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Qualità del tuorlo e contaminanti ambientali: gli effetti sullo sviluppo embrionale di *Caretta caretta*

Yolk quality and environmental contaminants: effects on the embryonic development of *Caretta caretta*

Tesi di Laurea Magistrale

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RIASSUNTO

La tartaruga marina comune *Caretta caretta* è una delle sette specie di tartarughe marine presenti negli oceani. Lungo il suo ciclo vitale, *C. caretta* affronta numerose minacce, tra cui fattori ambientali e pressioni antropiche, che possono influenzare il successo riproduttivo già nelle fasi embrionali. Infatti, già durante il suo sviluppo embrionale questa specie è particolarmente sensibile a variazioni ambientali e a diverse attività antropiche.

Inoltre, lo sviluppo embrionale è strettamente legato alla qualità del tuorlo che rappresenta anche l'unico nutrimento durante tutto il processo.

Il presente studio ha avuto l'obiettivo di analizzare la composizione proteica del tuorlo di embrioni non schiusi di *C. caretta* provenienti da nidi deposti lungo la costa tarantina (Puglia, Italia) tra il 2015 e il 2022. Attraverso l'analisi elettroforetica SDS-PAGE, è stata riscontrata una predominanza di proteine con peso molecolare compreso tra 100 e 30 kDa, mentre le frazioni a peso molecolare superiore (>100 kDa) e inferiore (<30 kDa) risultavano meno rappresentate. Successivamente, mediante Western blot, è stata valutata l'espressione delle catepsine D, B ed L, enzimi coinvolti nel processo di digestione del tuorlo da parte dell'embrione. I risultati hanno evidenziato una maggiore espressione della catepsina D, mentre le isoforme B ed L risultavano

presenti in quantità minori, suggerendo che il processo di assimilazione del tuorlo da parte dell'embrione non fosse ancora stato attivato.

Inoltre, questo studio ha indagato la presenza di inquinanti organici, in particolare gli idrocarburi policiclici aromatici (PAHs), noti per i loro potenziali effetti tossici sullo sviluppo embrionale. L'analisi dei campioni di tuorlo ha rivelato la presenza di diversi composti appartenenti a questa classe di inquinanti, suggerendo un possibile trasferimento materno durante la vitellogenesi.

ABSTRACT

The loggerhead sea turtle (*Caretta caretta*) is one of the seven species of sea turtles found in the oceans. Throughout its life cycle, *C. caretta* faces numerous threats, including environmental factors and anthropogenic pressures, which can affect reproductive success even at the embryonic stage. In fact, during its embryonic development, this species is particularly sensitive to environmental variations and various human activities. Furthermore, embryonic development is closely linked to the quality of the yolk, which represents the only nourishment throughout the process. In light of this, this study aimed to analyze the protein composition of the yolk in unhatched *C. caretta* embryos from nests laid along the Taranto coast (Puglia, Italy) between 2015 and 2022. Through SDS-PAGE electrophoretic analysis, a predominant presence of proteins with a molecular weight between 100 and 30 kDa was observed, while fractions with higher (>100 kDa) and lower (<30 kDa) molecular weights were less represented. Subsequently, Western blot analysis was used to assess the expression of cathepsins D, B, and L, enzymes involved in the digestion of yolk by the embryo. The results revealed a higher expression of cathepsin D, while the B and L isoforms were present in smaller amounts, suggesting that the process of yolk assimilation by the embryo had not yet been activated. Additionally, this study investigated the presence of organic pollutants,

specifically polycyclic aromatic hydrocarbons (PAHs), known for their potential toxic effects on embryonic development. The analysis of yolk samples revealed the presence of several compounds belonging to this class of pollutants, suggesting a possible maternal transfer during vitellogenesis.

1. INTRODUCTION

1.1 Biology of sea turtles

Sea turtles are an important component of the marine ecosystems. Up to now, only seven species of marine turtles are still distributed in our oceans, mainly in tropical and subtropical ones (Witt et al., 2010).

Gaffney and Meylan (1988) offer the following cladogram for marine turtles with well-developed flippers (Figure 1).

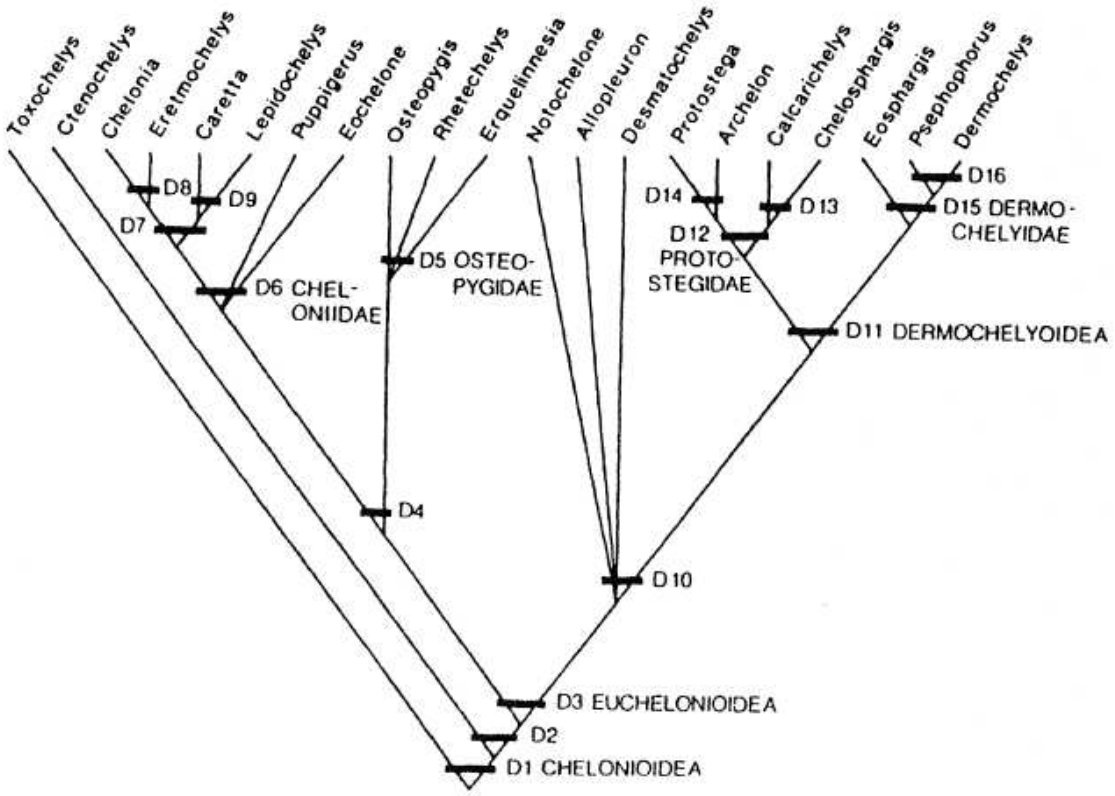

Figure 1: Cladogram "D", Chelonidea (Lutz & Musick, 1996).

Particularly the family of *Chelonidae*, which comprehend the *C. caretta* species, is a family of marine turtles characterized by an extensively roofed skull with well- developed rhamphothecae, secondary palate, incompletely retractile or nonretractile head, extremities in the form of nonretractile flippers covered with numerous small scales, the forelimbs have high elongate digits and are bound together by connective tissue, the claws are reduced to one or two on each limb, and the radius and ulna are immobilized against independent movement by juxtaposed rugose surfaces, shell covered with horny scutes variable in number (Lutz & Musick, 1996).

The largest known rookeries are in the southeast area of United States of America and Republic of Cape Verde, with nesting also occurring along the Brazilian coast within the South Atlantic basin. In the Mediterranean Sea, nesting was almost exclusively restricted to the Eastern basin, with notable aggregations occurring in Cyprus, Greece and Turkey (Witt et al., 2010).

