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Non-modal Scutes Patterns in the Green Turtle Hatchlings: a Possible Effect of Clutch Mass on the Frequency

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In order to gain insight into the field of embryonic developmental biology, the study of scute patterning in green turtles (*Chelonia mydas*) has been a subject of considerable attention. Numerous factors, including genetic, environmental, and physiological are known to affect the formation of scute patterns. The aim of this study was to investigate the relationship between estimated clutch mass (ECM), volume of the sand at the top of the eggs (sand volume), and nest depth (ND) with the number of individuals exhibiting non–modal scute patterns (NMSP) at Alata beach in the eastern Mediterranean during the 2009 nesting season. We also conducted an investigation into the prevalence of NMSP frequencies. Using generalized additive mixed model (GAMM) analyses, we evaluated 5 covariates, including ECM, sand volume, ND, distance from sea and vegetation, that could affect NMSP. A total of 1,064 hatchlings from 33 nests were examined, and 801 exhibited the modal scute pattern, while 263 exhibited NMSP. The prevalence of NMSP was found to be 24.8%. The GAMM model showed a significant relationship between ECM and the frequency of NMSP, which was supported by correlation. ECM was found to account for 26% of the observed variation in the model, while ND and sand volume were found to account for less. The limited effect of ND and sand volume on NMSP, when considered alongside the modest proportion of ECM alone in explaining NMSP frequency, suggests that ECM may play a role in NMSP frequency with other environmental factors. This is the initial study to provide preliminary information into the association between ECM and NMSP frequency. In future studies, experimental studies involving ECM and environmental factors will be crucial in terms of revealing the contribution and joint role of ECM on NMSP frequency.

Keywords: Clutch mass, Scute pattern, Prevalence, Green turtle, Alata, Mediterranean

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BACKGROUND

The green turtle (*Chelonia mydas*) is a species of significant ecological and conservation interest, particularly due to its vulnerable status and the threats it faces from habitat loss, climate change, and human activity (Poloczanska et al. 2009; Mazaris et al. 2023). Among the various aspects of green turtle biology, the patterns of scute formation have garnered attention for their potential implications in understanding developmental biology (Barcenas–Ibarra et al. 2015; Sönmez and Sağol 2024). Turtles have a unique shell pattern and are covered with large keratin scales, called scutes, whose number and arrangement form a species–specific mosaic (Zangerl and Johnson 1957; Kobayashi et al. 2017). The arrangement of the scutes is a conserved feature that provides taxonomic information despite showing great variability in hatchlings (Mast and Carr 1989; Maffucci et al. 2020). These variations or anomalies consist of differences in the shape, arrangement and number of scutes (misshapen, supernumerary, or subnumerary scutes) (Mast and Carr 1989; Barcenas–Ibarra et al. 2015; Zim et al. 2017; Sönmez and Sağol 2024). The presence of these variations, anomalies and asymmetries in wild animals may be an indicator of developmental instability (Velo–Anton et al. 2011).

The scute patterns variation in is the most common malformation in the carapace of hard–shelled sea turtles (Mast and Carr 1989; Barcenas-Ibarra et al. 2015; Martín–del–Campo et al. 2021; Sönmez and Sağol 2024). Previous hypotheses on scute variations include atavism (ancestral traits) (Gadow 1899; Newman 1906) and embryonic mutations (Coker 1910). Recent research has focused on mechanical defects during embryonic development (Moustakas-Verho et al. 2014; Zimm et al. 2017) and genetic factors (Velo–Anton et al. 2011; Kobayashi et al. 2017). Elevated incubation temperatures, especially in the last third of incubation (Zimm et al. 2017; Kobayashi et al. 2020;

Calderon Pena and Azanza Ricardo (2021), moisture and oxygen limitations (Hewavisenthi and Kotagama 1989), handling of eggs (Mast and Carr 1989; Sönmez et al. 2011), and environmental pollution (Bishop et al. 1998) have been suggested as possible causes of abnormal scute patterns. It has been also proposed that variability in the number of scutes may depend on environmental conditions during embryonic incubation through an epigenetic mechanism (Caracappa et al. 2016). In addition to the temperature and humidity the maternal origin, which encompasses both genetic and non–genetic factors, may affect the phenotype of hatchlings. The researchers demonstrated that egg mass, which is a maternal factor, had a more pronounced effect on carapace size than nest temperature in green turtles (Booth et al. 2013). The egg mass is a strong predictor of hatchling mass (Bandimere et al. 2021), and is positively associated with hatchling carapace length and width in leatherback turtles (Wallace et al. 2006). Given the correlation between egg mass and clutch mass in sea turtles (Gatto et al. 2020), it may be postulated that clutch mass may also exert an influence on morphology, e.g., size, a compressed carapace, and scute anomalies.

