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## Review

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# To freeze or not to freeze: adaptations for overwintering by hatchlings of the North American painted turtle

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### Summary

Many physiologists believe that hatchling painted turtles (*Chrysemys picta*) provide a remarkable, and possibly unique, example of ‘natural freeze-tolerance’ in an amniotic vertebrate. However, the concept of natural freeze-tolerance in neonatal painted turtles is based on results from laboratory studies that were not placed in an appropriate ecological context, so the concept is suspect. Indeed, the weight of current evidence indicates that hatchlings overwintering in the field typically withstand exposure to ice and cold by avoiding freezing altogether and that they do so without benefit of an antifreeze to depress the equilibrium freezing point for bodily fluids. As autumn turns to winter, turtles remove active nucleating agents from bodily fluids (including bladder and gut), and their integument becomes a highly efficient barrier to the penetration of ice into body compartments from frozen soil. In the absence of a nucleating agent or a crystal of ice to ‘catalyze’ the transformation of water from liquid to

solid, the bodily fluids remain in a supercooled, liquid state. The supercooled animals nonetheless face physiological challenges, most notably an increased reliance on anaerobic metabolism as the circulatory system first is inhibited and then caused to shut down by declining temperature. Alterations in acid/base status resulting from the accumulation of lactic acid may limit survival by supercooled turtles, and sublethal accumulations of lactate may affect behavior of turtles after the ground thaws in the spring. The interactions among temperature, circulatory function, metabolism (both aerobic and anaerobic), acid/base balance and behavior are fertile areas for future research on hatchlings of this model species.

Key words: turtle, *Chrysemys picta*, hibernation, freezing, supercooling.

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### Introduction

Hatchlings of the North American painted turtle [Family Emydidae: *Chrysemys picta* (Schneider 1783)] typically spend the first winter of their life in the shallow, subterranean nest where they completed incubation and hatched the preceding summer (Ultsch, 1989; Ernst et al., 1994). This behavior commonly causes neonates in populations from Nebraska (Packard, 1997; Packard et al., 1997a; Costanzo et al., 2004), northern Illinois (Weisrock and Janzen, 1999) and northern 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  $-10^{\circ}\text{C}$  (Fig. 1). Hatchling painted turtles are small (~3–6 g), ectothermic animals whose body temperature closely tracks that of their immediate surroundings (Claussen and Zani, 1991), so temperature in the nest is a close approximation to that of the turtles themselves. Many hatchlings withstand such extremes and emerge from their nest when the soil finally thaws in the spring (Woolverton, 1963;

Storey et al., 1988; DePari, 1996; Weisrock and Janzen, 1999; Nagle et al., 2000; Costanzo et al., 2004).

How do neonatal painted turtles survive in the cold? Early research on this subject revealed that hatchlings are able to withstand freezing by as much as 50% of their bodily water, and this discovery led in turn to the proposition that turtles spend some part of the northern winter in a frozen state (Storey et al., 1988; Churchill and Storey, 1992). The case was quite convincing (Storey, 1990; Storey and Storey, 1992, 1996), and the concept of ‘natural freeze-tolerance’ in hatchling painted turtles quickly found its way into textbooks of animal physiology (e.g. Schmidt-Nielsen, 1997). Work performed more recently, however, indicates that the correlation between overwintering by hatchlings in their nest and the indisputable ability of the animals to recover from limited freezing (see Rubinsky et al., 1994) is probably spurious and that a tolerance for freezing is not a general means by which hatchling painted turtles withstand exposure

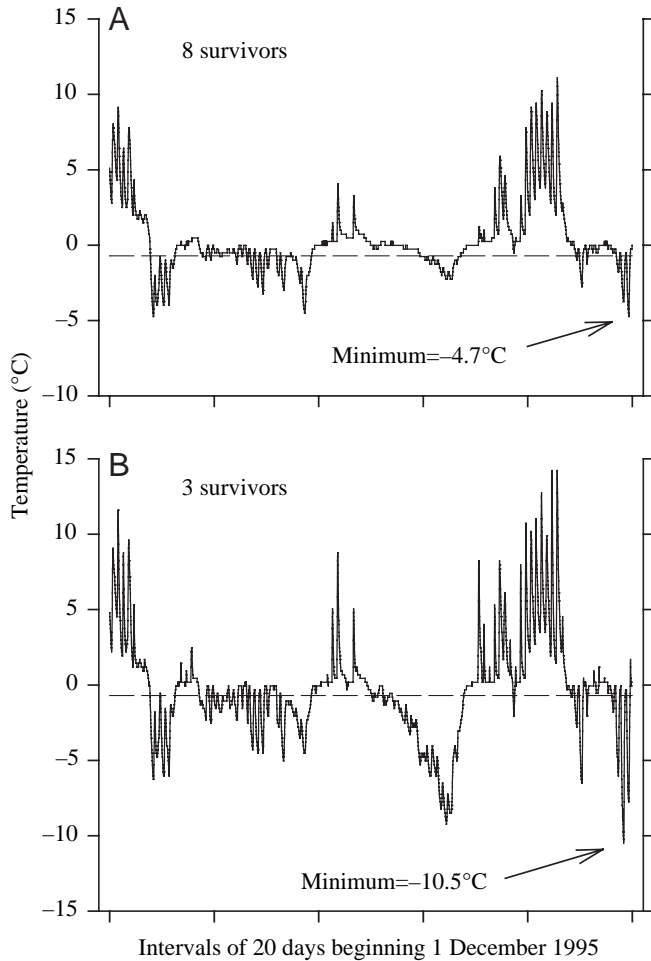


Fig. 1. Temperature profiles for representative nests at our study site in north-central Nebraska. Both nests contained eight painted turtles that were hatched in the laboratory and then placed into the nests in late October. (A) A relatively warm nest; (B) a relatively cold nest. The broken horizontal lines mark the equilibrium freezing point for bodily fluids of hatchlings (Storey et al., 1991; Packard and Packard, 1995; Costanzo et al., 2000b). Animals in this study hatched on substrates of vermiculite and consequently had a somewhat greater capacity for supercooling than turtles hatching in the field (Packard et al., 2001). Data are from Packard et al. (1997a).

to low temperatures in the field (Packard and Packard, 2003a; Packard et al., 1997b, 1999a).

The preceding contention is based on numerous reports that turtles are able to withstand freezing – but only when said freezing occurs under a set of very restrictive conditions that are likely to have limited relevance to the natural history of the animals. For example, if hatchlings are to survive freezing, they must begin to freeze at a temperature that is only slightly below the equilibrium freezing point for their bodily fluids (Packard et al., 1999b), that is, at a temperature only marginally lower than  $-0.7^{\circ}\text{C}$  (Storey et al., 1991; Packard and Packard, 1995; Costanzo et al., 2000b). The initiation of freezing at such a high subzero temperature apparently protects turtles from the osmotic shock to cells that accompanies the

'flash freezing' of more deeply supercooled animals (Claussen et al., 1990; Storey and Storey, 1996; Lee and Costanzo, 1998). Osmotic shock associated with flash freezing is usually fatal (Claussen et al., 1990), even to hatchling painted turtles (see Packard et al., 1999b).

