Salinity and Egg Shape Variation: A Geometric Morphometric Analysis

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ABSTRACT.—The harvest and incubation of American Alligator (*Alligator mississippiensis*) eggs is an important component to the commercial alligator harvest industry in the southeastern United States. As a result, various methodologies have been used to monitor alligator populations including abundance counts, stress quantification, and nesting surveys. Past studies have dismissed the importance of egg shape in crocodilians, Squamates, and turtles and deemed egg shape in birds and other amniotes as similar, in relation to functionality. The complexity of crocodilian eggs has been examined, and both turtle and Squamate eggs have been regarded recently as physiologically more intricate than bird eggs. This study introduces a physiological approach to monitor alligator populations in freshwater and low salinity environments by quantifying egg shape in correlation with varying salinity. We introduce a fractional semilandmark-shape template method to quantify egg shape within a geometric morphometric framework. This approach is beneficial because it allows for the quantification of shape for curved structures, such as eggs, which lack homologous landmarks. The results from this study suggest that alligator egg shape is correlated with varying salinity levels, such that variation in alligator egg shape at low salinities changes in gradient-like fashion, whereas salinities high enough to be deemed stressful result in reversion back to a low salinity egg shape or desiccation. This study elucidates a correlation that can be implemented in management and breeding techniques and opens the door to in-depth physiological examination of the system.

The American Alligator (*Alligator mississippiensis*), like most crocodilian species, has become an economically valuable species with an extensive commercial market. As a result, the number of commercial ranches has increased over the last couple of decades, and many of these ranches are sustained by the harvest of alligator eggs from wild populations. The management of alligator populations has been professionally discussed publicly for more than 60 yr, as indicated by Giles and Childs (1949), Allen and Neill (1949), Taylor and Neal (1984), and Rice et al. (1999). Currently, numerous management protocols are in place to protect and sustain wild populations in the United States. Such protocols were founded on numerous academic endeavors (many listed in Joanen and McNease, 1980), and populations are consistently modeled and their biology investigated (e.g., Taylor and Neal, 1984; Taylor et al., 1991). In addition to standard approaches, novel monitoring practices are also developed frequently and implemented (Rice et al., 1999). Investigation of egg shape and abiotic correlation, in a physiological context, may provide important information regarding management practices related to egg harvesting for crocodilian species.

The amniotic egg is an essential biological structure in reptiles, birds, and monotremes and has been studied extensively in a developmental and evolutionary context (e.g., Packard et al., 1977; Packard and Seymour, 1997). However, eggs have been vastly understudied from the perspective of shape and, more crucially, the relationship between shape and physiological processes and environmental conditions. Hoyt (1976) noted that physiological variation is not related to the variation in bird egg shape. However, Lutz et al. (1980) observed that the eggshell thickness and water conductivity of eggs of the American Crocodile, *Crocodylus acutus*, were twice that of bird eggs. Eggs of *C. acutus* were also found to exhibit a strong permeability–oxygen conductivity trade-off, and Lutz et al. (1980) noted that dehydration via transpiration is common in crocodile eggs. Given the physiological differences between bird

eggs and crocodilian eggs, applying the same shape–physiology relationship in bird eggs from Hoyt (1976) to crocodilian eggs may be a faulty comparison. Rose et al. (1996), in a study that predicted egg volume and related egg shape to maternal ovum compression in the turtle *Pseudemys texana*, confirmed that osmotic attributes of eggs in varying salinities is a "fruitful area for detailed investigation." Predictions persist that spherical eggs are favored in species with large clutch size or when the retardation of water loss is necessary (Iverson and Ewert, 1991; Rowe, 1994). Rowe (1994) revealed that *Chrysemys picta* populations with larger egg wet mass to maternal body size ratios had more elongate eggs. While prescribing future directions, Rowe (1994) stated that "the influence of egg shape and relative surface area on gas exchange (potentially greater in elongate eggs) and water exchange (retarded water loss in spherical eggs) during incubation should also be addressed (Iverson and Ewert, 1991) [citation his]."

Regarding external shape, eggs are composed of continuous curves, being ellipsoidal or spherical, and are difficult to quantify morphometrically, because they possess no homologous landmarks for use in shape analyses. Over the past few decades, geometric morphometric analyses have become a popular and useful tool in quantifying shape variation. Biologically, this approach has been put into practice within the realms of ecology, evolution, and medicine (Zelditch et al., 2004). Geometric morphometric analyses have been developed in an attempt to move away from the problems associated with traditional morphometrics, such as limited sample directions disregarding homologous anatomical landmarks, and allometric issues. Previous operational developments from Kendall (1977), Strauss and Bookstein (1982), and Bookstein (1989) have paved the way to modern methodologies (Rohlf, 1990; Rohlf and Marcus, 1993; Adams et al., 2004) that incorporate the isolation of shape analysis from size and the extraction of information pertaining to an organism's geometric structure.

