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# Morphological comparison of green turtle hatchlings from a different perspective: geometric morphometry

Serhat SERTTAŞ [1], Bektaş SÖNMEZ [2] and Can YILMAZ [3,4,*]

1. Department of Biology, Graduate School of Education, Hakkari University, 30000 Hakkari, Turkey
2. Suşehri Timur Karabal Vocational School, Sivas Cumhuriyet University 58600 Suşehri, Sivas, Türkiye
3. Medical Laboratory Techniques, Vocational School of Health Services,
Hakkari University, 30000 Hakkari, Turkey
4.Biodiversity Application and Research Centre, Hakkari University, 30000 Hakkari, Turkey
* Corresponding author: C. Yilmaz, E-mail: canyilmaz@hakkari.edu.tr

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**Abstract.** Many ecological and evolutionary studies have attempted to explain patterns of shape variation and its covariation with other variables using geometric morphometrics. This approach has been limited to morphological comparisons of marine turtle hatchlings between sexes. Therefore, we used geometric morphometrics to investigate the effect of nest temperature and depth on the morphology of green turtle (*Chelonia mydas*) hatchlings on Akyatan Beach in the eastern Mediterranean during the 2020 nesting season. A total of 19 nests (10 for temperature and 9 for depth) were used. Nest temperature was divided into two groups as: < 30 °C and ≥ 30 °C, and nest depth as: ≤ 70 cm and ≥ 71 cm. The shape analysis of the carapace and plastron regions of a total of 95 hatchlings was carried out. Geometric morphometrics showed a significant difference between the two nest temperatures for carapace shape and between the two nest depths for plastron shape. However, PCA did not confirm this difference. Also, the supra caudal notch, the third costal scute, and the femoral and anal regions showed more variation in the transformation grids. In studies on the morphology of marine turtle hatchlings, it can be said that geometric morphometrics can be an alternative to classical morphometrics studies, which may cause long processing times and stress to the hatchling by the researcher.

**Keywords**: *Chelonia mydas*, geometric morphometrics, nest depth, nest temperature, morphology.

## Introduction

With a long history, morphological studies are an integral and dynamic part of paleontology and biology. In addition, morphological studies provide fundamental information on animal development, evolution, biodiversity, biomechanics, behavior, ecology, and physiology. Marine turtles are ideal model organisms for comparative studies of life history variation because they move across very different ecological zones (i.e., from foraging to nesting areas) (Tiwari & Bjorndal 2000). Researchers have used the morphology of marine turtles to determine inter-regional and/or inter-nesting beach variation, adults' sexual dimorphism (Mortimer et al. 2022), growth rate (Omeyer et al. 2018), hatchling swimming performance (Mueller et al. 2019), hatchling health indices, and hatchling locomotor performance (Fleming et al. 2020, Page-Karjian et al. 2022, Steenacker et al. 2023).

In marine turtle hatchlings, morphology is also important as it affects locomotor and swimming performance (i.e., crawling and post-emergence migration) (Mueller et al. 2019, Steenacker et al. 2023) and, thus survival. Many studies have investigated the effects of biotic and abiotic nest characteristics and protective measures such as relocation on hatchling morphology in different marine turtle species (Sim et al. 2015, Mueller et al. 2019, Fleming et al. 2020, Matthews et al. 2021, Page-Karjian et al. 2022, Steenacker et al. 2023). Most studies reported that marine turtle hatchlings emerging from nests with low nest temperatures had slightly larger carapace sizes than hatchlings emerging from nests with high nest temperatures (Booth et al. 2013, Sim et al. 2015, Fleming et al. 2020, Page-Karjian et al. 2022). Similarly, studies have reported that incubation duration, a proxy for incubation temperature, influences the carapace size of hatchlings (Sönmez et al. 2011, Steenacker et al. 2023). However, hatchlings emerging from nests with high moisture content were reported to have smaller carapace sizes (Matthews et al. 2021). On the other hand, nest moisture content has been reported to affect hatchling carapace size and limb length (Sönmez et al. 2011). In contrast to nest temperature and moisture content, studies on nest depth and hatchling morphology are limited. Salleh et al. (2022) found that hatchlings emerging from deep nests were larger than those emerging from shallow nests.

