The Role of Python Eggshell Permeability Dynamics in a Respiration-Hydration Trade-Off

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Accepted 3/1/2010; Electronically Published 5/17/2010

ABSTRACT

Parental care is taxonomically widespread because it improves developmental conditions and thus fitness of offspring. Although relatively simplistic compared with parental behaviors of other taxa, python egg-brooding behavior exemplifies parental care because it mediates a trade-off between embryonic respiration and hydration. However, because egg brooding increases gas-exchange resistance between embryonic and nest environments and because female pythons do not adjust their brooding behavior in response to the increasing metabolic requirements of developing offspring, python egg brooding imposes hypoxic costs on embryos during the late stages of incubation. We conducted a series of experiments to determine whether eggshells coadapted with brooding behavior to minimize the negative effects of developmental hypoxia. We tested the hypotheses that python eggshells (1) increase permeability over time to accommodate increasing embryonic respiration and (2) exhibit permeability plasticity in response to chronic hypoxia. Over incubation, we serially measured the atomic and structural components of Children's python (*Antaresia childreni*) eggshells as well as in vivo and in vitro gas exchange across eggshells. In support of our first hypothesis, *A. childreni* eggshells exhibited a reduced fibrous layer, became more permeable, and facilitated greater gas exchange as incubation progressed. Our second hypothesis was not supported, as incubation O_2 concentration did not affect the shells' permeabilities to O_2 and H_2O vapor. Our results suggest that python eggshell permeability changes during incubation but that the alterations over time are fixed and independent of environmental conditions. These findings are of broad evolutionary interest because they demonstrate that, even in relatively simple parental-care models, successful parent-offspring relationships depend on adjustments made by both the parent (i.e., egg-brooding behavioral shifts) and the offspring (i.e., changes in eggshell permeability).

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Physiological and Biochemical Zoology 83(4):576–586. 2010. 1522-2152/2010/8304-9137$15.00
DOI: 10.1086/652425

Introduction

Since its appearance in the fossil record 350 million years ago (Paton et al. 1999), the amniotic egg has allowed animals to adopt a fully terrestrial life history, and it thus represents a momentous reproductive adaptation. In addition to maintaining embryonic water balance, eggshells and extraembryonic membranes reduce microbial and invertebrate infiltration (Packard and Packard 1980) while facilitating greater respiratory gas exchange than the gelatinous coat of nonamniotic amphibian eggs (Packard and Seymour 1997; Stewart 1997). However, as incubation progresses, the shell must provide for an increasing demand for respiratory gas exchange while maintaining embryonic water balance. This balance can be mediated by biotic (e.g., egg size or embryonic metabolism) or abiotic (e.g., incubation humidity, water potential, and temperature) factors (reviewed in Deeming and Ferguson 1991; Deeming 2004).

This egg respiration-hydration trade-off is not unique to amniotes; the diffusive conductance of oxygen (G_{O_2}) in common hawkmoth (*Manduca sexta*) egg coats increases as incubation progresses (Zrubek and Woods 2006). Among amniotes, the avian eggshell has been most widely studied (Seymour 1985; Deeming and Ferguson 1991) and represents an extreme trade-off, given the high metabolic needs of avian embryos and the relatively exposed nature of most avian incubation environments. For example, over the course of incubation in birds, many eggs develop a highly vascularized chorioallantoic membrane (Andrews 2004), and some reduce shell thickness via embryonic incorporation of shell-derived calcium (Booth and Seymour 1987). While both of these changes accommodate the increased respiratory demands of the embryo, the latter strategy also increases eggshell water vapor conductance (G_{H_2O}, the H_2O flux rate after correction for pressure gradient) and increases the rate of egg water loss (Ar 1991).

In addition to birds, increased eggshell G_{O_2} during incubation has also been demonstrated in the amniotic eggs of Johnston's crocodiles (*Crocodylus johnstoni*) and the pleurodiran turtle *Emydura macquari* (Thompson 1985; Whitehead 1987). Birds, crocodilians, and some turtles, including *E. macquari*, produce calcium-rich rigid-shelled eggs that have a high resistance to water (Thompson and Speake 2004). However, most

oviparous squamates, which represent 96% of living reptile species, lay parchment-shelled eggs (Pough et al. 2001; Deeming and Unwin 2004). Parchment shells are made up of a minimal calcareous layer interfaced with a fibrous inner layer (Thompson and Speake 2004) and exhibit a G_{H_2O} more than 700 times that of avian eggshells (Deeming and Unwin 2004). To avoid desiccation, parchment-shelled eggs are typically buried in moist substrate and actually gain water throughout incubation (Belinsky et al. 2004).

Pythons (family Pythonidae) are atypical squamates in that females usually brood their eggs throughout the incubation period (Wilson and Swan 2003). During brooding, eggs often have little if any contact with the substrate and thus absorb little to no water. Python egg brooding enhances embryonic temperature (Vinegar et al. 1970; Slip and Shine 1988; Stahlschmidt and DeNardo 2009*a*), maintains embryonic water balance (Aubret et al. 2005; Lourdais et al. 2007), and reduces egg predation (Madsen and Shine 1999). Brooding is a dynamic process wherein the egg-brooding female spends most of the time tightly coiled around her eggs but periodically loosens her coils (Stahlschmidt and DeNardo 2008; Stahlschmidt et al. 2008). In Children's pythons (*Antaresia childreni* Gray 1842), we have shown that tight coiling maintains embryonic water balance at the cost of embryonic respiration, while postural adjustments facilitate embryonic respiration at the cost of embryonic water balance (Stahlschmidt and DeNardo 2008; Stahlschmidt et al. 2008). However, despite a five- to sixfold increase in embryonic O_2 requirements, the rate and duration of ventilating postural adjustments do not increase during incubation, and this leads to a hypoxic developmental environment that imposes costs to offspring (Stahlschmidt and DeNardo 2008, 2009*b*; Stahlschmidt et al. 2008). As a result, it is logical to hypothesize that python eggshells increase G_{O_2} or O_2 permeability (K_{O_2}, the O_2 flux rate after correction for pressure gradient and surface area) as incubation progresses.

