

SEASONAL CHANGE IN THE CAPACITY FOR SUPERCOOLING BY NEONATAL PAINTED TURTLES

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Accepted 19 February; published on WWW 5 April 2001

Summary

Hatchlings of the North American painted turtle (*Chrysemys picta*) typically spend their first winter of life inside the shallow, subterranean nest where they completed incubation the preceding summer. This facet of their natural history commonly causes neonates in northerly populations to be exposed in mid-winter to ice and cold, which many animals survive by remaining unfrozen and supercooled. We measured the limit of supercooling in samples of turtles taken shortly after hatching and in other samples after 2 months of acclimation (or acclimatization) to a reduced temperature in the laboratory or field. Animals initially had only a limited capacity for supercooling, but they acquired an ability to undergo deeper supercooling during the course of acclimation. The gut of most turtles was packed with particles of soil and

eggshell shortly after hatching, but not after acclimation. Thus, the relatively high limit of supercooling for turtles in the days immediately after hatching may have resulted from the ingestion of soil (and associated nucleating agents) by the animals as they were freeing themselves from their eggshell, whereas the relatively low limit of supercooling attained by acclimated turtles may have resulted from their purging their gut of its contents. Parallels may, therefore, exist between the natural-history strategy expressed by hatchling painted turtles and that expressed by numerous terrestrial arthropods that withstand the cold of winter by sustaining a state of supercooling.

Key words: painted turtle, *Chrysemys picta*, cold-tolerance, hibernation, supercooling, acclimation.

Introduction

Painted turtles (*Chrysemys picta*) are common residents of shallow lakes and marshes across much of North America east of the Rocky Mountains. They are so common, in fact, that the species has become a popular model for studies on the ecology and evolution of chelonians (Wilbur and Morin, 1988). The natural history of painted turtles, however, differs from that of most other species in an important respect: whereas neonates of other species of freshwater turtle usually emerge from their subterranean nest in late summer or autumn and move to a nearby marsh, lake or stream to spend their first winter, hatchling painted turtles typically remain inside their shallow (8–14 cm) nest throughout their first winter and do not emerge above ground until the following spring (Ernst et al., 1994). This behavior commonly causes neonatal painted turtles in populations from Nebraska (Packard, 1997; Packard et al., 1997a), northern Illinois (Weisrock and Janzen, 1999) and New Jersey (DePari, 1996) northward to the limit of distribution in southern Canada (Storey et al., 1988) to be exposed during winter to ice and cold, with temperatures in some nests falling below -10°C . Many hatchlings withstand such extremes and emerge from their nest when the soil finally thaws in the spring (Storey et al., 1988; DePari, 1996; Packard, 1997; Packard et al., 1997a; Weisrock and Janzen, 1999).

Hatchlings are able to withstand exposure to ice and cold in mid-winter by becoming supercooled, i.e. they remain unfrozen at temperatures below the equilibrium freezing point for their body fluids (Packard et al., 1997b; Packard et al., 1999; Costanzo et al., 2000a; Costanzo et al., 2000b). The animals usually do not contain heterogeneous nucleating agents capable of causing body fluids to freeze at moderate subzero temperatures such as those encountered most often in the field (Costanzo et al., 1998b; Costanzo et al., 2000a; Costanzo et al., 2000b; Packard and Packard, 1999; Hartley et al., 2000), and the integument of neonates resists the penetration of ice crystals into body compartments from the environment (Costanzo et al., 2000b; Willard et al., 2000). In the absence of a suitable organizing site to promote a change in phase from liquid to solid, the body fluids of the hatchlings remain unfrozen (Dorsey, 1948; Franks, 1985; Vali, 1995). Supercooled solutions are quite stable at temperatures above -20°C (Dorsey, 1948), so turtles can remain unfrozen and supercooled for extended periods during winter (Packard and Packard, 1997; Hartley et al., 2000).

Despite the impressive ability of hatchling painted turtles to undergo supercooling during the coldest months of winter, the animals actually have only a modest capacity for supercooling

in the first few weeks after hatching. Indeed, recently hatched turtles freeze spontaneously at temperatures near -6°C (Costanzo et al., 2000b), which is so high that few animals would survive winters at higher latitudes if this temperature represented their lower limit of tolerance (see Packard et al., 1997a). However, the animals acquire a capacity for 'deeper' supercooling as winter approaches (Costanzo et al., 2000b), and the new limits for supercooling are lower than the temperatures commonly encountered in nests at the coldest time of the year (Packard, 1997; Packard et al., 1997a).

