Cold-Tolerance of Hatchling Painted Turtles (Chrysemys picta bellii) from the Southern Limit of Distribution

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Painted turtles (Chrysemys picta) have a natural history unlike that of other chelonians from the northern United States and southern Canada. Although neonates of other freshwater turtles usually emerge from their subterranean nests in late summer or autumn and move to nearby marshes, lakes, or streams to spend their first winter, hatchling painted turtles typically remain inside their shallow (8–14 cm) nests 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 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 going below −10°C. Many hatchlings withstand such extremes and emerge from their nests when the ground thaws in the spring (Storey et al., 1988; DePari, 1996; Packard, 1997; Packard et al., 1997a; Weisrock and Janzen, 1999).

Hatchling painted turtles from northern populations withstand exposure to ice and cold by remaining unfrozen at temperatures below the equilibrium freezing point for their body fluids (Packard and Packard, 2001). This supercooled state occurs (1) because the body fluids of hatchlings usually do not contain the necessary organizing sites (+ nucleating agents) to initiate freezing at temperatures above −15°C (Costanzo et al., 1998, 2000; Packard and Packard, 1999) and (2) because the integument of the turtles resists the penetration of ice crystals into body compartments from frozen soil (i.e., "inoculation"; Costanzo et al., 2000; 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 hatchlings remain in a supercooled state (i.e., a metastable liquid state) at temperatures below their freezing point (Dorsey, 1948). Supercooled solutions are quite stable at subzero temperatures above −20°C (Dorsey, 1948), so turtles can remain unfrozen for extended periods during winter (Packard and Packard, 1997; Hartley et al., 2000).

The preceding generalizations are based, however, on studies of hatchling painted turtles from higher latitudes. Little is known about the tolerance for cold in animals from populations at lower latitudes, yet such information is key to reconstructing the evolutionary history of painted turtles (Bleakney, 1958; Ultsch et al., 2001) and to understanding the post-Pleistocene expansion of the range of the species (Holman and Andrews, 1994). Accordingly, we report here the results of three experiments on cold-tolerance of hatchling painted turtles from a disjunct population near the southern limit of distribution for the species in the Rio Grande Valley of central New Mexico. We find that neonates from New Mexico have the same means and capacity as hatchlings from North Dakota for dealing with the challenges of ice and cold, despite the fact that winters at the New Mexico site are likely to be substantially milder than those in North Dakota.

Animals.—We captured four gravid painted turtles in late May 2000 near San Marcial, in the Rio Grande Valley south of the Bosque del Apache National Wildlife Refuge, Socorro County, New Mexico. Animals from this population currently are assigned to the subspecies bellii (see Ultsch et al., 2001), which comprises animals that presumably are descended from turtles occupying a southwestern refugium during Pleistocene glaciation (Bleakney, 1958). We injected the turtles with oxytocin to induce them to lay their clutches of fully formed eggs, after which the eggs were packed in damp sand, transported to Iowa State University, and incubated to hatching on moist vermiculite (water potential −150 kPa) at 28.3°C. Twenty-two hatchlings from these clutches later were shipped by air express to Colorado State University where experiments on cold-tolerance were performed. When the turtles arrived in Colorado, they were placed immediately into a darkened environmental chamber at 20°C. Temperature in the chamber was reduced in steps of 1–2°C every two days to 4°C (non-
nal temperature), which then was maintained until
the animals were used in the following studies.

**Experiment 1.**—Six turtles (representing all four
clutches) were dried carefully and cleaned with a
small paint brush, after which a copper/constantan
thermocouple (26 gauge wire) was glued to the cara-
pace of each animal with epoxy resin. The turtles were
placed individually into pint-volume canning jars
where they rested on a surface of dry styrofoam to
prevent them from contacting ice and possibly being
inoculated. The closed jars were placed into a Percival
environmental chamber set at 2°C, and the thermo-
couples were attached to a Campbell CR-10 datalogger
that recorded temperature every 10 min.

We programmed the controller for the chamber to
reduce temperature linearly by 1°C/day. On reaching a
minimum of −20°C, the temperature was immedi-
ately returned to 2°C. Turtles were removed from the
jars the following morning, placed into paper cups
containing a small amount of water and given two
days to recover. The hatchlings then were judged to
be alive or dead on the basis of their spontaneous ac-
tivity, their responses to tactile stimuli, the appearance
of their eyes (wide open and focused vs fully closed
or partially closed and vacant), and their general level of alertness.

