



# Nitrogen excretion during embryonic development of the green iguana, *Iguana iguana* (Reptilia; Squamata)

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## ABSTRACT

Development within the cleidoic egg of birds and reptiles presents the embryo with the problem of accumulation of wastes from nitrogen metabolism. Ammonia derived from protein catabolism is converted into the less toxic product urea or relatively insoluble uric acid. The pattern of nitrogen excretion of the green iguana, *Iguana iguana*, was determined during embryonic development using samples from allantoic fluid and from the whole homogenized egg, and in hatchlings and adults using samples of blood plasma. Urea was the major excretory product over the course of embryonic development. It was found in higher concentrations in the allantoic sac, suggesting that there is a mechanism present on the allantoic membrane enabling the concentration of urea. The newly hatched iguana still produced urea while adults produced uric acid. The time course of this shift in the type of nitrogen waste was not determined but the change is likely to be related to the water relations associated with the terrestrial habit of the adult. The green iguana produces parchment-shelled eggs that double in mass during incubation due to water absorption; the eggs also accumulate 0.02 mM of urea, representing 82% of the total measured nitrogenous residues that accumulate inside the allantois. The increase in egg mass and urea concentration became significant after 55 days of incubation then were unchanged until hatching.

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## 1. Introduction

Reptiles are typified by their development within an amniotic membrane that in most species is enclosed within a cleidoic egg. The evolution of this trait, together with internal fertilization, enabled reproduction to be completed independent of the aquatic environment and was an essential step in the conquest of land by ancestral reptiles (Packard and Packard, 1980). During embryonic development inside a cleidoic egg, it is necessary that the nitrogenous wastes resulting from embryonic metabolism are stored until the time of hatching. The main end-products of protein metabolism in vertebrates are ammonia, urea and uric acid. The excretion of a specific waste is related to the availability of water in the environment (Packard, 1966) to the relative solubility or toxicity of the waste product and to the cost of its production (Wright, 1995). Deamination of protein stores initially yields ammonia that is converted into compounds of reduced toxicity and stored outside of the embryo proper in the allantoic sac (Romer, 1957). It has been suggested that the allantoic sac is an osmoregulatory organ (Vleck, 1991) and that the albumin present in the allantois is a reservoir for nitrogenous wastes and water (Packard, 1966).

Nitrogen excretion in avian eggs was thought to recapitulate the evolution of nitrogen excretion in ancestral vertebrates, with progressive shifts from production of ammonia to urea and to uric acid related to invasion of the terrestrial environment (Needham, 1931). However, Fisher and Eakin (1957) showed that the three main nitrogen compounds were present from the beginning of the embryonic development in chick embryos and no recapitulation of modes of excretion occurs. In reptilian eggs, a group less studied than the avian model, it was also proposed that a shift from urea to uric acid would occur at the end of the incubation period or following hatching as a mechanism to combat the osmotic problems associated with the accumulation of soluble urea (Clark, 1953) or an adaptation for life outside of the egg (Packard, 1966).

The aim of this study was to determine the type of nitrogenous waste of the lizard *Iguana iguana* that is produced during embryonic development and after hatching. The primary aim was the investigation of the qualitative and quantitative alterations in nitrogenous waste concentrations and content in iguana eggs. This is the first study of nitrogen excretion during development in a squamate lizard.

## 2. Materials and methods

*Iguana iguana*, a large arboreal lizard from the family Iguanidae that is distributed throughout Central and South America, was chosen

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as the model for this study because it lays large clutches of relatively large eggs. Eggs were collected from a captive bred population of *I. iguana* kept at Rio Claro, São Paulo State, Brazil, during the breeding season (September and October in 2009 and 2010). Each egg was numbered using a soft graphite pencil and incubated in moistened vermiculite inside a covered plastic box in a climatic chamber (ELO11 Eletrolab, São Paulo, SP, Brazil) held at  $30 (\pm 0.1)^\circ\text{C}$  (see Licht and Moberly, 1965). The eggs were checked for viability every 72 h and on each occasion the moisture content of the vermiculite was maintained high by spraying with dechlorinated water from a hand held atomizer. As the eggs have flexible shells, with scattered crystals of calcareous material among the fibers of the shell membrane (Packard et al., 1982), they are able to gain mass by absorbing water from the external environment. The progressive changes in the physical dimensions of the eggs and the mass of the egg, embryo, yolk and allantoic sac were followed during development.

