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Alternating egg-brooding behaviors create and modulate a hypoxic developmental micro-environment in Children's pythons (*Antaresia childreni*)

Zachary R. Stahlschmidt* and Dale F. DeNardo

School of Life Sciences, Arizona State University, Tempe, AZ 85287-460, USA

*Author for correspondence (e-mail: zs@asu.edu)

SUMMARY

Parental care is a widespread and ecologically relevant adaptation known to enhance the developmental environment of offspring. Parental behaviors, however, may entail both costs and benefits for developing offspring. In Children's pythons (*Antaresia childreni*), we monitored both maternal egg-brooding behavior and intra-clutch oxygen partial pressure (P_{O_2}) in real-time to assess the effects of various brooding behaviors on P_{O_2} in the clutch micro-environment at three stages of development. Furthermore, at the same developmental stages, we measured O_2 consumption rates ($\dot{V}_{O_2}$) of eggs at varying P_{O_2} to determine their critical oxygen tension (i.e. the minimal P_{O_2} that supports normal respiratory gas exchange) and to predict the impact that naturally brooded intra-clutch P_{O_2} has on embryonic metabolism. At all three stages of development, a tightly coiled brooding posture created an intra-clutch P_{O_2} that was significantly lower than the surrounding nest environment. Maternal postural adjustments alleviated this hypoxia, and the magnitude of such corrections increased with developmental stage. Mean intra-clutch P_{O_2} decreased with stage of development, probably because of increasing egg $\dot{V}_{O_2}$. Additionally, embryo critical oxygen tension increased with developmental stage. Together, these results suggest that python embryos are unable to maintain normal metabolism under brooded conditions during the final 10% of incubation. These results demonstrate that specific parental behaviors can impose obligatory costs to developing offspring and that balancing these behaviors can mediate deleterious consequences.

Key words: adjustable diffusive barrier, critical oxygen tension, hypoxia, metabolism, parental care, snake, trade-off.

INTRODUCTION

Parental care is a widespread reproductive strategy that provides for critical developmental needs including water balance, energy balance and thermoregulation (Clutton-Brock, 1991). Although beneficial to offspring, parental care is typically demanding to the parent(s) and thus parental care is classically assessed as a trade-off between the benefits to the offspring and the costs to the parent(s) (Clutton-Brock, 1991; Williams, 1966). However, another, less-studied cost-benefit tradeoff exists in parental care. Parental care is complex and typically entails a suite of specific parental behaviors with each of those behaviors associated with costs and benefits for offspring. For example, nest attendance and food acquisition represent familiar parental behaviors of birds; however, each has its own cost and benefit to the thermoregulation and energy budget of offspring (Liang et al., 2002; Weston and Elgar, 2005). Thus, independent of parental expenditure, functional parental care must balance behavioral components to address the needs of the offspring while minimizing any associated costs to the offspring.

Studies of parental care are complicated by both temporal and spatial complexity. Parental care often lasts for extended periods of time (weeks to months), and changes during ontogeny may require alterations in parental behaviors (Clutton-Brock, 1991; Cezilly et al., 1994). Moreover, the parent(s) often travel considerable distances to meet various offspring needs (Stauss et al., 2005). Additionally, parental behavior can be sensitive to disturbance associated with many assessment methods beyond that of simple observations (Blokpoel, 1981; Cooke et al., 2000).

Maternal egg-brooding in pythons provides an ideal opportunity to study the importance of balancing specific parental care behaviors to regulate critical developmental parameters (e.g. O_2, CO_2, H_2O, temperature). After oviposition, pythons tightly coil around their clutch and can remain so throughout incubation (Wilson and Swan, 2003). Although body movements are subtle relative to parental behaviors of other species, python egg-brooding is a dynamic process in which varying female body postures represent individual parental behaviors that entail different costs and benefits to offspring. Some pythons, but not all species, shiver during brooding to provide heat to their clutch (Vinegar et al., 1970; Honegger, 1970). Even in python species where facultative thermogenesis does not occur, brooding probably provides thermal benefits through thermal inertia and behavioral thermoregulation (e.g. basking).

