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Response of Embryos of the Red-Eared Turtle (*Trachemys scripta elegans*) to Experimental Exposure to Water-Saturated Substrates

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ABSTRACT. – **We exposed early-stage (19–22 day old) and late-stage (39–42 day old) embryos of the red-eared slider turtle (*Trachemys scripta elegans*) that began incubation on substrates of three different water potentials (dry, intermediate, and wet) to water-saturated substrates for five different exposure intervals (1, 6, 12, 24, and 48 hrs). Exposure of early-stage embryos had no significant effect on survivorship for any exposure interval regardless of initial substrate water potential. Exposure of late-stage embryos caused dramatically reduced survivorship when exposure exceeded 12 hrs. Survivorship of eggs exposed for 24 and 48 hrs regardless of substrate was 0%, whereas it was 100% for all other exposure intervals. Exposure of eggs containing early-stage embryos for periods as short as 1 hr allowed rapid uptake of water by the eggs. Water uptake was most pronounced among eggs incubated on the driest substrate even though a narrow range of relatively wet substrates was used. Eggs containing late-stage embryos exposed for less than 24 hrs also took up water, but these changes in egg mass did not translate into increased hatchling mass. Our results support the hypothesis that nest inundation at later stages of embryogenesis is more detrimental to embryonic survival than nest inundation at earlier stages. The nesting season for turtles in the study area coincides with the end of the spring flood pulse as it existed historically prior to modifications of the hydrologic regime by dams. When coupled with unpredictable natural events such as the 1993 flood, altered hydrologic regimes are detrimental to turtles that may time nesting to coincide with decreasing water levels.**

KEY WORDS. – **Reptilia; Testudines; Emydidae; *Trachemys scripta*; turtle; reproduction; incubation; eggs; embryos; embryonic survival; hydrologic regimes; flooding; Illinois; USA**

Although predation is the most commonly noted source of egg mortality among turtles and other reptiles, flooding may also be a significant contributing factor to nest failure. Nest flooding may result from a number of factors. Plummer (1976) noted greater mortality among smooth softshell turtles (*Apalone mutica*) associated with artificially high water levels in the Kansas River. Excessive rainfall has also been associated with nest failures in sea turtles (Hendrickson, 1958; Ragotzkie, 1959; Caldwell et al., 1959; Kraemer and Bell, 1980). Nest flooding due to a hurricane caused 100% mortality among sea turtle nests on beaches closest to the eye of the storm (Milton et al., 1994). Others have reported nest failures in sea turtles due to high tides (Bustard and Greenham, 1968), due to varying lake water levels for a freshwater turtle, *Trionyx sinensis* (Cherepanov, 1990), and due to sudden changes in river levels for another freshwater turtle, *Podocnemis expansa* (Alho and Pádua, 1982). Nest failure due to flooding has been reported for a diversity of other turtle species such as tropical sliders (*Trachemys scripta*, Moll and Legler, 1971), painted turtles (*Chrysemys picta*, Christens and Bider, 1987; Janzen, 1994), ornate box turtles (*Terrapene ornata*, Legler, 1960), and musk turtles (*Sternotherus minor*, Cox and Marion, 1978).

Even though nest flooding may be an important source of hatching failure, relatively few experimental studies adequately explore the variables associated with nest flooding. Some experiments, such as those by Legler (1960) for *Terrapene ornata* and Moll and Legler (1971) for *Trachemys scripta*, used small sample sizes and embryos of varying age, making their results difficult to interpret. In an early experiment using large sample sizes, Plummer (1976) found an incremental increase in mortality associated with increasing immersion intervals for 1- to 12-day-old embryos of *Apalone mutica*, with no survivors after 15 days of immersion.

More recently McGehee (1990) found that hatching success of embryos of *Caretta caretta* was lowest for eggs incubated in sand with 75 and 100% moisture saturation. Kam (1994) exposed 19-day-old embryos of *Pseudemys nelsoni* to immersion for varying periods and found mortality increased with increasing immersion interval.

Although immersion in water has been shown to reduce survivorship in both experimental and empirical studies, it is largely unknown whether embryonic age influences the response of turtle embryos to flooding. Consequently, we exposed early- and late-stage embryos of *Trachemys scripta elegans* to water-saturated substrates for five different exposure intervals, ranging from 1 to 48 hrs. Because hydric environment varies within the same nest and among different nests, we repeated the experiment three times using different substrate water potentials similar to those found in

natural nests in clay soils (Ratterman and Ackerman, 1989). The purpose of the present paper is to report the results of these experiments and to consider the implications that they might have on the ecology, life history, and conservation biology of this and other turtle species.

