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Focus sulla tartaruga comune (*Caretta caretta*) spiaggiate in Abruzzo: valutazione delle plastiche ingerite, dello stato di salute e della maturità sessuale

Focus on Loggerhead Sea turtle (*Caretta caretta*) stranded in Abruzzo: evaluation of the plastics ingestion, the health status and sexual maturation

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RIASSUNTO

La tartaruga comune *Caretta caretta* è considerata una specie vulnerabile secondo la Lista Rossa dell'Unione mondiale per la conservazione della natura (IUCN); perciò, è fondamentale prevenire ed evitare il collasso delle popolazioni e gli eventuali effetti diretti e indiretti che si ripercuoterebbero sugli ecosistemi marini a diversi livelli della rete trofica. La presenza della tartaruga comune influenza in diversi modi la vita di altre specie marine. Ad esempio, la tartaruga comune ospita più di 100 specie di epibionti (cirripedi, alghe, poriferi e cnidari) che ancorati al carapace si fanno trasportare e si nutrono dagli scarti del cibo della tartaruga, inoltre ha un ruolo fondamentale nel riciclo dei nutrienti all'interno delle zone di foraggiamento. Per evitare la compromissione di questi delicati equilibri, è necessario ottenere una conoscenza approfondita della biologia e dell'habitat di questi animali per poterli preservare e per limitare gli effetti negativi delle attività antropiche sulla salute di questi animali. Lo scopo di questa tesi è quello di descrivere e comprendere il fenomeno degli spiaggiamenti di *C. caretta* registrati negli ultimi tre anni (2019-2021), con un particolare focus sugli animali trovati lungo le coste dell'Abruzzo. Considerando diversi parametri come la stagionalità

dei ritrovamenti, il rapporto tra maschi e femmine ritrovati, si è potuto osservare una crescita costante di questo fenomeno.

Analisi necroscopiche effettuate su esemplari di *C. caretta* spiaggiati durante il 2021, hanno messo in evidenza la presenza di microplastiche nel tratto gastrointestinale di 9 animali (8 femmine e 1 maschio). Per valutare i possibili effetti che queste plastiche potrebbero esercitare sulla salute dell'animale, sono state effettuate analisi istologiche su campioni di fegato e milza per quantificare la presenza di melanomacrofagi, cellule pigmentate che hanno un ruolo di sintesi della melanina, rimozione e autolisi dei globuli rossi e internalizzazione di organismi procarioti, che nei vertebrati, si trovano fisiologicamente in questi organi.

Un altro obiettivo di questa tesi è stato quello di caratterizzare, per la prima volta, la struttura della gonade femminile e descrivere la maturazione follicolare

I dati e i campioni analizzati in questa tesi sono stati raccolti dall' Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise (con sede a Teramo) all'interno di un più ampio progetto il cui titolo è 'l'identificazione di biomarkers per la valutazione degli effetti tossici causati dai contaminanti, microplastiche comprese, e infezioni antropozoonotiche in *C. caretta* definendo uno stato di salute nella popolazione di tartarughe nel Mediterraneo".

INDICE

1. INTRODUCTION

1.1 General biology of Caretta caretta

The loggerhead sea turtle, *Caretta* (Linnaeus 1758), belongs to the order of *Testudines* that includes various species such as *Chelonia mydas* (green sea turtle), *Chelonia agassizii* (black sea turtle), *Eretmochelys imbricata* (hawksbill sea turtle), *Lepidochelys kempii* (Kemp's ridley sea turtle), *Lepidochelys olivacea* (olive ridley sea turtle), *Natator depressus* (Australian flatback sea turtle) and *Dermochelys coriacea* (leatherback sea turtle) (Lutz, Musick and Jeanette, 2003).

Although the loggerhead sea turtles are spread all over the ocean, especially in tropical and temperate areas, much information regarding their biology and reproduction is missing.

Like all the other sea turtle's species, the loggerhead turtle is an ectotherm, which means that its body temperature changes according to the outer environment. Although it spends almost the entire life in the water, it has lungs, and it can maintain a long period of apnea, up to a maximum of 5 hours (Lutz, Musick and Jeanette, 2003).

It is distinguished from the other sea turtle species by a big head with the presence of four prefrontal shields (P), five vertebral shields flanked by five coastal shields (C), three inframarginal shields (I) and one nuchal (N) in contact with the first coastal one (Figure 1) (Spotila, J.R., 2004).

Sea turtles' nutrition changes depending on the species, for example, the loggerhead turtles are omnivorous, preferring prey like crustaceans and jellyfish mainly, while others as the green sea turtle are herbivorous (they eat mainly algae). Moreover, their diet changes also depending on their life stages: hatchlings and juveniles feed on pelagic organisms (such as jellyfish), while the adults prey on benthic organisms (e.g. crustaceans and mollusks) (Donaton *et al.*, 2019).

Sea turtles spent their life in the water except for eggs deposition that occurs on the dry land where females deposit their eggs in holes dug with their anterior natatory fins. They are characterized by internal fecundation, males and females meet in the nesting sites of the beaches where the latter were probably born (Lutz and Musick, 1996). One female can mate with several males and can collect the sperm and use it at different times of the same reproductive season (Lutz and Musick, 1996).

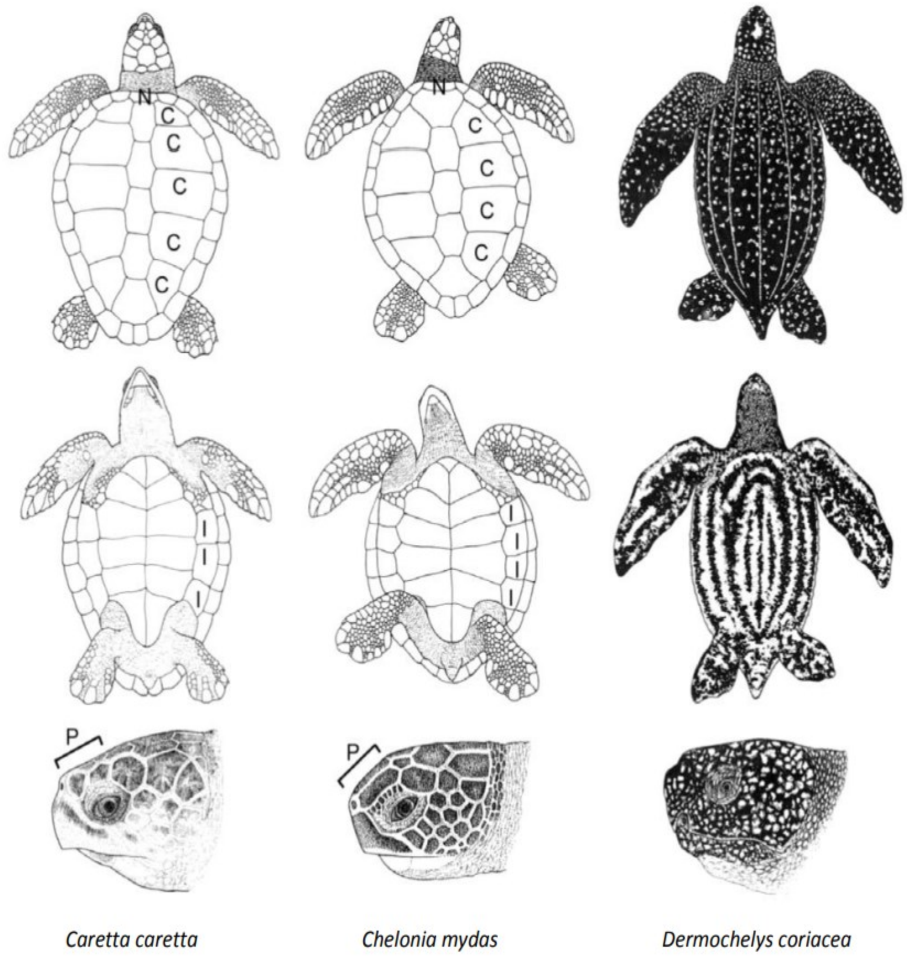

Figure 1: anatomical distinct tracts of three sea turtle species: *Caretta caretta, Chelonia mydas* and *Dermochelys coriacea*. P, prefrontal shield; C, costal shield; I, inframarginal shield and N, nuchal shield (tratto da: Scheda per la segnalazione di tartarughe marine , MSN Venezia, WWF).

1.2 Distribution of C. caretta in the Mediterranean and the Adriatic Sea

The Mediterranean Sea is a semi-enclosed basin divided into two parts: the western part and the eastern part. These two areas are divided by the strait of Sicily which limits the exchange of water between these two basins. From an oceanographic point of view, it is considered an evaporation basin which means that evaporation prevails over the freshwater input. Despite that, western and eastern parts have different hydrological conditions: the easter one is more saline and warmer (oligotrophic), while the western is eutrophic, with lower salinity and colder water. Moreover, the only two connections with the surrounding oceans are by the strait of Gibraltar (link with the Atlantic Ocean) and the Suez strait which represents the connection with the Indian Ocean through the Red Sea (Gasparini *et al.*, 2005). All these characteristics make the Mediterranean Sea a hotspot for both micro and macro-organisms (Bianchi and Morri, 2000).

Three of the seven species of sea turtle live in the Mediterranean Sea including *Dermochelys coriacea, Chelonia mydas and Caretta caretta.* The last two species are the only ones that nest along the Mediterranean coasts meanwhile, the leatherback turtle is more sporadic.

Thanks to the data collected from the sighting on the tourist's boats and the incidental capture in fishing gear it was possible to assume that *Caretta caretta* is the most abundant species and its presence has been documented in the whole Mediterranean basin (Storelli and Zizzo, 2014; Haywood *et al.*, 2020).

The Mediterranean Sea is crucial for these organisms both for its nesting and as foraging area, in particular, the loggerhead turtle nests are located mainly in the eastern part of the Mediterranean Sea along the coast of the Aegean Sea (Greece, Turkey), the Alboran Sea (Algeria, Morocco, Spain) Ionian Sea (Albania, Greece and Italy), Levantine basin (Greece, Cyprus, Turkey, Libya, Lebanon, Israel), Sicilian strait (Italy, Tunisia, Malta), Tyrrhenian Sea (France and Italy) and Balearic island (Spain) (Casale *et al.*, 2018).

Thanks to the use of satellite tags, it was possible to determine some important hotspot areas for sea turtles foraging and two main sites have been identified: one in the central Mediterranean which includes the area around the Pelagie Islands (Lampedusa and Linosa) in the neritic or edge zones and the Gulf of Gabès (Tunisia); and the other one in the northern part of the Adriatic Sea (Zbinden *et al.*, 2008; Casale *et al.*, 2012). Different studies have supported the importance of the Adriatic basin for marine sea turtles not only for foraging but also as overwintering areas (Bertuccio *et al.*, 2019).

The Adriatic Sea is an 800 km long and 150 km wide semi-enclosed basin communicating with the Mediterranean Sea through the Otranto Strait in the southern part. Due to its bathymetry, it can be divided into three sections: the North part, characterized by shallow, cold water and sandy seabed, the Central Part which includes Marche, Abruzzo and Molise and the first part of Puglia regions (Krasakopoulou *et al.*, 2005). It has an average depth of 270 m nearby Jabuka and

Pomo pit, (two continental shelf depressions) while the South part is characterized by a wide depression (South Adriatic Pit) more than 1200 m deep (Ferrarin *et al.*, 2019).

Its oceanographic characteristics and the rivers input, specifically the Po River, lead to a considerable concentration of nutrients and a consequent high primary productivity (Agostini and Bakun, 2002). This entails a consistent presence of benthic invertebrates that represent the main prey of adults' loggerhead turtles (Donaton *et al.*, 2019).

1.3 Threat: fishing, naval traffic, and pollution

The population dynamics of loggerhead sea turtles in the Adriatic Sea are deeply impacted by different anthropic activities.

In the last three years, from 2019 to 2021, the Middle Adriatic was characterized by a high presence of stranded adults identified during the whole year.

Unfortunately, stranded turtles are a growing widespread phenomenon and an urgent issue for marine conservation (Hamann *et al.*, 2010). The main causes of this anomaly are due to anthropic activities, not only related to accidental catches but also associated with pollution issues (Pulcinella *et al.*, 2019; Sala *et al.*, 2021). It seems that the number of stranded animals has increased in the years, even in

2020 when human marine activities were drastically reduced due to world Covid pandemic (Russo *et al.*, 2021).