1.2 The loggerhead life cycle

The seven species of sea turtles share a common life cycle with only minor variation (Lutz & Musick, 1996). In particular, Loggerheads spent their first years of life in an oceanic stage, during which they feed on planktonic invertebrates, such as gelatinous animals (Hatase et al., 2007). When they reach sexual maturity, loggerheads shift from an oceanic stage to a neritic stage,

getting closer to the natal beaches to approach the reproductive period (Hatase et al., 2007). Indeed, as other sea turtles species, *C. caretta* demonstrates natal philopatry, returning as an adult to their natal beach regions to breed (Witt et al., 2010). In this coastal stage, loggerhead turtles feed on benthic invertebrates, including crustaceans, mollusks and fishes (Witt et al., 2010). At the end of the breeding season females and males return to their oceanic areas to store energy for the next reproductive period (Hatase et al., 2007). Total length of the routes traveled by the Loggerhead turtle varies between 2554 and 7098 km, and the average travel rate is 1.2 km h–1 (Bentivegna, 2002) (Figure 2).

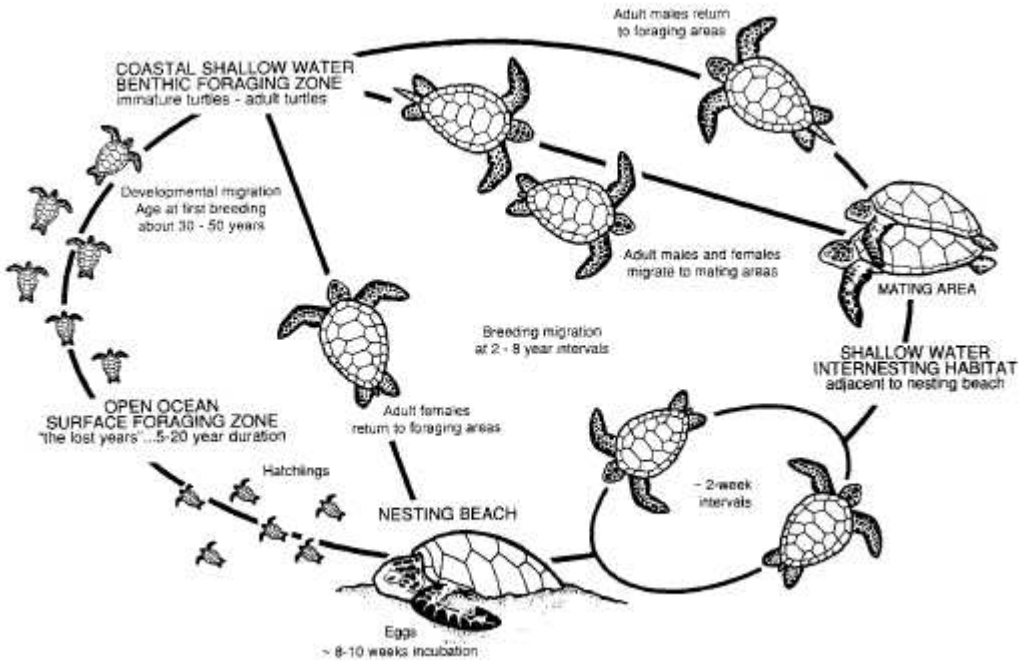

Figure 2: Generalized life cycle of sea turtles (Redraw from Lanyon et al., 1989).

Migration between breeding and foraging sites of adult animals often arises as a response to environmental heterogeneity, such as seasonal cycles in resource availability (Alerstam et al., 2003). In some populations, forage resources are so widely distributed that individuals may only aggregate for a limited period to breed before again dispersing (Miller, 1997).

Marine turtles do not begin to breed at a uniform or minimum size. Based on repeated gonads observations and growth rates of free-ranging turtles, the passage through puberty may require more than 10 years with turtle specimens at different sizes (Lutz & Musick, 1996).

1.3 Reproductive behavior

1.3.1 Periodicity

In general, female sea turtles do not reproduce every year, while males may breed every year or every two years (Lutz & Musick, 1996). The duration of the period between reproductive seasons is defined as the remigration interval with a range of 2-3 years (Henrhart et al., 2014) indeed the internesting intervals are between 10 and 14 days (Hays et al., 2002). During foraging period sea turtles accumulate the energy reserves required to support vitellogenesis over a variable period of several years, depending on the availability, quality and

quantity of food (Witt et al., 2010). When a set of exogenous and endogenous factors interact, the consequence is reproduction (Witt et al., 2010).

1.3.2 Courtship and mating

Courtship and mating are not gentle processes. Courtship is initiated by a male entering the visual range of a female, advancing quickly and biting at the carapace (Schofield et al., 2006). Males may spend about 30 days searching for a mate (Limpus, 1993), traveling considerable distances (Simmons et al., 1997). Although sea turtles have low visual acuity, males are thought to select a mate mainly using a visual cue based on size (Okuyama et al., 2014). The male attempts to get behind the female and mount her; however females can reject mounting attempts by males by raising their body vertically, resisting them in a face-to-face manner, or getting behind the males and biting at their tails to drive them away (Ye et al., 2020): maintaining head–head position prevent the male from circling, often trying to obtain a higher horizontal position in the water than the male (Schofield et al., 2010). During copulation a male mounts a female for at least 40 minutes. When a male mounted successfully, the female held its hind flippers together to refuse copulation (Schofield et al., 2010). In this circumstance, some suitors gave up temporarily, and some males attempted to conquer the breeding females by biting their necks and using their powerful tails to pry up their hind flippers (Ye et al., 2020). The

male hooks its fore-flipper claws over the anterior rim of the female carapace and was observed repeatedly biting at the necks of females (Schofield et al., 2010). Throughout the subsequent nesting season, the progressive healing of these injuries indicates that mating occurred during the pre-nesting season (Lutz & Musick, 1996). Males are not excluded from receiving damage while several males courting females. During mating, as a male sea turtle positions himself to mount the female or is already mounted, rival males often bite the trailing edges of his flippers and the dorsal part of his tail (Schofield et al., 2010). This biting may or may not unseat the mounted male at the time, but certainly, reduces his fitness to attempt mating with another female by causing severe damage to the flippers and the tail (Schofield et al., 2010). The male curls his long tail to bring their cloacae into contact, then his penis is erected into her cloaca (Lutz & Musick, 1996). The shape of the penis, with bifurcation of the sperm duct at the tip, allows for the transfer of semen into each oviduct without passing through the environment of the female's cloaca (Lutz & Musick, 1996). Females are in reproductive readiness for about 7 or 10 days, males appear to be sexually active for about one month (Schofield et al., 2010). Genetic studies have demonstrated that males mate with several females and females mate with several males (Lutz & Musick, 1996). At the end of the mating period, the males presumably return to their foraging areas while,

females disperse from the mating areas to nesting sites in the region to which they show fidelity (Lutz & Musick, 1996). Mating occurs in one or two months just before the ovipositional period of the season. Females can store sperm for several years in a small region of the oviduct's posterior portion between the infundibulum and the ovary (Gist & Jones, 1989). Sperm was identified in tubules of female turtles isolated from males for as long as 423 days (Gist & Jones, 1989).