One aspect of shape that the landmark-coordinate system ignores is the curvature of a structure (Zelditch et al., 2004).
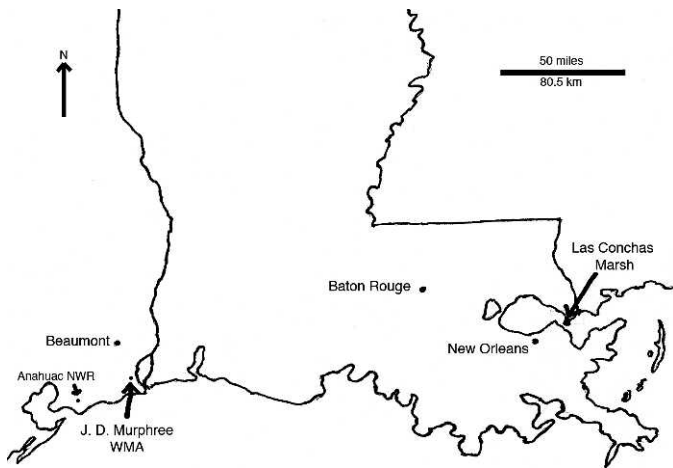
FIG. 1. Map showing the study sites. Las Conchas Marsh near Slidell, Louisiana, is highlighted to the east, whereas J. D. Murphree WMA and Anahuac NWR are highlighted to the west.

FIG. 2. The photographic setup used to collect egg-shape data. Nest and egg identification and scale bar are in picture.

Because curvature is of interest to biologists as a component of shape, some methods have been proposed to capture data associated with nonhomologous points along curves (Rohlf and Archie, 1984; Ferson et al., 1985; Lohman and Schweitzer, 1990; MacLeod and Rose, 1993; Zelditch et al., 2004). These points, termed semilandmarks, are defined in a number of ways, some of which have been illustrated by Zelditch et al. (2004, fig. 15.6). Aside from semilandmark designation, procedures exist to attempt to correct for the variance in statistical power between landmarks and semilandmarks. Two such corrections are differential weighting (Zelditch et al., 2004) and semilandmarks sliding along tangents to alleviate thin-plate spline bending energy (Green, 1996; Sampson et al., 1996; Bookstein, 1997). Specifications to statistical analyses and software packages may vary depending on how semilandmarks are incorporated.

The American Alligator occurs, primarily in freshwater swamps and backwater habitats in the south and southeastern United States. However, some populations in coastal regions occur in low salinity environments. Previous fieldwork and observations by the first author suggest that egg shape of alligators vary according to salinity. These cursory observations were based on a noticeable difference in egg shape among nests, and the background knowledge that salinity within each study site was a highly variable parameter. No specific direction of shape change was initially hypothesized. This study focused on investigating the relationship between egg shape and different natural salinity regimes. Specifically, the aims of the study are threefold: (1) to introduce a fractional semilandmark shape template (FSST) (similar to the "increments of the cord" method described in Zelditch et al., 2004:397) to allow for the digitization and subsequent geometric morphometric analysis of eggs or other structures with no homologous landmarks; (2) to explore the use of geometric morphometric landmark data in nonparametric statistical software and analyses; and (3) to test the hypothesis that egg shape variation in *A. mississippiensis* is correlated with the salinity of the water nearest the nest site. The hypotheses tested herein also provide new information regarding the developmental biology and life-history strategies of alligators using modern morphometric tools.

