

Intra-offspring Tradeoffs of Python
Egg-brooding Behavior

by

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ABSTRACT

Though it is a widespread adaptation in humans and many other animals, parental care comes in a variety of forms and its subtle physiological costs, benefits, and tradeoffs related to offspring are often unknown. Thus, I studied the hydric, respiratory, thermal, and fitness dynamics of maternal egg-brooding behavior in Children's pythons (*Antaresia childreni*). I demonstrated that tight coiling detrimentally creates a hypoxic developmental environment that is alleviated by periodic postural adjustments. Alternatively, maternal postural adjustments detrimentally elevate rates of egg water loss relative to tight coiling. Despite ventilating postural adjustments, the developmental environment becomes increasingly hypoxic near the end of incubation, which reduces embryonic metabolism. I further demonstrated that brooding-induced hypoxia detrimentally affects offspring size, performance, locomotion, and behavior. Thus, parental care in *A. childreni* comes at a cost to offspring due to intra-offspring tradeoffs (i.e., those that reflect competing offspring needs, such as water balance and respiration). Next, I showed that, despite being unable to intrinsically produce body heat, *A. childreni* adjust egg-brooding behavior in response to shifts in nest temperature, which enhances egg temperature (e.g., reduced tight coiling during nest warming facilitated beneficial heat transfer to eggs). Last, I demonstrated that *A. childreni* adaptively adjust their egg-brooding behaviors due to an interaction between nest temperature and humidity. Specifically, females' behavioral response to nest warming was eliminated during low nest

humidity. In combination with other studies, these results show that female pythons sense environmental temperature and humidity and utilize this information at multiple time points (i.e., during gravidity [egg bearing], at oviposition [egg laying], and during egg brooding) to enhance the developmental environment of their offspring. This research demonstrates that maternal behaviors that are simple and subtle, yet easily quantifiable, can balance several critical developmental variables (i.e., thermoregulation, water balance, and respiration).

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TABLE OF CONTENTS

	Page
CHAPTER	
1 INTRODUCTION	1
Family Pythonidae	2
Thermal dynamics of python brooding	3
Tradeoffs of python egg brooding	5
2 CONCLUSION	12
What are the ecological dynamics of python parental care?	12
What are the intrinsic mechanisms underlying python egg brooding?	13
How widespread and significant is post-paritive parental care in snakes?	15
What is the role of intra-offspring tradeoffs in parental care?	16
How should parental care research be conducted in the future?	17
REFERENCES	20

APPENDIX

I. POSTURAL SHIFTS DURING EGG-BROODING AND THEIR IMPACT ON EGG WATER BALANCE IN CHILDREN’S PYTHONS (*ANTARESIA CHILDRENTI*) 32

II. ALTERNATING EGG-BROODING BEHAVIORS CREATE AND MODULATE A HYPOXIC DEVELOPMENTAL MICRO-ENVIRONMENT IN CHILDREN’S PYTHONS (*ANTARESIA CHILDRENTI*) 42

III. OBLIGATE COSTS OF PARENTAL CARE TO OFFSPRING: EGG BROODING-INDUCED HYPOXIA CREATES SMALLER, SLOWER, AND WEAKER PYTHON OFFSPRING 49

IV. EFFECT OF NEST TEMPERATURE ON EGG-BROODING DYNAMICS IN CHILDREN’S PYTHONS 58

V. PARENTAL BEHAVIOR IN PYTHONS IS RESPONSIVE TO BOTH THE HYDRIC AND THERMAL DYNAMICS OF THE NEST 64

Chapter 1

INTRODUCTION

Parental care can be defined as any non-genetic contribution of an adult that increases the fitness of its offspring. Parental care represents an adaptation of paramount importance because it is a convergent trait used by a broad range of taxa (Clutton-Brock 1991). Despite its benefits to offspring, parental care often reduces aspects of parental fitness (e.g., future reproductive efforts and longevity), which allows natural selection to mediate this parent-offspring tradeoff (Clutton-Brock 1991). Also, parental care is often inextricably involved in other evolutionary processes (Clutton-Brock 1991). For example, the degree of parental investment often dictates the degree and direction of sexual selection (e.g., large, brightly colored male birds and female fish compete for mates with high parental investment) (Trivers 1972; Clutton-Brock 1991). Further, parental control of the developmental environment may play a role in the evolution of endothermy (Farmer 2000).

While birds typically use bi-parental care, female-only parental care is the predominant mode of care in other internally fertilizing vertebrates (e.g., reptiles and mammals, Clutton-Brock 1991), including species within major taxa in which external fertilization predominates (i.e., fish and amphibians, Gross and Shine 1981), as well as terrestrial arthropods (Zeh and Smith 1985). Nest attendance is a widespread, ancestral type of post-parity (i.e., after oviposition or parturition) parental care. Among other benefits, nest-attending parents can increase their fitness by reducing

embryonic predation (frogs: Townsend 1986), improving egg water balance (skinks: Somma 1989), thermoregulating embryos (bumblebees: Heinrich 1979), promoting embryonic respiration (fish: Lissaker and Kvarnemo 2006), reducing pathogen infiltration of eggs (crickets: West and Alexander 1963), and provisioning offspring with food (birds: Clutton-Brock 1991).

Within the broad context of female-only nest attendance, pythons have proven to be a useful and relevant taxon for studying the costs, benefits, and tradeoffs of parental care. After describing general aspects of python biology, I will discuss the thermal dynamics of python egg brooding, including embryonic thermoregulation through physiological and behavioral means. I will conclude with an overview of tradeoffs associated with python egg-brooding behavior and particularly focus on intra-offspring tradeoffs (i.e., those that reflect competing offspring needs, such as water balance and respiration).

Family Pythonidae

Pythons (Order Squamata) are members of a relatively basal family of snakes comprised of 33 species (Rawlings et al. 2008), which naturally ranges from western and southern Africa, through the tropical rainforests of Southeast Asia and New Guinea and into the cooler regions of southern Australia. They typically differ from boas (Family Boidae) in reproductive mode: viviparous (live bearing) boas and oviparous (egg laying) pythons. Pythons can be terrestrial, arboreal, fossorial, or semi-aquatic, and they

range in adult size from 0.6 m to 10 m in total length (i.e., the pygmy python [*Antaresia perthensis*] and reticulated python [*Python reticulatus*], respectively) (Minton and Minton 1973; Wilson and Swan 2008).

Thermal dynamics of python egg brooding

As early as the 19th century, scientists observed female pythons enhancing embryonic thermoregulation through internal means during egg brooding (Lamarre-Picquot 1835). The selection for physiological and behavioral traits that enhance egg temperature regulation is likely related to the thermal sensitivity of python embryos. Specifically, python embryos require a relatively high, stable incubation temperature (i.e., 30-33°C) for normal development, and deviations from this narrow range result in a combination of reduced hatching success, developmental rate, growth rate, body size, escape behavior, and willingness to feed (*P. bivittatus*, Vinegar 1973; *P. natalensis*, Branch and Patterson 1975; *M. s. spilota*, Harlow and Grigg 1984; water pythons [*Liasis fuscus*], Shine et al. 1997; *A. childreni*, DeNardo unpublished).

Facultative thermogenesis by egg-brooding, and otherwise ectothermic, pythons can raise clutch temperature as much as 7°C above ambient temperature (Vinegar et al. 1970). This phenomenon is frequently mentioned in introductory science textbooks and parental care reviews (e.g., Shine 1988; Farmer 2000; Somma 2003). However, facultative thermogenesis has thus far been convincingly documented (i.e., increased

metabolic rate or the maintenance of clutch temperature with decreasing nest temperature) in only three species (Burmese python [*Python bivittatus*], Indian python [*Python molurus*], Hutchison et al. 1966; Vinegar et al. 1970; diamond python [*Morelia spilota spilota*], Harlow and Grigg 1984; Slip and Shine 1988). Facultative thermogenesis has been either disputed or convincingly disproven in at least 10 other python species (reviewed in Stahlschmidt and DeNardo 2011). Despite the thermal benefits to offspring, variation in thermogenic capacity within Pythonidae may be driven by an interaction between biogeography and morphology (reviewed in Stahlschmidt and DeNardo 2011).

Although most species of pythons are not capable of significant heat production, they may use other behaviors to enhance the thermal environment around their eggs. For example, free-ranging black-headed pythons (*Aspidites melanocephalus*) and southern African pythons (*Python natalensis*) use heat radiated from the sun or conducted from substrate to warm their clutches (Johnson et al. 1975; Alexander 2007). Johnson and colleagues (1975) also noted that egg-brooding behavior is dynamic in that females adjust their posture (i.e., increase tight coiling behavior) to prevent egg cooling. In a more controlled environment, I have documented that Children's pythons (*Antaresia childreni*) spend more time tightly coiled around their eggs during nest cooling than during nest warming (Stahlschmidt and DeNardo 2009b [Appendix IV], 2010 [Appendix V]). Further, the amount of time that females spent tightly coiled during warming significantly affected the temperature gradient

between the nest and clutch environment (Stahlschmidt and DeNardo 2009b [Appendix IV]). Thus, although most female pythons are not facultatively thermogenic, they are likely capable of assessing the temperature gradient between the nest and clutch environment and making behavioral adjustments to enhance the thermal microenvironment of their developing offspring.

Tradeoffs of python egg brooding

Like other parental care systems, python egg brooding represent a parent-offspring tradeoff, where the costs to egg-brooding females are offset by the benefits to the developing offspring. Female pythons heavily invest body resources into reproduction because they typically (1) do not eat during reproduction, (2) allocate one-third (or more) of their body mass into their eggs, and (3) exhibit a prolonged brooding period (up to 50 of the ca. 100 total days of reproductive effort; Stahlschmidt and DeNardo 2011). Further, facultative thermogenesis represents a substantial portion of female energy expenditure during egg brooding at cool temperatures (*P. bivittatus*: 92%, *M. s. spilota*: 95%) (Vinegar et al. 1970; Harlow and Grigg 1984). Although the results of one study suggest that maternal costs of egg brooding in the ball python (*Python regius*) are minimal (Aubret et al. 2005a), other studies demonstrate that egg brooding independent of thermogenesis obligates substantial maternal costs. Egg brooding necessitates lost foraging time and is generally accompanied by anorexia (*L. fuscus*: Madsen and Shine 1999; *P. regius*: Aubret et al. 2005a).

Accordingly, in female *A. childreni* under laboratory conditions, brooding obligates significant epaxial muscle atrophy and reduces contraction strength (Lourdais and DeNardo unpublished; Stahlschmidt et al. unpublished), as well as increases susceptibility to oxidative stress (Stahlschmidt et al. unpublished). Under natural conditions, the duration of egg brooding is negatively related to reproductive frequency and post-reproductive maternal body condition in free-ranging *L. fuscus* (Madsen and Shine 1999).

While likely to confer some cost to the parent, egg brooding conveys benefits to offspring beyond those associated with thermoregulation. For example, egg brooding duration is negatively related to egg predation in free-ranging *L. fuscus* (Madsen and Shine 1999). Further, python eggshells have extremely high water vapor conductance, and eggs can desiccate in conditions as moist as 75% relative humidity when not maternally brooded (Lourdais et al. 2007; Stahlschmidt et al. 2008 [Appendix I]). Although some free-ranging female pythons cease brooding a week after oviposition (*L. fuscus*: Madsen and Shine 1999), experimental removal of females from their eggs results in reduced hatching success and altered hatchling phenotypes in *P. regius* (Aubret et al. 2005b) and 0% hatching success in *A. childreni* under some conditions (Lourdais et al. 2007). Using a less extreme manipulation, Aubret et al. (2003) showed that experimentally increasing clutch size by 50%, which was still within the natural range of variation, prohibited female *P. regius* from fully encompassing their

clutch, leading to desiccation of the eggs. As a result, embryos in these “enlarged” clutches were more likely to die before hatching or hatch later.

Although less studied, intra-offspring tradeoffs (i.e., those that reflect competing offspring needs) are important in parental care systems, wherein parental behaviors meet multiple developmental needs (Lissaker and Kvarnemo 2006; Stahlschmidt and DeNardo 2009a [Appendix III]). For example, *L. fuscus* females that nest in thermally superior sites abandon their clutches shortly after oviposition ($\bar{X} = 6.5$ d), while females that nest in thermally poorer sites brood their clutches for the entire incubation period ($\bar{X} = 53.8$ d) (Madsen and Shine 1999; but unpublished data from Stahlschmidt et al. demonstrates the contrary). Although *L. fuscus* nest-site selection mitigates a parent-offspring tradeoff (i.e., thermal benefits to offspring vs. lost foraging time for females), an intra-offspring tradeoff between embryonic temperature and predation also exists. Specifically, thermally favorable nests are more prone to predation than are thermally poor nests (Madsen and Shine 1999). The resolution of intra-offspring tradeoffs is important in other parental care systems, as well. For example, the behavior of nest-attending male sand gobies (*Pomatoschistus minutus*) mediates the broadly applicable tradeoff between egg predation risk and the respiratory environment, two key developmental variables (Lissaker and Kvarnemo 2006).

Intra-offspring tradeoffs associated with python egg brooding have also been detected at a finer scale of brooding behavior, and these relationships have been the focus of my dissertation. Python egg-brooding

behavior is dynamic and entails shifts in coiling posture (reviewed in Stahlschmidt and DeNardo 2011). Broadly, dynamics of egg-brooding behavior can be divided between (1) a tight coiling state, wherein the female is still and encompassing the clutch completely or nearly so, and (2) a postural adjustment state, wherein the female makes small movements that increases exposure of the eggs to the nest environment. These distinct brooding behaviors mediate several intra-offspring tradeoffs. During brooding, females predominately adopt the tightly coiled posture, and this positioning serves as an effective barrier to the exchange of gas and heat between the clutch environment (i.e., the space within the females coils) and the nest environment (i.e., the space immediately outside of the female's coils) (Stahlschmidt and DeNardo 2008 [Appendix II], 2009b [Appendix IV]; Stahlschmidt et al. 2008 [Appendix I]).

Although it is an effective barrier to gas exchange that dramatically reduces water loss from the eggs, tight coiling also limits the transfer of oxygen and carbon dioxide (Stahlschmidt and DeNardo 2008 [Appendix II], Stahlschmidt et al. 2008 [Appendix I]). Female *A. childreni* mediate this intra-offspring tradeoff between embryonic water balance and respiration by periodically performing postural adjustments to facilitate nest-clutch gas (O₂ and H₂O vapor) exchange to benefit respiration at the cost of embryonic water balance (Stahlschmidt and DeNardo 2008 [Appendix II], 2009a [Appendix III]; Stahlschmidt et al. 2008 [Appendix I]). However, while embryonic oxygen consumption increases dramatically over the course of development, female *A. childreni* do not alter the

relative frequency or duration of their postural adjustments, and this results in late-incubation developmental hypoxia that reduces offspring size, speed, and strength (Stahlschmidt and DeNardo 2008 [Appendix II], 2009a [Appendix III]; Stahlschmidt et al. 2008 [Appendix I]). Because these experiments measured of intra-clutch oxygen partial pressure in a convective, near normoxic nest environment, python embryos in fossorial nests may experience increased hypoxia. In other taxa, chronic hypoxia during development also detrimentally affects fitness-related traits, including decreased embryonic growth rate (crocodilians: Warburton et al. 1995; fish: Roussel, 2007), reduced hatchling mass (crocodilians: Crossley and Altimiras, 2005), delayed development of thermogenesis (birds: Azzam et al., 2007), and reduced predator avoidance ability of juveniles (fish: Roussel, 2007).

While oxygen depletion does not impact the relative frequency with which female *A. childreni* utilize tight coiling and postural adjustments, females do respond to other environmental conditions. As described above, females enhance the thermal environment of the eggs by performing more postural adjustments when ambient temperature is increasing than when it is cooling (Stahlschmidt and DeNardo 2009b [Appendix IV], 2010 [Appendix V]). Natural python nests exhibit similar thermal shifts due to diel temperature cycles (Madsen and Shine 1999; Stahlschmidt et al. unpublished). However, this response to environmental temperature is dependent on the nest's hydric condition. During moderate and high nest humidity treatments (23 and 32 g·m⁻³

H₂O, respectively) that are ecologically relevant (Stahlschmidt et al. unpublished), females show the previously described reduction in tight coiling during nest warming. However, in low humidity nest environments (13 g·m⁻³ H₂O) that are ecologically relevant (Stahlschmidt et al. unpublished), brooding females maintain a high frequency of tight coiling even when the nest is warming. Hence, females “choose” embryonic thermoregulation over embryonic water balance in relatively humid nest conditions, but *vice versa* during relatively dry nest conditions (Stahlschmidt and DeNardo 2010 [Appendix V]).

In sum, python egg brooding significantly impacts several critical developmental variables including embryonic predation (Madsen and Shine 1999), water balance (Aubret et al. 2005b; Lourdais et al. 2007; Stahlschmidt et al. 2008 [Appendix I]), respiration (Stahlschmidt and DeNardo 2008 [Appendix II]), and thermoregulation (Vinegar et al. 1970; Stahlschmidt and DeNardo 2009b [Appendix IV]). Further, python egg-brooding behavior is dynamic, with females assessing environmental conditions to adjust brooding behavior on both large scales (i.e., choosing to abandon or continue brooding the clutch) and fine scales (i.e., altering the frequency and duration of postural shifts during brooding). Additionally, gravid pythons also alter thermoregulatory behavior and adaptively select nest sites to enhance the thermal and hydric environment of their developing embryos (Shine and Madsen 1996; Lourdais et al. 2008; Stahlschmidt et al. 2011). Therefore, female pythons sense environmental temperature and humidity and utilize this information at

multiple time points (i.e., during gravidity, at oviposition, and during egg brooding) to enhance the developmental environment of their offspring. Thus, Family Pythonidae is an ideal taxon for studying parental care because pythons exhibit (1) a widespread mode of parental care (i.e., female-only care), (2) parent-offspring and intra-offspring tradeoffs, (3) control over several critical developmental variables, and (4) easily quantifiable behaviors (e.g., tight coiling or duration of nest attendance).

Chapter 2

CONCLUSION

Adaptive behavioral adjustments by parents are used by a broad range of taxa, but can vary from simple shifts in body posture to complex relocation of offspring to a more favorable site (Clutton-Brock 1991; Stahlschmidt and DeNardo 2011). My graduate research on parental care in pythons has contributed to our understanding of how parents use simple body movements to balance the complex and dynamic physiological needs of their offspring (Appendices I-V). Thus, my graduate work lays the conceptual and empirical foundation to address future questions regarding the role of intra-offspring tradeoffs (i.e., those that reflect competing offspring needs), the underlying mechanisms of parental care, the plasticity of parental care behaviors, and the adaptive significance of parental care.

What are the ecological dynamics of python parental care?

Aside from one long-term study in water pythons (*Liasis fuscus*, Madsen and Shine 1999), little is known in squamates about the effects of maternal decisions on both care-giving females and offspring in an ecological context. Thus, future field-based investigation of parental care in other pythons is particularly compelling. Pythons exhibit considerable variation in body size (0.6 m to 10 m in adult body length), geographical range (0 – 37.5° latitude), habitat use (semi-aquatic to arid-dwelling), and thermogenic capacity (ectothermy to facultative endothermy) (Minton and

Minton 1973; Rawlinson 1969; Stahlschmidt and DeNardo 2011; Wilson and Swan 2008). Therefore, the costs and benefits of parental care in pythons may depend on intrinsic (e.g., constraints in fecundity due to body size) and extrinsic factors (e.g., temperature profiles of available nest sites), which vary among species.