1.3.3 Nesting process

The general nesting process (beaching, digging, laying, filling-in, returning) is essentially the same in all species of sea turtles, although some differences could occur (Lutz & Musick, 1996). Typically, loggerheads nest on sandy wide, open beaches backed by low dunes and fronted by a flat sandy approach from the sea (Miller et al., 2003). Once a female has selected a beach to lay her eggs, she must choose the right location on the beach to dig the nest (Miller et al., 2003). There are serious outcomes from this choice. For instance, the eggs must be placed far enough from the tidal zone to avoid erosion by high tides, which may be lethal to embryonic development (Whitmore & Dutton, 1985). At the same time, the eggs must not be placed so far from the ocean to avoid a greater risk to land predators for the emerging hatchlings (Blamires & Guinea, 1998). In the first steps of oviposition, the turtle is particularly sensitive to external

disturbance, indeed it may scrutinize its surroundings to choose the right place where to lay its clutch (Pritchard & Trebbau, 1984). Lighting, movement, and/or obstacles may cause a change in direction or may even cause the turtle to abandon the nesting effort (Miller et al., 2003). Loggerhead turtles prepare the nesting site before digging the egg chamber by clearing away surface debris, using either simultaneous or alternating sweeps of the front flippers (Miller et al., 2003). Loose, dry substrate immediately under or behind the turtle may be cleared by its hind flippers (Hailman & Elowson, 1992). A body pit is created by movements of the plastron (Miller et al., 2003). The hind flippers are used to excavate the egg chamber (Schulz, 1975). The shape of the egg chamber has been described as "flask shaped" (Schulz, 1975) with a narrower neck and a wider bottom (diameter of neck ≅ 10–15 cm; chamber diameter ≅ 23–26 cm; depth to top of eggs ≅ 30 cm; depth to bottom of chamber ≅ 50 cm; (Miller et al., 2003). The sand is placed by the digging flipper to the side of the chamber. During oviposition both hind flippers are extended outwards on the sand (Miller et al., 2003). When the egg chamber is ready the eggs are laid singly or in small groups (two or three, sometimes four). During egg-laying loggerhead turtles are relatively tolerant of external stimuli (Miller et al., 2003). At the end of the oviposition, the nest chamber is filled by scraping the sand previously removed into the hole with the hind flippers (Hailman & Elowson, 1992). When

the neck of the chamber has been filled, the sand is compacted by alternating use of the hind limbs (Miller et al., 2003). This behavior was once interpreted as to camouflaging the nest, presumably from predators. The problem is that the predator can readily locate a nest within a few days of oviposition (Lutz & Musick, 1996). The primary function of this behavior is to reestablish the beach environment around the nest because the sand thrown on top of the nest provides insulation in terms of both temperature and moisture for the eggs (Lutz & Musick, 1996). All marine turtles lay white, spherical cleidoic eggs with flexible calcareous shells. The size of the eggs varies between species, *C. caretta* lays eggs of ≅ 4,0 cm, ≅ 36 g (Lutz & Musick, 1996). The number and size of eggs, and the number of clutches laid represent the result of an adaptive compromise for survival. For example, a female of *C. caretta* release an average of ≅ 100 eggs (Margaritoulis et al., 2003).

1.4 Embryonic development

The embryonic development is divided in 31 stages, but the first six stages have never been described because they occur in the mother's oviduct (Miller et al., 2017): the first stages analyzed are the seventh and eighth, where the blastopore is shaped as an inverted "U", head is fold shaped as a posteriorly opening crescent, but the somites appear only in the ninth. In the tenth stage, there are more than six somites and neural crests touch or fuse along the midline of the

head. During the next stages, the maxillary process is prominent, as the development of the facial components and limbs. Only from the 25th stage a complete embryo with a carapace is visible, with complete eyes and the interdigitation of the limbs (Miller et al., 2017).

Throughout this process, the egg exchanges heat, water, oxygen and carbon dioxide with other eggs in the clutch and with the beach surrounding the clutch (Miller et al., 2017). The extraembryonic parts of the egg act as an agent buffering or even regulating the embryonic environment (Miller et al., 2017). Yolk and albumen represent the two main matrices of freshly laid eggs (Wallace et al., 2006). Both matrices have a maternal origin and are produced during different processes, at different locations in the body, and over different timeframes. The synthesis of yolk from egg yolk precursor proteins (vitellogenin) originates from the liver of the mother before moving to the ovaries in a process (vitellogenesis) which can take months, potentially even years in sea turtles (Munoz & Vermeiren, 2020). During the development the temperature is a critical point because it must be as constant as possible (Reid et al., 2009). Indeed, the effects of nest temperature on total energy use are complex, higher temperatures may advance development and shorten the developmental time stimulate metabolic rate and increasing energy use (Reid et al., 2009). The thermal tolerance range (TTR) for development of sea turtles

embryos incubated at constant temperature appears to fall between about 25 to 27 degrees and 33 to 35 degrees, and is around 10 degrees wide (Lutz & Musick, 1996). In embryos of loggerhead sea turtles a 1°C rise in constant incubation temperature approximates to a reduction in incubation duration of about 8.5 day in natural nests (Mrosovsky, 1980). Meanwhile, the temperature is also an important feature to sexual determination. Indeed, as reptiles, sea turtles have a temperature-dependent sex determination (TSD) producing female hatchling at warm temperatures and males at cooler ones (Tomillo & Spotila, 2020). The pivotal temperature, corresponding to threshold temperature in which the 50% of hatchling are females and the 50% are males is around 29 to 30 degrees for loggerheads (Tomillo & Spotila, 2020).

1.5 Maternal yolk precursors synthesis during folliculogenesis

To support metabolic and reproductive costs, breeding females are strongly reliant on lipids that provides energy and maintain metabolic processes and reproduction, particularly during vitellogenesis (Whittier & Mason 1996). Lipids are stored in subcutaneous layers or as visceral fat that can be then mobilized toward liver and ovaries (Whittier & Mason 1996). She must allocate the energetic cost of nesting over her total number of clutches in a season, as well as allowing a residual for return migration (Hamann et al. 2002).

Based on previous examinations of ovarian follicular development in the Kemps Ridley turtle (Rostal et al., 1998), the lipid uptake by ovarian follicles will occur prior to the beginning of the nesting season, and the yolk lipid concentrations will be similar throughout the nesting season (Hamann et al., 2002). Atresia of large pre-ovulatory follicles is common in all vertebrate species (Guraya, 1989) and energy derived from the resorbed yolk material may aid survival during unfavorable conditions (Bradshaw, 1993). The increased triglyceride concentrations in vitellogenic females reflect behavioral and physiological adaptations related to resource accruement, lipid deposition, lipid mobilization and follicular development (Hamann et al., 2002).

Plasma triglyceride values in vitellogenin sea turtles appeared to increase over a similar seasonal time frame to plasma steroids in females with a similar reproductive state (Hamann et al., 2002). Corticosterone and triglyceride complete yolk deposition and follicular development prior to the breeding season and produce yolks with similar lipid content across clutches (Hamann et al., 2002). For instance, yolk is produced from yolk precursor proteins (vitellogenin) which are produced in the liver (Munoz & Vermeiren, 2020). Yolk is an important reservoir of proteins, carbohydrates and lipids to support the energetic demands during embryogenesis (Noble, 1991). Plasma triglyceride values only indicate mobilized lipid, reflecting temporal variation

within a nesting attempt, rather than the cumulative result of several unsuccessful attempts (Hamann et al., 2002). Values of triglyceride concentrations are similar throughout most nesting stages but significantly higher during pre-ovipositional nest digging (Hamann et al., 2002). Kuchling and Bradshaw (1993) suggested that, in the freshwater turtle *Pseudemydura umbrina*, atresia of mature sized follicles is an important mechanism for sparing lipid during periods of unfavorable conditions. Hammann et al. (2002) found that the total lipid concentration of adipose tissue from atretic *C. mydas* females was both lower than the total lipid concentration of adipose tissue from non-atretic females, and comparable to concentrations reported by Kwan (1994) in post-breeding females. Overall, it appears that atresia of mature follicles could be an important residual store of lipid that may be utilized to supplement unforeseen depletions of other lipid stores, such as adipose tissue (Hamann et al., 2002). Because follicular development in sea turtles is completed prior to the nesting season (Rostal et al., 1998), it is likely that the end of the nesting season is marked by ovulation of the last mature follicles. However, other physiological factors may underline the cessation of the breeding season (Miller, 2017). For example, plasma triglyceride concentrations in female *C. mydas* decreased towards the end of the nesting season, and plasma protein concentrations increased. This implies that a metabolic shift towards protein

catabolism may be involved in regulating the breeding season length (Hammann et al., 2002). Indeed, corticosterone levels tended to decline at the end of the nesting season in sea turtles (Hamann et al., 2002).

1.6 Sexual maturity acquisition and oogenesis of sea turtles

Marine turtles do not begin to breed at a uniform or minimum size. Based on repeated gonads observations and growth rates of free-ranging turtles, the passage through puberty may require more than 10 years with turtle specimens at different sizes (Lutz & Musick, 1996).

