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Use of portable ultrasonography as a nondestructive method for estimating reproductive effort in lizards

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Summary

Obtaining population-level life history data such as egg and clutch size in reptiles has most often required that individuals be sacrificed. This prevents a reexamination of individuals over intra-annual and inter-annual time scales, limiting insight into the effects of varying environmental conditions on reproductive output. Here, we test the use of a laptop-sized portable ultrasound imaging system as a nondestructive means for quantifying reproductive investment in five species of lizards with a range of body sizes, forms and life histories. Ultrasound scans produced egg counts that were accurate for clutch sizes of two to seven eggs, and provided good estimates (within 5.5±1.69 eggs, mean ± s.e.m., relative error 21%) for clutch sizes of between 18 and 41 eggs. Egg measurements using virtual calipers produced average egg volumes that deviated from actual volumes by 0.09±0.01 cm^3 (relative error 25.9%), and estimated clutch volumes that differed from actual volumes by 1.03±0.26 cm^3 (relative error 29.5%). We also monitored development in five lizard species and found that changes in follicle and egg size and degree of embryonic development can be measured over periods of just a few days.

Key words: ultrasound imaging, lizard, egg size, energetics, clutch size, life history, reproduction.

Introduction

Studies of animal life history and the allocation of energy to reproduction, somatic growth, and storage have been central questions in evolutionary biology for at least six decades (e.g. Cole, 1954; Brockelman, 1975; Stearns, 1976; Ballinger, 1983; Heppell, 1998; Goodwin et al., 2002). Lizards have been one of the more prominent model organisms used to develop and test life history theory (e.g. Tinkle, 1969; Tinkle and Ballinger, 1972; Dunham et al., 1988; Adolph and Porter, 1993). In most studies, the acquisition of energy and its allocation to life history parameters has been determined by sacrificing individuals taken from populations and calculating average clutch and egg size (e.g. Shine, 1983; Jones and Ballinger, 1987; Mendez de la Cruz et al., 1988; Ramirez-Bautista and Olvera-Becerril, 2004). Then, data were usually combined with data on the age at first breeding, instantaneous mortality, and growth rates to test predictions from life history theory and trade-off models (e.g. Tinkle and Ballinger, 1972; Shine, 2005).

Although these data provide a measure of average reproductive investment at the population level, they provide only limited insight into individual variation in reproductive effort over intra-annual and inter-annual time scales. Such longitudinal within-individual variation is particularly important in studying species that show plasticity in reproduction in response to changes in seasonal or inter-annual environmental conditions such as fluctuations in precipitation and resource availability (Doughty and Shine, 1998; Shine, 2005). Population-level data from sacrificed individuals also provides only limited insight into lifetime reproductive effort.

Several non-destructive methods, including palpation, radiography, temporary housing of gravid females, and ultrasound imaging have previously been used to investigate reproductive dynamics and effort in reptiles, but each has unique limitations. Palpation provides information about clutch size, egg size, and reproductive stage in species with small clutch sizes. However, palpation can result in false negative diagnosis and cause follicle and egg damage (DeNardo, 1996). In addition, the accuracy of measurements of egg and clutch size using this method may be highly variable (Cuellar, 1971), and in viviparous species pregnant females are often diagnosed as non-reproductive (Gartrell et al., 2002). Palpation also provides no visual record and limited measurable data that can be reviewed later. Radiography has been used to diagnose reproductive stage, egg size and clutch size, but this method poses potential risk to the operator and damage to the animal. More importantly, embryonic development is not visible until later stages of development when calcification of bones occurs

(Gartrell et al., 2002). Temporary housing of gravid females until oviposition provides information about offspring and allows re-introduction of females and offspring to the original capture sites, but it provides limited information about egg development and requires housing the animals for days or longer in an artificial environment, potentially affecting their biology (see Sinervo and Doughty, 1996; Sinervo et al., 2001; Sinervo and Zamudio, 2001; Sinervo et al., 2006).

The use of ultrasound imaging on animals began in the 1970s with veterinarians and its use has continued to expand with time and advances in technology (Hildebrandt et al., 1998). Studies published using ultrasound imaging have included such diverse taxa as chicken (Melnychuk et al., 2001), birds (Newton, 1993; Sears, 1998; Dietz et al., 1999; Dekinga et al., 2001), cattle (Chupin and Procureur, 1983; Perkins et al., 1992; Herring et al., 1994; Pierson and Ginther, 1998), pigs (Chiba, 1995), molluscs (Haefner et al., 1996), fish (Reimers et al., 1993; Evans et al., 2004) and badgers (Woodroffe, 1995) [see also Hildebrandt et al. (Hildebrandt et al., 1998) and Starck et al. (Starck et al., 2001) for review and technical information].