Non-thermal benefits provided by python brooding are much less understood. Recently, however, it was shown that brooding enhances egg water balance (Lourdais et al., 2007) and, more specifically, a tightly coiled posture beneficially reduces clutch water loss at the cost of reduced respiratory gas exchange between the clutch and nest environment (Z.R.S., unpublished data). Conversely, loosening of the coils and thereby partially exposing the clutch enhances clutch–nest respiratory gas exchange at the cost of increased clutch water loss (Z.R.S., unpublished data). These latter results suggest that minor brooding postural adjustments provide an adjustable diffusive barrier that allows for adequate embryonic respiratory ventilation while enhancing egg water conservation through discontinuous gas exchange. However, the

extent to which brooding creates a hypoxic clutch micro-environment and the degree to which postural adjustments alleviate the hypoxia are unknown.

Embryos could experience prolonged conditions of hypoxia since python incubation lasts 45–90 days (Wilson and Swan, 2003). Chronic hypoxia has been shown to decrease embryonic growth rate (Warburton et al., 1995; Crossley and Altimiras, 2005; Roussel, 2007), reduce hatchling mass (Crossley and Altimiras, 2005), delay the development of thermogenesis (Azzam et al., 2007), and reduce predator avoidance ability of juveniles (Roussel, 2007). Moreover, acute hypoxia can have immediate effects on embryos, including reduced metabolic rate (Kam, 1993a; Kam, 1993b) and increased cell death (Devoto, 2006). The significance of a given level of hypoxia is often determined by its relationship to an animal's critical oxygen tension (P_{O_2crit}), the minimal partial pressure of oxygen that supports normal respiratory gas exchange (Yeager and Ultsch, 1989; Kam, 1993a; Kam, 1993b). Reptile embryos are particularly tolerant of hypoxia and, thus, have low P_{O_2crit} relative to other amniotes (Kam, 1993a). As development progresses, some reptiles use several morphological strategies to promote respiratory gas exchange with their environments, such as rapid proliferation of chorioallantoic vasculature and eggshell thinning (Andrews, 2004). Despite such adaptations, reptile embryo P_{O_2crit} increases as the need for respiratory gas exchange increases during ontogeny (Kam, 1993a). The importance of maintaining proper respiratory conditions is clear, and postural adjustments by brooding pythons may vary with embryonic stage of development to meet these dynamic requirements.

We tested the hypothesis that python egg-brooding behaviors both create and modulate a potentially detrimental hypoxic clutch micro-environment. To test this hypothesis we serially monitored naturally and artificially brooded clutches of Children's pythons (*Antaresia childreni*) at their preferred incubation temperature. We predicted that: (1) tight brooding creates a hypoxic clutch micro-environment that is alleviated by female postural adjustments, (2) the level of hypoxia during tight brooding will become more severe as development progresses due to increased embryonic metabolism, and (3) the balance between tight brooding and postural adjustments will keep P_{O_2} in the clutch micro-environment above the critical oxygen tension of the developing embryos (i.e. intra-clutch $P_{O_2}>P_{O_2crit}$) throughout incubation. Support for these predictions would demonstrate the importance of balancing individual parental behaviors to meet the dynamic needs of the developing offspring using a simple, quantifiable parental care model.

MATERIALS AND METHODS
Study species and reproductive husbandry
We used a long-term captive colony of *Antaresia childreni* Gray 1842 maintained at Arizona State University Tempe, AZ, USA for this study. *A. childreni* are medium-sized (up to 1.2 m snout–vent length and 600 g body mass), non-venomous, constricting snakes of the Pythonidae family that inhabit rocky areas in northern Australia (Wilson and Swan, 2003). Husbandry and breeding of the animals followed that described previously (Lourdais et al., 2007).

A few days prior to 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. Because clutches of naturally brooding pythons have minimal (if any) contact with substrate (Wilson and Swan, 2003), females and their resultant clutches were not provided any substrate. We placed brooding chambers in an environmental chamber that had a 14 h:10 h

