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The Incubation Environment Shapes the Inflammatory Response and Enables Expression of Maternal Effects on Sea Turtle Hatchling Body Size

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ABSTRACT

Female turtles are believed to select nesting sites that optimize conditions for egg development and allocate resources accordingly. Although relocating clutches to shaded hatcheries enhances hatchling survival, growth, and immune configuration, the impact of these unexpected environments on maternal investment remains uncertain. Herein, the effects of maternal (body size, as well as hematological and biochemical indicators) and environmental (sand temperature and moisture in both unshaded and shaded nests) variables on local inflammation after a challenge (as a proxy of immune function) and offspring size were evaluated using a split-clutch design. The association of maternal parameters with reproductive investment, as well as the relationship of incubation conditions to survival indicators (hatching and emergence success), was also evaluated. Hatchlings from shaded nests showed less intense inflammation and were heavier and longer compared to offspring from unshaded conditions. The incubation conditions influenced inflammation in hatchlings, as well as their body mass, straight carapace width, and survival. Maternal leukocytes differentially interacted with the environment to determine hatchling length. Maternal amylase and creatinine concentrations were related to clutch size and mass, respectively, while shading enhanced survival indicators. The results indicate that the incubation condition is the primary factor influencing hatchling phenotypes, both directly and indirectly by facilitating the expression of maternal effects. These findings suggest that while optimal environmental conditions and maternal quality do not synergize to enhance offspring fitness, environmental conditions can override the effects of maternal investment. This highlights the relevance of the incubation environment to guarantee hatchling phenotypes.

1 | Introduction

In sea turtles, embryonic development is presumably influenced by a combination of genetics with the quantity and quality of egg nutrients, among other factors (Booth et al. 2012; Martín-delcampo et al. 2021; Stewart et al. 2019). Studies in other reptiles suggest that the maternal physical condition at nesting could determine hatchling immune competence through the transfer of immune modulators (Brown and Shine 2016; Virgin et al. 2022). Moreover, a relationship between the maternal physical condition and her reproductive investment (i.e., egg number and weight) to hatchling body size and immune competence has been suggested (Perrault et al. 2012).

Besides maternal contributions, the incubatory environment plays a pivotal role in the development of hatchlings. Among environmental factors, temperature is particularly influential, as it governs key processes such as organogenesis, hatchling body size, immune competence, and sex determination in reptiles (Dang et al. 2015; Fleming et al. 2020; Freedberg et al. 2008; Rivas et al. 2019). Studies have shown that *ex situ* incubation in shaded hatcheries can further enhance development. Specifically, this has been observed in *Lepidochelys olivacea*, where hatchlings from shaded hatcheries exhibited advanced developmental traits at nest emergence (Robledo-Avila et al. 2022, 2023). Presumptive optimal development was characterized by splenic differentiation as well as increased body size and better survival parameters (i.e., hatching and emergence success; Robledo-Avila et al. 2022). Indeed, hatchery incubation diminished prooxidant–antioxidant system activity and increased mRNA expression of heat shock protein 70 and toll-like receptor 4 in the spleen (Robledo-Avila et al. 2023).

In addition to altering the phenotype and fitness of hatchling turtles, incubation in shaded hatcheries also biases their sexual determination to males (Robledo-Avila et al. 2022, 2023), making the effects of incubation within hatcheries more challenging to study. Because incubation temperatures in shaded hatcheries are several degrees Celsius lower than those in natural nests, some of these effects have been attributed to temperature-induced sex differences (Reboul et al. 2021; Rivas et al. 2019; Robledo-Avila et al. 2022, 2023). This is especially true for traits such as prooxidant–antioxidant balance and transcriptional activity, as well as splenic development and body size (Robledo-Avila et al. 2023). However, the effects of incubation in shaded hatcheries on hatchling body size and splenic development were sex-independent (Robledo-Avila et al. 2022). This suggests that the incubation environment can determine turtle phenotypes by at least two alternative pathways: a sex-dependent pathway, potentially involving hormonal regulation, and a sex-independent pathway, possibly linked to stress responses or resource location.

As global warming accelerates, sea turtle hatchlings face increased exposure to pathogens and environmental stressors. Relocating sea turtle eggs to shaded hatcheries, while protective against predators and poachers, may expose embryos to environmental conditions not anticipated by nesting females, potentially compromising their optimal development. Alternatively, these novel environments with incubation conditions near optimal for sea turtle incubation could promote a better phenotype. Another possibility is that the combination of optimal maternal investment and shaded hatcheries with near-ideal conditions could synergistically enhance hatchling development, potentially leading to improved phenotypic traits. To evaluate these alternatives, entire clutches from nesting females were relocated to two contrasting incubation conditions: unshaded (simulating the natural condition) versus shaded. Maternal biochemical and hematological parameters related to metabolic and nutritional status were recorded, along with the temperature, grain size of the sand within nests, and their moisture levels. Their interaction with incubation conditions was evaluated in relation to local inflammation and hatchling body size. Additionally, the association between nesting female parameters and reproductive investment indicators, as well as the relationship between incubation conditions and survival success, were evaluated.