Ultrasound imaging has also been used on reptiles to estimate clutch sizes (Kuchling, 1989; Hellgren et al., 2000) and monitor egg development (Kuchling, 1989; DeNardo, 1996; Hellgren et al., 2000) and embryonic stage (DeNardo, 1996; Gartrell et al., 2002). Thus far, however, it has been used in the field only on a limited basis and none of the studies published have systematically investigated how well reproductive output can be estimated using ultrasound imaging (Robeck et al., 1990; Rostal et al., 1990; Love et al., 1996; Kuchling and Razandrimamilafiniarivo, 1999; Martinez-Torres et al., 2006). Additionally, although ultrasound imaging has been proved to be an accurate and reliable technique in many larger taxa, we felt it was important to investigate the accuracy of this technique in small lizards, given the unique challenges they present (i.e. potentially many eggs contained in a relatively small area). Finally, collections of large numbers of individuals are becoming more difficult to justify in some research environments, and this study provides insight into the effectiveness of this nondestructive alternative.

Given the recent introduction of portable laptop-sized ultrasound imaging systems, our goal was to test the effectiveness of ultrasound imaging as a field-worthy, non-destructive tool for quantifying follicle and egg size, as well as egg volume, clutch volume and clutch size in lizards. We were also interested in establishing if this technique was equally effective for lizards with differing body forms and clutch sizes, and how operator experience level affected the outcome of this approach. We thought that training level was an important aspect of understanding if ultrasonography could be used more widely in the field by biologists that may have not previously been exposed to the technology. Although our study was based in the lab to facilitate the dissections used for comparisons, all of the methods and equipment used are directly transferable to the field. In this study we compared measurements and quantities obtained from ultrasound images to actual values from dissection in individuals in five oviparous lizard species. We also monitored egg development nondestructively in individuals of three oviparous species and embryonic development in two viviparous species to determine in what time scale changes in reproductive development could be detected.

Materials and methods

We collected, scanned and dissected 45 lizards of five species during the spring and summer months of 2004 and 2005 to determine the accuracy of ultrasound estimated clutch sizes and volumes (University of New Mexico Animal Care Protocol Approval # A4023-01). These included 12 *Uta stansburiana* (Baird and Girard 1852), 12 *Crotophytus collaris* (Say 1823), six *Phrynosoma cornutum* (Harlan 1825), nine *Cophosaurus texanus* (Troschel 1850), and four *Aspidoscelis tesselata* (Say 1823) and two *Aspidoscelis tigris* (Say 1852; combined into one group because of morphological similarities between the two species). Lizards ranged in size from 41.1 to 103.0 mm SVL (snout-vent length) and had body masses that ranged from 1.80 to 90.06 g. Lizards were collected in central New Mexico (Bernalillo and Socorro counties) at sites of variable habitat. Animals were placed in cloth bags and transported live to the lab within 6 h of capture. Any lizards that were not immediately processed were housed in 40 l tanks filled with approximately 5–7 cm of sand, and were supplied with water, fed crickets, and placed on a 8 h:16 h light:dark cycle. All animals were scanned within 5 days of capture.

Ultrasound scans were performed on live animals from 10 June to 2 August 2004 and 28 April to 1 August 2005. Prior to scanning, lizards were weighed and snout–vent length was measured with calipers. Each lizard was placed on its back on a restraining board, which consisted of a 25×40 cm plastic cutting board with small holes drilled through the middle on a rectangular grid at 0.5 cm increments. The relative position of the each animal was indexed to a ruler embedded into the restraint board to provide relative position for comparing scanned images to dissections. Elastic shock-cord approximately 2 mm diameter was pulled in loops through the holes, the lizard's legs were placed in the loops, and the loops were tightened with spring-loaded cord locks on the underside of the board. This provided effective restraint for the ultrasound procedure and once in this position the lizards rarely moved.

Scans were performed using a SonoSite Titan Portable Ultrasound system and a Titan L38 5–10 MHz broadband linear array transducer with a lateral and axial resolution of 2.95–3.05 cm and $\leqslant$0.05 cm, respectively (SonoSite, Inc., Bothell, WA, USA). The depth settings on the instrument were varied from 2.8 to 4.6 cm to allow for the best visualization of the internal anatomy. We also varied screen contrast level to allow for optimal visualization. Because the ventral surfaces of lizards are convex and irregular when gravid, we applied a relatively thick layer of gel (0.25–0.5 cm) in order to allow for continuous scanning and minimal interference in the signal transmission. The ultrasound system was connected to a battery-powered portable Hi 8 digital video recorder with

screen (Sony Digital Video Walkman GV-D800, Sony Corporation, New York, NY, USA) through an S video connection and scans were recorded for future analysis. We made a series of ventral transverse, sagittal and coronal scans of each individual. These scans included the capture of continuous video and still images. Follicle and egg (hereafter written as egg for simplification, unless clarification is necessary) lengths and widths were recorded on still images using the virtual calipers provided as part of the Titan's software, and clutch size was recorded as observed.

