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SEASONAL CHANGES IN PHYSIOLOGY AND DEVELOPMENT OF COLD HARDINESS IN THE HATCHLING PAINTED TURTLE *CHRYSEMYS PICTA*

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Summary

Hatchling painted turtles (*Chrysemys picta*) commonly hibernate in shallow, natal nests where winter temperatures may fall below –10 °C. Although hatchlings are moderately freeze-tolerant, they apparently rely on supercooling to survive exposure to severe cold. We investigated seasonal changes in physiology and in the development of supercooling capacity and resistance to inoculative freezing in hatchling *Chrysemys picta* exposed in the laboratory to temperatures that decreased from 22 to 4 °C over a 5.5 month period. For comparison, we also studied hatchling snapping turtles (*Chelydra serpentina*), a less cold-hardy species that usually overwinters under water. Although *Chrysemys picta* and *Chelydra serpentina* differed in some physiological responses, both species lost dry mass, catabolized lipid and tended to gain body water during the acclimation regimen. Recently hatched, 22 °C-acclimated *Chrysemys picta* supercooled only modestly (mean temperature of crystallization –6.3±0.2 °C; *N*=6) and were susceptible to inoculation by ice nuclei in a frozen substratum (mean temperature of crystallization –1.1±0.1 °C; *N*=6) (means ± S.E.M.). In contrast, cold-acclimated turtles exhibited pronounced capacities for supercooling and resistance to inoculative freezing. The development of cold hardiness reflected the elimination or deactivation of potent endogenous ice nuclei and an elevation of blood osmolality that was due primarily to the retention of urea, but was not associated with accumulation of the polyols, sugars or amino acids commonly found in the cryoprotection systems of other animals. Also, *Chrysemys picta* (and *Chelydra serpentina*) lacked both antifreeze proteins and ice-nucleating proteins, which are used by some animals to promote supercooling and to initiate freezing at the high temperatures conducive to freezing survival, respectively.

Key words: painted turtle, *Chrysemys picta*, snapping turtle, *Chelydra serpentina*, cold hardiness, acclimation, supercooling, ice inoculation, antifreeze protein, ice-nucleation protein.

Introduction

Ectotherms survive exposure to temperatures below the equilibrium freezing point (FP_{eq}) of their body fluids by remaining supercooled, by tolerating the freezing of their tissues or, in a few cases, by using either mechanism. The physiological basis for cold hardiness in some ectothermic animals has been extensively studied (for a review, see Storey and Storey, 1988). Some species enhance their supercooling capacity by eliminating endogenous ice nuclei and/or by producing macromolecular antifreeze proteins (AFPs), or 'thermal hysteresis factors', which apparently prevent spontaneous freezing of the body fluids and may also inhibit inoculative freezing (DeVries, 1982; Zachariassen and Husby, 1982; Duman et al., 1991). AFPs may also be used by freeze-tolerant species to prevent damage from ice recrystallization (Knight and Duman, 1986; Tursman et al., 1994; Ramlov et al., 1996). Many freeze-tolerant species produce ice-nucleating proteins (INPs) that trigger freezing at a relatively high body temperature (T_b), thereby ensuring that the tissues freeze slowly and that ice is restricted to the extracellular spaces (Storey and Storey, 1988; Duman et al., 1991). In addition, many cold-hardy species accumulate polyols, sugars, amino acids and other compounds that function as cryoprotectants either to protect tissues against freezing injury or to facilitate supercooling (Storey and Storey, 1988).

It is commonly known that the seasonal development of cold hardiness in arthropods is linked to changes in ambient photoperiod, temperature and hydric conditions and to food quality and/or availability (Lee, 1991). Because the acquisition of cold hardiness in ectothermic vertebrates has received relatively little study, we undertook this investigation of the painted turtle (*Chrysemys picta*), an intriguing example of a cold-hardy reptile. Whereas adults of this species generally hibernate in ponds or streams, hatchlings usually overwinter within the natal nest chamber, approximately 10 cm or less below the ground surface (Ultsch, 1989). Although the soil adjacent to turtle nests freezes infrequently in areas where snow cover persists (Breitenbach et al., 1984; Nagle et al., 2000), in northern regions, where snow cover is sparse or

transient, these turtles may cool below the FP_{eq} of their body fluids (approximately –0.6 °C) (Costanzo et al., 1995; Packard, 1997; Packard et al., 1997a). Such chilling episodes are usually brief (e.g. several hours to a few days), but they sometimes persist for a week or longer.

The winter survival of hatchling *Chrysemys picta* exposed to subzero temperatures may be promoted by both freeze-tolerance and supercooling. Because turtles apparently tolerate freezing only if T_b remains above −4 °C (Storey et al., 1988; Churchill and Storey, 1992; Costanzo et al., 1995; Attaway et al., 1998), survival at temperatures lower than this is possible only if they remain supercooled (Packard et al., 1997b). Hatchling *Chrysemys picta* can supercool extensively in the laboratory, but in nature supercooling may be constrained by environmental ice nuclei that seed the freezing of their body fluids. Inoculative freezing may occur when turtles contact ice or any other ice nuclei (e.g. inorganic crystals, organic compounds and microorganisms) present in the nesting soil (Costanzo et al., 1998, 2000).

We investigated the seasonal development of cold hardiness in hatchling *Chrysemys picta* subjected to a laboratory regimen of acclimation to winter temperatures by tracking changes in their supercooling capacity and inoculation resistance. Because these turtles do not eat or drink during their 8 month stay within the nest, we also studied changes in body composition, hydro-osmotic balance and concentrations of metabolites in the blood. To investigate mechanisms of cold hardiness in hatchling *Chrysemys picta*, we also assayed turtles for the presence of common cryoprotectants, AFPs and INPs. For comparison, some experiments were performed on snapping turtles (*Chelydra serpentina*) which, unlike *Chrysemys picta*, usually emerge from their natal nests in autumn and avoid exposure to subzero temperatures by overwintering under water (Ultsch, 1989).

Materials and methods

Experimental animals and acclimation regimen

Approximately 160 eggs were obtained *via* oxytocin injection (see Etchberger et al., 1992) from approximately 12 *Chrysemys picta bellii* (Gray) collected in summer 1998 near Gimlet Lake, in Crescent Lake National Wildlife Refuge, Garden County, west-central Nebraska, USA (41°N, 102°W). The eggs were combined to form a single group, transported to our laboratory, and incubated at 29 °C in moist vermiculite (1.0 g water g^{-1} vermiculite; water potential approximately –150 kPa) until they hatched, in late July. To mimic conditions within natural nests, hatchlings were kept in darkened plastic boxes containing damp vermiculite (0.5 g water g^{-1} vermiculite; water potential approximately –350 kPa) and denied food and free water.