### 2.1. Nitrogen excretion in embryos

Measurement of the nitrogenous wastes stored in the eggs was done in two ways: (I) from samples taken directly from the allantois ( $n = 48$ ); (II) samples taken from homogenized eggs ( $n = 18$ ). Samples included eggs from three clutches (two or three eggs per clutch at each sampling period) starting at 25 days of incubation and then subsequently every 10 days until hatching. In 2009, incubation time was delayed and the last sample was taken at 85 days of incubation rather than the more usual 75 days seen in 2010. These samples were all treated as the last stage sampled. The growth curve (see Fig. 1) revealed that the embryos were growing at identical rates until 65 days, so any delay in development likely occurred at late stages of development.

At each sampling point, eight eggs were weighed to the nearest 0.01 mg using an analytical balance (Nagema Owa Labor, VEB Wägetechnik Rapido;  $\pm 0.01$  g) and placed in a closed chamber filled with carbon dioxide for 60 min to kill the embryos. Each egg was then opened and the allantois, yolk sac, membranes, eggshell and embryo were separated and weighed. The allantoic volume was measured with a graduated cylinder in the first period sampled and found to be the same value of the mass. This relationship between volume and mass was assumed to be maintained throughout development, so mass was used as an indicator of volume at all subsequent stages. Samples of the allantois were taken for analysis. Two eggs from each age were homogenized and centrifuged (as described in Packard and Packard, 1983), and the liquid fraction was used for detection of nitrogen content of the whole egg.

Samples of the allantois and egg homogenate were analyzed in duplicate for urea and uric acid using commercial enzymatic

colorimetric kits (Uric Acid Liquiform LABTEST and Urea CE LABTEST, Belo Horizonte, MG, Brazil). For determination of ammonia a modified technique of Verdouw et al. (1978) was used, which includes the addition of 400  $\mu\text{l}$  trichloride acetic to 100  $\mu\text{l}$  of each of the allantoic samples before proceeding to centrifugation (P.A. Wright, personal communication). For measurement of insoluble urates, six eggs immediately prior to hatching were homogenized with the addition of 25 ml of distilled water, following the method described by Packard et al. (1983). The samples were analyzed with the colorimetric enzymatic kit for uric acid (Liquiform, LABTEST). Samples prepared for analysis were measured using a spectrophotometer (FEMTO 600 Plus, São Paulo, SP, Brazil) at 600 nm for ammonia and urea and 550 nm for uric acid. Standard curves were used to confirm the linear relationship between concentration and absorbance. The total amount of nitrogen-ammonia, nitrogen-urea and nitrogen-uric acid within the allantois was calculated by multiplying the concentration of each metabolite by the volume of the allantois, taking into account the nitrogen content of each compound. The mean volume of iguana eggs as calculated from the linear dimensions of 30 eggs collected at oviposition was 15.65 ml. This relates to the mean mass of these eggs, which was 15.85 g by a factor of 1.02. This factor was used to calculate the volume of the homogenized eggs, and it is similar to the factor of 1.05 given for chelonian eggs by Tracy et al. (1978).

### 2.2. Nitrogen excretion in hatchlings and adult iguanas

Blood samples were taken from 10 newborn iguanas 7 days after hatching (body mass =  $9.67 \pm 0.15$  g) before having had their first meal. The newborn iguanas were killed by decapitation, and the blood collected was immediately centrifuged (Sanyo MSE, Jepson Bolton, Watford, Herts, UK) for 3 min at 16,000 g for plasma collection. For adult iguanas (mean body mass =  $1.87 \pm 0.18$  kg;  $n = 10$ ) 2 ml of blood was collected by caudal vein puncture, and centrifuged (Sigma 1-14K, Osterode am Harz, Germany) for plasma collection. The plasma samples were analyzed using the same procedures as those used for the allantois.