In addition to determining whether python eggshell conductance and permeability change over time, it is of interest to determine whether such changes are responsive to environmental conditions. During chronic hypoxia, reptile embryos enhance their access to ambient oxygen by increasing the surface area available for gas exchange (i.e., chorioallantoic vasculature; Corona and Warburton 2000) or the ability to pump blood to the periphery (Crossley and Altimiras 2005; Stahlschmidt and DeNardo 2009*b*). Similarly, the eggshell may be adaptively plastic, such that, for example, chronic exposure to hypoxia increases eggshell permeability. Pythons are ideal candidates to test such a response because egg brooding naturally creates a hypoxic developmental environment (Stahlschmidt and DeNardo 2008).

First, we used lab-reared *A. childreni* clutches to test the hypothesis that python eggshells change structurally over the course of incubation and that this results in an increase in their permeability to H_2O vapor and O_2. To test this hypothesis, we serially evaluated (1) eggshell structure and atomic composition, (2) in vitro eggshell permeability to O_2 and H_2O (K_{O_2} and K_{H_2O}, respectively), and (3) in vivo egg clutch O_2 consumption and water loss rates ($\dot{V}_{O_2}$ and $\dot{M}_{H_2O}$, respectively). We predicted that eggshell permeability, egg $\dot{V}_{O_2}$, and egg $\dot{M}_{H_2O}$ would increase as incubation progressed. Second, we tested the hypothesis that python eggshells exhibit morphological plasticity in response to chronic hypoxia. Thus, we compared eggshell permeabilities of late-stage *A. childreni* eggs reared in normoxia (NRM) with those reared in biologically relevant hypoxia (LOW). We predicted higher permeability in the LOW eggshells. Support for these hypotheses would demonstrate the compensatory ability of python eggs to respond to relevant intrinsic (increased O_2 requirements) and extrinsic (reduced O_2 availability) factors. Broadly, results from these experiments will increase our knowledge of reptile embryology and the coevolution of maternal and offspring adaptations of parental care.

Material and Methods

Study Species and Reproductive Husbandry

We used a long-term captive colony of *Antaresia childreni* maintained at Arizona State University, Tempe, for this study. *Antaresia childreni* are medium-sized (up to 1.2 m in snout-to-vent length and 600 g in body mass), nonvenomous, constricting, pythonid snakes that inhabit rocky areas in northern Australia (Wilson and Swan 2003). Husbandry, breeding, and incubation of the animals followed methods described previously (Lourdais et al. 2007; Stahlschmidt and DeNardo 2008). All live animal use in this study was approved by the Arizona State University Institutional Animal Care and Use Committee (protocol 05-792R). Briefly, a few days before oviposition, we moved each gravid python into a Teflon-coated 1.9-L brooding chamber that was opaque on the bottom and sides but transparent on the top to allow observation. We placed brooding chambers in an environmental chamber that had a 14L : 10D photoregime and maintained temperature at 31.5°C (range 31.2°–31.8°C), the species' preferred incubation temperature (Lourdais et al. 2008). Within 1 h of oviposition, eggshells were opaque and dry to the touch. The imperfect ellipsoid eggs were adhered to one another in a clutch conglomerate, which made conventional measurements of surface area infeasible. However, at oviposition, we briefly removed each female from her clutch to determine clutch size, clutch mass, and female postoviposition mass. Within 1 wk of oviposition, we removed clutches from females and artificially incubated them to term at 31.5°C (range 31.2°–31.8°C) in 1.0- or 1.4-L plastic containers with a moistened perlite substrate.

Structural and Atomic Analyses Experiment

To reduce desiccation in the female-free environment, we partially buried six recently laid (≤7 d) NRM clutches in 60–80 mL of moistened perlite suspended with fine mesh above 200–300 mL of distilled water in 1-L dual-ported plastic containers. We placed all of the clutch-housing containers in a 765-L incubator (model 3770, Forma Scientific, Marietta, OH) maintained at 31.5°C (range 31.2°–31.8°C) for the remaining incubation duration. We supplied clutches with compressed

outside air via a manifold system. We hydrated influent air supplies to near vapor saturation by bubbling the air through heated columns of distilled water and maintained the flow rate to each clutch at 50 mL min^{-1}, using an adjustable flow meter (FL-344, Omega Instruments, Stamford, CT) that was calibrated under experimental conditions.

Although intraclutch variation in eggshell characteristics in *A. childreni* is unknown, we used a subsampling repeated-measures experimental design for this experiment and the in vitro gas-exchange experiment (see below) because of the inability to measure serially the same egg (i.e., eggs had to be sacrificed to conduct the experiment) and the large interclutch variation in other *A. childreni* egg traits (Stahlschmidt and DeNardo 2009*b*; Z. R. Stahlschmidt and D. F. DeNardo, unpublished data). Specifically, we terminally sampled one egg from each clutch at each of three time points (7, 24, and 35 d postoviposition, i.e., mean 14%, 50%, and 72%, respectively, of the incubation duration). We removed one 1 × 2-cm section of eggshell from the upper hemisphere of each removed egg, and after removal from the egg, we killed the embryo via rapid decapitation. We gently removed the extraembryonic membranes from the removed section of eggshell and divided the section approximately in half. We immediately used one of the 1-cm^2 sections for the in vitro gas-exchange experiment (see below). The other sections from the early (7 d postoviposition) and late (35 d postoviposition) eggs were stored in 95% ethanol for structural and atomic analyses using scanning electron microscopy (SEM) and energy-dispersive spectroscopy (EDS), respectively. For SEM, we cut the 1-cm^2 samples longitudinally into several pieces using microsurgery scissors, air-dried these pieces at room temperature, mounted them with double-sided transparent tape onto brass stubs, coated the pieces with gold, using an ion sputter-coater (JFC 1100, JEOL, Tokyo), and examined them with an SEM (JSM 6301F, JEOL; Heulin et al. 2005). For each eggshell sample, we took photos of the outer surface (× 3,000) and of one or two cross sections (× 600). We estimated the thickness of each sample by averaging 15 different measurements from different sites spaced along the length of 5–10 cross sections. We determined the thickness of the fibrous layer, the thickness of the calcareous crust, and the total thickness (fibrous layer + crust) of each eggshell for early and late stages of incubation ($n = 6$). For the EDS analyses, we used a spectrometer (LINK INCA, Oxford Instruments, Abingdon, UK) connected to an SEM (JSM 6400, JEOL). We analyzed two or three cross sections for each eggshell and distinguished four regions: calcareous crust, outer fibrous layer (the most superficial third of the fibrous layer), middle fibrous layer, and inner fibrous layer (the deepest third of the fibrous layer). For each of these areas, we took repeated measures (7–10 specter points) and averaged them to determine each region's atomic composition.