Upon completion of the scanning procedure, the animal was left in place on the board and the ultrasound gel was carefully removed using paper towels and warm water. Once clean, the lizard was sacrificed by cardiac injection of 1.0 ml Sleep Away (26% sodium pentobarbital; Fort Dodge Animal Health, Fort Dodge, IA, USA). A ventral incision was made from the sternum to the vent and the abdominal wall was reflected and clamped with hemostats to expose the internal organs and eggs. Digital photographs were taken with the eggs in situ for later reference. The eggs were then removed, counted, weighed to ±0.01 mg (Mettler Toledo AX205, Mettler-Toledo International Inc., Columbus, OH, USA), and measurements of the height and diameter at the widest point taken to the nearest 0.01 cm with digital calipers (General Tools Mfg. Co., NY, USA). Egg volumes were calculated by using the mean egg lengths and widths (measured on two axes) for all eggs in each clutch, and either the standard ellipse ($4/3\pi a^2 b$) or spherical volume equation ($4/3\pi a^3$), as necessitated by the shape, where a is half the diameter and/or height, and b is half the length. Total clutch volumes were estimated by multiplying the average volume of the eggs from each individual by egg number.

We monitored reproductive development over time in one or two individuals of five species: one *Holbrookia maculata* (Girard 1851), two *U. stansburiana*, two *C. collaris*, one *Phrynosoma hernandesii* (Girard 1858), and one *Sceloporus poinsettia* (Baird and Girard 1852). Prior to scanning, we obtained a body mass and measured the snout–vent length of each animal, and then restrained and scanned them as described above making transverse and longitudinal still and live scans to obtain egg dimensions. Animals were housed individually or in groups of two under the same conditions listed previously. When shelled eggs were observed during scans, the oviparous species were provided with 10.2×14.6 cm plastic containers filled with at least 7.5 cm of moist Perlite to serve as nests. Animals were scanned and weighed at least weekly and were housed until oviposition or parturition. Animals were then released at their respective capture sites.

Results

Summaries of mass, snout–vent length, clutch size, clutch volume and egg length for each species group used in the validation study are listed in Table 1.

Egg counts, measurements and stage determination

In all species, pre-vitellogenic and vitellogenic follicles were spherical in shape and progressively echolucent (appearing dark) to echogenic (appearing light). Mid- and late-stage eggs were elongate and echogenic (see DeNardo, 1996). Shelling could be detected by the overall echogenic appearance of the eggs, and in later stages by the separation of the echolucent albumin from the echogenic yolk. In species with small clutch sizes, with increasing operator experience we were able to correctly categorize all ova as follicles or ovulated eggs simply based on their general appearance on the scans. Of the six *P. cornutum* we used for this validation, four had shelled eggs and two had early-stage vitellogenic follicles. Without any individuals containing late-stage follicles, we were not able to compare the appearance of late-stage follicles to ovulated eggs and could not determine if we would be able to correctly categorize late-vitellogenic *versus* early ovulation egg stage in this species.

We found that the easiest way to count both follicles and eggs was to hold the transducer in a transverse orientation and slowly move it from the chest to the pelvis and back again, and count how many eggs or follicles appeared and disappeared from view. Once a count was obtained, we repeated the process along the sagittal plane, moving the transducer from one side to the other and back again. If the counts did not match, we repeated this process until we were confident of our estimate. Occasionally, the stomach and intestines appeared similar to eggs and follicles in cross section. However, using the method above, we were able to distinguish the stomach and intestines, which are continuous structures, from the ova, which appear either spherical or ellipsoidal. This scanning method was useful in distinguishing ova from other anatomical structures in all but one lizard. One *C. collaris* had an unidentified structure that was counted as an egg because it appeared similar (in density,

Table 1. *Individuals used in the validation portion of the study*

Species	*N*	Mass (g)	*SVL* (mm)	Clutch size	Clutch volume (cm^3)	Egg length (cm)
Aspidoscelis tesselata/tigris	6	21.42±2.01 (15.73–27.68)	91.22±2.47 (81.8–97.60)	4±0.42 (2–5)	2.80±0.41 (1.41–4.44)	1.47±0.19 (0.88–2.04)
Cophosaurus texanus	9	8.59±1.45 (4.40–19.52)	59.06±1.14 (52.60–62.90)	4±0.31 (2–5)	1.06±0.15 (0.40–1.49)	1.12±0.13 (0.69–1.63)
Crotophytus collaris	9	27.45±1.89 (20.70–34.98)	88.43±1.69 (81.10–92.70)	5±0.48 (2–7)	4.56±1.08 (0.73–10.76)	1.64±0.21 (0.32–2.20)
Phrynosoma cornutum	6	55.22±11.02 (24.30–90.06)	96.80±2.74 (89.00–103.00)	26±3.74 (18–41)	11.61±3.70 (0.58–20.26)	0.94±0.20 (0.37–1.51)
Uta stansburiana	12	2.96±0.16 (1.80–3.82)	44.31±0.53 (41.10–47.40)	4±0.23 (2–5)	0.60±0.07 (0.07–1.12)	0.91±0.07 (0.32–1.14)

SVL, snout–vent length.

