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CONTROL OF METABOLISM AND GROWTH IN EMBRYONIC TURTLES: A TEST OF THE UREA HYPOTHESIS

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Summary

We performed two experiments to determine (1) whether the metabolism and growth of embryonic snapping turtles (*Chelydra serpentina*) incubating in wet and dry environments are correlated inversely with the concentration of urea inside their eggs, and (2) whether urea accumulating inside eggs might be the cause of reductions in metabolism and growth by embryos. Eggs in the first experiment were incubated in different hydric environments to induce different patterns of net water exchange between the eggs and their surroundings. Turtles hatching from eggs that were in positive water balance had larger carcasses, smaller residual yolks and lower concentrations of urea in their blood than animals emerging from eggs that were in negative water balance. Thus, we confirmed the existence of correlations among water exchange by eggs, concentrations of urea in fluid compartments inside eggs, and metabolism and growth of embryos. In the second experiment, eggs were injected with solutions of urea at the mid-point of incubation to induce different levels of uremia in developing embryos. The injection protocol induced variation in the concentration of urea in blood of hatchlings similar to that observed in the first experiment for turtles hatching in wet and dry environments. However, the injection protocol did not induce variation in size of hatchlings or in mass of their residual yolk. Thus, the reduction in metabolism and growth of chelonian embryos developing in dry environments does not result from an inhibition of intermediary metabolism caused by urea, and the 'urea hypothesis' for control of metabolism cannot be accepted in its present form.

Introduction

The eggs of many turtles have flexible, porous shells that allow water to be exchanged relatively freely between the interior of the eggs and their nest environment (Packard & Packard, 1988*a*). Eggs incubating in wet environments consequently absorb large quantities of water during incubation, whereas eggs incubating in dry environments lose water to their surroundings (Packard *et al.* 1983, 1985*a*, 1987). The resulting variation in availability of water to embryos is

Key words: embryos, metabolism, turtles, urea, water.

apparently important physiologically, because oxygen consumption is higher, mobilization of nutrients from the yolk is faster, and growth in body mass is swifter among embryos in wet environments than among those in dry conditions (Morris *et al.* 1983; Gettinger *et al.* 1984; Packard & Packard, 1986). Turtles hatching in wet environments consequently are much larger than those hatching in dry environments, and the former have smaller masses of unused (residual) yolk than the latter (Packard *et al.* 1983, 1985*a*, 1987, 1988).

How are the metabolism and growth of embryonic turtles modulated by the availability of water inside their eggs? One possible answer to this question lies in a consideration of the patterns of accumulation of urea in body fluids of embryos (Packard & Packard, 1984; Packard *et al.* 1984). Urea is the primary end-product of protein catabolism in chelonian embryos, and this solute accumulates inside eggs throughout incubation (Tomita, 1929; Packard & Packard, 1983; Packard *et al.* 1983, 1984, 1985*a*).

Embryos in wet environments have higher rates of metabolism than embryos in dry environments (Gettinger *et al.* 1984), so the former catabolize larger quantities of protein (Packard *et al.* 1988). Although the total amount of urea inside eggs consequently increases more rapidly in wet environments than in dry ones (Packard *et al.* 1984), the concentration of urea may actually increase faster in the dry environments (Packard *et al.* 1984). This unexpected possibility arises as a result of the different patterns of net water exchange characterizing eggs in wet and dry environments: eggs in wet environments contain more water in which to disperse urea than eggs in dry environments (see Morris *et al.* 1983; Packard *et al.* 1983).

We focus on concentrations of urea inside eggs, because this compound is a potent inhibitor of numerous metabolic enzymes that have been studied *in vitro* (Rajagopalan *et al.* 1961). Urea seemingly inhibits the activity of enzymes by destabilizing their tertiary and/or quaternary structure (Hand & Somero, 1983; Hochachka & Somero, 1984), and such inhibition can be detected at concentrations as low as 25–50 mmol l^{-1} (Giordano *et al.* 1962; Hand & Somero, 1982, 1983). Interestingly, these are the very concentrations of urea that have been predicted to characterize fluid compartments of turtle eggs incubating in relatively dry situations (Packard *et al.* 1984).