2 | Materials and Methods

2.1 | Study Site and Female Sampling

This study was conducted at Playa La Escobilla Sanctuary in Oaxaca, México (15°43′54.984″N, 96°44′16.008″W; Figure S1). *Lepidochelys olivacea* sampling and handling protocols were approved by an Animal Rights Committee, under License Number SEMARNAT: SGPA/DGVS/09122/21; in accordance with Mexican regulation (NOM-059-SEMARNAT-2010, 2012). Additionally, this study adheres to the ARRIVE guidelines, ensuring that all efforts were made to minimize pain, stress, and discomfort during the sampling process. Sampling of nesting females, clutch relocation, and nest construction were carried out in collaboration with experienced hatchery staff. Twenty female turtles were selected during the last days of February 2022, according to previously described criteria (Robledo-Avila et al. 2022). Briefly, nesting females were identified during four consecutive nights by beach patrolling before the start of oviposition. Once they initiated nest building, visual inspection to identify body lesions and parasites was performed. Maternal straight carapace length (SCL) and width (SCW) were measured using a calibrator (Titanium, max = 1 m, d = 0.02 mm). At the end of oviposition, 5 mL of blood was collected from the cervical venous sinus using a 21G × 1–1/4 in needle with previous asepsis and transferred into sodium heparin coated tubes (BD Vacutainer, Becton Dickinson). Female body weight was registered using a Roman tubular balance (Trupper, max = 50 kg, d = 0.5 kg), and the body condition index (BCI) was calculated according to the formula: $\frac{\text{Body mass(kg)}}{\text{SCL(cm)}^3} \times 10000$ (Espinoza-Romo et al. 2018). At the end of sampling, females were released and returned to the sea.

Microhematocrit was quantified using a 75-mm long, 1-mm diameter capillary tube, heat sealed and centrifuged at 5000 rpm for 5 min in a clinical centrifuge (Dewinner, H-XLJ). Additionally, a blood smear stained with Wright's hematoxylin (J. T. Baker) was used to quantify the leukocyte profile according to criteria previously published for *L. olivacea* (Zhang et al. 2011). Total leukocytes per microliter were estimated by counting white blood cells from 10 fields at 400× magnification and multiplying them by 1500 (Sykes and Klaphake 2008). Biochemical parameters were measured in blood plasma, which was obtained by centrifuging total blood at 4000 during 15 min. Plasma was

recovered into 1.6-mL cryogenic vials and stored at 4°C until laboratory processing at the Universidad del Mar (not more than 8 h after sampling). Sixteen biochemical analytes, including metabolic and nutritional indicators, were studied: albumin, total proteins, globulins, albumin/globulin ratio, glucose, urea nitrogen, creatinine, urea nitrogen/creatinine ratio, cholesterol, total bilirubin, alkaline phosphatase, amylase, alanine aminotransferase, creatinine kinase, calcium, and phosphorus. This was done using 100 μL plasma samples in an automated blood chemistry analyzer Celercare V5 (Kabla Veterinary DX) with a commercial kit (Mnchip, 41116105).

2.2 | Egg Relocation and Offspring Collection

Individual eggs were collected immediately after their release inside the nest and alternately placed in two cotton bags. Each half of the clutch was reburied at contiguous hatcheries in previously built nests, under contrasting conditions: unshaded ($n = 20$) and shaded (50% shade cloth; $n = 20$; Figure S2), with random allocation to each. Egg manipulation was kept to the bare minimum, and efforts were made to avoid rotation. Nests were built according to national norms for *L. olivacea*, approximately 40 cm deep (Sarti et al. 2006). The time between laying and reburial lasted less than 100 min. Nests were observed daily starting on incubation day 44 to identify signs of turtle emergence. Each nest was fenced with sieve mesh until hatchling emergence. The rationale for all experimental procedures was based on previous reports (Herrera-Vargas et al. 2017; Robledo-Avila et al. 2022; Unda-Díaz et al. 2022).

A total of 120 hatchlings were collected, with three hatchlings taken from each of 20 unshaded nests and 20 shaded nests. Body size was measured for all selected hatchlings. Two hatchlings per nest were used to assess PHA-triggered inflammation, while the third was euthanized immediately upon body size measurement for a separate experimental protocol. In total, 40 hatchlings (one per nest) were euthanized, and all remaining hatchlings were released.

Hatchling collection was previously described (Robledo-Avila et al. 2022). Briefly, three emerging turtles from each nest were collected as soon as they surfaced from each nest. Immediately after, hatchlings were weighed with a digital precision balance (OHAUS Scout Pro Sp 602, Max 600 g, $d = 0.01$ g). Their SCL and SCW (hereinafter body length and width, for easy differentiation from maternal variables) was measured using a digital Vernier caliper (Mitutoyo, Max 200 mm, $d = 0.03$ mm).

Two hatchlings per nest were subcutaneously inoculated (prior asepsis with 70% ethanol) in the left cranial flipper (between the 2nd and 3rd proximal scales) with 50 μg phytohemagglutinin (PHA, Sigma-Aldrich) reconstituted in 30 μL sterile phosphate-buffered saline (PBS). The right flipper was inoculated with 30 μL PBS as a control. Flipper thickness was measured using a digital micrometer (Mitutoyo, max = 200 mm, $d = 0.03$ mm) prior to inoculation (baseline), and the difference in inflammation was assessed 8, 12, 16, 20, and 24 h after challenge. This was calculated by subtracting baseline values from the data obtained after PHA inoculation. These values were standardized by taking the difference in inflammation after PHA challenge and dividing between baseline values (inflammation index, per hatchling).

