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Endogenous and exogenous ice-nucleating agents constrain supercooling in the hatchling painted turtle

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Summary

Hatchlings of the painted turtle (*Chrysemys picta*) commonly hibernate in their shallow, natal nests. Survival at temperatures below the limit of freeze tolerance (approximately –4°C) apparently depends on their ability to remain supercooled, and, whereas previous studies have reported that supercooling capacity improves markedly with cold acclimation, the mechanistic basis for this change is incompletely understood. We report that the crystallization temperature (T_c) of recently hatched (summer) turtles acclimated to 22°C and reared on a substratum of vermiculite or nesting soil was approximately 5°C higher than the T_c determined for turtles acclimated to 4°C and tested in winter. This increase in supercooling capacity coincided with elimination of substratum (and, in fewer cases, eggshell) that the hatchlings had ingested; however, this association was not necessarily causal because turtles reared on a paper-covered substratum did not ingest exogenous matter but nevertheless showed a similar increase in supercooling capacity. Our results for turtles reared on paper revealed that seasonal development of supercooling capacity fundamentally requires elimination of ice-nucleating agents (INA) of endogenous origin: summer turtles, but not winter turtles, produced feces (perhaps derived from residual yolk) that expressed ice-nucleating activity. Ingestion of vermiculite or eggshell, which had modest ice-nucleating activity, had no effect on the T_c, whereas ingestion of nesting soil, which contained two classes of potent INA, markedly reduced the supercooling capacity of summer turtles. This effect persisted long after the turtles had purged their guts of soil particles, because the T_c of winter turtles reared on nesting soil (mean ± S.E.M.=–11.6±1.4°C) was approximately 6°C higher than the T_c of winter turtles reared on vermiculite or paper. Experiments in which winter turtles were fed INA commonly found in nesting soil showed that water-soluble, organic agents can remain fully active for at least one month. Such INA may account for the limited supercooling capacity (T_c≈–7.5°C) we found in turtles overwintering in natural nests and may therefore pose a formidable challenge to the winter survival of hatchling *C. picta*.

Key words: painted turtle, *Chrysemys picta*, cold hardiness, hibernation, acclimation, supercooling, ice nucleation, gut, yolk.

Introduction

The hatchling painted turtle (*Chrysemys picta*) is a favorite study organism among investigators interested in the cold-hardiness adaptations of northern reptiles (for reviews, see Lee and Costanzo, 1998; Packard and Packard, 2001). Because these turtles commonly remain within the natal nest throughout their first winter of life, they may encounter conditions that cause them to freeze. These animals are freeze tolerant (to approximately –4°C; Storey et al., 1988; Churchill and Storey, 1992; Attaway et al., 1998); however, survival at very low nest temperatures (e.g. –12°C) is possible only if they remain supercooled (Packard et al., 1997).

Under idealized laboratory conditions, fully cold-hardened hatchlings can supercool to remarkably low temperatures (e.g. –20°C), and, in fact, their supercooling capacity is on par with that of a droplet of water (Lee and Costanzo, 1998). However, the hypothesis that these hatchlings survive every chilling episode by virtue of their innate supercooling capacity (e.g. Packard et al., 1997; Packard and Packard, 2001) is at odds with recent findings (Costanzo et al., 1998, 2000a, 2001) that the turtles are susceptible to inoculation by ice or ice-nucleating agents (INA) in the winter microenvironment. Environmental INA include particulates, such as sand grains, dust and other motes, and various organic entities, including certain microorganisms and amino acids. Even brief contact with nesting soil harboring such agents can markedly constrain the supercooling capacity of hatchling *C. picta* (Costanzo et al., 2000a).

A recent study of the seasonal development of cold hardiness in *C. picta* showed that, whereas fully cold-hardened turtles supercool extensively, recently hatched turtles spontaneously freeze at relatively high temperatures (Costanzo et al., 2000b). Packard and colleagues (2001) confirmed this

result and also reported that the guts of recently hatched turtles contained bits of eggshell and soil ingested during pipping and hatching. Finding that winter turtles had empty guts, these authors surmised that seasonal development of supercooling capacity requires elimination of ingested matter from the gastrointestinal tract. However, they did not determine whether the substrata and eggshell ingested by hatchlings actually expressed ice-nucleating activity, so this association remains conjectural. In addition, the possibility that hatchlings harbor INA of endogenous origin, as was earlier suggested by Costanzo et al. (2000b), remains untested.

Our goal was to rigorously test the hypothesis that seasonal development of supercooling capacity in hatchling *C. picta* is the result of attenuation or elimination of INA, of either endogenous or exogenous origin, that is present shortly after hatching. We also characterized the ice-nucleating activity of experimental substrata and eggshell in order to elucidate their influence on the supercooling capacity of hatchlings. Finally, by feeding INA commonly found in nesting soil to winter turtles, we directly evaluated the effect of these agents on hatchling cold hardiness.