The ovaries are paired organs attached by mesovarium to the dorsolateral wall of the abdominal cavity. The ovarian cortex is covered by a simple squamous epithelium and it is composed of germinal bed and follicles in different developmental stages and atresia (Perez-Bermudez et al., 2012).

The sexual maturation begins when the hypothalamus pituitary gonad axis is activated and it turns to induce gonadal steroidogenesis and folliculogenesis (Perez-Bermudez et al., 2012). Folliculogenesis consists in 8 stages:

Stage I: folliculogenesis. In this stage primary oocytes with fibrillar chromatin in different stages of prophase I of meiosis are observed adjacent to the oogonia. The oocytes begin the morphophysiological interaction with follicular cells inside the germinal beds (Perez-Bermudez et al., 2012).

Stage II: beginning of previtellogenesis. The oocytes still inside the germinal bed are more heterometric than in the previous stage, and they increase up to 10 times the diameter reached in the previous stage. The nucleus, with morphology and position identical to that in Stage I, grows in proportion to the oocyte and presents several peripheral and heterometric nucleoli. The lampbrush chromosomes become evident (Perez-Bermudez et al., 2012).

Stage III: previtellogenesis. The oocyte already outside of the germinal bed and as part of a follicle with external and heterometric thecal layers. It increases up to five times the diameter reached in the previous stage. The nucleus is spherical, eccentric, and granulated with lampbrush chromosomes that are less conspicuous than those in Stage II (Perez-Bermudez et al., 2012).

Stage IV: end of previtellogenesis. The oocyte increases up to four times the diameter reached in the previous stage and macroscopically it maintains the white-yellow coloration. The nucleus also maintains the morphology and position observed in Stage III but without lampbrush chromosomes (Perez-Bermudez et al., 2012).

Stage V: beginning of vitellogenesis. At the beginning of vitellogenesis the oocyte increases 1.5 the diameter. Macroscopically these follicles are intensely yellow and this is maintained in later stages. The nucleus presents the same morphology as that found in Stage IV and it is displaced to the animal pole.

Neutral lipid droplets are observed mixed with glycoprotein in yolk platelets, but spatially and chemically segregated. This distribution is maintained throughout vitellogenesis (Perez-Bermudez et al., 2012).

Stage VI: vitellogenesis. The oocyte doubles the diameter reached in the previous stage. The nucleus still has a similar position and morphology to that found in stage V. the ooplasm is completely occupied by spherical yolk platelets that increase its density. The quantity of granulated and pigmented glycoprotein platelets increase with respect to the previous stage (Perez-Bermudez et al., 2012).

Stage VII: vitellogenesis. The oocyte doubles the diameter reached in the previous stage. The spherical yolk platelets and lipid droplets increase their diameter drastically. These large platelets and lipid droplets are observed at the limit of the zona pellucida and the cortical ooplasm (Perez-Bermudez et al., 2012).

Stage VIII: end of vitellogenesis. The oocyte almost doubles in diameter as compared to the previous stage and it acquires the preovulatory condition (Perez-Bermudez et al., 2012). At its maximum of development, the oocyte reaches a diameter of 19-20 mm and the germinal vesicle is found flattened at the surface of the ooplasm and is ready for ovulation (Callebaut et al., 1997).

Within the body of the female, a short period after the oocyte has been ovulated from ovary, it enters the infundibulum of the oviduct; the oocyte then passes through the aglandular region into the magnum, where it is fertilized by sperm (Lutz & Musick, 1996). The oviduct is a long tube (>4 m) containing a lining of specialized secretory cells that produce the albumen, shell membrane, and shell (Miller et al., 2003). The female turtle arrives at her nesting area with more than enough mature follicles present in her ovaries (Miller et al., 2003). The follicles result from the metabolic processes of digestion of food, and the shell is constructed from calcium carbonate in the form of aragonite (orthorhombic CaCO3) (Miller et al., 2003). Because the sperm from all successful males are stored and mixed in the upper portion of the oviduct, the follicles in a clutch are fertilized by sperm from several different males (Lutz & Musick, 1996). The first external sign that development is progressing is the appearance of a white spot on the uppermost part of the egg; the embryo is situated just beneath the shell (Miller et al., 2003). As the white spot becomes progressively bigger to eventually encompass the entire shell, the embryonic membranes (vitelline, amnion, allantois, chorion) develop (Lutz & Musick, 1996).

1.7 Survival status of Loggerhead

Marine turtles are so important for coastal economies and native communities, because they attract a lot of tourists due to their commercial value, especially in countries where there's an illegal commerce of eggs and carcasses, as well as extreme vulnerability to mankind at least while nesting. The largest known rookeries are in the southeast area of United States of America and Republic of Cape Verde, with nesting also occurring along the Brazilian coast within the South Atlantic basin. In the Mediterranean Sea, nesting was almost exclusively restricted to the Eastern basin, with notable aggregations occurring in Cyprus, Greece and Turkey (Witt et al., 2010). Moreover, Global warming is affecting habitat quality and availability on our planet and some species are predicted or are now changing their distribution range. Among them, loggerhead turtles have already started to expand their nesting range into Western Mediterranean (Hochscheid et al., 2022). In the South of Italy, there has been a great expansion, especially in some regions, like Sicily and Calabria (Hochscheid et al., 2022). In particular, the global population of the loggerhead turtle (*C. caretta*) comprises 10 subpopulations that vary widely in population size, geographic range and population trends, and are the appropriate units for assessment of global conservation status for this species. To this, at global scale, *C. caretta* has been included in the IUCN Red List as a Vulnerable

species (Casale et al., 2015), while the Mediterranean population is population is listed as as “Least Concern” due to conservation efforts in recent years (Caminas et al., 2020). Further conservation measures are needed to maintain this status (Caminas et al., 2020).

1.8 Threats

1.8.1 Organic Pollutants

Persistent Organic Pollutants (POPs), including polychlorinated biphenyls (PCBs) and organochlorine pesticides (OCPs), have been present in the marine environment for the last 70 years. Some organophosphorous pesticides, polybrominated diphenyl ethers (PBDEs), polycyclic aromatic hydrocarbons (PAHs) toxaphenes and perfluorinated alkyl substances (PFAS) are now being recognized as POPs. As a result, a cocktail of persistent pollutants is insidiously and ubiquitously distributed throughout the marine environment. Sea turtles present a case study of threatened marine migratory species that can bioaccumulate chemicals over long periods across locations that might be hard to biomonitoring (Filippos et al., 2021). For example, migratory movements of sea turtles can span whole ocean basins and vary from short, seasonal movements to long migrations (Witherington, 2017). This lipid content makes yolk a highly suitable environment for lipophilic contaminants such as POPs. A further challenge, common to many wildlife species, is that concentrations

of many pollutants are relatively low in the environment, and that measurements of individual pollutant compounds within wildlife species might be below analytical detection limits (Kon Kam King et al., 2014). The relation to POP concentrations in maternal blood differed to the one found in yolk and albumen. The trophic transfer POPs can potentially be maternally transferred to offspring. Maternal transfer of accumulated POPs to offspring is of particular conservation importance because it confronts developing embryos with pollution legacy, even before they come into contact with the external environment (Kon Kam King et al., 2014). As Munoz & Vermeiren suggest in their article, egg and blood concentrations of PCBs, PBDEs and specific congeners from OCPs are positively correlated between maternal blood and eggs, between eggs and hatchling blood and between maternal blood and hatchling blood. Similar positive correlations were found for chlordane, DDT and DDE (Munoz & Vermeiren, 2023).

To understand the reasons for the observed selective toxicokinetic of individual compounds, we need to consider the way egg yolk is produced. Since yolk precursors (vitellogenin and fatty acids) are synthesized from liver, any POPs stored in liver could be available during vitellogenesis and mobilized with vitellogenin (Munoz & Vermeiren, 2023). Several processes in the vitellogenesis pathway could underlie the selective toxicokinetic of individual

compounds the bioaccumulation of POP and lipid mobilization are closely linked to each other. Consequently, the equilibrium between lipid formation and digestion will change the absorption/desorption dynamic of POPs (Kon Kam King et al., 2014). The transport of POPs throughout an organism is influenced by factors such as the anatomical location of the target tissue, the tissue volume and the blood flow. Recent insights suggest that the speed of diffusion during the initial transfer between tissues and the bloodstream can be compound-specific (Munoz & Vermeiren, 2023).