The above-mentioned studies on nest characteristics and hatchling morphology of marine turtles relied primarily on measurements using calipers and flexible tape. These studies typically measured straight carapace length and width, mass, self-righting, and limb size of hatchlings. However, Kircher & Wyneken (2017) note that these measurements are susceptible to errors by the researcher and the equipment. In addition, they require long processing times by the researcher, which may cause stress to the hatchling. Therefore, the ideal method for these studies should be quick and cost-effective, applicable to dead and live hatchlings in the field, and designed to minimize stress for the live hatchlings.

A common approach to shape analysis, geometric morphometrics, can provide an alternative method for morphological comparisons. This method records the relative positions of morphological points, boundary curves, and surfaces, using landmark coordinates as the basis for shape measurement (see Adams & Otárola-Castillo 2013 for details). In recent years, ecological and evolutionary studies have increasingly used geometric morphometrics, as they offer a more comprehensive measure of biological shape than alternative approaches. For instance, researchers have extensively utilized geometric morphometrics to uncover phenotypic changes linked to species interactions (Adams 2004), investigate phylogenetic and macro-evolutionary trends (Klingenberg & Gidaszewski 2010), and uncover ontogenetic patterns in human evolution (Bookstein et al. 2003). In recent years, geometric morphometrics has been widely used as a method for habitat determination and skull shape variation in extinct and extant turtles and for sexual dimorphism in the Testudinata group (Valenzuela et al. 2004, Benson et al. 2011, Foth et al. 2017, Sönmez et al. 2019, Duro et al. 2021). The use of geometric morphometry in marine turtles is limited, although it has mainly been used for sex discrimination (Ferreira-Junior et al. 2011, Kircher & Wyneken 2017, Sönmez et al. 2019). It has also been used in studies such as allometry and health status of adult and juvenile marine turtles (Casale et al. 2017, Maulida et al. 2017, Álvarez-Varas et al. 2019, Chatterji et al. 2022).

As mentioned above, since determining the effect of different nest characteristics on hatchling morphology is important for survival, this study aimed to compare the morphology of hatchlings emerging from different nest depths and temperatures in green turtles (*Chelonia mydas*). This study used geometric morphometry as opposed to the traditional approach (measurements using calipers and flexible tapes) and discussed the effectiveness of this method.

## Material and methods

Hatchlings emerging from different nest depths and incubation temperatures of green turtles were collected according to the standard protocol of the protection and monitoring study at Akyatan Beach in the eastern Mediterranean (Yılmaz et al. 2022). Successful nesting activities were recorded and protected at their original location (in situ).

The temperature was measured using temperature data loggers (Orion Components, Chichester, UK, TK-4014). Within a few hours of oviposition, data loggers were placed in the center of the clutch and synchronized to record the temperature every five minutes (Yalçın Özdilek et al. 2016, Türkozan et al. 2021, Yılmaz & Oruç 2022). Temperature data were downloaded from each data logger using Tinytaq software version 2.3.1. Data logger records during the first 24 hours, which did not include equilibration with the surrounding sand, were excluded (Türkozan et al. 2021, Yılmaz & Oruç 2022). When hatchling emergence was complete, nests were excavated (1 week after the first hatchling emerged) and the remains were examined (Yılmaz et al. 2022; Sönmez et al. 2024). Nest depth (ND) was measured with a tape measure as the straight vertical distance from the sand surface to the deepest point of the nest (Yılmaz & Oruç, 2022).

