Editorial Comment. – This section has been established as a forum for the exchange of ideas, opinions, position statements, policy recommendations, and other reviews regarding turtle-related matters. Commentaries and points of view represent the personal opinions of the authors, and are peer-reviewed only to the extent necessary to help authors avoid clear errors or obvious misrepresentations or to improve the clarity of their submission, while allowing them the freedom to express opinions or conclusions that may be at significant variance with those of other authorities. We hope that controversial opinions expressed in this section will be counterbalanced by responsible replies from other specialists, and we encourage a productive dialogue in print between the interested parties. Shorter position statements, policy recommendations, book reviews, obituaries, and other reports are reviewed only by the editorial staff. The editors reserve the right to reject any submissions that do not meet clear standards of scientific professionalism.

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How to Minimize Risk and Optimize Information Gain in Assessing Reproductive Condition and Fecundity of Live Female Chelonians

GERALD KUCHLING[1]

[1]*Department of Zoology, the University of Western Australia, Perth, Western Australia 6907, Australia*
[Fax: 618-9380-1029; E-mail: kuchling@cyllene.uwa.edu au]

Radiography has been used for over 30 years as a non-destructive method to study oviductal eggs with calcified shells in live chelonians (Burbidge, 1967; Gibbons and Green, 1979; Hinton et al., 1997). It's use for turtle life history studies was developed at the University of Western Australia during the mid 1960s for the non-destructive determination of clutch and egg size (Fig. 1) in wild populations of *Pseudemydura umbrina* (Burbidge, 1967). The sacrifice of individuals of this species for reproductive studies would have been unethical and illegal, since the world population of *P. umbrina* numbered then only about 200 — the species was and is one of the rarest and most endangered chelonians of the world. Since Gibbons and Green (1979) also started to apply radiography to turtle research in the USA, it has become a widely used tool in life history studies of chelonians. During late gravidity, radiography allows determination of clutch size with 100% accuracy (Gibbons, 1990) and oviductal egg width can also be measured, although a slight enlargement of image does occur for which corrections should be made (Graham and Petokas, 1989).

However, despite two decades of large-scale use of radiography on female chelonians, studies on the long-term effects of radiographs on fecundity, hatchling health, and survivorship are still scarce. Gibbons and Green (1979) reported that the hatching success of eggs from females which were X-rayed while gravid was statistically equal to that of a non-radiographed control group. This rather basic observation reassured many researchers and wildlife managers that this method is safe in chelonians. The technique was and is extensively used in many chelonian studies (listed by Hinton et al., 1997), including those of threatened species for which it was originally developed. For example, from 1979 until 1986, all known females of *P. umbrina* were annually radiographed during the breeding season (Burbidge, 1983). However, by 1986 the population of *P. umbrina* had declined to less than 50 individuals. In view of the potential risk to the last survivors of this species, I recommended cessation of radiography when I arrived in Western Australia in 1987, and instead introduced, for *P. umbrina* and other chelonians, monitoring of ovarian activity and oviductal eggs by ultrasound scanning (Kuchling, 1988, 1989). This method provided key data on the factors which impact reproduction and was and is an important component of the successful recovery program for *P. umbrina* (Kuchling et al., 1992; Kuchling and Bradshaw, 1993). In many respects, ultrasound scanning proved to be far superior to radiography for investigation of reproductive processes. In contrast to radiography, which only detects shelled oviductal eggs (Fig. 1), ultrasound provides data for most stages of the female reproductive cycle, including vitellogenic and atretic follicles in the ovaries and freshly ovulated ova in the oviducts (Fig. 2). It can provide year-round information on the reproductive performance of individual females and allows a more accurate assessment at which stage individuals reach maturity (Kuchling and Bradshaw, 1993). One drawback of the method is that the keratinous scutes and bone plates of the chelonian shell block ultrasound waves, restricting the "acoustic window" for scanning the body cavity to the soft skin of the inguinal area. This limits the accuracy of quantitative assessments (counting follicles or eggs; see below). Wide application of ultrasound scanning during human pregnancies suggests that it poses much less risk of damage to germ cells and embryos than radiography, which is generally contraindicated during pregnancies, especially during the first trimester.

