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Seasonal Variation of Embryonic Diapause in the Striped Mud Turtle (*Kinosternon baurii*) and General Considerations for Conservation Planning

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ABSTRACT. – **Embryonic diapause in turtles is a state of developmental arrest in which early embryos cease developing under otherwise favorable environmental conditions and await an additional environmental factor, particularly prolonged exposure to cool temperatures, that will terminate diapause and allow resumption of development. We determined that expression of diapause in *Kinosternon baurii* varied with nesting season and incubation temperature. Significantly fewer embryos expressed diapause in spring than in fall and fewer at warm temperatures than at moderately cool ones. Because the same individual females were gravid and visiting their field nesting area in the spring and in the fall, females probably do not vary phenotypically in the incidence of diapause expressed by their embryos. We postulate that every embryo is potentially capable of expressing diapause, depending on the season of oviposition. Embryos with development artificially activated during the fall did not die while overwintering in natural nesting habitat, but possibly, these embryos are less fit than those on a natural schedule that develop during the spring and summer. Embryonic diapause, when present, can engender problems for conservationists commencing controlled incubation programs. Current protocols for incubating turtle eggs may not incorporate a need to chill early embryos to terminate diapause. Most species of turtles that express diapause occupy warm temperate to peripherally tropical habitats, nest well into periods of declining daylength, and lay eggs with brittle shells. We suggest that these characteristics may be used to identify species potentially needing special protocols for incubation. Alternatively, for embryos that may have diapause, it may be safest to initiate incubation at cool temperatures rather than at warm ones.**

KEY WORDS. – **Reptilia; Testudines; Kinosternidae; *Kinosternon baurii*; turtle; development; diapause; incubation; ecology; reproductive cycle; nesting; overwintering; conservation biology; USA**

The embryonic development of turtles remains arrested in a late gastrula stage in the oviduct and resumes only after oviposition (reviews in Ewert, 1985; Miller, 1985). In addition to this obligate pre-ovipositional arrest, several types of post-ovipositional developmental arrest may also occur during subsequent incubation (Table 1). Our paper focuses on the type of post-ovipositional arrest called embryonic diapause as it is expressed in the striped mud turtle, *Kinosternon baurii*. Embryos of *K. baurii* express the two diagnostic characteristics of embryonic diapause: 1) they persist in arrest from oviposition to long after their eggshells have chalked (formed a white band), and 2) arrest is terminated by an external stimulus (e.g., chilling). Advanced embryos of *K. baurii* also can experience an arrest at the end of development (Ewert, 1985); however, the focus of our paper is on early arrest — embryonic diapause.

In this study we compare the expression of embryonic diapause in *K. baurii* among different nesting seasons and incubation temperatures. We explore whether individual adult, gravid females can produce only embryos with diapause, only embryos without diapause, or both of these types. Because avoidance of winter mortality could be an adaptive function of diapause, we evaluate experimentally whether embryos express diapause because they cannot survive the winter environment in an active state of development. We conclude with general management implications of diapause that hitherto have not been discussed.

Embryonic diapause and other forms of embryonic developmental arrest (Table 1) have been identified in several species of turtles (Ewert, 1991, see below; Kennett et al., 1993b). These phenomena, however, are only sparsely represented in the overall literature on chelonian biology and natural history (reviews in Ernst and Barbour, 1989; Swingland and Klemens, 1989; Gibbons, 1990; Das, 1991; Ernst et al., 1994). Further, implicit interpretation of the literature leads to inconsistencies on whether species obligately or only facultatively express diapause (e.g., *Deirochelys reticularia*, Congdon et al., 1983, vs. Jackson, 1988; *Rhinoclemmys pulcherrima*, Christensen, 1975, vs. Ewert, 1991). We propose an explanation for some of the inconsistencies as we attempt to focus greater attention on embryonic diapause in chelonian biology.

MATERIALS AND METHODS

Study Species. — The striped mud turtle, *Kinosternon baurii*, is a small, primarily aquatic turtle that ranges along the southeastern coastal plain of North America from the

Table 1. Characteristics of four types of embryonic developmental arrest in turtles.

Type of Arrest	Outer Eggshell (Appearance)	Eggshell-Egg Contents (Adhesion)[1]	Yolk (Appearance)	Embryo (Stage of Development)	Extra-embryonic Membranes (Condition)	Metabolism	Sustaining Environment[2]	Terminating Environment[3]	References
Extension of Pre-ovipositional Arrest[4]	Translucent	No Adhesion (Egg contents free)	Partitioned[5]	Pre-somite (Early)[6]	Rudimentary, Colorless	Minimal[7]	Very wet (Other?)[8]	Dry, Keep Warm	Kennett et al., 1993a,b Ewert unpubl.
Embryonic Diapause	Chalked (with white band or patch)[9]	Moderate to Full Adhesion[10]	Partitioned[5]	Pre-somite (Early)[6]	Pre-vitelline, with Blood Islands	Minimal[7]	Moderate Temperature[11]	Chill, then Warm[12]	Jackson, 1988 Ewert, 1991
Delayed Hatching	Completely White	Full Adhesion	Partially to fully Internalized[13]	Term (Ready to hatch)	Broken Amnion, Large Chorioallantois	Changing (Declining)	Dry	Wet, Anoxic	Webb et al., 1986
Embryonic Estivation	Completely White	Little or No Adhesion[14]	Fully Internalized[13]	Term (Ready to hatch)	Chorioallantois reduced, may be withered	Static (Low)	Dry	Wet, Anoxic	Ewert, 1985, 1991

[1] During post-ovipositional development, the membrane that encloses the yolk (the vitelline membrane) adheres to the inside of the eggshell membrane and renders the egg contents largely immobile relative to the eggshell (Webb et al., 1987).

[2] Environmental conditions that prolong the state of developmental arrest.

[3] Environmental conditions that allow transition into an active state of development.

[4] The egg and embryo retain the appearance of the fully mature condition within the oviduct.

[5] Prior to oviposition, the yolk within most eggs separates into an upper clear, subgerminal space and a lower opaque, yellow mass of yolk solids.

[6] The exact stage, usually a gastrula, has not been confirmed in post-ovipositional eggs with developmental arrest, except to note that somites are not present.

[7] The level of oxygen consumption is too small to measure even by methods in respirometry that will give readings for embryos with several somites.

[8] Although the best documented case is for eggs that are submerged underwater (*Chelodina rugosa*), additional candidates for this type of arrest seem to persist in just damp environments (e.g., *Chelodina mccordi*), or even in dry ones (e.g., *Malacochersus tornieri*).

[9] For details on white patch development, see Thompson (1985).

[10] Moderate adhesion allows the egg contents to shift gradually ("slow slippage") in relation to the eggshell (Ewert, 1985).

[11] Temperatures that normally sustain development (e.g., > 21–25°C).

[12] See our methods for some protocols.

