

and predation refuge, but considerable future work is needed as these ecological benefits have not been clearly demonstrated (Bohnsack and Sutherland, 1985; Bohnsack, 1989; Lindquist et al., 1994).

Alternatively, the suggested benefits of artificial reefs may be reduced by high fishing mortality around the structures, as indicated by the 57% fishing mortality of ultrasonic tagged red snapper by hook-and-line fishers. However, eight of 23 ultrasonic tag returns in this study were from well-known public reefs (barges 1 and 2) that probably receive higher fishing pressure compared to privately deployed reefs.

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- DEPARTMENT OF FISHERIES AND ALLIED AQUACULTURES, AUBURN UNIVERSITY MARINE EXTENSION AND RESEARCH CENTER, 4170 COMMANDERS DRIVE, MOBILE, ALABAMA 36615. E-mail: szedlma@acesag.auburn.edu. Submitted: 3 Aug. 1996. Accepted: 12 March 1997. Section editor: S. T. Ross.
- Reproductive ecology often focuses on relationships between reproductive parameters and female body size. With most crocodylians, data for clutch size or egg dimensions can be reliably associated with the female who protects the nest. However, that approach is impractical for free-ranging *Crocodylus johnstoni* because females display no nest-guarding tendencies (Compton 1981; Webb et al., 1983b). This complication prompted us to test diagnostic ultrasonography as an efficient field tool to investigate reproductive characteristics of gravid female crocodylians. The technique is well suited for *C. johnstoni* because the species has a small clutch size (4-20 eggs), moderate body size, and lays a single clutch per year in a well-defined reproductive season. Therefore, follicle size classes will be distinct in any given year.
- We used ultrasound to characterize appearance, size, and number of follicles or eggs in gravid *C. johnstoni*. Ultrasound has been successful in assessing reproductive status in tortoises (Robeck et al., 1990; Rostal et al., 1994), freshwater turtles (Kuchling, 1989, 1993), marine turtles (Rostal et al., 1990, 1996), snakes (Smith et al., 1989), and lizards (Sainsbury and Gili, 1991; Anderson et al., 1996; Love et al., 1996). Ultrasound has been used to follow follicular development in captive *Caiman latirostris* (Vac et al., 1992) and hatchling development of *C. siamensis* (Brazaitis and Watanabe, 1983), but no studies have quantified clutch size for crocodylians by means of ultrasound. This investigation is the first assessment of ultrasonography to determine reproductive parameters in non-captive crocodylians.
- Materials and methods.**—We examined 36 wild females from the Lynd River near Mount Surprise, Queensland, and eight captive females from the Queensland Reptile Park near Beerwah, Queensland. Investigations coincided with nesting activity in late August to September for the wild population and October for the captive animals. We obtained crocodiles by hand capture or with nets. We placed crocodiles on their backs to obtain measurements for snout-vent length (SVL, to the nearest 0.1 cm). We placed crocodiles in a rope sling attached to a Salter 50 kg scale to measure their mass to the nearest

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0.2 kg. After processing, all animals were returned to their capture location.

We used ultrasound equipment (Ausonics Sonovet 1, Apscan Pty. Ltd., Lane Cove NSW 2066, Australia) with a 5.0 or 7.5 MHz mechanical sector scanner to scan body depths of 10.0-12.5 cm, depending on female size. A portable generator provided power. We restrained females on their backs and inserted a finger into the cloaca while external pressure was applied to the lower abdomen to void all urine or feces (Limpus, 1984). Ultrasound coupling gel (Aquasonic 100, Parker Laboratories, Orange, NJ) was applied before the transducer was placed in a ventrolateral position on the abdomen to scan. Left and right sides were scanned independently to simplify counting and minimize acoustic shadows from gastralia in the belly or osteoderms in the flanks. Distinct circular or elliptical images were frozen on-screen and measured with built-in electronic calipers. Average dimensions of eggs or follicles were calculated from three measured images per female. Ultrasound scans were conducted before laparoscopy to avoid possible sonic interference from air remaining in the abdomen after laparoscopy.

