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Geometric Morphometric Sex Estimation for Hatchling Turtles: A Powerful Alternative for Detecting Subtle Sexual Shape Dimorphism

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Identifying sex of hatchling turtles is difficult because juveniles are not obviously externally dimorphic, and current techniques to identify sex are often logistically unfeasible for field studies. We demonstrate a widely applicable and inexpensive alternative to detect subtle but significant sexual dimorphism in hatchlings, using landmark-based geometric morphometric methods. With this approach, carapace landmarks were digitized from photographs of each hatchling, and shape variables were generated after variation in size, location and orientation were eliminated. These variables were then analyzed for sexual dimorphism, and used in discriminant function analysis to estimate sex of each hatchling. Using this approach on two species (*Chrysemys picta* and *Podocnemis expansa*), we found this method had high accuracy in assigning sex when compared with true sex (98% and 90%, respectively), and cross-validation revealed a correct classification rate of 85%. These correct classification rates were considerably higher than those found on the same species using linear distance measurements as data. We also explored two alternative statistical approaches for assessing sex (K-means clustering and multiple logistic regression) and found that these alternative approaches were accurate only 61% and 78% of the time, respectively, in *C. picta* and 69% and 77% of the time in *P. expansa*. These findings are similar to classification rates found for turtle species using approaches based on linear distance measurements. We also found that the observed sexual dimorphism differed between the two species. In *P. expansa*, males displayed relatively more expansion of the central region of the carapace relative to females, whereas in *C. picta* this pattern was reversed. We conclude that discriminant analysis of morphology quantified using geometric morphometrics provides researchers with a powerful tool to discriminate between male and female hatchling turtles.

SEX ratio is a fundamental life-history parameter essential for the study of population dynamics. Sex ratios at birth/hatching can differ from adult sex ratios, and their comparison can inform about other important parameters, such as differential mortality, migration and differential dispersal between sexes (Bulmer, 1994). Although many turtle species are highly dimorphic as adults, hatchling turtles typically exhibit little or no obvious dimorphism that allows straightforward identification of sex by external observation (Ernst and Barbour, 1989). Neonates typically grow for long periods of time (years) before external sex-specific differences are conspicuous; thus, researchers often employ intrusive or destructive techniques to estimate sex ratios at hatching. Among those techniques used are radioimmunoassay (RIA) to quantify circulating plasma steroid concentrations from the blood (Owens et al., 1978; Lance et al., 1992; Rostal et al., 1994) or from the egg chorioallantoic/amniotic fluid (Gross et al., 1995), gonadal histology (Yntema and Mrosovsky, 1980), and observation of external gonadal morphology either by laparoscopy of live animals (Wood et al., 1983; Rostal et al., 1994) or most commonly, by dissection of dead animals (Van der Heiden et al., 1985; reviewed in Wibbels et al., 2000). These techniques suffer from associated high costs, extensive training requirements, unfeasibility in remote field sites, and potential modification of the population structure under study. Because most turtles and many other reptiles have temperature-dependent sex determination, molecular sexing via comparison of sex-specific genetic sequences (reviewed in Wibbels et al., 2000) is precluded because these taxa lack sex chromosomes, and

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no consistent genetic differences are known to exist between males and females (Valenzuela et al., 2003).

An alternative to the above approaches is to use a sensitive morphometric technique for detecting subtle differences between hatchling males and females that are not noticeable to the naked eye. Typically, sets of linear distance measurements are obtained for each specimen, and statistical methods are used to attempt to discriminate males and females using these characteristics. This technique has been used successfully to identify hatchlings of the Olive Ridley Sea Turtle (*Lepidochelys olivacea*; Michel-Morfin et al., 2001). Unfortunately, similar attempts have proven unsuccessful for hatchlings of other species, including *Podocnemis expansa* (Hildebrand et al., 1997) and several chelonians (e.g., *Gopherus agassizii*; Burke et al., 1994; Boone and Holt, 2001). For instance, Hildebrand et al. (1997) analyzed 159 *P. expansa* hatchlings using 25 morphological characters, such as the length and width of the carapace, plastron, tail, limb, head, and the number of limb scales. They found no difference between males and females in these characteristics ($P > 0.8$), and using these data, fewer than 78% of the specimens could be correctly classified to sex. *Podocnemis expansa* is a tropical endangered freshwater turtle belonging to the chelonian suborder Pleurodira, and sex has been determined nonlethally using radioimmunoassay (Lance et al., 1992; Valenzuela et al., 1997; Valenzuela, 2001), but because of the difficulties mentioned above, this technique is not commonly used by researchers. Therefore, a more sensitive method using morphology is sorely needed.