According to the International Union for the Conservation of Nature (IUCN), *C. caretta* is a vulnerable species. The main threats affecting adults' survival are fishing, maritime traffic, and pollution (Lotze, Coll and Dunne, 2011; Giani *et al.*, 2012).

1.3.1 Fishing

The Adriatic Sea is very exploited from the point of view of fishing especially in the northern and central parts of the basin (Barausse *et al.*, 2009).

Sea turtles are often victims of bycatch and the type of fishing gear in which they are usually entangled are trammel nets, bottom trawling and pelagic longlines. Sometimes sea turtles survive with both none or superficial injuries other times they are seriously hurt and die.

According to FAO (www.fao.org), trammel nets consist of three layers: the two external ones have a larger mesh where the prey can easily penetrate, while the internal one has a slack small mesh in which fish will entangle. These nets are strings of single, double, or triple netting walls kept vertical and are occasionally set in strings. The death of sea turtles caught on these nets is caused by forced apnea due to the high period of immersion and the consequent drowning.

According to EU Data collection Framework set up in 2000, the average mortality rate reported is 55% of all the sea turtles belonging to the bycatch (Lucchetti, Vasapollo and Virgili, 2017), due to the bad weather conditions, operation damages and improper gear use, this type of fishing gear is lost or abandoned in the sea promoting a phenomenon called 'ghost fishing', whereby the gears continue to catch ample wildlife from different taxa (Stelfox, Hudgins and Sweet, 2016). Entanglement in this ghost gear can also cause abrasions or loss of extremities due to necrosis with the consequence of a reduction in the capacity of turtles to swim, feed efficiently or escape from danger that implicates death by starvation or drowning (Nelms *et al.*, 2016).

The mortality caused by bottom trawlers depends on the duration of nest tow (Sasso and Epperly, 2006) which also leads to a comatose status and eventually drowning if the turtles are forced to be submerged for too long (Lutz and Musick, 1996). In the north of the Adriatic Sea, a study conducted in 1999 and 2000 showed that about 10% of turtles accidentally captured were dead and within this number, about 44% of the animals were in a comatose status caused by the long-haul durations estimated at 130 minutes (Casale, Laurent and De Metrio, 2004). Furthermore, once sea turtles enter the cod end of the trawler net, they are unable to escape. A possible solution is the Turtle Excluder Device (TED) that was introduced in the early 1980s, it is a grid-like device that allows large animals (not only turtles but marine mammals as well) towards an exit located before the cod end (Sala, Lucchetti and Affronte, 2011).

Another potential trap for sea turtles is represented by the pelagic longlines used for tuna and swordfish fishing that unfortunately catch a great number of loggerhead and leatherback turtles (Gilman *et al.*, 2006). Indeed, sea turtles can be hooked while trying to ingest the bait from hooks or get caught when their flippers encounter the hooked branch or the mainlines. Moreover, when they eat the bait, they also swallow the hook and the line with high risks of grave damages to the gastrointestinal tract that could lead to death (Casale *et al.*, 2010).

1.3.2 Maritime traffic

Sea turtles reach the sea surface for three main reasons: first, they need to breathe regularly; secondly, to reproduce and third to bask in the sun. The main reasons why these animals show this atypical behavior are thermoregulation factor, delay of algal or fungal infestations, synthesis of vitamin D and probable improvement of the immune system (Hochseheid *et al.*, 2010; Maxwell *et al.*, 2014). During the exposure to the sea surface, they are more vulnerable to incidents caused by the impact with ships, especially in the north and central part of the Adriatic Sea since it is an intensively exploited basin characterized by the massive presence of both small scale and industrial vessels (Fortibuoni *et al.*, 2017; Russo *et al.*, 2020, 2021).

Thus, collisions with boats are frequent and they represent the second most common cause of mortality for *C. caretta*, the impact with the propeller or the keel can seriously damage the carapace, the flippers, and the head of the animals (Casale *et al.*, 2010).

1.3.3 Pollution

Marine pollution is a serious threat for sea turtles, different substances such as heavy metals, organic compounds, Persistent Organic Pollutants (POPs), and marine litter, including plastics and microplastics (MPs) could have negative effects on the organism when sea turtles are exposed to them (D'ilio *et al.*, 2011). Persistent pollutants tend to bio-magnify up to the food chain and their bioaccumulation became a matter of concern for its impact on wildlife including loggerhead sea turtles (Storelli, Barone and Marcotrigiano, 2007; D'ilio *et al.*, 2011).

These chemicals are lightly hydrosoluble, they occur in the aquatic environments adsorbed by particulate matter in the water that gradually precipitates and accumulates in the sediments. Consequently, high concentrations of these pollutants could be easily bio-accumulated by benthic organisms while feeding.

As sea turtles are long-lived reptiles (they can live up to 70 years) that mainly prey on benthic organisms (mollusks, gastropods, crustaceans and echinoderms), they could bio-accumulate large quantities of organic substances through the diet (Cammilleri *et al.*, 2017). Additionally, the presence of the Po River, which crosses a wide industrial and agriculture area, largely contributes to the nutrient and chemical load flushed into the sea (Lazar and Gračan, 2011). Many studies have investigated the presence of contaminants in sea turtles, but some of them refer to *C. caretta* (Campani *et al.*, 2013; Di Renzo *et al.*, 2021). It was highlighted that pollutants may induce adverse effects on endocrine and central nervous system: disrupting reproduction, osmoregulation, prey location, interspecific communication. Neuro-developmental disorders were observed after a chronic exposure to various contaminants in several species of freshwater turtles (Esposito *et al.*, 2020).

The term marine litter refers to "any disposed or abandoned non-biodegradable item in the marine and coastal environment made of persistent manufactured materials such as plastics, wood, glass, rubber, clothing or paper" (Galgani *et al.*, 2010). The great majority of marine litter is made of plastic and its ubiquity in the sea is extremely discussed. Indeed, many studies have revealed the presence of plastic and MPs fragments in all the water columns, from the surface to the seabed of all oceans and seas (Cannas *et al.*, 2017; Bonanno and Orlando-Bonaca, 2018). Due to mechanical and biological processes and UV radiations, plastic litter slowly breaks down into little fragments that are classified according to their dimension as macroplastics (Ø >5mm), microplastics (0.1µm < Ø <5mm) and nanoplastics (Ø <0.1µm) (Galgani *et al.*, 2010).

Marine litter could derive from ships, fishing gears, recreational boats and garbage carried into the sea from highly populated and industrialized areas. The Adriatic Sea is a hotspot regarding the presence of pollution. The northern part of the basin is extremely populated, with four million inhabitants that reach eighteen million tourists in summer bringing a heavy input of land-based pollutants into the sea (Taffi *et al.*, 2014).

Marine litter is classified into three different groups: pollutants, which include slag and oil; plastic, for example, industrial plastic pellets and user plastics such as sheetlike, threadlike and foamlike disposable plastics, and fragments; others like cigarettes filters. Seven types of plastics have different densities and can be found in several products as it is shown in Table 1 (Galgani *et al.*, 2010).

The presence of microplastics in the stomach may cause a series of consequences on sea turtle's health and survival.

Classification	Abbreviation	Density (g/L^{-1})	Products
Polyester	PET	1.37	Soft drink, water, juice, and beer bottles
High-density polyethylene	HDPE	0.94	Milk jugs, juice bottles; bleach, detergent and household cleaner bottles; butter and yoghurt containers
Polyvinyl chloride	PVC	1.38	Window cleaner and detergent bottles, shampoo bottles, cooking oil bottles; clear food packaging, medical equipment, boots
Low-density polyethylene	LDPE	0.91-0.93	Plastic bags, six-packs rings, netting, drinking straws, wire cables
Polypropylene	PP	0.85-0.93	Ropes, bottle caps, netting cars bumpers, flowerpots, folders
Polystyrene	PS	1.05	Disposable plates and cups, meat trays, eggs cartons, carry-out containers, aspirin bottles
Others			DVDs, sunglasses, iPod and computer cases, signs and displays, nylon, baby bottles, 'bullet-proof' materials

Table 1: different types of plastic for different needs (Gesamp, 2019).

1.4 Ingestion of plastic

Scientific literature has already reported many studies about plastics ingestion by sea turtles at a global level (Stamper *et al.*, 2009; White *et al.*, 2018; Petry *et al.*, 2021). While for the Mediterranean Sea the literature is more consistent (Hamann *et al.*, 2010; Cannas *et al.*, 2017; Sala *et al.*, 2021), quantitative and qualitative data are poor for the Adriatic Sea (Di Renzo *et al.*, 2021).

The principal effects of plastics on sea turtles are related to their size and shape and to the dangerous chemicals added during the manufacturing process to increase the performance of the final products (Di Renzo *et al.*, 2021).

Plastic debris can cause several problems to wildlife; however, the main effects could be divided into two categories: effects deriving from entanglement and effects due to ingestion. As mentioned above referring to fishing gears, entanglements could cause drowning, inability to feed normally or to escape from the prey, while ingestion usually leads to death after irreversible damages of the digestive system (Schuyler *et al.*, 2012).

Firms apply additives such as chemicals during plastic production to make products more resistant, but many of them are potentially toxic. Contamination of both biota and the environment is extremely high (Campanale *et al.*, 2020).

Ingestion of micro and macro plastics can cause severe consequences by simply passing through the gastrointestinal tract, or they can cause the death of the animals by reducing the feeding stimulus and stomach capacity or leading to gut perforation.

“The structure of the sea turtle gastro-intestinal tract and the number of anthropogenic debris items they ingest play a crucial role in the relationship between plastic ingestion and the chance of mortality. The alimentary canal in sea turtles is particularly prone to plication and accumulation of debris items due to the inability of turtles to regurgitate items and their convoluted gastrointestinal tracts” (Wilcox *et al.*, 2018).

The amount of plastic material ingested could also alter the swimming behavior and cause buoyancy problems. When sea turtles are unable to dive, they are more exposed to human threats such as collision with boats, also their ability to hunt decreases while their susceptibility to predators increases. Finally, the ingestion of plastics could also be related to the decrease of growth rate, low fecundity, and low fertility. All the aspects that compromise the stability and survival of sea turtles’ populations (Nelms *et al.*, 2016).

Moreover, the longer the plastic stays in the intestine, the greater is the chance of releasing toxic compounds, such as POPs. Among the POPs, polychlorinated biphenyls (PCB) are the most studied: they include a great variety of congeners divided into dioxin-like (DL-PCBs) and non-dioxin-like (NDL-PCBs), which prevail in animal tissues (Cammilleri *et al.*, 2017). These compounds showed toxicological effects on the neuronal cells and on other biological tissues and systems (such as the liver and the immune and endocrine systems) interfering with calcium homeostasis and decrease of dopamine neurotransmitters (Tilson *et al.*, 1998). Bisphenol A (BPA), a common plasticizer and the additive para-phthalic acid (PTA) were observed to negatively affect the reproduction system and the hormonal functions in rats (Tilson *et al.*, 1998).

Plastics ingestion can impact the health status of sea turtles altering the correct functioning of the immune system increasing the susceptibility to disease, such as fibropapillomatosis and interfering with lipid metabolism (Guzzetti *et al.*, 2018). The consequences of debris ingestion, as before mentioned, can affect sea turtles at all lifecycle stages, from hatchlings to adults. Depending on the stage, they eat both in the neritic and benthonic habitats: due to the ubiquity of plastics that can be found both on the surface and in the sediments, the probability to ingest it is extremely high. Sea turtles are mostly attracted by floating debris and ingestion is not determined by sex or age (neither by dimension) (Lazar and Gračan, 2011). One hypothesis suggested to explain accidental plastic ingestion is that they might confuse white floating plastic bags with jellyfish, one of their favorite preys (Witherington, 2002).

1.5 Health status biomarkers

The effects caused by the presence of plastics and MPs in the organism were investigated in both invertebrates and vertebrates with a major focus on the formers. Fish were the most studied (182 species) vertebrates while other groups such as mammals, amphibians and reptiles were less debated. In sea turtles, it was considered only the effects of potential ingestion, whereas information on bioaccumulation and other effects is still missing. Long-lived species are good models to determine how plastics could manifest their toxicity after chronic exposure (Prokić *et al.*, 2021). Usually, to evaluate the health status of an organism exposed to a potential toxic substance are used different biomarkers. For sea turtles, for example, in a study conducted in 2018, they evaluate the effects of organochlorine compounds (OC) analyzing the activity of antioxidant enzymes in blood samples of hawksbill sea turtles (Salvarani *et al.*, 2018).