Materials and methods

Experimental animals and acclimation regimen

Eggs of *Chrysemys picta bellii* (Gray) were collected in summer 2001 near Gimlet Lake in Crescent Lake NWR, Garden County, west-central Nebraska, USA (41°N, 102°W). They were obtained from females administered oxytocin (Etchberger et al., 1992), transported to our laboratory and incubated at 29°C in moist vermiculite (1.0 g water g vermiculite^{-1}; water potential, approximately –150 kPa) until near hatching, in early August. At this time, eggs were assigned to one of three groups, such that each group contained only one egg from any given clutch. Eggs in one group were transferred to plastic boxes containing moist nesting soil, collected from the study site in summer 2001; eggs in another group were transferred to boxes containing moist vermiculite, the substratum most commonly used in studies of hatchling cold hardiness (e.g. Costanzo et al., 1998; Packard et al., 2001). These eggs were permitted to hatch on their respective substrates. By contrast, we manually extricated turtles from eggs in the third group in order to prevent them from ingesting exogenous matter. This procedure was performed on unpipped eggs and was timed to coincide with the hatching of other eggs in the same clutch. Turtles in this group were transferred to boxes lined with a sheet of moist paper towel, and the shells from their eggs were reserved for later analysis. One of these hatchlings died, but all others appeared to be healthy and resembled turtles that hatched naturally.

Hatchlings were kept in darkness, denied food and free water, and progressively acclimated to temperatures corresponding to those occurring in *C. picta* nests during late summer, autumn and winter (see Costanzo et al., 1995). Upon hatching (or manual extrication from the shell), in mid-August, they were exposed to 22°C, but on 1 October they were placed in an environmental chamber that was set initially at 15°C and then changed to 10°C on 1 November. Turtles were exposed to 4°C on 1 December and were thereafter held at this temperature. We remoistened substrata as necessary to prevent turtles from desiccating.

We also examined *C. picta* that had hatched and overwintered inside natural nests located near Gimlet Lake. Nests were found by observing the nesting forays of females and were protected from predators by installing a piece of hardware cloth over each one. We excavated these nests on 6 April 2002, at which time 4–5 hatchlings per nest were briefly rinsed with water, blotted dry with paper towel, placed in plastic bags and shipped under refrigeration to Miami University, where they were kept chilled (4°C) for 1–2 days before being used in supercooling trials. Upon completion of the trials, we dissected the turtles and isolated the gut, which was later examined for the presence of endogenous and exogenous matter (see below). Temperatures experienced by these turtles during winter were recorded by miniature data loggers (Onset Computer, Tidbit; Pocasset, MA, USA) placed in the soil column adjacent to the nest cavity.

Supercooling capacity of summer and winter turtles

The primary purpose of this experiment was to elucidate the association between the anticipated increase in supercooling capacity with cold acclimation and elimination of ingested matter in hatchling *C. picta*. Our experimental approach was to determine the supercooling capacity of hatchlings reared on paper, vermiculite or nesting soil, both shortly after hatching (summer) and after cold acclimation (winter). Trials with summer turtles were conducted in late August, approximately two weeks after turtles had emerged from their eggs and were transferred to 22°C; trials with winter turtles were conducted in late January, after hatchlings had been exposed to 4°C for eight weeks.

Following Costanzo et al. (1998), we determined supercooling capacity by progressively cooling hatchlings until they spontaneously froze. Turtles were prepared for testing by gently brushing away any adherent vermiculite or soil and then holding them in a sheltered box for 24 h. This procedure, performed in darkness at the prevailing acclimation temperature, permitted evaporation of any surface moisture that otherwise might freeze and inoculate the tissues. Turtles were placed separately in 50 ml plastic tubes and covered with a piece of plastic foam, which insulated the hatchling and anchored a 30-gauge thermocouple (copper/constantan) in position, with the sensing junction nearly touching the carapace. The tubes were suspended in a refrigerated ethanol bath (Neslab, model RTE 140; Portsmouth, NH, USA) programed to cool turtles from the acclimation temperature to –0.4°C, at which temperature they were held for 1 h before being further cooled (3°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 data logger (Omega, model RD3752; Stamford, CT, USA). We then

determined the temperature of crystallization (T_c) from the recording and took this value to represent the supercooling limit.

We investigated somatic compartmentalization of INA by measuring the T_c of the isolated gut, internalized yolk sac (which, in summer turtles, contained residual yolk and hereafter is termed 'yolk') and carcass. Turtles used in the supercooling trials described above were removed from the bath upon appearance of the exotherm, thawed briefly on ice, euthanized by decapitation and dissected under virtually aseptic conditions. The gastrointestinal tract, from esophagus to rectum, and the yolk sac were removed and placed in tared, 0.5 ml microcentrifuge tubes, which were then weighed to the nearest 0.01 g. Samples were coated with mineral oil (in order to prevent them from dehydrating) and instrumented with a thermocouple, whose tip was placed next to, but not touching, the tissue. We chilled the tubes in a refrigerated bath, measuring the T_c of the samples as we did for intact turtles. The guts were reserved for examination of their contents (see below).

An important assumption inherent in our experimental design was that ice-nucleating activity of turtle tissues was unaltered by the nucleation event and brief freezing associated with the initial supercooling trial. Reasoning that an increase in ice-nucleating activity in any compartment necessarily diminishes the supercooling capacity of the entire turtle, we validated our assumption by comparing the T_c values determined for turtles (hatched and reared on vermiculite) subjected to two successive supercooling trials. After the first trial, conducted as described above, turtles were euthanized and left intact (rather than being dissected), coated with oil and used in a second supercooling trial. Because the mean difference between the pair of T_c values did not differ from zero (paired *t*-test: t=0.977, d.f.=4, P=0.384, N=5), we concluded that brief freezing, in the context of our experimental protocol, did not alter ice-nucleating activity of the tissues.