In Vitro Gas-Exchange Experiment

Simultaneously with the NRM clutches described above, we incubated seven *A. childreni* clutches under LOW conditions, using a manifold system that supplied a controlled mixture of compressed N_2 and outside air. This treatment mimicked the biologically relevant O_2 partial pressure (Po_2) profile of brooded *A. childreni* eggs in the absence of maternal postural adjustments (as in Stahlschmidt and DeNardo 2009*b*; Fig. 1). Other than the Po_2 of the supply air, NRM or LOW treatment clutches were incubated identically (i.e., at 31.5°C and supplied with near vapor-saturated air at 50 mL min^{-1}). We determined the Po_2 of each treatment's supply air daily by using a syringe pump (model 230, Stoelting, Wood Dale, IL) to flow air samples at a controlled rate through an O_2 analyzer (S 3-A, Applied Electrochemistry, Sunnyvale, CA). We converted the $\%O_2$ displayed on the analyzer to Po_2 ($\%O_2/100$ × barometric pressure), using barometric pressure recorded daily from a gas analyzer (FC-1B, Sable Systems, Las Vegas, NV) located nearby in the lab. The Po_2 for the LOW treatment supplies was changed daily by adjusting the flow meters for N_2 and outside air (Fig. 1).

To determine the effect of incubation stage on eggshell conductance and permeability, we used the 1-cm^2 eggshell sections taken from the NRM clutches at 7, 24, and 35 d postoviposition as described above. On removal from the source clutch, the 1-cm^2 sections were stored in a vapor-saturated container for 20–30 min and then placed into a two-cell, closed-system diffusion chamber at 31.5°C (Fig. 2). The upper "gas-sample" cell interfaced with 28.3 mm^2 of the outer side of the eggshell, while the lower, liquid-water cell interfaced with the inner surface of the same 28.3 mm^2 of eggshell. We equipped the nylon upper cell (2.73 cm^3) with two ports, a downloadable hygrometric sensor (DS1923, Maxim Integrated Products, Sunnyvale, CA), and a fiber-optic O_2 sensor (OxyMini, World Precision Instruments, Sarasota, FL), and we recorded the resulting data every 10 min for 2 h (Fig. 2). Under the conditions used, the hygrometer had a resolution of 0.04% relative humidity (RH),

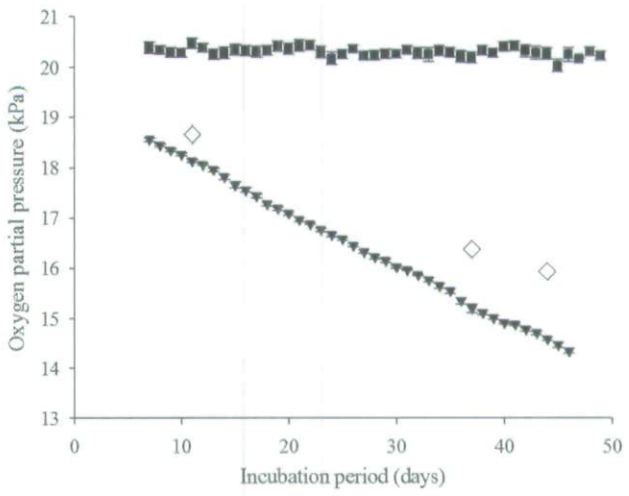

Figure 1. Oxygen regimes for normoxic (NRM, *squares*) and hypoxic (LOW, *triangles*) *Antaresia childreni* eggs during incubation. The diamonds represent intraclutch oxygen partial pressures during maternal egg brooding (from Stahlschmidt and DeNardo 2008). Data are reported as means ± SEM.

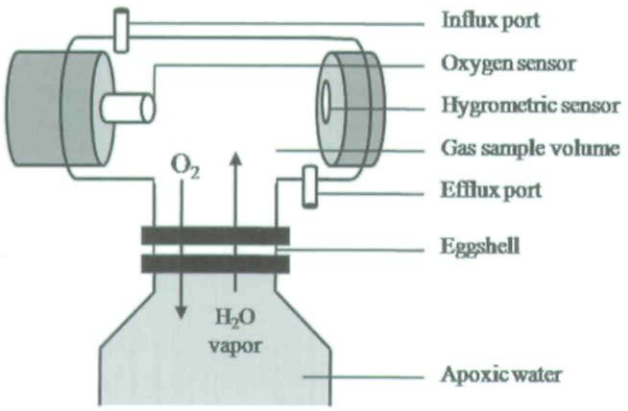

Figure 2. Two-cell, closed-system diffusion chamber used to measure *Antaresia childreni* eggshell permeability to O_2 and H_2O vapor (K_{O_2} and K_{H_2O}, respectively). To summarize, by injecting dry O_2 into the gas-sample volume of the top cell, we created steep O_2 and H_2O vapor gradients across the eggshell. The reduction of these gradients over time elucidated each eggshell's K_{O_2} and K_{H_2O}. Note: the lower cell consisted of only apoxic liquid water and interfaced directly with the inner surface of the eggshell.

and the O_2 sensor had a resolution of 0.06–0.12 kPa. We used two-point calibrations for the hygrometer (0% and 100% RH) and O_2 sensor (0 and 20.7 kPa) before each trial. The lower cell consisted of a 100-mL glass Erlenmeyer flask filled entirely with a 1% Na_2SO_3 aqueous solution and submerged in a water bath maintained at 31.5°C (range 31.3°–31.7°C; Fig. 2). Before the study, we confirmed that this concentration of Na_2SO_3 maintained an anoxic solution in a shell-less (i.e., zero-resistance) chamber for >24 h.