Turtles were gradually acclimated to temperatures occurring in soil adjacent to *Chrysemys picta* nests during late summer, autumn and winter (Fig. 1; see also Costanzo et al., 1995). The hatchlings were exposed to 22 °C in mid-August and then transferred on 1 October to an environmental chamber set at 15 °C. The temperature inside the chamber was changed to 10 °C on 1 November and to 4 °C on 1 December. We sampled the turtles three times during the acclimation regimen: in mid-August, approximately 3 weeks after hatching and 1 week after transfer to 22 °C; in late November, after exposure to 10 °C for 4 weeks; and in late January, after exposure to 4 °C for

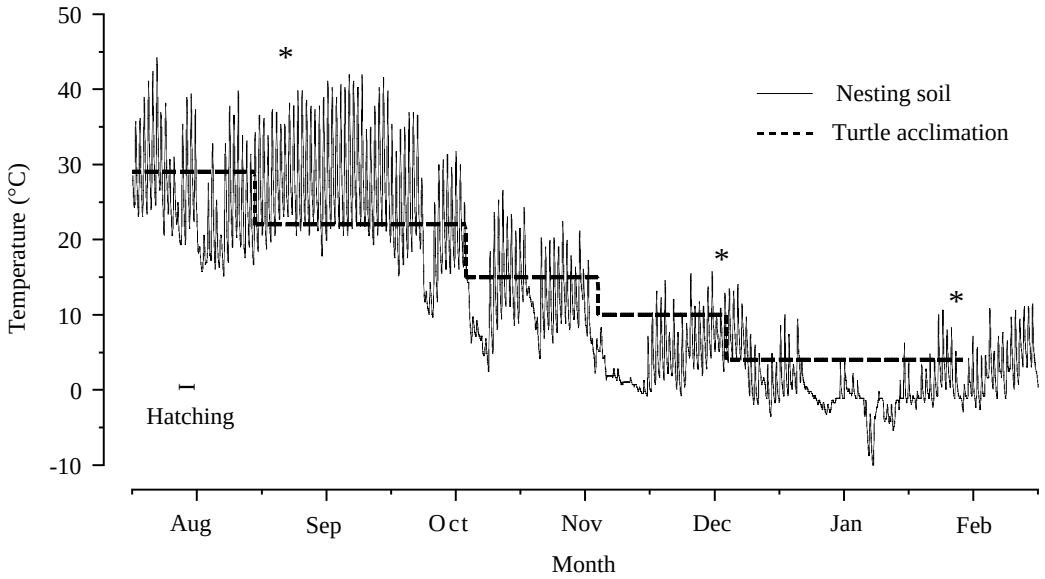

Fig. 1. Seasonal changes in soil temperature at a *Chrysemys picta* nesting area in comparison with the laboratory regimen used to acclimate turtles to winter conditions. Asterisks indicate times when experiments were conducted. Soil temperatures were measured in 1997–1998 near Gimlet Lake, Crescent Lake NWR, Garden County, west-central Nebraska, USA, using a single-channel temperature logger (Onset Computer, Tidbit; Pocasset, MA, USA). The device was placed in a sandhill, 10 cm beneath the soil surface, near a group of recently constructed *Chrysemys picta* nests.

approximately 8 weeks (Fig. 1). At each sampling, turtles were selected at random from the available pool of animals.

Over 500 eggs of snapping turtles, *Chelydra serpentina* (Linnaeus), inhabiting Gimlet Lake were obtained from approximately 10 nests within 12 h of deposition and incubated as described above. Hatchling *Chelydra serpentina* were cold-acclimated and sampled following the schedule used for *Chrysemys picta*. However, because *Chelydra serpentina* hatched in mid-August, approximately 2 weeks later than *Chrysemys picta*, the timing of the initial sample was offset by an equal period.

Supercooling and ice inoculation trials

Supercooling capacity was measured by cooling hatchlings in the absence of external ice nuclei until they spontaneously froze (N=6 for each acclimation group). Turtles were prepared for testing by gently brushing adherent vermiculite from their surfaces and then holding them in sheltered boxes for 24 h (Costanzo et al., 1998). This procedure, performed in darkness at the prevailing acclimation temperature, permitted evaporation of surface moisture which otherwise might freeze and inoculate the tissues. Each turtle was instrumented with a 30 gauge thermocouple (copper–constantan) glued to its carapace, placed individually in a 50 ml plastic tube, and insulated by loosely filling the space above it with plastic foam. The tubes were then suspended in a refrigerated ethanol bath (Neslab, model RTE 140; Portsmouth, New Hampshire, USA) programmed to cool the turtles from the holding temperature to –0.4 °C. The turtles were held at –0.4 °C for 1 h before being cooled further (at 0.5 °C h^{-1}) until each produced a freezing exotherm. During cooling, turtle temperature, as registered by the thermocouple, was logged at 30 s intervals on a datalogger (Omega model RD3752; Stamford, Connecticut, USA), and the temperature of crystallization (T_c) of each hatchling was determined from the recordings.

Inoculation trials were conducted by measuring the T_c values of turtles cooled in contact with a frozen substratum (see below) intended to seed the freezing of body fluids. Hatchlings (N=6 for each acclimation group) were cleaned, dried and fitted with a thermocouple probe, as described above, and then placed individually in a 50 ml plastic tube containing approximately 12 g of prepared substratum. To ensure that the turtles were uniformly exposed to ice nuclei, more or less substratum was used to achieve a substratum:body mass ratio of 4:1, and the material was firmly tamped around the hatchling. A piece of plastic foam placed in the tube above the turtle aided the retention and detection of its exotherm. Turtles were habituated to the substratum, in darkness and at the prevailing acclimation temperature, for 24 h before trials commenced. The trials were initiated by suspending the tubes in the refrigerated ethanol bath, which cooled the turtles from the holding temperature to –0.4 °C. After the turtles had attained thermoequilibrium, the substratum was inoculated with small ice crystals and permitted to freeze for 1 h. Subsequently, the temperature inside the tube was reduced by 0.5 °C h^{-1} and the T_c of the turtle was determined as above.

The substratum used in inoculation trials was a composite of soil samples collected at our field site in September 1996. Soil was sampled at a depth of 10 cm, from seven locales, each less than 0.5 m from a *Chrysemys picta* nest constructed the previous year (see Costanzo et al., 1998). Samples were stored at 4 °C in covered containers and combined in equal quantities before use. The pooled material (loamy sand) was sieved with a 2 mm^2 mesh, mixed (9:1) with fine clay, and then autoclaved to destroy any constituent organic ice nuclei. The substratum was then dried in an oven at 65 °C and hydrated (0.075 g water g^{-1} dry mass; water potential –400 kPa) with autoclaved, ultrapurified water. Most winter-conditioned hatchlings cooled in contact with this substratum resist inoculative freezing (Costanzo et al., 1998).

Blood sampling and analyses

At each of the three sample times, turtles (N=6 for both *Chrysemys picta* and *Chelydra serpentina*) were removed from their holding boxes, weighed to within 0.01 g, cleaned by swabbing the skin on the head and neck with ethyl alcohol and killed by severing the spinal cord near the cranium with a scissors. Blood was drawn from severed neck vessels into heparinized microcapillary tubes that were centrifuged (2000 ***g***, 5 min) to pack the erythrocytes. We measured the hematocrit and harvested approximately 70 or 100 µl of plasma from each *Chrysemys picta* or *Chelydra serpentina*, respectively. Blood plasma was stored frozen (–80 °C) in polypropylene microfuge tubes until used. To guard against contaminating the blood with ice nuclei, all surgical instruments, sample containers and dispensing tools were thoroughly cleaned and autoclaved before use.