These considerations lead to the following hypothesis for modulation of metabolism in embryonic turtles developing in wet and dry environments. The concentration of urea increases more rapidly during early stages of incubation in eggs in dry environments than in eggs in wet conditions, because eggs in dry conditions lose water to their surroundings whereas eggs in wet conditions absorb water from the environment. Consequently, urea attains a concentration sufficient to inhibit key metabolic enzymes earlier in incubation in dry environments than in wet ones. The metabolism of embryos in dry environments is inhibited accordingly. Although the rate of formation of urea is subsequently reduced in eggs in dry environments, the rate of increase in the concentration of this solute in fluid compartments of these eggs still exceeds that in eggs in wet settings, because of the

differences in patterns of net water exchange (Packard & Packard, 1984; Packard *et al.* 1984). Thus, the initially small differences in concentration of urea between wet and dry environments are amplified as incubation continues, and the temporal increase in metabolism of embryos in dry environments does not keep pace with that of embryos in wet conditions (see Gettinger *et al.* 1984). Embryos in dry environments therefore mobilize less of their yolk in support of metabolism, and they also grow more slowly than embryos in wet environments. Consequently, turtles hatching in dry environments are smaller than turtles hatching in wet settings, and the smaller hatchlings contain larger masses of residual yolk in their abdomen than the larger hatchlings.

The present investigation assesses a critical assumption of the urea hypothesis and then tests a prediction of the hypothesis. The first part of the study tests the assumption that the concentration of urea in fluid compartments inside eggs is higher for turtles developing and hatching in dry environments than for those developing and hatching in wet conditions. The second part is an experiment testing effects of altered levels of urea in fluid compartments of embryos on size of turtles at hatching. It is predicted that turtles hatching from eggs injected with relatively large quantities of urea will be smaller than animals hatching from eggs receiving little or no urea.

Materials and methods

Plasma urea in hatchlings from wet and dry environments

Eggs of common snapping turtles (*Chelydra serpentina*) were collected on 5 June 1987 from three newly constructed nests located in the Valentine National Wildlife Refuge, Cherry County, Nebraska. The eggs were packed in damp peat moss and then stored in a cool cellar to arrest development of embryos until the eggs could be transported to Colorado State University on 14 June (Christian *et al.* 1986). We were careful not to invert eggs or to jar them unduly during handling and transport (Limpus *et al.* 1979).

On 15 June, 24 viable eggs (identified by the presence of a white spot on the uppermost surface; Ewert, 1985) were selected from each clutch and assigned randomly to wet or dry substrata for incubation (i.e. 12 eggs were placed in each treatment). Wet substrata were prepared by mixing 337·5 g of water into 300 g of dry vermiculite (Terralite, grade 3) to create media having water potentials of approximately −150 kPa; dry substrata were prepared by mixing 51·2 g of water into 300 g of the vermiculite to produce media having water potentials near −950 kPa (Packard *et al.* 1987). The eggs were placed on the substrata so that approximately half of their eggshell contacted the medium and half was exposed to air trapped inside the covered containers. Two boxes were required for each clutch (i.e. one wet, one dry), and six boxes were therefore required for the full experiment.

The boxes containing eggs were placed in an environmental chamber maintaining a diel cycle in temperature ranging from 23·5°C (at 04.00 h) to 29·5°C (at

16.00 h). Eggs incubating in natural nests are exposed to similar cycles in temperature (Packard *et al.* 1985*b*), so this facet of our protocol is ecologically relevant. The boxes were shifted once each day according to a predetermined schedule to minimize possible effects of gradients in temperature inside the chamber (Lee & Rawlings, 1982). Moreover, all eggs were weighed every 7 days, and water was added to substrata to replace that which had been absorbed by eggs or which had escaped from boxes (Packard *et al.* 1987).

We anticipated that incubation would require approximately 63 days under conditions used here. This period of incubation was divided into trimesters of 21 days. At the end of the first trimester, half the eggs in each container were transferred to the other box holding eggs from the same clutch, and a similar transfer of eggs was made between wet and dry substrata at the end of the second trimester. The transfers caused eggs to be exposed to different hydric conditions at different times in incubation, and eight treatments were identified accordingly (i.e. wet–wet–wet, wet–wet–dry, wet–dry–dry, etc.). The primary reason for making the transfers was to induce a range in patterns of net water exchange between eggs and their surroundings (see Packard & Packard, 1988*b*). Additionally, such transfers simulate the variable and changing hydric conditions that eggs encounter in natural nests (Packard *et al.* 1985*b*), increasing the probability that phenomena observed in the laboratory have parallels in nature.

