UNIVERSITÀ POLITECNICA DELLE MARCHE

**DIPARTIMENTO SCIENZE DELLA VITA E DELL'AMBIENTE**

**Corso di Laurea Magistrale**

Biologia Marina

# Valutazione degli inquinanti in *Caretta caretta*: trasferimento dal tuorlo all'intestino negli embrioni in stadio avanzato provenienti dai nidi del Mar Tirreno e Mar Ionio

# Pollutant assessment in *Caretta caretta*: yolk-to-intestine transfer in late-stage embryos from nests in the Tyrrhenian and Ionian Seas.

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## RIASSUNTO:

La tartaruga marina comune, *Caretta caretta,* è ampiamente distribuita nel Mar Mediterraneo, ma a causa dei cambiamenti ambientali e delle minacce antropiche è attualmente classificata come specie vulnerabile a livello globale. A causa della continua interazione tra questa specie e il marine litter è stata selezionata come bioindicatore ufficiale per il descrittore 10, Marine Litter, nella direttiva della Strategia Marina europea. Questo studio ha indagato le differenze delle caratteristiche morfologiche e fisiologiche di embrioni all'ultimo stadio di sviluppo (stadio 30) di *C. caretta* provenienti da nidi deposti lungo le coste del Mar Ionio e del Mar Tirreno nel 2022. Lo studio ha avuto come obiettivo la valutazione degli inquinanti in embrioni all'ultimo stadio di sviluppo (stadio 30) di *C. caretta* provenienti da nidi deposti lungo le coste del Mar Ionio e del Mar Tirreno nel 2022, mettendo in luce un trasferimento di inquinanti di idrocarburi aromatici policiclici (PAHs) dal tuorlo all'intestino. La presenza di PAHs nel tuorlo e nell'intestino è stata valutata tramite cromatografia HPLC. La funzionalità intestinale è stata valutata tramite valutazione della numerosità delle cellule mucose intestinali tramite analisi istologica. L'analisi degli inquinanti ha identificato concentrazioni significative di PAHs, in particolare fluoranthene, acenaphthylene e benzo-b-fluoranthene, sia nel tuorlo che nell'intestino di questi embrioni indicando un possibile

assorbimento da parte dell’intestino di inquinanti accumulati nel tuorlo, e. Mentre, l'analisi delle cellule mucipare intestinali ha evidenziato una regolare preparazione allo sviluppo della flora intestinale e alla nutrizione autonoma successiva la schiusa.

## ABSTRACT

The loggerhead sea turtle, *Caretta caretta*, is widely distributed in the Mediterranean Sea, but due to environmental changes and anthropogenic threats, it is currently classified as a vulnerable species globally. Due to the ongoing interaction between this species and marine litter, it has also been selected as an official bioindicator for Descriptor 10, Marine Litter, under the European Marine Strategy Framework Directive. This study aimed to assess pollutants in *Caretta caretta* embryos, highlighting the transfer of polycyclic aromatic hydrocarbons (PAHs) from the yolk to the intestine of embryos at the final stage of development (stage 30) of C. caretta from nests along the coasts of the Ionian and Tyrrhenian Seas in 2022. The presence of PAHs in both the yolk and intestine was evaluated using HPLC chromatography. Intestinal functionality was assessed by analyzing the number of mucosal cells in the intestine through histological analysis. The pollutant analysis identified significant concentrations of PAHs, particularly fluoranthene, acenaphthylene, and benzo-b-fluoranthene, in both the yolk and intestine of these embryos, indicating a possible absorption of pollutants accumulated in the yolk by the intestine. Furthermore, the analysis of mucosal cells highlighted a regular preparation for the development of intestinal flora and subsequent independent nutrition after hatching.

# 1. INTRODUCTION

## 1.1 Biology and ecology.

The loggerhead sea turtle, *Caretta caretta* (Linneaus, 1758), is one of the sea turtle species found in the ocean and one of the three species present in the Mediterranean Sea basin, along with *Chelonia mydas* and *Dermochelys coriacea*. There are currently 7 species of sea turtles, which belong to the order Testudines, divided into two families: *Dermochelyidae* and *Cheloniidae*. The loggerhead sea turtle, *C. Caretta*, belongs to the family *Cheloniidae*, which includes other species found in the ocean such as *Dermochelys coriacea* (leatherback sea turtle), *Lepidochelys kempii* (Kemp's Ridley sea turtle), *Lepidochelys olivacea* (olive Ridley sea turtle), *Natator depressus* (flatback sea turtle), *Eretmochelys imbricata* (hawksbill sea turtle), and *Chelonia Mydas* (Green turtle) (Figure 1) (Pritchard, 1996).

This species has a distinguishable carapace of reddish-brown color. The carapace has four prefrontal scales, one nuchal scale in contact with the first costal scale, five vertebral scales, five costal scales, and three inframarginal scales (Figure 2) (Wyneken, 2005).

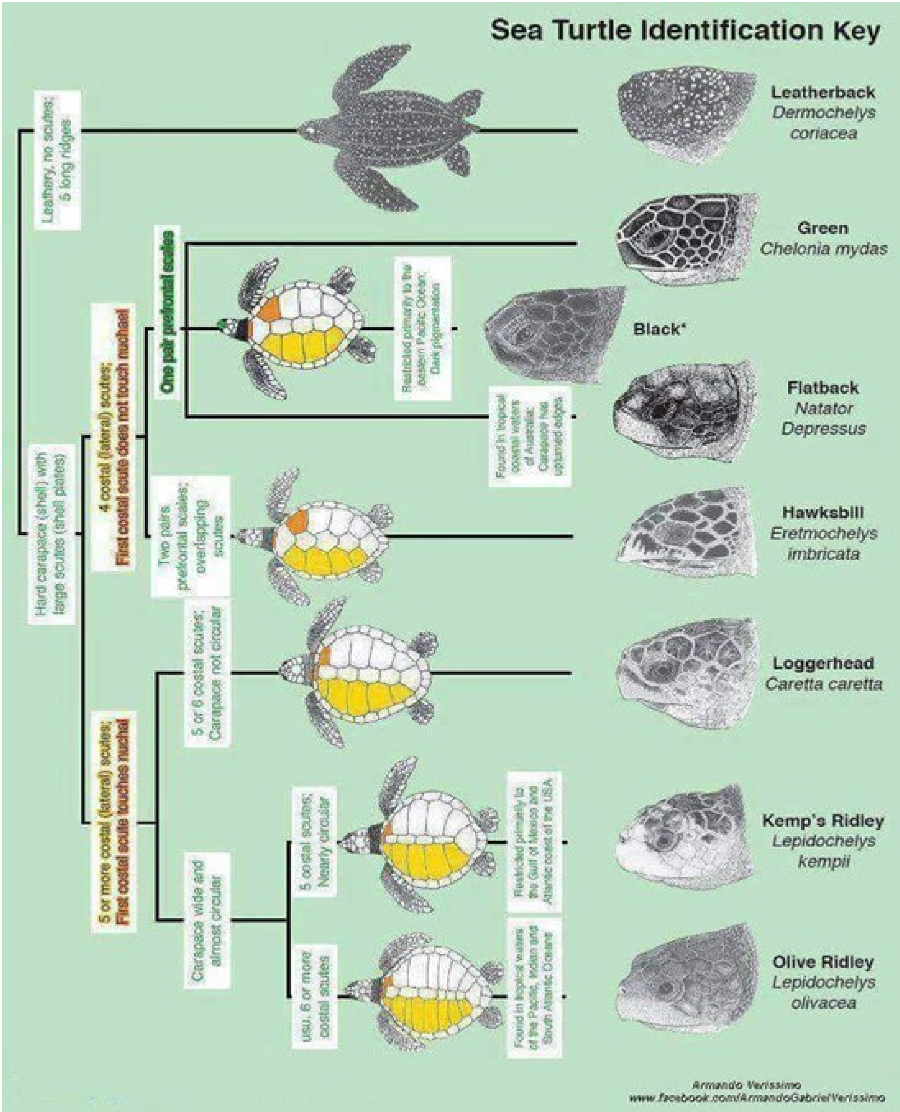


**Figure 1**: Diagram of the phylogenetic relationships of the seven existing turtles (Source: Seaturtle.org).

The head has a yellow-brownish shades coloration, as well as the limbs and plastron. The structure of mouth consists of a slightly curved and sharp horny sheath called the *rhamphotheca*, which forms the beak and is used primarily for prey such as mollusks, algae, jellyfish, echinoderms, and crustaceans

(Wyneken, 2005). Both the plastron and carapace are keratinized, protecting against environmental and human threats such as predation, and collisions with boats and rocks (Dodd, 19).