Hinton et al. (1997) provided a hypothetical discussion of possible impacts of radiography on chelonians, although they did not use most of their own data. They reviewed various radiation doses to which female chelonians and their eggs are routinely subjected during population studies, compared them with literature data on radiation sensitivities of various vertebrates and hypothesized that large scale radiographic screening to assess fecundity of female chelonians does not place adults, embryos, or populations in jeopardy.

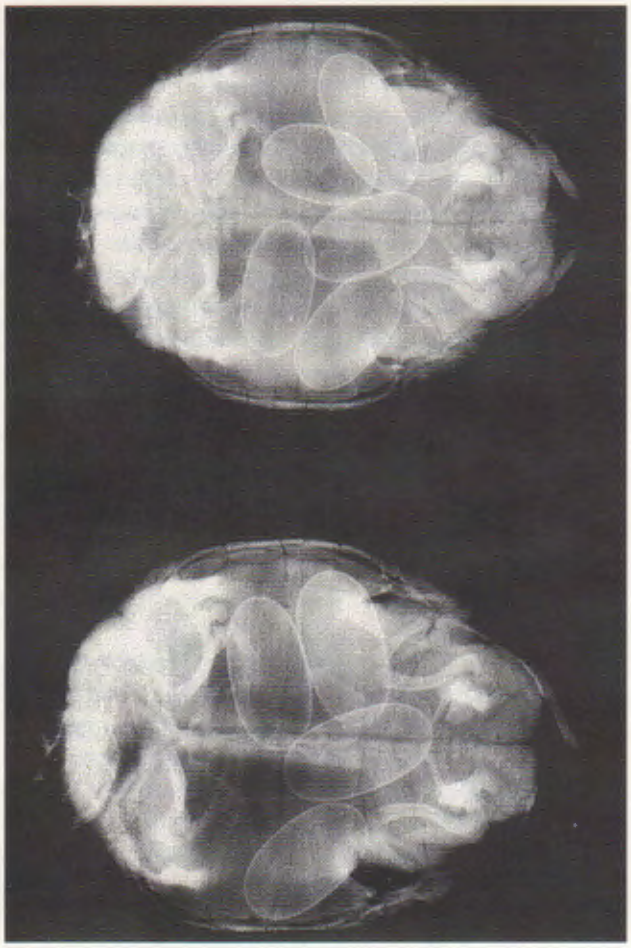

Figure 1. X-rays of two gravid *Pseudemydura umbrina* females with four (bottom) and five (top) oviductal eggs with calcified shells; 31 October 1966 (from Burbidge, 1967; with permission).

The main argument for their assertion that radiography does not pose risks to embryos was that "embryogenesis in turtles is delayed while the egg is in the female and does not resume until oviposition. Thus, concern for heightened radio-sensitivity to the eggs because they are undergoing rapid cell division is negated" (Hinton et al., 1997:412). However, I believe that this reasoning to justify routine radiography of chelonians is faulty and based on doubtful assumptions and wishful thinking rather than on facts. The five co-authors are long-term and extensive users of radiography in chelonians, but they failed to analyse or consider the state of oogenesis in routinely radiographed turtles.

If radiography is used on females intercepted during nesting excursions, as is the case in population studies of freshwater turtles using terrestrial drift fences and pit falls surrounding aquatic habitats (e.g., Gibbons, 1990), many captured females may carry shelled eggs ready for oviposition. In that specific case, a reasonable percentage of females may be in late gravidity with eggs in developmental arrest, as suggested by Hinton et al. (1997) — although multiple clutching females may, in addition, still carry oocytes in other stages of development (see below). It would have been informative to read how many of the turtles collected in that way over the last 20 years actually did have eggs ready for oviposition when radiographed. If females are captured during population studies rather than selectively during their nesting movement, e.g., in field studies of terrestrial species with widely dispersed nesting sites, routine screening by radiography needs to be done frequently in order not to miss clutches. In such studies, the percentage of radiographed females which are in late gravidity, with eggs in developmental arrest, certainly drops significantly.

