The use of ultrasonography to assess reproductive investment and output in pythons

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Reproductive investment and output are integral fitness components, often incorporated into life-history trade-off models and important to population dynamics. The trade-offs associated with reproduction can be dramatic in species such as snakes that make especially large investments into reproduction. Unfortunately, traditional methods used to determine reproductive investment and output are effective in many (but not all) situations. Thus, we used portable ultrasonography to serially estimate reproductive investment and reproductive output in three python species that exhibit significant variation in phylogeny, geographic range, body size, egg size, and clutch size: ball pythons (*Python regius*), Children's pythons (*Antaresia childreni*), and water pythons (*Liasis fuscus*). At each time point of measurement (range: 1–49 days pre-oviposition), ultrasound estimates of viable clutch size were highly accurate in all three species. However, ultrasound estimates of mean viable egg mass, and thus viable clutch mass, significantly differed from the actual values (range: 23–73% error). Interestingly, this error was considerably smaller as females approached oviposition, suggesting that female pythons transfer a significant amount of water into their eggs during the week before oviposition. Thus, water balance during late-stage egg development may be an integral part of reproductive success. The results obtained in the present study form the foundation for future assessments of reproductive investment, and also provide insight into the use of ultrasound technology to assist such efforts. © 2011 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2011, **103**, 772–778.

ADDITIONAL KEYWORDS: egg – ova – population biology – reproduction – effort – snake.

INTRODUCTION

Reproduction is a principal fitness component, and thus its investigation spans centuries, taxa, and disciplines (Beard, 1898; Craig-Bennett, 1931; O'Donnell *et al.*, 2001). In particular, reproductive trade-offs (e.g. allocation of resources to reproduction at the expense of other body needs) are central to life-history theory (Stearns, 1976; Linden & Moller, 1989; Shine, 2005). Although reproduction is often examined as a single event, understanding the impact of reproduction-induced trade-offs would benefit from finer-scale examinations among reproductive stages (e.g. mating, vitellogenesis, gravidity/pregnancy, and parental care), as well as within reproductive stages (e.g. examination of changes over time during gravidity).

To conduct such work, the means by which reproductive activity is evaluated must be effective and non-invasive, at the same time as also allowing for serial assessments. Ultrasonography has recently undergone significant technological advances to give it greater resolution and make it more portable. Ultrasonography has been used to determine shifts in reproductive morphology (e.g. increasing follicular size) and demonstrate the relationships between such shifts and other reproductive events, such as changes in circulating hormone concentration or the timing of reproductive behaviors (e.g. rattlesnakes: Taylor & DeNardo, 2005; turtles: Rostal, 2005; Manire *et al.*, 2008; wolves: McNay, Stephenson & Dale, 2006; seals: Adams *et al.*, 2007; sharks: Daly *et al.*, 2007; horses: Botha *et al.*, 2008; mice: Mircea *et al.*, 2009; monkeys: Monteiro *et al.*, 2009; fish: Swanson *et al.*, 2008).

Reproduction in reptiles has long been a source of interest because they exhibit tremendous variability

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in reproductive mode (e.g. income to capital breeding and oviparity to viviparity), and many species make substantial investments into single reproductive events (Shine, 2005). In particular, reproduction in pythons (Family Pythonidae) has received considerable scientific attention of late, because they: (1) make extremely high reproductive investments, allocating 33–52% of their body mass into their eggs (Aubret *et al.*, 2003; Stahlschmidt, Hoffman & DeNardo, 2008) and (2) dynamically brood their eggs, making them an emerging model for examining parental care trade-offs (Stahlschmidt & DeNardo, 2011).

Unfortunately, most reptiles (including pythons) use secluded and inaccessible nesting sites, which make determination of reproductive output difficult unless the animal is being maintained in captivity at the time of parition (i.e. oviposition or parturition). Fortunately, gravid reptiles remain active and may increase their surface activity to promote thermoregulation through basking (e.g. snakes: Bonnet, Naulleau & Shine, 1999). Thus, it is critical to have an effective and non-invasive method to predict reproductive output in reptiles.