The first eggs were found to be pipped on day 61 of incubation. Cylinders formed by removing the bottoms from waxed drinking cups then were placed around each of the eggs so that hatchlings could be identified with certainty. The boxes were checked daily thereafter, and the dates of pipping and hatching were recorded. The number of days elapsing between day 0 (15 June) and pipping of the eggshell was used as the measure of length of incubation, because this interval is more useful than the interval between day 0 and hatching in assessing time required for an embryo to reach a point in development where it is capable of surviving outside the egg (Gutzke *et al.* 1984).

Hatchlings were cleaned of adhering vermiculite and killed by decapitation. Blood was collected from severed vessels in the neck directly into capillary tubes treated with sodium heparin. Unused (residual) yolk was dissected from the body of each animal, and the yolk and carcass were dried to constant mass at 50 °C.

Plasma was isolated from the blood samples by centrifugation. The osmolality of plasma was measured with a Wescor vapor-pressure osmometer, and the concentration of urea in each sample was measured colorimetrically (Fawcett & Scott, 1960; Chaney & Marbach, 1962) using reagents purchased from Sigma Chemical Company (St Louis, Missouri, USA). Urea passes with relative ease through membranes of cells, so we assumed that concentrations of urea in blood plasma reflected urea concentrations in interstitial and intracellular compartments (Arieff *et al.* 1977). We also assumed that the concentration of urea in plasma at hatching reflected the general level of uremia experienced by embryos during later stages of incubation.

Data were examined by mixed-model analyses of covariance (Snedecor &

Cochran, 1980), where the hydric environments to which eggs were exposed in the first, second and third trimesters of incubation were fixed treatment effects and clutch was a random factor. The mass of eggs at the beginning of the experiment was used as a potential covariate, but we reverted to analyses of variance when the covariate was not a statistically significant source of variation. All computations were performed using program 2V in the BMDP statistical library (Jennrich *et al.* 1981).

Manipulation of urea concentration inside eggs

Eggs of snapping turtles used in this experiment were collected on 7 June 1988 from four nests located on the Valentine National Wildlife Refuge. None of the nests was more than 12 h old at the time it was opened by us, so all the embryos can safely be assumed to have been in the gastrula stage of development (Ewert, 1985). The eggs were packed in damp peat moss and stored in a cool cellar until they could be transported to Colorado State University on 10 June.

Thirty viable eggs were selected randomly from each clutch and placed in the experiment on 12 June. The eggs were divided evenly between two covered boxes assigned to different environmental chambers at $29{\cdot}0 \pm 0{\cdot}4$ °C so as to replicate the study fully. For the first half of incubation, eggs rested on wire platforms above moistened vermiculite having a water potential of approximately -550 kPa (see Packard *et al.* 1987). This procedure caused eggs to be in slight negative water balance (see Morris *et al.* 1983) and thus prevented their contents from being under a positive pressure at the mid-point of incubation when experimental manipulations were performed.

A small hole was drilled in the shell of all eggs in replicate 1 on day 30 of incubation and in all eggs in replicate 2 on day 31, and 45 μl of a solution of urea was injected by Hamilton syringe through the hole and into the allantoic sac. The injectate was a freshly prepared solution of urea at a concentration of 0, 2·5, 5·0, 7·5, or 10·0 mmol l^{-1}. The hole was then sealed with a drop of Super-glue, and the egg was returned to incubation. However, eggs were placed in contact with moistened vermiculite having a water potential of -150 kPa for the remainder of incubation, so that large quantities of water would not be lost to the environment during later stages of development (see Packard & Packard, 1988*b*). This step was taken to prevent unduly high, non-physiological concentrations of urea from being caused by the loss of solvent.

Eggs were weighed every 7 days, and sufficient water was added to substrata to replace that which had been absorbed by eggs or had escaped from the containers (Packard *et al.* 1987). The boxes containing eggs were shifted inside the environmental chambers once each day to minimize possible effects of thermal gradients inside the chambers (Lee & Rawlings, 1982).