Recently with results still pending, I investigated the role of several factors (e.g., clutch size, parasite load, body condition and size, and nest temperature and humidity) in the large-scale maternal decision-making of *L. fuscus* in a field-based collaboration with Drs. Richard Shine and Dale DeNardo. Yet, to better understand the ecological dynamics of parental care within the Pythonidae, future research should similarly investigate other python species. In particular, field-based parental care research on python species that are facultatively endothermic, invasive, or both is particularly warranted. For example, the Burmese python (*Python molurus*) is facultatively endothermic and has successfully invaded into Everglades National Park, Florida, U.S.A. (Meshaka et al. 2004). Research investigating the ecology of parental care in *P. molurus* may provide insight into broad evolutionary concepts (e.g., the evolution of endothermy) and the success of invasive species.

What are the intrinsic mechanisms underlying python egg brooding?

Although my research and that of others has demonstrated that pythons exhibit two simple yet sensitive and effective parental behaviors,

the endogenous control mechanisms underlying maternal expression of these traits are unknown. First, facultative endothermy has been demonstrated by a few python species, and muscular contractions (i.e., “shivering”) likely contribute to the generation of heat by egg-brooding females of these species. However, poor correlations between contraction frequency and both oxygen consumption and the clutch-nest temperature gradient suggest that thermogenic pythons may also incorporate non-shivering heat generating processes such as the use of uncoupling proteins (Van Mierop and Barnard 1976, 1978). Future research should investigate other mechanisms that may contribute to facultative endothermy in pythons (e.g., up-regulation of uncoupling proteins or thyroid hormones during egg brooding) in addition to the role of skeletal muscle contraction. Understanding these proximate mechanisms may elucidate how some, but not all, python species are facultatively thermogenic, which may provide insight into the role of parental care in the evolution of endothermy (see Farmer 2000).

Second, care-giving female Children’s pythons (*Antaresia childreni*) exhibit adaptive thermo- and hygrosensation, but the mechanisms underlying these parental care traits are unknown. Recent research in *Drosophila* and mammals has implicated the role of transient receptor potential channels in these sensory dynamics (Romanovsky 2007; Montell 2008). Thus, future research on thermo- and hygrosensation in python parental care could build upon the conceptual framework established by research on traditional model systems.

How widespread and significant is post-paritive parental care in snakes?

When combined with previous research, my graduate research demonstrated that parental care within Pythonidae exhibits significant variation with regard to physiology (ectothermy to facultative endothermy) and behavior on large- and fine-scales (e.g., egg-brooding duration and amount of time spent tightly coiled) (reviewed in Stahlschmidt and DeNardo 2011). Expanding detailed examinations of the variation in parental care among snakes, in general, will only increase the value of snakes as contributors to understanding the evolution of parental care. Snakes provide a diversity of parental behaviors: from shifts in thermoregulatory behavior during embryo retention to egg and neonate attendance. Yet, even in its most complex form, I have shown that snake parental care can be quite amenable to studying tradeoffs. Post-paritive (i.e., after oviposition or parturition) parental care by snakes serves to balance multiple tradeoffs, and the factors involved (e.g., temperature, water balance, respiration) are easily quantifiable. Thus, researchers should continue to examine other snake lineages to clarify the independent or interactive effects of phylogeny, morphology, physiology, and environment on parental care in snakes.

One such lineage is the pit vipers (Crotalinae), where parental attendance is nearly ubiquitous but divided between oviparous species that invest long periods of time in attending their eggs and viviparous

species that limit attendance predominantly to the relatively short period prior to post-birth ecdysis (skin shedding). These two parental attendance behaviors likely present considerably different costs to the parents and benefits to the offspring, such as enhanced thermoregulation. Regardless, it is of interest to examine whether these behaviors are similarly regulated by intrinsic or extrinsic factors (e.g., hormones or environmental temperature, respectively).

The Colubridae are a diverse family of snakes, and parental attendance either occurs throughout egg incubation, is limited to the immediate post-paritive period, or is non-existent. What factors drive the selection for these different strategies, and are the regulators of parental care in those species that provide it similar to the regulators in the lineages where parental attendance is the norm? I specifically investigated parental care tradeoffs in species whose parental behaviors have been most frequently observed but mostly unstudied (Somma 2003), including both colubrids (e.g., Taiwan beauty snake [*Orthriophis taeniurus friesi*], mud snake [*Farancia abacura*], skaapstekers [*Psammophylax* spp.], keelbacks [*Xenochrophis* spp.]) and elapids (e.g., shield-nosed cobras [*Aspidelaps* spp.], kraits [*Bungarus* spp.], cobras [*Naja* spp.], and king cobra [*Ophiophagus hannah*]).

What is the role of intra-offspring tradeoffs in parental care?

Although less studied than parent-offspring tradeoffs, intra-offspring tradeoffs (i.e., those that reflect competing offspring needs) are

important to parental care dynamics in some systems. In pythons, maternal decision-making mediates tradeoffs between embryonic predation risk and thermoregulation (Madsen and Shine 1999), and tradeoffs among embryonic thermoregulation, water balance, and respiration (reviewed in Stahlschmidt and DeNardo 2011). Further, some male fish mediate a tradeoff between embryonic predation risk and respiration during nest attendance (e.g., sand gobies [*Pomatoschistus minutus*], Lissaker and Kvarnemo 2006). Thus, the resolution of intra-offspring tradeoffs may be an important, general component of parental care evolution. Future research should investigate the role of intra-offspring tradeoffs across taxa, particularly in taxa that have been traditionally examined. For example, nestling attendance by birds can enhance offspring thermoregulation. Yet, nest-attending parents cannot forage, which comes at a cost to offspring energy balance. Therefore, birds use a suite of parental care behaviors (i.e., nest attendance and foraging) to meet the varied needs of their offspring. Researchers investigating avian parental care could explicitly examine intra-offspring tradeoffs using experimental approaches similar to those described in my dissertation, a meta-analysis approach, or both.

How should parental care research be conducted in the future?

Animal behaviorists have a long history of investigating the ultimate and proximate mechanisms of parental care (reviewed in Clutton-Brock 1991). To best understand the dynamics of parental care, I propose

research should operate on two tenets. First, researchers should explore parental care across animal taxa. Although parental care has traditionally been viewed as a primarily vertebrate trait (Clutton-Brock 1991), recent research is beginning to demonstrate that parental care may be remarkably widespread. For example, *Drosophila* is a member of an incredibly speciose family (Diptera, true flies: ca. 150,000 species; Wiegmann and Yeates 2005) relative to vertebrates (ca. 58,000 species; Baillie et al. 2004), and they exhibit adaptive oviposition-site selection (Montell 2008; Dillon et al. 2009). Further, investigation of parental care in the burying beetle (*Nicrophorus vespilloides*, Order Coleoptera: ca. 400,000 species; Hammond 1992) may provide further insight into the evolution of parent-offspring signaling (Smiseth et al. 2003, 2010), a co-adaptation traditionally examined in higher vertebrates. Among lesser-studied vertebrates, recent research on amphibians demonstrated the role of ecological factors in the evolution of bi-parental care and monogamy (Brown et al. 2010). Future research should continue to explore the costs and benefits associated with the dynamics of parental care across taxa to provide a more complete conceptual framework of the evolution of parental care.

The second tenet guiding parental care research is that researchers should devote relatively more attention to taxa that are ideal models to examine broad aspects of the evolution of parental care (e.g., universal costs and benefits). I argue that pythons meet both of the criteria of ideal parental care models: (1) tractability (easy to locate, obtain, and

manipulate), and (2) generality (exhibit a mode or type of parental care of broad significance). My research has demonstrated that egg-brooding pythons use simple behaviors to balance the complex and dynamic physiological needs of their offspring in easily controlled environments that are ecologically relevant (Appendices I-V; Stahlschmidt et al. unpublished). Further, pythons exhibit female-only parental care, which is the predominant mode of care by other internally fertilizing vertebrates (e.g., mammals and reptiles). It is also most prevalent among species within major taxa in which external fertilization predominates (e.g., fish and amphibians), as well as terrestrial arthropods (Gross and Shine 1981; Zeh and Smith 1985; Clutton-Brock 1991). Thus, research on pythons and other taxa fulfilling these criteria should be particularly encouraged in the future because it may provide considerable insight into parental care in general.

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APPENDIX I

POSTURAL SHIFTS DURING EGG-BROODING AND THEIR IMPACT
ON EGG WATER BALANCE IN CHILDREN'S PYTHONS (*ANTARESIA
CHILDRENI*)

Postural Shifts During Egg-Brooding and Their Impact on Egg Water Balance in Children's Pythons (*Antaresia childreni*)

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Abstract

Parental care typically consists of distinct behavioral components that are balanced to address the multiple needs of offspring. Female pythons exhibit post-oviposition parental care in which they coil around their parchment-shelled eggs throughout incubation (40–80 d). Subtle postural shifts during egg-brooding facilitate embryonic gas exchange but may entail hydric costs to the clutch. This study used a simple behavioral model to (1) further quantify the costs and benefits of specific parental behaviors to developing offspring and (2) determine the influence that developmental stage and relative clutch mass have on parental behavior. Although previous research has demonstrated that egg-brooding as a whole reduces clutch water loss, we hypothesized that egg-brooding female pythons specifically adopt a tightly coiled posture to conserve embryonic water, but must make postural adjustments to enhance gas exchange between the clutch and nest environments at the cost of increased clutch water loss. We measured rates of water loss in brooding Children's pythons (*Antaresia childreni*) and their respective clutches (i.e., brooding units) and monitored changes in brooding posture. We conducted serial trials to elucidate the effect of developmental stage on postural adjustments and water loss. Results demonstrated that the proportion of time females spent in a tightly coiled posture was inversely related to mean water loss from the brooding unit. Analyses indicated that slight adjustments in posture led to bursts in brooding unit water loss. Indeed, brooding unit water loss during postural adjustments was significantly higher than during tight coiling. These findings imply that python egg-brooding provides an adjustable diffusive barrier that leads to discontinuous gas exchange, which minimizes clutch water loss. Because females with larger relative clutch masses spent more time tightly coiled, egg-brooding female pythons may use a 'water first' strategy in which they intentionally conserve clutch water at the cost of reduced embryonic respiratory gas exchange.

Introduction

Parental care is an ecologically significant behavior adopted by many vertebrates, especially endothermic ones, and has thus received considerable attention

(Clutton-Brock 1991; Farmer 2000). Parental care provides offspring with many requirements for development, such as water balance, energy provisioning, and temperature regulation (Clutton-Brock 1991; Deeming, 2004). Because the needs of

offspring are numerous and complex, parental care tends to comprise a repertoire of behaviors with each specific behavior probably associated with both costs and benefits to the offspring. For example, both nest attendance and food acquisition represent parental care behaviors in many birds; however, each has its own cost and benefit to the offspring (Clutton-Brock 1991). Furthermore, the relative importance and subsequent dedication to a specific parental behavior can vary with such factors as environmental temperature (Weston & Elgar 2005), availability of food (Dewey & Kennedy 2001), and age of offspring (Clutton-Brock 1991; Cezilly et al. 1994).

While widespread among endotherms and common in fish and amphibians, parental care is limited among reptiles (Clutton-Brock 1991). Among most squamates (lizards and snakes), parental care is usually restricted to pre-birth or pre-hatching events which include but are not limited to nest-site selection, altered thermoregulation during pregnancy, and deposition of energy-dense yolk reserves that can be utilized after hatching or birth (Shine 1983; Clutton-Brock 1991; Somma 2003). One exceptional, yet understudied, case of squamate post-oviposition parental care is the egg-brooding behavior exhibited by all pythons (family Pythonidae), wherein the female coils around her clutch during incubation (Vinegar et al. 1970; Sliip & Shine 1988; Pearson et al. 2003; Fig. 1). Reptile eggs are greatly affected by changes in developmental temperature (Angilletta et al. 2000; Birchard 2004), and brooding

provides a buffer against changes in environmental temperature. In fact, some pythons use facultative thermogenesis to raise clutch temperature as much as 7°C above ambient temperature (Vinegar et al. 1970; Sliip & Shine 1988).

While the effects of temperature on development have been the best documented, other factors, such as water balance (Shine & Brown 2002) and gas exchange (Kam 1993; Warburton et al. 1995), also influence development and offspring quality. While the porosity of the nest allows for gas exchange (Walsberg 1980) during egg-brooding in birds, the tightly coiled nature of egg-brooding pythons limits gas exchange. Thus python brooding can conserve embryonic water (Lourdais et al. 2007) but limit oxygen availability to the embryos (Stahlschmidt & DeNardo 2008). However, python egg-brooding behavior is dynamic and involves two primary postures: tight coiling and postural adjustment (Stahlschmidt & DeNardo 2008). While postural adjustments serve to reduce intra-clutch hypoxia (Stahlschmidt & DeNardo 2008), they may impose a hydric cost to the parchment-shelled eggs. In this study, we document the occurrence of postural shifts by egg-brooding pythons and correlate those movements with changes in water loss from the brooding unit (i.e., female and associated clutch).

Python egg-brooding provides a simple model that can be used to quantitatively assess the implications of distinct parental care behaviors on physiologic parameters that are critical to embryonic development (e.g., temperature, CO₂, O₂, and H₂O). We used serial physiologic and behavioral observations of egg-brooding female Children's pythons (*Antaresia childreni*) to address several predictions. First, we predicted that tight coiling greatly reduces water loss rate of brooding units. Second, periodic postural adjustments facilitate rapid exchange of all gases including water vapor between the clutch and the nest environment. Support for these two predictions would provide further evidence that python egg-brooding serves as an adjustable diffusive barrier that minimizes clutch water loss while maintaining a viable clutch gaseous micro-environment. Previous work has shown that artificially increasing clutch size in ball pythons (*Python regius*) leads to decreased hatching success, presumably due to critically high water loss through the parchment-shelled eggs (Aubret et al. 2003). Thus, we predict that clutch characteristics such as size, absolute mass, and relative mass (i.e., clutch mass divided by maternal mass) are positively correlated with brooding unit water loss.



Fig. 1: A female *Antaresia childreni* brooding her clutch in the opening adjustment (PA) posture. Note that even in the PA posture, there is very close association between the female and her clutch. Photograph by D. F. DeNardo.

Methods

Study Animals and Maintenance

Antaresia childreani are medium-sized (up to 1.2 m snout-vent length, 600 g body mass), constricting snakes that inhabit rocky areas in northern Australia (Wilson & Swan 2003) and do not use facultative endothermy during brooding (DeNardo & Stahlschmidt, unpubl. data). *Antaresia childreani* nest at the end of a warm dry season (Jul.–Sep., Australian winter). Compared to the buried eggs of other squamates, maternally brooded python eggs have little, if any, contact with the substrate.

The snakes we used for this study are part of a long-term captive snake colony maintained at Arizona State University (ASU). A few days prior to oviposition, we moved gravid pythons into dual-ported, Teflon-coated, 1.9-l cylindrical brooding chambers which we then placed in an environmental chamber that had a 14:10 L:D photoperiod and maintained temperature at $30.5 \pm 0.3^\circ\text{C}$ (which approximates the species' preferred incubation temperature, Lourdais et al. 2008). Females were not provided food or drinking water from this point until the end of the incubation to the best mimic natural conditions and minimize disturbance associated with feeding the females and cleaning any excrement from the chamber. At oviposition, we briefly removed females and their clutches from their respective brooding chambers to determine clutch size, clutch mass, and female post-oviposition mass. Each female re-coiled on her clutch within minutes of returning to her brooding chamber. Chambers were opaque on the bottom and sides, but the tops were transparent to allow observation. To prevent desiccation during non-experimental periods, we supplied each brooding chamber with 20–40 ml/min of hydrated air (absolute humidity, AH = $25.1\text{--}26.7\text{ g/m}^3$; relative humidity, RH = 80–85%) using a valve-controlled aeration system that combined saturated air (bubbled through a water-filled column) with dry air. We housed animals in these chambers throughout the study to minimize disturbance.

Experimental Procedure

We put each brooding unit through a sequence of respiro-hygrometric trials at 30.5°C during two periods of post-oviposition development (i.e., day of incubation divided by total incubation duration): the first week after laying (EARLY, mean = 16% of post-oviposition) and between 38- and 40-d post-laying

(LATE, mean = 73%). This allowed us to elucidate the effect that embryonic development and, possibly, increased clutch metabolism had on postural adjustments. To avoid disturbance, we monitored trials in darkness with an infrared camera (EC-PC-Cam; Elyssa Corp., Briarcliff Manor, NY, USA) and image recording software (v2.0.13, ImageSaks, Data Crunch Technologies, Folsom, CA) in real-time. We collected brooding images and respirometric data for 12 h, starting >60 min after the beginning of the trial. We imposed this delay to account for any female-metabolic effect of disturbance associated with starting the trial. We humidified influent air to 23.5 g/m^3 absolute humidity (i.e., 73% RH at 30.5°C) by combining dry CO_2 -free air (CDA 1112; PureGas, Broomfield, CO, USA) with vapor-saturated CO_2 -free air (bubbled through a water-filled column). We made continuous, automatic adjustments to the fluxes of the respective gases via a feedback-controlled system that included an input-output datalogger (23X; Campbell Scientific Instruments, Logan, UT, USA), precision hygrometer (RH100; Sable Systems, Las Vegas, NV, USA), and mass flow controller (Unit Instruments, Inc., Yorba Linda, CA, USA). We maintained flow of the humidified air to the experimental brooding chamber at 560 ml/min with a mass flow controller. Given this flow rate and the functional brooding chamber volume (i.e., 1.9 l minus brooding unit volume), the 99% equilibration period was approx. 9 min (Lasiewski et al. 1966). We passed air exiting the brooding chamber (effluent) 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, USA) and an O_2 analyzer (FC-1B; Sable Systems). We analyzed a baseline sample immediately before and after brooding trials to determine the composition of the influent. We compared H_2O , CO_2 , and O_2 levels of influent and effluent air samples (recorded using a 21X datalogger; Campbell Scientific Instruments) to obtain an accurate, minute-to-minute assessment of each brooding unit's contribution to changes in the fractions of those three gases. For a summary of how we converted these raw data to rates of H_2O loss ($\dot{V}_{\text{H}_2\text{O}}$), and oxygen consumption (\dot{V}_{O_2}), and carbon dioxide production (\dot{V}_{CO_2}), see Equations 1–7 in Walsberg & Hoffman (2006).

To estimate total water loss and metabolic gas exchange of non-brooded clutches, we removed each clutch from its respective mother after the brooding trial. We weighed each clutch, counted viable eggs, and then placed the clutch into a 1.3-l test chamber. Then, we supplied each clutch chamber with the

same influent described above (i.e., 0% CO₂, 21% O₂, 23.5 g/m³ AH, 560 ml/min), waited for the effluent dew point to stabilize (25–60 min), and determined clutch \dot{V}_{H_2O} as above. Next, we used closed-system respirometry to determine clutch metabolic rate. We sealed the clutch chamber, passed dried air samples from the chamber ($T_{initial}$ and T_{final}) through an O₂ analyzer (S-3A; Applied Electrochemistry, Inc., Sunnyvale, CA, USA) and a CO₂ analyzer (FoxBox-C; Sable Systems), and used Equations 5, 6, and 11 in Vleck (1987) to determine \dot{V}_{O_2} and \dot{V}_{CO_2} . We then returned each clutch to its respective mother and moved them back to the temperature controlled housing chamber. We repeated the entire process 30–32 d later for LATE incubation measurements.