[13] Withdrawn from outside the body cavity to within the body cavity.

[14] The chorioallantois may retain small areas of close local contact with the eggshell and the turtle may so fully fill the interior space that little movement is possible without breaking (pipping) the eggshell.

Florida Keys north into Virginia (Iverson, 1992). Individual adult females (Fig. 1) typically produce several clutches of 1–6 (usually 2–4) elliptical, brittle-shelled eggs each year. Nesting occurs under or near vegetation in upland habitats from early September through June, with a break in July and August (review in Wilson, in press).

Study Sites and Capture Techniques. — We collected gravid females during various seasons from early 1988 to late 1994 from two sites in central Florida: one in Seminole County (28°45'N, 81°13'W) and the other 130 km SW of the first, in Hillsborough County (28°00'N, 82°23'W).

At the Seminole County site, original habitat appears to have been palm and hardwood hammock but currently is cattle pastures and orange groves with drainage ditches along their borders. Several ditches contain permanently flowing water (see Etchberger and Ehrhart, 1987) and others usually contain water but occasionally become dry. Adults of *K. baurii* were found crawling or swimming in the ditches or hidden in partially submerged pockets within the banks of the ditches. A few females were found nesting along trails or in shrubby vegetation near the ditches.

The Hillsborough County site is the Ecological Research Area of the University of South Florida. The predominant habitat is a natural, intermittently flooded baldcypress and hardwood swamp forest, which extends to the Hillsborough River. The swamp forest borders turkey oak and longleaf pine sandhill upland habitat. A drift fence, approximately 1.6 km in length, intercepted adult females of *K. baurii* as they travelled from the swamp forest to the sandhill habitat to nest. Turtles were retrieved from pitfall buckets spaced at 10 m intervals along the fence or were hand captured near the fence (see Mushinsky and Wilson, 1992).

Testing for Diapause. — To detect diapause we exposed eggs to controlled laboratory conditions that normally foster development and examined the eggs almost daily for evidence of either diapause or development. We induced oviposition in gravid females in the laboratory by injecting them with oxytocin (Ewert and Legler, 1978) and placed the oviposited eggs in plastic containers with moist vermiculite (a vermiculite to water mixture of 1:1 by weight). Each container was covered with a loose-fitting lid to allow some gas exchange and still retain high humidity within the container. We distributed the containers among five incubators, each one set at a different constant temperature. We designated these constant temperatures as "challenge temperatures" because they challenge the embryos either to develop or to remain in diapause. A summary of our protocol is given in Fig. 2. Prior experience with diapausing embryos has shown that excessive exposure to challenge temperatures, especially warm ones, can cause permanent arrest and eventual embryonic death (Ewert, 1985:227, 1991:180). We therefore exposed eggs to relatively warm challenge temperatures for shorter periods of time than we exposed eggs to relatively cool challenge temperatures (Table 2). We used five different challenge temperatures because temperatures in shallow natural nests fluctuate (Pieau, 1981; Bull, 1985; Plummer et al., 1994), and we wanted to examine a range of temperatures that embryos of *K. baurii* could experience while in diapause in natural nests.

Figure 1. Top: A young adult female *Kinosternon baurii* from the Hillsborough study area. Photo by D.S. Wilson; see journal cover for color. **Bottom:** Detail of the head of another young adult female from the same area. Photo by Julian C. Lee.

Incubation and Observation of Embryos. — During the challenge period, eggs were examined almost daily by candling them with a fiberoptic lamp in a dimly lit room. We classified an embryo as developing when it reached developmental stage 8+ (Yntema, 1968). This stage is characterized by a small, often nearly circular, red-bordered, pink disk, consisting of the recently established area of extra-embryonic vitelline circulation with the elongate, many-somite embryo near the center. Embryos not expressing diapause usually develop to this stage by 1 to 3 weeks of incubation (Ewert, 1985, unpublished data). We classified an embryo as in diapause when it displayed no evidence of vitelline circulation by the end of the challenge period, and yet the shell of its egg was chalked and the system was clearly still alive (most visibly, a vitelline sac with sharply delineated, clear subgerminal space above opaque yellow yolk) .

We terminated diapause because we wanted the post-diapause embryos for additional research. Preliminary work has shown that chilling at 15–21.5°C for at least 90 days is usually necessary to terminate diapause in *K. baurii*. (Ewert, unpublished data). Embryos still in diapause at the ends of their challenge periods were chilled (in plastic containers) for 90 days. At the end of this period the embryos were warmed to 27–28°C until they reached developmental stage 8+.

To contrast laboratory-incubated embryos with others developing in cool natural environments, some embryos that had reached stage 8+ at 28°C were transferred to a cool constant temperature (21.5, 22.5, or 24°C) for the duration of incubation. At hatching we transferred eggs to plastic cups that contained a wet paper towel or shallow (2–3 mm) water. We topped the cups with a wad of damp paper towel to maintain high humidity. Hatchlings (Fig. 3) were housed in this environment at room temperature (24–27°C) and were inspected every other day for 1–2 months.

Gravid Females and Seasonal Incidence of Diapause. — Because we collected eggs from gravid females during both spring and fall nesting seasons, we were able to test for differences in the occurrence of diapause between seasons (Table 2). Based on preliminary results (Ewert, 1991), we expected the proportion of embryos expressing diapause to differ between spring and fall. We asked if females that oviposit in spring belong to different cohorts from females that oviposit in fall. If so, the differences in the expression of diapause by embryos could be associated with distinct cohorts of individuals and perhaps have a hereditary basis. We compiled all records for females captured three or more times along the wetland side of the drift fence at the Hillsborough County site from September 1991 to November 1994. Because all 100 such female captures and recaptures at this site between 1991 and 1992 proved by radiography to be gravid females (Mushinsky and Wilson, 1992), we assumed that females thus captured in subsequent years were also gravid.

Field Experimentation. — To determine whether diapause is essential for embryos to survive the long, cool environment of winter, we exposed actively developing embryos to winter temperatures in a nearly natural environment in the field. Eggs for this testing were oviposited by females from the Hillsborough study area during the fall seasons of 1992 and 1993. Eggs were incubated as described previously; however, the periods of exposure to specific challenge temperatures were more variable than the periods used in our basic protocols (Table 2). As we wanted to obtain actively developing embryos and we knew early in our study that warm challenge temperatures were more effective than cool ones in preventing diapause, we used mostly warm challenge temperatures (27–30°C). Some of the embryos that did not express diapause were allowed to develop past stage 8+ at the challenge temperature. By allowing this variability in development we introduced embryos of different stages to our field environment.