Barcenas–Ibarra et al. (2015) noted that the pressure exerted by both overlying eggs and sand should not be ignored and that this issue is worthy of further investigation. Compared to artificial incubation (e.g., incubation of eggs in a Styrofoam box packed with sand) in Styrofoam boxes where eggs are evenly distributed and experience minimal sand pressure, natural nests (i.e., those left in their original places where the female deposited eggs) are likely to provide a more limited area (Barcenas–Ibarra et al. 2015). Consequently, eggs in natural nests are subjected to greater vertical pressure from the accumulated mass of eggs and sand above them. This pressure is related to clutch size, hence mass, and becomes more marked when large numbers of eggs are deposited (Barcenas–Ibarra et al. 2015). Sönmez and Sağol (2024) reported that nests with malformations (most observed supernumerary scute) exhibited significantly larger clutch sizes than nests without malformations. Taking a different perspective, it can be concluded that artificial incubation may reduce scute anomalies by reducing pressure. However, some researchers have recorded a higher prevalence of scute anomalies in artificial nests (such as relocated and hatchery nests, i.e., nests moved to suitable locations on the beach that are close to the characteristics of the original nest) compared to natural nests (Özdemir and Türkozan 2006; Durmuş et al. 2010; Tanabe et al. 2021). This inconsistency may be due to biological mechanisms that lead to different outcomes in different nest protection protocols. This is because in artificial incubation and nests, various factors such as temperature, moisture, and oxygen in the nest microenvironment (Sönmez et al. 2011; Kobayashi et al. 2020; Calderon Pena and Azanza Ricardo 2021; Tanabe et al. 2021), as well as the handling of eggs (Mast and Carr 1989), may have affected scute patterns to varying degrees. However, there are currently no reports on the possible consequences of the pressure exerted by the eggs and the overlying sand on the scute pattern, either in natural nests or in artificial incubation and nests.

As well as the pressure, clutch size may alter the overall nest developmental environment by increasing nest temperature via metabolic heating. Studies on green turtles demonstrate a positive correlation between clutch size and metabolic heat, which increases during the middle and last third of incubation, peaking in the last third and leading to elevated nest temperature (Önder and Candan 2016; Sönmez 2018). Given the documented relationships between nest temperature and carapace scute pattern (Telemeco et al. 2013; Zimm et al. 2017; Kobayashi et al. 2020; Calderon Pena and Azanza Ricardo 2021), the effect of clutch mass on carapace scute pattern seems highly likely but is yet undocumented. To date, no study has examined the relationship between clutch mass (or related clutch size and egg mass) and scute abnormalities. In consideration of the aforementioned background information, we hypothesize that clutch mass and the volume of the sand at the top of the eggs (hereafter referred to as sand volume) may exert an influence on the carapace scute pattern. Nevertheless, while all studies have concentrated on the underlying causes of carapace scute deviations, there is a limited number of studies that consider the number of individuals exhibiting this deviation in each nest (Telemeco et al. 2013; Kobayashi et al. 2020). Therefore, it is important to investigate the number of hatchlings with abnormalities that join the population. It was also noted that the survival of these hatchlings entering the population may be jeopardised by abnormalities in their development (Wyneken and Salmon 2020). In contrast, given that abnormalities in the carapace of green and loggerhead turtles do not affect their ability to survive (Bentley et al. 2021), if these malformations are inherited, they may have an impact on the future gene pool. Therefore, the aim of this study is to determine the relationship between estimated clutch mass and sand volume in green sea turtle nests and the number of individuals exhibiting deviations in the carapace scute pattern (number of individuals with a non–modal scute pattern). It also aims to determine the prevalence of non-modal scute patterns according to the sample size examined.

MATERIALS AND METHODS

Study area

The data were collected at Alata Beach located on the eastern Mediterranean coastline of Türkiye, during the 2009 nesting season. Alata Beach was found to be one of the important nesting sites for green turtles. This beach, 30 km from the center of Mersin province in Türkiye, is located within the borders of the Alata Horticultural Research Institute. It extends across 3 km from the marine resort on the eastern side of the Institute (36°37′930″ N, 34°21′187″ E) to the Topraksu camping site, also belonging to the Institute (36°36′868″ N, 34°19′711″ E) on the western end of the beach (Aymak et al. 2017). This beach is designated as a first degree protected natural site; it has natural sand dunes and is also sheltered, as it is within the boundaries of the Institute (Aymak et al. 2017).

Monitoring protocol

The data on the scute pattern of hatchlings were collected in accordance with the standard protocol that was followed during the protection and monitoring study at Alata beach (Aymak et al. 2005; Ergene et al. 2009). The protection and monitoring study was conducted under the licence and supervision of the Ministry of Agriculture and Forestry of the Republic of Turkey, 7th Regional Directorate, Mersin Province Section. No animal experiments were used in this study. The monitoring study was conducted from the middle of May to the end of September. The whole beach was patrolled both night and day with a team of five people. The successful nesting activities were marked and recorded with GPS. All nests were protected in their original place (in situ) and no cage was used. Nest parameters such as the distance from the sea and vegetation were also collected during daily monitoring patrols. Distance from the sea and vegetation for each nest were measured using a flexible tape (± 1 cm).

Sampling protocol

The nests were checked during the night patrol. Upon the emergence of the hatchlings, the scute patterns were examined, and then the hatchlings were safely released from the original nest site. All hatchlings emerging from the nests were checked for scute pattern. However, in some cases, such as instances where the emergence was missed, it was not possible to access all of the hatchlings. In the present study, a two–pronged approach was adopted for the sampling of hatchlings. Initially, the scute patterns of hatchlings were examined on the first day of emergence. The nests of these hatchlings were then monitored. Subsequently, if hatchling scute patterns from these nests were examined on a daily basis (until the end of emergence), they were included in the analysis. Conversely, if the scute patterns of hatchling emerging from a particular nest were checked on different days (non–consecutive), the data from these nests were excluded from the analyses. This approach was adopted to ensure a homogeneous distribution of hatchlings from each nest across days and to avoid potential biases in the identification of individuals with NMSP.

