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Article

Pathology of Free-Living Loggerhead Turtle (*Caretta caretta*) Embryos on the Island of Linosa (Italy)

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Simple Summary: The loggerhead turtle (*Caretta caretta*) is a pelagic species found in all temperate oceans, including the Mediterranean Sea. In Italy, the main nesting areas are located on the islands of Linosa and Lampedusa. Despite conservation efforts, the Mediterranean subpopulation of *Caretta caretta* is classified as „Vulnerable.” The main threats to the species are bycatch in fishing, habitat degradation, and climate change. In particular, the rise in environmental temperatures negatively impacts egg incubation and development. This study examined the factors contributing to mortality in unhatched embryos on Pozzolana di Ponente beach, Linosa, with a focus on renal alterations. The results showed signs of liver and kidney damage, likely due to environmental toxins and high temperatures. Global warming is increasing the sand temperature, compromising hatching success and contributing to issues like renal calculosis in embryos. Conservation actions should include protecting the nests and studying the impacts of environmental contaminants to safeguard endangered sea turtles in the Mediterranean.

Abstract: On the beach of Linosa Island (Italy), forty-three loggerhead sea turtle (*Caretta caretta*) unhatched eggs were recovered from nests, formalin-fixed and necropsied. The tissue samples were stained with Haematoxylin-Eosin (HE), Grocott, von Kossa, Periodic Acid-Schiff (PAS), and Movat pentachrome stains. Histologically, vacuolar degeneration (100.0%) and increased numbers of melanomacrophages (18.6%) in the liver, and edema (14.0%) in the lungs were observed. Twenty-five kidneys (58.1%) showed deposition of blue amorphous material with HE staining, which also appeared PAS-positive and black with von Kossa staining, allowing a diagnosis of calcium oxalate, confirmed by transmission electron microscopy. The hepatic lesions may be indicative of toxicosis, infection, or a defense mechanism. A statistically significant association between the nest position and renal calculosis was observed. Renal calculosis was probably due to the exceptionally high summer temperatures, which were statistically higher compared to the temperatures recorded in the previous two years

Keywords: *Caretta caretta*; embryos; necropsies; renal calculosis; high temperatures

1. Introduction

The loggerhead turtle (*Caretta caretta*) is a pelagic species found in all the oceans of the temperate zone, including Mediterranean Sea [1]. Several studies have documented the presence of nesting areas in Italy, along the coasts of Puglia, Basilicata, Campania, Calabria and Sicily [2] but the only consistent nesting sites are the 'Pozzolana di Ponente' beach on Linosa Island and the 'Spiaggia dei Conigli' beach on Lampedusa Island [3].

The International Union for Conservation of Nature (IUCN) identified seven sea turtle species facing endangerment, with a specific focus on three species in the Mediterranean Sea classified as at risk of extinction. In particular, *Caretta caretta* appeared for the first time in 1975 in the category of the IUCN Red List of Italian Vertebrates as „critically endangered“. After over three decades of conservation initiatives, the Mediterranean loggerhead turtle sub-population has been designated as „Vulnerable“ according to the latest assessment using the IUCN Red List criteria [4]. Several studies have been carried out to ascertain the main causes of death during the life of these species [5], but there is a lack of studies on the earlier stages, such as incubation and hatching. In fact, the loss of laid eggs results in the loss of a significant number of potential future adults already at risk of extinction. Nevertheless, this classification relies on conservation efforts, as substantial threats persist, including fishery bycatch, degradation of marine and terrestrial habitats, climate change, and marine pollution [6]. In comparison with other Mediterranean nesting sites, Italian regions exhibit heightened human activities, including tourism, fishing, and marine traffic, all of which could potentially impact the utilization of coastal habitats by turtles [7,8]. These human-induced activities, combined with natural threats, may jeopardize various stages of sea turtle reproduction, including egg-laying, embryonic development, the occurrence of carapacial abnormalities, and the survival of hatchlings on the beach [5]. For example, during the embryonic stages, the success of hatchlings may be compromised by a range of factors such as human disturbances, with a critical impact on the embryonic development, reducing hatchling success and leading to embryonic mortality [9–11]. In recent years, the primary causes described for loggerhead turtles' mortality are the sea turtle egg fusariosis (STEF) [12,13], organic pollutants contamination [14] and microplastic ingestion [15,16]. In particular, STEF is a recently identified fungal disease associated with mortality in the eggs of endangered sea turtle nests on a global scale [17]. Moreover, the rise in global temperatures as a result of climate change poses a threat to biodiversity worldwide [18]. Non-avian reptiles are more vulnerable to heat stress during incubation due to the absence of parental care and limited behavioural thermoregulation [19]. This has spurred increased research attention to comprehend the consequences of elevated nest temperatures on turtle development. Therefore, the purpose of this work is to evaluate the cause of death of unhatched *Caretta caretta* embryos from Pozzolana di Ponente beach on Linosa Island.