In the field we constructed an artificial, simulated "nest" in the same sandhill habitat where the turtles normally nest. On 11 and 30 January 1993 and again on 18 December 1993, we placed actively developing embryos and still-arrested embryos (as a control) in the nest. Eggs were buried approximately 5–7 cm deep in the soil (Fig. 4), the average depth for nest eggs of *K. baurii* (Wilson, 1995). During winter 1993–94 the local (12 x 12 m) nesting area contained 12 temperature probes at depths of 5–7 cm among *K. baurii* eggs belonging to another nest study and attached to a datalogger (Campbell Scientific CR10) which recorded 48 soil temperatures per day. Because we did not have a probe available for direct placement within our simulated nest, we

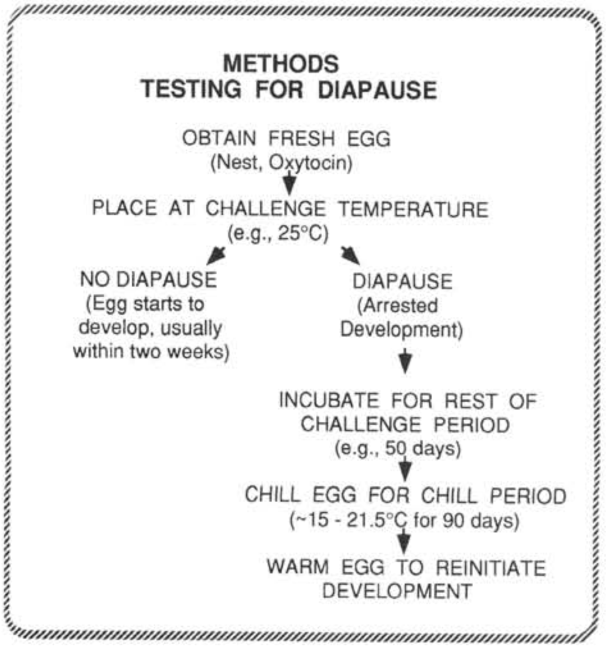

Figure 2. A protocol for the early incubation of turtle eggs suspected of having embryonic diapause. Within an incubation facility, the eggs should be in contact with air (i.e., a gas phase) as it passes through the incubation substrate (e.g., sand, moss, straw, vermiculite) or across egg surfaces that may be exposed above the substrate. The necessary content of the incubation substrate will vary with species and eggshell characteristics.

selected data from a probe at a slightly more shaded site and another probe at a slightly more open site to provide estimates of the thermal conditions at our site. To monitor viability and progress in development during field incubation, eggs were removed briefly several times from the simulated nest, candled (using a flashlight) under a sheet of black plastic, and then returned to the nest.

We wished to be able to interpret our field results relative to winter severity. Had the winters of our field experiment (1992–93 and 1993–94) been warmer or cooler than average winters and thus have provided a possibly biased set of results? Winters in Florida usually vary appreciably from one year to the next. We compiled 17 years (1977–94) of NOAA records of soil temperatures (recorded at 10 cm depth) from agricultural experimentation stations near Bradenton and Lake Alfred, 40 km south and 33 km east, respectively, of our study area.

Organization and Analysis of the Data. — We were primarily interested in making comparisons among eggs produced in the spring and in the fall, although *K. baurii* nests during all seasons (Mushinsky and Wilson, 1992; Wilson, 1995; unpublished). For analytical purposes we assigned "spring" to gravid females collected between 15 March and 10 June and "fall" to gravid females that we collected between 30 September and 10 December. Females collected from 11 December to 14 March were allocated to "winter." Gravid females collected during "spring" provided eggs with embryos designated as "spring embryos." Similarly, gravid females collected during "fall" provided our "fall embryos." We also used these dates to classify individual females that nested during more than one of our designated seasons as "spring-fall," "fall-winter," etc., according to their drift fence records in Hillsborough.

Table 2. Testing for diapause in *Kinosternon baurii*: temperatures, incubation times, and sample sizes.

Challenge Temp. (°C)	Challenge Period (Days)	Chill Period* (Days at 21.5°C)	Spring Eggs (Number)	Fall Eggs (Number)
22.5	60	90	31	19
24.0	60	90	8	23
25.0	50	90	11	20
27.0	40	90	17	16
30.0	30	90	10	15

*Followed by incubation at 28°C for initiation of development.

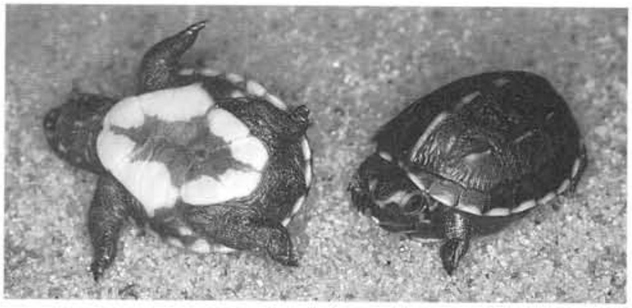

Figure 3. Hatchlings of *Kinosternon baurii* hatched from eggs produced in Seminole Co. Photo by M.A. Ewert.

To detect trends at the level of the population, we wanted to remove variation among siblings (clutchmates) from each seasonal sample (see Bull et al., 1982; Bobyn and Brooks, 1993). Some entire clutches of eggs may express diapause, while other clutches from the same season may not express diapause (Ewert, 1991). In most cases, therefore, we used just one embryo per clutch per season to represent a sample at each given challenge temperature. In these comparisons, then, the unit of analysis for each challenge temperature-by-season combination becomes the clutch.

To achieve economy in use of eggs, we distributed many clutches across challenge temperatures, to provide one egg at each of two, three, or four different challenge

Figure 4. Eggs of *Kinosternon baurii* in a partially excavated shallow nest. The eggs measure approximately 29 x 17 mm and contain near term embryos. Photo by D.S. Wilson.

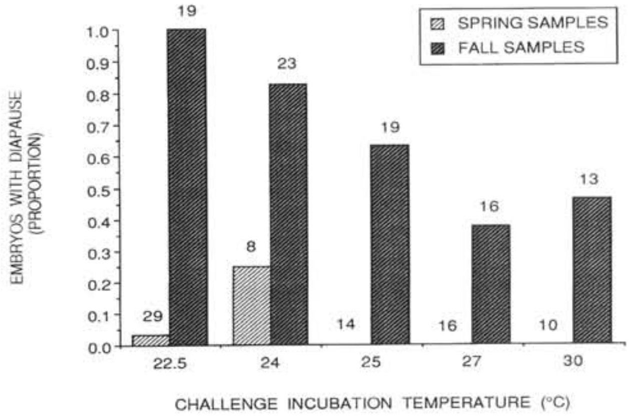

Figure 5. Seasonality of diapause by embryos of *Kinosternon baurii* according to challenge temperature. The numbers above the vertical bars refer to the numbers of embryos that survived the challenge period at the challenge temperature.