Why are newly hatched painted turtles less able than slightly older animals to undergo supercooling, and what happens during the period after hatching to enhance the capacity of neonates for supercooling? The answers to these questions are key to understanding the unusual natural-history strategy expressed by the species. We addressed these important questions in the current study and simultaneously expanded on other recent investigations on this subject (Costanzo et al., 2000a; Costanzo et al., 2000b).

Materials and methods

Nests, eggs and hatchlings

We observed gravid painted turtles, *Chrysemys picta* (Schneider, 1783), in June 2000 as they searched for suitable nesting sites in lawns and gardens and along roadsides inside the headquarters compound at the Valentine National Wildlife Refuge (NWR), Cherry County, Nebraska. We drove a small stake into the ground adjacent to several of these nests so that we could relocate them later. Other nests were opened immediately, and the eggs were removed, packed in damp vermiculite and transported to the laboratory at Colorado State University.

On arrival at the laboratory, the eggs were cleaned as completely as possible of adhering soil, placed into boxes of damp vermiculite (417 g of water per 300 g of vermiculite; water potential -100 kPa) and incubated at 27°C . At the end of 5 weeks, four eggs from each of six clutches (which form the basis for the laboratory investigation) were cleaned again and then transferred to freshly prepared boxes of damp vermiculite, and another four eggs from the same clutches were cleaned and transferred to boxes containing a damp, loamy sand from a single site inside the headquarters compound at the Valentine NWR. The boxes were returned to the environmental chamber, and the eggs were allowed to complete incubation at 27°C . The temperature was changed to 25°C on 1 September (i.e. after all the eggs with viable embryos had hatched).

A sample of turtles was taken on 13 September so that we could determine the limit of supercooling for unacclimated hatchlings from the laboratory incubation. Ten hatchlings ($N=1-2$ per clutch) came from vermiculite and 11 from soil. The turtles were prepared for study and immediately placed into the protocol for measuring the limit of supercooling (see below). The remaining (unsampled) turtles were then acclimated over the next 18 days to 4°C by reducing the temperature in the environmental chamber by 2°C every

second day. The acclimated animals were held at 4°C , in complete darkness and without food or water, until 11 November, at which time the remaining 1–2 clutchmates from each of the substrata ($N=12$ on vermiculite and 11 on soil) were prepared for study and their limit of supercooling was measured.

We recovered hatchlings from four of the marked nests at the Valentine NWR on 10 September, and removed turtles from another four nests on 10 November (by which time the weather had cooled appreciably and a light snow had fallen, although the temperature actually reached in the nests is not known). The animals were transported to the laboratory and prepared for study on 13 September and 11 November, respectively (i.e. at the same times that turtles hatched in the laboratory were being prepared for study). Three hatchlings were used from each clutch sampled in September, and four turtles were taken from each clutch sampled in November. The limit of supercooling for these turtles was determined according to the following protocol.

Measuring the limit of supercooling

The turtles were first cleaned as thoroughly as possible with a small paint brush to remove vermiculite or soil adhering to their shell or filling spaces in the axillary and inguinal pockets. A copper/constantan thermocouple (26 gauge) was then glued to the carapace of each animal with epoxy resin, and the hatchling was placed onto a platform of dry styrofoam in a pint-volume canning jar. The dry environment in the jar ensured that the turtle would not contact ice and be inoculated during its subsequent exposure to subzero temperatures. The jars were closed and placed inside an environmental chamber at a holding temperature of $1-2^{\circ}\text{C}$. The free ends of the thermocouples were attached *via* a multiplexor to a Campbell CR-10 datalogger programmed to record temperature every 10 min. The microprocessor controlling the environmental chamber was then set to reduce the temperature by 1°C per day to a minimum near -20°C , at which point temperature was immediately shifted back to $1-2^{\circ}\text{C}$. We removed the turtles the next day (i.e. after they had thawed); none of them survived the procedure.

We downloaded data from the datalogger to a PC and constructed a temperature profile for each hatchling. We then examined the profiles for spikes in temperature (exotherms) caused by the release of latent heat of fusion by water in the bodies of the turtles changing phase from liquid to solid. The temperature recorded on the surface of the carapace immediately preceding the appearance of a temperature spike was taken as the limit of supercooling.