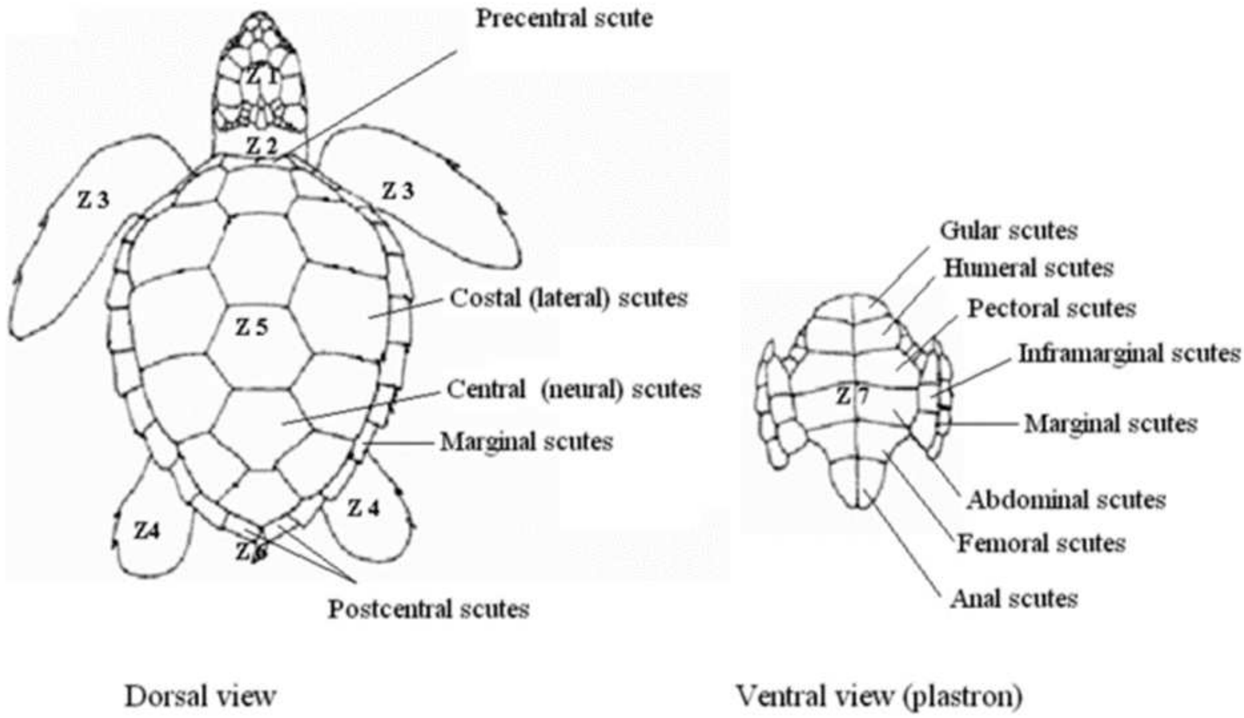


**Figure 2**. Various parts of the body of loggerhead sea turtle (Karaa, Jribi, Bounai, 2012).

The loggerhead sea turtle, *C. caretta*, is a species with a wide distribution; it is widely spread in temperate, subtropical, and tropical waters across the Atlantic, Indian, and Pacific Oceans, as well as within the Mediterranean Sea. This wide range of distribution is allowed thanks to their migratory capacity that allows them to swim for many kilometers during the day.

Their movement is adjusted in distance and mode by the stage of the life cycle they are facing. (Hughes, 1989; Luschi et al. 2003).

In the Mediterranean, *C. caretta* nesting has become much more frequent since 2010, the year when a database was created for nesting sites in Italy, France, Spain, and Tunisia (Hochscheid et al., 2022).

In Italy, beaches were monitored following the SWOT (2011) descriptors, and occasional monitoring was also conducted using drones, especially in Calabria. The areas with the highest nesting activity are the regions of Campania, Tuscany, and Calabria in the Tyrrhenian coast (Figure 3) (Hochscheid et al., 2022).Within ecosystems, the *C. caretta* turtles are a key species both as consumers and predators and have a key role in the trophic chain itself (Mingozzi et al., 2006).

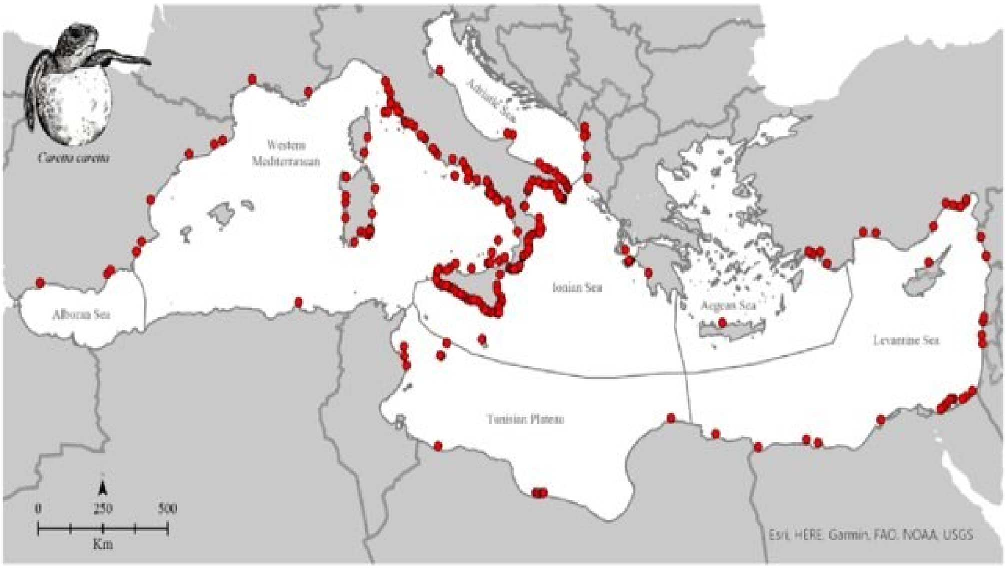


**Figure 3.** Map of different nest of loggerhead sea turtle in the Mediterranean Sea (red dots), from 1963 to 2020 (image from © Fao; NOAA)

## 1.2. Reproduction and life cycle

The life cycle of the *C. caretta* turtle consists of a set of ontogenic phases (Figure 4), which occur both on an ecological and geographical level and correspond in several stages: the first stage is spawning, embryonic development and hatching (Briscoe et al., 2016). The cycle begins following oviposition which occurs on the beaches by the females (Briscoe et al., 2016). During the incubation phase, the yolk sac provides the primary energy necessary for them to head towards the sea and swim away from the coast. After the embryonic incubation period, hatchlings reach the sea (Briscoe et al. 2016; Parker et al., 2016).

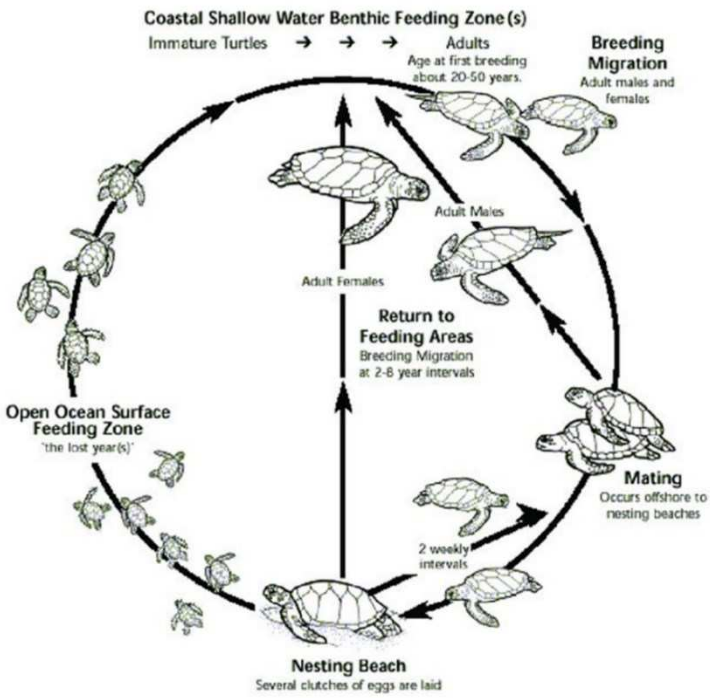


**Figure 4.** A generalized life cycle of sea turtles, from Lanyon et al. (1989).

Subsequentially, we have the transition stage, where the hatchlings actively feed, spending most of their time on the surface and making the transition from the neritic zone to the oceanic zone, a shift influenced by currents (Miller, 1996). The juvenile stage begins when the turtles are in the furthest area from the coast, in which they have active and passive swimming phases. During this phase they are defined as epipelagic, meaning they spend most of their time in the first 5m of the surface, in oceanic areas, feeding and occasionally descending to greater depth (Limpus et al., 2019). Finally, when turtles reach sexual maturity, they perform migrations towards the foraging area and the reproduction area in neritic areas, in which they feed from the seabed. This latest phase corresponds to the adult stage (Miller, 1996). Sea turtles are iteroparous breeders, oviparous with internal fertilization and with deposition of many eggs in a nest that is often built in the same place (Miller, 1996). The reproductive period of *C. caretta* can vary, influenced by various endogenous and exogenous factors, as well as the energy factors and fat reserves on the part of the female, which are then used to complete vitellogenesis (Miller et al., 1996). Females have the activation of follicular growth, resulting in an increase in hormones for vitellogenesis and adipose tissue storage, approximately 10 months before the breeding season (Miller et al., 2003).

The breeding season begins when adults migrate to neritic areas and mating areas, where males and females meet. Females, as reported by several studies, can mate with different males generating genetic diversity with variability in the offspring (Miller, 1996; Gist and Congdon, 1998). Once the mating process is over, the males return to the foraging areas, while the females begin their migratory process towards the beaches for spawning. Usually, the females return to coasts where they have previously spawned, in temperate climate or subtropical areas, and with a soft and humid sand (Miller, 1996; Miller et al., 2003).

Nesting takes place during the night, the turtle comes out of the water and heads along the surf line, to the beach where it looks for a place to dig the nest (Hailamn et al., 1992).

The nest is dug through the movements that the female makes using her plastron and hind limbs. The nest is complete when it reaches a depth of about 60 cm and inside the female lays between 90 and 150 eggs. At the end of laying, the turtle covers the chamber with sand with the movement of its hind limbs (Hailman et al., 1992). During the reproductive season, the female can lay up to four nests, with an interval called internesting of about fifteen days, adopting this strategy probably to have more offspring and ensure hatching (Hamann et al, 2003).

### 1.2.1 Embryological development

Following the deposition by the female *C. Caretta* we have the incubation phase, which lasts between 40 and 60 days, at the end of which the turtle breaks the egg and hatching occurs (Zbinden et al., 2011).

Being a reptile, the environment around the nest influences Environmental Sex Determination (ESD) of *C. caretta* embryos. This causes ovaries and testes to develop based on the environmental conditions to which the eggs are exposed during the development period (TSD, Temperature-dependent sex). Usually in turtles the middle third of embryonic development corresponds to the most sensitive period to temperature variations (Zbinden et al., 2011). Indeed, in this period, male gonads develop at lower temperatures, while female gonads develop at higher temperatures.