Indeed, the total number of nests sampled was 133, with a minimum of one and a maximum of 93 hatchlings. Nests and hatchlings that did not meet the aforementioned conditions were excluded from the analysis. In this context, the scute pattern of a minimum of 15 and a maximum of 70 hatchlings, with an average of 32 hatchlings from a total of 33 nests, was examined. A previous study (Ergene et al. 2011) revealed the most frequently observed scute pattern on Alata beach, which was accepted as the modal scute pattern. The modal scute pattern was identified as comprising five vertebral, four pairs of costal, and eleven pairs of marginal for *C. mydas* hatchlings on Alata beach (Fig. 1b) (Ergene et al. 2011). A deviation in at least one of these scute patterns was considered to be a non–modal scute pattern (NMSP) (Fig. 1a). The prevalence index indicates the ratio of individuals with at least one NMSP to the sample size examined.

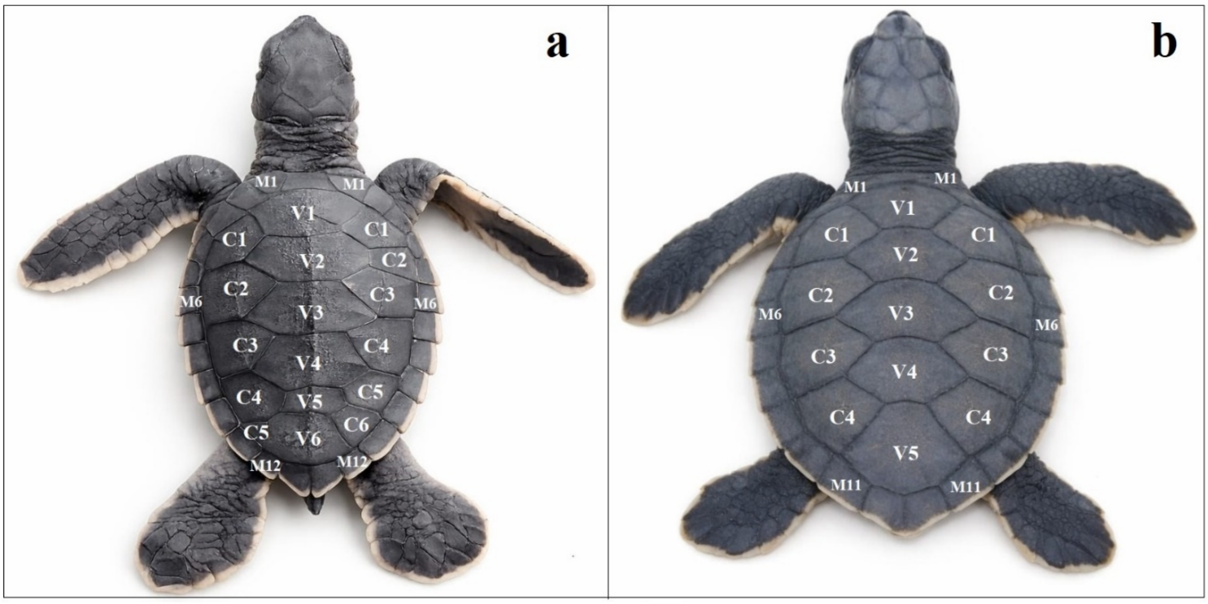

Fig. 1. The images presented depict a hatchling individual exhibiting two distinct carapacial scute patterns. The first pattern is characterized by a non–modal scute pattern (a), while the second individual displays a modal scute pattern (b). Vertebral scutes (V) are constituted of five elements that are arranged longitudinally along the midline, and do not form pairs. Costal scutes (C) are defined as those surrounded by a series of paired scutes (4 pairs) on either side of the vertebral scutes. Marginal scutes (M) are defined as those surrounded by an additional series of paired scutes (11 pairs) on the outer side.

Estimation of clutch mass and sand volume

The nests were excavated to examine their contents 1 week after the first hatchling emerged from the nest (Aymak et al. 2005; Ergene et al. 2009). Clutch size and nest depth were measured during nest excavation. Clutch size was determined by counting the number of unhatched eggs and hatch fragments (shell formation of more than 50% was accepted as 1 hatch (Miller 1999)). Estimated clutch mass was calculated by multiplying the mean egg mass by the number of eggs in the clutch (clutch size). The egg mass was obtained from previously published studies on the Mediterranean green turtle population. The mean egg mass for green turtles was reported as 34.79 g in Cyprus (Kaska and Downie 1999) and 33.25 g in Samandağ beach (Sönmez 2016). The egg mass was accepted as 34.02 g by taking the mean of both studies.

Nest depth was measured from the sand surface to the bottom of the nest using a flexible tape (± 1 cm). First, the nests were excavated to the top of the eggs during the process of excavating the nest. A cylindrical shape was then created between the surface and the eggs, covering the top of the eggs. The vertical distance between the top of the eggs and the sand surface was measured with a flexible tape (± 1 cm), and also the diameter of this cylindrical shape was measured from two different points. This provided the height and radius (r) of the cylindrical shape (the mean of two measurements). The sand volume of the cylinder was calculated as $V = \pi * r^2 * h$ in cubic centimeters. Where V is the sand volume of the cylinder, π (pi number) is 3.14, r is the base radius, and h is the height.