1.8.2 Microplastics

The loggerhead sea turtle is particularly inclined to accidentally ingest plastic and microplastic due to its long-life cycle features (Caron et al., 2016). Two factors are likely to increase the risk of plastic debris ingestion by sea turtles compared to other marine species: their visual feeding strategy, which confuses soft floating plastics and analogous structures for jelly-fish (especially during the juvenile pelagic phase), and backward-facing esophageal papillae which inhibit regurgitation and facilitate particle accumulation in the gut (Caron et al., 2016). Damage to the digestive system, alteration of swimming behavior, buoyancy difficulty and at worst animal death have been reported in sea turtles that ingested plastic debris (Senko et al., 2020). Following Matiddi and colleagues' (2021) definition, the term "micro-plastics" (MPS) identifies all the

small solid plastic particles less than 5 mm in two of the three dimensions or diameter. They can be either of primary or secondary origin. Primary MPs are produced in their micro-size to find applications in several industrial products (Cincinelli et al., 2019). On the other hand, secondary MPs derive from the fragmentation of greater plastic debris which is subjected to the synergistic effects of chemical, physical, biological, photic and thermal degradation (Cincinelli et al., 2019). In fact, the possible transfer of microplastics from female to the eggs has been demonstrated. The study made by Chemello et al. (2023) investigated the presence of microplastics in yolk and liver samples evaluating also the possible effect at the hepatic level. This study underlined that the number of melanomacrophages in the embryonic hepatic tissue correlated with the abundance of MP's in the same tissue assessing a potential impact on the embryonic health status. Indeed, all the embryos analyzed were found to be contaminated by MPs, a total number of 21 MPs, with dimensions lower than 5 micrometers that can be included in two shape categories: sphers and fragments. Seven different polymers were identified, the most frequent polymers were polyethylene (PE), polyvinyl chloride (PVC) and acrylonitrile butadiene styrene (ABS) (Chemello et al., 2023). The majority of the MPs identified were found in the yolk of sea turtle embryos. The MPs shape and the distribution of polyhmers and colors support the maternal transfer theory.

Concerning the route that led MPs into egg, the vitellogenesis process should be considered (Chemello et al., 2023). As hypothesized in a previous study, a possible pathway for mobilization of small-size MPs within the organism is the circulatory system through which MPs are relocated in different target tissues such as liver (Chemello et al., 2023). Once in the liver, MPs could move to the egg yolk during vitellogenesis binding yolk precursors (Chemello et al., 2023). The presence of the same polymer in the yolk and liver of the embryos analyzed, suggested that MPs could be internalized by the embryo during the yolk absorption. One possibility suggested by the authors is the affinity of MPs to the lipidic component of the yolk which is almost completely absorbed after hatching (Chemello et al., 2023). Considering the significant release of these contaminants from plastic, it has been hypothesized that maternal transfer could be the more plausible route of exposure that explains the presence of MPs in both yolk and embryos' liver. Indeed, Cheloniidae embryos are surrounded by an eggshell made of crystalline calcium carbonate in form of aragonite characterized by a selective permeability to water and gases (Phillott & Permenter, 2006). This solid barrier, after a calcification process within the oviduct, prevents external material from entering the egg. MPs buoyancy and sink could be altered by biofouling plastic degradation and leaching of additives (Cincinelli et al., 2019). Therefore, several types of MPs such as the

dense plastics PVC and PET are commonly found at the same depth of floating lower-density polymers (such as PE and PP also herein detected) after experiencing degradation in smaller particles (Horton and Dixon, 2018). Other than density-based distribution, also MPs shape influences their occurrence in the aquatic environment highlighting the complexity of predicting MPs fate and transport (Horton and Dixon, 2018). To this, in the Mediterranean Sea, the loggerhead sea turtle (*C. caretta*) is considered as a bio-indicator of plastic pollution and it's the official descriptor for marine litter of the European Marine Strategy (Matiddi et al., 2017).

2. **AIM**

Loggerhead sea turtles face numerous threats throughout their life cycle, including habitat degradation, climate change, and pollution that could menace the survival of this species (Camiñas et al., 2020). Among these, embryonic development represents a particularly vulnerable phase, as it is influenced by both endogenous and exogenous factors (Barraza et al, 2021). Endogenous factors, such as yolk composition and nutrient availability, play a crucial role in supporting proper development, while exogenous factors, including environmental variations and the presence of contaminants, can significantly impact embryonic viability and hatchling success (Craven, 2001; Alava et al., 2006; Muñoz et al., 2023). In light of this, this study aimed to characterize the yolk protein profile in unhatched *C. caretta* embryos from nests laid along the Taranto coast (Puglia, Italy) between 2015 and 2022, using SDS-PAGE and to assess through western blot the expression of key proteolytic enzymes, such as cathepsins D, B, and L, which play a crucial role in yolk absorption and embryonic nutrition. Moreover, through High Performance Liquid Chromatography HPLC were detected and evaluated the presence of polycyclic aromatic hydrocarbons (PAHs) in yolk samples to determine the potential maternal transfer of these contaminants during vitellogenesis.

3. MATERIAL AND METHODS

3.1 Data sample collection

In collaboration with WWF Policoro Herakleia, 109 *C. caretta* eggs were collected from Taranto beach, Italy nests in different years, 2015, 2017, 2019, 2021 and 2022.

Figure 3: Sampling area, Taranto, Apulia, Italy

3.2 Sampling

The collected eggs were transferred to the Laboratory of Reproduction and Developmental Biology, Department of Life and Environmental Science at the Polytechnic University of Marche (Ancona, Italy). Each egg was weighed and the diameter was measured. Then the eggs were opened, the presence of the embryo was assessed. Following the embryonic stages described by Miller (2017), for each egg, a developmental stage was recorded. Where no visible

embryo was present, a <10 embryonic stage was assigned due to the uncertainty of the embryo's presence (N=30 eggs). In the present study, only <10 embryonic stage embryos were used (N=85) and from those samples the yolk weight was recorded. Yolk was then stored at -80 °C for further analysis of proteins and pollutants.

3.3 Statistical analysis

Average values of embryos' biometric parameters were analyzed through a one-way ANOVA test followed by Tuckey's test to perform multiple comparisons, using the GraphPad Software Prism8 for Windows. For analysis the significance was set at $p \leq 0.05$.

3.4 Protein extraction

0,1 g of yolk sample was used to analyze the proteins expression. Under chemical hood the protein extraction was performed adding 500 µl buffer Hanna, (Tris HCl o,125 M, SDS 4%, Glycerol 20%, β-mercaptoethanol) and 50 µl of anti-proteolytic cocktail (Protease Inhibitor Cocktail, P2714, Sigma-Aldrich). The samples were homogenized with a pestle, previously sterilized. Then the samples were boiled at 100 °C for five minutes. At the end all the samples were centrifugated at 12.000 x g for 10 minutes at 4 °C and the

supernatant was extracted. Protein lysate were stored in 0,6 ml Eppendorf at -20 °C.

3.5 Protein quantification BCA assay

The detergent-compatible formulation based on bicinchoninic acid (BCA) has been used for the colorimetric detection and quantitation of total protein, using a plate with 96 cells. First of all the reagent A has been mixed with reagent B in relation 50:1 to create the Working reagent. Then the dilutions must be done, starting from 1000 µl/ml, then 500 µl/ml, 250 µl/ml, 125 µl/ml, 62.5 µl/ml and 31.25 µl/ml, plus the blank to create the calibration line. To quantify it's needed to do the dilutions 1:1000 of the sample. Then, into the plate, it's charged 25 µl of sample, in two replicates + 200 µl of the Working reagent. After an incubation period of 30 minutes the concentration of samples was read with a BioTek Synergy HTX Multimode Reader.

3.6 Western Blot

According to the BCA assay, the same quantity in terms of concentration of protein extracts were submitted to electrophoretic run. Samples were centrifuged at 1200 g for 10 min before adding the sample buffer (1:1 ratio) (Bromophenol blue 0,1%, β-mercaptoethanol, Glycerol); And the solutions were homogenized using a stirrer.