Identification of gut contents

Guts isolated from lab-reared and field-collected turtles were placed on translucent dissecting trays and examined with a dissecting scope (Olympus America, SZH10 Research Stereo; Melville, NY, USA**)**. Viewing the gut under low magnification, we mapped areas of concentration of matter on an enlarged diagram of the digestive tract. Having been familiarized with the magnified image of reference samples of nesting soil, vermiculite and eggshell, we then characterized any matter as being of exogenous (substratum and/or eggshell) or endogenous origin.

Ice-nucleating activity in experimental substrata, eggshell and feces

We established indices of ice-nucleating activity associated with materials that may occur in turtle gastrointestinal tracts. Samples of vermiculite and nesting soil were collected, in August, from the boxes housing the hatchlings and kept refrigerated until analyzed. Eggshells of turtles reared on paper were stored at –20°C for this purpose. Eggshells were thawed, rinsed with water, dried at 65°C and pulverized with a glass rod before use in the assay.

We measured the ice-nucleating activity expressed in bulk samples of these materials, and also in washings prepared from them, because water-soluble INA may be retained in the body even after solids have been eliminated. In addition, we characterized the INA as to whether the agents were derived from organic matter. Interpretation of the assay results was predicated on the facts that small volumes of pure (i.e. INA-free) water supercool extensively and that organic INA are deactivated by high heat and pressure (Vali, 1995). Water used in the assays was obtained from a reverse-osmosis ultrapurification system (0.2 μm filter, Dayton Water Systems; Dayton, OH, USA) and sterilized in an autoclave. To guard against contaminating samples with ambient INA, all vessels and utensils used in the assays were thoroughly cleaned and autoclaved before use. Potency of constituent INA was gauged relative to the T_c of samples of water.

Following Costanzo et al. (1998), we measured ice-nucleating activity expressed in natural (non-autoclaved) and autoclaved samples of vermiculite, nesting soil and eggshell. A 100 mm^3 sample of air-dried material was placed in a 0.5 ml polypropylene microcentrifuge tube to which we added 12.5 μl of water. The contents were mixed by vortexing and then consolidated by gentle centrifugation (180 ***g***, 3 min). We taped the sensing junction of a 36-gauge copper/constantan thermocouple to the exterior of each tube, which was then inserted into a dry, 20 ml test tube. Samples (N=5 replicates) of each material were chilled to 0°C in a refrigerated ethanol bath and then further cooled (1.5°C min^{-1}) until the water within them froze. The T_c of each sample was read from the output of a data logger to which the thermocouples were connected.

We also measured ice-nucleating activity expressed in washings prepared from natural and autoclaved samples of vermiculite, nesting soil and eggshell (N=5 replicates of each material). Washings were prepared by adding water to a 100 mm^3 sample of each material, vortexing the mixture for 60 s and isolating the particulates by centrifugation (2000 ***g***, 5 min). To avoid excessive dilution of INA, samples were washed with the smallest volume of water, which, beyond that absorbed by the sample, yielded the minimum volume of washing needed for assay (150–300 μl). The supernatant was expressed through a 5 μm disk filter to remove any fine particulates, and a 10 μl aliquot of the filtrate was drawn into the center of a 20 μl glass microcapillary tube, such that the fluid column was bounded by equal volumes of air. The ends of the tube were sealed with clay and the sensing junction of a 36-gauge copper/constantan thermocouple was taped to its center. Five tubes prepared from each washing were chilled, as described above, until each exhibited a freezing exotherm. We took the average of the five T_c values to represent each sample.

During their first month of life, hatchlings reared on paper produced feces that we weighed, placed individually in a microcentrifuge tube and stored at –20°C for subsequent

analysis of ice-nucleating activity. No droppings were found after 10 September. Feces may have been produced by turtles reared on vermiculite or soil, but we could not confirm their presence. Ice-nucleating activity associated with the feces was determined as described for other materials.

Experimental ingestion of INA

We inoculated the guts of live hatchlings with two distinct types of INA commonly found in nesting soil in order to compare their effects on the supercooling capacity of winter turtles. Turtles were administered autoclaved nesting soil, which contained soil particles but no organic INA, or a soil washing, which contained organic INA but no soil particles. Control turtles ingested only the water vehicle, or were sham treated (i.e. they ingested nothing). We conducted the experiments in mid-winter, using cold-acclimated turtles reared on vermiculite; hatchlings cultured under these conditions have an extensive capacity for supercooling (Costanzo et al., 2000b; Packard et al., 2001).