A pipped egg was discovered on day 58 of incubation. Paper cylinders were immediately placed around the eggs to confine hatchlings with remains of their eggshell. The numbers of days required for embryos to develop to pipping and to hatching were recorded. Hatchlings were removed from containers on the day

they emerged from their eggshell and were killed by decapitation. Blood was drawn into heparinized capillary tubes, and the concentration of urea in plasma was measured as described previously. The residual yolk and carcass of each hatchling were then dried separately to constant mass.

Data were examined by analyses of covariance in a mixed-model design (Snedecor & Cochran, 1980), using concentration of urea in the injectate as a fixed treatment effect and using clutch and replicate as random factors. The mass of eggs at the outset of the study was a potential covariate to compensate for variation in the data stemming from variation in the size of eggs. We again reverted to analyses of variance when the covariate was not a significant source of variation in the data being examined.

Interpretation of statistical analyses

Our interpretation of results from the analyses of variance and covariance was guided by simultaneous consideration of (1) the sizes of the *F*-ratios, (2) the associated levels of significance (i.e. the probabilities), (3) the magnitude of differences between means, and (4) the number of degrees of freedom. We concluded that an *F*-ratio less than 4 for any treatment effect (as either a main effect or an interaction) identified an unimportant source of variation in the data, and that an *F*-ratio between 4 and 9 characterized a relatively minor source of variation. Only when an *F*-ratio was greater than 9 were we convinced that the test identified a biologically important source of variation. Our interpretations consequently are based on a subjective evaluation of several factors, and not simply on a rigid criterion for statistical and biological significance (see Cox, 1977; Warren, 1986).

Results

Plasma urea in hatchlings from wet and dry environments

The clutch of origin had no influence on either the net change in mass of eggs during incubation or the length of incubation (Table 1). However, substantial variation was detected among clutches in data for dry mass of residual yolks and carcasses from hatchlings and for plasma urea concentration, and clutch was a source of minor variation in data for plasma osmolality (Table 1). Despite the existence of variation among clutches in the mean levels of response for several of the variables, the eggs and hatchlings in each of the clutches exhibited similar patterns of response to the treatments. We therefore pooled data across clutches to assess the effects of the experimental manipulations.

Two patterns of response to experimental manipulations were apparent in the analyses (Table 1). First, the only important sources of variation in data for length of incubation, plasma urea level and plasma osmolality were the hydric conditions to which eggs were exposed in the second and third trimesters of incubation (Table 1). Hydric conditions during the first trimester had no apparent influen[illegible] on any of these variables (Tables 1, 2). In general, embryos developing on wet

Table 1. *F-ratios and levels of significance (in parentheses) from analyses of variance (or covariance) on data for snapping turtles hatching from eggs incubated in different hydric environments in different trimesters (T)*

Source of variation	Data set					
	Change in egg mass	Days till pip	Yolk dry mass	Carcass dry mass	Plasma urea	Plasma osmolality
Clutch	1·80	1·25	29·50	115·77	13·35	8·49
	(0·17)	(0·29)	(<0·001)	(<0·001)	(<0·001)	(0·001)
T1	29·94	0·05	24·44	15·79	0·11	2·54
	(<0·001)	(0·82)	(<0·001)	(<0·001)	(0·74)	(0·11)
T2	217·91	17·51	111·71	85·57	15·20	54·50
	(<0·001)	(<0·001)	(<0·001)	(<0·001)	(<0·001)	(<0·001)
T3	91·59	16·87	80·59	51·56	27·00	69·01
	(<0·001)	(<0·001)	(<0·001)	(<0·001)	(<0·001)	(<0·001)
T1×T2	2·81	0·60	13·18	7·89	0·29	5·68
	(0·10)	(0·55)	(0·001)	(0·007)	(0·60)	(0·02)
T1×T3	0·00	0·15	7·06	4·98	2·98	3·17
	(0·96)	(0·70)	(0·01)	(0·03)	(0·09)	(0·08)
T2×T3	0·03	1·54	5·22	5·95	4·38	0·79
	(0·86)	(0·22)	(0·02)	(0·02)	(0·04)	(0·62)
T1×T2×T3	1·32	0·10	0·48	0·94	0·95	0·94
	(0·25)	(0·75)	(0·50)	(0·66)	(0·66)	(0·66)
Covariate	8·36	–	4·57	–	–	–
	(0·006)		(0·03)			

The error term in the analyses of variance had 57 degrees of freedom, and that in the analyses of covariance was based on 56 degrees of freedom. The slope used to adjust data for change in mass of eggs was −0·512, whereas that used to adjust data for mass of yolks was 0·064.

substrata in the second or third trimesters incubated longer than embryos developing concurrently on dry substrata, and plasma from turtles exposed to wet media in the second or third trimesters had lower osmolalities and urea concentrations than that from animals exposed concurrently to dry media (Table 2).