Values shown are means and s.e.m. Ranges are shown in parentheses.

shape and size) on the scans to the other eggs in the abdomen. However, upon dissection, we found this structure was not an egg, but a tumor-like growth in the intestine.

Measuring eggs can be difficult, given their compressible nature (see Discussion), however, we found that the most accurate measurements could be obtained by first moving the transducer transversely and sagittally (and occasionally diagonally) to visually 'trace' the outline of an egg, before attempting to use the calipers. This approach allowed us to get oriented to the egg shape, and more accurately measure the longest and widest dimensions of the egg. It also allowed us to get a feel for the overall size and shape of all the eggs in the abdomen, so we could avoid measuring any eggs that appeared to be compressed.

Operators should also be aware that ova within a single individual may be in different stages of development. For example, two individuals (*Aspidoscelis*) had both shelled eggs and vitellogenic follicles that were clearly distinguishable on the scans (see Fig. 1). Two individuals (*C. collaris* and *C. texanus*) had two distinct groups of vitellogenic follicles, and in the *C. collaris* the two sizes of follicles (N=5, ~6.3 mm; N=2, ~3.8 mm) were clearly visible on the scans. However, in some individuals, the close proximity of small follicles to larger ones may make the smaller follicles difficult to distinguish. For example, we were unable to detect the single smaller follicle (3.2 mm) found during dissection of the *C. texanus* female because it was attached to a larger follicle (6.85 mm), and did not appear as a discrete ovum.

Analyses

In three *C. collaris* that were scanned but not included in the statistical analyses, large amounts of material were found in the digestive tract. No follicles were observed during the scanning procedure of two of these females, although dissection revealed that follicles were present and were in the size range that should have been visible on the scans (~4.5 mm). In the third individual, follicles were observed, but were difficult to count and measure. Dense materials, such as bones or exoskeletons of prey, reflect an ultrasound signal, and cause a shadowing effect on the structures underneath (Starck et al., 2001). The large amounts of material in the digestive tracts of all three females blocked the transmission of the ultrasound, and prevented us from visualizing the ova. The three sets of scans were done early in the study, before we were aware that large amounts of food in the intestines could be a potential challenge with this species (see Discussion).

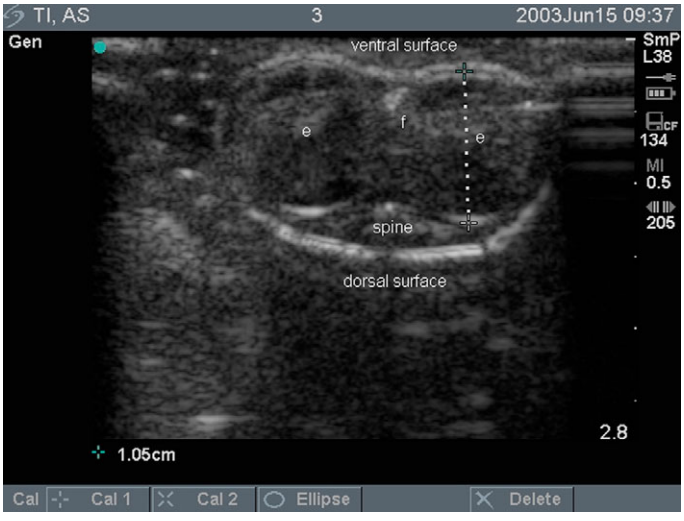

Fig. 1. Transverse scan of one female *A. tesselata*. Dense tissues (skin, bone, egg shell) appear lighter (echogenic) on the scan. This individual was observed to have two large, shelled eggs (e) and a single vitellogenic follicle (f) located between them, close to the ventral surface. Virtual calipers (dotted white line) show the height of one of the eggs (height in cm is shown to the bottom left of the image).

The relationship between clutch size estimated using ultrasound images and actual clutch size from dissection for all individuals was highly significant (Fig. 2A). Because the species groups fell into two distinct categories of clutch size (2–7 eggs for all species except *P. cornutum*, and 18–41 eggs for *P. cornutum*), we evaluated these categories separately. There was no significant difference in the relative discrepancies ($|1-i_{estimated}/i_{actual}|\times100$, absolute relative difference between estimated and observed values, where i is the value for each individual) between estimated and actual clutch sizes between years (Mann–Whitney P=0.5548), or between the first 21 and last 21 animals used for the study (Mann–Whitney P=0.8097), so the data for both years were combined. For smaller clutch sizes (2–7 eggs) ultrasound-based egg counts deviated from the true counts by 0.39±0.11 eggs (mean ± s.e.m. of the absolute differences), which represented a relative difference of 10.1% (Table 2). For large clutch sizes (18–41 eggs), the difference between ultrasound and true counts was 5.50±1.69 eggs (mean and s.e.m. of the absolute differences, which represented a relative difference of 21.1%).

