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DELL'AMBIENTE**

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Biologia marina

**Uno studio pilota sulla presenza di microplastiche in
embrioni di tartaruga marina comune (*Caretta caretta*) e
possibili effetti sullo stato di salute**

**A pilot study on the presence of microplastics in
loggerhead sea turtle (*Caretta caretta*) embryos and
possible effects on the health status**

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Riassunto

La tartaruga marina comune (*Caretta caretta*) è una delle sette specie di tartarughe marine presenti negli oceani, è largamente diffusa nell'oceano Atlantico, Pacifico e Indiano e nelle acque del Mar Mediterraneo e del Mar Nero. La variazione dei fattori ambientali e la sempre maggior presenza antropica negli oceani ne stanno minacciando la sopravvivenza, tanto da essere inserita come specie vulnerabile nella lista rossa della IUCN (Unione Internazionale della Conservazione della Natura). L'innalzamento di temperatura causato dal riscaldamento globale sta portando ad uno spostamento delle tartarughe marine, animali eterotermi, verso aree marine dove le minori temperature stagionali sono più ottimali per le loro attività. Inoltre, l'inquinamento antropico presente nei mari si sta rivelando una delle principali minacce emergenti per la conservazione della tartaruga marina comune. Una delle minacce di origine antropica di più recente interesse è la presenza di plastiche nell'ambiente acquatico. Si stima che almeno il 52% delle tartarughe marine possa aver ingerito almeno una volta nella loro vita del materiale plastico, scambiandolo per cibo o assimilandolo sotto forma di microplastiche. L'ingestione di plastica da parte di questi animali è tale che le tartarughe marine sono state dichiarate bioindicatore ufficiale per la presenza di rifiuti in mare

(descrittore 10 “Marine Litter”), la cui maggior parte è di natura plastica, della Strategia Marina dalla Commissione Europea.

Per questo, diversi studi sulla presenza e l’effetto di plastiche e microplastiche sono stati effettuati negli ultimi anni su individui giovanili, sub-adulti e adulti di *C. caretta*, ma ad oggi non sono stati condotti studi a livello embrionale.

Questa tesi ha lo scopo di analizzare lo stato di salute di embrioni di *C. caretta* provenienti da uova deposte in due nidi lungo le coste toscane nell’agosto del 2020, di identificare la presenza di eventuali microplastiche all’interno del tuorlo e del fegato di questi embrioni e di individuarne possibili correlazioni con alcuni bioindicatori dello stato di salute dell’animale.

Tramite analisi macroscopiche sono stati determinati gli stati di sviluppo degli embrioni e sono state raccolte le principali biometrie. Successive analisi istologiche hanno permesso di analizzare lo stato di salute dell’animale tramite l’individuazione e la quantificazione di biomarker, come i melanomacrofagi, cellule pigmentate fagocitarie con il ruolo di rimozione ed autolisi dei globuli rossi, e la percentuale di lipidi internalizzata nel fegato necessaria per sostenere il costo energetico dello sviluppo embrionale. Infine, tramite protocolli di estrazione delle microplastiche e analisi di micro-spettroscopia Raman sono state identificati e classificati i polimeri ed i pigmenti delle microplastiche rilevate all’interno di campioni di tuorlo e di fegato. Il ritrovamento di

microplastiche nei campioni analizzati ha dato credito all'ipotesi di un trasferimento materno nel tuorlo e la successiva assimilazione da parte degli embrioni nell'uovo.

I risultati ottenuti ci mostrano un quadro generale in cui il numero di microplastiche/g di tuorlo e fegato è molto più alto rispetto a studi effettuati su altri organismi marini, come i pesci; la presenza delle microplastiche è associata, a livello embrionale, ad un aumento nel numero dei melanomacrofagi a livello epatico e un diverso assorbimento del tuorlo.

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1. Introduction

1.1 Biology and ecology of *Caretta caretta*

The loggerhead sea turtle, *Caretta caretta* (Linnaeus, 1758), is one of the most abundant and widespread species of sea turtles living in the oceans (Pritchard, 1996). *C. caretta* is the only species belonging to the genus *Caretta* within the order Testudines (family Cheloniidae) that includes other species such as *Chelonia mydas* (green sea turtle), *Eretmochelys imbricata* (Hawksbill Sea Turtle), *Dermochelys coriacea* (Leatherback Sea turtle), *Lepidochelys kempii* (Kemp's Ridley Sea Turtle), *Lepidochelys olivacea* (Olive Ridley Sea Turtle), *Natator depressus* (Flatback Sea Turtle) (Pritchard, 1996). *C. caretta* is morphologically distinguishable from the other sea turtle species for the typical shape and color of its reddish-brown carapace, its large yellow-brown head and yellowish plastron and limbs (Figures 1A and 1B).



Figure 1. Adult specimen of *Caretta caretta*. (A) dorsal view; (B) ventral view.

Considering their anatomical structure, loggerhead turtles have specific features which differ from the other species such as four prefrontal scutes, five vertebral scutes flanked by five coastal scutes, three inframarginal scutes and one nuchal scutes in contact with the first coastal one (Figure 2) (Wyneken, 2003). The carapace and plastron of the loggerheads are keratinized to efficiently protect them against predators and environmental threats such as collisions with boats (Dodd, 1988).

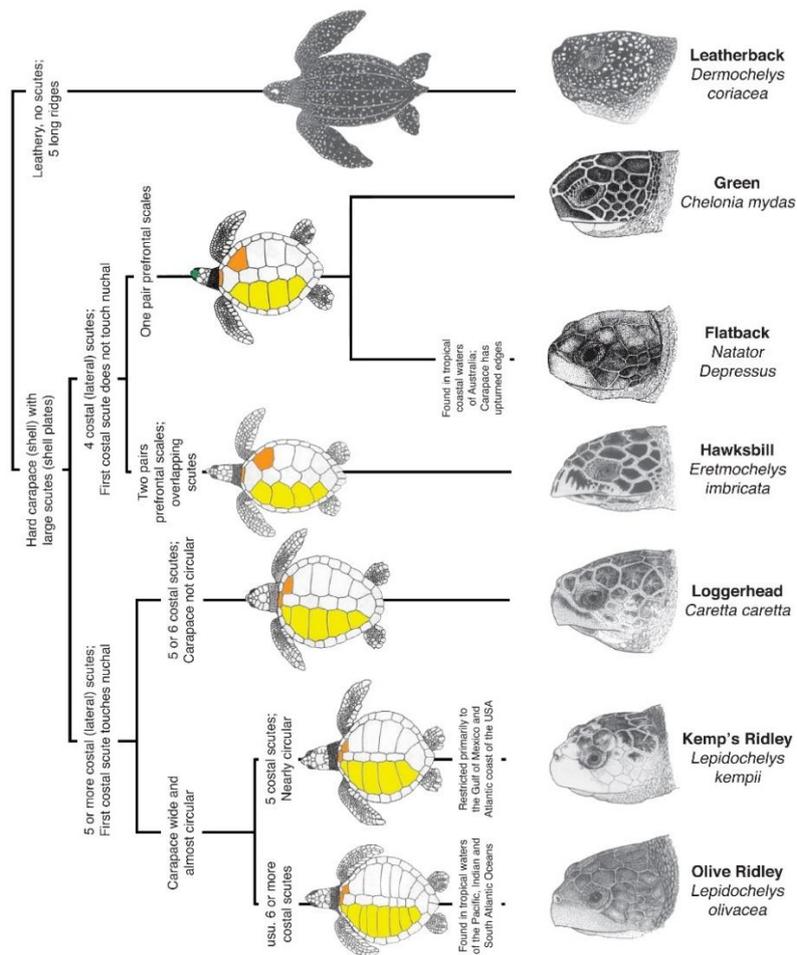


Figure 2 Scute patterns and shell morphology of 7 different sea turtle species. (seaturtle.org)

Sea turtles, including *C. caretta*, have morphological and physiological modifications that allow them to keep apnea for a long time, indeed they are the breath-hold vertebrates with the longest and deepest apnea ranges. They possess an efficient and rapid gas exchange at sea surface and a maximized oxygen storage and tissue oxygen transport during the submersion period. Thanks to these characteristics, they can dive from 2 to 5 hours and reach more than 200 mt of depth resting on the surface only for the 3-6% of the day (Lutcavage and Lutz, 1996). As reptiles, loggerhead turtles are ectotherms, they regulate their inner temperature with the environmental one. The loggerhead turtles are widely distributed in subtropical and temperate waters of the Atlantic, Pacific and Indian Oceans, and the Mediterranean Sea. They are solitary migratory animals able to move for thousands of kilometers during their lifetime (Plotkin et al., 2003).

Loggerheads turtles play an important key role in the trophic chain and marine ecosystem as consumers, ecosystem engineers, nutrient transporters, prey and facilitators (Hamann et al., 2010). Indeed, they could represent the habitat for a lot of epibionts, cleaning organisms and fishes which feed on dead skin, parasites and algae in their carapace. Loggerheads can be considered nutrient transporters and bioturbators since they reduce in particle sizes the shell of their prey and resuspend them in the water column making them available for

microorganisms and primary producers. Finally, sea turtles are in turn the prey for different marine species at all development stages (Heithaus, 2013).

1.2 Life cycle and reproduction of *Caretta caretta*

The complex life cycle of loggerhead sea turtles starts when the hatchlings leave the nest located on sandy beaches to reach oceanic areas where they will spend their juvenile life (from 7 to 12 years) feeding on epi-pelagic prey (mainly cnidaria and cephalopods) (Bolten, 2010). Successively, the juveniles move toward neritic areas where they feed on benthic preys (mollusks and crustaceans) and reach sexual maturity at the age of circa 16-18 years old for males and from 20 to 30 years old for females depending on the different population of *C. caretta* (Bolten, 2010; Guarino et al., 2020). During the reproductive season, mature *C. caretta* specimens start a natal homing migration to come back to the breeding sites close to the natal beaches of the female *C. caretta* (Figure 3) (Bolten, 2010; Chimienti et al., 2020).

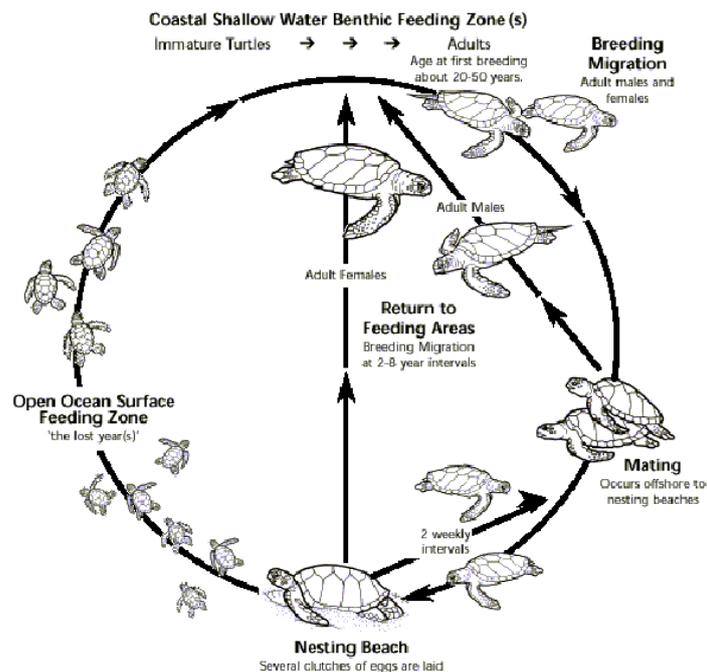


Figure 3. The life cycle of sea turtles. (Lanyon et al., 1989)

In all sea turtle species, the reproductive process is characterized by typical features such as iteroparous reproduction, oviparity with internal fertilization, stereotyped nesting behavior, laying of a large number of eggs and loyalty to the nesting site (Miller, 1996).

C. caretta could breed every year or every two years, depending on endogenous (hormone levels) and exogenous factors (photoperiod, temperature and resource availability) for the timing and period of reproduction. All these factors influence the energy budget and the fat reserves that females, in particular, can accumulate and use to complete the vitellogenesis (~9 months) and ovulation process (Whittier et al, 1997; Patel et al., 2015). The reproductive season starts 8-10 months before the breeding season with the activation of physiological mechanisms that promote follicular growth and, in females, the

increasing of adipose tissue and plasma hormones for the vitellogenesis (Miller, 1996).

The breeding season begins with the migration of adult loggerheads from neritic foraging sites to mating areas, where female and male loggerheads meet. Genetic studies have demonstrated that females can mate with several males within the same mating period with a consequence of multiple paternity, an increased genetic diversity and increased viability and variability of offspring and ensuring sperm viability following long-term storage in the oviduct (Miller, 1996; Gist and Congdon, 1998). The precopulatory and copulatory behaviors are vehement processes, during mating, the male could injure the female's flippers, neck and/or head (Miller, 1996). At the end of the mating period, males come back to foraging areas while females start their journey to the nesting site, with a high degree of accuracy to return to their birth area (with a mean site fidelity of <50 km) which are located typically in warm temperate and subtropical areas (Miller, 1996; Miller et al., 2003). The selection process of the nesting beach is not fully understood, however, some characteristics of the beach (e.g. width, slope and vegetation, salinity, pH, organic content) have been stated to possibly influence the female choice. Moreover, it has been observed that the optimal substrate for the nesting should be moist and fine to

prevent the collapse of the egg chamber during the construction of the nest and allow the gas diffusion (Karavas et al., 2005; Kaska et al., 2010).