Statistical analyses

A nonparametric Wilcoxon signed–rank test was employed to ascertain whether there were significant differences in the number of individuals exhibiting modal and NMSP among green turtle nests in terms of hatchlings. The Wilcoxon signed–rank test visualizations presented here, including all relevant statistical details, were generated using the 'ggstatsplot' package in R (Patil 2021). Correlations between the number of individuals exhibiting a NMSP and the independent variables were performed using the "sjPlot" package V2.8.16 in R and employing the Spearman method with listwise–deletion (Lüdecke 2018).

Generalized additive non–parametric regression models, referred to as generalized additive mixed models (GAMM) with fixed and random or mixed effects, were used to investigate factors and/or covariates that affect the number of individuals with NMSP (Wood and Scheipl 2017). This modelling technique enables the flexible specification of both error and link functions, thus enabling the arbitrary definition of the functional form for each continuous covariate included in the model. The GAMM analyses included a single response variable (NMSP individuals) and five fixed effects, as well as one random effect (nest number). The five fixed effects comprised five continuous covariates, including estimated clutch mass (ECM), sand volume, nest depth (ND), distance from sea (DFS), and distance from vegetation (DFV). Given that the number of NMSP individuals in the GAMM model is a counting variable, the Poisson distribution is employed. The Poisson distribution is a statistical model frequently employed in the analysis of data concerning the number of events characterized by dimensions greater than one. It has been used in many studies of sea and freshwater turtles to compare the number of live hatchlings and adults, the number of non–viable eggs, and the total number of eggs (*i.e.*, clutch size) between years, individuals, and nests (Pradhan and Leung 2006; Sönmez 2019; Arcanjo–Oliveira et al. 2024; Hamilton et al. 2024). The GAMM models were fitted using the R package gamm4, via a version of the function "gamm()" from the "mgcv" package using the "lme" package (Wood and Scheipl 2017). The R code for the number of non–modal individuals is as follows: gamm4(non–modal ~ s(ECM) + s(SVolume) + s(ND) + s(DFV) + s(DFS), + random = ~(1| Nestname), + family = poisson, data = data).

RESULTS

The abiotic and biotic characteristics of the nests in which the carapace scute pattern was counted are presented in table 1. A total of 1,064 green turtle hatchlings from 33 nests were examined for the consistency of their scute pattern. Of the hatchlings, 801 (75.2%) exhibited a modal scute pattern, while 263 exhibited a NMSP. The prevalence for the sample size was 24.8%. An illustrative visualization of the numbers of modal and NMSP individuals in each nest is presented in figure 2. The graph, which includes paired counts within the same nest, provides details of both modal counts in terms of scute patterns of individuals. Moreover, the comparison of the modal and the NMSP individual counts revealed a statistically significant difference between the green turtle nests ($V_{\mathrm{Wilcoxon}} = 499.5$, $p < 0.001$, $CI_{95\%} = 0.57, 0.89$). That is, the NMSP individual counts showed a significantly lower frequency than modal individual counts in the green turtle nests. This result indicates that the two groups are independent of each other and that the data are suitable for further statistical analyses for NMSP individuals.