After placing an eggshell section into the diffusion chamber, we opened both ports on the gas-sample cell, flushed the cell with 60 mL of bottled O_2, and closed both ports. This created steep gradients for water vapor (100% RH, or 4.6 kPa) and O_2 (97.5 kPa) across the eggshell, and these gradients degraded over time. We used the following equations, modified from Wangesteen et al. (1970/1971), to calculate eggshell conductance and permeability:

$$K = \frac{1.868V}{t_{0.5}T},$$

$$K = \frac{G}{A},$$

where G is the conductance to O_2 or H_2O (cm^3 STP d^{-1} kPa^{-1}), K is the permeability (area-specific conductance) to O_2 or H_2O (cm^3 STP d^{-1} cm^{-2} kPa^{-1}), V is the volume of gas-sample cell (cm^3), $t_{0.5}$ is the time required for the P_{O_2} (or P_{H_2O}) in the gas-sample cell to reach 50% of the lower-cell P_{O_2} (or P_{H_2O}; d), A is the area of the shell available for gas exchange (cm^2), and T is the absolute temperature (K).

With this method, we simultaneously measured eggshell permeability to both H_2O vapor and O_2 while mimicking natural conditions (i.e., liquid inside/gas outside, preferred incubation temperature, and diffusive gas exchange only). The value of G is directly related to K in our study because surface area (28.3 mm^2) and volume (2.73 cm^3) were fixed throughout the trials. Determining the surface area of imperfect ellipsoid eggs was not feasible; thus, we do not provide results for G because we did not determine eggshell diffusive conductance of whole individual eggs, as in previous studies (reviewed in Deeming and Thompson 1991). Rather, we present results for K, area-specific conductance, or permeability.

To determine the effect of developmental P_{O_2} on eggshell conductance and permeability, we similarly processed late-stage LOW eggshells (i.e., at 72% postoviposition development) and compared their K_{O_2} and K_{H_2O} with those of late-stage NRM eggshells. We used late-stage eggshells because late-stage *A. childreni* embryos are more sensitive than early-stage embryos to LOW conditions (Stahlschmidt and DeNardo 2008, 2009*b*) and because the developmental microenvironment during egg brooding becomes progressively more hypoxic during incubation (Stahlschmidt and DeNardo 2008).

In Vivo Gas-Exchange Experiments

We used six artificially incubated *A. childreni* clutches and measured embryonic oxygen consumption rates ($\dot{V}_{O_2}$) at 31.5°C (range 31.2°–31.8°C) during three periods: 10–16, 36–38, and 43–45 d after oviposition (i.e., mean 26%, 76%, and 90%, respectively, of incubation duration). Closed-system respirometry was necessary to determine $\dot{V}_{O_2}$ at the early incubation stage, so we used it at all stages for consistency. During a trial, we kept the clutch in a 1.2-L dual-ported airtight respirometry chamber with no substrate and supplied the clutch chamber with influent air of known composition by hydrating a controlled flow of acapnic air (CDA 1112, PureGas, Broomfield, CO). We controlled flow with a mass-flow controller (Unit Instruments, Yorba Linda, CA) that we calibrated by using soap-film flow meters. After allowing the chamber to reach equilibrium (i.e., minimally a 99% turnover of chamber air; Lasiewski et al. 1966), we collected an initial 60-mL air sample ($T_{initial}$) from the clutch chamber and stopped the influx air. We then sealed the clutch chamber for a recorded duration (64 ± 8 min) and collected a final 60-mL air sample (T_{final}) from the chamber, which created negative pressure within the chamber for only a brief period of time. We then used a syringe pump (Stoelting model 230) to pass the dried $T_{initial}$ and T_{final} samples at a controlled rate through an O_2 analyzer (S-3A) that we had calibrated with dried outside air at the same controlled flow rate <30 min before analyses. We used equations (5), (6), and (11) in Vleck (1987) to determine clutch $\dot{V}_{O_2}$, and we divided clutch $\dot{V}_{O_2}$ by clutch size to determine mean egg $\dot{V}_{O_2}$.

Less than 2 h after the $\dot{V}_{O_2}$ trial, we measured clutch water loss rate ($\dot{M}_{H_2O}$), using flow-through hygrometry at 31.5°C, by combining dry, acapnic air (CDA 1112, PureGas) with water-vapor-saturated air (produced by bubbling dry air through a water-filled hydrating column) in a feedback-controlled system. Resulting influent air was humidified to 22.6 g m^{-3} absolute humidity (69% RH) and maintained at a flow rate of 560 mL

min^{-1} with a mass-flow controller (Unit Instruments). We passed air exiting the test chamber (effluent air) through a hygrometer (RH200, Sable Systems) and then dried it with anhydrous $CaSO_4$ before flowing it through a CO_2 analyzer (LI-6252, Li-Cor Biosciences, Lincoln, NE) and an O_2 analyzer (FC-1B, Sable Systems). We calibrated all equipment before experimentation. During trials, we recorded O_2 concentration, CO_2 concentration, and dew point of effluent air every minute, using a data logger (23X, Campbell Scientific Instruments, Logan, UT), and we used equations (1)–(7) in Walsberg and Hoffman (2006) to determine clutch $\dot{M}_{H_2O}$. We calculated individual egg $\dot{M}_{H_2O}$ by dividing clutch $\dot{M}_{H_2O}$ by clutch size.