2. Materials and Methods

Samples collection

Forty-three unhatched loggerhead sea turtle eggs (*Caretta caretta*) were found on the beach of Pozzolana di Ponente on Linosa Island at the end of the summer season. A total of 5 unhatched nests were part of this study. In particular, the total number of eggs and the depth of eggs in the sand were recorded for each nest.

When the nest was opened, an external visual examination of the eggs was carried out, and they were subsequently opened for the extraction of the embryos. The samples were then fixed immediately by immersion in 10% buffered formalin (pH 7) to confer stability to the samples and inactivate the enzymes responsible for the autolytic processes. An initial selection was made on the specimens collected to exclude subjects clearly in a state of decomposition and therefore unsuitable for subsequent examinations. Fixed samples were sent to the Department of Veterinary Science, University of Turin.

Biometric data collection

For each sample, the following biometric data were recorded: standard carapace length (SCL) was measured from the midpoint of the nuchal plate to the caudal portion of the supracaudal scutes of the carapace; standard carapace width (SCW) was obtained by measuring the widest part of the carapace; head length (HL) was measured along the midline, from the posterior tip of the supra-occipital crest to the rostral part of the head (the rhaphotheca of the upper jaw); and head width (HW) was obtained by measuring the widest part of the head. These data were compared with Miller’s tables to evaluate age at death. [20].

Gross and histological examination

After an external examination of each specimen to search for any lesions or alterations, a necropsy was performed on each turtle embryo following the procedures described by Wyneken [21]. However, the protocol for necropsy was partly modified since the dissected specimens were small in size and still contained some embryonic structures, such as the yolk sac. After gross examination, organs (liver, stomach, intestine, heart, lungs, kidneys, and central nervous system) were paraffin-embedded according to routine histological procedures. Representative sections of each sample were stained with Haematoxylin-Eosin (HE), Grocott, Periodic Acid Schiff (PAS), von Kossa, and Movat pentachrome stain. All slides were observed using a Nikon Eclipse E600 light microscope (Nikon Corporation, Tokyo, Japan).

Electron microscopy

Paraffin-embedded kidney blocks were cut into small pieces and deparaffinized in Sub X (Leica Biosystems) for 48 h. Samples were retrimmed to pieces no larger than 1 mm, rehydrated in an ethanol series of descending concentrations, and washed in distilled water. Then they were fixed in 2.5% glutaraldehyde (TAAB) in PBS pH 7.4 for 2 h at 4°C and post-fixed in 1% osmium tetroxide (OsO₄) (Next Chimica, South Africa) in PBS for 2 h at 4°C. The tissues were dehydrated through ascending grades of ethanol, incubated in propylene oxide (TAAB) for 5 min at room temperature, and embedded in Epon 812. Resin blocks were solidified at 60°C for 48 h. Semi-thin sections (1 µm) were cut and stained with 1% toluidine blue (w/v) pH 3.5. Silver-colored ultrathin sections (60-70 nm) were collected onto copper grids coated with a Formvar layer (Electron Microscopy Sciences) and double-stained with uranyl acetate and lead citrate. Samples were examined using and photographed at 80 kV on a CM12 STEM electron microscope (Philips, Eindhoven, The Netherlands).

Temperature collection

Temperatures during July and August, when oviposition occurred and when the eggs should have hatched, on Linosa Island, from two years before to two years after the egg sampling, were monitored and recorded by the weather station on Lampedusa Island.

Statistical analysis

The collected data were analyzed using GraphPad Prism software version 4.00 for Windows (GraphPad Software, San Diego, California, USA). Fisher’s test was applied to evaluate the association between the depth at which the eggs were laid and the presence or absence of renal lesions.

The statistical significance of the association between the nests and the presence or absence of renal lesions was evaluated using the chi-square test. The Kruskal-Wallis test (nonparametric ANOVA) and the corresponding Dunn’s post-test were used to evaluate the differences in the distribution of average temperatures in the months of July and August over five consecutive years, using the year of deposition of the examined samples as the reference.

3. Results

Egg Depth Distributions and Biometric Data

The unhatched eggs were distributed in the sand at different depths, as reported in Table 1.