We downloaded data from the datalogger to a PC
and then constructed a temperature profile for each
turtle. These temperature profiles were examined for
the presence of freezing exotherms (i.e., for the abrupt
temperature increases in temperature resulting from the release of
latent heat of fusion by water changing phase from
liquid to solid). The temperature on the carapace of
an animal immediately before the appearance of a
freezing exotherm was taken to be the limit of super-
cooling for the turtle.

One turtle in this test froze spontaneously at the
relatively high temperature of −12.0°C, but values for
the limit of supercooling for the other five animals
were clustered between −17.1°C and −18.6°C. The
arithmetic mean for the limit of supercooling was
−17.0°C (SD = 2.5°C) for the six turtles in our sample,
and the median was −17.8°C. None of the hatchlings
survived the treatment.

**Experiment 2.**—When none of the turtles survived
the preceding experiment, we set out to determine
whether neonatal animals are able to withstand ex-
posure to moderate subzero temperatures above their
limit for supercooling. Accordingly, the six hatchlings
(again representing all four clutches) in this second
experiment were treated in the same way as animals
in the preceding experiment, except that temperature
in the chamber was lowered only to −6.5°C. This min-
umum was maintained for 24 h, after which temper-
ature was returned to 2°C and the condition of the
animals (i.e., alive or dead) was assessed. Temperature
profiles for the turtles again were examined for
the presence of freezing exotherms.

Our protocol caused the turtles to be exposed for 8
days to temperatures below the equilibrium freezing
point for their body fluids (approximately −0.7°C;
Storey et al., 1991; Packard and Packard, 1995; Cos-
tanzo et al., 2000) and for the last 24 h to minima
averaging −8.7°C (SD = 0.2°C; range, −8.5°C to
−8.9°C). The variation in minima recorded in different
jars is merely a reflection of the fact that environmen-
tal chambers seldom maintain uniform conditions
throughout their interior (Measures et al., 1973). None
of the turtles froze during the course of this experi-
ment, and all the hatchlings survived their exposure.

**Experiment 3.**—The remaining 10 turtles (represent-
ing all four clutches) were prepared for study as de-
scribed previously, but for this experiment the hatch-
lings were placed individually into artificial nests con-
structed in jars of damp, loamy sand (water content,
25 g/100 g dry soil; water potential, approximately
−50 kPa as estimated by thermocouple hygrometry).
Soil was tamped gently into spaces around each
hatchling to maximize its contact with the substratum
and thereby maximize the probability that the turtle
would be inoculated when water in the soil was sub-
sequently caused to freeze (Salt, 1963).

The closed jars were placed into the Percival envi-
ronmental chamber, which was set to bring tempera-
ture in the jars to approximately −0.4°C. This tem-
perature is below the equilibrium freezing point for
water in moist soils (Bodman and Day, 1943) but
above that for body fluids of baby painted turtles
(−0.7°C; Storey et al., 1991; Packard and Packard,
1995; Costanzo et al., 2000). Each jar then was opened;
a few pieces of shaved ice were placed onto the sur-
faced of the soil; and the jar was closed and quickly
placed back into the environmental chamber. Super-
cooled water in the soil began to freeze immediately,
as was indicated by a sudden increase in temperature
(i.e., by exotherms) in every jar to approximately 0°C
(Fig. 1). The temperature in the chamber was held at
the nominal level of −0.4°C for four days so that water
in the soil could freeze to an equilibrium.

After four days, temperature in the chamber was
lowered linearly at a rate of 1°C/day to a minimum
near −4.5°C. This minimum was maintained for seven
days before temperature in the chamber was reset to
2°C and the jars (and turtles) were allowed to rewarm.
The turtles then were removed and their condition
(alive or dead) was assessed from their appearance
and behavior. The minimum temperature and the du-
ration of exposure used here were the same as were
used in an earlier investigation of neonatal *P. bellii*
from northern North Dakota (Packard et al., 1997b),
thereby to enable us to compare responses by animals from
a southerly population with those of turtles from near
the northern limit of distribution. Later we download-
ed data from the datalogger to a PC and constructed
a temperature profile for each jar (i.e., for the turtle
and surrounding soil).

Temperature in the jars averaged −0.5°C (SD = 0.1°C;
range, −0.2°C to −0.6°C) after water in the soil
had frozen to a thermal equilibrium (Fig. 1), so none
of the animals was at risk of freezing at this early
point in the experiment (because temperature in all
the jars was above the equilibrium freezing point for
body fluids of the turtles). Temperature then was re-
duced to a minimum averaging −4.5°C (SD = 0.2°C;
range, −4.2°C to −4.7°C), which was maintained for
the requisite seven days. Temperature profiles re-
vealed that three turtles froze during their exposure
(Fig. 1A) but that remaining hatchlings remained un-
frozen (Fig. 1B). In all instances where an animal
froze, ice began to form in its body fluids only after
the turtle already had been in contact with ice and at
temperatures below the equilibrium freezing point for
Fig. 1. Temperature profiles for hatchling painted turtles confined in artificial nests in jars of damp, loamy sand. The left-hand arrow in each panel identifies the time at which ice was added to the jar to induce freezing of supercooled water in the soil. The right-hand arrow identifies the time at which temperature began to decline linearly at the rate of $1^\circ C/11034$ days.

