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PRESENZA DI INQUINANTI NEL FEGATO DI EMBRIONI DI *C. CARETTA*: EFFETTI SULLO STATO DI SALUTE

PRESENCE OF POLLUTANTS IN THE LIVER OF *C. CARETTA* EMBRYOS: EFFECTS ON HEALTH STATUS

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A causa del lungo ciclo di vita, della maturità sessuale tardiva e della migrazione, la tartaruga *Caretta caretta* è particolarmente vulnerabile a diverse minacce antropogeniche, tra cui l'inquinamento da microplastiche e altri contaminanti chimici associati, come gli idrocarburi policiclici aromatici (IPA). La tartaruga *C. caretta* è considerata un efficace bio-indicatore per il monitoraggio dell'inquinamento da plastica, ma i suoi effetti sullo stato di salute e sulla riproduzione delle tartarughe deve essere approfondito.

In questo studio è stata analizzata la presenza di microplastiche e IPA nel fegato di embrioni a diversi stadi di sviluppo (28 e 30), prelevati da uova non schiuse deposte lungo le coste ioniche italiane. Le biometrie embrionali e l'istologia del tessuto epatico sono state analizzate tramite microscopia, immunoistochimica e Western blot, valutando i potenziali effetti degli inquinanti sulla crescita e su biomarcatori come i melanomacrofagi, il citocromo CYP450 e la proteina HSP70.

La presenza di microplastiche è stata confermata nel 75% degli embrioni analizzati, confermando quanto emerso da un precedente studio pilota e sostenendo la teoria del trasferimento materno, secondo la quale esse vengono incorporate nel tuorlo durante la vitellogenesi e successivamente assorbite

dall'embrione durante lo sviluppo. Le microplastiche rilevate erano principalmente frammenti di dimensioni inferiori a 10μm, con colori variabili. Diversi IPA sono stati rilevati e quantificati per la prima volta nel fegato e nel tuorlo del 75% degli embrioni analizzati, suggerendo la loro particolare abbondanza e persistenza già nella fase embrionale e sostenendo il passaggio materno come via di esposizione anche per questi inquinanti.

Non sono emerse differenze nella presenza di contaminanti tra gli stadi di sviluppo 28 e 30, né nei livelli di biomarcatori come melanomacrofagi, CYP450 e HSP70, suggerendo un livello di stress duraturo in entrambi gli stadi di sviluppo.

Ulteriori studi sono necessari per esplorare gli effetti negativi sinergici della presenza di microplastiche e IPA sulla salute dell'embrione e sviluppare strategie di conservazione più efficaci.

ABSTRACT

Due to its long life cycle, late sexual maturity and migration, the loggerhead turtle *C. caretta* is particularly vulnerable to various anthropogenic threats, including microplastic pollution and other chemical contaminants. *C. caretta* turtle is considered an effective bio-indicator for plastic pollution monitoring, but its effects on turtle health and reproduction need to be further investigated. In this study, the presence of microplastics and polycyclic aromatic hydrocarbons (PAHs) was analyzed in the liver of embryos at different developmental stages (28 and 30) collected from unhatched eggs laid along the Ionian coast of Italy. Embryonic biometrics and liver tissue histology were analyzed by microscopy, immunohistochemistry and Western blot analysis, assessing the potential effects of pollutants on growth and on biomarkers such as melanomacrophages, cytochrome CYP450 and HSP70 protein.

The presence of microplastics and PAHs was confirmed respectively in 75% and 70% of the embryos analyzed, supporting the theory of maternal transfer for these contaminants and indicating their abundance and persistence in the embryonic stage. There were no differences in their presence between developmental stages 28 and 30, nor in the levels of biomarkers such as melanomacrophages, CYP450 and HSP70, suggesting a sustained level of stress. Further studies are needed to explore the synergistic negative effects of the presence of microplastics and PAHs on embryonic health.

1. INTRODUCTION

1.1 Model overview: biology and ecology of *C. caretta*

1.1.1 Taxonomy

Turtles play a crucial role in marine ecosystems, even if only seven species of marine turtles survive today (Pritchard, 1996). The model species considered for this study is the loggerhead turtle *Caretta caretta*, first described by Linnaeus in 1758 with the name *Testudo caretta* (Conant et al., 2009). The monotypic genus *Caretta* belongs to the family Cheloniidae, which also

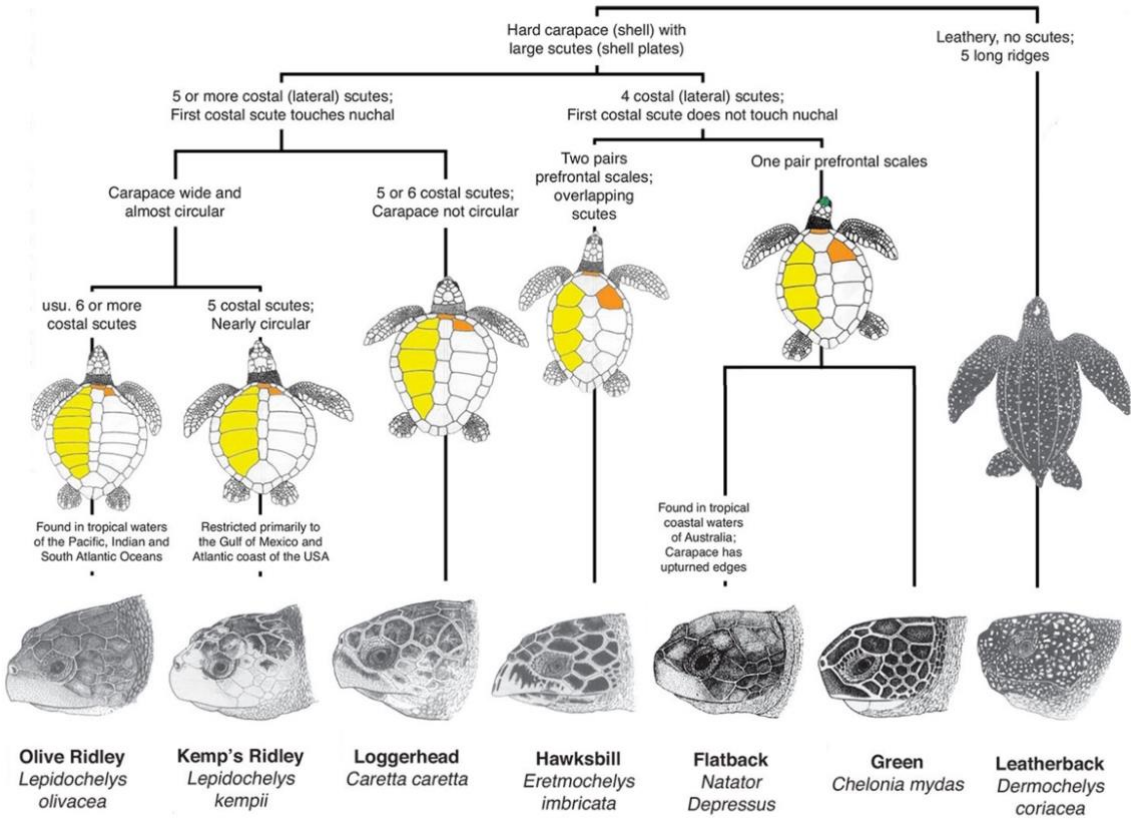

***Figure 1**: phylogenetic tree and identification features of all seven sea turtle species. Marine Turtle Specialist Group (iucn-mtsg.org) and illustrations by Tom McFarland.*

includes the green turtle *Chelonia mydas*, the flatback turtle *Natator depressus*, the hawksbill turtle *Eretmochelys imbricata*, the Kemp's ridley turtle *Lepidochelys kempi* and the olive ridley turtle *Lepidochelys olivacea*; the leatherback turtle *Dermochelys coriacea* belongs to the monotypic family Dermochelyidae instead (Pritchard & Mortimer, 1999; Meylan & Meylan, 1999) (Figure 1).

1.1.2 Morphology

The shape and color of marine turtle's carapace are distinguishing features that set different species apart. The loggerhead turtle is morphologically characterized by a moderately wide carapace, with a thickened area at the base of the tail (Pritchard & Mortimer, 1999); both the carapace and the plastron are heavily keratinized (Dodd, 1988), consistent with their primary function as protective structures and reducing frictional resistance in water (Solomon et al., 1986). In adults, the carapace has five pairs of costal shields, of which the anterior one is the smallest and touches the nuchal scute (Conant et al., 2009); coloration is reddish-brown in adults and light or dark brown in juveniles. The head is relatively large, triangular and it has two pairs of prefrontal scales. The anterior fins are relatively short compared to other species, each equipped with two claws (Pritchard & Mortimer, 1999). Adults generally reach 180kg in weight and 105cm in carapace length (Gavilán, 2001), but in the Mediterranean

the average size is smaller (up to 90cm in length) (Pritchard & Mortimer, 1999) (Figure 2).

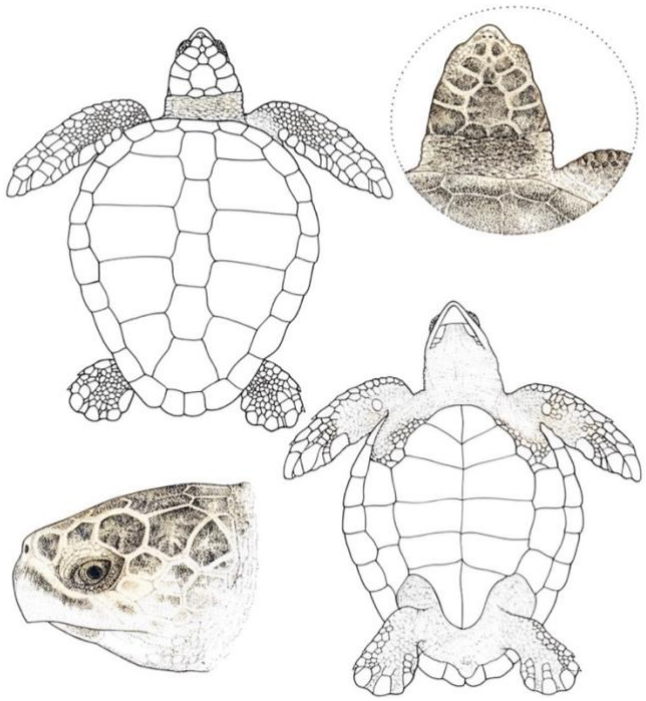

***Figure 2**: C. caretta morphologic features (colorized from Pritchard & Mortimer, 1999)*

1.1.3 Distribution

The loggerhead turtle is widely distributed in the temperate, subtropical and tropical oceans (Pritchard & Mortimer, 1999), with populations inhabiting continental shelves, bays, lagoons and estuaries in the Atlantic, Pacific and Indian Oceans. Unlike most other sea turtle species, the main nesting areas are located in warm temperate and subtropical regions (except for Masirah Island, Oman) (Miller, 1996). Nests laid on tropical beaches represent only a small fraction of the species' total nestings (Dodd, 1988), which occur mainly between 19 and 36 degrees latitude in each hemisphere (Witherington et al.,

2006). Turtles nesting in temperate zones often migrate to tropical waters to feed in foraging areas (Dodd, 1988).

C. caretta is the most abundant sea turtle species in the Mediterranean (Margaritoulis et al., 2003). Its Mediterranean population nests mainly in the eastern Mediterranean basin, especially along the coasts of Cyprus, Greece, Turkey and Libya, where more than 96% of clutches are laid (Casale et al., 2018). Recently, an expansion of the loggerhead turtle nesting range has been reported, with an increase in the number of nests on the western Mediterranean coasts (Hochscheid et al., 2022). Nesting, previously sporadic, has become regular in some areas of Italy, Spain, France and Tunisia (Hochscheid et al., 2022). Some studies explore possible correlations between this areal shift, global warming and anthropogenic variables (Witt et al., 2010; Pike, 2013; Mancino et al., 2022).

1.1.4 Life cycle

Like many other chelonids, loggerhead turtles are long-lived, highly migratory, slow-growing and late-maturing animals (Mansfield & Putman, 2013). They display a complex life cycle, which develops through different ecosystems: from terrestrial habitats (oviposition and embryonic development) to neritic zone habitats (waters less than 200m deep) and oceanic zone habitats (water depths greater than 200 m) (Bolten, 2003) (Figure 3).

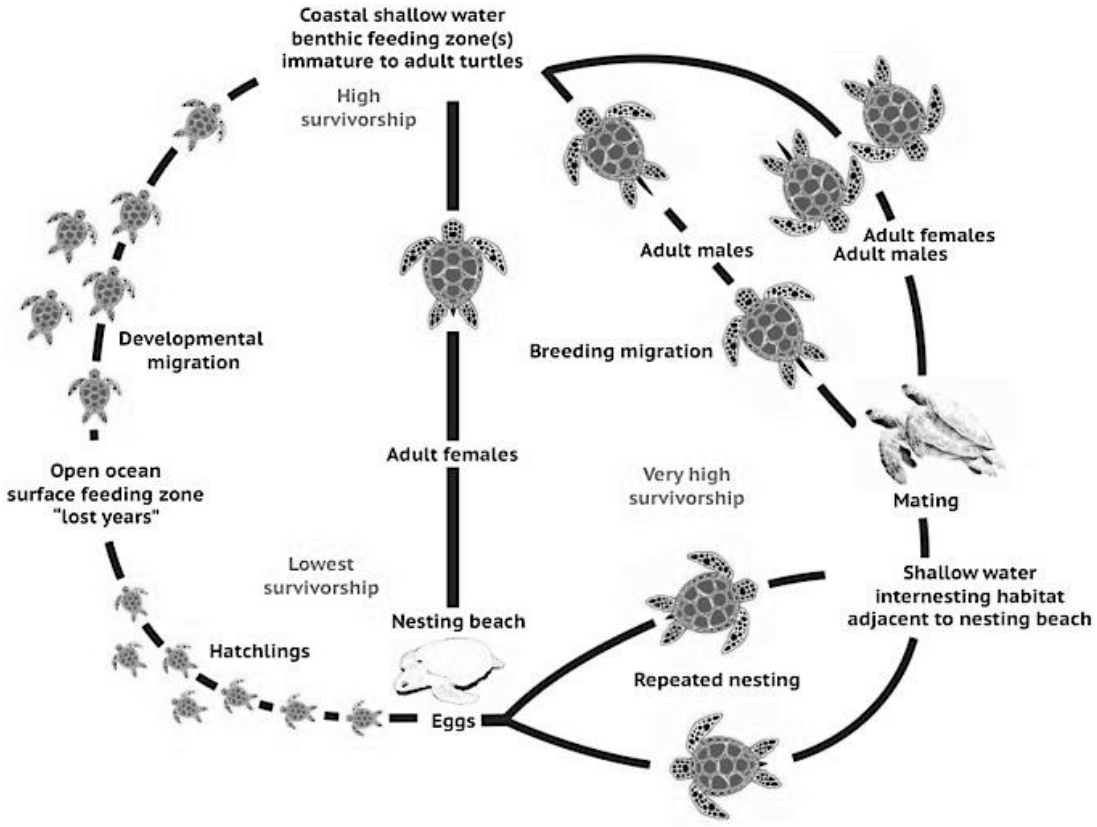

***Figure 3**: the complex life history of sea turtles (from Jones et al., 2016)*

Loggerhead turtle's life history begins on wide sandy beaches with low dunes, where the female has dug her nest (Miller et al., 2003). After an embryonic developmental process lasting 40-60 days, hatchlings emerge from the eggs in 1-3 days, leaving the nest in 2-4 days (Christens, 1990). Emergence from the nest occurs almost exclusively at night, probably stimulated by lower sand temperatures (Moran et al., 1999). Ambient light seems to guide the hatchlings towards the ocean (Lohmann et al., 1996). During this phase, post-hatchlings move frenziedly (Conant et al., 2009) and are vulnerable to predation by mammals, birds, crustaceans and fish (Stewart & Wyneken, 2004). Once far from the coast, hatchlings begin to feed on hydroids, copepods and pleuston,

no longer relying on the retained yolk (Witherington, 2002). This neritic phase may last for weeks or months (Witherington, 2002), after which post-hatchlings move into ocean waters: the transition is mainly driven by oceanographic factors (currents and winds) (Bolten, 2003; Casale et al., 2018), but recent studies also show the relevance of swimming and the active dispersal of post-hatchings (Putman & Mansfield, 2015; Briscoe et al., 2016). This oceanic dispersal period is metaphorically known as "lost years" (Carr, 1972; Casale & Mariani, 2014), as epipelagic individuals live in open waters near floating resources and are difficult to observe (Casale & Mariani, 2014). This oceanic phase forced by the limited diving capacity (Casale et al., 2008) can last from 6,5 to 11,5 years in the Atlantic population (curved carapace length, CCL, between 46 and 64cm) (Bjorndal et al., 2000).