Statistical Analyses

Data met the appropriate assumption of parametric statistics or were transformed as necessary, and we analyzed data with SPSS Statistics (ver. 15.0, SPSS, Chicago). We determined experimentwise two-tailed significance at $\alpha < 0.05$ for all tests and performed power $(1 - \beta)$ analyses for nonsignificant results. We used two-sample *t*-tests to determine the effect of treatment among individuals (e.g., comparing shell permeability of eggs reared in NRM and LOW conditions). To determine within-individual effects (e.g., the effect of incubation stage on eggshell permeability), we used repeated-measures ANOVA (rmANOVA) tests. In rmANOVA analyses with significant sphericity, we used χ^2 tests with ϵ-adjusted Greenhouse-Geisser or Huynh-Feldt tests. For post hoc analyses, we used Bonferroni-corrected paired *t*-tests to correct for experimentwise Type I error rate. All values are given as mean ± SEM.

Results

Structural and Atomic Analyses

Using SEM, we showed that *Antaresia childreni* eggshells are primarily made up of a fibrous layer, similar to eggshells of other squamates (Fig. 3; Table 1). Further, we demonstrated that the fibrous layer and total eggshell thickness decreased as incubation progressed (Table 1). Because the eggs did not increase in mass throughout incubation (Table 2), we assume that this reduction in thickness was not simply the result of shell stretching because of swelling. Eggshell thinning was functionally significant because individual values were correlated with both K_{O_2} and K_{H_2O} for early (K_{O_2}: $F_{1,4} = 27.94$, $P = 0.006$, $r^2 = 0.88$; K_{H_2O}: $F_{1,4} = 8.14$, $P = 0.046$, $r^2 = 0.67$) and late (K_{O_2}: $F_{1,4} = 25.00$, $P = 0.007$, $r^2 = 0.86$; K_{H_2O}: $F_{1,4} = 8.62$, $P = 0.043$, $r^2 = 0.68$) stages of incubation (Fig. 4). Using EDS, we characterized the composition of 10 elements in four eggshell layers (Table 3). Although such data violate the assumption of independence, we determined a change only in the percentage of magnesium in the calcareous crust during incubation ($t_5 = -3.6$, $P = 0.016$; Table 3). Not unexpectedly, we demonstrated differences in several elements among eggshell layers (e.g., the calcareous layer had higher percentage of calcium, while the fibrous layers had higher percentage of carbon; Table 3).

In Vitro Gas-Exchange Experiments

We determined that eggshell *K* increased with incubation for both O_2 and H_2O vapor during NRM conditions (Table 2). With post hoc Bonferroni analyses, we demonstrated a significant difference due to incubation stage for *K*: early-stage *K* was less than late-stage *K* (both $P < 0.015$), but middle-stage *K* was not significantly different from either early- or late-stage values.

Regarding the plasticity of eggshell permeability to hypoxia, we did not detect a significant effect of developmental Po_2 on eggshell K_{O_2} (NRM: $8.6 \times 10^{-5} \pm 3.0 \times 10^{-6}$ cm^3 d^{-1} cm^{-2} kPa^{-1}; LOW: $8.6 \times 10^{-5} \pm 3.3 \times 10^{-6}$ cm^3 d^{-1} cm^{-2} kPa^{-1}; $t_{11} = -0.66$, $P = 0.95$, $1 - \beta = 0.95$) or K_{H_2O} (NRM: 206.2 ± 5.7 cm^3 d^{-1} cm^{-2} kPa^{-1}; LOW: 210.9 ± 4.4 cm^3 d^{-1} cm^{-2} kPa^{-1}; $t_{11} = -0.66$, $P = 0.52$, $1 - \beta = 0.60$). Hatching success for the remainder of the clutches from which the eggs used in this experiment were taken was not significantly different between NRM (85% ± 4%) and LOW (92% ± 3%) clutches ($t_{11} = -1.4$, $P = 0.18$, $1 - \beta = 0.53$).

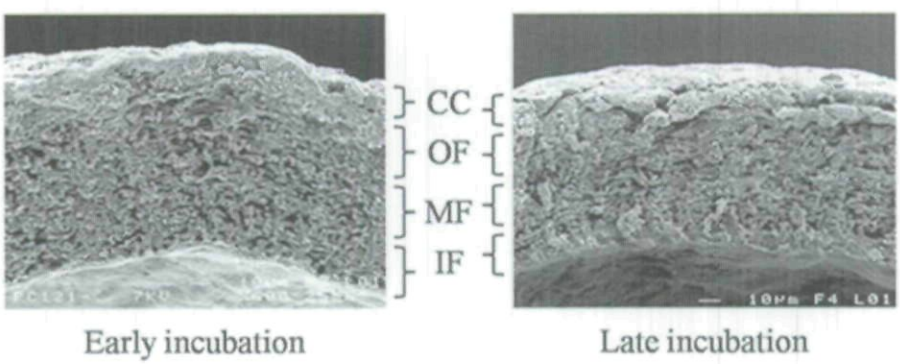

Figure 3. Cross-section scanning electron micrograph of *Antaresia childreni* eggshells from the same clutch during early (16% of incubation duration) and late (74% of incubation duration) stages of incubation. *CC* = calcareous crust, *OF* = outer fibrous layer, *MF* = middle fibrous layer, and *IF* = inner fibrous layer.

Table 1: Total and compositional thickness of *Antaresia childreni* eggshells during early (mean = 15% of incubation duration) and late (mean = 72% of incubation duration) stages of incubation ($n = 6$)

Incubation Stage	Calcareous Layer Thickness (μm)	Fibrous Layer Thickness (μm)	Total Thickness (μm)	Proportion of Fibrous Layer (%)
Early	5 ± 0	84 ± 4[a]	89 ± 4[a]	94.3 ± .4
Late	5 ± 0	73 ± 4	77 ± 4	93.8 ± .4

[a] Significantly greater than that at late stage.