Plasma osmolality was measured using a vapor pressure osmometer (Wescor, model 5500; Logan, UT, USA) and appropriate NaCl standards. We used enzymatic assays to determine plasma concentrations of glucose (Sigma, no. 510; St Louis, MO, USA), glycerol and triglycerides (Sigma, no. 337) and lactate (Sigma, no. 735). Urea nitrogen in the plasma was measured using a urease/nitroprusside method (Sigma, no. 640). Total protein was measured using the Bradford procedure (BioRad, Richmond, CA, USA), with bovine serum albumin as the standard. To conserve plasma, the above procedures used samples diluted (1:1) with ultrapurified water. Preliminary tests indicated that dilution of the samples did not introduce error into the determination of plasma osmolality (see Sweeney and Beuchat, 1993).

Portions of the plasma samples were shipped to the Biopolymer Facility, Ohio State University, for analysis of amino acid concentrations. The samples were lyophilized, derivatized as phenylthiocarbamamoylamino acids using phenylisothiocyanate as the reagent, and separated for quantification by high-performance liquid chromatography (HPLC). Sample peaks were referenced to peaks of amino acid standards for identification and calculation of concentrations.

Additional samples of plasma from some *Chrysemys picta* (N=3 in each acclimation group) were used for HPLC analysis of polyols, sugars and other carbohydrates commonly found in

animal cryoprotectant systems. These samples were mixed with ethyl alcohol solution (80 %, v/v), frozen to precipitate the proteins, and filtered through a Whatman GF/F filter to remove fine solids. The filtrate was incubated with finely divided charcoal, isolated by GF/F filtration, and evaporated under vacuum at 40 °C. Remaining particulates were removed using a nylon 0.2 µm centrifugal filter, and the clear filtrates were then lyophilized. The residue was resuspended in 60 µl of water for routine separation and quantification of carbohydrates by HPLC (Hendrix and Salvucci, 1998). Sample peaks were referenced to peaks of various carbohydrate standards for identification and calculation of concentrations. Peak identity was also confirmed by analysis of samples following ion-exchange, chemical and/or enzymatic treatment. Treatment with 0.5 mol l^{-1} NaOH at 100 °C was used to distinguish reducing from nonreducing sugars (Bedford, 1977).

Body composition

At the conclusion of the supercooling and inoculative freezing trials, all turtle carcasses (N=12) were weighed to the nearest 0.1 mg and dried for approximately 1 week in an oven at 65 °C. The carcasses were weighed again, and the body water content was calculated by subtracting the dry mass from the fresh mass.

Carcasses of turtles used in hematological studies were oven-dried (at 65 °C) and finely ground in a coffee mill. A 100 mg sample of the homogenized material was analyzed for nonpolar lipids using the methanol/chloroform-based Folch extraction procedure (Tietz, 1970). An additional sample (500 mg) was placed in a porcelain crucible and incinerated at 550 °C in a furnace (Thermolyne, type 1400; Dubuque, IA, USA) for approximately 18 h, and the organic content was determined from the change in mass of the sample during incineration.

Thermal hysteresis activity in blood plasma

We compared the melting point (MP) and freezing point (FP) of individual plasma samples to determine whether the blood of *Chrysemys picta* and *Chelydra serpentina* exhibits thermal hysteresis activity indicative of the presence of antifreeze proteins. We measured the MP and FP of 2–4 samples of undiluted plasma from each turtle using a nanoliter osmometer (Clifton Technical Physics; Hartford, NY, USA). Droplets (approximately 1 nl) were frozen by cooling them rapidly in the sample chamber and then viewed at 42× under a microscope during slow (0.05 °C min^{-1}) warming. The temperature at which the smallest observable ice crystal disappeared was recorded as the MP. The droplets were again frozen, warmed and held momentarily at a temperature slightly below the observed MP. The samples, each now containing a small (approximately 50 µm diameter) ice crystal, were then cooled slowly (at 0.05 °C min^{-1}) until the ice crystal spontaneously enlarged, and the associated temperature was recorded as the FP. Preliminary tests of the procedure used osmolality standards or saline solutions containing antifreeze glycoprotein extracted from Antarctic fish in place of turtle plasma.

Ice-nucleating activity in blood plasma

We examined turtle blood for the presence of ice-nucleating proteins by measuring ice-nucleating activity in the blood plasma of *Chrysemys picta* and *Chelydra serpentina*. A 3 µl sample of undiluted plasma was drawn from its storage vessel into the center of a 10 µl glass capillary tube. The sample was positioned such that that an equal volume of air bounded the fluid column, and the opening at each end of the tube was then plugged with clay. After taping the sensing junction of a 36 gauge copper–constantan thermocouple to each tube, the samples were inserted into a 20 ml test tube submerged in a refrigerated ethanol bath. Samples were allowed to attain thermoequilibrium at approximately 0 °C and were then cooled at 1.5 °C min^{-1} until each produced a freezing exotherm. Results from five replicates were averaged to produce a single T_c value for each turtle. Control trials in which an approximately isotonic saline solution (150 mmol l^{-1} NaCl) was substituted for plasma were conducted at each sample time. Saline solutions were prepared with water obtained from a reverse-osmosis ultrapurification system (0.2 µm filter; Dayton Water Systems, Dayton, OH, USA) and sterilized by autoclaving. To mimic the blood sampling and storage protocol, small samples of each saline solution were drawn into heparinized capillary tubes and then expelled into a polypropylene microcentrifuge tube. Six samples prepared in this way were stored frozen (–80 °C) before testing.

We tested the hypothesis that exposure of hatchling *Chrysemys picta* to nesting soil elevates the ice-nucleating activity in their blood. Three hatchlings, acclimated to 4 °C, were cleaned of adherent vermiculite, air-dried for 24 h and placed in plastic cups containing damp nesting soil. The soil, collected from our field site as described above, had been air-dried at 4 °C and then hydrated (0.05 g water g^{-1} soil) with autoclaved, ultrapurified water. The turtles were exposed to the soil for 7 days at 4 °C. We gently cleaned the turtles before collecting their blood; however, it was not possible to remove all traces of soil from their body surfaces. Blood was sampled from severed neck vessels, and plasma was separated by centrifugation, stored frozen and tested for ice-nucleating activity as before. We also determined the activity of ice nuclei in the nesting soil to which the three hatchlings had been exposed by measuring the T_c of 10 µl samples (N=24) of a filtered washing prepared from the soil (Costanzo et al., 2000).

Statistical inferences

Sample means were compared using one- or two-factor analysis of variance (ANOVA). Student–Newman–Keuls tests were used to make *post hoc* comparisons among the means. Significance of statistical analyses was accepted at $P \leqslant 0.05$. Mean values are reported ± S.E.M.

Results

Supercooling capacity and inoculation susceptibility

The supercooling capacity of *Chrysemys picta* varied markedly during the acclimation regimen ($F_{2,15}$=1013.5,

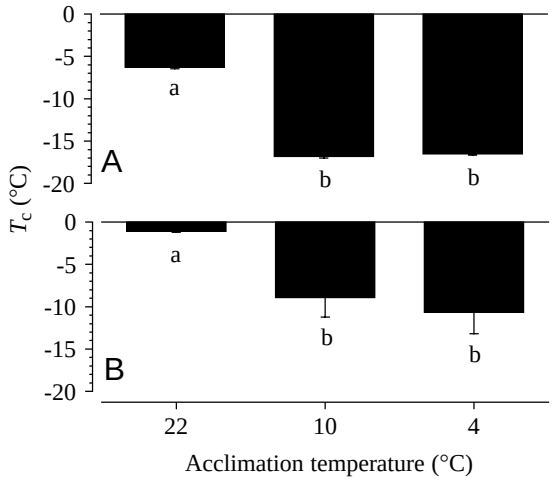

Fig. 2. Temperatures of crystallization (T_c) of hatchling *Chrysemys picta* when cooled (A) in an environment lacking ice nuclei and (B) in contact with a frozen substratum. These results indicate the intrinsic capacity for supercooling and the ability to resist inoculative freezing, respectively. Within each plot, mean values (+ S.E.M., N=6 replicates per group) identified by different letters were statistically distinguishable (P<0.05).