2.3 | Survival Indicators and Environmental Parameters

Nests were inventoried 72 h after offspring departure to determine hatching and emergence success, as previously described (Robledo-Avila et al. 2022). Briefly, the number of eggs per nest was estimated (from fragments representing >50% of the egg and unhatched eggs). Hatching success (%) was determined: 100 × (eggs − unhatched eggs)/eggs. Emergence success (%) was also calculated: 100 × [eggs−(unhatched eggs + dead hatchlings + live hatchlings)/eggs.

Nest temperature was registered by fourteen data loggers (Onset HOBO Bluetooth Pendant MX2200 and UA-002-64; accuracy ± 0.2°C) placed inside selected nests (approximately 30 cm deep; Figure S2). Care was taken to place the data loggers in clutches from the same mother under both incubation conditions. However, due to technical failures, two loggers in shaded nests did not record data. As a result, temperature was reliably recorded every hour during incubation from seven unshaded nests and five shaded nests. Sand particle size and moisture were registered as previously reported (Unda-Díaz et al. 2022). Briefly, 100 g of sand surrounding the eggs was immediately collected after nest inventory in a sealed plastic bag, weighed, dried at 110°C in a standard oven, and weighed again. The humidity content was calculated as the ratio of wet to dry sand mass (Head 1992). Grain size analysis was carried out by particle sieving, according to the American Society for Testing Material norms (medium sand: ≥ 1.68; medium/fine sand: 1.67 − 0.42; fine sand: 0.41 − 0.149; silt: 0.148 − 0.074; fine silt: 0.073 − 0.038 mm) and subsequent weighting with an analytical balance.

2.4 | Statistical Analysis

A main interest herein was to evaluate any differences between the two contrasting incubation conditions (unshaded vs. shaded). Thus, appropriate statistical tests (Student's *t*, Wilcoxon rank-sum, Fisher's exact) were performed comparing the inflammation index at 8, 12, 16, 20, and 24 h post-inoculation, as well as hatchling body size (body length, width, and mass), days of incubation, and hatching and emergence success in addition to nest parameters (temperature, moisture, and sand particle sizes). Data normality and equal variance were analyzed by Shapiro–Wilk and Levene tests.

Another chief concern herein was to investigate whether maternal variables contributed to hatchling phenotypes. This study evaluated the effect of interactions between maternal variables and incubation conditions on inflammation (measured at 8 and 12 h post-PHA administration, where significant differences between incubation conditions were observed) and hatchling body size, using linear mixed models. Maternal ID was used as a random factor to account for the statistical dependence of the split-clutch design. Models with the lowest conditional Akaike Information Criterion are shown. Model residuals were

tested for normality and homoscedasticity. Linear regression slopes relating maternal indicators to hatchling parameters were compared between shaded and unshaded conditions. For these analyses, the experimental units were the turtle hatchlings nested within their mom (maternal ID).

Additionally, the associations of maternal parameters to BCI (defined by the formula above, i.e., the link between body mass and SCL), as well as maternal variables to reproductive investment parameters, were also assessed. For these analyses, the nests and female turtles served as the experimental units, respectively.

Finally, the effect of the nest environment on hatchlings was evaluated. All these latter analyses were done using multiple linear regressions. All analyses were done with R (Team R 2020). The following packages were used: readxl (Wickham et al. 2019), car (Fox and Weisberg 2019), coin (Hothorn et al. 2008), janitor (Firke 2024), lme4 (Bates et al. 2015), lmerTest (Kuznetsova et al. 2017), cAIC (Säfken et al. 2021), emmeans (Lenth 2023), DHARMa (Harting 2022), performance (Lüdecke et al. 2021), and sjPlot (Lüdecke et al. 2023). Statistical significance was defined as $p < 0.05$. The data used herein are available from the corresponding author on request.

3 | Results

3.1 | Nesting Female Parameters

All turtles appeared healthy upon physical examination and had no apparent external lesions. Maternal body size, as well as hematological and biochemical parameters, are shown in Table S1. No relationships were found between BCI and hematological or biochemical indicators, except for the percentage of heterophils, which showed a negative association (Figure 1A; Tables S2 and S3). A linear negative relationship between maternal blood amylase and clutch size was identified (Figure 1B; Table S2), as well as between maternal creatinine and total clutch (egg) mass (Figure 1C; Table S2). The other maternal parameters did not show a relationship with reproductive investment indicators.

3.2 | Incubation Parameters, Hatchling Survival Success, and Sex Ratios

Incubation temperatures in shaded nests were lower than those in unshaded conditions during the whole incubation period, as well as during the early, middle, and late incubation intervals (Figure 2A; Table 1). Moisture, as well as the proportion of medium and medium/fine sand particles, was higher at shaded hatcheries, while fine sand and silt proportions were lower (Figure 2B; Table 1). The incubation duration for hatchlings from shaded clutches was longer than for turtles incubated in unshaded conditions (Table 1). Incubation in shaded hatcheries increased hatching and emergence success (Figure 2C,D), as well as skewing sex determination toward males compared to the unshaded incubation (Table 1).

3.3 | Flipper Inflammation

Inflammation was less intense in hatchlings from shaded nests in comparison to offspring from unshaded conditions at 8 and 12 h post PHA inoculation (Figure 3A; Table 1). Analysis using linear mixed models revealed that the incubation condition contributed significantly to inflammation 12 h after challenge (Table 2). The best models for hatchling inflammation at 8 and 12 h explained 29% and 19% of the data variation, respectively (Table 2).