In Vivo Gas-Exchange Experiments

Hatching success for the clutches used in this experiment was similar to that measured for the clutches used in the in vitro experiment and that of previous *A. childreni* egg-brooding studies (80% ± 8%; Lourdais et al. 2007; Stahlschmidt and DeNardo 2008). We detected increased clutch $\dot{V}O_2$ and egg $\dot{V}O_2$ as embryos grew and developed (Table 2). Further, we determined that mean egg mass decreased significantly with incubation (Table 2), which suggests that eggshell surface was either diminishing or, at most, static throughout incubation. Thus, an increase in clutch $\dot{M}H_2O$ and egg $\dot{M}H_2O$ with incubation (Table 2) was not simply the result of an increase in surface area available for gas exchange or a reduced shell thickness because of swelling. Further, the metabolic heat production of even late-stage *A. childreni* embryos is minuscule (i.e., assuming that 1 mL of O_2 consumed is equivalent to 20 J, as in Brouwer 1957, late-stage *A. childreni* embryos produced only 0.010 more watts than early-stage embryos). Thus, embryonic heat production, in the presence of the 560–mL min^{-1} air flow through the chamber, would not significantly affect temperature or internal water-vapor pressure during $\dot{M}H_2O$ measurements. With post hoc Bonferroni analyses, we demonstrated significant differences due to incubation stage for clutch $\dot{V}O_2$, egg $\dot{V}O_2$, clutch $\dot{M}H_2O$, and egg $\dot{M}H_2O$ (i.e., early < middle < late; all $P < 0.015$). Further, mean eggshell KH_2O was strongly related to mean egg $\dot{M}H_2O$ over incubation ($r^2 = 0.99$), although the data were taken during different experiments.

Discussion

We have demonstrated structural and functional changes in python eggshells during incubation that are consistent with our first hypothesis, that python eggshells change structurally over the course of embryonic development to increase permeability. As the embryos' respiratory needs increased, eggshells exhibited a reduced fibrous layer (Table 1). This reduction in shell thickness in turn led to increased KO_2 and KH_2O as incubation progressed (Table 2; Fig. 4). Because python eggs typically lose or at most do not gain mass (i.e., water) during maternal incubation (Stahlschmidt and DeNardo 2008; Stahlschmidt et al. 2008), increased eggshell permeability obligated an increase in egg water loss. Thus, increasing permeability entailed both costs (increased water loss) and benefits (increased respiratory gas exchange) to developing embryos.

This trade-off between respiratory gas exchange and water balance may be adaptive, because biologically relevant levels of developmental hypoxia lead to smaller, weaker, and slower *Antaresia childreni* offspring (Stahlschmidt and DeNardo 2008, 2009*b*). Hypoxia in other species can decrease embryonic growth rate (*Alligator mississippiensis*: Crossley and Altimiras 2005; *Salmo trutta*: Roussel 2007), reduce hatchling mass (Crossley and Altimiras 2005), delay the development of thermogenesis (*Gallus gallus*: Azzam et al. 2007), reduce predator avoidance ability of juveniles (Roussel 2007), and impair sexual development (*Danio rerio*: Shang et al. 2006). Given these deleterious effects, many species have acquired adaptations to enhance gas flux across the eggshell as embryonic metabolism increases. For example, bird and reptile eggs develop a highly vascularized chorioallantoic membrane that increases the surface area available for gas exchange (Andrews 2004), while embryonic incorporation of shell-derived calcium deposits reduces shell thickness (Booth and Seymour 1987). Further, as incubation progresses, liquid is removed from the eggshell pores of *A. mississippiensis* because of drying. Because the diffusion coefficient of O_2 through gas is several orders of magnitude greater than that through liquid, eggshells with dry pores exhibit increased diffusive conductance and thus increased flux across the eggshell (Kern and Ferguson 1997). Also, as embryos grow, their natural increase in metabolic rate serves to increase the O_2 concentration gradient (Seymour 1985). Thus, eggs can mediate the respiration-hydration trade-off in a number of ways by promoting respiratory gas flux as incubation progresses.

Although they both serve as mediating barriers, archosaur (i.e., birds and crocodilians) and squamate eggshells exhibit marked morphological differences. Bird eggshells are much thicker (365 μm) and are predominately made up of a calcareous outer layer (82% of total thickness; Paganelli 1991). Alternatively, squamate eggs are thinner and are largely composed of a fibrous layer (*Lacerta vivipara*: 50–70 μm total, 87%–89% fibrous layer; *A. childreni*: 67–99 μm, 92%–96% fibrous layer; Heulin et al. 2002; Table 1). This difference is functionally significant because colubrid snake eggshells contribute only 23%–28% of hatchling calcium requirements, with the remainder being supplied by yolk (Packard and Packard 1984; Stewart et al. 2004). Conversely, bird eggshells meet 62%–92% of hatchling calcium requirements (Packard and Packard 1984). Heavy deposits of calcium in the shell drastically reduce permeability and allow birds to use a broad range of nest sites (Paganelli 1991). Alternatively, the high eggshell permeability of squamate eggs may occur concurrently with longer durations

Table 2: *Antaresia childreni* clutch, egg, and eggshell characteristics at multiple stages of development for in vivo ($n = 7$) and in vitro ($n = 6$) experiments (mean ± SEM)