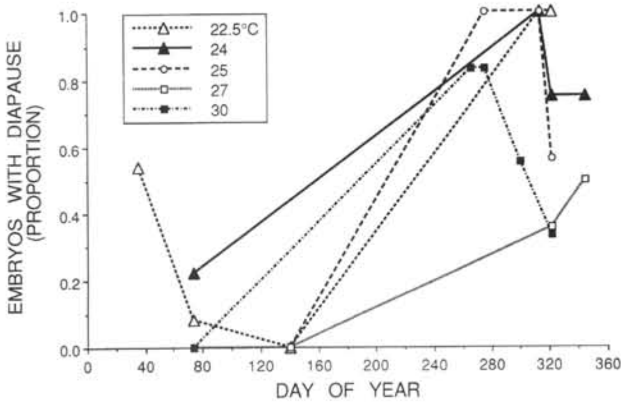

Figure 6. Seasonal incidence of diapause in embryos of *Kinosternon baurii* from Seminole Co. according to day of year and challenge temperature. The plotted points represent dates of capture of gravid females with shelled eggs. Sample sizes vary from 2–35 embryos representing two or more clutches per point.

temperatures. Although this allocation did not yield a completely random design for comparisons of embryonic responses among challenge temperatures within season, we do not regard this shortcoming as critical to our results. We applied the G-test of independence (Sokal and Rohlf, 1981) in various contingency analyses throughout this paper.

RESULTS

Within-Season Effects of Different Challenge Temperatures. — The five different challenge temperatures (Table 2) induced significantly different levels of diapause response among the samples of fall embryos (Fig. 5); however, these temperatures did not clearly differ in their effects on the samples of spring embryos. With an increase in challenge temperature, the proportions of fall embryos that expressed diapause decreased significantly ($G = 24.36$, df = 4, $P < 0.001$; Pearson's rho = -0.9, $P = 0.036$). The overall fall sample represents observations combined from four different fall seasons; but the effect also can be demonstrated within a single year by examining a single large series (63 embryos) obtained during November 1992. The challenge temperatures followed by the numbers of embryos with and without diapause and the proportion with diapause (in parentheses) are 22.5°C, 12, 0 (1.0); 24°C, 9, 3 (0.75); 25°C, 9, 7 (0.56); 27°C, 5, 9 (0.36); and 30°C, 3, 6 (0.33). The proportions of embryos that expressed diapause varied significantly among challenge temperatures ($G = 19.5$, $P < 0.001$) and decreased with increase in challenge temperature (Pearson's rho = -1, $P = 0.023$). Challenge temperature did not significantly influence the proportions of spring embryos that responded with diapause ($G = 8.11$, df = 4, $0.1 > P > 0.05$). However, this analysis is affected by the complete absence of any spring embryos with diapause at the three warmest challenge temperatures (Fig. 5). The two coolest challenge temperatures revealed a few spring embryos with diapause (1 embryo at 22.5 and 2 at 24°C). This result is comparable to cool challenge temperatures also showing the highest incidence of diapause among the samples of fall embryos.

Seasonal Incidence of Diapause. — Only 3 of 77 (3.9%) embryos produced in the spring expressed diapause, whereas 62 of 90 (68.9%) embryos produced in the fall expressed diapause (Fig. 5). When the five incidences of diapause representing the five challenge temperatures were averaged, the incidences became 5.7% of spring embryos and 65.9% of fall embryos.

Expression of diapause varied throughout the year (Fig. 6), being least prevalent in May (calendar days 121–151) and most prevalent between mid-September and mid-Octo-

Table 3. Significance testing of the seasonality of diapause.

Challenge Temperature (°C)	Seasons Compared[1]	Condition	Spring Clutches[2]	Fall Clutches[2]	G-statistic[3]	Probability that Spring = Fall
22.5	S'88–F'91	Diapause	1	7		
		No Diapause	10	0	18.03	< 0.001
22.5	S'93–F'92	Diapause	0	12		
		No Diapause	18	0	40.38	< 0.001
24.0	S'88–F'92	Diapause	2	9		
		No Diapause	6	3	5.03	0.025
25.0	S'93–F'92	Diapause	0	9		
		No Diapause	14	7	14.73	< 0.001
27.0	S'88 & S'93–F'89 & F'92	Diapause	0	6		
		No Diapause	16	10	9.72	< 0.003
30.0	S'88–F'92 & F'93	Diapause	0	6		
		No Diapause	10	7	8.46	< 0.005

[1] S = spring, F = fall. Some comparisons involve consecutive seasons and others do not. We did not assume that this variable would be critical to the outcome. In two contingency tables, we combined the results from two springs, two falls, or both so as to increase the robustness of these comparisons.
[2] Each clutch is represented by one egg or one decision per clutch (see text).
[3] Computations follow Sokal and Rohlf (1981).

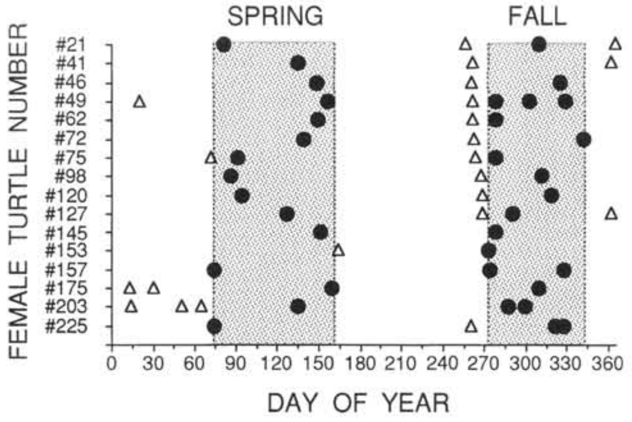

Figure 7. Drift fence captures of 16 of 27 females of *Kinosternon baurii* in Hillsborough that nested in both spring and fall. In each case, gravidity was confirmed by radiography or with oxytocin injection. The shaded areas indicate the spring and fall sampling periods for the gravid females from Seminole that provided embryos for determining seasonal incidences of diapause (Table 2, Fig. 5). Solid circles represent captures within the spring and fall sampling periods; open triangles represent other captures of the same females.

ber (calendar days 258–288). These intervals include the spring minimum incidence of diapause at 22.5°C, the challenge temperature most conducive to diapause (Fig. 5), and the fall maximum incidence at 30°C, the temperature least conducive to diapause.

Diapause at each of the five challenge temperatures was expressed significantly less often in clutches produced in the spring than in clutches produced in the fall (Table 3). Perhaps because no more than two embryos ever represented a given clutch at a given temperature, no spring or fall clutches from Seminole ever included embryos both with and without diapause at the same temperature. We did, however, challenge seven complete fall clutches (2–4 eggs each) from Hillsborough at 30°C. Three of these clutches expressed mixed responses (13 of 18 embryos with diapause overall). Additionally, we challenged 14 complete winter clutches from Seminole at 22.5°C (females gravid on 4 February; 2–3 eggs per clutch). Four of these clutches gave mixed responses, six showed only diapause, and four showed only direct development without diapause. Thus, some independence of response can occur within a single clutch, but most often all of the embryos respond alike.