Developing a method for sex estimation of hatchlings from morphology requires two important considerations. The first is choosing the best method for quantifying morphology, and the second is determining the most suitable statistical approach for analyzing these data. Here we show that quantifying morphology using geometric morphometric (GM) methods is a feasible and inexpensive approach based on external morphological characteristics, which can successfully detect sexual dimorphism in hatchlings of *P. expansa*, where other morphological techniques have failed. Additionally, GM methods allow the classification of hatchlings into sex categories with high accuracy. We also explore various statistical methods for estimating differences between males and females based on these characteristics and show that linear discriminant analysis is the most useful approach. We present an identical analysis in another turtle species, *Chrysemys picta*, a widely distributed temperate freshwater turtle representing the other suborder of chelonians (Criptodira).

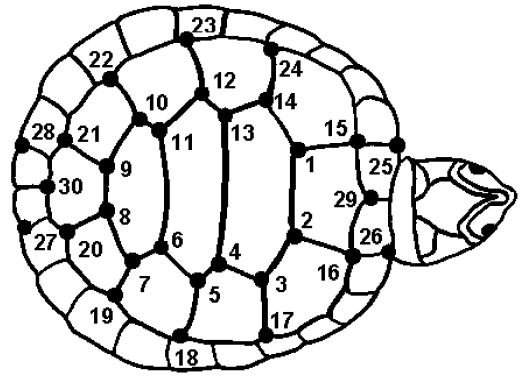


Fig. 1. *Podocnemis expansa* hatchling illustrating the location of the 30 landmarks used in this study. For statistical analyses, only landmarks on the right-half of the carapace were used.

Materials and methods

Specimens.—Hatchlings of *P. expansa* were collected from a population along the Caquetá River in Colombia. They were also used during a previous study of sex determination in the field and laboratory and correspond to several natural and experimental nests differing in depth, shading, and temperature conditions (Valenzuela, 2001). All *P. expansa* specimens used for this study were 1–2 weeks old. *Chrysemys picta* eggs were collected in June 2001 from a population that nests on the northern end of an island in the Mississippi River near Thomson, Illinios. Once collected, eggs were brought to a laboratory at Iowa State University and placed into plastic boxes containing moist vermiculite (-150 kPa) for incubation. Eggs were incubated at a constant temperature (28 C), and the boxes were rotated regularly to minimize any effects of thermal gradients within the incubator. A total of 230 *P. expansa* and 87 *C. picta* hatchlings were photographed at < 2 weeks and ~ 2 months of age, respectively (Fig. 1). All of these hatchlings presented a normal number of scutes and had fully unfolded after hatching. Hatchlings were sexed by radioimmunoassay (*P. expansa*; Valenzuela, 2001) or by macroscopic examination of gonadal morphology after dissection (*C. picta*; Ewert and Nelson, 1991; Bowden et al., 2000).

Data collection and analysis.—Developing a method for sex estimation of hatchlings from morphology requires two important considerations. The first is choosing the best method for quan-

tifying morphology, and the second is determining the most suitable statistical approach for analyzing these data. Previous work has shown that linear distance measures have had only limited success in distinguishing hatchling males and females (e.g., Michel-Morfin et al., 2001). Therefore, we used an alternative approach and quantified morphology from the positions of biologically repeatable (operationally homologous) anatomical points, which were then subjected to a geometric morphometric (GM) analysis. GM methods quantify the shape of an object after the effects of nonshape variation (position, orientation, and scale) have been mathematically held constant (Rohlf and Slice, 1990). These methods are preferable to linear distance methods because they retain the geometry of shape throughout the analysis (Rohlf and Marcus, 1993). Additionally, when compared to other approaches (including linear distance methods), simulation studies have shown that Procrustes-based GM methods outperform other approaches and have higher statistical power, appropriate type I error rates, low bias and error of estimating mean shapes, and do not introduce patterns of covariation to the data (see Rohlf, 1995, 2000a,b, 2003). Therefore, when compared to linear distance methods, quantifying morphological shape using landmark-based GM approaches will provide the best opportunity to capture subtle differences between male and female hatchlings.