The pivotal temperature is the temperature at which the sex ratio between male and female is the same, and the sex ratio can shift from sex to another based on temperature values for *C. caretta* this temperature is around 29°C (Lolavar et al., 2021).

The eggs are spherical and white in color, surrounded by a porous calcareous shell. Inside the egg there is a subdivision of the components into external and internal parts, the embryo is immersed in the amniotic fluid, surrounded by the amniotic sac, the yolk sac, the allantoic membranous sac and in the outermost part by the chorionic membrane and from albumen (Figure 5) (Spotilla,2004). Each internal part of the egg plays a significant role in embryonic development. The amniotic fluid sac allows for a stable internal environment while the yolk component provides nourishment to the embryo with the vitelline vessels (Moncada, 2021). The allantoic sac allows a gaseous exchange, allowing the diffusion of gases through the calcareous shell (Gabrielli and Accili, 2010).

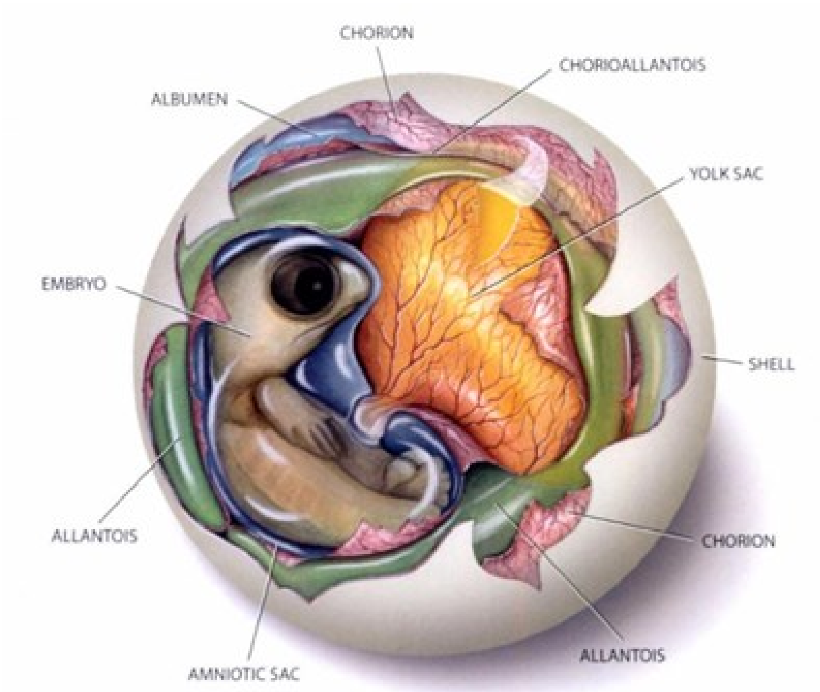


**Figure 5.** Internal structure of a sea turtle egg. (Spotila, 2004)

The chorioallantoic membrane is used for the reabsorption of ions and $H_2O$ by the allantoic fluid before the first lungs breathe at the time of hatching (the lungs are fully formed at the end of embryonic development) (Spotila, 2004; Gabrielli and Accili, 2010).

The embryonic development is composed of a total of 31 stages, which develop from fertilization to hatching (Figure 6). The process of embryonic development from one phase to another takes various times which vary due to the temperature, with an increase in temperature the process takes a shorter time (Miller et al., 2017).

Development Process:

- 76-79 days: 26°C.
- 54-58 days: 29°C.
- 46-49 days: 32°C.

Following the last stage of development, turtles break their shells and come out to the surface, usually in small groups; Indeed, the synchronous effort of many individuals to leave the nest could save a bit of the energy stored by the hatchlings to reach the sea and, at the same time, it decreases the percentage of predation attempts on each hatchling (Rusli et al., 2016). The hatching event is also influenced by the external environment, light and temperature. Elevated temperatures inhibit stopovers and allow the pump to emerge at night, while light influences its progress toward the sea.

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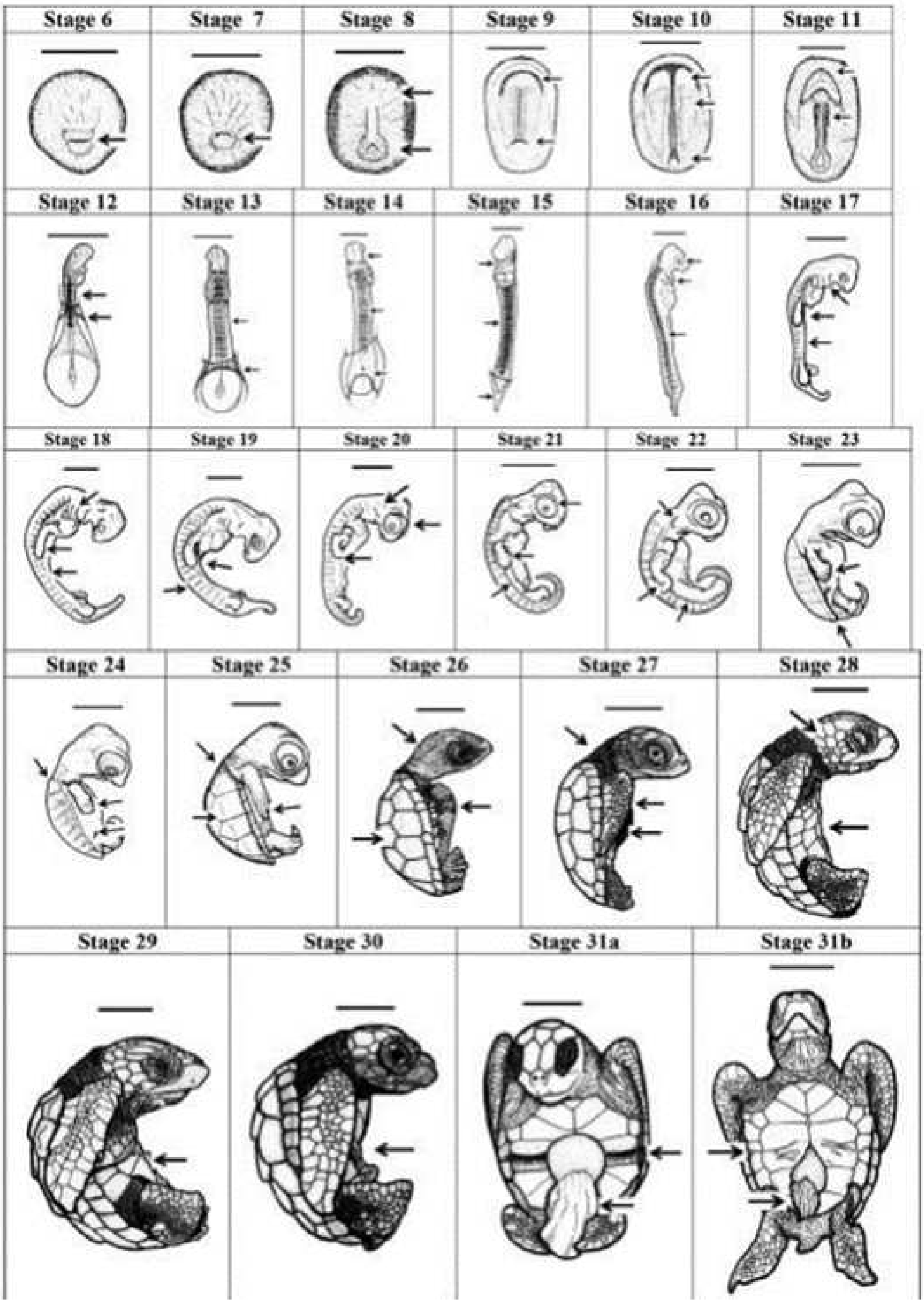


**Figure 6.** Sea turtles' development of embryonic process table (Miller et al, 2017).

## 1.3 Threats

The *C. caretta* turtles are exposed, during their life cycle, to several threats, both anthropogenic and natural, both in marine and non-marine habitats. Since 2020, the subpopulation of *C. caretta* present in the Mediterranean Sea has been included in the red list of List concern species by the IUCN, the International Union for Conservation of Nature (Camiñas et al., 2020). The Barcelona Convention, which has adopted a protection plan for specimens in the Mediterranean since 1989, has recognized fishing activity as one of the most impactful anthropic factors responsible for the death of sea turtles (Camiñas et al., 2020). It is also considered due to the high vulnerability for plastic pollution within the Marine Strategies Framework Directive (MSFD), a European-level directive, as a bioindicator for the presence of marine litter in the sea (Matiddi et al.; 2017).

The loggerhead sea turtles encounter abiotic and biotic conditions, during their life and reproductive cycle, which could compromise the conservation of this species (Martins et al., 2022). Threats can be of several types, both natural and anthropogenic, which very often do not only concern tourism and disturbance during the reproductive phase.

### 1.3.1 Anthropogenic threats

The main anthropogenic threats to the *C. caretta* population are marine debris, pollution and oil spills, microplastic, bycatch and ghost fishing, light pollution, illegal poaching and chemical pollution. Oil spills and pollution from sea discharges and ghost fishing (Figure 7) have direct effects on the loggerhead sea turtle population, leading to high mortality. Fishing activity and ghost fishing, especially in the Mediterranean Sea, make the stock of *C. caretta* susceptible. In fact, these activities cause high mortality and strandings, also due to the wounds often sustained by the turtles themselves (Álvarez de Quevedo et al., 2013). The high incidence of injuries caused by maritime traffic and fishing gear, combined with exposure to heavy metals and the presence of subclinical *Chelonid herpesvirus 5* (ChHV5) infections, were significant findings. Over 60% of the turtles exhibited injuries attributable to maritime traffic or fishing activities, while 38.5% had ingested marine debris, including plastic and fishing hooks, which caused severe and, in some cases, fatal lesions (Serra et al., 2023).