We prepared for the experiment by cleaning and air drying the turtles (as described above). We filled a 25 µl syringe (Hamilton; Reno, NV, USA) with water obtained from a reverse-osmosis ultrapurification system and sterilized in an autoclave, expelling 15 µl of this volume into a length of polyethylene tubing (PE50) attached to the hub. An aliquot of soil slurry (1.0 g autoclaved nesting soil mixed with 300 µl water), soil washing (prepared by vortexing 1.0 g nonautoclaved soil with 500 µl water, centrifuging the mixture and passing the supernatant through a 0.5 µm filter) or water was then drawn into the tube. Using broad forceps, we withdrew the head from the shell, stabilizing the extended neck by placing the thumb and forefinger behind the skull. The blade of a second pair of forceps was inserted between the maxilla and mandible, and, by applying gentle downward pressure, the mandible yielded to expose the buccal cavity. We inserted the tubing and dispensed 10 µl of the prescribed material (air, in the case of sham-treated animals) into the pharynx. Turtles were returned to their cages and used in supercooling trials, in general the following day. However, one group was tested for supercooling capacity 33 days after turtles were fed soil washing. We observed no ill effects of forced ingestion on any of the turtles used in this experiment.

Statistical inferences

Sample means were compared using one- or two-factor analysis of variance (ANOVA) followed by appropriate multiple comparisons tests. Significance of statistical analyses was accepted at $P \leq 0.05$. Mean values are reported ± S.E.M.

Results

Supercooling capacity of summer and winter turtles

Supercooling capacity was strongly influenced by the substratum on which turtles were reared ($F_{2,54}$=37.51, P<0.0001) and by the season of testing ($F_{1,54}$=72.53, P<0.0001). In all treatment groups, ice nucleation occurred at higher temperatures in the summer turtles than in their winter counterparts (Fig. 1). For turtles reared on vermiculite or paper, the increase in supercooling capacity coincident with cold acclimation was in the order of 5°C. For turtles reared on nesting soil, the magnitude of the increase was similar (factor interaction: $F_{2,54}$=0.01, P=0.992); however, supercooling capacity in these turtles was comparatively limited, both shortly after hatching and after cold acclimation (Fig. 1).

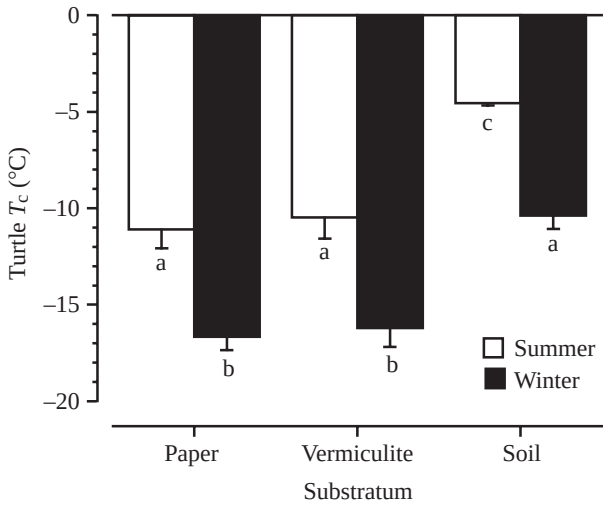

Fig. 1. Temperatures of crystallization (T_c) of *Chrysemys picta* reared on different experimental substrata and tested shortly after hatching (summer) or after acclimation to 4°C (winter). Mean values (± S.E.M., N=10 replicates per group) identified by different letters are statistically different (ANOVA/Student–Newman–Keuls multiple comparisons test; P<0.05).

Experiments in which we measured the T_c of the isolated yolk and gut, and the carcass were intended to elucidate compartmentalization of INA within the live turtle. However, because the ice-nucleating activity expressed in the carcass and gut was generally higher than that found in the intact turtle (Table 1), these samples must have acquired INA that were not present in life. This result is not readily explained, but perhaps the novel activity is a consequence of tissue damage or contamination with ambient INA sustained during the dissection (e.g. Baust and Zachariassen, 1983). Furthermore, we cannot exclude the possibility that samples of yolk also expressed unnaturally high levels of ice-nucleating activity. These cautions aside, the experimental results yielded some useful information. For instance, the greater supercooling capacity in winter turtles, as compared with summer turtles, was invariably reflected in the results for yolk, gut and carcass (Table 1). In addition, because the T_c values determined for yolk corresponded with those determined for live turtles, ice-nucleating activity associated with this compartment may effectively set the limit of supercooling. However, ice-nucleating activity associated with the gut and carcass more closely matched the T_c of the summer turtles reared on nesting soil (Table 1). These qualitative assessments were corroborated by the results of multiple regression analyses

Table 1. *Temperatures of crystallization (T_c) of isolated yolk, gut and carcass of* Chrysemys picta *reared on different experimental substrata and tested shortly after hatching (summer) or after acclimation to 4°C (winter)*

	Temperature of crystallization (°C)					
	Paper		Vermiculite		Soil	
	Summer	Winter	Summer	Winter	Summer	Winter
Yolk	–9.7±1.0	–16.2±1.1	–11.8±1.3	–17.6±1.1	–6.3±0.3*	–11.6±1.4
Gut	–7.7±0.3*	–11.8±1.3*	–7.9±0.4	–11.7±1.3*	–4.2±0.1	–8.4±0.6
Carcass	–6.5±0.1*	–7.6±0.3*	–6.3±0.2*	–7.6±0.2*	–4.7±0.1	–5.5±0.2*
Live turtle	–11.1±1.0	–16.7±0.7	–10.5±1.1	–16.2±1.0	–4.6±0.1	–10.4±0.7