1.5.1 Melanomacrophages

Melanomacrophages (MMs) are pigmented cells present in the hematopoietic and other soft tissues of lower vertebrates. From a histological point of view, they appear dark brown due to their pigments content such as lipofuscin, hemosiderin, and melanin. The MMs are involved in the melanin synthesis, in free radicals capture, removal and autolysis of red blood cells, internalization prokaryotic organisms, and vary in density depending on the species and the individual and state of nutrition (Hur and Lee, 2010).

In fish, they can be normally found in these tissues, however, they are more numerous in physiologically abnormal states related to disease or stress. In this case, the number, size, and shape of MMs vary depending on the species, ages, organs, and nutrition status (Hur and Lee, 2010).

MMs are morphologically and biochemically different in different taxonomic groups and different organs of the same species, phagocytizing melanin in some tissues and synthesizing it in others (Rund, Christiansen and Johnson, 1998).

They are considered biomarkers for evaluating the general health status of animals: they are often measured and estimated analyzing blood, urine, or soft tissue samples. There are several reports on the immunotoxic effects of starvation, potassium dichromate, ulcer disease, cadmium chloride, foreign bodies, toxic plants, and heavy metals on MMCs in several fish species (Sayed and Younes, 2017).

1.5.2 Liver

To date, few studies exist concerning the reptile's liver function and structure. Reptile's liver has the same functions as in mammals, it is involved in the metabolism of fats, proteins, glycogens and bile, acid uric and coagulation factors.

The liver is the biggest visceral organ in sea turtles, it is located ventrally between the heart and the stomach in a central position, it fills the entire middle portion of the coelomic cavity, surrounding the pancreas, stomach and duodenum it is composed of two lobes connected by one or more strips of liver tissue. Its color ranges from dark brown to reddish (Moura *et al.*, 2012).

The histological structure of the reptile's liver was well studied in the freshwater tortoise *Trachemys scripta elegans*, highlighting the presence of a thin connective tissue capsule around the organ (Marycz K *et al.*, 2011). The hepatic cells were polygonal or pyramidal and nuclei with noticeable nucleoli within the hepatocyte are situated centrally or eccentrically (Marycz K *et al.*, 2011). As already mentioned before, the reptiles' liver could contain a large number of MMs (Hanley and Hernandez-Divers, 2003). Interestingly, hepatic MMs aggregation were found to increase in size and number with age in turtles (Christiansen, Grzybowski and Kodama, 1996). In reptiles, the presence and activity of MMs are usually evaluated in the spleen and kidney, and no studies considered their presence in *C. caretta* liver, although their high concentration observed in the liver of freshwater tortoise suggests that they may have an important biological relevance that should be considered also in the study of loggerhead sea turtle (Johnson *et al.*, 2005).

1.5.3 Spleen

The spleen is the largest and most important organ in immunological defense, it is the only organ specialized in blood filtration and also involved in the response action against particulate antigens in the blood. This organ has been studied in many classes of reptiles, including lizards, tuatara and tortoise but very little information on the presence and function of MMs is known for sea turtles including *C. caretta.* Having information is important to understand its immunology role and for analysis of several infectious diseases (Bao *et al.*, 2009). All the studies on MMs activity in the spleen were conducted in freshwater tortoise: they were isolated in the Yellow Mud Turtle, *Kinosternon flavescens* and it was demonstrated that the size and number of their aggregation may serve as a marker for senescence in different healthy turtles (Christiansen, Grzybowski and Kodama, 1996). Another experiment was conducted in three divergent turtle families, the Chelydridae (*Snapping Turtles*), the Emydidae (*Pond Turtles*), and the Trionychidae (*Softshell Turtles*) where the morphology of MMs was observed in cell cultures (Rund, Christiansen and Johnson, 1998). The most recent article dealing with MMs underline the utility of MMCs as a broadly applicable histological indicator of the fish (as well as amphibian and reptilian) immune response in both laboratory and wild populations of both model and non-model vertebrates. Factors like sex, pollution exposure, stress and stocking density should be considered when using MMCs to study immunity in non-model vertebrates in wild populations (Steinel and Bolnick, 2017).

In loggerhead sea turtles, the spleen was identified as a small, spherical, or triangular structure located between the stomach and the pancreas. Information is still missing from both histological and functional points of view.

1.6 Gonads

The gonads of *C. caretta* are in the dorsal cavity of the body, posterior to the lungs, on the ventral base of the kidneys and enclosed in the peritoneal membrane. At the end of the embryonic phase, the remaining yolk can be found attached to the gonads. Sexual differentiation into testes or ovaries occurs through the modification of the epithelial mass of the gonads (Hidayatulloh, Dhamayanti and Purnama, 2021). Sexual differentiation is affected by the incubation temperature of the eggs: it was demonstrated that cooler incubation temperatures determined a differentiation into males while warmer temperatures produce more females.

There is a transitional range of temperatures in which sex ratio shifts from 100% males to 100% females and within this range, there is a temperature referred to as the pivotal temperature at which the embryos sex ratio would be 1:1 (Wibbels, 2003)

Pivotal temperatures reported for *C. caretta* differ from 27.7 °C in the Australian coast to 29.7 °C for nests located in South Africa, suggesting that pivotal temperatures could vary among and even within populations of the space (Wibbels, 2003).

The structure of the gonadal primordia is similar in both presumptive sexes. It has two regions: the cortex (outer), which is characterized by a single layer of cuboidal epithelial cells with embedded germinal primordial cells; the medulla (inner), which is derived from mesenchyme cells within the middle part of the undifferentiated gonad.

As sexual differentiation starts during stages 24–29, that means after 30-45 days of incubation (Miller, Mortimer and Limpus, 2017), the cells of the gonadal primordia develop into two distinct and opposite patterns. Changes in both the cortex and medulla are visible. Most of the primordial germ cells are situated in the cortex; both nerve and blood vessels penetrate the mass of mesenchyme cells in the medullary area. In presumptive females, the ovary results from the simultaneous proliferation of cells in the cortex and regressive modification in the medulla. The cells of the cortex become more columnar, and the layer becomes thicker; primordial germinal cells are distributed among the cells of the cortex. The medullary area shows some differentiation of cells form primitive medullary (sex) cords, but for the most part, this regress and the area remains a thick mass of undifferentiated cells that will be penetrated by blood vessels (Lutz, Musick and Jeanette, 2003).

Ovaries and oviducts change in shape and size with age and during all the life cycle depending on reproductive period. In immature animals, the ovary appears pink and with a granular appearance due to the presence of immature follicles. Following the increase in age and weight, the follicles increase in size and accumulate yolk. The position of the opening cloacal is used as a sign of sex dimorphism in mature animals: in females, this orifice is located approximately equidistant from the tip of the tail and the anal shield of the plastron, while in males the orifice is closer to the tip of the tail. Furthermore, males can also be recognized for having a longer and more muscular tail than females (Lutz, Musick and Jeanette, 2003). To date, detailed information on ovarian structure and oogenesis of *C. caretta* is still lacking.

2. AIM

C. caretta is evaluated as vulnerable species according to the Red List of IUCN; therefore, it is very important to prevent the collapse of their populations and its indirect consequences on the marine environment. This species plays a key role in marine ecosystems and their presence influence the life of numerous species both directly and indirectly. As an example, loggerhead sea turtles provide habitats for more than a hundred species of epibionts (such as barnacles, algae, poriferans and cnidarians). Moreover, they can play an important role in moving nutrients across ecosystem boundaries as well as in nutrient dynamics within foraging habitats (Wyneken *et al*, 2013). To prevent these delicate equilibria from being compromised, it is fundamental to obtain a deepened knowledge of their biology and habits and to take proper actions to limit the effects of human activities on sea turtles health and to assure a safe interaction with their environment. For this reason, the aim of the present thesis is to present a general overview on the trend of stranded *C. caretta* specimens registered during the last three years, with a special focus on animals found along the Abruzzo coasts. Considering different parameters such as seasonality of the recoveries and the male-female ratio, it was observed the evolution of this phenomenon up to the present.

Preliminary necroscopy analysis performed during the last year on stranded *C. caretta* specimens, identified plastic materials in the gastrointestinal tract of 9 animals (8 female and 1 male) collected between June and October 2021 along the Abruzzo coasts. To evaluate the possible effects of plastics on the health of these animals, liver and spleen samples were collected and analyzed.

Moreover, another focus of the present thesis was the characterization of *C. caretta* ovarian structure and oogenesis since, to our knowledge, no studies exist that characterized the structures of the ovaries at different maturation stages. Data and samples analyzed in the present thesis were collected by the "Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise" (sede di Teramo, Italy) within the project: "Identification of biomarkers for the evaluation of the toxicological effects caused by contaminants, including microplastics, and anthropozoonotic infections in *Caretta caretta* by defining a health status indicator of the Mediterranean Sea turtle population".

3. MATERIAL AND METHODS

3.1 Data and samples collection

From January 2019 to October 2021, 215 marine turtles stranded or caught dead along the Adriatic coast of Abruzzo region, were recovered by the official regional network for stranded animals (DCA 16/02/2018 n.12 n.d.). This network includes the Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise "G. Caporale" (IZSAM), Centro Studi Cetacei (Rescue Center), Coast Guard, local veterinarians and municipal offices. For each animal, the status of carcass conservation, sex, curved carapace length (CCL) and weight were recorded in the Geocetus database (www.Geocetus.it). Based on their conservation status, 53 specimens were selected, excluding animals still alive or mummified, and then necropsied by IZSAM, according to an optimized protocol 'INDICT' from existing published guidelines (Flint et al. 2009; Poppi and Di Bello 2015). According to 'INDICIT' protocol, different scores corresponding to specific conservation status (or decomposition levels) were assigned to each animal:

- 1 indicates live animals.
- 2 for fresh animals recently dead and in good status.
- 3 corresponds to internal organs in good condition, although signs of autolysis (swollen), bad smell and color changes in skin occurs.

- 4 represents an advanced level of decomposition, skin scales are raised or lost, it is still possible to record CCL and the presence of ingested plastic.
- 5 is assigned when the animal is mummified: part of the skeleton or the body is missing, and internal organs are exhibited.

Considering the CCL measurements, turtles were subdivided into three groups: young juveniles (CCL< 40 cm), sub-adults (40 < CCL < 70 cm) and adults (CCL > 70 cm), according to Casale, Mazaris and Freggi (2011) and August *et al.*, (2018).

The intestinal content of the 53 animals necropsied was sampled and stored in glass containers at + 4 °C for microplastics (MPs) content determination. Liver, spleen and gonad samples (3 cm^3) were collected and properly stored for the histological analysis.

3.2 Microplastics Intestinal Extraction Protocol (MEP)

The extraction protocol of MPs (MEP) from the gastrointestinal content stored at + 4 °C was arranged as described by Di Renzo *et al.*, (2021) and summarized below. Litters were washed with tap water throughout metallic mesh of 1 mm; MPs bigger than 1 mm were detected, counted, and classified according to the INDICIT protocols (Table 2) (Matiddi *et al.*, 2019).

TYPE	CODE	DESCRIPTION
Industrial plastic	IND PLA	Industrial plastic granules, usually cylindric but also sometimes oval spherical or cubical shapes, or suspected industrial item, used for the tiny spheres (glassy, milky...)
Use sheet	USE SHE	Remains of sheet, e.g. from bag, cling-foil, agricultural sheets, rubbish bags
Use threadlike	USE THR	Threadlike materials, e.g. pieces of nylon wire, net-fragments, woven clothing…
Use foam	USE FOA	All foamed plastics, e.g. polystyrene foam, foamed soft rubber (as in mattress filling…)
Use fragment	USE FRAG	Fragments, broken pieces of thicker type plastics, can be a bit flexible, but not like sheet materials
Other use plastics	USE POTH	Any other plastic type of plastics, including elastics, dense rubber, cigarette filters, balloon pieces, soft air gun bullets… specify in the column "Notes"
Litter other than plastic	OTHER	All non-plastic rubbish and pollutant
Natural food	FOO	Natural food for sea turtles (e.g. pieces of crabs, jellyfish, algae…)
Natural no food	NFO	Anything natural but which cannot be considered as normal nutritious food for sea turtle (stone, wood. Pumice, etc.)

Table 2: MPs classification according to INDICIT protocol (Matiddi *et al.*, 2019).