L:D photo regime and maintained temperature at 31.5±0.3°C, the species' preferred incubation temperature (Lourdais et al., 2008), to preclude the need for any behavioral thermoregulation by the females. Additionally, we plumbed brooding chambers with two, three-way stopcocks on opposite sides of the chamber and used a valve-controlled aeration system that combined saturated air (produced by bubbling air through a water-filled hydrating column) with dry air to deliver 20–40 ml min^{-1} of hydrated air (absolute humidity, AH=25.1–26.7 g m^{-3}; relative humidity, RH=80–85%) to each brooding chamber. We housed brooding females in these chambers during and between experimental trials to minimize disturbance and avoid clutch abandonment. At oviposition, we briefly removed each female from her clutch to determine clutch size, clutch mass, and female post-oviposition mass. Then, we randomly assigned clutches to one of two experimental groups: naturally brooding or critical oxygen tension. Females we assigned to the brooding experiment quickly recoiled around their clutches when we returned them to their respective brooding chambers. Critical oxygen tension experimental trials required the clutch to be separated from its mother for >8 h and, thus, would have led to clutch abandonment if naturally brooded. Therefore, we artificially incubated clutches used in the critical oxygen tension experiment to term in 1.4 l plastic containers with a moistened Perlite substrate at 31.5°C.

Statistical analyses
Data met the appropriate tests of statistical assumptions or were transformed as necessary, and were analyzed using JMP IN (version 5.1.2, SAS Statistical Institute, Inc., Cary, NC, USA). Significance was determined at α<0.05 for all tests. Unpaired *t*-tests were used when comparing characteristics of the brooded and critical oxygen tension clutches. To determine the effect of time or treatment, repeated-measures analysis of variance (RMANOVA) tests were used. In analyses with significant sphericity, χ^2-tests with epsilon-adjusted Greenhouse–Geisser tests were used. *Post-hoc* analyses used Bonferroni-corrected paired *t*-tests to correct for experiment-wise type I error rate. To test relationships within individuals, we used simple linear regression analysis. All values are given as mean ± s.e.m.

Brooding experiment
To assess the extent to which brooding behavior affects the P_{O_2} of the clutch micro-environment, we measured real-time brooding behavior, nest P_{O_2} (P_{O_2nest}; in kPa), and intra-clutch P_{O_2} ($P_{O_2clutch}$) of six *A. childreni* brooding units (i.e. female and associated clutch) at 31.5°C. For each brooding unit, we conducted trials three times: 9–14 days (early), 36–39 days (middle) and 43–45 days (late) post-oviposition (mean=22%, 78%, and 91% post-oviposition development). We conducted serial trials to determine the effect of embryonic metabolic rate on the clutch–nest oxygen gradient. Because reptile egg metabolic rate increases significantly but non-linearly during incubation (Ar et al., 2004), we selected trial timepoints that would provide progressive increases in metabolic rate rather than timepoints that were equally spaced temporally. To avoid disturbance, we monitored trials in darkness with an infrared camera and recorded real-time video of behaviors for later analysis of brooding behavior variables. To accommodate any initial change in female metabolic rate resulting from disturbance, we collected behavioral and $P_{O_2clutch}$ data >60 min after the beginning of the trial, and trials lasted 12 h.

Influent air of known composition and flow rate was created by combining dry, acapnic air (CDA 1112, PureGas, Broomfield, CO,

USA) with water vapor-saturated air (produced by bubbling dry air through a water-filled hydrating column) using a feedback-controlled system. Resulting influent air was humidified to 23.5 g m^{-3} absolute humidity (73% RH) and maintained at a flow rate of 560 ml min^{-1} with a mass flow controller (Unit Instruments, Inc., Yorba Linda, CA, USA) that we calibrated before the study using soap film flow meters. We analyzed a baseline sample of influent air immediately before and after brooding trials and averaged the two to determine the composition of influent air. Air exiting each chamber (effluent air) was dried with anhydrous CaSO$_4$ before flowing through an O$_2$ analyzer (FC-1B, Sable Systems, Las Vegas, NV, USA) that we calibrated prior to experimental use with dried outside air. During trials, we recorded the O$_2$ level of effluent air every minute using a 23X datalogger (Campbell Scientific Instruments, Logan, UT, USA) to determine O$_2$ consumed by the brooding unit. The difference between influent and effluent P_{O_2} was relatively small (0.034–0.12 kPa), so P_{O_2nest} was calculated as the mean of influent and effluent P_{O_2} (20.10±0.01 kPa). We measured intra-clutch P_{O_2} in real-time for the duration of trials with a fiber optic O$_2$ probe and meter (OxyMini, World Precision Instruments, Sarasota, FL, USA) and we recorded resultant data every minute with a 23X datalogger. We inserted a 4 mm diameter O$_2$ probe through a port in the floor of each brooding chamber 1.5–2 cm into the intra-clutch space. Under the conditions used, the O$_2$ probe had an accuracy of 0.19 kPa, a resolution of 0.06–0.12 kPa, a response time of 40 s, and consumed no O$_2$. In addition to the calibration procedure recommended by the manufacturer, the O$_2$ probe was calibrated immediately prior to each trial with the FC-1B O$_2$ analyzer and three gas mixtures (i.e. air stripped of CO$_2$ and H$_2$O combined with bottled N$_2$ to achieve P_{O_2} of ~19.5, 15.0 and 12.3 kPa). To determine the degree to which postural adjustments reduced intra-clutch hypoxia, we randomly chose 12 adjustments from each trial and analyzed the $P_{O_2clutch}$ immediately before (i.e. during tight posture) and 3 min after each adjustment. The lowest intra-clutch P_{O_2} recorded during each 12 h trial represented the absolute minimum $P_{O_2clutch}$.