Tourism and coastal development linked to an ever-increasing anthropogenic demand generate degradation in the habitats chosen by turtles for nesting (MASE, 2024). Even the light pollution generated by the lighting of beaches constitutes a cause of failure for the hatching of broods (MASE, 2024). At the time of hatching, baby turtles tend to orient themselves and reach the sea with the dominant light sources. The artificial lights cause disorientation that leads them in opposite directions and certain death (Chapman et al., 2016)

In other countries, another major anthropogenic threat is illegal poaching, which can take various forms, from the illegal harvesting of animals and eggs to the illegal consumption of food. According to some studies, turtle meat is consumed in 75% of IOSEA signatory states, while the trade in shell products seems to be predominant in East Asian countries (IOSEA Marine Turtles, 2014).

### 1.3.2 Anthropogenic contaminants

The anthropogenic contaminants that most affect the life of sea turtles are chemical pollutants and plastics (Nelms et al., 2016).

The analyses carried out in the Mediterranean Sea basin have underlined how chemical pollution caused by human and industrial activities determines the release into the marine environment of non-essential toxic metals such as arsenic (As), cadmium (Cd), mercury ( Hg) and lead (Pb) (Valavanidis and Vlachogianni, 2011; Esposito et al., 2020; Lambiase et al., 2022), which are accumulated by sea turtles mainly through the ingestion of contaminated prey, but also through marine sediments and sea water (Figure 14) (Storelli et al., 2014; Bucchia et al., 2015; Cammilleri et al., 2017; Lambiase et al., 2022).The accumulation of these chemicals, as already reported for other marine pollutants (González-Fernandez and Cuesta, 2022), could weaken the immune system of sea turtles (Chaousis et al., 2023), making them more vulnerable to bacterial or viral diseases (Fiorito et al., 2020; Serra et al., 2023). In 2023, a study highlighted the critical role of anthropogenic and chemical factors in the health of loggerhead sea turtles (*C. caretta*) in the Mediterranean. Heavy metals, including arsenic, cadmium, lead and mercury, were detected in the liver, kidneys, and muscles (Figure 7: box plot concentration), with a significant accumulation of arsenic particularly observed in turtles from Calabria (Serra et al., 2023).

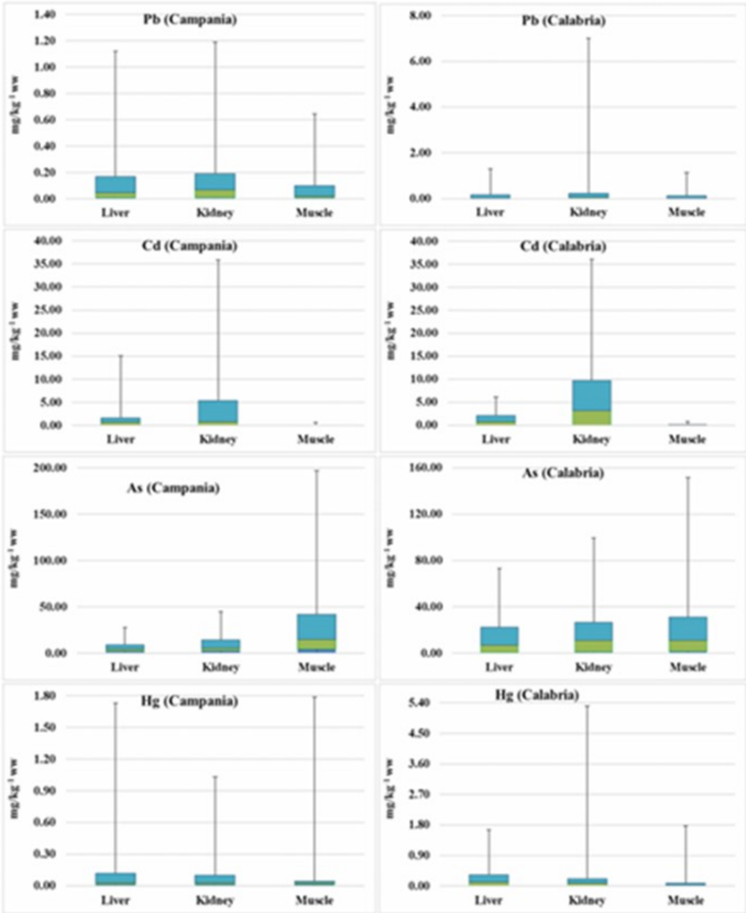


**Figure 7**. Boxplots of As, Hg, Pb, Cd and Pb concentrations (mgkg−1ww) in turtles along the Italians coast. The lines in the middle of the box plots represent the medians (Serra et al., 2023).

The study underscores the urgency of targeted conservation measures. The findings provide a scientific foundation for developing strategies to mitigate human impacts on marine ecosystems and to protect this vulnerable species (Serra et al., 2023).

### 1.3.3 Plastic and microplastic pollution

Plastic and microplastic pollution are one of the most expanding threats globally since 1950 (Barnes et al., 2002; Andray, 2011; Gall et al, 2015). Plastic debris impacts marine ecosystems, becoming entangled in or ingested by marine organisms, acting as a vector for invasive species, and absorbing persistent organic pollutants (Barnes et al., 2002; Andray, 2011; Gall et al, 2015). Plastic differs in size which are classified based on their size into four main categories: macroparticles (larger than 25 mm), mesoplastics (ranging from 5 mm to 25 mm), microplastics (smaller than 5 mm), and nanoplastics (smaller than 0.001 mm). Plastic pollution in the Mediterranean Sea is extremely high, due to its nature as an almost closed basin which retains most of the debris inside, even if compared to some areas of the Ocean it has no accumulation points (Cózar et al., 2015). *C. caretta* actively ingests plastics, mistaking them for food (Meaza et al.; 2021). As a result, plastic debris is regularly found in the intestinal contents of turtles and within the embryos. (Fukuoka et al., 2016; Nelmas et al., 2016, Duncan et al., 2017; Lynch, 2018).

In addition to the danger of ingestion itself with potential physical damage, the ingestion of debris and microplastics increases the exposure of turtles to organic pollutants deriving from plastic (OPE) (Teuten et al., 2009; Engler, 2012). To date there are few comprehensive studies on the presence of OPEs and microplastics. For example, in the study conducted by Duncan et al., (2024) they analyze how *C. caretta* is currently a bio-indicator for plastic pollution in the Mediterranean. The study, carried out between 2012 and 2022 on 131 turtle specimens, found that 42.7% of the individuals examined had ingested macroplastics (≥ 5 mm). The frequency of ingestion did not vary over time, nor in relation to body size, type of turtle (stranded or bycaught), or the amount of material ingested. The main characteristics of the ingested plastic were thin sheets, predominantly light-colored (transparent or white), and the most common polymers identified were polypropylene and polyethylene. Furthermore, a preference for certain types, colors, and shapes of plastic was observed (Duncan et al., 2024).

Instead, other studies, such as the one conducted by Chemello et al., (2023) explore the presence and impact of microplastics in the transfer from mother to eggs, investigating how these particles affect embryonic health. The researchers analyzed yolk and liver samples from embryos collected from two different nests, using Raman microscopy to identify microplastics after alkaline digestion. Microplastics smaller than 5 μm were found in both the yolk and liver of loggerhead sea turtles at the 30th stage of embryonic development. The most common polymers identified were polyethylene, polyvinyl chloride, and acrylonitrile butadiene styrene. Although the number of microplastics was higher in the yolk compared to the liver, a significant positive correlation was observed between the number of microplastics in the liver and the number of melanomacrophages (a biomarker of tissue damage), suggesting that microplastics may affect the hepatic health of the embryos (Chemello et al., 2023).

What emerges from the few studies conducted on adults and embryos is that very often microplastics are not only directly consumed, but also that turtles are contaminated through prey such as squid and jellyfish (Sala et al., 2021).

### 1.3.4 Organic compound

Polycyclic aromatic hydrocarbons (PAHs) are a class of complex organic compounds primarily generated during the incomplete combustion of organic materials such as coal, petroleum, natural gas, and biomass. These compounds are widespread in the environment and, due to their persistence and ability to accumulate in marine sediments, pose a significant risk to aquatic ecosystems (Wang et al.,2023).

PAHs can enter marine ecosystems through both natural and anthropogenic sources, including oil spills, industrial discharges, and wastewater effluents, thereby constituting a serious threat to marine biodiversity (Wang et al., 2023).

Among the species most exposed to the ecotoxicological effects of PAHs, marine turtles such as *C. caretta* are particularly threatened (Dias et al., 2024). Recent studies have demonstrated that marine turtles absorb PAHs through multiple pathways, such as the ingestion of contaminated food, accidental consumption of plastic debris, and direct contact with polluted water (Casini et al., 2018).

PAH contamination can originate from various sources, including chemical pollution and solid waste in the marine environment. These contaminants tend to bioaccumulate in the tissues of marine turtles, causing adverse effects on their overall health and reproductive success (Arienzo, 2023).

PAHs can be used as biomarkers; indeed, for *C. caretta* in 2018, a study was conducted to evaluate the exposure of juvenile specimens to them, by analyzing the expression of specific genes related to their presence. The study highlights how exposure to these contaminants can impact the health status and physiological mechanisms of the turtle, stimulating the activity of genes involved in oxidative stress response, detoxification processes, and DNA regulation. Although the article does not explicitly address embryos, the results imply that similar exposure during embryonic development could have analogous effects, negatively influencing the development and survival of the embryos (Cocci et al., 2019).

The integration of chemical and biological analyses has further highlighted the vulnerability of *Caretta caretta* to PAHs, emphasizing the need for urgent conservation measures. The use of non-invasive approaches for monitoring these contaminants represents an innovative methodology, which can also be applied to other marine species, providing crucial tools for assessing the impact of chemical pollution on a global scale.