Mean values (± S.E.M., N=10 replicates per group) identified by an asterisk differed significantly (ANOVA/Dunnett multiple comparisons test; $P<0.05$) from the mean for live turtles.

testing the effects of three independent variables (yolk T_c, gut T_c and carcass T_c) on the T_c of live turtles. This model was significant for turtles reared on paper ($F_{3,14}$=5.37, P=0.011, r^2=0.535), vermiculite ($F_{3,16}$=3.59, P=0.037, r^2=0.402) and nesting soil ($F_{3,16}$=19.68, $P<0.0001$, r^2=0.787). Yolk T_c was the best predictor of live turtle T_c for hatchlings reared on paper or vermiculite; however, gut T_c was the most important factor for turtles reared on nesting soil.

Morphometric variables

Because turtles assigned to the summer and winter groups did not differ in carapace length ($F_{1,54}$=0.43, P=0.52) or plastron length ($F_{1,54}$=0.08, P=0.78), any difference in response variables can be ascribed to a particular experimental treatment, rather than the influence of body size (Table 2). Comparing values for summer turtles and winter turtles indicated that body mass decreased ($F_{1,54}$=26.74, $P<0.0001$) by 15–24% during cold acclimation, and that this change was, in part, due to yolk consumption ($F_{1,54}$=47.23, $P<0.0001$) and a decrease in the mass of the gastrointestinal tract ($F_{1,54}$=32.06, $P<0.0001$). The latter, which chiefly reflected elimination of ingested matter, was significant only for the turtles reared on vermiculite or nesting soil (Table 2).

Identification of substances in turtle guts

Examination of the dissected guts, isolated from the specimens used in the supercooling trials, showed that the summer turtles reared on vermiculite or nesting soil had ingested large amounts of substrata (Table 3). Approximately 50% of them also had ingested eggshell fragments. Hatchlings purged their guts during cold acclimation, as neither substratum nor eggshell was found in the guts of winter turtles.

Except for one summer turtle, which had ingested a few paper fibers, the guts of turtles reared on paper were devoid of exogenous matter (Table 3). The intestines of eight of the 10 hatchlings in this group contained a small quantity of a yellowish-green, amorphous substance that may have been derived from yolk. We found this material, in trace amounts, in only three of the 10 hatchlings examined in winter.

Ice-nucleating activity of experimental substrates, eggshell and turtle feces

Bulk samples of nesting soil catalyzed the freezing of water at –5°C, indicating that they contained potent INA (Table 4). By contrast, samples of ultrapurified water supercooled to approximately –18°C. Ice-nucleating activity was also expressed in vermiculite and eggshell, but the activity temperature was lower than that determined for soil. Autoclaving, which destroys organic INA, reduced the activity in soil (t=3.08, P=0.015, N=10) but not in vermiculite (t=1.15, P=0.28, N=10) or eggshell (t=0.46, P=0.66, N=10). Washings prepared from nesting soil contained potent INA, whereas washings of vermiculite or eggshell supercooled extensively and therefore lacked INA (Table 4). Autoclaving the nesting

Table 2. *Morphometric measurements of* Chrysemys picta *reared on different experimental substrata and tested shortly after hatching (summer) or after acclimation to 4°C (winter)*

	Paper		Vermiculite		Soil	
	Summer	Winter	Summer	Winter	Summer	Winter
Carapace length (mm)	21.9±0.6	23.4±0.4	24.5±0.3	23.9±0.5	24.7±0.3	23.5±0.4
Plastron length (mm)	21.7±0.5	22.7±0.4	23.1±0.3	23.2±0.6	24.2±0.4	23.4±0.4
Body mass (g)	3.3±0.2	2.8±0.2	4.2±0.2	3.2±0.2*	4.7±0.2	3.6±0.2*
Yolk mass (mg)	177.0±39.0	43.0±7.8*	117.0±20.0	28.0±5.7*	150.0±26.6	15.0±3.1*
Gut mass (mg)	77.0±6.0	73.0±5.2	123.0±6.2	83.0±6.3*	163.0±14.1	99.0±5.0*

Values are means ± S.E.M.; N=10 replicates per group.

Asterisk denotes that the value for the winter group differed significantly from the corresponding value for the summer group within the same treatment. Statistical significance is taken at the level of $P<0.05$.

Table 3. *Incidence of ingested substratum or eggshell, determined by microscopic examination of isolated gastrointestinal tracts, in* Chrysemys picta *reared on different experimental substrata and tested shortly after hatching (summer) or after acclimation to 4°C (winter)*

Rearing substratum	Summer (*N*=10)		Winter (*N*=10)	
	Substratum	Eggshell	Substratum	Eggshell
Paper	1	0	0	0
Vermiculite	9	6	0	0
Soil	10	6	0	0

Table 4. *Ice-nucleating activity of natural (nonautoclaved) and autoclaved samples of experimental substrata, eggshell and turtle feces, as determined by measuring the temperature of crystallization (T_c) of bulk samples containing a small volume of water and of washings prepared from samples*

	Temperature of crystallization (°C)			
	Bulk		Washing	
	Natural	Autoclaved	Natural	Autoclaved
Vermiculite	–6.1±0.2	–6.5±0.3	–18.7±0.2	–18.7±0.6
Nesting soil	–4.9±0.3	–6.1±0.2*	–5.4±0.1	–13.2±0.7*
Eggshell	–8.4±0.3	–8.8±0.7	–17.3±0.5	–20.0±0.4*
Feces	–9.1±1.1	–13.7±0.6*	–15.3±1.2	–16.8±0.9
Water (control)	–	–17.8±1.2	–	–22.7±0.3

Values are means ± S.E.M.; *N*=10 replicates per group.