Table 1. Number and depth of the eggs for each nest.

Depth	Nest 1	Nest 2	Nest 3	Nest 4	Nest 5
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13-18 cm	-	-	1	-	-
19-23 cm	3	-	-	-	1
24-28 cm	3	-	8	3	-
29-33 cm	6	2	3	1	-
34-38 cm	-	3	1	2	-
39-43 cm	-	6	-	-	-
TOTAL	12	11	13	6	1

Temperatures recorded in July and August, when oviposition occurred and when the eggs should have hatched, on Linosa Island, from two years before to two years after the egg sampling are summarized in Table 2.

Table 2. Average monthly temperatures referred to the months of July and August in the island of Linosa.

JULY		AUGUST	
2 years before	25.7°C	2 years before	26.1°C
1 year before	25.8°C	1 year before	25.8°C
Sampling year	30.2°C	Sampling year	30.7°C
1 year after	25.7°C	1 year after	27.2°C
2 years after	26.8°C	2 years after	27.1°C

The biometric data showed that 17/43 (39.5%) animals had an SCL between 3.5 and 4.9 cm. These animals were classified as ready to hatch (also referred to as hatchlings) [20], with an approximate range between the 28th and 30th days, corresponding to the very late stage of embryonic development.

All the remaining animals, 26/43 (60.5%), had SCL values between 2.3 cm and 3.5 cm and were accordingly classified as being in the last third of development (in the range between the 22nd and 27th days of development), according to Miller [20].

Gross and Histological Examination

Histological examination of gonads and the central nervous system was not significant due to the heavy degree of autolysis. Stomach and intestine did not show any alterations. In 1/43 (2.3%) cases, a focal non-suppurative infiltration of the heart was observed. An increasing number of melanomacrophages (8/43 cases; 18.6%), haemorrhages (3/43 cases; 7.0%), and vacuolar degeneration (43/43 cases; 100%) were present in the liver (Figure 1a). Oedema was observed in the lungs of 6/43 (14%) cases (Figure 1b), and 25/43 (58.1%) animals showed glomerular and tubular calculosis, identified by von Kossa staining as calcium carbonate crystals, involving more than half of the renal parenchyma in 19 out of 25 (76%) animals (Figure 1c).

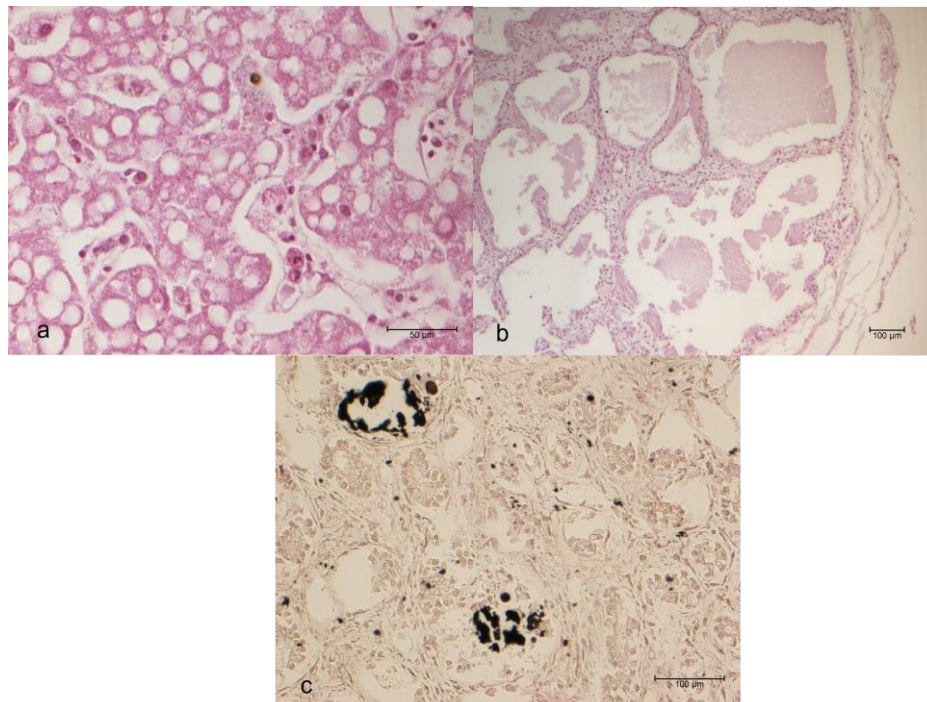


Figure 1. **a:** liver: of melanomacrophages and vacuolar degeneration (HE); **b:** lung: oedema (HE); **c:** kidney, glomerular and tubular calculosis (Von Kossa).