The nesting process required ~1/2 hours and occurs mainly at night. The female loggerhead turtle emerges from the water, climb up to the beach, perpendicularly to the surf line, wandering around the beach, until they find a suitable nesting site (Hailman et al., 1992).

The female starts to move, rubbing the plastron on the sand in a typical way forming a “body pit” and begins to dig the egg chamber with alternate movements of the hindlimbs. The excavation of the nest is completed when the chamber reaches a depth of ~50/60 cm. Finally, the female loggerhead starts laying the eggs. For every nest, 80-170 eggs can be laid and at the end of the deposition, the turtle moves the forelimbs to cover the chamber with sand to protect it from possible predators (Hailman et al., 1992).

Every year, during the nesting season, each female can build up to 4 nests with an inter-nesting period of 12-16 days (Hamman et al, 2003).

The laying of several clutches of relatively small eggs during one nesting season may be a strategy to enhance the offspring’s survival against unexpected environmental events (Hamman et al., 2003).

1.3 Embryonic development

The loggerhead sea turtle's eggs appear white and spherical surrounded by a porous calcareous shell. Inside, the egg different components are distinguishable from the inside to the outside part: the embryo submerged in the amniotic fluid surrounded by the amniotic sac, the yolk sac, the allantois membranous sac, the chorion membrane and the albumen. (Figure 4) (Spotila, 2004). Each component of the egg plays a key role in the development of the embryo. The sac of amniotic fluid allows the maintenance of a stable inner environment, while the yolk gives nourishment to the embryo through the vitelline vessels (Blackburn, 2021). The allantois sac allows the gas exchange between the embryo and the albumen which is in contact with the internal side of the eggshell allowing gas diffusion through the calcareous shell (Gabrielli and Accili, 2010). During the development process, chorion and allantois fuse to form the chorioallantois membrane that facilitates the respiratory gas exchange, the maintenance of acid-base homeostasis in the embryo and the calcium transport from the eggshell (Gabrielli and Accili, 2010). Moreover, the chorioallantois membrane is responsible of ion and H₂O reabsorption from the allantoic fluid before the first lungs breathing at hatching time (lungs are completely formed at the end of the embryonic development) (Spotila, 2004; Gabrielli and Accili, 2010).

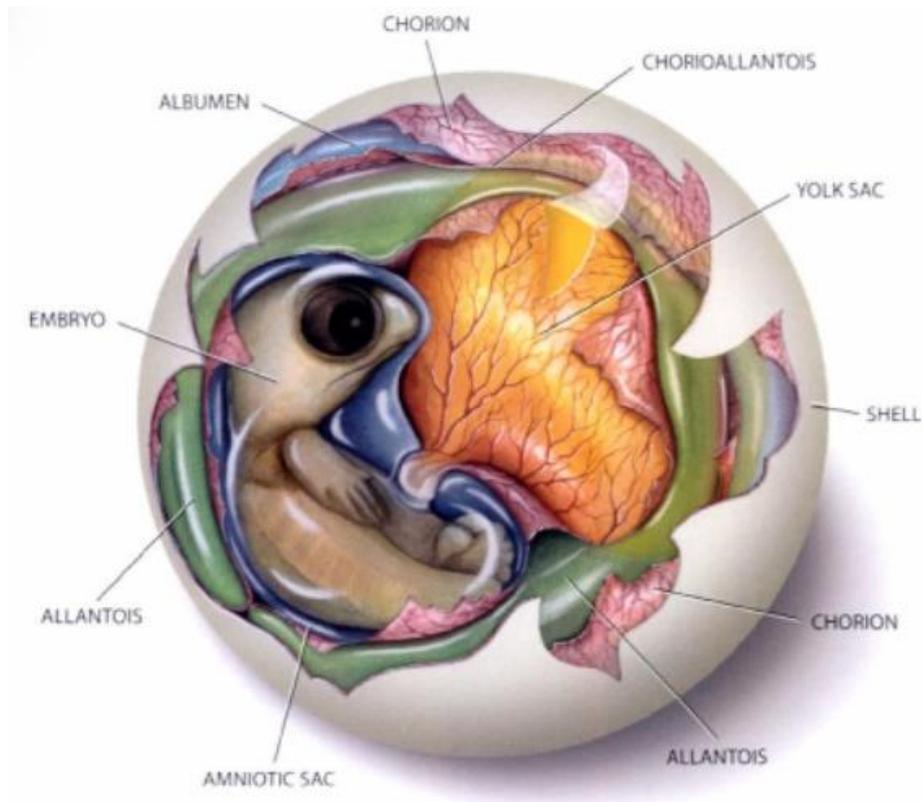


Figure 4. Internal structure of a sea turtle egg. (Spotila J.R., 2007)

The embryonic development in sea turtle was extensively described by Miller et al., (2017) through the identification of 31 embryonic stages from fertilization to hatchling emergence (Figure 5). Briefly, the development of embryos begins in the oviduct (stages 1-5) and at the moment of the oviposition the eggs are in the middle of the gastrulation phase (stage 6). In the first post-ovipositional stages (6-10) the formation of blastopore occurs together with the differentiation of the notochord, neural folds and head folds occurs (Miller et al., 2017). During the 11-22 stages, the head differentiates, the pigmentation of the eyes takes place, and the pharyngeal clefts develop. In the last stages (23-31) the carapace formation is completed with the pigmentation and the

differentiation of the scales. In these stages the volume of the embryo changes related to the yolk volume, reaching the maximum size at the 30-31 stages when the embryo reaches too large dimension and breaks the eggshell (Miller et al., 2017). The development process can last for 40 to 60 days and the transition from one stage to the next one may require different times, from a few hours (stage 7-8) to more than 1 day (stages 14-15) (Miller et al., 2017).

However, all the embryonic developmental processes are closely influenced by temperature. At $26^{\circ}\text{C} \pm 0,5$ the incubation of the eggs takes 76-79 days, at $29^{\circ}\text{C} \pm 0,5$ it lasts 54-58 days and at $32^{\circ}\text{C} \pm 0,5$ the incubation last for 46-49 days (Miller et al., 2017).

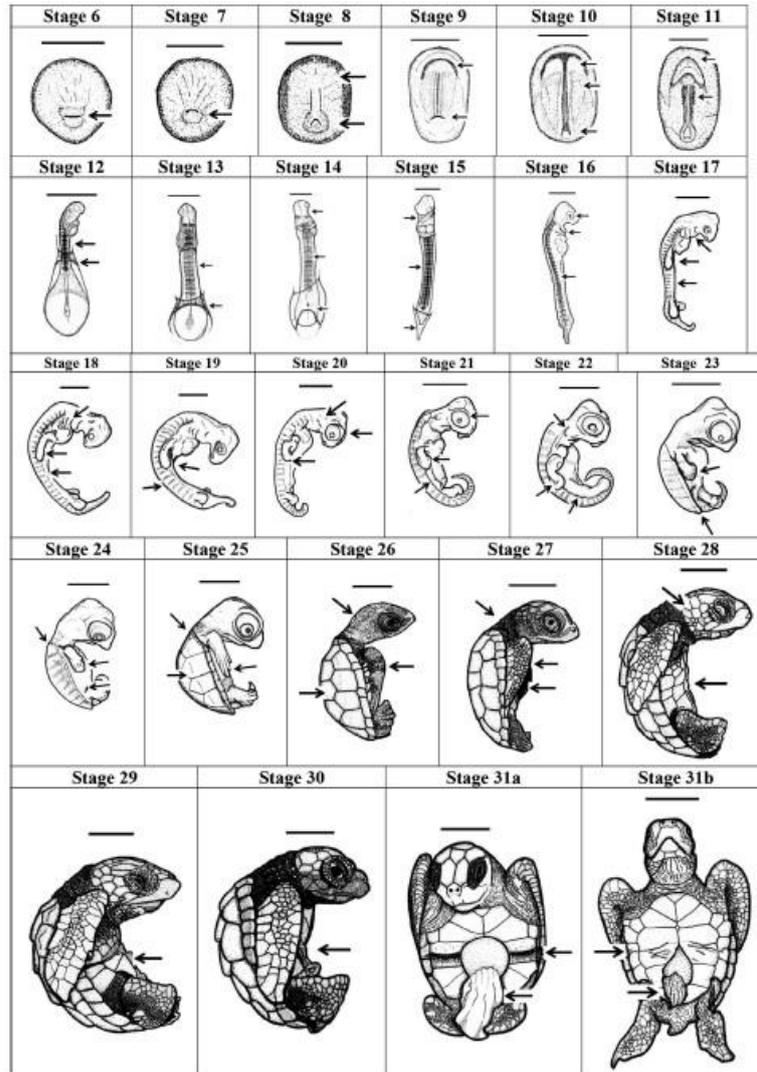


Figure 5. Sea turtles' embryonic development table (Miller et al, 2017).

Like other sea turtle species, *C. caretta* is characterized by an Environmental Sex Determination (ESD) that depends on the incubation temperature and the metabolic heating produced by the embryos inside the nest (temperature-dependent sex determination, TSD) (Zbinden et al., 2006). The sex is irreversibly determined during the middle third of embryonic developmental stages when the embryos are more sensitive to the temperature variations (temperature-sensitive period, TSP). At low temperatures, the embryos will

develop male gonads, at higher temperatures they will be female (Lolavar et al., 2020). The pivotal temperature is the temperature at which the sex ratio between male and female is the same, and the sex ratio can shift from one sex to another based on temperature values (figure 6) (Lolavar et al., 2020). For *C. caretta* the pivotal temperature is estimated at $29^{\circ}\text{C} \pm 0,67$ (Woolgar et al., 2013).

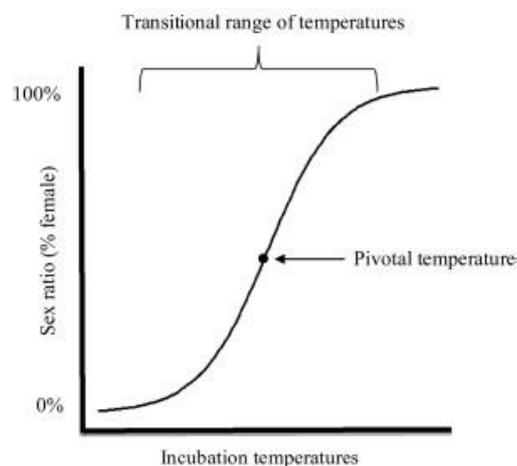


Figure 6. Identification of pivotal temperature based on the generalized curve of incubation temperature and consequence sex ratio. (Lolavar et al., 2020)

At the end of embryonic development, the hatchlings break the egg's shell and move to the surface of the nest chamber. Generally, the hatchling emersion is synchronized in groups of hatchlings probably for a "social facilitation". Indeed, the synchronous effort of many individuals to leave the nest could save a bit of the energy stored by the hatchlings to reach the sea and, at the same time, it decreases the percentage of predation attempts on each hatchling (Rusli et al., 2016).

The hatching event is mainly influenced by the temperature and light. High temperatures inhibit the digging activity. Therefore, the hatchling emergences occur usually at night, early morning or during diurnal rainstorms. In addition, the light direction seems to influence the “sea-finding” behavior of hatchling; however, this aspect is still unclear (Ackerman, 1996).

1.4 Threats sources

On a global scale, *C. caretta* is listed as vulnerable species in the RED LIST of the IUCN (International Union for Conservation of Nature).

At all life stages, the loggerhead sea turtles may encounter several natural and anthropogenic threats that could compromise the conservation of this species (Hamman et al., 2010). Specifically, the reproductive success and the survival rate of embryos and hatchlings are closely related to the biotic and abiotic conditions encountered (Martins et al., 2022).

1.4.1 Natural threats

Temperature, predation, tidal inundation, diseases, and the alteration of nesting ground are the main environmental factors that influence the hatching success of a nest. Among them, temperature is the most important environmental factor for the survival of the eggs (Spotila and Stendora, 1985). Excessively low or high incubation temperatures alter embryos development or cause their death (Mahdy et al., 2020).

In the perspective of a climate change scenario leading to the constant increase of global temperature, the effects of this environmental parameter could affect considerably the conservation of this species (Laloë et al., 2017).

Another effect related to climate change is the sea-level rise which could alter the nesting habitats causing tidal inundations, for example. It was already demonstrated that, during the incubation period, tidal inundations can cause hypoxia that has lethal and sublethal effects such as the death of embryos or the alterations of neurological functions, behavior, learning or spatial orientation in hatchlings (Pike et al., 2015).