Due to the high variability and plasticity found among different populations, a new life history model was proposed in 2009 by the Turtle Expert Working Group (TEWG), consisting of five life stages. According to this model, after the oceanic phase, there is a transitional juvenile stage, where sea turtles use both oceanic and neritic habitats (Mansfield & Putman, 2013). During the oceanic phase, young loggerheads are opportunistic diurnal predators, primarily feeding on gelatinous plankton (jellyfish and tunicates), fish and squid (Casale et al., 2018). Approaching neritic areas, they begin to frequent

and feed in benthic habitats, adapting their feeding strategy: here they complete their development, reaching sexual maturity and adult stage (Mariani et al., 2023). During this latter phase, loggerheads mainly feed on invertebrates such as crabs, hermit crabs, bivalves, gastropods, cephalopods and, in some areas, fish discarded by fishing vessels (Casale et al., 2018; Mariani et al., 2023). Habitat transitions are thus coupled with changes in turtles' feeding ecology, also varying their exposure to different threats (Schuyler et al., 2014).

The age and size at which transitions between different life stages occur can differ considerably between and within populations (Dodd, 1988). Focusing on the Mediterranean, Casale et al. (2008) proposed a more relaxed model, with a shorter obligate epipelagic stage followed by an opportunistic amphi-habitat stage, as soon as their benthic foraging efficiency improves. Here, the achievement of sexual maturity varies among populations (Casale et al. 2018) but appears to be reached at similar sizes both in males and females (Casale et al. 2005). Based on the average size of breeding females, length at sexual maturity in the Mediterranean is estimated between 66,5 and 84,7cm (CCL) (Casale et al., 2009). The average age at sexual maturity has been estimated to be 25 yr (range: 21-34 yr) (Casale et al. 2018), although Guarino et al. (2020) reported mature females to be 69,5cm long (estimated age 20 yr) and mature males to be 65cm long (estimated age 16 yr).

Loggerheads are highly migratory animals, periodically traveling hundreds to thousands of kilometers between foraging and breeding areas (Plotkin, 2002), up to a maximum recorded migration distance of 2150 km (Hays & Scott, 2013). In the Mediterranean, the main feeding grounds for loggerheads are located off Tunisia, in the Adriatic Sea, in the Levantine Basin (Turkey, Syria and Egypt), in the Aegean Sea, in western Greece and south-western coasts of Italy (Casale et al., 2018).

Females and males asynchronously reach the breeding grounds several weeks to months before the nesting season (Plotkin, 2003), with males anticipating females by a few weeks (Henwood, 1987). In the Mediterranean, males tend to migrate to breeding grounds more often than females (on average, in a 1-1,8 year range); females, on the other hand, migrate to nest every 2-3,35 years (Casale et al., 2018). The mating period is around April/May (Casale et al., 2018) and occurs in courtship areas, which are usually located close to the nesting beaches. After mating, males return to the foraging areas and females move to their nesting beaches (Miller, 1996). The peak of the nesting season in the Mediterranean corresponds to June and July (Casale et al., 2018). Females show a tendency to return to nest in the same geographical areas they came from: this behavior is called natal homing. This ability seems to be linked to a learning process known as imprinting, based on the perception of the magnetic

field and/or distinctive chemical cues associated with their natal beach (Lohman et al., 2013). After the nesting site selection, females lay 1 to 5 clutches (Casale et al., 2018), with an average interval of 12,7-19,9 days between one laying and the next. During this inter-nesting period, they remain resident near the nesting beaches (Musick & Limpus, 1996).

After completing their reproductive migration, *C. caretta* adults return to feeding grounds (Musick & Limpus, 1996) via migratory corridors, which have been identified using satellite telemetry and tagging; the consistency of these routes is variable (Casale et al., 2018). Loggerheads exhibit a high level of foraging and nesting site fidelity over multiple years: they usually return to the same foraging ground after the breeding season (Broderick et al., 2007). However, it has been observed that some adults also frequent other mating sites, with possible consequences for gene flow among different rookeries (Casale et al., 2018).

1.1.5 Reproductive cycle

Miller (1996) reported the reproductive features common to all species of sea turtles: they show iteroparous reproduction with stereotypical nesting behavior, laying a large number of eggs multiple times during the breeding season and relatively strong attachment to a particular nesting site, with variations between populations. Sea turtles are capital breeders: reproduction is financed using

stored energy (capital), that can be mobilized to cover the reproductive effort (Stearns, 1989; Stephens et al., 2009). During the foraging phase, loggerheads accumulate the energy reserves required to support gametogenesis: the foraging phase length varies between populations and depends on the quantity and quality of food available (Miller, 1996).

Both mature males and females exploit this quiescent reproductive period to grow and prepare for reproduction (Miller & Limpus, 2003). Mature females begin vitellogenesis approximately 8-10 months prior to the reproductive event: vitellogenesis requires the mobilization of stored fat and its modification through the liver, before being inserted into various groups of pre-vitellogenic follicles (Miller & Limpus, 2003). Lipid deposition and follicular development are completed before the nesting season (Hamann et al., 2003). Also, spermatogenesis starts around 9 months before reproduction and it is completed before the breeding period (Hamann et al., 2003), reaching the peak of sperm production during migration (Miller & Limpus, 2003). A combination of exogenous factors (such as photoperiod) and endogenous factors (such as hormone and fat levels) interact, triggering reproductive migration (Miller, 1996).

Once in the breeding grounds, courtship and mating begin. Signals of receptiveness and precopulatory behaviors are not yet well understood but may

be more important for females than for males (Miller, 1996). Adult males and females display sexual dimorphism: males have a longer tail and more developed claws on their flippers, used during mating (Dodd, 1988). Mating can be a rather violent event, during which females may be injured (Miller, 1996). Schofield et al. (2006) reported direct in-water observations of the courtship: the male tries to position behind the female to mount her, holding her with the claws of the front and rear flippers and biting her carapace and neck. The male uses his tail to keep the cloaca in contact and inserts his bifurcated penis, allowing the sperm transmission to both oviducts (Miller, 1996). Male-male competition has been observed in many mating areas (Hamann et al., 2003): attendant males often bite the mounting male in an attempt to dislodge him (Miller, 1996; Schofield et al., 2006).

Sea turtles are generally promiscuous breeders (Hamann et al., 2003): genetics confirmed that males mate with several females and vice versa (Miller, 1996). Sperm competition has been found, but no sperm storage appears to occur between different reproductive seasons (Sakaoka et al., 2011; Sakaoka et al. 2013). Multiple paternity analyses were carried out studying the largest Mediterranean population of *C. caretta*: Zbinden et al. (2007) showed high levels of multiple paternity, suggesting that polyandry has important consequences on increasing effective population size. Direct observations and

the healing of mating wounds support the thesis that mating only occurs during the pre-nesting season (Miller, 1996), while during the inter-nesting period females show strong male avoidance behaviors (Schofield et al., 2006).

At the end of the breeding season males return to their foraging territories, while females head for their native area to nest (Miller, 1996). The features the beach must have to be suitable for nesting are not yet clear. It must be sandy, accessible from the sea and high enough to prevent the nest inundation (Miller, 1996). The chemical and physical characteristics of the substrate are also relevant: it must be sufficiently moist and fine to allow the construction of the egg chamber without collapsing and it must allow gas exchanges (Mortimer, 1995); it seems that well-sorted sand grains favor nesting activity (Karavas et al., 2005), which is instead discouraged by the presence of obstacles and coarse and dry sand (Mortimer, 1990). Other factors that could influence choice are the distance from the nearest human settlement, substrate temperature, organic content, salinity, pH, artificial lighting and vegetation (Miller et al., 2003; Karavas et al., 2005). Females have often been observed emerging and testing the sand compactness by thrusting their heads inside, in a behavior known as sand smelling. If conditions are unfavorable or disturbance occurs, females return to the sea and try again later often on the same night and at the same

beach (Margaritoulis, 1985; Miller, 1996); between 10 and 75% of loggerhead nesting attempts are unsuccessful (Dodd 1988).

To avoid high diurnal temperatures, usually the nest is excavated during the night and the whole process takes about 1-2 hours in *C. caretta* (Miller, 1996; Margaritoulis, 1985). Pritchard and Trebbau (1984) and Hailman and Elowson (1992) accurately described the nesting behavior of *C. caretta*. The female emerges from the water in search of the ideal nesting spot, often stopping to rest and observe the surroundings. Loggerhead uses an alternating gait, moving only the two opposite flippers at the same time (Miller et al., 2003). After preparing the nesting site by clearing away surface debris, the female starts to move forming the body pit. At this point she digs the egg chamber using her hind flippers: the flask-shaped chamber has a thick neck and a wider bottom, the dimensions of which are correlated with female size and her reproductive output (Miller et al., 2003). When the chamber has reached a depth of about 60cm and the sand can no longer be removed, stereotyped alternating digging stops and oviposition starts (Hailman & Elowson, 1992). The eggs are laid singly or in small groups of two, three or four and during laying the females are rather tolerant to external disturbances (Hailman & Elowson, 1992). Reproductive output varies widely between populations considered, depending on various endogenous and exogenous factors (Hamann et al., 2003) and the

female's experience (Limpus, 1985). Generally, in the Mediterranean, between 64 and 126 eggs are laid in each clutch (Casale et al., 2018). Once oviposition is complete, the nest is filled in by scraping sand into the hole and compacting it with the hind flippers (Hailman & Elowson, 1992), before returning to the water.

Loggerhead turtles lay several clutches of eggs during the nesting season (Miller et al., 2003): generally, in the Mediterranean, the number is between 1 and 5 and the inter-nesting period varies between 12 and 19 days, but depends on various factors (Casale et al., 2018). Inter-seasonal renesting generally occurs very close to the first nest (Miller, 1996) but can range up to 290 km (Bjorndal et al., 1983). The separation of groups of eggs in time and space can be considered an evolutionary advantage to reduce the impact of an unpredictable environment on hatchling production (Bjorndal et al., 1983). Other turtle species have been proven to be primarily aphagic during the nesting season: sufficient lipid reserves to allow for the whole breeding season must be allocated during the feeding phase (Hamann et al., 2003). It is possible that the end of the nesting season is triggered by an insufficient amount of mature follicles to generate another clutch (Hamann et al., 2003) and females migrate back to the feeding grounds, entering a resting and quiescent phase (Miller & Limpus, 2003).

1.1.6 Embryonic development

C. caretta eggs are white, spherical, cleidoid and are protected by a flexible, porous shell composed of aragonite (Miller et al., 2003). On average, the egg size is 4.0cm in diameter and 36g in weight; the size is intermediate compared to other turtle species (Miller et al., 2003). Inside the egg there is the embryo, surrounded by a series of structures aimed at supporting the embryo's needs during development, such as respiration, nutrition, protection and excretion (Ferner & Mess, 2011) (Figure 4).

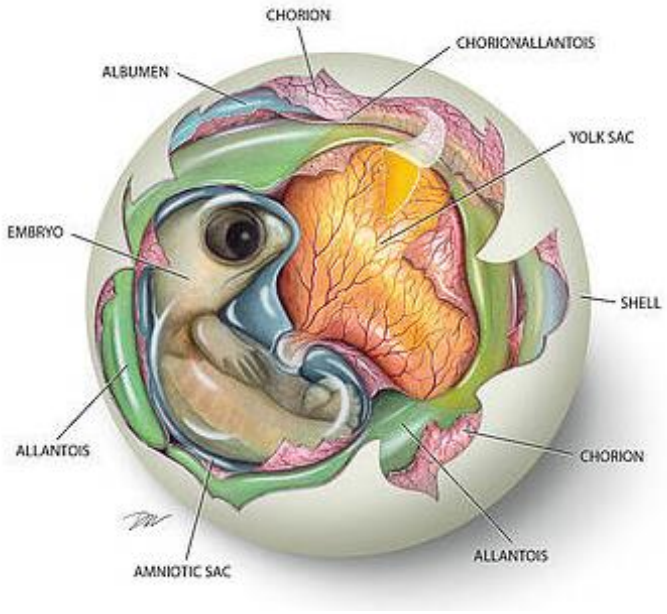

Figure 4: *illustration of the structure of a cleidoic egg and supporting embryonic membranes (Spotila, 2004)*

The embryo is surrounded by amniotic fluid, which is contained in a sac called the amnion: its function is to provide a stable aquatic environment and to protect the embryo from impacts (Spotila, 2004). The embryo is then connected to the yolk sac: it regulates and mobilizes the nourishment present in the yolk.

The yolk sac encloses the yolk and is irrigated by several blood vessels, which transport fats, sugars, starches and proteins to the embryo (Spotila, 2004; Ferner & Mess, 2011; Blackburn, 2021). The yolk content depends on the food consumed by the female in her foraging area, not in her nesting area (Miller et al., 2003). The posterior part of the embryo's gut is connected to the allantois: this is a membrane that allows gas exchange and the elimination of waste such as ammonia and urea; during development this membrane grows by accumulating waste substances (Spotila, 2004). The whole is surrounded by a membrane called the chorion. The chorion and allantois fuse at the beginning of embryonic development, forming the chorioallantoic membrane (Cruze et al., 2013). This membrane is the location of gaseous exchanges, transport of essential inorganic ions such as calcium, potassium and phosphorus from the eggshell, acid-base homeostasis and water reabsorption (Cruze et al., 2013; Gabrielli & Accili, 2010). External to the chorion is the albumen, an aqueous substance involved in gas exchange and water balance (Spotila, 2004). The external shell is composed of calcium carbonate in the form of aragonite (orthorhombic $CaCO_3$) (Miller et al., 2003); it is thin and porous, allowing gases to pass through (Spotila, 2004).

In Miller et al. (2017), an accurate description of the embryonic developmental stages of sea turtles is presented, in the form of a dichotomous key consisting of 31 developmental stages (Figure 5).