MATERIALS AND METHODS

We used eggs from 26 clutches, induced by injection of oxytocin (Ewert and Legler, 1978), from red-eared turtles (*Trachemys scripta elegans*) collected while on their nesting excursions in Jersey County, Illinois. We used 120 eggs from 10 clutches on dry substrate, 120 eggs from 7 clutches on intermediate substrate, and 120 eggs from 9 clutches on wet substrate. We uniquely numbered the eggs with carbon ink and determined egg mass (to 0.01 g) with a Sartorius electronic balance. Because we collected the eggs in two groups, the first group began incubation four days before the second group.

For each experiment 120 eggs were randomly divided among 12 Sterilite brand storage boxes (32.5 x 18.1 x 10.6 cm). Boxes were randomly assigned to various exposure intervals, including a control and five experimental intervals (1, 6, 12, 24, and 48 hrs) to a water-saturated substrate (see below) at two different embryonic stages: early-stage = embryos of 19 or 22 days incubation and late-stage = embryos of 39 or 42 days incubation.

We mixed three different perlite substrates by adding various amounts of water to 150 g perlite to produce wet (170 ml water added; –60 kPa), intermediate (85 ml water added; –92 kPa), or dry (27 ml water added; –189 kPa) substrates. Eggs that began incubation on Julian day 155 were kept on wet, intermediate, or dry substrates until day 159 when they, along with eggs started on day 159, were assigned to different exposure intervals. All experimental boxes had freshly made perlite substrate at the start of the experiment (day 159).

We measured initial egg mass on Julian date 155 or 159. We reweighed all early-stage eggs on days 167, 178, 181, 195, and 207, and all late-stage eggs on days 167, 181, 198, 201, and 207. We measured pre-placement egg mass on day 178 for early-stage embryos and day 198 for late-stage embryos, and then reweighed all eggs 36 hrs after the placement in water-saturated substrates began (i.e., day 181 for early-stage embryos and day 201 for late-stage embryos).

We maintained hydration in each box by first determining mass of the box, eggs, and substrates on day 159. We then added sufficient water at each remeasurement date to bring each box back to the original mass after subtracting water taken up by eggs in the box and any losses to the atmosphere. We rehydrated boxes by adding water onto the substrate as evenly as possible without allowing water to directly contact the eggs. All boxes had an aluminum foil covering between the box and lid to retard moisture loss.

We prepared water-saturated substrates by adding 3.7 liters water to 600 g vermiculite into a Sterilite brand box (40 x 27.5 x 15 cm). We used vermiculite for water-saturated substrates because preliminary trials with non-experimental eggs indicated that eggs floated out of comparable water and perlite mixtures. This mixture of vermiculite and water resulted in liquid water at the surface of the vermiculite but not above it. We prepared this mixture freshly for each exposure time. We then placed all eggs for all five exposure intervals (1, 6, 12, 24, and 48 hrs) into the box simultaneously. We positioned eggs so as to completely cover them with water-saturated substrate and did not allow individual eggs to touch each other. At the end of the exposure interval, we brushed off any adhering vermiculite, patted each egg dry, and returned each to its original box. Handling of turtle eggs may (Limpus et al., 1979) or may not (Marcellini and Davis, 1982; Feldman, 1983) influence survivorship. In order to ensure that we subjected experimental and control eggs to equal handling, we also moved control eggs to boxes containing vermiculite mixed in the same proportions as the perlite in their original boxes and returned them to their original boxes after 48 hrs.

During incubation we kept all boxes at the same height and horizontally rotated them once weekly to reduce the effects of temperature gradients. We did not control temperature but recorded it daily beginning on day 161 with minimum-maximum thermometers placed next to the boxes. Estimated mean incubation temperature was 28.9°C determined using the method of Godfrey and Mrosovsky (1994).