The second pattern of response was manifested in data for change in mass of eggs between days 0 and 56 of incubation (which is an index to net water exchange between eggs and their environment), for dry mass of the residual yolk and for dry mass of the carcass (Table 1). The primary sources of variation in these analyses were hydric conditions encountered by eggs during the first, second and third trimesters of incubation (Table 1). The statistical interaction between trimester 1 and trimester 2 was an important source of variation in data for dry mass of yolk (Table 1), and indicates that the response by embryos to conditions encountered during the second trimester was influenced by conditions encountered during the first trimester. None of the other statistical interactions was important, however. In general, exposing eggs to a wet environment during any one of the trimesters

Table 2. *Means (pooled across other treatments and clutches) for data gathered from eggs and hatchlings of snapping turtles exposed to wet or dry environments during the first, second or third trimesters of incubation*

	Trimester 1		Trimester 2		Trimester 3	
Variable	Wet	Dry	Wet	Dry	Wet	Dry
Sample size	35	32	33	34	35	32
Change in egg mass, days 0–56 (g)	−0·584	−1·020	−0·139	−1·426	−0·407	−1·213
Days till pip	64·0	64·0	64·7	63·2	64·7	63·2
Yolk dry mass (g)	0·299	0·365	0·252	0·407	0·268	0·399
Carcass dry mass (g)	1·496	1·440	1·535	1·406	1·525	1·409
Plasma urea (mmol l^{-1})	32·1	31·9	27·7	36·2	27·3	37·1
Osmolality of plasma (mosmol kg^{-1})	324	331	310	344	310	346

Data for eggs exposed to wet or dry conditions during a given trimester are pooled irrespective of conditions to which they were exposed during the other trimesters.

caused the eggs to be in a more positive water balance overall than eggs exposed in the same trimester to a dry environment, and hatchlings from eggs exposed to wet conditions during a given trimester had larger carcasses and smaller residual yolks than did clutchmates exposed concurrently to dry conditions (Table 2).

Mean values for change in mass of eggs between days 0 and 56 are ranked in descending order in Table 3, from those gaining the most mass during incubation to those losing the most mass. Corresponding means for other variables reveal a general relationship between water exchanges experienced by eggs and several characteristics of hatchlings (Table 3). However, the most important relationships from the perspective of the current investigation are those that exist between water exchanges by eggs and masses of carcasses, masses of residual yolks and concentrations of urea in blood plasma. Large hatchlings from eggs in positive water balance tended to have small residual yolks and low concentrations of urea in their plasma, and the opposite was true for small hatchlings from eggs in negative water balance.

Manipulation of urea concentration inside eggs

The clutch of origin was an important source of variation in all the data sets, and important differences in plasma urea levels were detected between the two replicates (Table 4). However, responses to the injection regime followed the same pattern in all clutches and in both replicates. We therefore pooled data across clutches and replicates in order to examine the common patterns of response to the experimental manipulations.

Injecting different solutions of urea into eggs at the mid-point of incubation w[illegible] sufficient to elicit different degrees of uremia in developing embryos (Table 4).

The urea concentration was 31·9 mmol l^{-1} in plasma of turtles hatching from eggs receiving the lowest level of injection, and 50·4 mmol l^{-1} in plasma of animals hatching from eggs receiving the highest level of injection (Table 5). Concentrations of urea were intermediate in plasma of animals receiving intermediate

Table 3. *Means (pooled across clutches) for data gathered from eggs and hatchlings of snapping turtles exposed to wet or dry environments at different times in incubation*

Treatment	Change in egg mass (g)	Days till pip	Yolk dry mass (g)	Carcass dry mass (g)	Plasma urea (mmol l^{-1})	Plasma osmolality (mosmol kg^{-1})
WWW ($N = 9$)	0·469	65·0	0·211	1·579	19·4	290
DWW ($N = 8$)	0·064	65·4	0·191	1·582	22·9	299
WWD ($N = 8$)	−0·470	64·2	0·284	1·508	37·3	333
DWD ($N = 8$)	−0·694	64·2	0·328	1·465	32·1	320
WDW ($N = 9$)	−0·782	64·3	0·291	1·500	32·3	315
DDW ($N = 9$)	−1·327	64·0	0·371	1·444	34·2	335
WDD ($N = 9$)	−1·539	62·4	0·409	1·400	40·0	359
DDD ($N = 7$)	−2·237	62·0	0·599	1·243	38·8	374
S.D.	0·360	1·4	0·061	0·063	8·1	18