Asterisk denotes significant difference between natural and autoclaved samples within the pair. Statistical significance is taken at the level of $P<0.05$.

soil greatly diminished (t=10.39, $P<0.0001$, N=10) the ice-nucleating activity expressed in washings prepared therewith, suggesting that the water-soluble INA in nesting soil was organic.

Feces produced by the summer turtles reared on paper contained weak INA that were sensitive to autoclaving (t=3.82, P=0.005, N=10; Table 4). Given that the washings prepared from the feces expressed little ice-nucleating activity, the constituent INA were probably insoluble.

Supercooling capacity of hatchlings ingesting INA in nesting soil

Experimental ingestion of INA strongly influenced the supercooling capacity of winter turtles ($F_{4,24}$=208.50, $P<0.0001$; Fig. 2). Whereas the sham-treated turtles and the turtles ingesting water supercooled extensively (as did other winter turtles reared on vermiculite; Fig. 1), turtles ingesting a small quantity of autoclaved soil, or washing prepared from unadulterated soil, froze at relatively high temperatures. Soil washing was the more potent INA (Fig. 2). The mean T_c of turtles tested 33 days after ingesting soil washing (–7.5±0.1°C) was similar to that determined for turtles tested 24 h afterwards (–6.8±0.04°C).

Supercooling capacity of hatchlings collected from natural nests

We determined the T_c for 2–4 live turtles collected from each of nine nests on 6 April 2002. T_c values averaged for turtles in each nest ranged from –6.4°C to –9.1°C, and the mean T_c for all 32 turtles was –7.5°C. The intestines of 22 (69%) of the turtles contained a white, caseous material, apparently of endogenous origin, that was not observed in our laboratory-reared animals. This material did not influence the supercooling limit, as the T_c of the affected turtles was identical (t=0.007, d.f.=30, P=0.99) to that of the turtles lacking it. The mean mass of the gut (101 mg) and yolk sac (40 mg) of the field-collected turtles was comparable with values determined for the laboratory-reared winter turtles (Table 2). The thermal minimum recorded in each nest ranged from –6.1°C to –15.0°C (mean, –9.5°C); mortality ranged from 0 to 33.3% (mean, 12.6%).

Discussion

With the approach of winter, many temperate ectotherms undergo marked physiological changes that enable them to survive the ensuing cold. Mechanisms promoting seasonal development of cold hardiness are best known among the invertebrates (Lee, 1991; Loomis, 1991; Oswood et al., 1991). In some insects, for example, supercooling capacity is enhanced by production of antifreezes (thermal hysteresis

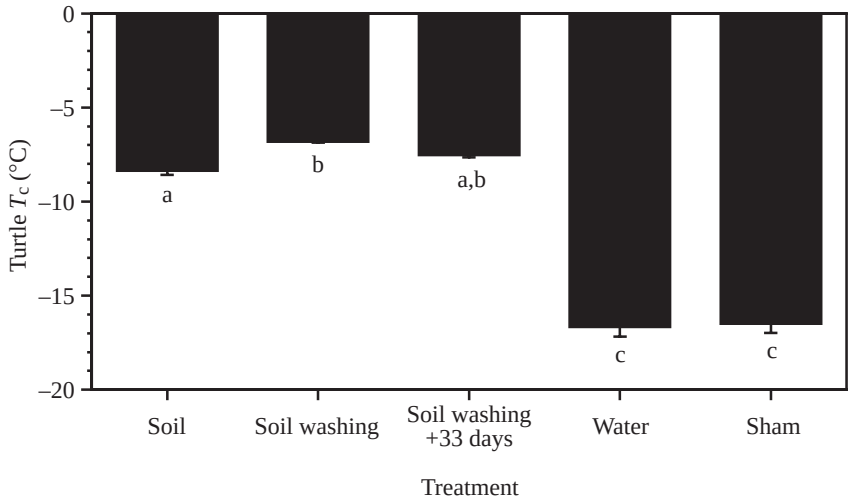

Fig. 2. Temperatures of crystallization (T_c) of *Chrysemys picta*, reared on vermiculite and acclimated to 4°C, after ingesting a small amount of autoclaved nesting soil or a washing of non-autoclaved nesting soil. Control turtles ingested vehicle (water) or nothing (sham-fed). Turtles were tested 24 h or 33 days after treatment. Mean values (± S.E.M., N=5 replicates per group) identified by different letters are statistically different (ANOVA/Student–Newman–Keuls multiple comparisons test; $P<0.05$).

proteins) or accumulation of low-molecular-mass carbohydrates. Cold acclimation or acclimatization to winter conditions often coincides with elimination of ingested food and other potential INA, which is required for development of supercooling capacity (Block and Zettel, 1980; Sømme, 1982; Zachariassen, 1985; Bale et al., 1989; Duman et al., 1995). Evidence for seasonal variation in cold hardiness among ecothermic vertebrates is much less compelling (Costanzo and Lee, 1995), although recent studies (Costanzo et al., 2000b; Packard et al., 2001) of hatchling *C. picta* have demonstrated an enhancement of supercooling capacity during cold acclimation. This change apparently involves neither antifreeze proteins nor any of the cryoprotectants commonly found in cold-hardy animals (Costanzo et al., 2000b). Rather, our present study suggests that elimination of endogenous and exogenous INA is requisite to achieving the full measure of supercooling capacity in these turtles.