Once the first egg pipped, we placed a bottomless waxed paper cup over each egg (Janzen, 1993). We recorded pip date and defined incubation period as pip date minus initial date (Gutzke et al., 1984). Once a turtle left the eggshell, we considered it a survivor. We recorded hatchling mass (to 0.01 g). We opened all eggs that failed to hatch. We did not detect an embryo in any egg that initially failed to whiten (Ewert, 1985). We defined an embryo as killed if its stage of development was consistent with the embryonic age at or after the exposure interval (Yntema, 1968; Kam, 1994). We considered hatchlings that pipped but then died as killed. We prepared a hydrograph using the methods of Nelson et al. (1994), with the nesting season for turtles collected in 1994 superimposed.

Differential mortality could be due either to exposure to water-saturated substrates (Kam, 1994) or to water potential of the incubation substrates (reviewed by Packard, 1991). We addressed mortality caused by exposure effects by comparing survivorship of the subsample of eggs that whitened in combination with an estimation of the age of embryonic death in relation to age at exposure to water-saturated substrates. We staged dead embryos following Yntema (1968).

Statistical Procedures. — Statistical analysis was performed using SAS (SAS Institute, 1988). We used Fisher's exact test (two-tailed) to evaluate differences between survivorship due to substrate and exposure interval effects. We calculated least significant difference (LSD) for each substrate and embryonic age (Figs. 1A–F), using harmonic means because cell sizes differed among exposure intervals

(Snedecor and Cochran, 1986). We used Type III sum of squares to calculate mean squares for both repeated measures analysis and three-way analyses of covariance (ANCOVA).

For tests of hypotheses for between-subject effects in repeated measures analysis and three-way analyses of covariance, one of the variables (i.e., clutch) was a random effect. Consequently, we used the Satterthwaite approximation of denominator degrees of freedom to calculate the expected mean square and F-ratio (SAS Institute, 1988; Janzen et al., 1995).

Exposure interval, embryonic age, and the interaction between them were fixed effects, whereas clutch and the interactions between clutch and the fixed effects were random for both repeated measures analyses and mixed models of covariance. Initial egg mass was the covariate in all cases. In some cases the covariate was not a significant source of variation. However, without analysis of covariance (ANCOVA), clutch effects due to egg size differences among clutches are overestimated. Consequently, the covariate was used in all analyses.

For multiple comparisons involved in repeated measure ANCOVA, three-way ANCOVA, and comparisons of least square means, we used the sequential Bonferroni method (Rice, 1989) to identify apparently significant p values insufficient to exclude type I errors at the 0.05 level. For comparisons of the amount of change in egg mass between pre- and post-exposure weighing, we used the REGWQ procedure in SAS analysis of variance (GLM procedure).

RESULTS

Exposure of early-stage embryos to water-saturated substrates had no significant effect on survivorship at any exposure interval regardless of original substrate water potential (dry substrate: $\chi^2 = 4.10$, $p = 1.00$, 5 df; intermediate substrate: $\chi^2 = 7.38$, $p = 0.29$, 5 df; wet substrate: $\chi^2 = 5.11$, $p = 0.48$, 5 df). Survivorship for embryos on dry substrate ranged from 87.5% for eggs exposed to water-saturated substrates for 48 hrs to 100% for all other exposure intervals and the control. Survivorship for embryos on intermediate substrate ranged from 87.5% for eggs exposed for 12 hrs to 100% for control eggs and all other exposure intervals except for eggs exposed for 24 hrs, of which 90% of the eggs hatched. Survivorship for embryos on wet substrates ranged from 80% for eggs exposed to water-

