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A Comparison of Reproductive Assessment Techniques to Determine the Reproductive Status of Female Diamondback Terrapins (*Malaclemys terrapin*)

Accurately assessing the reproductive activity of wild chelonians is a critical factor in evaluating the reproductive potential of individuals and populations. Such evaluations contribute to a better understanding of the status of populations, with implications for conservation and management of species (Cagle and Tihen 1948; Congdon et al. 1994; Horne et al. 2003). A variety of techniques have been used to determine clutch size and reproductive frequency, including manual palpation (e.g., Bertolero and Marín 2005) to more technologically advanced imaging methods such as radiographs (e.g., Gibbons and Greene 1979) and ultrasound (e.g., Rostal et al. 1990). Palpation is the cheapest method and involves digitally inspecting the inguinal region of the turtle for any palpable eggs or follicles (Congdon et al. 1987). This method is not always accurate because uncalcified eggs, or eggs located more anterior in the body cavity, may not be detectable. Radiography involves using medical X-ray machines to take images of the internal structures of the specimen, with radio-dense structures (e.g., bones, eggs) becoming apparent on film (Jackson and Fasal 1981). This method allows for clutch size estimates and egg size measurements. However, only shelled eggs can be detected, which may lead to false assumptions of reproductive activity if unshelled eggs are not detectable. Ultrasonography involves using echoes from the production of sound pulses to depict structures within the body cavity (Rostal et al. 1990; Penninck et al. 1991). Ultrasonography is costly, but it can provide a substantial amount of data because it allows assessment of additional reproductive structures including preovulatory, unshelled follicles. This method does not allow for an accurate estimate of clutch size, however, and may be difficult to perform based on the size of the chelonian.

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We compared the accuracy of these three reproductive assessment techniques to determine their applicability and accuracy in field studies. We also assessed the monetary costs of each method in order to provide researchers a general idea of the cost associated with each.

We performed this study as part of an ongoing study of Diamondback Terrapins (*Malaclemys terrapin*) from saltmarsh sites at the Rockefeller Wildlife Refuge (Vermilion Parish, Louisiana, USA) and from the lower Mermentau River (Cameron Parish, Louisiana, USA). For information about the site description and trapping techniques, see Selman et al. (2014) and Selman and Baccigalopi (2012), respectively. We captured 15 adult female terrapins in May

Table 1. A comparison of reproductive detectability and price among techniques.

Terrapin ID	Palpation Eggs detected? (Y/N)	Radiography Eggs detected? (Y/N)	Ultrasonography Eggs detected? (Y/N)
9,11-1,11	Y	Y	Y-Shelled
9,11-1,12	Y	Y	Y-Shelled
9,11-2,10	Y	Y	Y-Shelled
9,11-2,11	Y	N	Y-Not fully shelled
9,11-3,9	Y	Y	Y-Shelled
9,11-3,11	Y	Y	Y-Shelled
9,11-3,12	Y	Y	Y-Shelled
9,11-8,9	Y	Y	Y-Shelled
9,11-8,10	Y	Y	Y-Shelled
9,11-8,11	Y	Y	Y-Shelled
9,12-1,9	Y	Y	Y-Shelled
9,12-1,12	Y	Y	Y-Shelled
9,12-1,10	N	N	Y-Not fully shelled
2,11-11	N	N	N-Preovulatory follicles
9,11-9,10	Y	N	N-Preovulatory follicles
Cost per individual exam/rental (~USD)	0	20–100	100–200
Cost for purchase (~USD)	0	1000–4700	1000–20,000