Analyses of Egg-Brooding

We recorded egg-brooding behavior from a viewpoint directly above the brooding female. We took digital still images every 5 s and analyzed them after all trials were completed. We determined tight brooding to be when a female was motionless and tightly coiled around her clutch to the point where none of the clutch was visible. We considered postural adjustments as individual behavioral events only if they were >30 s removed from any other postural adjustment. We categorized subtly distinct postural adjustments into three simple types: (1) non-opening adjustments (NA) were those in which motion was detected but no part of the clutch was visibly exposed, (2) opening adjustments (OA) involved visible exposure of the clutch, lasted <5 min, and did not entail a female's snout breaching the perimeter of her outermost coil, and (3) exploratory adjustments (EA) were postural adjustments that also involved visible exposure of the clutch. However, they were characterized by lasting more than 5 min or including a female's snout breaching the perimeter of her outermost coil. Also, during many EA bouts, the female inserted her head between her eggs and her coils, suggesting a different behavioral motivation (e.g., possibly egg inspection) than that of OA.

Postural adjustments were immediately followed by an increase in \dot{V}_{H_2O} by the brooding unit (Fig. 2). To calculate the mean \dot{V}_{H_2O} of the 'tight' posture for a given trial, we removed any behavior-induced peaks in \dot{V}_{H_2O} (i.e., duration of postural adjustment + 13-min equilibration period) and averaged the remaining real-time \dot{V}_{H_2O} . To calculate the \dot{V}_{H_2O} during an 'adjusting' posture, we divided the volume of H₂O lost during the postural adjustment (V_a) by

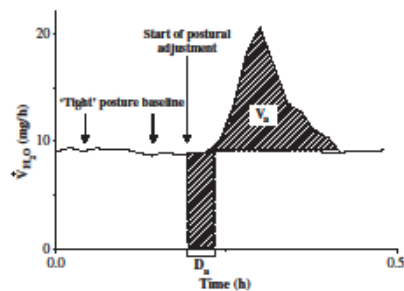


Fig. 2: Sample graph of a single adjustment-induced spike in brooding unit \dot{V}_{H_2O} . D_a (bracketed component of the x-axis) is the duration of the postural adjustment, while V_a (entire shaded area) is the volume of H₂O exchanged during the postural adjustment.

the duration of the adjustment (D_a) (Fig. 2). We calculated the mean \dot{V}_{H_2O} of 'adjusting' posture by averaging the postural adjustment rates of a given trial. Often, several postural adjustments would take place during the 13-min equilibration period; thus, we could not distinguish the physiologic impact of individual adjustments in many instances.

Statistical Tests

For statistical tests, we used the JMP IN statistical package (version 5.1.2; SAS Statistical Institute, Inc., Cary, NC, USA). In EARLY-LATE and $\dot{V}_{H_2O, tight} - \dot{V}_{H_2O, adjust}$ comparisons, we used paired t-tests and used \log_{10} or arcsine-transformations to normalize any non-normal data. To test the relationships of variables within individuals (e.g., clutch characteristics, water loss, and brooding behavior), we used simple linear regression analysis. All values are displayed as $\bar{x} \pm SE$. For negative results, we provide the results of a power analysis ($1 - \beta$).

Results

Brooding Unit Characteristics

Among EARLY ($16 \pm 1\%$ post-oviposition development) and LATE ($73 \pm 1\%$ post-oviposition development) trials, maternal mass (341.9 ± 18.9 and 340.7 ± 18.7 g, respectively) was not significantly different ($n = 7$, $t_6 = -0.21$, $p = 0.084$, $1 - \beta = 0.086$). Also, clutch size was not significantly different among EARLY and LATE trials (10.4 ± 0.5 and 9.7 ± 0.6 ,

respectively; $n = 7$, $t_6 = -2.0$, $p = 0.094$, $1 - \beta = 0.79$). However, due to a combination of removing dead eggs and mass lost as water, mean clutch mass was significantly less for the LATE trials (120.0 ± 7.4 vs. 109.8 ± 9.5 ; $n = 7$, $t_6 = -2.7$, $p = 0.035$). Hatching success was similar to that measured in previous python egg-brooding studies ($80 \pm 8\%$) (Aubret et al. 2003; Lourdais et al. 2007) and was not related to any maternal or clutch variables, as well as variables displayed in Tables 1 and 2 for either EARLY or LATE trials (R^2 : $\bar{x} \pm SE = 0.16 \pm 0.02$, range = 0.004–0.53).

Table 1: Summary of behavioral analyses for EARLY and LATE trials ($\bar{x} \pm SE$; $n = 7$)

	EARLY	LATE	t_6	p-value	$1 - \beta$
Percentage of time spent tightly coiled	94.2 \pm 1.2	94.7 \pm 0.7	0.51	0.63	0.68
Non-opening adjustment (NA) rate (NA/h)	4.3 \pm 0.5	5.0 \pm 0.5	0.47	0.38	0.57
NA duration (s)	29 \pm 3	23 \pm 2	-1.6	0.0038	
Opening adjustment (OA) rate (OA/h)	0.6 \pm 0.3	0.6 \pm 0.3	0.52	0.62	0.63
OA duration (s)	84 \pm 26	91 \pm 17	0.26	0.80	0.81
Exploratory adjustment (EA) rate (EA/h)	0.05 \pm 0.03	0.04 \pm 0.02	-0.91	0.88	0.89
EA duration (s)	440 \pm 20	373 \pm 28	-0.80	0.81	1.0

Table 2: Summary of physiologic data for EARLY and LATE trials ($\bar{x} \pm SE$; $n = 7$)

	EARLY	LATE	t_6	p-value	$1 - \beta$
Brooding unit (female coiled on eggs)					
Mass (g)	469.0 \pm 21.8	451.6 \pm 21.1	-2.8	0.033	
CO ₂ production rate (ml/h)	9.11 \pm 0.57	12.36 \pm 0.52	4.7	0.0033	
O ₂ consumption rate (ml/h)	12.28 \pm 0.72	16.34 \pm 0.69	6.8	0.0048	
Total H ₂ O loss rate (mg/h)	19.4 \pm 3.0	19.8 \pm 4.2	0.20	0.85	0.85
H ₂ O loss rate during tight coiling (mg/h)	17.5 \pm 3.0	17.9 \pm 3.9	0.27	0.79	0.79
H ₂ O loss rate during postural adjustment (mg/h)	44.6 \pm 7.7	40.2 \pm 5.4	-1.0	0.35	0.42
Clutch separated from female					
CO ₂ production rate (ml/h)	3.32 \pm 0.20	8.42 \pm 0.64	8.6	0.00040	
O ₂ consumption rate (ml/h)	4.3 \pm 0.3	11.8 \pm 1.1	8.2	0.00039	
H ₂ O loss rate (mg/h)	130.1 \pm 14.3	162.3 \pm 7.2	2.7	0.036	
Individual egg separated from female ^a					
Mass (g)	11.6 \pm 0.9	11.3 \pm 0.7	-2.0	0.097	0.11
CO ₂ production rate (ml/h)	0.32 \pm 0.02	0.87 \pm 0.05	12	<0.0001	
O ₂ consumption rate (ml/h)	0.40 \pm 0.02	1.09 \pm 0.07	11	0.00013	
H ₂ O loss rate (mg/h)	13.23 \pm 1.00	16.92 \pm 1.20	4.1	0.0090	

^aIndividual egg values were calculated by dividing clutch values by clutch size.

Egg-Brooding Behavior

Females spent, on average, approx. 94% of brooding time tightly coiled around their eggs. Among the adjustment behaviors, there was a negative relationship between the frequency of a given adjustment behavior type and the mean duration of that type of adjustment (Table 1). The mean NA frequency was relatively high, but the mean duration of an NA was short (Table 1). Conversely, the mean EA frequency was roughly 100-fold less (Table 1). In fact, fewer than 43% of the 12-h trials contained an EA event. However, when EA occurred, the duration was long relative to the other types of postural adjustments (Table 1). Relative to NA and EA behaviors, OAs were intermediate in both frequency and duration.

Interestingly, mean NA duration was the only behavioral variable that was statistically different between EARLY and LATE trials (Table 1). Because the percentage of time spent adjusting included the total frequency and duration of postural adjustments, it was the most inclusive behavioral metric, and it was not significantly different between EARLY and LATE trials (EARLY: $5.8 \pm 1.2\%$; LATE: $5.3 \pm 0.7\%$, $t_6 = -0.51$, $p = 0.63$, $1 - \beta = 0.62$).

Brooding Unit and Clutch Physiology

Predictably, mean clutch \dot{V}_{CO_2} and \dot{V}_{O_2} increased approx. 2.5-fold between trial periods (Table 2). We found a significant, negative relationship between mean brooding unit \dot{V}_{H_2O} and the percentage of time

spent in the tightly coiled posture (Fig. 3). Also, $\dot{V}_{H_2O, tight}$ was significantly lower than $\dot{V}_{H_2O, adjust}$ for brooding units during both EARLY and LATE trials (Fig. 4). The mean non-brooded clutch \dot{V}_{H_2O} was higher in LATE trials compared to EARLY trials (Table 2). However, brooding unit \dot{V}_{H_2O} did not significantly differ between EARLY and LATE trials, indicating that egg-brooding prevents the enhanced water loss from the eggs as development progresses.

Aside from a negative relationship with clutch size in LATE trials [$R^2 = 0.63$, $F(1,5) = 8.2$, $n = 7$, $p = 0.035$], mean brooding unit \dot{V}_{H_2O} was not significantly related to our other predicted independent

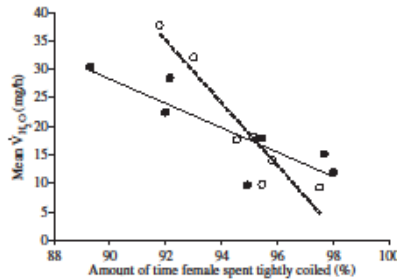


Fig. 3: Relationship between the percentage of time spent in the tightly coiled posture and mean brooding unit \dot{V}_{H_2O} for EARLY (solid line; $y = -2.1x + 220.7$, $R^2 = 0.73$, $F(1,5) = 13$, $n = 7$, $p = 0.014$) and LATE (dashed line; $y = -5.5x + 541.1$, $R^2 = 0.89$, $F(1,5) = 40$, $n = 7$, $p = 0.0014$) trials.

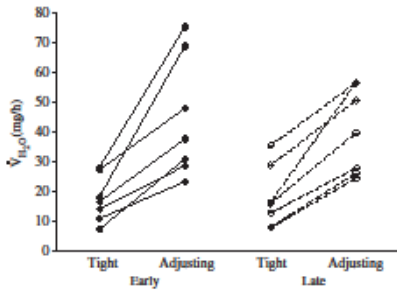


Fig. 4: Comparison between brooding unit $\dot{V}_{H_2O, tight}$ and $\dot{V}_{H_2O, adjust}$ for EARLY and LATE trials (EARLY, $t_6 = 4.7$, $p = 0.0034$; LATE, $t_6 = 7.1$, $p = 0.0004$, $n = 7$). Each line connects the tight and adjusted values of an individual female.

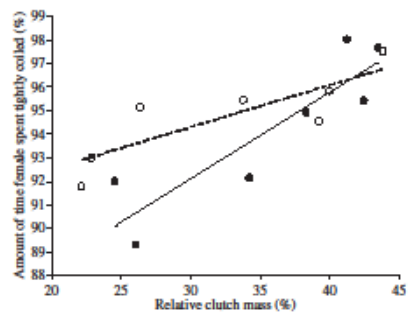


Fig. 5: Relationship between relative clutch mass (clutch mass divided by maternal mass) and the percentage of time spent in the tightly coiled posture for EARLY (solid line; $y = 36.6x + 81.1$, $R^2 = 0.79$, $F(1,5) = 19$, $n = 7$, $p = 0.0074$) and LATE (dashed line; $y = 32.5x + 86.8$, $R^2 = 0.71$, $F(1,5) = 12$, $n = 7$, $p = 0.018$) trials.

variables, i.e., clutch mass and relative clutch mass (R^2 -range: 0.032–0.52; p -range: 0.066–0.71). However, relative clutch mass was related to the amount of time females spent tightly coiled in both EARLY and LATE trials (Fig. 5).

Discussion

Our data further demonstrate that python egg-brooding is dynamic, combining a predominant tight-coiling behavior with varying types of postural adjustments that differ in frequency and duration. Several results from our study suggest that through these postural adjustments egg-brooding provides an adjustable diffusive barrier between the clutch and the nest environment that limits the exchange of water vapor. Postural adjustments were tied to bursts in \dot{V}_{H_2O} (Fig. 2), as well as \dot{V}_{CO_2} and \dot{V}_{O_2} by the brooding unit.

Despite 30+ d between our EARLY and LATE trials, brooding female *A. childreni* did not show a reduction in body mass, and this result is consistent with published data for other egg-brooding pythons (Aubret et al. 2005b; Stahlschmidt & DeNardo 2008). These results suggest that python egg-brooding, unlike parental care behaviors of many other vertebrate taxa, entails very little direct energy costs. However, this does not imply that egg-brooding comes without costs to females. Madsen & Shine (1999) found that female water pythons (*Liasis fuscus*) that brooded their eggs for the entire incubation were in poorer

body condition and were less likely to reproduce the following year compared to females that abandoned their clutches early in incubation. Thus, egg-brooding may impose an energy cost through the loss of foraging time.

Our data support those of Lourdaïs et al. (2007) in demonstrating that brooding, as a whole, drastically reduces clutch water loss in *A. childreni*. However, our finding that $\dot{V}_{\text{H}_2\text{O,spht}}$ is approx. two- to threefold $< \dot{V}_{\text{H}_2\text{O,adj}}$ for brooding units demonstrates that tight brooding specifically is important to egg water balance. In further support, the proportion of time spent tightly coiled was inversely correlated to mean brooding unit $\dot{V}_{\text{H}_2\text{O}}$ (Fig. 3).

In contradiction of our third prediction and previous research in *P. regius* (Aubret et al. 2003), *A. childreni* brooding unit $\dot{V}_{\text{H}_2\text{O}}$ was not affected by female and clutch characteristics. The discrepancy between our results and those of Aubret et al. (2003) may result from the fact that clutch size variation was a natural non-manipulated variable in our study, while Aubret et al. (2003) manipulated clutch size by adding or subtracting eggs. Nonetheless, prior to manipulation, *P. regius* in Aubret et al. (2003) had considerably larger relative clutch masses than *A. childreni* [*P. regius*: $51.7 \pm 2.9\%$ at oviposition; *A. childreni*: $35.8 \pm 2.9\%$ (EARLY) and $32.8 \pm 3.3\%$ (LATE)], possibly challenging the female's ability to fully encompass her clutch. Regardless, it is noteworthy that *A. childreni* females with larger relative clutch masses adopt a tightly coiled posture more often than females with smaller relative clutch masses, possibly representing some compensatory behavior to limit water loss from the larger clutch (Fig. 5).

The idea that egg-brooding may provide an adjustable diffusive barrier is not novel. Several researchers have suggested that some birds are capable of behaviorally modifying nest humidity to benefit their eggs (Rahn et al. 1977; Morgan et al. 1978). For example, water to humidify the nest environment could be provided by the nest-attending parent via respiratory and cutaneous water loss rather than by diffusion from the eggs. Most avian eggs lose 20–22% of their mass through water loss during the incubation period (Deeming & Ferguson 1991), so an increase in nest humidity by the parent could significantly benefit offspring (Rahn et al. 1977; Morgan et al. 1978). However, Walsberg (1980, 1983) rejected this notion that bird parental behavior significantly regulates egg dehydration due in large part to the combination of low resistance and high convection characterized by most avian nests. Additionally, prairie skinks, *Eumeces septentrionalis* and *P. regius* exhibit nest-attending

behaviors that significantly reduce egg mortality due to desiccation (Somma & Fawcett 1989; Aubret et al. 2005a). However, in none of these studies have the effects on water loss rates of specific behavioral components of parental care been quantified nor has the potential impact of the behaviors on embryonic CO_2 and O_2 exchange been explored. Thus, to the best of our knowledge, python egg-brooding represents the only established example of dynamic parental care by vertebrates that significantly mediates the fluxes of water vapor, CO_2 , and O_2 in the developmental environment.

When compared to other parchment-shelled squamate eggs, *A. childreni* eggshells have very high conductance to water vapor (Lourdaïs & DeNardo, unpubl. data) that appears to be related to maternal egg-brooding (but which led to the other is unclear). High eggshell conductance to gases (e.g., O_2 , CO_2 , and H_2O vapor) or 'thinning' probably occurs concurrently with longer durations of egg retention and prior to the evolution of viviparity (live-bearing) (Packard et al. 1977; Shine 1985). Interestingly, *A. childreni* lay eggs which contain embryos at stage 35 of 40 (Lourdaïs et al. 2008) whereas other squamates lay eggs near stage 30 and rarely beyond stages 32–33 (Shine 1983; Andrews & Mathies 2000). Thus, maternal egg-brooding coupled with eggshell thinning may serve as either a pre-adaptation to viviparity or an alternative reproductive strategy to viviparity.

The discontinuous gas exchange exhibited by some insects is somewhat analogous to the described python egg-brooding system. Some insects utilize a discontinuous gas exchange cycle (DGC) and periodically close their spiracles, which is a highly effective barrier to diffusion between their endotracheal systems and the external environment (Lighton 1996; Quinlan & Gibbs 2006). DGC have been confirmed in 59 species, primarily within the Hymenoptera and Coleoptera (Quinlan & Gibbs 2006) and may have evolved as a means to reduce evaporative water loss (Buck 1958; Chown & Davis 2003). When spiracles are closed (C), spiracular water loss is reduced and internal CO_2 concentration is elevated. This 'C' phase of the typical DGC is comparable to the tight postural phase exhibited by brooding pythons, although pythons in the tightly coiled posture do not completely prevent gas exchange between the clutch and nest environments. In DGC, spiracles are periodically fluttered or opened (O) to allow respiratory gas exchange at the expense of a short-term increase in water loss. The 'O' phase in DGC is similar to the postural adjustment phase of python egg-brooding (Fig. 2).

Researchers have generally concluded that spiracle control during DGC is regulated by CO₂ detection (Kaars 1981; Harrison et al. 1995). Similar to other species, python egg \dot{V}_{CO_2} increases substantially throughout the incubation period. However, as in Stahlschmidt & DeNardo (2008), nearly all metrics of python brooding behavior showed no change between EARLY and LATE trials. Thus, it appears that the frequency and duration of postural adjustment respond to neither changes in embryonic metabolic rate nor developmental stage.

Because water loss rate across the eggshell increases as incubation proceeds, an increase in the frequency or duration of postural adjustments in response to increased embryonic metabolism might lead to fatal egg desiccation. Indeed, the absence of maternal egg-brooding is lethal to *A. childressi* embryos in conditions of relative humidity as high as 75–100% (Lourdals et al. 2007). Thus, egg-brooding female pythons may use a ‘water first’ strategy in which they intentionally conserve clutch water at the cost of hypoxic clutch conditions that can lead to reduced embryonic metabolism (Stahlschmidt & DeNardo 2008). Larger clutches have higher \dot{V}_{O_2} and \dot{V}_{CO_2} than smaller clutches and likely would require more frequent postural adjustments to maintain normal respiratory gas exchange. Instead, females with larger relative clutch masses more frequently adopted a tightly coiled posture (Fig. 5) in support of the ‘water first’ strategy.