Ultrasound diagnoses were confirmed qualitatively by cloacal examination or laparoscopy for the wild population. Examinations consisted of inserting a finger via the cloaca into the lower intestine, voiding any fluids, and palpating through the intestinal wall for follicles or eggs (Limpus, 1984). For laparoscopic examinations, the lateral body wall anterior to the right hind limb was cleaned with alcohol and a 1-cm incision made in the body wall. A 7-mm O. E. trocar and cannula with valve were inserted through the incision. A Storz 26031B Hopkins telescope (O. D. 6.5 mm, forward-oblique viewing, 30° wide angle, incorporating fiber optic light transmission) connected to a Storz 482B cold light source was inserted through the cannula. The coelomic cavity was inflated after visually checking that the tip of the scope was between coils of intestine. Internal structures were described following Limpus (1984) and recorded on videotape. Chromic gut (3.0) was used to close the incision. Laparoscopies were performed without prior knowledge of the ultrasound results. Laparoscopy provided qualitative

information on reproductive status but no counts of egg numbers because of the physical restrictions of the viewing field.

Results acquired by ultrasound were compared with data from nesting surveys of the Lynd River (C. J. Limpus and K. McDonald, unpubl. data). Additional validation was obtained from captive females in the Queensland Reptile Park. Females were intercepted during exploratory nesting attempts in their enclosure and scanned. Confirmations of reproductive status or egg counts were obtained later from direct counts of nest contents.

Electronic measurements were checked against preserved reproductive tissues from a gravid female that died at the Lynd study site (3574†). The intact reproductive tract was placed in a water bath to scan and measure the diameter of each follicle with the on-screen electronic calipers. Follicle measurements were repeated with Vernier calipers and compared by a paired two-tailed *t*-test.

Results.—We examined a total of 45 cases, including a preserved reproductive tract and seven females that were examined twice each (Table 1). Once the cloaca and lower digestive tract were voided, recently ovulated (less calcified) and fully developed (well calcified) oviductal eggs appeared as echogenic ellipsoidal images (Fig. 1). Egg dimensions averaged 70×37 mm with ranges of 62–83 mm in length and 32–42 mm in width (Table 1). Eggs were more echogenic than vitellogenic or preovulatory follicles and thus easier to count (18 of 18 females). Vitellogenic and preovulatory follicles appeared as clusters of round echogenic images. Two general size classes of larger follicles were distinguished because some females were further advanced in vitellogenesis than others. The largest follicles were 32–38 mm diameter ($n = 11$ females) and regarded as mature preovulatory follicles (Table 1). Follicles less than mature-sized but regarded as in vitellogenesis for the present breeding season ($n = 3$ females) ranged from 22–27 mm in diameter (Table 1). A few smaller follicles (10–19 mm) were occasionally seen, but this size class was believed to be too small to mature in the same reproductive season, given that scans were conducted so close to the onset of nesting. Ten of 13 females with vitellogenic follicles were imaged well enough to provide a confident count (Table 1).

Laparoscopy ($n = 27$) or cloacal examinations ($n = 46$) confirmed that all gravid females were correctly diagnosed by ultrasound although two situations warranted comment. The first situation was a procedural difficulty when

three females were scanned before all intestinal fluids were voided. Follicles were detected and measured after a second effort was made to void all urine, but reliable counts were not possible. The second situation was in distinguishing between nonreproductive females and postoviposition females. For example, no remaining follicles were detected in female 263, but we could verify that the female was postoviposition rather than not breeding for that season after viewing corpora lutea via laparoscopy and noticing a loose cloaca that resulted from oviposition (Table 1). The same conclusion was reached for a captive female that retained a few enlarged follicles after nesting (see below).