Eggs were exposed to wet (water potential = −150 kPa = W) or dry (water potential = −950 kPa = D) substrata during the first, second and third trimesters of incubation. The notation indicates the combination of conditions to which eggs in each of the treatment groups were esposed.

Estimates of the standard deviations within samples were computed from the within-sample variances in the analyses of variance/covariance.

Table 4. F-*ratios and levels of significance (in parentheses) from analyses of variance (or covariance) on data for snapping turtles hatching from eggs injected with different solutions of urea*

Source of variation	Data set			
	Plasma urea	Days till pip	Dry mass of yolk	Dry mass of carcass
Injection	23·70 (<0·001)	0·96 (0·56)	1·42 (0·23)	2·02 (0·10)
Clutch	11·37 (<0·001)	17·56 (<0·001)	18·28 (<0·001)	13·36 (<0·001)
Replication	31·24 (<0·001)	0·53 (0·52)	0·22 (0·65)	1·58 (0·21)
Covariate	9·01 (0·004)	–	–	11·38 (0·002)

The error term in the analyses of variance had 85 degrees of freedom, and that in the analyses of covariance was based on 84 degrees of freedom. The slope used to adjust values for urea was 0·3, whereas that used to adjust carcass mass was 0·505.

Table 5. *Means (pooled across clutches and replicates) for plasma urea, for length of incubation, for dry mass of residual yolks and for dry mass of carcasses for snapping turtles hatching from eggs injected with different solutions of urea*

Solution injected	Plasma urea ($mmol\,l^{-1}$)	Days till pip	Dry mass of yolk (g)	Dry mass of carcass (g)
0 ($N = 21$)	31·9	63·1	0·423	1·527
2·5 ($N = 23$)	36·2	62·5	0·465	1·514
5·0 ($N = 16$)	43·6	62·2	0·411	1·613
7·5 ($N = 17$)	45·5	62·8	0·440	1·535
10·0 ($N = 17$)	50·4	61·9	0·528	1·464
S.D.	6·8	1·6	0·139	0·128

Estimates of the standard deviations within samples were computed from the within-sample variances in the analyses of variance/covariance.

levels of injection, and the order of increase in the concentration of urea in their plasma followed the same order as the concentration of urea in the injectate (Table 5).

Despite the apparent influence of the injection protocol on levels of urea in plasma of developing embryos, the injections had no demonstrable effect on the duration of incubation or on the dry mass of residual yolk or carcass (Tables 4, 5). Turtles pipped their eggshell after approximately 62·5 days of incubation irrespective of treatment, and residual yolks and carcasses weighed an average of 0·453 g and 1·529 g, respectively.

Discussion

Plasma urea levels in hatchlings from wet and dry environments

The first experiment confirms and extends findings of an earlier study on the water relations of embryonic snapping turtles (Packard & Packard, 1988*b*). Water exchanges by eggs are affected by water potential of the substratum during each of the trimesters of incubation, with eggs that are exposed to wet media in any given trimester having a more positive water balance overall than eggs exposed during the same trimester to dry media (Table 2). Moreover, embryos in wet environments in any given trimester consume more of their yolk and attain a larger size before hatching than embryos exposed simultaneously to dry environments (Table 2). The larger size of hatchlings from wet environments presumably reflects higher rates of metabolism and growth by embryos in these settings (Morris *et al.* 1983; Gettinger *et al.* 1984; Packard & Packard, 1986), but the longer incubation by embryos exposed to wet conditions in the second and third trimesters is also a factor (Tables 2, 3).

