J. exp. Biol. **108**, 195–204 (1984)
Printed in Great Britain © The Company of Biologists Limited 1984

INFLUENCE OF HYDRATION OF THE ENVIRONMENT ON THE PATTERN OF NITROGEN EXCRETION BY EMBRYONIC SNAPPING TURTLES (*CHELYDRA SERPENTINA*)

BY GARY C. PACKARD, MARY J. PACKARD AND THOMAS J. BOARDMAN

Department of Zoology and Entomology and Department of Statistics, Colorado State University, Fort Collins, CO 80523, U.S.A.

Accepted 18 July 1983

SUMMARY

Flexible-shelled eggs of common snapping turtles were incubated on wet and dry substrates. More than 70 % of the waste nitrogen accumulating in eggs on both substrates was in the form of urea, and less than 25 % was in the form of ammonia. Low levels of soluble urate were detected in eggs late in incubation, but insoluble urate was never present. Accumulation of ammonia and soluble urate was unaffected by the hydration of the environment, but more urea accumulated late in incubation in eggs on the wet substrate than in those on the dry substrate. Thus, embryos are ureotelic throughout development, and the pattern of accumulation of the primary nitrogenous waste is influenced by hydration of the environment.

INTRODUCTION

One of the most important generalizations to emerge from the field of chemical embryology concerns the patterns of nitrogen excretion characterizing embryos of oviparous vertebrates (Needham, 1931; Baldwin, 1964). According to this paradigm, embryos of species that develop in standing water (e.g. teleost fishes and amphibians) excrete the ammonia produced in catabolism of proteins directly into the environment, where this toxic waste is diluted below levels that could prove harmful. However, among species that develop on land (e.g. reptiles and birds), excretion of ammonia into the environment is precluded, so the compound is detoxified by embryos before being stored within developing eggs. If there is no shortage of water inside eggs (e.g. reptiles), ammonia is detoxified by converting it to urea, a highly soluble but relatively innocuous compound. On the other hand, if the water inside eggs is conserved for direct support of embryogenesis (e.g. birds), the end product of detoxification is an insoluble substance, uric acid.

Despite the wide acceptance of this paradigm, it is not based on complete and unequivocal evidence. Relatively few studies of nitrogen excretion have been conducted on embryonic vertebrates in general or on embryonic reptiles in particular

Key words: Embryos, excretion, turtles.

(Packard, Tracy & Roth, 1977). Moreover, studies on reptilian embryos have frequently yielded conflicting results (Clark, 1953; Athavale & Mulherkar, 1967), or have been based on too few individuals to support generalizations (Clark, Sisken & Shannon, 1957; Haggag, 1964). We therefore undertook the present study of common snapping turtles (*Chelydra serpentina*), to determine whether its embryos are ammonotelic, ureotelic or uricotelic, and thereby to contribute new information on the relationship between nitrogen excretion of embryos and the availability of water.

A second objective was to discover whether the pattern of nitrogen excretion by embryos varies with the hydration of the environment. Although the flexible-shelled eggs of these turtles contain as much water at oviposition as do those of birds (Packard & Packard, 1980), the turtle eggs exchange water rather freely with their environment (Packard, Packard & Boardman, 1982; Morris *et al.* 1983). Thus, the water in the yolk and albumen may be supplemented or depleted depending on the degree of hydration in the incubation chamber. For instance, when eggs are incubated in moist conditions, absorption of liquid from the substrate exceeds transpirational losses of vapour to the air (see Packard, Packard & Boardman, 1981), and there is a net increase in the amount of water available to support embryogenesis (Morris *et al.* 1983). On drier substrates, however, absorption of liquid does not keep pace with transpiration (see Packard *et al.* 1981), and the amount of water present in the yolk and albumen decreases (Morris *et al.* 1983). These differences are physiologically important, because the rates of consumption of yolk by embryos and of growth in dry mass of their tissues are directly related to the reserve of water available to support development (Morris *et al.* 1983). It might therefore be expected that the availability of water in the environment would influence the pattern of nitrogen excretion by developing embryos.

