*, Jacqueline D. Litzgus, Richard E. Lee Jr

Department of Zoology, Miami University, Oxford, OH 45056, USA

Received 10 November 1998; received in revised form 6 January 1999; accepted 20 January 1999

Abstract

We monitored behavioral responses of cold-acclimated hatchling painted turtles (*Chrysemys picta*) indigenous to Nebraska and hatchling snapping turtles (*Chelydra serpentina*) indigenous to Nebraska and Arkansas during cooling (0.1°C/min) to temperatures as low as −19°C. All turtles made exploratory movements during cooling and locomotion occurred at temperatures as low as −2 to −4°C, but *C. picta* maintained relatively higher levels of locomotor activity than *C. serpentina*, and no differences in motility occurred between northern and southern groups of *C. serpentina*. Slow movements of the head and limbs were observed in supercooled hatchling *C. picta* at temperatures as low as −10°C, whereas at about −5°C, *C. serpentina* exhibited an increase in spontaneous motor activity followed by muscle contracture, immobility, and spontaneous freezing. *C. picta* spontaneously froze at about −16°C without exhibiting cold contracture, suggesting that they are better adapted to survive exposure to extreme cold.

Keywords: Behavior; *Chelydra serpentina*; *Chrysemys picta*; Cold adaptation; Locomotion; Supercooling

1. Introduction

Painted turtles (*Chrysemys picta*) hatch in late summer but overwinter within the nest chamber, about 10 cm below the ground surface (Gibbons and Nelson, 1978). In areas where snow cover persists, nest temperatures rarely fall below freezing (Breitenbach et al., 1984). However, in cold regions where snow cover is sparse or transient, hatchlings are commonly exposed to temperatures below the equilibrium freezing point of their tissues (about −0.6°C), and occasionally to temperatures < −10°C. Most of these chilling episodes are brief, lasting from several hours to a few days, although they may last a week or more and turtles may be exposed to many such events over the course of winter (Costanzo et al., 1995; Packard, 1997).

Lee and Costanzo (1998) reviewed the adaptations promoting survival of hatchling *C. picta* at subzero temperatures. Only relatively high temperatures (e.g. −4°C) may be tolerated in the frozen state, although these turtles may survive exposure to markedly lower temperatures by remaining supercooled (see Packard et al., 1997). Indeed, the supercooling capacity of *C. picta* (to −20°C) is the best known among vertebrates and rivals that of some invertebrates (Lee and Costanzo, 1998; Costanzo et al., 1999).

The snapping turtle (*Chelydra serpentina*) also

* Corresponding author. Tel: +1-513-529-3173; Fax: +1-513-529-6900.

E-mail address: costanjp@muohio.edu (J.P. Costanzo)

0306-4565/99/$ - see front matter
PII: S0306-4565(99)00006-6

occurs in northern regions, but in contrast to *C. picta*, hatchlings of *C. serpentina* emerge from nests in late summer and move to permanent water. By hibernating under water, *C. serpentina* is protected from extreme temperatures (Ultsch, 1989).

Despite the recent attention given to cold hardiness in hatchlings of *C. picta* (e.g. Packard et al., 1997), and of other northern chelonians (Costanzo et al., 1995), behaviors of these animals during cooling at subzero temperatures have not been investigated. Comparing chilling responses of *C. picta* and other northern species, and of turtles from northern and southern populations, may provide clues concerning the evolutionary development of cold hardiness and life history traits of chelonians. We thus undertook an observational study to determine whether behavioral responses during cooling differed between *C. picta* and *C. serpentina*, and between specimens of *C. serpentina* indigenous to northern and southern locales.

2. Materials and methods

2.1. Animals

Eggs of *C. picta bellii* and *C. serpentina* were collected in summer 1997, near Gimlet Lake, Crescent Lake National Wildlife Refuge, Garden County, west-central Nebraska (41°N, 102°W), from oxytocin-treated females. Additional eggs of *C. serpentina* were recovered from a dead female in Lonoke County, Arkansas (34°N, 91°W). Eggs were transported to the laboratory and incubated in moist vermiculite (1.0 g water/g dry vermiculite; about −150 kPa), at approximately 29°C, until they hatched in late August (Costanzo et al., 1998, 1999).

