

## Non-invasive sex identification of juvenile gopher and desert tortoises (genus *Gopherus*)

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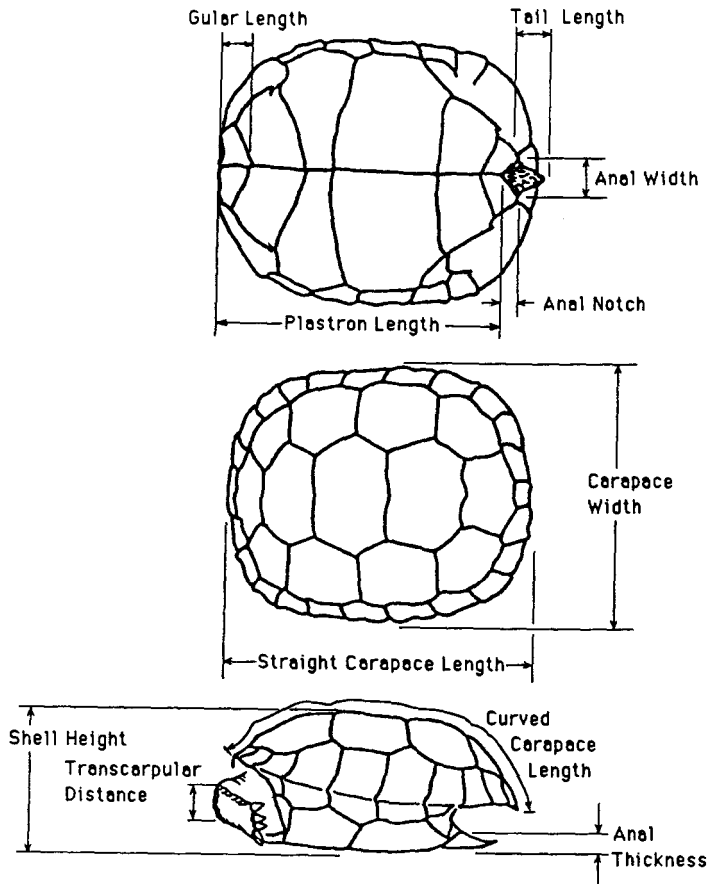
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**Abstract.** Using previously preserved specimens, we attempted to develop multivariate discriminating functions that could accurately identify the sex of hatchling *Gopherus agassizii* and juvenile *G. polyphemus* based on external morphometrics. A simple and successful function was determined for *G. polyphemus*, but not for *G. agassizii*. The failure in the latter case may have been due to the use of individuals from morphologically distinct populations.

### Introduction

While many chelonians have sexually dimorphic external characters as adults, juveniles are typically monomorphic. This is unfortunate, because non-invasive sex identification of young turtles could be useful for a variety of reasons. For example, the possibility of temperature sex determination (TSD) has numerous implications important to conservation of desert tortoises (*Gopherus agassizii*; Spotila and Standora, 1986). The standard approach to identifying the sex of hatchlings requires sacrificing numerous individuals for direct examination of the gonads (e.g. Ewert and Nelson, 1991). Killing individuals of this species is unacceptable under most circumstances, because of their low population numbers.

Behavioral and ecological studies of many species may be enhanced by the ability to analyze for sex-related differences (Graham 1979). Finally, as Gibbons (1990) has noted, it has not yet been demonstrated for any turtle that the gonadal sex identification used as evidence of TSD at hatching necessarily indicates the sex of the adult that would have resulted had that individual survived. Resolution of this point has been difficult, partially because age to maturity is generally long, but also because hatchling turtles do not generally exhibit strongly dimorphic external characters. Thus, identification of the sex of an individual as both a hatchling and adult has not been reported.



**Figure 1.** Characters used to test for sexual dimorphism in hatchling *G. polyphemus* and *G. agassizii*.

Some populations of desert (*G. agassizii*) and gopher (*G. polyphemus*) tortoises are federally protected under the Endangered Species Act (of the USA) and both species have legal protection throughout their ranges. Conservation measures primarily consist of habitat protection, but artificial incubation of eggs has been proposed as part of a head-starting program in the most northerly population of *G. polyphemus* (J. McLemore, pers. comm.). Concern over possible sex-biasing due to TSD has led to a need for non-invasive sex identification in trial studies. Unfortunately, juvenile *Gopherus* have no conspicuous sexually dimorphic external features to facilitate such identification. While TSD has been reported in the congener *G. agassizii* (listed as "*Gopherus*" in Vogt and Bull, 1982), this was based on data now considered unreliable (J. Bull, pers. comm., R. Vogt, pers. comm., K. Berry, pers. comm.).