For our analyses we quantified carapace shape in the following manner. First, digital images of the carapace of each specimen were obtained using a Nikon DXM-1200 high-resolution digital camera (*C. picta*) or by scanning high-resolution slide color photographs (*P. expansa*). For all images, a ruler was included for scale. From each image, the x,y coordinates of 30 anatomical landmarks (Fig. 1) were then recorded using TpsDig software (F. J. Rohlf, unpubl.). The landmarks included the intersections of the lines delineating the vertebral and the marginal scutes, as well as the perimeter of the carapace at the boundary between the first and second marginals (anterior) and between the 11th and 12th marginals (posterior). For shape analyses, 16 landmarks from the right half of each carapace were subjected to a Generalized Procrustes Analysis (GPA), to superimpose the specimens to a common coordinate system, and mathematically remove the effects of digitizing position, orientation, and scale (Rohlf and Slice, 1990). Half of the carapace was used to generate shape variables because use of landmarks obtained from both sides of a symmetric object (e.g., a carapace) generates linear dependencies among shape variables that cause statistical difficulties (for a discussion see Bookstein, 1996a). For graphical depictions of results however, all 30 landmarks were used to facilitate biological interpretation. From the aligned specimens, a total of 28 shape variables were calculated, as partial warp scores from the thin-plate spline (Bookstein, 1989, 1991) and the two standard uniform components (Bookstein, 1996b; Rohlf and Bookstein, 2003). These variables describe the shape change necessary to transform the landmark coordinates of the mean specimen to the locations of the landmarks for that particular specimen. Together, they capture the linear and nonlinear aspects of shape variation and can be used to test hypotheses of shape variation and covariation within and among groups using standard multivariate statistical procedures (see e.g., Caldecutt and Adams, 1998; Adams and Rohlf, 2000; Rüber and Adams, 2001). All superimposition and thin-plate spline computations for generating morphometric shape variables were performed in TPSRelw (F. J. Rohlf, unpubl.).

Using the shape data described above, morphological variation was assessed in several ways. First we performed a two-factor multivariate analysis of variance (MANOVA) to determine whether there were differences in carapace shape between species, between the sexes (i.e., sexual dimorphism), and to determine whether sexual dimorphism was consistent between the species. We then assessed sexual dimorphism for each species individually by performing MANOVA separately on each species. To estimate the sex of hatchlings, we used three statistical methods and examined their performance by comparing the percent of hatchlings whose estimated sex correctly matched their true sex (i.e., the percent correct classification). The first approach was *K*-means clustering. With this approach, a set of specimens are partitioned into K-groups such that the objects within the K-groups are more similar to one another than objects in the other clusters, thereby minimizing within-group variation (Wong and Lane, 1983; Legendre and Legendre, 1998). One can then assess how well the specimens partitioned into two groups correspond to the two sexes. This approach is closest in spirit to what is desired when biologists ask, ''How many groups are present in my data?'' The second approach was multiple logistic regression. Here the set of shape variables were treated as independent variables, and sex was the dependent variable. The third approach was discriminant function analysis (DFA). Here the set of shape variables were treated as independent variables, and a multi-

TABLE 1. RESULTS TWO-FACTOR MANOVA COMPARING CARAPACE SHAPE BETWEEN SPECIES AND BETWEEN THE SEXES.

Source	Exact *F*	df	*P*
Species	172.1083	27, 287	<0.0001
Sex	1.9037	27, 287	0.0005
Species × Sex	1.5758	27, 287	0.0104

variate equation (function) was defined such that males and females were maximally discriminated.