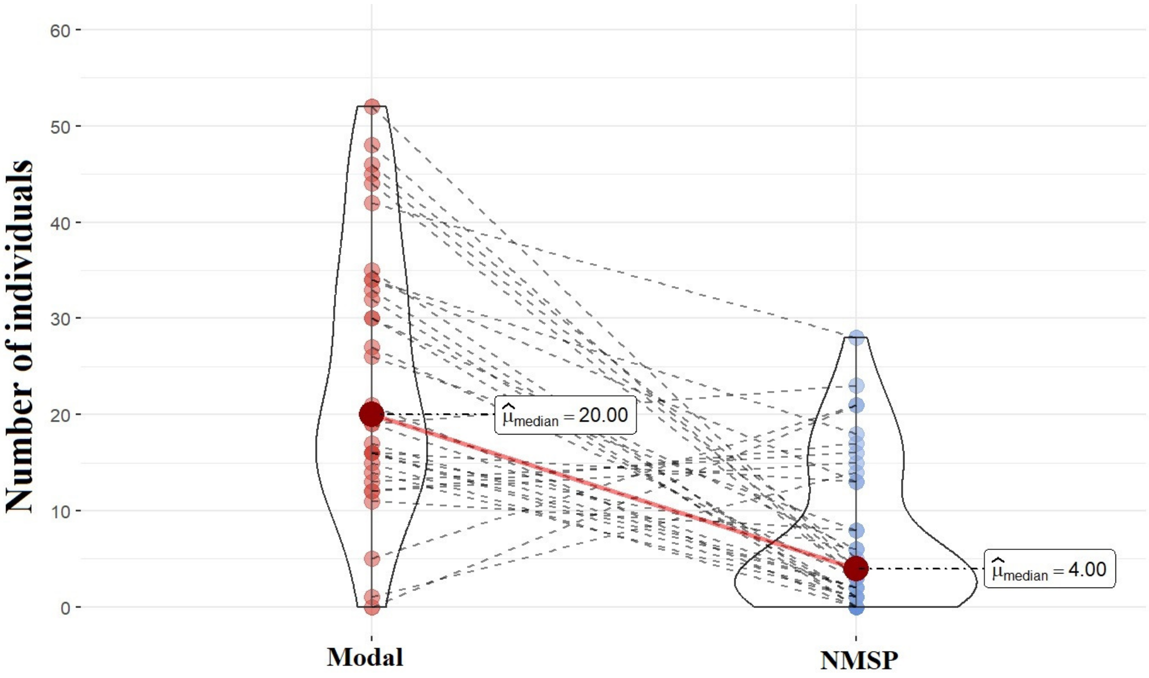

Fig. 2. Comparison of modal and non–modal scute pattern individuals in each nest of green turtle hatchlings. The each dashed lines demonstrate the discrepancies between modal and NMSP (non–modal scute pattern) individuals in the same nest.

Table 1. The abiotic and biotic properties of the nests in which the carapace scute pattern was counted, and the parameters used in calculating nest cylinder volume. * These values are used to calculate the volume of the cylindrical area of the nest. They are not referring to the nest's diameter or depth. (DFS: distance from sea, DFV: distance from vegetation, ND: nest depth)

Parameters		Mean ± Sd	Min–Max	Median	1st Quartile	3rd Quartile
Abiotic properties	DFS (m)	13.2 ± 3.7	6.7–23.8	13.4	10.8	15.8
	DFV (m)	3.2 ± 3.4	0–13.7	2.5	0	4.8
	ND (cm)	75.2 ± 13.2	55–101	71	66	85
Nest cylindrical volume	Sand Volume (cm^3)	29999 ± 10778	12874–60350.8	30902.3	21178.5	37486.1
	Height (cm)*	56.1 ± 14.6	34–87	52	46	67
	Mean Diameter (cm)*	25.8 ± 2.7	20–31	26	24	27.5
Biotic properties	Clutch Size (count)	118.5 ± 32.9	39–173	120	95	130
	Estimated Clutch Mass (g)	4030.9 ± 1121.5	1326.8–5885.5	4082.4	3231.9	4728.8
	Eggshell (count)	96.2 ± 28.4	38–149	91	78	120
	Hatching Success (%)	82 ± 12.8	51.1–97.8	85	76.4	91.9

The correlations between the number of individuals exhibiting a NMSP and the independent variables are shown in figure 3. A significant positive correlation was found between the number of NMSP individuals and estimated clutch mass ($r = 0.418$, $p = 0.016$,) and nest depth ($r = 0.349$, $p = 0.046$). However, no significant correlation was found between the number of NMSP individuals and the other variables ($p > 0.05$). Furthermore, nest depth exhibited a significant positive correlation with both estimated clutch mass ($r = 0.48$, $p = 0.005$) and sand volume ($r = 0.68$, $p = 0.0001$).