The relationship between estimated average egg volumes from ultrasound images and actual average egg volumes from dissection for all individuals was highly significant (Fig. 2B). Average egg volumes for individual clutches ranged from 0.02 to 3.30 cm^3 (0.54±0.09 cm^3; ± s.e.m.). There was no significant difference in the relative discrepancies between estimated and actual average egg volumes between years (two sample t-test P=0.495), or between the first 21 and last 21 animals used for the study (two sample t-test P=0.636), so the data for both years was combined. Estimated average egg volumes differed from actual volumes by 0.10±0.02 cm^3 (mean ± s.e.m. of the absolute differences), which was about a 26% difference relative to the volume of the eggs.

The relationship between estimated clutch volumes using ultrasound imaging and actual clutch volumes from dissection for all individuals was highly significant (Fig. 2C). Total clutch volumes ranged from 0.07 to 20.26 cm^3 (3.52±0.79 cm^3 mean ± s.e.m.). There was no significant difference in the relative discrepancies between estimated and actual clutch volumes between years (two sample t-test P=0.958), or between the first 21 and last 21 animals used for the study (two sample t-test P=0.696), so the data for both years was combined. Estimated

clutch volumes differed from actual volumes by 1.03±0.26 cm^3 (mean ± s.e.m.), a difference of about 30% relative to the volume of the clutches.

Reproductive development over time

The two *U. stansburiana* had eggs that progressed as expected from early-stage follicles (0.40 and 0.41 cm average diameter), through ovulation, to shelled eggs (1.04 and 0.94 cm average length, *N*=4 eggs each), over a 32- and 24-day period, respectively (Fig. 3). Weekly increases in egg diameter and changes in egg shape (e.g. from round follicle to elongated ovulated egg) were clearly visible. The single *H. maculata* followed had eggs that initially progressed as expected, increasing in size from 0.22 cm average diameter to 0.69 cm average length (*N*=5). However, shortly following ovulation, we were unable to discern the boundaries of the individual eggs (i.e. the clutch appeared as a mass with no distinct dimensions). This continued until the eggs became shelled, at which point the egg lengths were again measurable, although only once, with an average length of 0.91 cm. Scans for this individual continued for 68 days, at which point the animal was sacrificed and the eggs dissected out. The average length of the dissected eggs was 0.99 cm. Both *C. collaris* had vitellogenic follicles that initially increased in size (from 0.75 and 0.93 to 1.08 and 1.12 cm average diameter) but shortly before the estimated ovulation date (based on the size of the follicles) the follicles began to regress (to 1.02 and 0.93 cm average diameter) and were reabsorbed. This process occurred over 11 days for one lizard and 52 days for the other individual. Because we believed these animals would not reproduce in captivity during the study period they were released at their capture sites.

Because the *C. collaris* did not lay their eggs in captivity, we were unable to compare the estimated number of follicles to the actual number. However, with both *C. collaris*, the number of follicles observed on the scans remained constant throughout the study, indicating a great likelihood that our estimations were correct. We were unable to count the number of follicles in the *H. maculata* because they were too small to distinguish individually until 2 weeks into the study when they had reached 0.37 cm average diameter. We were also unable to