Acrylamide gel was prepared by overlaying 4% stacking gel (1,55 ml deionized water, 0,625 ml TRIS HCl 0.5 M pH 6, 350 µl Acrylamide 30%, 25 µl SDS 10%, 5 µl TEMED, 25 µl APS 10%) for sample loading and 15% running gel (2,5 ml deionized water, 2,5 ml TRIS HCl 0.5 M pH 8.8, 5 ml Acrylamide 30%, 100 µl SDS 10%, 20 µl TEMED, 50 µl APS 10%). The gel was placed in the electrophoresis chamber submerged in 1L of Electrode buffer (3g TRIS, 14,4 g glycine, 1g SDS, volume deionized water) and each sample was loaded into a different well. One well was allocated for loading 3 µl standard molecular weights (Precision Plus Protein Standards, Bio Rad). The electrophoretic run was set at 30mA for about 30 minutes, then 60 mA until the end. Once proteins ran in the gel, they were transferred to a nitrocellulose membrane. The transfer was done in a transfer chamber using sponges and sheets of paper to hold the gel and the membrane in place for 2 hours at 250 mA. The all was submerged in 1L of cold transblotting buffer (6g TRIS, 28,8g Glycine, 200 mL 100% ethanol, deionized water to volume), continuously stirred with a magnet. The buffer was kept cold during the entire transfer by surrounding the transfer chamber with ice. The membrane was then rinsed with deionized water and stained using Ponceau Red, marking the molecular weight bands of interest with a pencil. After a was in deionized water and a wash in T-TBS (TWEEN 20%, TBS 1X in 1:100 ratio), the membrane was blocked in EveryBlot

Blocking Buffer solution (12010020, Biorad) oscillating at room temperature for 30 minutes. After that, the membrane were incubated inside Falcon tubes containing Antigen Cathepsin D, Antigen Cathepsin B and Antigen Cathepsin L primary antibodies (1:1000 in the same blocking solution) at 4 °C overnight. The following day after 5 washes with T-TBS for 5 min each in oscillator, the membranes were incubated inside Falcon tubes containing the secondary antibodies Anti-Rabbit IgG (a-0545, Sigma) (1:5000 concentration in the same blocking solution) in incubator at 30° C for 1 h. After 5 washes with T-TBS for 5 min each in oscillator, the membranes were incubated with ClarityTM Western ECL Substrate (170-5061, Biorad) (500 µl solution A, 500 µl solution B) for 5 min; after which the membranes were developed and read at Chemi Doc MP Imaging System. The bands were quantified with the program Fiji ImageJ (ImageJ, NIH, USA, https://imagej.nih.gov/ij/).

3.7 PAH extraction

The egg yolk sampled at Laboratory of Reproduction and Developmental Biology, Department of Life and Environmental Science at the Polytechnic University of Marche (Ancona, Italy), were then transported at the School of Biosciences and Veterinary Medicine at Camerino University (Macerata, Italy) for the analysis of PAH (polycyclic aromatic hydrocarbons). Organic contaminants were extracted *via* a QuEChERS (quick, easy, cheap, effective,

rugged, and safe) methodology used in previous research with sea turtles (Finlayson et al., 2020). QuEChERS is non-targeted and extracts chemicals with a range of polarities across many tissue types (Escher et al., 2021). Homogenised yolk samples were thawed, and 5 mL were aliquoted into 50 mL centrifuge tubes and homogenised with ceramic homogenisers for 30 s. MilliQ water (10 mL) was then added to each sample and vortexed for 1 min, followed by addition of 15 mL of acetonitrile and vortexed for 1 min. Five grams of magnesium sulfate and 1 g of sodium chloride were added into each centrifuge tube and shaken by hand for 2 min. The resulting mixtures were centrifuged at 4500 rpm (1900 ×*g*) for 8 min at 10 °C. The acetonitrile layer was aliquoted into glass vials and evaporated with nitrogen gas. Dried extracts were then reconstituted in 1 mL methanol, transferred into glass amber vials with Teflon lids, and stored at −20 °C until further analysis (Barraza et al., 2023).

4. RESULTS and DISCUSSION

4.1 Embryonic developmental stages

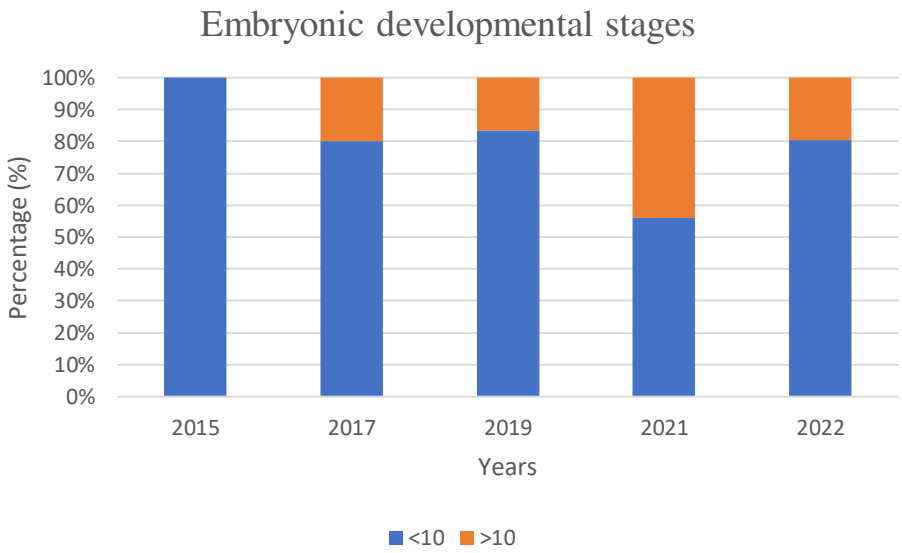

Figure 4: Percentage (%) of developmental stages (classified as <10 and >10 stages) of *Caretta caretta* embryos collected from 109 unhatched eggs in different years.

In all years analyzed, the majority of unhatched eggs were found at developmental stage <10 (Figure 4). In particular in 2015 all the unhatched eggs collected were <10. These results are in agreement with those already described for this species. In fact, embryonic mortality of *C. caretta* is mainly observed during early development, supporting the hypothesis that the first days of incubation are critical in embryonic sea turtle development (Blank & Sawyer, 1981; Withmore & Dutton, 1985). Embryos of both freshwater and

marine turtles undergo a period of suspended development following gastrulation, with further morphological development resuming only after egg laying (Rafferty & Reina, 2012). The embryonic arrest before oviposition likely serves as an adaptive mechanism, giving female turtles more flexibility in their reproductive timing. Additionally, it offers a survival advantage for the offspring by synchronizing their development after egg laying (Santos et al., 2016). This pre-ovipositional development arrest is thought to be caused and maintained by the low-oxygen conditions in the turtle oviduct. Once the eggs are laid, the exposure to atmospheric oxygen levels prompts the resumption of development (Williams et al., 2017). This characteristic makes the first days after laying a critical period, highly susceptible to inducing embryo death. Moreover, embryonic development halts at a very early stage before oviposition, meaning that ontogenetic growth is expected to contribute only minimally to the overall consumption of yolk. For this reason, the composition of the yolk in embryos at stage <10 closely reflects the composition of the same yolk before fertilization.