MATERIALS AND METHODS

Gravid snapping turtles were captured on 13–14 June 1982 at the Valentine National Wildlife Refuge, Cherry County, Nebraska, as they were digging nests prior to oviposition. The animals were placed in damp sacks and transported to Colorado State University, where oviposition was induced by injecting them with synthetic oxytocin (Ewert & Legler, 1978). One group was injected on 19 June, and another on 21 June. Embryonic snapping turtles do not develop beyond the late gastrula stage within the maternal oviducts (Lynn & von Brand, 1945; Yntema, 1968), so all of the eggs were at approximately the same stage of development at oviposition.

Eggs from 15 females were placed randomly in plastic boxes containing 300 g (dry mass) of vermiculite to which different amounts of distilled water had been added to produce wet and dry substrates. At the outset, each of the four wet substrates contained 333·8 g of water at a chemical potential of −150 kPa, whereas each of the four dry substrates held 56·5 g of liquid at a chemical potential of −800 kPa. Water potentials of the vermiculite were verified by dewpoint hygrometry using a Wescor HR-33T microvoltmeter and C-52 sample chamber (Campbell, Campbell & Barlow, 1973).

All eggs were half buried in substrates, to allow for an exchange of liquid water across the bottom of each eggshell and for transpiration across the top (see Packard *et al.* 1981). The boxes were covered with aluminium foil to maintain high humidities

in air around eggs (Tracy, Packard & Packard, 1978) and to minimize water loss from containers (Packard *et al.* 1981; Morris *et al.* 1983). Boxes with eggs obtained on 19 June were placed in a controlled-temperature chamber at 29 ± 0·4 °C, and containers with eggs obtained on 21 June were placed in a second cabinet at the same temperature.

Eggs were weighed using a Sartorius electronic balance on days 1 and 6 of incubation and at weekly intervals thereafter until day 48. They were also weighed on the day that they were analysed for nitrogenous wastes (see below). Substrates were rehydrated twice weekly to prevent water potentials from changing appreciably during the course of incubation (Packard *et al.* 1981).

Three eggs were removed from the dry environment and four eggs from the wet environment on day 1 of incubation; six eggs with viable embryos were sampled from each environment on days 23, 35, 43, 47, 51 and 55 of development. Contents of these eggs were analysed for accumulated ammonia and urea, as detailed below. On days 1, 35, 47, 51 and 55, two of the eggs from each treatment were analysed further for soluble and insoluble urates. Unused eggs began to pip on day 55 of incubation, and hatching was completed after an average of 59·2 days for eggs on the dry substrate and 62·2 days for eggs on the wet one (see Morris *et al.* 1983).

Nitrogenous wastes were determined by a composite of methods used by others (Tomita, 1929; Haggag, 1964; Minnich, 1972; McNabb & McNabb, 1975). Briefly, eggs and their contents were homogenized individually in approximately 25 ml of distilled water using a mini-chamber for a Waring blender. Each homogenate was washed into a large tube and then centrifuged for 10 min at 1550 ***g***. After a thin film of lipid had been skimmed from the surface of the supernatant, the solution was transferred to a volumetric flask (usually 50 ml). The pellet was then washed with distilled water and centrifuged again, the wash was added to the flask, and the flask was brought to volume with distilled water. Aliquots (10 μl) of this solution were analysed for ammonia and urea using a modification of the Berthelot procedure (Fawcett & Scott, 1960; Chaney & Marbach, 1962), as detailed in Sigma Technical Bulletin 640. Each analysis was performed in duplicate, and final readings of absorbance were made at 570 nm with a Bausch & Lomb Spectronic 21 digital spectrophotometer.

Soluble urate in the aqueous solution was determined using a colourimetric test based on reduction of phosphotungstate (Henry, Sobel & Kim, 1957), as described in Sigma Technical Bulletin 680. A digestion with uricase was used to correct for foreign substances that also reduce phosphotungstate (Blauch & Koch, 1939). Readings of absorbance were taken on deproteinized samples at a wavelength of 650 nm.

For insoluble urate, the pellet remaining after the earlier centrifugation was suspended in approximately 25 ml of 0·4 % lithium carbonate and allowed to stand for 1–6 h to dissolve precipitated urate. After the suspension had been centrifuged, the supernatant was transferred to a volumetric flask (50 ml), which was then brought to volume with distilled water. Aliquots of this solution were analysed by the phosphotungstate procedure.