A short recapitulation of some processes of egg formation will highlight the fact that, during routine non-targeted screening of chelonians, radiography is, indeed, overwhelmingly performed at critical times of gametogenesis and embryogenesis, the stages most sensitive to irradiation.

First of all oogenesis, the formation of oocytes from oogonia, occurs throughout the reproductive life of chelonians (and other reptiles), in contrast to Petromyzontia, Elasmobranchii, a few Teleostei, Aves, and Mammalia in which oogenesis is restricted to the embryonic phase (Blüm, 1986). Adult *Trachemys scripta* ovaries show several germinal beds in which preoogonia and oogonia are localized at the surface of the ovary (Callebaut et al., 1997). The oogonia divide mitotically and eventually form oocytes which are then surrounded by follicle cells and form primordial follicles. Oogonial proliferation in chelonians seems to be a seasonal event. For example, in the box turtle (*Terrapene carolina*), a peak of mitotic activity of oogonia was observed during July and August following the period of ovulation and egg laying (Altland, 1951). Although individual turtles may be less susceptible to gross X-ray damage than mammals (Altland et al., 1951), it is wrong to extrapolate from this finding that the same relationship might also apply to germ cells in adult females (since multiplication of oogonia occurs in adult reptiles, but not in adult mammals and birds). Thus, the statement that "the data available give no indication that reptiles should be especially sensitive to radiation when compared to other organisms" (Hinton et al., 1997:412) is misguided in regard to the germ cells of adult females, since data on reptilian oogenesis indicate a higher sensitivity to radiation due to cell multiplications and have been available since the studies of Waldeyer (1870). Adult chelonian females, in contrast to post-embryonic mammalian females, including humans, are not more or less immune to X-ray damage to their germ line, because oogonial divisions and oocyte formation take place throughout their life.

Before the chelonian oocyte matures, it develops through three successive stages which have been described in detail for *Trachemys scripta* (Callebaut et al., 1997): 1) the prelampbrush stage with chromosomes in diplotene; 2) the lampbrush stage which begins when the oocyte has a diameter of 600-700 µm; 3) the postlampbrush stage (phase of vitellogenesis) which commences when the oocyte has a diameter of 3–4 mm. During the lampbrush stage (also described in *Emys orbicularis* [Loyez, 1906] and *Lissemys punctata* [Guraya, 1989]), chromosomes are greatly extended and present the lampbrush chromosome configuration with lateral loops. These form the site for synthesis of RNAs and permit a continuous flow of RNAs of high