We placed groups of hatchlings in darkened plastic boxes containing damp vermiculite (0.5 g water/g dry vermiculite; about −350 kPa) and denied them food and free water. Turtles were exposed to 22°C for several weeks before subjecting them to an acclimation regimen intended to mimic a progressive, seasonal exposure to low temperature. In this regimen, turtles were transferred to an environmental chamber, on 1 October (*C. picta*, northern *C. serpentina*) or 15 October (southern *C. serpentina*), in which they were exposed to 15°C. After 1 month, the chamber temperature was reduced to 10°C and the turtles remained at this temperature for an additional month. Finally, hatchlings were exposed to 4°C in the chamber until used in cooling trials during late winter.

2.2. Cooling trials

We cooled turtles individually inside a 1000 ml glass beaker that was almost completely submerged in a refrigerated ethanol bath (RTE 140, Neslab; Portsmouth, New Hampshire). Within this beaker, turtles were confined within a chamber fashioned by placing an inverted transparent plastic cup (diameter = 9 cm, height = 3 cm) on a sheet of rigid plastic foam. The foam provided a lightly textured surface (64 cm^2) over which turtles could easily move. An array of lines radially emanated from a central point, dividing the floor into eight equal-sized segments. A 30 gauge copper–constantan thermocouple, which monitored turtle temperature during cooling, was passed through the top of the chamber. Perforations in the upper sides of the chamber promoted exchange of air between the chamber and the beaker.

Turtles inside the cooling chamber were videotaped using a flexible-necked camera (FlexCam, Videolabs; Minneapolis, MN) and a time-lapse cassette recorder (model AG-6730, Panasonic; Secaucus, NJ). The camera was positioned inside the beaker, directly above the chamber. The beaker's opening was closed with plastic foam, which reduced heat input to the chamber and excluded most incident light. Despite the darkness, high quality video images were obtained.

Thermal dynamics within the empty chamber during cooling were studied by instrumenting various regions of the chamber floor, ceiling, and air spaces with several thermocouples. The greatest vertical and lateral differentials in temperatures recorded by these thermocouples were 1.7 and 0.6°C, respectively. Preliminary study also confirmed that the temperature registered by a thermocouple affixed to a turtle's carapace provided a reasonably good representation of core body temperature. Core temperature could not be directly measured because the presence of the thermocouple in a body orifice may induce freezing and hamper movements. Pilot work was conducted using *C. serpentina* (southern) carcasses instrumented with a thermocouple inserted 2.8 cm into the cloaca and another epoxied onto the anterior surface of the carapace, near the head. Even with these relatively large hatchlings, core-to-surface temperature differentials were < -0.3°C at the cooling rate (0.1°C/min) used in the experiments. Thus, data obtained using surface mounted thermocouples approximated core (and brain) temperatures under the conditions of our experiments.

Turtles to be used in cooling trials were removed from their holding box, cleaned of adhering vermiculite using a fine-haired brush, and held in open cups in an environmental room set at 4°C for 24 h to permit evaporation of surface moisture. After drying, which was necessary to inhibit inoculative freezing of the tissues (see Costanzo et al., 1999), a thermocouple's junction was epoxied to its carapace and the turtle was permitted to habituate for 60–90 min within the cooling chamber. During this period, the turtle attained thermoequilibrium (4°C) with the chamber. Turtles