Data for total nitrogen in the form of ammonia, urea and urates were examined by separate analyses of covariance (Snedecor & Cochran, 1967), where substrate water potential and time during incubation were the treatment effects of interest. Mass of

eggs on day 1 of incubation was used as a potential covariate to assess variation in the data stemming from individual differences in size of eggs at the outset of study. Also, the data were grouped by temperature cabinet to assess the possibility that systematic differences existed between eggs in the two chambers and, more importantly, to increase the power of our statistical analyses.

RESULTS

Eggs on the wet substrate increased in mass during the first half of development and declined in mass thereafter, so that their mass toward the end of incubation was similar to that at oviposition (Table 1). In contrast, eggs on the dry substrate declined in mass throughout development (Table 1). Similar patterns of change in mass have been reported previously for eggs of this species (Packard *et al.* 1982; Morris *et al.* 1983). Assuming that changes in mass reflect net fluxes of water across eggshells (Packard *et al.* 1981), eggs in wet and dry environments therefore experienced substantial differences in net water exchange.

The size (mass) of eggs at the outset of study had a significant effect on amounts of ammonia and urea present at different stages in development (Table 2), with large eggs containing more of these substances than small eggs in the same samples. Consequently, data for these variables were adjusted by the covariance procedure prior to final analysis (Snedecor & Cochran, 1967). However, the initial mass of eggs had no effect on amounts of urate accumulating in eggs (Table 2), so no adjustment of these values was required. There were no significant differences between eggs incubated in the two temperature chambers in amounts of ammonia, urea and urate accumulated by the several sampling dates (Table 2).

Approximately 0·86 mg of urea-nitrogen was found in eggs at the start of incubation (Fig. 1), which presumably indicates that some urea was transmitted from female turtles to their eggs. Urea subsequently accumulated in eggs throughout incubation, and accounted for more than 70% of the waste nitrogen produced through 55 days of development (Fig. 1). However, the pattern of increase was different for eggs held on wet and dry substrates (Table 2). Comparisons of means using the Least Significant Difference (Snedecor & Cochran, 1967) indicate that significantly more urea-nitrogen was present on days 51 and 55 of incubation in eggs on the wet substrate than was present in eggs on dry vermiculite (Fig. 1).

Very small quantities of ammonia-nitrogen also were present in freshly laid eggs (Fig. 1), and the amount of nitrogen in this form increased in eggs during development (Fig. 1). The significant interaction component in the analysis of covariance (Table 2) was presumably caused by unusually high values for ammonia-nitrogen in eggs on the wet substrate on day 35 and by unusually low values for eggs on the dry substrate on day 47 (Fig. 1). However, the absence of significant differences between means on days 51 and 55 (Fig. 1), coupled with the absence of a treatment effect for ammonia (Table 2), leads us to believe that the interaction component has little biological importance. We therefore emphasize the overall similarity in patterns of increase in ammonia-nitrogen in eggs exposed to both wet and dry environments (Fig. 1). In both environments, ammonia accounted for less than 25% of the waste nitrogen accumulating in eggs through 55 days of incubation.

Table 1. *Average masses (g) of eggs of snapping turtles on the day of sampling*

Day	Wet substrate	Dry substrate
1	11·98	11·91
23	12·86	11·74
35	13·33	11·26
43	13·09	10·87
47	12·88	10·34
51	12·59	9·93
55	12·24	9·50

Analysis of covariance indicated that there is significant variation among the samples ($P < 0{\cdot}001$ for substrate, time and the interaction of substrate × time). Means differing by the Least Significant Difference = 0·35 g are significantly different at alpha = 0·05 (Snedecor & Cochran, 1967).

Table 2. *Summaries of analyses of covariance of data for nitrogenous wastes accumulating in eggs of snapping turtles incubated for different times on wet and dry substrates*

Source of variation	Degrees of freedom	Mean square	F-ratio	*P*
		Urea		
Substrate	1	1·4736	5·02	0·028
Time in incubation	6	23·7720	81·04	<0·001
Interaction time × substrate	6	0·3297	1·12	0·359
B.O.D. cabinet	1	0·6820	2·32	0·132
Covariate: mass of eggs day 1	1	1·6368	5·58	0·021
Residual	63	0·2934		
		Ammonia		
Substrate	1	0·1359	2·92	0·093
Time in incubation	6	3·0088	64·57	<0·001
Interaction time × substrate	6	0·1698	3·64	0·004
B.O.D. cabinet	1	0·0530	1·14	0·290
Covariate: mass of eggs day 1	1	0·4621	9·92	0·003
Residual	63	0·0466		
		Soluble urate		
Substrate	1	0·0075	12·27	0·008
Time in incubation	4	0·0514	84·34	<0·001
Interaction time × substrate	4	0·0037	6·00	0·016
B.O.D. cabinet	1	0·0005	0·77	0·407
Covariate: mass of eggs day 1	1	0·0002	0·26	0·622
Residual	8	0·0006		