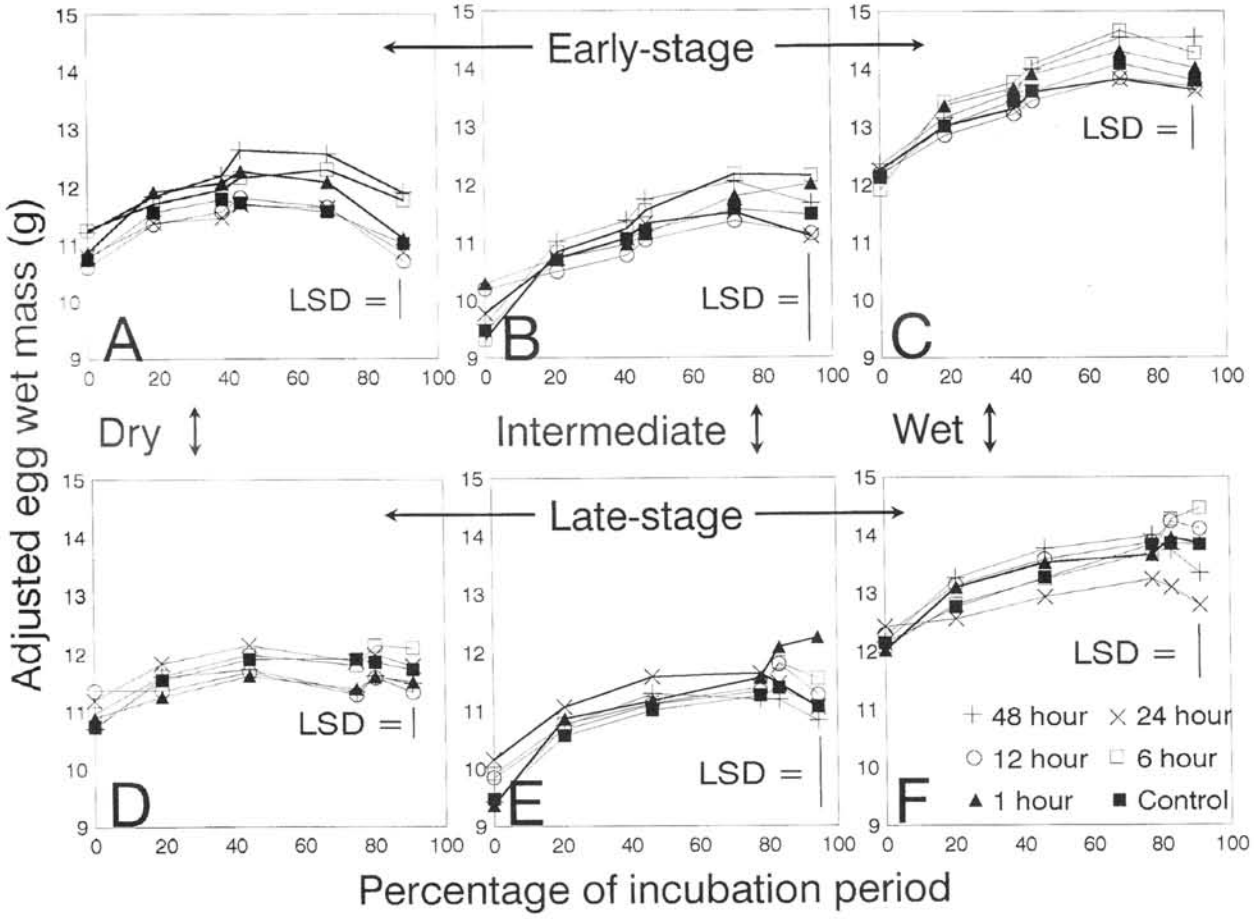

Figure 1. Comparison of changes in egg mass (adjusted wet mass in g) over incubation period (number of days) for exposure intervals to water-saturated substrates at two embryonic ages incubated on three substrates of differing water potentials. **A:** early-stage embryos on dry substrate; **B:** early-stage embryos on intermediate substrate; **C:** early-stage embryos on wet substrate; **D:** late-stage embryos on dry substrate; **E:** late-stage embryos on intermediate substrate; and **F:** late-stage embryos on wet substrate. LSD = least significant difference; except for initial egg mass (at 0% incubation period) all other egg masses are adjusted by initial egg mass.

saturated substrates for 48 and 24 hrs to 100% for all other exposure intervals and the control. Early-stage embryos presumably killed by exposure to water-saturated substrates were all between stages 14 and 15. Exposure of late-stage embryos to water-saturated substrates caused dramatically reduced survivorship when exposure time exceeded 12 hrs. None of the 60 embryos among the 24 and 48 hr exposure intervals survived to emerge from the egg. One individual pipped but died prior to emerging. All other embryos that we identified as killed were between stages 21 and 23. Late-stage embryos were more sensitive to exposure to water-saturated substrates than were early-stage embryos. Survivorship among exposure intervals including the control was different for all three experiments (dry substrate: $\chi^2 = 41.00$, $p < 0.0001$, 5 df; intermediate substrate: $\chi^2 = 50.00$, $p < 0.0001$, 5 df; wet substrate: $\chi^2 = 51.00$, $p < 0.0001$, 5 df).