Predation and diseases should be also considered as natural threats affecting eggs and hatchlings' survival. Crabs, dogs, foxes, pigs, and raccoons are the main predators of *C. caretta* eggs. It was demonstrated that, in some cases, such predation led to the loss of up to 96% of nests (Butler et al., 2020).

Infectious diseases caused by a large number of fungi are new emerging main threats for sea turtle eggs. The Sea Turtle Egg Fusarios (STEF) (Figure 7) is a newly lethal pathology caused by *Fusarium solani* (Gleason et al., 2020). Moreover, other 8 species of genus *Fusarium* and other fungi (*Aspergillus*, *Emericella*, *Rhizopus*, *Actinomucor* and *Apothysomyces*) have also been observed inside some nests causing the same effects. The warm temperature and the organic matter inside the clutch provide a suitable environment for the

growth of the fungi that could cause the embryos' death and the decomposition of eggs (Gleason et al., 2020). Indeed, pathogenic fungi growing in the nests, produce enzymes and organic acids which degrade the shells and penetrate inside the embryonic tissues.

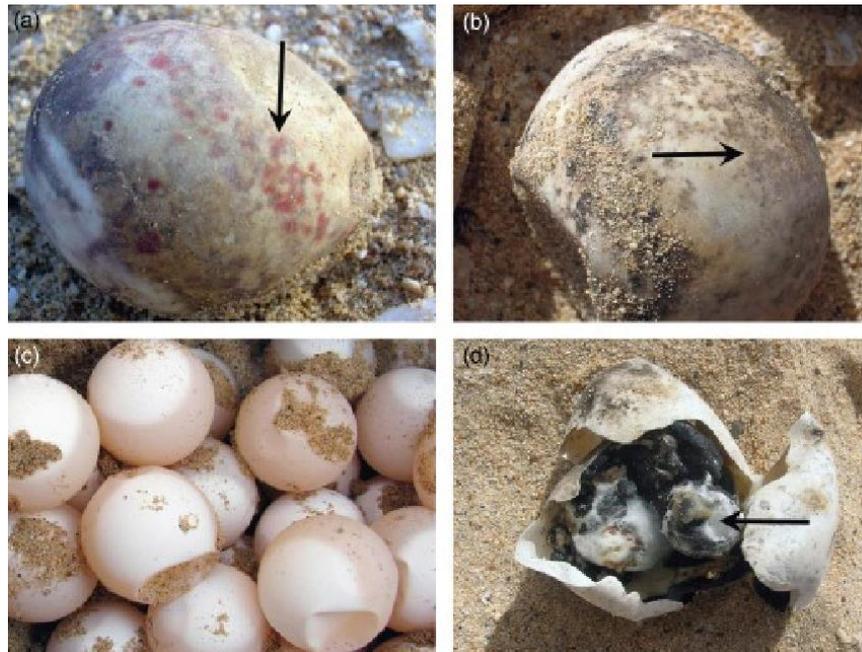


Figure 7. Example of *Caretta caretta* infected eggs by *Fusarium solani*. (a) reddish and (b) bluish spots symptoms of the disease; (c) healthy eggs; (d) dead embryo infected by *F. solani* (Sarmiento-Ramirez et al., 2010).

1.4.2 Anthropogenic threats

Costal development, tourism, consumption, and pollution are the main threats related to human activity which negatively interact with sea turtles and their habits.

Habitat degradation is one of the main threats for sea turtles linked to tourism and urbanization of beaches and coastal areas. During the last years, in the

breeding season, female turtles had to choose the right site for the nest on beaches that are increasingly overexploited by tourist activities. The loss of a nesting site could have a permanent effect on the sea turtle population and the ecosystem. Indeed, if female turtles do not find a suitable site in their fidelity area, they have to reach a new suitable nesting site consuming more energies normally invested in the nest building (Welicky et al., 2012). Other anthropogenic threats are linked to the consumption of sea turtle products. Despite national laws prohibiting their commercialization, sea turtle products (meats, adipose tissue, organs and eggs) are a common food in many countries worldwide (Aguirre et al., 2006).

The consumption of sea turtles and their products not only damages the turtles' population, but it also affects the health of people eating them. They could be carriers of environmental pollutants, such as organochlorines and heavy metals that unfortunately bioaccumulate in sea turtle tissues (Aguirre et al., 2006).

1.5 Contaminants' exposure

1.5.1 Heavy metals

Heavy metals (HMs) are a group of metals and metalloids present in nature having high atomic weights. HMs are usually divided into essential and non-essential. The essential HMs are Cu, Fe, Mn, Co, Zn and Ni and they are required in the fundamental biological processes like growth, metabolism and

development of living organisms (Esposito et al., 2020). Conversely, non-essential HMs (Cd, Pb, Hg, Cr and Al) are not involved in the metabolic pathways of living organisms and often induce toxicity manifestation when the organism absorbs them (Tchounwou et al., 2012). Heavy metals are also produced by human industrial activities and typically, the use of fertilizers, pesticides and oil, and gas production contributes to the release of HMs in the environment (Varol, 2011).

As well as organic and inorganic pollutants, HMs released in the aquatic environment could be ingested by sea turtles through the trophic network or from direct contact with the water and soil (Esposito et al., 2020). Through the food web, in particular, HMs can be accumulated by sea turtles which feed on benthic and pelagic preys which are themselves bio-accumulator of these chemical compounds (Esposito et al., 2020).

Non-essential HMs can have different effects on the turtle's health status, e.g. Mercury (Hg) was observed to induce negative effects on the endocrinal and central nervous system, affecting reproduction, osmoregulation, prey location and interspecific communication, while lead (Pb) caused neuro-development diseases (Esposito et al., 2020).

1.5.2 Plastic pollution

To date, plastic pollution represents one of the most severe global threats. It consists of the production and release, in the environment, of plastic debris which are differentiated depending on their size in macroplastics (>200 mm), mesoplastics (dimension between 200 mm and 5 mm), microplastics (MPs) (between 5 mm and 1µm) and nanoplastics (<1 µm) (Barnes et al., 2009). The main sources of plastic debris in the ocean have a land-based origin, coming from river runoff, inadequate waste management and industrial activities; or ocean-based origin, from the fishing industry, nautical activities and aquaculture (Sheavly, 2007). The main plastic polymers discharged in the marine environment are polyethylene (PE), polyethylene terephthalate (PET), polypropylene (PP), polyvinyl chloride (PVC), polystyrene (PS), polycarbonate (PC) and nylon (Andrady, 2011). Once plastics reach the sea, they can persist for years and be ingested by marine organisms with different feeding strategies, from deposit-, suspended- and filter- feedings to carnivorous (Guzzetti et al., 2018). It was estimated that more than 52% of sea turtles have ingested plastic debris during their life, possibly mistaking plastic for food (like jellyfish) (Schuyler et al, 2016; McCauley and Bjorndal, 1999) and probably this percentage has raised in the last few years following the increasing presence of plastic debris in the aquatic compartment. For this reason, starting

from 2017, sea turtles have been chosen as official bioindicators for marine litter (Descriptor 10) by the European Union Marine Strategy Framework Directive (MSFD). The ingestion of plastic debris has both direct and indirect effects (Savoca et al., 2021). The direct effects are strictly connected with the blockage of the gastrointestinal tract and internal lacerations that are even lethal. Instead, the indirect effects are due to the reduction of feeding rate and the release of toxic chemical compounds used as additives during plastic production or like components of plastic debris (McCauley and Bjorndal, 1999, Savoca et al., 2021). Sometimes, the effects worsen if considering the consequence of microplastics (MPs) exposure. Indeed, thanks to their small size and widespread in the environment, MPs can be easily ingested by marine organisms, bioaccumulated in tissues, and biomagnified through the trophic chain (Wright et al., 2013). MPs derive mainly from personal care products and textile fibers or can be originated from the degradation of macro/meso-plastics by the effect of wind, ocean waves and sun (Andrady, 2011),

In marine organisms the potential effects of MPs were observed to be connected with the induction of oxidative stress, effects on reproduction, decreased neurofunctional activity, development impairment and mortality (de Sà et al., 2018).

Moreover, some of the chemical compounds used during plastic production are recognized as endocrine-disrupting chemicals (EDCs) which interfere with those hormonal processes essential for growth and embryonic development (Savoca et al., 2021). For instance, Phthalates, also known as phthalic acid esters (PAEs), are used as additives in the plastic industry and for other industrial products and unfortunately, they are widely present in the marine environment (Habert et al., 2009). Studies performed using fish, amphibians, reptiles, birds and mammals as experimental models have highlighted that exposure to PAEs causes acute and chronic toxic effects (Habert et al., 2009; Vos et al., 2000). The most well-known effects are related to reproduction, with endocrine, testicular, ovarian, neural, hepatotoxic and cardiotoxic effects (Savoca et al., 2021).

1.6 Health status biomarkers

As already mentioned above, the presence of pollutants, plastics and MPs in marine organisms may have different effects depending on the species, the life-cycle stage, and the interaction with the abiotic and biotic factors of different ecosystems. Therefore, to evaluate the health status of an organism exposed to different toxic substances several biomarkers can be used (Handy et al., 2003). In *C. caretta*, for instance, in a study conducted in 2018, the evaluation of the health status of juvenile organisms exposed to polycyclic aromatic

hydrocarbons (PAH) was performed by analyzing the expression of a specific set of genes potentially dependent on PAHs presence (Cocci et al., 2018).

1.6.1 Liver

The liver is the biggest visceral organ in sea turtles, it is located ventrally between the heart and the stomach in a central position in the middle portion of the coelomic cavity, dorsally covered by lungs (Figure 8). It is directly connected with the gut and the pancreas (Herbst and Jacobson, 2003).



Figure 8. Dorsal view of the coelomic cavity of a *C. caretta* embryo. (1) lungs; (2) stomach; (3) kidneys; (4) liver.

Turtles' liver is involved in the metabolism of fats, proteins, glycogens and bile, acid uric, and coagulation factors (Silva et al., 2011). In sea turtle embryos, liver analysis could be of particular interest to assess the health status and the

metabolism of embryos. Therefore alteration in the liver structure could be associated with its disfunction and also could have consequence on liver absorption activity of nutrients.

Another physiological liver function is detoxification. Studies performed on *C. mydas* and *C. caretta* have demonstrated that the highest levels of some non-essential heavy metals and chemical compounds associated with microplastics are recorded in the liver of adult specimens (Andreani et al., 2008; Savoca et al., 2018; Ribeiro et al., 2019). Therefore, the sea turtle liver can be considered of important biological relevance in the study of pollutants effects on sea turtles. To date, no studies have considered the toxicity effects of pollutants in embryos' livers.

1.6.2 Melanomacrophages

Melanomacrophages (MMs) are highly pigmented phagocytes found in kidney, liver and spleen of ectothermic vertebrates and fish. MMs can be isolated cells or can form aggregates as melanomacrophages centers (MMCs) (Steinel et al., 2017).

Melanomacrophages are involved in immune defense mechanisms and normal physiological processes. Their primary function is the phagocytosis of cellular degraded materials (Agius and Roberts, 2003). The internalization of unmetabolized materials gives MMs their characteristic dark-brown

pigmentation with the high lipofuscin, melanin and haemosiderin content (Agius and Roberts, 2003). The phagocytosed materials can have both endogenous and exogenous origins. The endogenous one derives from the phagocytosis of dead or damaged cells, mainly erythrocytes. Instead, exogenous materials are represented by different organic environmental pollutants (Steinel et al., 2017). Several studies in fish have shown that the MMs are important health status biomarkers (Stosik et al., 2019; Passantino et al., 2014). Indeed, the MMs increase in number and size as a direct effect of the exposure to chemical pollutants and environmental stress (Passantino et al., 2014). Moreover, their presence in the liver was already analyzed to evaluate the health status of stranded adult specimens of *Caretta caretta* which have ingested different types of plastic debris (Zonta et al., 2022).

1.6.3 Yolk

The yolk is mainly composed of lipids and proteins and inorganic components such as K, Ca, Mg, Fe and Zn and its formation occurs during the vitellogenesis process through which these macromolecules are stored in the developing oocytes of all the oviparous reptile species (Prieto et al., 2019; Guraya, 1989). Vitellogenin (VTG) is the main protein involved in vitellogenesis. VTG is synthesized in the liver as a yolk precursor and transported to the ovary through the plasma as a lipoprotein complex (Heck et al, 1997). The VTG accumulation

in ovarian follicles is stimulated by estrogen production (Heck et al, 1997). In vitellogenic sea turtle females (feeding females), the levels of plasma hormones (corticosterone, testosterone and estrogen) triglyceride and adipose tissue lipids increase. Instead, at the end of the vitellogenesis (during courtship and nesting season) the levels of VTG and estrogens decrease, while testosterone, corticosterone and triglyceride increase with the reach of maximal follicle size (Hamman et al. 2002). Same levels of hormones and triglyceride are found in egg yolk during the early, middle and late nesting season (Hamman et al. 2002). Studies on *C. caretta* eggs have detected in the yolk some toxic compounds (Stoneburner et al., 1980; Savoca et al., 2021). In a study conducted in the western Atlantic, egg yolk analysis revealed high levels of non-essential heavy metals (Hg and Pb) (Stoneburner et al., 1980). Similarly, in the eggshell, albumen and yolk of *C. caretta* samples were found phthalates commonly used in plastic production (Savoca et al., 2021). The presence of these compounds shows could be explained by the possible maternal transfer of them during the vitellogenesis process (Savoca et al, 2021).