Soluble urate was not present in detectable levels for the first 35 days of development (Fig. 1). However, this substance was detectable on day 47 of incubation and continued to accumulate in eggs thereafter (Fig. 1; Table 2). There may have been a significant difference between eggs held in wet and dry environments in amounts of soluble urate accumulated by day 55 (Table 2), but only two eggs were represented in each sample and the amounts of urate present were very low. Thus, the outcome of the statistical analysis may be misleading. Moreover, the magnitude of the apparent

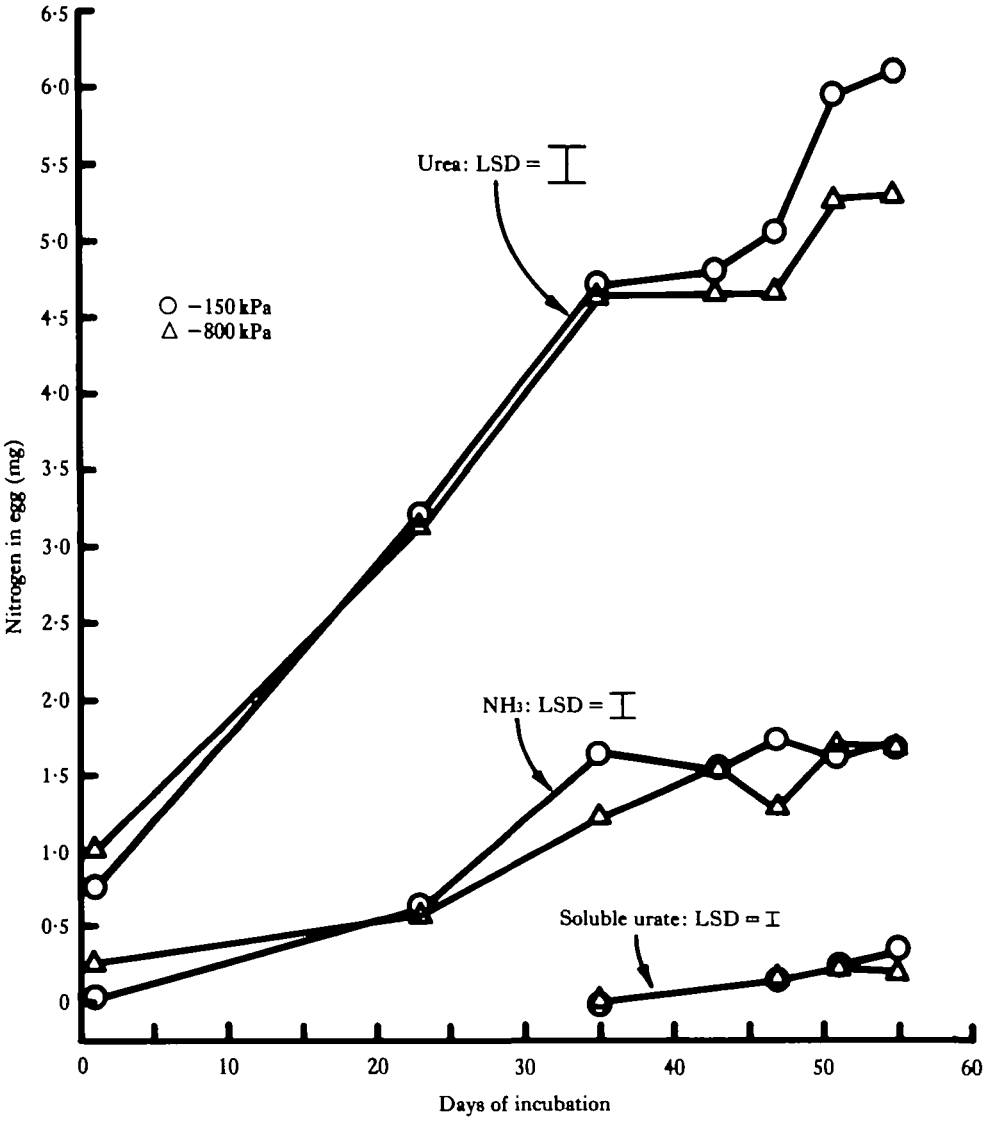

Fig. 1. Mean values for masses of excretory nitrogen accumulating inside eggs of snapping turtles while incubating on wet and dry substrates. Soluble urate was below the limits of detection on day 1, and is omitted from this graph to preserve clarity of presentation. A Least Significant Difference (LSD) is provided for each set of data to permit multiple comparisons; means differing by the LSD are significantly different at alpha = 0·05 (Snedecor & Cochran, 1967).

difference between eggs in the two environments is so small as to be unimportant biologically (Fig. 1).

Insoluble urate was never detected by our colourimetric procedure, and we never noted accumulations in the allantoic sac of the white, crystalline material that is so characteristic of avian eggs prior to hatching. Thus, we conclude that embryonic snapping turtles do not store insoluble urate in the allantois or in any other compartment inside eggs.

DISCUSSION

Pattern of nitrogen excretion

Earlier studies of nitrogen excretion by reptilian embryos indicate that some waste may be voided from eggs as gaseous ammonia (Nakamura, 1929; Clark, 1953). However, these reports are suspect because of the nature of the analyses, which did not take into account such factors as variation among samples in size of the eggs, differences among clutches in the nitrogen content of eggs, or production of ammonia by microbes living in the incubation chambers. Indeed, other studies indicate that there is little or no loss of gaseous ammonia from incubating eggs (Haggag, 1964).

Assuming that gaseous ammonia does not account for significant amounts of excretory nitrogen, our data indicate that the embryos of snapping turtles are ureotelic throughout development (Fig. 1). Indeed, urea may be even more important than the present results indicate, because values for ammonia-nitrogen may be overestimates resulting from decomposition of labile amides (Brown, Duda, Korkes & Handler, 1957) and soluble urate may have been produced in catabolism of purines instead of proteins. In either case, the proportion of waste nitrogen from protein catabolism that is committed to urea would be underestimated.