Coelomic palpation is the most widely used method to assess reproductive state and anticipated clutch size in squamate reptiles. Although effective in many instances, it can be traumatic, is difficult in heavy-bodies species, and provides limited information on egg size (D. DeNardo, unpubl. results). Researchers have begun to examine the dynamics of reptile reproduction with ultrasonography (Kuchling, 1989; Gartrell *et al.*, 2002; Rostal, 2005; Taylor & DeNardo, 2005; Gilman & Wolf, 2007; Manire *et al.*, 2008). Most of these studies did not quantify reproductive investment (Kuchling, 1989; Gartrell *et al.*, 2002; Rostal, 2005; Taylor & DeNardo, 2005; Manire *et al.*, 2008). However, Gilman & Wolf (2007) examined reproductive effort in six species of lizards with varying maternal, egg, and clutch size characteristics. In their study, mean estimates of clutch size, egg size, and clutch volume deviated from the actual values (by 13%, 26%, and 30%, respectively); yet, inter-specific variation as a result of clutch size existed (Gilman & Wolf, 2007).

To clarify the quantitative value of ultrasonography in other reptiles, we conducted a study to ascertain the efficacy of ultrasonography in predicting reproductive investment and output in three python species. Pythons have a relatively heavy-bodied build, which makes them poor candidates for palpation. Unlike lizards, the elongated cylindrical shape of snakes dictates a mostly linear arrangement of eggs within females. Also, female snakes tend to be anorectic during gravidity, eliminating the likelihood of gut contents interfering with ultrasonic imaging of the eggs. Using Children's pythons (*Antaresia childreni*, Gray, 1842), ball pythons (*Python regius*, Shaw, 1802), and water pythons (*Liasis fuscus*, Peters, 1873), we asked: (1) how accurate are ultrasound estimates of the number of viable eggs (viable clutch size), number of nonviable ova, mean viable egg mass, and total viable egg (clutch) mass in pythons and (2) does the stage of gravidity affect reproductive output estimates? The latter question is important for both practical reasons and physiological understanding. From a practical perspective, it is important because gravid snakes are found opportunistically and thus ultrasound estimates may occur at different time points of post-ovulatory egg development. Physiologically, ultrasonic imaging might enable the examination of serial changes in egg size or composition during gravidity; thus, it can provide insight into the dynamics of reproductive allocations and trade-offs.

MATERIAL AND METHODS

STUDY SPECIES AND REPRODUCTIVE HUSBANDRY

The Pythonidae comprises eight genera and 33 species, all of which naturally occur in the Old World (Rawlings *et al.*, 2008). Pythons are characterized by oviparity (egg laying) and exhibit a large range of adult sizes (i.e. 0.5–10 m) (Minton & Minton, 1973; Wilson & Swan, 2008). We used three python species that are taxonomically diverse and represent considerable variation in body size, habitat preference, clutch size, and egg mass (Rawlings *et al.*, 2008; Wilson & Swan, 2008). *Python regius* is a 1–2 m, relatively heavy-bodied python that is endemic to western Africa, typically inhabiting open fields and secondary forest (Aubret *et al.*, 2003), *Antaresia childreni* is a small (< 1 m), relatively slender python found largely in rocky outcrops and escarpments of northern Australia (Wilson & Swan, 2008). *Liasis fuscus* is sympatric with *A. childreni*, although it is a semi-aquatic species of moderate girth and 2–3 m in length (Wilson & Swan, 2008).