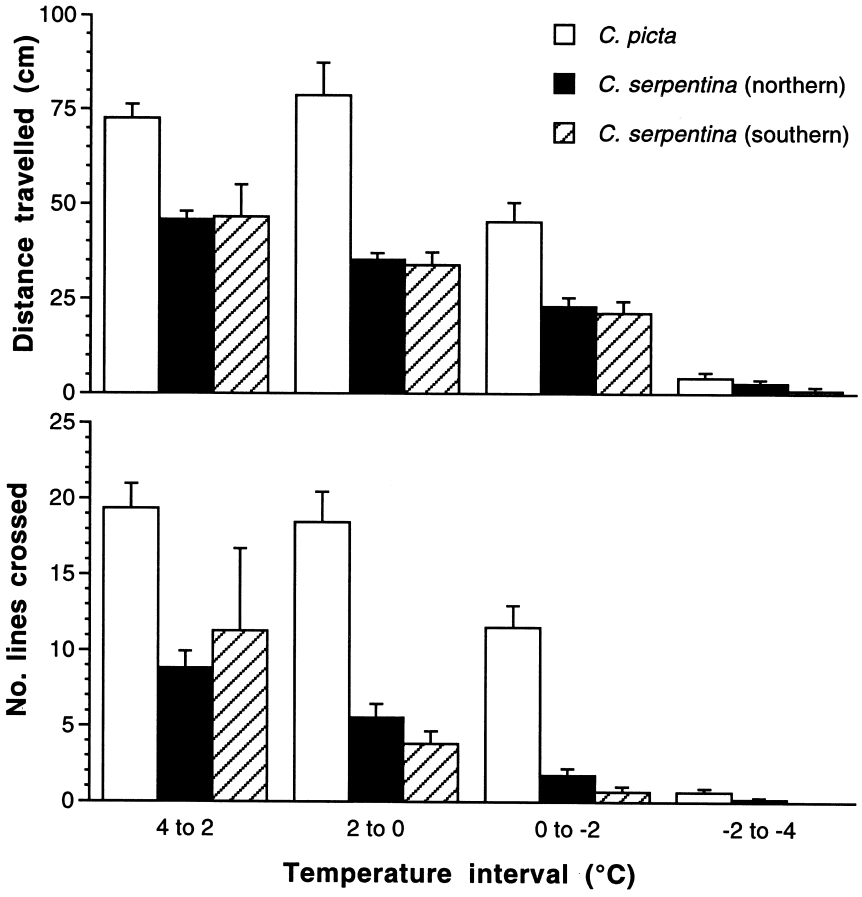

Fig. 1. Behavioral responses of hatchling painted turtles (*Chrysemys picta*) from Nebraska and snapping turtles (*Chelydra serpentina*) from Nebraska (northern) and Arkansas (southern), during cooling to low temperatures, showing distance traveled (top) and number of lines on the cooling chamber floor crossed by turtles during each temperature interval (bottom).

were then cooled at 0.1°C/min until they spontaneously froze, although seven of the 11 *C. picta* were removed from the chamber once they had cooled to −10°C. During cooling, turtle temperature, as registered by the thermocouple, was logged at 30 s intervals on a chart recorder (RD3752, Omega; Stamford, Connecticut). Turtles used in trials that culminated in freezing (and death) were subsequently weighed to 0.01 g and dried at 65°C to constant mass to determine carcass moisture content. We do not know if these data accurately reflect water contents of the turtles which remained unfrozen.

2.3. Data collection and analyses

Behavioral observations of each turtle during cooling were grouped by temperature intervals that spanned two degrees Celsius and lasted approximately 20 min. Evaluations of motility were based upon: (1) measurements of distance traveled within the chamber; and (2) the number of lines on the chamber floor crossed by turtles within each interval. Distance traveled was quantified using image analysis software (NIH Image Analysis software, v.1.61) on paths of turtles traced on acetate laid over the monitor's screen during playback of the videotape. The number of lines the turtles crossed was determined by summing the instances in which the turtle's head passed over a line drawn on the chamber's floor. The effect of cooling on these indices of motility was compared among the three turtle groups using repeated measures ANOVA, followed by a Bonferroni post-test. Other experimental variables were compared among the three turtle groups using ANOVA/Tukey–Kramer. Data are presented as mean ± SEM.