### 1.3.5 Natural threats

The natural factor that most influences the nest and hatching is certainly the temperature, which can compromise the development and incubation of the embryos with excessive lowering or raising (Mahdy et al., 2020).

Climate change has a compromising role in brood survival, especially with rising temperatures. One of the most studied solutions is the phenological adaptation of *C. caretta* which would allow the species to capture a thermal window like what they experience nowadays during the incubation period (Mazaris et al., 2017). Floods are also linked to climate change, which causes rising seas, leading to hypoxia or damage at the embryonic level to the nest, causing lethal effects or dysfunctions at the level of spatial and neurological orientation (Pike et al., 2015).

Predation is another problem that affects nesting success and hatching. If during the development of the nest the greatest predation occurs from foxes, pigs, raccoons, and dogs, once hatching occurs predation occurs both on the beach and in the sea, first with crabs and birds and then with cetaceans (Kurz et al., 2012; Wyneken, 2007). Finally, there are diseases that can develop inside the nest, especially caused by fungi (Pietrolungo et al., 2023; Lockley et al., 2020)

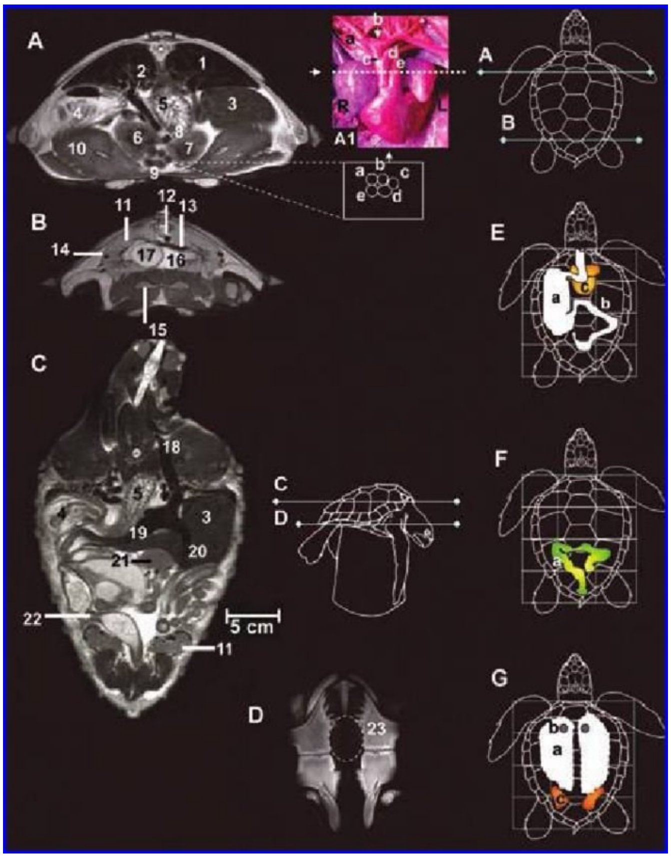


**Figure 8.** Results of MRI and schematic drawings of topographic anatomic features of a live loggerhead sea turtle. Views A and B—Caudal transverse T2-weighted images. View A1 is a ventral view of an anatomic dissection of the heart injected with latex; notice the right pulmonary artery (a), right aorta (b), brachiocephalic trunk (c), left aorta (d), and the left pulmonary artery (e). Views C and D—Dorsal T2-weighted images; the circular area on View D indicates the cardiac region. View E—Schematic drawing of the stomach (a), duodenum (b), and heart (c). View F— Schematic drawing of the large intestine (a). View G—Schematic drawing of the lungs (a), bronchi (b), and kidneys (c). 1 = Right lung. 2 = Left bronchus. 3 = Liver. 4 = Stomach. 5 = Esophagus. 6 = Left atrium. 7 = Right atrium. 8 = Left aorta. 9 = Transverse section of large vessel of cardiac base. 10 = Pectoral musculature. 11 = Left kidney. 12 = Dorsal aorta. 13 = Renal vein. 14 = External iliac vein. 15 = Pubis and pelvic muscles. 16 = Urinary bladder. 17 = Descending colon. 18 = Right cranial vena cava. 19 = Left hepatic vein. 20 = Caudal vena cava. 21 = Spleen. 22 = Narrowing of large intestine. 23 = Hyoplastron bone. (Valente et al, 2007).

## 1.4. Gut

In reptiles, and so in loggerhead sea turtles, the gastrointestinal tract (Figure 8 and 9) is made up of the oral cavity, the esophagus, the stomach and the small and large intestine (Figure 9) (Wyneken, 2001). The small intestine covers approximately 50% and is divided into the duodenum, jejunum and ileum (Thompson, 1988). The wall of the small intestine was thicker than that of the large intestine (Valente et al., 2007). Chemical digestion begins in the stomach and continues into the small intestine, where enzymes are added that break down carbohydrates and proteins, specializing in the absorption of amino acids, minerals such as calcium and phosphorus, and water (Wyneken, 2001).

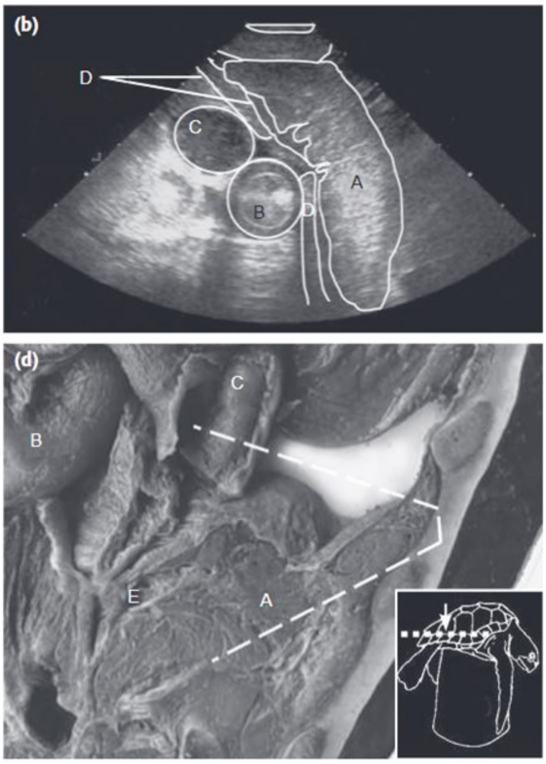


**Figure 9.** Ultrasound images of the kidney and intestines of loggerhead sea turtle (*Caretta caretta*) (b). Images taken through the right femoral acoustic window. (d) Frontal anatomical section. (Valente et al.,2007).

## 2. AIM

The *Caretta caretta* is a species threatened by several natural and anthropogenic factors throughout its long-life cycle, from the embryonic stage to the adult one in the sea (Hamann et al., 2010).

Recently, the most common threats undoubtedly come from anthropogenic activities, both due to waste and plastic debris as well as various chemical and metallic pollutants (Nelms et al., 2016). To date, several studies focused primarily on the presence and the potential harmful effects of plastics and other pollutants in juveniles and adult stage of *C. caretta,* while just few studies were performed on the presence and the effects of these threats in an embryonic stage (Sousa et al., 2023; Chemello et al., 2023).

This study aimed to assess pollutants in *Caretta caretta* embryos, highlighting the transfer of polycyclic aromatic hydrocarbons (PAHs) from the yolk to the intestine of embryos at the final stage of development (stage 30) of *C. caretta* from nests along the coasts of the Ionian and Tyrrhenian Seas in 2022. The presence of PAHs in both the yolk and intestine was evaluated using HPLC chromatography. Intestinal functionality was assessed by analyzing the number of mucosal cells in the intestine through histological analysis.

# 3. MATERIAL AND METHODS

## 3.1 Samples collection

The unhatched eggs of *C. caretta* were collected from various nests found on the beaches of the Ionian Sea and Tyrrhenian Sea coasts (Figure 10) during the summers of 2022 in collaboration with TartAmare and WWF Policoro Herakleia.

According to ISPRA guidelines, at the end of the incubation period, the nests were excavated, and unhatched eggs were collected. The eggs were then stored at -20°C until following analysis.

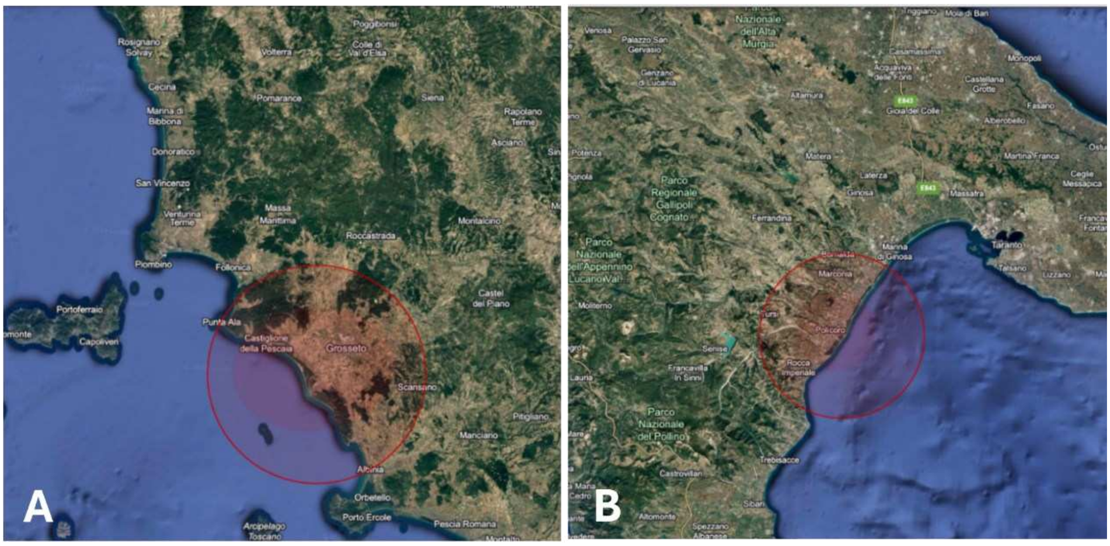


**Figure 10.** Location of nests along the coast of Tyrrhenian (A) and Ionian (B) sea (images from © Google Earth)

## 3.2 Sampling

The unhatched eggs, 77 for the Tyrrhenian coast and 108 for the Ionian coast were sampled at the Laboratory of Reproduction and Developmental Biology, Department of Life and Environmental Science (DISVA) at the Polytechnic University of Marche (Ancona, Italy). Before sampling activities, the integrity of the eggshells was assessed, and any broken eggs were excluded. Each egg was weighed, and its diameter was measured.