Parental Contribution to Seasonality. — We recorded 65 presumably gravid females with 3–8 captures at the drift fence each, a total of 270 records. Each one of these females was encountered at the drift fence at least once during the fall. Fewer females were encountered during the spring (28 spring vs. 174 fall); still, 27 of the 65 females (42%) were encountered during both seasons (Fig. 7). Only 10 females had just fall encounters; the remaining 28 had fall-winter or summer-fall encounters. If an individual female was recaptured more often, she was more likely to be a spring-fall visitor to the nesting area. The frequency of encountering a spring-also female was less (< 0.35) for 3, 4, or 5 drift fence records and more (> 0.70) for 6, 7, or 8 records (G = 8.45, $P < 0.005$). Probably, all females are capable of nesting in both seasons, but they do not nest as often in spring as in fall.

Table 4. Trends in simulated nests in the field by *Kinosternon baurii* embryos previously challenged by incubation temperatures in the laboratory.

Status when Placed in the Field	Time of Placement	Number of Eggs	Number Developed
Developing	Winter '92, '93	12	12
	Late Fall '93	5	5
Arrested	Winter '92, '93	10	10
	Late Fall '93	13	1*

*All eggs depredated after check in April.

Winter Soil Temperatures. — Our historical survey of regional data on soil temperatures indicated that winter 1992–93 was unusually warm, with January ranking as second warmest in 17 consecutive winters and the overall winter mean temperature as third warmest, and that winter 1993–94 was cooler, an average winter, with January as well as the overall winter ranking as the eighth warmest in the 17 winters. During 1993–94 the two temperature probes at the nesting site indicated a 50-day period of cool temperatures (18 December – 6 February), with 71–76% of the readings <17°C, and 85–89% of the readings <19°C. During this period the temperature rose above 21.5°C only 2.6–3.3% of the time, or 1.3–1.7 days cumulatively, mostly toward the end of the period. Later in February conditions became appreciably warmer and 31–36% of the records exceeded 21.5°C.

Winter Field Survival of Post-Diapause Embryos. — Sixteen embryos that had broken diapause in the laboratory ranged from early somite (ca. stage 6 of Yntema, 1968) to early carapacial scutellation (ca. stage 18) when placed in the field "nest." These embryos survived the remainders of their respective winters (Table 4), which extended from 11 January 1993 and from 18 December 1993.

Ten embryos that had not broken diapause in the laboratory and were placed in the field "nest" on 30 January 1993 subsequently broke diapause and hatched. Seven of these embryos broke diapause after 5 May 1993. Thirteen diapausing embryos were placed in the field on 18 December 1993, and only one broke diapause by 1 April 1994 (Table 4). However, it had appeared on 1 April that four of the embryos might have been dead, perhaps from an overlong challenge at 30°C (see methods), and all 13 subsequently suffered depredation.

Cool Laboratory Survival of Post-Diapause Embryos. — Development at constant 21.5°C resulted in several late embryonic and early post-hatching deaths (Table 5). The incubation time from stage 8+ to pipping at 21.5°C averaged 176 days ± 16 SD days (median 172–173 days; $n = 22$ embryos) but was quite variable (range 156–231 days). Many of the hatchlings from incubations at 21.5°C appeared to have neurological defects and were unable to right themselves. Hatchlings from 22.5 and 24°C survived for several weeks with less evidence of neurological defects (Table 5).

DISCUSSION

Seasonality of Diapause. — We applied the same protocols of collection and incubation to eggs of *K. baurii* collected during different nesting seasons of the year and observed seasonally dependent responses in embryonic development. Replicated observations at the same and different challenge temperatures revealed almost no embryonic diapause in the spring and prevalent diapause in the fall (Table 3). Although prior data (Ewert, 1991:179) had led us to anticipate a seasonal factor in developmental arrest, we believe now that our evidence is robust and perhaps the first unambiguous demonstration of a seasonally dependent expression of diapause in reptiles.

The expression of diapause in embryos of *K. baurii* under different thermal conditions has been misinterpreted if not underestimated (Ewert, 1991:176) or overlooked altogether (Ewert, 1985:189). These oversights resulted from numerous observations on eggs obtained during the late winter and spring when diapause is weak or absent (Fig. 6), and no observations on development in eggs from early to midway through the fall when diapause is strong.

Oversights pertaining to seasonality in the expression of diapause may explain additional discrepancies in the literature and with personal experience. It once seemed likely that expression of embryonic diapause was diagnostic of some populations or species while being absent in others (Ewert, 1985). Embryos of the chicken turtle (*Deirochelys reticularia*) from Florida that were initially challenged at different temperatures (including 25 and 30°C) almost always expressed diapause, requiring substantial chilling before warm temperatures would foster continuous development (Ewert, 1985; Jackson, 1988). In contrast, embryos of *D. reticularia* from South Carolina appeared not to express diapause, developing at 29°C without an initial period of chilling (Congdon et al., 1983). Although the geographic factor (Florida vs. South Carolina) could explain this difference, we now view seasonality as a strong alternative explanation. All eggs of *D. reticularia* represented in Ewert (1985) were fall eggs and all eggs incubated by Jackson (1988; *pers. comm.*) were fall or winter eggs. By contrast, all eggs in Congdon et al. (1983) were obtained in the spring. Together, these observations suggest the pattern of limited diapause in the spring and prevalent diapause in the fall that we have identified in *K. baurii.*

We had hypothesized that the seasonal occurrence of diapause could be an inherited trait if some females nested only in the spring and others only in the fall. In this scenario, females in the spring-nesting cohort would never nest in the fall and would never produce embryos with diapause, and the converse would be true for the fall-nesting females. We found, rather, that all of the spring-nesting females also nested in the fall. Given that the response of embryos to cool challenge temperatures (22.5–25°C) is nearly always with diapause in the fall and without diapause in the spring (Figs. 2 and 3), females must be capable of producing both types of embryos because the females nest in both seasons.

The Diapause Factor. — Reptilian embryos may express diapause because they lack a stimulatory factor or because they possess only a precursor to one. It is conceivable that females normally supply their egg albumen with some factor, such as a hormone, that activates development upon exposure to air and thereby terminates pre-ovipositional arrest. Perhaps in embryos with diapause, the stimulatory factor is synthesized or activated only during a chill. Alternatively, the eggs may possess an inhibitory factor that can only be deactivated during a chill. Although we cannot distinguish among these alternatives, there is anecdotal evidence that the developmental decision for or against diapause occurs late in oogenesis in *K. baurii*, possibly soon after ovulation and fertilization. In one fall case and one spring case, females that were not gravid when collected subsequently ovulated in captivity. The fall-collected female was lightly chilled in captivity before she ovulated. Her two eggs developed without diapause. The spring-collected female ovulated during warm maintenance, and her two eggs expressed diapause. Perhaps the diapause factor was reversed during the brief period in captivity in both cases.