We categorized subtly distinct postural adjustments into three simple types. (1) Non-opening adjustments (NA) were those in which female movement was noted but none of the clutch was visibly exposed. (2) Opening adjustments (OA) involved female movement with visible exposure of some of the clutch, lasted less than 5 min, and did not entail a female's snout breaching the perimeter of her outermost coil. (3) Exploratory adjustments (EA) were postural adjustments that also involved visible exposure of the clutch; however, they lasted more than 5 min and/or entailed the female's snout breaching the perimeter of her outermost coil. Exploratory adjustments were distinguished from OA because an activity bout longer than 5 min involved a significant increase in female metabolic and evaporative water loss rates. Also, during EA females often inserted their heads between their eggs and their coils suggesting a different behavioral motivation (e.g. possibly egg inspection) than that of OA.

We used unpaired and paired t-tests to determine if $P_{O_2clutch}$ during the tightly coiled brooding posture was statistically indiscernible from P_{O_2nest} or $P_{O_2clutch}$ during postural adjustments, respectively. We used RMANOVA to determine if the stage of incubation (i.e. time) had an effect on the difference between $P_{O_2clutch}$ during tight coiling and P_{O_2nest}, the difference between $P_{O_2clutch}$ during tight coiling and $P_{O_2clutch}$ during postural adjustments, or brooding behavior variables. We used simple linear regression to determine if clutch size and clutch mass were related to maternal mass, $P_{O_2clutch}$, or brooding behavior.

Critical oxygen tension experiment

We selected six artificially incubated *A. childreni* clutches for trials that measured embryonic oxygen consumption rate ($\dot{V}_{O_2}$) under varying P_{O_2} at 31.5°C during three periods: 10–16 days, 36–38 days, and 43–45 days after laying (mean=26%, 76% and 90% post-oviposition development). We used serial measurement to elucidate the effect of increasing embryonic metabolic rate on P_{O_2crit} and to compare P_{O_2crit} to $P_{O_2clutch}$ at similar stages of development. During trials, we kept the clutches in 1.2 l dual-ported airtight respirometry chambers and exposed them to five P_{O_2} levels (20.12±0.21 kPa, 17.36±0.13 kPa, 14.34±0.18 kPa, 12.31±0.13 kPa and 10.30±0.03 kPa) in an order determined by randomized draw. We supplied clutch chambers with influx air of known composition by combining and hydrating controlled flows of dry, acapnic air (CDA 1112, PureGas, Broomfield, CO, USA), and bottled N$_2$. Flows were controlled using two mass flow controllers (Unit Instruments, Inc., Yorba Linda, CA, USA) that we calibrated using soap film flow meters. We determined the P_{O_2} of the mixed gas using an O$_2$ analyzer (FoxBox-C, Sable Systems, Las Vegas, NV, USA). After estimating a 99% turnover of chamber air using flow rate and chamber volume values (Lasiewski et al., 1966), we collected initial air ($T_{initial}$) samples from each clutch chamber and stopped influx air. We then sealed the clutch chambers for a recorded duration (64±8 min), collected end air (T_{end}) samples, and passed dried $T_{initial}$ and T_{end} samples through an O$_2$ analyzer (S-3A, Applied Electrochemistry, Inc., Sunnyvale, CA, USA) that we calibrated with dried, outside air <30 min prior to analyses. We used eqns 5, 6 and 11 in Vleck (Vleck, 1987) to determine clutch $\dot{V}_{O_2}$ and divided clutch $\dot{V}_{O_2}$ by clutch size to determine mean egg $\dot{V}_{O_2}$.