Electron Microscopy

Transmission Electron Microscopy (TEM) revealed mineral deposits characterized by concentric, spherically shaped multilayered rings of crystals with alternating light and dark appearance, often embedded in an organic layer, consistent with calcium oxalate (Figure 2).

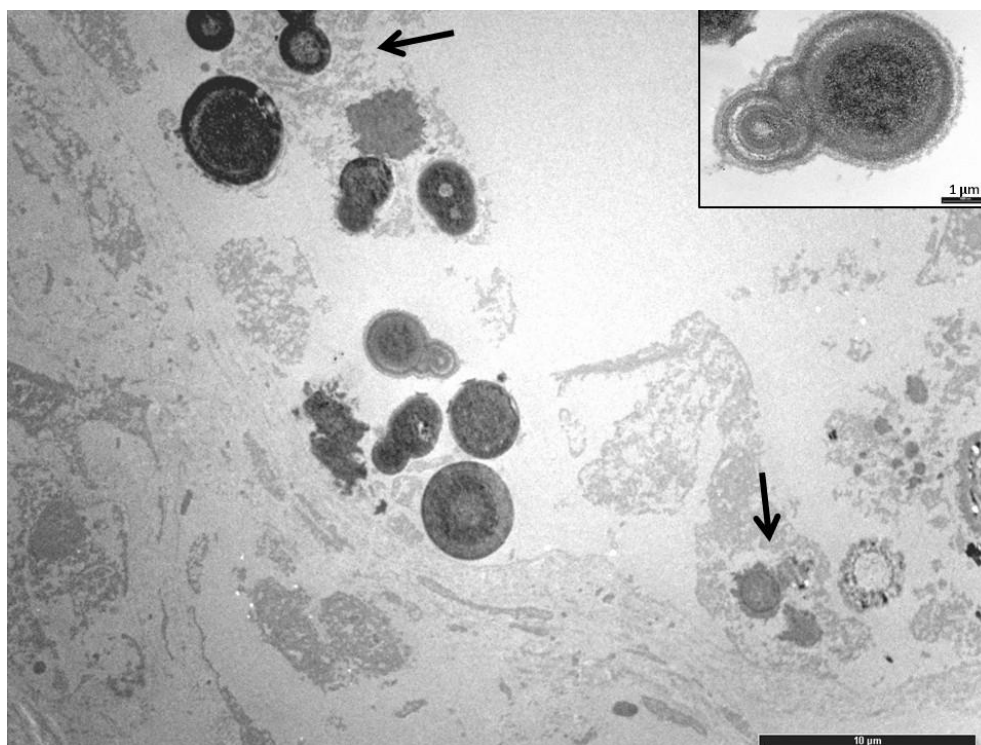


Figure 2. Mineral deposits in the tubular lumen seen as electron dense spherically shaped particles with alternating light and darks rings (inset). Some of them are encased in an organic matrix (arrows). (TEM).

Histochemical Staining

Grocott staining did not detect any fungal infection.

Statistical Analysis

Statistical analysis revealed an association between nest position and renal calculosis ($p < 0.05$) and between the differences in the average temperatures of July and August in the sampling year and the other years considered ($p < 0.001$). No correlation was found between egg depth and kidney lesions.

4. Discussion

The most relevant findings in this study were observed in liver and kidney, primarily consisting of vacuolar degeneration of hepatocytes, increasing amount of melanomacrophages and renal calculosis.

The vacuolar lesions appear to be attributable to a chronic process, since the degeneration appears widespread and uniform throughout the parenchyma. These types of lesions have been previously observed for *Caretta caretta* in previous studies and are compatible with a pattern of toxicosis. Merendi and others [22] have observed this degeneration in the liver of *Caretta caretta* stranded on the coast of Emilia Romagna; in that study it was hypothesized that the high concentration of arsenic in the liver, along with traces of other heavy metals, were responsible for the alterations. Moreover, Prearo and others [23] observed that high concentrations of these substances were present in liver and kidneys of adult specimen of *Caretta caretta*. Further studies on the presence of persistent organic pollutants have been conducted: Storelli and Marcotrigiano [24] have shown that the concentration of organo-chlorinated pesticides in the organs, particularly liver and kidneys, were higher in juveniles than in adults, while Alam and others [25] described the presence of these substances in eggs of turtles. Environmental pollutants accumulated in female can play an important role during egg development of its eggs. Furthermore, sea turtles are animals at the top of the food chain, hence these substances can accumulate in the fatty tissue, before being transferred to the egg during its formation, as a result of the mobilization of the mother's energy reserves [26,27]. Moreover, if the sand is further polluted, exposure to contaminants can continue after the deposition, through the gaseous exchanges occurring through the pores of the shell and the shell membranes [28].