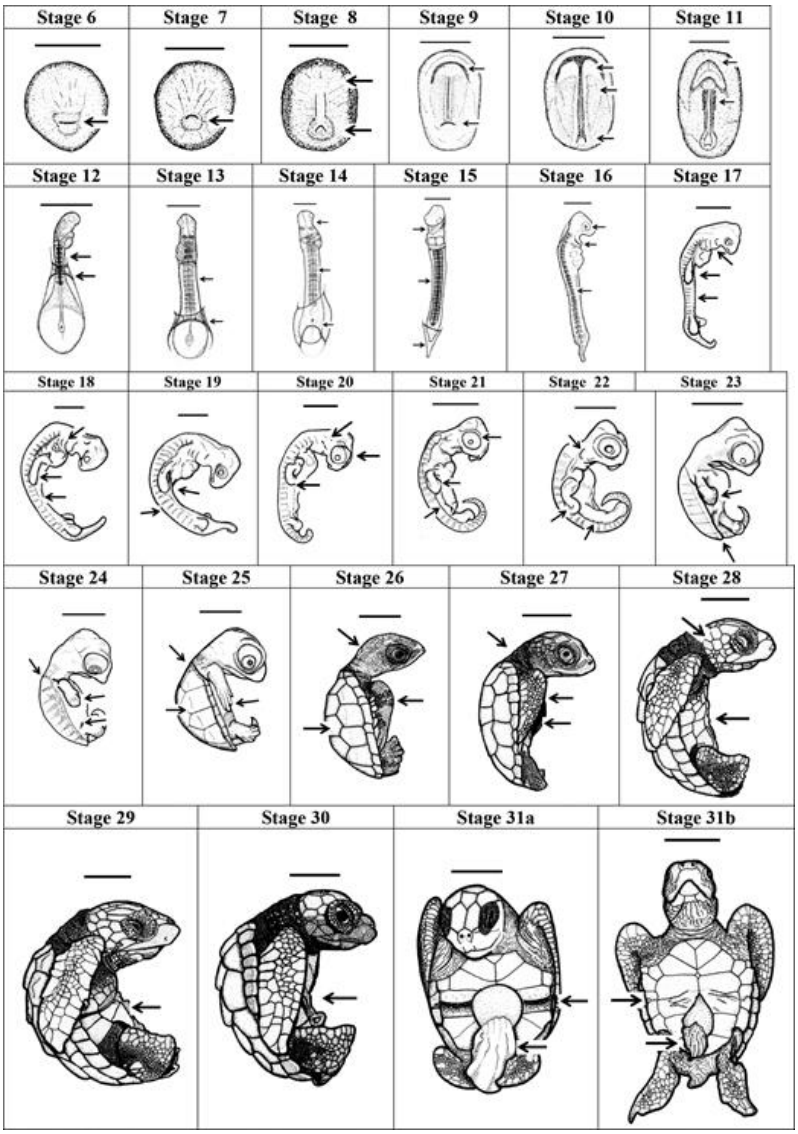

Figure 5: *stages of C. caretta turtle embryonic development (from Miller et al., 2017)*

Egg formation begins with ovulation, when eggs are expelled from the ovary and fertilized in the anterior part of the oviduct (Miller & Limpus, 2003). Here, embryonic development stops mid-gastrulation and resumes in 4-8 hours

(depending on temperature) after oviposition: this stage corresponds to stage 6 (Miller, 1996). Stages 6 - 10 are characterized by changes in the shape of the blastopore and the differentiation of notochord, neural folds and head folds. Stages 11 - 22 are distinguished by characters such as the number of somites, head differentiation, eye pigmentation and pharyngeal fissures. During stages 23 - 31, we observe the formation of carapace and scales, a change in pigmentation and a variation in the ratio of embryo to yolk volume (Miller et al., 2017). Complete embryonic development is reached at stage 31a, where the embryo is quite large compared to the remaining yolk and is about to break the shell. At stage 31b, the yolk has already been reabsorbed, the embryo has broken out of the shell using the 'egg tooth' (which will be lost in a few days) (Alibardi, 2020) and is straightening (Miller et al., 2017).

Environmental conditions inside the nest can affect embryonic development: the main parameters to consider (alone or synergistically) are humidity, gas exchange and temperature (Miller et al., 2003). In particular, the incubation temperature is the main factor regulating the duration of embryonic development and the sex ratio of the brood.

The incubation period (the time between oviposition and hatching) shows a strong negative correlation with the temperature recorded in the nest (Mrosovsky & Yntema, 1980; Matsuzawa et al., 2002; Miller et al., 2003). In

the Mediterranean, the temperature range compatible with development appears to be between 26°C and 33°C: at these limiting temperatures, the duration of incubation varies from a minimum of 36 to a maximum of 89 days (Casale et al., 2018). In this range, a change of 1°C increases or decreases the incubation period by approximately 5 days (Mrosovsky & Yntema, 1980).

As in other reptiles, sex determination in turtles is environmental: specifically, it is temperature-dependent sex determination or TSD (Lolavar & Wyneken, 2020). Cooler incubation temperatures produce males, while warmer temperatures produce females (Wibbels, 2003); intermediate temperatures produce clutches with a mix of males and females (Spotila, 2004). Bull & Vogt (1981) identified the middle third of embryonic development (stages 21-26) as the temperature-sensitive period when differentiation of the embryonic gonad occurs, and sex is irreversibly determined. The sex ratio of the clutch concerning the temperature is described by a logistic curve (Lolavar & Wyneken, 2020). The transitional range of temperatures (TRT) is the range of temperatures within which sex ratio varies from 100% male to 100% female (Wibbels, 2003). The pivotal temperature is the temperature at which males and females are produced in equal numbers (Mrosovsky & Godfrey, 2010). In addition to external temperature, the heat generated by metabolic processes in the eggs also influences the sex ratio, with eggs at the center more likely to

produce females than those at the margins (Spotila, 2004). In the Mediterranean Sea the pivotal temperature for loggerheads is around 29 to 29,3°C (Casale et al., 2018).

Once embryonic development is complete, hatching takes place. Generally, *C. caretta* eggs have a hatching success of over 80%, excluding variables such as predation, unfavorable environmental conditions and microbial infections (Miller et al., 2003). Hatching and emerging from the nest are cooperative processes, where the upward movement of hatchlings is stimulated by the activity of others, in a process known as 'social facilitation' (Carr & Hirth, 1961).

1.1.7 Ecological role

Loggerhead turtles have always been important in maintaining marine systems, which they inhabit as consumers, prey, competitors, hosts of parasites, substrates for epibionts, transporters of nutrients and landscape modifiers (Bjorndal & Jackson, 2003). During their life cycle, loggerhead turtles prey on a large number of species (such as gelatinous plankton, benthic invertebrates and teleosts), keeping their population abundance under top-down control (Bjorndal & Jackson, 2003; Heithaus, 2013; Patel et al., 2022). Especially in their early life stages, sea turtles are preyed upon by a wide range of predators, including insects, birds, mammals, large lizards, crocodiles, different teleosts,

sharks and even squid (Heithaus, 2013). In addition, sea turtles act as substrates and transporters for many epibionts and parasites: dozens of species of epibionts and algae have been found attached to their bodies (Frick, 2013). Sea turtles play an important role in moving nutrients within the ecosystem, transferring energy between rich foraging areas and poor nesting grounds (Bjorndal & Jackson, 2003). *C. caretta* turtles can modify the physical structure of sandy seabed habitats, acting as bioturbators: actively feeding on benthic mollusks, they crush the shells of their prey into small fragments (Lazar et al., 2011). They also represent a resource for various cleaning organisms and can facilitate the foraging of several fish species (Heithaus, 2013).

1.1.8 Conservation status, protection and legislation

In addition to their ecological role, turtles also provide other important ecosystem services that benefit humans. The cultural (identity and symbolism) and economic (tourism, trade, food, decoration) aspects of turtles should not be forgotten (Patel et al., 2022; Campbell, 2003). Loggerhead turtles are vulnerable to many anthropogenic threats, both in terrestrial habitats (coastal development, erosion, beach armoring and nourishment, recreational activities, climate change, artificial light) and in marine habitats (interaction with fisheries, human exploitation, marine debris and pollution, climate change, entanglement) (Lutcavage et al., 1997; Casale et al., 2018). In recent centuries,

interactions with humans have drastically reduced the global abundance of turtles, requiring conservation efforts (Lutcavage et al., 1997). Globally, *C. caretta* turtle is divided into regional management units (RMUs), identified through mitochondrial (mtDNA) and nuclear (nDNA) DNA (Vella & Vella, 2023). The philopatry of loggerhead turtles has genetically differentiated the Mediterranean subpopulation from the Atlantic subpopulation (Carreras et al., 2011) and this required the establishment of an independent RMU (Hochscheid et al., 2022). Globally, *C. caretta* is listed as 'vulnerable' on the IUCN Red List (Casale & Tucker, 2017). Recently, the Mediterranean subpopulation has been downgraded to 'least concern' (Casale, 2015) due to an increase in nesting, showing the success of conservation projects. However, improvements seem to be dependent on conservation efforts (Hochscheid et al., 2022) and several conservation measures are needed to maintain this status (Camiñas-Hernández et al., 2020). *C. caretta* is also protected by other international conventions such as the Barcelona Convention (Appendix II), the Convention on Migratory Species (CMS, Appendix I), the Bern Convention (Appendix II) and the Convention on International Trade in Endangered Species (CITES, Appendix I) (Vella & Vella, 2023).

To date, the loggerhead turtle is the most abundant sea turtle species in the Mediterranean (Broderick et al., 2002) with approximately 10 000 nesting females, but this number may be underestimated (Hochscheid et al., 2022).

1.2 Threats to loggerhead turtle populations

Due to their long life cycle, late sexual maturity, migrations and habitat changes, marine turtles are particularly vulnerable to several threats, both natural and anthropogenic (Abalo-Morla et al., 2022). Each life cycle stage and each habitat visited is characterized by a different pattern of threats that can affect individuals (Bolten et al., 2011). A deeper understanding of the threats and their effects is crucial for developing appropriate mitigation and conservation strategies (Hamann et al., 2010).

1.2.1 Natural threats

Environmental variables such as temperature variations (excessive overheating or cooling), sediment granulometry, salinity, humidity, natural catastrophes and inundation caused by tides or storms are some of the factors that most influence the survival of turtles in their early life stages (Bolten et al., 2011; Limpus et al., 2020; Pietroluongo et al., 2023). However, in many areas it has been observed that the main threats of natural origin are interactions with other organisms, such as predation of nests by native or alien species, plant root invasion of the nest, diseases, parasites and pathogenic infections (Butler et al.,

2020; Bolten et al., 2011). For example, an emerging natural threat is a disease called sea turtle egg fusariosis (SEFT): it is caused by species of the *Fusarium solani* complex (FSSC) that infect turtle eggs, leading to the death of embryos and increasing mortality within nests (Gleason et al., 2020). Human activity can indirectly influence these natural threats, worsening their consequences: the introduction of non-native predator species, the rising temperatures and sea levels and the increasing frequency of extreme weather events linked to anthropogenic climate change are having an even greater effect on the survival of the hatchlings (Reece et al., 2013; Bolten et al., 2011).

1.2.2 Anthropogenic threats

Many of the drivers threatening the survival of *C. caretta* are mainly related to human activities (Casale et al., 2010; Casale & Tucker, 2017). The main threats are interactions with fishing gear (bycatch) (Casale, 2011), entanglement (in ghost nets or debris), collision with vessels, habitat alteration related to coastal development (human presence, tourism, beach armoring and nourishment, sand mining, coastal erosion, dredging), pollution (marine debris ingestion, macro and microplastics, oil pollution, artificial light pollution, noise pollution, presence of contaminants and other chemical pollutants), direct human exploitation for food and ornaments, climate change and its consequences

(Fuentes et al., 2011; Bolten et al. , 2011; Casale & Tucker, 2017; Lutcavage et al., 1997; Casale et al., 2018).

1.3 Plastic pollution: characteristics, origin and classification

Marine litter is increasing worldwide, representing an emerging threat to marine biodiversity (Barnes et al., 2009). Marine litter is defined as 'any persistent, manufactured or processed solid material discarded, disposed or abandoned in the marine and coastal environment' (UNEP). Most marine litter is composed of plastic (Deudero & Alomar, 2015), a mix of synthetic organic polymers (Worm et al., 2017). Plastic (and other petroleum-based products) pollution is a widely recognized problem in the marine environment, and it is still far from being solved (Borrelle et al., 2020; Ryan et al., 2009; Williams & Rangel-Buitrago, 2022). In 2018, global plastic production reached 360 million tons (PlasticsEurope, 2019) and it is expected to increase further (Lebreton & Andrady, 2019); 79% of the plastic produced ended up in landfills or the natural environment (Geyer et al., 2017). Plastics are versatile in countless applications due to their durability, flexibility, hydrophobic nature, low production cost and resistance to degradation (Lebreton & Andrady, 2019); however, these properties make them difficult for nature to assimilate (Geyer et al., 2017). 80% of the plastic present in the sea originates from land-based sources (beach use,

rivers and sewage); however, in some environments, marine sources (ship traffic and platforms) may have a greater impact (Ivar do Sul et al., 2009).

Plastics are highly persistent and durable (Deudero & Alomar, 2015), potentially remaining chemically intact for centuries (Worm et al., 2017). Plastic objects decompose slowly through processes of photodegradation, thermo-oxidation, biodegradation, hydrolysis and mechanical abrasion (Andrady, 2011; Zhang et al., 2021); in marine environments these processes occur even more slowly than on land (Andrady, 2011). Plastic fragments can be classified according to size: macroplastics (>5mm), microplastics (between 5mm and 1μm) and nanoplastics (<1μm), although this classification is still debated (Frias & Nash, 2019). Other attributes used to classify plastics are polymer type, color, shape, origin and toxicity (Frias & Nash, 2019). The most common polymers found in marine environments are polyethylene (PE), polypropylene (PP), polyester, polyamide and acrylic (PP&A) and polystyrene (PS) (Erni-Cassola et al., 2019). According to their origin, microplastics are divided into: primary microplastics, intentionally produced for commercial and industrial uses (exfoliants, cosmetics, shipbreaking, abrasives) and introduced into the marine environment through run-off; secondary microplastics,

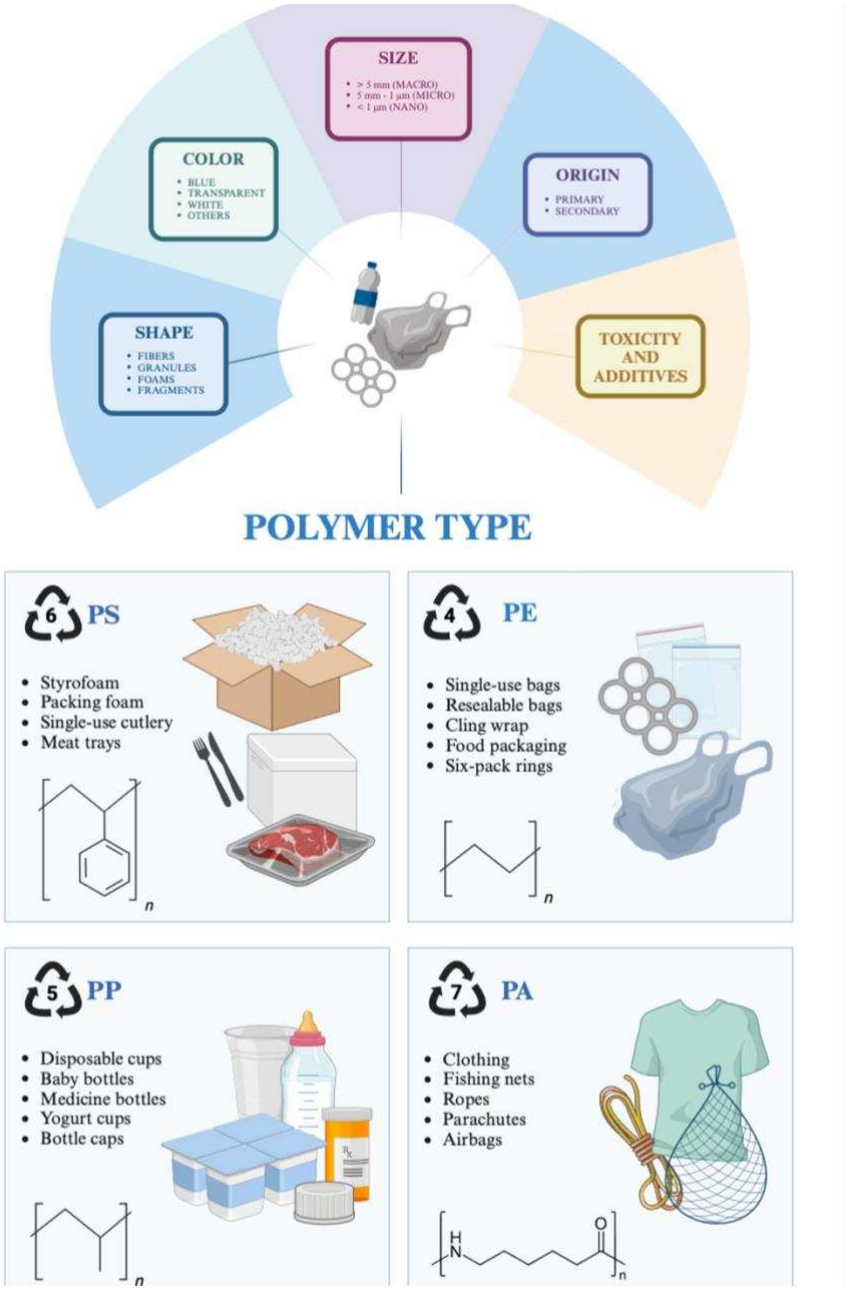

***Figure 6**: microplastic classification (modified from Ekanayaka et al., 2022)*

produced by the degradation of larger plastic fragments (Andrady, 2011; Ramkumar et al., 2022) (Figure 6).