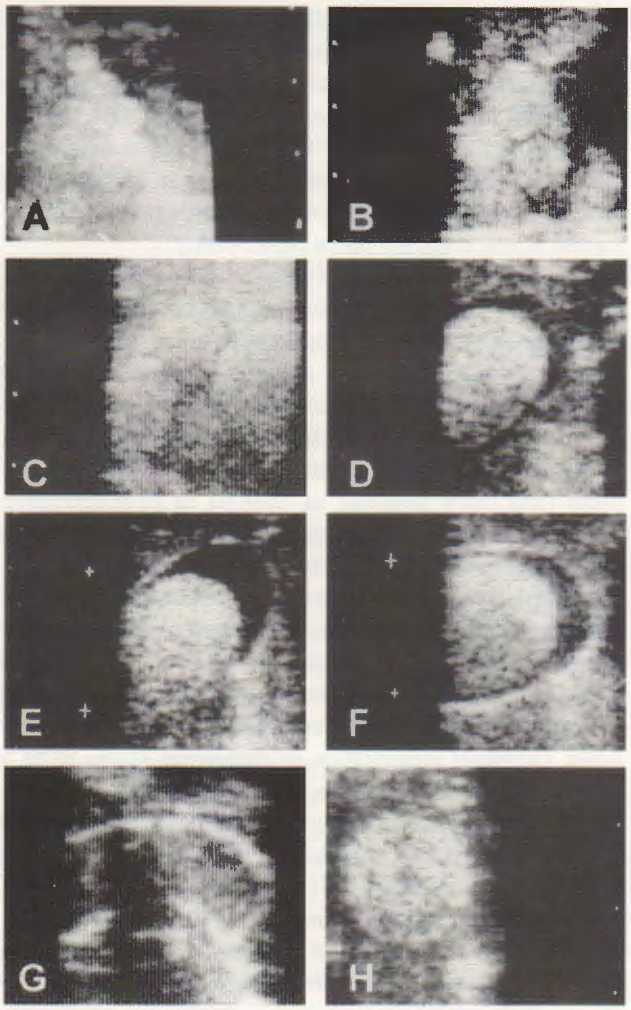

Figure 2. Ultrasound scans of various stages of the female reproductive cycle of *Pseudemydura umbrina*; the scales (white dots) represent cm. **A:** ovarian follicles with a diameter of 4 mm; February, early vitellogenesis. **B:** vitellogenic follicles with a diameter of 5–9 mm; April. **C:** preovulatory follicle with a diameter of 18 mm; September. **D:** freshly ovulated ovum in oviduct, start of albumen secretion (anechoic dark area surrounding echodense white yolk); October. **E:** ovum in oviduct, start of shell membrane secretion, 48–60 hrs after ovulation; yolk echodense, albumen anechoic, thin shell membrane echodense; October. **F:** soft-shelled egg in oviduct, shell membrane secretion completed, start of calcium secretion (yolk and albumen become increasingly diffuse when calcium accumulates in the shell); October. **G:** fully calcified egg in oviduct; yolk and albumen are not clearly defined due to ultrasound reflection from the calcium layer; November. **H:** follicle of preovulatory size in early atresia; variable echodensity indicates the degeneration of yolk globules; December.

complexity to the ooplasm. During lampbrush chromosome activity the germinal vesicle increases strongly in size. An extensive development of nucleoli also takes place inside the germinal vesicle which is related to the amplification of ribosomal genes (rDNA; Guraya, 1989). During these transcription processes, the uncoiled, extended chromosomes may be more sensitive to radiation than during other phases when the chromosomes are condensed.

During the vitellogenic growth period (Fig. 2A, B) oocytes are arrested in prophase I and resume meiosis at or near the end of their growth (Fig. 2C). In chelonians, this happens prior to the breeding season and, in species laying multiple clutches, repeatedly over the entire breeding season. The oocytes must complete the first meiotic division to become fertilizable. The process by which oocytes arrested in prophase I resume meiosis and reach the second meiotic metaphase is called maturation; this may occur some days or weeks before each ovulation event. In most vertebrates, meiosis is again arrested at the metaphase II stage and does not resume until after ovulation, but as far as I know this has not been investigated in chelonians. Shortly thereafter the oocytes are ovulated. The meiotic process leading to extrusion of the second polar body may resume at the time of fertilization, immediately after sperm penetration. Fertilization must occur in the proximal part of the oviduct between ovulation and the onset of the secretory processes to envelop the ovum. In *P. umbrina*, albumen secretion (Fig. 2D) starts 12–48 hrs after ovulation (Kuchling and Bradshaw, 1993). If chelonians are radiographed some days (or weeks) before or after ovulation, their eggs may well be exposed to irradiation during the sensitive cell division phases of meiosis and fertilization.

In the early days or weeks of the oviductal period, presumably before the egg shells are fully calcified (Fig. 2E, F) and the eggs are ready for oviposition, the embryos undergo an active period of cell division until they reach the late gastrula stage. It is not known how much time it takes embryos to reach this stage and this may differ between species and environmental conditions, but presumably it takes several days or weeks. Therefore, only if females with oviductal eggs ready for oviposition (Fig. 2G) are radiographed, can it be assumed that embryogenesis is in an arrested state. Most females that are routinely subjected to radiography before, during, or after the breeding season may not be in that state and, with a high probability, their germ cells and embryos may be in a stage where they may be highly sensitive to X-ray exposure and prone to irradiation damage.