3.3 Histological analysis

Liver, spleen and ovary samples collected by IZSAM (as mentioned in paragraph 3.1) were fixed in formalin solution to preserve the integrity of tissues structures and stored at 4°C for 24 h. Successively, the samples were washed in ethanol (70%) three times (15 minutes each) and stored in the same ethanol solution at

4°C until the inclusion process. Samples were dehydrated in increasing ethanol solutions (80, 95 and 100%), washed with xylene (Bio-Optica, Milano, Italy) and embedded in paraffin (Bio-Optica). Solidified paraffin blocks were cut with a microtome (Leica RM2125 RTS, Nussloch, Germany) and 5 μm sections were stained following Mayer haematoxylin and eosin Y (Merck KGaA) protocol. Sections were observed using a Zeiss Axio Imager.A2 (Oberkochen, Germany) and images were acquired using a combined color digital camera Axiocam 503 (Zeiss, Oberkochen, Germany).

To evaluate the presence of MMs and their size in liver and spleen samples the ImageJ software was used to count the number of MMs and their area percentage proportional to each section analyzed, the results were reported as the percentage of area occupied by the MMs counted on the total area of the section.

Ovaries were fully sectioned and observed to determine the characteristics of follicles and gonads maturity stages of each animal sampled.

3.4 Statistical analysis

The statistical analysis was performed using the software package Prism6 (GraphPad Software) for Windows. All data collected from the 9 animals sampled were analyzed by Pearson's Correlation. Significance was set at $p \leq 0.05$.

4. RESULTS

4.1 Data of recorded C. caretta along the Abruzzo coast

According to the data collected within the 'Geocetus project' (www.Geocetus.it), along the Abruzzo coast during the last three years (2019-2021), a total number of 345 *C. caretta* specimens was recorded, 222 were already dead and 123 were still alive. During this period, 213 sea turtles were found stranded while a total of 132 specimens represent the bycatch portion. The total number of registered loggerhead sea turtles has progressively increased (103, 110 and 132 in 2019, 2020 and 2021 respectively) (Figure 2). Stranded animals have lightly increased during the three-years period considered. Conversely, the number of turtles from the bycatch, after a slight decrease during the biennium 2019-2020 (43 animals in 2019, 38 animals in 2020), reached the highest number of registered individuals in the 2021 (51 animals). In each year, stranded animals were always more abundant than those from bycatch (Figure 2).

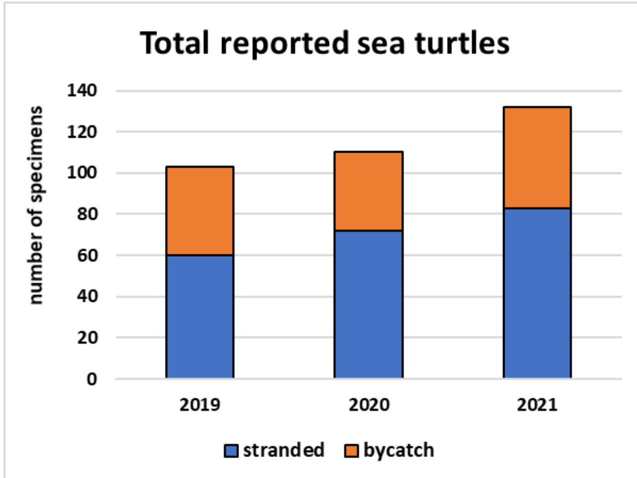

Figure 2: Number of *C. caretta* both stranded and from bycatch along the Abruzzo coast during the period 2019-2021.

Concerning the sex of stranded turtles, the number of females has doubled through the years, from 11 in 2019 to 23 in 2021 as shown in Figure 3. The number of males doubled as well (from 6 to 10 animals). Nevertheless, the sex of a great portion of stranded animals was not detected (Figure 3).

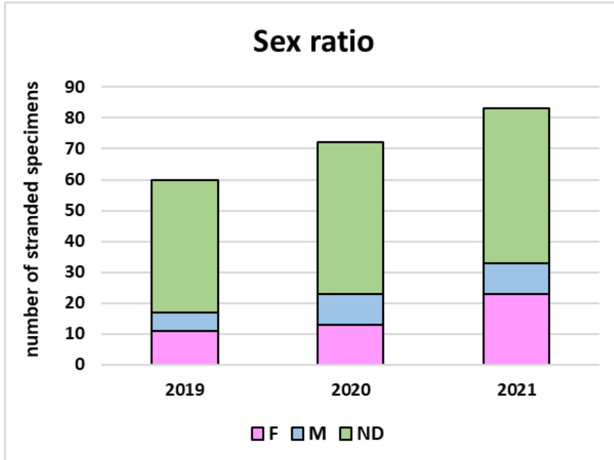

Figure 3: number of stranded *C. caretta*, between 2019-2021, divided by sex identification. **F**, female; **M**, male; **ND**, sex no detected.

Considering the temporal distribution of stranded animals within each year, no clear trend could be observed (Figure 4A, B, C). No stranded animals were found in May 2020 and December and January 2021 (Figure 4B, C). The highest number of total stranded animals have been detected in January (> 10 animals) in 2019 (Figure 4A), in June, July and November (≥ 10 animals) in 2020 (Figure 4B) and in July, September and October (≥ 10 animals) in 2021 (Figure 4C).

The lowest number of stranded animals were recorded during February, April, May, June and October (less than 3 stranded animals) (Figure 4A).

In 2020, the highest numbers of stranded animals were recorded from June to November (summer and autumn), while a drastic decrease was reported from December to May (winter and spring) (Figure 4B).

In 2021, a similar situation of 2020 was recorded, the majority of animals were found stranded from May to November (Figure 4C).

Observing the animals whose sex was determined, females represent the great majority of stranded animals except for November 2020 and 2021, when males represent 35% of total stranded animals while females were only the 21% of the total (Figure 4B, C).

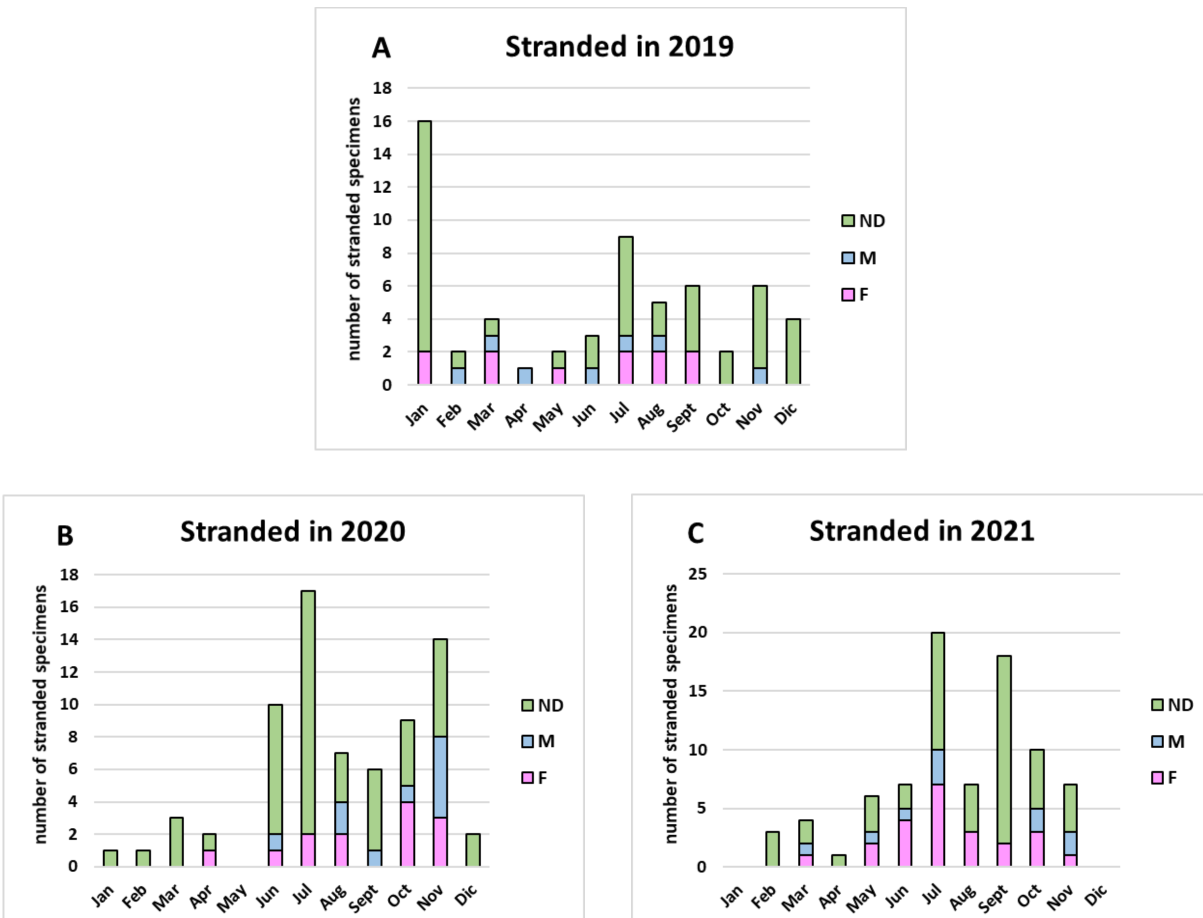

Figure 4: number of stranded *C. caretta*, during each year: 2019 (**A**); 2020 (**B**); 2021 (**C**). **F**, female; **M**, male; **ND**, sex no detected.

In Figure 5A, B and C, stranded turtles are represented into three groups based on their CCL (curved carapace length): young juveniles (CCL< 40 cm), sub-adults (40 < CCL < 70 cm) and adults (CCL > 70 cm).

In the three years considered, the majority of animals are classified as sub-adults, in 2020 and 2021 the second category more represented were adults (16 and 27 animals respectively), while in 2019 young juveniles prevail over adults (17 out of 12 animals).

Among the identified female, the number of sub-adult females increased over the three years analyzed (4, 7 and 12 in 2019, 2020 and 2021 respectively) while young juveniles females decreased in their number (3, 2 and 2 in 2019, 2020 and 2021 respectively). On the contrary, the highest number of animals recognized as males could be included in the adults' category (3, 4 and 6 in 2019, 2020 and 2021 respectively) (Figure 5A, B and C).

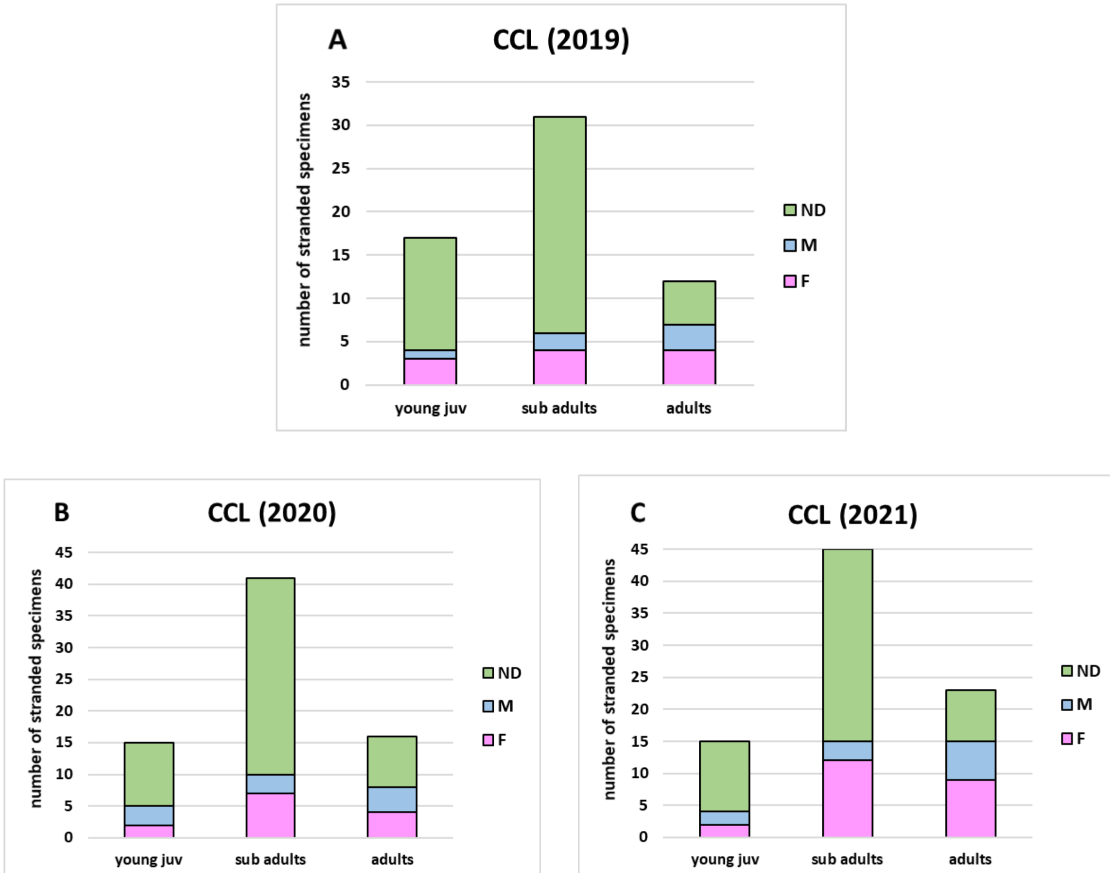

Figure 5: number of stranded *C. caretta* during each year: 2019 (**A**); 2020 (**B**); 2021 (**C**), divided into young juveniles, sub adults and adults based on their CCL (curved carapace length). **F**, female; **M**, male; **ND**, sex no detected.