Phytohemagglutinin-triggered inflammation at 8 h post-inoculation was influenced by moisture percentage (Figure 3B; Table S4) and the temperature during the first third of incubation (Figure 3C; Table S4), while the percentage of medium/fine sand temperature during the second and last third of incubation affected inflammation at 12 h post-PHA challenge. The best models for hatchling inflammation at 8 and 12 h post-PHA challenge accounted for 47% and 50% of the variation in the data, respectively (Figure S3 A–C; Table S4).

3.4 | Hatchling Body Size

Consistent with previous findings, hatchlings incubated in shaded hatcheries showed greater body size than offspring incubated in unshaded nests (Figure 3D–F; Table 1). The incubation condition was the only parameter that explained body mass and SCW (Table 2), while maternal total leukocytes and incubation condition, as well as their interaction, explained body length in hatchlings (Figure 3D; Table 2). The relationship between maternal leukocytes and hatchling body length was influenced by the incubation condition, as indicated by the significant interaction term in the best model based on AIC (additionally, the slopes were also different; Figure 3D). The best models for hatchling body mass and length, as well as for SCW, explained between 19%, 30%, and 58% of the data variation, respectively (Table 2).

Environmental variables affecting body length included nearly all sand size particles (Figure 3E; Figure S4A–C and Table S4) and the temperature during the second-third of incubation (Figure 3F; Table S4). Moisture, medium/fine, fine sand, as well as silt particles, influenced SCW in addition to the temperature within nests during the first period (Figure S5A–E; Table S4). In turn, body mass was explained by moisture content, medium, medium/fine, and fine sand, as well as temperature, during the second and last thirds of incubation (Figure S6 A–F; Table S4). The best models for hatchling body length, as well as for SCW and body mass, explained between 75%, 57%, and 60% of the data variation, respectively (Table S4).

4 | Discussion

This study examined how maternal traits (body size and blood biochemical markers) and incubation conditions (shaded vs. unshaded hatcheries) impact inflammation triggered by PHA, as well as hatchling size. Additionally, it assessed how maternal traits influence reproductive investment and how hatchery

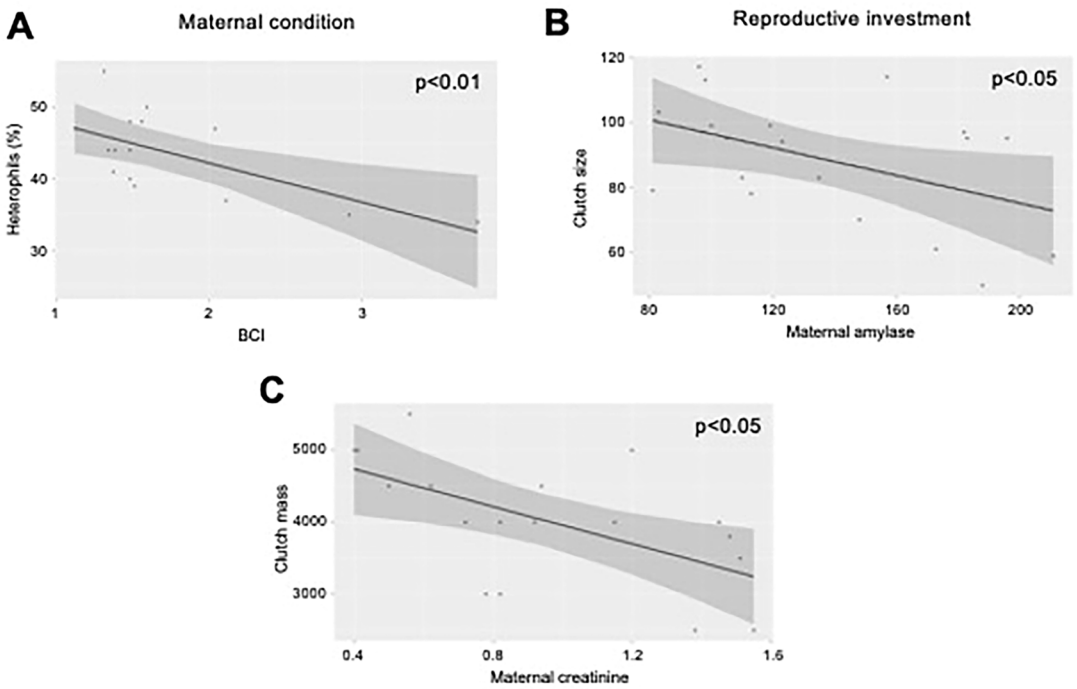

FIGURE 1 | Maternal blood amylase and creatinine are negatively related to reproductive investment parameters in *Lepidochelys olivacea* sea turtles. Graphs show the linear relationship between (A) body condition index (BCI) and heterophil percentage in nesting females. (B,C) Blood amylase and creatinine concentration to clutch size and mass in nesting females. Each point represents an individual female. Shaded areas show the confidence intervals for the regressions.

conditions affect offspring survival. The results indicate that the incubation environment is the primary factor influencing hatchling inflammation, body size, and survival, while maternal traits modify clutch attributes. Additionally, the findings suggest that incubation conditions are essential for facilitating the expression of maternal effects on hatchling body length.

4.1 | Higher Plasma Amylase and Creatinine Concentrations of Nesting Females Are Associated With Lower Reproductive Investment

Standard nutritional parameters for *L. olivacea* females do not exist; however, maternal body size and blood biochemistry indicators showed that nesting female values were within species-specific reference parameters (Brenes et al. 2013). Nevertheless, even though biochemical parameters reflect nutritional and metabolic conditions, there were no relationships between BCI and biochemistry indicators in *L. olivacea* females. Heterophil percentage was the only hematological parameter negatively related to maternal BCI. A possible explanation for this relationship is that larger, better-conditioned organisms may allocate more resources to adaptive immune responses managed by leukocytes. Previous studies have shown a negative association between the percentage of heterophils and body size in reptiles (Brown and Shine 2022).