MATERIALS AND METHODS

Eggs were collected from two primary study sites and one additional site: J. D. Murphree Wildlife Management Area (WMA) southwest of Port Arthur, Texas; Las Conchas private marsh east of Slidell, Louisiana; and Anahuac National Wildlife Refuge near Anahuac, Texas, respectively. The J. D. Murphree WMA is a 24,250-acre area of marsh habitat with salinities ranging from fresh to brackish water from northwest to southeast. Las Conchas Marsh is an approximately 4,000-acre tract of intermediate marsh habitat bisected by the freshwater salt bayou and extending south to the saline Rigolet's (Fig. 1). One nest from Anahuac NWR in southeastern Texas was also analyzed for use in this study in 2009, and these data are included in the J. D. Murphree WMA dataset for all analyses. Sites in southeastern Texas showed higher salinity ranges across both years (0 to 14 ppt) than in Slidell, Louisiana (0 to 4 ppt) because of hurricane Rita in 2009 and anthropogenic inflow in 2010. Five- hundred fifty-five eggs were analyzed from 22 nests across two reproductive seasons (2009 and 2010). The reproductive season is defined as the onset of mating (April through mid-May) to hatching roughly 66 days post-deposition (August) (Lance, 1989). Three hundred eggs across 11 nests from Slidell, Louisiana, and 255 eggs across 11 nests from southeastern Texas were analyzed. Data were collected from any and all nests that were found and accessible by boat. Eggs were removed from each nest and not rotated to avoid lethal developmental disruption. A minimum of 10 eggs from each nest were photographed with scale bar and ID in the picture (Fig. 2), and every attempt was made to collect all data within one week of egg deposition. All eggs were photographed from the majority of nests. If poor weather or equipment failure threatened data collection, 10 eggs were randomly selected from the clutch. This was the case in eight of the 22 nests. Photographs were taken with a Panasonic Linux Digital Camera in the field. Salinity was measured using a Quanta HydroLab at
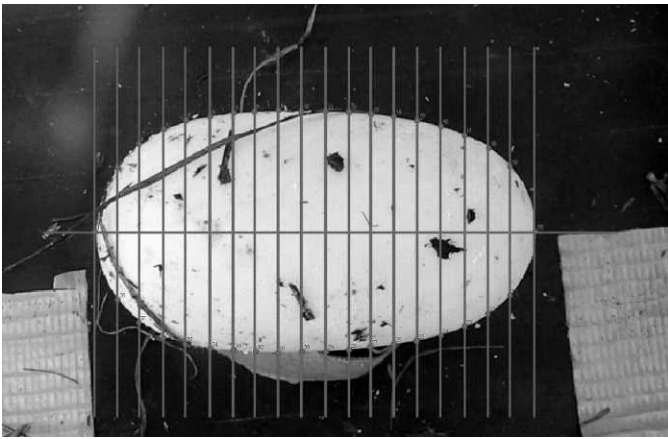
Fig. 3. The fractional semilandmark shape template overlaid on an alligator egg displaying all 38 semilandmarks at the intersection of the eggs circumference with horizontal centerline and 19 perpendicular verticals.

the water's edge nearest to the nest site. Aggressive mothers were captured and temporarily processed for use in another study and to conveniently avoid disruption of within nest data collection. Damaged eggs were not photographed and, thus, not incorporated into analyses because shape was confounded.

Photographs were cropped and eggs were digitally aligned such that conical eggs pointed to the right side of the photograph to standardize specimen orientation using Microsoft Office Picture Manager. A fractional semilandmark shape template (FSST) was created and overlaid onto each egg using Adobe Photoshop CS2 Version 9.0.2 (Adobe Systems, San Jose, CA) (Fig. 3). The FSST consists of one horizontal line and 20 vertical lines equidistant from each other and perpendicular to the one horizontal line. The FSST was oriented such that the horizontal line bisected the egg at its greatest length. The farthest left and farthest right vertical lines were also oriented such that they bisected the points at the eggs greatest length. The FSST was resized to fit each egg without distortion and, thus, maintain the same scale in every photo. The ratio between height of the FSST and width (aspect ratio) did not change. The purpose of the FSST was to allow for two semilandmarks to be positioned along equal proportions of an egg's circumference, one on the top and one on the bottom at the intersection of the vertical lines and the circumference (every 1/19th of the egg's circumference in this case). Two additional semilandmarks were positioned at the tips of each egg's length where the horizontal line intersects the farthest left and farthest right vertical lines.

Thirty-eight semilandmarks were digitized for each egg using TPSDig2 (Rohlf, 2004), and scale was designated using the ruler in each photograph. Data files were combined, and grouping files were obtained using TPSUtil (Rohlf, 2000). TPSRegr (Rohlf, 2009) was used to obtain consensus, aligned data, centroid size, and weight matrix files. A classifier was made by transposing the weight matrix into Microsoft Excel (Professional Edition 2003). Egg ID, site, and salinity range were designated as classifier variables. Nest site salinity was allocated a classifier range between the nearest 2 ppt. For example, a nest site salinity of 1.83 ppt was allocated a "1–2 ppt" salinity classifier. One nest at 14 ppt and two at 9 ppt were designated classifier variables "14" and "9," respectively.