4.2 Egg and yolk weight and egg diameter

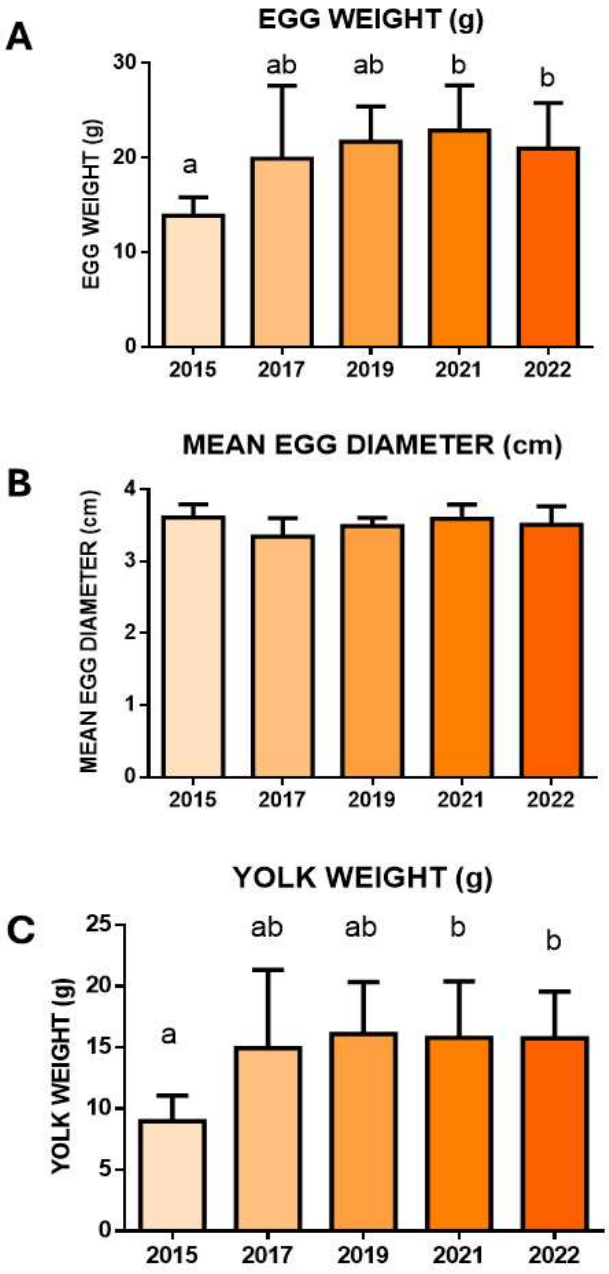

Figure 5: Egg weight (A) egg diameter (B) and yolk weight (C) differences among different years.

The analysis of the biometric characteristics of the unhatched eggs sampled in different years shows that the eggs sampled in 2015 have the lowest total weight and yolk weight (Figure 5). In the following years (2017-2019), these characteristics show an increasing trend, which becomes significant in 2021 and 2022. Notably, there was no variation in the diameter of the eggs across the different sampling years. A difference in the weight of eggs laid by sea turtles has often been attributed to variations in feeding areas or quantities. Additionally, it has been shown that egg size also depends on the size of females that lay them. However, regardless of the cause, embryos from smaller eggs have a lower hatching success rate (Hatase et al., 2014). It means that it's necessary to assess yolk components to understand the meaning of these changes. That's why it's been analyzed the yolk proteins pattern of unhatched eggs laid in different years.

4.3 Yolk protein pattern

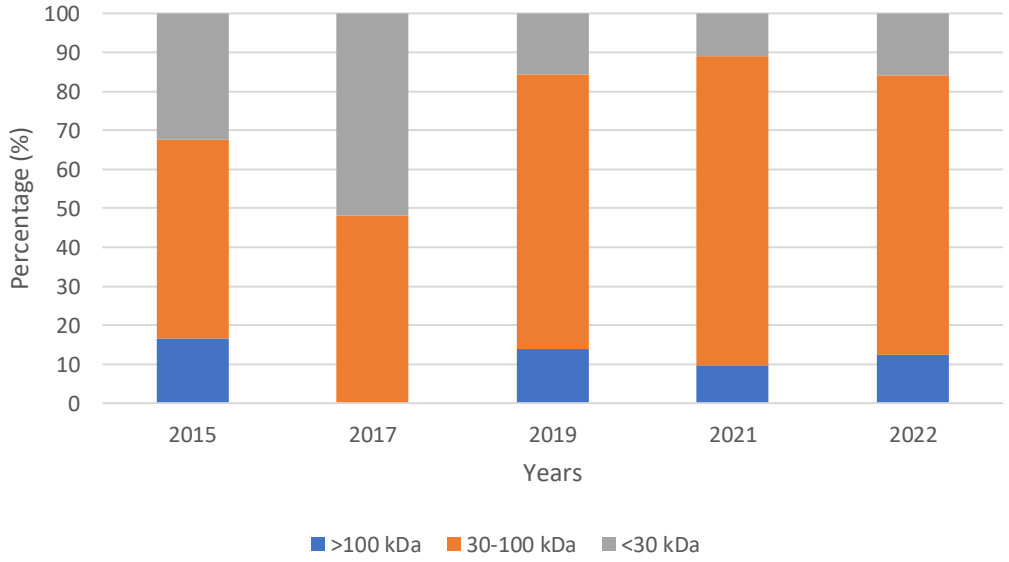

Figure 6: Mean yolk proteins pattern (classified in high molecular weight >100 kDa, medium molecular weight between 30 and 100 kDa and low molecular weight <30 kDa) of unhatched eggs laid in different years.

The highest percentage of proteins present in yolk of embryos at developmental stage <10 was the one between a molecular weight of 30 and 100 kDa (in 2015 it's 51%, in 2017 it's 48%, in 2019 it's 70%, in 2021 it's 79%, in 2022 it's 71%), mainly represented by lipovitellin (Figure 6). The lipovitellins represent fundamental power supply for embryonic development. There is approximately half the amount of lipid as there is protein (Thompson & Speake, 2002). Much of the lipid in these eggs is present in the form of yolk droplets originating from a specialised type of low density lipoprotein (LDL*y*) which is secreted by the

maternal liver (Speake and Thompson, 1999). LDL*y* is a triacylglycerol-rich lipoprotein containing two proteins, apo B and apo-LDL-II (Walzem, 1996). The apo B component of LDL*y* binds to specific receptors on the oocyte plasma membrane resulting in receptor-mediated uptake (Schneider et al., 1998). Vitellogenin is taken up into the oocyte via this same receptor (Elkin et al., 1995). The adoption of LDL*y* as a yolk precursor has enabled reptiles and birds to incorporate far greater amounts of lipid into their eggs than would have been possible by relying solely on vitellogenin as a lipid carrier (Thompson & Speake, 2002). Since lipid is a highly concentrated source of metabolic energy, the lipid-rich amniote egg enables the embryo to develop to a relatively advanced state, thus avoiding the need for the aquatic larval stage required by the ancestral tetrapods (Speake and Thompson, 1999). Indeed, as displayed in Figure 5, the percentage of these proteins in all years was around more than 50%. Conversely, the lowest percentage was represented by the high density lipovitellins, with a molecular weight >100 kDa. Only the years 2017 didn't present high density proteins. The rest is represented by the presence of phosvitin, with a molecular weight <30. Egg yolk phosphoproteins are composed of phosvitin and phosvettes (Wallace, 1985). Phosvitin accounts for 60% of total egg yolk phosphoproteins and holds about 90% of the egg yolk phosphorous (Samaraweera et al., 2011). Abe and others (1982) identified 2

main components of phosvitin in sodium dodecyl sulfate polyacrylamide-gel electropherogram as α and β. The detailed analysis of amino acid sequence indicated that serine accounts for more than 55% of the total amino acids of phosvitin and β-phosvitin contains more serine residues than α-phosvitin (Samaraweera et al., 2011). α-Phosvitin is rich in glycine, alanine, lysine, glutamine, and thrionine; and β-phosvitin is rich in histidine. Almost all the serine residues in phosvitin molecules are phosphorylated (Samaraweera et al., 2011). Phosvitin is the most phosphorylated protein found in nature, and due to its chemical composition, phosvitin is capable of easily capturing Ca^{2+} and Fe^{2+} ions and it possesses good preservative, antioxidant and emulsifying properties (Lesnierowski & Stangierski, 2018). This analysis has been done to understand which causes could interrupt the embryonic development, because all the elements are necessary for a correct embryonic development: in fact, one of the causes of these interruptions could be the low presence of high density lipovitellins, that are important especially at the beginning of development.