4.2 Intestinal microplastics identification

The necroscopy analysis performed on the 53 stranded *C. caretta* highlighted the presence of different types of microplastics debris in the gastrointestinal tract of animals (Table 3).

ID	SHE	THR	POTH	FRA	TOT
ABASCC3121	2		2		4
ABASCC3021	1			3	4
ABASCC1621		1			1
ABASCC1321		1			1
ABASCC2521	2			1	3
ABASCC1521		1			1
ABASCC2121	1				1
ABASCC2721	1				1
ABASCC2421	1				1

Table 3: number of different types of microplastics found in 9 stranded *C. caretta.* **ID**, identification number; **SHE**, use sheet; **THR**, use threadlike; **POTH**, other use plastics and **FRAG**, use fragments.

The type of microplastic fragment more frequently found is SHE. It was found in the gastrointestinal tract of 6 animals. POTH was found as the only type in the gastrointestinal tract of the animal ABASCC3121 with a total of four pieces ingested, while three fragmented, broken pieces of thicker type microplastics (FRA) were found in one specimen (ABASCC3021) (Table 3).

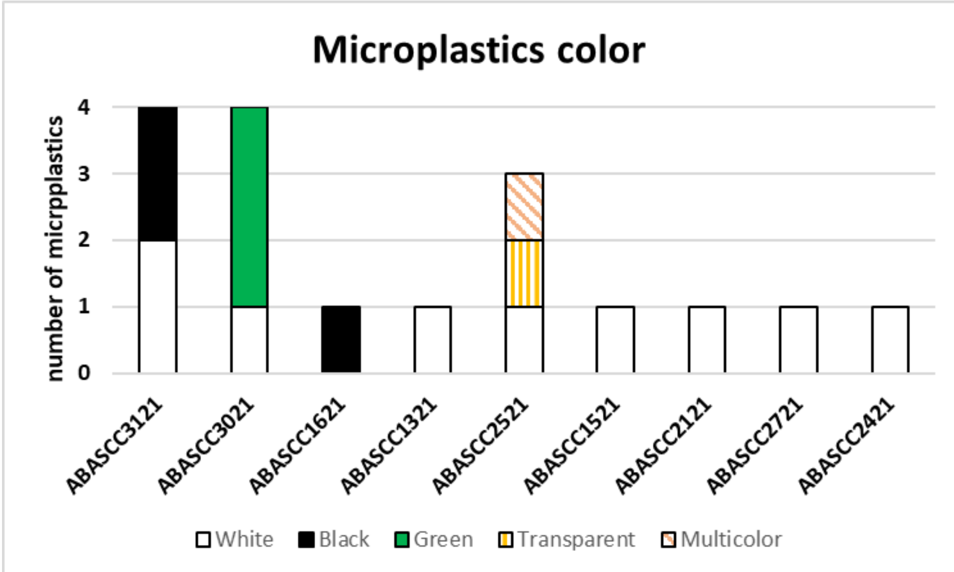

Figure 6: color of microplastic fragments ingested by 9 stranded *C. caretta.*

The prevalent color ingested is white, as 8 out of 9 ingested fragments of this color were identified. ABASCC3031 was the only animal that ingest green fragments; black pieces were found in two specimens, while transparent and multicolor was detected in only the same one animal (ABASCC2521).

ABASCC3121 ingested the highest number of white and black fragments (2 pieces of both color type) (Figure 6).

4.3 Pearson's Correlation analysis

ID	Status	CCL	Weight	Sex	Data	N of plastic	MMs liver area	N° MMs liver	% MMs liver	MMs spleen area	N° MMs spleen	% MMs spleen
ABASCC3121	2	19.2	0.7	F	18.10.21	4	42.40	6.5	0.03	37.57	1.50	0.03
ABASCC3021	2	29.8	2.0	F	1.10.21	4	96.60	9.66	0.06	80.76	1.16	0.07
ABASCC1621	3	41	13.0	M	5.07.21	1	46.63	3.16	0.03	22.52	0.66	0.03
ABASCC1321	4	50	24.3	F	21.06.21	1	107.49	13.66	0.07	72.54	1.66	0.06
ABASCC2521	4	57	x	F	5.08.21	3	165.79	2	0.12	x	x	x
ABASCC1521	2	59.6	38.0	F	4.07.21	1	239.98	10.5	0.16	83.96	8.66	0.06
ABASCC2121	2	65.5	37.1	F	23.07.21	1	217.56	7	0.14	138.74	3.16	0.09
ABASCC2721	2	67	31.4	F	3.09.21	1	199.71	3	0.13	76.26	6.66	0.05
ABASCC2421	3	88	88.7	F	3.08.21	1	295.85	8.16	0.01	130.73	1.30	0.10

Table 4: values data set. **ID**, official identification number; **Status**, conservation status or decomposition level; **CCL**, curved carapace length; **Weight**, weight of the whole carcass express in Kg; **Sex**, F female, M male; **N° of plastic**, number of plastic found in the gastrointestinal tract of sampled turtles; **MMs liver area**, average area of melanomacrophages in liver samples; **N° MMs liver**, average number of melanomacrophages in liver samples in 1,523x10^5 μm^2; **% MMs liver**, percentage of melanomacrophages average area in the liver samples; **MMs spleen area**, average area of melanomacrophages in the spleen samples; **N° MMs spleen**, average number of melanomacrophages in spleen samples in 1,523x10^5 μm^2; **% MMs spleen**, percentage of melanomacrophages average area in spleen samples.

Table 4 shows the data collected from the 9 animals with plastics fragments in the gastrointestinal tract. To the majority of specimens, it was assigned the score 2 (5 samples), while a score of 3 and 4 was assigned to 2 samples respectively. CCL expressed in cm is included between 19 and 88 cm, while weight expressed in kg is included between 0.2 and 88.7 kg. The X represent the lack of data due to technical problems. The average number of MMs counted in both liver and spleen samples is highly various among the animals, as well as the MMs average area. The percentage of MMs average area is under the 1 % for all the samples analyzed. Pearson's correlation analysis highlights that there is no significant correlation between CCL and the status of decomposition (Table 5).

A strong, significantly positive correlation is highlighted between CCL and weight (r=.93, p=0.001), CCL and MMs liver area (r=.91, p=0.001), while between N° MMs spleen and % MMs spleen a strongly significant negative correlation could be observed (r=-.01, p=0.000104).

The correlation coefficients obtained between weight and liver MMs average area (r=.88, p=0.004), MMs liver area and MMs spleen area (r=.84, p=0.009) and the % MMs liver and N° MMs spleen (r=.84, p= 0.0026) suggested a moderate significantly positive correlation. Finally, a low significant positive correlation is described between CCL and MMs spleen area (r=.74, p=0.035), CCL and % MMs spleen (r=.72, p=0.46), weight and MMs spleen area (r=.72, p=0.044), weight and % MMs spleen (r=.72, p=0.045) and MMs liver area and % MMs in spleen (r=.72, p=0.026) (Table 5).

	Status	CCL	Weight	N° of plastic	MMs liver area	N° MMs liver	% MMs liver	MMs spleen area	N° MMs spleen
CCL	.17	—							
Weight	.22	**.93*****	—						
N° of plastic	-.14	-.72	-.62	—					
MMs liver area	-.08	**.91*****	**.88****	-.52	—				
N° MMs liver	.01	-.05	.09	-.10	.08	—			
% MMs liver	-.22	.26	-.02	-.26	.40	-.06	—		
MMs spleen area	-.11	**.74***	**.72***	-.33	**.84****	.28	.33	—	
N° MMs spleen	-.45	.34	.16	-.37	.51	-.02	**.84****	.17	—
% MMs spleen	.03	**.72***	**.72***	-.30	**.77***	.32	.16	.96	**-.01*****

Table 5: Pearson's correlation coefficients (n=9). **Status**, conservation status or decomposition level; **CCL**, curved carapace length; **Weight**, weight of the whole carcass express in Kg; **N° of plastic**, number of plastic found in the gastrointestinal tract of sampled turtles; **MMs liver area**, average area of melanomacrophages in liver samples; **N° MMs liver**, average number of melanomacrophages in liver samples in 1,523x10^5 μm^2; **% MMs liver**, percentage of melanomacrophages average area in the liver samples; **MMs spleen area**, average area of

melanomacrophages in the spleen samples; **N° MMs spleen**, average number of melanomacrophages in spleen samples in 1,523x10^5 μm^2; **% MMs spleen**, percentage of melanomacrophages average area in spleen samples. *= $p \leq 0.05$; **= $p \leq 0.01$; ***=$p \leq 0.001$.

4.4 Characterization of oocytes and follicles maturation stages

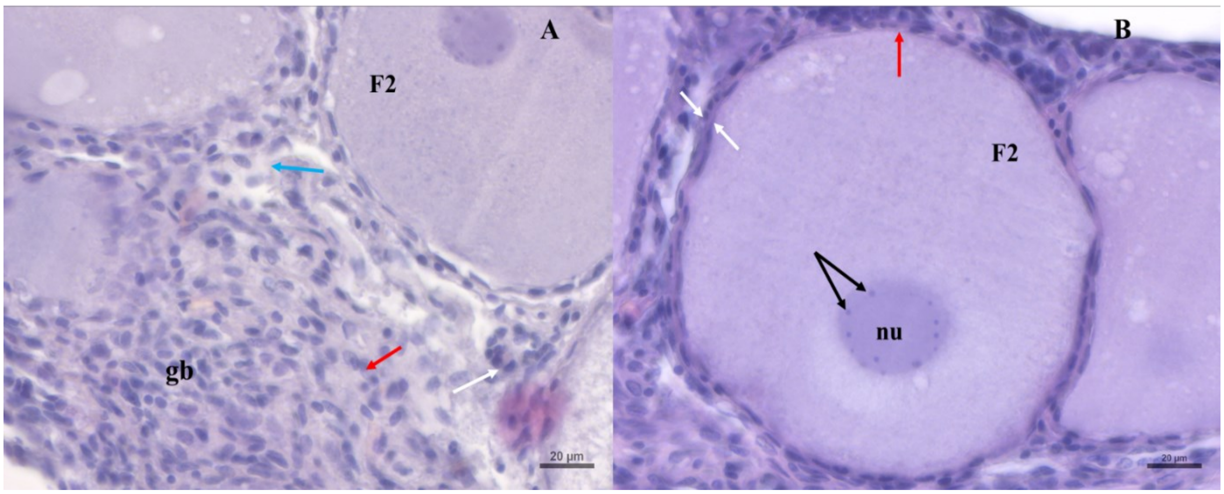

Figure 7: example of *C. caretta* ovary images. **(A): gb**, germinative beds; **F2**, secondary follicle; **white arrow**, primordial follicles; **red arrow**, primary follicles; **blue arrow**, batch of oogonia. Scale bar: 20 µm **(B): white arrow,** bilayer of follicular cells; **red arrow,** zona radiata, **F2**, secondary follicle; **black arrows**, nucleoli; **nu,** nucleus. Scale bar: 20 µm.

Figure 7A represents an ovary of *C. caretta,* in which is visible the germinative bed (gb) randomly distributed in the cortex area, sufficiently vascularized and surrounded by connective tissues. In the gb, a batch of oogonia (blue arrow) could be identified. Each oogonium is small, round with a round and central nucleus and a clear cytoplasm. Both primordial and primary follicles (white and red arrows respectively) can be found. Primordial follicles are characterized by an oocyte (with a similar oogonia's dimension) surrounded by somatic cells. Primary follicles are bigger than the primordial ones, surrounded by a single layer of somatic cells.

During follicles maturation, the size of the oocytes progressively increases.