Finally, we dissected each of the turtles and removed the entire gastrointestinal tract. The gut was cut into several segments, and the contents were expressed with the blunt end of a pair of forceps. The expressed material was then examined with the aid of a dissecting microscope. We scored each gut for the presence or absence of particles of soil and/or eggshell; turtles commonly ingest large quantities of such material in the process of hatching (Packard et al., 2000).

Statistical analyses

Values for the limit of supercooling by turtles hatched on soil or vermiculite in the laboratory (first experiment) were submitted to factorial analysis of variance (ANOVA) (Proc Mixed in SAS version 8.1) in which treatment (i.e. unacclimated animals sampled in September *versus* acclimated turtles sampled in November) and medium used for incubation (vermiculite *versus* native soil) were fixed effects and the clutch of origin was a random factor. Data for turtles recovered from natural nests (second experiment) were examined by hierarchical ANOVA in which sampling time (month) was a fixed effect and clutch was a random factor nested within sampling time. Degrees of freedom for the error terms in these mixed-model analyses were estimated using the Satterthwaite procedure, and tests of significance were based on Type 3 sums of squares.

Finally, we compared the limit of supercooling for turtles hatching on native soil in the laboratory with that for animals hatching on native soil in the field by using sampling date and incubation site as fixed effects in ANOVA in a factorial design. We did not include clutch as a term in this last analysis because of the resulting imbalance in design. Tests of significance for treatment effects were again based on Type 3 sums of squares, and multiple comparisons of least-squares means were by *t*-tests.

Results

Turtles hatching in the laboratory

Analysis of variance indicated that acclimation ($F_{1,35.2}=39.77$, $P<0.001$) and the type of substratum on which turtles hatched ($F_{1,36.1}=15.77$, $P<0.001$) both influenced their ability to undergo supercooling, but the interaction between the fixed effects was not an important source of variation ($F_{1,35.2}=1.61$, $P=0.213$). The limit of supercooling was 6°C lower, on the average, in November than in September, and the limit was approximately 4°C lower for turtles hatching on vermiculite than for those hatching on native soil (Fig. 1).

The gastrointestinal tract of most of the unacclimated turtles studied in September contained particles of soil and/or eggshell, whereas none of the acclimated animals sampled in November had material in its gut. Thus, the lower limit of supercooling in November was associated with an emptying of the gut. Interestingly, the gut of four turtles (all hatched on vermiculite) studied in September was also empty, and the limit of supercooling for these four animals (mean -12.9°C, range -8.0 to -15.3°C) was substantially lower than that for six other turtles hatched on vermiculite (mean -7.1°C, range -5.9 to -8.2°C).

Turtles hatching in the field

Statistical analysis revealed that the limit of supercooling for turtles from natural nests differed between animals sampled in September and those sampled in November ($F_{1,6.8}=10.09$, $P=0.016$). The limit was approximately 2°C lower in November than in September (Fig. 2), and this difference was

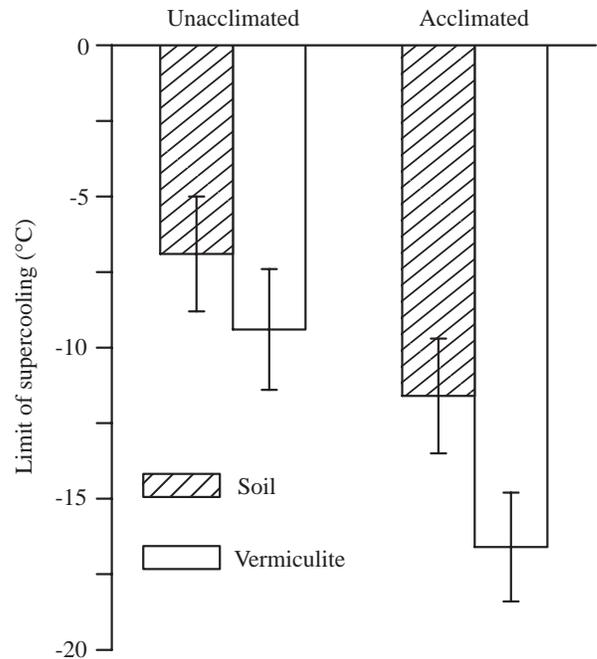


Fig. 1. Least-squares means (± 2 S.E.M.) for limits of supercooling for painted turtles hatching in the laboratory on substrata of vermiculite ($N=10$ for September; $N=12$ for November) or native soil ($N=11$). Unacclimated turtles were studied in mid-September, whereas acclimated animals were studied in mid-November.