MorphoJ software (Klingenberg, 2011) was used to perform ordination statistical tests and a Procrustes ANOVA to assess egg shape and centroid size variation in varying salinities. Canonical variance analyses (CVA) by site and salinity variables were conducted and transformation grids showing the sources of egg shape variation at varying salinities were obtained using TPSRegr (Rohlf, 2009). Canonical variates are one of any designated landmark that undergo shape change across samples based on x- and y-axes. For instance, the axis "canonical variate 1" is a landmark that undergoes a proportion of change along one axis, and specimens are placed within the plot area along that axis based on directionality of change. Each CVA performed has 38 covariates because there are 38 semilandmarks per egg and all covariates are similar in their response to variation.

CVAs were performed to assess the variation in egg shape by salinity classifier within each site to ensure that our follow up analyses were not confounded by a simple difference between study sites and that similar trends were occurring in both sites. One nest at 14 ppt was omitted from this analysis to elucidate the separation between lesser and more similar salinity levels. One CVA was performed using data only from the 2009 sampling period. A combined 2009 and 2010 CVA was conducted with more general classifiers (such as "0–2 ppt") such that separation in the ordination could be more adequately assessed. Further, the first five and later 20 partial warp scores (Cartesian coordinates for each landmark) were obtained from the W file (weight matrix of Procrustes-residuals and partial warp eigenvectors) and realigned in Microsoft Excel (Professional Edition 2003). These 20 scores represent the top half of every egg, whereas five provide data for the top left corner of every egg. All 38 partial warp scores were not used to avoid redundancy, because all eggs are symmetrical. These two data sets (5 and 20 partial warp scores) were imported into PRIMER 6 (Clarke and Gorley, 2001), and Euclidean distance resemblance matrices were obtained. Factors for each specimen were designated as the salinity ranges mentioned previously, and the dataset was averaged over for this factor. Multi Dimensional Scaling (MDS) analyses were performed to assess variation in egg shape between salinities. The relationship of the points to one another indicates the amount of similarity between them in two-dimensional space.

RESULTS

Within-site canonical variance analyses (Fig. 4) revealed a similar trend in both study sites. Southeastern Texas revealed variation in egg shape in correlation with nest-site salinity in gradient-like fashion (Fig. 4A). Separation exists between 1-, 2-, 3-, and 4-ppt salinity levels. Eggs at less than 1 ppt and 9 ppt group similarly to each other and disobey the gradient-like change seen in eggs from other salinity levels. Las Conchas Marsh shows distinct separation between <1, 1–2 (labeled as 1), and 3–4 ppt (labeled as 3) (Fig. 4B).

Transformation grids (Fig. 5) reveal the source of egg shape variation at differing salinities. Eggs from nests at higher salinities are short and wide (Fig. 5A), whereas eggs deposited in lower salinity nests are long and narrow (Fig. 5B). We illustrate the 10-times exaggerated egg shape at both salinity extremes using red lines to show the direction of shape change from an averaged consensus egg (Figs. 5, 6).