Ultrasound detected no previtellogenic follicles in pubescent females ($n = 11$) or in nonbreeding adult females ($n = 1$) nor did it provide information on developmental state of the oviduct, i.e., immature animals could not be distinguished from adults. Other organs down to 10 mm diameter were imaged (e.g., loops of intestine and heart ventricles); therefore we concluded that previtellogenic follicles (2–4 mm in the preserved reproductive tract) were anechoic or below the equipment's resolution limit. Corpora lutea were visible with laparoscopy (10–15 mm diameter) but undetected by ultrasound, perhaps for the same reason. No atretic follicles were detected by either ultrasound or laparoscopy. It was unknown whether any follicles that remained in postovulatory and postoviposition captive females would become atretic or be retained until the next reproductive cycle.

In the wild population, clutch size increased significantly with female SVL ($F_{1,21} = 9.42$, $P = 0.006$) and mass ($F_{1,23} = 13.46$, $P = 0.001$), but there was no relationship between egg dimensions and SVL or mass (P -values ranged from 0.16–0.80). Linear relationships of SVL and mass against reproductive output (Fig. 2) were clutch size = $0.17(\text{SVL}) - 1.332$ and clutch size = $0.32(\text{mass}) + 8.95$, but these relationships explained only 32% and 38% of the variance, respectively. A condition index of mass to length ($\text{kg}/\text{cm SVL}$) gave minor improvement in explaining reproductive output (clutch size = $46.03[\text{condition index}] + 6.22$, $r^2 = 0.42$, $F_{1,21} = 14.18$, $P = 0.001$).

Average clutch sizes were similar between the ultrasound study ($\bar{x} = 13.8$, $SD = 2.4$, $n = 21$) and a Lynd nesting survey ($\bar{x} = 11.8$, $SD = 2.5$, $n = 19$), indicating that ultrasound gave a reasonable estimation of the average reproductive output. However, this small difference was statistically significant ($t = 2.58$, 28 df, $P < 0.05$), and given the relationship between body size and reproductive output (Fig. 2A), a difference

TABLE 1. COUNTS AND DIMENSIONS OF EGGS AND FOLLICLES FROM FEMALE AUSTRALIAN FRESHWATER CROCODILES (*Crocodylus johnstoni*) AS DETERMINED BY ULTRASOUND. Females are identified by numbers for the wild population, letters for the captive population, and † for the necropsied animal. Developmental states are: 0 = pubescent or not breeding that year, 1 = vitellogenic follicles, 2 = preovulatory follicles, 3 = recently ovulated, less calcified eggs, 4 = fully developed, well-calcified eggs, and 5 = postoviposition. Dimensions are the means ($n = 3$) of follicle diameters or average lengths and widths of shelled eggs. * indicates unreliable clutch count resulting from an incompletely voided bladder. ** designates a postovulatory female (5) diagnosed as having no follicles or eggs by ultrasound (0) but bearing corpora lutea when examined by laparoscopy and a loose cloaca when examined by cloacal palpation.