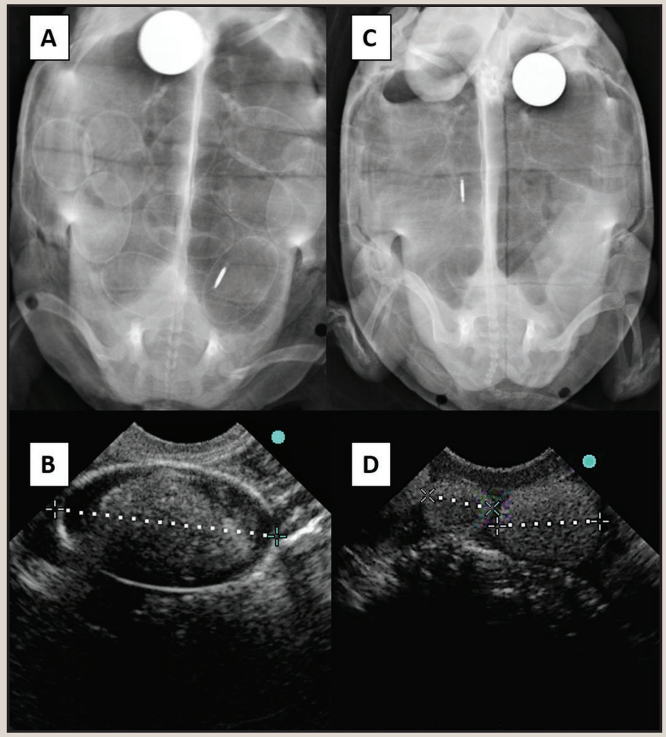

Fig. 1 Radiographs (top) and ultrasonography (bottom) of two Diamondback Terrapin (*Malaclemys terrapin*) females from Louisiana. A female (9,12-1,9) is positive for shelled eggs via both radiographic (A) and ultrasonography (B) methods. A second female (2,11-11) is radiographic negative for shelled eggs (C) but ultrasonography positive for developing follicles (D).

2015 and performed reproductive assessments using all three methods. Two researchers assessed terrapins in the field via inguinal palpation (~15 yr of total experience using this method). This provided two independent assessments of the presence of eggs. For radiographic imaging, we took all 15 females to a local veterinary clinic (Bayou South Animal Hospital, Lake Charles, Louisiana) to determine the presence of shelled eggs. Radiographic settings were 30.0 mAs, mA 300, and kVp 74, which resulted in high-quality digital images. JD performed an ultrasound examination using a portable SONOSITE Titan Ultrasound instrument with an 8-5MHz convex transducer, using Parker brand aqausonic transmission gel.

Results of the examination of 15 females are given in Table 1. Using inguinal palpation, we determined that 13 of the 15 females were gravid, with 11 females having calcified eggs and two additional females having large preovulatory follicles or eggs before shell deposition occurred (interpretations which we confirmed via ultrasound and radiographs). Using radiography, 11 of 15 females had fully calcified eggs (Fig. 1A), while the two females with large preovulatory follicles and the two females palpated as non-gravid were negative using this method (Fig. 1C). Using ultrasound, we determined all 15 females to be reproductively active due to the presence of eggs in 11 (Fig. 1B), or large preovulatory follicles in the other four (Fig. 1D).

Our results suggest that palpation by experienced researchers is a reliable, though limited, form of reproductive assessment. We had no false positives using inguinal palpation (i.e., no females palped as gravid were determined not gravid using another method) and we were able to detect both shelled eggs and large unshelled follicles (87% detection rate of shelled eggs and large follicles). However, two individuals deemed gravid via palpation lacked sufficient calcium deposition on the eggs to be detected via radiograph. Interestingly, of the two individuals that we palpated as non-gravid, one had unshelled eggs undergoing calcification, and the other had large preovulatory follicles (Fig. 1C, 1D). At this stage of follicular development, researchers might expect to see radio-dense eggs during radiographic examination, but such was not the case in the current study. Therefore, this scenario may confuse an investigator without access to an ultrasound, leading to an inaccurate representation of reproductive status.

Radiographs were more limited than the other two methods in terms of egg and follicle detection, allowing for the detection of only radio-dense calcified eggs (11 of 15 females; 73.7% detection rate). Although limited in the ability to detect follicles and developing eggs, radiography allows for the counting of clutch sizes and estimates of egg size. This method has been used extensively by researchers across chelonian taxa (e.g., Congdon and Gibbons 1987; Gibbons and Greene 1979; Kern et al. 2016; Mueller et al. 1998), but our study indicated that using only radiography is likely to underestimate the number of reproductively active females because it lacks the ability to detect uncalcified or developing ova.