Using each of the three approaches, sex was estimated for each hatchling, and the overall correct classification rate was observed by contrasting the estimated sex with the true sex (as assigned by RIA or gonadal inspection). These values were qualitatively compared to examine the relative performance of the three statistical approaches in estimating hatchling sex. Additionally, because classification rates can be inflated when the same specimens are used to generate the DFA and assess its classification rate (see Krzanowski, 1988), we performed a cross-validation analysis. For this analysis, 20 specimens (10 males and 10 females) of *P. expansa* were excluded from the dataset, the discriminant function was obtained for the remaining specimens, and the sex of the 20 excluded hatchlings were estimated using the discriminant function. The 20 excluded specimens were then used to assess the DFA classification rate by comparing their estimated sex to their true sex (as found by RIA). Smaller sample size precluded a similar cross-validation procedure for *C. picta*. Finally, to visualize sexual dimorphism, we generated graphical representations of shape for males and females along the discriminant axis using thin-plate spline deformation grids. These plots are analogous to D'Arcy Thompson's (1917) transformation grids and provide a graphical representation of shape difference among groups. All statistical analyses were performed using NTSYS-pc (F. J. Rohlf, unpubl.) and JMP 5.0.1 (SAS Institute, unpubl.).

RESULTS

Examining carapace shape variation using a two-factor MANOVA, we found significant effects for species, sex, and species by sex interaction (Table 1). This implied that *P. expansa* and *C. picta* differ in carapace shape, that there is significant sexual dimorphism of carapace shape, and that the sexual dimorphism in shape is not consistent between the two species. When the two species were examined separately, significant sexual dimorphism was confirmed (*P. expansa*: exact $F = 3.39$, df = 27,202, P 0.00001; *C. picta*: exact $F = 1.63$, df = 27,59, $P = 0.07$). When comparing sex estimation methods, we found that discriminant analysis greatly outperformed alternative techniques. Using *K*-means clustering, we found that 69% of *P. expansa* hatchlings and 61% of *C. picta* hatchlings were correctly classified. Multiple logistic regression performed slightly better, correctly classifying 77% and 78% of the hatchling turtles, respectively. Using DFA, however, posthoc classification revealed an extremely high degree of separation of the two sexes. For *P. expansa*, 90% (207 of 230) of the individuals were classified to the correct sex, and for *C. picta*, 98% (85 of 87) of the individuals were classified to the correct sex. When cross-validation analysis was performed for *P. expansa*, the classification rate of the included specimens was identical (90%), and that of the specimens excluded from the analysis was 85% (17 of 20).

To describe the sexual dimorphism visually, we generated graphical representations of shape for males and females along the discriminant axis for each species using thin-plate spline deformation grids, such that anatomical differences between the sexes could be assessed. Using this approach, differences in carapace shape between species, as well as the different sexual dimorphism in each species, were evident (Fig. 2). When comparing the species, the most noticeable differences were in the cranial region of the carapace, where there was more flaring of these scutes in *C. picta*, and more relative compression of this region in *P. expansa* (Fig. 2). With respect to sexual dimorphism, both species exhibited differences in the anal region of the carapace, which was more flared in males and relatively more compressed in females. Additionally, males and females differed in the central vertebral region, although the male-female pattern was in the opposite direction for the two species. In *P. expansa*, males displayed relatively more expansion of the central region of the carapace relative to females, whereas in *C. picta* this pattern was reversed (Fig. 2). This provided graphical confirmation that the sexual dimorphism in carapace shape