Embryos of all turtles studied to date are ureotelic, and in no instance do urates comprise an important fraction of their excretory nitrogen (Tomita, 1929; Packard & Packard, 1983; Packard *et al.* 1983; present study). Unfortunately, it is not yet possible to make a general statement concerning patterns of nitrogen excretion by embryos of other species of reptiles, owing to unduly small numbers of animals studied (Clark *et al.* 1957; Haggag, 1964), inappropriate sampling protocol (Athavale & Mulherkar, 1967) and contradictory findings (Clark, 1953; Haggag, 1964; Athavale & Mulherkar, 1967).

Sources of energy for embryos

Protein catabolism by embryonic snapping turtles can be assessed by considering the present data together with an earlier report that dry mass of yolks in eggs on a wet substrate declined from approximately 2·020 g at the beginning of incubation to 0·432 g on day 55 (Morris *et al.* 1983). During the same interval, dry mass of embryos increased from 0 g to 1·216 g (Morris *et al.* 1983). Thus, there was an overall decline in dry mass of contents of eggs amounting to 372 mg, which probably is a reasonable approximation to the combined amount of lipid and protein that was oxidized (von Brand & Lynn, 1947).

We can estimate the amount of protein catabolized during incubation on the wet substrate by converting values for the total amount of nitrogen accumulated as urea and ammonia into equivalent values for protein (Natelson, 1971). This procedure leads to an estimate of 43 mg of protein consumed by embryos during 55 days of incubation. If eggs used in this study experienced changes in dry mass of yolks and embryos similar to those reported earlier (Morris *et al.* 1983), approximately 12 % of the decline in mass of the contents of eggs can be attributed to consumption of proteins. The other 88 % presumably was lipid.

Thus, our data indicate that embryonic snapping turtles rely primarily on lipid as

substrate for energy transformations, and that protein satisfies only a small part of the overall requirement. However, an earlier study of eggs and embryos of this species by von Brand & Lynn (1947) indicated that 50% of the organic material consumed during incubation is protein. Unfortunately, we are unable to resolve the apparent discrepancy between the present investigation and work reported by von Brand & Lynn (1947). The question of metabolic substrates deserves further study, however, because it has important bearing on theories of chemical evolution (Needham, 1931; Packard & Packard, 1980).