One of the main objectives to achieve Good Environmental Status (GES) is to monitor the concentration of pollutants in marine environments and their

impact on marine biota (Sbrana et al., 2023). To do this, marine litter have been listed as descriptor 10 of the Marine Strategy Framework Directive (MSFD, European Commission, 2008), selecting some sentinel species to survey its effects on biota. To assess the impact of plastic pollution on organisms, bioindicators such as large marine vertebrates (Fossi et al., 2012) and *Fulmarus glacialis* (Van Franeker et al., 2011) have been used. In 2012 loggerhead turtle *C. caretta* was proposed as a target-indicator species in the Mediterranean (Camedda et al., 2012) for quantitative and qualitative monitoring of marine litter (Di Renzo et al., 2021; Galgani et al., 2014). Loggerhead turtles fulfill the requirements to be a valid bioindicator: they have feeding habits involving different habitats and prey from various trophic levels, strong inclination to regularly ingest debris, wide spatial and temporal distribution, and availability of samples (Matiddi et al., 2017; Baudouin et al., 2019). *C. caretta* as a bioindicator has been validated by several studies conducted at the European level (Indicit project) and several GES scenarios have been proposed, assessing their suitability (Matiddi et al., 2024). To date the more appropriate and conservative threshold value to be adopted to achieve GES is: values greater than 0,05g of ingested plastic should be detected in less than 33% of turtles, as no lethal effects have been observed at these concentrations (Matiddi et al., 2024).

1.4 Polycyclic aromatic hydrocarbons (PAHs)

Polycyclic aromatic hydrocarbons (PAHs) are a ubiquitous class of environmental pollutants found in air, water, sediment, and soil (Zychowski & Godard-Codding, 2017; Camacho et al., 2012). They are a large group of different substances characterized by the presence of at least two benzene rings (Duran & Cravo-Laureau, 2016); the different chemical structures (different molecular weight, substituents, number, position and arrangement of aromatic rings) have different consequences in terms of interactions and effects on biological systems (Hylland, 2006; Hutchinson & Simmonds, 1991). PAHs have a natural origin, but human activities have considerably increased their environmental level in marine ecosystems (Duran & Cravo-Laureau, 2016). There are 4 sources of PAHs: they can be produced by organisms (biogenic), derived from transformation processes in soils and sediments (diagenic), derived from incineration processes (pyrogenic) or from fossil fuels (petrogenic); only pyrogenic and petrogenic PAHs are quantitatively relevant in marine ecosystems (Hylland, 2006). PAHs reach marine ecosystems through sewage, road runoff, oil spills, smelter industry, offshore activities, outboard motor exhaust (Hylland, 2006), volcanic activity and forest fires (marginal) (Bamforth & Singleton, 2005).

US Environmental Protection Agency listed 16 PAHs as priority compounds for monitoring due to their potential toxicity (ATSDR, 2007). PAHs are highly toxic, mutagenic, immunotoxic, carcinogenic and teratogenic to many organisms (Arienzo et al., 2023). Sea turtles are one of the main targets of PAHs (Meador et al., 1995) and are therefore considered sentinel species and good indicators of marine contamination (Perugini et al., 2006). Their exposure to PAHs is considered chronic and can occur through skin contact, inhalation, direct ingestion of oil or consumption of contaminated prey and sediment (Cocci et al., 2019).

1.5 Effect of plastics and related chemical contaminants on marine organisms

Several studies have shown the impacts caused at various levels by chemical pollution on sea turtles (see Dias et al., 2024 for a review), also highlighting possible synergistic effects exacerbated by exposure to plastics (see Sun et al., 2022 for a review).

Plastics in marine environment can interact with organisms in multiple ways, including habitat degradation, entanglement, carcinogenesis, toxicity, endocrine disruption, internal abrasion, clogging, spread of invasive species and carriage of other persistent contaminants (Deudero & Alomar, 2015). Entanglement in macroplastic debris or ghost nets (abandoned or lost fishing

nets) (Baudouin et al., 2019) can cause injury and infection due to abrasion, reduced mobility, and even drowning and death (Laist, 1997). Debris ingestion affects at least 208 species (Gall & Thompson, 2015) and can occur accidentally, if these are mixed with natural food or because they are encrusted by prey, or voluntarily, actively selected following visual or olfactory cues because they are similar to natural prey (Casale et al., 2016; Worm et al., 2017). Microplastics are within the size of many planktonic organisms and thus they can be ingested by low trophic level organisms such as filter and deposit feeders, detritivores and planktonic organisms (Wright et al., 2013; Eastman et al., 2020). In this way, microplastics become bioavailable to a wide range of organisms (Wright et al., 2013) and they can be bioaccumulated and transferred through the trophic web in a process called biomagnification: this also represents a possible route of indirect exposure to contaminants associated with plastics (Wright et al., 2013; Worm et al., 2017; Kershaw & Rochman, 2015). Once in the marine environment, also PAHs become bioavailable to marine organisms, associating with suspended particles and sediments (Sinaei & Zare, 2019). They can be bioaccumulated in the tissues of invertebrate organisms (Meador et al., 1995). However, for PAHs biomagnification through the marine food web is considered insignificant (Roscales et al., 2011; Camacho et al., 2012), due to the efficient metabolization mechanism that occurs in the liver of

vertebrates (Rossi et al., 2023). However, some authors argue that the metabolization of PAHs can lead to the production of more toxic compounds (Varanasi et al., 1989).

Ingestion has been documented in all sea turtle species and in all ocean basins (Casale et al., 2016) and it is estimated that 52% of sea turtles have ingested plastic litter (Schuyler et al., 2016). Sea turtles show a higher risk of plastic ingestion compared to other species (Moon et al., 2022), probably due to the keratinized papillae in their esophagus, which inhibit regurgitation (Müller et al., 2012). Due to their visual hunting, sea turtles can ingest plastic items (Camedda et al., 2014) mistaking them for zooplanktonic prey (jellyfish, siphonophores, euphausiids, larvae and fish juveniles) (Deudero & Alomar, 2015). This occurs when feeding in both neritic and pelagic habitats (Camedda et al., 2014), but early life stages are potentially more vulnerable (Eastman et al., 2020), as the oceanic convergence zones where they live also concentrate debris (Rees et al., 2016). In the Mediterranean, it appears that exposure to plastic pollution remains similar in all life stages, occupying approximately the same trophic niche (Mariani et al., 2023; Matiddi et al., 2024).

Debris ingestion can cause obstruction, inflammation or damage to the digestive tract, often leading to the death of the animal (Casale et al., 2016; Bjorndal et al., 1994). Ingestion of debris can also cause sub-lethal effects such

as nutrient dilution, which occurs when non-nutritive elements displace food in the gut (McCauley & Bjorndal, 1999). Another side effect is floating syndrome, making the turtle more vulnerable to bycatch, collisions and malnutrition (Casale et al., 2016; Nelms et al., 2016). These effects can result in poor health, reduced growth and reproduction (McCauley & Bjorndal, 1999; Rees et al., 2016).

Microplastics are also associated with other indirect harms, such as chemical toxicity (Rees et al., 2016). Due to the large size of their polymers, they have been considered biochemically inert materials (Teuten et al., 2009). However, plastics in the environment are associated with a complex mixture of chemicals (Rochman, 2019): 78% of the chemicals listed as priority pollutants by the US Environmental Protection Agency (EPA) are associated with plastic debris (Rochman et al., 2013). These are residual monomers, additives and plasticizers used during production (such as phthalates and bisphenol A, S and F) and intermediates from the partial degradation or combustion of plastics (styrene and other aromatics) (Andrady, 2011). Furthermore, due to their large surface area-to-volume ratio and hydrophobicity, microplastics can transport, accumulate and release Persistent Organic Pollutants (POPs), concentrating them up to six orders of magnitude more than the surrounding water (Wright et al., 2013). These pollutants include, for example, polychlorinated biphenyls

(PCBs) (Zarfl & Matthies, 2010), polycyclic aromatic hydrocarbons (PAHs) (Teuten et al., 2009), organochlorine pesticides such as DDT (Monagas et al., 2008; Do Sul & Costa, 2014) and heavy metals (Godley et al. 1999, Maffucci et al. 2005, García-Fernández et al. 2009).

The ecotoxicological effects of chemical contaminants in sea turtles are not yet well documented (Finlayson et al., 2016), but several studies show how they can have relevant effects on the health of different marine organisms: pollutants have been associated with immune system suppression (Keller et al., 2006), carcinogenesis and endocrine disruption (Oehlmann et al., 2009; Rochester & Bolden, 2015), degeneration of hatchlings' organs and low emergence success (Perrault et al, 2011), decrease in hatching success (De Andrés et al., 2016), potentially altered physiological and metabolic conditions (Camacho et al., 2013), altered gut microbiome (Iurk et al, 2024), hepatic stress (including glycogen depletion, lipidosis and cellular death in fish) (Rochman et al., 2013), behavioral changes in fish (Oehlmann et al., 2009), inflammation processes in many invertebrates (Avio et al., 2017) and vertebrates, including humans (Amran et al., 2022). In particular, chronic exposure to PAHs can cause effects such as cancer, developmental and reproductive abnormalities, respiratory problems, genotoxicity, immunotoxicity, negative alterations in eating behavior (Zychowski & Godard-Codding, 2017), oxidative stress, alteration of

endocrine regulation (Hylland, 2006), alteration of the degree of DNA methylation (Cocci et al., 2018), alteration of enzyme activity (Rossi et al., 2023), DNA fragmentation (Casini et al., 2018), possible mortality (Figure 7). High values of microplastics have been demonstrated in many species of invertebrates and fish that serve as prey for loggerhead turtles (Curl et al., 2024). Once inside the organism, small microplastics can cross cell membranes and reach through the circulatory system different target body tissues in a process called translocation (Curl et al., 2024). This translocation has been demonstrated in invertebrates and fish and appears to vary with fragment size, duration of exposure, and species characteristics (Brennecke et al., 2015; Lu et al., 2016; Cattaneo et al., 2023). Likewise, PAHs can reach different target tissues inside the organisms: they have been highlighted in blood plasma (Cocci et al., 2018), liver (Vilca et al., 2018), pectoral muscle and adipose tissue (Perugini et al., 2006), stomach, small intestine, large intestine (Athey et al., 2016) and salt gland (Harms et al., 2019). The fact that microplastics and other chemical contaminants can be mobilized within the organism supports the hypothesis of maternal transfer as a possible route of exposure for embryos (yolk). This has recently been demonstrated in zebrafish (*Danio rerio*) (Pitt et al., 2018), humans (Ragusa et al., 2021) and proposed to explain the presence of microplastics in cuttlefish (*Sepia officinalis*) yolk (Chemello et al., 2022).

The actual presence of microplastics within turtle eggs has been little studied so far. Recently, microplastics have been found for the first time in the yolk and liver of *C. caretta* late-stage embryos (Chemello et al., 2023) and within unviable, undeveloped loggerhead turtle eggs (Curl et al., 2024).

Several works have also proven the presence within turtle eggs of chemical contaminants related to plastic pollution: POPs found include PCBs and PBDEs (De Andrés et al., 2016; van de Merwe et al., 2010), DDT and pesticides (Alava et al., 2006), phthalates (Savoca et al., 2018; Savoca et al., 2021), PAHs (Holliday et al., 2008; Alam & Brim, 2000) and others (Muñoz & Vermeiren, 2020).

The effects of microplastics and plastic-related chemical contaminants on the health and development of the embryo are still unclear. A positive correlation between microplastic presence and the number of melanomacrophages in the liver has been observed, suggesting a hepatic stress response (Chemello et al., 2023). PAHs exposure in turtle could be related to reduction of egg fertilization and hatching success (Sinaei & Zare, 2019), occurrence of embryo deformations and reduced embryo survival rates (Van Meter et al., 2006). Other POPs exposure was linked with a reduction in hatching success (Andrés et al., 2016) and a decrease in mass:length ratio (van de Merwe et al., 2010). Studies in other oviparous reptile species have shown that POPs can have negative

effects on embryonic development, such as increased egg mortality in American alligator (*Alligator mississippiensis*) (Guillette & Gunderson, 2001) and embryonic mortality and deformities in freshwater snapping turtle (*Chelydra serpentina*) (De Solla et al., 2008) (Figure 7).

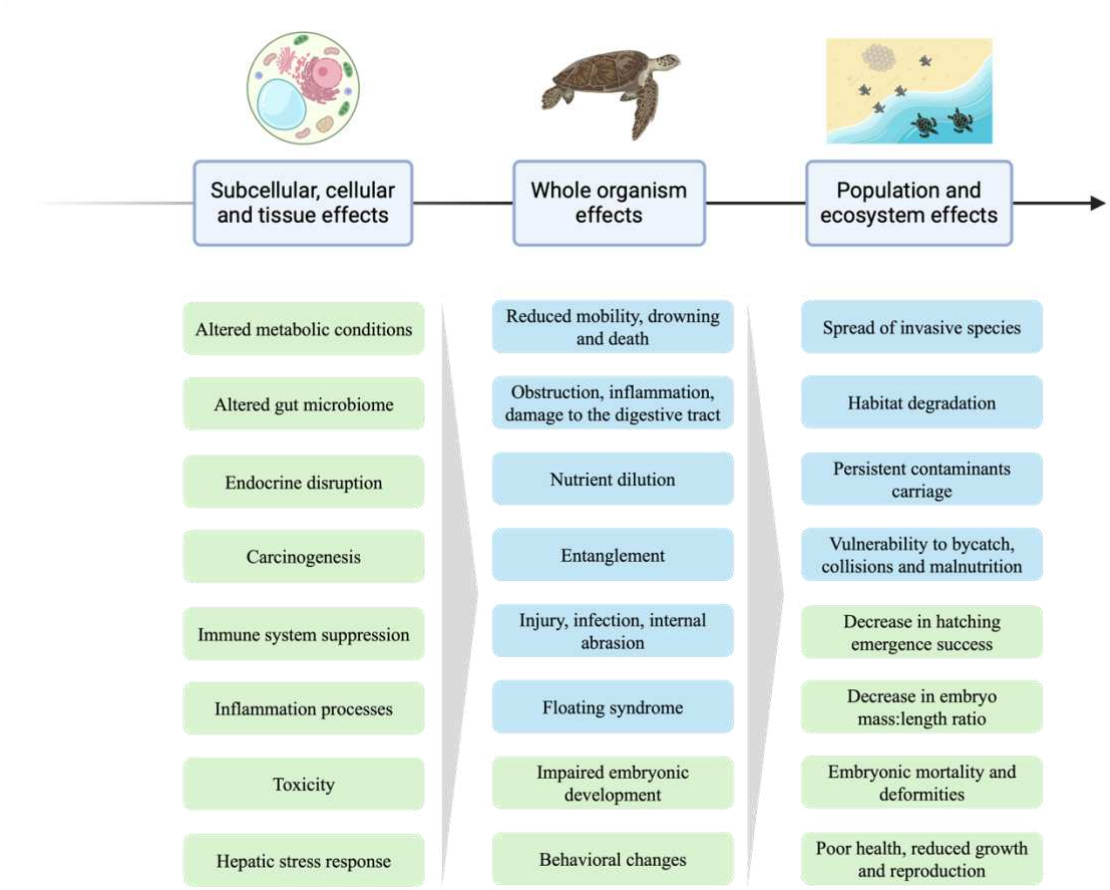

Figure 7*: macroplastics (in blue) and microplastics and related pollutants (in green) effects on marine organisms*

Contamination of eggs appears to occur by maternal route through yolk: several studies argue that this is the most probable explanation for the transfer of microplastics (Chemello et al., 2023; Curl et al., 2024), POPs (van de Merwe et al., 2010; Muñoz & Vermeiren, 2020; Stewart et al., 2011; Savoca et al.,

2021), heavy metals (Sinaei & Bolouki, 2017) and neurotoxins (Perrault et al., 2016). Some authors have also shown possible contamination of eggs through uptake from the environment surrounding the nest (Sousa-Guedes et al., 2023).