(A) Profile for a turtle that froze after eight days in contact with ice and at temperatures below the equilibrium freezing point for body fluids; the spike in temperature on day 13 of the test is a freezing exotherm for the hatchling. (B) Profile for a turtle that remained unfrozen for the duration of its exposure.

The body fluids for 4–8 days (Fig. 1A). Frozen turtles were dead at the end of the experiment, but all the unfrozen animals were alive (Table 1).

Discussion.—Turtles in the first experiment were prevented from making contact with crystals of ice that might have penetrated their integument and caused their body fluids to freeze. Thus, freezing of these animals must have been initiated by heterogeneous nucleation (i.e., by nucleation caused by appropriately configured contaminants or inclusions), because homogeneous nucleation (i.e., spontaneous formation of suitable organizing sites by water molecules themselves) rarely occurs at temperatures above $-20^\circ C$ (Dorsey, 1948; Franks, 1985). Also, the nucleating agents in question were not overly efficient, because the animals typically did not freeze spontaneously until their body temperature was near $-17^\circ C$. This value is indistinguishable from those reported for hatchling bellii from more northerly populations in Nebraska (Packard and Packard, 1999; Costanzo et al., 2000).

Turtles in the second experiment also were prevented from making contact with crystals of ice that might have penetrated their integument and caused their body fluids to freeze. None of these animals froze, and all survived their exposure to temperatures near $-8.5^\circ C$. These findings reflect a level of cold-tolerance in hatchlings from central New Mexico similar to that of neonates from more northerly populations of bellii in Nebraska (Packard and Packard, 1999), Minnesota (Packard et al., 1999), and North Dakota (Packard et al., 1999b).

The three turtles that froze during their exposure to subzero temperatures in the third experiment presumably were caused to freeze by ice penetrating into body compartments from the surrounding soil, because none of the animals was exposed to a temperature low enough to elicit spontaneous freezing of its body fluids by heterogeneous nucleation. However, the integument of these animals afforded some resistance to the inward growth of ice crystals, because the turtles did not freeze until they had been in contact with ice (and at temperatures below the equilibrium freezing point for their body fluids) for several days. Additionally, the other seven turtles in this third experiment remained unfrozen for the duration of their exposure. Had the integument of the 10 animals in this test not resisted the penetration of ice into body compartments, the turtles surely would have frozen soon after their body temperature went below the equilibrium freezing point, much as occurs when frogs are caused to freeze by inoculation (Layne et al., 1990; Layne, 1991; Costanzo et al., 1999). A cutaneous barrier to penetration of ice also is characteristic of hatchling painted turtles from northerly populations of bellii (Packard et al., 1999b, 1999; Costanzo et al.,

<table>
<thead>
<tr>
<th>Turtle froze</th>
<th>New Mexico</th>
<th>North Dakota</th>
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<tbody>
<tr>
<td>Alive</td>
<td>0</td>
<td>3</td>
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<tr>
<td>Dead</td>
<td>7</td>
<td>8</td>
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Table 1. Survival by hatchling painted turtles confined in artificial nests in jars of damp, loamy sand and then exposed to $-4.5^\circ C$ for seven days. A freezing exotherm appeared in the temperature profile for each of the turtles that is said to have frozen, but no exotherm was detected in the profile for any other animal. Data for hatchlings from New Mexico are from the current study; those for neonates from North Dakota were taken from Packard et al. (1997b). The frequencies for freezing by animals in the two studies could not be distinguished statistically (Fisher’s Exact Test, $P = 1.0$).
2000; Willard et al., 2000); indeed, turtles from New Mexico had the same resistance to inoculation as animals from North Dakota (Table 1).

Finally, the animals that survived the third experiment were the ones that remained unfrozen, and the turtles that froze were the ones that died (Table 1; P = 0.008 by Fisher's Exact Test). Virtually identical results again were reported for hatchling painted turtles from populations of belii in Nebraska (Packard and Packard, 1997), North Dakota (Packard et al., 1997b), and Minnesota (Packard et al., 1999).