To summarize, we have demonstrated that python egg brooding consists of distinct behaviors that significantly affect egg water balance. Combined with previous data, it appears that maternal egg-brooding behavior exhibited by pythons serves as an adjustable diffusive barrier that balances the hydric and respiratory needs of developing embryos. Furthermore, 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. Although parental care provides an overall benefit to offspring, we have demonstrated that individual parental care behaviors entail both costs and benefits for developing offspring. Specifically, the tightly coiled python brooding posture enhances egg water balance at a cost to embryonic metabolism (Stahlschmidt & DeNardo 2008), whereas postural adjustments enhance clutch respiratory gas exchange at a cost to egg water balance. Future studies should elucidate the long-term consequences of tight coiling on offspring (e.g., reduced hatching success or offspring quality) and potential environmental cues used by the female to regulate parental care behavior.

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APPENDIX II

ALTERNATING EGG-BROODING BEHAVIORS CREATE AND
MODULATE A HYPOXIC DEVELOPMENTAL MICRO-ENVIRONMENT
IN CHILDREN'S PYTHONS (*ANTARESIA CHILDRENI*)

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

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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 Akimiras, 2005; Roussel, 2007), reduce hatchling mass (Crossley and Akimiras, 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 ($P_{O_{2,crit}}$), the minimal partial pressure of oxygen that supports normal respiratory gas exchange (Yeager and Utsch, 1989; Kam, 1993a; Kam, 1993b). Reptile embryos are particularly tolerant of hypoxia and, thus, have low $P_{O_{2,crit}}$ 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 $P_{O_{2,crit}}$ 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 $P_{O_2} > P_{O_{2,crit}}$) 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 $31.5 \pm 0.3^\circ\text{C}$, 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 $20\text{--}40\text{ ml min}^{-1}$ of hydrated air (absolute humidity, $AH=25.1\text{--}26.7\text{ g m}^{-3}$; relative humidity, $RH=80\text{--}85\%$) 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 $>8\text{ h}$ 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 $\alpha < 0.05$ 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 \pm 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} ($P_{O_{2,nest}}$; in kPa), and intra-clutch P_{O_2} ($P_{O_{2,clutch}}$) 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 $P_{O_{2,clutch}}$ data $>60\text{ min}$ after the beginning of the trial, and trials lasted 12 h.

Influent air of known composition and flow rate was created by combining dry, a capnic 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 CaSO₄ before flowing through an O₂ 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 O₂ level of effluent air every minute using a 23X datalogger (Campbell Scientific Instruments, Logan, UT, USA) to determine O₂ consumed by the brooding unit. The difference between influent and effluent P_{O₂} was relatively small (0.034–0.12 kPa), so P_{O₂} was calculated as the mean of influent and effluent P_{O₂} (20.10±0.01 kPa). We measured intra-clutch P_{O₂} in real-time for the duration of trials with a fiber optic O₂ 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 O₂ 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 O₂ 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 O₂. In addition to the calibration procedure recommended by the manufacturer, the O₂ probe was calibrated immediately prior to each trial with the FC-1B O₂ analyzer and three gas mixtures (i.e. air stripped of CO₂ and H₂O combined with bottled N₂ to achieve P_{O₂} 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_{O₂} immediately before (i.e. during tight posture) and 3 min after each adjustment. The lowest intra-clutch P_{O₂} recorded during each 12 h trial represented the absolute minimum P_{O₂}.

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_{O₂} during the tightly coiled brooding posture was statistically indiscernible from P_{O₂} or P_{O₂} 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_{O₂} during tight coiling and P_{O₂}, the difference between P_{O₂} during tight coiling and P_{O₂} 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_{O₂}, or brooding behavior.

Critical oxygen tension experiment

We selected six artificially incubated *A. childreani* clutches for trials that measured embryonic oxygen consumption rate (\dot{V}_{O_2}) under varying P_{O₂} 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_{O₂} and to compare P_{O₂} to P_{O₂} 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_{O₂} 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 N₂. 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_{O₂} of the mixed gas using an O₂ 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 O₂ 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_{O₂} had an effect on egg \dot{V}_{O_2} . To determine P_{O₂}, we used *post-hoc* analyses to identify the experimental P_{O₂} (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_{O₂} of 20.1 kPa) and termed such P_{O₂} normoxia. Then, we sorted data into two groups: (1) those less than or equal to P_{O₂} normoxia, (2) those greater than P_{O₂} normoxia. We then constructed linear trend lines for the two data sets, and the intersection of these lines represented the P_{O₂} (Yeager and Ultsch, 1989) (Fig. 1).

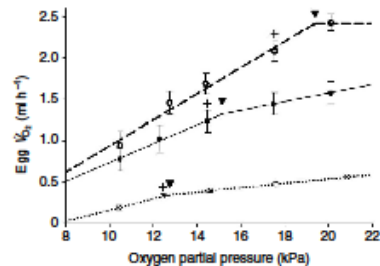


Fig. 1. Egg oxygen consumption rate (\dot{V}_{O_2}) from six *A. childreani* clutches under multiple oxygen tensions during early (dotted line), middle (solid line) and late (broken line) stages of development. Crosses indicate P_{O₂} sub-normoxia, and inverted triangles indicate critical oxygen tension for each stage of development.

Table 1. *Antaresia childreni* clutch and maternal characteristics (mean \pm 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 \pm 0.7	10.2 \pm 0.7	8.5 \pm 1.2	8.5 \pm 1.2	0.067	0.81
Clutch mass (g)	138.7 \pm 9.9	130.7 \pm 10.7	102.8 \pm 14.8	102 \pm 15.8	0.044	0.85
Maternal mass (g)	306.5 \pm 15.1	305.2 \pm 14.8	303.5 \pm 14.6	303.2 \pm 14.7	0.0031	0.99
Critical oxygen tension experiment						
No. of fertile eggs	11.0 \pm 0.6	10.7 \pm 0.3	10.5 \pm 0.4	10.5 \pm 0.4	0.025	0.88
Clutch mass (g)	141.3 \pm 8.6	137.3 \pm 6.1	125.3 \pm 9.2	120.2 \pm 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 $P_{O_2,clutch}$ immediately prior to postural adjustment was significantly lower than $P_{O_2,nest}$ at all stages of development (early: $t=1.1$, 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 $P_{O_2,clutch}$ (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 $P_{O_2,clutch}$ 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 $P_{O_2,clutch}$.

At all three stages of development, $P_{O_2,clutch}$ 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' $P_{O_2,clutch}$ and 'adjusting' $P_{O_2,clutch}$ increased with incubation stage ($F_{2,10}=5.7$, $P=0.023$; Table 2). Thus, postural adjustments alleviated intracutch hypoxia, but mean $P_{O_2,clutch}$ 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 $P_{O_2,crit}$ to be 12.8 kPa, 15.1 kPa and 19.4 kPa for early, middle and late trials, respectively. Analysis of $P_{O_2,clutch}$ data indicated that $P_{O_2,clutch}$ was below $P_{O_2,crit}$ for a mean 0:10%, 16.5 \pm 15.6%, and 100:10% of the time for early, middle and late trials, respectively. When $P_{O_2,clutch}<P_{O_2,crit}$, 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 \pm s.e.m.) at three stages of development

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

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

	Early	Middle	Late	F	P
Time spent tightly coiled (%)	89.3 \pm 4.5	91.8 \pm 2.3	93.4 \pm 1.4	1.2	0.32
Non-opening adjustment (NA) rate (h ⁻¹)	3.8 \pm 0.7	3.9 \pm 0.4	4.2 \pm 0.4	0.35	0.71
NA duration (s)	15.5 \pm 2.2	17.1 \pm 4.1	10.6 \pm 1.3	1.8	0.22
Opening adjustment (OA) rate (h ⁻¹)	1.4 \pm 0.3	1.2 \pm 0.3	1.9 \pm 0.8	0.55	0.55
OA duration (s)	26.8 \pm 3.3	36.2 \pm 11.8	72.7 \pm 38.4	1.5	0.28
Exploratory adjustment (EA) rate (h ⁻¹)	1.1 \pm 0.6	0.6 \pm 0.4	0.6 \pm 0.2	0.39	0.69
EA duration (s)	298.3 \pm 64.4	747.0 \pm 337.2	208.8 \pm 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 $P_{O_{2,clutch}}$ 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 $P_{O_{2,clutch}}$ and $P_{O_{2,clutch}}$ 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 Altamirano, 2005; Roussel, 2007). Both embryonic growth and development are adversely affected by prolonged periods of hypoxia (Crossley and Altamirano, 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

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

We have shown that $P_{O_{2,emb}}$ for *A. childreni* embryos increases with development similar to other reptile embryos (Kam, 1993a). However, unlike previous research, we monitored real-time $P_{O_{2,clutch}}$ to determine if and when the embryos' micro-environment was below $P_{O_{2,emb}}$. We determined that *A. childreni* embryos are probably unable to maintain normal metabolism under brooded conditions during the final 10% of incubation (i.e. $P_{O_{2,clutch}} < P_{O_{2,emb}}$ 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 $P_{O_{2,clutch}}$ 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

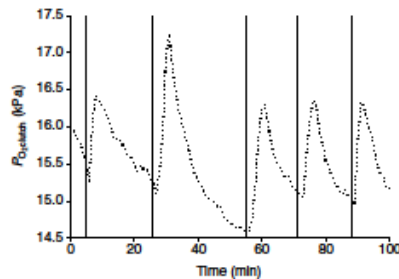


Fig. 2. Example of intra-clutch P_{O_2} ($P_{O_{2,clutch}}$) during brooding at the late stage of development in *A. childreni*. Vertical lines denote the beginning of female postural adjustments.

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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APPENDIX III

OBLIGATE COSTS OF PARENTAL CARE TO OFFSPRING: EGG
BROODING-INDUCED HYPOXIA CREATES SMALLER, SLOWER, AND
WEAKER PYTHON OFFSPRING

Obligate costs of parental care to offspring: egg brooding-induced hypoxia creates smaller, slower and weaker python offspring

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Python egg brooding typifies parental care because it consists of multiple behaviours that provide for multiple developmental needs. For example, tightly coiling around the eggs benefits embryonic water balance, but periodic female postural adjustments improve embryonic gas exchange. Regardless of these postural adjustments, egg brooding creates a hypoxic intra-clutch environment that constrains embryonic metabolism. We further examined this novel and useful parental care model to determine: (1) any fitness-related costs of egg brooding to offspring; (2) whether any long-term costs are alleviated by postural adjustments. We artificially incubated Children's python (*Antaresia childreni*) clutches and modulated oxygen partial pressure (PO_2) to create three treatments: normoxic (NRM, 20.3 kPa O_2), brooding [BRD, PO_2 profile typical of clutch PO_2 ($PO_{2\text{clutch}}$) in maternally brooded clutches, 15.8–19.3 kPa O_2] and low (LOW, predicted PO_2 profile of maternally brooded $PO_{2\text{clutch}}$ if females did not make postural adjustments, 14.4–18.6 kPa O_2). Using various metrics from ~12 days pre-hatching to 14 days post-hatching, we demonstrated that NRM offspring were larger, faster and stronger than BRD offspring. As only hatchling heart mass differed between BRD and LOW treatments (LOW > BRD), postural adjustments may not alleviate hypoxia-related costs to embryos. Our results demonstrate that parental care may represent a compromise between competing developmental needs and thus entails obligate costs to the offspring. © 2009 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2009, 98, 414–421.

ADDITIONAL KEYWORDS: adaptive significance – Children's python (*Antaresia childreni*) – offspring quality – respiration – snake – trade-off.

INTRODUCTION

Parental care is a widespread behaviour adopted by many vertebrates, especially endothermic ones, and has consequently received considerable attention (Clutton-Brock, 1991; Farmer, 2000; Huang, 2006). Parental care meets many critical needs of offspring during development, such as water balance, energy acquisition and temperature regulation (Clutton-Brock, 1991; Deeming, 2004). Given these multiple needs, trade-offs between competing developmental variables often exist. Although likely important to understanding the adaptive significance of parental behavioural suites, such intra-offspring trade-offs are less studied than parent-offspring trade-offs.

One exceptional, yet understudied, case of post-oviposition parental care among squamates (lizards and snakes) is egg-brooding behaviour exhibited by all pythons (family Pythonidae), in which the female coils around her clutch during incubation (Vinegar, Hutchison & Dowling, 1970; Slip & Shine, 1988; Pearson, Shine & Williams, 2003). Python egg 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 & DeNardo, 2008; Stahlschmidt, Hoffman & DeNardo, 2008). 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 & DeNardo, 2008; Stahlschmidt *et al.*, 2008). Although maternal egg brooding is critically

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important to offspring survival and phenotype in biologically relevant nest environments (Aubret *et al.*, 2005; Lourdaï, Hoffman & DeNardo, 2007), it creates a hypoxic developmental environment that reduces embryonic metabolism (Stahlschmidt & DeNardo, 2008). However, the long-term, fitness-related costs to embryos associated with hypoxia induced by python egg brooding are unknown.

Chronic hypoxia often elicits compensatory changes in developmental morphology, such as cardiac hypertrophy (*Alligator mississippiensis*, Daudin 1802, Crossley & Altamiras, 2005). Despite such plasticity, hypoxia can decrease embryonic growth rate (*Alligator mississippiensis*, Warburton, Hastings & Wang, 1995; Crossley & Altamiras, 2005; *Salmo trutta*, Linnaeus 1758, Roussel, 2007), reduce hatchling mass (Crossley & Altamiras, 2005), delay the development of thermogenesis (*Gallus gallus*, Linnaeus 1758, Azzam, Szizuy & Mortola, 2007), reduce predator avoidance ability of juveniles (Roussel, 2007) and reduce sexual development (*Danio rerio*, Hamilton-Buchanan, 1822, Shang, Yu & Wu, 2006). Although insightful, these studies reared embryos in arbitrary oxygen concentrations, such as 14% (Shang *et al.*, 2006), 26% (Roussel, 2007), 48% (Crossley & Altamiras, 2005), 72% (Crossley & Altamiras, 2005; Azzam *et al.*, 2007) or 81% (Warburton *et al.*, 1995) of normoxia. However, in at least one study, experimenters determined the detrimental effects of developmental hypoxia (e.g. reduced embryonic growth rate and survival) on embryos by creating an artificial oxygen tension (PO_2) environment that models *in vivo* developmental PO_2 (Parker & Andrews, 2006). Similarly, in this study we used previously quantified intra-clutch oxygen tensions ($PO_{2\text{clutch}}$) in Children's pythons (*Antaresia childreni*, Gray 1842, Stahlschmidt & DeNardo, 2008) to determine the hypoxia-related costs of egg brooding to *A. childreni* embryos.

Experimental manipulation of relevant proximate factors (e.g. developmental PO_2) may elucidate the adaptive significance of traits (e.g. parental behaviour). Thus, we incubated *A. childreni* clutches in different PO_2 treatments and hypothesized that python egg brooding represents an adaptive trade-off between embryonic water balance and respiration that is enhanced by modulating behaviours (i.e. tight coiling and postural adjustment). Specifically, we predicted that (1) hypoxia induced by python egg brooding entails fitness-related costs to offspring (e.g. reduced hatchling mass and strength) and (2) these costs are mitigated, at least partially, by hypoxia-alleviating postural adjustments. Support for these predictions would demonstrate the importance of balancing individual parental behaviours to meet the dynamic needs of the developing offspring using a simple, quantifiable parental care model that modu-

lates several critical developmental variables, such as embryonic temperature (Z. Stahlschmidt & D. DeNardo, unpubl. data), respiration (Stahlschmidt & DeNardo, 2008) and water balance (Lourdaï *et al.*, 2007; Stahlschmidt *et al.*, 2008).

MATERIAL AND METHODS

STUDY SPECIES AND CLUTCH INCUBATION

We used a long-term captive colony of *A. childreni* maintained at Arizona State University (ASU) for this study. *Antaresia childreni* are relatively small (< 1 m) pythons from northern Australia (Wilson and Swan, 2003) and they do not employ facultative thermogenesis while egg brooding (Stahlschmidt & DeNardo, in review). Husbandry and breeding of the animals followed that described previously (Lourdaï *et al.*, 2007). In 2007, we allowed seven females to brood their respective clutches for the entire incubation period in 1.9-L egg-brooding chambers supplied with hydrated air and maintained at optimal incubation temperature (31.5 °C, Lourdaï *et al.*, 2007) (herein, MAT clutches). In 2008, 22 females similarly incubated their clutches for the first 6 days as they underwent other non-invasive, behavioural studies. During these 6 days, clutches were not oxygen-limited because early stage *A. childreni* embryos have < 20% of the metabolic requirements for oxygen consumption of late-stage embryos and exhibit a very low critical oxygen tension (i.e. the minimal partial pressure of oxygen that supports normal respiratory gas exchange) (Stahlschmidt & DeNardo, 2008).

On incubation day 7 (ID7), we separated the 2008 clutches from their respective females, as well as counted the eggs and weighed the clutches. We then assigned each clutch to one of the following treatments: normoxic (NRM, $N = 7$), brooding hypoxic (BRD, $N = 8$) and low hypoxic (LOW, $N = 7$). To reduce desiccation in a viable micro-environment, we partially buried clutches in 60–80 mL of moistened Perlite and suspended them 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 (no. 3770; Forma Scientific Inc., Marietta, OH, USA) maintained at 31.5 ± 0.2 °C for the remaining incubation duration. Clutches in the NRM treatment received compressed room air. Clutches assigned to the BRD treatment received a mixture of compressed N_2 and air to mimic the PO_2 profile of brooded *A. childreni* eggs (Fig. 1; Stahlschmidt & DeNardo, 2008). During tight coiling, $PO_{2\text{clutch}}$ decreases in a logarithmic fashion until a postural adjustment causes it to rapidly increase up to 1 kPa O_2 (Stahlschmidt & DeNardo, 2008). We used data from our previous study (Stahlschmidt &

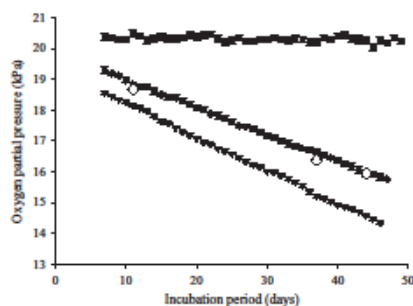


Figure 1. Oxygen regimes for normoxic (NRM, ■), brooded (BRD, ●) and low (LOW, ▼) *Antaresia childreni* clutches during incubation. The BRD oxygen regime was modelled after maternally egg-brooded intra-clutch oxygen partial pressures (◇) from Stahlschmidt & DeNardo (2008). Displayed symbols are $\bar{x} \pm \text{SEM}$ error bars.

DeNardo, 2008) to estimate the points at which PO_{clutch} would reach a diffusion equilibrium in the absence of postural adjustments throughout incubation. Thus, we created the LOW treatment using a mixture of N_2 and air to approximate what we predicted a clutch would experience if females did not use postural adjustments during egg brooding (Fig. 1).

We hydrated influent air supplies to near vapour saturation by bubbling them through heated columns of distilled water and maintained the flow rate to each clutch at 50 mL min^{-1} using adjustable flow meters (FL-344; Omega Instruments, Stamford, CT, USA) that were calibrated under experimental conditions. Daily, we determined the % O_2 of each treatment's supply air by running gas samples through an O_2 analyser (S-3A; Applied Electrochemistry Inc., Sunnyvale, CA, USA). We converted % O_2 to PO_2 (% $O_2/100$ barometric pressure) using barometric pressure recorded daily from a gas analyser (FC-1B; Sable Systems, Las Vegas, NV, USA). Oxygen tension for the BRD and LOW treatments supplies was changed daily by adjusting the flow meters for the N_2 and air (Fig. 1). For simplicity, this experimental design did not incorporate the O_2 fluctuations experienced by maternally brooded clutches during postural adjustments. However, as postural adjustments generally entail fluctuations of $< 0.5 \text{ kPa } O_2$ (Stahlschmidt & DeNardo, 2008), we feel that neglecting these periodic fluctuations in our experimental design did not affect our results.