Additionally, hatchling painted turtles recover from freezing only in the event that their exposure to cold is relatively brief and that temperature does not go below  $-4^{\circ}\text{C}$  (Churchill and Storey, 1992; Costanzo et al., 1995; Attaway et al., 1998; Packard et al., 1999b; Packard and Packard, in press). Frozen turtles typically withstand exposure to  $-2^{\circ}\text{C}$  for as long as 4 days, but most animals that are held frozen at this temperature have died from unknown causes by the end of 6 days and none survives for as long as 8 days (Fig. 2; also Churchill and Storey, 1992). Moreover, when hatchlings are frozen over 24 h to a thermal equilibrium at  $-2^{\circ}\text{C}$  and then

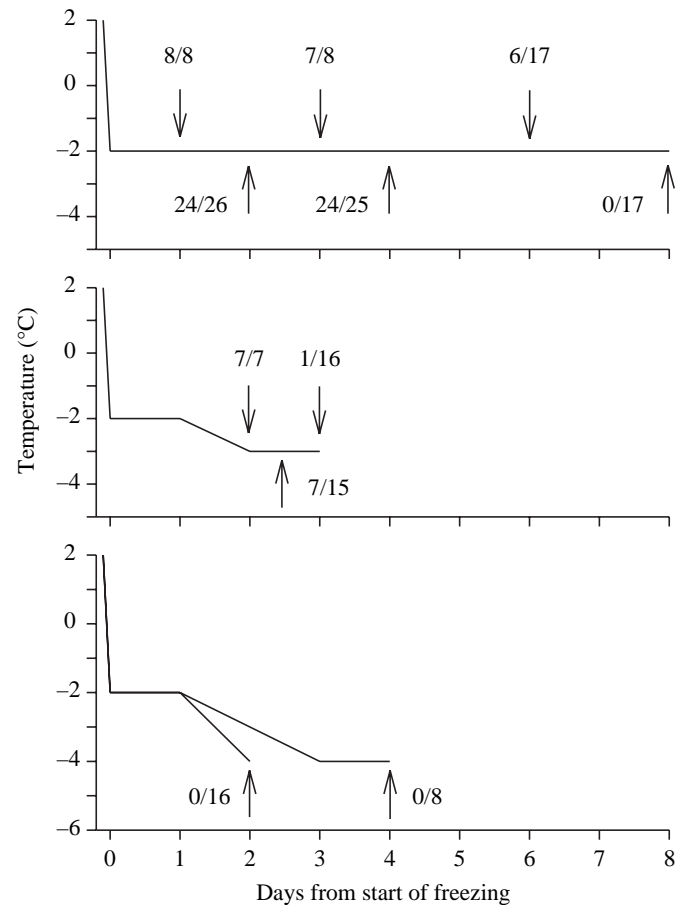


Fig. 2. Hatchling painted turtles were inoculated with ice at temperatures only slightly below the equilibrium freezing point for bodily fluids (approx.  $-0.7^{\circ}\text{C}$ ). After the animals had frozen to a thermal equilibrium at  $-2^{\circ}\text{C}$ , they were exposed for varying periods to temperatures between  $-2$  and  $-4^{\circ}\text{C}$  (as indicated by lines representing the different temperature profiles). Samples were removed at times designated by arrows, and the turtles were allowed to thaw slowly. The number to the left of each solidus is the number of survivors; the number to the right of each solidus is the sample size. Data are from Packard et al. (1999b) and Packard and Packard (in press).

exposed to slightly lower subzero temperatures, the animals recover from a very brief exposure to  $-3^{\circ}\text{C}$ , but mortality increases rapidly thereafter and is nearly complete after 24 h (Fig. 2; also Costanzo et al., 1995). Finally, frozen hatchlings typically are unable to withstand even brief exposures to  $-4^{\circ}\text{C}$ , which represents the absolute lower limit of tolerance (Fig. 2; also Churchill and Storey, 1992; Costanzo et al., 1995).

Two exceptions to the preceding generalizations merit brief mention. First, Churchill and Storey (1992) reported that two hatchlings studied at the end of hibernation (April) survived freezing at  $-2.5^{\circ}\text{C}$  for 11 days; that is, the turtles survived in a frozen state for longer than has been reported in any other investigation (see Fig. 2). This finding, which is unusual even in comparison with other results from the same investigation, could not be confirmed by research performed more recently (Packard and Packard, in press). Second, Costanzo et al. (2004) reported that animals collected during winter from nests in the field withstood freezing at  $-3.0^{\circ}\text{C}$  for 3 days, which again is a survival time that exceeds expectation (Fig. 2). We suspect, however, that many of the turtles studied by Costanzo et al. did not freeze in the intended way at the outset of study and that most of these turtles remained unfrozen for the duration of their exposure to subzero temperature (see Packard and Packard, 1993a). Thus, while we acknowledge the potential importance of these deviant findings, we do not attach significance to them at this time.

Accordingly, we take the weight of evidence to indicate that hatchling painted turtles may survive freezing in nature, but only in the event that (1) they begin to freeze at a temperature near the equilibrium freezing point for their bodily fluids (i.e. without supercooling appreciably) and (2) the exposure is limited in duration and to temperatures above  $-4^{\circ}\text{C}$ . However, turtles that freeze in the laboratory under conditions like those they encounter in natural nests usually are supercooled by several degrees at the instant of nucleation and the experience is usually fatal (Packard and Packard, 1993b, 1995; Packard et al., 1997b, 1999a). Moreover, temperatures in natural nests at northern localities generally go below  $-2^{\circ}\text{C}$  for longer than 4 days, and they commonly go below the minimum tolerable temperature of  $-4^{\circ}\text{C}$  at some point during the winter (Woolverton, 1963; DePari, 1996; Packard, 1997; Packard et al., 1997a; Weisrock and Janzen, 1999; Nagle et al., 2000; Costanzo et al., 2004). These considerations alone render it highly unlikely that hatchlings overwintering in the field typically survive until spring by withstanding the physiological challenges of freezing. Moreover, even when temperatures in nests are sufficiently benign that hatchlings could conceivably survive a short bout of freezing (e.g. Nagle et al., 2000; Costanzo et al., 2004), the turtles are unlikely to be frozen (Packard and Packard, 1995, 2003a,b; Willard et al., 2000) for reasons detailed below.

The physiology of hatchlings also has bearing on the question of natural freeze-tolerance in painted turtles. For example, neonates do not produce any of the nucleating agents, thermal hysteresis factors or cryoprotectants that enable some arthropods and anurans to withstand freezing by water in the

extracellular space (Storey et al., 1988, 1991; Churchill and Storey, 1991; Costanzo et al., 2000b), and acclimation of hatchling painted turtles to low (but non-freezing) temperatures elicits a suite of physiological and behavioral responses that actually are appropriate to an alternative adaptive strategy for withstanding exposure to ice and cold (see below). Such findings, when taken together with the ecological factors mentioned earlier, indicate that tolerance of hatchlings for freezing simply reflects a limited capacity to withstand the physiological stress associated with the formation of ice in bodily fluids (Baust, 1991). This ability to survive the stress of limited freezing is not unique to hatchling painted turtles but instead is a trait that is shared with neonates of several other species of turtle, most of which have distributions or life histories that effectively prevent neonates from being exposed to the threat of freezing (Costanzo et al., 1995; Packard et al., 1997c, 1999b, 2000b). Consequently, the ability of hatchling painted turtles to withstand limited freezing probably is a correlate of some as-yet unidentified process that is widespread in turtles, and it is no more an adaptation to cold than the limited capacity for humans to recover from serious burns is an adaptation to mishaps involving fire (see Baust, 1991). This is not to say that hatchlings in the field never recover from shallow freezing (Costanzo et al., 2004); it is simply to say that recovery from freezing probably is not a phenomenon of general importance to the species and that the capacity is not an outcome of natural selection enhancing the ability for turtles to recover from freezing by water in the extracellular compartment.

A more likely explanation for how neonatal painted turtles typically withstand the rigors of winter in the northern United States and southern Canada is that they remain unfrozen and that they do so without the benefit of an antifreeze (Costanzo et al., 2000b; Packard and Packard, 2003a; Packard et al., 1997b, 1999a). The secret to such an adaptive strategy is to remove nucleating agents from all body compartments and simultaneously to prevent ice crystals from entering the body from frozen soil (Packard and Packard, 2003a). In the absence of an organizing site to facilitate the change in phase from liquid to solid, bodily fluids cannot freeze at high subzero temperatures (Franks, 1985; Wilson et al., 2003). Hatchling map turtles [*Graptemys geographica* (Le Sueur 1817)] probably manifest the same adaptive strategy for overwintering that we advance here for neonatal painted turtles (Baker et al., 2003).