Prolonged retention of eggs in the oviducts may influence diapause. Oviductal eggs in captive *K. baurii* normally remain viable for 40–60 days when females are housed at room temperature (ca. 24–26°C). The schedule of induced oviposition of eggs from a series of Seminole females sometimes lasted a few weeks, regardless of season, even though the period for field collection of females was often brief, normally a day or two. In accord with the hypothesis that oviductal retention reduces instances of diapause, comparisons of fall eggs induced early vs. later approached statistical significance ($P = 0.07$) but only at challenge temperatures of 25 and 27°C and not at 22.5, 24, or 30°C, nor across all challenge temperatures. In one case, a fall gravid female was induced to oviposit two eggs, and then, with the third egg retained, she was chilled at 15°C for 30 days. The chilled egg (following laying) as well as the two unchilled eggs expressed diapause.

Table 5. Developmental successes of *Kinosternon baurii* at cool, constant incubation temperatures.

Incubation Temperature (°C)	Total Number	Number Lived	Number Died	Problems with Coordination (*n*)	Percent with Coord. Problems
21.5	26	16	10	8 (18)	44
22.5	11	11	0*	3 (11)	27
24.0	25	25	0*	1 (25)	4

* The probability of survival success being the same at 21.5°C vs. a warmer temperature is $P < 0.005$ (G = 8.53) relative to 22.5°C, and $P < 0.001$ (G = 10.22) relative to 24°C.

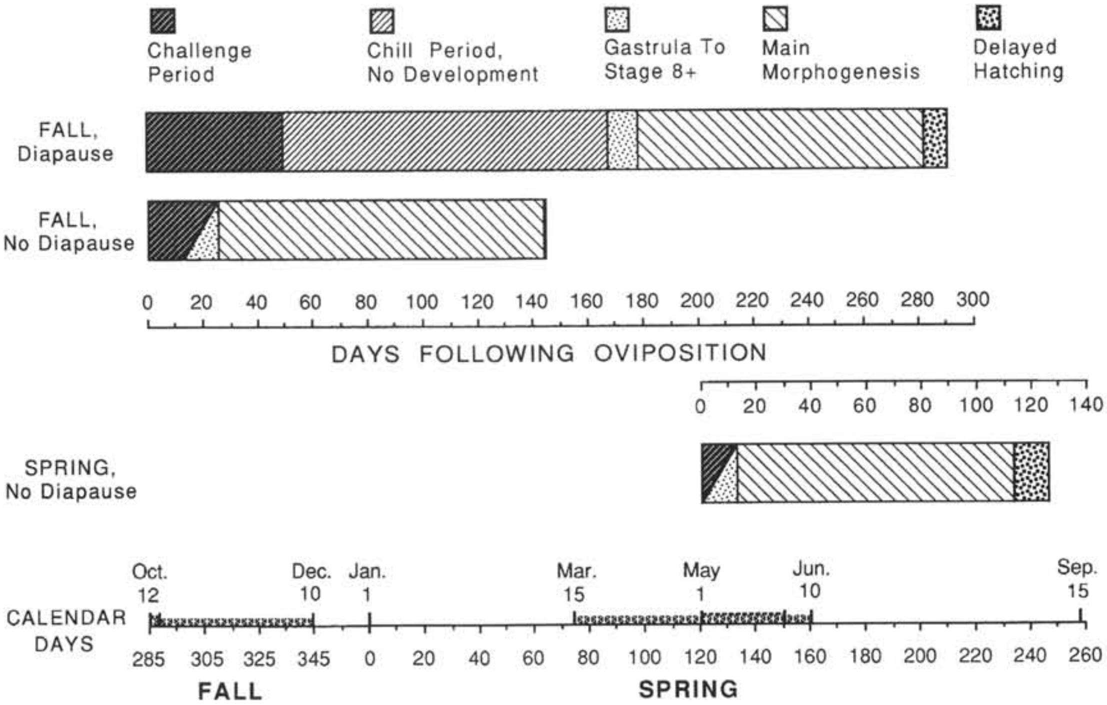

Figure 8. Incubation timelines of embryos of *Kinosternon baurii* from Seminole Co., with and without diapause. Timelines are fitted to a hypothetical seasonal progression (bottom axis with arbitrary spring and fall intervals highlighted with cross-hatching), with dates of oviposition arbitrarily set on 12 October for fall embryos and 1 May for spring embryos. All challenge periods and the main morphogenesis occurred at 25°C. In the "fall, diapause" series post-chilling incubation to developmental stage 8+ occurred at 28°C. Sample sizes, mean total incubation times (through hatching), standard deviations, and ranges are: for "fall diapause," 8, 290.9 ± 9.7, 271–303 days; "spring, no diapause," 11, 125.2 ± 9.1, 108–135 days; "fall, no diapause," 3, 144.7 ± 8.3, 138–154 days. The condition "fall, no diapause" probably does not occur in nature because early fall embryos have strong diapause and would not commence development at any temperature, whereas late fall embryos may have weaker diapause but would experience only temperatures more conducive to sustaining diapause (i.e., 22.5°C or cooler; see Fig. 3 and text). It is noteworthy that the obligatory duration of chilling is quite long (ca. 90 days) because this duration greatly facilitates the passage of winter.

We believe that fall eggs are ovulated during the fall and that spring eggs are ovulated during the spring. Our evidence assumes that formation of the calcareous eggshell (Packard et al., 1984) takes only a short time (Ewert, 1985:219). We also assume that a clutch of mature eggs must be oviposited before ovarian follicles for a second clutch are ovulated, because the alternative leads to adverse consequences (Ewert et al., 1984). Some of the females of *K. baurii* collected from ditches in Seminole have incompletely formed, soft oviductal eggs during palpation in the field. A few females collected in the spring and others collected in the fall laid incompletely shelled eggs following early post-capture injection of oxytocin. Drift fence records show that females in the Hillsborough study area occasionally produce more than one clutch in a single season. Thus, present observations do not strongly implicate oviductal retention as a factor in the expression of diapause, but experiments are needed to confirm this hypothesis.

Adaptive Significance. — Fall embryos naturally progress into winter in diapause (Wilson, unpublished). Further, according to estimates from laboratory incubation (Fig. 8), diapause in fall embryos may delay embryonic development until it partially overlaps the active development occurring in spring embryos from the following spring. We therefore hypothesized that the cool temperatures of winter would be lethal to actively developing embryos. We found, however, that none of our 17 experimental embryos died (Table 4). We must qualify our observation in that neither winter was colder than average, and the embryos were not in the field for the entire period of cool temperatures (i.e.., from 15 November to 1 March). Still, we tested a broad array of embryonic stages and for most of the cooler of the two winters (1993–94). Our laboratory observations on development and hatchling behavior at 21.5°C (Table 5) suggest that 50 days of almost continuously cool temperatures in the field would not enhance hatchling fitness (see Janzen, 1993), even if this exposure lasted less than a third as long as the time needed to complete development at 21.5°C. Development by fall embryos that is uninterrupted by diapause, that is, development that would occur during the winter (Fig. 8) could render hatchlings from fall eggs less fit than hatchlings from spring eggs. It may be appropriate, therefore, to compare performance tests on field hatchlings with a history of development that was artificially initiated during the fall with hatchlings that developed during the spring.