At hatching, we recorded fitness-related measurements of offspring, transferred them into individual

500-mL plastic containers maintained at 31.5°C and provided them drinking water *ad libitum* for the duration of the study.

OFFSPRING FITNESS-RELATED MEASUREMENTS

In 2008, we made the following morphometric and performance measurements during incubation (duration: $\bar{X} \pm \text{SEM} = 47.1 \pm 0.6$ days) and after hatching:

Incubation day 7 (ID7). Clutch mass was measured ($\pm 0.01 \text{ g}$, V-200; Acculab, Edgewood, NY, USA) and mean egg mass was determined (clutch mass/clutch size). At random, we terminally sampled one egg per clutch to determine embryo mass and sampled egg mass ($\pm 0.0001 \text{ g}$, AG245; Mettler-Toledo, Columbus, OH, USA). These measurements validated the similarity of the clutches prior to treatment.

Incubation day 35 (ID35). As on ID7, we randomly sampled one egg from each clutch to determine egg mass and embryo mass. After killing the embryo (via decapitation), we isolated, removed and weighed the heart within 2 min of removal from each sampled embryo to determine heart wet mass ($\pm 0.0001 \text{ g}$).

Day of hatching (H). We determined mean incubation duration, hatching success [(number of hatchlings/(clutch size at ID7-2)) $\times 100$], hatching duration (incubation duration of last hatchling – incubation duration of first hatchling) and average hatchling body mass at hatching. We determined snout-vent length (SVL) by temporarily immobilizing each hatchling between -5 cm of open-cell foam and a -3-mm sheet of transparent acrylic, tracing its outline with a dry-erase marker and using a cloth tape ($\pm 0.5 \text{ mm}$) to measure the tracing. We measured head length (i.e. the distance from the snout to the posterior process of the quadrate bone) using digital calipers ($\pm 0.0005 \text{ mm}$, FB-2400; Frei and Borel, Oakland, CA, USA). We also determined body condition for each hatchling in two ways: (1) residuals from a SVL vs. body mass regression line (derived from the best fit of pooled data) and (2) using analysis of covariance (ANCOVA) to determine the effect of treatment on hatchling body mass after controlling for SVL. We used a digital scanner (Scanjet 3670; Hewlett-Packard, Palo Alto, CA, USA) to capture images of the ventral scale patterns of each hatchling that we later analysed for abnormalities (e.g. significant scale asymmetry or extra scales). Lastly, as on ID35, we determined heart wet mass for a single hatchling killed from each clutch.

Also at H and 1 days post-H, we agitated each hatchling with a padded copper tube for 1 min at room temperature ($25.9 \pm 1.0^\circ\text{C}$). We counted the strikes for each 1-min session and used the higher count to determine maximum strike frequency (a metric of defensive behaviour) for each snake. After

each strike session, we transferred hatchlings to an environmental chamber maintained at 31.5 °C. After acclimation, we forced hatchlings to undergo three sequential swimming trials in 3 m × 2 cm troughs maintained at 31.5 °C. We used the fastest of the six trials (i.e. three sequential trials for two consecutive days) to determine maximum swimming speed (an estimate of predation avoidance).

In 2007, we measured incubation duration, hatching success, hatchling body condition and maximum strike frequency data for the MAT clutches. We compared the MAT, BRD and NRM clutch data to validate that the BRD treatment adequately mimicked the naturally brooded developmental environment.

Fourteen days post-hatching (14DPH). As at H, we determined average hatchling body mass, SVL, body condition, heart wet mass, relative wet mass of heart and head length. We compared H and 14DPH measurements to determine SVL growth rate ((14DPH SVL - H SVL)/14 days), body mass loss rate ((H mass - 14DPH body mass)/14 days) and head length growth rate ((14DPH head length - H head length)/14 days) for each hatchling. Daily, we checked all hatchlings in individual 500-mL containers for sheds to determine the duration to first shed for each hatchling.

After morphometric processing and at room temperature (25.9 ± 1.0 °C), we attached one end of a string to the vent of each hatchling using medical tape and the other end of the string to a force transducer (FT-100; iWorx Systems Inc., Dover, NH, USA) that was calibrated daily using an interfacing software (LabScribe; iWorx Systems Inc.). Then, we pulled each hatchling taut, held their heads and allowed them to contract for 1 min. We recorded measurements using the LabScribe software and conducted a second trial immediately after the first. For each hatchling, we determined maximum contraction strength (an estimate of prey acquisition ability and predation avoidance) to be the greatest contraction elicited during either trial. We used the trial with the higher rate of contractions to determine contraction rate for each hatchling.

STATISTICAL ANALYSES

We analysed data using JMP IN (version 5.1.2; SAS Statistical Institute, Inc., Cary, NC, USA) and determined experiment-wide significance at $\alpha < 0.05$ for all tests. To determine any significant differences among treatments, we used single-factor analysis of variance (ANOVA) tests on parametric data. To determine the effect of time within treatment groups (e.g. wet mass of heart), we used repeated measures ANOVA (rmANOVA) tests on parametric data with non-significant sphericity. To determine the treatment

effect between specific predicted comparisons (e.g. NRM vs. BRD and BRD vs. LOW), we used sequential Bonferroni-corrected two-sample Student's *t*-tests on parametric data and similarly corrected Mann-Whitney *U*-tests on non-parametric data (Holm, 1979). As some variables varied significantly among treatment groups (e.g. hatchling body mass) and likely contributed to other offspring variables (e.g. hatchling contractile strength), we used ANCOVA tests on parametric data to further determine the treatment effect between specific predicted comparisons. ANCOVA tests are more appropriate than comparing 'relative variables' (e.g. relative contractile strength = contractile strength/body mass) as most variables exhibit allometric, not isometric, relationships with one another (Packard & Boardman, 1988). We performed power analyses on non-significant parametric data and displayed all results as $\bar{X} \pm \text{SEM}$.

RESULTS

At the onset of the experiment (i.e. ID7), we detected no significant differences among treatments for clutch size ($\bar{X} = 8.5 \pm 0.4$, $P = 0.47$), clutch mass ($\bar{X} = 99.6 \pm 3.7$ g, $P = 0.06$), mean egg mass ($\bar{X} = 11.7 \pm 0.4$ g, $P = 0.19$), sampled egg mass ($\bar{X} = 11.8 \pm 0.6$, $P = 0.18$) and embryo mass ($\bar{X} = 0.669 \pm 0.044$, $P = 0.20$). Thus, treatment-dependent corrections for subsequent dependent variables (e.g. ANCOVA tests with egg mass as a covariate) were not necessary.

At ID35, significant differences in heart mass were detected between NRM and BRD clutches, indicating that hypoxia associated with egg brooding creates cardiac hypertrophy in late-stage python embryos (Table 1). By H (i.e. -12 days later), NRM and BRD offspring exhibited significantly different body mass, heart mass, head size, locomotor ability and defensive behaviour (Table 1). Significant differences in morphometric (e.g. mass and body condition) and performance (i.e. swimming speed) measurements were again detected at 14DPH (i.e. mass and contractile strength) (Table 1). Throughout the experiment, the only difference between BRD and LOW clutches was in the wet mass of embryo hearts on the day of hatch (Table 1). Upon further comparison of NRM and BRD clutches, we found non-significant effects of treatment on hatchling head length at hatching and body mass 14 days post-hatch after controlling for the effect of body mass at hatching (Table 1). Thus, hypoxia-induced differences in hatchling mass had significant carryover effects on other morphometric variables.

In addition to variables described in Table 1, differences existed among groups for incubation duration ($F_{2,12} = 4.5$, $P = 0.03$, NRM: 48.7 ± 0.6 days, BRD:

Table 1. Summary of all fitness-related offspring metrics in comparisons of *Araucaria chilensis* clutches in treatments of normoxia (NRM; N = 7) and hypoxia equivalent to maternal egg-brooding (BRD; N = 8), as well as comparisons between clutches in treatments of BRD and estimated hypoxia that would result if egg brooding occurred without postnatal adjustments (LOW; N = 7) at incubation day 35, day of hatch and 14 days post-hatching. NRM, BRD and LOW values are displayed as $\bar{X} \pm \text{SEM}$

	NRM			BRD			LOW			NRM vs. BRD			BRD vs. LOW				
										t-test			ANCOVA				
	\bar{X}	SEM		\bar{X}	SEM		\bar{X}	SEM		t	P	1- β	P	1- β	P		
Day 35 (DD35)																	
Egg mass (g)	13.0 ± 0.9			11.6 ± 0.5			11.9 ± 0.9			0.23	0.53	-	-	0.78	0.86	-	
Embryo mass (g)	4.551 ± 0.410			4.826 ± 0.209			4.264 ± 0.267			0.6	0.65	-	-	0.19	0.52	-	
Heart mass (mg)	22.2 ± 2.0			38.6 ± 0.9			38.1 ± 3.8			< 0.0001	-	-	-	0.296*	-	-	
Day of hatch (H)																	
Incubation duration (day)	48.7 ± 0.6			46.8 ± 0.8			45.9 ± 0.6			0.075	0.52	0.13†	0.32†	0.37	> 0.99	0.27†	0.19†
Hatching duration (day)	1.9 ± 0.3			1.8 ± 0.3			1.1 ± 0.3			0.23	0.52	0.61†	0.077†	0.11	0.67	0.062†	0.39†
Hatching success (%)	87.1 ± 3.7			80.0 ± 10.1			96.7 ± 3.1			0.39	0.56	0.64†	0.073†	0.36	0.88	0.43†	0.056†
Hatching mass (g)	8.55 ± 0.37			7.43 ± 0.35			6.92 ± 0.43			0.047	0.56	0.041†	-	0.36	0.88	0.43†	0.056†
Wet mass of heart (mg)	24.2 ± 2.0			25.7 ± 2.7			34.8 ± 2.1			0.66	0.81	0.005†	-	0.02	0.51	0.042†	-
Snout-vent length (SVL, mm)	221.7 ± 2.9			217.5 ± 9.5			210.8 ± 4.7			0.38	0.55	0.28†	0.19†	0.26	0.51	0.52†	0.066†
Body condition (residual g body)	0.44 ± 0.57†			-0.28 ± 0.15			-0.15 ± 0.19			0.04	-	-	-	0.61	0.66	-	-
Head length (mm)	14.14 ± 0.15			13.60 ± 0.10			13.52 ± 0.09			0.01	-	0.063†	0.47†	0.59	0.81	0.92†	0.051†
Maximum atrial frequency (per min)	15.1 ± 1.8			8.4 ± 2.2			7.7 ± 2.2			0.068	-	0.034†	-	0.82	0.82	0.53†	0.091†
Maximum swimming speed (mls)	0.14 ± 0.03			0.11 ± 0.01			0.13 ± 0.01			0.016	-	0.037†	-	0.16	0.15	0.23†	0.21†
14 day post-hatch (14DPH)																	
Hatching mass (g)	7.99 ± 0.33			6.82 ± 0.33			6.48 ± 0.36			0.027	-	0.18†	0.26†	0.5	0.59	0.29†	0.18†
Wet mass of heart (mg)	23.3 ± 1.0			25.6 ± 2.1			24.4 ± 3.3			0.36	0.54	0.081†	0.49†	0.75	0.76	0.51†	0.10†
SVL (mm)	237.2 ± 3.4			227.2 ± 6.4			225.6 ± 3.6			0.2	0.52	0.83†	0.51†	0.83*	0.63	-	-
Body condition (residual g body)	0.48 ± 0.17			-0.11 ± 0.36			-0.36 ± 0.38			0.19	0.53	-	-	0.56	0.63	-	-
Hatching SVL growth rate (mm day ⁻¹)	1.106 ± 0.159			0.889 ± 0.422			1.054 ± 0.105			0.4	0.56	0.62†	0.076†	0.82*	0.82*	-	-
Hatching mass loss rate (g day ⁻¹)	0.040 ± 0.006			0.044 ± 0.005			0.032 ± 0.007			0.61	0.73	0.34†	0.15†	0.15†	0.49	0.30†	0.17†
Head length (mm)	14.68 ± 0.11			13.91 ± 0.15			14.12 ± 0.08			0.0014	-	0.016†	0.73†	0.24	0.24	-	-
Head length growth rate (mm day ⁻¹)	0.028 ± 0.004			0.022 ± 0.010			0.043 ± 0.005			0.17*	-	-	-	0.11	0.56	0.19†	0.25†
Maximum contraction strength (N)	1.46 ± 0.10			0.82 ± 0.09			0.99 ± 0.12			0.00002	-	0.0000†	-	0.37	0.62	0.19†	0.23†
Contraction rate (min ⁻¹)	8.0 ± 0.5			9.4 ± 0.4			10.9 ± 0.9			0.76	0.79	0.86†	0.058†	0.15	0.49	0.17†	0.26†
Ventral scale abnormality (% of hatchlings)	19.0 ± 5.1			6.3 ± 6.3			6.9 ± 5.6			0.14	0.516	0.19†	0.25†	0.94	0.94	0.90†	0.051†
Duration to first shed (day)	11.2 ± 0.9			9.9 ± 0.6			9.9 ± 0.5			0.28	0.52	0.31†	0.17†	0.5	0.59	0.50†	0.097†

*Mann-Whitney U-test results.
 †ANCOVA covariate body mass at H.
 ‡ANCOVA covariate snout-vent length at H.
 §ANCOVA covariate snout-vent length at 14DPH.
 ¶ANCOVA covariate body mass at 14DPH.
 † Not applicable.

46.8 ± 0.8 day. LOW: 45.9 ± 0.6 day) and 14DPH body condition ($F_{2,12} = 4.5$, $P = 0.03$, NRM: $0.034 \pm 0.001 \text{ g mm}^{-3}$, BRD: $0.030 \pm 0.001 \text{ g mm}^{-3}$, LOW: $0.029 \pm 0.001 \text{ g mm}^{-3}$). See Supplementary Table 1 in Supporting Information for complete results of NRM, BRD and LOW comparisons from ID35 to 14DPH.

Within treatments, wet heart mass of BRD and LOW clutches decreased significantly among ID35, H and 14DPH time points (BRD: $F_{2,4} = 11$, $P = 0.0067$; LOW: $F_{2,4} = 4.1$, $P = 0.04$).

At H, there were no differences between BRD clutches and MAT clutches for incubation duration (BRD: 46.8 ± 0.8 day, MAT: 48.6 ± 0.7 day, $t_{12} = -1.8$, $P = 0.10$), hatching success (BRD: $82 \pm 4\%$, MAT: $87 \pm 4\%$, $t_{12} = -0.46$, $P = 0.65$), body condition (BRD: $0.034 \pm 0.001 \text{ g mm}^{-3}$, MAT: $0.037 \pm 0.001 \text{ g mm}^{-3}$, $t_{12} = -1.6$, $P = 0.13$) and strike frequency (BRD: 8.4 ± 2.2 , MAT: 5.9 ± 2.0 , $t_{12} = 0.83$, $P = 0.42$), suggesting that our BRD treatment adequately mimicked the maternally brooded developmental environment. Further, variables significantly different between NRM and BRD clutches (Table 1) were similarly different between NRM and MAT clutches (body condition: $t_{12} = 2.2$, $P = 0.049$; strike frequency: $t_{12} = 3.4$, $P = 0.0053$), which suggests that the NRM treatment did not mimic the maternally brooded environment.

DISCUSSION

In support of our first prediction, we demonstrated that hypoxia associated with python egg brooding entails significant negative effects to offspring despite compensatory alterations in developmental morphology (i.e. cardiac hypertrophy). Hatchlings incubated in hypoxic conditions relevant to egg brooding were smaller, slower and weaker than hatchlings incubated under normoxic conditions (Table 1). We further predicted that periodic postural adjustments during egg brooding serve to minimize hypoxia-related costs to embryos. However, our second prediction was generally not supported by our results as only a difference in the wet mass of hatchling hearts existed between BRD and LOW treatment clutches. Thus, our results do not fully support an adaptive behaviour hypothesis regarding the use of multiple behaviours (i.e. tight coiling and postural adjustments) to balance embryonic water balance and respiration in egg brooding *A. childreni*.

The detrimental effects of hypoxia on embryonic development have been widely documented among oviparous taxa (birds: Azzam *et al.*, 2007; crocodilians: Warburton *et al.* 1995; fish: Roussel, 2007; flies: Frazier, Woods & Harrison, 2001). 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 & Warburton, 2000) or, like *A. childreni*, the ability to pump blood to the periphery (Crossley & Altimiras, 2005). Notably, in addition to differences in *A. childreni* heart mass among PO_2 treatments, there was also an effect of time on heart mass within BRD and LOW treatment groups. That is, when reared in biologically relevant levels of PO_2 , embryos had relatively larger hearts when in the egg than after hatching despite significant body growth. These results suggest that the oxygen diffusion capability of an eggshell with very high gas conductance (Thompson & Speake, 2004) forces morphological compensation by the embryos. Bird embryos are similarly oxygen-limited during incubation (Ar, Girard & Rodeau, 1991); thus, amniotic development across many taxa may obligate respiration-related costs or, minimally, morphological compensation that is outweighed by benefits to egg water balance.

Regardless of morphological compensation in *A. childreni* embryos, PO_2 levels typical of brooding reduced embryonic metabolism (Stahlschmidt & DeNardo, 2008) and altered offspring traits. Although many quantifiable metrics are termed 'fitness-related variables', empirically verifying the link between phenotype, particularly hatchling phenotype, and fitness is often logistically difficult (Shine, 2004). While we did not validate this link to *A. childreni* fitness, we demonstrated significant effects on an array of metrics that are known to be fitness-related in other species – hatchling body mass: turtles (Janzen, Tucker & Paukstis, 2000; Paitz *et al.*, 2007), snakes (Brown & Shine, 2005); locomotion: turtles (Delmas *et al.*, 2007) lizards (Husak, Fox & Lovern, 2006); predator defence behaviour: spiders (Person, Walker & Rypstra, 2002); gape size: larval salamanders (Kohmatsu, Nakano & Yamamura, 2001). In water pythons (*Liasis fuscus*, Peters 1873), a short incubation duration has a strongly positive association with yearling survivorship in the field (Madsen & Shine, 1999) and hatchling size in the laboratory (Shine *et al.*, 1997). However, our results indicate that under hypoxic conditions python embryos hatch earlier but are less fit than those incubated in normoxia, which supports a variation of Shine's 'safe harbour' hypothesis (Shine, 1978). Accordingly, incubation duration is dictated by the relative costs of embryonic and juvenile developmental stages where adverse incubation conditions expedite hatching.

Notably, we found no difference in offspring fitness between our BRD and LOW treatment groups, which is counter to our adaptive behaviour hypothesis. This result raises the question: If postural adjustments during egg brooding are detrimental to embryonic water balance and do not significantly reduce the respiration-related costs of egg brooding, why do egg-brooding female pythons periodically adjust their

posture? Explanations may be biological, logistical or both. First, postural adjustments may be hard-wired or solely as a result of female restlessness. However, this possibility is refuted by the fact that female *A. childreni* alter their egg-brooding behaviour to significantly enhance clutch temperature (Z. Stahlschmidt & D. DeNardo, unpubl. data).