2. Aim

The conservation of *Caretta caretta* population is endangered by several threats that organisms face during their entire lifetime. Recently, the most widespread threat for loggerhead turtles is considered the interaction (and its consequence) with marine litter (e.g. plastic debris) and chemical pollutants (such as non-essential heavy metals). In recent years, several studies have investigated the presence of plastic debris and their potential effects on the health status of juvenile-adult loggerhead turtles. Previous studies have demonstrated the presence of some chemical compounds, used in the plastic industry, in loggerhead turtles' eggs and how they could affect the embryo's survival (Savoca et al.,2021). However, in *C. caretta* eggs, the presence of MPs has not yet been demonstrated.

This thesis aim is to investigate, for the first time to our knowledge, the presence of microplastics, related to a possible maternal transfer, in the yolk and embryos of *C. caretta* with particular focus on liver samples.

Moreover, the MPs presence has been correlated to different embryonic biometric parameters and with the presence of melanomacrophages in embryos liver as promising biomarkers for the evaluation of the health status. This study was performed in collaboration with the association tartAmare which provided the biological samples.

3. Material and methods

3.1 Data and samples collection

C. caretta eggs were collected by volunteers of tartAmare Onlus from two nests which were re-located, to protect them from possible tidal inundation, at 3.5 km apart along the beach of Tuscany coast (Italy) during the summer of 2020. The first nest of 96 eggs, was laid on August 10th and relocated on the Baratti beach (42°59'49.192" N 10°30'54.764" E). In the second nest, 64 eggs were laid on August 15th and relocated on the beach of Rimigliano (42°01'31.727" N 10°31'25.734" E)(Figure 9).



Figure 9. Location of Rimigliano and Baratti nests

The internal temperature of both nests has been constantly monitored using data loggers (iButton® temperature loggers, DS1922L/DS1922T) for the first 40 days of the incubation period. At the end of the expected incubation time (90

days), the nests were dug and after verifying the failure of hatching (100% of hatching failed), the eggs were collected and placed at -20° C for further analysis.

3.2 Sampling

100 eggs (50 from Rimigliano nest and 50 from Baratti nest) were sampled at the laboratory of Reproduction and Developmental Biology, Department of Life and Environmental Science (DISVA) at the Polytechnic University of Marche (Ancona, Italy). Before sampling, the integrity of the eggshell was evaluated, and broken eggs were excluded from the sampling. Each egg was weighted, and the diameter was measured. The eggs were then opened, the presence of the embryo assessed, and the following biometric parameters recorded (Figure 10A, B):

- a. Embryo weight
- b. Yolk weight
- c. Total Tail Length (TTL)
- d. Straight Carapace Length (SCL)
- e. Straight Carapace Width (SCW)
- f. Forelimb Length dx/sx (FLL)
- g. Hindlimb Length dx/sx (HLL)

Successively, each embryo was sampled (Figure 10C) (9 and 10 animals from Rimigliano and Baratti nest respectively) and a portion of the liver was weighed and stored in formalin fixative at 4 °C to preserve the tissue integrity for histological analysis. The other portion of 5 livers was placed in an aluminum box for the microplastics detection analysis and stored at -20 °C. Also, the yolk from the 5 animals sampled (each nest) was collected and stored in aluminum boxes at -20°C for the microplastics detection analysis.

To avoid MPs contamination, the sampling procedure was conducted using plastic-free tools carefully and repeatedly washed with pre-filtered 70% ethanol after each sample following the protocol already developed by Di Renzo et al (2021).

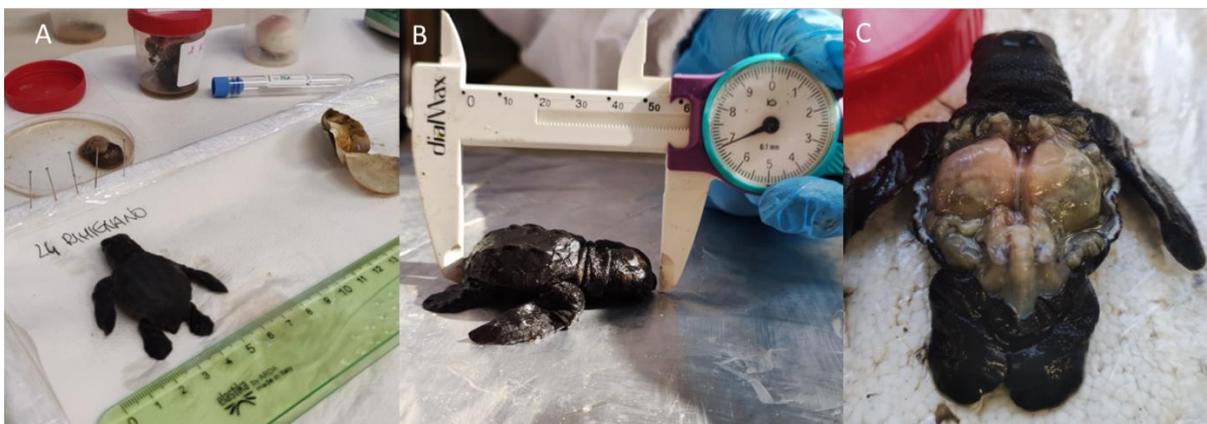


Figure 10. (A, B) eggs sampling and embryonic biometric measurement, (C) embryos' liver collection.

3.3 Histological analysis

The liver samples stored in formalin at 4°C were successively prepared for the inclusion process. They were washed in ethanol (70%) three times (15 minutes each), dehydrated in increasing ethanol solution (80, 95 and 100%), washed with xylene (Bio-Optica, Milano, Italy) and included in paraffin (Bio-Optica). Solidified paraffin blocks were cut into 5µm sections with a microtome (Leica RM2125 RTS, Nussloch, Germany). The sections were spread on a microscope slide, stained following Mayer haematoxylin and eosin Y (Merck KGaA) protocol and observed using an optic microscope (Zeiss Axio Imager.A2, Oberkochen, Germany). Images were acquired using a combined color digital camera Axiocam 503 (Zeiss, Oberkochen, Germany).

To evaluate the presence and number of melanomacrophages and the lipid percentage in liver sections 6 images per sample were analyzed using ImageJ software.

3.4 Microplastics extraction and filtration protocol

The digestion of 5 liver and 5 yolk samples (each nest) was performed at the laboratory of Reproduction and Department of Life and Environmental Science (DISVA) at the Polytechnic University of Marche (Ancona, Italy). Yolk and liver samples were stored at -20°C and weighted before the digestion in a pre-filtered 10% KOH solution (fiber-glass filter, 1.6 µm pore-size, Whatman

GF/A) made with deionized water and KOH tablets (Sigma-Aldrich). The solution was added to each sample (ratio 1:10 w/v) and incubated at 40 °C for 24 h modifying the existing protocol described by Di Renzo et al., 2021.

Considering the substantial amount of fat in yolk samples, 1 h before the filtration, a pre-filtered solution (fiber-glass filter, 1.6 µm pore-size, Whatman GF/A) of Et-OH 100% (Bio-Optica) was added to each yolks' digestive solution (1:10 Et-OH:KOH ratio) to remove the saponified gel formed on its surface following the protocol suggested by Dawson et al., (2020).

After 24 h, the digestates were filtered in fiber-glass filters (1.6 µm pore-size, Whatman GF/A) using a vacuum pump connected to a filter funnel. The filter membranes were dried at room temperature and placed into glass petri dishes until the visual inspection for the identification and characterization of plastic particles. To prevent plastic contamination, plastic materials were avoided when possible, and work surfaces and laboratory tools were carefully and repeatedly washed with pre-filtered 70% ethanol during the entire digestion and filtration process. Cotton laboratory coats, face masks, and single-used latex gloves were worn during all the procedures.

Three procedural blanks (KOH solution with no yolk nor liver samples) were treated as the biological samples and three glass fiber filters (placed into glass petri dishes) were kept close to the samples during the entire analysis and then

tested to monitor and correct potential contamination. Moreover, an environmental blank was prepared each day: a filter membrane soaked with 1.6 μm -filtered deionized water was placed into an uncovered Petri dish and positioned next to the stereomicroscope and the Raman microscope during all the analyses.

3.5 Raman microspectroscopy analysis

The preliminary inspection of the filters for the quantitative and qualitative assessment of MPs was performed at the optical microscopy of XploRA Nano Raman Microspectrometer (Horiba Scientific) at x100 magnification. Each filter was observed moving laterally from the top to bottom, from left to right, and in the opposite direction to cover all the filter's surface. Microparticles that were assumed by eye to be MPs were registered according to number, color and maximum diameter. Then, Raman Microspectroscopy analysis was performed by using a XploRA Nano Raman Microspectrometer (Horiba Scientific), at the ARI Laboratory of Polytechnic University of Marche (Ancona, Italy). All filter membranes, including the procedural and the environmental blanks, were inspected by visible light using a $\times 10$ objective (Olympus MPLAN10x/0.25) and by using the results of the stereomicroscope as a guide. The thorough morphological characterization was performed by a $\times 100$ objective (Olympus MPLAN100x/0.90) and then analyzed directly on the

filter by RMS (spectral range 400–1800 cm^{-1} , 532 nm or 785 nm laser diode). Raw Raman spectra were polynomial baseline-corrected, and vector normalized (Labspec 6 software, Horiba Scientific). The chemical composition of the detected particles was identified by comparing the collected Raman spectra with several spectral libraries of polymers and pigments (KnowItAll software, John Wiley & Sons, Inc.) (Dong et al.,2020; SLOPP Library of Microplastics). Similarities higher than 80 of Hit Quality Index (HQI) were considered satisfactory.

3.6 Statistical analysis

Statistical analyses were performed using the software GraphPad Prism 8 for Windows. The analysis of biometric parameters at all the developmental stages was performed through one-way ANOVA followed by Tukey's posthoc test. Lipid percentage and analysis of MMs in liver samples were analyzed using the Student's t-test as well as morphological biometrics, lipid percentage and MMs in liver samples of embryos at stage 30. Significance was set at $p \leq 0.05$. The data obtained by biometric, histological and RAMAN analysis were analyzed using Pearson Correlation considering both the embryos at different developmental stages and data collected from embryos at stage 30. Significance was set at $p \leq 0.05$.

4. RESULTS

4.1 Temperature ranges

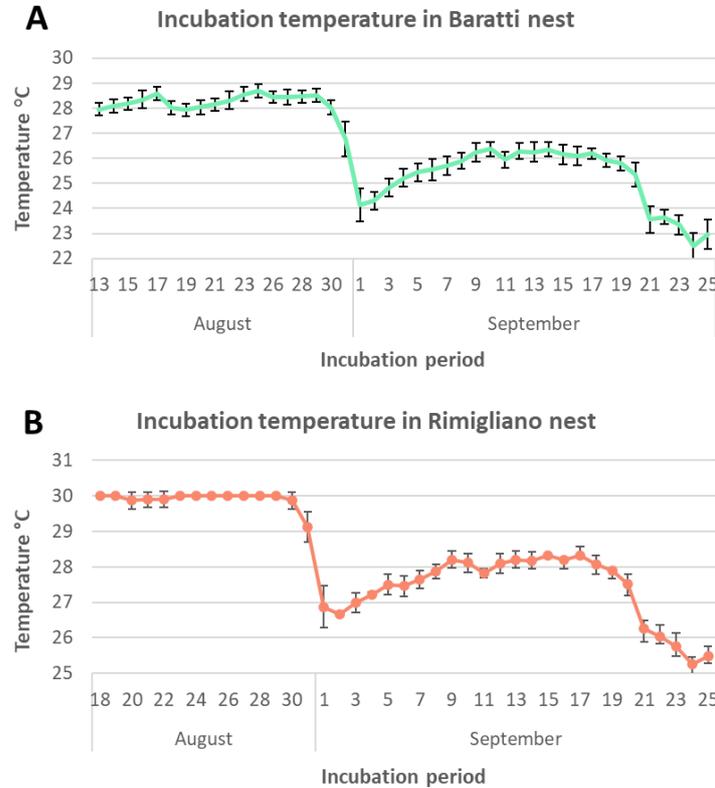


Figure 11. Incubation temperatures (°C) recorded by dataloggers located in the chamber of Baratti (A) and Rimigliano (B) nests for c.a. 37 days. Values are represented as mean \pm standard deviation.