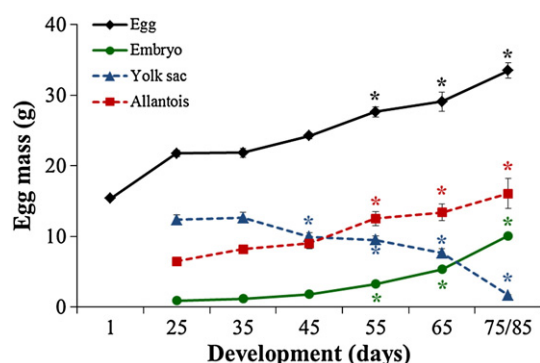
### 2.3. Statistical analysis

The significance of differences in concentrations of nitrogenous waste among embryonic developmental stages was analyzed using a one-way ANOVA, and for pairwise comparisons, Student–Newman–Keul's post hoc test was used. For progressive changes in mass and nitrogen content of the egg and embryo, an ANOVA on ranks test was used, followed by post hoc Dunn's test. To detect differences in mean concentrations found in the allantois and in the homogenized egg, the Man–Whitney test was used. For comparisons between hatchlings and adults, a two-way ANOVA was used, with age and type of nitrogenous waste as factors. Analyses were performed using the SigmaPlot package. Significance was attributed at a 95% confidence level ( $P = 0.05$ ) for all comparisons.

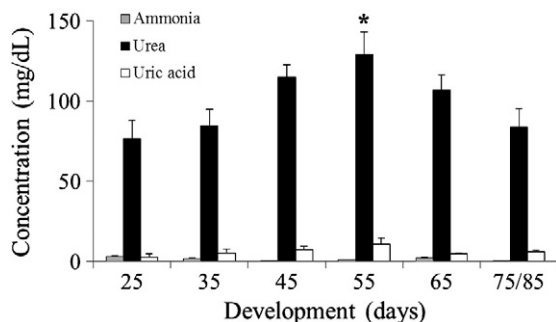
## 3. Results

### 3.1. Changes in mass of eggs and embryos

Fig. 1 illustrates the changes in mass of eggs from oviposition until prior to hatching, and the masses of the embryos, allantois and yolk sac from day 25 until the last period of incubation sampled. The mean initial mass of the eggs from different clutches immediately after laying was  $15.65 \pm 0.07$  g. Prior to hatching, the mean mass of the eggs had virtually doubled to  $33.5 \pm 1.67$  g. The embryos' mean mass increased nonlinearly during development, with 8.9% of the growth occurring in the first third of incubation time, 16.2% in the second third, and 74.9% in the last third. The mean embryo mass



**Fig. 1.** Increase in mass (mean  $\pm$  SEM) of eggs (from oviposition; black diamonds) and embryos (green circles), yolk sac (blue triangles) and allantois (red squares) (from 25 days) until the end of the incubation period. Egg, embryo and allantois masses became different from 25 days values after 55 days then were unchanged until the last period sampled. Yolk sac values were different of the initial values from 45 days (ANOVA on ranks and Dunn's,  $P < 0.05$ ). The color-coded asterisks denote significant differences.



**Fig. 2.** Concentration in mg/dL of the different nitrogen metabolites in the allantois (values are represented as the mean  $\pm$  SEM). The asterisk denotes significant difference relative to the initial value (ANOVA on ranks and Dunn's  $P < 0.05$ ).

was  $0.90 \pm 0.09$  g at 25 days of incubation and grew to  $10.07 \pm 0.35$  g before hatching, which represents an increase of more than 10-fold. The allantois progressively increased in mass, from  $6.45 \pm 0.47$  g at 25 days to  $16.05 \pm 2.14$  g prior to hatching. The yolk sac decreased gradually in mass from  $12.34 \pm 0.73$  g to  $1.65 \pm 0.12$  g.