Maternal amylase and creatinine were inversely associated with clutch size and mass, respectively. Amylase is an enzyme that participates in carbohydrate digestion. Previous studies have related high blood amylase levels to nesting in *Eretmochelys imbricata* (Ehsanpour et al. 2015), as well as to dietary preferences in *Dermochelys coriacea* (Perrault et al. 2012). Creatinine, a byproduct of muscle metabolism, showed a negative correlation with survival success in *Dermochelys coriacea* (Perrault et al. 2012). Recent studies have associated reduced creatinine blood levels with prolonged catabolism or intense physical activity in turtles (Gradela et al. 2020). Elevated maternal creatinine and amylase levels may indicate an emaciated condition in nesting females, as previously suggested (Chandavar et al. 2013; Perrault et al. 2012; Wrobel et al. 2011). Such suboptimal physiological states could result in negative trade-offs in resource allocation to developing eggs, thereby reducing clutch size and mass.

4.2 | Egg Incubation in Shaded Nests Improves Survival Indicators in *L. olivacea* Hatchlings

In this study, the incubation environment in shaded hatcheries was characterized by lower temperatures, higher moisture levels, and a greater proportion of medium and medium/fine sand particles. Shading likely explains the temperature and moisture differences between incubation conditions, while variations in sand particle size may result from the slight differences in slope, as previously reported (McFall 2019). Together, these parameters have been previously associated with reduced developmental stress, a lower risk of abnormalities, increased energy reserves, and improved locomotor performance (Fleming et al. 2020; Tezak et al. 2020; Unda-Díaz et al. 2022). Although the precise physiological mechanisms are not fully clarified, previous studies on *L. olivacea* at the same beach suggest that incubation

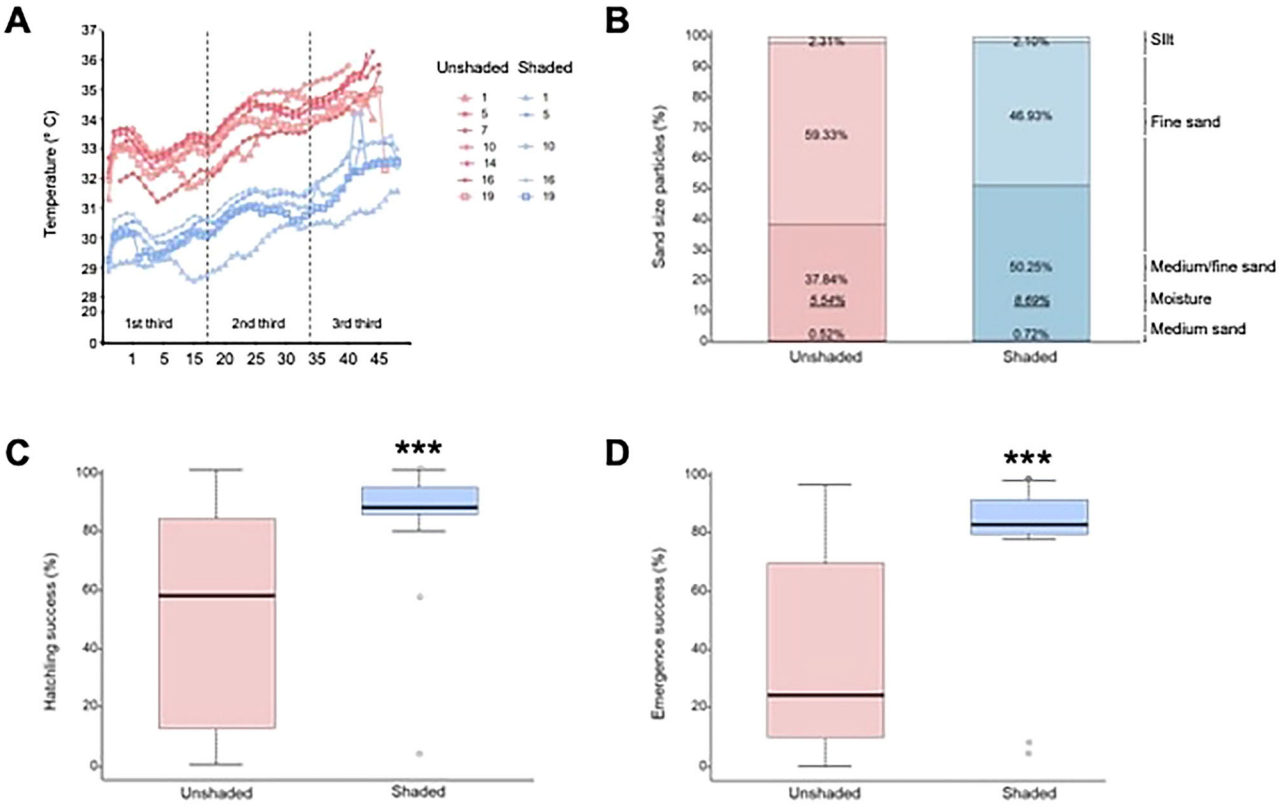

FIGURE 2 | The incubation environment in shaded nests differs from unshaded hatcheries and enhances survival success. (A) The dotted line graph displays the mean temperature per nest during three incubation periods in unshaded (red) and shaded (blue) nests. (B) Graphs represent sand particle sizes and moisture percentage for both incubation conditions. (C,D) Boxplots illustrate the median (central lines), interquartile ranges, as well as maximum and minimum percentages for hatching (C) and emergence success (D) in unshaded and shaded conditions. Asterisks indicate significant effects at $p < 0.001$.