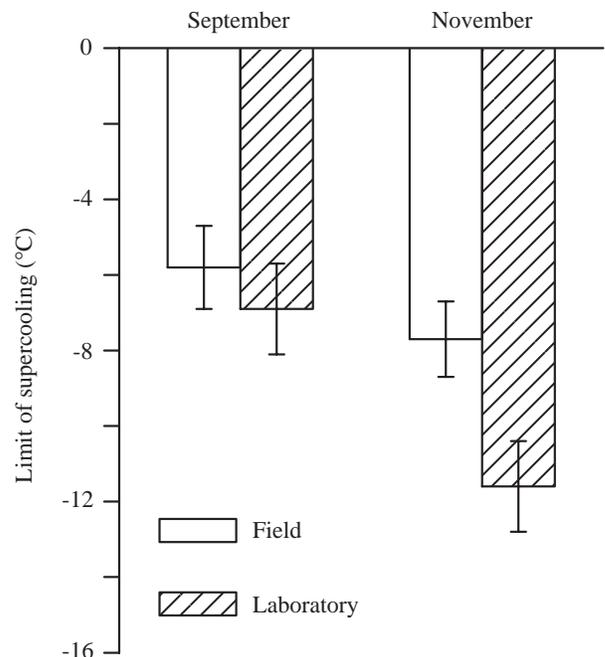


Fig. 2. Least-squares means (± 2 S.E.M.) for limits of supercooling for painted turtles hatching on native soil in the laboratory ($N=11$) and in natural nests in the field ($N=12$ for September; $N=16$ for November).

again associated with emptying of the gut. Only one of 12 turtles had an empty gut in September, but none of the 16 animals had particulate matter in its gut in November.

Turtles hatching on soil in the laboratory versus soil in the field

We also compared data for turtles hatching on soil in the laboratory with those for animals hatching on soil in the field. The significant interaction term in the ANOVA ($F_{1,46}=6.45$, $P=0.015$) indicates that effects of season (or acclimation/acclimatization) were not the same for turtles hatching in the laboratory as for those hatching in the field (Fig. 2). The limit of supercooling in September did not differ between animals hatched in the laboratory and those hatched in the field ($P=0.185$), but a substantial difference between sites was detected in November ($P<0.001$). Animals in both groups were able to supercool to a greater extent in November (Fig. 2), but the limit of supercooling was reduced more in turtles hatched in the laboratory than in those hatched in the field (Fig. 2).

Discussion

Painted turtles hatching on substrata of vermiculite are able to supercool to substantially lower temperatures than animals hatching on native soils (Fig. 1; see also Costanzo et al., 2000a). This difference in capacity for supercooling has been ascribed to the different kinds of nucleating agents present in the respective media. Native soils contain a multitude of nucleating agents (Costanzo et al., 1998b; Costanzo et al., 2000a; Costanzo et al., 2001), the most efficient of which is a variety of bacterium that freezes spontaneously at high subzero temperatures (Lindow, 1983; Hirano and Upper, 1995), whereas vermiculite typically contains nucleating agents (possibly inorganic) that function only over a lower range of temperatures (Costanzo et al., 2000a). Costanzo et al. (Costanzo et al., 2000a, p.357) suggested that nucleating agents in soil or vermiculite contaminate the surface of the skin of hatchlings and somehow gain access to body compartments 'via orifices or wounds, or by directly transiting the integument'. The idea that the nucleators pass through the integument directly was presumably based on the proposal that this type of mechanism applies to freezing by plants (Lindow, 1983), even though it is unclear how the nucleators would pass through the lipids in the α -keratin layer of the epidermis of painted turtles (Willard et al., 2000). Animals that are penetrated by the nucleating agents supposedly freeze by heterogeneous nucleation when the temperature becomes low enough, and such spontaneous freezing occurs at higher subzero temperatures in turtles hatching on native soil than in those hatching on vermiculite because of the difference in effectiveness of the nucleators investing the two media.