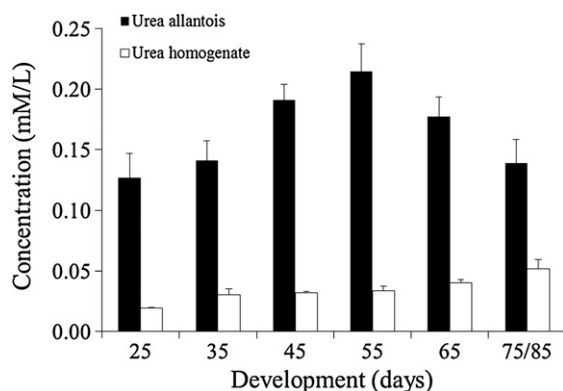
### 3.2. Concentration and nitrogen content of nitrogenous wastes

The predominant nitrogenous waste stored in the allantois was urea found in higher concentrations than the other two metabolites of nitrogen throughout the incubation time (Fig. 2). The maximum urea concentration in the allantois was  $1292.5 \pm 138.3$  mg/l or  $0.21 \pm 0.02$  mM/l at 55 days of incubation. Compared with allantois, homogenized egg had a lower urea concentration of  $0.04 \pm 0.01$  mM/l at the same age (Fig. 3). However, ammonia and uric acid concentrations were similar in homogenized egg and in allantois.

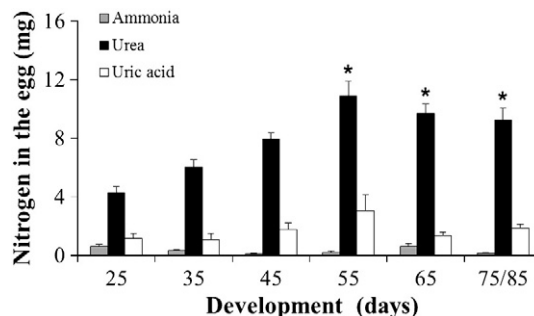
The N-ammonia, N-urea and N-uric acid content were calculated based on proportional molecular weight of nitrogen, and the values obtained are illustrated in Fig. 4. A total of  $11.25 \pm 0.79$  mg of the measured nitrogen was stored in the allantois by the end of the incubation period, approximately 82% of which in the form of urea; ammonia and uric acid represented 1% and 8% of stored nitrogen, respectively. Compared with the initial values, urea-nitrogen content was significantly higher at 55 days ( $P < 0.05$ ). It then remained at this elevated level until the end of incubation period. The content of the other nitrogen compounds remained unchanged throughout development.

### 3.3. Hatchlings and adults

Analysis of blood from 7-day-old hatchling and adult iguanas revealed a change in the excretion pattern during ontogeny. Adults had



**Fig. 3.** Urea concentration (mean  $\pm$  SEM) calculated in mmol/l in the allantois and in the whole homogenized egg. The mean concentration found in the allantois was significantly higher than in the homogenized egg throughout development (Mann-Whitney;  $P = 0.002$ ).



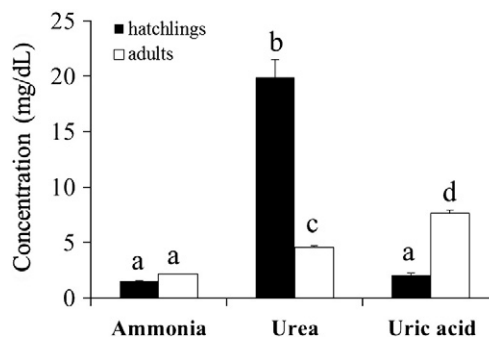
**Fig. 4.** Nitrogen content (mg) of the egg in the form of ammonia, urea and uric acid, relative to days of development (mean  $\pm$  SEM). The asterisk denotes that the content was significantly different from the initial value (ANOVA on ranks and Dunn's,  $P < 0.05$ ).

higher plasma concentrations of uric acid than hatchlings. The concentration in adults was  $76.3 \pm 3.1$  mg/l while in hatchlings it was  $20.6 \pm 2.1$  mg/l. Urea plasma concentration in adults was  $45.5 \pm 1.6$  mg/l while in hatchlings it was four-fold higher at  $199.0 \pm 15.6$  mg/l. Ammonia concentrations were not different between hatchlings and adults (Fig. 5).