The eggs were then opened, the presence and developmental stage of the embryo assessed, and the following biometric parameters recorded (Figure 11):

- Embryo weight
- Yolk weight
- Straight Carapace Length (SCL) and Width (SCW)
- Straight Head Length (SHL) and Width (SHW)
- Forelimb Length dx/sx (FLL) and Hindlimb Length dx/sx (HLL)

**Figure 11**. Analysis of sea turtle embryos *C. caretta*, including measurements of biometric parameters. (© personal photo)

Subsequently, from stage 30 embryos (N=17) intestine was sampled, (when available) were weighed and in part preserved in formaldehyde/glutaraldehyde solution ($NaH_2PO_4$-$H_2O$ + NaOH + Formaldehyde (36,5%) + Glutaraldehyde (25%) + $H_2O$) and stored at 4°C to maintain tissue integrity for histological analysis, and in part stored at - 80°C for HPLC analyses. In addition, the entire yolk from each embryo was stored at -80°C for HPLC analysis.

## 3.3 Histological analysis

Following the inclusion protocol (Chemello et al., 2023), intestine samples were washed three times in ethanol (Et-OH) 70% (15minutes each), then dehydrated in increasing ethanol solutions (80, 95, and 100%), washed with xylene (Bio-Optica, Milan, Italy) and embedded in paraffin (Bio-Optica). Later, solidified paraffin blocks were cut with a microtome (Leica RM2125 RTS, Nussloch, Germany) into 5 $\mu$ m sections. For each sample, 3 slides, each one containing 3 sections were collected. Subsequently, the sections were stained following the Hematoxylin and Eosin Y protocol and the Alcian Blue staining protocol (Alcian Blue 1%, pH 2.5 Stain Kit, BioOptica, Milan, Italy). These staining protocols allow the identification of several types of cells and tissues, providing essential information about the pattern, shape, and structure of cells in the tissue sample. The Alcian Blue staining used in intestines allows the identification of mucopolysaccharides, which are key components of the mucous secretions and glycoproteins present in the intestinal walls. At the end, samples were observed using an optical microscope (Zeiss Axio Imager.A2, Oberkochen, Germany), with which sections images have been acquired using a combined color digital camera Axiocam 503 (Zeiss, Oberkochen, Germany). The abundance of mucous cells was classified in low, medium and high concentration following (Tlachi et al., 2021).

## 3.4 PAHs assessment

The toxicological analyses were performed in collaboration with the School of Biosciences and Veterinary Medicine, Università di Camerino (San Benedetto del Tronto, Italy). A QuEChERS (quick, easy, cheap, effective, rugged, and safe) methodology, previously described by Barraza and colleagues (2023), was followed to extract PAHs in intestines. The samples were homogenized in 5 mL of MilliQ water each one, then sonicated for 1 minute and vortexed for 1 minute. Then 4mL of Acetonitrile were added in each sample and vortexed for 1 minute. 3,6 grams of magnesium sulfate and 1,2 gram of sodium chloride were then added to each tube and shaken by hand for 3 minutes. The resulting mixtures were centrifuged at 4500 rpm (1900 ×g) for 8 minutes at 10°C. The acetonitrile layer was aliquoted into glass vials and evaporated with nitrogen gas. The dried extracts were then reconstituted in 1 mL of methanol, transferred to amber glass vials with Teflon lids, and stored at -20°C until chromatography analysis. Analysis by an HPLC system (Variant ProStar 240 equipped with a Varian ProStar 310 UV-VIS Detector) was performed using appropriate reference standards from Sigma-Aldrich.

# 4. RESULTS

## 4.1 Stages of embryonic development

The evaluation during sampling did not report any structural abnormalities of the unhatched eggs and, subsequently, after macroscopic analysis once opened, no abnormalities were found in the embryos of the analyzed nests. As described in Figure 12A, in the Ionian area most eggs (78%) presented a not eye-visible embryo categorized as <10 developmental stage. In this area, just 5% of the sampled eggs presented an embryo at stage 30, which represents the latest developmental stage before hatch. Similarly, in the nests laid in Tyrrhenian coast the 83% of embryos were categorized as <10 developmental stage and just the 13% of sampled embryos reached the 30 developmental stage (Figure 12B).

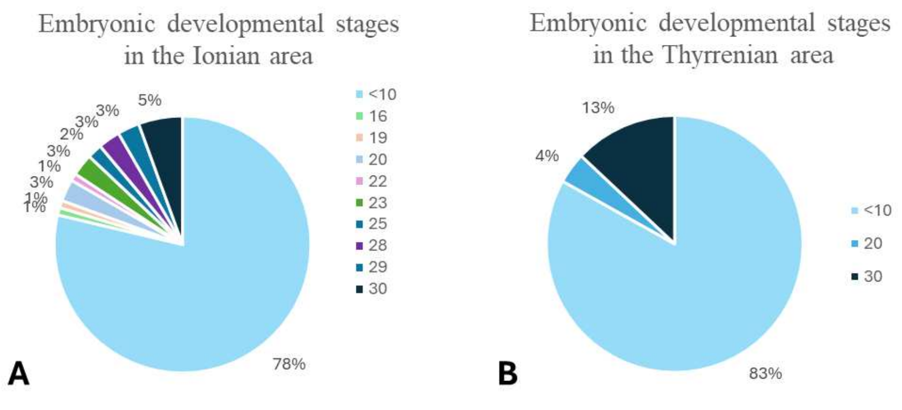


**Figure 12**: Percentage (%) of different *Caretta caretta* embryonic developmental stages. A: embryonic developmental stage in the Ionian area. B: embryonic developmental stages in the Tyrrhenian area.

## 4.2 Biometric measurements

Biometric measurements are reported in the following graphs. When comparing the weight of eggs collected from the two sampling areas, a significant difference was observed, with eggs from the Ionian Sea (mean value: 14,90 g ±2,196) being significantly (p value: 0,0208) smaller than those collected from the Tyrrhenian Sea (mean value: 20,62 g± 6,025) (Figure 13).

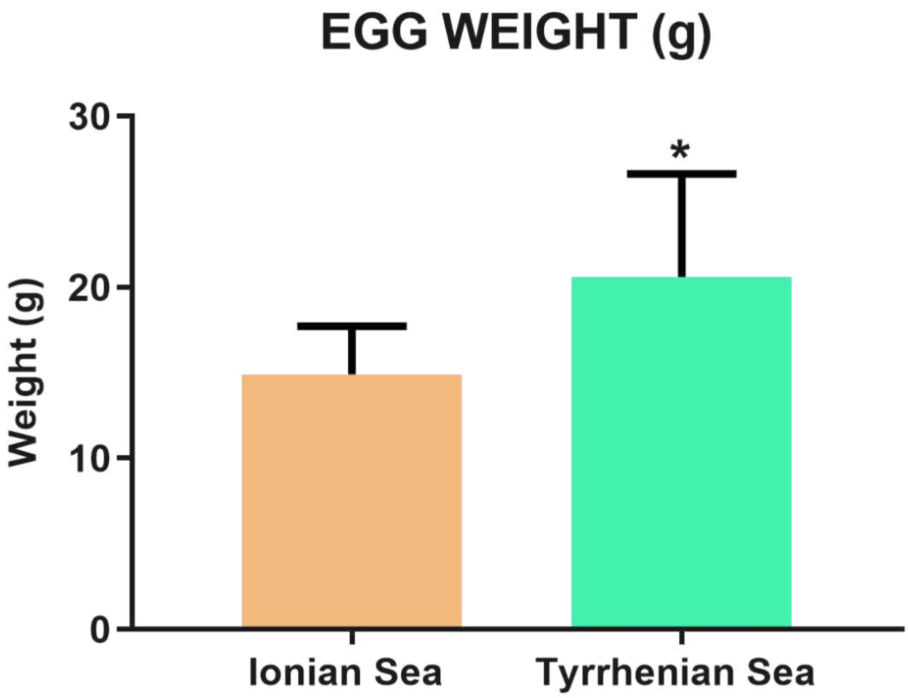


**Figure 13:** Unpaired t-test performed on egg weight of samples collected in the Ionian and the Tyrrhenian Sea. Values are represented as mean ± standard deviation (sd). Significance: *= $p \leq 0.05$;

The comparison of yolk and embryo weight of embryos at 30 developmental stages are described in Figure 14. Regarding the yolk weight (A), the Ionian Sea presented a higher mean value (0,3497±0,1972) while for the Tyrrhenian sea the mean yolk value was 0,7600±0,36. A significative difference (p value:0,0008) was detected in the comparison of embryo weight (B), with the embryos from the Ionian Sea (mean value: 10,96±0,7827) smaller than the ones from the Tyrrhenian Sea (mean value: 15,07±1,63).