Characteristic	Early	Middle	Late	F	P
In vivo experiment:					
Postoviposition development (%)	25.6 ± 2.1	76.2 ± 1.7	89.9 ± 1.9	...	...
Clutch size	10.7 ± .3	10.5 ± .4	10.4 ± .4	1.00	.363
Mean egg mass (g)	12.9 ± .6	11.9 ± .7	11.5 ± .6	9.07	.012
Clutch $\dot{V}O_2$ (mL h^{-1})	5.9 ± .3	16.3 ± 1.6	23.7 ± 2.1	57.6	<.001
Egg $\dot{V}O_2$ (mL h^{-1} egg^{-1})	.56 ± .03	1.58 ± .14	2.44 ± .10	192	<.001
Clutch $\dot{M}H_2O$ (mg h^{-1})	241.6 ± 14.1	264.3 ± 13.2	313.9 ± 14.0	27.6	.001
Egg $\dot{M}H_2O$ (mg h^{-1} egg^{-1})	22.7 ± 1.2	25.3 ± 1.4	29.5 ± 1.5	36.6	<.001
In vitro experiment:					
Postoviposition development (%)	14.5 ± .2	50.0 ± .7	72.3 ± 1.0	...	...
Sampled egg mass (g)	13.0 ± .4	13.7 ± .5	13.2 ± .6	2.32	.149
Eggshell O_2 permeability (K_{O_2}; cm^3 d^{-1} cm^{-2} kPa^{-1})	$6.6 \times 10^{-5} \pm 2 \times 10^{-6}$	$7.5 \times 10^{-5} \pm 4 \times 10^{-6}$	$8.6 \times 10^{-5} \pm 3 \times 10^{-6}$	11.9	.009
Eggshell H_2O permeability (K_{H_2O}; cm^3 d^{-1} cm^{-2} kPa^{-1})	168.8 ± 6.2	184.3 ± 4.3	206.2 ± 7.5	17.6	.008

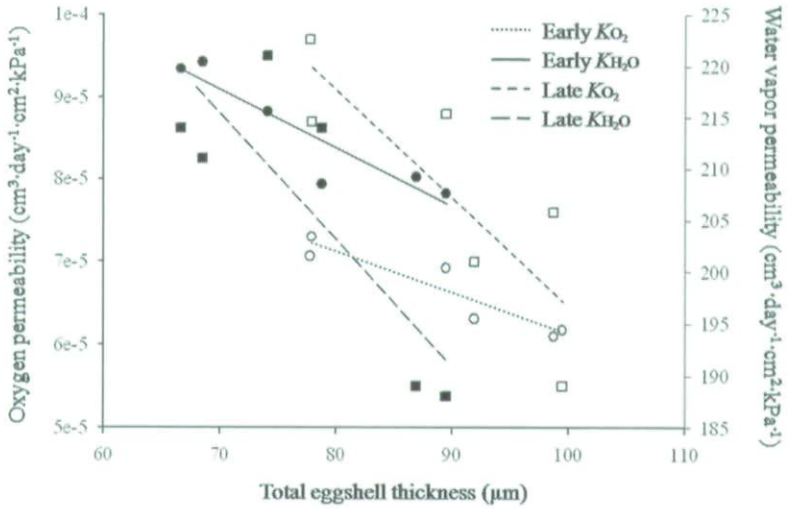

Figure 4. Significant inverse relationships between *Antaresia childreni* eggshell thickness and permeability to O_2 (K_{O_2}) and H_2O vapor (K_{H_2O}) during early (mean = 15% of incubation duration) and late (mean = 72% of incubation duration) stages of incubation. *Open circles* = early-stage K_{O_2}, *filled circles* = late-stage K_{O_2}, *open squares* = early-stage K_{H_2O}, and *filled squares* = late-stage K_{H_2O} ($n = 6$).

of egg retention and before the evolution of viviparity (Packard et al. 1977).

Comparatively, *A. childreni* eggshell K_{H_2O} (169–206 $cm^3\ d^{-1}\ cm^{-2}\ kPa^{-1}$) fits into the range of the reported values for similarly sized parchment-shelled eggs of other snakes (117–227 $mg\ d^{-1}\ cm^{-2}\ kPa^{-1}$; reviewed in Deeming and Thompson 1991). Because of different media (i.e., O_2 diffused into liquid H_2O while H_2O vapor diffused into gas), we measured drastic differences between K_{O_2} and K_{H_2O} at each incubation stage (Table 1). Although our eggshell K_{O_2} measurements were ecologically relevant and useful in distinguishing shifts in K_{O_2} over time, they cannot be readily compared with eggshell K_{O_2} values acquired with more traditional methods (e.g., O_2 diffusing into gas; reviewed in Deeming and Thompson 1991). Further, the proportion of *A. childreni* eggshell K_{H_2O} to K_{O_2} (mean = 2.5×10^6) is 230 times the proportion of the H_2O vapor diffusion coefficient to the O_2 diffusion coefficient under similar conditions (1.1×10^4; Montgomery 1947; Wilke and Chang 1955). Thus, as reported in other reptiles and some birds (Deeming and Thompson 1991; Paganelli 1991), H_2O and O_2 permeabilities are not coupled in the parchment-shelled eggs of pythons. The mechanism of this phenomenon at the proximate level is not fully understood, but it may ultimately be related to reptiles' relatively humid nest environments and low embryonic metabolic rates (Deeming and Thompson 1991).

To the best of our knowledge, we provide the first evidence of eggshell thinning independent of egg swelling in squamate reptiles. Other squamate eggs absorb water from surrounding substrate throughout incubation (Belinsky et al. 2004). The resultant swelling serves to both increase the surface area available for gas exchange and reduce the thickness of the eggshell. Our results suggest that pythons and other squamates seem to accomplish the same goal (i.e., eggshell thinning to reduce late-stage embryonic respiratory constraints) through different means. That is, pythons reduce the thickness of the eggshell's fibrous layer independent of egg swelling, while other squamate eggshells stretch because of swelling from water influx. Like other pythons, *A. childreni* oviposit at the end of a prolonged dry season (Wilson and Swan 2003). Thus, in contrast to many other squamates, pythons may not have access to a nest site with moist substrate, which in turn precludes the eggs from reducing eggshell thickness because of swelling with water. The ability to reduce eggshell thickness independent of extrinsic moisture, combined with egg-brooding behavior, may confer certain advantages to pythons by expanding the temporal and spatial flexibility of reproduction.

The mechanism for eggshell thinning in pythons is less straightforward. Recent research suggests that osteopontin, an eggshell matrix glycoprotein, may regulate eggshell growth rate and calcium flux in bird eggs (Chien et al. 2009). However, squamate eggshells are minimally calcified, and we did not detect significant shifts in thickness or atomic composition in the calcareous crusts of *A. childreni* eggs. Nonetheless, another eggshell matrix compound may similarly regulate the catabolism of the fibrous layer components (e.g., keratin, collagen, or elastin fibers) of python eggshells.