## 4. Discussion

Urea was found to be the predominant nitrogenous waste product during the entire incubation period in the *I. iguana* embryos. This was the first study of nitrogen excretion in an embryonic lizard. Embryos of other reptiles studied to date were also found to be ureotelic, e.g., the snakes *Coluber constrictor constrictor* (Clark, 1953; Packard and Packard, 1987), *Thamnophis s. sirtalis* (Clark and Siskin, 1956) and two species of the genus *Psammophis* (Haggag, 1964) plus the turtles *Trionyx spiniferus* (Packard and Packard, 1983), *Chelydra serpentina* (Packard et al., 1984) and *Terrapene ornata* (Packard et al., 1985). The only known exception to ureotelicity is the crocodilian species *Alligator mississippiensis* (Clark et al., 1957), which excretes equal proportions of urea and uric acid.

Iguana hatchlings also showed a predominance of urea as the main product of nitrogen excretion, and thus, hatching in green iguana is not an event that, per se, modifies the nitrogenous waste from urea to uric acid. In contrast, the snakes of the genus *Psammophis*, which are ureotelic throughout the embryonic period, showed a rise in the production of uric acid 2 days after eclosion (Haggag, 1964). Additionally, in eggs of the snake *Coluber c. constrictor*, production of urea at the end of development decreases while production of uric acid increases proportionally (Clark, 1953). The predominance of urea excretion in iguana hatchlings may relate to catabolism of the remaining yolk in the abdominal cavity that may provide metabolic water as well as an energy resource, obviating the need to



**Fig. 5.** Concentration of nitrogenous wastes in the blood of hatchlings and adult iguanas (mean  $\pm$  SEM). Hatchlings had higher values of urea while adults had higher values of uric acid denoted by the different letters a, b, c and d (two-way ANOVA;  $P < 0.05$ ).

change the type of waste generated. Comparison of the concentrations of nitrogenous wastes found in embryos and adults showed that a change from urea to uric acid production occurred at some point following hatching. Although the timing of this shift was not determined, this change is likely to be related to the transition from the aquatic environment provided by the chorioallantoic membrane, where the embryo develops, to the terrestrial environment faced in the post embryonic phase. Adult iguanas are typically arboreal, and so their behavior removes them from a direct contact to water. The transition between the types of waste produced would have involved changes in the utilization of enzymatic pathways for the conversion of ammonia into uric acid rather than urea.

In the iguana embryos urea was concentrated in the allantois, while ammonia and uric acid were distributed with similar relatively low concentrations in the whole egg. Fisher and Eakin (1957) and Clark (1953) also found that urea is stored mainly in the allantoic sac in chicken and snake eggs, respectively. It would be important to the embryo to accumulate urea in the allantois as this compartment is left behind in the egg after hatching. This contrasts with avian embryos that reabsorb it at the end of incubation (Clark, 1953). Urea active transporters are used in marine elasmobranch fishes (Fines et al., 2001) and frogs adapted to hyperosmotic conditions or dehydrated (Lacoste et al., 1991) to maintain higher concentrations of urea in tissues and body fluids or to increase urea plasma retention. The allantoic epithelium in chicken eggs serve as a selectively permeable barrier (Gabielli and Accili, 2010) and although we did not test it in iguana eggs, the same mechanism may be used to maintain higher urea levels in the allantoic sac compared to the other compartments of the egg.

Egg mass increased by a factor of 2.14 from the start of incubation in moist vermiculite. Water uptake by reptilian eggs has been reported in several studies, e.g., the diamond back turtle *Malaclemys centrata* and the fence lizard *Sceloporus undulatus* (Cunningham and Hurwitz, 1936); the painted turtle *Chrysemys picta* and the snapping turtle *Chelydra serpentina* (Cunningham and Huene, 1938). The mechanism for water absorption is dependent on the existence of an osmotic gradient between the egg and the incubation medium. Reptile eggs are thought to exchange water with their surroundings in both vapor and liquid forms (Packard and Packard, 1987). Total exchange depends on the hydraulic conductivity and the water vapor conductivity of the media (Ackerman, 1991). Differences in water potential between the egg contents and the surrounding medium determine the direction of water flow. Thus, eggs incubated in substrates with high water potentials generally experience a net absorption of water over the course of incubation and consequently increase in mass (Packard, 1991).