For each exposure interval among early-stage embryos, egg mass immediately after exposure increased, suggesting that exposure to water-saturated substrates for periods as short as 1 hr allowed rapid uptake of water by the eggs (Fig. 1A–C; Table 1). This effect was most pronounced on the dry substrate group where the change in egg mass for all exposure intervals was significantly greater than for the control, and the change in egg mass for the 48 hr exposure interval was significantly greater than all other exposure intervals (Table 1). The changes in mass were less pronounced on wetter substrates, but even so, the control on each of the substrates changed the least whereas the mass of eggs in the 48 hr exposure interval changed the most. The sensitivity of the response of eggs to increased water availability is underscored by the fact that the range of water potentials used was narrow (–60 to –189 kPa).

Eggs containing late-stage embryos exposed to water-saturated substrates for less than 24 hrs also showed evidence of water intake (Fig. 1D–F; Table 1). However, the pattern of mass changes for eggs exposed to water-saturated substrates for 24 or 48 hrs differed between substrates (Table 1). For eggs on wet and intermediate substrates, eggs lost mass in the post-exposure weighing (Fig. 1D, E; Table 1). Eggs exposed for 24 and 48 hrs on dry substrate, on the other hand, increased in mass at the same rates as did those exposed to shorter exposure intervals and at greater rates than the control (Fig. 1F; Table 1).

We performed separate repeated measures ANCOVA on egg mass measurements taken at various stages in incubation for each substrate to examine between-subject and within-subject effects on egg mass suggested by the changes in egg mass apparent in Fig. 1. Clutch (for the intermediate substrate, $F = 13.53$, $p = 0.0019$) and the interaction between exposure interval and embryonic age (for dry substrate, $F = 11.59$, $p < 0.0001$) were the only significant sources of between-subject variation. This reflected the larger amounts of water taken up by early-stage eggs with longer exposure intervals compared to late-stage eggs (Table 1).

Within-subject effects were most pronounced on dry substrate with all effects (i.e., exposure interval, embryonic age, clutch, and their interactions), except the covariate, being significant ($p < 0.0056$, for nine comparisons). In other words, mass changes through time were greater for late- and early-stage eggs subjected to longer exposure intervals. Only exposure interval (for wet and intermediate substrates, $F = 4.04$, $p < 0.0001$ and $F = 10.62$, $p < 0.0001$, respectively) and the interaction between exposure interval and embryonic age (for wet substrate, $F = 3.25$, $p < 0.0001$) were significant sources of variation on the other two substrates.

We also compared hatchling mass and incubation period with mixed model ANCOVA. Among fixed effects including embryonic age, exposure interval, and their interactions, only embryonic age accounted for a significant amount of the variance for hatchling mass and then only for the intermediate substrate ($F = 12.95$, $p = 0.0041$). Hatchlings from eggs on the intermediate substrate that were exposed to water-saturated substrates early in incubation were heavier than those exposed late in incubation. Clutch, a random effect, and its interaction with the fixed effects had no significant influence on variance in hatchling mass ($p > 0.0658$).

In contrast, exposure interval had a significant effect on variance in incubation period for eggs on both the wet and intermediate substrates ($F = 5.38$, $p = 0.0010$ and $F = 8.32$, $p < 0.0001$, respectively). For the wet substrate, incubation period for eggs exposed to water-saturated substrates was longer than for the control. Mean incubation period ranged from 54.8 days for the 6 hr exposure interval to 57.3 hrs for the 24 hr interval. Mean incubation period for the control was 53.8 days. On the intermediate substrate, the significant

Table 1. Comparison of pre- to post-exposure changes in egg mass (g) for embryos on dry, intermediate, and wet substrates.

	Substrate								
Early-stage	Dry			Intermediate			Wet		
Interval	A	B	C	A	B	C	A	B	C
48 hour	0.46			0.36			0.36		
24 hour		0.24			0.25			0.29	
12 hour		0.24			0.27				0.23
6 hour		0.21			0.26			0.31	
1 hour		0.21			0.18				0.24
control			0.02			0.13			0.15
				A	B		A	B	
					B	C		B	C

Late-stage	Dry					Intermediate			Wet				
Interval	A	B	C	D	E	A	B	C	A	B	C	D	E
48 hour		0.27						-0.02					-0.26
24 hour				0.11				-0.15				-0.13	
12 hour		0.30				0.43				0.37			
6 hour	0.33					0.47			0.57				
1 hour			0.22			0.51				0.30			
control					-0.07			0.14			0.02		
	A	B									C	D	
		B	C										