Using adult *G. polyphemus*, McRae et al. (1981) developed a discriminant function that accurately identified the sex of individuals based on external features alone. Unfor-

tunately, they did not specify the minimum body size for which their function could be accurately applied. Here we describe a quick and easy sexing technique for *G. polyphemus* similar to that used by McRae et al., but suitable for juvenile tortoises. We also describe our attempt to develop a similar technique for *G. agassizii* specimens known to be less than one year old.

## Material and methods

### *Gopherus polyphemus*

We initially examined 58 preserved museum specimens. All specimens had been previously collected in central or northern Florida, and all were estimated to be age 4 years or less (based on the average number of annuli on three carapacial scutes). Straight carapace length (CL), carapace width (CW), shell height (SH), plastron length (PL), gular length (GL), anal width (AW), anal match (AN), and anal thickness (AT) were measured on each specimen (see fig. 1 and McRae et al. [1981] for specific details on these characters). In addition, curved carapace length (CC), tail length (from cloaca to end of tail) (TL), and maximum transcarpular distance (MT) (fig. 1) were also measured, as these characters are thought to be sexually dimorphic in juvenile *Gopherus* and other tortoise species (W. Auffenberg, personal communication, I. Swingland, personal communication). Plastron concavity, a sexually dimorphic feature in adult *G. polyphemus* (McRae et al., 1981), was either very small or nonexistent in each of these specimens.

### *Gopherus agassizii*

The procedure for *G. agassizii* was similar to that described above for *G. polyphemus*, though the source of the specimens was different. Hatchlings were donated by members of the California Turtle and Tortoise Clubs and by the Arizona-Sonoran Desert Museum. These hatchlings were produced as a result of captive matings from parents of generally unknown origin. Hatchlings that died naturally or accidentally under captive care were measured, preserved, dissected, and gonadal tissues examined as described below.

### Histology

After measurements, the cranial poles of the kidneys and adjacent gonadal tissue from each specimen were removed, embedded in paraffin, sectioned at 6 $\mu$ m, and stained with hematoxylin and eosin (H&E) or periodic acid Schiff (PAS) method. Stained tissue sections were examined by light microscopy.

### Statistical analysis

The gonads of 27 *G. polyphemus* specimens were too poorly preserved to identify sex and these specimens were eliminated from further analysis. Twenty-six of the identifiable *G.*

*polyphemus*, and 33 *G. agassizii*, were used to develop two species-specific discriminating functions, whereas five additional specimens of each species were reserved to allow an independent test of the resulting function for that species. Several aggregate variables were created by multiplying  $CW \times CL \times SH$  as a correlate of shell volume (VOL), and making body-size corrections by creating variables  $GL/VOL$ ,  $AN/VOL$ ,  $TL/VOL$ ,  $PL/VOL$ ,  $CC/VOL$ ,  $MT/VOL$ ,  $AW/VOL$ ,  $AT/VOL$ ,  $CW/CL$ , and  $CW/SH$ . Both the raw and size-corrected data were used as potential independent variables in regression analysis. In a separate set of analyses, all data were log-transformed before analysis. In all analyses sex was the binary dependent variable.

Stepwise multiple logistic regression (SYSTAT) with entry and removal levels of 0.10 was initially used. In addition, an "all possible combinations" multiple regression procedure in the BMDP statistical package was utilized. This procedure compared all possible combinations of variables in models containing one, two, three, and four independent variables. We would have preferred to use an all possible combinations approach with either discriminant function analysis or logistic regression instead of the multiple regression, but were unable to locate a computer program with this ability. However, these techniques are based on the same assumptions (Kleinbaum et al., 1988, Afifi and Clark, 1984) and should give the same results.

Functions were compared by their ability to accurately identify the sex of the original specimens. For each species, the function that had identified more of the original specimens correctly than any other was then used to classify the five additional specimens that had not been used in the development of the function.

## Results

Of the various regression functions developed using the *G. polyphemus* data,  
 $Y = 38.1029 + 0.733627 (CW) - 1.15358 (SH) - 0.000010213 (VOL) - 23.113 (CW/SH)$

was clearly the best function for classifying the 26 original specimens. Here Y varies from 0 to 1; Y values greater than 0.5 indicate males and values less than 0.5 indicate females. This function correctly classified individuals by sex in 21/26 (80.8%) of the original specimens, and in all five of the independent test cases. The next most successful function correctly classified 20/26 (76.9%) of the original specimens, and all of the test cases.