P<0.0001). Turtles acclimated to 22 °C and tested shortly after hatching supercooled little (T_c −6.3±0.2 °C), whereas turtles acclimated to 10 °C or 4 °C supercooled extensively (Fig. 2A). Turtles acclimated to either of the lower temperatures froze at approximately −17 °C, suggesting that maximal supercooling capacity was attained during acclimation to 10 °C.

We also found marked seasonal changes in the susceptibility of turtles to inoculative freezing ($F_{2,15}$=6.6, P=0.009). Turtles acclimated to 22 °C exhibited little resistance to inoculation, freezing at temperatures (T_c −1.1±0.1 °C) only slightly below the FP_{eq} of their body fluids. In contrast, turtles acclimated to 10 or 4 °C remained supercooled until they had reached –8.9±2.3 and −10.7±2.5 °C, respectively (Fig. 2B).

Changes in body composition and physiology

Changes in body mass and body composition during the acclimation regimen were compared between *Chrysemys picta* and *Chelydra serpentina* using the interaction term (species × sample time) of a two-factor ANOVA constructed for each response variable. Variation among the sample groups was then analyzed separately for each species using one-factor ANOVAs. *Chrysemys picta* and *Chelydra serpentina* showed similar changes in fresh body mass, dry body mass, body water content and hematocrit (Table 1). However, they differed with respect to changes in carcass organic content ($F_{2,30}$=5.2, P=0.011), carcass lipid concentration ($F_{2,30}$=8.2, P=0.001) and plasma osmolality ($F_{2,30}$=6.7, P=0.004).

Turtles acclimated to 10 or 4 °C generally weighed less than turtles acclimated to 22 °C (Table 1). Given that body water content increased in *Chrysemys picta* and tended to increase (albeit non-significantly) in *Chelydra serpentina*, the decrease in fresh body mass did not reflect desiccation of the turtles; rather, it probably reflected the 3–6 % loss of organic matter (Table 1). Both species lost approximately 50 % of their nonpolar lipids during acclimation to 10 °C. Subsequent acclimation to 4 °C caused no further loss of lipids in *Chelydra serpentina*; however, *Chrysemys picta* catabolized additional lipids and ultimately consumed two-thirds of the amount present after hatching (Table 1). Among recently hatched turtles, plasma osmolality was approximately 100 mosmol kg^{-1} higher in *Chelydra serpentina* than in *Chrysemys picta* (Table 1). However, during the acclimation regimen, plasma osmolality increased by 60–70 mosmol kg^{-1} in *Chrysemys picta*, but tended to decrease in *Chelydra serpentina*.

Table 1. *Somatic characteristics of hatchling painted turtles and snapping turtles acclimated to different temperatures*

	Acclimation temperature (°C)		
	22	10	4
Chrysemys picta			
Fresh body mass (g)	4.7±0.1^a	4.2±0.09^b	4.1±0.1^b
Dry body mass (g)	1.1±0.02^a	0.9±0.02^b	0.9±0.02^b
Body water content (g g^{-1} dry mass)	3.3±0.08^a	3.6±0.09^b	3.6±0.07^b
Organic content (mg g^{-1})	888±3^a	869±4^b	868±4^b
Nonpolar lipid content (mg g^{-1})	142±7^a	68±4^b	46±5^c
Hematocrit (%)	25±1^a	28±2^a	30±2^a
Plasma osmolality (mosmol kg^{-1})	328±10^a	399±5^b	388±10^b
Chelydra serpentina			
Fresh body mass (g)	7.8±0.2^a	7.8±0.3^a	7.3±0.3^a
Dry body mass (g)	1.8±0.1^a	1.6±0.1^a	1.6±0.03^a
Body water content (g g^{-1} dry mass)	3.2±0.1^a	3.5±0.1^a	3.4±0.1^a
Organic content (mg g^{-1})	865±4^a	815±4^b	825±9^b
Nonpolar lipid content (mg g^{-1})	91±8^a	47±5^b	43±4^b
Hematocrit (%)	25±1^a	28±2^a	30±2^a
Plasma osmolality (mosmol kg^{-1})	427±18^a	363±33^a	349±34^a

Values are means ± S.E.M.; N=6 turtles per group, or N=18 turtles per group for measurements of fresh body mass, dry body mass and body water content of *Chrysemys picta*.

Within a row, values indicated by different superscripts are statistically different (P<0.05; Student–Newman–Keuls test).

Changes in metabolite concentrations

Two-factor ANOVAs showed that changes in *Chrysemys picta* and *Chelydra serpentina* during the acclimation regimen differed markedly with respect to plasma concentrations of glucose ($F_{2,30}$=13.8, P<0.0001), lactate ($F_{2,30}$=13.8, P<0.0001) and urea ($F_{2,27}$=22.4, P<0.0001), but not of glycerol or proteins. Mean plasma glucose levels were 5–6 mmol l^{-1} in all sample groups except hatchling *Chrysemys picta* acclimated to 10 °C, which had a concentration of twice this value (Table 2). We found low concentrations of glycerol in the

Table 2. *Concentrations of key metabolites in plasma of hatchling painted turtles and snapping turtles acclimated to different temperatures*

	Acclimation temperature (°C)		
	22	10	4
Chrysemys picta			
Glucose	5.2±0.4[a]	11.2±0.9[b]	6.3±0.5[a]
Glycerol	0.2±0.03[a]	0.3±0.1[a]	0.4±0.1[a]
Lactate	4.1±0.6[a]	4.3±0.8[a]	5.3±0.7[a]
Protein	19.7±0.9[a]	21.3±1.8[a]	18.7±1.2[a]
Triglycerides	1.0±0.1[a]	0.6±0.1[b]	0.9±0.2[a,b]
Urea	34.3±4.9[a]	73.9±9.9[b]	77.6±14.7[b]
Chelydra serpentina			
Glucose	6.1±0.7[a]	5.2±0.3[a]	4.7±0.9[a]
Glycerol	0.1±0.03[a]	0.1±0.04[a]	0.3±0.04[b]
Lactate	4.0±0.7[a]	13.2±2.1[b]	3.9±0.8[a]
Protein	24.0±0.8[a]	18.9±1.1[a]	20.9±2.1[a]
Triglycerides	0.5±0.1[a]	0.7±0.2[a]	1.2±0.3[a]
Urea	84.2±14.9[a]	3.1±0.4[b,*]	2.9±0.2[b,**]

Values are means ± S.E.M.; N=6 turtles per group, except as noted: *N=5, **N=4.

Metabolite concentrations are in mmol l^{-1} (or mg ml^{-1} for protein only).