A total of 18 nests with data loggers were installed, but 8 of these could not be used because not enough hatchlings were captured or the hatchlings that were captured had deformities or anomalies. A total of nine nests were also selected for comparison by depth. The selected nests were divided into two groups: the nest temperature as up to 30 °C (except 30 °C) and above (≥ 30 °C), and the nest depth as 70 cm (≤70 cm) and above (≥ 71 cm). A total of 163 nests in Akyatan Beach (2014-2020) had mean temperatures higher than 30.0°C during the entire incubation and sex determination period (see for details, Yılmaz & Oruç 2022). Therefore, this value was chosen to see the effect of temperature on hatchling morphology. Moreover, since the mean nest depth for Akyatan Beach is around 70 cm, this depth was selected as the pivotal depth (unpublished data).

Five hatchlings were randomly selected from each of the studied nests. The hatchlings were chosen to ensure that there were no deformities or abnormalities on the carapace or plastron. Digital images of the body region of each hatchling analyzed were then taken using a high-resolution digital camera. Attention was paid to the light quality of the environment during photography. The camera was held parallel to the image and photographed from the same distance. Once photographed, each hatchling was safely released to the sea.

Photographs of the hatchlings were transferred to 'tpsDIG2' software (Rohlf 2015). The x and y coordinates of 48 anatomical landmarks (36 and 12 on the carapace and plastron, respectively) were recorded from each image (Figure 1). These landmarks are the locations of biologically repeatable (operationally homologous) anatomical points (Valenzuela et al. 2004). The landmarks were then exported to an MS Office Text document, and the numerical coordinates in the text were imported into the 'MorphoJ' package software (Klingenberg 2011) for statistical analysis. Superimposition, translation, rotation, and scale changes were removed using a full Procrustes fit (Rohlf & Slice 1990), and a covariance matrix was created for each data set transferred into the software package. After this process, the datasets were prepared for statistical analysis. General Procrustes Analysis (GPA) was used for geometric morphometric analysis. The Procrustes ANOVA test was used to compare differences in shape, and discriminant function analysis (DFA) was used to test whether these differences were statistically significant. Principal component analysis (PCA) was also used to determine formal appearance, using the first two principal components. Geometric morphometric analysis was performed in 'MorphoJ' version 1.02d. A comparison of nests that have temperature and depth was carried out by a non-parametric Mann-Whitney U test.