### The innate capacity for supercooling

Newly hatched painted turtles taken from natural nests in the field can be cooled to approximately  $-5.5^{\circ}\text{C}$  before they begin to freeze spontaneously by heterogeneous nucleation (left column in Fig. 3). Measured values are tightly clustered, which points to relatively high concentrations of nucleating agents in bodily fluids, bladder or gut of the animals (Salt, 1970). Only 2 months later, however, hatchlings collected from similar nests can be cooled to approximately  $-7.5^{\circ}\text{C}$  before they freeze

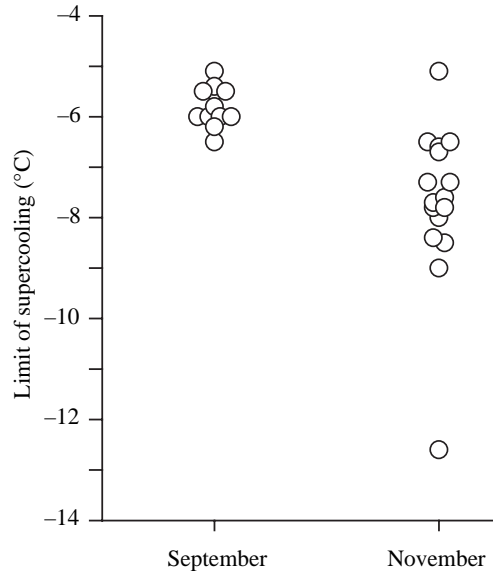


Fig. 3. Values for the limit of supercooling for individual painted turtles collected at our field site in north-central Nebraska in September (four hatchlings from each of three clutches) and November (four hatchlings from each of four clutches). The turtles were placed into the experimental protocol the day after they were removed from the nests in which they completed incubation and hatched. The distribution of values for animals studied in September differs from that for turtles studied in November (Wilcoxon two-sample test,  $z=3.86$ ,  $P<0.001$ ). Data are from Packard et al. (2001).

spontaneously (right column in Fig. 3), and values for individual turtles exhibit the increased scatter that usually is associated with generally lower, but more variable, concentrations of nucleators (Salt, 1970). This enhanced capacity for supercooling among turtles collected in late autumn is of apparent benefit to animals that soon will be exposed to subzero temperatures (Fig. 1). Questions arise, however, concerning the stimulus for the increase in capacity for supercooling and the means by which this increase is achieved.

Key to understanding this process is an appreciation of one of the many peculiarities of turtles. As baby turtles are breaking out of their egg, they typically ingest quantities of soil and fragments of eggshell (Packard et al., 2000a, 2001; Costanzo et al., 2003; Packard and Packard, 2003c). Such geophagy is of uncertain function in any animal (Dominy et al., 2004), but the behavior has important consequences for creatures such as hatchling painted turtles. Natural soils typically contain potent nucleating agents – probably bacteria such as *Pseudomonas syringae* (Cochet and Widehem, 2000) – capable of initiating the formation of ice at relatively high temperatures (Costanzo et al., 1998b, 2000a, 2001c). If water in the gut begins to freeze, however, the resulting crystals of ice penetrate the wall of the intestine and initiate ('seed') freezing by water in the extracellular compartment, in much the same manner that ice forming in the gut of insects propagates across the wall of that organ and into the hemolymph (Salt, 1966; Shimada, 1989;

Block, 1990). Thus, the nucleating agents in the gut of newly hatched painted turtles set the limit of supercooling at temperatures between approximately  $-5$  and  $-6^{\circ}\text{C}$  (Costanzo et al., 2003).

Baby painted turtles purge their gut of particulate matter before the start of winter (Packard et al., 2001; Costanzo et al., 2003; Packard and Packard, 2003c), and the purging is accompanied by a reduction in the population of bacterial nucleators (Fig. 3). However, emptying the gut of particulate matter is not, by itself, sufficient to remove the nucleators. Turtles that are held at a relatively high ambient temperature for 6 weeks following hatching purge most or all of the particulate matter from their gut, but this purging does not result in a major reduction in the limit for supercooling, and the apparent concentration of nucleating agents remains high (as indicated by the low variability for data in column 2 of Fig. 4). By contrast, turtles that are acclimated to approximately  $3^{\circ}\text{C}$  over the same 6-week period purge all particulate material from their gastrointestinal (GI) tract, yet the animals also achieve a substantial reduction in the apparent concentrations of nucleators (note the generally lower values and increased variability for data in column 3 of Fig. 4). Consequently, the limit of supercooling for acclimated turtles is considerably lower than that of recent hatchlings as well as that of turtles held at high ambient temperature (Fig. 4). Full development of the capacity for supercooling seems, therefore, to require that hatchlings be exposed to declining temperatures like those to which animals in natural nests are exposed as autumn turns to winter (Costanzo et al., 2000b; Packard and Packard, 2003c).

It is unclear exactly how acclimation to low temperature elicits a reduction in the populations of bacterial nucleators in the guts of hatchling painted turtles, but the findings for turtles have parallels among freeze-intolerant terrestrial insects, many of which also purge their gut of food and nucleating agents before the start of winter (Lee et al., 1996). Such purging of the gut of particulate material seldom enables the insects in question to remove all the nucleating agents, which persist in variable numbers in an otherwise empty GI tract (Strong-Gunderson et al., 1990; Costanzo et al., 1998a; Castrillo et al., 2001) and which elicit spontaneous freezing over relatively wide ranges of temperature (Salt, 1970; Strong-Gunderson et al., 1990).

### The role of the integument

If overwintering hatchlings are to exploit their full capacity for supercooling (see Fig. 4), they must avoid being penetrated by crystals of ice growing inward from the frozen soil, because penetrating ice would provide organizing sites for bodily water to change phase from liquid to solid (Franks, 1985). This consideration alone points to an important role for the integument in an adaptive strategy that has supercooled hatchlings spending the winter in the frost zone of the soil. Evidence supporting this idea comes from several experiments demonstrating that neonates whose body surface makes

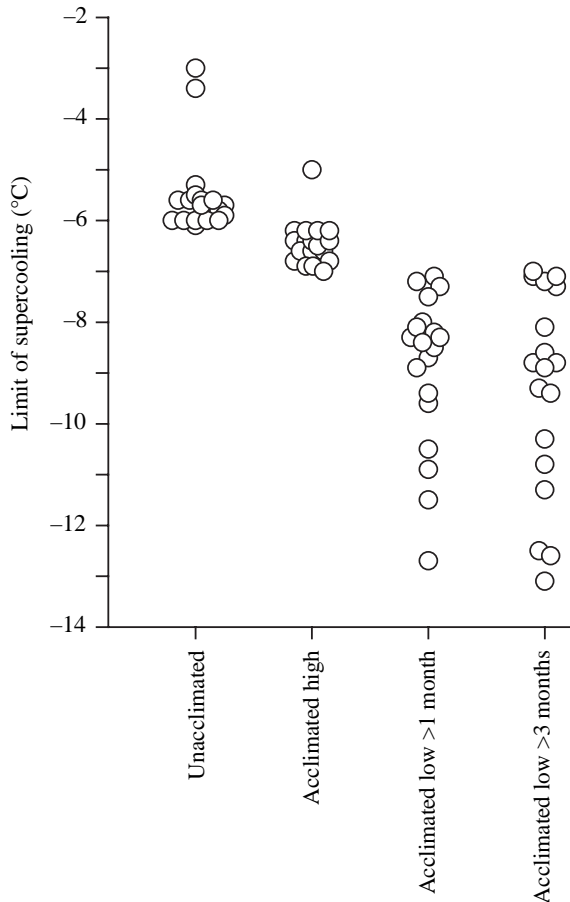


Fig. 4. Values for the limit of supercooling for individual painted turtles that hatched from eggs incubated on natural soil in the laboratory. Unacclimated animals were studied within days of the time they hatched; animals in the high acclimation group were held at 24°C for 6 weeks; turtles in the low acclimation groups were acclimated to 3°C over periods of 6 weeks and 15 weeks. The distribution of values for unacclimated animals differs from the distribution for turtles acclimated at high temperature (Wilcoxon two-sample test,  $z=4.87$ ,  $P<0.001$ ), and the distribution for the latter differs from that for hatchlings acclimated at low temperature for 6 weeks (Wilcoxon two-sample test,  $z=5.40$ ,  $P<0.001$ ). Data are from Packard and Packard (2003c).

intimate contact with wet soil seldom are inoculated when water in the soil subsequently freezes (Packard and Packard, 1993a,b, 1995, 2003a,b; Packard et al., 1997b, 1999a). Moreover, damage to the integument greatly increases the risk of freezing by hatchlings (Willard et al., 2000), presumably by providing a pathway for ice to grow through what is otherwise a highly resistant barrier.