This experiment also confirms our earlier finding that the moisture regime to which eggs are exposed during the first trimester has no influence on the length of incubation by embryos (Packard & Packard, 1988*b*), but also yields the unexpec-

ted outcome that neither the concentration of urea in plasma in hatchlings nor the osmolality of their plasma is influenced by hydric conditions encountered by eggs during the first trimester (Tables 1, 2). These latter findings are enigmatic because eggs in wet and dry environments differ appreciably in the water exchanges they experience during the first trimester (Tables 1, 2; also Packard & Packard, 1988*b*). Water absorbed by (or lost from) eggs during the first trimester seemingly contributes to the pool of water available to support developing embryos (Morris *et al.* 1983; Packard *et al.* 1983) and, therefore, to the amount of solvent available to disperse urea and other solutes. Further study is needed to determine where water is stored inside eggs and whether water exchanged during early stages of incubation is added to (or drawn from) the same compartments as are affected by water exchanges occurring later in incubation.

The most important finding from this experiment, however, is that plasma urea concentration is higher in turtles hatching in generally dry conditions than in animals hatching in wetter settings (Tables 2, 3). We assume that urea concentrations in interstitial fluids and in the intracellular compartment were similar (or identical) to those recorded for plasma (Arieff *et al.* 1977), so putative effects of urea on metabolic enzymes clearly should have been more pronounced in animals incubating in generally dry conditions than in those incubating on wetter substrata.

In summary, data from this experiment indicate that an association exists between wetness of the substratum, the pattern of water exchange between eggs and their environment, the concentration of urea in body fluids and the growth of embryos. This association is a fundamental requirement for the urea hypothesis, and led to the second part of the investigation.

Manipulation of urea concentration inside eggs

The experimental protocol followed in the second part of the current investigation produced different levels of uremia in embryos, and these uremias were reflected in hatchlings (Table 5). The range of uremias that we induced by injection was similar to that induced earlier by exposing eggs to wet and dry environments at different times in incubation (Table 3). The range of values in the injection experiment was shifted upwards by approximately 10 mmol l^{-1} compared to values from the transfer experiment, but eggs in the injection experiment were held on platforms for the first half of incubation to prevent them from absorbing water from the substratum. Accordingly, none of the eggs in the injection experiment was in positive water balance for the duration of incubation, and none is comparable to eggs in the WWW or DWW groups of the transfer experiment (see Table 3).

Although the injection procedure elicited important differences among groups in the concentration of urea in plasma of hatchlings, it had no effect on the duration of incubation or on the dry mass of residual yolks and carcasses from hatchlings. Thus, we found no evidence that elevated urea concentration in embryonic turtles elicits an inhibition of metabolism and growth. Although a correlation was apparent in earlier studies (including experiment 1 in this

investigation) between the concentration of urea in fluid compartments inside eggs and the size of turtles hatching from those eggs, the correlation does not reflect a causal relationship between the variables. In other words, the small size of carcasses of turtles hatching in dry environments is not due directly to higher levels of urea in their body fluids. Thus, we must reject the urea hypothesis in its current form; alternative hypotheses must be formulated and tested.

The results of this investigation cast doubt on the widely accepted theory of 'counteracting solutes' that inspired our work (e.g. Yancey *et al.* 1981; Hochachka & Somero, 1984; Somero, 1986). This theory is based on observations that the activity of numerous metabolic enzymes *in vitro* is inhibited by urea, but that activity of these enzymes can be maintained if a counteracting solute (e.g. trimethylamine oxide, or TMAO) also is present in the system. When urea and a counteracting solute are present in the correct proportions, the destabilizing effects of the urea are seemingly compensated by the stabilizing influence of the other solute, and conformation and function of the enzyme are thereby preserved. The adaptive strategy of matching a stabilizing solute with a destabilizing solute has presumably been adopted by elasmobranchs, coelacanths and a few anurans that use urea (and TMAO) to increase the osmotic activity of their body fluids and thereby minimize problems of osmotic exchange in marine or brackish environments. In contrast, urea retained by estivating lungfish and desert anurans is not accompanied by a counteracting solute, and the accumulating urea presumably elicits an inhibition of metabolism that serves to conserve metabolic reserves in these animals during long periods of inactivity.

Despite the elegance and appeal of the theory of counteracting solutes, the theory is based only on studies of enzymes *in vitro* and on correlational data gathered from intact animals. Similar data were the basis for the urea hypothesis that was tested – and rejected – in the current investigation. We therefore emphasize that the theory of counteracting solutes has yet to be tested explicitly, and suggest that the theory should be viewed with caution until supporting evidence has been reported from experiments manipulating the physiology of intact animals.

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