in shaded nests may mitigate oxidative stress and enhance immune function, potentially through the differential activation of temperature-regulated metabolic, antioxidant, and endocrine pathways (Robledo-Avila et al. 2022, 2023). Here, the use of clutches from the same mother and the placement of unshaded nests adjacent to shaded hatcheries underscores the significance of incubation conditions for optimal hatching, particularly for hatchling emergence.

4.3 | Incubation in Shaded Nests Reduces Sea Turtle Flipper Inflammation in Response to a Local Challenge

Inflammation is a critical immune response triggered by infectious challenges or tissue damage. For it to serve as a homeostatic mechanism, the inflammatory response must be rapid in onset, of high intensity, and efficiently resolved (Serhan and Petasis 2011). Phytohemagglutinin is a mitogen that recruits local T cell populations to the site of inoculation, and the resulting edema serves as an indicator of immune response strength (Demas et al. 2011). Herein, incubation in shaded nests reduced inflammation in response to PHA challenge when compared to offspring incubated in the unshaded conditions. Offspring from unshaded nests exhibited an elevated inflammatory response between 8 and 16 h, which returned to near-basal levels by 20 h. In contrast, hatchlings from shaded nests showed a delayed inflammatory response, reaching its peak at 16 h, followed by a subsequent decline.

Warmer incubation temperatures have been associated with increased activation of metabolic, inflammatory, and prooxidant pathways (Castelli et al. 2021; Singh et al. 2020). Specifically, these temperatures in hatchling sea turtles are linked to heightened inflammation (Fleming et al. 2020) and increased activity of both prooxidant (e.g., hydrogen peroxide) and antioxidant (e.g., total antioxidant capacity; Robledo-Avila et al. 2023) systems. The findings suggest that warmer temperatures, such as those found in unshaded nests, might promote a more intense inflammatory response due to enhanced metabolism and immune activation. This idea is partially supported by the negative association between nest temperature during the first and second third of incubation with the inflammation observed at 8 and 12 h post-challenge.

Because shaded incubation tends to produce more male hatchlings while unshaded nests skew toward female development, it is difficult to disentangle the effects of the incubation environment from sex on the observed inflammatory response. Previous studies in *Chrysemys picta* have reported a higher PHA-induced inflammatory response in nesting females compared to males, potentially associated with hormonal activity or differences in environmental exposure and social interactions (Sanchez and Refsnider 2017). An opposite pattern has been observed in

TABLE 1 | Summary of tests evaluating the effect of the incubation condition (unshaded vs. shaded) on nest temperature, moisture, and sand particle sizes, as well as hatchling sex, incubation duration, survival success, inflammation, and body size (mass, length, and width).

	Incubation condition			
Parameter	**Unshaded**	**Shaded**	**Test statistic** $_{df}$	***p***
Temperature (°C)	**median ± IQR**	**median ± IQR**		
Whole incubation period	33.85 ± 0.65	30.89 ± 0.51	$W = 504$	**< 0.001**
1st third	32.80 ± 1.27	29.82 ± 0.36	$W = 504$	**< 0.001**
2nd third	33.89 ± 1.12	30.86 ± 0.48	$W = 504$	**< 0.001**
Last third	34.71 ± 0.55	32.00 ± 0.70	$W = 504$	**< 0.001**
Moisture (%)	**median ± IQR**	**median ± IQR**		
	1.55 ± 0.91	2.32 ± 0.37	Fisher's exact	**< 0.001**
Sand particle sizes (%)	**median ± IQR**	**median ± IQR**		
Medium sand (1.68 mm)	0.23 ± 0.70	0.70 ± 0.55	Fisher's exact	**< 0.001**
Medium/fine sand (0.42 mm)	35.99 ± 6.25	49.50 ± 3.56	Fisher's exact	**< 0.001**
Fine sand (0.149 mm)	59.88 ± 5.64	45.19 ± 4.05	Fisher's exact	**< 0.001**
Silt (0.074 mm)	2.20 ± 0.41	2.00 ± 0.25	Fisher's exact	**< 0.001**
Fine silt (0.038 mm)	0.44 ± 0.19	0.49 ± 0.07	Fisher's exact	**< 0.001**
Sex (%)	Female (100)	Male (100)		
Incubation days	**median ± IQR**	**median ± IQR**		
	43.98 ± 1.22	48.21 ± 0.84	$W = 0$	**< 0.001**
Hatching success (%)	**median ± IQR**	**median ± IQR**		
	64.00 ± 71.18	87.76 ± 8.91	Fisher's exact	**< 0.001**
Emergence success (%)	**median ± IQR**	**median ± IQR**		
	44.00 ± 58.74	83.33 ± 11.53	Fisher's exact	**< 0.001**
Inflammation index (h)	**mean ± SEM**	**mean ± SEM**		
8	0.60 ± 0.04	0.22 ± 0.05	$t_{64} = 3.81$	**< 0.001**
	median ± IQR	**median ± IQR**		
12	0.68 ± 0.61	0.42 ± 0.67	$W = 939.5$	**< 0.01**
16	0.59 ± 0.43	0.52 ± 0.45	$W = 702$	0.201
20	0.43 ± 0.48	0.26 ± 0.53	$W = 497.5$	0.794
	mean ± SEM	**mean ± SEM**		
24	0.33 ± 0.05	0.33 ± 0.05	$t_{52} = -0.03$	0.974
Body mass (g)	**median ± IQR**	**median ± IQR**		
	15.68 ±1.58	17.01 ± 1.69	$W = 1477$	**< 0.001**
Body length (mm)	**median ± IQR**	**median ± IQR**		
	41.33 ± 1.96	42.76 ± 1.69	$W = 1303.5$	**< 0.001**
Straight carapace width (mm)	**median ± IQR**	**median ± IQR**		
	31.61 ± 2.10	34.70 ± 1.52	$W = 397$	**< 0.001**