It is noteworthy, however, that the integument affords little resistance to the penetration of ice into turtles in the days immediately after hatching (Costanzo et al., 2000b; Packard and Packard, 2003b), at which time neonates are highly susceptible to freezing by inoculation (Fig. 5). The cutaneous barrier to penetration of ice – like the capacity for supercooling – is enhanced in the weeks leading up to winter, and the process

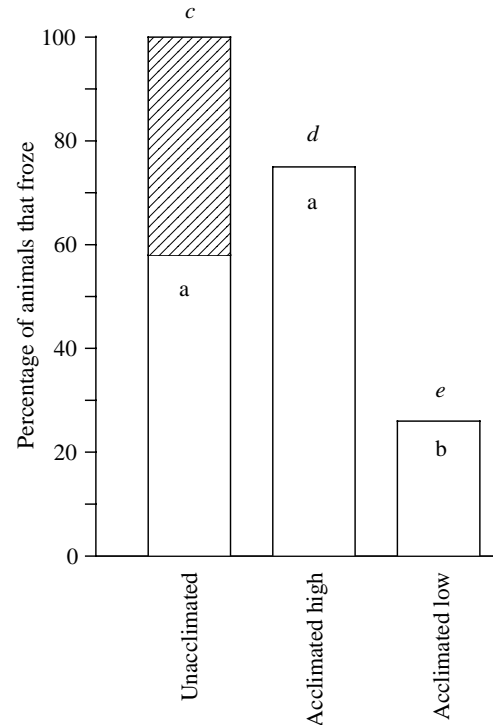


Fig. 5. The risk to hatchling painted turtles of freezing by inoculation was assessed in animals that had just completed incubation at 27°C (unacclimated) and in neonates that were acclimated at a high temperature (24°C) or a low temperature (3°C). Turtles were placed individually into artificial nests where they came into intimate contact with moist soil. Water in the soil first was caused to freeze at a temperature above the equilibrium freezing point for bodily fluids of hatchlings, so turtles came into contact with ice before they were susceptible to freezing. Temperature then was lowered to  $-2^{\circ}\text{C}$ , which is above the temperature at which hatchlings freeze spontaneously by heterogeneous nucleation, and this temperature was maintained for 7 days. Spikes (exotherms) in the temperature profiles yielded evidence for freezing by the turtles themselves. A malfunction of an environmental chamber caused background noise to be extraordinarily high in temperature profiles for unacclimated turtles, and some freezing exotherms consequently may have escaped detection. Indeed, all the animals for which no exotherm was detected (hatched bar in left column) are thought to have frozen. Letters displayed in italics above the bars are from one statistical analysis, and those within the bars are from another. Bars that share a letter from the same analysis cannot be distinguished statistically. Figure reproduced from Packard and Packard (2003b).

again requires that hatchlings be exposed to low temperatures like those to which they would be exposed in natural nests (Fig. 5). The specific changes leading to enhancement of the cutaneous barrier are currently unknown, but an explanation probably lies in a better understanding of the epidermis, which is the site of barrier functions in amniotes generally (Alibardi, 2003).

Two kinds of epidermis cover the body of neonatal painted turtles. Epidermis overlying the head, neck and limbs (and including that in the axillary and inguinal pockets) is limited

externally by an  $\alpha$ -keratin layer comprised of distinct cells and intercellular spaces (Landmann, 1986; Alibardi, 1999, 2002). By contrast, epidermis covering the shell (i.e. the carapace, plastron and bridges) is bounded on the external surface by a compact  $\beta$ -keratin layer in which intercellular spaces are reduced or absent (Alibardi and Thompson, 1999; Alibardi, 2002; Alibardi and Sawyer, 2002). The outermost cells of the epidermis are flattened, dead structures containing filaments of either  $\alpha$ -keratin or  $\beta$ -keratin, as is implied by the names assigned to the respective layers. The  $\alpha$ -keratin layer is what confers flexibility to skin on the head, neck and limbs, while the  $\beta$ -keratin layer causes scutes on the shell to be hard and inflexible.

The  $\alpha$ -keratin layer of epidermis from flexible skin is similar in its general appearance to the stratum corneum of mammalian integument, which has been likened to a wall formed from 'bricks and mortar'. The 'bricks' in the stratum corneum of mammalian epidermis are the corneocytes, and the 'mortar' is the lipid occupying the intercellular domain (Elias, 1983; Elias and Menon, 1991; Menon and Ghadially, 1997). Moreover, lipids in the extracellular matrix of mammalian stratum corneum limit the loss of water from body compartments to the surrounding atmosphere (Elias and Friend, 1975; Grubauer et al., 1989; Elias and Feingold, 1992), whereas lipids in the  $\alpha$ -keratin layer of flexible skin from exposed surfaces of neonatal painted turtles seemingly resist the penetration of ice crystals into body compartments from frozen soil (Willard et al., 2000). The parallels in structure and function between flexible skin of turtles and the integument of mammals are apparent.

Flexible skin from cold-acclimated hatchlings of the painted turtle varies from place to place over the surface of the body in the amount of lipid that is present in the  $\alpha$ -keratin layer, and this variation also seems to be correlated with regional variation in effectiveness of the barrier to inoculation. The  $\alpha$ -keratin layer of skin from exposed surfaces of the forelimbs, for instance, has dense deposits of lipid in the inner domain whereas the  $\alpha$ -keratin layer of integument from more protected sites at the base of the neck lacks such deposits (Willard et al., 2000). The exposed sites on the forelimbs are suspected to be resistant to inoculation while skin at the base of the neck and limbs is thought to be penetrated more readily by crystals of ice (Packard and Packard, 1995). Thus, resistance of flexible skin to inoculation is positively correlated with lipid in the  $\alpha$ -keratin layer. Parallels with mammals again are apparent, because mammalian skin exhibits regional variation in lipid content and in efficacy of the integument in resisting the passage of water (Elias et al., 1981; Lampe et al., 1983; Law et al., 1995).

The similarities between the stratum corneum of mammals and the  $\alpha$ -keratin layer of flexible integument from hatchling painted turtles also afford a plausible explanation for how the cutaneous barrier of neonates becomes enhanced by cold acclimation. For example, the stress of desiccation elicits an increase in formation and deposition of lipid (especially ceramides) in the stratum corneum of mammalian skin (Denda et al., 1998; Kömüves et al., 1999). We speculate, therefore,

that acclimation of hatchling painted turtles to cold entails an increase in the amount of lipid in the  $\alpha$ -keratin layer of epidermis overlying exposed surfaces of the head and limbs and that this accounts for the increase in effectiveness of the cutaneous barrier to inoculation (Fig. 5).