Python regius and *A. childreni* used in the present study were part of long-term captive colonies at Arizona State University (ASU), and procedures involving these species were approved by the ASU Institutional Animal Care and Use Committee (IACUC; protocol #08-967R). We individually housed these animals in cages ($91 \times 71 \times 46$ cm) maintained at room temperature under a 12 : 12 h light/dark cycle. We provided continuous access to supplemental heat using a sub-surface heating element (Flexwatt, Flexwatt Corp.) under one side of each cage. Breeding occurred in February 2009 and 2010 after a 2-month wintering period, and we conducted ultrasound scans from late March to mid-July. Oviposition occurred

between early April and late July 2009 and 2010. In August 2010, we caught gravid *L. fuscus* near Beatrice Hill, which is situated on the Adelaide River floodplain in the Northern Territory, Australia. After capture, we conducted an ultrasound examination and then housed each female in a translucent container ($58 \times 39 \times 35$ cm) in a building maintained at 26–34 °C under dimly lit to dark conditions until oviposition. Procedures involving *L. fuscus* were approved by the ASU IACUC (protocol #08-968R) and the Northern Territory (Australia) Parks and Wildlife Commission (permit #37045).

At oviposition, we temporarily removed *P. regius* and *A. childreni* females from their respective clutches of eggs to measure actual reproductive investment and output. We ran female *L. fuscus* through egg brooding behavioral trials during the first week after oviposition (as part of another study), and removed females from their clutches immediately thereafter to measure actual reproductive investment and output. For all species, we recorded the mass of each female and clutch (± 0.1 g) and counted the number of viable, shelled eggs (i.e. clutch size), as well as nonviable ova (i.e. nonshelled yolk masses).

ULTRASOUND PROCEDURE

We performed brief (1–5 min) scans on non-anaesthetized *P. regius* and *A. childreni* that were gently manipulated but otherwise unrestrained. To limit struggling by the wild-caught *L. fuscus* females, we briefly anaesthetized each female with isoflurane to conduct the ultrasound examinations within 18 h of capture. For *P. regius* and *A. childreni*, we used a portable ultrasound system and a 7.5-MHz linear array transducer (Concept/MCV, Dynamic Imaging). For *L. m. fuscus*, we used a newer portable ultrasound system and a 7.5-MHz linear array transducer with a lateral and axial resolution of 2.95–3.05 cm and ≤ 0.05 cm, respectively (SonoSite, Inc.). We adjusted the depth and screen contrast settings on the instruments to allow for the best visualization of the internal anatomy.

We liberally applied a layer of conductive gel to the snake's body and made a series of ventral and lateral scans of the lower third of each individual's body. We determined the presence of eggs by the soft-tissue echogenicity of the egg yolks, as well as the more echogenic shells (Fig. 1). We counted the number of viable eggs and nonviable ova (as determined by their smaller size and/or lack of a shell) during every procedure. Because the length (anteroposterior axis) of *P. regius* and *L. fuscus* eggs often exceeded the width of the ultrasound transducer, estimates of mean viable egg mass (and, thus, clutch mass) were often unfeasible. Thus, we made a single estimate of egg number for each *P. regius* in 2009 (mean ± SE: 24 ± 6 days pre-oviposition) and *L. fuscus* (mean ± SE: 33 ± 2 days pre-oviposition). In 2009, we serially measured *A. childreni* ($N = 14$) to determine the effect of gravidity stage on estimates of viable egg number, nonviable ova number, mean viable egg mass, and clutch mass (mean ± SE: 15 ± 1 days and 7 ± 1 days pre-oviposition). Similarly, we made weekly estimates of clutch size and nonviable ova number in two *P. regius* in 2010 (1–49 days pre-oviposition).

For estimates of mean viable egg and clutch mass in *A. childreni*, we captured still images of three randomly chosen viable eggs and measured their lengths and widths using the virtual calipers provided as part of the ultrasound system's software (± 0.1 mm) (Fig. 1). In 2010, we used a modified scanning technique (as opposed to measuring egg dimensions solely from still images) to estimate the length of viable eggs for the two *P. regius* because egg length often exceeded the width of the transducer and viewing screen. Similar to estimates in *A. childreni*, we used the digital calipers to determine the dimensions of three randomly chosen viable eggs. However, we slowly moved the transducer along the anteroposterior axis and used the echolucent shadow of a rib as an additional point of measurement. We then
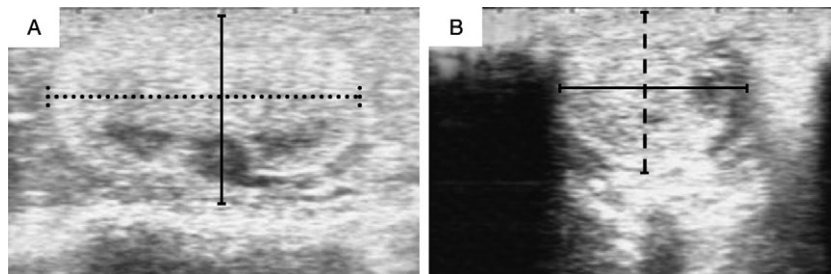