Changes in supercooling capacity

Our finding that supercooling capacity was greater in winter turtles than in summer turtles concurs with a previous report (Costanzo et al., 2000b). In addition, our finding that the guts of nearly all turtles reared on vermiculite or nesting soil were distended and packed with these materials accords with the observations of Packard et al. (2001), who found that *C. picta* commonly ingested substratum during hatching or shortly thereafter. On the other hand, relatively few of our turtles ingested eggshell, which purportedly provides a supplemental source of calcium needed for ossification of the maturing hatchling (Packard et al., 2000, 2001). The contention that recently hatched *C. picta* consume eggshell for this reason is inconsistent with the appearance of the ingested eggshell fragments, which were dense and sharp-edged, rather than eroded from mineral leaching. Furthermore, eggshell constituted a small proportion of the volume of the matter ingested, and the guts of some turtles contained substratum but no eggshell (Table 3). We believe that hatchlings intend to ingest primarily substratum (i.e. nesting soil), rather than eggshell, although the adaptive value of doing so is unclear. Perhaps the passage of soil aids in the elimination of feces or otherwise promotes proper gut function. In addition, ingesting soil (and eggshell) may serve to inoculate hatchlings with normal intestinal flora. Turtles urinate on their nests after ovipositing (see Carr, 1952), possibly introducing beneficial microbes, flushed from the cloaca, into the nest chamber. Coprophagy permits some neonatal reptiles to inoculate themselves with the symbiotic gut flora required for proper digestive efficiency (Troyer, 1983; Lance and Morafka, 2001).

Among the hatchlings reared on vermiculite, greater supercooling capacity in winter turtles, as compared with summer turtles, was strongly associated with elimination of ingested substratum and eggshell. Packard et al. (2001) also found this association, reporting that recently hatched turtles harboring these materials in the gut froze at –7.1°C, whereas cohorts having empty guts supercooled to –12.9°C. Nevertheless, our finding that hatchlings reared on vermiculite supercooled to the same degree as the hatchlings reared on paper, which consumed nothing, argues that the presence of vermiculite and eggshell in the gut has no effect on supercooling capacity. This conclusion is bolstered by results of a *post-hoc* analysis of the data for vermiculite-reared turtles: there was no difference (t=0.85, d.f.=8, P=0.38) in T_c between the hatchlings that had ingested eggshell and the hatchlings that had not. Vermiculite and eggshell fragments are moderately active INA (Table 4); however, these materials did not limit supercooling in summer hatchlings.

Evidence for endogenous INA in summer turtles

Our finding that supercooling capacity improved with cold acclimation in the paper-reared turtles revealed that seasonal development of cold hardiness derives from elimination of endogenous INA that are present in summer turtles. The exact identity of the INA is unknown, although our results suggest they are associated with residual yolk. We found good accord between ice-nucleating activity in isolated yolk and the T_c values determined for live turtles reared on paper or vermiculite. Also, egested feces, probably derived from yolk, which is heavily mobilized after hatching (Table 2; see Filoramo and Janzen, 1999; Lance and Morafka, 2001) contained INA whose activity also matched the supercooling limit of these summer turtles (Tables 1, 4). Given their apparent insolubility, defecation should eliminate these INA from the body, and, indeed, supercooling capacity was markedly improved in the winter turtles, which had voided their guts. Therefore, seasonal development of cold hardiness in hatchling *C. picta* apparently depends on the elimination of INA that would otherwise constrain supercooling capacity rather than production of cryoprotective solutes or specialized proteins (see Costanzo et al., 2000b).

Our results provide few clues about the factors triggering gut evacuation in recently hatched *C. picta*, although some evidence suggests that gut clearance may be stimulated by cold exposure. For example, hatchlings reared on vermiculite and subjected to a naturalistic cold-acclimation regimen apparently developed their full capacity for supercooling in late November, after a four-week exposure to 10°C, because the T_c of 4°C-acclimated turtles, tested in mid-winter, was virtually unchanged (Costanzo et al., 2000b). On the other hand, gut evacuation might be promoted by maturational changes, as at least some of our turtles defecated within two weeks of hatching, even though ambient temperature remained high.

Effect of ingested INA on supercooling capacity

Our results suggest that ingestion of exogenous matter may or may not influence the supercooling capacity of hatchling *C. picta*. The effect may be negligible if, as was the case with vermiculite and eggshell, the ice-nucleating activity of the ingesta is similar to, or less than, the activity of any endogenous INA. However, in the case of nesting soil, which commonly harbors a host of relatively potent INA (Costanzo et al., 1998, 2000a, 2001), the effect may strongly influence winter survival. Purging the gut of nesting soil during cold

acclimation may improve supercooling capacity modestly, but ultimately the degree of cold hardiness the hatchlings attain is inferior to that achieved by turtles that do not ingest soil.