Influence of environmental hydration levels on nitrogen excretion

Embryonic snapping turtles consume nutrient reserves in the yolk faster and grow more rapidly when their eggs are incubated in wet environments than they do when their eggs are held in drier settings (Morris *et al.* 1983). Such findings may indicate that embryos in wet environments have higher rates of metabolism than embryos in dry settings (Morris *et al.* 1983). Data from the present study are consistent with this interpretation. Because protein forms part of the substrate for energy transformations (see preceding section), higher rates of metabolism in embryos in wet environments should be accompanied by higher rates of production of urea. This prediction is confirmed by our analysis (Fig. 1).

Our findings have even more important implications, however. Eggs in wet and dry environments exhibit different patterns of net water exchange with their surroundings (Packard *et al.* 1982; Morris *et al.* 1983), so different amounts of liquid are present inside eggs at different points in incubation to serve as solvent for nitrogenous wastes (Morris *et al.* 1983). Amounts of solvent actually present in eggs used in the present investigation can be estimated by assuming that 70% of their mass on day 1 of incubation was water (Packard & Packard, 1980) and by further assuming that changes in mass of eggs between day 1 and the day of sampling reflect net uptake or loss of water (Table 1). Finally, if we assume also that urea was distributed proportionately among all of the water compartments occurring inside developing eggs (see Clark, 1953; Clark & Fischer, 1957; Clark *et al.* 1957), concentrations of this metabolite can readily be computed.

Concentrations of urea increased inside eggs throughout incubation, but the pattern of increase differed between eggs in wet and dry environments (Fig. 2). Whereas concentrations did not differ significantly over the first 23 days of development, they were significantly higher in eggs on dry substrates by day 35 of incubation. The differences in concentration became more pronounced thereafter (Fig. 2).

Urea is a competitive inhibitor of numerous enzymes catalysing key reactions in intermediary metabolism (Rajagopalan, Fridovich & Handler, 1961) – even at concentrations as low as 25–50 mM (Giordano, Bloom & Merrill, 1962; Hand & Somero, 1982). We therefore suggest that the water exchanges experienced by eggs of snapping turtles may affect concentrations of urea attained in body fluids of embryos (Fig. 2), and that metabolism of embryos may be subject to slight inhibition in later stages of incubation in consequence of rising titres of this waste. However, the inhibition of metabolism may be greater among eggs held on dry substrates than among those incubated on wet media, because concentrations of urea are higher in the former than in the latter (Fig. 2). This hypothesis provides a simple explanation for the apparent