Figure 1. Ultrasound scans of one viable Children's python (*Antaresia childreni*) egg from lateral (A) and ventral (B) aspects of the female. Dense tissues (e.g. eggshell and bone) are echogenic and appear lighter in the scan. Linear measurements of egg length (dotted), width (solid), and height (dashed) were used to estimate viable egg volume and mass.

Table 1. Actual and estimated reproductive characteristics of ball pythons (*Python regius*, N = 7 after pooling 2009 and 2010 data), Children's pythons (*Antaresia childreni*, N = 14), and water pythons (*Liasis fuscus*, N = 10)

	Gravid female mass (g)	Number of viable eggs (clutch size)	Number of nonviable ova	Mean viable egg mass (g)	Mass of viable eggs (clutch mass, g)
Python regius					
Actual	1729.9 ± 279.2	5 ± 1	0 ± 0	82.6 ± 6.4	381.3 ± 35.1
	–	5 ± 1	0 ± 0	–	–
Estimate*		(3)	(0)		
Antaresia childreni					
Actual	450.7 ± 24.5	8 ± 1	2 ± 1	10.8 ± 0.4	83.3 ± 8.4
	–	8 ± 1	1 ± 0†	5.0 ± 0.3†	46.3 ± 3.4†
Early estimate (approximately 15 days pre-oviposition)		(12)	(68)	(54)	(38)
	–	8 ± 1	2 ± 1	6.9 ± 0.4†	55.1 ± 5.9†
Late estimate (approximately 7 days pre-oviposition)		(5)	(0)	(36)	(34)
Liasis fuscus					
Actual	2139.6 ± 169.1	13 ± 1	1 ± 0	48.8 ± 2.4	627.9 ± 81.6
	–	13 ± 1	0 ± 0	–	–
Estimate*		(3)	(43)		

Values are displayed as the mean ± SEM, and the relative error [100–100 × (estimated/actual)] is displayed parenthetically in italics as a percentage.
*Because the dimensions of *P. regius* and *L. fuscus* eggs often exceeded the width of the ultrasound transducer, estimates of mean viable egg mass (and, thus, clutch mass) were often unfeasible. However, for error in weekly ultrasound estimates of mean viable egg mass for two *P. regius*, see Fig. 3.
†Statistically different than the actual values.

determined viable egg length (anteroposterior axis) as the sum of the two line segments: (1) distance from the anterior edge of the egg to the rib and (2) distance from the rib to the posterior edge of the egg. To estimate the volume of each viable egg, we used the following volumetric equation for a scalene ellipsoid:

$$V = \frac{3}{4}\pi lwh$$

Where V = egg volume, l = egg length, w = egg width (left-right axis), and h = egg height (dorsoventral axis). After oviposition, python eggs adhered to one another and formed a clutch conglomerate composed of irregularly-shaped eggs. This aspect made linear and, thus, volumetric measurements of post-oviposition eggs improbable by conventional means. Hence, we used ultrasound estimates of egg volume as proxies for egg mass because snake eggs are composed primarily of H_2O (i.e. 70–76%; 1 g mL^{-1}) and lipoprotein (1–1.2 g mL^{-1}) (Mahley, 2001; Thompson & Speake, 2004). We determined estimates of clutch mass as the product of estimated clutch size and mean estimated egg volume. After their respective final ultrasound scans, we weighed gravid females (±0.1 g).