Our study confirmed previous findings (Costanzo et al., 2000a; Packard et al., 2001) that *C. picta* hatched and reared on nesting soil supercool modestly as compared with turtles kept on vermiculite. Turtles are readily contaminated with environmental INA, as immersing vermiculite-reared hatchlings in nesting soil for as little as 48 h markedly reduces their supercooling capacity (Costanzo et al., 2000a). Ingestion is probably an important means by which INA gain access to the body fluids of turtles; however, transmission through non-oral apertures (cloaca, nares, ocular openings) or the integument may occur in recently hatched and older hatchlings (Costanzo et al., 2000a). Our results for turtles reared on nesting soil, showing limited supercooling in both the isolated gut and the carcass (Table 1), imply that contamination with exogenous INA occurred *via* multiple routes.

Nesting soil contains at least two classes of INA that demonstrably impact cold hardiness in hatchling turtles. These include various mineral particulates, which are water insoluble and are largely unaffected by high temperature and pressure, as well as water-soluble, autoclave-sensitive agents, which may be derived from ice-nucleating microorganisms (Costanzo et al., 2000a). Experimental ingestion of either type dramatically constrained supercooling, although the effect of the organic INA was more pronounced, consistent with its greater ice-nucleating activity (Tanaka, 1994). Hatchlings reared on nesting soil eliminated particulate INA through defecation, and, apparently, this process improved their supercooling capacity. However, they probably retained at least some organic INA, because the winter turtles were unable to supercool to the same extent as turtles in the other groups. Their solubility and small size (see Costanzo et al., 2000a) may render these agents highly vagile and, thus, especially difficult to purge from the body. Indeed, the relatively high T_c determined for the yolk isolated from soil-reared turtles (Table 1) suggests that this organ was contaminated with INA, which may have dispersed from the gut *via* the yolk stalk, Meckel's diverticulum (Ewert, 1985; Lance and Morafka, 2001). Furthermore, results of our experimental ingestion trials indicate that organic INA found in nesting soil can long retain their ice-nucleating activity in the internal milieu.

Ecological implications of contamination by soil INA

In reviewing the available literature, Costanzo et al. (2000a) identified a dichotomous pattern in which *C. picta* hatching under natural conditions (or those otherwise exposed to nesting soil) supercooled much less extensively than laboratory-reared turtles hatched on vermiculite, an essentially INA-free, artificial substratum, attributing the variation to contamination of the field-collected turtles with INA present in the nesting soil. Our present work not only bolsters this explanation but also confirms ingestion of nesting soil as a primary avenue of INA contamination. Furthermore, our finding that hatchlings purge particulate INA from their guts, as well as the similarity between the T_c of turtles experimentally ingesting organic INA and the hatchlings extricated from natural nests in winter (Packard et al., 2001; present study), may indicate that the organic class of soil INA is of greater importance to the cold hardiness of hatchling *C. picta*.

Some of the results for naturally hibernating turtles were perplexing. For example, we cannot explain why the average T_c for these animals was approximately 2°C higher than the average minimum temperature recorded within their nests. In addition, unexpectedly, we found that supercooling capacity differed between the field-collected turtles ($T_c \approx -7.5$°C) and the winter turtles reared on nesting soil ($T_c \approx -10$°C). Packard and colleagues (2001) obtained the same result, speculating that their field-collected turtles, sampled in November, had not yet experienced a level of cold sufficient to induce full development of supercooling capacity. However, our field-collected turtles, sampled at winter's end, had experienced temperatures much lower than those encountered by our lab-reared turtles. An alternative explanation is that the organic INA influencing turtle T_c, being sensitive to changes in their thermal environment (Costanzo et al., 2000a), lost potency under static laboratory conditions, but retained, or even increased, their ice-nucleating activity under the more favorable thermal and trophic conditions in natural nests (Lindow et al., 1982; Nemecek-Marshall et al., 1993; Fall and Fall, 1998).

Our conclusion that supercooling is constrained by INA that persist in the body, even after ingested soil has been eliminated from the gut, raises new questions about the efficacy of supercooling as a cold-hardiness strategy in hatchling *C. picta*. Whereas some authors (e.g. Packard and Packard, 2001) have maintained that their supercooling capacity meets or exceeds the demands imposed by the winter thermal environment, this argument derives from studies of hatchlings reared on a vermiculite substratum, and it is now clear that such experiments grossly overestimate the supercooling capacity of this species. Furthermore, recent work has established that, under certain environmental conditions, hatchling *C. picta* are highly susceptible to inoculation by ice or INA within the nest microenvironment (Costanzo et al., 1998, 2000a, 2001). Considering the high efficacy of endogenous and exogenous INA, and the fact that the lower limit for freeze tolerance is approximately –4°C, it is remarkable that these turtles can survive winter inside nests that attain minimum temperatures below –12°C (Packard et al., 1997; Packard and Packard, 2001) and even –15°C (present study).

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