3. Results

Hatchlings moved freely about the chamber, loco-

Table 1
Somatic and behavioral response variables of hatchling turtles used in cooling trials. Mean values are shown ±1 SE; sample sizes are indicated in parentheses

	C. picta	*C. serpentina* (northern)	*C. serpentina* (southern)	*F*	p[1]
Body mass (g)	3.7 ± 0.1^{a} (11)	8.5 ± 0.2^{b} (11)	10.5 ± 0.2^{c} (6)	533.8	< 0.0001
Water content (%)	79.8 ± 0.3^{a} (4)	81.1 ± 0.4^{a} (7)	80.2 ± 0.4^{a} (4)	3.1	0.08
Burst activity onset (°C)	—	-5.1 ± 0.2^{a} (8)	-3.1 ± 0.3^{b} (5)	5.8	< 0.0001
Contracture onset (°C)	—	-5.4 ± 0.1^{a} (7)	-3.8 ± 0.3^{b} (5)	5.2	0.0004
T_c (°C)[2]	-16.1 ± 0.8^{a} (4)	-5.7 ± 0.7^{b} (10)	-6.2 ± 1.2^{b} (5)	34.8	< 0.0001

[1] Means within the same row identified by dissimilar superscripted characters differed significantly (ANOVA/Tukey–Kramer).
[2] Temperature of crystallization.

moting normally at temperatures as low as −2 to −4°C (Fig. 1A). Distance traveled by turtles during cooling was influenced by species/origin of the turtles ($p < 0.0001$) and temperature ($p < 0.0001$). *C. picta* were more motile than *C. serpentina* from northern ($p < 0.0001$) or southern ($p < 0.0001$) populations at all temperature intervals, although the responses were similar ($p > 0.9$) between the two groups of *C. serpentina*. A similar pattern of response was obtained in analyses of the number of lines on the chamber floor that were crossed by turtles during cooling (Fig. 1B), with the mean value depending on both turtle species/origin ($p < 0.0001$) and temperature ($p < 0.0001$). Again, higher values were obtained for *C. picta* than for *C. serpentina* from either locale ($p < 0.0001$), and responses of northern and southern *C. serpentina* were statistically indistinguishable ($p > 0.5$). Additional trials, in which we observed two *C. serpentina* confined within the test chamber kept at 4°C, showed no differences in the distance traveled ($p > 0.5$) or number of lines crossed ($p > 0.5$) during four sequential 20-min periods. Thus, the loss of motility exhibited by turtles during cooling likely was due to temperature, rather than due to the duration of confinement within the chamber.

Locomotion ultimately ceased after the turtles cooled to < −4°C, although turtles usually exhibited other, more subtle movements (e.g. flexure of the limbs, extension/retraction of the head) at remarkably low temperatures. In *C. picta*, such movements were observed in eight of the ten specimens at temperatures as low as −6 to −8°C, and in one turtle cooling between −8 to −10°C. At lower temperatures, *C. picta* became inanimate.

Unlike *C. picta, C. serpentina* exhibited non-locomotory movements at temperatures only as low as −4 to −6°C. Their response to cooling in this interval was marked by distinctive behavioral changes that were not observed in *C. picta*. *C. serpentina* showed a sudden increase in alertness and vigor that contrasted markedly with the quiescence that had been developing. This spontaneous burst of activity rarely resulted in locomotion, but instead resembled a scratching or digging motion directed at the floor of the chamber. The onset temperature for this behavior ranged from −4.6 to −5.0°C in northern *C. serpentina* ($N = 8$), and from −2.4 to −4.3°C in southern *C. serpentina* ($N = 5$); mean onset temperatures differed between the populations (Table 1). This activity lasted 3.0 ± 0.6 and 7.6 ± 0.7 min in northern and southern *C. serpentina*, respectively, and culminated in an apparent state of contracture (Gasser, 1930). Upon entering contracture, turtles characteristically extended their limbs laterally with a smooth sweeping motion that brought them caudally. Once their limbs were in the fully extended position, the turtles thenceforth were inanimate. This posture was assumed at temperatures ranging from −5.0 to −5.9°C in northern *C. serpentina* ($N = 7$) and from −3.1 to −4.8°C in southern *C. serpentina* ($N = 5$). The mean temperature of contracture onset was strongly dependent on the origin of the specimens (Table 1).