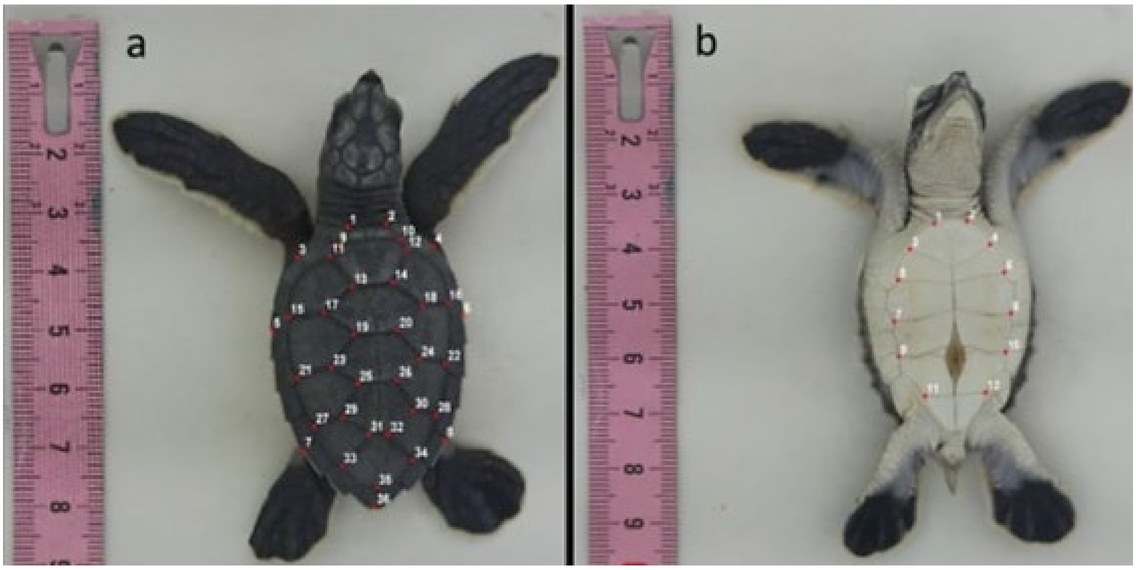

Figure 1. The location of the 48 landmarks (a = carapace, b = plastron) in green turtle hatchlings on Akyatan Beach.

## Results

Table 1 provides descriptive statistics on the nests' temperature and depth. The nests divided into two groups showed statistically significant differences (Table 1).

In the two body regions of the hatchling obtained at two different nest temperatures, the carapace shape analysis showed a significant difference in the Procrustes shape ANOVA (F=3.52, df =68, P=0.001), while the plastron did not (F=0,93, df=20, P=0,5541). Similarly, in the DFA test, the carapace showed a significant separation (p>0.001), but the plastron did not (P=0,4780). The PCA generated 48 principal components (PCs) for the carapace and 20 PCs for the plastron, with the first and second PCs accounting for 38.31% and 51.48% of the total variance, respectively. The transformation grids produced due to the PCA for the carapace and plastron are shown in Figures 2a and b.

Although the Procrustes shape ANOVA and discriminant function analysis were significant (for the carapace only) due to the low total variance and clustered over the elliptical figure with a 95% confidence interval, the hatchlings obtained from different temperatures were not separated and clustered on each other for both body regions (Figure 3a and b).

Table 1. Descriptive statistics of nests in terms of temperature and depth, and statistical comparison results between both groups.

| | Nest temperature (°C) | | Nest depth (cm) | |
|---|---|---|---|---|
| | < 30 °C | ≥ 30 °C | ≤ 70 cm | ≥ 71 cm |
| n | 5 | 5 | 4 | 5 |
| mean | 29.5 | 30.9 | 66.3 | 80.6 |
| Sd | 0.3 | 0.6 | 3.8 | 5.4 |
| Min | 29.2 | 30.3 | 63 | 74 |
| Max | 29.8 | 31.7 | 70 | 87 |
| Mann-Whitney U | Z= -2.611 | p=0.009 | Z=-2.460 | p=0.014 |

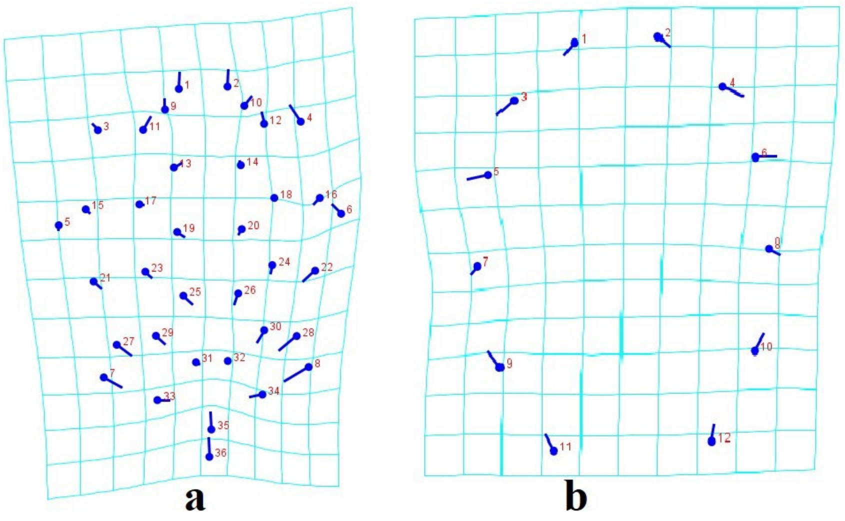

Figure 2. Transformation grids of hatchlings' carapace (a) and plastron (b) obtained at two different nest temperatures.