1.6 Assessing health status: biomarkers

In order to obtain an early warning of the biological effects caused by contaminants in the marine environment and to assess the health status of organisms, indicators called biomarkers are used (Lam & Gray, 2003). Biomarkers of xenobiotic contamination are quantitative measures of biological changes in response to exposure and/or doses of xenobiotic substances; these changes can occur at cellular, biochemical, molecular or physiological levels (Lam & Gray, 2003). The most sensitive sampling matrices to pollutant exposure used to test the health status of sea turtles are blood, cell cultures (primary fibroblasts), liver, carapace, adipose tissue and eggs (Dias et al., 2024).

1.6.1 Liver as sampling matrix

The liver is the largest organ located within the coelomic cavity, organized into two lobes (Silva et al., 2011). The liver performs several functions, including metabolism of proteins, carbohydrates and fats, waste management and removal of toxic substances (Silva et al., 2011). It is the main organ responsible for the metabolism and detoxification of xenobiotics, which is why many health

disorders are associated with impaired liver functions (Dias et al., 2024). In females, the liver also plays a key role in yolk production: it is involved as a reserve source for the synthesis of vitellogenin (main yolk precursor protein) and lipids that will be transferred to the ovarian follicles during vitellogenesis, and that will support energy requirements during embryogenesis (Muñoz & Vermeiren, 2023). The quality of the yolk inserted into the eggs reflects the quality of the maternal diet during the foraging phase (White, 1991): it is during this period that vitellogenesis occurs; many hydrophobic and lipophilic contaminants such as POPs could be assimilated and bind to the hepatic lipid component, which will then be transferred into the yolk (Savoca et al., 2021; Muñoz & Vermeiren, 2023). The embryo's liver is then the organ responsible for assimilating the lipids present in the yolk (Noble, 1991). Embryos could therefore be exposed to the presence of contaminants transferred maternally via the yolk (Savoca et al., 2021; Muñoz & Vermeiren, 2020). This makes the liver a sensitive matrix in which to study the health status of turtles (Gu & Manautou, 2012).

1.6.2 Cytochrome P450 (CYP450)

Once absorbed by the organism, environmental pollutants such as PAHs and halogenated hydrocarbons (e.g. PCBs, chlorinated dibenzofurans and chlorinated dibenzodioxins) (Yawetz et al., 1997) are readily metabolized

through a biotransformation process: this occurs thanks to biotransformation enzymes that are mainly present in the liver (Sinaei & Zare, 2019). The enzymatic transformation involves two phases: during phase I, a polar group is generally introduced into the xenobiotic molecule through oxidative, reductive or hydrolytic processes (Sinaei & Zare, 2019), generally leading to an increase in the hydrophilicity of the compound (Richardson et al., 2010); during phase II, the resulting metabolites are conjugated with endogenous polar molecules, obtaining water-soluble conjugates that can more easily be excreted from the body (Sinaei & Zare, 2019) (Figure 8).

Cytochrome P450 (CYP450) represents a highly conserved family of hemoproteins involved in phase I of the biotransformation of hydrophobic chemicals (Yawetz et al., 1997). These enzymes are mainly found in the endoplasmic reticulum of hepatocytes (Coppock & Dziwenka, 2019). It has been shown that several pollutants (including PAHs) can cause induction of the

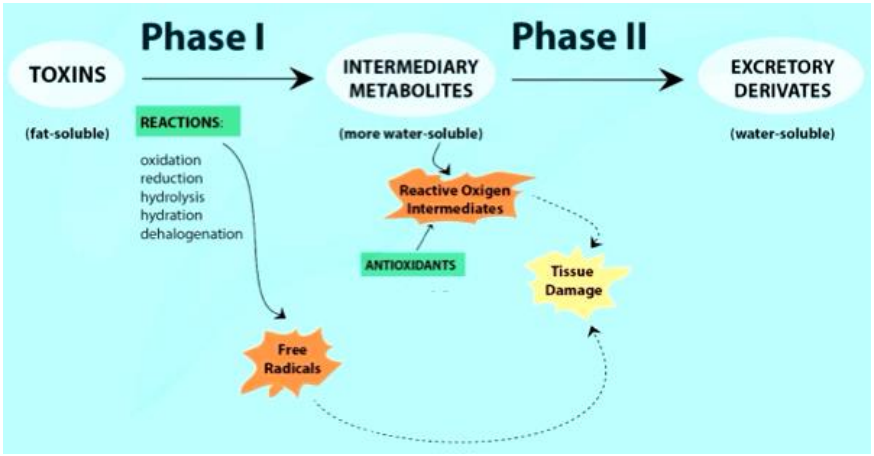

***Figure 8**: liver detoxification system (modified from Liska, 1998)*

CYP450 biotransformation system, especially the enzyme CYP450-1A (Yawetz et al., 1997). Exposure to PAHs and other compounds stimulates the upregulation of *de novo* synthesis of this enzyme (Coppock & Dziwenka, 2019), whose hepatic activity in fish increases within a week after the start of exposure (Hylland, 2006). Induction occurs when the xenobiotic binds to the cytosolic aryl hydrocarbon receptor (AhR), causing a signal transduction cascade and protein synthesis (Coppock & Dziwenka, 2019). Co-exposure to PAHs and microplastics was observed to increase CYP1A induction in fish liver compared to exposure with PAHs alone, suggesting possible synergistic effects of multiple contaminants on this biomarker (Takai et al., 2023). CYP450 enzymatic activity may also be toxic due to the production of reactive oxygen by-products (ROS): biotransformation of contaminants such as PAHs may also be linked to oxidative stress (Coppock & Dziwenka, 2019). For these reasons, CYP450 enzyme upregulation is considered a good biomarker to study the presence and effects of many contaminants (Coppock & Dziwenka, 2019).

1.6.3 Melanomacrophages

Melanomacrophages are pigmented phagocytes found in many non-mammalian vertebrates, mainly located in the kidney, pronephros, mesonephros, spleen and liver (Steinel & Bolnick, 2017; Wolke, 1992). They are visible under the optical microscope and recognizable as they are darkly

pigmented: this coloration results from the high content of lipofuscin, melanin and hemosiderin (Agius & Roberts, 2003). Melanomacrophages can be solitary (Wolke, 1992) or be found clustered in nodular aggregates known as melanomacrophage centers (MMCs) (Steinel & Bolnick, 2017). Under normal conditions, MMCs have the function of phagocytosing endogenous or exogenous material, including damaged cells (especially erythrocytes), debris, bacteria and fungi (Johnson et al., 1999; Steinel & Bolnick, 2017). They are also involved in iron recycling (Wolke, 1992) and storage of indigestible and/or toxic materials (Steinel & Bolnick, 2017). Moreover, MMCs have a function in both the innate and adaptive immune response: their phagocytic action makes them an important mechanism of elimination and detoxification of pathogens, free radicals and exogenous debris, possibly acting as long-term antigen retention sites (Agius & Roberts, 2003). Several studies performed on fish show how changes in the size, number and density of MMCs occur in relation to the presence of pollutants, spoilage, stress, chronic inflammation and chronic bacterial infection (Flint et al., 2009; Agius & Roberts, 2003). This makes MMCs a potential response (effect) biomarker for measuring the effects of exposure to different types of stress, including environmental pollutants (Basilone et al., 2018; Agius & Roberts, 2003; Passantino et al., 2014). This is

a response that can be easily visualized and quantified under an optical microscope, without using species-specific reagents (Steinel & Bolnick, 2017).

1.6.4 Heat Shock Protein 70 (HSP70)

Heat Shock Proteins (HSPs) are a group of proteins belonging to the molecular chaperone family (Moreira-de-Sousa et al., 2018) and are extremely conserved in every organism, from bacteria to mice to soybean (Richter et al., 2010). There are different classes of HSPs, classified according to their molecular weight (e.g. HSP100s, HSP90s, HSP70s, HSP60s, etc.) (Richter et al. 2010). Among these, the most studied are HSP70s (molecular weight of 70 kDa), which are characterized by high sensitivity and abundance and are expressed in all subcellular compartments (Moreira-de-Sousa et al., 2018). Under physiological conditions, HSP70 are involved in the proper de novo folding of proteins (Richter et al. 2010) and are expressed at low levels (De Toda & De la Fuente, 2015). Under stress conditions, they act as an emergency cellular response, eliminating protein misfolding and aggregation by folding proteins correctly (Bentley et al., 2017; Richter et al. 2010). Many pathological and physiological stressful stimuli can induce a significant increase in the synthesis of HSPs, in a process called the heat shock response (Verbeke et al., 2001). These stimuli include increased environmental temperature and exposure to chemical, physical or biological substances such as metals, metabolic

inhibitors, inflammatory and infectious processes, cell death or division processes and growth factors (Moreira-de-Sousa et al., 2018). HSP70 is also involved in cellular homeostasis, translocation of proteins across membranes, protection of neurons from apoptosis (Tedeschi et al., 2015), cytoprotection from oxidative-induced damage (Polla et al., 1996) and in anti-inflammatory and immune reactions (De Toda & De la Fuente, 2015). Several studies have used HSP70 as a biomarker to assess the effects of heat stress (in *C. caretta*: Bentley et al., 2017; Tedeschi et al, 2015), aging (De Toda & De la Fuente, 2015), oxidative stress (El Golli-Bennour & Bacha, 2011), non-halogenated hydrocarbon pollution (Rios-Sicairos et al., 2010), exposure to microplastics (Jaikumar et al., 2021) and other environmental stresses (see Moreira-de-Sousa et al., 2018 for a review).

2. AIM OF THE STUDY

The loggerhead sea turtles *Caretta caretta* are particularly vulnerable to various threats due to their long life cycle, late sexual maturity and migration (Mansfield & Putman, 2013). Anthropogenic impacts have increased in recent decades and are threatening the conservation of this species (Lutcavage et al., 1997; Casale et al., 2018). Some of the most widespread threats are entanglement and ingestion of plastic waste (macro- and microplastics) and chemical contaminants (e.g. PAHs), either free in the sea or associated with plastic fragments (Casale et al., 2018). Several studies have demonstrated the effectiveness of using *C. caretta* turtle as a bio-indicator species for qualitative and quantitative monitoring of these threats (Di Renzo et al., 2021).

Recent studies have demonstrated the presence of microplastics and chemical contaminants within turtle eggs from possible maternal transfer. However, effects of microplastic and pollutants on the development, health and survival of the embryo need to be further investigated to develop appropriate mitigation and conservation strategies.

The aim of this thesis was to deepen the knowledge on the effects of microplastics and contaminants on the health of *C. caretta* turtle embryos, focusing on yolk and liver samples. In particular, the presence of these pollutants was investigated through microplastics and PAH extraction. Their

potential effects on embryo health status were assessed through histological, immunohistochemistry and western blotting analysis to evaluate the expression of selected biomarkers: melanomacrophages and lipid percentage, Cytochrome P450 (CYP450), Heat Shock Protein 70 (HSP70).

3. MATERIAL AND METHODS

3.1 Data and sampling collection

The *C. caretta* eggs used in this study were taken from different beaches located in the Italian provinces of Matera (Basilicata, Italy), Cosenza (Calabria, Italy) and Taranto (Puglia, Italy). At the end of the maximum time expected for hatching, a total of 23 nests were sampled: we obtained 5 unhatched eggs from each nest apart from nests 45 TA (4 eggs), 37 TA (3 eggs), 11 TA (3 eggs), and 08 CS (3 eggs), for a total of 108 eggs. Eggs were stored in fridge at -20 °C until analysis began.

3.2 Sampling

A total of 108 eggs were sampled at the Laboratory of Reproductive and Development Biology, Department of Life and Environmental Sciences (DISVA), Polytechnic University of Marche (Ancona, Italy). Before sampling, the working area was cleaned and interdicted in order to minimize the risk of environmental contamination. Furthermore, the tools used (scissors, tweezers, aluminum containers) were made of non-plastic materials and were thoroughly cleaned before and after each use with deionized water and 70% pre-filtered ethanol. The eggs were taken from the fridge at -20 °C where they were stored and cleaned from the remaining sand using a brush. During this operation, the integrity of the eggshell was checked: eggs with cracks were also sampled, but

they were excluded from the analyses for microplastics and contaminants, as external contamination could not be excluded. Each egg was weighed, and the diameter was measured using a caliper; the average diameter was obtained as the mean value between the largest and smallest diameter. Using tweezers and scissors, the eggs were opened to assess the presence of the embryo. Where the embryo was present, it was separated from the shell and the following weight data were recorded:

1. Weight of mass embryo + residual yolk
2. Weight of the embryo alone
3. Weight of the residual yolk

Afterwards, the embryo was unfolded, and the following biometric measurements were recorded (Figure 9):

4. Crown-Rump Length (CRL): represents the straight-line length from the cranial to the caudal end of the body
5. Straight Carapace Length (SCL): represents the straight-line length of the carapace
6. Straight Carapace Width (SCW): represents the maximum straight-line width of the carapace
7. Straight Head Length (SHL): represents the straight-line length of the head, from the apex of the rhamphotheca to the nape of the neck

8. Straight Head Width (SHW): represents the maximum straight-line width of the head
9. Straight Plastron Length (SPL): represents the straight-line length of the plastron
10. Left and Right Forelimb Length (FLL left, FLL right): represents the straight-line length of the left and right forelimbs
11. Left and Right Hindlimb Length (HLL left, HLL right): represents the straight-line length of the left and right hindlimbs
12. Number of scutes present in the carapace, counted by distinguishing the three columns left, middle and right.

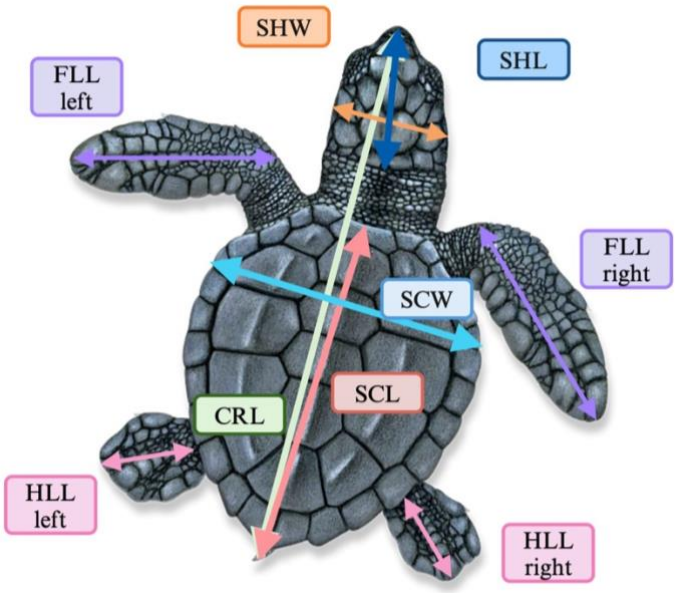

***Figure 9**: embryo biometric measurements*

A stage of embryonic development from <10 to 30 was then assigned to each embryo, following the guidelines provided by the dichotomous key of turtle

embryonic development elaborated by Miller et al. (2017). Any notes, such as the presence of molds or fungi, were also reported.

At this point, embryos sufficiently developed and in good preservation condition were opened by removing the carapace; the liver was removed and weighed. Liver samples were collected only for embryos at a developmental stage greater than or equal to 28. A portion of the liver was weighed, placed in an aluminum container for microplastic analysis and stored in the fridge at -20 °C. Another portion of the liver was weighed, placed in a 1,5mL Eppendorf tube and stored in formaldehyde/glutaraldehyde solution (NaH_2PO_4-H_2O + NaOH + Formaldehyde (36,5%) + Glutaraldehyde (25%) + H_2O) at 4 °C for subsequent histological analysis of the tissue. A third portion of liver was divided into two 1,5mL Eppendorf tubes for Western blot analysis and chemical analysis of contaminants (PAHs), respectively; both were stored in a refrigerator at -80 °C.