Ultrasonography was the only method to exhibit 100% accuracy in detecting reproductive status, allowing for the detection of calcified and developing eggs, as well as multiple size classes of follicles. Thus, this method provides a more complete and accurate assessment of reproductive status without being limited to detection of only calcified eggs via radiography, or only determining gravid/non-gravid status via palpation. In various turtle species, the ovulation and calcification process takes anywhere from 12–72 h (Kuchling and Bradshaw 1993; Licht 1982; Mahmoud and Licht 1997; Owens 1980). As a result, reproductive assessment by radiography and palpation may miss this limited timeframe and lead to an inaccurate representation of individual reproductive status within a population. Ultrasonography, however, can detect non-egg evidence such as developing follicles. Furthermore, radiographs require a standard scaling device (e.g., U.S. quarter; Graham and Petokas 1989), or post-image software to measure egg width, whereas an ultrasound machine allows for real time measurements via built-in software.

Even though ultrasonography provided a better reproductive assessment, a complete picture is often unobtainable. Ultrasounds generally are only able to predict a minimum clutch size due to the location of the oviducts in chelonians. Some eggs situated in the anterior portion of the oviducts will go undetected by the probe. Ultrasound imaging of follicles could also potentially lead to overestimates of reproductive effort, depending on the time of sampling. It is unlikely that all follicles observed will be ovulated and become eggs, as follicles often become atretic near the end of the reproductive season (Atland 1951; McPherson and Marion 1981; Myre et al. 2016). Thus, follicles identified in early portions of a reproductive cycle may not culminate in eggs before being broken down during atresia.

The inguinal palpation method works well once it is learned and is a suitable method for a general assessment of gravidity in chelonians; however, palpation does limit the scope of study to assessing gravidity because the detection of small follicles and clutch sizes are not possible through palpation alone. There is no expense associated with this method and with proper training and experience, the assessment of gravidity could be more accurate than radiography due to the ability to detect large, unshelled follicles.

Both imaging techniques allow for the detection of both fully and faintly shelled eggs and radiography allows for determination of clutch size. However, both techniques require funds to implement. Radiographs may require additional transport and potential stress of the animal if it has to be taken to a veterinary facility for examination. In this study, the veterinary clinic charged a reduced rate of US $20 per female for digital radiographs, and other clinics may reduce or waive fees (P. Lindeman, pers. comm). If a discounted rate is not approved by the veterinary clinic, each radiograph may cost ~ US $40–100. If a clinic is not near the study site, biologists can use portable radiograph machines (similar to those used in dental offices) that are readily available and effective in egg detection in several species of turtle (Turner et al 1986; Miller 2001; Germano 2010). The price of portable machines is also reasonable, with some machines starting as low as US $1000, but the price may increase depending on size and model (Denshine.com; Dentalplanet.com).

Ultrasound exams also may require transport of turtles to a clinic and the costs range from ~US $100–150 per ultrasound, depending on the size of animal examined. However, discounted rates may also apply depending on the situation. Portable ultrasounds come at a much higher cost than other methods, with new units ranging from US $1000–$20,000 depending on the brand and type of transducer that is sold with it (e.g., http://www.contecmed.com; http://www.providianmedical.com). Recently, Phillips electronics and other companies have released portable probes that link to mobile devices (www.lumify.philips.com). They come complete with monthly rental plans starting at $200 USD. This offers an alternative to full purchases if imaging equipment is only needed for a short period of time (e.g., a 3-month field study). Transducer size in this model may be limited to studies of larger chelonians because it may not be able to fit into the tight space of the inguinal region in smaller species like terrapins. Indeed, we found this true with at least one female omitted from the study. This female could not be assessed with ultrasound because her inguinal cavity was too small for the transducer to fit. Therefore, researchers need to account for the size of the study animal when selecting a transducer for study, which could incur additional costs.

Currently, no other study has assessed both the efficacy and costs of inguinal palpation, radiography, and ultrasonography. Of the two imaging methods used, we found ultrasound to be the best method for assessing all aspects of reproductive status (i.e., shelled/unshelled eggs and follicles), while palpation was unable to detect small follicles, and radiography was unable to detect unshelled follicles of any size. Even though there are clear benefits to using radiography (e.g., the ability to count eggs and reasonable costs), many prior studies relying solely on radiography likely underestimated the number of reproductively active females (i.e., females radiographed before eggs are shelled or after oviposition). Therefore, we recommend that researchers investigating turtle reproductive ecology consider using ultrasonography or a combination of ultrasonography and radiography in future studies (if funding is available). Using multiple techniques would greatly contribute to more accurately assessing the reproductive potential of individuals in a population.

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