Female ID	W	SVL (cm)	Mass (kg)	Count (L, R)	Developmental state	Dimensions (mm)
3574†	95	72.1	8.8	6, 6	2	—
1905	95	72.4	7.3	0, 0	0	—
1987	95	73.2	7.5	0, 0	0	—
1889	95	75.0	8.0	5, 5	3	65 × 36
6288	94	76.2	8.2	0, 0	0	—
549	95	76.2	9.0	0, 0	0	—
653	95	76.9	8.5	0, 0	0	—
2955	95	77.4	8.7	*	2	33
877	95	78.1	9.9	0, 0	0	—
244	94	78.2	9.5	6, 8	2	32
3556	95	78.4	8.5	0, 0	0	—
1549	95	78.5	9.8	6, 6	2	34
3156	95	78.7	13.5	7, 5	2	34
249	94	79.7	8.8	0, 0	0	—
634	95	83.0	11.0	8, 6	2	35
226	94	83.6	10.0	0, 0	0	—
226	95	83.5	10.0	0, 0	0	—
271	95	84.0	11.5	0, 0	0	—
265	95	84.5	12.5	5, 5	2	—
578	94	84.9	11.0	*	1	35
578	95	84.8	11.2	6, 8	1	27
221	95	87.5	12.1	7, 6	2	28
627	94	87.8	14.5	8, 10	3	37
340	95	88.5	13.7	7, 6	2	76 × 37
263	94	88.5	13.0	0, 0	0/5**	35
976	94	90.5	15.5	5, 7	4	—
976	95	90.0	16.0	8, 7	3	83 × 39
3170	94	90.8	14.8	7, 7	4	69 × 37
212	94	91.0	14.5	6, 6	1	76 × 41
687	94	91.1	15.1	6, 8	3	24
251	95	92.1	17.0	6, 6	3	76 × 37
212	95	92.1	16.0	7, 7	2	38
655	95	93.1	16.5	*	3	66 × 34
MA	94	93.4	20.0	7, 8	2	38
MA	96	93.5	—	8, 9	4	72 × 35
2635	95	94.6	18.8	8, 8	3	65 × 35
GO	96	96.0	—	—	—	66 × 38
TA	94	96.0	22.5	5, 5	4	62 × 32
TA	96	98.1	—	6, 8	4	67 × 36
17	94	99.8	23.0	8, 9	4	68 × 36
17	94	99.8	23.0	8, 10	4	77 × 41
17	95	99.8	23.0	10, 9	4	—
3196	94	100.9	19.8	7, 6	4	66 × 38
ME	96	101.0	—	7, 7	4	81 × 42
AS	96	104.2	—	0, 0	3	65 × 34
TE	96	109.1	—	0, 0	5	—
				0, 0	0	—



Fig. 1. Ultrasound image (lateral view) of a soft-shelled oviductal egg in a female *Crocodylus johnstoni*. The white elliptical border outlines the calcified shell surrounding the egg contents. The sphere centered in the dark elliptical area of the egg contents shows a well-defined yolk. Egg dimensions are 7.8 cm x 3.9 cm.

among years in female body size may have contributed to a minor difference in reproductive output. Mean body size of breeding females in the ultrasound sample (88.5 cm SVL, SD = 7.72, $n = 21$) was larger than for the nesting survey (84.2 cm SVL, SD = 6.41, $n = 12$) although the difference was not statistically significant ($t = 1.63$, 28 df, $P = 0.10$) because of low statistical power (power was 0.36 for Cohen's effect size $d = 0.59$ and $\alpha = 0.05$).

All captive females were correctly diagnosed, whether gravid ($n = 6$), postoviposition ($n = 1$), or not breeding ($n = 1$). Most nests were destroyed before eggs could be counted; two females laid 16 and nine eggs after 15 and 10 eggs had been estimated by ultrasound. Average egg measurements by ultrasound for these clutches were not significantly different from average egg dimensions in length ($P > 0.05$) or width ($P > 0.05$). A female (AS) examined one week after nesting retained a preovulatory-size follicle (41 mm) and three vitellogenic-size follicles (30–32 mm) and, on this basis, was correctly diagnosed as a female postoviposition. One female (ME) was bearing 14 shelled eggs but also retained three follicles (each 37 mm). Only two follicles (13, 19 mm) were found in the largest female (TE), and she did not breed that year.

The preserved reproductive tract examined in vitro contained vitellogenic follicles from 19–29 mm, a smaller size class from 9–17 mm, and previtellogenic follicles of 2–4 mm. There was no difference between vitellogenic follicle diameters measured by electronic calipers and Vernier calipers (paired t -test, 11 df, $t = 0.64$, $P = 0.54$).