However, a substantial (arguably the largest) fraction of the epidermis making contact with frozen soil is that of the shell. Certainly this is so when hatchlings withdraw their head and limbs into the shell, as we have observed them to do when they are exposed to low temperatures (also Storey et al., 1988). The resistance of the shell to penetration by growing crystals of ice has not been studied, but we predict that  $\beta$ -epidermis is an effective barrier to inoculation right from the time of hatching. This prediction is based on studies indicating (1) that scutes covering the shell are largely impermeable to water (Rose, 1969), (2) that water traverses the epidermis of mammalian integument *via* intercellular spaces (Elias and Friend, 1975; Nemanic and Elias, 1980; Simonetti et al., 1995; Meuwissen et al., 1998) and (3) that intercellular spaces are absent from the  $\beta$ -keratin layer overlying the shell of turtles (Alibardi and Thompson, 1999; Alibardi, 2002). Assuming that crystals of ice grow through the same channels that are followed by water, we hypothesize that ice is unable to penetrate integument overlying the shell of hatchling painted turtles owing to the absence of pathways through which the crystals might grow.

In summary, we believe that  $\beta$ -epidermis overlying the shell is impermeable to ice from the moment of hatching, and that the  $\alpha$ -epidermis on exposed surfaces of head and limbs has a low permeability to ice in acclimated hatchlings (but not in unacclimated ones). The part of the  $\alpha$ -epidermis that is in more concealed sites at the base of the neck and limbs apparently affords less protection against inoculation, even in acclimated turtles, but these sites are also shielded somewhat from exposure to ice in the soil, thereby reducing the risk that animals will be inoculated across these surfaces.

### Physiological consequences of supercooling

#### *Differences in vapor pressure*

The vapor pressure for bodily fluids of supercooled turtles is higher than that of ice at the same temperature (Zachariassen, 1991), so overwintering hatchlings of the painted turtle must experience a loss of water by evaporation for the entire time that they are exposed to subzero temperatures in frozen soil (see Lundheim and Zachariassen, 1993). Unfortunately, the extent of dehydration by animals overwintering in the field is virtually unstudied (Costanzo et al., 2001b), so little can be said about the potential for differential losses of water by turtles subjected to different thermal regimes in different nests. Nonetheless, the loss of water by overwintering animals conceivably affects their locomotor performance when they emerge above ground in the spring and begin their overland trek to water, because the performance of desiccated turtles lags behind that of fully hydrated ones (Miller et al., 1987; Finkler, 1999).

Of more immediate concern, however, is the likelihood that

losses of water across the integument actually increase the risk to overwintering hatchlings of freezing by inoculation (Packard and Packard, 1993b). By this hypothesis, vapor escaping across the skin (or from a bodily orifice) condenses on the nearest crystal of ice in the environment, thereby causing the crystal to grow toward the source of the vapor (Salt, 1963). If temperature in the nest is low enough (thereby increasing the transcutaneous gradient in vapor pressure), or if the exposure to subzero temperature is long enough, the crystal penetrates into and through the integument and seeds the formation of ice in the extracellular space (Packard and Packard, 1993b). Such delayed inoculation usually results in the death of the hatchling (Packard and Packard, 1993a,b, 1995; Packard et al., 1997b, 1999a).

#### Circulation, metabolism and acid/base balance

Total metabolic activity has yet to be measured in supercooled hatchlings, but it seems reasonable at this juncture to assume that metabolism and temperature are positively correlated. However, the oxygen-dependent component of metabolism seems to be affected more than total metabolic activity by declining temperature, owing to the influence of temperature on cardiac activity. The heart of a hatchling painted turtle contracts about once each minute at 0°C, but heart rate declines rapidly as temperature goes lower and reaches zero between -9 and -10°C (Birchard and Packard, 1997). The resultant reduction in delivery of oxygen to peripheral tissues means that those tissues have to increase their reliance on anaerobic pathways to generate ATP, despite the fact that overall demand for ATP is also lower (Hartley et al., 2000; Costanzo et al., 2001a).

Patterns of accumulation of lactic acid in bodies of hatchling painted turtles are shown in Fig. 6, where the slopes of the lines reflect rates of accumulation of lactate in bodies of supercooled animals held at different temperatures. Turtles at 0°C did not accumulate lactate during 25 days of exposure, indicating that these animals were able to meet requirements for ATP by aerobic respiration; this means, in turn, that the already low rate of circulation was still sufficient to supply peripheral tissues with oxygen. Neonates at -4°C and -8°C, however, were unable to support peripheral tissues by oxidative respiration alone, and lactate consequently accumulated. The rate of accumulation of lactate was higher in hatchlings held at -8°C than in those held at -4°C.

All the turtles that were held in a supercooled state at -8°C had died by day 25. The amount of lactate in bodies of these animals was only slightly higher than the quantity of lactate recovered from hatchlings held at the same temperature for 15 days. We take this finding to mean that turtles survived in a supercooled state for 15 days at -8°C but that they died soon after – perhaps as a result of shifts in pH resulting from the presence of the lactic acid.

The concentration of lactic acid in the dead hatchlings was only about half to two-thirds that which can be tolerated by adult painted turtles held in cold, anoxic water (Hartley et al., 2000). Why might a hatchling succumb to a lactic acidosis that

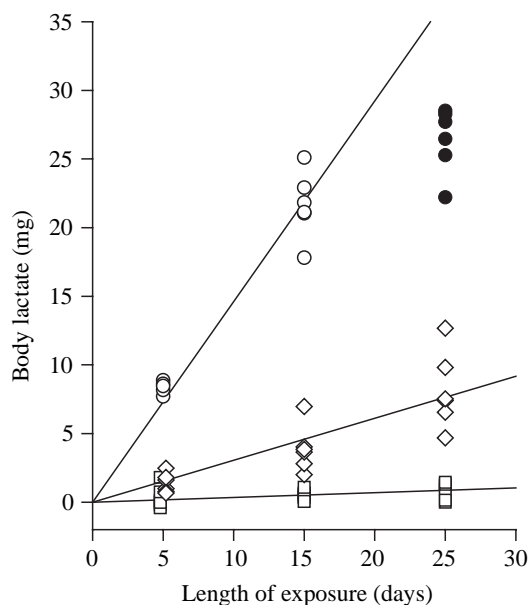


Fig. 6. Straight lines fit to the displayed values for total lactate in bodies of supercooled hatchlings of the painted turtle describe rates of accumulation of this metabolic end-product at 0°C (□), -4°C (◇) or -8°C (○). Animals that were held for 25 days at -8°C (●) were probably dead at the point in time when they were sampled, so data for these turtles were not used in computing the regression line for that temperature. The regression lines, which were forced through the origin, have the following equations: lactate at 0°C=0.035 mg×day; lactate at -4°C=0.306 mg×day; lactate at -8°C=1.462 mg×day. Figure reproduced from Hartley et al. (2000).

can be readily tolerated by an adult animal? We propose that the difference in levels of tolerance stems, in part, from differences in the perfusion of peripheral tissues with blood and, in part, from differences in the degree of calcification of the bony shell.

Adult painted turtles subjected to anoxic water buffer the lactic acid by sequestering a portion of the acid in the shell itself and by mobilizing calcium carbonate from the shell to buffer lactate in other parts of the body (Jackson, 2000). These processes depend on functioning of the circulatory system (Herbert and Jackson, 1985), which is impaired in supercooled neonates and which ceases to function altogether as temperature approaches -10°C. Additionally, the shell of hatchlings is not fully calcified (Zangerl, 1969; Ewert, 1985), so the animals do not have as large a reserve of mineral as adults to buffer the lactate. With a more limited supply of mineral to buffer acid, and with an impaired (or non-functioning) circulation to move around both the buffer and the acid, hatchlings apparently are more susceptible to a fatal lactic acidosis.

Even when lactate does not accumulate to levels that are life-threatening, the accompanying impairment of acid/base status may have important consequences. Supercooled hatchlings that have survived long exposures at low temperatures in laboratory tests commonly exhibit a prolonged lethargy after

they have been rewarmed to room temperature (Hartley et al., 2000; Costanzo et al., 2001a). If the lethargic state is a result of the lactic acidosis, it is easy to imagine that hatchlings might delay their emergence from their nest in the spring until the lactic acid has been metabolized or otherwise removed from the system (Jackson et al., 1996). Moreover, if the lactic acid debt has not been repaid fully by the time hatchlings dig their way out of their nest, their behavior might be affected adversely during the trek to water.