A more convincing explanation for the lack of a difference in fitness between BRD and LOW treatment offspring is a logistical one – our modelled PO_2 incubation regime for LOW clutches may have been too conservative. Our PO_2 profile for the BRD treatment was based on an extrapolation of empirical data (Fig. 1) and the effectiveness of this approach was validated by the lack of differences between MAT and BRD clutches for fitness-related variables. However, for the LOW treatment, we had to estimate the PO_2 profile for a fictitious situation (constant tight coiling throughout incubation). As it is difficult or impossible to quantitatively estimate the cumulative effect of postural adjustments, it is highly possible that continuous coiling would lead to far greater oxygen suppression than that which we provided in our LOW treatment. Conversely, our modelled LOW treatment may have been realistic, whereby python egg-brooding behavioural adjustments balance thermal and hydric, but likely not respiratory, developmental needs.

Our experimental design focused on the impact of hypoxia, but, in doing so, the impact of developmental hypercapnia remains unknown. Brooding-induced elevations in P_{CO_2} (i.e. partial pressure of CO_2) may play a role in the adaptive significance of egg-brooding postural adjustments. Studies examining the effect(s) of hypercapnia on amniotic development are scarce. However, in early stage chicks, developmental hypercapnia often entails minimal costs or confers benefits, such as increased hatching success (Willemsen *et al.*, 2008) or increased embryonic growth rate (Bruggeman *et al.*, 2007).

Because of its physiologically and behaviourally quantifiable nature, python egg brooding continues to serve as a useful model for the study of intra-offspring and parent-offspring trade-offs associated with parental care. Current and previous studies have evaluated proximate factors, such as developmental temperature (Z. Stahlschmidt & D. DeNardo, unpubl. data), water balance (Lourdais *et al.*, 2007; Stahlschmidt *et al.*, 2008) and hypoxia (Stahlschmidt & DeNardo, 2008; Table 1), to help elucidate the adaptive significance of python egg brooding. Results demonstrate that the hydric and thermal benefits of egg brooding to developing offspring are provided at a respiratory cost that has long-term consequences to the offspring. Future studies to better understand the significance of python parental care should examine

the extent to which environmental conditions and behavioural responses can diminish costs.

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APPENDIX IV
EFFECT OF NEST TEMPERATURE ON EGG-BROODING DYNAMICS IN
CHILDREN'S PYTHONS



Effect of nest temperature on egg-brooding dynamics in Children's pythons

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ABSTRACT

Parental care meets several critical needs of developing offspring. In particular, egg brooding in pythons is an exceptional model for examining environmental influences on specific parental behaviors because brooding behaviors are dynamic yet simple and modulate embryonic temperature, respiration, and water balance. We used captive Children's pythons (*Antaresia childreni*) to assess their facultative endothermic capability, the influence on egg-brooding behaviors of the gradient between the nest temperature (T_{nest}) and clutch temperature (T_{clutch}), and the effect of these behaviors on the developmental micro-environment. We monitored maternal egg-brooding behavior, rates of brooding unit (i.e., female and associated clutch) respiratory gas exchange, T_{nest} , T_{clutch} , and intra-clutch oxygen tension (PO_2 clutch) during acute changes among four incubation temperature conditions: constant preferred temperature (31.5 °C); 'cooling' ($T_{nest} < T_{clutch}$); constant cool temperature (25.6 °C); and 'warming' ($T_{nest} > T_{clutch}$). We demonstrated that *A. childreni* are not facultatively endothermic because brooding unit temperature coefficient (Q_{10}) for V_{O_2} and V_{CO_2} was similar to other ectothermic boid snakes (1.9–5.7) and T_{clutch} conformed to T_{nest} at the constant, cool temperature treatment. Females coiled tightly around eggs more often during cooling than during warming. Further, the amount of time that females spent tightly coiled during warming significantly affected the $T_{nest} - T_{clutch}$ gradient. Together these results indicate that, although female *A. childreni* are not facultatively endothermic, they are capable of assessing the $T_{nest} - T_{clutch}$ gradient and making behavioral adjustments to enhance the thermal micro-environment of their developing offspring.

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1. Introduction

Parental care is a widespread behavior adopted by many vertebrates, especially endothermic species, and has thus received considerable attention [12]. Parental care meets many critical needs of the offspring during development, such as water balance, energy, and temperature regulation [13]. Therefore, parental care usually consists of a suite of distinct parental behaviors that can vary in relative importance with environmental factors like temperature [4], availability of food [5], season [6], and hydric conditions [7].

Although common in fishes and amphibians, parental care is relatively limited among reptiles [1,8]. One exceptional, yet understudied, case of post-oviposition parental care among squamates (lizards and snakes) is egg-brooding behavior exhibited by all python species (family Pythonidae), in which the female coils around her clutch during incubation [9–11]. Python egg brooding is a dynamic process, wherein the brooding female spends most of the time tightly coiled around her eggs, but periodically loosens her coils [12]. This balance between tight coiling and postural adjustments during brooding limits embryonic water loss while allowing sufficient respiratory gas exchange between the clutch and nest environment [12,13]. However, despite a more than 5-fold increase in respiratory gas exchange requirements by developing

embryos during incubation, female Children's pythons (*Antaresia childreni*) do not increase the frequency or duration of postural adjustments with incubation stage [12,13]. This lack of an adjustment to the changing metabolic needs of the embryos suggests that parental behaviors of female pythons are fixed or that behavior is dependent on competing critical incubation variables (e.g., respiration and water balance).

In some pythons (e.g., *Python molurus* and *Morone spilota*), females are facultatively endothermic during egg brooding and this enhanced metabolic capability provides considerable thermal benefits to the developing embryos [9,14]. However, not all pythons are facultatively endothermic, and the extent to which egg brooding alone provides a thermal benefit through insulation or thermal inertia has not been quantified. Additionally, nest temperature can influence a female python's decision whether or not to brood her eggs for the full incubation period [15]. Although nest-attending bumble bees (*Bombus bifarius nearcticus*) [16] and hooded plovers (*Thinornis rubricollis*) [4] modulate their parental behaviors in response to nest temperature (T_{nest}), it is not known if T_{nest} alters the frequency or duration of postural adjustments of egg-brooding pythons.

The relationship between temperature and egg-brooding behavior is of great interest because reptile eggs are greatly affected by changes in developmental temperature [17,18]. For example, water python (*Liasis fuscus*) eggs incubated in ecologically relevant diel temperature cycles (i.e., 27.1°–32.9 °C and 24.3°–32.9 °C) exhibit reduced developmental,

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morphological, and locomotor performance metrics relative to those reared in warm, constant thermal regimes (i.e., 32.0 °C) [19]. Thus, our study aimed to (1) confirm that our study species, *A. childreni*, is non-endothermic, (2) identify behavioral or physiological responses elicited by changes in the thermal environment of the nest, and (3) determine the extent to which egg brooding influences clutch temperature during shifts in nest temperature. We simultaneously monitored maternal behavior, rates of brooding unit (i.e., female and associated clutch) respiratory gas exchange, nest temperature (T_{nest}), clutch temperature (T_{clutch}), and intra-clutch oxygen tension (PO_2 clutch) during four incubation temperature conditions: constant preferred temperature (31.5 ± 0.1 °C) [20]; ‘cooling’ ($T_{nest} < T_{clutch}$); constant cool temperature (25.6 ± 0.1 °C); and ‘warming’ ($T_{nest} > T_{clutch}$) (Fig. 1).

In this study, we tested a hypothesis based on embryonic thermoregulation to explain female brooding behavior. We propose that egg-brooding pythons will alter their posturing patterns to enhance the thermal environment of their developing embryos. We predict that female pythons will be more tightly coiled around their eggs during the cooling phase to reduce heat loss from the clutch, and less tightly coiled during the warming phase to facilitate embryonic heat gain from the warming nest environment. Because there will be no heat loss or gain from the clutch when nest temperature is constant (assuming *A. childreni* are not facultatively endothermic), we predict that behavior variables would not significantly differ between the two constant temperature conditions despite their nearly 6 °C difference. Alternatively, our null hypothesis is that temperature, differential or absolute, does not influence egg-brooding behavior. Support for null hypothesis would imply that python egg-brooding behavior patterns are fixed and the female is incapable of sensing or responding to changes in environmental conditions. Results can clarify the adaptive significance of python egg brooding, a parental care model that can elucidate the proximate factors involved in more complicated nest-attending parental care systems (e.g., rodents, birds, crocodylians, teleost fish, and social insects).

2. Materials and methods

2.1. Study species and reproductive husbandry

We used a long-term captive colony of *A. childreni* maintained at Arizona State University (ASU) for this study. *A. childreni* are non-venomous, constricting snakes that inhabit rocky areas in northern

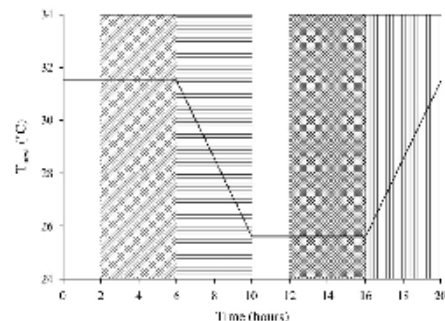


Fig. 1. Nine *A. childreni* brooding units were monitored during a sequence of four experimental temperature conditions: constant preferred incubation temperature (diagonal shading); cooling (horizontal shading); constant cool temperature (cross-hatched shading); and warming (vertical shading). We did not use the first 2 h of data for the two constant temperature conditions in order to allow time for stabilization of that condition.

Australia [21]. Husbandry and breeding of the animals followed that described previously [22]. All procedures used in this study were approved by the ASU Institutional Animal Care and Use Committee (protocol #05-792R).

As described previously [12], 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 [21] and because substrate would provide a sink for gases that would interfere with data interpretation, females and their resultant clutches were not provided any substrate. We placed brooding chambers in an environmental chamber that had a 14:10 LD photo regime and maintained temperature at 31.5 ± 0.3 °C to preclude the need for any behavioral thermoregulation by females. Additionally, we plumbed brooding chambers with two 3-way stopcocks on opposite sides of the chamber and used a valve-controlled aeration system that combined vapor-saturated air (via bubbling air through a water-filled hydrating column) with dry air to deliver $20\text{--}40$ ml·min⁻¹ of hydrated air (absolute humidity, AH = $25.1\text{--}26.7$ gm⁻³; relative humidity, RH = 80–85%) to each brooding chamber. We housed brooding females in these chambers prior to and during 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.

2.2. Experimental procedure

To test our hypothesis, we evaluated real-time egg-brooding behavior, rates of brooding unit (i.e., female and respective clutch) oxygen consumption (V_{O_2}) and carbon dioxide production (V_{CO_2}), T_{nest} , T_{clutch} , and PO_2 clutch of nine *A. childreni* brooding units (mean female mass = 33.49 ± 11.0 g, mean clutch size = 10 ± 1 eggs, mean absolute clutch mass = 125.3 ± 9.7 g, mean relative clutch mass (i.e., 100·clutch mass/maternal mass) = $37.9 \pm 3.5\%$) using a flow-through system during a 16-h sequence through four temperature conditions: 31.5 °C, cooling (1.5 °C·h⁻¹), 25.6 °C, and warming (1.5 °C·h⁻¹) (Fig. 1). Because field data do not exist for *A. childreni* nests, we created a thermal regime that represents a contracted diel cycle of the temperatures measured in the nests of *L. fuscus* [15], a python species sympatric with *A. childreni* (Fig. 1). We conducted all trials 5 to 7 d post-oviposition. To avoid disturbance, we monitored trials in darkness with an infrared camera and recorded real-time video for later analysis of brooding behavior variables as previously described [12,13].

Experimental trials were conducted in a modified 150 l cooler controlled by a datalogger (21X, Campbell Scientific Instruments, Logan, UT) that produced the thermal cycle. As previously described [12,13], influent air of known gas composition and flow rate was created by combining dry, acapnic air (CDA 1112, Pure Gas, Broomfield, CO) 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 a 25.0 °C dewpoint and maintained at a flow rate of 560 ml·min⁻¹ with a mass flow controller (Unit Instruments, Inc., Yorba Linda, CA) 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 (the difference between initial and final baseline samples was negligible at $0.0039 \pm 0.00078\%$). Air exiting each chamber (effluent air) was passed through a precision hygrometer (RH200, Sable Systems, Las Vegas, NV), and dried by anhydrous CaSO₄ before flowing through a CO₂ analyzer (LI-6252, Li-Cor Biosciences, Lincoln, NE) and an O₂ analyzer (FC-1B, Sable Systems, Las Vegas, NV) that we calibrated to the manufacturer's specifications prior to experimental use. During trials, we recorded the O₂, CO₂, and H₂O concentrations of effluent air every minute using a Campbell 23X datalogger. We converted these

raw data to \dot{V}_{O_2} and \dot{V}_{CO_2} using Eqs. (1)–(6) in Walsberg and Hoffman [23]. This flow-through respirometry system has demonstrated an accuracy of 0.4% in determining steady-state \dot{V}_{O_2} and \dot{V}_{CO_2} [24].

We measured PO₂ clutch in real-time for the duration of trials with a fiber optic O₂ probe and meter (OxyMini, World Precision Instruments, Sarasota, FL), and we recorded resultant data every minute with the 23X datalogger. We inserted a 4 mm diameter O₂ 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 O₂ 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 O₂. Prior to each trial, we used the two-point (i.e., 0% and 20.95% O₂) calibration procedure recommended by the O₂ probe manufacturer. We fixed a Type-T thermocouple to the O₂ probe, interfaced it with the 23X datalogger, and used it to measure real-time T_{clutch} . We positioned another Type-T thermocouple 1–2 cm inside each brooding chamber's influx port, interfaced it with the 23X datalogger, and used it to measure T_{inlet} in real-time. Together, this allowed us to determine the T_{inlet} – T_{clutch} gradient (i.e., the difference between mean T_{inlet} and mean T_{clutch}) at each temperature condition.

2.3. Metabolic, thermal, and behavioral analyses

We used two methods to determine if *A. childreni* females were facultatively endothermic during egg brooding. The energetic cost of endothermy is substantially higher than that of ectothermy, and early stage *A. childreni* eggs comprise just one-third of brooding unit respiratory gas exchange [12]. Thus, in direct contradiction to the metabolic sensitivity to ambient temperature of obligate ectotherms, brooding unit \dot{V}_{O_2} and \dot{V}_{CO_2} would be significantly higher when T_{inlet} is 25.6 °C compared to when it is 31.5 °C if egg brooding *A. childreni* were facultatively endothermic. To indirectly determine the endothermic capability of egg-brooding *A. childreni*, we measured the temperature coefficient (Q_{10}) of metabolism, the factor by which \dot{V}_{O_2} and \dot{V}_{CO_2} increase for every 10 °C rise in T_{inlet} . In addition, we directly determined the females' abilities to regulate the temperature of their respective clutches when they experience a cool temperature by measuring T_{clutch} when T_{inlet} was held constant at 25.6 °C.

As described previously [12,13], we categorized egg brooding into several behaviors. We defined tight brooding to be when a female was motionless and tightly coiled around her clutch. We considered postural adjustments as individual behavioral events only if they were >30 s removed from another postural adjustment. Subtly distinct postural adjustments are categorized into three types. (1) Non-opening adjustments (NA) are those in which female movement is noted but none of the clutch is visibly exposed. (2) Opening adjustments (OA) involve female movement with visible exposure of some of the clutch, last less than 5 min, and do not entail a female's snout breaching the perimeter of her outermost coil. (3) Exploratory adjustments (EA) are postural adjustments that also involve visible exposure of the clutch; however, they last longer than 5 min or entail the female's snout breaching the perimeter of her outermost coil. EA are distinguished from OA because during EA females often insert their heads between their eggs and their coils suggesting a different behavioral motivation (e.g., possibly egg inspection) than that of OA. Although not noted in previous studies, we define loose coiling to be when a female is motionless and coiled around her clutch in such a manner that two or more eggs and an area of the clutch larger than the size of her head (3–4 cm²) is exposed.

2.4. Statistical analyses

Data met the appropriate statistical assumptions of parametric statistics or were transformed as necessary, and were analyzed using JMP IN (version 5.1.2, SAS Statistical Institute, Inc., Cary, NC). Significance was determined at $\alpha < 0.05$ for all tests. To determine the overall effect of treatment (i.e., temperature condition), repeated-

measures analysis of variance (rmANOVA) tests were used. Post-hoc analyses used Bonferroni-corrected paired *t* tests. To determine the treatment effect between specific predicted pairs (e.g., tight coiling between constant 31.5 °C and 25.6 °C treatments), we used paired *t* tests. To test relationships within individuals, we used simple linear regression analysis. All results refer to comparisons within nine individuals (i.e., $n = 9$), and values are displayed as mean \pm S.E.M.

3. Results

Respiratory exchange ratios (i.e., RER, $\dot{V}_{CO_2}/\dot{V}_{O_2}$) did not significantly differ ($t_8 = 0.76$; $P = 0.47$) between the constant 25.6 °C (RER: 0.79 ± 0.03) and 31.5 °C (RER: 0.82 ± 0.04) conditions. Mean rates of respiratory gas exchange for brooding units increased with T_{inlet} (Fig. 2, Table 1). To further refute facultative endothermy by egg-brooding *A. childreni*, the T_{inlet} – T_{clutch} gradient during the constant 25.6 °C phase was miniscule (i.e., 0.11 ± 0.02 °C).

Temperature condition significantly affected the amount of time that females spent tightly coiled around their clutches (Table 1). Post-hoc analyses revealed no difference for any metric of behavior between constant 25.6 °C and 31.5 °C conditions. However, females spent more time tightly coiled during cooling than during warming, while constant temperature conditions were intermediate (Fig. 3). Paired analysis ($t_8 = 3.3$; $P = 0.011$) revealed that females spent more time adjusting

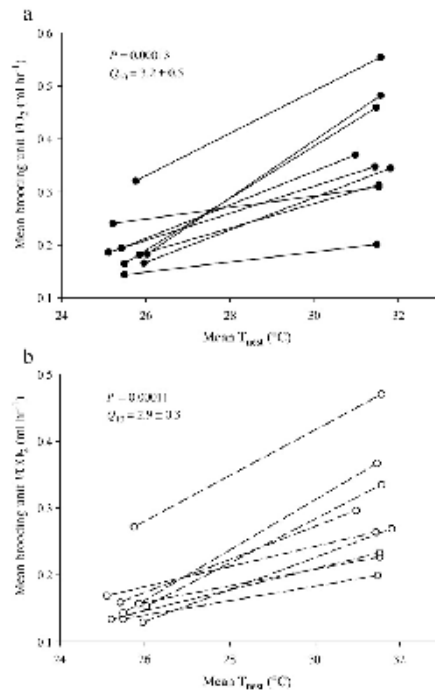


Fig. 2. Effect of constant temperature condition on mean brooding unit: a) Oxygen consumption rate (\dot{V}_{O_2}) and associated temperature coefficient (Q_{10}); and b) carbon dioxide production rate (\dot{V}_{CO_2}) and associated Q_{10} ($n = 9$).

Table 1
Anastrepha childreni maternal brooding behavior, nest-to-clutch thermal relations, respiratory gas exchange rates, and intra-clutch oxygen partial pressure during four temperature conditions (mean \pm S.E.M.).