Within a row, values indicated by different superscripts are statistically different (P<0.05; Student–Newman–Keuls test).

plasma of *Chrysemys picta* and *Chelydra serpentina* from all sample groups, but the level tended to increase during the acclimation regimen. With few exceptions, plasma concentrations of lactate, proteins and triglycerides were largely unchanged (Table 2). Levels of lactate in *Chelydra serpentina* acclimated to 10 °C were three times higher than in conspecifics acclimated to 22 or 4 °C. We noted trends towards a decrease in plasma protein levels and an increase in plasma triglyceride levels in *Chelydra serpentina*, although these changes were not quite significant (P=0.08 and 0.12, respectively). In *Chrysemys picta*, plasma triglyceride concentrations were lowest in the 10 °C-acclimated turtles (Table 2).

Urea was an important organic osmolyte in recently hatched turtles (Table 2). In *Chrysemys picta*, urea levels increased 2.3-fold during the acclimation regimen, with concentrations in individual 4 °C-acclimated turtles ranging from 48 to 142 mmol l^{-1}. In these animals, urea made up approximately 20 % of the osmotically active solutes and contributed more to total osmotic pressure than all the other measured solutes combined. Conversely, plasma urea levels in *Chelydra serpentina* fell precipitously during the acclimation regimen, and concentrations in cold-acclimated turtles were below 4 mmol l^{-1}, with the exception of one individual in the 10 °C group (250 mmol l^{-1}) and two individuals (75 and 224 mmol l^{-1}) in the 4 °C group. These extraordinarily high values, which we presume were obtained from specimens that had not urinated, were eliminated from calculations of the group mean concentrations presented in Table 2. Excluding osmolality data from these same individuals would lower mean values to 330±5 mosmol kg^{-1} (N=5) for the 10 °C-acclimation group and to 304±8 mosmol kg^{-1} (N=4) for the 4 °C-acclimation group.

Changes in amino acid and carbohydrate levels in turtle blood

Interaction terms of two-factor ANOVAs identified differences between *Chrysemys picta* and *Chelydra serpentina* with respect to changes in plasma concentrations of ten amino acids: serine, glycine, histidine, threonine, tyrosine, valine, methionine, isoleucine, leucine and phenylalanine. Among recently hatched turtles, levels of these amino acids were generally higher in *Chelydra serpentina* than in *Chrysemys picta* (Table 3). During the acclimation regimen, the total amino acid pool decreased by approximately 70 % in *Chelydra serpentina*, but it was unchanged in *Chrysemys picta*.

With few exceptions, plasma concentrations of amino acids in *Chrysemys picta* did not vary seasonally (Table 3). Hydroxyproline level was reduced in these cold-acclimated turtles. Acclimation to 10 °C was accompanied by a 10-fold increase in methionine level, but levels of this compound decreased during subsequent exposure of the turtles to 4 °C (Table 3). In *Chelydra serpentina*, plasma levels of amino acids generally tended to decrease during the acclimation regimen. With the sole exception of arginine, concentrations of amino acids were nominally lower in 4 °C-acclimated turtles than in recently hatched turtles; in most cases, these differences were statistically significant (Table 3). Simple regression analysis showed that total amino acid concentration was positively correlated with plasma osmolality in *Chelydra serpentina* (r^2=0.30, $F_{1,17}$=6.9, P=0.018), but not in *Chrysemys picta* (r^2=0.04, $F_{1,17}$=0.7, P=0.428). Total amino acid concentration was also positively correlated with plasma protein concentration in *Chelydra serpentina* (r^2=0.44, $F_{1,17}$=12.4, P=0.003), but not in *Chrysemys picta* (r^2=0.03, $F_{1,17}$=0.4, P=0.524).

HPLC analysis of carbohydrates in *Chrysemys picta* plasma revealed the presence of at least 15 compounds, several of which occurred exclusively in warm- or cold-acclimated specimens. Because some of the peaks did not match the standards we selected, ion-exchange procedures, enzymatic digestion and other treatments were used to aid identification of some compounds. Nevertheless, some compounds could not be identified. Other than glycerol, none of the polyols commonly found in animal cryoprotectant systems, including mannitol, sorbitol, erythritol and threitol, was present in turtle plasma. HPLC analyses of glucose concentrations in *Chrysemys picta* gave results similar to those obtained with enzymatic assays, confirming that plasma levels in 10 °C-acclimated turtles were higher ($F_{2,6}$=10.4, P=0.01) than in turtles acclimated to either 22 or 4 °C.

Variation in fructose concentrations exhibited a similar pattern: turtles acclimated to 10 °C had significantly ($F_{2,6}$=7.0, P=0.027) higher concentrations (0.5±0.1 mmol l^{-1}) than turtles acclimated to either 22 °C (0.1±0.05 mmol l^{-1}) or 4 °C

(0.2±0.03 mmol l^{-1}). Concentrations of an NaOH-resistant (i.e. non-reducing) compound, tentatively identified as inositol, increased during the acclimation regimen: the levels in turtles acclimated to 10 °C (2.2±0.1 mmol l^{-1}) or 4 °C (2.0±0.2 mmol l^{-1}) were significantly ($F_{2,6}$=6.4, P=0.03) higher than those (1.3±0.1 mmol l^{-1}) in recently hatched turtles. Concentrations of maltose and maltotriose tended to increase during the acclimation regimen, although they were very low (<0.04 mmol l^{-1}) in all samples analyzed.

Ribose and two unidentified compounds were present in most warm-acclimated turtles but absent from turtles acclimated to 10° or 4 °C. Conversely, six late-eluting compounds that were found in low concentrations in plasma of turtles acclimated to 10 or 4 °C were not detected in turtles acclimated to 22 °C. These compounds were retained by Dowex and/or anion-exchange resin, suggesting that they might be phosphorylated sugars. However, their apparent insensitivity to phosphatase contradicted this supposition, and the identities of these compounds were not determined.

Table 3. *Concentrations of amino acids in plasma from painted turtles and snapping turtles acclimated to different temperatures*

	Acclimation temperature (°C)		
	22	10	4
Chrysemys picta			
Aspartate	35±11[a]	28±11[a]	26±5[a]
Glutamate	254±133[a]	253±104[a]	168±30[a]
4-Hydroxyproline	12±2[a]	2±1[b]	2±1[b,*]
Serine	13±5[a]	23±15[a,*]	78±61[a]
Glycine	32±8[a]	39±9[a]	38±10[a]
Histidine	81±16[a]	89±11[a]	70±19[a]
Arginine	90±14[a]	155±31[a]	163±35[a]
Threonine	30±8[a]	27±11[a,*]	30±10[a]
Alanine	64±23[a]	111±46[a]	56±13[a]
Proline	39±16[a]	27±18[a,*]	15±5[a]
Tyrosine	20±6[a]	53±18[a]	70±19[a]
Valine	34±10[a]	28±10[a]	41±12[a]
Methionine	25±9[a]	301±107[b]	73±29[a]
Cysteine	3±1[a]	6±3[a]	5±2[a]
Isoleucine	12±3[a]	9±3[a]	13±4[a]
Leucine	19±6[a]	22±8[a]	24±10[a]
Phenylalanine	6±3[a]	5±2[a]	2±1[a,*]
Lysine	24±7[a]	27±10[a]	14±4[a]
Tryptophan	12±3[a]	14±9[a]	10±6[a]
Total	804±197[a]	1312±218[a]	918±146[a]
Chelydra serpentina			
Aspartate	25±5[a]	24±5[a]	16±4[a,*]
Glutamate	271±94[a]	169±75[a]	88±24[a,*]
4-Hydroxyproline	11±3[a]	9±2[a]	4±3[a,*]
Serine	104±27[a]	7±2[b]	6±1[b,*]
Glycine	69±11[a]	39±8[b]	12±1[c,*]
Histidine	198±49[a]	85±24[b]	28±12[b,*]
Arginine	141±26[a]	140±45[a]	157±82[a]
Threonine	76±21[a]	12±4[b]	3±2[b,*]
Alanine	166±67[a]	48±25[a]	14±3[a,*]
Proline	34±13[a]	18±12[a]	6±2[a,*]
Tyrosine	30±11[a]	93±24[b]	19±12[a,*]
Valine	48±9[a]	10±1[b]	8±1[b,*]
Methionine	26±12[a]	15±3[a]	12±2[a,*]
Cysteine	5±2[a]	3±2[a]	0±0[a,*]
Isoleucine	23±4[a]	4±1[b]	3±0.2[b,*]
Leucine	41±8[a]	8±2[b]	5±1[b,*]
Phenylalanine	28±6[a]	7±2[b,*]	4±1[b,*]
Lysine	26±5[a]	11±2[b]	8±2[b,*]
Tryptophan	32±9[a]	7±2[b]	3±0.4[b]
Total	1354±277[a]	757±142[b]	396±82[b,*]