Discussion.—Ultrasound diagnosis was correct in assessing reproductive status if crocodiles were

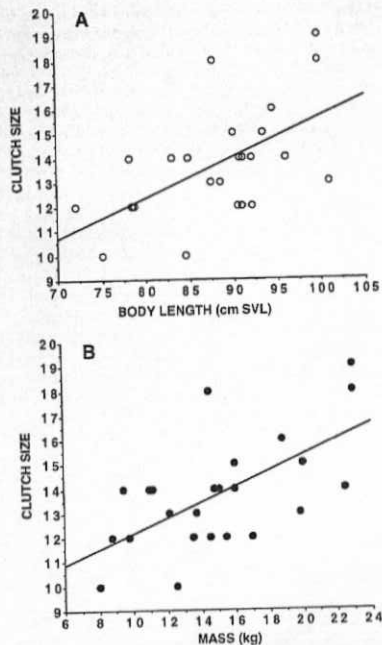


Fig. 2. Clutch size plotted against (A) body size and (B) body mass for breeding female *Crocodylus johnstoni* in the Lynd River. Slopes are least-squared regressions reported in the text.

examined before oviposition and with due care taken to void the intestine. Egg or follicle counts were obtained for 91% of gravid females. Difficulties in counting eggs or follicles (as opposed to detecting them) were related to incompletely voided intestines. Similar precautions are needed for correct assessment of reproductive status during cloacal examinations (Limpus, 1984). This result differed from findings in turtles in which bladder urine was not a source of visual impairment on scanned images (Rostal et al., 1990; Kuchling, 1989).

We did not resolve images for previtellogenic follicles, corpora lutea, or late stage atretic follicles by ultrasound, possibly because of equipment limitations. Corpora lutea measured several months after oviposition were approximately 17 mm in *C. johnstoni* (Webb et al., 1983a), 10 mm diameter in *C. niloticus* (A. Graham, Kenya Game Dept., 1968, unpubl.), and less than

10 mm in *Alligator mississippiensis* (Guillette et al., 1995). Only one of two postovulatory females had large follicles remaining, and these were not atretic at the time of scanning. Atretic follicles in *A. mississippiensis* are seldom greater than 20 mm, and postovulatory females generally have from 1–4 atretic follicles. However, the low number of atretic follicles suggests that most follicles that enter vitellogenesis are ovulated (Lance, 1989). Late-stage atretic follicles in turtles have an anechoic center bordered by a hyperechoic layer (Rostal et al., 1990). If similar atretic follicles occur in *C. johnstoni*, resolving the size and composition should not be problematic. A linear array probe would be more effective to detect small structures than the sector scanners we used (Rostal et al., 1990).

In *C. johnstoni*, previtellogenic follicles were 1–4 mm (Webb et al., 1983b; this study), and intermediate-sized follicles measured from 7–17 mm in nonbreeding adults (Webb et al., 1983b; this study). We found that vitellogenic follicles ranged from 22–38 mm with the preovulatory size class from 32–38 mm. Intermediate follicle sizes are also noted for *C. niloticus*, *A. mississippiensis*, and *C. latirostris* (A. Graham, Kenya Game Dept., 1968, unpubl.; Lance, 1985; Vac et al., 1992). Preovulatory follicles are 40–45 mm in *A. mississippiensis* (Lance, 1985, 1989), 30–35 mm in *C. latirostris* (Vac et al., 1992), and 40–50 mm in *C. niloticus* (A. Graham, Kenya Game Dept., 1968, unpubl.). The similar gradients in follicle size suggest that any follicles remaining after ovulation (Webb et al., 1983b; Vac et al., 1992; this study) either become follicles for a subsequent season or undergo atresia. In only one female were we able to observe follicles when shelled eggs were also present. We cannot say whether this was a general result of all follicles being ovulated in the single clutch per year or whether strong acoustic reflections from calcified eggs obscure the images of any remaining follicles.