4.4 Cathepsins protein levels in eggs yolk

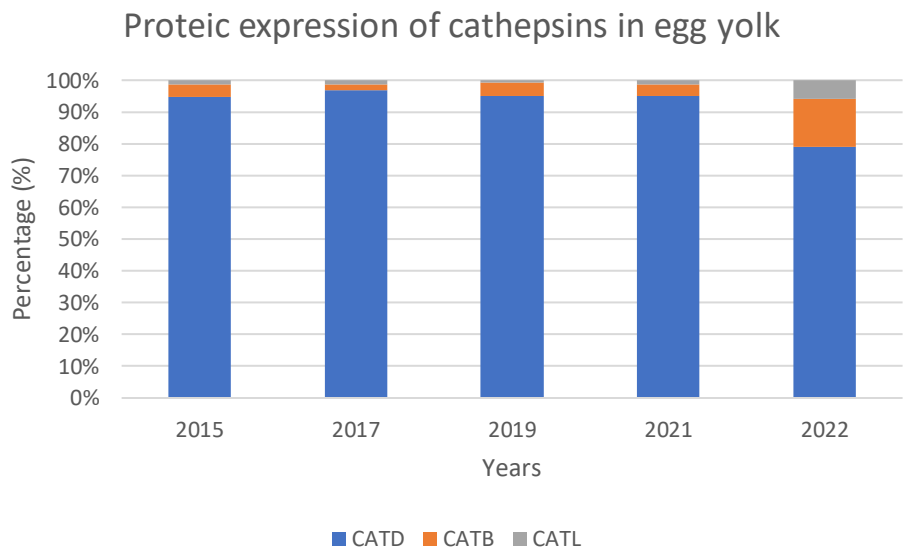

Figure 7: Mean concentration of cathepsins D, B and L (expressed in percentage) in the yolk of unhatched eggs laid in different years

Figure 7 show the percentage of Cathepsins found in samples analyzed. Cathepsin D was the most expressed (more than 80%) in all years. The only year where cathepsin B and L can be considered is 2022, where cathepsin B represents 10 % and cathepsin L represents 5%. In the rest of the years there's a very low percentage, but they are still present (in 2015 CATB is 5% and CATL is 1,4%, in 2017 CATB is 3% and CATL is 1.5%, in 2019 CATB is 6% and CATL is 1,5%, in 2021 CATB is 4% and CATL is 2%). The role of Cathepsins was already evinced during oogenesis. Changes in cathepsin B, D,

and L activity were found depending on the oocyte maturation stage; cathepsin B and D were found to be at maximum level in early vitellogenesis oocytes, and cathepsin L in mid-vitellogenesis ones (Carnevali et al., 1999). High activity of Cat B in the late vitellogenic stage suggests its involvement in the activation of Cat D proenzyme into active Cat D, which then cleaves incorporated vitellogenin into egg-yolk components (Sharma et al., 2022). The ability of cathepsin L to process the lipovitellins in small peptides is shown with the disappearance of the higher molecular weight components (Carnevali et al., 1999). The low concentration of CATB and CATL evidenced in unhatched eggs laid in different years, could reduce the degradation of HDL and LDL in small peptides and therefore interrupting the embryonic development.

4.5 PAH (Polycyclic Aromatic Hydrocarbons) analysis

	2015	2017	2019	2021	2022
Naphthalene	40% (8,7)	17% (1,6)	60% (6,8)	17% (1,4)	60% (5,1)
Acenaphthene	20% (1,9)	0%	40% (1,5)	16% (8,2)	0%
2-Bromonaphthalene	40% (1,5)	30% (1)	40% (5,5)	0%	0%
Acenaphthylene	0%	30% (1,9)	100% (1,5)	83% (4,8)	30% (1,8)
Fluorene	40% (5,1)	66% (8,5)	15% (10,6)	0%	50% (8,7)
Phenanthrene	60% (6,1)	30% (16)	0%	15% (4,4)	66% (7,4)
Anthracene	60% (6,1)	33% (1,5)	100% (17,1)	0%	84% (3)
Fluoranthene	0%	15% (1,3)	60% (7,2)	0%	16% (2,5)
Pyrene	80% (1,2)	50% (2,9)	60% (1,7)	0%	33% (0,8)
Benzo (a) anthracene	20% (0,6)	15% (5)	0%	0%	0%
Chrysene	20% (2,4)	15% (3)	0%	0%	15% (1,2)
Benzo (b) fluoranthene	0%	0%	15% (0,8)	0%	0%
Benzo (a) pyrene	0%	0%	0%	0%	0%
Indeno (1, 2, 3) PYRENE	0%	0%	15% (2,2)	0%	0%
Dibenz (a, h) anthracene	0%	0%	0%	0%	0%
Benzo (g, h, i) perylene	0%	0%	0%	0%	0%

Table 1: Percentage of PAHs in yolk of unhatched eggs laid in different years. In brackets are indicated the highest concentration (ng/g) detected in a single egg.

The presence of PAH (Polycyclic Aromatic Hydrocarbon) in the egg yolk is extremely evident from Table 1. It's evident that the Naphthalene is the only pollutant present in all the years, but in different percentages and

concentrations. In fact, in both 2017 and 2021 was detected the lowest Naphthalene concentration in the lowest percentage of contaminated eggs. Benzo (b) fluoranthene and Indeno (1, 2, 3) pyrene were detected only in 2019 samples. Acenaphthylene and Anthracene are present in all the eggs in 2019. It's interesting to notice that the year 2021 has lower percentages of contaminated eggs and lower maximum concentrations, in fact only four pollutants are present, Naphthalene, Acenaphthene, Acenaphthylene and Phenanthrene, with low percentages, except for Acenaphthylene with 83%. Notably in 2020, the COVID-19 pandemic led to a significant slowdown in industries activities and transportation. This could had a positive impact on the environment, allowing it to recover from the intense pollution it had been subjected to. A statistically significant decline in beach litter concentration on Mediterranean beaches during the pandemic was observed (Segal et al., 2022), which suggest a considerable reduction in the pollution ingested by sea turtles during their oceanic phase. PAHs are classified as significant widespread organic pollutants due to their persistence and bioaccumulation in the environment with moderate to high toxicity and carcinogenicity, which has raised significant concerns for both marine organisms and human (Baali & Yahyaoui, 2019). The toxicity of polycyclic aromatic hydrocarbons (PAHs) and similar pollutants varies across species and is influenced by factors such as

the kind of the contaminant, the exposure route, and the organism's life stage (Ball & Truskewycz, 2013). Prolonged or high-level exposure to PAHs or crude oil has been linked to health and reproductive issues in various wildlife species, including loggerhead sea turtles (*Caretta caretta*) (Alam & Brim, 2000). High levels of PAH are found in the egg yolk, due to the high lipid content and lengthy duration of yolk formation (Pouyande et al., 2024). Inter-nest variations in levels and profiles of PAHs are evident, which might be related to the turtle's diets and migration patterns, due to the fact that sea turtles are wide-ranging and long-lived migratory species with high trophic levels, and are exposed to various contaminants over long periods from various regions (Pouyande et al., 2024). Considering only the egg yolk, it's evident the maternal transfer of PAHs into the egg before the deposition, and given this maternal transfer of pollutants, eggs serve as an ideal tissue for biomonitoring of maternal chemical burdens and provides a practical approach to assessing pollutants in migratory species that are hard to access (Muñoz & Vermeiren, 2023).

5. Conclusions

In conclusion, the embryonic development of sea turtles is a complex and delicate process, highly influenced by both intrinsic and extrinsic factors. The critical stages, such as those before stage 10, highlight the vulnerability of embryos to environmental conditions, particularly in terms of nutritional deficiencies and pollutant exposure. The changes in yolk composition during these stages, including alterations in protein, enzymes and the accumulation of contaminants, underline the profound impact that the mother's health and the surrounding environment can have on embryo survival and development. Future research into the effects of pollutants, combined with a deeper understanding of the mechanisms behind embryonic development, will be essential in improving conservation efforts. Protecting the marine habitat and ensuring the optimal health of adult turtles are key to preserving the future of these endangered species. Ultimately, further studies on this subject will contribute not only to our understanding of sea turtle biology but also to the development of effective strategies to safeguard their populations in an increasingly polluted world.

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