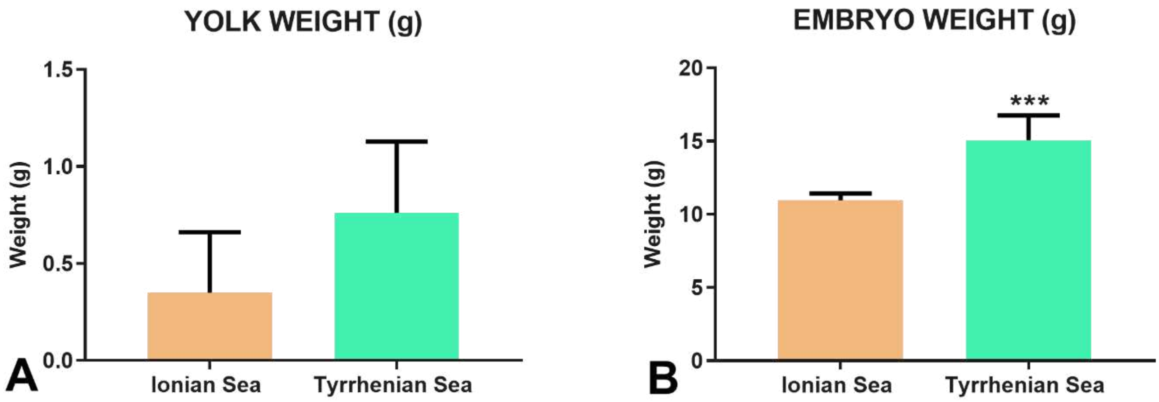


**Figure 14:** Unpaired t-test performed on embryo weight (A) and yolk weight (B) of samples collected in the Ionian and the Tyrrhenian Sea. Values are represented as mean ± standard deviation (sd). Significance: ***=p ≤ 0.001.

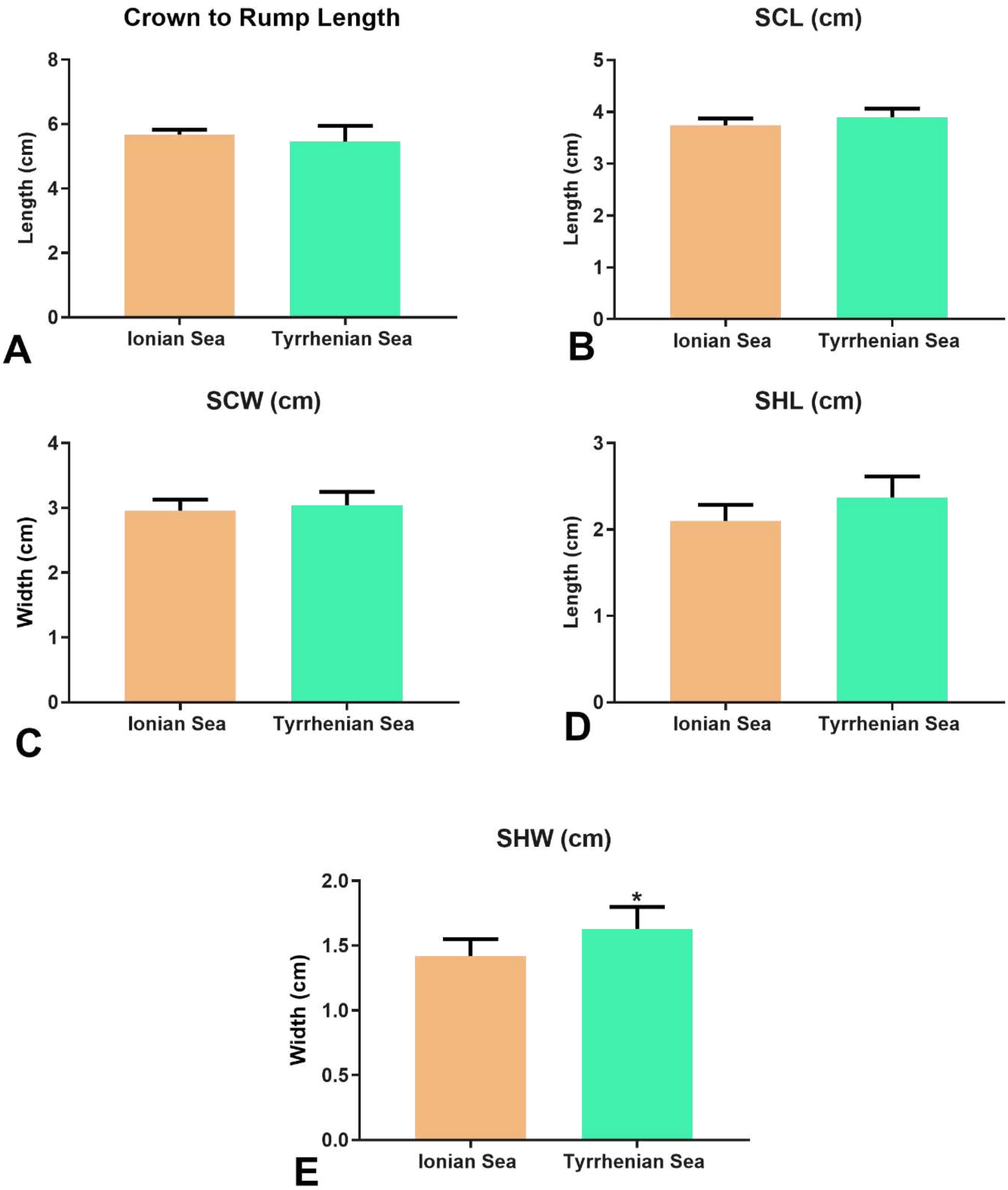


**Figure 15:** Unpaired t-test performed on Crown to Rump Length (CRL) (A), Straight Carapace Length (SCL) (B), Straight Carapace Width (SCL) (C), Straight Head Length (SHL)(D) and Straight Head Width (SHW) (E) of samples collected in the Ionian and the Tyrrhenian Sea. Values are represented as mean ± standard deviation (sd). Significance: *= $p \leq 0.05$.

Biometric parameters such as Crown to Rump Length (CRL) (Figure 14A), Straight Carapace Length (SCL) (Figure 15B) and Straight Carapace Width (SCW) (Figure 15C), Straight Head Length (SHL) (Figure 15D) and Straight Head Width (SHW) (Figure 15E) showed similar trends, with a smaller size in embryos from the Ionian Sea. Concerning the CRL, the SCL, the SCW and the SHL no significant differences were detected between the two areas. SHW showed significant difference with the embryos from the Ionian Sea significantly smaller than the Tyrrhenian ones (p value: 0,0451).

## 4.4 Histological analysis on gut

The histological analysis of gut sections performed following the Alcian blue staining highlighted the full development of intestinal fold and mucosa cells on all samples from both nests (Figure 16). The intestinal sections were also classified based on the density of mucous cells (white circle (Figure 16 A and B).

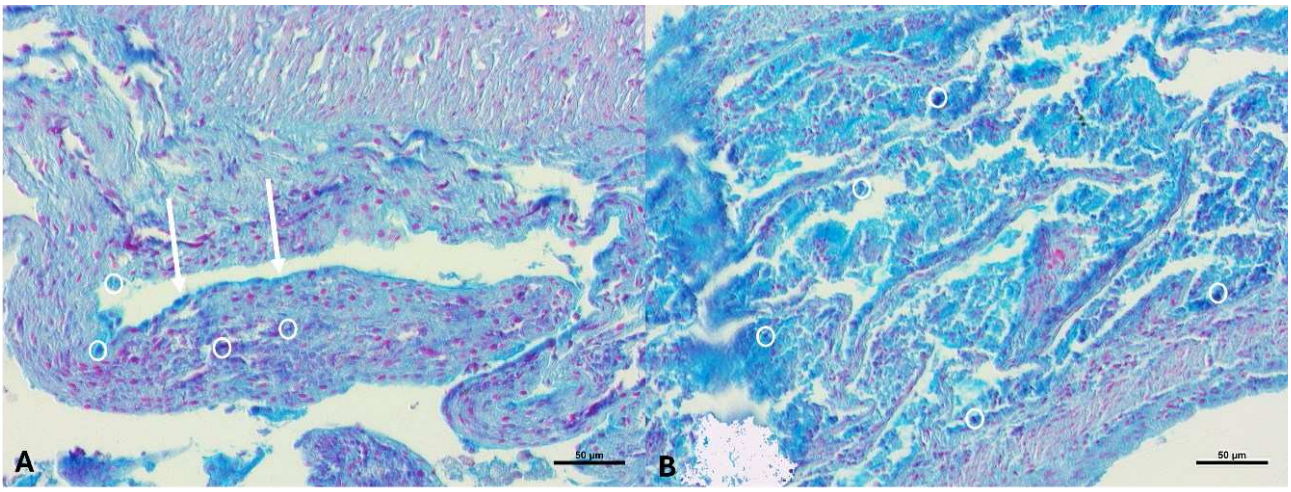


**Figure 16:** (A) Gut' histological section of embryos from the Ionian nests (A) and the Tyrrhenian one. The white circles highlight the mucous cells and the white arrows highlight the brush border. Scale bars: 50 µm.