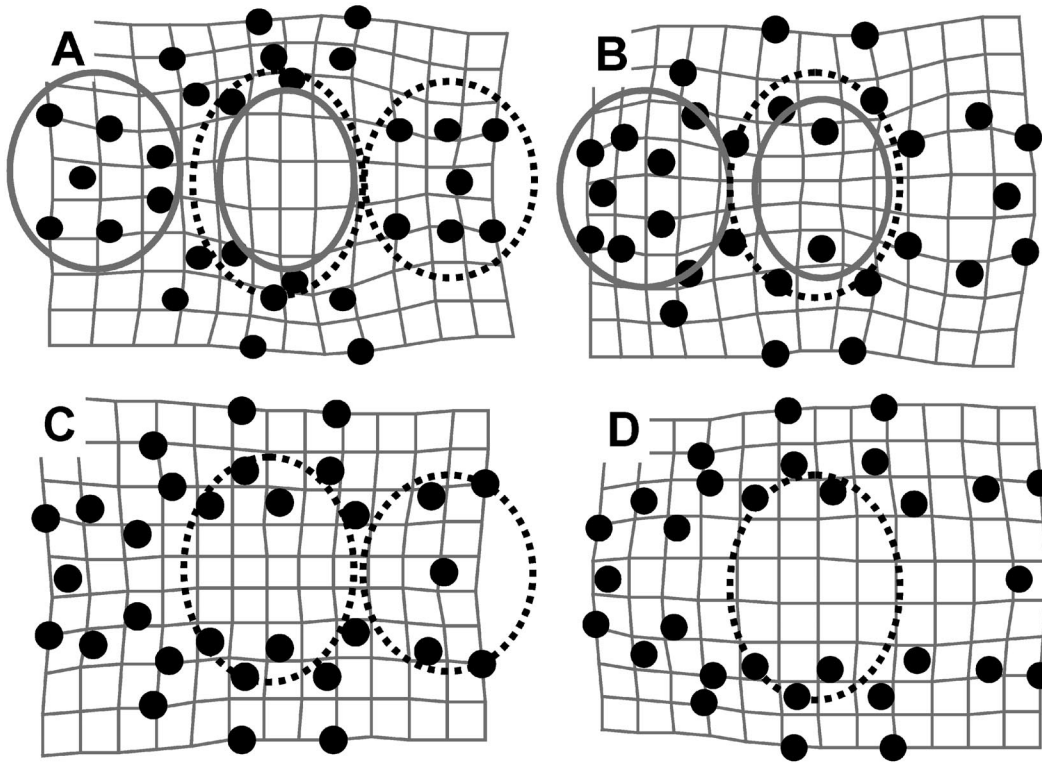


Fig. 2. Thin-plate spline deformation grids representing the mean shape of (A) male *Podocnemis expansa* (B) female *P. expansa* (C) male *Chrysemys picta* and (D) female *C. picta*, as found along the discriminant function axis between sexes (grids are displayed at 3× magnification to emphasize group differences). Orientation is as in Figure 1 with the head of the animals to the right. Solid gray ovals highlight regions of the carapace with significant differences between the sexes, whereas dashed black ovals denote regions were significant differences between species were more pronounced.

was present in both species but was not consistent between them.

Discussion

Because the sex of hatchlings of most turtle species are not identifiable by the naked eye, a more sophisticated method is required to distinguish males from females. When morphology is used, it is logical that one quantifies morphology using the most powerful and sensitive approach possible. Computer simulations have shown that shape data from geometric morphometric methods have higher power, better type I error rates, lower bias, and lower mean squared error when compared to variables generated from sets of linear distances (Rohlf, 1999, 2000a,b, 2003). Therefore, from a theoretical perspective, GM methods are preferable to distance methods for morphological quantification. Furthermore, these methods are quite powerful in discriminating male and female hatchling turtles. In our study, we found higher correct classification rates than are typically found using sets of linear distance measures, and these results were consistent for two turtle species of different suborders. One of these species (*P. expansa*) also has been examined using more traditional methods of DFA on a set of linear distances, and this method was not able to distinguish male and female hatchlings (Hildebrand et al., 1997). Therefore, a direct comparison of two methods for quantifying morphology on the same species has revealed that the GM protocol is capable of identifying subtle morphological dimorphism between the sexes that linear distances could not.

In this study, we were able to reveal significant sexual dimorphism in the external morphology of both *P. expansa* and *C. picta* hatchlings, a dimorphism that was undetected using other morphometric techniques (Hildebrand et al., 1997). This technique is not species-specific and is expected to be widely applicable because it was implemented with equal success in two highly divergent species. Second, this methodology