In Figure 7B a secondary follicle (F2) is shown in which somatic cells are elongated and organized in a bilayer structure around the follicle (white arrows). The zona radiata (zr) starts to appear in this stage (red arrow), the cytoplasm is uniform. The nucleus (nu) is well defined with numerous clearly visible nucleoli, and it is moving from the central part of the oocyte to the peripheric zone.

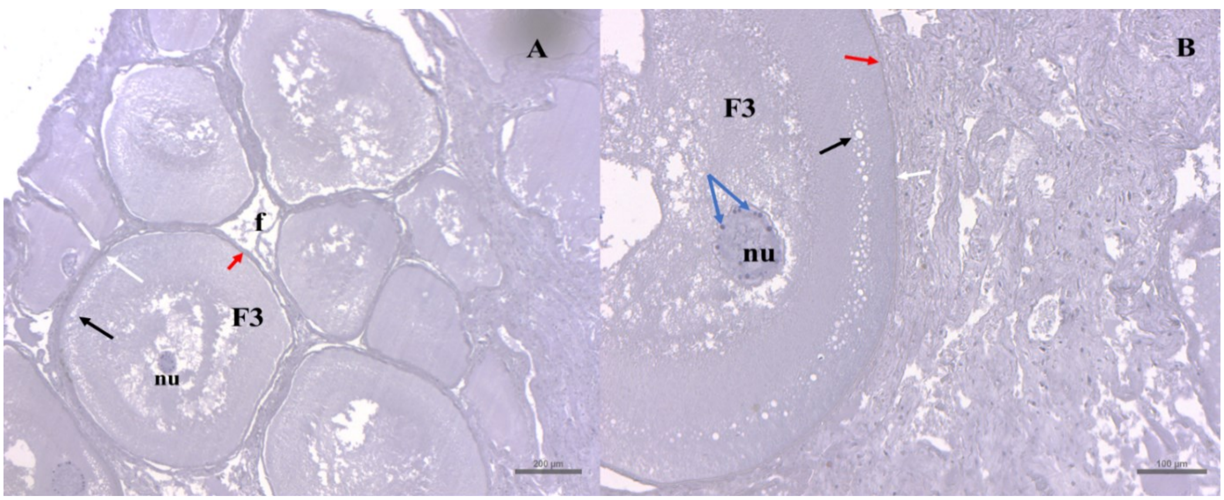

Figure 8: example of follicular previtellogenic stage. **(A): F3**, tertiary follicle; **nu**, nucleus; **f**, fat; **red arrow**, zona radiata; **white arrow**, bilayer; **black arrow**, lipid droplets. Scale bar: 200 µm **(B) F3**, tertiary follicle; **nu**, nucleus; **blue arrow**, nucleoli; **black arrow**, oil droplets; **red arrow**, zona radiata. Scale bar: 100 µm.

The tertiary follicle (F3) is characterized by two defined follicular layers (white arrows), the zona radiata (red arrow) begins to be more evident although it is not completely structured (Figure 8A, B). At this stage, oil droplets are internalized through endocytosis and at the beginning of the process are visible in the cortical area (black arrow) (Figure 8A, B). The nucleus maintains the morphology and position observed in the previous stage, nucleoli are still clearly visible (blue arrows) (Figure 8B). In the interstitial area between follicles is evident the presence of small fat accumulations (Figure 8A).

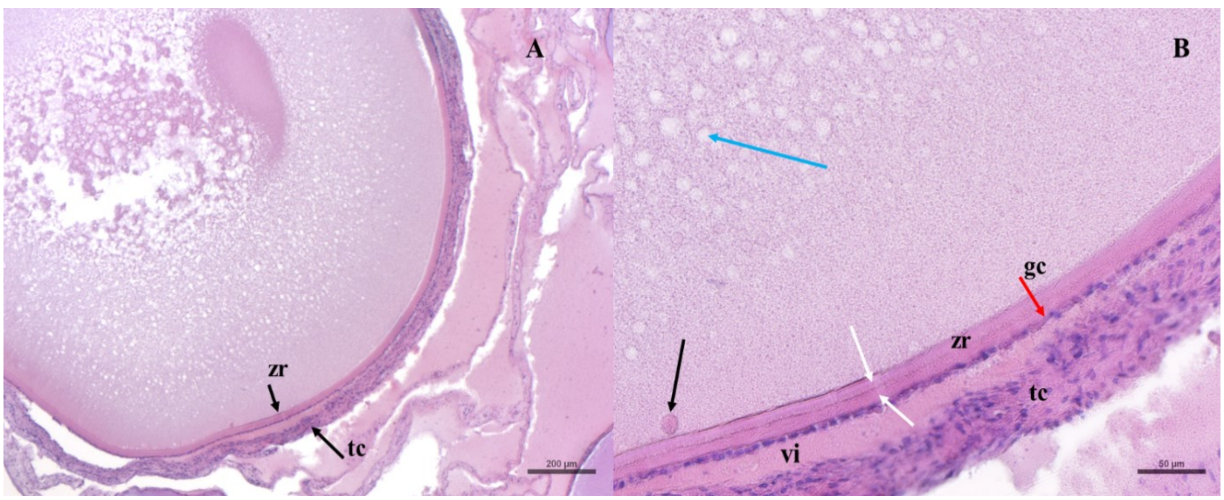

Figure 9: example of early follicular vitellogenic stage. **(A): tc**, theca cells; **ZR**, Zona Radiata; Scale bar: 200 µm **(B): vi**, vitellogenin; **white arrow**, inner and outer layer of Zona Radiata; **ZR**, zona radiata; **tc**, theca zone; **gc**, granulosa cells; **blue arrow**, oil droplets: **black arrow**, vitellogenin droplet. Scale bar: 50 µm.

The vitellogenic stage (Figure 9) is defined by the accumulation of vitellogenin.

At the beginning of early vitellogenesis, the follicle is characterized by elongated and multilayer theca cells (tc) and a single layer of granulosa cells (gc) (Figure

9B). The zona radiata (zr) is distinguished in the outer and inner layers (white arrows) (Figure 9A, B). Between the tc layers and the gc layer, a presume accumulation of vitellogenin (vi) is visible. The cortical region of the oocyte is granulated due to the internalization of vitellogenin droplets (black arrow) (Figure 9B). Oil droplets are now numerous and clearly distributed in the entire cytoplasmatic area and yolk globules coalesce together in the oocyte center (Figure 9A). Also, oil droplets form bigger droplets approaching the oocyte center (blue arrow) (Figure 9B).

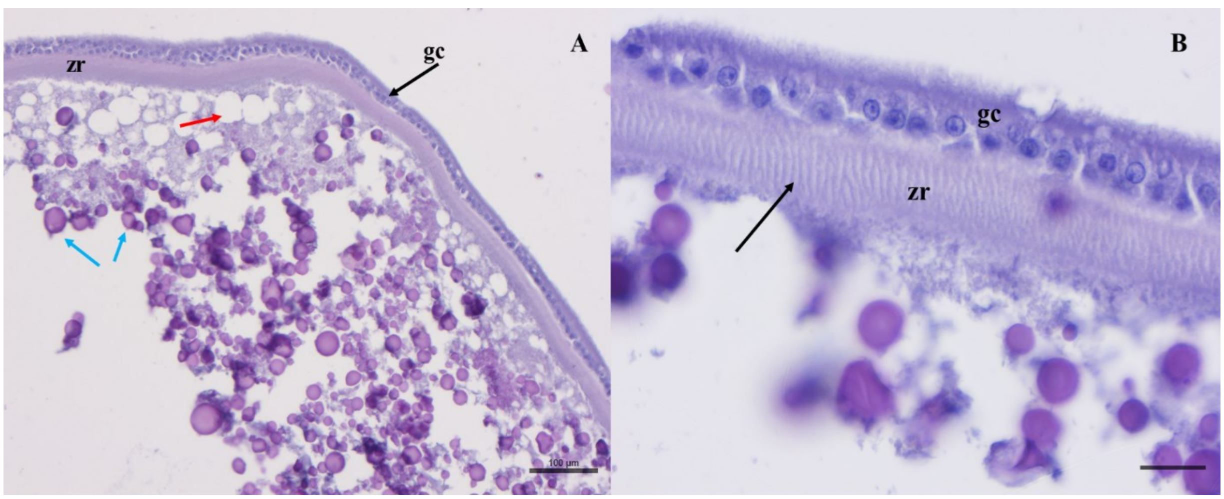

Figure 10: example of follicular late vitellogenic stage. **(A) zr,** zona radiata, **gc**, granulosa cells; **red arrow**, oil droplets; **blue arrow**, yolk granules. Scale bar: 100 µm **(B) gc**, granulosa cell; **zr,** zona radiata; **black arrow**, cytoplasmic bridges. Scale bar: 50 µm

The late vitellogenic stage is characterized by a bilayer of granulosa cells (gc) and a thick and differentiated zona radiata (zr) (Figure 10A, B). At this stage, the zr is organized with many cytoplasmatic bridges connecting the granulosa cells with the oocyte cytoplasm (black arrow) (Figure 10B). The oocyte cytoplasm is completely occupied by spherical yolk granules (blue arrows) and oil droplets (red arrow) (Figure 10A).

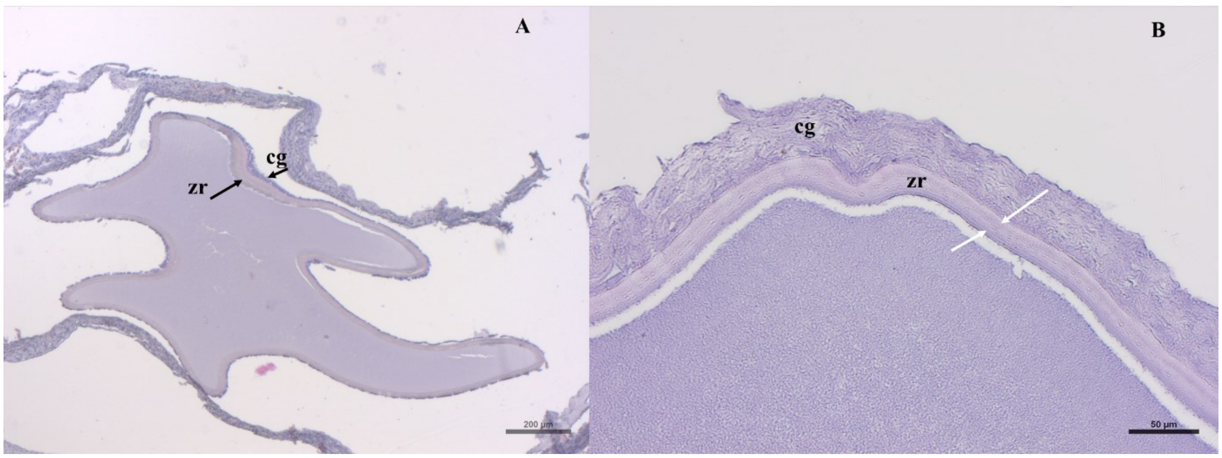

Figure 11: example of follicular mature stage. **(A)**, **(B): gc,** granulosa cells; **zr,** Zona Radiata; **white arrows**, outer and inner membrane of the zona radiata. Scale bar: 200µm (A), 50 µm (B).

Figure 11A represents a mature oocyte: granulosa cells (gc) have a bilayer organization, the zona radiata (zr) is thick and differentiated in the outer and inner membrane (white arrows) (Figure 11B), the cytoplasm is homogeneous: yolk coalescence has occurred (Figure 11B). The cytoplasmic bridges that characterized the vitellogenin stage are not visible anymore (Figure 11B).

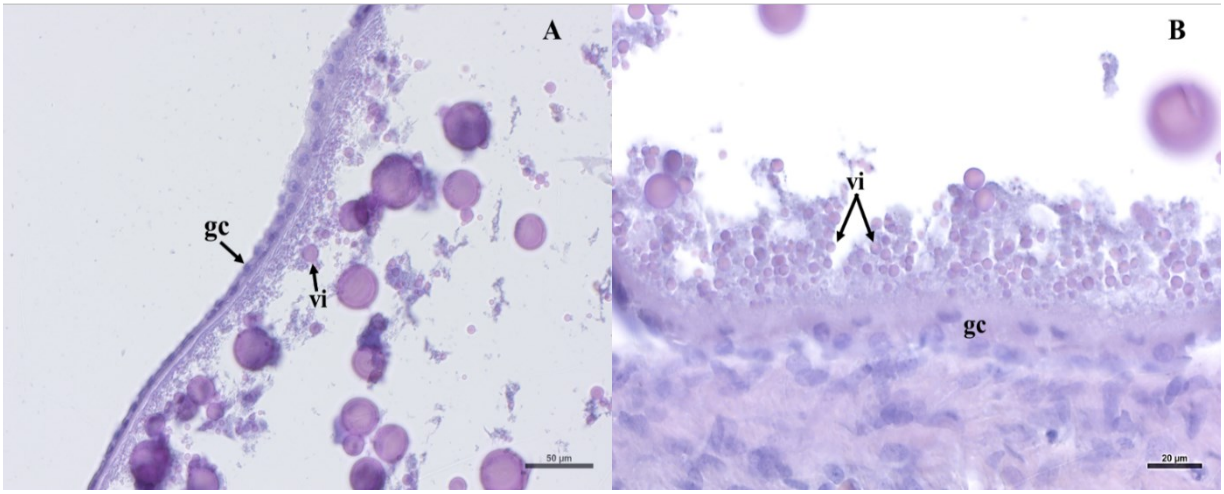

Figure 12: example of first stage of follicular atresia. **(A), (B): gc**, granulosa cells; **vi**, vitellogenin. Scale bar: 50 µm (A), 20 µm (B).