Significance levels interconnected by a bar do not differ statistically from each other, levels not directly connected by a bar differ statistically ($p < 0.05$). For example, in early-stage embryos on dry substrate, levels A vs. B, B vs. C, and A vs. C all differ significantly, but the changes within level B do not differ from each other; on intermediate substrate, levels A vs. C differ significantly, but A vs. B, B vs. C, and within B do not.

effect due to exposure interval was the result of an unusually long mean incubation period for eggs in the 6 hr interval. Mean incubation period for these eggs was 56.8 days compared to the next longest mean incubation period of 55.1 hrs for 48 hr interval eggs.

DISCUSSION

No previous study has compared the relative sensitivity to flooding of early- and late-stage embryos of any chelonian species. Therefore, we cannot know whether our finding of reduced survivorship among late-stage embryos is typical. If our results are typical for other chelonian species, then nest inundation at later stages of embryogenesis should be more detrimental to embryonic survival than nest inundation at earlier stages.

Flooding and inundation of nests are common enough phenomena that one turtle, *Chelodina rugosa*, has adapted its life cycle to take advantage of flooding. This Australian species lays its eggs underwater where they remain arrested in an early state of development until seasonally flooded billabongs recede (Kennett et al., 1993a, 1993b). Other turtles may select elevated nest sites and avoid possible failure due to flooding (Plummer, 1976; Cox and Marion, 1978). *Trachemys scripta* in Panama apparently time their nesting to take advantage of wet season rains to allow the escape of hatchlings from the nest as well as to avoid inundation during embryonic development (Moll and Legler, 1971).

Other tropical turtles (*Podocnemis unifilis* and *P. expansa*) nest on sand beaches exposed by receding rivers during the dry season, but have short incubation periods that allow emergence of hatchlings before river levels rise in response to seasonal increases in rainfall (Alho and Pádua, 1982; Thorbjarnarson et al., 1993). In contrast, *Dermatemys mawii* from Belize nests at or close to the waterline during the flood season, but has a long incubation period (Polisar, 1996). The early-stage eggs of this species may be unusually tolerant of submergence because one clutch survived simulated nest flooding for 36 days (Polisar, 1996).

Consequently, adaptations in nest timing to local, predictable hydrologic regimes may be expected when other environmental factors allow successful incubation. Ideally, turtles should time nesting so that embryogenesis, particularly its later stages, occurs at a time when nest flooding is least likely. Studies of the timing of nesting relative to hydrologic patterns are few (e.g., Moll and Legler, 1971; Alho and Pádua, 1982; Kushlan and Jacobsen, 1990; Kennett et al., 1993a, 1993b; Thorbjarnarson et al., 1993; and Polisar, 1996).

We plotted the nesting season for *T. scripta* from our study area on a hydrograph for the Mississippi River near its confluence with the Illinois River where most of our turtles were collected (Fig. 2). Although undoubtedly also associated with other environmental variables, nesting coincided closely with the end of the spring flood pulse for hydrological data collected prior to the impoundment of the river by Lock and Dam 26 in 1938.

The impact of the alteration of the natural hydrologic regime was clearly demonstrated during the flood of 1993 (Fig. 2). Normally, maintenance of a 9-foot channel for navigation effectively keeps the river at artificially high levels and suppresses the flood pulse. During 1993 the combination of the effects of unusually heavy rainfall (a natural event) and the preceding maintenance of a 9-foot