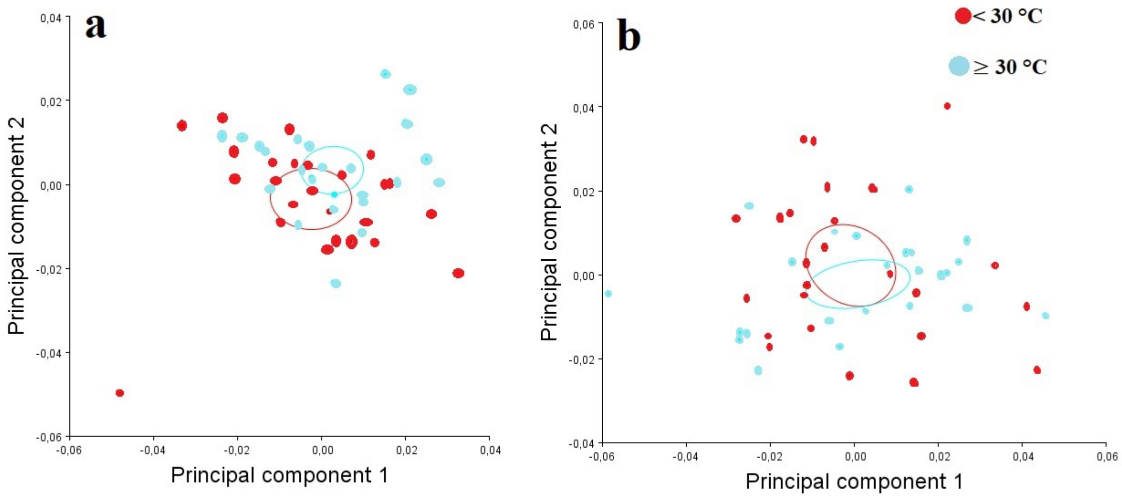

Figure 3. PCA plot with 95% confidence ellipses for carapace (a) and plastron (b) according to the different nest temperature

In the two body regions of the hatchling obtained at two different nest depths, the plastron shape analysis showed a significant difference in the Procrustes shape ANOVA (F=5.53, df=20, P=0.0001), while the carapace did not (F=0.70, df=68, P=0.97). Similarly, in the DFA test, the plastron showed a significant separation (p>0.001), but the carapace did not (P=0.88). The PCA generated 43 PCs for the carapace and 20 PCs for the plastron, with the first and second PCs explaining 87.4% and 43.7% of the total variance for the carapace and plastron, respectively. The transformation grids produced due to the PCA for the carapace and plastron are shown in Figures 4a and b.

Although the Procrustes shape ANOVA and discriminant function analysis were significant (for the plastron only) due to the low total variance and clustered over the elliptical figure with a 95% confidence interval, the hatchlings obtained from different depths were not separated and clustered on each other for both body regions (Figure 5a and b).

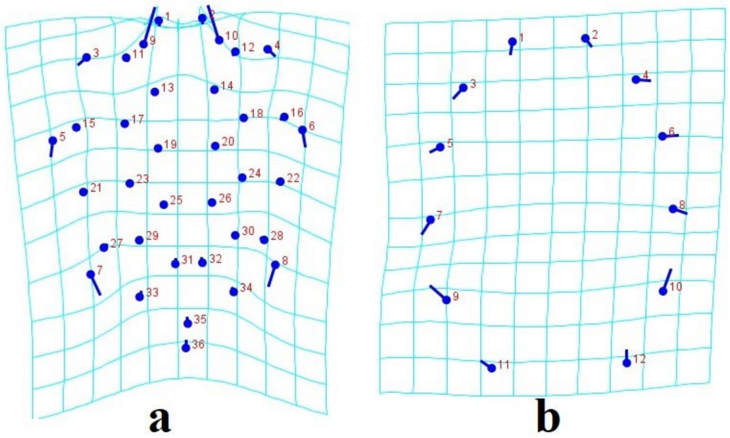

Figure 4. Transformation grids of hatchlings' carapace (a) and plastron (b) obtained at two different nest depths