3.3 Histological analysis

Samples preserved in formaldehyde/glutaraldehyde solution were included in paraffin using the following protocol. The tissue fragment underwent 3 washes in 70% ethanol for 15 minutes each and stored in 70% ethanol overnight. The samples were then dehydrated in alcohol of increasing concentration: 45 minutes in 80% ethanol, 45 minutes in 95% ethanol, 60 minutes in 100%

ethanol (solution I) and 60 minutes in 100% ethanol (solution II). They then underwent two washes in xylene (Bio-Optica, Milan, Italy) for 15 and 30 minutes and were finally included in paraffin (Bio-Optica). Once the paraffin had solidified, the blocks were cut using a microtome (Leica RM2125 RTS, Nussloch, Germany) into 5μm thick sections.

In order to be able to observe liver sections at different levels, three sections for each sample were obtained 10 cuts apart (one section selected every 50μm). The three sections for each sample were laid out, placed on a microscope slide and dried. Tissue staining was done following the Mayer hematoxylin and eosin Y protocol (Merck KGaA) and then observed under an optical microscope (Zeiss Axio Imager.A2, Oberkochen, Germany). Several color pictures were acquired for each sample using the Axiocam 503 digital camera (Zeiss, Oberkochen, Germany). 6 images for each sample were then analyzed using ImageJ software to obtain the number and area occupied by melanomacrophages and the percentage lipid present in the tissue.

3.4 Immunohistochemistry protocol

The same protocol was used for the inclusion and cut of the sections used for immunohistochemical analysis. The microscope slides on which the tissue sections were laid were previously covered with gelatin. Two tissue sections were placed on each slide for each sample: one section will be incubated with

the antibody, and one will serve as a negative control. The staining protocol started with 2 different washes in xylene (Bio-Optica, Milan, Italy) of 10 minutes each. It is then soaked in different ethanol solutions at decreasing concentrations: 2 solutions of 100% ethanol for 5 min each, 5 min in 95% ethanol, 5 min in 80% ethanol, 5 min in 70% ethanol. Finally, it was rinsed for 5 min in running deionized water. Then, slides were incubated in an Antigen Retrieval solution (10mm sodium citrate and 0,05% (v/v), Tween-20, pH 6,6) for 20 minutes at 100 ºC. After a 5-min wash in deionized water, the slides were let to dry, and the samples were delimited using a hydrophobic pen. Membrane permeabilization was achieved by placing the slides in a humid chamber at room temperature for 20 min, treating each section with 25µL of (1% (v/v) Triton 48-100 in PBS 1X; pH 7,4). After 3 washes with PBS 1X of 5 min each, the slides were placed in a humid chamber at room temperature for 1 h, treating each section with 25µL of Blocking Solution (3% (w/v) of BSA, 0,1% (v/v) of Tween-20 and PBS; pH 7,4). Afterwards, the samples were incubated overnight in a humid chamber at 4 °C, treating the sections with 25µL of Rabbit anti-fish CYP1A peptide Polyclonal antibody, (CP-226, Biosense laboratories) antibodies in 1:100 concentration in blocking solution; one section was not incubated with the antibody and served as a negative control. The next day, the sections were rinsed with three PBS washes, in the same way as those described

above, and the secondary antibody (Goat anti-Rabbit IgG (H+L) Highly CrossAdsorbed Secondary Antibody, Alexa Fluor™ Plus 647, Thermofisher) was added and incubated at 37 °C for one hour. Once the PBS washes were repeated, a coverslip was mounted thanks to a mounting solution with fluorochrome that colors the nuclei (Mounting Medium with DAPI - Aqueous, Fluoroshield (ab104139, Abcam). The slides were seen under the Nikon A1R confocal microscope, and the analysis of the images was carried out with the program Fiji ImageJ (ImageJ, NIH, USA, https://imagej.nih.gov/ij/).

3.5 Liver digestion protocol and filtration of microplastics

The 10 liver samples selected for microplastics analysis were digested according to the following protocol. Before starting, the working area was cleaned and delimited to minimize the risk of external microplastic contamination; all instruments used were washed with deionized water and pre-filtered 70% ethanol and were kept covered with aluminum foil during the procedure. In addition, the use of plastic objects was avoided, favoring the use of non-synthetic clothes and disposable gloves. In order to be able to eliminate and correct any contamination during the procedure, a procedural blank was used, which underwent all the steps of the protocol, without, however, containing any liver samples inside; an environmental blank consisting of a fiberglass filter was also placed inside an open aluminum container near the

working table where the protocol was performed. The samples were weighed and a prefiltered 10% KOH digestion solution was prepared (glass fiber filter, 1.6μm pore size, Whatman GF/A); the solution was obtained by dissolving KOH tablets (Sigma-Aldrich) in deionized water. The solution was placed in glass Beckers containing the sample in a 1:10 weight/volume ratio and each Becker was covered with aluminum foil. The samples were then incubated at 40 °C for 24 hours, following a protocol similar to the one used by Di Renzo et al. (2021). The solutions were then filtered using glass fiber filters (1,6μm pore size, Whatman GF/A) placed on a funnel connected to a vacuum pump. The filters were dried and stored in closed aluminum containers until microscopic analysis.

3.6 Assessment of microplastic color, shape and size

The filters deriving from microplastic extraction, including environmental and procedural blanks, were firstly inspected by Olympus microscope (Horiba Scientific). All filters were examined using a ×10 objective (Olympus MPLAN10x/0,25). Each filter was observed by moving from left to right and in the opposite direction from top to bottom to analyze the entire surface of the filter. Once a microparticle of consistent size was detected, it was inspected with a ×100 objective (Olympus MPLAN100x/0,90). Once microplastics were recognized, they were morphologically characterized by recording the shape,

color, and size. Only microplastics smaller than 10μm were considered in order to exclude any external contamination.

3.7 Western blot analysis

Liver samples preserved for Western blot were weighed and where possible 0,1g was taken and placed inside Eppendorf tubes. Hanna buffer (Tris HCl 0,125 M, SDS 4%, Glycerol 20%, β-Mercaptoethanol 10%) at a ratio of 1:5 to the weight of the sample taken and antiproteolytic (Protease Inhibitor Cocktail, P2714, Sigma-Aldrich) at a ratio of 1:10 to the amount of Hanna buffer were added. All steps were done while keeping the tubes on ice to avoid degradation of the samples. The tissue fragments were homogenized using a pestle, previously washed in deionized water and ethanol. The tubes were placed in a stand and submerged in boiling water at 100 °C for 5 min. The samples were then centrifuged at 12000 g for 10 min using the centrifuge. Where the fat content was high, 5μL of 10% SDS was added at the end of centrifugation and centrifuged again at 12000 g for 10 min. Finally, the supernatant containing protein was extracted and placed in Eppendorf tubes.

The protein extracts were submitted to electrophoretic run according to the following Western blot protocol. 10μL of protein lysate for each sample was centrifuged at 12000 g for 10 min before adding 10μL of sample buffer (1:1 ratio) (Bromophenol blue 0,1%, β-Mercaptoethanol, Glycerol); solutions were

homogenized using a stirrer. Acrylamide gel was prepared by overlaying 4% stacking gel (1,55mL deionized water, 0,625mL TRIS HCl 0,5 M pH 6, 350µL Acrylamide 30%, 25µL SDS 10%, 5µL TEMED, 25µL APS 10%) for sample loading and 15% running gel (2,5mL deionized water, 2,5mL TRIS HCl 0,5 M pH 8,8, 5mL Acrylamide 30%, 100µL SDS 10%, 20µL TEMED, 50µL APS 10%). The gel was placed in the electrophoresis chamber submerged in 1L of Electrode buffer (3g TRIS, 14,4g glycine, 1g SDS, volume deionized water) and each sample was loaded into a different well. One well was allocated for loading 3µL standard molecular weights (Precision Plus Protein Standards, Bio Rad). The electrophoretic run was set at 30 mA for about 30 min, then at 60 mA until the end. Once the 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 membrane in place for 2 hours at 250mA. The all was submerged in 1L of cold transblotting buffer (6g TRIS, 28,8g Glycine, 200mL 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 wash 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 min. After that, the membranes were incubated overnight inside Falcon tubes containing the antibody HSP70 (sc66048, Santa Cruz Biotechnology) (1:1000 concentration in the same blocking solution) in incubator at 4 °C overnight. After 5 washes with T-TBS for 5 min each in an oscillator, the membranes were incubated inside Falcon tubes containing the secondary antibodies Anti-Mouse IgG peroxidase conjugate (a-9044, Sigma Aldrich) (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 developed in the dark with ClarityTM Western ECL Substrate (170-5061, Bio-rad) (500µL solution A, 500µL 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/).

3.8 PAHs extraction and quantification protocol

Extraction and quantification of PAHs were performed at the laboratories of the Department of School of Biosciences and Veterinary Medicine at the University of Camerino. PAHs in liver samples were extracted by a QuEChERS (quick, easy, cheap, effective, rugged, and safe) methodology, a non-targeted extraction methodology that allows the extraction of chemicals

with a range of polarities across many tissue types. Samples were thawed, homogenized, and 5mL of homogenized tissue was placed in 50mL tubes. MilliQ water (10mL) was added to each sample and stirred for 1 min; 15mL of acetonitrile was then added, stirring for 1 min. 5g of magnesium sulfate and 1g of sodium chloride were added to each tube, stirring manually for 2 min. The samples were centrifuged at 4500rpm for 8 min at 10 °C. The acetonitrile layer was placed in glass vials and evaporated with nitrogen gas. The dry extracts were dissolved in 1mL methanol, transferred to amber glass vials with Teflon lids and stored at -20 °C for further analysis. Identification and quantification of PAHs were performed using an HPLC system (Ultimate 3000, Thermo Fisher Scientific, Waltham, MA, USA). Where PAHs were detected (concentration greater than the LOD detection threshold), they were quantified if present above the LOQ quantification threshold; otherwise, their unquantifiable presence was reported as <LOQ.

3.9 Statistical analysis

Statistical analyses were performed using GraphPad Prism 8 software. Several variables were compared between the two experimental groups (embryonic developmental stages 28 and 30) using unpaired t test. Significance was set at $p \leq 0.05$.

4. RESULTS

4.1 Embryonic developmental stages

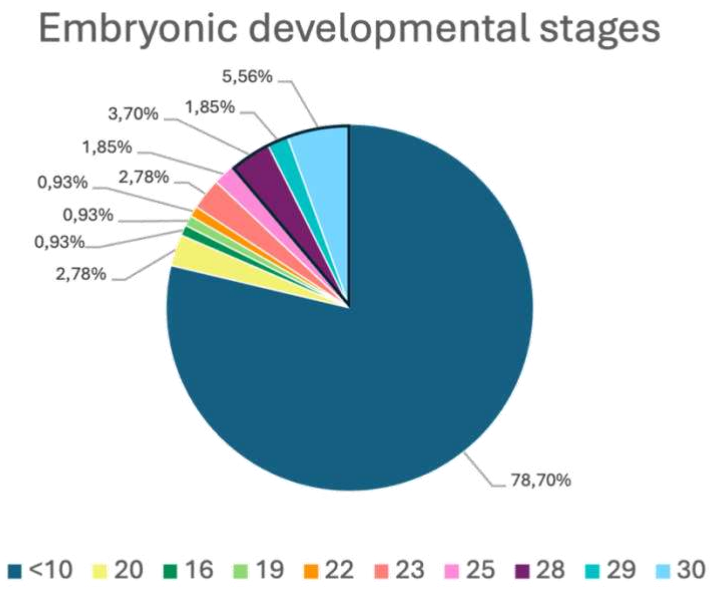

***Figure 10**: percentage frequency (%) of different embryonic developmental stages*

The majority of the eggs sampled (78,70%) was in a developmental stage lower than 10 (Figure 10), which included unfertilized eggs and early developmental stages after oviposition, where the embryo is barely distinguishable. All other stages above stage 10 were present in lower percentage, in ascending order: 11 embryos (10,19%) were at developmental stages between 10 and 27 and a total of 12 embryos (11,11%) were at developmental stages greater than or equal to 28, respectively stage 28 (4; 2,78%), stage 29 (3; 2,78%) and stage 30 (6; 5,56%) (Figure 10). Five of the six embryos at stage 30 belonged to the same nest (45MT).

Both embryos at developmental stage 29 were excluded from the analyses due to the poor state of preservation and the presence of breaks in the shell. After the evaluation of eggs integrity and embryos conditions, only 10 liver samples were suitable for analysis: 4 embryos at stage 28 and 6 at stage 30.

4.2 Biometric measurements of eggs and embryos

The comparison of biometric parameters between eggs at 28 and 30 developmental stages showed that egg weight, embryo + yolk weight and yolk weight were significantly lower ($p \leq 0{,}001$, $p \leq 0{,}01$, $p \leq 0{,}001$, respectively) in stage 28 respect to stage 30 (Figure 11A, B, C). Differently, embryo weight did not significantly differ between stages 28 and 30 (Figure 11D).

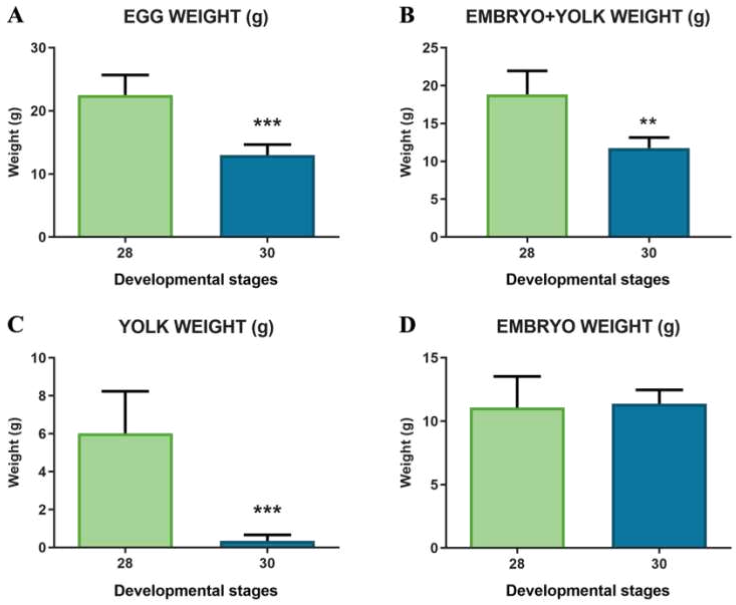

***Figure 11**: (**A**) egg, (**B**) embryo + yolk, (**C**) yolk and (**D**) embryo weight (g) comparison between 28 and 30 developmental stages. Data are reported as mean values ± standard deviation and significance was set at $p \leq 0{,}05$. *= $p \leq 0{,}05$; **= $p \leq 0{,}01$; ***=$p \leq 0{,}001$*

In Figure 12, measurements of Crown-Rump length (CRL, Figure 12A), carapace length (SCL, Figure 12B) and width (SCW, Figure 12C) were compared between stages 28 and 30. Statistically significant differences ($p \leq 0,01$) between embryos at different developmental stages were observed in all biometrics (Figure 12A, B and C) with higher average values in stage 30 compared to stage 28.