A large temporal window exists for detecting breeding status via ultrasound because vitellogenesis extends for several months in crocodilians (Lance, 1989; Vac et al., 1992), and the period of ovulation to oviposition lasts an additional 3–4 weeks (A. Graham, Kenya Game Dept., 1968, unpubl.; Joanan and McNease, 1980). However, corpora lutea were undetected by ultrasound even though 15-mm scars were visible by laparoscopy. Therefore, late in the nesting season, scans do not distinguish between pubescent females, nonbreeding adult females, or females that have already oviposited unless enlarged follicles are noticed (as in captive female AS).

Egg counts validated from the captive females suggest that ultrasound provides a reasonable but not always accurate estimate of clutch size. Larger samples are clearly needed, but if count discrepancies for captive females are representative, biases of the order of 6–9% can occur if eggs are difficult to image or lie within an acoustic shadow. Vitellogenic follicles that are not ovulated can also inflate estimates of reproductive output if follicle counts rather than egg counts are used to estimate reproductive output. Kuchling (1989) found a similar discrepancy (~7%) by ultrasound when comparing numbers of preovulatory follicles, oviductal eggs, and eggs oviposited by the turtle *Chelodina oblonga*. We consider ± 1 egg or follicle to be a reasonable error estimate for the range of clutch sizes produced by *C. johnstoni* or *C. oblonga*. Other researchers report difficulty in achieving clutch counts for turtles with large clutches or multiple clutches per season (Rostal et al., 1990) compared with turtles that have a single small clutch (Kuchling, 1989). We anticipate similar difficulty in achieving precise egg counts for crocodile species with high fecundity. There are alternative methods to assess reproductive status in crocodiles (Gibbons and Greene, 1979; Webb et al., 1983a; Limpus, 1984). For our study, the combination of diagnostic ultrasound and laparoscopy had distinct advantages in rapid field assessment (Table 2).

We tested standard veterinary equipment that required limited training because similar equipment is available to most researchers. Improved resolution can be expected with equipment of improved quality or if a linear convex probe is used (cf. Rostal et al., 1996). A 5.0 or 7.5 MHz transducer was appropriate for *C. johnstoni*, but a 3.5 MHz probe would provide greater scanning depths for larger crocodilians. However, scanning heavily ossified crocodilians such as *Paleosuchus* or *Osteolemus* may prove difficult if osteoderms cause acoustic shadowing. For example, lateral positions for scanning provided the best resolution in *C. latirostris* because of the heavy ossification of belly scales (Vac et al., 1992).

Diagnostic ultrasound is commonly used by captive breeding programs and zoological parks, but our investigation illustrates its practical use for studies of ecology and life history. Because female *C. johnstoni* were seldom encountered during nest-finding surveys, size-related reproductive information was previously lacking. The existing information on size-related reproductive output in a *C. johnstoni* consisted of four females from the McKinlay River, two from the Katherine River, and two from the

TABLE 2. TECHNIQUES FOR DIAGNOSIS OF REPRODUCTIVE STATUS IN REPTILES.

Technique	Cost	Portability	Training	Detects	Disadvantages
Dissection	low	high	low	all stages	requires sacrifice of animal
Surgery	low	high	high	all stages	low processing rate, not well suited as a field technique
RIA	high	low	high	hormonal	samples taken in the field levels only but not a field diagnosis
Cloacal exam	0	high	low	all stages	qualitative information only
Laparoscopy	high	high	high	all stages	qualitative information only
x-ray	high	limited	moderate	shelled eggs	does not detect follicles
NMR	very high	limited	high	all stages	not widely available, impractical for field diagnosis?
Ultrasound	moderate	high	moderate	all breeding	limits of image resolution

Mann River, pooled to derive a tentative relationship for female size and clutch parameters (Smith, 1987). In contrast, ultrasound provided a rapid means (10–15 min per crocodile) to determine that female reproductive output was scaled with body size in *C. johnstoni*, a trend in common with other crocodylians (Thorbjarnarson, 1996). Given the uniformity of crocodylian ovarian anatomy, ultrasound equipment may be useful to investigate similar questions with crocodylians that have small clutches or where nest-guarding is uncommon.

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