A further interesting result found in this study is the increase of melanomacrophages in 18.6% of liver samples. Factors that may contribute to the increase in number and/or size of these cells are represented by seasonal changes in temperature (as a defense mechanism in adaptive cold-blooded animals) [29], weakness, wasting, stress, chronic inflammation and chronic diseases caused by bacteria [30]. These data suggest that the increase in melanomacrophages could be due either to a physiological adaptive mechanism against exceptionally hot temperatures during the month of August, when the eggs were about to hatch, or to a defence mechanism against the exposure to toxic substances.

Kidneys showed in 58.1% of cases the deposition in the glomeruli and tubules of amorphous material, identified as crystals of calcium oxalate. It has been shown that in reptiles the most common causes of renal calculosis include dehydration [31], excessive intake of protein and oxalate, vitamin and calcium deficiency, and bacterial infections [32]. Considering that samples were represented by not hatched turtles, their metabolic energy is satisfied by the transfer of nutrients from the mother to the egg [33]. The loggerhead turtle has an omnivorous-carnivorous diet, mainly based on the assumption of shellfishes, molluscs, and at a lesser extent algae [34]. It is therefore unlikely that the renal oxalosis in embryos is due to the vertical transfer of food substances containing calcium oxalate. According to Ackerman [35], the success of incubation for eggs of sea turtle depends on the presence of suitable conditions of temperature, humidity and salinity of the sand. Packard and Packard [36] have demonstrated that a weight loss of more than 40% of the initial weight of the egg (for example due to dehydration), causes a considerable risk for the successful hatching. An analysis of

environmental temperatures recorded in the months of July and August (Table 2), in which the eggs were laid, revealed that the average temperature was statistically higher than the previous two and the next two years. Moran and others [37] observed that the critical temperature of the sand beyond which the eggs of *Caretta caretta* do not hatch is 32.4°C. The sand temperature depends both on the environmental temperature and on its colour. Inasmuch, as the sand of the Pozzolana di Ponente beach is very dark, being of volcanic origin, a great heat radiation and a high nest temperature are present. The temperature of the sand around the nest also increases in the last stage of embryonic development, following the increases of the metabolic rate of the embryos [38]. Considering that 39.5% of the samples analyzed were hatchlings, while the remaining were in the last stage of incubation, and renal oxalosis can result from dehydration, it can be speculated that the renal alterations were caused by the loss of water from the eggs, due to the abnormally high environmental temperatures.

5. Conclusions

So far, discussions on the effects of global warming on sea turtle populations have primarily focused on the loss of egg-laying sites due to rising sea levels and tides, as well as alterations in sex ratios caused by the resulting increase in sand and nest temperatures [39]. In summary, climate change and human-induced impacts are considered among the most significant threats to the well-being of sea turtles, and should therefore be prioritized in research and conservation management efforts. Furthermore, identifying potential pathogens that pose a threat to endangered sea turtle species, influenced by both global warming and human activities, is essential for developing effective conservation strategies. The findings of this study may indicate a potential threat to loggerhead sea turtle nesting areas in the Mediterranean basin, a phenomenon likely linked to global warming.

Since the loggerhead sea turtle is an endangered species and Linosa is a crucial site in the Mediterranean for the birth of nearly all female individuals, more detailed studies are necessary to assess the critical temperature for successful hatching. Therefore, preventive measures should be implemented to protect the nests. Additional research on sand pollution and its toxicity to the turtles is also needed to identify corrective and protective actions to safeguard nesting sea turtles in the Mediterranean Sea.

Author Contributions: FES was the mentor and principal advisor and proposed the concept of the study, EB, EL and MC performed histological examination and diagnosis, EM performed electron microscopy, PP performed statistical analysis, DZ, SA performed sampling and monitoring of the nests, SN, ADL, MP, MG performed laboratory analysis, FRS, PP, MC were involved in the drafting and writing of the manuscript. All authors have read, commented and approved the final article.

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Abbreviations

The following abbreviations are used in this manuscript:

IUCN International Union for Conservation of Nature
SCL Standard carapace length
SCW standard carapace width
HL head length
HW head width

HE Haematoxylin-Eosin stain
 PAS Periodic Acid Schiff
 TEM Transmission Electron Microscopy

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