To determine whether the contracture we observed was associated with a lethal condition, some turtles were held at the contracture onset temperature for various periods, rewarmed to 4°C, and assessed for survival. In these trials, cooling of the turtle was interrupted by resetting the thermostat on the refrigerated bath to a temperature that maintained the animal at the temperature at which contracture was induced. One southern *C. serpentina* rapidly recovered normal behaviors and locomotor capacity after being held in contracture at −3.1°C for 30 min. Two northern *C. serpentina* fully recovered after remaining in contracture at temperatures of −5.0 and −5.3°C, for 5 and 8 min, respectively. The first of this pair was retested in the chamber, after recovering for about 4 h in the 4°C incubator, this time entering contracture at a body temperature of −5.5°C. It recovered after being held at this temperature for 10 min.

Hatchling *C. serpentina* that had undergone spontaneous nucleation of the body fluids at relatively high

temperatures (i.e. $> -4°C$) did not exhibit the spontaneous burst of activity or enter contracture. Rather, these turtles underwent several convulsive body movements, afterwards remaining quiescent until they were removed from the test chamber. For the specimens of *C. serpentina* that attained contracture, freezing often occurred after additional cooling. The time elapsed between the onset of contracture and nucleation of the body fluids was statistically indistinguishable (Student's t-test: $t=1.4$; df$=7$, $p=0.19$) between the two *C. serpentina* groups (northern: 17.2 ± 6.1 min; southern: 30.1 ± 6.5 min). The mean temperature of crystallization (T_c) for *C. serpentina* was about 10°C higher than that for *C. picta* (Table 1). None of the turtles in which nucleation occurred showed vital signs after thawing. However, all seven of the *C. picta* that were removed, unfrozen, from the test chamber survived being supercooled to a minimum temperature of −10°C.

4. Discussion

Despite the recent attention given to mechanisms of cold tolerance in hatchling *C. picta* (e.g. Packard et al., 1997; Lee and Costanzo, 1998), observations of these turtles during cooling at subzero temperatures were heretofore lacking. Behaviors of turtles in our cooling chamber may not match those occurring during natural hibernation because *C. picta* overwintering within the nest chamber are surrounded by other hatchlings and soil. Also, our turtles were cooled at rates higher than those occurring under field conditions (Costanzo et al., 1995). Nevertheless, our study provided a means for systematically comparing fundamental responses of turtles of different species, and from different geographic origins, to acute cooling at very low temperatures.

Our finding that *C. picta* and *C. serpentina* were capable of motion while supercooled agrees with previous observations of other reptiles (Fitch, 1956; Vincent, 1971; Spellerberg, 1972; Viitanen, 1974; Costanzo, 1988). Notably, one study showed that garter snakes (*Thamnophis sirtalis*) maintained locomotor function during cooling at temperatures as low as −4°C (Costanzo, 1988). Spellerberg (1972) suggested that cold-hardy reptiles might conduct routine activities in the supercooled state. This is the case with certain coldwater fishes, although in these species the supercooled state is stabilized by the presence of antifreeze glycoproteins and peptides (DeVries, 1982).