Conservation Planning for Embryonic Diapause

Programs to incubate eggs in protected and possibly artificial environments and then release the hatchlings promptly might be justified when a high percentage of natural nests are lost to predators or other factors.

Recommendations to manipulate incubation temperature relative to natural regimes have addressed the control of sex ratio (Vogt, 1994) and enhancement of hatchling vigor (Packard and Phillips, 1994). However, naive application of incubation technology (e.g., constant temperature) can cause serious problems for species that express embryonic diapause. Application of any single temperature or narrow range of temperatures will not suffice! Early application of temperatures that are warm enough to bias sex ratio favorably (Vogt, 1994) and to produce vigorous hatchlings, regardless of sex (Janzen, 1993), can lock embryos in diapause until they die. Alternatively, chilling embryos that do not express diapause can also cause embryonic death, especially when subtropical species are involved. Conservation workers have the choice of protecting nests *in situ*, of moving the eggs to protected surroundings at ambient temperatures, or of learning whether embryos of the species of concern express diapause.

Candidate Species. — Known species of turtles that express diapause tend to share common features of natural history and geographic distribution (Ewert, 1985, 1991; Jackson, 1988). Some aspects of diapause in 11 taxonomically diverse species are provided in Table 6. These species tend to have warm temperate to peripherally tropical distributions. India, Mexico, Argentina, and peninsular Florida all have two or more species that express diapause (Jackson, 1988; Ewert, 1991 and unpublished). No species north of 35°N in North America, Europe, or eastern Asia has yet been found that expresses diapause. Among turtles with approximately equatorial distributions (see Iverson, 1992), several species may express developmental arrest (e.g., *Chelodina rugosa*, Kennett et al., 1993; *Geochelone carbonaria*, *Malacochersus tornieri*, Ewert, unpublished, Table 6), but only in some *Phrynops geoffroanus* does development appear to have been activated by a change in temperature (Ewert, 1991, unpublished).

Eggs of species with diapause tend to have rigid to brittle shells. Of the 23 species of turtles with confirmed diapause, only *Deirochelys reticularia* lays eggs with flexible eggshells.

Species or populations that express diapause usually nest well into a period of declining daylength. For example, in *K. baurii* the maximum expression of diapause occurs during the fall period of declining daylength. For many species the fall or onset of a winter dry season are times to expect eggs with diapause. However, the association of diapause with declining daylength may not always apply. For example, *K. flavescens arizonense* lays brittle-shelled eggs and peaks in nesting late in July (Iverson, 1989), but its embryos do not express diapause. Even though *K. hirtipes* and *K. sonoriense* peak in their nesting in late July or August (Iverson et al., 1991) and thus fit our proposed conditions of declining day length, eggs produced on 17 and 29 June, respectively, still expressed diapause (Ewert, unpublished). Diapause may be obligatory in these two species.

Cool Temperature Challenge. — In zoos, species with unknown natural histories may still produce fertile eggs, and attempts to hatch these eggs using alternative strategies (whether to initiate warm or cold incubation) may prove lethal. In such cases we recommend beginning incubation at moderately cool temperatures (< 25°C). Most species tolerate cool incubation early in development, and some non-diapausing species as well as diapausing species appear to require it (e.g., < 30°C for some *Elseya novaeguineae*). Most non-diapausing embryos will develop sufficiently far in about three weeks to allow diagnosis by candling (except in some tortoises). Eggs that have begun to develop can be warmed, whereas eggs that do not show development and yet appear fertile can be chilled. Unfortunately, even an initial three weeks at 25°C may be too warm for diapausing eggs of some species (e.g., *Phrynops hilarii*, Table 6). If sufficient numbers of eggs are available, application of diverse protocols to small groups of eggs may be appropriate. Because non-diapausing embryos are likely to develop quickly under warm protocols, these protocols can be eliminated from further consideration if development is not evident within two weeks.

Once diapause is indicated, it is necessary to choose a temperature and a duration for chilling. In general, chilling for 90 days at 20–21.5°C works well for most species, although some embryos respond dependably only to lower temperatures (e.g., *Kinosternon sonoriense* to 15°C; Ewert, 1991; Table 6). Regardless, workers overseeing the incubation must be prepared for a long period of attendance (Fig. 8).

Meeting Warm Incubation Objectives. — After chilling, the eggs of most species may be transferred to almost any temperature that will sustain active development. A few species have difficulty completing early somite development to stage 8+ at > 29°C (e.g., *Staurotypus* spp., *Melanochelys trijuga coronata*; Ewert, 1985), and an initial period at a moderately warm temperature before a warmer one may be necessary. For example, consider the objective of controlling sex ratio in turtles that have temperature-dependent sex determination (Vogt, 1994). If advancing from an initial post-chilling temperature to a desired sex-determining temperature requires a shift in incubation temperature (e.g., from 28 to 30°C), then the worker must be attentive to the embryonic development in order to perform the temperature shift promptly after the embryo has developed to stage 8+. Otherwise, there is risk of development advancing into the temperature-sensitive period for sex determination (Mrosovsky and Pieau, 1991; Wibbels et al., 1991) at the wrong temperature and producing the undesired sex. In *K. baurii* initiation of post-chilling development in different eggs can proceed fairly synchronously at moderately warm temperatures. For example, 34 chilled fall embryos advanced to stage 8+ in 5–13 days (mean: 9.6, $s^2 = 4.4$ days) after warming to 28°C. Nine unchilled fall embryos, designated as non-diapause because they began developing before the end of their challenge periods at 27°C, varied from 13 to 34 days ($s^2 = 56.5$ days) to advance to stage 8+. Ten unchilled spring embryos at 27°C varied from 9 to 21 days, with $s^2 = 14.9$ days. For management purposes, the synchronization of developmental timing obtained by chilling gave

Table 6. Some diverse species of turtles that express embryonic diapause and recommendations for incubation protocols.