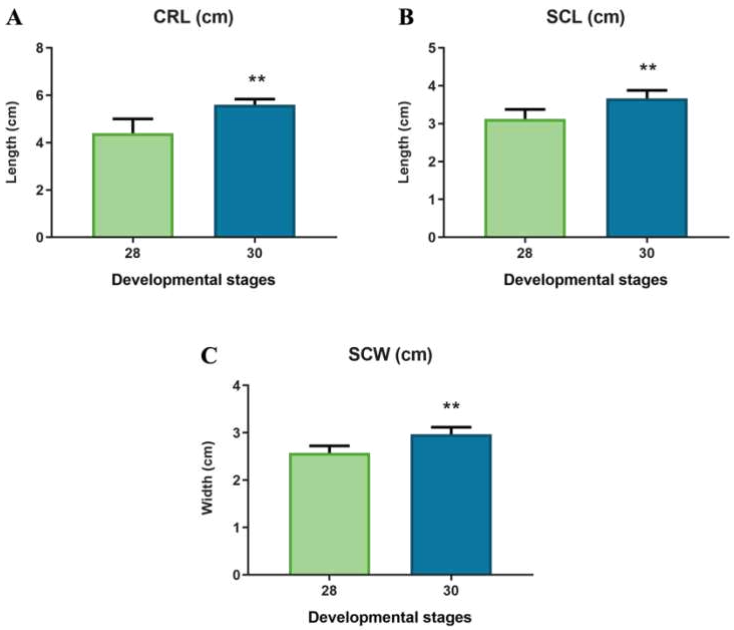

***Figure 12**: (**A**) Crown-Rump Length (CRL), (**B**) Straight Carapace Length (SCL) and (**C**) Straight Carapace Width (SCW) (cm) comparison between 28 and 30 developmental stages. Data are reported as mean values ± standard deviation and significance was set at $p \leq 0,05$. *= $p \leq 0,05$; **= $p \leq 0,01$; ***=$p \leq 0,001$*

Differently, when considering forelimb and hindlimb length (Figure 13) no significant differences were observed between embryos at stage 28 and 30 (Figure 13A, C and D), except for the right forelimb length which appeared

significantly higher in embryonic stage 30 compared to values of stage 28 ($p \leq 0,01$) (Figure 13B).

Counting the number of scutes for each column in the carapace (from left to right), different patterns were observed: the most common was 5-5-5 (60%), followed by 5-6-5 (20%), 5-7-5 (10%) and 5-5-4 (10%).

In total, 40% of the embryos had a carapace scutes pattern different from the modal pattern (5-5-5), which is considered the most common one (Sim et al., 2014).

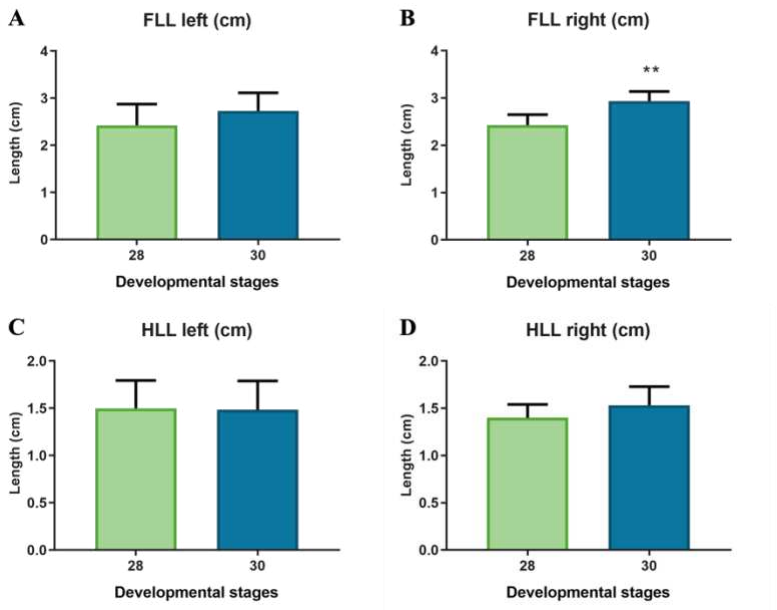

***Figure 13**: (**A**) Left and (**B**) Right Forelimb Length and Left (**C**) and Right (**D**) Hindlimb Length (cm) comparison between 28 and 30 developmental stages. Data are reported as mean values ± standard deviation and significance was set at $p \leq 0,05$. *= $p \leq 0,05$; **= $p \leq 0,01$; ***=$p \leq 0,001$*

4.3 Microplastics analysis

The analysis to detect microplastics in liver samples revealed the presence of microplastics in the 75% of samples analyzed. All microplastics observed were fragments of different colors: orange (25%), pink (12,5%), brown (12,5%), green (12,5%), and black (12,5%). No statistically significant differences were observed in the number of microplastics per gram of digested tissue between the two developmental stages considered (Figure 14).

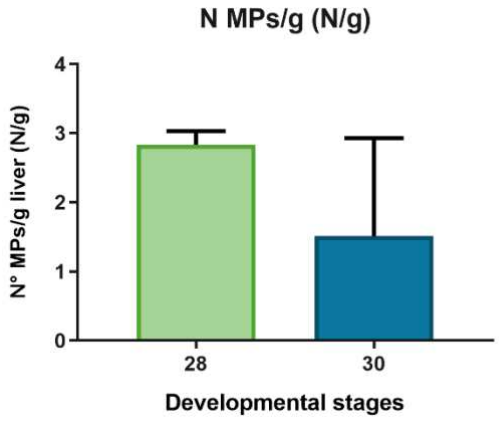

***Figure 14**: comparison between the number of microparticles per g of digested tissue in developmental stages 28 and 30. Data are reported as mean values ± standard deviation and significance was set at p ≤ 0,05. *= p ≤ 0,05; **= p ≤ 0,01; ***=p ≤ 0,001*

4.4 PAHs analysis

***Table 1**: PAHs detected (>LOQ) and relative concentration in ng/g for each sample*

ID	24PZ_5	29TA_2	29TA_5	34TA_2	31TA_4	45MT_1	45MT_2	45MT_3	45MT_4	45MT_5
PAHs ng/g	Acenaphthylene 9,906		Acenaphthylene 7,545		Acenaphthylene 1,759	Acenaphthylene 0,979	Acenaphthylene 0,334		Acenaphthylene 2,338	Benzo (a) pyrene 2,699
	Anthracene 8,380				Fluorene 5,666	Fluoranthene 2,436	Fluoranthene 2,085		Anthracene 1,707	
	Fluoranthene 23,710						Benzo (a) pyrene 2,659			
	Pyrene 0,986									
	Benzo (a) anthracene 2,397									
	Benzo (g, h, i) perylene 1,492									
MPS presence	X		X	X		X			X	X

Table 1 reports the concentrations of PAHs detected and the simultaneous presence of microplastics for each sample.

Table 2 shows the relative percentage frequency, and the maximum concentration of each PAH detected in liver and yolk samples. Acenaphthylene, Anthracene and Fluoranthene were detected in both liver and yolk samples from embryos at the developmental stage 28. Considering stage 30, only Fluorene was found in both liver and yolk samples.

Table 2: *frequency percentage of each PAH detected in liver and yolk samples from 28 and 30 developmental stages. For each PAH, the maximum concentration in ng/g is reported in parentheses. The double-line border separates low molecular weight PAHs (above) from high molecular weight PAHs (below)*

	Liver (ng/g)		Yolk (ng/g)	
	Stage 28	Stage 30	Stage 28	Stage 30
Naphthalene	0%	0%	0%	20% (4,9)
Acenaphthene	0%	0%	0%	20% (12)
2-Bromonaphthalene	0%	0%	0%	20% (1)
Acenaphthylene	30% (9,9)	67% (2,3)	100% (4,1)	0%
Fluorene	0%	17% (5,7)	0%	20% (0,8)
Phenanthrene	0%	0%	30% (1,7)	20% (1,4)
Anthracene	30% (8,4)	17% (1,7)	30% (4)	0%
Fluoranthene	30% (23,7)	33% (2,4)	30% (7,4)	0%
Pyrene	30% (1)	0%	0%	0%
Benzo (a) anthracene	30% (2,4)	0%	0%	0%
Chrysene	0%	0%	30% (1)	0%
Benzo (b) fluoranthene	0%	0%	0%	0%
Benzo (a) pyrene	0%	33% (2,7)	0%	0%
Indeno (1, 2, 3) PYRENE	0%	0%	0%	0%
Dibenz (a, h) anthracene	0%	0%	0%	0%
Benzo (g, h, i) perylene	30% (1,5)	0%	0%	0%

No statistically significant differences were observed in the sum of low molecular weight, high molecular weight, and total PAHs between developmental stages 28 and 30.

4.5 Histological analysis

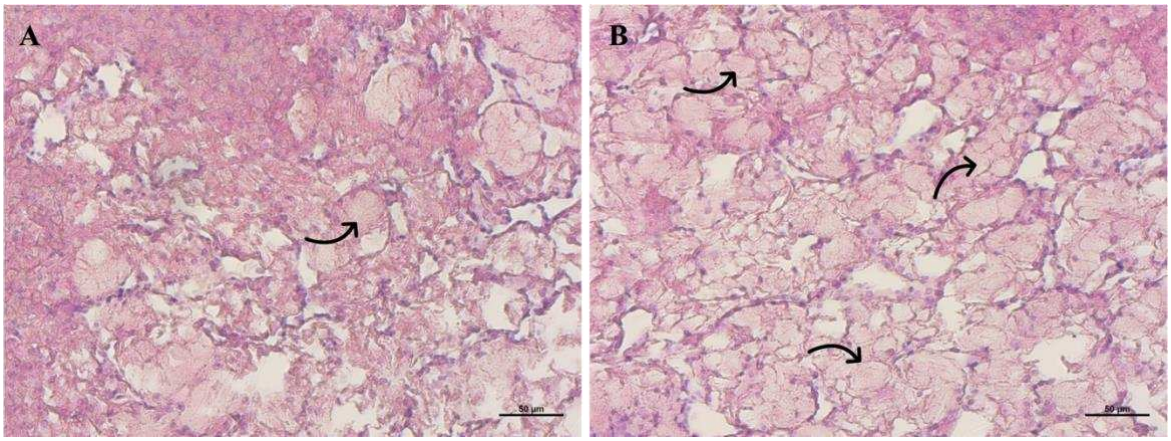

***Figure 15**: exemplifying histological images of embryonic liver sections with low (**A**) and high (**B**) lipid content. Black arrows indicate lipid granules. Scale bar: 50µm*

The number of lipid granules appeared higher in samples with a high mean lipid percentage respect to samples with low levels of lipid content (Figure 15).

Liver samples form embryos at the developmental stage 30 had a significantly higher lipid percentage ($p \leq 0.001$) respect to those from embryos at stage 28 (Figure 16).

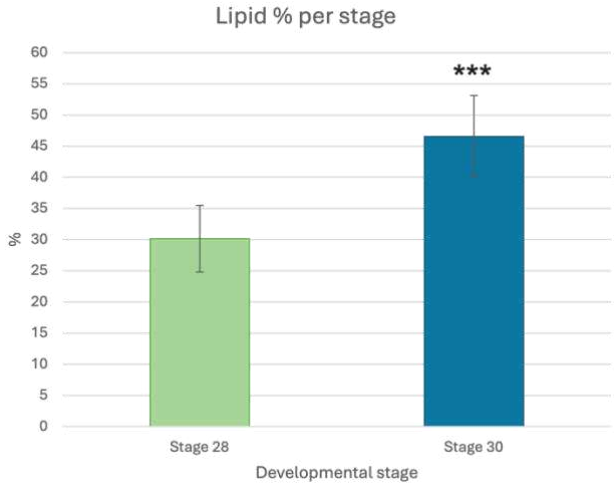

***Figure 16**: average lipid percentage in liver samples of embryos at developmental stages 28 and 30. Data are reported as mean values ± standard deviation and significance was set at $p \leq 0,05$. *= $p \leq 0,05$; **= $p \leq 0,01$; ***=$p \leq 0,001$*

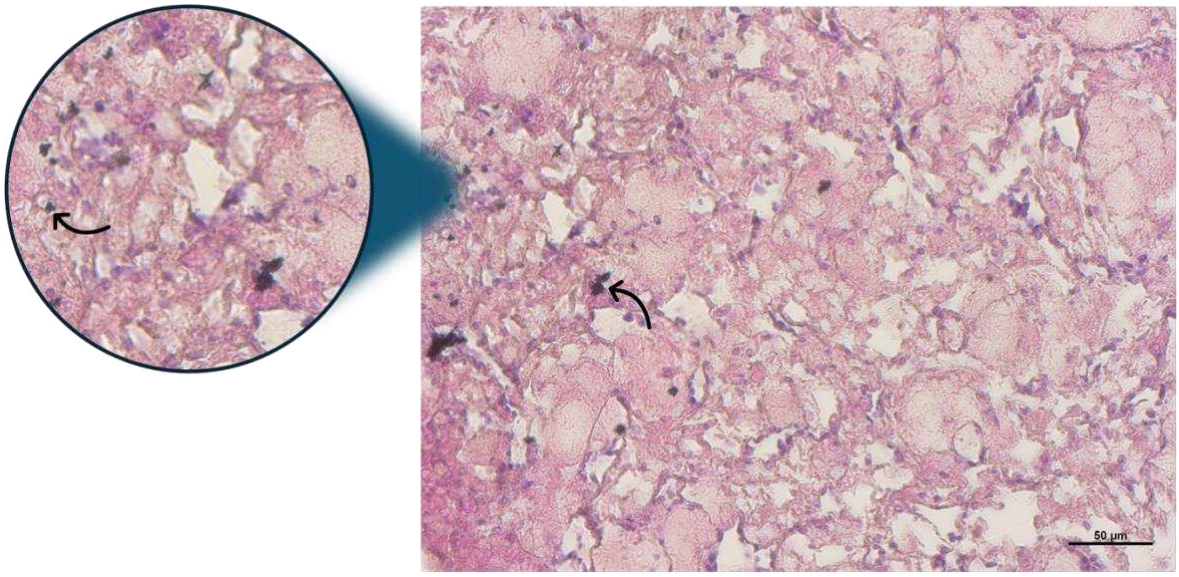

***Figure 17**: exemplifying histological images of embryonic liver sections. Black arrows indicated examples of melanomacrophages. Scale bar: 50µm*

No significant differences were observed concerning the number and size of melanomacrophages analyzed in liver samples from embryo at developmental stages 28 and 30 (Figure 18A and B).

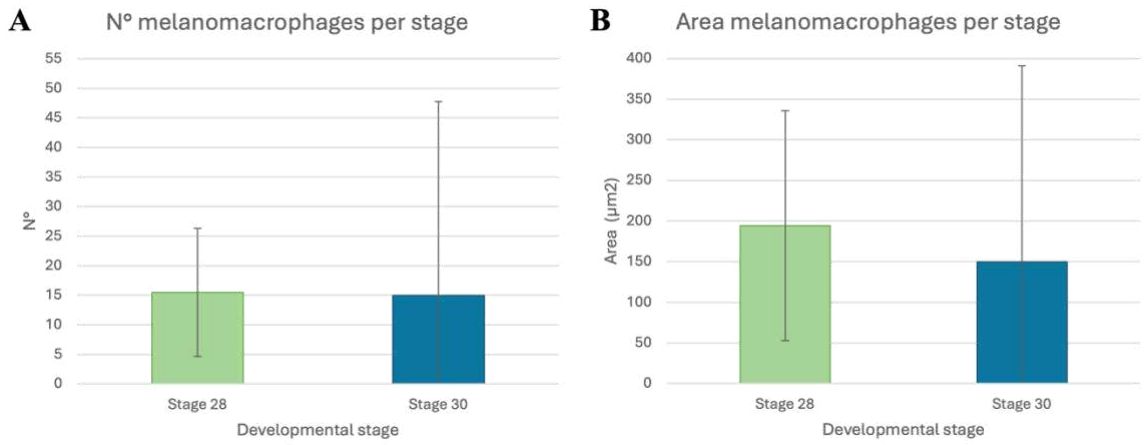

***Figure 18**: (**A**) average number of melanomacrophages and (**B**) average area occupied by melanomacrophages at developmental stages 28 and 30. Data are reported as mean values ± standard deviation and significance was set at $p \leq 0,05$. *= $p \leq 0,05$; **= $p \leq 0,01$; ***=$p \leq 0,001$*

4.6 Immunohistochemistry analysis

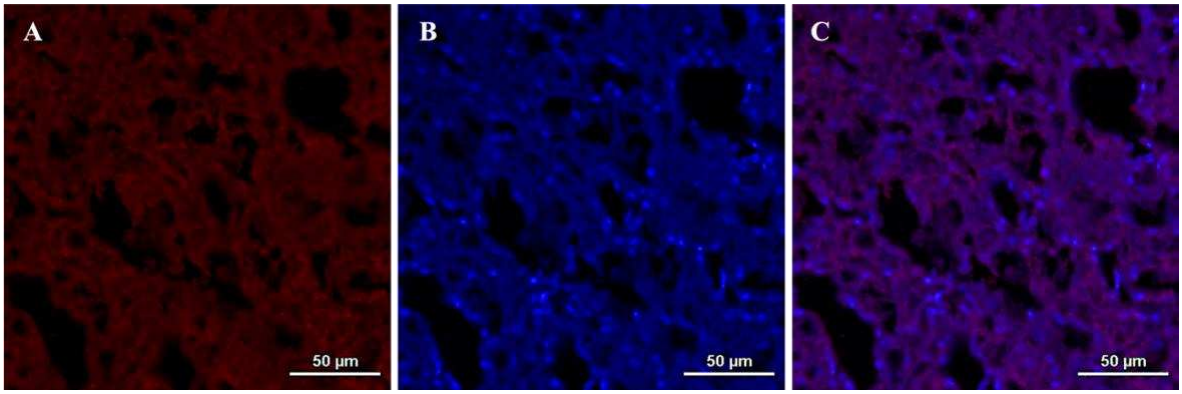

Figure 19: *example of immunohistochemistry images of liver section. Image **A** shows in red fluorescence CYP450 signal present in the tissue. Image **B** shows the nuclei and cytoplasm by DAPI staining (blue light). Image **C** shows the overlapping fluorescent signal of CYP450 and DAPI. Scale bar: 50µm*

Quantification of the CYP450 signal in samples at different developmental stages showed no significant difference between stage 28 and stage 30 (mean values 7.540 ± 0.743 a.u. and 5.902 ± 3.970 a.u., respectively) (Figure 20).