STATISTICAL ANALYSIS

Data met the appropriate assumption of parametric statistics or were transformed as necessary, and were analyzed using SPSS, version 15.0 (SPSS, Inc.). We determined experiment-wise, two-tailed significance at $\alpha < 0.05$ for all tests. We used paired *t*-tests to determine whether ultrasound estimates significantly differed from the actual values. To test relationships between estimated and actual values within individuals, we used simple linear regression analysis (e.g. estimated egg mass versus actual egg mass). All values are displayed as the mean ± SEM.

RESULTS

The estimates of viable clutch size for *P. regius*, *A. childreni*, and *L. fuscus* were highly accurate (Table 1). However, the results for *P. regius* in 2010 and *A. childreni* suggest that the timing of scans may influence the accuracy of viable clutch size estimates (Table 1). Estimates of nonviable ova number during early-stage gravidity in *A. childreni* significantly differed from the actual values at oviposition. However, there was no difference between nonviable ova
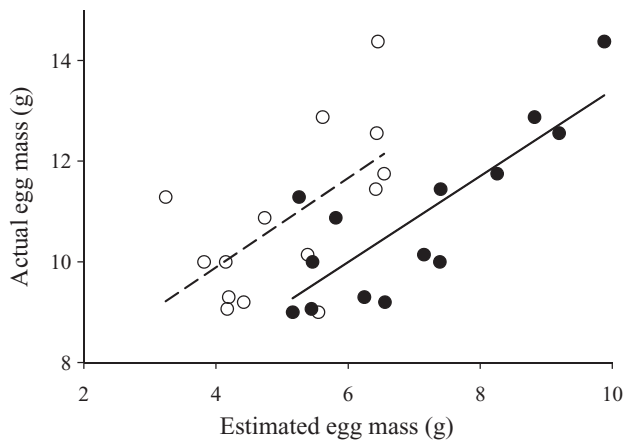
Figure 2. Although ultrasound estimates of mean egg mass were significantly lower than the actual values in Children's pythons (*Antaresia childreni*), significant relationships between estimated and actual values existed during two stages of gravidity: early (approximately 15 days pre-oviposition; ○, $F_{1,12} = 7.0$, $P = 0.021$, $R^2 = 0.37$) and late (approximately 7 days pre-oviposition; ●, $F_{1,12} = 24.6$, $P < 0.001$, $R^2 = 0.67$) ($N = 14$).

estimates made during late-stage gravidity and actual nonviable ova at oviposition (Table 1). As in Gilman & Wolf (2007), estimates of viable clutch size in small-clutching species (*P. regius* in the present study) were highly accurate, despite the relatively early sampling point (approximately 24 days pre-oviposition).

Although estimated viable egg mass was correlated to actual viable egg mass, our estimates of mean viable egg and viable clutch mass deviated significantly from the actual values (Fig. 2, Table 1). The slopes of estimated versus actual regressions for mean viable egg mass during early and late measurements were almost identical (early: 0.89, late: 0.86), although the *y*-intercepts deviated significantly (i.e. early: 6.4, late: 4.9) (Fig. 2). Furthermore, weekly ultrasound scans of *P. regius* in 2010 exhibited error in a nonlinear, yet predictable trend (Fig. 3). Thus, the timing of measurement clearly affected the estimated values of mean viable egg mass.