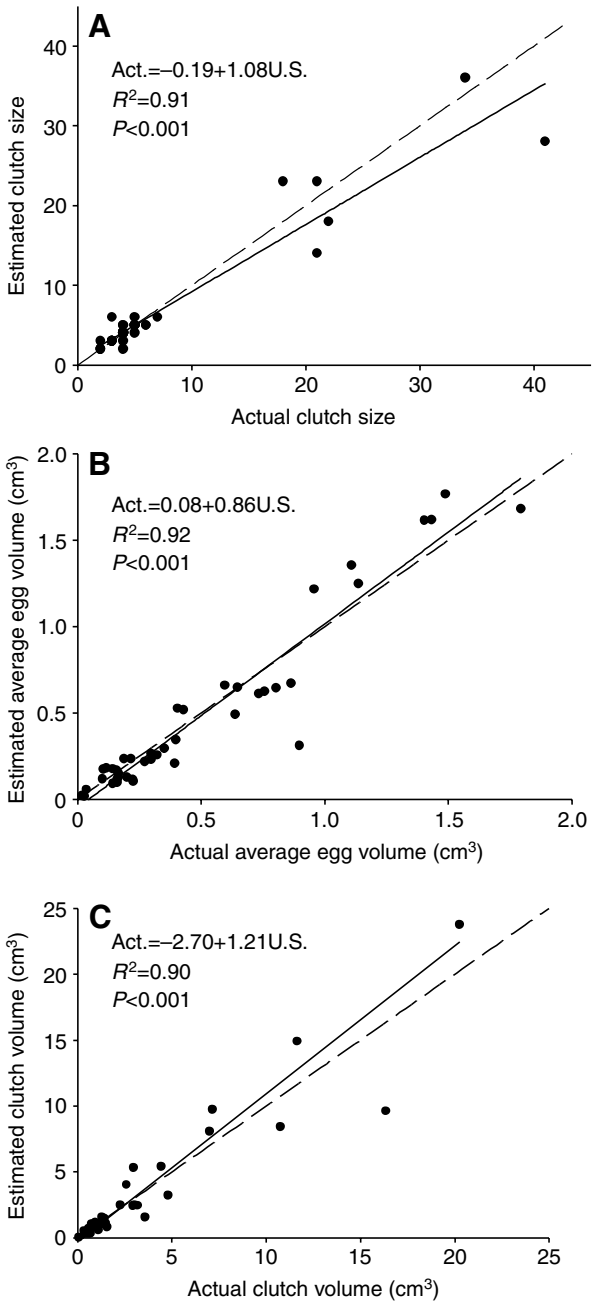

Fig. 2. Relationships between ultrasound-estimated values and actual values determined from dissection for (A) clutch size, (B) average egg volume and (C) clutch volume. Each graph shows values for all individuals (*N*=42). The dotted lines indicate the isometric reference and the solid lines represent the regression equations fit to the data. Act., actual value; U.S., ultrasound estimate.

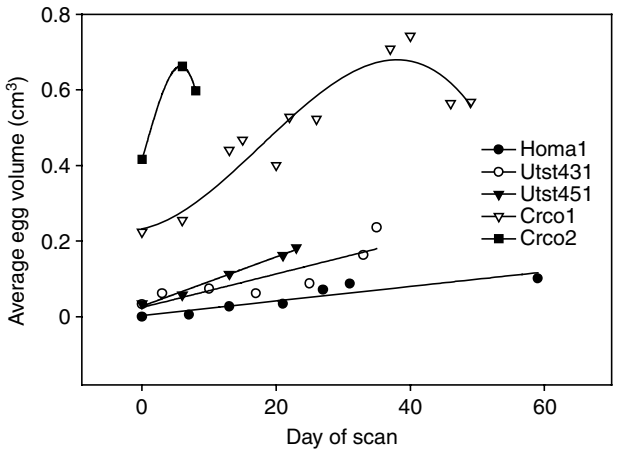

Fig. 3. Changes in average egg volume for each clutch throughout the duration of the study. Data shown are for one *H. maculata* (Homa1) and two *U. stansburiana* (Utst431 and Utst451), all of whom had clutches that progressed from small follicles to shelled eggs, and two *C. collaris* (Crco1 and Crco2), whose follicles increased in volume initially but then decreased prior to ovulation. Lines are fit solely to illustrate the trends.

Table 2. *Absolute and relative differences in clutch size, average egg volume and total clutch volume between values estimated using ultrasound and actual values from dissection*

		Clutch size		Average egg volume		Total clutch volume	
Species group	*N*	Absolute difference (no. of eggs)	Relative difference (%)	Absolute difference (cm^3)	Relative difference (%)	Absolute difference (cm^3)	Relative difference (%)
Aspidoscelis tesselata/tigris	6	0.67±0.49	20.8	0.15±0.03	18.3	0.65±0.21	21.9
Cophosaurus texanus	9	0.22±0.15	5.0	0.07±0.02	22.3	0.28±0.07	23.9
Crotophytus collaris	9	0.67±0.17	15.8	1.20±0.05	27.2	1.44±0.31	36.0
Phrynosoma cornutum	6	5.50±1.69	21.1	0.07±0.04	23.9	3.61±1.32	29.5
Uta stansburiana	12	0.17±0.22	4.2	0.05±0.01	32.3	0.19±0.04	32.7
All species except *P. cornutum*	36	0.39±0.11	10.1	–	–	–	
All species	42	–	–	0.10±0.02	25.9	1.03±0.26	29.5

Absolute values are given as mean and s.e.m., and relative differences are given as means. *N* = number of individuals per species group.

count eggs in that individual during the time period in which the lengths were not clearly visible. Our estimations of egg number were accurate to ±0.3 mean eggs over six scans for the single *H. maculata*, ±0.2 mean eggs for one *U. stansburiana* over five scans, and correct for the other *U. stansburiana* in five scans.

In both viviparous females studied, we observed embryonic development as expected. The female of *S. poinsetti* was scanned over a period of 32 days. On the initial day of scanning we were able to see distinct fetuses and attached yolk masses. Fetal skeletons appeared as echogenic dots within the echolucent mass of the fetus. We used the technique outlined above to count yolk sacs and attached fetuses. Yolks appeared as echogenic masses spanning the length of the fetuses. 8 days later, we were able to visualize and count heartbeats of each fetus using the Titan's Doppler mode. Fetal size increased throughout the study as yolk size decreased. The structure of each skeleton became increasingly more detailed and identifiable (Fig. 4). At this point we used the individual heartbeats and distinguishable skeletal features (primarily the spine) of the fetuses to estimate the number of actual fetuses. At the end of 32 days, this female gave birth to six young. The mother died within a day of giving birth, and a necropsy revealed that there was one retained fetus (*N*=7 total young). We overestimated the number of fetuses by about three throughout the study (*N*=9.88±0.48 estimated fetuses), and actual and estimated counts were significantly different (paired *t*-test: $P<0.001$, *N*=7 scans).