The interactions among temperature, circulatory function, metabolism, acid/base balance and behavior are an area of research that promises to yield new insights into the adaptive strategy for overwintering by these animals. One of the more intriguing of the many unanswered questions has to do with the possibility that the stagnant hypoxia induced by exposure of hatchlings to subzero temperature elicits the same kind of metabolic depression that has been reported for adult painted turtles subjected to anoxia (Jackson, 2000). Such a depression would help to conserve the reserves of glycogen that are needed to fuel metabolism of such critical organs as heart and brain, and it would also serve to minimize the disruption of acid/base balance resulting from the formation of lactic acid.

### Evolutionary considerations

Several investigators have asked why neonatal painted turtles spend their first winter of life inside the nest where they completed embryogenesis the preceding summer (Gibbons and Nelson, 1978; Packard and Packard, 2001). After all, hatchlings of other aquatic turtles typically emerge from their nest in late summer or autumn and go to a nearby body of water where they spend the winter in the unfrozen depths (Ernst et al., 1994), so why don't neonatal painted turtles behave similarly? Speculation about why baby painted turtles overwinter where they do has focused on hypotheses (1) that food for neonates is in limited supply in autumn so that no benefit accrues to animals that emerge from their nest and go to water before the start of winter; (2) that turtles reduce their exposure to predators by delaying their emergence from the nest until spring and (3) that remaining inside the subterranean nest allows hatchlings to avoid osmotic problems that attend overwintering in cold water. Unfortunately, none of these speculations provides a wholly satisfactory answer to the question.

Another outstanding question has to do with factors that underlie the acquisition of traits enabling hatchling painted turtles to undergo supercooling while they are overwintering in the frost zone of the soil (Packard et al., 2002). One possibility is that the cutaneous barrier to penetration of ice, as well as the physiological or behavioral means for inactivating nucleating agents in bodily fluids, was acquired as a result of directional (natural) selection exerted by ice and cold during the northward expansion of the species at the end of Pleistocene glaciation. Alternatively, these attributes of morphology, physiology and behavior may have been acquired before the end of the Pleistocene in response to different

selection pressures altogether, in which case the role of the characters in promoting supercooling was entirely serendipitous. Phylogeographic analysis has the potential to distinguish between these competing hypotheses, albeit the one such analysis that has been performed to date failed to resolve the issue (Starkey et al., 2003).

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### References

- Alibardi, L. (1999). Differentiation of the epidermis of neck, tail and limbs in the embryo of the turtle *Emydura macquarii* (Gray, 1830). *Belgian J. Zool.* **129**, 391-404.
- Alibardi, L. (2002). Immunocytochemical observations on the cornification of soft and hard epidermis in the turtle *Chrysemys picta*. *Zoology* **105**, 31-44.
- Alibardi, L. (2003). Adaptation to the land: the skin of reptiles in comparison to that of amphibians and endotherm amniotes. *J. Exp. Zool.* **298B**, 12-41.
- Alibardi, L. and Sawyer, R. H. (2002). Immunocytochemical analysis of beta ( $\beta$ ) keratins in the epidermis of chelonians, lepidosaurians, and archosaurians. *J. Exp. Zool.* **293**, 27-38.
- Alibardi, L. and Thompson, M. B. (1999). Epidermal differentiation during carapace and plastron formation in the embryonic turtle *Emydura macquarii*. *J. Anat.* **194**, 531-545.
- Attaway, M. B., Packard, G. C. and Packard, M. J. (1998). Hatchling painted turtles (*Chrysemys picta*) survive only brief freezing of their bodily fluids. *Comp. Biochem. Physiol. A* **120**, 405-408.
- Baker, P. J., Costanzo, J. P., Iverson, J. B. and Lee, R. E., Jr (2003). Adaptations to terrestrial overwintering of hatchling northern map turtles, *Graptemys geographica*. *J. Comp. Physiol. B* **173**, 643-651.
- Baust, J. G. (1991). The freeze tolerance oxymoron. *Cryo-Lett.* **12**, 1-2.
- Birchard, G. F. and Packard, G. C. (1997). Cardiac activity in supercooled hatchlings of the painted turtle (*Chrysemys picta*). *J. Herpetol.* **31**, 166-169.
- Block, W. (1990). Cold tolerance of insects and other arthropods. *Philos. Trans. R. Soc. Lond. B* **326**, 613-631.
- Castrillo, L. A., Lee, R. E., Jr, Wyman, J. A., Lee, M. R. and Rutherford, S. T. (2001). Field persistence of ice-nucleating bacteria in overwintering Colorado potato beetles. *Biol. Control* **21**, 11-18.
- Churchill, T. A. and Storey, K. B. (1991). Metabolic responses to freezing by organs of hatchling painted turtles *Chrysemys picta marginata* and *C. p. bellii*. *Can. J. Zool.* **69**, 2978-2984.
- Churchill, T. A. and Storey, K. B. (1992). Natural freezing survival by painted turtles *Chrysemys picta marginata* and *C. picta bellii*. *Am. J. Physiol.* **262**, R530-R537.
- Claussen, D. L. and Zani, P. A. (1991). Allometry of cooling, supercooling, and freezing in the freeze-tolerant turtle *Chrysemys picta*. *Am. J. Physiol.* **261**, R626-R632.
- Claussen, D. L., Townsley, M. D. and Bausch, R. G. (1990). Supercooling and freeze-tolerance in the European wall lizard, *Podarcis muralis*, with a revisional history of the discovery of freeze-tolerance in vertebrates. *J. Comp. Physiol. B* **160**, 137-143.
- Cochet, N. and Widehem, P. (2000). Ice crystallization by *Pseudomonas syringae*. *Appl. Microbiol. Biotechnol.* **54**, 153-161.
- Costanzo, J. P., Iverson, J. B., Wright, M. F. and Lee, R. E., Jr (1995). Cold hardiness and overwintering strategies of hatchlings in an assemblage of northern turtles. *Ecology* **76**, 1772-1785.
- Costanzo, J. P., Humphreys, T. L., Lee, R. E., Jr, Moore, J. B., Lee, M. R. and Wyman, J. A. (1998a). Long-term reduction of cold hardiness following ingestion of ice-nucleating bacteria in the Colorado potato beetle, *Leptinotarsa decemlineata*. *J. Insect Physiol.* **44**, 1173-1180.
- Costanzo, J. P., Litzgus, J. D., Iverson, J. B. and Lee, R. E., Jr (1998b). Soil hydric characteristics and environmental ice nuclei influence supercooling capacity of hatchling painted turtles *Chrysemys picta*. *J. Exp. Biol.* **201**, 3105-3112.
- Costanzo, J. P., Litzgus, J. D., Iverson, J. B. and Lee, R. E., Jr (2000a).