The current hypothesis proposes that embryos developing in wet environments experience more water absorption into the egg, diluting its urea concentration (Packard et al., 1984). Such water absorption could be the reason for the present study's finding that urea concentration remained constant from 55 days until the end of incubation, although the embryo continued to grow and metabolize, generating nitrogenous residues. Although nitrogen, in the form of urea had increased 2.5 times, the rise in concentration was only to 1.7 times of the initial value measured at 25 days incubation. Urea never rose above 0.20 mM, which is far below the toxic limit of 26 mM for mammals, (Hand and Somero, 1982). Studies of incubation at low water potentials suggested that urea may increase to toxic levels (Packard et al., 1985; Packard and Packard, 1989). However, injections of urea at different concentrations inside developing eggs did not affect the metabolism and size of turtle hatchlings (Packard et al., 1985).

Urea storage may provide an osmolyte that creates a negative water potential inside the egg, preventing water loss to the environment, in the same fashion as in aestivating frogs (Hoffman et al., 1990) or in elasmobranch fishes (Forster and Goldstein, 1976). In

the case of the anuran *Eleutherodactylus coqui*, urea is one of the possible osmolytes that accumulates in the gelatinous layer of eggs to induce water transference from the parental male blood. This difference in water potential is a possible explanation to the maintenance of the hydric balance of developing embryos (Taigen et al., 1984). Similar to the skin of amphibians, the flexible eggshells of reptiles can easily lose water through evaporation. Then, the accumulation of urea, as suggested for *E. coqui*, could be used to prevent desiccation by reptile eggs incubating in situations of low water potential.

Iguana hatchlings had a mean mass equivalent to approximately 30% of the total mass of their egg just prior to hatching but this was equivalent to about 60% of the mass of the freshly laid egg. These data suggest that, although the embryo showed a significant increase in mass due to growth, the water absorbed was only partially incorporated into the tissue. Previous studies have shown that a plentiful supply of water to the eggs of the painted turtle resulted in larger hatchlings, and also greater use by the embryo of the energy reserves held within the egg (Packard et al., 1981). So, water absorption may be necessary to supply room within the expanded eggshell for healthy development. Parchment-shelled eggs, that are permeable to water, have relatively little albumen content. This reduces their individual size and may enable females to produce a large clutch of eggs within her limited body cavity. These eggs then increase in size by absorbing water, allowing the embryo to grow to full size prior to hatching. Rigid-shelled eggs are provided with large quantities of albumen, and the embryos utilize this initial reserve plus small quantities of metabolic water formed during the development, growing to fill the space provided in the freshly laid egg (Tracy, 1980).

As only a few species of reptiles have been studied, it is not possible to relate nitrogen excretion to the type of eggshell, but some differences can be highlighted. Crocodilians, which have a very rigid calcareous eggshell, similar to avian eggs, excrete low amounts of urea during incubation corresponding to 44% of total nitrogenous wastes, while uric acid represents 50% (Clark et al., 1957). These eggs also have little exchange of water with the environment but have a large amount of albumen and the embryos rely on this initial reservoir of water for their subsistence (Packard et al., 1983). Three species of turtles, three snakes, and the iguana, which have eggs ranging from calcareous to parchment eggshells, excrete predominantly urea from 60% to 83% of the total nitrogenous wastes (Haggag, 1964; Packard and Packard, 1983; Packard et al., 1984, 1985; Packard and Packard, 1987; this study). Therefore, in these species the type of eggshell seems not to play an exclusive role in characterizing the form of nitrogenous waste and ureotely could be the synapomorphic condition in reptile embryos.

Lizards represent a very diverse group of species that inhabit a wide range of habitats, so it is necessary to study more species for a broader comparison of patterns of nitrogen excretion and water balance with other reptile groups. The capacity of parchment-shelled eggs to absorb large amounts of water give rise to questions relating to the predominance of ureotely and its role in the growth and survival of embryos in environments where water potential may vary.

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