We used RMANOVA to determine if ambient P_{O_2} had an effect on egg $\dot{V}_{O_2}$. To determine P_{O_2crit}, we used *post-hoc* analyses to identify the experimental P_{O_2} (i.e. 17.4 kPa, 14.3 kPa, 12.3 kPa or 10.3 kPa) at which egg $\dot{V}_{O_2}$ decreased significantly below egg $\dot{V}_{O_2}$ at normoxia (i.e. P_{O_2} of 20.1 kPa) and termed such $P_{O_2sub-norm}$. Then, we sorted data into two groups: (1) those less than or equal to $P_{O_2sub-norm}$, (2) those greater than $P_{O_2sub-norm}$. We then constructed linear trend lines for the two data sets, and the intersection of these lines represented the P_{O_2crit} (Yeager and Ultsch, 1989) (Fig. 1).
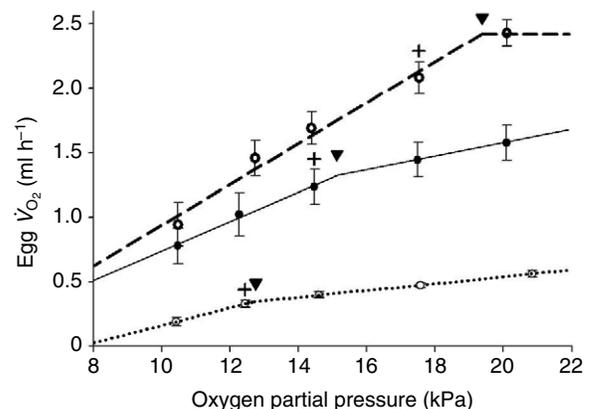

Fig. 1. Egg oxygen consumption rate ($\dot{V}_{O_2}$) from six *A. childreni* clutches under multiple oxygen tensions during early (dotted line), middle (solid line) and late (broken line) stages of development. Crosses indicate P_{O_2} sub-norm, and inverted triangles indicate critical oxygen tension for each stage of development.

Table 1. *Antaresia childreni* clutch and maternal characteristics (mean ± s.e.m.) at four stages of incubation

	Initial	Early	Middle	Late	F	P
Post-oviposition development (%)	0	22	78	91		
Brooding experiment						
No. of fertile eggs	10.5±0.7	10.2±0.7	8.5±1.2	8.5±1.2	0.067	0.81
Clutch mass (g)	138.7±9.9	130.7±10.7	102.8±14.8	103±15.8	0.044	0.85
Maternal mass (g)	306.5±15.1	305.2±14.8	303.5±14.6	303.2±14.7	0.0031	0.99
Critical oxygen tension experiment						
No. of fertile eggs	11.0±0.6	10.7±0.3	10.5±0.4	10.5±0.4	0.025	0.88
Clutch mass (g)	141.3±8.6	137.3±6.1	125.3±9.2	120.2±6.3	2.7	0.13

RESULTS

At oviposition, the differences in female mass (t=1.5, d.f.=10, P=0.17), clutch size (t=0.52, d.f.=10, P=0.61) and clutch mass (t=0.20, d.f.=10, P=0.84) between the two experimental groups were not significant. As incubation progressed, a few embryos died and females lost mass due to typical brooding-associated anorexia. However, clutch size, clutch mass and maternal mass did not significantly decrease with developmental stage (Table 1). Thus, time-dependent corrections for these variables were not necessary prior to temporal analyses.