Family, species[1]	Nesting season[2]	Sample sizes[3]	Embyro develops without diapause (incidence)[4]	Diapause ends without chilling (incidence)[5]	Safe vs. potentially hazardous challenge temperatures[6]	Minimally effective chilling period[7]	Recommended chilling period[8]	Safe vs. potentially hazardous temperatures for early post-diapause embryos[9]	Days following chilling until development is obvious[10]	References[11]
Chelidae										
Acanthochelys pallidopectoris	Late summer (c)	11,6,3	none	none	to 30°C,?	unknown[12]	>100 d @ 18-20°C[12,13]	24°C, unknown	20-40 d	K,L
Phrynops hilarii	Fall (c)	86,77,73	none	none	to 24, >25°C	50 d @ 21.5°C	90 d @ 21°C	25-27, 30°C	15-25 d	K,L
Kinosternidae										
Kinosternon baurii	Fall-winter-spring (w)	>200	many (see text)	5 in 74	to 30, >32°C?	60 d @ 15-21.5°C	90 d @ 15-21°C	23-28, 32°C	5-20 d	G,N,P
K. creaseri	Summer-fall-winter (c)	59,56,39	none	2 in 59	to 28, >29°C	35 d @ 22.5°C	50 d @ 21°C	23-28, 30°C	10-20 d	C,K
K. sonoriense	Summer (w)	83,81,77	none	none tolerated[14]	to 32°C	90 d @ 21.5°C	90 d @ 15°C	25-28, 32°C	11-18 d	C,I,K
Staurotypus salvinii	Fall-winter (c)	97,92,82	none	42 in 97	to 27, >30°C	25 d @ 22.5°C	30 d @ 18°C	23-27, 30°C	15-25 d	B,C,E,K
Emydidae										
Deirochelys reticualria[15]	Fall-winter (w)	133,83,67	none	3 in 133	to 25, 30°C	45 d @ 15-21°C	90 d @ 15-21°C	23-30, >30°C	9-20 d	B,F,K
Melanochelys t. coronata	Fall-winter (c and w)[16]	113, 91,80	1 in 113	49 in 112	to 27, >30°C	25 d @ 21.5°C	30 d @ 21°C	23-28, >30°C	11-20 d	B,C,D,K,O
Rhinoclemmys pulcherrima	Fall (c)	69,63,57	1 in 69	2 in 68	to 27, >28°C?	40 d @ 22.5°C	60 d @ 20°C	25-27, >30°C	11-25 d	C,K
Testudinidae										
Malacochersus tornieri	Fall (c)	52,51,46	none[17]	51 in 52	to 30, >32°C?	unknown[18]	30 d @ 22°C[19]	27-30, 32°C	30-40 d?	A,J,L
Trionychidae										
Aspideretes gangeticus	Fall (c and w)	>30,3,2	unknown[20]	unknown[20]	to 30, >32°C	19 d @ 10°C[21]	longer, warmer?[22]	28-30, ?	unknown	C,H,M,O

[1] Listings emphasize diverse chelonian families and some of the species showing strong or consistent embryonic diapause.

[2] Listings emphasize peak periods of nesting activity or egg production in (c) captive groups or (w) in the wild.

[3] Fertile eggs under observation, embryos achieving vitelline circulation (stage 8+), embryos achieving near term or hatching (stages 25–26), respectively.

[4] Here, the term "diapause" is not applied according to its strictest definition, but refers to arrested embryos (that eventually develop) in eggs with adhesion (Table 1, footnote 1) and subtle to distinct eggshell chalking (Table 1, column 1). Estimates are only suggestive because challenge conditions varied non-uniformly within and across species.

[5] As with footnote 4. Qualifying embryos have early developmental arrest and yet resume development without a period of chilling and after delays of four or more fold the times that related species require to complete the same early embryonic stages. This may be viewed as a weak or only partially expressed form of diapause.

[6] Evaluation applies to an immediate post-ovipositional period of 2–3 weeks. All embryos indefinitely arrested in diapause eventually deteriorate at any temperature.

[7] Protocols that terminate diapause in some embryos but not in many.

[8] Protocols that terminate diapause in nearly all challenged embryos (except see column footnotes).

[9] Applies to somite stages prior to stage 8+. Perseverance at the higher temperature varies among species but some individuals succumb at the higher temperatures.

[10] Refers to the warm period subsequent to chilling that is required to achieve stage 8+, generally at > 24°C. An observer must wait this long to be sure that a protocol of chilling has been a success.

[11] References: A: Darlington and Davis, 1994, B: Ewert, 1985, C: Ewert, 1991, D: Goode, 1991, E: Goode, 1994, F: Jackson, 1988, G: Mushinsky and Wilson, 1992, H: Rao, 1986, I: Rosen, 1987, J: Schmalz and Stein, 1994, K: M.A. Ewert, unpubl. data, L: M.A. Ewert, R.E. Hatcher, and J.M. Goode, unpubl. data, M: K. Vasudevan, unpubl. data, N: D.S. Wilson, unpubl. data, O: Annual records of the Madras Crocodile Bank, 1986–93, P: present study.

[12] Chilling for 105–185 days at 21.5°C has terminated diapause but subsequent development by two of four embryos was abnormal. Chilling for 65 days at 15°C has not been adequate and additional incremental chillings (15–30 days at 15–22.5°C) have been necessary to terminate diapause. Clearly more research is needed to terminate diapause effectively.

[13] A suggested, not proven protocol (see footnote 12).

[14] All of seven embryos that were challenged for 50 days at 32°C and then chilled eventually hatched.

[15] All data refer to populations in northern Florida.

[16] The "w" refers to a captive group of several subspecies of *Melanochelys trijuga* maintained in captivity but under natural ambient conditions. *Melanochelys t. coronata* and *M. t. thermalis* nest concurrently in captivity.

[17] Generally following oviposition, the egg contents persist for 20–60 days prior to adhesion and then for another 30–80 days prior to the onset of stage 8+. The latter period approximates a diapause condition.

[18] Chilling for 30 days at 24°C has given ambiguous results.

[19] Suggested protocol, not proven. For practical purposes, these eggs almost always commence development even if chilling is not attempted.

[20] Despite strong evidence for diapause, more precise observation is needed.

[21] The attempted protocol was attended with complications. More observation is needed.

[22] Because most embryos do not appear to escape diapause in southern India and the species occurs naturally where winters are cooler, some chilling seems logically requisite to normal development

the most homogeneous embryonic development for conducting additional temperature shifts. Chilling can provide an additional benefit. Without damaging the embryos, the duration of chilling can be extended beyond the minimum period necessary to break diapause. Eggs can therefore be accumulated in a chilling environment over a period of several weeks and then rewarmed together. If all of the embryos have been chilled at least long enough to break diapause, then all should develop synchronously, despite the asynchronous timing of their origins.

Application to Kinosternon baurii. — Many Florida populations of *K. baurii* appear to be robust except, perhaps, a portion of those in the lower Florida Keys (Monroe Co.; Wilson, in press and unpublished). While management plans should always address the basic biology of a species of concern, the difficult prospect of managing the incubation of eggs expressing diapause in this case probably only underscores the need for management to address more obvious concerns for conservation, such as habitat protection.

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