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

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APPENDIX 1

*Specimens Examined*

All specimens examined were from the Museum of Vertebrate Zoology (MVZ), University of California, Berkeley, California, the Natural History Museum of Los Angeles County (LACM), California, and the museum at Arizona State University (ASU), Arizona, USA. Unless noted otherwise, all localities of specimens are from California, USA.

*Crotalus viridis oreganus*

MVZ: 3818, San Luis Obispo; 10537, San Mateo; 14597, Trinity; 17584, Mendocino; 18407, Siskiyou; 21379, 21381, San Benito; 21574, San Mateo; 28214, 28216, 28217, Stanislaus; 34824, Tuolumne; 42653, 42662, 42669, Madera; 92684, 92685, Shasta; 191367, San Joaquin; 193427, 193428, Siskiyou; 29238, Grant (Oregon).

*Crotalus viridis helleri*

MVZ: 391, 55780, San Diego; 33588, 34643, 35357, Santa Barbara; 35427, Ventura; 55780, San Diego. LACM: 3074, 20083, 20088, 28029, 28031, 59179, Los Angeles. ASU; 30984, Los Angeles.

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## 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 an unfrozen, liquid state (Dorsey, 1948; Franks, 1985). 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 in 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 (nom-

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inal 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 carapace 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 thermocouples 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 immediately 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 activity, 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 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 supercooling 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°) 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 exposure 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 −8.5°C. This minimum was maintained for 24 h, after which temperature 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; Costanzo et al., 2000) and for the last 24 h to minima averaging −8.7°C (SD = 0.2°; range, −8.5°C to −8.9°C). The variation in minima recorded in different jars is merely a reflection of the fact that environmental chambers seldom maintain uniform conditions throughout their interior (Measures et al., 1973). None of the turtles froze during the course of this experiment, and all the hatchlings survived their exposure.

*Experiment 3.*—The remaining 10 turtles (representing all four clutches) were prepared for study as described previously, but for this experiment the hatchlings were placed individually into artificial nests constructed 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 subsequently caused to freeze (Salt, 1963).

The closed jars were placed into the Percival environmental chamber, which was set to bring temperature in the jars to approximately −0.4°C. This temperature 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 surface of the soil; and the jar was closed and quickly placed back into the environmental chamber. Supercooled 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 duration of exposure used here were the same as were used in an earlier investigation of neonatal *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 downloaded 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 reduced 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 revealed that three turtles froze during their exposure (Fig. 1A) but that remaining hatchlings remained unfrozen (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

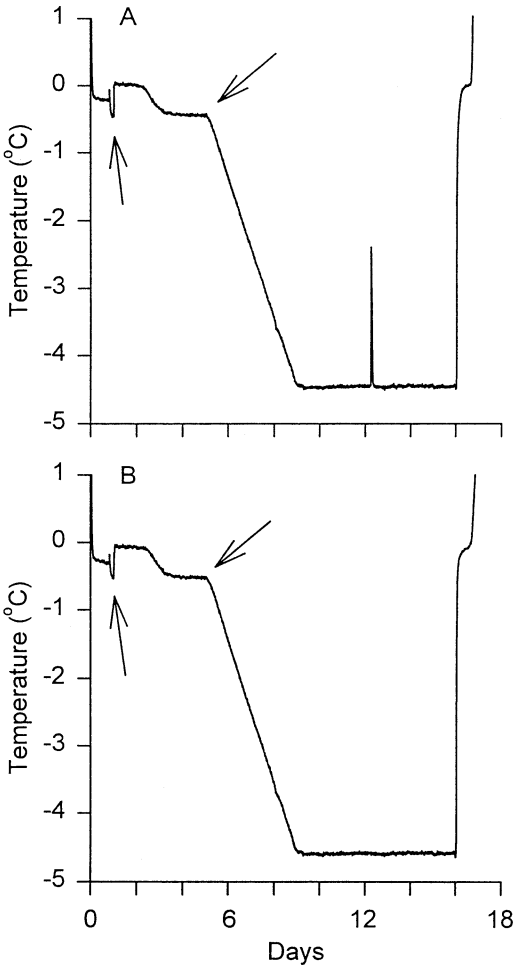


FIG. 1. Temperature profiles for hatchling painted turtles confined in artificial nests in jars of damp, loamy sand. The left-hand arrow in each panel identifies the time at which ice was added to the jar to induce freezing of supercooled water in the soil. The right-hand arrow identifies the time at which temperature began to decline linearly at the rate of 1°C/day. (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°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°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).

TABLE 1. Survival by hatchling painted turtles confined in artificial nests in jars of damp, loamy sand and then exposed to −4.5°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, $P = 1.0$).

| Turtle froze | New Mexico | | North Dakota | |
|---|---|---|---|---|
| | Alive | Dead | Alive | Dead |
| Yes | 0 | 3 | 0 | 8 |
| No | 7 | 0 | 24 | 0 |

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°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., 1997b).

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., 1997b, 1999; Costanzo et al.,

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 *bellii* 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 bellii* 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 preadapting neonates for overwintering in nests at higher latitudes.

*Acknowledgments.*—Our procedures were considered and approved by the Committee for Care and Use of Animals in Research at Iowa State University (protocol 12-9-4382-1-J) and by the Committee for Animal Care and Use at Colorado State University (protocol 00-101A-01). Female turtles were collected and handled under authority of permit 3040 from the New Mexico Department of Game and Fish. The research was supported in part by a Grant-in-Aid-of-Research from the Society of the Sigma Xi (to CM), by a predoctoral fellowship from the National Science Foundation (to CM), and by grant IBN-9612562 from the National Science Foundation (to GCP). We also thank M. Knutzen for helping to care for the eggs.

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## 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 rare 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 secondary diapause stage (Booth, 1998, 2000). Other turtle species are reported to have extended incubation periods because of developmental attest late in incubation (Ewert, 1985, 1991; Webb et al., 1986), but in *C. expansa* patterns of oxygen consumption indicate that development is continuos 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 ovipostion 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