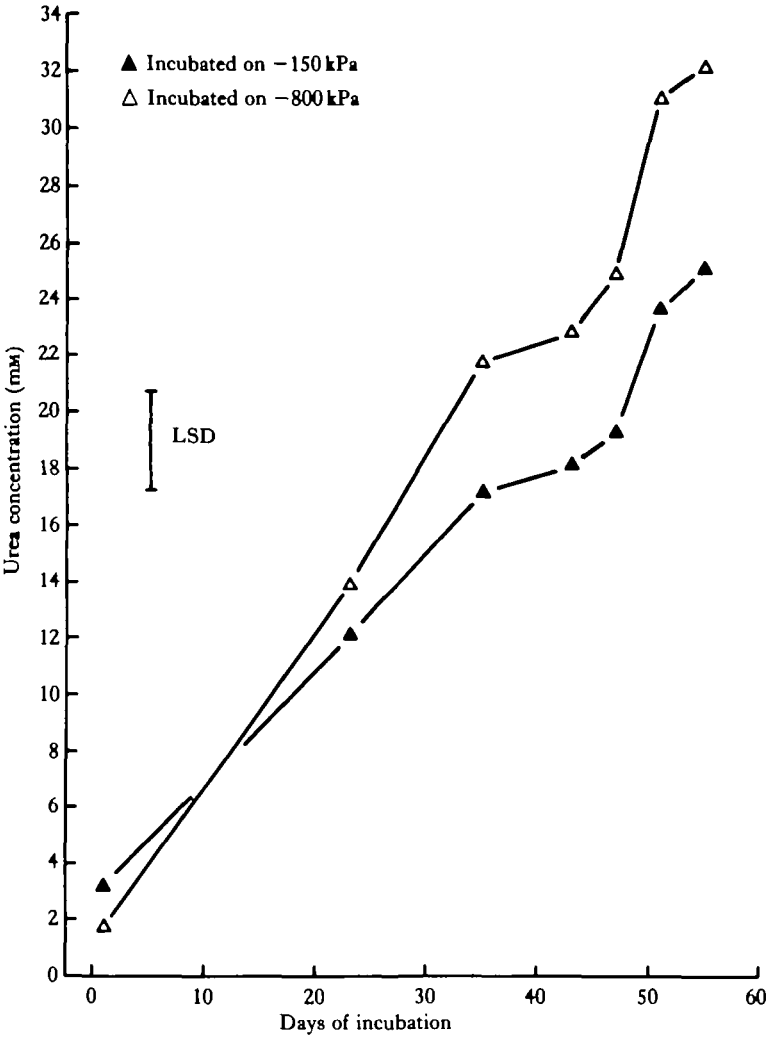

Fig. 2. Means for estimates of concentrations of urea accumulating in fluid compartments inside eggs of snapping turtles incubating on wet and dry substrates. Analysis of covariance indicated that the temporal increase in concentration differs between the experimental treatments ($F_{6,63} = 2{\cdot}70$, $P = 0{\cdot}021$, for the interaction of substrate × time). Means differing by the Least Significant Difference (LSD) are significantly different at alpha = 0·05 (Snedecor & Cochran, 1967).

differences in rates of metabolism of embryos in wet and dry environments (Morris *et al.* 1983), and also provides a plausible mechanism for coupling metabolism of developing young to the hydration of the environment in the nest cavity.

Female turtles were collected under authority of permit No. 82-29 issued by the Nebraska Game and Parks Commission. We thank R. M. Ellis and L. McDaniel for permission to do field work on the Valentine National Wildlife Refuge, and G. L. Paukstis and W. H. N. Gutzke for assistance collecting animals. A draft of this report was critically examined by R. D. Gettinger. This investigation was supported, in part, by the National Science Foundation (DEB 79-11546).

REFERENCES

ATHAVALE, M. V. & MULHERKAR, L. (1967). Studies in nitrogenous excretion during the development of *Calotes versicolor* (Daud). *J. Anim. Morphol. Physiol.* **14**, 89–97.

BALDWIN, E. (1964). *An Introduction to Comparative Biochemistry*, 4th ed. London: Cambridge University Press.

BLAUCH, M. B. & KOCH, F. C. (1939). A new method for the determination of uric acid in blood, with uricase. *J. biol. Chem.* **130**, 443–454.

BROWN, R. H., DUDA, G. D., KORKES, S. & HANDLER, P. (1957). A colorimetric micromethod for determination of ammonia; the ammonia content of rat tissues and human plasma. *Arch. Biochem. Biophys.* **66**, 301–309.

CAMPBELL, E. C., CAMPBELL, G. S. & BARLOW, W. K. (1973). A dewpoint hygrometer for water potential measurement. *Agr. Meteorol.* **12**, 113–121.

CHANEY, A. L. & MARBACH, E. P. (1962). Modified reagents for determination of urea and ammonia. *Clin. Chem. Winston-Salem N. C.* **8**, 130–132.

CLARK, H. (1953). Metabolism of the black snake embryo. I. Nitrogen excretion. *J. exp. Biol.* **30**, 492–501.

CLARK, H. & FISCHER, D. (1957). A reconsideration of nitrogen excretion by the chick embryo. *J. exp. Zool.* **136**, 1–15.

CLARK, H., SISKEN, B. & SHANNON, J. E. (1957). Excretion of nitrogen by the alligator embryo. *J. cell. comp. Physiol.* **50**, 129–134.

EWERT, M. A. & LEGLER, J. M. (1978). Hormonal induction of oviposition in turtles. *Herpetologica* **34**, 314–318.

FAWCETT, J. K. & SCOTT, J. E. (1960). A rapid and precise method for the determination of urea. *J. clin. Path.* **13**, 156–159.

GIORDANO, C., BLOOM, J. & MERRILL, J. P. (1962). Effects of urea on physiologic systems. I. Studies on monoamine oxidase activity. *J. Lab. clin. Med.* **59**, 396–400.