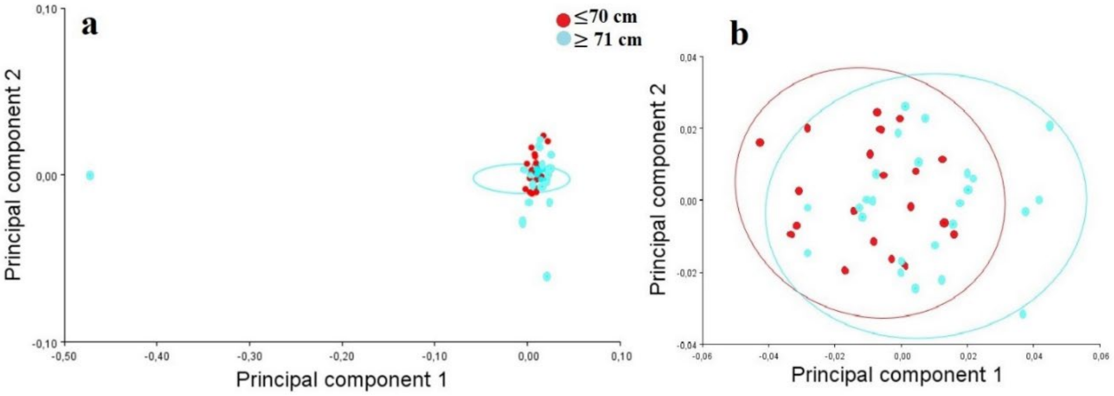

Figure 5. PCA plot with 95% confidence ellipses for carapace (a) and plastron (b) according to the different nest depth.

## Discussion

In marine turtles, nest temperature directly affects the biochemical reaction rate that drives physiological and developmental processes (Booth 2017). Lower incubation temperatures result in the conversion of more yolk material into hatchling tissues, leading to a larger yolk-free hatchling mass and a smaller residual yolk mass in hatchlings from cooler temperatures (Burgess et al. 2006, Booth et al. 2008). In this case, nest temperature appears to adjust a trade-off between hatchling morphological dimensions and energy reserves in the residual yolk (Booth 2017).

Our geometric morphometrics results showed that Procrustes shape ANOVA and DFA analyses revealed significant differences in carapace shape between the two nest groups. However, PCA has not confirmed this. Geometric morphometrics studies in marine turtles have mostly focused on sex discrimination (Ferreira-Junior et al. 2011, Kircher & Wyneken 2017, Sönmez et al. 2019). In their geometric morphometric study, Ferreira-Junior et al. (2011) reported that hatchlings from nests with a longer incubation period (i.e., cooler nests) tended to have longer carapace and plastron. In contrast, hatchlings from shorter nests (i.e., warmer nests) tended to be rounder in loggerhead turtles. However, Kircher & Wyneken (2017) reported that female green turtle hatchlings' supra caudal notch region showed more variation, although the Procrustes shape ANOVA did not show significant differences. Ceballos et al. (2014) reported in their geometric morphometric analyses that the anal notch of the plastron became increasingly sexually dimorphic (deeper in males than females) from 5 months of age in a non-sea turtle, *Podocnemis expansa*, produced at different nest temperatures (cooler and warmer).

In our study, the supra caudal notch and the 3rd costal scute regions showed more variation in the transformation grids. Considering the effect of nest temperature on sex (more females in warmer nests) (Mrosovsky 1994), perhaps these landmarks are the first signs of sex differentiation. However, the temperature <29 °C required for male production (Mrosovsky 1994) was unavailable in our study. This difference in landmarks may be explained by the fact that nest temperature has different effects even on the morphology of the same sex. In contrast, Kircher & Wyneken (2017) noted that the landmarks provided by scute patterns may be too unstable to reveal signals specific to morphological differences (e.g., sexual differences).