Sudden increase in locomotor activity and behavioral hyperexcitability during chilling, as exhibited in our hatchling *C. serpentina*, has been observed in various ectothermic vertebrates (Prosser and Nelson, 1981). In *T. sirtalis*, for example, a resurgence in locomotor activity occurs near 0°C (Stewart, 1965; Vincent, 1971; Costanzo, 1988). A hyperactive response occurs in the tropical sea turtle (*Lepidochelys kempi*) during cooling, albeit at relatively high temperatures (Moon et al., 1997). Goldfish (*Carassius auratus*) also exhibit hyperexcitability and increased swimming upon cold exposure, apparently a manifestation of the effect of cold on the cerebellum (Friedlander et al., 1976). Behavioral excitability may stem from failure of inhibitory synapses, which are more thermally labile than excitatory ones (Prosser and Nelson, 1981). One possible benefit of this behavior is that the animal may be stimulated to remove itself from an environment in which conditions may be incipiently lethal. Hatchling *C. serpentina*, which usually overwinter under water where they avoid exposure to subzero temperatures, typically froze (and died) within 20–40 min of exhibiting the increase in behavioral activity. In contrast, hyperexcitability was not observed in hatchling *C. picta*, which remain within the natal nest during winter and readily tolerate exposure to subzero temperatures.

Differences in the overwintering habits of these species were also reflected in the temperatures at which the turtles spontaneously froze. The T_c for *C. serpentina*, about −6°C, was markedly higher than that for *C. picta* (−16°C), a value similar to that previously reported for Nebraskan *C. picta* (Costanzo et al., 1998). Supercooling capacity is influenced by biophysical and physiological factors such as body size and body water content (Lee and Costanzo, 1998). Although differences in T_c between these species may partly reflect variation in body mass (Table 1), the basis for the exceptional supercooling capacity exhibited by hatchling *C. picta* as compared to other species, such as *C. serpentina* (Birchard and Packard, 1997; Costanzo et al., 1999), is as yet unknown (Lee and Costanzo, 1998).

Cooling to −4 or −5°C induced contracture in both groups of hatchling *C. serpentina*, but not in *C. picta*. The onset temperature was about 1°C lower in turtles indigenous to Nebraska than in those from Arkansas (Table 1), suggesting that northern turtles are more tolerant of low temperature. Although we determined that onset temperatures varied little within each group, and that turtles maintained in contracture for up to 30 min recovered fully upon warming, the actual physiological cause of the contracture was not determined. This response may reflect loss of the integrating functions of the neuromuscular junction, which is more susceptible to blockage than either nerve or muscle (Prosser and Nelson, 1981). It may also stem from disruption of metabolic regulation, the attendant loss of ATP-production capacity, and failure to sustain critical ion gradients (Hochachka, 1986).

Our finding that *C. picta* hatchlings exhibited oc-

casional movements of the head and limbs at temperatures as low as −10°C, and that contracture did not ensue at temperatures as low as −16°C, suggests that this species is better adapted to function at very low temperature relative to *C. serpentina*. Birchard and Packard (1997) reported that cardiac function, and presumably tissue perfusion, persists in both *C. picta* and *C. serpentina* hatchlings supercooled to as low as −9°C. However, we note that the Q_{10} for heart rate at subzero temperatures was markedly lower in *C. picta* than in *C. serpentina* (see Fig. 3 in Birchard and Packard, 1997). The relatively higher metabolism exhibited by *C. picta* at such temperatures might partially account for the interspecific differences in behavior observed in the present study. Function of nervous and other systems at extremely low temperatures is critical to the survival of hatchling *C. picta* overwintering within the natal nest.

Acknowledgements

We gratefully acknowledge technical assistance by C. Kostizen. J. B. Iverson generously provided turtles for this study, and D. L. Claussen and J. R. Layne, Jr, commented on a draft of the manuscript. The research protocol (No. 381) was approved by the Institutional Animal Care Committee. This work was supported, in part, by grants from the National Science Foundation (IBN 9507437) and National Institutes of Health (1 R15 DK48067-01A1) to J.P.C.

References

Birchard, G.F., Packard, G.C., 1997. Cardiac activity in supercooled hatchlings of the painted turtle (*Chrysemys picta*). J. Herpetol. 31, 166–169.