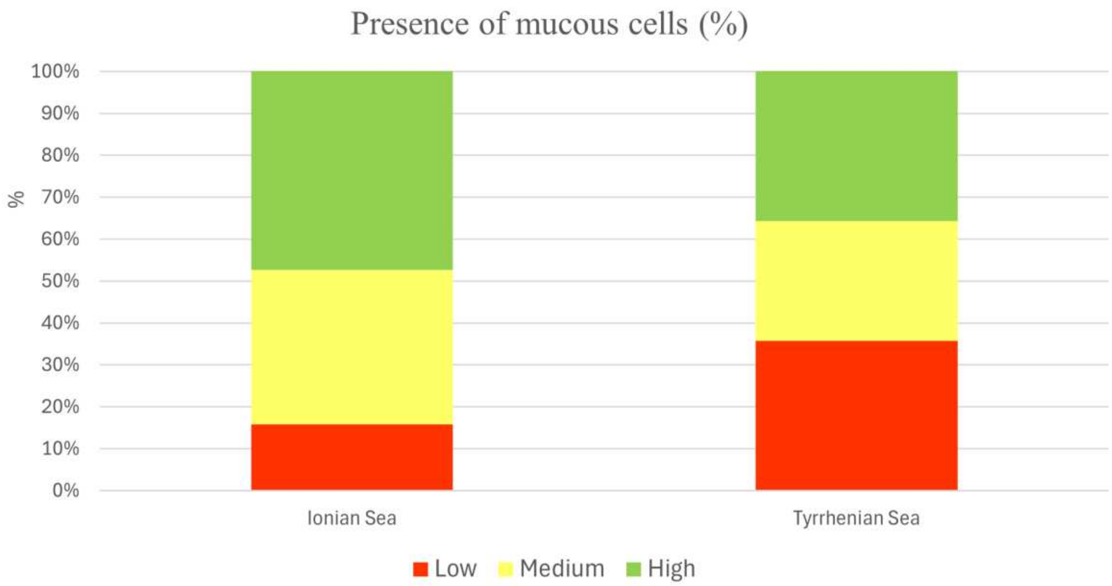


**Figure 17:** Graph representing percentage of density (low, medium and high) of mucous cells within the intestines of stage 30 embryos from their respective nests. Ionian Sea: Low 17%, Medium 35%, and High 48%. Tyrrhenian Sea: Low 36%, Medium 28%, and High 36%

The 83% of intestine from embryos collected in Ionian nests presented medium-high density of mucus cells. On the contrary, 63% of intestine from embryos collected in Tyrrhenian nests presented a medium-high density of mucus cells.

## 4.5 HPLC analysis for PAHs assessment

The study on the presence of PAHs and toxic agents within the embryos of *C. caretta* was conducted in collaboration with the University of Camerino (Camerino, Italy), where analyses were carried out to identify various chemical pollutants. The study analyzed and compared the presence of PAHs within yolk (below stage 10) and intestine (from embryos at stage 30). The main PAHs identified, particularly between yolk and intestine (Fig. 18), include naphthalene, acenaphthene, 2-bromonaphthalene, acenaphthylene, fluoranthene, and benzo(b)fluoranthene. These pollutants are commonly present in the marine environment, particularly in the Mediterranean Sea, including the Tyrrhenian and Ionian Seas, due to industrial activities, maritime traffic, and emissions from incomplete combustion of fossil fuels.

| | Ionian Sea | | Tyrrhenian Sea | |
|---|---|---|---|---|
| | YOLK (ng/g) | INTESTINE (ng/g) | YOLK (ng/g) | INTESTINE (ng/g) |
| Naphthalene | 20% (4,9) | 0% | 0% | 0% |
| Acenaphthene | 20% (12) | 0% | 0% | 0% |
| 2-Bromonaphthalene | 20% (1) | 0% | 20% (3,8) | 0% |
| Acenaphthylene | 0% | 40% (7,02) | 20% (4,1) | 20% (7) |
| Fluorene | 20% (0,8) | 0% | 0% | 0% |
| Phenanthrene | 20% (1,4) | 0% | 20% (1,2) | 0% |
| Anthracene | 0% | 0% | 0% | 0% |
| Fluoranthene | 0% | 40% (5,5) | 0% | 20% (4,3) |
| Pyrene | 0% | 0% | 0% | 0% |
| Benzo (a) anthracene | 0% | 0% | 0% | 0% |
| Chrysene | 0% | 0% | 0% | 0% |
| Benzo (b) fluoranthene | 0% | 0% | 0% | 40% (2,3) |
| Benzo (a) pyrene | 0% | 0% | 0% | 0% |
| Indeno (1, 2, 3) PYRENE | 0% | 0% | 0% | 0% |
| Dibenz (a, h) anthracene | 0% | 0% | 0% | 0% |
| Benzo (g, h, i) perylene | 0% | 0% | 0% | 0% |

**Figure 18:** PAHs concentration (ng/) in Yolk and Intestine of embryos from Ionian and Tyrrhenian nests.

The Figure. 18 show the percentage of yolk and liver presenting at least one PAHs. The maximum concentration found in a single sample is indicated in parentheses (ng/g). Concerning the Ionian Sea, in the yolks Naphthalene (4,9 ng/g), Acenaphthene (12ng/g), and 2-bromonaphthalene (1ng/g), Fluorene (0,8ng/g) and Phenanthrene (1,4ng/g) were detected and as described in the Figure, 20% of the eggs collected in that area presented these contaminants. In intestines the Acenaphthylene and the Fluoranthene were the only PAHs found in significant quantities 7,02 and 5,5 ng/g respectively. Conversely, in the embryos collected in the Tyrrhenian Sea, the 2-bromonaphthalene, the Acenaphthylene and the Phenanthrene were detected at maximum concentration of 3,8; 4,1 and 1,2 ng/g in the yolks and the Acenaphthylene, Fluoranthene and Benzo (b) fluoranthene were detected in intestines at maximum concentration 7, 4,3 and 2,3 ng/g respectively. Notably, in the Tyrrhenian area, the same PAH, Acenaphthylene, was detected both in yolk and intestine of the same embryos. Finally, it is noteworthy that benzo(b)fluoranthene was found only in the intestine of nest from Tyrrhenian Sea.

# 5. DISCUSSION

In recent years, *C. caretta* has expanded into the north-western Mediterranean along the Italian coast in search of new nesting sites (Hochscheid et al 2022). This new migratory pattern is likely related to rising water temperatures, which are pushing marine turtles further north in search of cooler and more suitable temperatures (Bentivegna et al., 2010; Witt et al., 2010; Hochscheid et al 2022). As a result, the number of nests in Italy, including in northern regions such as Tuscany, increased.

Focusing on morphological and physiological characteristics, this study compared embryos laid in the same year (2022) on the Tyrrhenian coast with the ones laid on the Ionian coast. Firstly, all the embryos analyzed in the present study were at 30 developmental stages, the final embryonic stage before hatching. In this stage the embryo completed most of its morphological development and accumulated the necessary energy reserves for post-hatching life (Miller et al., 2017). Embryonic growth is a process strictly influenced by both external environmental factors and the physical characteristics of the mother (Margaritoulis et al., 2003; Wallace et al., 2006).

The environment, such as temperature and sand quality can influence both egg size and weight (Wallace et al., 2006). Higher temperatures may lead to moisture loss within the egg, thereby reducing its weight (Wallace et al., 2006). Meanwhile, day-to-night temperature fluctuations and sand composition could influence humidity inside the egg and consequently the weight (Wallace et al., 2006). Nevertheless, the differences in the egg weight recorded in our study cannot reflect differences in temperature. Indeed, all the embryos analyzed in this study were laid in 2022, and the average summer temperature in the two areas were nearly the same. Meanwhile, as suggested by Margaritoulis and colleagues (2003), maternal characteristics could affect egg weight and size. Indeed, nutrition and maternal physical condition are dependent on the habitat and are crucial for the weight and size of the laid eggs. Turtles nesting in food rich environments tend to have larger and heavier eggs (Margaritoulis et al., 2003). Comparing the physiological status of embryos, the histological analysis performed in this study revealed an abundant presence of mucous cells within them. The presence of mucous cells and chylomicrons in the intestines of *C. caretta* hatchlings may be linked to preparation for high cholesterol consumption, associated with their first feeding on diet rich in crustaceans (Kakizoe, 2004; Ernst & Lovich, 2009; Tlachi et al., 2014). Unfortunately, the small intestines of turtles are a unique organ, primarily due to the pseudostratified epithelium and numerous lymphatic vessels (Kakizoe et al. 2004; Ernst & Lovich, 2009; Tlachi et al. 2014). In our study, we highlighted a difference in mucous cells' presence between the two sampling areas. Indeed, the Ionian Sea presented a higher presence of this cellular type in the embryonic intestines suggesting an increasing development of mucous cells as a preparatory and defensive mechanism for the development of bacterial flora (Kakizoe et al. 2004; Ernst & Lovich, 2009; Tlachi et al. 2014). This alteration could reflect an interaction with compounds that can alter the physiological processes and the health status of embryos. Indee, the absorption of chemical compounds such as PAHs can alter reproduction, growth, and behavior in the species with which they interact (Arienzo et al. 2023). Being lipophilic, these pollutants tend to accumulate in the adipose tissues of organisms. Toxic effects include damage to the central nervous system, endocrine disruption, genetic damage, and the risk of carcinogenicity (Arienzo et al. 2023). The toxicological analyses performed in this study identified the presence of several PAHs in yolks and intestines. The main compounds detected were naphthalene, acenaphthene, 2-bromonaphthalene, acenaphthylene, fluoranthene, and benzo(b)fluoranthene. In the marine environment, PAHs are widespread pollutants; however, their concentrations tend to be higher in shallow and coastal waters, particularly in areas heavily influenced by anthropogenic inputs, such as river mouths, harbors, wastewater discharge points, and urban or industrial runoff (Baumard et al, 1998; Latimer and Zheng, 2003). These compounds are not bioaccumulated in vertebrates, but a chronic exposure could produce serious toxic effects (López-Berenguer et al., 2023). Notably, just few contaminants were found in the intestines of embryos at higher stages (30), likely due to an hepatic and kidneys absorption and detoxification process (Arienzo et al., 2023; Dias et al., 2024). Among the PAHs detected, the acenaphthylene and fluoranthene were present in both areas. Finally, benzo(b)fluoranthene was found only in samples from the Tyrrhenian coast. It is a compound that causes damage to the nervous and immune systems, impairing growth and development (Shi et al., 2023). The findings of the same compound in both yolk and intestine of embryos not only confirm a maternal transfer as previously seen for other chemical compounds (Muñoz-Baquero et al., 2023) but also their yolk-intestine transfer.

In conclusion, this study highlights significant findings on the development of *C. caretta* embryos along the Italian coasts. Overall, this preliminary study underscored the impact of environmental and chemical stressors on marine turtle embryos, highlighting the need for further monitoring and conservation efforts.

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