Another explanation, however, emerges from a consideration of the 'adaptive strategy' for overwintering exploited by numerous species of terrestrial arthropod, and this alternative has the added advantage of affording an explanation for the seasonal increase in capacity for supercooling. The arthropods in question commonly possess only a limited capacity for supercooling in late summer and autumn, but acquire an impressive capacity for supercooling before the arrival of the most severe cold of mid-winter (Salt, 1966;

Tanaka, 1994). Animals studied in late summer have usually been feeding, and the food contains an abundance of nucleating agents that elicit heterogeneous nucleation at relatively high subzero temperatures (Salt, 1966; Strong-Gunderson et al., 1990; Lee et al., 1991; Costanzo et al., 1998a; Castrillo et al., 2000). When the contents of the gut begin to freeze, ice propagates across the lining of the gut and seeds the formation of ice in extracellular fluids. With the approach of winter, however, these same arthropods purge their gut of its contents (Salt, 1966; Costanzo et al., 1998a) and, in the process, they commonly remove the most effective of the nucleating agents (Strong-Gunderson et al., 1990; Costanzo et al., 1998a). Some animals achieve a more complete purging than do other individuals, so that some continue to be relatively susceptible to freezing at relatively high subzero temperatures while others become quite resistant (Costanzo et al., 1998a; Castrillo et al., 2000). Nevertheless, many of these animals have acquired a greater capacity for supercooling by mid-winter, and they are thereby able to withstand the most severe conditions in an unfrozen state.

The parallels between these terrestrial arthropods and hatchling painted turtles are apparent. The turtles have a limited capacity for supercooling in late summer (Figs 1, 2), when the gut of these animals is also full. In contrast to the arthropods, which have filled their gut in the course of feeding, the turtles have filled their gut with particles of soil and eggshell ingested in the course of hatching (Packard et al., 2000). The ingested eggshell may provide a supplementary supply of calcium to support the mineralization of bone during the neonatal period (Packard et al., 2000), but the material presumably contains a host of nucleating agents as well. If the turtles hatched on native soil, ice-nucleating bacteria are likely to be among the ingested nucleating agents, producing a relatively high limit of supercooling (Fig. 1). If the turtles hatched instead on vermiculite, the ingested nucleating agents are likely to be largely of mineral origin, and the limit of supercooling of these turtles is, therefore, lower (Fig. 1).

As the turtles become acclimatized to lower temperatures with the approach of winter, they, like the arthropods, purge their gut of its contents. Many of the nucleating agents would thus be removed, and the turtles acquire a greater capacity for supercooling (Figs 1, 2). Some residual effect of the nucleators nonetheless persists, because the limit of supercooling continues to be lower for turtles hatched on vermiculite than for those hatched on a native soil (Fig. 1). However, this same retention of nucleating agents occurs among the arthropods (Costanzo et al., 1998a; Castrillo et al., 2000).

We favor the second hypothesis for explaining seasonal changes in the capacity of hatchling painted turtles to undergo supercooling and for different limits of supercooling in animals hatching on vermiculite and native soil, but we also recognize that important issues remain to be addressed. For example, we have shown that a correlation exists between the capacity for supercooling by neonates and the presence of soil in their gut, but this correlation does not establish a causal relationship between the factors. Is the enhanced capacity for supercooling

that animals achieve before the onset of winter actually caused by purging the gut of its contents, or is the enhancement a result of some other, more subtle, change in the physiology or condition of the animals?

We have also shown that the capacity for supercooling is greater among acclimated (or acclimatized) turtles in November than among unacclimated animals in September, but this again is a correlation that does not establish causality. Do changes in the capacity for supercooling result from thermal acclimation or do they result simply from maturational changes in the animals? The enhancement of the capacity for supercooling in the current investigation was not as great for turtles acclimatized in the field as for those acclimated in the laboratory, which suggests to us that the process is more likely to be influenced by temperature than by simple maturation (because animals from the laboratory and the field were of similar ages). We do not know the temperatures to which turtles were exposed prior to their being removed from nests in the field, but the soil temperature at our study site in Nebraska is commonly between 5 and 15 °C for the entire month of November (G. C. and M. J. Packard, unpublished observations). Thus, turtles hatched in the laboratory may have been exposed for several weeks to temperatures several degrees lower than those to which neonates were exposed in the field, in which case differential acclimation to low temperature could account for the difference in their capacity for supercooling (Fig. 2).

Our work was supported by the National Science Foundation (IBN-9612562). The protocol for the study was considered and approved by the Animal Care and Use Committee at Colorado State University (00-101A-01). Work at the Valentine National Wildlife Refuge was performed under authority granted by Special Use Permit VLT-00-005 from the US Fish and Wildlife Service and Collecting Permit 2000-89 from the Nebraska Game and Parks Commission.

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