During the first 40 days of the incubation period, temperatures were daily recorded, and the two nests showed a similar trend of temperature fluctuation (Figure 11A, B). However, for the first 17 days, the internal mean temperature of the Baratti nest (Figure 11A) was lower ($28^{\circ}\text{C} \pm 0.56$) than the mean temperature recorded within the Rimigliano nest ($30^{\circ}\text{C} \pm 0.30$) (Figure 11B). Successively, between August 30th and September 1st, the temperature in both nests dropped to 4°C and 3°C respectively in Baratti and Rimigliano nests (Figure 11A, B). For the last 24 days, the temperature in both nests raised again

reaching an average higher temperature in Rimigliano nest ($27^{\circ}\text{C} \pm 0.88$) than Baratti nest ($25^{\circ}\text{C} \pm 1.22$). Finally, during the last six days, the temperature decreased constantly reaching the lowest mean temperature recorded in both nests, $24^{\circ}\text{C} \pm 1.22$ and $26^{\circ}\text{C} \pm 1.01$ in Baratti and Rimigliano nests respectively.

4.2 Embryonic developmental stages

At sampling time, the macroscopical evaluation of eggs and embryos did not detect any structural and morphological abnormalities in all embryos from both nests.

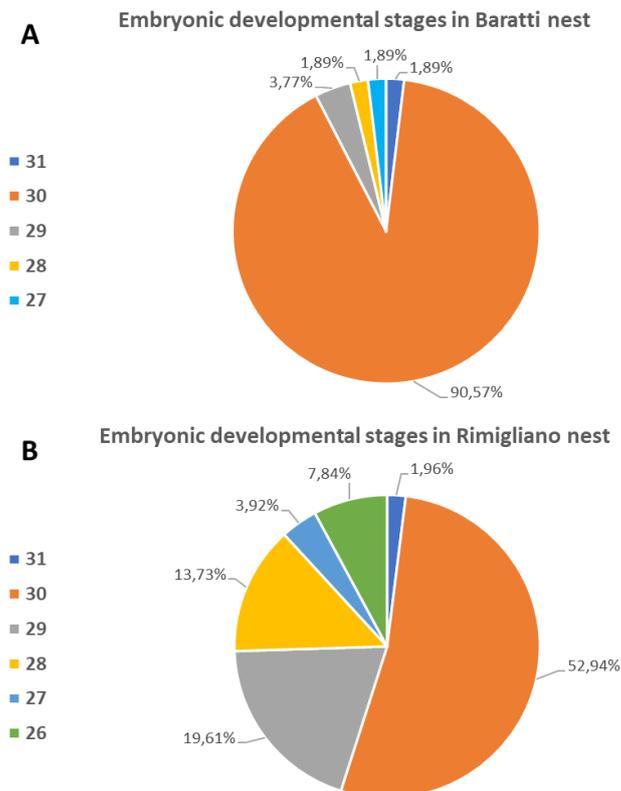


Figure 12. Percentage (%) of different *Caretta caretta* developmental embryonic stages in Rimigliano (A) and Baratti nest (B).

In both Baratti and Rimigliano nests, the majority of embryos were at developmental stage 30 representing more than 90% and 50% of total embryos in Baratti and Rimigliano nests respectively (Figure 12A, B). In Baratti nest lower developmental stages were represented in lower percentages (< 4%) (Figure 12A). The lowest development stage observed in this nest was the 27 (1.89%). Differently, in Baratti nest, lower stages occurred in higher percentages (19.61%, 13.73%, 7.84% and 3.92% at 29, 28, 26 and 27 respectively) than in Rimigliano nest (Figure 12B). Moreover, the 26 developmental stage was the lowest found in this nest.

4.3 Biometric measurements

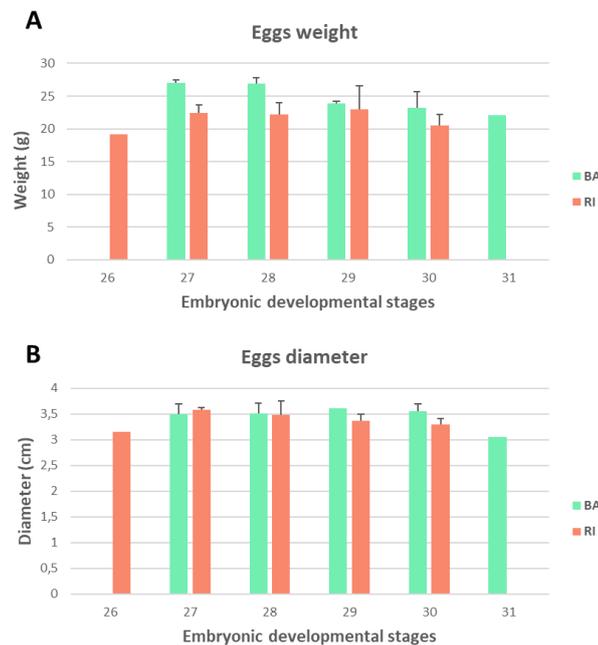


Figure 13. Weight (g) (A) and diameter (cm) (B) of *C. caretta* eggs at different embryonic development stages in Baratti and Rimigliano nests. Values are represented as mean \pm standard deviation when it was possible to calculate. Significance was set at $p \leq 0.05$. *= $p \leq 0.05$; **= $p \leq 0.01$; ***= $p \leq 0.001$.

Biometric measurements showed that average eggs weight values (Figure 13A) and the mean eggs' diameter (Figure 13B) no significant differences can be observed between the two nests.

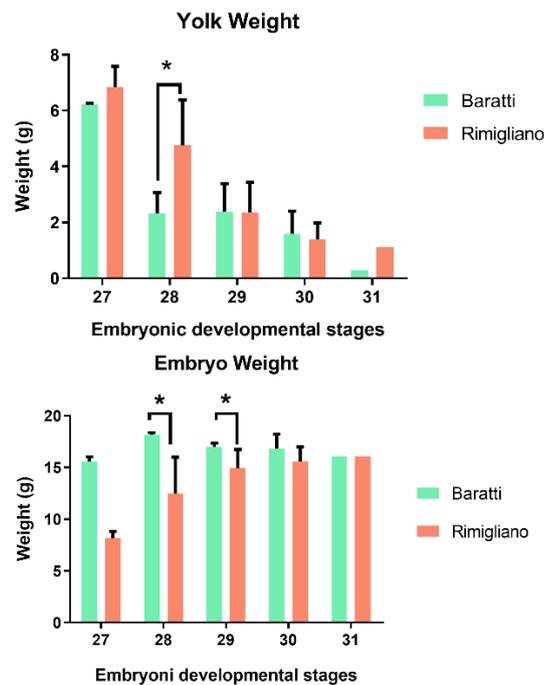


Figure 14. Yolk (A) and embryo (B) weight (g) at different embryonic developmental stages in Baratti and Rimigliano nests. Values are represented as mean \pm standard deviation when it was possible to calculate. Significance was set at $p \leq 0.05$. *= $p \leq 0.05$; **= $p \leq 0.01$; ***= $p \leq 0.001$.

In figure 14(A, B) embryos and yolks' weight at different embryonic developmental stages were compared between the two nests. In Baratti nest, the maximum mean value of embryos weight was recorded at the 28 developmental stages, while the mean embryos' weight slightly decreased following the increase of embryonic stages (Figure 13B). Conversely, in Rimigliano nest, the mean egg weight increased from the lowest embryonic

stages to the highest (Figure 14B). Concerning the yolk weight, a decreasing trend was visible in both nests following the increase in the developmental stages (Figure 14A). However, comparing the two nests, in Baratti nest the decrease of yolk weight was significant between stages 27 and 28 with respect to the same stage interval in Rimigliano nest.

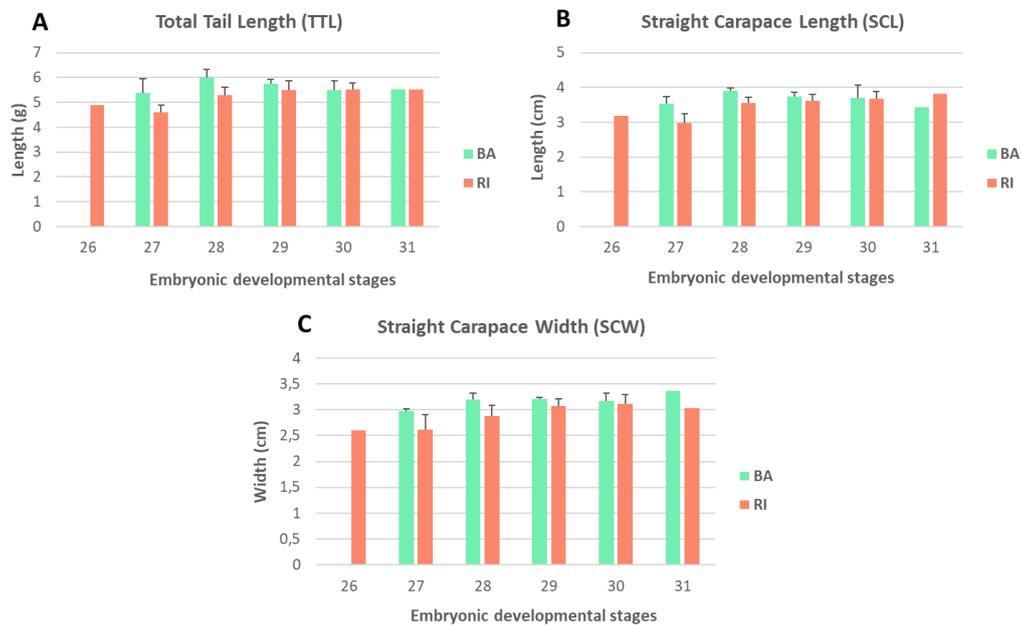


Figure 15. Total tail length (TTL) (A), straight carapace length (SCL) (B) and straight carapace width (SCW) in *C. caretta* embryos at different developmental stages, from Baratti and Rimigliano nests. Values are represented as mean \pm standard deviation when it was possible to calculate. Significance was set at $p \leq 0.05$. * = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$.

Concerning the TTL, SCL and SCW values no evident differences had been observed between the two nests (Figure 15A, B, C). In both nests, these measurements increased with the increasing of developmental stages. A similar

situation can be observed considering the forelimb (Figure 16A, B) and hindlimb length (Figure 16C, D).

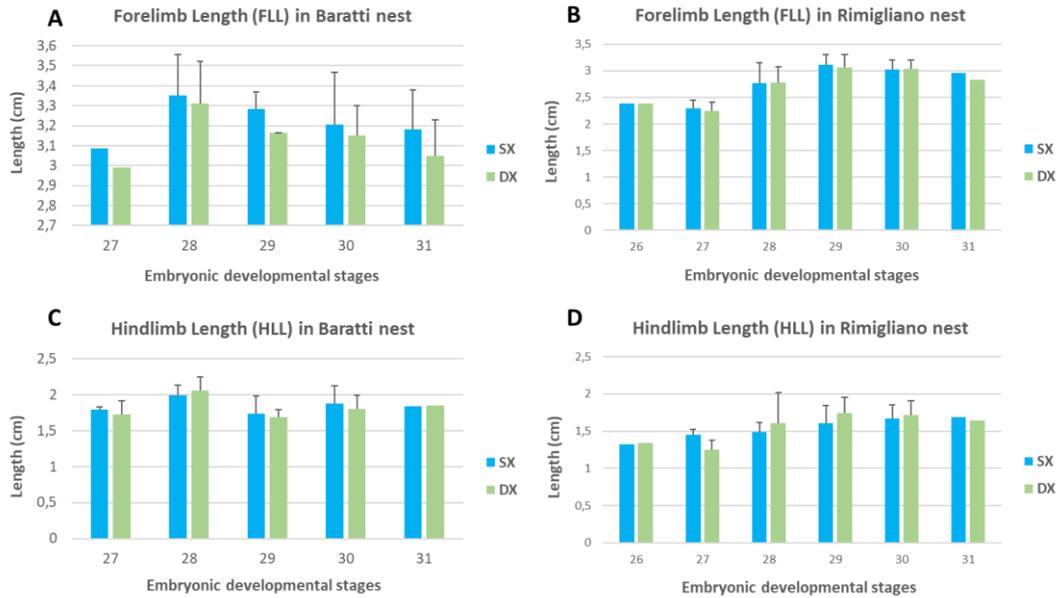


Figure 16. Forelimb and Hindlimb length in *C. caretta* embryos at different developmental stages from Baratti (A, C) and Rimigliano (B, D) nests. Values are represented as mean \pm standard deviation when it was possible to calculate. Significance was set at $p \leq 0.05$. * = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$.

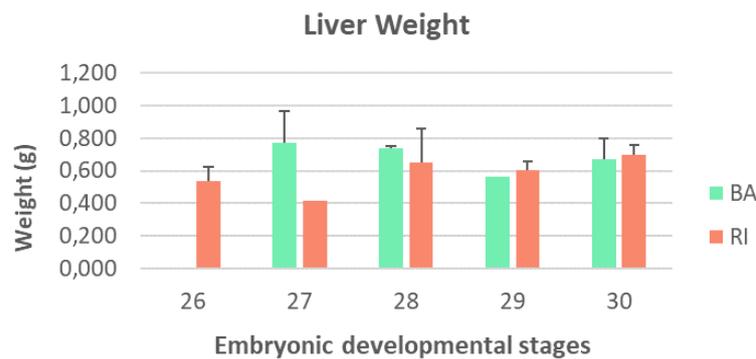


Figure 17. Liver weight of *C. caretta* embryos at different developmental stages from Baratti and Rimigliano nests. Values are represented as mean \pm standard deviation when it was possible to calculate. Significance was set at $p \leq 0.05$. * = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$.