Values are means ± S.E.M.; N=6 turtles per group, except as noted: *N=5.

Concentrations are in nmol ml^{-1}.

Within a row, values indicated by different superscripts are statistically different (P<0.05; Student–Newman–Keuls test).

Ribose and two unidentified compounds were present in most warm-acclimated turtles but absent from turtles acclimated to 10° or 4 °C. Conversely, six late-eluting compounds that were found in low concentrations in plasma of turtles acclimated to 10 or 4 °C were not detected in turtles acclimated to 22 °C. These compounds were retained by Dowex and/or anion-exchange resin, suggesting that they might be phosphorylated sugars. However, their apparent insensitivity to phosphatase contradicted this supposition, and the identities of these compounds were not determined.

Thermal hysteresis activity in turtle blood

We examined the blood of *Chrysemys picta* and *Chelydra serpentina* for thermal hysteresis activity indicative of the presence of AFPs by comparing measured values of MP and FP for individual plasma samples. Because FP was only marginally lower (approximately 0.1 °C) than the MP in all samples (Table 4), we concluded that turtle blood lacked thermal hysteresis of biological relevance. Plasma MP tended to decrease during the acclimation regimen in *Chrysemys picta* and to increase in *Chelydra serpentina*, in accord with observed changes in plasma osmolality determined by vapor-pressure osmometry (Table 1).

Ice-nucleating activity in turtle blood

We examined the blood of *Chrysemys picta* and *Chelydra serpentina* reared in the absence of potent external ice nuclei (i.e. on a sterilized vermiculite substratum) and sampled after acclimation to 22, 10 or 4 °C for the presence of INPs. Ice-nucleating activity in turtle plasma or in freshly prepared NaCl

Table 4. *Melting point and freezing point of plasma from painted turtles and snapping turtles acclimated to different temperatures*

	Acclimation temperature (°C)		
	22	10	4
Chrysemys picta			
Melting point (°C)	–0.68±0.02[a]	–0.78±0.01[a]	–0.73±0.05[a]
Freezing point (°C)	–0.78±0.02[a]	–0.86±0.01[a]	–0.82±0.06[a]
Hysteresis	0.09±0.01[a]	0.07±0.01[a]	0.09±0.01[a]
Chelydra serpentina			
Melting point (°C)	–0.86±0.02[a]	–0.70±0.08[b]	–0.68±0.05[a,b]
Freezing point (°C)	–0.98±0.02[a]	–0.80±0.08[b]	–0.77±0.05[b]
Hysteresis	0.11±0.01[a]	0.09±0.01[a]	0.09±0.01[a]

Values are means ± S.E.M.; N=6 turtles per group.

Hysteresis is the difference between the freezing point and melting point.

Within a row, values indicated by different superscripts are statistically different (P<0.05; Student–Newman–Keuls test).

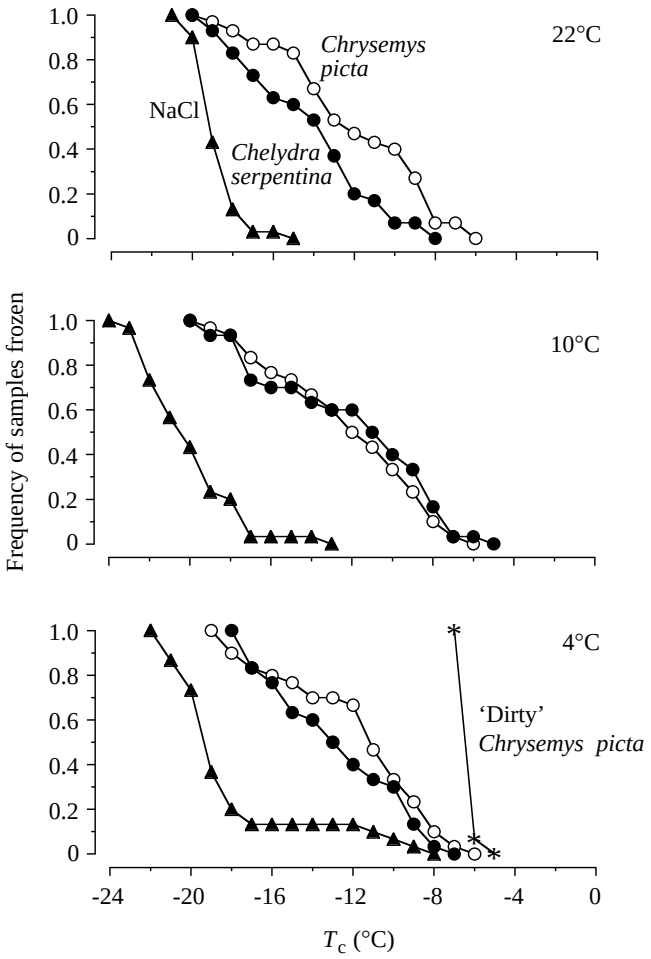

Fig. 3. Ice nucleation spectra for plasma from hatchling *Chrysemys picta* (open circles) and *Chelydra serpentina* (filled circles) hatched and reared on vermiculite and sampled after acclimation to 22, 10 or 4 °C. Spectra for approximately isotonic saline solutions (150 mmol l^{-1} NaCl; triangles) are given for comparison. Data for an additional group of 4 °C-acclimated *Chrysemys picta* (asterisks), exposed to nesting soil containing potent ice nuclei ('dirty'), are also shown. In each data set, points represent the cumulative frequency distribution of mean crystallization temperatures (T_c) for five portions of a plasma sample collected from each of six turtles.

solutions was not influenced by sample group (P=0.2). However, ice-nucleating activity was higher in plasma than in the saline solutions ($F_{2,45}$=82.6, P<0.0001; Fig. 3). Mean T_c values of the three preparations of saline solution were −20.0±0.2, −20.3±0.7 and −18.4±0.7 °C. Whereas some portions of the plasma samples also supercooled extensively (Fig. 3), others froze at temperatures as high as −5.4 °C (*Chrysemys picta*) or −6.3 °C (*Chelydra serpentina*). Overall, these results suggested that potent ice nuclei were lacking because mean T_c values for plasma of *Chrysemys picta* acclimated to 22, 10 and 4 °C were −14.5±0.7, −12.3±1.2 and −13.1±0.5 °C, respectively. Corresponding values for *Chelydra serpentina* were −12.1±0.5, −12.4±0.9 and −12.0±0.9 °C.