HAGGAG, G. (1964). Nitrogenous excretion in snakes embryo. *Z. vergl. Physiol.* **48**, 462–466.

HAND, S. C. & SOMERO, G. N. (1982). Urea and methylamine effects on rabbit muscle phosphofructokinase. Catalytic stability and aggregation state as a function of pH and temperature. *J. biol. Chem.* **257**, 734–741.

HENRY, R. J., SOBEL, C. & KIM, J. (1957). A modified carbonate-phosphotungstate method for the determination of uric acid and comparison with the spectrophotometric uricase method. *Am. J. clin. Path.* **28**, 152–160.

LYNN, W. G. & VON BRAND, T. (1945). Studies on the oxygen consumption and water metabolism of turtle embryos. *Biol. Bull. mar. biol. Lab., Woods Hole* **88**, 112–125.

MCNABB, F. M. A. & MCNABB, R. A. (1975). Proportions of ammonia, urea, urate and total nitrogen in avian urine and quantitative methods for their analysis on a single urine sample. *Poult. Sci.* **54**, 1498–1505.

MINNICH, J. E. (1972). Excretion of urate salts by reptiles. *Comp. Biochem. Physiol.* **41A**, 535–549.

MORRIS, K. A., PACKARD, G. C., BOARDMAN, T. J., PAUKSTIS, G. L. & PACKARD, M. J. (1983). Effect of the hydric environment on growth of embryonic snapping turtles (*Chelydra serpentina*). *Herpetologica* (in press).

NAKAMURA, Y. (1929). Über das Verhalten des im Reptilienei vorhandenen Reststickstoffes bei der Bebrütung. *J. Biochem., Tokyo* **10**, 357–360.

NATELSON, S. (1971). *Techniques of Clinical Chemistry*, 3rd ed. Springfield, Illinois: C. C. Thomas.

NEEDHAM, J. (1931). *Chemical Embryology*. London: Cambridge University Press.

PACKARD, G. C. & PACKARD, M. J. (1980). Evolution of the cleidoic egg among reptilian antecedents of birds. *Am. Zool.* **20**, 351–362.

PACKARD, G. C. & PACKARD, M. J. (1983). Patterns of nitrogen excretion by embryonic softshell turtles (*Trionyx spiniferus*) developing in cleidoic eggs. *Science, N.Y.* **221**, 1049–1050.

PACKARD, G. C., PACKARD, M. J. & BOARDMAN, T. J. (1981). Patterns and possible significance of water exchange by flexible-shelled eggs of painted turtles (*Chrysemys picta*). *Physiol. Zool.* **54**, 165–178.

PACKARD, G. C., PACKARD, M. J., BOARDMAN, T. J., MORRIS, K. A. & SHUMAN, R. D. (1983). Influence of water exchanges by flexible-shelled eggs of painted turtles *Chrysemys picta* on metabolism and growth of embryos. *Physiol. Zool.* **56**, 217–230.

PACKARD, G. C., TRACY, C. R. & ROTH, J. J. (1977). The physiological ecology of reptilian eggs and embryos, and the evolution of viviparity within the class Reptilia. *Biol. Rev.* **52**, 71–105.

PACKARD, M. J., PACKARD, G. C. & BOARDMAN, T. J. (1982). Structure of eggshells and water relations of reptilian eggs. *Herpetologica* **38**, 136–155.

RAJAGOPALAN, K. V., FRIDOVICH, I. & HANDLER, P. (1961). Competitive inhibition of enzyme activity by urea. *J. biol. Chem.* **236**, 1059–1065.

SNEDECOR, G. W. & COCHRAN, W. G. (1967). *Statistical Methods*, 6th ed. Ames: Iowa State University Press.

TOMITA, M. (1929). Beiträge zur Embryochemie der Reptilien. *J. Biochem., Tokyo* **10**, 351–356.

TRACY, C. R., PACKARD, G. C. & PACKARD, M. J. (1978). Water relations of chelonian eggs. *Physiol. Zool.* **51**, 378–387.

VON BRAND, T. & LYNN, W. G. (1947). Chemical changes in the developing turtle embryo. *Proc. Soc. exp. Biol. Med.* **64**, 61–62.

YNTEMA, C. L. (1968). A series of stages in the embryonic development of *Chelydra serpentina*. *J. Morph.* **125**, 219–251.