	31.5 °C	Cooling	25.6 °C	Warming	F	P
Time spent loosely coiled (%)	9.9 \pm 7.3	3.1 \pm 2.9	20.5 \pm 12.5	15.9 \pm 7.7	1.6	0.23
Time spent adjusting posture (%)	5.7 \pm 1.5	4.4 \pm 1.5	9.3 \pm 2.6	18.9 \pm 4.2	3.6	0.086
Non-opening adjustment (NA) rate (per h)	2.3 \pm 0.4	2.5 \pm 0.4	10 \pm 0.2	3.0 \pm 0.6	3.6	0.087
NA duration (s)	21.6 \pm 5.7	15.3 \pm 2.8	19.5 \pm 6.2	18.2 \pm 5.3	0.25	0.86
Opening adjustment (OA) rate (per h)	12 \pm 0.4	12 \pm 0.3	12 \pm 0.3	1.9 \pm 0.4	2.3	0.38
OA duration (s)	49.3 \pm 10.2	51.7 \pm 9.6	44.0 \pm 9.4	75.5 \pm 20.0	1.2	0.38
Exploratory adjustment (EA) rate (per h)	0.4 \pm 0.1	0.4 \pm 0.3	0.5 \pm 0.2	1.2 \pm 0.3	2.0	0.22
EA duration (s)	2470 \pm 44.8	175.0 \pm 38.8	640.0 \pm 134.0	458.0 \pm 117.8	0.15	0.93
O ₂ consumption rate (ml h ⁻¹)	22.5 \pm 2.1	23.1 \pm 1.9	11.9 \pm 1.1	14.8 \pm 2.1	30	<0.0001
CO ₂ production rate (ml h ⁻¹)	17.7 \pm 1.7	15.6 \pm 1.8	9.7 \pm 0.9	13.1 \pm 0.9	25	<0.0001
T _{nest} -T _{clutch} gradient (°C)	0.03 \pm 0.01	-1.52 \pm 0.13	0.11 \pm 0.02	1.42 \pm 0.11	110	<0.0001
PO ₂ clutch (kPa)	18.78 \pm 0.30	19.14 \pm 0.09	19.53 \pm 0.11	19.29 \pm 0.13	71	<0.0001

their posture during warming (18.9 \pm 4.2%) than during cooling (4.4 \pm 1.5%). Because embryonic metabolic rate increases with temperature and females did not change their rate of ventilating behaviors between 25.6° and 31.5 °C, PO₂clutch was negatively related to T_{nest} (Table 1).

During warming, brooding behavior impacted developmental temperature since the amount of time that females spent tightly coiled was negatively correlated to the T_{nest}-T_{clutch} gradient (R² = 0.45; F_{1,7} = 5.7; P = 0.049). Absolute clutch mass was positively correlated to the absolute value of the T_{nest}-T_{clutch} gradient during both cooling (R² = 0.77; F_{1,7} = 2.3; P = 0.0019) and warming (R² = 0.55; F_{1,7} = 8.5; P = 0.023) conditions, demonstrating that the clutch itself provides thermal inertia. Relative clutch mass was also positively correlated to the T_{nest}-T_{clutch} gradient during both cooling (R² = 0.53; F_{1,7} = 7.8; P = 0.027) and warming (R² = 0.52; F_{1,7} = 7.7; P = 0.028) conditions.

4. Discussion

Often referred to as shivering thermogenesis, facultative endothermy during egg brooding and its thermal benefits have been demonstrated in only two python species: *P. molurus* (17.1 kg body mass) [9] and *M. spilota* (6.6 kg body mass) [14]. We have clarified the temperature-related patterns of metabolism and behavior in egg-brooding *A. childreni*. The Q₁₀ values for V_{O₂} and V_{CO₂} in *A. childreni* brooding units (mean: 3.1; range: 1.9–5.7) approximate those of other, non-reproductive boa and python species (mean = 2.6) [25]. In further support of our prediction that *A. childreni* are not facultatively endothermic, we demonstrated that egg-brooding females were unable to maintain an elevated T_{clutch} when experiencing a constant cool T_{nest} (Table 1). In larger python species, endothermy comprises a substantial portion of females' energy expenditure while egg brooding at cool temperatures (*P. molurus*: 92%, *M. spilota*: 95%) [9,14]. Thus, the tremendous additional energetic cost of maintaining homeothermy at cool ambient temperatures for small-bodied pythons with high surface area-to-volume ratios and less reserve energy, such as *A. childreni*, may make facultative endothermy unfeasible.

Parental care provided by female pythons modulates critical and often competing developmental variables such as clutch temperature [9,10,15], water balance [12,22], and respiration [13]. A tightly coiled posture is central to regulating these variables and, thus, integral to the embryonic thermoregulation hypothesis we proposed and tested with *A. childreni*. Because the amount of time that females spent tightly coiled changed with shifts in temperature, the hard-wired (null) hypothesis was not supported (Table 1), which suggests that egg-brooding pythons use external information to enhance the embryonic micro-environment. Not unexpectedly, the ability of parents to use cues from the embryonic micro-environment for behavioral modification has been shown in other taxa as well [26,27]. In particular, nest-attending thermoregulatory behaviors are influenced by T_{nest} in a number of species, such as bumble bees (*Bh. nearcticus*) [16], greater snow geese (*Anser caeruleus atlantica*) [28], and hooded plovers (*T. rubricollis*) [4]. In fact, the embryonic thermoregulation hypothesis, which asserts that

egg-brooding behavior is influenced by the T_{nest}-T_{clutch} gradient, was supported by our results. Females coiled tightly more often during cooling and less often during warming (xFig. 3). In addition to the effect of temperature on the relative use of different brooding behaviors in the laboratory, free-ranging female *I. fuscus* use T_{nest} to determine the duration of egg brooding ('cool' nests: mean = 53.8 d; 'warm' nests: mean = 6.5 d) [15]. Thus, it appears that both short- (e.g., postural adjustments) and long-term (e.g., duration of clutch attendance) maternal decisions are influenced by thermal conditions in egg-brooding pythons.

In addition to incubation temperature, other variables are critical to embryonic development and could thus influence egg-brooding behavior in pythons. Prairie skinks (*Eumeces septentrionalis*) and Malayan pit vipers (*Calloselasma rhodostoma*) modulate their egg-attending behavior in response to shifts in incubation humidity [7,29]. Further, non-brooded *A. childreni* eggs are extremely sensitive to desiccation with relative humidity (RH) as high as 75–80% leading to no successful hatching of non-brooded eggs [22]. However, egg brooding increases hatching success at 75–80% RH to 80% [22]. By maintaining a constant 25.0 °C nest dewpoint during all of the temperature conditions, our study created high (i.e., 31.5 °C: 1.45 kPa) and low (i.e., 25.6 °C: 0.11 kPa) clutch-nest vapor pressure gradients. However, because both temperature and vapor pressure were altered between treatments, the influence of nest humidity on egg-brooding behavior was not directly tested. Future studies should hold T_{nest} constant and alter nest dewpoint to better examine the role of the clutch-nest vapor pressure gradient on egg-brooding behavior in pythons. Like python egg-brooding postural adjustments [13], fin-fanning parental behavior exhibited by some fish increases oxygen in the eggs' micro-environment [26,27]. However, while fin-fanning is negatively correlated with dissolved oxygen levels

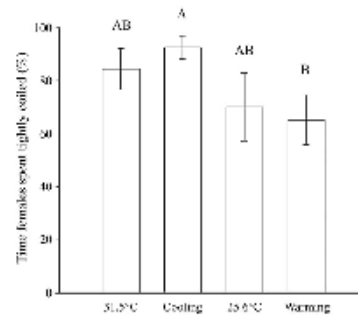


Fig. 3. Effect of temperature condition on the amount of time females spent tightly coiled (n = 9).

[27], egg-brooding female *A. childreni* do not alter their tight coiling behavior as incubation progresses and as embryonic metabolic rate increases substantially [12,13]. As a result, oxygen concentration within the clutch diminishes as incubation progresses and the low PO_2 entails short-term costs (i.e., reduced embryonic metabolism) [13] and long-term costs (i.e., smaller, weaker offspring) [30]. The longer term effects of such hypoxic conditions remain unknown and warrant study. Thus, although water balance, respiration, and thermoregulation are each important to development, temperature appears to be the primary variable upon which python egg-brooding behavior is regulated.

The role of temperature in regulating parental behaviors is not unexpected since metrics critically related to offspring fitness in reptiles (e.g., hatchability and hatchling growth rate), as well as sex determination in many species, are significantly affected by changes in incubation temperature [18,31]. We have quantified the degree of thermal buffering between the clutch and nest environment provided by egg brooding. Though not dramatic, the mean (1.47 °C) and maximum (3.39 °C) $T_{nest} - T_{clutch}$ gradient during warming and cooling periods likely have a significant impact on development. For example, incubation duration is temperature dependent and considered to be the strongest indicator of neonate survival in free-ranging *L. fuscus* [15]. Also, incubation duration is significantly affected by a 1 °C change in incubation temperature in our colony of *A. childreni* (e.g., incubation at 30.5 °C compared to 31.5 °C extends incubation duration an average of 6 days; Stahlschmidt and DeNardo, unpublished). Thus, the ability of egg-brooding females to evaluate the $T_{nest} - T_{clutch}$ gradient to regulate behavior and enhance T_{clutch} could significantly benefit developing offspring. Although clutch mass better explains the variance in the $T_{nest} - T_{clutch}$ gradient during changes in T_{nest} , we have demonstrated that shifts in egg-brooding behavior during warming also significantly impact the $T_{nest} - T_{clutch}$ gradient in *A. childreni*.

Our results have further defined the physiological effects of T_{nest} on python brooding units, the role of the $T_{nest} - T_{clutch}$ gradient on egg-brooding behaviors, and the influence of these behaviors on the developmental micro-environment. The thermally, physiologically, and behaviorally quantifiable nature of the python egg-brooding system allowed us to perform a multi-faceted assessment of a simple parental care model. Farmer's reproductive model postulates that parental care played an integral role in the transitions from obligate ectothermy to facultative endothermy to obligate endothermy [2]; thus, a better understanding of python egg brooding may elucidate the role of parental care in the evolution of endothermy. Future studies should continue to examine the costs and benefits associated with python egg brooding for both ectothermic and facultatively endothermic species. Ecologically, further field studies are necessary to clarify the role of relevant factors (e.g., relative clutch characteristics, the $T_{nest} - T_{clutch}$ gradient, and nest humidity) on maternal python decision-making.

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APPENDIX V

PARENTAL BEHAVIOR IN PYTHONS IS RESPONSIVE TO BOTH THE
HYDRIC AND THERMAL DYNAMICS OF THE NEST

Parental behavior in pythons is responsive to both the hydric and thermal dynamics of the nest

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SUMMARY

Parental behavior contributes to the success of a diverse array of taxa, and female-only nest attendance is particularly widespread. Python egg-brooding behavior is an intriguing example of female-only nest attendance because it significantly influences several critical developmental variables, namely embryonic predation, hydration, respiration and temperature. During brooding, females predominately adopt a tightly coiled posture that reduces the exchange of heat, water vapor, O₂ and CO₂ between the nest and clutch environment, which benefits egg water balance at the cost of respiration. To determine the plasticity of this important behavior, we manipulated nest temperature and humidity while monitoring nest-clutch thermal, hydric and respiratory relationships to test the hypothesis that female Children's pythons (*Antaresia childreni*) modify their egg-brooding behavior due to an interaction between environmental thermal and hydric conditions. During moderate and high nest humidity treatments (23 and 32 g m⁻³ H₂O, respectively), females spent more time coiling tightly when the nest was cooling than when it was warming, which benefited clutch temperature. However, brooding females in low-humidity nest environments (13 g m⁻³ H₂O) showed a high frequency of tight coiling even when the nest was warming; thus, nest temperature and humidity had an interactive effect on egg-brooding behavior in support of our hypothesis. Our results also suggest that certain egg-brooding behaviors (i.e. postural adjustments) are more energetically costly to females than other behaviors (i.e. tight coiling). In sum, we provide empirical support for the adaptive plasticity of python egg-brooding behavior, which offers insight into the general significance of female-only nest attendance in animals.

Key words: adaptive plasticity, life history trade-offs, metabolism, parental care, snake, thermoregulation, water balance.

INTRODUCTION

Parental care is instrumental to the success of a diverse array of taxa, and its broad evolutionary significance has been the motivation for substantial investigation (Williams, 1966; Trivers, 1972; Clutton-Brock, 1991; Gross, 2005). Among other benefits, nest-attending parents can increase their fitness by reducing embryonic predation [frogs (Townsend, 1986)], improving egg water balance [skinks (Somma, 1989)], thermoregulating embryos [bumblebees (Heinrich, 1979)], promoting embryonic respiration [fish (Lissaker and kVarnemo, 2006)], reducing pathogen infiltration of eggs [crickets (West and Alexander, 1963)] and provisioning offspring with food [birds (Clutton-Brock, 1991)]. As a result of this wide assortment of benefits, nest-attending behavior is taxonomically widespread (Clutton-Brock, 1991). While birds typically use bi-parental care, female-only parental care is the predominant mode of care in other internally fertilizing vertebrates [e.g. reptiles and mammals (Clutton-Brock, 1991)], including species within major taxa in which external fertilization predominates [i.e. fish and amphibians (Gross and Shine, 1981)], as well as terrestrial arthropods (Zeh and Smith, 1985).

Within the broad context of female-only nest attendance, python egg brooding has recently proven itself as a useful and relevant parental care model. Python egg brooding is simple, yet it affects several critical and quantitatively measurable developmental variables, including embryonic predation (Madsen and Shine, 1999), thermoregulation (Vinegar et al., 1970; Stahlschmidt and DeNardo, 2009a), water balance (Aubret et al., 2005a; Lourdaïs et al., 2007; Stahlschmidt et al., 2008) and respiration (Stahlschmidt and DeNardo, 2008). While the benefits of python egg brooding have

been well established, egg brooding limits nest-clutch respiratory gas exchange, which entails short-term metabolic and long-term phenotypic costs to offspring (Stahlschmidt and DeNardo, 2008; Stahlschmidt and DeNardo, 2009b). Thus, python egg brooding can be used to examine the physiological trade-offs of parental care.

We have shown in Children's pythons [*Antaresia childreni* (Gray 1842)] that python egg brooding is dynamic. During egg brooding, females predominately adopt a tightly coiled posture that reduces the exchange of heat, water vapor, O₂ and CO₂ between the nest and clutch environment that benefits water balance at the cost of embryonic respiration (Stahlschmidt and DeNardo, 2008; Stahlschmidt and DeNardo, 2009a; Stahlschmidt et al., 2008). Hence, females periodically adjust their egg-brooding postures to facilitate nest-clutch gas (O₂ and H₂O vapor) exchange to benefit respiration at the cost of embryonic water balance (Stahlschmidt and DeNardo, 2008; Stahlschmidt et al., 2008).

While postural adjustments are used to balance the various embryonic needs even under static environmental conditions, incubation conditions vary over time (e.g. diel temperature shifts). Thus, it would seem beneficial to the developing offspring if females adjusted the timing and duration of postural adjustments based on environmental inputs. However, while embryonic oxygen consumption increases dramatically over the course of development, female *A. childreni* do not alter the relative frequency or duration of their postural adjustments, which results in developmental hypoxia that reduces offspring size, speed and strength (Stahlschmidt and DeNardo, 2008; Stahlschmidt et al., 2008). Contrarily, we have recently shown that egg-brooding females are capable of assessing

the nest-clutch temperature gradient, and they make behavioral adjustments to enhance the thermal micro-environment of their developing offspring. That is, females coil tightly around eggs less often when the nest is warming compared with when it is cooling (Stahlschmidt and DeNardo, 2009a).

Because of the contrasting results found in female response to variation in environmental temperature and respiratory gas concentrations, we designed an experiment to evaluate female response to another critical variable associated with brooding – water balance. Thus, we designed a multifactorial experiment to assess the relative importance of environmental temperature and hydric condition, two variables critically important to embryonic python development (Shine et al., 1997; Lourdis et al., 2007). We manipulated nest temperature and humidity while monitoring nest-clutch thermal, hydric and respiratory relationships to test the hypothesis that females modify their egg-brooding behavior due to an interaction between environmental thermal and hydric conditions. Our results will specifically provide further understanding of the dynamic nature of python egg brooding and, in general, build upon existing knowledge of the environmental influences on parental behaviors.

MATERIALS AND METHODS

Study species and reproductive husbandry

For this study, we used a long-term captive colony of *A. childreni* maintained at Arizona State University (ASU), Tempe, AZ, USA. *Antaresia childreni* are non-venomous, constricting snakes that inhabit rocky areas in northern Australia (Wilson and Swan, 2003). Husbandry and breeding of the animals followed that described previously (Lourdis et al., 2007). All procedures used in this study were approved by the ASU Institutional Animal Care and Use Committee (protocol #08-967R).

As described previously (Stahlschmidt et al., 2008; Stahlschmidt and DeNardo, 2009a), we housed brooding females in substrate-free, Teflon-coated 1.9 l chambers a few days prior to oviposition through the completion of all experimental trials to minimize disturbance, avoid clutch abandonment and ensure the exchange of respiratory gases (i.e. H₂O vapor, O₂ and CO₂) to and from the brooding unit (i.e. female and associated clutch). When not being used in an experimental trial, these brooding chambers were kept in an environmentally controlled room that had a 14 h:10 h L:D photo regime and maintained temperature at the species' preferred developmental temperature (31.5±0.3°C) (Lourdis et al., 2008) to preclude the need for any behavioral thermoregulation by females. Also, we delivered 20–40 ml min⁻¹ of hydrated air [absolute humidity=25.1–26.7 g m⁻³; relative humidity (RH)=80–85%] to each brooding chamber using methods described previously (Stahlschmidt et al., 2008; Stahlschmidt and DeNardo, 2009a). At oviposition, we briefly removed each female from her clutch to determine clutch size, clutch mass and female post-oviposition mass.

Experimental procedure

To test our hypothesis, we evaluated real-time egg-brooding behavior, rates of brooding unit oxygen consumption (\dot{V}_{O_2}), carbon dioxide production (\dot{V}_{CO_2}) and water loss (M_{H_2O}), nest temperature (T_{nest}) and clutch temperature (T_{clutch}) of eight *A. childreni* brooding units [female mass (means ± s.e.m.): 359.5±20.4 g; clutch size: 9±1 eggs; absolute clutch mass: 101.6±7.3 g; relative clutch mass (i.e. 100 × clutch mass / maternal mass): 28.4±1.7%] using a flow-through system. We evaluated each brooding unit during consecutive 4-h treatments of four temperature conditions [31.5°C, cooling

(1.4°C h⁻¹), 26.0°C, and warming (1.4°C h⁻¹)], and we repeated these trials at three influent humidity conditions assigned in random order [dew points (DP) of 31°C, 25°C and 16°C (13, 23 and 32 g m⁻³ H₂O, respectively)] (Fig. 1). Because field data do not exist for *A. childreni* nests, we used a thermal regime that represents a contracted diel thermal cycle from nests of water pythons, *Liasis fuscus*, which are sympatric with *A. childreni* (Madsen and Shine, 1999). Further, we used humidity information from a database maintained by the Commonwealth of Australia's Bureau of Meteorology to create ecologically relevant nest humidity treatments. Previously, we determined that *A. childreni* brooding behavior does not have a diel cycle (Stahlschmidt et al., 2008), so the effect of the sequential treatments would not be confounded by time of day. Also, as egg-brooding behavior does not change over incubation (Stahlschmidt and DeNardo, 2008; Stahlschmidt et al., 2008), we conducted all trials <1 week post-oviposition. To avoid disturbance, we monitored trials in darkness with an infrared camera and recorded real-time video for later analysis of brooding behavior variables as previously described (Stahlschmidt et al., 2008; Stahlschmidt et al., 2009a).