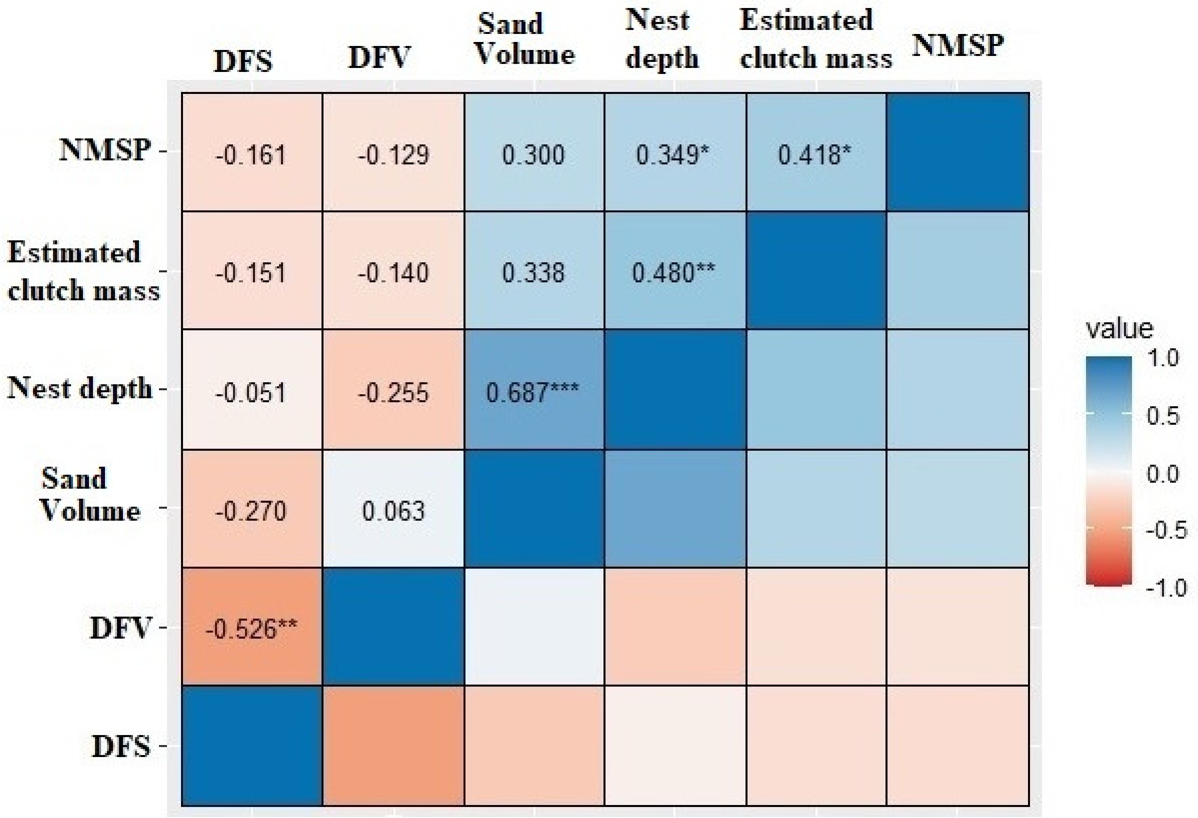

Fig. 3. The correlations between the number of individuals exhibiting a non–modal scute pattern and the independent variables (NMSP: non–modal scute pattern, DFS: distance from sea, DFV: distance from vegetation).

The statistical output from GAMMs is presented in table 2. The number of NMSP individuals was analyzed across six models, and the results demonstrated that only three models, *i.e.*, estimated clutch mass, sand volume and nest depth, were effective in terms of the number of NMSP hatchlings (Table 2 and Fig. 4). The estimated clutch mass model was found to explain 26% of the model deviance (Table 2) and provided an adequate fit to data with significant non–linear effects. The addition of four covariates (sand volume, nest depth, distance from sea and vegetation) and one random effect (nest number) to the model resulted in a reduction of the explained deviance to 20% and the failure to provide a statistically significant result.

Table 2. Statistical output from generalized additive mixed models (GAMMs) used to investigate the potential influence of different covariates on the non–modal scute pattern response of green turtle hatchlings (ECM: estimated clutch mass, SVolume: Sand Volume, ND: nest depth, DFS: distance from sea, DFV: distance from vegetation, edf: estimated degrees of freedom)

	Adjusted r^2	Variable	edf.	Chi.sq	p	Variable	Estimate	Std. Error	Z value	Pr(> |z|)
$\text{GAMM4}_{(\text{non–modal} \sim s(\text{ECM}))}$	0.26	ECM (g)	1	8.555	0.003	Intercept	1.546	0.19	8.135	< 0.001
$\text{GAMM4}_{(\text{non–modal} \sim s(\text{SVolume}))}$	0.09	Sand Volume (cm^3)	1	3.933	0.047	Intercept	1.530	0.20	7.408	< 0.001
$\text{GAMM4}_{(\text{non–modal} \sim s(\text{ND}))}$	0.19	ND (cm)	1	5.671	0.017	Intercept	1.537	0.20	7.669	< 0.001
$\text{GAMM4}_{(\text{non–modal} \sim s(\text{DFS}))}$	– 0.03	DFS (m)	1	0.149	0.699	Intercept	1.529	0.21	7.012	< 0.001
$\text{GAMM4}_{(\text{non–modal} \sim s(\text{DFV}))}$	– 0.01	DFV (m)	1	0.982	0.322	Intercept	1.530	0.21	7.132	< 0.001
$\text{GAMM4}_{(\text{non–modal} \sim s(\text{ECM}) + s(\text{SVolume}) + s(\text{ND}) + s(\text{DFV}) + s(\text{DFS}))}$	0.20					Intercept	1.544	0.18	8.241	< 0.001
		ECM (g)	1	3.467	0.062					
		Sand Volume (cm^3)	1	0.163	0.686					
		ND (cm)	1	0.031	0.860					
		DFS (m)	1	0.002	0.965					
		DFV (m)	1	0.152	0.696					