The atresia process (the consequence of a failed ovulation) is characterized by different stages: initially, the atretic follicles maintain the size and shape of mature follicles while the granulosa cells (gc) lose their normal cuboidal appearance and shift from a single layer organization to a 2-4 cell layers structure as the hyperplasia process begins (Figure 12A). The zona radiata is disorganized and yolk granules are accumulated along the cortex area (Figure 12B).

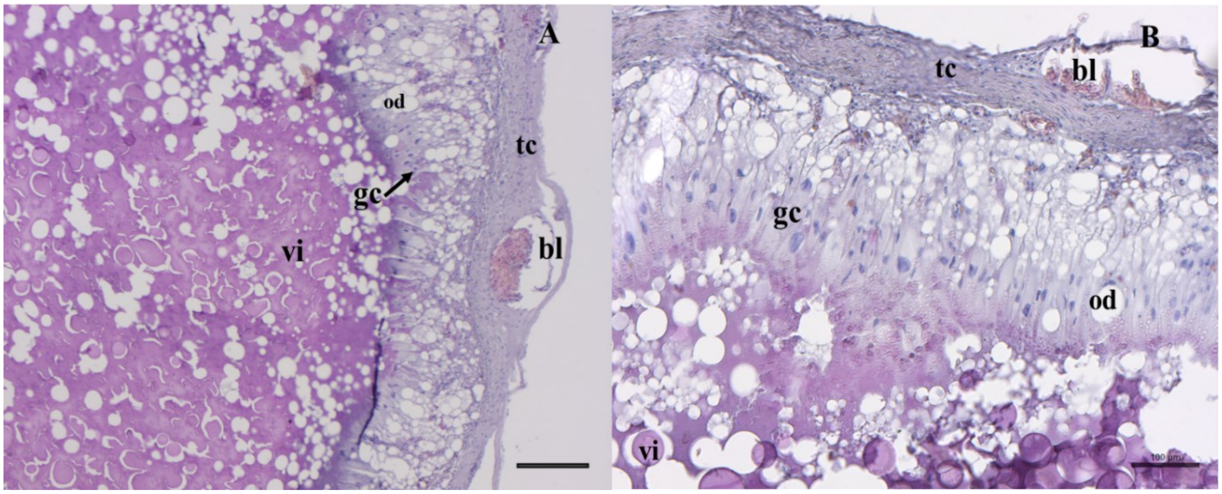

Figure 13: second stage of follicular atresia. **(A), (B) bl,** blood vessel; **tc**, theca cells; **od**, oil droplet; **gc**, granulosa cells; **vi**, vitellogenin. Scale bar: 200 μm (A), 100 μm (B).

The second stage of atresia is characterized by an increase of the vascularity of the connective tissue surrounding the follicle and blood vessels are clearly visible (bl) (Figure 13A, B). Granulosa cells (gc) start to penetrate inside the follicles appearing more elongated with clearly visible nucleus and are identified as 'giant cells' (Figure 13A). Meanwhile, oil droplets (od) and yolk granules (vi) are reabsorbed by the follicle cells (Figure 13B).

The amount of ooplasm decreases leading to increased vacuolization. Externally, the cells of theca layers are considerably hypertrophied (tc).

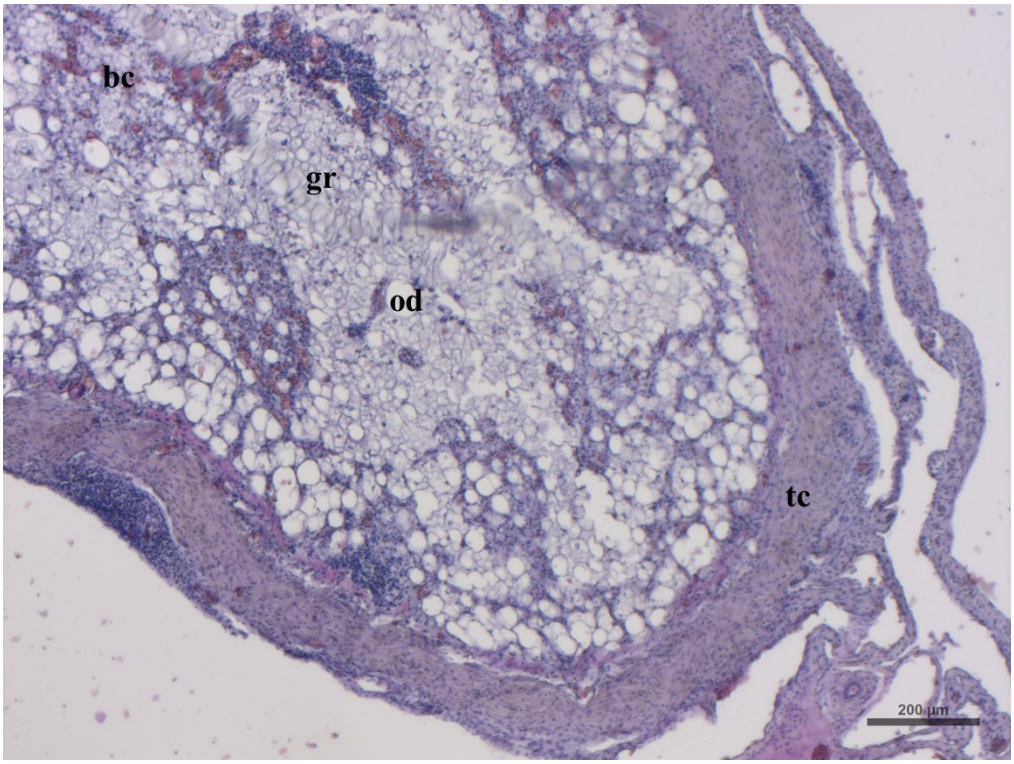

Figure 14: example of advanced stage of follicle atresia. **gr**, granulosa cells; **od**, oil droplets; **tc**, theca cells. Scale bar: 200 μm.

Internally, an atretic follicle at the advanced stage is now completely filled with enlarged granulosa (gr) cells and blood cells (bc) and it is surrounded by a thick layer of thecal cells (tc) (Figure 14). Very few yolk globules still occur in the cytoplasm (not visible). The cytoplasm vacuoles (od) further increase both in size and number. The theca cells (tc) are highly hypertrophied and vascularized (Figure 14). Successively, in the final stage of atresia, follicles will lose their shape and will be completely filled with the granulosa and theca cells and thick connective tissue. All ooplasm will be gradually reabsorbed.

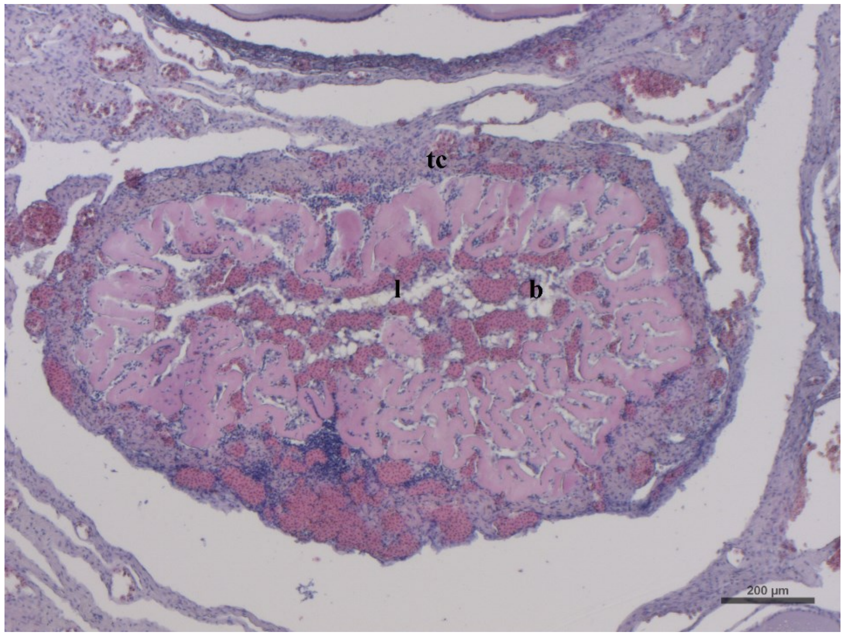

Figure 15: example of corpus luteum. **l**, lipid; **b**, blood; **tc**, theca cell. Scale bar: 200 µm.

The corpus luteum is the result of post-ovulatory follicles transformation that continue to secrete progesterone after ovulation. It has a compact structure characterized by a central lipids (l) aggregation and a great accumulation of blood (bl) (Figure 15).

4.5 Ovarian maturation stages

Based on the description of oocytes and follicles development (paragraph 4.4), different stages of gonad maturation were identified and described. Three different stages have been detected analyzing 8 gonad samples out of the 9 animals investigated (animal ABASCC2521 was not provided with gonad sample).

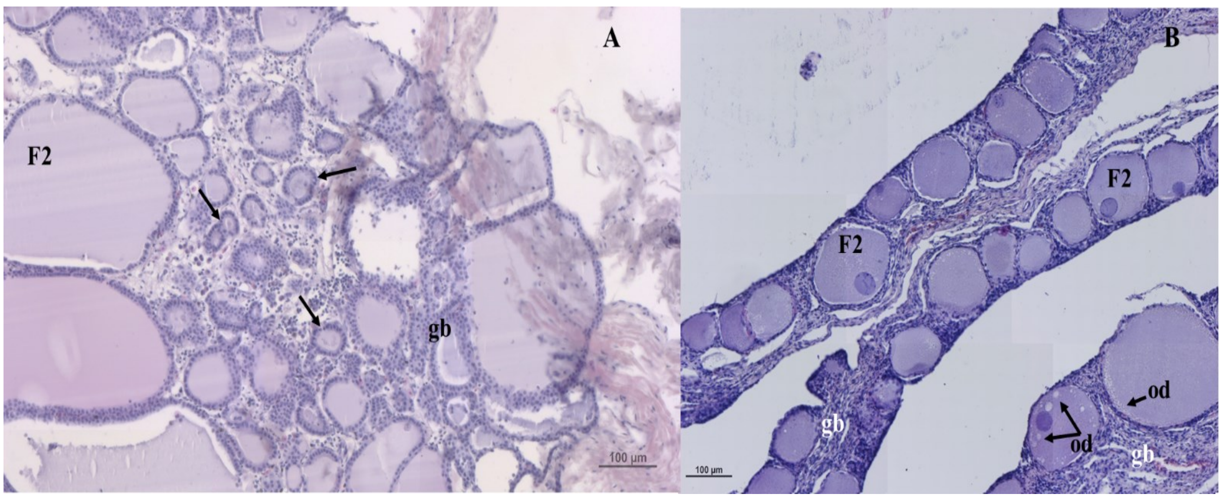

Figure 16: example of gonad of juveniles animals. **(A) gb**, germinative bed; **F2**, secondary follicle; **black arrow**, primary follicle. Scale bar: 100 µm. **(B) gb**, germinative bed; **od**, oil droplets; **F2**, secondary follicle. Scale bar: 100 µm.

Immature gonads were found in two juvenile specimens (ABASCC3021, CCL= 19.2 stranded on October, 1 and ABASCC3121, CCL= 29.8 stranded on October, 23). They could present follicles at different stages of maturation. In this stage, germinative beds (gb) are clearly visible in the cortex area. Many previtellogenic follicles, both primary (black arrow) and secondary (F2) ones, are distributed in the gb (Figure 16A). Primary follicles are surely distinguishable as they are surrounded by a single layer of somatic cells. In Figure 16B it is clear the presence of secondary follicles (F2) with a defined nucleus in the peripherical zone and visible nucleoli and in some of them, it is visible the internalization of oil droplets (od). Gb is still delocalized in the ovary.