Breitenbach, G.L., Congdon, J.D., van Loben Sels, R.C., 1984. Winter temperatures of *Chrysemys picta* nests in Michigan: effects on hatchling survival. Herpetologica 40, 76–81.

Costanzo, J.P., 1988. Ecophysiological adaptations to overwintering in the eastern garter snake, *Thamnophis sirtalis sirtalis*. Ph.D. Dissertation, Miami Univ., Oxford, OH.

Costanzo, J.P., Iverson, J.B., Wright, M.F., Lee, R.E., 1995. Cold hardiness and overwintering strategies of hatchlings in an assemblage of northern turtles. Ecology 76, 1772–1785.

Costanzo, J.P., Litzgus, J.D., Iverson, J.B., Lee, R.E., 1998. Soil hydric characteristics and environmental ice nuclei influence supercooling capacity of hatchling painted turtles, *Chrysemys picta*. J. Exp. Biol. 201, 3105–3112.

Costanzo, J.P., Litzgus, J.D., Iverson, J.B., Lee, R.E., 1999. Ice nuclei in soil compromise cold hardiness of hatchling painted turtles, *Chrysemys picta*, Ecology (in press).

DeVries, A.L., 1982. Biological antifreezes in coldwater fishes. Comp. Biochem. Physiol. 73A, 627–640.

Fitch, H.S., 1956. Temperature responses in free-living amphibians and reptiles of northeast Kansas. Univ. Kansas. Publ. Mus. Nat. Hist. 8, 417–476.

Friedlander, M.J., Kotchabkakdi, N., Prosser, C.L., 1976. Effects of cold and heat on behavior and cerebellar function in goldfish. J. Comp. Physiol. 112, 19–45.

Gasser, H.S., 1930. Contractures of skeletal muscle. Physiol. Rev. 10, 35–109.

Gibbons, J.W., Nelson, D.H., 1978. The evolutionary significance of delayed emergence from the nest by hatchling turtles. Evolution 32, 297–303.

Hochachka, P.W., 1986. Defense strategies against hypoxia and hypothermia. Science 231, 234–241.

Lee, R.E., Costanzo, J.P., 1998. Biological ice nucleation and ice distribution in cold-hardy ectothermic animals. A. Rev. Physiol. 60, 55–72.

Moon, D-Y., Mackenzie, D.S., Owens, D.W., 1997. Simulated hibernation of sea turtles in the laboratory: I. feeding, breathing frequency, blood pH, and blood gases. J. Exp. Zool. 278, 372–380.

Packard, G.C., 1997. Temperatures during winter in nests with hatchling painted turtles (*Chrysemys picta*). Herpetologica 53, 89–95.

Packard, G.C., Lang, J.W., Lohmiller, L.D., Packard, M.J., 1997. Cold tolerance in hatchling painted turtles (*Chrysemys picta*): supercooling or freeze tolerance? Physiol. Zool. 70, 670–678.

Prosser, C.L., Nelson, D.O., 1981. The role of nervous systems in temperature adaptation of poikilotherms. A. Rev. Physiol. 43, 281–300.

Spellerberg, I.F., 1972. Temperature tolerances of southeast Australian reptiles examined in relation to reptile thermoregulatory behavior and distribution. Oecologia (Berlin) 9, 23–46.

Stewart, G.R., 1965. Thermal ecology of the garter snakes *Thamnophis sirtalis concinnus* (Hallowell) and *Thamnophis ordinoides* (Baird and Girard). Herpetologica 21, 81–102.

Ultsch, G.R., 1989. Ecology and physiology of hibernation and overwintering among freshwater fishes, turtles, and snakes. Biol. Rev. 64, 435–516.

Viitanen, P., 1974. Hibernation and seasonal movements of the viper, *Vipera berus berus* (L.), in southern Finland. Annales Zoologici Fennici 4, 472–546.

Vincent, T.K., 1971. Resistance to cold stress in the red-sided garter snake *Thamnophis sirtalis parietalis*. M.Sc. University of Manitoba.