To eliminate the potential confounding effects of intraclutch variation in eggshell characteristics, the best experimental approach would have been to measure traits on the same eggs at two time points. Unfortunately, certain experimental methods (e.g., SEM morphological assessment and in vitro assessment of conductance) require egg sacrifice; thus, repeated measures are not possible. Rather, we chose to pair eggs from the same clutch because data on *A. childreni* offspring characteristics demonstrate that intraclutch variation is much less than interclutch variation (Stahlschmidt and DeNardo 2009*b*; Z. R. Stahlschmidt and D. F. DeNardo, unpublished data). Not only was the difference between the time points statistically signif-

Table 3: Atomic composition of *Antaresia childreni* eggshells during early (mean = 15% of incubation duration) and late (mean = 72% of incubation duration) stages of incubation in four layers ($n = 6$)

	Calcareous Layer		Outer Fibrous Layer		Middle Fibrous Layer		Inner Fibrous Layer	
Element	Early	Late	Early	Late	Early	Late	Early	Late
Carbon[a,b]	49.66 ± 1.46	46.58 ± 1.67	74.21 ± .95	71.65 ± 2.46	76.32 ± 1.31	74.40 ± .80	74.85 ± 1.82	74.04 ± .56
Nitrogen[a,b]	6.78 ± 1.12	5.05 ± .77	13.04 ± .43	13.03 ± 1.22	11.94 ± .93	12.75 ± .38	11.43 ± 1.00	11.97 ± .44
Oxygen[a,b]	32.02 ± 1.41	34.77 ± 1.64	10.45 ± .61	12.39 ± 1.03	9.57 ± .55	10.94 ± .69	12.29 ± 1.05	11.97 ± .75
Sodium[a]	.11 ± .03	.04 ± .01	.02 ± .01	.02 ± .01	.02 ± .00	.02 ± .01	.03 ± .00	.04 ± .01
Magnesium[a,b]	.23 ± .02[c]	.30 ± .01	.04 ± .01	.05 ± .01	.02 ± .01	.03 ± .01	.04 ± .01	.04 ± .01
Phosphorus	.03 ± .00	.03 ± .01	.02 ± .01	.01 ± .00	.01 ± .00	.02 ± .01	.04 ± .03	.03 ± .01
Sulfur[a,b]	.35 ± .03	.39 ± .08	1.52 ± .34	1.50 ± .35	1.78 ± .40	1.50 ± .30	.98 ± .13	1.48 ± .45
Chlorine	.10 ± .04	.07 ± .02	.09 ± .04	.12 ± .04	.10 ± .04	.09 ± .04	.05 ± .01	.17 ± .09
Potassium	.05 ± .02	.03 ± .00	.01 ± .00	.04 ± .02	.02 ± .00	.02 ± .00	.02 ± .01	.04 ± .02
Calcium[a,b]	10.67 ± 1.21	12.75 ± 1.19	.60 ± .41	1.20 ± .80	.21 ± .08	.27 ± .13	.28 ± .14	.23 ± .06

[a] Significantly different among layers at early developmental stage.
[b] Significantly different among layers at late developmental stage.
[c] Significantly different from the late stage in same layer.

icant, but all paired samples (i.e., an early-stage egg compared with a late-stage egg from the same clutch) showed the same directional effect: thinner shell and higher conductance during the latter stage of development. Thus, presenting the results of these three experiments in one article ensures that the conclusions on the effects of time on egg shell characteristics are well supported as an accurate assessment of a biological phenomenon and not merely statistical aberration.

Our results do not support our second hypothesis, that python eggshells exhibit functional plasticity in response to a hypoxic environment, because eggshell permeability was nonresponsive to the level of O_2 in the incubation environment. This result suggests that eggshell thinning and egg-brooding behavioral adjustments may have coevolved in pythons. Postural adjustments may partially alleviate constraints on early-stage embryonic respiration, while eggshell thinning may alleviate late-stage constraints. Increased permeability associated with the thinning of the eggshell facilitates greater O_2 diffusion. Thus, females need not alter the frequency of their postural adjustments, thus preserving egg water balance late in incubation. Other examples of synergistic adaptations to parental care exist. For instance, parental birds often adjust food provisioning to their chicks on the basis of specific chick begging behaviors (reviewed in Smiseth et al. 2008). This coevolution serves to enhance chick energy balance, whereas the coevolution of python egg brooding and eggshell thinning may serve to preserve water balance throughout incubation.

Through the use of several techniques, we have demonstrated that *A. childreni* eggshells contribute to balancing respiration and hydration demands throughout embryonic development but do not exhibit functional plasticity in response to chronic hypoxia. Future studies should further examine the proposed hypotheses in an ecological context. For example, the respiratory microenvironment of wild python nests is unknown and would prove valuable in quantifying the proposed respiration-hydration trade-off. Also, our hypotheses should be tested in other parchment-shelled systems (i.e., other squamates) as well as in pliable-shelled (i.e., some turtles) and rigid-shelled (i.e., some turtles, crocodilians, gekkonine lizards) systems. Finally, the regulatory mechanism of eggshell thinning independent of egg swelling should be further examined. Such studies would continue to fill a large gap in our current knowledge of amniote embryology, particularly the proximate mechanisms and constraints to adaptation.

Acknowledgments

We thank Glenn Walsberg, Ty Hoffman, and members of Jon Harrison's lab for technical support and Ernest Nigro for animal husbandry support. We are grateful to the engineers of the Centre de Microscopie Électronique à Balayage et Microanalyse (CMEBA) at Rennes University for their help in SEM and EDS analyses. We also thank the National Science Foundation (grant IOS-0543979 to D.F.D.) and the Graduate and Professional Students' Association at Arizona State University for financial support. We further appreciate Glenn Walsberg, Kevin McGraw, and two anonymous reviewers for comments on the manuscript. Finally, we thank Olivier Lourdais for mediating transportation logistics.

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