We scanned the female *P. hernandesii* over a period of 54 days. The initial scans of this individual showed large echogenic masses, similar in appearance to shelled eggs, although they appeared asymmetrical as opposed to elongate. Two weeks later, the yolk masses contained small areas of echolucent material (embryos). Embryos increased in size throughout the study as yolk masses decreased. Fetal heartbeats were first observed on day 18 of the study. Skeletal components and movement were first observed on day 31 of the study. We estimated the number of young by counting the number of visible yolk sacs and attached embryos, or heartbeats, depending on the development of the embryos. Embryos continued to grow and anatomical structures became more identifiable until the 54th day of the study, when this individual gave birth to 22 young. The estimated number of fetuses (*N*=17.71±2.67, mean ± s.e.m.) was not statistically different from the actual number (paired *t*-test: *P*=0.08, *N*=7 scans).

Discussion

The small physical size, durable weather-resistant case, long battery life and high imaging resolution of current ultrasound imaging systems make it possible to use this highly accurate non-destructive method for the quantification of reproductive parameters in small animals in the field. Scans can be saved to a flash-disc and/or recorded to video for later review in the lab. These scans can, with high accuracy, provide life history data such as clutch size, clutch volume, egg and embryonic developmental stage. If used repeatedly on the same individuals, they provide longitudinal time series data and insight into lifetime reproductive effort.

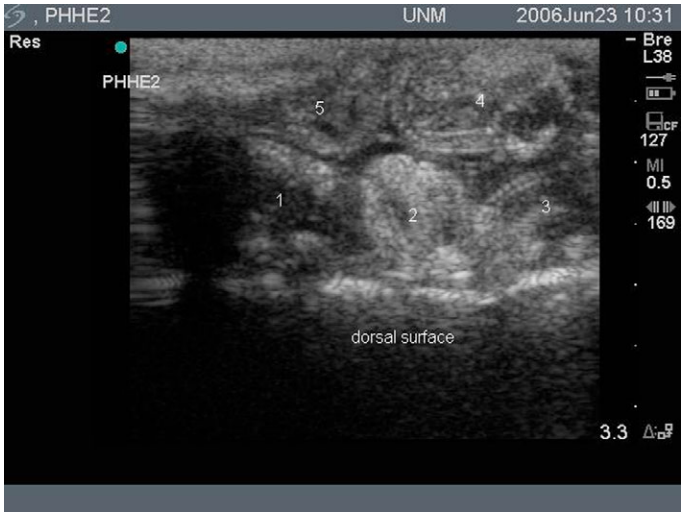

Fig. 4. Sagittal scan of one *P. hernandesi* female, showing partial skeletons of at least five well-developed embryos. This scan was made 42 days after the initial scan and 12 days before the young were born.

Our lab-based study shows that this nondestructive method has several advantages over other invasive and non-invasive approaches. It produces estimations of clutch size, average egg size, and clutch volume that are reasonably accurate, and this level of accuracy holds over a range of species that vary in size, shape, and life history. Additionally, ultrasound imaging allows for the detection of changes in egg size and shape and embryonic development over short periods of time, making this tool useful for re-examining individuals to measure changes in reproductive activity over the course of a breeding season. We were able to follow egg development (from small follicles through shelled eggs) in three of our five gravid individuals. In the remaining two lizards (*C. collaris*), we were able to document the initial increase and subsequent decrease in follicle size prior to the anticipated ovulation date. The ability to rescan individuals over time to detect a decrease in vitellogenic follicle size or to compare the number of vitellogenic follicles to ovulated eggs will be useful in field studies of species that show a high incidence of later-stage follicle atresia (e.g. Lemos-Espinal et al., 1999). Because this method can be used nondestructively to re-examine individuals not only over the same season, but over years, it could be particularly effective in areas where populations are small and perhaps endangered or where there is substantial variability in the reproductive output of individuals from year to year (e.g. Ballinger, 1977; Doughty and Shine, 1998; Lemos-Espinal et al., 1999; Shine, 2005). Another benefit of using this method is the possible identification of both vitellogenic follicles and ovulated eggs, as observed in two *Aspidoscelis.* Although we were unable to count the total number of follicles because not all of them were clearly visible with the large eggs present, we were able to clearly identify the potential for two clutches in a breeding season.

We found that clutch size estimations were accurate for species with small clutch sizes (Table 2). For example, in *U. stansburiana* and *C. texanus* the relative clutch size discrepancies were 4.2 and 5%, respectively. The larger discrepancies in *C. collaris* and *A. tesselata* (15.8 and 20.8%), can be attributed to one individual in each group. Both of these individuals were scanned early in the study when the operator had only limited experience with the system. Removing those individuals from the analyses reduced the relative discrepancies to 11.6 and 11.2%. The discrepancies in *P. cornutum* were simply due to our inability to estimate the number of eggs when the clutch sizes were extremely large (clutch sizes ranged from 18–41 eggs). The layering of so many eggs on top of and next to each other made visualization and counting difficult (see Fig. 5). Although ultrasound can still provide valuable information in species with large clutch sizes (i.e. egg and embryonic developmental stages), exact measurements of clutch size may not always be possible.