The mean T_c of plasma collected from *Chrysemys picta* exposed to nesting soil, −6.4±0.1 °C (N=3), was markedly higher ($F_{1,7}$=90.3, P<0.0001) than the value for 4 °C-acclimated turtles reared in vermiculite, −13.1±0.5 °C. All samples of plasma from 'dirty' turtles froze at temperatures of −6.8 °C or above (Fig. 3), indicating that potent ice nuclei were present in these samples. This result probably reflected contamination of the blood by residual soil nuclei present on the skin during the sampling procedure. A washing prepared from the nesting soil exhibited high nucleating activity (T_c=−5.3±0.03 °C) relative to that of a sample of sterilized, ultrapurified water (T_c=−18.9±0.3 °C), indicating that the soil did indeed contain potent ice nuclei.

Discussion

Our study documented seasonal changes in the physiology and in the development of cold hardiness in hatchling painted turtles during an acclimation regimen that mimicked the dynamic thermal conditions in natal nests. We cannot determine whether the responses of the turtles resulted from their exposure to progressively decreasing ambient temperature, to ontogenetic development or to the effects of aphagia. Our results demonstrate, nevertheless, that hatchlings undergo profound seasonal changes in physiology and cold hardiness and that such changes are necessary for these turtles to survive exposure to low, subzero temperatures during hibernation within the natal nest.

Seasonal development of supercooling capacity and inoculation resistance

Supercooling is an important survival strategy of many ectotherms that are seasonally or chronically exposed to subzero temperatures. The supercooling capacity of hatchling *Chrysemys picta*, which can remain unfrozen at a body temperature as low as −20 °C (Costanzo et al., 2000), is exceptional among vertebrates (Lee and Costanzo, 1998). Previous investigations of freeze avoidance in these turtles have used only cold-acclimated specimens. In the present study, the lower limit for supercooling decreased from −6 °C in turtles acclimated to 22 °C and tested shortly after hatching to −17 °C in turtles acclimated to 4 °C and tested in winter. Thus, cold acclimation may be required for the full development of supercooling capacity in these turtles. This finding is important because, with the exception of certain coldwater fishes (DeVries, 1982), there is little evidence that supercooling capacity varies seasonally among vertebrate ectotherms. Our results contrast with those of Churchill and Storey (1992), who found no difference in supercooling capacity of Canadian *Chrysemys picta* collected in autumn (T_c −3.7 °C) and spring (T_c −4.8 °C). However, the limited supercooling exhibited by hatchlings in both seasons suggests that their turtles, which were field-collected from their nests, were contaminated by environmental ice nuclei.

The innate capacity for supercooling may not be realized in animals that are susceptible to inoculative freezing. For

terrestrial hibernators, inoculative freezing is influenced by animal T_b, physiology, morphology and behavior and by environmental factors such as the texture, composition and water content of the substratum (Packard and Packard, 1997; Costanzo et al., 1998; Lee and Costanzo, 1998). The development of resistance to inoculative freezing usually occurs in autumn so that animals are protected against (lethal) freezing once cold weather arrives (Fields and McNeil, 1986; Gehrken, 1992; Rojas et al., 1992). Such was also the case with our *Chrysemys picta*. These turtles were highly susceptible to inoculative freezing after hatching, but developed resistance to inoculative freezing by the onset of winter because they remained supercooled in the presence of external ice nuclei to T_b values near −11 °C. This resistance was imperfect, however, because they did not supercool as extensively (i.e. to −16.5 °C; Fig. 2) as winter hatchlings tested in the absence of environmental ice nuclei. Nevertheless, winter conditioning appears to be crucial to the development of cold hardiness in this species. Further research is needed to determine whether such conditioning also improves freeze tolerance in *Chrysemys picta*, as is the case with many ectotherms (Storey and Storey, 1988).

Changes in body composition and physiology

A primary objective of this study was to investigate the physiological basis for cold hardiness in hatchling *Chrysemys picta*, a species that apparently relies on both supercooling and freeze-tolerance for winter survival. As an aid to identifying adaptive responses in *Chrysemys picta*, we also studied hatchling *Chelydra serpentina*, a largely sympatric species that commonly hibernates under water (Ultsch, 1989).

Little is known about the physiology of turtles during the time between hatching in late summer and emergence from the hibernaculum in spring (Ultsch, 1989). Turtles that remain on land during this period presumably have no access to food or free water, and they must depend upon stored nutrients derived from embryonic yolk. Cold acclimation in hatchling sliders (*Trachemys scripta*) is associated with a decrease in yolk mass and a net increase in dry body mass, suggesting that somatic growth may occur during winter (Filoramo and Janzen, 1999). In contrast, our *Chrysemys picta* (and *Chelydra serpentina*) lost dry body mass during the acclimation regimen, with 40–45 % of the associated decrease in organic matter being represented by nonpolar lipids. In hatchling *Chrysemys picta*, lipid may be important not only as an energy substrate but also as a source of metabolic water. Notably, body water content increased in *Chrysemys picta* during the acclimation regimen despite the fact that they did not eat or drink. The larger fat reserve in *Chrysemys picta*, compared with *Chelydra serpentina* (Table 1; Congdon et al., 1983), may be adaptive for this reason.

Plasma osmolality in our winter *Chrysemys picta* was similar to that reported for conspecifics from another Nebraska population (Packard and Packard, 1995), but slightly higher than values reported for Canadian *Chrysemys picta* (Storey et al., 1991). Our finding that plasma osmolality increased seasonally, largely due to the retention of urea, suggests that *Chrysemys picta* avoid urinating inside their natal nest, perhaps as a water conservation measure necessitated by chronic aphagia and exposure to low environmental water potential. The mechanism triggering urea accumulation in *Chrysemys picta*, although clearly not dehydration, is unknown. Water conservation may be relatively unimportant in *Chelydra serpentina*, which apparently urinated during the acclimation regimen, because these hatchlings usually hibernate under water (Ultsch, 1989; Costanzo et al., 1995).

Does urea retention promote cold hardiness in hatchling *Chrysemys picta*? By depressing the FP_{eq} of the body fluids, increased concentrations of urea or other solutes may indirectly promote supercooling and enhance resistance to inoculative freezing. Urea accumulates in the tissues of some fishes during cold acclimation, apparently for this purpose (Gordon et al., 1962; Raymond, 1994), but otherwise this compound is an unlikely component of cryoprotectant systems, perhaps because of its tendency to destabilize proteins (Somero and Yancey, 1997). Some animals sequester methylamines (e.g. trimethylamine oxide, glycerophosphoryl choline and betaine) or ‘counteracting solutes’, which ameliorate the perturbing effects of urea (Somero and Yancey, 1997); others do not (Grundy and Storey, 1994). Among the latter, the reversibly inhibiting effect of urea on enzyme function may play an adaptive role in depressing metabolism during dormancy (Yancey et al., 1982). Whether this is the case in *Chrysemys picta* remains to be determined.