Concerning the liver weight, no significant differences had been detected among the embryonic developmental stages in both nests except at the 27 stage characterized by a higher mean value in Baratti nest than in Rimigliano (Figure 17).

4.4 Histological analysis

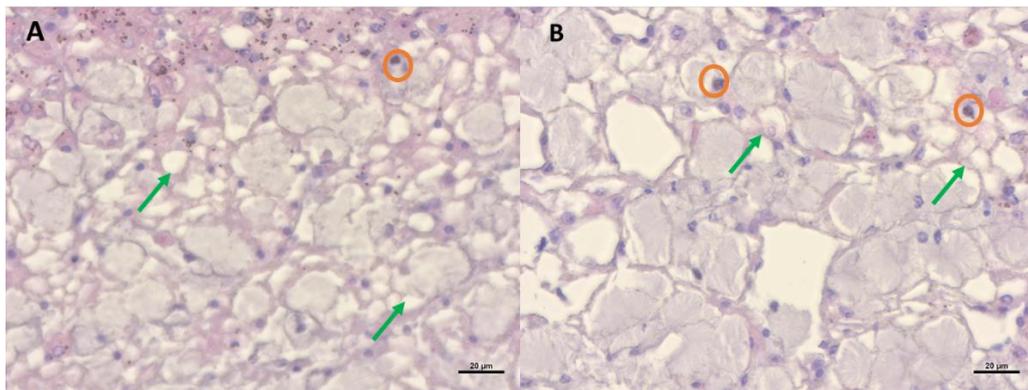


Figure 18. Example images of liver histological sections of *C. caretta* embryos in Baratti (A,) and Rimigliano (B) nests. Green Arrows indicate lipid vacuoles, orange circles indicate melanomacrophages. n= 17 samples analyzed in all embryonic developmental stages detected. Scale bars: 20 μ m.

The histological analysis of liver sections (Figure 18) let evaluate the lipid percentage and the presence of melanomacrophages in *C. caretta* embryos from Baratti (Figure 18A) and Rimigliano (Figure 18B) nests.

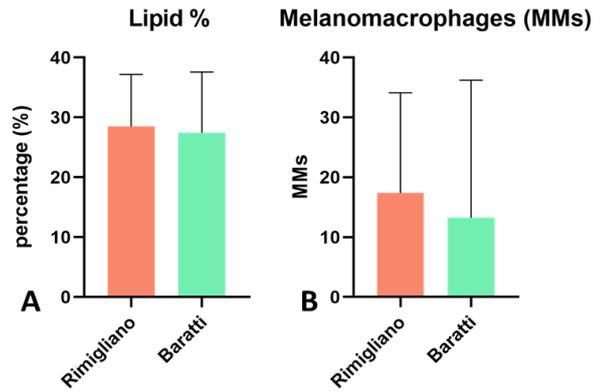


Figure 19. T-tests analysis on lipid percentage (%) (A) and melanomacrophages numbers (MMs) (B) in *C. caretta* liver embryos from Baratti and Rimigliano nests. n= 17 samples analyzed in all embryonic developmental stages. Values are represented as mean \pm standard deviation. Significance was set at $p \leq 0.05$.

The lipid percentage analysis in liver sections did not show any significant difference between Baratti and Rimigliano nests (Figure 19A). While the number of MMS appeared lower in Baratti samples than in Rimigliano (Figure 19B) but the difference between the two nests was not statistically significant.

4.5 Pearson's correlation analysis

Tables 1 represent the data set for Rimigliano and Baratti nest used for correlation analysis.

ID	stage	egg weight	egg diameter	embryo weight	yolk weight	TTL	SCL	SCW	lipid	MMs
RI1	30	20.40	3.18	16.74	1.24	5.39	3.79	2.94	31.15	9.00
RI2	30	20.62	3.25	16.65	1.26	5.45	3.59	3.12	22.18	4.00
RI3	30	22.80	3.20	17.18	1.37	5.82	3.84	3.10	21.54	10.00
RI4	29	25.35	3.32	17.08	2.61	6.05	3.86	3.26	35.54	3.00
RI5	29	26.98	3.41	11.78	2.35	5.44	3.59	2.90	37.03	3.00
RI6	28	24.41	3.68	17.55	3.76	5.25	3.71	3.04	40.10	31.00
RI7	28	20.69	3.76	12.41	2.68	5.05	3.56	2.72	23.41	1.67
RI8	27	21.61	3.55	7.73	6.31	4.42	2.77	2.65	15.86	46.00
RI9	26	23.52	3.37	10.06	8.51	5.00	3.22	2.61	24.41	33.00
BA1	30	28.28	3.71	20.68	1.53	5.22	4.04	3.32	23.96	65.00
BA2	30	24.06	3.71	16.29	2.62	5.77	3.90	3.16	21.83	4.00
BA3	30	19.93	3.52	17.13	0.81	5.80	3.88	3.26	33.03	2.00
BA4	30	26.23	3.67	18.60	1.86	5.62	3.96	3.22	21.82	85.00
BA5	30	18.88	3.29	13.73	0.78	5.80	3.85	3.24	35.34	1.00
BA6	30	23.58	3.50	17.56	2.06	5.52	3.70	3.12	20.29	29.00
BA7	30	23.39	3.42	15.83	3.92	5.50	3.83	3.06	48.65	2.00
BA8	28	27.57	3.65	18.32	2.85	5.79	3.86	3.11	29.92	2.00
BA9	27	26.78	3.64	15.90	6.19	4.99	3.37	2.95	47.42	2.00
BA10	27	27.36	3.37	15.23	6.25	5.78	3.68	3.00	13.58	1.00

Table 1. Data set for Rimigliano nest. **ID**, official identification number; **stage**, embryonic developmental stage; **egg weight**, weight (g) of the whole egg; **egg diameter**, mean diameter of the egg; **embryo weight**, weight (g) of the embryo; **yolk weight**, weight (g) of the yolk; **TTL**, total tail length (cm); **SCL**, straight carapace length (cm); **SCW**, straight carapace width (cm); **lipid**, average lipid fraction percentage (%) in liver samples; **MMs**, average number of melanomacrophages in liver samples.

	stage	egg weight	egg diameter	embryo weight	yolk weight	TTL	SCL	SCW	lipid	MMs
stage										
egg weight	-0.126									
egg diameter	-0.571	0.038								
embryo weight	0.749*	-0.003	-0.373							
yolk weight	-0.957***	0.124	0.326	-0.730*						
TTL	0.701*	0.363	-0.545	0.798**	-0.654					
SCL	0.736*	0.188	-0.337	0.898***	-0.766*	0.893**				
SCW	0.765*	0.238	-0.441	0.881**	-0.717*	0.870**	0.785*			
lipid	0.191	0.668*	0.072	0.475	-0.257	0.484	0.596	0.457		
MMs	-0.734*	-0.043	0.302	-0.533	0.804**	-0.727*	-0.759*	-0.549	-0.280	

Table 2. Pearson's correlation coefficients (n=8) for Rimigliano nest. **stage**, embryonic developmental stage; **egg weight**, weight (g) of the whole egg; **egg diameter**, mean diameter of the egg; **embryo weight**, weight (g) of the embryo; **yolk weight**, weight (g) of the yolk; **TTL**, total tail length (cm); **SCL**, straight carapace length (cm); **SCW**, straight carapace width (cm); **lipid**, lipid average percentage (%) in liver samples; **MMs**, average number of melanomacrophages in liver samples. * = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$.

For Rimigliano nest, Pearson's correlation analysis (Table 2) highlighted a strong significant positive correlation between SCL and embryo weight ($r=0.898$, $p=0.00997$) and a strong negative correlation between stage and yolk weight ($r=-0.957$, $p=5.021E-05$). The correlation coefficient obtained between embryo weight and total tail length and straight carapace width (TTL and SCW) ($r=0.798$, $p=0.009$ and $r=0.881$, $p=0.0016$ respectively), total tail length (TTL) and straight carapace length and width (SCL and SCW) ($r=0.893$, $p=0.0011$ and $r=0.87$, $p=0.0022$ respectively); and yolk weight and number of melanomacrophages (MMs) ($r=0.804$ and $p=0.0089$) suggested a moderate positive correlation. Finally, a low significant positive correlation was described between stage, embryo weight, TTL, SCL and SCW ($r=0.749$, $p=0.02$; $r=0.701$, $p=0.035$; $r=0.736$, $p=0.023$ and $r=0.765$, $p=0.016$ respectively); egg weight and %lipid ($r=0.668$, $p=0.049$), while a low negative significant correlation coefficient was described between stage and MMs ($r=-0.73$, $p=0.024$) between yolk weight and embryo weight, SCL and SCW ($r=-0.730$, $p=0.025$; $r=-0.766$, $p=0.016$; $r=-0.717$, $p=0.029$ respectively) and between MMs and TTL and SCL ($r=-0.727$, $p=0.026$; $r=-0.759$, $p=0.017$ respectively).

	stage	egg weight	egg diameter	embryo weight	yolk weight	TTL	SCL	SCW	lipid	MMs
stage										
egg weight	-0.539									
egg diameter	0.029	0.571*								
embryo weight	0.229	0.558*	0.741**							
yolk weight	-0.844***	0.537	-0.066	-0.313						
TTL	0.224	-0.440	-0.374	-0.293	-0.366					
SCL	0.715**	-0.108	0.186	0.479	-0.744**	0.452				
SCW	0.769**	-0.324	0.175	0.458	-0.918***	0.254	0.843***			
lipid	-0.045	-0.283	-0.125	-0.311	0.147	-0.421	-0.358	-0.265		
MMs	0.386	0.377	0.466	0.721**	-0.352	-0.267	0.475	0.510	-0.387	

Table 3. Pearson's correlation coefficients (n=9) for Baratti nest **Stage**, embryonic developmental stage; **Egg weight**, weight (g) of the whole egg; **Egg diameter**, mean diameter of the egg; **Embryo weight**, weight (g) of the embryo; **Yolk weight**, weight (g) of the yolk; **TTL**, total tail length (cm); **SCL**, straight carapace length (cm); **SCW**, straight carapace width (cm); **lipid**, average percentage (%) lipid area in liver samples; **MMs**, average N° of melanomacrophages in liver samples. *= $p \leq 0.05$; **= $p \leq 0.01$; ***= $p \leq 0.001$.

For Baratti nest, Pearson correlation coefficient (Table 3) obtained highlight a strong significant positive correlation between SCL and SCW ($r=0.843$, $p=0.00082$) and a strong negative significant correlation between stage and yolk weight and yolk weight and SCW ($r=-0.844$, $p=0.001$ and $r=-0.918$, $p=8.75E-05$ respectively). A moderate positive correlation is obtained from correlation coefficient between stage and SCW and SCL; between egg diameter/embryo weight and MMs/embryo weight ($r=0.769$, $p=0.00117$; $r=0.715$, $p=0.0100$; $r=0.741$, $p=0.007$; $r=0.721$, $p=0.0092$ respectively) while a moderate negative correlation is obtained between yolk weight and SCL ($r=-0.744$, $p=0.0067$). Finally, a low significant positive correlation is suggested from the correlation coefficient between egg weight and egg diameter and embryo weight ($r=0.571$, $p=0.042$ and $r=0.558$, $p=0.0467$).

4.6 Biometric and histological analysis of 30° embryonic developmental stage

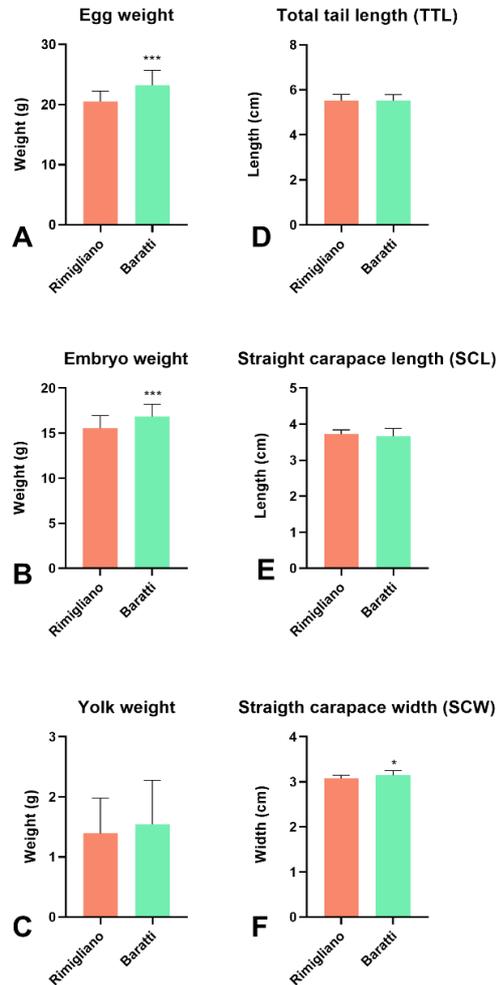


Figure 20. T-test analysis performed on different biometrics, egg weight (A), embryo weight (B), yolk weight (C), total tail length (TTL) (D), straight carapace length (SCL) (E) and straight carapace width (SCW) (F) in *C. caretta* embryos at the 30 embryonic developmental stages from Baratti and Rimigliano nest. Values are represented as mean \pm standard deviation. Significance was set at $p \leq 0.05$. * = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$.