The Procrustes ANOVA revealed a significant difference in egg shape among varying salinities ($P < 0.0001$) and no
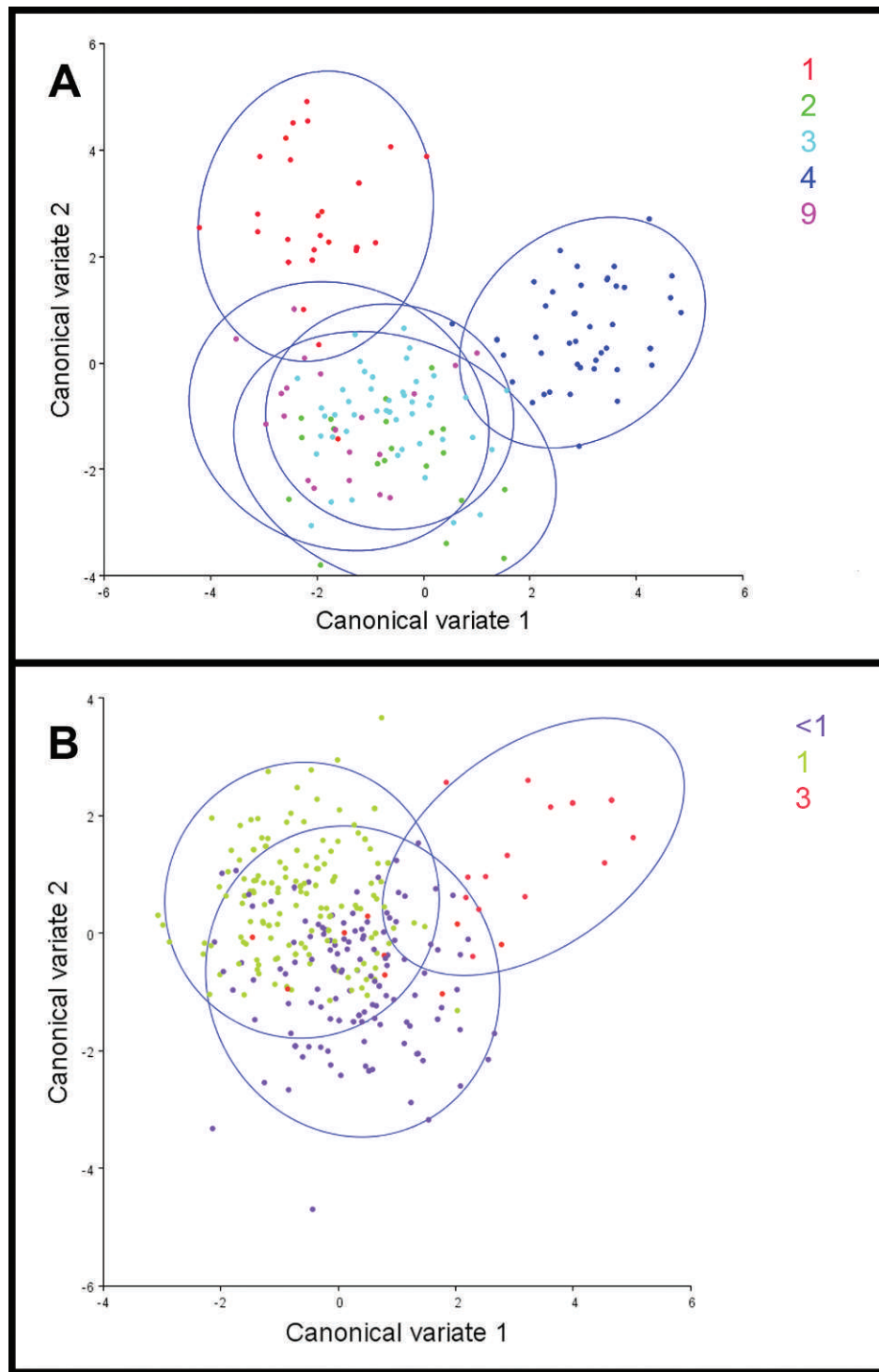
Fɪɢ. 4.   Within site canonical variance analyses depicted similar trends in correlation between egg shape and nest-site salinity. (A) Canonical variance analysis performed in MorphoJ using 2009 and 2010 data from J. D. Murphree WMA with one nest at 14 ppt omitted to elucidate gradient-like change in egg shape between varying salinities. (B) CVA performed in MorphoJ using 2009 and 2010 data from Las Conchas Marsh.

significant difference in centroid size among the same salinity ranges. The CVA using 2009 data from both sites used five salinity classifiers: <1 ppt, 1–2 ppt (2), 3–4 ppt (3), 4–5 ppt (4), and 14–15 ppt (14) (Fig. 6A). The ordination reveals separation between each salinity cluster and continuous shape change in a gradient-like fashion from 0 to 5 ppt. A cluster at 14 ppt is less similar to every other group than all other groups are to each other.

The CVA analyses of both 2009 and 2010 data revealed separation between 0–2.9 ppt and 3–5 ppt (Fig. 6B). These two clusters were oriented near each other with minimal overlap. A 14-ppt egg shape cluster was again noticeably different from all other egg shapes at all other salinities. Interestingly, eggs at 9 ppt overlapped both the 0–2 ppt and 3–5 ppt clusters and were diverse in egg shape. Multidimensional scaling (MDS) using partial warp scores from the first five designated landmarks also
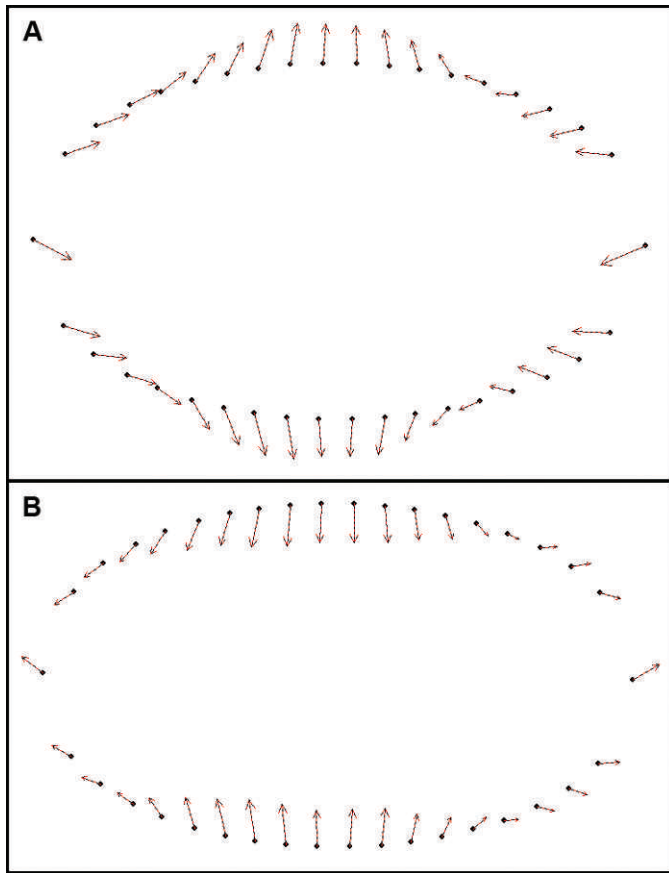
FIG. 5. Transformation grids depicted in TPSRegr at 10× exaggerated shape change from average consensus egg. The grids show the elongate narrow shape of eggs at lower relative salinities (A) and the shorter wider shape of eggs at higher salinities (B). Arrows indicate the directionality of shape change for each landmark in eggs from a specific salinity.