Brooding experiment

Mean $P_{O_2clutch}$ immediately prior to postural adjustment was significantly lower than P_{O_2nest} at all stages of development (early: t=11, d.f.=5, P<0.0001; middle: t=6.4, d.f.=5, P=0.0014; late: t=7.1, d.f.=5, P=0.0009; Table 2) and lower than the overall mean $P_{O_2clutch}$ (early: t=4.8, d.f.=5, P=0.0049; middle: t=5.7, d.f.=5, P=0.0024, late: t=2.7, d.f.=5, P=0.045; Table 2) indicating that tight brooding created a significant barrier to O_2 diffusion between the clutch and nest environments. After examining the $P_{O_2clutch}$ data for entire trials, we determined that the overall mean clutch–nest P_{O_2} gradient and the clutch–nest P_{O_2} gradient during tight brooding significantly increased with stage of incubation ($F_{2,10}$=8.0, P=0.0085 and $F_{2,10}$=8.0, P=0.0075, respectively). Because female oviposition mass was not significantly correlated with clutch size (R^2=0.27, P=0.070, N=36) or clutch mass (R^2=0.36, P=0.15, N=36) in our *A. childreni* colony, we used clutch size and clutch mass as independent variables rather than clutch-to-female ratios. Clutch characteristics

also affected the mean clutch–nest P_{O_2} gradient during later trials as clutch size (middle: R^2=0.77, P=0.021; late: R^2=0.89, P=0.0050) and clutch mass (middle: R^2=0.77, P=0.021; late: R^2=0.81, P=0.014) were both negatively related to $P_{O_2clutch}$.

At all three stages of development, $P_{O_2clutch}$ was significantly lower immediately prior to postural adjustment than at 3 min after the adjustment (early: t=9.0, d.f.=5, P=0.0003; middle: t=7.3, d.f.=5, P=0.0007; late: t=4.8, d.f.=5, P=0.0049; Table 2). The difference between 'tight' $P_{O_2clutch}$ and 'adjusting' $P_{O_2clutch}$ increased with incubation stage ($F_{2,10}$=5.7, P=0.023; Table 2). Thus, postural adjustments alleviated intraclutch hypoxia, but mean $P_{O_2clutch}$ still decreased during development (Table 2).

Brooding females did not shiver at any point during the study. The frequency and duration of brooding behaviors did not increase as development progressed (Table 3) and brooding behavior was also not influenced by clutch characteristics.

Critical oxygen tension experiment

Environmental P_{O_2} affected *A. childreni* egg $\dot{V}_{O_2}$ at all three stages of development (early: $F_{4,16}$=24, P<0.0001; middle: $F_{2,8}$=7.6, P=0.013; late: $F_{4,16}$=14, P<0.0001; Fig. 1). We determined P_{O_2crit} to be 12.8 kPa, 15.1 kPa and 19.4 kPa for early, middle and late trials, respectively. Analysis of $P_{O_2clutch}$ data indicated that $P_{O_2clutch}$ was below P_{O_2crit} for a mean 0±0%, 16.5±15.6%, and 100±0% of the time for early, middle and late trials, respectively. When $P_{O_2clutch}$<P_{O_2crit}, embryos were probably unable to maintain normal metabolism under brooded conditions and, thus, were considered to be metabolically conforming.

Table 2. Intra-clutch P_{O_2} (kPa) of six brooding *A. childreni* (mean ± s.e.m.) at three stages of development

	Early	Middle	Late	F	P
Absolute minimum	17.54±0.14	15.35±0.55	15.07±0.54	12	0.0026
During tight coiling	17.78±0.15	16.08±0.50	15.76±0.49	8.4	0.0074
Overall	18.66±0.12	16.38±0.51	15.94±0.53	8.0	0.0085
During postural adjustment	18.03±0.15	16.57±0.48	16.37±0.46	7.0	0.013

Table 3. *Antaresia childreni* brooding behavior (mean ± s.e.m.) at three stages of development

	Early	Middle	Late	F	P
Time spent tightly coiled (%)	89.3±4.5	91.8±2.3	93.4±1.4	1.2	0.32
Non-opening adjustment (NA) rate (h^{-1})	3.8±0.7	3.9±0.4	4.3±0.4	0.35	0.71
NA duration (s)	15.5±2.2	17.1±4.1	10.6±1.3	1.8	0.22
Opening adjustment (OA) rate (h^{-1})	1.4±0.3	1.2±0.3	1.9±0.8	0.55	0.55
OA duration (s)	26.8±3.3	36.2±11.8	72.7±38.4	1.5	0.28
Exploratory adjustment (EA) rate (h^{-1})	1.1±0.6	0.6±0.4	0.6±0.2	0.39	0.69
EA duration (s)	298.3±64.4	747.0±337.2	208.8±28.9	1.2	0.36

NA, postural adjustments during which the clutch was not visibly exposed; OA, postural adjustments during which some part of the clutch was exposed for <5 min; EA, postural adjustments during which part of the clutch was exposed for >5 min or the female's snout breached the perimeter of her outermost coil.