- Ice nuclei in soil compromise cold hardiness of hatchling painted turtles (*Chrysemys picta*). *Ecology* **81**, 346-360.
- Costanzo, J. P., Litzgus, J. D., Iverson, J. B. and Lee, R. E., Jr** (2000b). Seasonal changes in physiology and development of cold hardiness in the hatchling painted turtle *Chrysemys picta*. *J. Exp. Biol.* **203**, 3459-3470.
- Costanzo, J. P., Jones, E. E. and Lee, R. E., Jr** (2001a). Physiological responses to supercooling and hypoxia in the hatchling painted turtle, *Chrysemys picta*. *J. Comp. Physiol. B* **171**, 335-340.
- Costanzo, J. P., Litzgus, J. D., Iverson, J. B. and Lee, R. E., Jr** (2001b). Cold-hardiness and evaporative water loss in hatchling turtles. *Physiol. Biochem. Zool.* **74**, 510-519.
- Costanzo, J. P., Litzgus, J. D., Larson, J. L., Iverson, J. B. and Lee, R. E., Jr** (2001c). Characteristics of nest soil, but not geographic origin, influence cold hardiness of hatchling painted turtles. *J. Therm. Biol.* **26**, 65-73.
- Costanzo, J. P., Baker, P. J., Dinkelacker, S. A. and Lee, R. E., Jr** (2003). Endogenous and exogenous ice-nucleating agents constrain supercooling in the hatchling painted turtle. *J. Exp. Biol.* **206**, 477-485.
- Costanzo, J. P., Dinkelacker, S. A., Iverson, J. B. and Lee, R. E., Jr** (2004). Physiological ecology of overwintering in the hatchling painted turtle: multiple-scale variation in response to environmental stress. *Physiol. Biochem. Zool.* **77**, 74-99.
- Denda, M., Sato, J., Masuda, Y., Tsuchiya, T., Koyama, J., Kuramoto, M., Elias, P. M. and Feingold, K. R.** (1998). Exposure to a dry environment enhances epidermal permeability barrier function. *J. Invest. Dermatol.* **111**, 858-863.
- DePari, J. A.** (1996). Overwintering in the nest chamber by hatchling painted turtles, *Chrysemys picta*, in northern New Jersey. *Chelon. Conserv. Biol.* **2**, 5-12.
- Dominy, N. J., Davoust, E. and Minekus, M.** (2004). Adaptive function of soil consumption: an *in vitro* study modeling the human stomach and small intestine. *J. Exp. Biol.* **207**, 319-324.
- Elias, P. M.** (1983). Epidermal lipids, barrier function, and desquamation. *J. Invest. Dermatol.* **80**, 44s-49s.
- Elias, P. M. and Feingold, K. R.** (1992). Lipids and the epidermal water barrier: metabolism, regulation, and pathophysiology. *Sem. Dermatol.* **11**, 176-182.
- Elias, P. M. and Friend, D. S.** (1975). The permeability barrier in mammalian epidermis. *J. Cell Biol.* **65**, 180-191.
- Elias, P. M. and Menon, G. K.** (1991). Structural and lipid biochemical correlates of the epidermal permeability barrier. *Adv. Lipid Res.* **24**, 1-26.
- Elias, P. M., Cooper, E. R., Korc, A. and Brown, B. E.** (1981). Percutaneous transport in relation to stratum corneum structure and lipid composition. *J. Invest. Dermatol.* **76**, 297-301.
- Ernst, C. H., Barbour, R. W. and Lovich, J. E.** (1994). *Turtles of the United States and Canada*. Washington, DC: Smithsonian Institution Press.
- Ewert, M. A.** (1985). Embryology of turtles. In *Biology of the Reptilia*, vol. 14, *Development A* (ed. C. Gans, F. Billett and P. F. A. Maderson), pp. 75-267. New York: Wiley.
- Finkler, M. S.** (1999). Influence of water availability during incubation on hatchling size, body composition, desiccation tolerance, and terrestrial locomotor performance in the snapping turtle *Chelydra serpentina*. *Physiol. Biochem. Zool.* **72**, 714-722.
- Franks, F.** (1985). *Biophysics and Biochemistry at Low Temperatures*. Cambridge: Cambridge University Press.
- Gibbons, J. W. and Nelson, D. H.** (1978). The evolutionary significance of delayed emergence from the nest by hatchling turtles. *Evolution* **32**, 297-303.
- Grubauer, G., Feingold, K. R., Harris, R. M. and Elias, P. M.** (1989). Lipid content and lipid type as determinants of the epidermal permeability barrier. *J. Lipid Res.* **30**, 89-96.
- Hartley, L. M., Packard, M. J. and Packard, G. C.** (2000). Accumulation of lactate by supercooled hatchlings of the painted turtle (*Chrysemys picta*): implications for overwinter survival. *J. Comp. Physiol. B* **170**, 45-50.
- Herbert, C. V. and Jackson, D. C.** (1985). Temperature effects on the responses to prolonged submergence in the turtle *Chrysemys picta bellii*. II. Metabolic rate, blood acid-base and ionic changes, and cardiovascular function in aerated and anoxic water. *Physiol. Zool.* **58**, 670-681.
- Jackson, D. C.** (2000). Living without oxygen: lessons from the freshwater turtle. *Comp. Biochem. Physiol. A* **125**, 299-315.
- Jackson, D. C., Toney, V. I. and Okamoto, S.** (1996). Lactate distribution and metabolism during and after anoxia in the turtle, *Chrysemys picta bellii*. *Am. J. Physiol.* **271**, R409-R416.
- Kömüves, L. G., Hanley, K., Jiang, Y., Katagiri, C., Elias, P. M., Williams, M. L. and Feingold, K. R.** (1999). Induction of selected lipid metabolic enzymes and differentiation-linked structural proteins by air exposure in fetal rat skin explants. *J. Invest. Dermatol.* **112**, 303-309.
- Lampe, M. A., Burlingame, A. L., Whitney, J., Williams, M. L., Brown, B. E., Roitman, E. and Elias, P. M.** (1983). Human stratum corneum lipids: characterization and regional variations. *J. Lipid Res.* **24**, 120-130.
- Landmann, L.** (1986). The skin of reptiles/epidermis and dermis. In *Biology of the Integument/ 2 Vertebrates* (ed. J. Bereiter-Hahn, A. G. Matoltsy and K. Sylvania Richards), pp. 150-187. Berlin: Springer.
- Law, S., Wertz, P. W., Swartzendruber, D. C. and Squier, C. A.** (1995). Regional variation in content, composition and organization of porcine epithelial barrier lipids revealed by thin-layer chromatography and transmission electron microscopy. *Arch. Oral Biol.* **40**, 1085-1091.
- Lee, R. E., Jr and Costanzo, J. P.** (1998). Biological ice nucleation and ice distribution in cold-hardy ectothermic animals. *Annu. Rev. Physiol.* **60**, 55-72.
- Lee, R. E., Jr, Costanzo, J. P. and Mugnano, J. A.** (1996). Regulation of supercooling and ice nucleation in insects. *Eur. J. Entomol.* **93**, 405-418.
- Lundheim, R. and Zachariassen, K. E.** (1993). Water balance of overwintering beetles in relation to strategies for cold tolerance. *J. Comp. Physiol. B* **163**, 1-4.
- Menon, G. and Ghadially, R.** (1997). Morphology of lipid alterations in the epidermis: a review. *Microsc. Res. Tech.* **37**, 180-192.
- Meuwissen, M. E. M. J., Janssen, J., Cullander, C., Junginger, H. E. and Bouwstra, J. A.** (1998). A cross-section device to improve visualization of fluorescent probe penetration into the skin by confocal laser scanning microscopy. *Pharmaceut. Res.* **15**, 352-356.
- Miller, K., Packard, G. C. and Packard, M. J.** (1987). Hydric conditions during incubation influence locomotor performance of hatchling snapping turtles. *J. Exp. Biol.* **127**, 401-412.
- Nagle, R. D., Kinney, O. M., Congdon, J. D. and Beck, C. W.** (2000). Winter survivorship of hatchling painted turtles (*Chrysemys picta*) in Michigan. *Can. J. Zool.* **78**, 226-233.
- Nemanic, M. K. and Elias, P. M.** (1980). In situ precipitation: a novel cytochemical [sic.] technique for visualization of permeability pathways in mammalian stratum corneum. *J. Histochem. Cytochem.* **28**, 573-578.
- Packard, G. C.** (1997). Temperatures during winter in nests with hatchling painted turtles (*Chrysemys picta*). *Herpetologica* **53**, 89-95.
- Packard, G. C. and Packard, M. J.** (1993a). Hatchling painted turtles (*Chrysemys picta*) survive exposure to subzero temperatures during hibernation by avoiding freezing. *J. Comp. Physiol. B* **163**, 147-152.
- Packard, G. C. and Packard, M. J.** (1993b). Delayed inoculative freezing is fatal to hatchling painted turtles (*Chrysemys picta*). *Cryo-Lett.* **14**, 273-284.
- Packard, G. C. and Packard, M. J.** (1995). The basis for cold tolerance in hatchling painted turtles (*Chrysemys picta*). *Physiol. Zool.* **68**, 129-148.
- Packard, G. C. and Packard, M. J.** (2001). The overwintering strategy of hatchling painted turtles, or how to survive in the cold without freezing. *BioScience* **51**, 199-207.
- Packard, G. C. and Packard, M. J.** (2003a). Natural freeze-tolerance in hatchling painted turtles? *Comp. Biochem. Physiol. A* **134**, 233-246.
- Packard, G. C. and Packard, M. J.** (2003b). Cold acclimation enhances cutaneous resistance to inoculative freezing in hatchling painted turtles, *Chrysemys picta*. *Funct. Ecol.* **17**, 94-100.
- Packard, G. C. and Packard, M. J.** (2003c). Influence of acclimation and incubation medium on supercooling by hatchling painted turtles, *Chrysemys picta*. *Funct. Ecol.* **17**, 611-618.
- Packard, G. C., Fasano, S. L., Attaway, M. B., Lohmiller, L. D. and Lynch, T. L.** (1997a). Thermal environment for overwintering hatchlings of the painted turtle (*Chrysemys picta*). *Can. J. Zool.* **75**, 401-406.
- Packard, G. C., Lang, J. W., Lohmiller, L. D. and Packard, M. J.** (1997b). Cold tolerance in hatchling painted turtles (*Chrysemys picta*): supercooling or tolerance for freezing? *Physiol. Zool.* **70**, 670-678.
- Packard, G. C., Tucker, J. K., Nicholson, D. and Packard, M. J.** (1997c). Cold tolerance in hatchling slider turtles (*Trachemys scripta*). *Copeia* **1997**, 339-345.
- Packard, G. C., Lang, J. W., Lohmiller, L. D. and Packard, M. J.** (1999a). Resistance to freezing in hatchling painted turtles (*Chrysemys picta*). *Can. J. Zool.* **77**, 795-801.
- Packard, G. C., Packard, M. J., Lang, J. W. and Tucker, J. K.** (1999b). Tolerance for freezing in hatchling turtles. *J. Herpetol.* **33**, 536-543.
- Packard, G. C., Packard, M. J. and Birchard, G. F.** (2000a). Availability of water affects organ growth in prenatal and neonatal snapping turtles (*Chelydra serpentina*). *J. Comp. Physiol. B* **170**, 69-74.
- Packard, G. C., Packard, M. J. and Lang, J. W.** (2000b). Why hatchling