revealed separation between eggs at 14 ppt and eggs at all other salinities (Fig. 7A). Two other groups were also revealed, strictly based on data from the top left corner of each egg. Eggs from 3–4 ppt and 4–5 ppt grouped similarly to each other but differently from the other grouping consisting of 0–1 ppt, 1–2 ppt, 2–3 ppt, and 9 ppt. These lower salinity groupings and 9 ppt were all very similar to one another. The stress necessary to conform this three-dimensional analysis to two-dimensional space was 0.01. A more robust MDS based on data from the first 20 partial warp scores (top half of egg) revealed similar results (Fig. 7B). Eggs from 0–1 ppt, 1–2 ppt, and 2–3 ppt grouped out similar to each other along some axis, whereas those from 3–4 ppt, 4–5 ppt, and 9 ppt grouped similarly to each other along a similar axis as the lower salinities. These two groups were minimally separated from each other, but both were different from eggs at 14 ppt. Stress on this MDS was 0.

DISCUSSION

Methodology.—The fractional semilandmark shape template (FSST) proved to be a successful method for identifying semilandmarks along egg curvature. The method provided a substantial amount of data and multiple analyses reveal similar results based on FSST data. Transformation grids show that the FSST did elucidate subtle variation in overall egg shape. It is important to note that the number of vertical lines (20 in our case); in turn, the fraction of an egg's circumference at which a semilandmark is placed (1/19th in our case) is arbitrary. We acknowledge that more data are better, and more lines would provide more data. An ideal method would implement a drawn line around the circumference of the egg using every marked adjacent pixel as a semilandmark. However, a method of this nature is impractical for hundreds of eggs. The MorphoJ (Klingenberg, 2011) Procrustes ANOVA revealed no significant difference in centroid size. The scaling adjustments of the FSST to fit each egg did not confound centroid size and appeared to have isolated shape.

The nonparametric analyses using partial warp score landmark Cartesian coordinates also proved to be a valid statistical representative of shape. The ability of geometric morphometric data to be analyzed using a diversity of more rigorous statistical tests is beneficial to the geometric morphometric program as a tool. Both Euclidean distance-based Multidimensional scaling (MDS) tests performed in PRIMER 6 (Clarke and Gorley, 2001) (using 5 partial warp scores and then 20 partial warp scores) mimicked the spatial patterns seen in the Canonical Variance Analyses done on MorphoJ (Klingenberg, 2011). In our opinion MDS analyses are a more rigorous assessment of data and elucidate these geometric morphometric data to finer detail. The use of partial warp scores as environmental data, based on this test, is an appropriate way to analyze geometric morphometric data.

Hypothesis Testing.—The majority of work dealing with the quantification of egg shape has been performed using birds as model organisms. Preston (1953) developed an "eccentric angles" algorithm to assess interspecific variation in bird egg shape using 24 species. This algorithm was later applied to snake egg shape (Maritz and Douglas, 1994) without concern for physiological implications. Hoyt (1976) introduced a surface to volume index method for egg-shape quantification using 29 bird species that was intraspecifically used by Kern and Cowie (1996). The hypothesis that variation in alligator egg shape is correlated to the salinity of the water nearest the site of deposition cannot be falsified by these data and the aforementioned analyses. This study suggests that there is some physiological advantage to changes in alligator egg shape, potentially related to surface area to volume ratios allowing for variation in the number of pores present on the surface of eggs. This implication is bolstered by the complexity of physiological attributes of reptile eggs (Lutz et al., 1980), as well as the documented effect that egg shape has on meeting and maintaining necessary moisture and oxygen requirements (Rose et al., 1996). It would be irresponsible, however, to ignore the possibility that there is no physiological advantage or adaptive value to alligator egg-shape plasticity, and these data and abiotic correlations are coincidental (Gould and Lewontin, 1979).