The results for *G. agassizii* were more ambiguous. The function with the best ability to classify the sex of 33 specimens was correct in 31/33 cases (93%). However, it was correct for only 3/5 (60%) of the test cases. Therefore, it is not clear whether this function would be valuable in classifying additional specimens.

## Discussion

Some other techniques for non-invasive sex identification have been developed for turtles, and these might be considered for species that are grossly sexually monomorphic

as juveniles as well. These techniques include laparoscopy (Wood et al., 1983), cytotoxicity assay (Wellins, 1987), DNA screening (Demas et al., 1990), univariate analysis of morphology (Wibbels et al., 1987), and radioimmunoassay (Owens et al., 1978; Wibbels et al., 1987). However, each has drawbacks in comparison to the technique described here. Laparoscopy and the biochemical techniques require relatively expensive equipment and at least one incision in the animal. Additionally, the small size of hatchlings limits the usefulness of these techniques for these turtles. Laparoscopy also requires the use of anesthesia. Although laparoscopy may work for young *Chelonia mydas* which have grossly dimorphic gonads (Wood et al., 1983), the gonads of juvenile gopher tortoises show little external dimorphism (Burke, personal observation) and so laparoscopy may not be useful. Wellins (1987) emphasized both the high cost of materials and labor as well as the poor discrimination power of cytotoxicity assays. The serum testosterone-radioimmunoassay used by Wibbels et al. (1987) is both relatively expensive and requires specialized equipment. The univariate analysis of tail length and shell measurements carried out by Wibbels et al. was unsuccessful in correctly identifying sex of immature *Caretta caretta*, and these authors did not report trying multivariate analyses. Owens et al. (1978) used a challenge radioimmunoassay to sex young *Chelonia mydas*, and found that multiple injections of follicle stimulating hormone were necessary to obtain satisfactory results for animals less than 4.8 years old.

The morphological approach used here has some advantages over other methods in that once a reliable function is developed, non-invasive sex identification is easily made, even under field conditions. An inexpensive programmable hand-held calculator could be used to quickly produce a reliable identification. As with the other techniques, verification with known sex animals is necessary, and so some animals must be killed for gonadal inspection. Unlike some of the methods described above, this need not require the killing of additional animals if sufficient numbers of museum specimens are available. The statistical analysis is fairly straightforward and clearly can identify otherwise inconspicuous dimorphism.

However, if there are important morphological differences between populations, it is possible that this technique will be inaccurate if tortoises from morphologically distinct populations are combined for analysis. This could explain our failure to develop a useful discriminating function for *G. agassizii*, because the specimens used were from numerous populations throughout the species' range, and some may have been the result of matings between members of different populations. Germano (1989) has demonstrated that dramatic morphological differences exist among different populations of *G. agassizii*. Differences between populations may have swamped differences between sexes, and we are confident that analysis of additional material from a single population will result in a good classifying function.

We did not test our function on *G. polyphemus* from outside of north Florida, but we feel that reliability of even a highly successful function should be retested before use on different populations. This limitation probably does not exist with either the laparoscopy or the biochemical techniques.

### *Implications for experimental sample size*

The classifying function developed here for *G. polyphemus* was incorrect in assigning sex to 19.1% of the original specimens; of these five specimens misidentified, two were males and three were females. Thus it probably misidentifies each sex approximately equally. To illustrate the effect of the estimated error rate on experimental designs to be based on its results, sample size calculations are presented here for a hypothetical test for TSD. *Testudo graeca* is the only tortoise species for which sex determination mode data have been reported, and it has TSD (Pieau, 1975). Of 38 eggs incubated at 29.6°C, 37 (=97.4%) developed testicular tissue and were presumed to be males (Pieau, 1975). If *G. polyphemus* has a similar sex skewing tendency, a minimum of 20 eggs would be required at any one incubation temperature to demonstrate TSD. This is because of the 20 eggs, 19 would actually become males, but four ( $0.191 \times 19$ ) of these would be mistakenly identified as females. The single real female is likely to be identified correctly. The perceived sex ratio would be 0.75 which would be recognized as significantly skewed if compared to a sex ratio of 0.50 using a standard one-tailed binomial test ( $\alpha=0.05$ ).

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