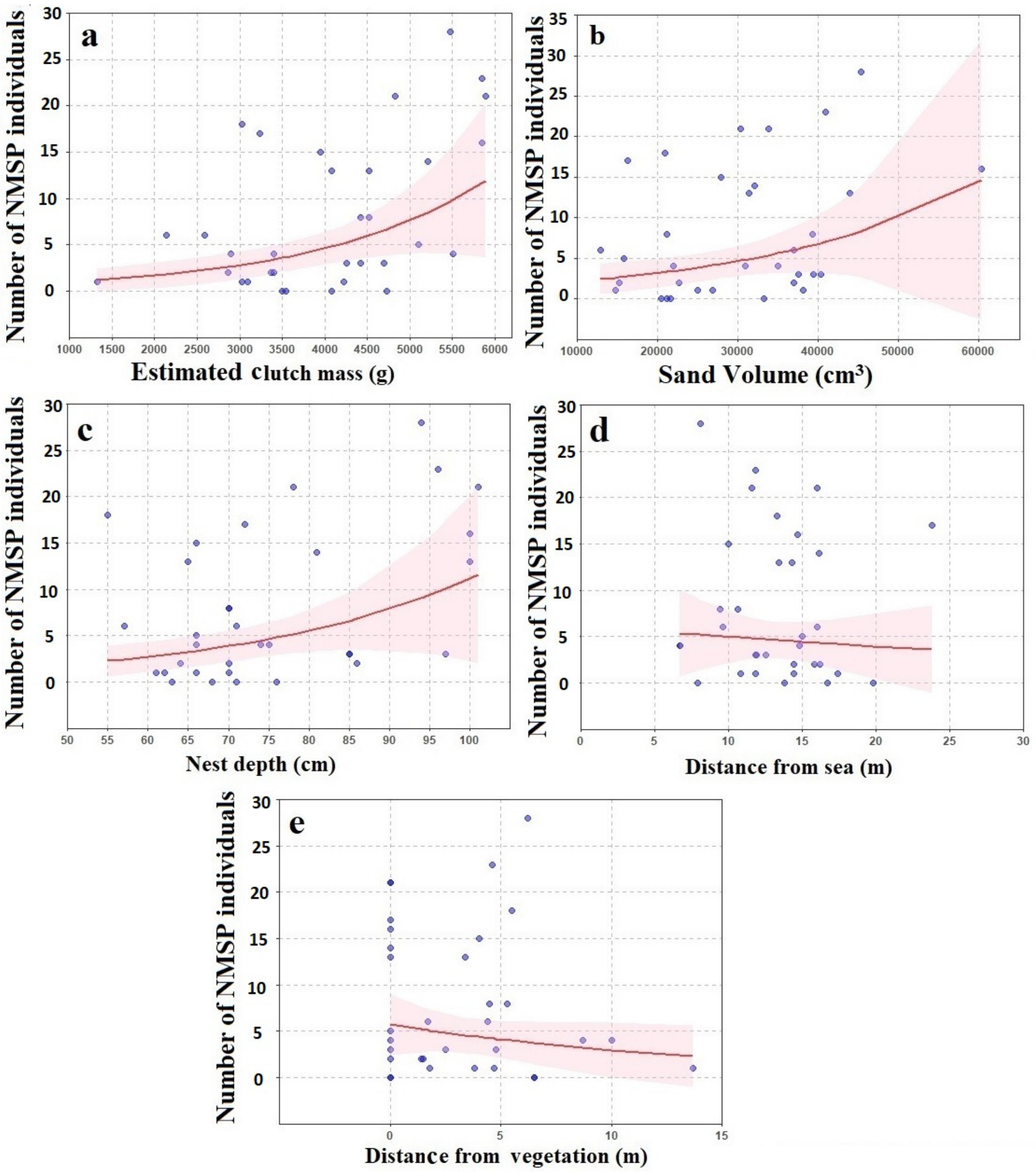

Fig. 4. Graphical summary of GAMM analysis. The response variable (number of non–modal scute pattern individuals) is shown on the y–axis. The covariates are shown on the x–axis: (a) estimated clutch mass (g) (b) sand volume (cm^3) (c) nest depth (cm), (d) distance from sea (m) (e) distance from vegetation. Shaded areas are bounded by pointwise 95% confidence curves around the fits (NMSP: non–modal scute pattern).

DISCUSSION

The number of individuals exhibiting the modal scute pattern was significantly higher than the number of individuals exhibiting an NMSP. Furthermore, the prevalence of NMSP individuals was found to be 24.8%. In a study conducted at Alata beach in 2003, the prevalence of NMSP in live hatchlings was found to be 21.9% (*n* = 917 hatchlings) (Ergene et al. 2011). Durmuş et al. (2010) reported a prevalence of 7.8% (*n* = 644 hatchlings) in nests that were protected in situ and 9.8% (*n* = 536 hatchlings) in nests that were relocated at Kazanlı beach, which is an area of significant importance for green turtle nesting in the Mediterranean. In Cyprus, the prevalence of individuals exhibiting a NMSP in relocated green turtle nests was reported as 40.3% (*n* = 718 hatchlings) (Özdemir and Türkozan 2006). Similarly, the prevalence of individuals exhibiting a NMSP in relocated nests was reported as 25.4% (*n* = 760 hatchlings), and 8.5% (*n* = 1313 hatchlings) in nests protected in situ at the green turtle nesting beach of Regand Island, Peninsular Malaysia (Tanabe et al. 2021). Barcenas–Ibarra et al. (2015) reported a prevalence of congenital malformations (including head, neck, tail, flippers, and carapace abnormalities) of 11% per nest at El Cuyo beach, Mexico. The prevalence of congenital malformations (including head, neck, tail, flippers, and carapace abnormalities) per nest was reported as 39.9% in live and dead hatchlings at Samandağ beach in the eastern Mediterranean (Sönmez and Sağol 2024).

The prevalence of scute pattern anomalies varied considerably between nesting beaches, even within the Mediterranean. These discrepancies can be attributed to a number of factors, including environmental conditions (*e.g.*, temperature and humidity) (Telemeco et al. 2013; Zimm et al. 2017), conservation procedures (e.g., in situ or relocation) (Mast and Carr 1989; Sönmez et al. 2011), and maternal effects (*e.g.*, egg mass) (Booth et al. 2013) specific to each nesting beach. However, while these approaches are more specific to the underlying etiological causes of carapace scute deviation, they may be limited in explaining the number of individuals that possess carapace scute deviation This may be because the time and duration of action of the aforementioned etiologies on the hatchlings are more important. Telemeco et al. (2013) have previously reported that natural nests of painted turtles (*Chrysemys picta*) exposed to extremely high temperatures for > 60 h produced more hatchlings with scute abnormalities. It can be hypothesized that such prolonged temperatures during the developmental period, when the scute is particularly sensitive to environmental factors (embryonic stage 23 and later are characterized by the appearance and development of the scutes) (Miller et al. 2017), may have led to an increase in the number of individuals with NMSP. Similarly, green turtle nests that produced a higher frequency of hatchlings with NMSP in unshaded areas were reported to have nest temperatures exceeding 31°C within 20 days of laying (Kobayashi et al. 2020). Furthermore, this result was demonstrated in laboratory conditions, where a significant increase in hatchlings with NMSP was observed when incubation was conducted at constant temperatures exceeding 30°C (Zimm et al. 2017).