We tracked changes in concentrations of amino acids in our turtles because certain of these compounds are known to function as cryoprotectants (Storey and Storey, 1988) and/or compatible osmolytes (Somero and Yancey, 1997). In *Chrysemys picta*, the amino acid pool showed little seasonal change. In contrast, amino acid concentrations generally decreased in *Chelydra serpentina*, suggesting that protein metabolism was inhibited. Maintenance of the amino acid pool in *Chrysemys picta* may help curb cellular dehydration during episodes of somatic freezing. Furthermore, given that the amino acid pool, but not necessarily the glycogen reserve, diminishes during freezing episodes (Churchill and Storey, 1992), these compounds may constitute an important source of energy in frozen, anoxic turtles (Van Waarde, 1988).

Levels of plasma glucose, glycerol and lactate in our winter *Chrysemys picta* were generally similar to those reported previously (Storey et al., 1988; Churchill and Storey, 1992). We found no evidence that *Chrysemys picta* (or *Chelydra serpentina*) accumulated large quantities of these compounds during the acclimation regimen. Furthermore, our HPLC analyses confirm earlier findings that cryoprotectants such as glycerol, mannose, sorbitol and fructose were absent (Storey et al., 1988), or present only at low concentrations (Churchill and Storey, 1992), in winter turtles. Cold acclimation of our *Chrysemys picta* was associated with a 50–70 % increase in inositol concentration and the appearance of six presumably carbohydrate compounds, although only low levels were present. Some cryoprotectants are effective at low

concentrations (Storey and Storey, 1988), but whether these compounds are involved in the cold hardening of *Chrysemys picta* is unknown.

Seasonal development of cold hardiness: possible mechanisms

Cold hardening commonly involves the elimination or deactivation of endogenous ice nuclei that would otherwise diminish supercooling capacity (Duman et al., 1991; Lee, 1991). This was apparently the case with our *Chrysemys picta*, which harbored potent ice-nucleating activity shortly after hatching, but not after acclimation to 10 or 4 °C. Our finding that the blood plasma of recently hatched turtles supercooled extensively indicates that the endogenous ice nuclei were restricted to intracellular and/or interstitial compartments. These agents may have been associated with the yolk or extraembryonic membranes because their disappearance coincided with developmental maturation of the turtles (see Bale et al., 1989).

In some animals, the seasonal development of supercooling capacity and resistance to inoculative freezing are promoted by changes in hydro-osmotic balance resulting from the accumulation of osmolytes and/or dehydration of the body (Zachariasen and Hammel, 1976; Storey and Storey, 1988; Lee, 1991). The plasma osmolality of our *Chrysemys picta* increased by approximately $60\,\text{mosmol}\,\text{kg}^{-1}$ during the acclimation regimen; however, the effect of the increased osmotic pressure on supercooling capacity, if considered on a colligative basis, would be modest (Zachariasen and Hammel, 1976; Lee et al., 1981). Given that experimental dehydration of hatchling *Chrysemys picta* enhances their supercooling capacity (Costanzo et al., 1995), we were surprised to find that the body water content of our turtles increased, rather than decreased, during the acclimation regimen. Perhaps these turtles also underwent a seasonal redistribution of body water, such as an increase in the proportion of osmotically inactive or 'bound' water that accounts for their enhanced capacity for supercooling. Further study is needed to determine whether such changes, together with the elimination of potent endogenous ice nuclei and a seasonal increase in the osmotic pressure of body fluids, can explain the exceptional supercooling capacity of winter *Chrysemys picta*.

The seasonal development of inoculation resistance in *Chrysemys picta* probably has a physiological basis. The concomitant increase in plasma osmolality may improve resistance to inoculative freezing inasmuch as the activity of ice nuclei is sensitive to the osmotic pressure in the nucleating environment (Zachariasen and Hammel, 1976; Lee et al., 1981). However, it is doubtful that this change alone can account for the marked increase in inoculation resistance. We are currently testing the possibility that cold acclimation influences the permeability of the skin, the principal barrier between the internal and external environments, by influencing the quantity and/or quality of waxes or lipids in the integument (e.g. Olsen et al., 1998).

Some invertebrates and fishes use AFPs to stabilize the supercooled state and to prevent inoculative freezing (DeVries, 1982; Duman et al., 1991). AFPs are reportedly absent from the blood of wood frogs *Rana sylvatica* (Wolanczyk et al., 1990) and of hatchling *Chrysemys picta* (Storey et al., 1991). Although our results also indicate that the blood plasma of *Chrysemys picta* (and of *Chelydra serpentina*) lacks hysteresis activity, we cannot rule out the possibility that AFPs exist solely in the extravascular compartments or intracellular spaces of these turtles (e.g. Olsen et al., 1998). Furthermore, it is possible that AFPs are present at concentrations too low to induce hysteresis activity. At low concentrations, AFPs serve to protect frozen animals against recrystallization injury (Knight and Duman, 1986; Tursman et al., 1994; Ramlov et al., 1996).

We examined turtles for the presence of INPs, which are used by some freeze-tolerant animals to initiate freezing and thereby to guard against damage resulting from spontaneous nucleation of deeply supercooled tissues (Storey and Storey, 1988; Duman et al., 1991). INPs reportedly occur in the blood of freeze-tolerant vertebrates, including *R. sylvatica* (Wolanczyk et al., 1990) and hatchling *Chrysemys picta* (Storey et al., 1991), although their role in the cold hardiness of these species is equivocal (Costanzo and Lee, 1996; Lee and Costanzo, 1998).

Our results showed not only that *Chrysemys picta* hatched and reared in a 'clean' environment (sterilized vermiculite) lacked potent blood INPs but also suggested that blood is readily contaminated with environmental ice nuclei during the sampling procedure (see also Bale et al., 1989). The nucleating activity discovered by Storey et al. (1991) in the blood of *Chrysemys picta* collected from natural nests, which commonly harbor potent ice nuclei (Costanzo et al., 2000), was remarkably similar to that of the blood of our 'dirty' turtles. Thus, the commonly held idea that hatchling *Chrysemys picta* synthesize INPs may be based on artifact.

The absence of INPs (and other endogenous ice nuclei) in *Chrysemys picta*, as found in the present study, must be reconciled with the fact that freezing survival in this species, and in other freeze-tolerant animals, is promoted by mechanisms that reliably initiate nucleation at a relatively high T_b. In freeze-tolerant vertebrates, including hatchling *Chrysemys picta*, inoculative freezing serves this function (Lee and Costanzo, 1998). An important benefit of using inoculative freezing, rather than INPs, is that turtles can also employ supercooling as a winter survival strategy. Whether freeze-tolerance or supercooling is invoked during a given chilling episode depends on prevailing physiological and environmental conditions (Costanzo et al., 1995, 1998, 2000); however, supercooling permits survival over a much broader range of T_b values.

In summary, we found that hatchling *Chrysemys picta* exhibit a seasonal development of cold hardiness exemplified by increases in supercooling capacity and resistance to inoculative freezing. These changes coincided with an increase in plasma osmolality due primarily to urea retention, but were

not associated with an accumulation of polyols, sugars or amino acids. The development of cold hardiness derived, in part, from the elimination or deactivation of potent endogenous ice nuclei present in the tissues, albeit not blood plasma, of recently hatched turtles, and the increase in osmotic pressure in winter turtles. We found no evidence that the cold hardiness strategy of *Chrysemys picta* involves AFPs. INPs were also absent, although their role in these turtles is played by environmental ice nuclei.

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