Within-site canonical variance analyses suggest that the same trend is occurring in both sites and that variation in egg shape cannot be attributed to a difference in study sites (Fig. 4). Combined site canonical variance analyses suggest strong correlation between egg shape and nest-site salinity across spatial and temporal boundaries. Variation among eggs at lower salinities, such as 0 ppt to 5 ppt, is apparent, and shape change follows a gradient pattern across minute salinity changes. The 2009 CVA illustrates shape change along a salinity gradient the best in a lower salinity range (Fig. 6A), whereas the 2009 and 2010 combined CVA (Fig. 6B) reveals variation in egg shape between differing salinity ranges using twice the amount of
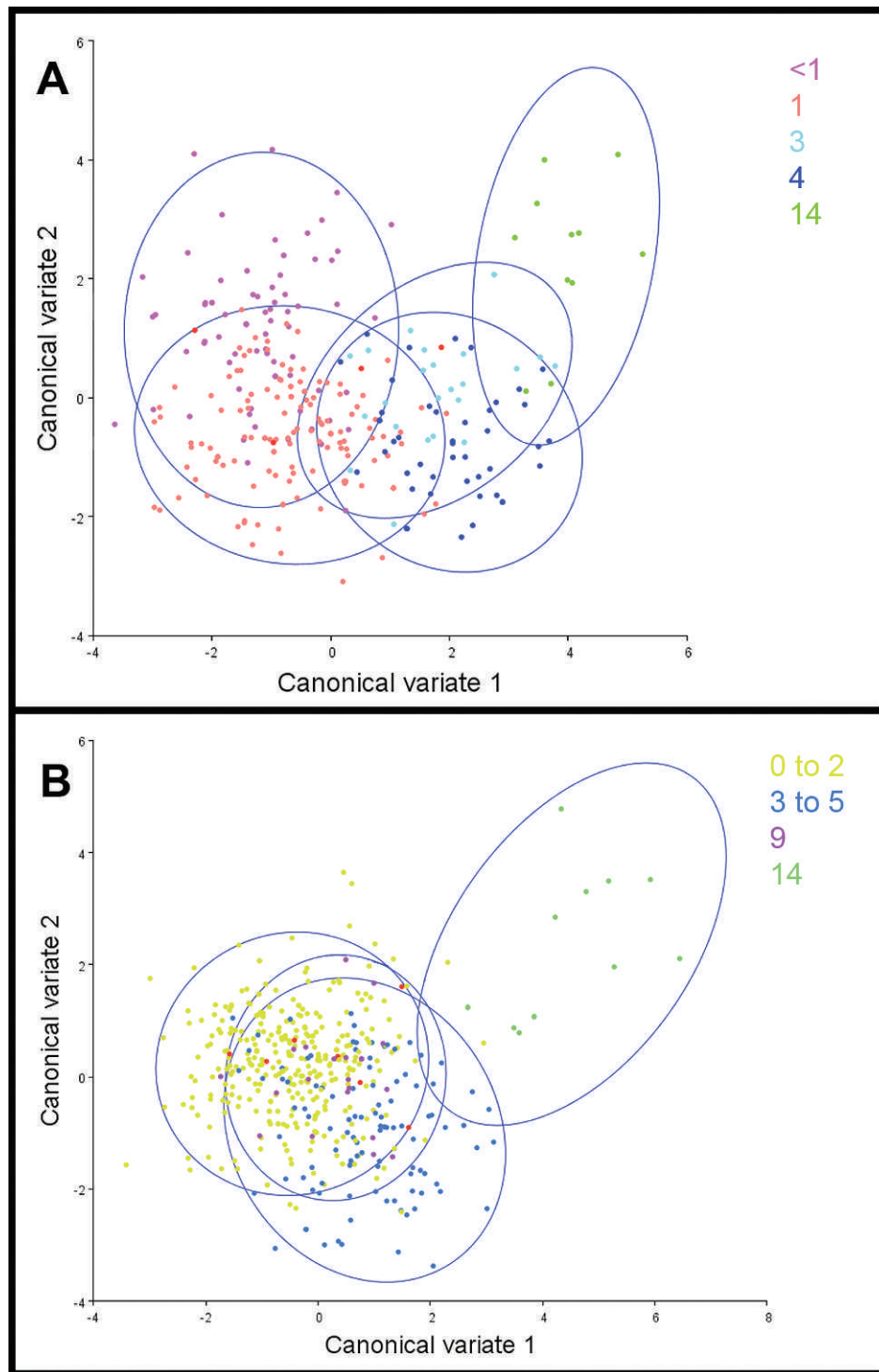
F<small>IG</small>. 6. (A) Canonical variance analysis using 2009 data from both study sites with five salinity classifiers: <1 ppt, 1–2 ppt (1.2), 3–4 ppt (3), 4–5 ppt (4), and 14–15 ppt (14). The ordination reveals separation between each salinity cluster and continuous shape change in a gradient-like fashion from 0 to 5 ppt with 14 ppt being less similar to every other group than all other groups are to each other. (B) Canonical variance analysis of 2009 and 2010 data reveal separation between 0–2 ppt (including all nests from 0 ppt to 2.99 ppt) and 3–5 ppt. These two clusters are oriented near each other with minimal overlap. A 14-ppt egg shape cluster is noticeably different from all other egg shapes at all other salinities. Eggs at 9 ppt overlapped both the 0–2-ppt and 3–5-ppt clusters and were diverse in egg shape.