Abbreviations: *t*, Student *t*-test statistic; W, Wilcoxon signed-rank test statistic; IQR, Interquartile range; *p*, *p* value.
Bold values denote significant effects at $p < 0.05$.

green anoles, where males exhibited a stronger inflammation than females (Husak et al. 2016). Thus, although sex-related differences in PHA-triggered inflammatory responses have not been specifically studied in hatchlings, the potential influence of hormones on the immune response cannot be ruled out (Foo et al. 2017). Nevertheless, it is clear that shaded hatcheries impact the immune response in sea turtles, with potential broader implications for other facilities using similar incubation practices.

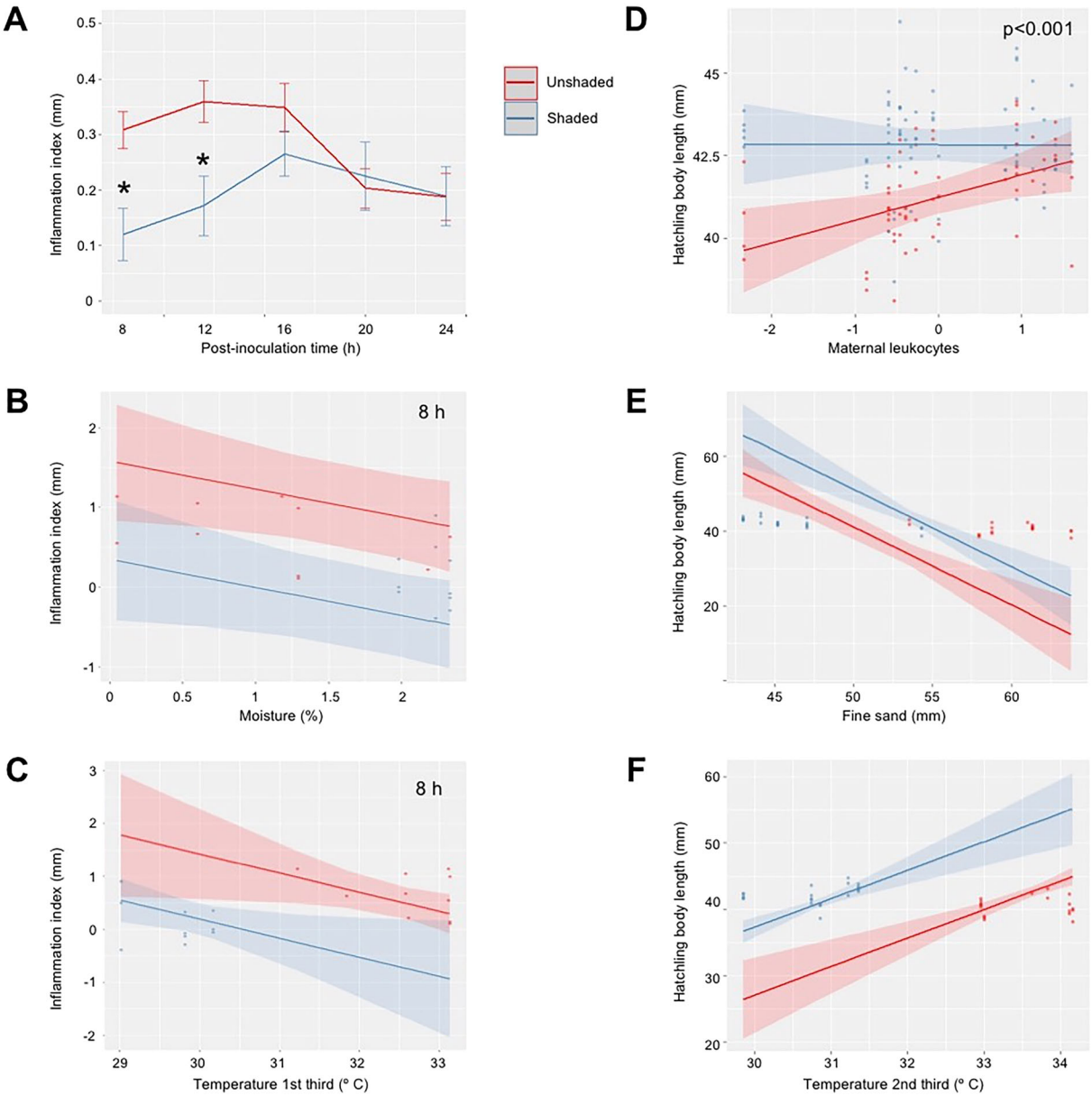

FIGURE 3 | Incubation in shaded nests reduces flipper inflammation in sea turtles in response to local challenges and overrides maternal effects on hatchling body length. Graphs showing: (A) the time course of flipper inflammation after phytohemagglutinin (PHA) challenge in *Lepidochelys olivacea* hatchlings incubated in unshaded (red) and shaded (blue) nests; (B,C) linear relationship of moisture percentage (B) and temperature (C) to flipper inflammation at 8 h post challenge, respectively; (D) relationship between maternal leukocytes and body length in offspring from unshaded and shaded nests; (E,F) linear relationship between fine sand (B) and temperature (C) to hatchling body length. Asterisks denote significant effects at $p < 0.05$. Mean ± SEM. Each point represents an individual hatchling. Shaded areas show the confidence intervals for the regressions; note the significantly different slope values ($p < 0.001$).

4.4 | Incubation in Unshaded Nests Enhances Maternal Effects on Hatchling Body Length

Hatchlings incubated in shaded nests were heavier, longer, and wider than those incubated in unshaded hatcheries. Although body size has been associated with sex in turtle hatchlings (Sönmez et al. 2016), previous work in *L. olivacea* incubated in the same conditions has shown that the effect of shaded hatcheries on weight and length is sex-independent (Robledo-Avila et al. 2022). In addition to the incubation condition, the results showed that maternal total leukocytes explained offspring body length. A differential interaction between maternal leukocytes and body length was observed across the two incubation conditions: offspring from unshaded nests exhibited a positive link, while those from shaded nests showed a flattened or negligible relationship. The observed maternal effect, exclusively in hatchlings from unshaded nests, suggests that its expression is influenced by the incubation environment. The significance of this effect on hatchling body length is highlighted by the fact that longer hatchlings from unshaded nests achieve body lengths comparable

TABLE 2 | Summary of the best linear mixed models explaining inflammation index at 8 and 12 h post-phytohemagglutinin challenge, as well as body size (mass, length, width) in *Lepidochelys olivacea* hatchlings in relation to the maternal variables.

	β	*t* (df)	*p*	R^2