Thus, neonates from the southern limit of distribution for Chrysemys picta belii have a level of cold-tolerance that is indistinguishable from that of hatchlings from populations at the northern limit of distribution. Animals from northern and southern populations are able to resist the penetration of ice into body compartments from frozen soil, and they also have similar limits for supercooling. We do not know, however, whether the resistance to ice and cold manifested by hatchlings from New Mexico is an adaptation to conditions encountered in nests during winter or whether it derives from a suite of characters pre-adapting neonates for overwintering in nests at higher latitudes.

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LITERATURE CITED


The Breaking of Diapause in Embryonic Broad-Shell River Turtles (Chelodina expansa)

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The Australian broad-shelled turtle (Chelodina expansa) typically constructs its nests during the Austral autumn or early winter when soil temperatures are decreasing (Goode and Russell, 1968; Legler, 1985; Booth, 1998). Eggs laid in early autumn may experience warm temperatures for a month or two before soil temperature drops below 20°C, eggs laid in winter experience cool soil temperatures immediately, and eggs laid in late season nests experience warm temperatures (>20°C) throughout incubation (Booth, 1998). Embryos of C. expansa normally experience two periods of developmental diapause during ontogeny (Booth, 2000). The first is a preovipositional diapause (primary diapause) termed “extension of preovipositional arrest” by Ewert and Wilson (1996) that is common to all turtles (Ewert 1985, 1991), and in C. expansa this may extend for up to six weeks after oviposition (Booth, 2000). Once primary diapause is broken and the white-patch has developed to cover half to three-quarters of the eggshell, embryos invariably enter a second diapause period termed “embryonic diapause” by Ewert and Wilson (1996).

Embryos of C. expansa have an exceptionally long incubation period not only because embryonic development is inherently slow (Goode and Russell, 1968; Legler 1985) but also because embryos enter a second diapause stage (Booth, 1998, 2000). Other turtle species are reported to have extended incubation periods because of developmental arrest late in incubation (Ewert, 1985, 1991; Webb et al., 1986), but in C. expansa patterns of oxygen consumption indicate that development is continuous during the latter phase of incubation and that developmental arrest is confined to early incubation (Booth, 2000). If eggs of C. expansa are artificially incubated at a high and constant temperature immediately after oviposition, embryos still enter secondary diapause, but a large proportion fail to break out of this secondary diapause phase and perish (Booth, 2000). The failure to break secondary diapause when incubated at high and constant temperature appears to be a feature of turtle species that experience a secondary diapause period during embryonic development (Ewert 1985, 1991). Those embryos that break diapause do so asynchronously so that eggs from the same clutch hatch over a large period of time (up to 70 days; Booth, 2000). Asynchronous hatching is probably maladaptive in natural nests (Booth, 2000), but embryos in natural nests appear to hatch at a similar time (Booth, 1998). In natural nests of C. expansa, both daily and seasonal changes in nest temperature occur (Booth, 1998), so temperature is a likely cue for the synchronous breaking of secondary diapause in this species. Indeed changes in temperature appear to break arrested development in other turtle species (Ewert, 1991; Ewert and Wilson, 1996). In a closely related species Chelodina rugosa, which has the unusually habit of depositing its eggs underwater in drying swamps, the stimulus for breaking primary diapause is drying of mud which then allows oxygen to enter the egg (Kennett et al. 1993). However, embryos of C. rugosa have never been reported to enter secondary diapause during embryonic development. I artificially incubated eggs of C. expansa under three different thermal regimes in order to investigate the role change in temperature has in breaking secondary diapause.

I collected two clutches of eggs of C. expansa immediately after natural oviposition on 26 May 2000. Eggs were transported to the laboratory, rinsed briefly in tap water to remove soil adhering to the eggshell, and weighed. Eggs had the clutch and egg number marked on the eggshell with graphite pencil, and eggs from each clutch were evenly distributed across three plastic incubation boxes. Eggs were incubated half-buried in vermiculite with a water potential of ~150 kPa and sealed in boxes with a loose-fitting lid. Temperature data loggers that recorded temperature twice per hour were placed in boxes at this time. Eggs and boxes were weighed periodically throughout incubation and water lost from the vermiculite was replaced to ensure relatively stable water potential throughout incubation (Packard et al., 1981). Each of the boxes was assigned to one of three treatments (Fig. 1). In the first treatment, which was designed to imitate eggs laid in early autumn, eggs were incubated at 25°C for 30 days, transferred to 18°C for 67 days and then incubated until hatching at 28°C. In the second treatment, which was designed to imitate eggs laid during winter, eggs were incubated for 97 days at 18°C then incubated until hatching at 28°C. In the third treatment, which was also designed to imitate eggs laid during winter, eggs were incubated for 97 days at 18°C, then...