Estimations of egg volume and clutch volume were less accurate, although still relatively good. Relative discrepancies in egg volume ranged from 18.3 to 32.3%. Variability in the accuracy of our estimations of clutch volume is largely due to the fact that clutch volume is a product of clutch size and average egg volume, so that any error in these estimations will be multiplied when calculating clutch volume. Removing the two individuals listed above (*C. collaris* and *A. tesselata*) reduced the relative discrepancies in clutch volume estimation in these groups from 36 and 22% to 31 and 15%. The error in *P. cornutum* clutch volume estimation is due both to the difficulty in estimating clutch size in lizards with large clutch sizes and error due to egg compression (discussed below).

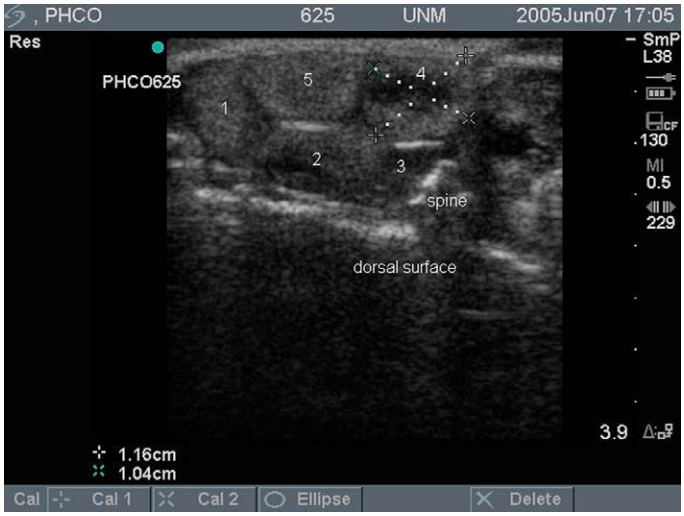

Fig. 5. Transverse scan of one *P. cornutum* female, showing five shelled eggs. Notice the asymmetry of the eggs caused by egg overlap and compression. Virtual calipers (dotted white lines) show the width of egg 4. Scan is focused on one side of the animal because the animal's width exceeded that of the transducer, as was the case in all individuals of *P. cornutum*.

We believe that there are other factors that may affect the accuracy of using ultrasound imaging. There is a learning curve associated with this method and the experience of the operator, including the ability to correctly identify anatomical structures and effective use of the instrument (e.g. placement of the transducer, applying the most effective amount of gel, and speed of live scans) can influence the amount of error. For example, we found that when scanning *C. collaris* in particular, there is a greater tendency to compress the eggs than in other species. This compression can result in variable egg measurements within the same individual. A better practice for some species may be to take at least two sets of measurements and averaging the values. However, we feel that once initial validations are performed for a study, the experience level of the operator will not necessarily alter the overall accuracy. This can be seen in the lack of significant difference in error over the two years of the study.

In addition to the learning curve that determines skill level, we found several species-specific challenges. The presence of large amounts of prey in the intestines of two *C. collaris* prevented us from being able to visualize the underlying follicles. Without validation this could have resulted in no vitellogenic follicles being recorded in these individuals. This was the only species in our study that we found ingested

enough food to cause a problem. Fasting the animal or scanning from the dorsal surface of the animal if food is noted in the gut might help to overcome this obstacle (although we did not attempt this at the time of the scans). Another example of a species-specific challenge was seen in the single *H. maculata*. We scanned this individual for 68 days, which is an unusually long reproductive period. It is unclear if our inability to count and measure the eggs following ovulation was a unknown physiological side effect of captivity, is typical for this species, or was unique to this individual. Further work with this species would be necessary to answer this question.

There is one last source of error worth noting, which can occur in any species. Lizard eggs are quite plastic, and compression and overlap of these eggs on each other can make measuring full lengths and widths difficult, especially in large clutches, such as those of *P. cornutum.* Even in smaller clutches, if the eggs are large and densely packed, the ends can overlap making length measurements difficult. However, we found that in most cases, enough eggs were sufficiently visible to estimate average length for the clutch, and that manipulation of the abdomen and rescanning the individual may allow for additional length measurements to be taken.

Even with the limitations described, we found that this approach for measuring reproductive effort in lizards can be quite accurate, and provide many types of life-history data. Additional advances in portable ultrasonography such as 3D capabilities will clearly add to the accuracy and utility of this approach for quantifying reproductive effort. Our study shows that ultrasonography has great potential as an alternative to destructive methods for quantifying reproductive effort of lizards in the field. We also believe that in single-species studies, where the operator becomes familiar with the specific challenges posed by a particular species, the level of accuracy will exceed that of our study.

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