Inflammation index 8 hours				0.29
Intercept	0.39	$1.33_{(23.66)}$	0.196	
Incubation condition	0.37	$0.86_{(56.98)}$	0.394	
Alkaline phosphatase	0.01	$0.74_{(24.16)}$	0.488	
Alkaline phosphatase: Incubation condition	−0.04	$-1.84_{(56.54)}$	0.072	
Inflammation index 12 hours				0.19
Intercept	0.97	$3.08_{(20.76)}$	**0.006**	
Incubation condition	−1.05	$-2.89_{(66.00)}$	**0.005**	
Amylase	−0.002	$-0.88_{(22.61)}$	0.387	
Amylase: Incubation condition	0.005	$1.94_{(64.29)}$	0.057	
Body mass (g)				0.19
Intercept	16.00	$10.88_{(24.29)}$	**<0.001**	
Incubation condition	2.40	$2.32_{(116.60)}$	**0.022**	
Glucose	−0.002	$-0.12_{(23.88)}$	0.906	
Glucose: Incubation condition	−0.013	$-1.22_{(116.48)}$	0.227	
Straight carapace length (mm)				0.30
Intercept	41.23	$166.74_{(26.26)}$	**<0.001**	
Incubation condition	1.59	$8.27_{(119.71)}$	**<0.001**	
Total leukocytes (scaled)	0.69	$2.74_{(24.85)}$	**0.011**	
Total leukocytes: Incubation condition	−0.70	$-3.61_{(118.83)}$	**<0.001**	
Straight carapace width (mm)				0.58
Intercept	31.96	$114.81_{(29.65)}$	**<0.001**	
Incubation condition	−2.59	$9.63_{(114.60)}$	**<0.001**	
Creatinine kinase	$-4.02e^{-4}$	$-1.39_{(29.11)}$	0.176	
Creatinine kinase: Incubation condition	$4.84e^{-4}$	$1.65_{(114.80)}$	0.101	

Abbreviations: β, regression coefficient; df, degrees of freedom; *t*, Student *t*-test indicator; *p*, Satterthwaite's *p* value; R^2, marginal coefficient of determination. Bold values denote significant effects at $p < 0.05$. Note that all the mixed effects models include an interaction with maternal variables.

to those from shaded nests. Previous studies have linked maternal leukocyte profiles to hatchling length in both snakes and humans (Brown and Shine 2016; McDade et al. 2019).

Here, temperature during the middle third of incubation and sand particle size accounted for differences in body length. Previous studies have demonstrated a negative relationship between warmer temperatures and the body length of sea turtle hatchlings (Fleming et al. 2020; Rivas et al. 2019). Indeed, larger sand particles in incubation environments have been associated with increased body size (Unda-Díaz et al. 2022).

5 | Conclusion

The results indicate that the incubation environment is the primary determinant of inflammatory responses to local challenges and hatchling body size in sea turtles, both directly and indirectly. Temperature, moisture, and sand particle size were key factors influencing inflammatory responses and body size. Additionally, the environment facilitated maternal investment in hatchling body length. These findings do not support a synergistic effect between optimal environmental conditions and maternal quality for producing "better" offspring. Instead, they suggest that unexpected environmental conditions may override or neutralize maternal investment in offspring.

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Conflicts of Interest

This work is not being considered for publication elsewhere, and there are no known conflicts of interest associated with its publication.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.