DISCUSSION

Taken together with the results previously reported by Gilman & Wolf (2007), the results of the present study provide strong support for the reliable use of ultrasonography to estimate clutch size in squamates, which represent 96% of living non-avian reptile species (Pough *et al.*, 2001). However, ultrasound estimates of viable egg and viable clutch mass deviated significantly from the actual values. Furthermore, estimates of viable egg and viable clutch mass were significantly affected by the timing of measurement.
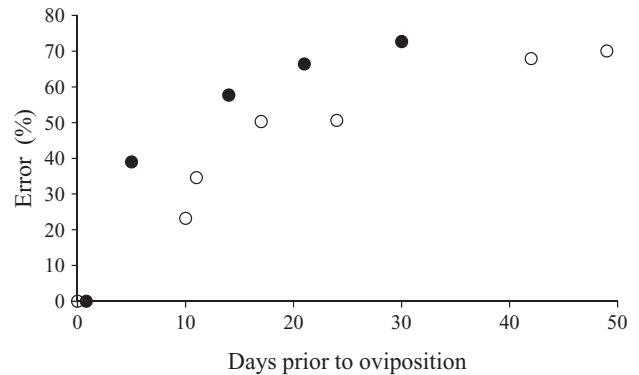

Figure 3. The error (i.e. the difference between estimated and actual values, %) of weekly ultrasound estimates of mean fertile egg mass throughout gravidity in two ball pythons (*Python regius*), denoted by (○) and (●).

We offer several explanations for the discrepancies between estimated and actual measures of mean viable egg and viable clutch mass. First, systematic error as a result of either operator inexperience or equipment limitations (e.g. resolution or accuracy) may have deflated estimates of viable egg size and, thus, viable clutch mass. Next, the shape of viable eggs *in utero* may have been overly irregular and non-ellipsoid, and this may have resulted in an underestimation of viable egg volume. Although we used the longest axes available in our estimates of viable egg volume, egg compression (as a result of the proximity of eggs to one another and abdominal volume constraints) may have confounded our ultrasound estimates, *sensu* Gilman & Wolf (2007). Third, our assumption that viable egg volume is equal to viable egg mass may have been inappropriate. Yet, even the most liberal estimate of viable egg density (i.e. 70% H_2O at 1 g mL^{-1} and 30% lipoprotein at 1.2 g mL^{-1}) yields only a 6% difference between viable egg volume and mass. Last, and most intriguing, the discrepancy between estimated and actual egg mass, along with the variation in estimated mass over time, might be attributable to real changes in the eggs during gravidity. Although nutrient deposition into eggs is limited to the vitellogenic stage, python eggs may undergo substantial influx of H_2O during gravidity, especially during the week before oviposition, as indicated by the dramatic late-stage changes in egg volume estimates in the serially evaluated *P. regius*. Gilman & Wolf (2007) noted a similar increase in egg volume over time in lizards.

Pythons may be under increasing selection to mobilize water into their eggs because their oviposited eggs are highly prone to desiccation and, unlike many other squamates, do not necessarily gain water during incubation (Ackerman & Lott, 2004;

Stahlschmidt *et al.*, 2008; Stahlschmidt, Heulin & DeNardo, 2010). Delaying a large proportion of the water investment into eggs until late-stage egg development would shorten the period of potential water stress for gravid females. However, a massive influx of water into the eggs just before oviposition could create an acute challenge to water balance. Furthermore, female pythons choose a nesting site during late-stage gravidity and then typically remain in the nest to brood their eggs for the duration of incubation (40–75 days depending on species). Thus, if free water is not accessible, the incurred water stress would last throughout the brooding period. Clearly, the timing of water investment into eggs could have a considerable impact on female homeostasis and therefore survival, as well as on the quality and survival of the offspring. Although our data, along with those of Gilman & Wolf (2007), provide some support for predominantly late-stage water uptake by squamate eggs, this phenomenon merits further consideration and experimentation that specifically assesses the timing of egg water accumulation.

The use of portable ultrasonagraphy has proven itself an effective means of non-invasively assessing some important aspects of reproductive investment and output in a range of reptile species (tortoises: Kuchling, 1989, lizards: Gilman & Wolf, 2007, and snakes: present study). Therefore, it can be a useful tool for examining population dynamics, life-history trade-offs, and conservation efforts in reptilian systems. As demonstrated by the results obtained in the present study, as well as by those previously reported by Gilman & Wolf (2007), it is vital that ultrasonography, similar to any other methodology, is assessed for its accuracy and repeatability before being specifically used in studies of species ecology, evolutionary life-history biology, population dynamics, physiology or conservation biology.

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