DISCUSSION

We have demonstrated that python egg-brooding provides a barrier to respiratory gas diffusion between the clutch and nest environments. Moreover, our results support the hypothesis that python egg-brooding behaviors both create and relieve a potentially detrimental hypoxic developmental micro-environment. During tight coiling, the clutch–nest P_{O_2} gradient was significantly greater than the overall mean clutch–nest P_{O_2} gradient at each developmental stage, in support of our first prediction that tight brooding creates a hypoxic clutch micro-environment. Previous research has demonstrated that this particular brooding posture also minimizes clutch evaporative water loss (Lourdais et al., 2007) (Z.R.S., unpublished data). Together, these results quantitatively demonstrate that brooding behavior provides a hydric benefit at a cost to embryonic respiratory gas exchange.

Postural adjustments caused brief disruptions to the diffusive barrier created by tight coiling, which supports our first prediction (Fig. 2). Using absolute minimum $P_{O_2clutch}$ data, we estimated that without postural adjustments, the amount of time embryos were metabolically conforming would have increased from 16.5% to 50% for middle trials under brooded conditions. Also, the effectiveness of postural adjustments at reducing the clutch–nest P_{O_2} gradient created by tight coiling increased with incubation. However, in support of our second prediction that the level of hypoxia during tight brooding will become more severe as development progresses, developmental stage affected both mean overall $P_{O_2clutch}$ and $P_{O_2clutch}$ during tight coiling (Table 2). Thus, despite hypoxia-reducing postural adjustments, intra-clutch hypoxia increased with time as embryonic metabolism and respiratory gas exchange increased.

Our results demonstrate that *A. childreni* embryos experience chronic hypoxia, the effects of which have been studied in a variety of vertebrate taxa (Taylor et al., 1971; Crossley and Altimiras, 2005; Roussel, 2007). Both embryonic growth and development are adversely affected by prolonged periods of hypoxia (Crossley and Altimiras, 2005; Azzam et al., 2007; Roussel, 2007). Chronic hypoxia negatively affects hatching success (Taylor et al., 1971) as well as post-hatching fitness-related variables including predator avoidance (Roussel, 2007), sexual development (Shang et al., 2006) and development of an unfavorable sex ratio (Shang et al., 2006). Notably, some reptile eggs exhibit significant morphological responses, such as increased chorioallantoic vasculature, to

Fig. 2. Example of intra-clutch P_{O_2} ($P_{O_2clutch}$) during brooding at the late stage of development in *A. childreni*. Vertical lines denote the beginning of female postural adjustments.

prolonged hypoxia that successfully diminish the costs of their low oxygen environments (Kam, 1993b; Corona and Warburton, 2000).

We have shown that P_{O_2crit} for *A. childreni* embryos increases with development similar to other reptile embryos (Kam, 1993a). However, unlike previous research, we monitored real-time $P_{O_2clutch}$ to determine if and when the embryos' micro-environment was below P_{O_2crit}. We determined that *A. childreni* embryos are probably unable to maintain normal metabolism under brooded conditions during the final 10% of incubation (i.e. $P_{O_2clutch} < P_{O_2crit}$ for 100% of the time in late trials). Thus, similar to avian embryos (Ar et al., 1991), python embryos probably become metabolically conforming later in incubation in contradiction to our third prediction that postural adjustments will keep P_{O_2} in the clutch micro-environment above the critical oxygen tension of the developing embryos. The effect was most severe late in development presumably because females maintain relatively stable frequencies and durations of postural adjustments throughout incubation despite increased embryonic metabolism.