It must be stressed that irradiation effects may not be limited to the possibility of abrupt mortality (the LD_{50} ratios presented by Hinton et. al., 1997), or the production of gross abnormalities in developing embryos, or a reduced hatching rate. Potential damages may also include the emergence of pathological conditions many years after exposure, or reduced fertility in the next generation, effects which are notoriously difficult to detect. The only abnormality which Hinton et al. (1997) briefly discussed as having been possibly caused by radiography was the unusual number of carapacial shields in a hatchling, but they concluded that it was not possible to determine if this was really X-ray induced. However, based on a large data set of *Testudo graeca*, Lapid and Robinzon (1997) recently suggested a ten-fold increase in shell deformities of hatchlings (from 1.8% to 21.6%) after females were repeatedly X-rayed during the breeding season. Even if, in many cases, causal relationships are hard to prove scientifically, I believe it is irresponsible to dismiss these possible impacts of radiography simply by negation.

Current protocols to routinely radiograph individual radiotracked females over several years at biweekly or

monthly intervals are not restricted to studies of abundant, well-established chelonian species or populations, they are also used to study threatened, terrestrial ones (e.g., *Testudo kleinmanni*: Geffen and Mendelssohn 1991; *Testudo graeca* in Spain: Díaz-Paniagua et al., 1996; *Psammobates geometricus*: Baard and Hofmeier, *pers. comm.*; *Gopherus agassizii*: Karl, 1997; Lovich, *pers. comm.*). With a very high likelihood, most or even all of these females have been or will be repeatedly exposed to X-rays during critical, highly radiation sensitive phases of gametogenesis, such as oogonial proliferation, oocyte maturation including meiosis, ovulation, and fertilization, as well as during the highly radiation sensitive phase of early embryogenesis.

The best procedure to avoid the risk of radiographing chelonian females during these highly sensitive times is to assess the reproductive condition of every single female before radiography is even contemplated. This is imperative if threatened and endangered species are studied. An excellent, simple, and non-invasive method to do this is ultrasound scanning, which was evaluated a decade ago as a tool to assess the reproductive condition of female chelonians (Kuchling, 1989) and which is now accepted and used by several turtle researchers (e.g., Casares, 1995; Gumpenberger, 1996a, b; Penninck et al., 1991; Plotkin et al., 1997; Robeck et al., 1990; Rostal et al., 1990, 1994, 1996). Hinton et al. (1997:409) dismissed ultrasound scanning in a single sentence by stating: "ultrasound has also been used to assess ovarian status, but this technique is not accurate when used on females carrying large numbers of eggs." In my opinion, ultrasound scanning is the best and safest method for assessment of ovarian status of live chelonians, no matter how many eggs the female carries. Hinton et al. (1997) seem to equate "assessment of ovarian status," with "counting shelled oviductal eggs." The latter, in fact, has very little to do with the ovaries.

Ultrasound scanning does not allow 100% accurate counting of oviductal eggs, in particular not if females carry large numbers of eggs. This is because the scanning occurs from the two inguinal pockets and some eggs or follicles will remain undetectable in the shadow of other eggs. A quantitative assessment is impossible in large chelonians (giant tortoises, sea turtles) in which only a portion of the ovaries or oviducts can be visualized, but this still allows the qualitative assessment of the reproductive state (e.g., if ovarian follicles or soft- or hard-shelled oviductal eggs are present). A quantitative assessment (although not 100% accurate) of follicles and eggs by ultrasound scanning is feasible in turtles of intermediate size (about 200-4000 g) and small or intermediate egg numbers per clutch (up to about 20). And the best results are obtained in species with relatively large shell openings; e.g., a 92% accuracy in counting follicles and eggs was found in *Chelodina oblonga* which has a clutch size of 8–16 eggs (Kuchling, 1989). In large-bodied species with large clutch sizes of 100+ (e.g., sea turtles) in which eggs cannot be counted by ultrasound scanning, clutch size information is already routinely gained by counting eggs in the easily accessible nests rather than by radiography.