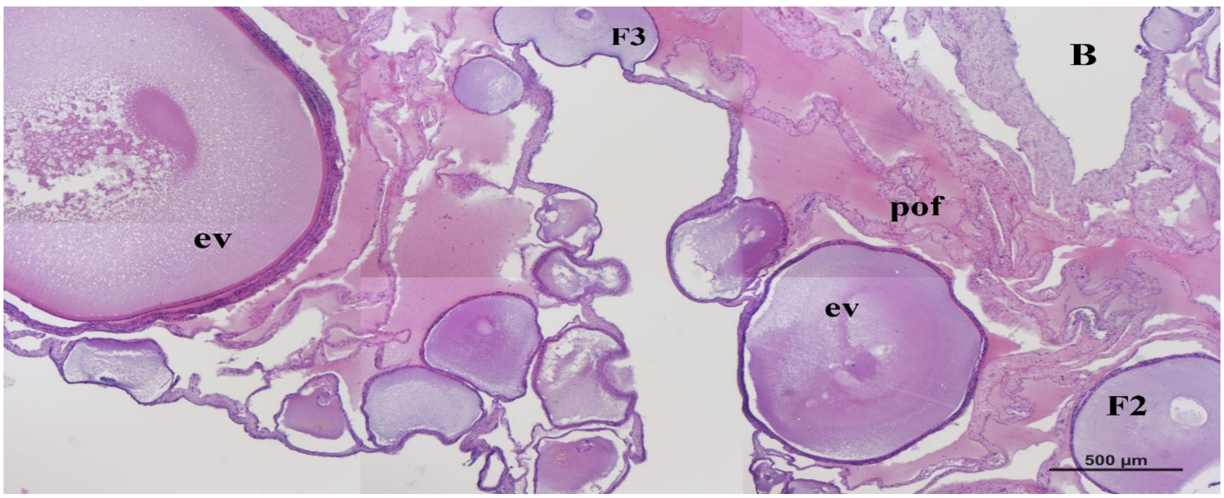

Figure 17: example of a gonad of a sub-adult animal. **ev**, early vitellogenic; **pof**, post-ovulatory follicle; **F2**, secondary follicle; **F3**, tertiary follicle. Scale bar: 500 µm.

Figure 17 represents the gonad of a sub-adult animal in which an early vitellogenic follicle (ev) is evident: the radiate zone is now completely formed, and oil droplets are copiously distributed in the cytoplasm. Vitellogenin droplets is penetrating inside the oocyte, and it is evident how they gradually fuse together while approaching the center (Figure 17). In this stage, it is possible to find secondary

(F2) and tertiary follicles (F3) as well and post ovulatory follicles (pof). This stage was identified in three sub-adult animals ABASCC2121 (CCL=65.5 cm, stranded on July, 23), ABASCC1521 (CCL= 59.6 cm, stranded on July, 4) and ABASCC1321 (CCL=50 cm, stranded on June, 21).

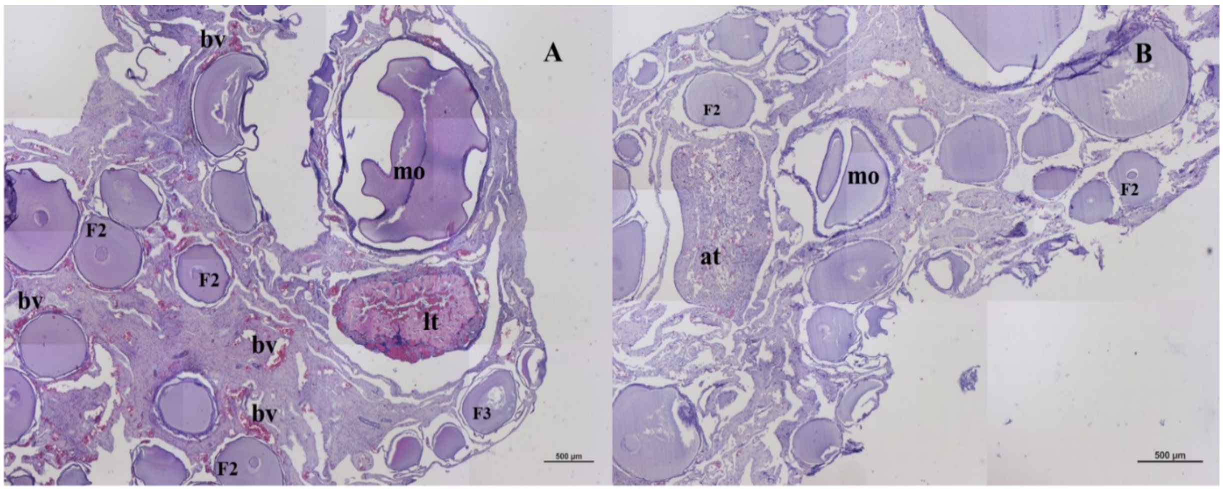

Figure 18: example of a gonad of adult animals. **(A) F2**, secondary follicle; **F3**, tertiary follicle; **bv**, blood vessel; **lt**, corpus luteum; **mo**, mature oocyte. Scale bar: 500 µm**. (B) mo,** mature oocyte; **F2**, secondary follicle; **at**, atretic follicle. Scale bar: 500 µm.

The ovary of the only adult animal analyzed, ABASCC2721 (CCL=67 cm, stranded on September, 3) was reported in Figure 18. In this gonad, oocytes at different developmental stages are visible, from the previtellogenic (F2 and F3) to the mature one (mo). The mature oocyte presents an irregular shape due to the hydration stage that occurs before ovulation. The connective tissue is very vascularized, and this is clear with the high presence of blood vessels (bv) (Figure

18A). It is possible to see an advanced state of atretic follicle (at) that follows the mature oocyte step (Figure 18B) and a corpus luteum (lt) (Figure 18A).

5. DISCUSSION

According to the Marine Strategy Framework directive, the loggerhead sea turtle *Caretta caretta* is the official bio-indicator for quantitative and qualitative monitoring of marine litter ingested by marine animals in the Mediterranean Sea (European Commission, 2013).

The presence of marine debris is a serious issue that needs to be studied and controlled, often the interactions between the marine biota (both vertebrates and invertebrates) with the marine litter could permanently damage the formers. In particular, microplastics became an important matter of discussion due to their high and constant presence in the Mediterranean Sea and particularly in the Adriatic basin (Vianello *et al.*, 2018). Investigating the effects that microplastic ingestion has on these animals is fundamental to protecting the species from extinction in the near future. Moreover, a special focus should be placed on both direct and indirect effects that microplastics ingestion could generate in different aspects of reproduction. Indeed, some dysfunctions were already observed in reptiles, such as developmental abnormalities, altered sexual morphology, and sex reversal (Barraza *et al.*, 2021).

5.1 Data of recorded C. caretta along the Abruzzo coast

The increasing number of *C. caretta* recorded (stranded and from bycatch) between 2020-2021 is not in line with the trend that could been foreseen considering the widespread of Covid pandemic during these years. As Russo *et al.* (2021) stated, a decrease in fishing effort was registered, in the Adriatic Sea, during the period of pandemic, therefore, the accidental contact with the fishing vessels and fishing nets, considered as the main threat for loggerhead sea turtles, should have reduced visibly. Conversely, during the biennial 2020-2021, not only the number of stranded turtles increase, but the majority of them were found in a time frame that coincide with the reproduction period (from the beginning of June to the end of August) (Patel *et al.*, 2016). The offshore area along the Abruzzo coast is not a typical nesting area: Adriatic Sea is a common foraging site (Bertuccio *et al.*, 2019) even though in 2021 an unusual deposition along the Venetian coast (Jesolo lido) was recorded (www.arpa.veneto.it/arpavinforma/comunicati-stampa/anno-2021).

The high number of stranded animals, during the summertime (June-August), could be related to the massive touristic affluence on the shore and the intense traffic of touristic boats that increase the probability to encounter a stranded animal or the chance of collision offshore.

More likely, the phenomenon observed, may be associated with the higher presence of loggerhead sea turtles in the Adriatic Sea due to the climate and environmental change occurring in the last decades. For example, the increase of water temperature in the Eastern Mediterranean Sea, together with the rivers' mouth inputs lead to a concentration of nutrients that through the trophic net creates an ideal foraging area (Patel *et al.*, 2016).

However, it should not be excluded other causes that could lead to the sea turtles stranding. For example, it has been demonstrated that the ingestion of plastics debris provokes some serious damages in the gastrointestinal tract compromising sea turtles survival (Wilcox *et al.*, 2018; Di Renzo *et al.*, 2021).

The results obtained in this study confirmed that feeding preference may affect the probability of debris ingestion, small size turtles have a propensity to ingest more plastic fragments that can be confused as one of their pelagic preys. Differently, adults appear less susceptible to debris ingestion due to a shift to a benthonic-based diet (Di Renzo *et al.*, 2021). The color of microplastics is also an important element that influence their ingestion. The predominant presence of white plastic fragments among all samples analyzed could be associated with the similarity of white floating plastics pieces to jellyfish, one of the typical preys of both juvenile and adults *C. caretta* (Milisenda *et al.*, 2014). This confirmed that turtles have an excellent vision color as already highlighted by Lutz, Musick and Jeanette (2003). However, more studies are necessary to fully understand the underwater perception also considering the shapes and movements of plastics debris.

Microplastics ingestion could be potentially toxic at different physiological levels, therefore, it is important to identify a proper biomarker to assess the health status associated to the presence of MPs. In the present study, MMs were evaluated as possible biomarkers of the general health status associated with the presence of MPs in the gastrointestinal tract, as they have already been related to the exposure to different environmental pollutants in blue fin tuna (*Thunnus thynnus*) (Passantino *et al.*, 2014).

The results obtained suggested that i) MMs activity could be exclusively related to changes in the physiological process occurring in live animals since a lack of correlation was observed between their presence in both liver and spleen) and the animals decomposition status. ii) The MMs observation is comparable in both liver and spleen, therefore analysis concerning only one organ could be assessed. iii) As already shown in a previous study (Johnson *et al.*, 2005), MMs number and size increase with age of the animals. This could clarify the negative, although not significant, correlation between MPs numbers and the number and size of MMs. Indeed these results suggested the suitability of a different approach that analyzed the MMs within the same class of age. To obtain valid data that unequivocally associate MPs and MMs, animals with MPs should be compared to animals with no MPs in their gastrointestinal tract and belonging to the same age class.

5.2 Ovarian characterization

Through the histological analysis, the present study identified the features that characterized the follicular development and allowed the determination of the maturity stage of the animals.

At date, the official method used to determine the sexual maturity of sea turtles is based on the measurements of the CCL (Margaritoulis, 2003). Following this method that divided stranded *C. caretta* into three dimensional classes (young juveniles, sub adults and adults) the majority of animals belonged to the sub adult class that includes the young turtles starting the puberty process to reach the sexual maturity (Casale, Mazaris and Freggi, 2011; Ishihara *et al.*, 2011). However, the histological analysis of the ovary performed on a reduced sample of females, highlighted how CCL may not reflect the real maturity status of the animals. Indeed the "sub adults" females presented the features of a mature animal such as the presence of corpora lutea observed in the ovary of a sea turtle identified as not mature for CCL standard. The corpus luteum is a structure deriving from post ovulatory follicles indicating a mature gonad that has already experienced the follicles maturation and the final ovulation. Several studies have stated turtles reproduce every two-three years and corpora lutea persist in the gonad with their excretory function for a long period after ovulation (Gemmell, 1995; Guarino *et al.*, 1998). Moreover, in 4 animals, identified as sub adults, it was possible to identify primary, secondary, and tertiary follicles together with corpus lutea and atretic follicle that are typical components of an asynchronous ovary present in mature animals that nest three or four times during the reproductive season (Canbolat, 2004).

6. CONCLUSION

Although sea turtles are very common in our seas, a lot of information about their biology, especially from the reproduction point of view, are still missing. To the best of our knowledge, this is the first study aimed to describe the follicle maturation in *C. caretta*. The present study suggested that the CCL method used to discriminate the sexual maturity of sea turtles is not always representative of the real development stage. Further analysis should be performed also to evaluate the effectiveness of the CCL method in male specimens. Finally, this thesis lays the groundwork for further characterization and quantification of melanomacrophages as validate biomarkers to assess the health status of sea turtles exposed to the presence of microplastics.

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