- Blanding's turtles don't overwinter inside their nest. *Herpetologica* **56**, 367-374.
- Packard, G. C., Packard, M. J. and McDaniel, L. L.** (2001). Seasonal change in the capacity for supercooling by neonatal painted turtles. *J. Exp. Biol.* **204**, 1667-1672.
- Packard, G. C., Packard, M. J., Morjan, C. L. and Janzen, F. J.** (2002). Cold-tolerance of hatchling painted turtles (*Chrysemys picta bellii*) from the southern limit of distribution. *J. Herpetol.* **36**, 300-304.
- Packard, M. J. and Packard, G. C.** (in press). Accumulation of lactate by frozen painted turtles (*Chrysemys picta*) and its relationship to freeze tolerance. *Physiol. Biochem. Zool.*
- Rose, F. L.** (1969). Desiccation rates and temperature relationships of *Terrapene ornata* following scute removal. *Southwest. Nat.* **14**, 67-72.
- Rubinsky, B., Hong, J. and Storey, K. B.** (1994). Freeze tolerance in turtles: visual analysis by microscopy and magnetic resonance imaging. *Am. J. Physiol.* **267**, R1078-R1088.
- Salt, R. W.** (1963). Delayed inoculative freezing of insects. *Can. Entomol.* **95**, 1190-1202.
- Salt, R. W.** (1966). Factors influencing nucleation in supercooled insects. *Can. J. Zool.* **44**, 117-133.
- Salt, R. W.** (1970). Analysis of insect freezing temperature distributions. *Can. J. Zool.* **48**, 205-208.
- Schmidt-Nielsen, K.** (1997). *Animal Physiology/Adaptation and Environment*. 5th edition. Cambridge: Cambridge University Press.
- Shimada, K.** (1989). Ice-nucleating activity in the alimentary canal of the freezing-tolerant prepupae of *Trichiocampus populi* (Hymenoptera: Tenthredinidae). *J. Insect Physiol.* **35**, 113-120.
- Simonetti, O., Hoogstraate, A. J., Bialik, W., Kempenaar, J. A., Schrijvers, A. H. G. J., Boddé, H. E. and Ponc, M.** (1995). Visualization of diffusion pathways across the stratum corneum of native and in-vitro-reconstructed epidermis by confocal laser scanning microscopy. *Arch. Dermatol. Res.* **287**, 465-473.
- Starkey, D. E., Shaffer, H. B., Burke, R. L., Forstner, M. R. J., Iverson, J. B., Janzen, F. J., Rhodin, A. G. J. and Ultsch, G. R.** (2003). Molecular systematics, phylogeography, and the effects of Pleistocene glaciation in the painted turtle (*Chrysemys picta*) complex. *Evolution* **57**, 119-128.
- Storey, K. B.** (1990). Life in a frozen state: adaptive strategies for natural freeze tolerance in amphibians and reptiles. *Am. J. Physiol.* **258**, R559-R568.
- Storey, K. B. and Storey, J. M.** (1992). Natural freeze tolerance in ectothermic vertebrates. *Annu. Rev. Physiol.* **54**, 619-637.
- Storey, K. B. and Storey, J. M.** (1996). Natural freezing survival in animals. *Annu. Rev. Ecol. Syst.* **27**, 365-386.
- Storey, K. B., Storey, J. M., Brooks, S. P. J., Churchill, T. A. and Brooks, R. J.** (1988). Hatchling turtles survive freezing during winter hibernation. *Proc. Natl. Acad. Sci. USA* **85**, 8350-8354.
- Storey, K. B., McDonald, D. G., Duman, J. G. and Storey, J. M.** (1991). Blood chemistry and ice nucleating activity in hatchling painted turtles. *Cryo-Lett.* **12**, 351-358.
- Strong-Gunderson, J. M., Lee, R. E., Jr, Lee, M. R. and Riga, T. J.** (1990). Ingestion of ice-nucleating active bacteria increases the supercooling point of the lady beetle *Hippodamia convergens*. *J. Insect Physiol.* **36**, 153-157.
- Ultsch, G. R.** (1989). Ecology and physiology of hibernation and overwintering among freshwater fishes, turtles, and snakes. *Biol. Rev.* **64**, 435-516.
- Weisrock, D. W. and Janzen, F. J.** (1999). Thermal and fitness-related consequences of nest location in painted turtles (*Chrysemys picta*). *Funct. Ecol.* **13**, 94-101.
- Willard, R., Packard, G. C., Packard, M. J. and Tucker, J. K.** (2000). The role of the integument as a barrier to penetration of ice into overwintering hatchlings of the painted turtle (*Chrysemys picta*). *J. Morphol.* **246**, 150-159.
- Wilson, P. W., Heneghan, A. F. and Haymet, A. D. J.** (2003). Ice nucleation in nature: supercooling point (SCP) measurements and the role of heterogeneous nucleation. *Cryobiology* **46**, 88-98.
- Woolverton, E.** (1963). Winter survival of hatchling painted turtles in northern Minnesota. *Copeia* **1963**, 569-570.
- Zachariassen, K. E.** (1991). The water relations of overwintering insects. In *Insects at Low Temperature* (ed. R. E. Lee, Jr and D. L. Denlinger), pp. 47-63. New York: Chapman and Hall.
- Zangerl, R.** (1969). The turtle shell. In *Biology of the Reptilia, vol. 1, Morphology A* (ed. C. Gans), pp. 311-339. London: Academic Press.