Radiography is clearly an excellent, although not risk free, method of counting fully shelled oviductal eggs in live chelonians with 100% accuracy. Egg width can be accurately measured by radiography as well as by ultrasound scanning. For other kinds of data, ultrasound scanning provides more and better quality information on reproductive processes (Fig. 2; Kuchling and Bradshaw, 1993). The phase during which fully shelled eggs are in the oviducts is at best 3–12% of the year in actively breeding females, depending on species and population; although a very few exceptions exist where females carry eggs for many months. During this phase those eggs may indeed be in a state of lessened radiosensitivity, as suggested by Hinton et al. (1997). But this possibility does not justify routine radiographic screening of females with unknown reproductive condition. Why radiograph females without knowing if they even carry shelled oviductal eggs? No information on fecundity (except for the purely negative information that there are no shelled eggs) or reproductive state can be gained by radiographing (in contrast to ultrasound scanning) females which do not carry shelled eggs. In addition, the probability of radiation damage to germ cells or embryos is greater in adult females without shelled oviductal eggs, because it is more likely that they are in one of the sensitive stages of egg formation. Although it is often possible to detect shelled eggs by palpation, for many population studies this method may not be accurate enough to assess the presence of oviductal eggs, especially if eggs are small or few and/or if the inguinal shell opening is narrow. Since a well-established, harmless technique is available in the form of ultrasound scanning to accurately assess the occurrence of shelled oviductal eggs (as well as other stages of egg development), the routine radiographic screening of female chelonians is not justified.

Clearly targeted radiography, as opposed to routine screening radiography, does have a sound role in some turtle studies, but females should only be selected for radiography after and if shelled oviductal eggs are demonstrated by ultrasound screening or palpation and then only if accurate counting of oviductal eggs is really needed. Eggs can also be counted and measured after oviposition. Incomplete or split clutches can be detected by ultrasound scanning as well as by radiography. It is debatable if the number of eggs in the oviducts or the number of eggs in the nest is the better parameter for fecundity. Retention of single eggs after nesting has for example been reported for Indian turtles of the genus *Kachuga* (Gupta, 1987). I have also observed that one or two eggs occasionally fail to be deposited with the remainder of the clutch in wild *P. umbrina*, *Chelodina steindachneri*, and *Erymnochelys madagascariensis*. These eggs are then simply dropped into the water a few days after nesting (Kuchling, unpublished) and are lost for reproduction. This suggests that the number of eggs in the nest is a better parameter for fecundity than the number of eggs in the oviducts.

The precise measurement of reproductive output in turtles (e.g., the 100% accuracy of radiography versus the 90% or less accuracy of ultrasound scanning) may be of importance to some aspects of life history theory, in which case ultrasound scanning can be complemented by radiography. However, this difference in accuracy between the two methods seems to be of only marginal relevance to conservation biology (e.g., to the modelling of population dynamics and viability) and to the management of turtles — Congdon et al. (1993, 1994) demonstrated that population stability in turtles is relatively insensitive to fecundity, but highly sensitive to larger juvenile and adult survival. These results were confirmed by other modelling studies of chelonian population dynamics (see review in Chaloupka and Musick, 1997). Therefore, in regard to our understanding of chelonian population dynamics and viability, the efforts expended in precisely measuring insignificant variables such as clutch size and egg width, simply because they can be measured precisely (by radiography), may be largely wasted relative to any research which helps quantify the important variables (e.g., mortality rates) more precisely (Webb, 1997).

The fact that chelonian populations have been routinely screened by radiography for two decades makes this practice neither safe nor acceptable, nor does the wish for a continuity of data justify routine continuation of that practice. Females in which shelled eggs are found by ultrasound examination (or, if accuracy of detection is of less concern, by palpation) can always be targeted for radiography. In those cases, the suggestion of Hinton et al. (1997) should be followed to reduce radiation doses during radiography by using cassette films with rare earth screens instead of Ready Pack films. To argue that the assessment of the reproductive condition of females by ultrasound scanning prior to radiography should not be done because of expense is to place the "costs" of radiation-related damage on the chelonians themselves. Even brave followers of the philosophy that only populations are important and that we do not have to be concerned with individuals (Hinton et al., 1997) should think twice before needlessly burdening chelonian species or populations with the danger of damage to their germ lines and long-term future.