The effects of sand albedo (Hays et al. 2003) and air temperature (Laloe et al. 2014) as an environmental factor on incubation temperature should not be ignored. This is because Laloe et al.

(2014) emphasised that there is a positive relationship between sand temperature and air temperature in sea turtle nests. This provides important clues to support the hypothesis that nesting date (i.e. the beginning, middle and end of the nesting season) may also be an important factor on NMSP. Although the relationship between the number of individuals with NMSP and nesting time does not cover our main hypothesis, the nesting date and its relationship with the number of individuals with NMSP can be suggested as an important research topic in future studies.

The GAMM analysis indicated that there was a statistically significant relationship between ECM and the number of NMSP individuals. In green turtles, metabolic heating is correlated with clutch size, hence mass, and metabolic heating increases during the middle to late incubation period (Booth and Astill 2001; Önder and Candan 2016; Sönmez 2018). The period of development when the scute is particularly sensitive to environmental factors coincides with a period of increased metabolic heating in the nest (Miller et al. 2017). This may result in embryos being exposed to prolonged higher incubation temperatures. The extent to which egg mass, clutch size, and mass contribute to nest temperature through metabolic heating and the association of this on scute pattern can be investigated in further studies for the eastern Mediterranean green turtle.

The number of individuals with NMSP was positively correlated with nest depth, and also sand volume increased as nest depth increased in this study. Given the negative correlation between nest depth and temperature documented in green turtles (Booth and Astill 2001), it can be considered that the nest depth, through the sand volume, could have a contribute. However, the opposing effects of nest depth and thus nest temperature (a deeper nest means lower nest temperature) and increased mass on top of the eggs may suggest that nest depth acts in conjunction with methylation variance in differentially methylated sites (DMS) as an epigenetic effect (Yen et al. 2024). Indeed, Yen et al. (2024) found that methylation variance in DMS in loggerhead turtle nests showed a strong discrimination between hatchlings in deeper (i.e., cooler) and shallower (i.e., warmer) nests.

Furthermore, the results of the GAMM analysis indicated a significant interaction between the number of NMSP individuals and three explanatory variables: estimated clutch mass, sand volume, and nest depth. However, the model was best explained by ECM, while nest depth and sand volume had a relatively minor impact (see Table 2 for details). Upon the introduction of continuous covariates (sand volume, nest depth, distance from sea and vegetation) to the independent clutch mass model, no significant relationship was observed on the number of NMSP individuals. It is possible that there is collinearity between these additional variables, in that some variables may be correlated with each other and thereby mask each other's effect. For instance, nest depth is correlated with estimated clutch mass (see Fig. 3 for details), which may have resulted in the observed effect of estimated clutch mass being underestimated in the model. Furthermore, each variable was modelled with a spline (s), which attempts to capture the non–linear effects of the variables. In this case, the effect of estimated clutch mass may have been masked by the other spline terms in the model.

Barcenas–Ibarra et al. (2015) reported that clutch size, through pressure, can lead to carapace compression in hatchlings. Based on our field observations, nearly all of the hatchlings exhibiting carapace compression also displayed scute deviation. An increased clutch mass is generally associated with a greater number of embryos and hatchlings. An increase in the number of hatchlings is a means of a greater probability of detecting anomalies.

A significant limitation of this study is the absence of investigation into nest temperature and metabolic heating in the sampled nests. Previous studies have associated NMSP with high nest temperatures. Kobayashi et al. (2017; 2020) stated that exposing nests to high temperatures during the scute formation period of the embryonic development may result in a higher proportion of NMSP hatchlings. Furthermore, Calderon Pena and Azanza Ricardo (2021) noted a correlation between the temperature during the last third of incubation and scute anomalies. It is known that nest temperature increases from the middle third to the last third of incubation due to metabolic heat (Booth and Astill 2001; Önder and Candan 2016; Sönmez 2018). Considering the correlation between metabolic heat and clutch size, hence mass, embryos may be exposed to high incubation temperatures due to clutch mass. This suggests that clutch mass together with nest temperature or metabolic heat, may be potential confounding factor for NMSP. The limited impact of nest depth and sand volume on NMSP, coupled with the moderate proportion of estimated clutch mass alone in explaining NMSP frequency, indicates that clutch mass may play a role in NMSP frequency, potentially in conjunction with temperature (Zimm et al. 2017).

CONCLUSIONS

It can be concluded that estimated clutch mass is significantly associated with NMSP, with an increase in the number of NMSP individuals observed as clutch mass increases. We believe that this study as a preliminary can provide a basis for further studies to identify that ECM may play a role in NMSP frequency when considered in conjunction with other environmental factors (such as temperature and metabolic heating). In addition, more detailed information is needed on parameters that act on NMSP under controlled conditions which would enable a more accurate assessment of their contribution to NMSP frequency.

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