data. Of interest is the shape of eggs at 9 ppt. Eggs from multiple nests at 9 ppt, all in J. D. Murphree WMA, have high within-classifier variation and group with eggs from lower salinities (Figs. 4A, 6B, 7).

Multidimensional scaling using landmark partial warp scores examined the variation in egg shape with salinity variation along two portions of egg circumference: the top left corner and the top half. Both analyses reveal separation between 14 ppt and all other salinities and grouped eggs at 9 ppt with considerably lower salinities.

Documentation exists that pertains to a maximum salinity for healthy nesting and alligator health in general (Lance et al.,

FIG. 7. A. Multidimensional scaling (MDS) using partial warp scores from the first five designated landmarks. Note the separation between eggs at 14 ppt and eggs at all other salinities as well as overlap between eggs at 9 ppt and 0–1, 1–2, 2–3 ppt. (B) Multi-dimensional scaling based on data from the first 20 partial warp scores (top half of egg) from all eggs. Eggs at 0–1 ppt, 1–2 ppt, and 2–3 ppt group out similarly to each other along some axis, whereas those at 3–4 ppt, 4–5 ppt, and 9 ppt group similarly to each other along a similar axis as the lower salinities.

2010). This observed maximum salinity for healthy nesting density is around 8–10 ppt (Joanen and McNease, 1989). Such physiological boundaries suggest that high salinities are not only stressful but alter life-history strategies (C. M. Murray, unpubl. data) and provide abiotic circumstances that negatively affect an individual's fitness. Because egg shape occurs within the mother, eggs deposited in high salinity environments (in this case >9 ppt) may be too costly to shape advantageously, because the mother may experience a somatic versus reproduc-

tive energy allocation conflict. Egg shape then reverts back to a "default" freshwater egg shape. Eggs deposited at 14 ppt have a different shape than those deposited at all lesser salinities. A single nest documented this nesting anomaly in 2009, and egg shape here may be attributed to some amount of desiccation. No banding, indicative of successful fertilization and development (Masser, 1993), was observed in these eggs, and the nest was poorly constructed in a sand substrate.

This study helps serve as a pathway for numerous future directions regarding alligator reproductive physiology. Alligator eggs are held within the mother and then deposited into a constructed nest on land. Eggs never touch the water nearest the site of deposition, which is where this study acquired its abiotic data. This begs the question, how and where, and at what stage in the mother, is shape variation occurring? Further, does egg-shape variation correlate to within-nest soil/debris salinity levels and humidity? This invites questions regarding temporal variation in individual egg shape, perhaps occurring in synchrony with abiotic variation, embryonic development, or some combination of the two. If and how variation in egg shape can maximize water and gas exchange in varying salinities is now an area in need of examination. The hypothesis that, at some salinity, maternal variation in egg shape is too energy expensive or futile for reproductive allocation, needs to be tested.

The harvest and sale of products derived from *A. mississippiensis*, including eggs, hatchlings, and adult parts, is a multimillion dollar industry along the southern Atlantic and Gulf Coasts of the United States. This study presents crucial information regarding the developmental and reproductive physiology of alligators and their eggs. Correlation between varying abiotic factors and alligator egg shape variation in a physiological context is of importance when considering and implementing management practices related to nest harvesting. This study, with the addition of future physiological hypothesis testing, aims to participate in the maintenance of alligator population stability in balance with a successful Gulf Coast alligator industry.

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