Like python egg-brooding postural adjustments, fin-fanning parental behavior exhibited by some fish (Hale et al., 2003; Green and McCormick, 2005; Lissaker and Kvarnemo, 2006) increases oxygen in the eggs' micro-environment. However, unlike python postural adjustments, fin-fanning is negatively correlated with dissolved oxygen levels (Hale et al., 2003; Lissaker and Kvarnemo, 2006). Therefore, an important question arises as to why brooding female pythons do not similarly increase the rate or frequency of postural adjustments to accommodate the decreasing $P_{O_2clutch}$ created by the increasing $\dot{V}_{O_2}$ of their developing embryos? Python egg-brooding behavior may be a 'hard-wired' process and, thus, brooding pythons lack the ability to use external cues for behavioral modification. This possibility is unlikely, however, as it represents a maladaptive behavior and contradicts many studies that have examined how the suite of parental care behaviors adjusts to changes in the embryonic micro-environment (Hale et al., 2003; Lissaker and Kvarnemo, 2006) and offspring development (Cezilly et al., 1995; Koskela, 2000; Green and McCormick, 2005).

Alternatively, egg-brooding female pythons may have the ability to process environmental or temporal information but choose to use a 'water first' strategy: compromising embryonic respiratory gas exchange to conserve embryonic water loss. The benefit of python egg-brooding to *A. childreni* egg water balance is dramatic and critical to embryo survival (Lourdais et al., 2007), as well as ecologically relevant because females generally oviposit during the dry season (Wilson and Swan, 2003). Although alligator embryos reared in 17% O_2 (i.e. ~16.5 kPa) exhibit significant growth retardation (Warburton et al., 1995), such effects of hypoxia on python egg hatchability or offspring quality have not been demonstrated and are in need of study.

Lastly, an external cue other than embryonic developmental stage and water balance may regulate postural adjustment by pythons. In particular, temperature is critical to embryonic development (Angilletta et al., 2000; Rodriguez-Munoz, 2001; Birchard, 2004) and was not variably manipulated in our study. Temperature is also of particular interest because increasing it directly increases both $\dot{V}_{O_2}$ and water loss in eggs. Ambient temperature is known to affect nest-attending behavior in birds (Hoset et al., 2004; Weston and Elgar, 2005) and thus may influence python egg-brooding behavior. To complement maternal brooding effects on the clutch micro-environment, perhaps *A. childreni* eggs may change during ontogeny to enhance respiratory gas exchange. Over the course of incubation, crocodilian and turtle eggs develop a highly vascularized chorioallantoic membrane and reduce shell thickness by embryonic
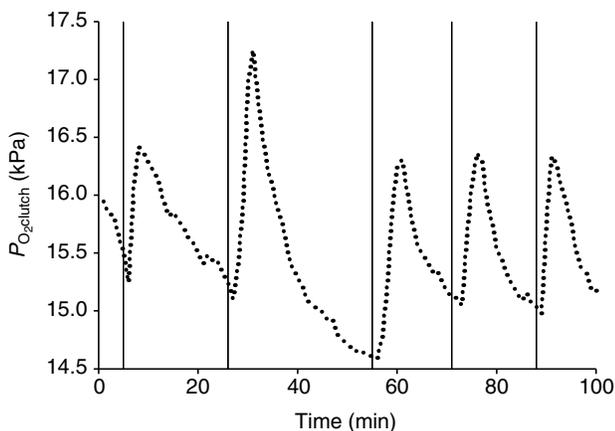
incorporation of shell-derived calcium deposits to meet increased metabolic demand for gas exchange (Andrews, 2004). However, the latter strategy increases eggshell water vapor conductance and, thus, increases the rate of egg water loss (Ar, 1991). Parchment-shelled squamate eggs are characterized by a very limited calcified layer (Thompson and Speake, 2004), and thus diffusion in late incubation may be enhanced through a reduction in the thickness of the fibrous, keratin-based layer of *A. childreni* eggshells. Regardless, our results indicate that even embryos of parchment-shelled eggs may be metabolically conforming during later stages of development.

Our results have further defined the physiological impact of python egg-brooding behaviors on developing offspring. The physiologically and behaviorally quantifiable nature of the python brooding system allowed us to perform a multi-faceted assessment of a simple parental care model. Parental care is often viewed as an adaptation that benefits offspring, however, we have shown that individual parental care behaviors can entail associated obligatory costs to developing offspring as well. Future studies should consider the presence and significance of acclimation to hypoxia in *A. childreni* embryos since other reptile embryos can acclimate to hypoxia (Kam, 1993b). Also, whether the brooding-associated constraint on python embryonic metabolic rate has deleterious effects on hatching success, morphology, performance and fecundity is unknown and warrants further study.

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