It should also be noted that our study population included only hatchlings and is limited by the small number of nests and hatchlings. Nevertheless, studies with small sample sizes have found fairly accurate results for mean size, standard deviation of size, and shape variance in geometric morphometry (Cardini & Elton, 2007). However, due to the number of samples in geometric morphometry, the mean shape and angle may be affected by the sampling error (Cardini & Elton 2007).

Nest depth affects temperature, humidity, incubation period, sex ratio, hatching success, and hatchling phenotype (Sao Miguel et al. 2022). Many researchers have reported that nest temperature gradually decreases with depth, and deeper nests have lower nest temperatures (Laloe et al. 2016, Lamont et al. 2020, Sao Miguel et al. 2022). Given this relationship between nest depth and temperature, deeper nests are expected to produce larger hatchlings. There are limited studies on the relationship between nest depth and hatchling morphology, particularly those that do not compare hatchling morphology to geometric morphometry. One study reported that the SCL and weight of hatchlings emerging from deeper nests were larger and heavier in green turtles (Salleh et al. 2022). Our study is the first geometric morphometric comparison based on nest depth. Although a Procrustes ANOVA and DFA did not reveal significant differences between the two nest depths for the carapace, landmarks 9-10 (first costal scute area) and 7-8 (9th marginal scute area) of the carapace showed particularly high variation. In contrast, the plastron shape analysis showed that the femoral and anal regions (landmarks 9-10) in particular showed more variation as a result of Procrustes ANOVA and DFA.

In marine turtle hatchlings, morphology is important for survival. Larger size may allow hatchlings to escape gape-limited predators and swim faster (bigger is a better hypothesis) (Booth et al. 2004). The ability of hatchlings to escape from predators is directly related to their locomotor performance (Booth 2017). Laboratory and field studies show that nest temperatures <27 °C decrease self-righting, crawling, and swimming ability, reaching a maximum at 28-32 °C and decreasing again at >32 °C (Booth 2017). While we did not investigate locomotor performance or survival in our study, we can assert that significant variations in the carapace and plastron of hatchlings produced at different temperatures and depths, resulting from geometric morphometrics measurements, may have influenced size differences and, consequently, survival. Similarly, classical morphometric studies in marine turtles (based on measurements using calipers and flexible tape) have reported that different nest temperatures and depths can affect hatchling size and hence survival (Sim et al. 2015, Fleming et al. 2020, Salleh et al. 2022, Steenacker et al. 2023). This demonstrates that geometric morphometry can serve as a viable alternative to classical morphometric studies, which may result in extended processing times and stress for the hatchling during the researcher's involvement in marine turtle hatchling studies. It minimizes researcher and equipment error and favors a quick, economical, and less stressful method. Nevertheless, geometric morphometry studies may also have some difficulties compared to classical morphometry studies. For example, data collection is more difficult. Whereas in classical morphometry, measurements can be made with calipers, in geometric morphometry, this is done with three-dimensional digitization tools or special software. This may also pose some difficulties. However, some researchers have suggested that digital image technologies such as geometric morphometry may be more advantageous than classical morphometry in non-marine turtle species (Valenzuela et al. 2004, Panda et al. 2024).

In conclusion, geometric morphometric analysis showed that nest temperature and nest depth caused variations in hatchling morphology in green turtles. These variations manifested in the carapace for nest temperature and the plastron for nest depth. We can say that variations in both body regions occurred in the posterior parts of the hatchling turtle. Similar studies in different populations are recommended. Thus, similar results can be discussed with the same method in other populations. In addition, using digital imaging technology such as geometric morphometry in the morphology of hatchlings may be more advantageous than classical morphometry in that it favors a quick, economical, and less stressful method.

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