The t-test analysis performed for the embryos belonging to the most represented developmental stage (30), highlighted a significant difference

between the two nests only if considering the mean egg weight, embryo weight and SCW (Figure 20A, B, F respectively). The values of these three parameters were significantly higher in Baratti nest than in Rimigliano. Conversely, the yolk weight (Figure 20C), TTL (Figure 20D) and SCL (Figure 20E) values showed no significant differences between Baratti and Rimigliano embryos.

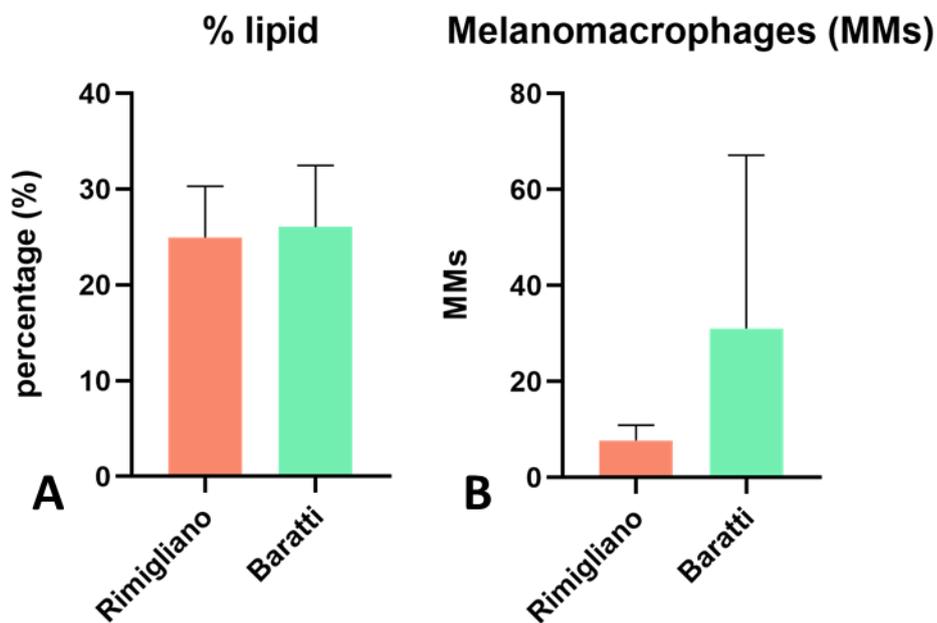


Figure21. T-tests analysis on lipid fraction percentage (%) (A) and melanomacrophages numbers (MMs) (B) in liver samples of the 30-stage embryos from Baratti and Rimigliano nests (n=9). Values are represented as mean \pm standard deviation. Significance was set at $p \leq 0.05$.

The lipid percentage analysis in liver sections of embryos at the 30 developmental stage did not show any significant difference between Baratti and Rimigliano nests (Figure 21A). Similarly, the number of MMS did not show any significant difference between the two nests, although the section

analyzed in Baratti samples reported high variability in the presence of melanomacrophages (Figure 21B).

4.7 MPs detection analysis

	n° MPs/yolk	n° MPs/liver	n° MPs/yolk (n/g)	n° MPs/liver (n/g)	polymer/yolk	polymer/liver
BA1	1	2	0.73	4.09	1 ABS	1 ABS, 1 PP blu
BA2	1	0	0.26	0.00	PVC yellow	ND
BA3	1	2	0.73	4.09	PP blu	2 PC yellow
BA4	1	1	0.73	2.04	ABS	1 PU
BA5	2	0	0.70	0.00	2 PE	ND
RI1	1	0	0.37	0.00	1 PE BLU	ND
RI2	2	1	0.53	1.76	1 BLU PHTALO, 1 PVC yellow	1 BLU PHTALO
RI3	2	0	1.47	0.00	1 PVC yellow, 1 PE orange	ND
RI4	2	0	1.58	0.00	1 PET grey, 1 PVC yellow	ND
RI5	2	0	0.81	0.00	1 PE BLU, 1 PE green	ND

Table 4. Number and type of MPs in yolk and liver samples of embryos from Baratti and Rimigliano nests. **n° MPs/yolk**, number of microplastics detected in yolk samples; **n° MPs/liver**, number of microplastics detected in liver samples; **n° MPs/yolk (n/g)**, number of microplastics detected for g of yolk; **n° MPs/liver (n/g)**, number of microplastics detected for g of liver; **polymer/yolk**, polymer and pigment of microplastics in yolk samples; **polymer/liver**, polymer and pigment of microplastics in liver samples. **ABS**, Acrylonitrile butadiene styrene; **PP**, polypropylene; **PVC**, Polyvinyl chloride; **PC**, Polycarbonate; **PU**, polyurethane; **PE**, Polyethylene; **BLU PHTALO**, phthalocyanine blue; **ND**, no detectable.

The Raman microspectroscopy analysis performed in this study revealed that MPs number in yolk samples from Rimigliano nest was higher than in yolks from Baratti nest ($1.8 \pm 0,447$; 1.2 ± 0.447 respectively) (Table 4). Conversely, the number of MPs in liver samples was higher in Baratti than Rimigliano nest (1 ± 1 ; 0.2 ± 0.447 respectively). In both nests were found a higher number of MPs in the yolk than in the embryos' liver. Considering the types of MPs detected, none of the polymers identified was present in predominant number over the others in Baratti nests, while in Rimigliano nest yellow PVC and PE (of different colors) were the more abundant polymers. In Figure 22 some examples of the polymers individuated during Raman microspectroscopy analysis.

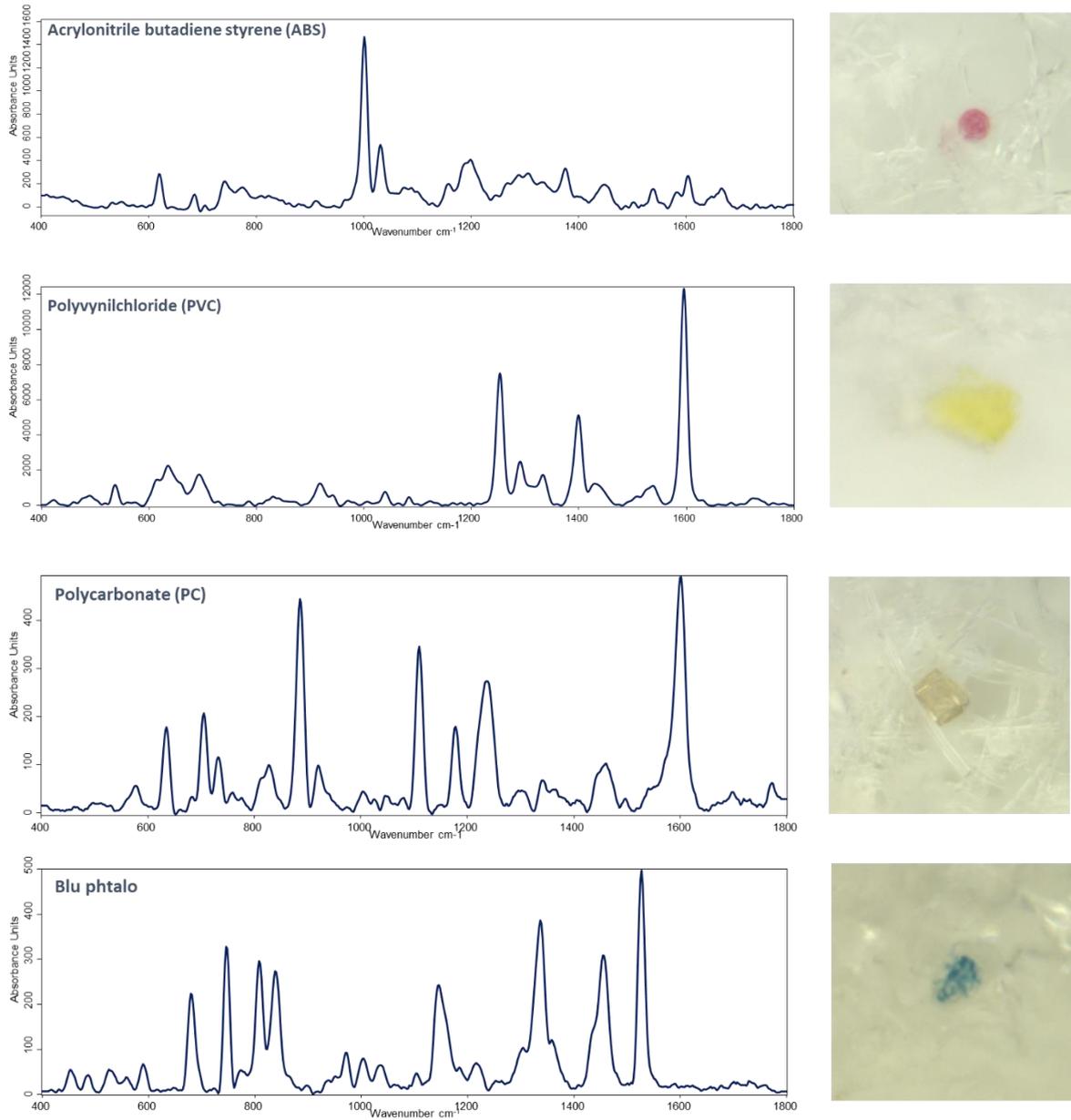


Figure 22. Examples of polymers and pigments observed in yolk and liver samples of Baratti and Rimigliano nests during Raman microspectroscopy analysis.

4.7.1 Pearson's Correlation analysis

ID	stage	egg weight	egg diameter	embryo weight	yolk weight	TTL	SCL	SCW	lipid	MMs	liver weight	n° MPs/yolk	n° MPs/liver
BA1	30	28.28	3.71	20.68	1.53	5.22	4.04	3.32	23.96	65	0.638	0.73	4.09
BA2	30	23.39	3.42	15.83	3.92	5.5	3.83	3.06	48.65	2	0.682	0.26	0
BA3	30	26.23	3.67	18.6	1.86	5.62	3.96	3.22	21.82	85	0.678	0.73	4.09
BA4	30	23.58	3.5	17.56	2.06	5.52	3.7	3.12	20.29	29	0.528	0.73	2.04
BA5	30	27.57	3.65	18.32	2.85	5.79	3.86	3.11	29.92	2	0.749	0.7	0
RI1	30	20.69	3.76	12.41	2.68	5.05	3.56	2.72	23.41	1.67	0.555	0.37	0
RI2	30	24.41	3.68	17.55	3.76	5.25	3.71	3.04	40.1	31	0.96	0.53	1.76
RI3	30	22.8	3.2	17.18	1.37	5.82	3.84	3.1	21.54	10	0.728	1.47	0
RI4	30	20.62	3.25	16.65	1.26	5.45	3.59	3.12	22.18	4	0.69	1.58	0
RI5	30	20.4	3.18	16.74	1.24	5.39	3.79	2.94	31.15	9	0.615	0.81	0

Table 5. Data set of Baratti (BA) and Rimigliano (RI) values. **ID**, official identification number; **stage**, embryonic developmental stage; **egg weight**, egg's weight expressed in g; **egg diameter**, mean diameter of the egg; **embryo weight**, embryo's weight expressed in g; **yolk weight**, yolk's weight expressed in g; **TTL**, total tail length (cm); **SCL**, straight carapace length (cm); **SCW**, straight carapace width (cm); **lipid**, average lipid fraction percentage in liver samples; **MMs**, average number of melanomacrophages in liver samples; **liver weight**, liver's weight expressed in g; **n° MPs/yolk**, number of microplastics (MPs) in yolk samples (n°/g); **n°MPs/liver**, number of microplastics in liver samples (n/g).