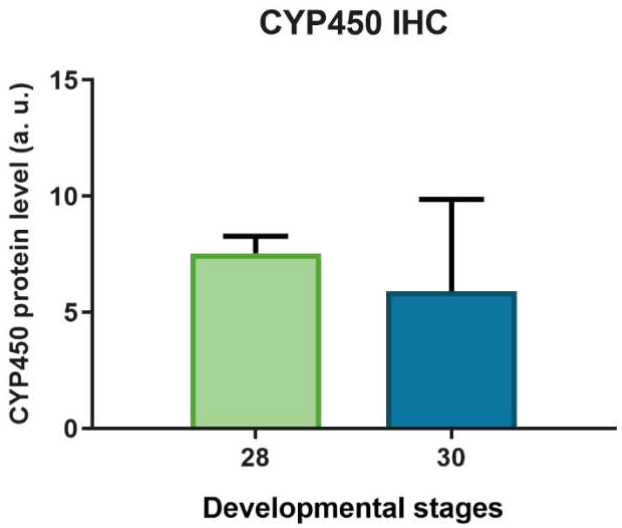

Figure 20*: comparison between CYP450 protein levels (expressed in arbitrary unit, a.u.) at developmental stages 28 and 30. Data are reported as mean ± standard deviation and significance was set at p ≤ 0,05. *= p ≤ 0,05; **= p ≤ 0,01; ***=p ≤ 0,001*

4.7 Western Blot analysis

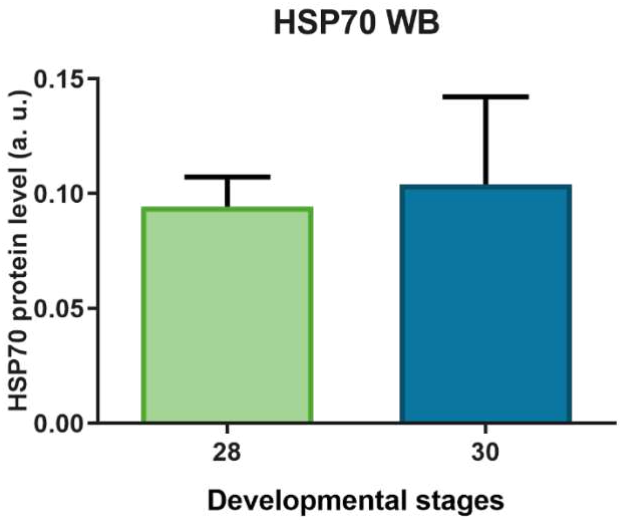

Figure 21*: comparison between HSP70 protein levels (expressed in arbitrary unit, a.u.) at developmental stages 28 and 30. Data are reported as mean ± standard deviation and significance was set at p ≤ 0,05. *= p ≤ 0,05; **= p ≤ 0,01; ***=p ≤ 0,001*

Quantification of HSP70 protein obtained by Western blot analysis showed no significant differences between stage 28 and stage 30 (mean values 0.094 ± 0.013 a.u. and 0.104 ± 0.038 a.u., respectively) (Figure 21).

5. DISCUSSION

Recently, the population of the loggerhead turtle *C. caretta* has been expanding its distribution range towards the north-western Mediterranean basin, leading to an increase in nesting on the Italian coast (Hochscheid et al., 2022). The eggs analyzed in this study came from nests laid in the Ionian coasts of Italy, in the years 2021 and 2022. The Mediterranean Sea turtle population is threatened by several natural and anthropogenic impacts, which could have synergistic effects on the health status of individuals (Casale et al., 2018). *Caretta caretta* is also particularly vulnerable to marine pollution by plastic waste, which is why this species has proven to be an excellent bio-indicator for monitoring this threat (Di Renzo et al., 2021). The effects of plastic pollution and other contaminants on embryos and their transfer via the maternal route have been hypothesized, but not yet further explored (Chemello et al., 2023). This study aims to deepen knowledge on the hepatic effects of microplastics and contaminants on the health of *C. caretta* turtle embryos.

The sharp decrease in yolk weight from developmental stage 28 to stage 30 suggests that the embryo is consuming it from the yolk sac, in agreement with what Miller et al. (2017) observed. At stage 30, the amount of yolk remaining is minimal (0.350 ± 0.313g), as most of it has been internalized in the embryo's abdomen (Miller et al., 2017). Egg weight also decreases as developmental

stage increases, while embryo weight remains constant between stages 28 and 30: this suggests that the embryo at the final stages of development continues nutrient uptake by consuming the yolk, in preparation for hatching. Some of the energy thus obtained supports the energetic costs of development, while some is invested in somatic growth, as evidenced by the increase in total length (CRL), length (SCL) and width (SCW) of the embryo's carapace from stage 28 to stage 30. At stage 30, however, the embryos analyzed appear to be smaller in size than those measured in other studies conducted in the Mediterranean, both in terms of body weight (generally > 15g) and carapace length (generally > 4cm) (Reid et al., 2009; Trotta E., 2022). Egg weight at the end of development also appears to be lower than found in these studies. Previous studies have shown how differences in embryo size at hatching may be related to the incubation temperature of the nest and consequently to different energy demand or energy allocation by the embryo during development (Reid et al., 2009; Glen et al., 2003).

The percentage of embryos showing abnormalities in the pattern of carapace shields (40%) was higher than reported in other studies conducted on hatchlings in the Mediterranean (20,5 %) (Maffucci et al., 2020). In contrast, other studies show similar frequencies to those found in this analysis (Caracappa et al., 2016), but this could be due to the small number of samples analyzed compared

to Maffucci et al. (2020). Although the mechanism through which the environment influences the scute pattern remains unclear, several possible causes have been proposed to explain these anomalies, including temperature, moisture and oxygen during incubation and environmental pollution (Maffucci et al., 2020). Since the percentage of abnormalities is reduced in adults, it is assumed that this may be indirectly associated with a reduced survival rate; this is probably related to morphological or physiological abnormalities that result from environmental stressors (Maffucci et al., 2020).

A high fraction (75%) of analyzed embryos had microplastics within the liver samples taken, with no significant differences between stages 28 and 30. The finding of microplastics in the liver of *C. caretta* turtle embryos confirms what has already been found by Chemello et al. (2023). The most plausible hypothesis suggested to explain the presence of microplastics in the yolk and liver was that of maternal transfer, rather than external contamination through the eggshell (Curl et al., 2024; Chemello et al., 2023). According to this hypothesis, small microplastics and other contaminants could reach the mother's liver via the bloodstream, then reach the interior of the follicles during vitellogenesis and thus be incorporated into the yolk; the embryo would then absorb these pollutants during the final stages of development, probably related to the lipid component (Chemello et al., 2023; Muñoz & Vermeiren, 2020;

Sinaei & Bolouki, 2017). Histological analyses of lipid percentage confirmed that the latter increases significantly between stage 28 and 30. However, the presence of microplastics as early as developmental stage 28 suggests that their uptake from the yolk may occur earlier than this stage.

The only shape of microplastic found was the fragment: this is consistent with evidence from a global scale characterization study of environmental plastics (Lim et al., 2022). Fragments are the most abundant shape found in the sea (Lim et al., 2022) and are often found inside fish and other organisms commonly preyed upon by turtles (Duncan et al., 2019). Most of the plastic microparticles ingested by adult turtles appear to be fibers (Duncan et al., 2019). However, they are generally quite large in size, making them more difficult to be mobilized; this is probably the reason why they were not found within the embryonic liver samples. From this analysis conducted on a few samples, color does not appear to be a relevant variable.

A high proportion (70%) of the liver samples analyzed contained at least one of the 16 PAHs analyzed and 50% had at least two different PAHs, up to a maximum of 6 within the same embryo. This suggests that the presence of pollutants within *C. caretta* turtle embryos is quite frequent, and that multiple PAHs are often present simultaneously. To our knowledge, there are no studies in the literature that provide values of PAHs measured in the liver of *C. caretta*

turtle embryos with which to compare those resulting from the present study. Therefore, for the first time the presence and concentration of 8 PAHs has been recorded in *C. caretta* embryos. Arienzo et al. (2023) measured the levels of PAHs in the liver of adult individuals stranded along the southern Italian shores, finding very high total concentrations (139 ± 55.10ng/g), while other studies conducted in different basins have reported results that are often lower and highly variable depending on the population considered (see Rossi et al., 2023 for a summary of different matrix in *C. mydas* and *C. caretta*). The maternal transfer theory has also been proposed for many contaminants, probably related to the lipid component given their affinity (Muñoz & Vermeiren, 2020).

All PAHs found within embryos were also found in the livers of adult individuals analyzed by Arienzo et al. (2023), except for pyrene. Similar concentrations are reported for fluorene, B(a)p and B(a)A, while other PAHs are present in very different concentrations such as naphthalene, present in high concentration in adults (125 ± 57.2ng/g) and detected in unquantifiable concentrations (<LOQ) only in two embryo samples. Although probably not from the same population, the presence of the same PAHs in adult individuals and embryos in the southern coasts of Italy is additional information that could support the maternal transfer hypothesis. It must be considered, however, that

the exposure of individuals could vary greatly depending on the population considered and the foraging territories used, as shown by the variability summarized in Rossi et al. (2023). In addition, it should be noted that even eggs from the same nest and therefore laid by the same mother show variability: in embryos belonging to the same nest different contaminants are present (when present) and at different concentrations. However, this is consistent with maternal transfer, suggesting that contaminants could be incorporated into the follicles at different times during vitellogenesis, which lasts several months (Miller & Limpus, 2003).

Comparing the presence of different PAHs within the yolk and liver of the embryo at different stages of development can provide us with information on the transfer of contaminants through the yolk and the timing of uptake by the embryo. For example, naphthalene, acenaphthene, 2-bromonaphthalene, phenanthrene, and chrysene are present exclusively in yolk and never in embryos, suggesting that these pollutants remain in the yolk until hatching and are fully absorbed. Benzo (g,h,i) perylene, benzo (a) anthracene, and pyrene were found exclusively in the liver and only at stage 28: this might suggest that their uptake from the yolk occurs at earlier developmental stages and that is why they are no longer present in the yolk; furthermore, the fact that they are not detected at stage 30 might suggest that they are transferred from the liver

to other body districts in the final stages of development. Acenaphthylene is the most common PAH and was found in 100% of the yolks analyzed, only at stage 28 and not at stage 30: the increase in its presence in the liver (from 30% to 67%) between stages 28 and 30 could indicate that the embryo begins its uptake at earlier stages and that it is completely absorbed before reaching stage 30. Fluoranthene appears to have a similar distribution, but with frequencies that differ only slightly (30% at stage 28 and 33% at stage 30). A similar trend could apply to anthracene, but the decrease in its presence in the liver (from 30% to 17%) could also suggest simultaneous mobilization to other districts. Fluorene was detected in both liver and yolk only at stage 30: its presence was recorded only in one embryo, which explains its absence in the stage 28 frequencies; in this case, uptake might have started early and by the end of development not all the contaminant was internalized. Benzo (a) pyrene, on the other hand, is present only at stage 30 in the liver and never in the yolk: since it is present in only two embryos, it might have been absorbed at early developmental stages and that is why it is no longer present in the yolk; another possible explanation could be that the pollutant was absorbed early from other body districts and only at the end of development was it transported within the liver. More samples could clarify the timing and pattern of its uptake.

Several studies show that low molecular weight PAHs tend to be more bioavailable, less metabolizable, and highly associated with genotoxic properties (Korfmacher et al., 1980; Matson et al., 2005). However, no significant differences were observed between the two stages in the concentration of low- and high-molecular-weight PAHs or in total PHAs.

In 83,33% of the samples in which microplastics were found, at least one PAH was also present. All PAHs detected in the liver samples were also found to be associated with microplastics, at different concentrations based on affinity to different polymer types (Tan et al., 2019). This might suggest that some of the pollutants detected in the liver of *C. caretta* turtle embryos may have been concentrated and transported by the plastic fragments. Furthermore, the simultaneous presence of different pollutants and microplastics in the same embryo could suggest the occurrence of synergistic adverse effects, which could also differ from those of the single contaminant.

Exposure to some of the PAHs analyzed here has been correlated with the increase in global DNA methylation and increased gene expression of biomarkers such as HSP60 and CYP1A in adult turtle blood cells (Cocci et al., 2018). In this study, the levels of some biomarker proteins (HSP70 and CYP450) were analyzed by western blot and immunohistochemistry, respectively. The level of HSP70 protein showed no significant differences

between developmental stages 28 and 30. There are no basal levels of HSP70 measured in the liver of turtle embryos in the literature with which to compare these results. However, since there were no significant differences between stages 28 and 30 in the presence of microplastics or PAHs, the similar expression levels of this protein could be consistent with a similar level of stress between the stages. Immunohistochemical analysis conducted in this study also found no significant differences in the level of CYP450 protein: again, this is consistent with the presence of PAHs in almost all embryos at both stages.

Histologically, no significant differences were found in the number of melanomacrophages and the area they occupied between the two developmental stages analyzed. This could be consistent with the fact that no differences were found between the stages in the content of PAHs and microplastics, or the differences could be masked by the high standard deviations due to the high variability among samples.

6. CONCLUSION

The loggerhead sea turtle *C. caretta* is considered a valid bioindicator of plastic pollution, although the effects of microplastics and other associated pollutants on its health and reproduction remain poorly understood. Its protected status, which is crucial for the conservation of the species, makes molecular analyses difficult to perform due to the poor conservation status of dead specimens available for research.

Despite these limitations, this study provided new evidence on the presence of microplastics in the liver of turtle embryos, supporting the theory of maternal transfer through the yolk. For the first time, several PAHs were also detected and quantified within embryonic liver tissue and in the yolk, suggesting maternal exposure also for these pollutants.

Microplastics and PAHs were detected respectively in 75% and 70% of the embryos analyzed, indicating the particular abundance, spread and persistence of these pollutants in the marine environment and their ability to reach even the embryonic stages of sea turtles. Considering embryonic developmental stages 28 and 30, there were no significant differences in the presence of microplastics and PAHs, nor in the levels of biomarkers such as melanomacrophages, CYP450 and HSP70, suggesting that the induced stress might be similar at both stages. Although this study did not demonstrate immediate and significant

effects on embryo health, the simultaneous presence of these contaminants as early as the embryonic phase could have synergistic and long-term deleterious implications.

This research explores the effects of marine pollution on *C. caretta* turtle embryos, supporting the hypothesis of maternal transfer of contaminants as a route of exposure. Further studies are needed to understand the long-term consequences and develop more effective conservation strategies.

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