We conducted experimental trials in a modified 150 l thermoelectric cooler controlled by a datalogger (21X, Campbell Scientific Instruments, Logan, UT, USA) that produced the thermal cycle. We created influent air of known humidity by bubbling building supply air through a heated water column and then sending the air through a condensation chamber held at the desired DP (e.g. 16°C, 25°C or 31°C DP). For the 31°C DP trials, the condensation chamber temperature mimicked that of the trial thermal cycle to maintain a saturated influent air without condensation on the eggs (Fig. 1). We verified the humidity of influent air with a precision hygrometer (RH100, Sable Systems, Las Vegas, NV, USA) positioned immediately upstream of the brooding chamber. We maintained an influent flow rate of 500 ml min⁻¹ with an upstream pressure regulator, an adjustable rotameter and a flow tube (FL-344, Omega Instruments, Stamford, CT, USA) that was calibrated under experimental conditions.

We analyzed a baseline sample of influent air immediately before and after brooding trials and averaged the two to determine the O₂ and CO₂ concentrations of influent air (the difference between initial and final baseline samples was negligible at 0.0042±0.00081%). Baseline air and air exiting each chamber (effluent air) was passed through a precision hygrometer (RH300, Sable Systems) and dried

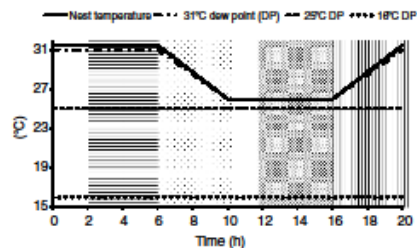


Fig. 1. Eight *Antaresia childreni* brooding units were monitored during a continuous sequence of four experimental temperature conditions [constant preferred incubation temperature (horizontal shading), cooling (dotted shading), constant cool temperature (diagonal shading) and warming (vertical shading)], and we repeated these trials under three humidity conditions [31°C dew point (DP) or vapor-saturated, 25°C DP and 16°C DP].

Table 1. The effects of temperature and humidity treatments on the percentage of time female *A. childeni* spent tightly coiled around their clutches (means \pm s.e.m., $N=8$)

	Nest temperature condition				Nest-clutch temperature gradient		
	31.5°C	Cooling	26.0°C	Warming	Negative	Zero	Positive
Nest humidity							
16°C dew point (DP)	95.9 \pm 1.4	95.8 \pm 1.4	98.5 \pm 0.3	94.7 \pm 1.9	95.8 \pm 1.4	97.2 \pm 0.79	94.7 \pm 1.9
25°C DP	91.5 \pm 3.7	95.6 \pm 0.8	90.6 \pm 6.8	82.3 \pm 6.4	95.6 \pm 0.8	91.1 \pm 4.5	82.3 \pm 6.4
31°C DP	92.2 \pm 2.8	95.2 \pm 0.9	91.1 \pm 3.6	70.5 \pm 9.7	95.2 \pm 0.9	91.7 \pm 2.4	70.5 \pm 9.7
Nest-clutch vapor pressure gradient							
Low (<0.01 kPa)	92.2 \pm 2.8	95.2 \pm 0.9	90.9 \pm 3.7	70.5 \pm 9.7	92.2 \pm 2.8	91.4 \pm 4.1	70.5 \pm 9.7
Medium (0.01–1.45 kPa)	91.5 \pm 3.7	95.6 \pm 0.8	98.5 \pm 0.3	82.3 \pm 6.4	95.6 \pm 0.8	95.0 \pm 1.7	82.3 \pm 6.4
High (1.46–2.82 kPa)	95.9 \pm 1.4	95.8 \pm 1.4	*	94.7 \pm 1.9	95.8 \pm 1.4	95.9 \pm 1.4	94.7 \pm 1.9

*Could not be determined because animals did not experience this treatment.

by anhydrous CaSO₄ before flowing through a CO₂ analyzer (LI-6252, Li-Cor Biosciences, Lincoln, NE, USA) and an O₂ analyzer (FC-1B, Sable Systems) that we calibrated to manufacturer specifications prior to experimental use. During trials, we recorded the O₂, CO₂ and H₂O concentrations of effluent air every minute using a Campbell 23X datalogger. We converted these raw data to \dot{V}_{O_2} , \dot{V}_{CO_2} and M_{H_2O} using equations 1–7 in Walsberg and Hoffman (Walsberg and Hoffman, 2006). This flow-through respirometry system has a demonstrated accuracy of 0.4% in determining steady-state \dot{V}_{O_2} and \dot{V}_{CO_2} (Walsberg and Hoffman, 2005).

To measure real-time clutch temperature (T_{clutch}), we positioned a Type-T thermocouple 1–2 cm into the clutch using an access port in the bottom of the brooding chamber, and interfaced it with the 23X datalogger (Campbell Scientific Instruments). We positioned another Type-T thermocouple 1–2 cm inside each brooding chamber's influx port, interfaced it with the Campbell 23X datalogger, and used it to measure nest temperature (T_{nest}) in real-time. Together, these temperatures allowed us to determine the T_{nest} – T_{clutch} gradient (i.e. the difference between mean T_{nest} and mean T_{clutch}) at each treatment. Given the dynamic nature of our experimental design, we were able to determine the effect(s) of several thermal and hydric variables on egg-brooding behavior. In addition to T_{nest} condition (i.e. 31.5°C, cooling, 26.0°C and warming) and nest humidity (31°C, 25°C and 16°C DP), we evaluated the effect(s) of the T_{nest} – T_{clutch} gradient (i.e. negative, zero and positive) and the nest-clutch vapor pressure gradient [i.e. low (<0.01 kPa), medium (0.01–1.45 kPa) and high (1.46–2.82 kPa)] on brooding behavior. This multi-factor approach allowed us to determine the relative importance of each variable on brooding behavior.

As described previously, we categorized egg brooding into two behavior types that are strongly associated with nest-clutch thermal, hydric and respiratory dynamics (Stahlschmidt and DeNardo, 2008; Stahlschmidt and DeNardo, 2009a; Stahlschmidt et al., 2008). We defined tight brooding to be when a female was motionless and tightly coiled around her clutch. We considered postural adjustments as individual behavioral events only if they were >30 s removed from another postural adjustment.

Statistical analyses

We verified that our data met the appropriate statistical assumptions of parametric statistics, or we transformed them as necessary. We analyzed all data with SPSS (version 15, SPSS, Inc., Chicago, IL, USA) and determined significance at $\alpha < 0.05$ for all tests. To determine the independent and interactive effects of treatment (i.e. temperature condition, T_{nest} – T_{clutch} gradient, nest humidity and the nest-clutch vapor pressure gradient), we used two-factor repeated-measures analysis of variance (RMANOVA) tests. If sphericity was significant, we used Huynh-Feldt epsilon adjustments. We used Bonferroni-corrected paired *t*-tests for *post-hoc* analyses. To test relationships among individuals within treatments (e.g. the effect of tight coiling on brooding unit \dot{V}_{O_2} during 31.5°C temperature and 26°C DP conditions of the nest), we used simple linear regression analysis. We present all results as means \pm s.e.m., and they refer to comparisons among eight individuals (i.e. $N=8$).

RESULTS

Coiling behavior was significantly influenced by all temperature and humidity treatments, and there was an effect of the temperature

Table 2. Repeated-measures analysis of variance results for the independent and interactive effects of temperature and humidity treatments on the percentage of time female *A. childeni* spent tightly coiled around their clutches ($N=8$)

Nest temperature condition and nest humidity	d.f.	<i>F</i>	<i>P</i>
Nest temperature condition	3	7.28	<0.01
Nest humidity	2	4.64	0.029
Temperature condition \times nest humidity*	4,4	3.11	0.026
Nest-clutch temperature gradient and nest humidity			
Nest-clutch temperature gradient	2	8.32	<0.01
Nest humidity	2	5.32	0.019
Temperature gradient \times nest humidity	4	3.99	0.011
Nest-clutch temperature and vapor pressure gradients			
Nest-clutch temperature gradient	2	8.88	<0.01
Nest-clutch vapor pressure gradient	2	5.20	0.020
Temperature gradient \times vapor pressure gradient*	2,7	4.30	0.021

*Huynh-Feldt epsilon adjusted.

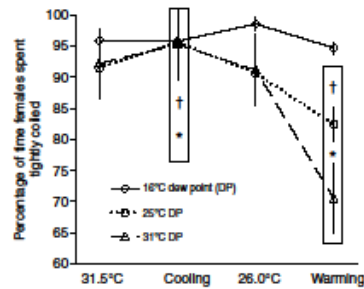


Fig. 2. The effect of humidity on the mean percentage of time female *Antaresia childreni* spent tightly coiled (% TC) during trials utilizing four sequential temperature conditions. Error bars represent s.e.m., and significant between-temperature differences for the 25°C DP and 31°C DP trials are denoted by boxed * and †, respectively ($N=8$). Note the significant decrease in % TC during the warming stage for both the 25°C DP and 31°C DP trials but the constant high rate of % TC during the drier 16°C DP trials.

× humidity interaction (Tables 1, 2; Fig. 2). Because animals did not experience a high nest–clutch vapor pressure gradient when nest temperature was 26.0°C, we could not determine the effects of the nest–clutch vapor pressure gradient + temperature condition on coiling behavior. *Post-hoc* analyses support previously reported findings in that brooding females held at a 25°C DP spent more time coiling tightly when the nest was cooling than when it was warming (Stahlschmidt and DeNardo, 2009a) (Fig. 2). We obtained similar results for brooding females during vapor-saturated nest conditions (i.e. the 31°C DP treatment, Fig. 2). However, this pattern did not persist under dry conditions, as brooding females at 16°C DP showed a high frequency of tight coiling even when the nest was warming (Fig. 2).

In agreement with a previous study (Stahlschmidt et al., 2008), tight coiling (TC) conserved embryonic water because the percentage of time that a female spent tightly coiled (% TC) was negatively related to brooding unit \dot{M}_{H_2O} during 16°C DP and 25°C DP nest conditions (Table 3) [note: exposed python eggs lose water ~10-fold faster than females alone so the vast majority of brooding unit \dot{M}_{H_2O} is derived from the eggs during postural adjustments

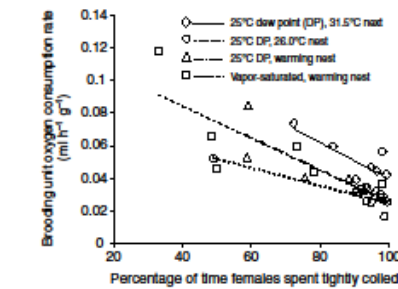


Fig. 3. Significant relationships between the amount of time female *Antaresia childreni* ($N=8$) spent tightly coiled (% TC) and brooding unit oxygen consumption rate. Relationships during other treatments were non-significant possibly due to low sample size and low among-individual variation in % TC (Tables 3 and 4).

(Stahlschmidt et al., 2008)]. As in Stahlschmidt and DeNardo (Stahlschmidt and DeNardo, 2009a), % TC was significantly and positively related to mean $T_{nest}-T_{clutch}$ gradient during warming at 25°C and 31°C DP treatments (Table 4). Low sample size and low among-individual variation in % TC may have contributed to the lack of significance during other warming and cooling treatments as % TC was positively, although non-significantly, related to mean $T_{nest}-T_{clutch}$ gradient in all of these instances (Table 4).

Respiratory exchange ratios (i.e. RER, $\dot{V}_{CO_2}/\dot{V}_{O_2}$, 0.82 ± 0.04) were not affected by temperature condition ($F_{3,21}=1.5$, $P=0.25$, $1-\beta=0.33$), DP ($F_{1,7}=0.011$, $P=0.92$, $1-\beta=0.051$) or the temperature × DP interaction ($F_{2,8,10,5}=2.4$, $P=0.11$, $1-\beta=0.49$). Postural adjustments seemed to come with an energetic cost to females as tight coiling behavior was negatively related to brooding unit \dot{V}_{O_2} during several treatments (Fig. 3), particularly when among-individual variance of % TC was high (Tables 3 and 4). The disparity in brooding unit \dot{V}_{O_2} is primarily due to changes in maternal \dot{V}_{O_2} because early-stage *A. childreni* embryos are not O_2 limited and the clutch consumes ~50% less O_2 than the brooding female (Stahlschmidt and DeNardo, 2008; Stahlschmidt et al., 2008). Again, low sample size and low

Table 3. Relationships between the amount of time female *A. childreni* ($N=8$) spent tightly coiled (% TC) and brooding unit water loss rates (\dot{M}_{H_2O} , $mg\ h^{-1}\ g^{-1}$) during 16°C dew point (DP) and 25°C DP nest conditions

Treatments	R^2	F	P	Equation	Variance of % TC
16°C DP					
31.5°C	0.59	8.6	0.026	$y=-0.0042x+0.47$	16.0
Cooling	0.54	7.0	0.039	$y=-0.0025x+0.30$	16.0
26.0°C	0.63	10.3	0.018	$y=-0.0136x+1.37$	0.70
Warming	0.80	23.5	0.0029	$y=-0.0019x+0.23$	30.0
25°C DP					
31.5°C	0.68	13.0	0.011	$y=-0.0025x+0.29$	84.5
Cooling	0.63	10.4	0.018	$y=-0.0027x+0.29$	18.6
26.0°C	0.73	16.2	0.0069	$y=-0.0014x+0.11$	287.6
Warming	0.55	7.3	0.036	$y=-0.0028x+0.30$	253.2

Note: relationships during 31°C DP are not presented because the nest–clutch vapor pressure gradient and, thus, \dot{M}_{H_2O} approximated zero in most instances (i.e. the air was vapor-saturated or nearly so).

Table 4: Relationships between the amount of time female *A. childreni* ($N=6$) spent tightly coiled (% TC) and the mean nest-clutch temperature gradients during warming and cooling nest conditions

Treatments	R^2	F	P	Equation	Variance of % TC
16°C Dew point (DP)					
Cooling	0.38	3.7	0.10	$y=0.050x-5.80$	16.0
Warming	0.20	1.5	0.26	$y=0.028x-1.64$	30.0
25°C DP					
Cooling	0.39	3.8	0.10	$y=0.034x-4.17$	18.6
Warming	0.66	11.5	0.015	$y=0.013x-0.33$	253.2
31°C DP or vapor-saturated					
Cooling	0.33	3.0	0.14	$y=0.048x-5.44$	11.2
Warming	0.61	9.4	0.022	$y=0.0084x-0.029$	571.3

among-individual variation in % TC may have contributed to the lack of significance during other treatments as % TC was similarly, although non-significantly, related to brooding unit $\dot{V}O_2$ in all of these instances (all non-significant $R^2=0.19-0.48$; $P=0.057-0.28$).

DISCUSSION

We experimentally demonstrate that both T_{nest} and humidity conditions influence python egg-brooding behavior, and that these two effects significantly interact with one another. Also, in agreement with previous studies, the tightly coiled brooding posture reduced nest-clutch exchange of water vapor and heat, particularly during unfavorable thermal and hydric nest conditions (Stahlschmidt and DeNardo, 2009a; Stahlschmidt et al., 2008). Thus, python egg-brooding postural adjustments are functionally significant to embryonic thermoregulation and water balance because these movements modulate and respond to these two important developmental variables.

Not unexpectedly, many animals significantly invest into the thermoregulation and water balance of their offspring because these aspects are crucial to development and survival (Clutton-Brock, 1991). Although taxonomically and functionally (i.e. behaviorally and physiologically) diverse, parental care adaptations that enhance these variables tend to fall within two simple categories – buffering and provisioning. First, parents can improve the fitness of their offspring by simply providing resistance to embryonic water or heat loss through building nests and synthesizing eggshells or egg coats (Clutton-Brock, 1991). Second, parents can enhance embryonic thermoregulation and water balance by providing heat or water to their embryos. Examples include viviparity (i.e. live-bearing, which allows parents to directly regulate embryonic temperature and hydration), endothermic brooding and parental feeding (Clutton-Brock, 1991). To optimize offspring fitness, pythons use either buffering alone [e.g. *A. childreni* (Stahlschmidt and DeNardo, 2009a); this study] or both buffering and provisioning [e.g. *Python molurus* (Hutchison et al., 1966; Vinegar et al., 1970)].

Given embryos' sensitivity to incubation conditions (Deeming and Ferguson, 1991; Deeming, 2004), parental behaviors are often modified in response to environmental thermal or hydric dynamics. For example, ambient temperature (T_a) influences the percentage of time female little stints (*Calidris minuta*) spend brooding and foraging (i.e. females brood more when T_a is low) (Tulp et al., 2009). To reduce egg desiccation, female prairie skinks (*Eumeces septentrionalis*) increase their egg-brooding behaviors when the nest substrate is relatively dry (Somma and Fawcett, 1989). In addition to shifts in brooding behaviors, several reptile species demonstrate adaptive nest site selection related to thermal or hydric conditions (e.g. lizards (Shine and Harlow, 1996), turtles (Belinsky et al., 2004), snakes (Brown and Shine, 2004)). The role of hygrosensation in

adaptive nest site selection is taxonomically widespread. For example, Montell demonstrated that fruit flies (*Drosophila melanogaster*) enhance the fitness of their offspring by preferentially ovipositing on moist substrate (Montell, 2008). Our results indicate that python egg-brooding behavior is adaptively plastic in response to both nest thermal and hydric dynamics.

While highly beneficial to the offspring, parental care generally entails substantial energy-related costs to the parent(s) (Clutton-Brock, 1991). In pythons, egg brooding is generally accompanied by lost foraging time and anorexia (Madsen and Shine, 1999; Aubret et al., 2005b). Accordingly, egg brooding obligates significant epaxial muscle atrophy and reduces contraction strength in female *A. childreni* under laboratory conditions (O. Lourdaïs and D.F.D., unpublished). However, other research suggests that brooding-related maternal costs are minimal (Aubret et al., 2005b). Interestingly, some water pythons nest in root boles where they brood their eggs for the duration of incubation (mean: 58 days) while other females nest in more thermally stable varanid burrows and only brood their eggs for the first week of incubation (Madsen and Shine, 1999). Females that use root boles and thus brood throughout incubation have reduced reproductive body condition and survival rate relative to those females that nest in burrows (Madsen and Shine, 1999).

Similar to large-scale decisions (i.e. to brood or not to brood the clutch), finer-scale egg-brooding decisions (i.e. the frequency of postural adjustments) also entail energetic costs. In fact, of the brooding units in this study, the one with the most behaviorally active female (i.e. lowest % TC) consumed >3-fold more $O_2 g^{-1}$ body mass than the brooding unit that had the least active female when the nest was warming and vapor-saturated. The energetic cost of postural adjustments is probably relatively low compared with the energy demands of other aspects of parental investment (e.g. yolk deposition and lost foraging time) but warrants further investigation.

To conclude, our results combined with those of previous studies (e.g. Madsen and Shine, 1999) demonstrate that female pythons make both large-scale and fine-scale parental decisions. In addition to deciding where to brood and for how long, female pythons can also alter their brooding behavior. Thus, while providing a less complex form of parental care compared with that of mammals and birds, female pythons assess and respond to specific nest conditions to optimize the developmental environment experienced by their offspring. Although python egg brooding is emerging as a simple yet valuable parental care model, many critical questions regarding this system remain unanswered. Future research should focus on the proximate sensory mechanisms of egg-brooding decision-making such as neuronal pathways [e.g. transient receptor potential channels (Romanovsky, 2007; Montell, 2008)] and hormonal regulation (e.g. estradiol, progesterone, thyroxin and prolactin). Additionally, while challenging, field studies that integrate the

various levels of parental decision-making (e.g. nest site selection, brooding duration and postural adjustment frequency) would provide critical insight.

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