proved to be a suitable tool to estimate the sex of hatchlings with high accuracy, thus providing researchers with a noninvasive and inexpensive alternative to current sexing techniques. However, some words of caution are in order. As field biologists, we desire a simple and straightforward technique that enables us to quickly identify male and female hatchlings using only a few measurements with little or no uncertainty. Unfortunately, no such method exists. First, it must be recognized that all existing methods except inspection of gonadal histology are little more than methods of sex estimation, rather than being methods of true sex identification. Therefore, all methods require empirical validation by comparing the estimated sex to the sex confirmed through gonadal inspection, at least for a subset of individuals. Second, even when a sex estimation method is found to be useful for a given population, extrapolation of its use to other populations and species is ill advised, even when the resulting sexual dimorphism is similar between species. Further, because all existing methods for sex estimation are data dependent, use of a different set of hatchings will result in a slightly (or greatly) different sex estimation function. Therefore, treating the resulting discriminant functions as sex estimation ''rules'' to be applied to other data (and to be compared among species) is tenuous at best, because they are not generalizeable beyond the data for which they were optimized. Only through empirical evaluation in additional populations (both geographic and generational) can it be determined whether a particular discriminant function is useful for additional samples of the same species. If the same (or similar) discriminant function is obtained for data from multiple populations, and across multiple years, this implies that hatchling sexual dimorphism is consistent within a particular species. To our knowledge, this has never been empirically demonstrated for any sex identification rule based on morphology.

Although significant, the difference in shape between males and females found in this study are subtle enough to pass undetected to the naked eye, hence requiring sensitive geometric morphometric techniques for sex estimation. Additionally, we failed to find a subset of diagnostic linear measurements that captured the essence of the variation in the regions of the carapace that changed the most between males and females, as determined by the geometric morphometric analysis (results not shown). This indicates that, although changes in parts of the carapace eliminated during the search for diagnostic cues were smaller, they are fundamental components of the overall shape dimorphism between the sexes, such that they cannot be removed without losing the dimorphic signal. This may also explain why previous discriminant analysis using distance measurements have been unsuccessful in *P. expansa* (Hildebrand et al., 1997).

The higher level of misclassification in the case of *P. expansa* compared to *C. picta* may be, in part, caused by the potential error associated with the radioimmunoassay itself. That is, if some individuals were misclassified by the RIA, they will appear in the ''incorrect'' sex category using morphometrics. The classification error may be smaller in *C. picta* because hatchlings were sexed by gonadal inspection. However, the RIA technique, as originally standardized for *P. expansa*, gave a 100% match to true sex as determined by histology (Lance et al., 1992). Thus, an alternative explanation is that, although shape differences appear evident for *P. expansa*, male and female hatchlings exhibit a larger overlap in shape space that results in a higher misclassification rate when compared to *C. picta*.

Finally, the biological significance of the pattern of sexual dimorphism in shape detected in either of these two species remains unclear. Only one other turtle species has been examined with GM methods, *Trachemys scripta* (Slice, 1993), to study the pattern of sexual dimorphism in adults, and a somewhat similar but not fully consistent pattern was found to that detected in hatchlings of *P. expansa*. The carapace of female *T. scripta* is centrally deeper, anteriorly broader, and posteriorly narrower, with a more laterally contracted midregion than the carapace of males (Slice, 1993). In many other chelonian species, one main adult dimorphic characteristic is the higher domed carapace of females relative to males (Ernst and Babour, 1989). However, direct shape comparisons with our results or those of Slice (1993) await future study because, to our knowledge, the sexual dimorphism of no other taxa have yet been examined with GM methods. One could speculate that the dimorphisms detected in this study may be a functional difference linked to reproduction. For example, females may be more constrained laterally in the midregion to produce a more domed carapace associated with larger abdominal volume for egg carrying, and the wider posterior end may also facilitate the passage of eggs during oviposition. Consistent with this idea, a flatter carapace constrains the clutch size of female *Natator depressa* when compared to females of similar size from other Cheloniidae species (Hays, 2001). However, it is difficult to

reconcile our results with this hypothesis because, although female *P. expansa* exhibited the more laterally constrained midregion, *C. picta* females did not, and males in both species displayed the wider posterior end relative to females.

The dimorphism we found was subtle in both species, but significant sexual dimorphism in adults can also be subtle. Slice (1993) suggested that, as detected for adult *T. scripta*, important sexual differences can result from slight shape differences, small in magnitude as compared to overall shape variation, but related to reproductive function directly or indirectly. More important, the onset of sexual dimorphism of a different trait related to reproduction of adult *Chelydra serpentina* (namely, precloacal length) is known to be as early as hatching (de Solla et al., 2002). Thus, our finding of a carapace shape dimorphism in *C. picta* and *P. expansa* at such an early life stage may not be completely unexpected. The biological significance of such dimorphism, however, remains an open question.

Acknowledgments

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