BARATTI	stage	egg weight	egg diameter	embryo weight	yolk weight	TTL	SCL	SCW	lipid	MMs	liver weight	n° MPs/yolk	n°MP s/liver
stage													
egg weight	-0.44												
egg diameter	-0.27	0.94*											
embryo weight	-0.04	0.87	0.92*										
yolk weight	-0.24	-0.52	-0.73	-0.85									
TTL	-0.70	-0.09	-0.08	-0.43	0.37								
SCL	0.08	0.79	0.76	0.72	-0.41	-0.41							
SCW	0.30	0.71	0.81	0.93*	-0.84	-0.62	0.81						
lipid	-0.05	-0.40	-0.64	-0.69	0.93*	0.07	-0.14	-0.60					
MMs	0.52	0.39	0.63	0.66	-0.81	-0.40	0.63	0.83	-0.65				
Liver weight	-0.65	0.48	0.30	-0.01	0.41	0.48	0.44	-0.11	0.43	-0.21			
n° MPs/yolk	-0.19	0.58	0.76	0.76	-0.89*	0.04	0.21	0.60	-0.97**	0.56	-0.23		
n°MPs/liver	0.56	0.40	0.63	0.73	-0.88*	-0.54	0.60	0.89*	-0.71	0.98**	-0.35	0.60	

Table 6. Pearson's correlation coefficients (n=5) for Baratti samples. **stage**, embryonic developmental stage; **egg weight**, weight (g) of the whole egg; **egg diameter**, mean diameter of the egg; **embryo weight**, embryo's weight expressed in g; **yolk weight**, yolk's weight expressed in g; **TTL**, total tail length (cm); **SCL**, straight carapace length (cm); **SCW**, straight carapace width (cm); **lipid**, average lipid fraction percentage in liver samples; **MMs**, average number of melanomacrophages in liver samples; **liver weight**, liver's weight expressed in g; **n° MPs/yolk**, number of MPs per g of yolk; **n°MPs/liver**, number of MPs per g of liver. *= $p \leq 0.05$; **= $p \leq 0.01$; ***= $p \leq 0.001$.

RIMIGLIANO	stage	egg weight	egg diameter	embryo weight	yolk weight	TTL	SCL	SCW	lipid	MMs	liver weight	N° MPs/yolk	N° MPs /liver
stage													
egg weight	-0.397												
egg diameter	-0.991**	0.311											
embryo weight	0.490	0.502	-0.576										
yolk weight	-0.940*	0.650	0.897*	-0.162									
TTL	0.777	0.185	-0.800	0.649	-0.620								
SCL	0.472	0.379	-0.573	0.664	-0.277	0.719							
SCW	0.581	0.397	-0.615	0.896*	-0.301	0.753	0.437						
lipid	-0.469	0.584	0.361	0.395	0.678	-0.360	0.201	0.040					
MMs	-0.408	0.887*	0.299	0.586	0.691	-0.036	0.345	0.343	0.882*				
liver weight	-0.282	0.915*	0.197	0.678	0.588	0.137	0.269	0.577	0.697	0.937*			
N° MPs/yolk	0.837	-0.124	-0.800	0.511	-0.739	0.830	0.254	0.787	-0.568	-0.300	-0.015		
N° MPs /liver	-0.612	0.834	0.527	0.385	0.844	-0.279	0.055	0.192	0.874	0.955**	0.902*	-0.431	

Table 7. Pearson's correlation coefficients (n=5) for Rimigliano samples. **stage**, embryonic developmental stage; **egg weight**, weight (g) of the whole egg; **egg diameter**, mean diameter of the egg; **embryo weight**, embryo's weight expressed in g; **yolk weight**, yolk's weight expressed in g; **TTL**, total tail length (cm); **SCL**, straight carapace length (cm); **SCW**, straight carapace width (cm); **lipid**, average lipid fraction percentage in liver samples; **MMs**, average number of melanomacrophages in liver samples; **liver weight**, liver's weight expressed in g; **n° MPs/yolk**, number of MPs per g of yolk; **n°MPs/liver**, number of MPs per g of liver. *= $p \leq 0.05$; **= $p \leq 0.01$; ***= $p \leq 0.001$.

Table 5 shows the data set used to perform Pearson's correlation analysis in both nests. In Baratti nest (Table 6), the correlation coefficient obtained between the MMs and the n° of MPs/liver ($r=0.955$, $p=0.0046$) suggested a significant positive correlation, while the correlation between the lipid percentage and the number of MPs in yolk samples highlighted a significant moderate negative correlation ($r=-0.9657$, $p=0.0076$). A significant strong positive correlation was described between the egg diameter and the egg and embryo weight ($r=0.915$, $p=0.029$ and $r=0.940$, $p=0.0172$ respectively), between embryo weight and SCW ($r=0.92$, $p=0.0228$), yolk weight and %lipid ($r=0.933$, $p=0.0205$) and between SCW and N°MPs/liver ($r=0.891$, $p=0.0423$). Conversely, significant strong negative correlations were observed between the yolk weight and the N° of MPs/yolk and /liver ($r=-0.889$, $p=0.0435$ and $r=-0.8822$, $p=0.048$ respectively).

For Rimigliano nest (Table 7), Pearson's correlation analysis highlighted a significant moderate negative correlation between stage and egg diameter ($r=-0.991$, $p=0.00105$). The correlation coefficients obtained suggested a significant strong positive correlation between: egg diameter/ yolk weight ($r=0.897$, $p=0.039$), egg weight /MMs and egg weight/liver weight ($r=0.887$, $p=0.0448$ and $r=0.915$, $p=0.02957$ respectively), weight embryo/SCW ($r=0.896$, $p=0.039$); MMs/%lipid, liver weight/N° MPs/liver ($r=0.882$,

p=0.047; r=0.937,p=0.019 and r=0.955,p=0.0114 respectively) and between N°MPs/liver and liver weight (r=0.902,p=0.03613). Finally, the only significant strong negative correlation was observed between embryos stage and yolk weight (r=-0.940, p=0.0176). .

5. Discussion

Until recently, loggerhead turtles' nests were found mainly in the south-east area of the Mediterranean Sea, from Cyprus, Greece and Turkey, to Linosa and Lampedusa islands off the southern Sicilian and Ionian coasts, while the northern part of the Mediterranean Sea is recognized mainly as a foraging area (Margaritoulis et al., 2003). Only in the last few years, *C. caretta* has moved to the north-western part of the Mediterranean along the Italian coast in search of new nesting sites (Bentivegna et al., 2005). This new migratory pattern is presumably related to the increase in water temperature that drives sea turtles further north to find lower temperatures more suitable for their activities and thus expanding their distribution range (Bentivegna et al., 2005; Witt et al., 2010; Bentivegna et al., 2010). Indeed, on the Tuscan coast, the number of nests found is growing each year. The eggs from the two nests analyzed in this study were laid in the middle of August and thus represent a late deposition if considering that in the Mediterranean area the peak of the nesting period occurs between June and July (Casale et al., 2018).

It should be restated that the purpose of this study was to analyze the morphological and physiological features of embryos that have reached the last stages of development with no visible alterations or disease often associated with embryos death, in addition, it was investigated the presence of

microplastics in embryos of *C. caretta* and its possible effects associated to physiological alterations. Nevertheless, it was considered that when eggs are laid at the end of the reproductive season, embryos are exposed to the decrease in temperature and the increase in rainfall events which are typical of the autumn season.

This environmental scenario usually affects the incubation temperature in the nest causing an extension of the incubation period and influencing the rate of embryonic development and sex ratio (Booth and Freeman, 2006). Probably, the late deposition time of the two nests analyzed in this study is the cause of the mean lower embryos' size observed if compared to the standard measurements reported in the literature. Indeed, comparing the mean SCL of embryos at the last and most representative stage (30) from both nests (3.6 ± 0.35 cm and 3.7 ± 0.21 cm in Baratti and Rimigliano nests respectively), they resulted slightly lower than SCL values of *C. caretta* embryos from other Italian and Mediterranean nests that usually reach values higher than 4.0 cm at hatching time (Glen et al., 2003; Bentivegna et al., 2010). Previous studies observed that smaller body size (SCL, SCW) of hatchlings is usually associated with higher nest incubation temperature (Glen et al., 2003; Reid et al., 2009). However, it is also stated that the nest temperature may play a key role in the energy use, with cooler temperature increasing the overall energy demands

placed on developing embryos (Reid et al., 2009). The higher demands of energy during the incubation period could be the cause of the slower growth of embryos.

In both nests, the embryos reached the latest developmental stages, despite the different frequency of each stage between the two clutches and different embryos weight between the same developmental stages. In particular, at the last stage before hatching (30), the egg weight and the embryo weight recorded in Baratti nest were higher than those in Rimigliano nest. These disparities could reflect a different growth rate between the two nests. Although the rate of yolk consumption was comparable between the Rimigliano and Baratti nests, once nutrients were absorbed, they could have been for different purposes than growth, in this way the reason for different embryos size between the two nests could be related to a different energy allocation.

It is important to consider that the developmental process strictly depends on the energy deposited in the yolk which is used by the embryo to support the energetic cost of development and as nourishment by the hatchling until they can actively feed in the sea (Troyer, 1983; Vleck and Hoyt, 1991). Indeed, the poor quality of yolk may be another cause of the different growth of embryos in the two nests. To date, the absorption and internalization mechanisms of yolk by sea turtle embryos are still poorly understood. Nevertheless, among its

functions, the liver has a key role in providing energy to the whole body and managing the systemic supply of nutrients (Gu and Manautou, 2012).

Therefore, through histological analysis of the liver sections was possible to evaluate the lipid storage in the embryos to support the developmental process. In both nests, the lipid percentage in the liver of embryo at the last stage before hatching (30), was almost similar highlighting that energetic resources were accumulated and used during the development process in the same way in both clutches and that should not be the cause of the different growth in the two nests.

Through the histological analysis was also possible to evaluate the presence of MMs in liver samples to better understand the health status of the embryos. As shown in studies performed on fishes, MMs are more numerous if associated with pathological conditions due to disease or stress (Hur and Lee, 2010). In this study was evidenced that in Rimigliano nest the number of MMs was strongly negatively correlated with the developmental stage, suggesting that, in the early developmental stages, stresses and a general not optimal health status had not allowed embryos to reach the end of the embryonic development.

Concerning the microplastic analysis, for the first time, several MPs were detected in yolk and liver samples in both nests. The polymers ABS, PVC, PE, PU, PET and PP collected are commonly used and their presence is widely

reported in the marine environment (Yuan et al., 2022). ABS is common in telephone handsets, rigid luggage, domestic appliance housings, electroplated parts, radiator grills, handles and computer housings; PC is used in blends due to its compatibility with a range of polymers; PET, PE and PVC are exploited in the manufacture of a wide range of components, including packaging, single-use plastics and medical devices; PP is widely used in domestic stuff such as microwaves and coffee pots (<https://www.bpf.co.uk/>). The identification of the polymer and the pigment of the microplastics found in the samples could be useful to hypothesize their possible origin. These MPs reach the sea through rivers and urban runoffs and they could be transported for long distances by marine currents and wind (Li et al., 2020). Once in the marine environment, MPs can be ingested by marine organisms. Several studies have been made to understand the interaction between *C. caretta* and marine plastic debris, enough to consider loggerhead turtle as official bioindicators for marine litter (Descriptor 10) by the European Union Marine Strategy Framework Directive (MSFD). The presence of MPs in organisms cause different effects, such as cytotoxicity, oxidative stress, intracellular uptake, produce immune response, alter gene expression, and induce weak embryotoxicity and hemolysis (Meaza et al., 2021). To our knowledge, this study is the first study detecting MPs in yolk and embryos of sea turtle. The process behind their presence in both yolk

and embryos of sea turtles, could be associated to the MPs maternal transfer. Presumably, MPs could accumulate in female's liver where during the vitellogenesis they bind to different proteins precursors of the yolk, and thus could be transferred to the embryos, as already described in studies performed on fish species (Pitt et al., 2018). In addition, the microplastics found in the liver samples of embryos suggested an absorption by the embryos of these MPs. Moreover, the MPs found in the yolk and liver are selectively different and characteristics of the two nests, supporting our thesis of the maternal transfer because if they were derivatives of environmental contamination probably, they would have been homogeneously distributed between the two nests laid few kilometers apart from each other.

The Pearson's correlation analysis suggested some possible effects of the MPs on embryo development.

The number of MPs identified in our study was higher than that found in other studies conducted on fishes' tissues, perhaps because sea turtles are subjected to a higher accumulation of microplastics given by their high level in the trophic chain (Abbassi et al., 2018; Meaza et al., 2021).

Moreover, the statistical analysis performed in embryos from Baratti nest showed an increasing of MPs number in yolk associated to a decrease of the yolk weight and the lipid percentage in liver section. To date, the transfer

mechanisms of MPs in tissues of organisms is still poorly explored, but our results could suggest a transport of MPs through the lipid components that were internalized at the end of development process for a selective absorption process of the yolk as also reported by Alava and colleagues (2006). Furthermore, in Baratti nest, the increasing in number of microplastics in the liver was associated to the rise in number of melanomacrophages. Similarly, a higher presence of MPs in liver samples caused a growing on the number of MMs in liver section in Rimigliano nest, proving that variation of number of MMs is a valid biomarker for the presence of pollutants in organisms, as already seen in studies performed in fishes (Passantino et al., 2014).

In conclusion to the present study, the detection of MPs was associated to variation of physiological parameters in *Caretta caretta* embryos, such as an increasing of number of MMs and an altered yolk absorption, which might cause impairment in the embryonic development and contribute to make embryos weaker and more susceptible to environmental threats such as temperature, pH and bacterial and fungal infections. Further analysis should be performed to understand the possible effects of MPs on the health status of the embryos and on the embryonic developmental process and to focus on the mechanisms of yolk internalization and absorption as the principal pathway of MPs internalization.

6. References

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