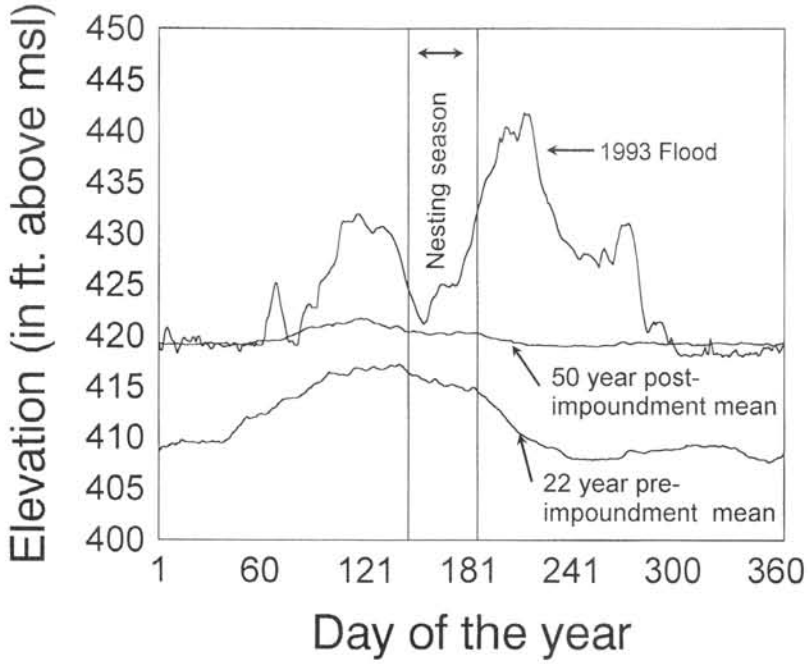

Figure 2. Hydrograph of river levels of the Mississippi River near the study site. Separate tracings reflect: 1) 1993 flood levels; 2) 50-year (1939–89) mean levels post-impoundment Lock and Dam 26 in 1938; 3) 22-year (1915–37) mean levels pre-impoundment. Nesting season for *Trachemys scripta* in 1994 is superimposed. Elevation is in feet above mean sea level; day of year is Julian date.

navigation channel made the resulting flood levels higher than they would have been, given the historically natural pattern in river levels prior to impoundment. Several nesting localities in the study area were inundated, and the nests in those localities were destroyed. The dramatic increase in mortality that we observed among our experimental eggs during the later stages of development underscores the importance of timing nesting to avoid inundation. The alteration of natural hydrologic patterns because of navigation and flood control dams may increase nesting failures even in years without extreme flood events, although this has not been studied. Clearly, investigations of nesting patterns in relation to hydrologic regimes will be important in predicting the effects of stream modification on chelonians.

The primary purpose of our study was to compare responses of early- and late-stage embryos to water-saturated substrates, because nearly all previous studies concentrated on the effects of flooding on early-stage embryos. Our study does not consider the possible impact of embryonic diapause (Ewert, 1985) on the response of embryos to flooding or water-saturated substrates.

Embryonic diapause is a mechanism that prolongs the egg stage making the egg a refuge for the embryo (see Ewert, 1985; Ewert and Wilson, 1996). Ewert (1985) suggested that a diapausing embryo should be able to escape certain crises such as flooding. At present no experimental study has examined the effect of diapause on embryonic survival of flooding. However, early-stage embryos of *T. scripta*, a species without embryonic diapause, are more resistant to flooding than are older embryos. Diapause in those species that display it occurs at a relatively early embryonic stage (Ewert, 1985; Ewert and Wilson, 1996). Consequently, separation of the effect of diapause *per se* from that of embryonic age on survival of flooding will be difficult.

The structure of the egg shell (i.e., brittle-shelled vs. flexible-shelled) may also be an important variable. Because the embryos of species with brittle-shelled eggs are less affected by variations in the hydric environment during incubation than are species with flexible-shelled eggs (see Packard, 1991), the response to flooding of species that lay brittle-shelled eggs may differ fundamentally from those that lay flexible-shelled eggs. Although no experimental study has examined interspecific survivorship of embryos of equivalent age, species that lay brittle-shelled eggs may be more resistant to flooding than those that lay flexible-shelled eggs. For instance, at least some 1- to 12-day-old embryos of *Apalone mutica* survived immersion for 8 days (Plummer, 1976). In contrast, all 19-day-old embryos of *Pseudemys nelsoni* died after immersion for 6 days, and few survived immersion for 3 days (Kam, 1994).

Although access to environmental water is critical to embryonic development in flexible-shelled eggs (Ewert, 1985; Packard, 1991), adsorption of excess amounts of water during immersion may interfere with development and even cause bursting of eggs (Ewert, 1985). Importantly, some of the embryos of *P. nelsoni* immersed for longer intervals did not die during immersion but continued development and then died prior to hatching (Kam, 1994). Whether such delayed mortality also occurs among species laying brittle-shelled eggs is not known but could be investigated experimentally. We did not observe any delayed mortality among the early-stage eggs of *T. scripta elegans* that we studied. However, we did not expose them to as long an immersion interval as did Kam for the *P. nelsoni* eggs that he studied.

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