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Age Determination in Turtles: Evidence of Annual Deposition of Scute Rings

DAVID J. GERMANO[1] AND R. BRUCE BURY[2]

[1]Department of Biology, California State University, Bakersfield, California 93311 USA [Fax: 805-589-7846; E-mail: DGermano@csubak.edu];
[2]U.S. Geological Survey, Forest and Rangeland Ecosystem Science Center, Biological Resources Division, 3200 SW Jefferson Way, Corvallis, Oregon 97331 USA

Determining the age of individuals in a turtle population is a useful tool for understanding their ecology (e.g., demography, growth rates, age at sexual maturity, senescence). Even recording age of only a portion of a population is important, especially if the age of younger individuals can be determined accurately. Most useful for long-term studies is a technique that does not require individuals to be killed or harmed. Counting the number of rings formed by deposition of epidermal scute layers in turtles has been used by many researchers to determine age without harming individuals. Several reviews (e.g., Gibbons, 1976; Graham, 1979; Castanet, 1988; Zug, 1991) have supported this technique to determine the age of young turtles, but recent papers have questioned its use (Stott, 1988; Cox et al., 1991; Tracy and Tracy, 1995; Kennett, 1996; Brooks et al., 1997). Although there are an impressive number of studies that have used scute annuli to estimate age of turtles, Kennett (1996) stated "growth annuli on many species have proved unreliable in determining ages of individuals." Further, there is concern that researchers do not validate the use of scute layers (Galbraith and Brooks, 1987; Brooks et al., 1997). The underlying concern is whether or not growth rings on scutes represent layers that are deposited annually or not. We provide a current review to investigate the evidence for and against the use of scute rings for age determination and compare its advantages and limitations.

Historical Use of Scute Annuli

The use of scute annuli to determine ages of turtles extends from Agassiz (1857) who used them to determine ages of *Chrysemys picta*. Discussing the general nature of scute layering in turtles, Agassiz (1857:259) stated "hence it follows that we find upon the surface of each scale, around a small angular central plate, (the scale of the first years' growth,) a smaller or greater number of concentric stripes or regular annual rings, as they are exhibited on a transverse section of an old tree." He also discussed the use and appearance of scute annuli in several tortoise species, including *Gopherus polyphemus*, *Geochelone radiata*, and *Psammobates geometricus*, as well as several aquatic species. Coker (1906) was the next to use scute annuli to determine age of a turtle species, *Malaclemys terrapin*. Other early pioneers of this technique were Benedetti (1926) working on *Testudo graeca*, Storer (1930) on *Clemmys marmorata*, Townsend (1931) on *Geochelone vicina*, Risley (1933) on *Sternotherus odoratus*, Sergeev (1937) on *Emys orbicularis*, Ewing (1939) and Nichols (1939) on *Terrapene carolina*, and Liu and Hu (1940) on *Chinemys reevesii*. Cagle was the first to extensively use scute annuli as a means of determining age of *Trachemys scripta* (1946, 1948a, 1948b, 1950), *Chrysemys picta* (1954a), *Malaclemys terrapin* (1952b), and several species of *Graptemys* (1952a, 1953, 1954b). Sexton (1959) showed how to determine age of *C. picta* even when some of the early annuli were missing due to wear. Carr (1952) pointed out some of the problems associated with using scute annuli to determine age of turtles, but believed that they were a useful tool.

Multiple authors have used scute annuli to determine age of at least some portion of populations of turtle or tortoise species (Table 1). The most frequently studied species were *Chrysemys picta*, *Clemmys insculpta*, *Trachemys scripta*, *Testudo graeca*, *Chelydra serpentina*, and *Emydoidea blandingii*. We have not presented this table to justify the use of scute annuli merely because others have used this method. We recognize that the hypothesis that scute rings are formed annually has not been tested in all of these studies, but it has been verified for numerous