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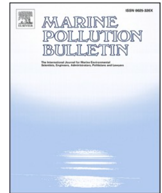

Parasitological investigation and potential effects of parasitism on hematological, genotoxic and immune parameters in free ranging sea turtles rescued along the Southern Adriatic coast (Eastern Mediterranean Sea)

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ABSTRACT

Sea turtles can host a wide range of parasitic taxa, some of which may lead to severe diseases, weakening and endangering their health. Assessing free-ranging sea turtles' responses to these potential pathogens using biomarkers can provide valuable insights into the cellular and molecular impacts of parasites. This information can serve as a crucial tool for conservation efforts. During rehabilitation, hematological, genotoxic and innate immune parameters were evaluated in 70 free-ranging sea turtles rescued along the Southern Adriatic coast (Eastern Mediterranean Sea) from 2021 to 2023, 24 of which were found to have parasitic infections. *Hapalotrema mistroides*, *Sulcascaris sulcata* and Neospirorchis sp. were identified by coprological and molecular techniques. Some significant differences for biomarkers were associated with these parasites. These findings highlight the importance of considering the infection status of free-ranging sea turtles when evaluating biomarker results, as major pathogens like parasites can influence certain parameters.

1. Introduction

The Mediterranean Sea is considered a hotspot for marine biodiversity, hosting approximately 17,000 species (Coll et al., 2010), including two vulnerable and one endangered sea turtle species: the loggerhead turtle (*Caretta caretta*; Cheloniidae), which is the most widespread, the green turtle (*Chelonia mydas*; Cheloniidae), and the leatherback turtle (*Dermochelys coriacea*; Dermochelyidae), although only a small number of individuals (large juveniles or adults) of the latter species enter the Mediterranean from the Atlantic, without breeding in the basin (Casale et al., 2011). Sea turtles are reptiles perfectly adapted to aquatic life; however, they rely on terrestrial environments for spawning, making them vulnerable to a range of threats from both the aquatic and terrestrial environments. Indeed, a wide range of anthropogenic and environmental factors can negatively impact the survival of sea turtle populations. Accidental catch due to fishing gear (commonly known as bycatch), including gillnets, longline hooks and shrimp trawl nets (Alió et al., 2010; Parga et al., 2015; Pingo et al., 2017), the alteration and loss of nesting habitats (Mazaris et al., 2009; McClenachan et al., 2006), injuries due to collisions with boats (Casale et al., 2010; Chaloupka et al., 2008; Denkinger et al., 2013; Orós et al., 2005), marine pollution (Bugoni et al., 2001; Casini et al., 2018; Flint et al., 2015; Morão et al., 2024; Orós et al., 2005), marine litter (Campani et al., 2013; Hahladakis, 2024; Matiddi et al., 2017), climate change (Chaloupka et al., 2008) and infectious diseases (Orós et al., 2005), have been recognized as the most important threats to sea turtle species (Fuentes et al., 2023).

In particular, infectious diseases caused by pathogenic organisms,

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such as some parasitic helminths, are a growing concern for sea turtle conservation, as they may further endanger these species (Chapman et al., 2019; Flint et al., 2010; Gračan et al., 2012; Marchiori et al., 2020; Morick et al., 2023; Santoro et al., 2017, 2019, 2020a,b; Stacy et al., 2010). Two key parasites infecting sea turtles are digenean trematodes, belonging to the paraphyletic blood fluke family Spirorchiidae (Chapman et al., 2019), and the nematode *Sulcascaris sulcata*, belonging to the Anisakidae family (Santoro et al., 2020a,b). The adult stages of *Hapalotrema mistroides* and Neospirorchis sp. (Spirorchiidae), whose intermediate hosts are represented by gastropods (Mollusca) (Corner et al., 2022; de Buron et al., 2018) live in the circulatory system (aorta, celiac artery, gastric artery, heart chambers, liver vessels, mesenteric arteries) of sea turtles. These parasites lay embryonated eggs in the bloodstream of infected animals and sometimes also into the intestinal tract through vessels. Embryonated eggs can embolize in internal organs causing serious functional and anatomical alterations at the sites of localization with arteritis, thrombosis and aneurysms of the large vessels, granulomas widespread in all areas of the body (kidneys, intestine, liver, lungs, pancreas, spleen, bladder), and even encephalitis (Flint et al., 2010; Santoro et al., 2017). The nematode *S. sulcata,* whose intermediate/paratenic hosts are represented by crustaceans, fish and molluscs (Santoro et al., 2022, 2020a,b; Marcer et al., 2020), live free in the gastric lumen or fixed with the cephalic end to the stomach wall of sea turtle where it is known to cause ulcerative gastritis with increasing degrees of severity as the parasite load increases (Santoro et al., 2020a, b).

Hematological parameters are useful tools for evaluating the health conditions of turtles (Manire et al., 2017), as they can be used as physiological indicators of disease (Fazio et al., 2012; Swimmer, 2000), stress (Knotkovà et al., 2005), hypothermia (Anderson et al., 2011), or contaminant exposure (Keller et al., 2004; Lutcavage et al., 1995), as well as to evaluate the degree of dehydration (Christopher et al., 2003; Peterson, 2002). Parasites can cause chronic physical stress in some animal species and this type of stress elicits a response that has been associated with adverse consequences for the host (Seguel et al., 2019). Molecular tools, such as biomarkers, provide sensitive, specific insights into the cellular and molecular effects of parasites, aiding in health assessments. For instance, several pathogens as parasitic helminths have been shown to cause genotoxicity in infected animals through the production of excretory-secretory metabolic products, leading to DNA and chromosome damage, which may result in mutations and even cancer (Chelomina et al., 2021).

Studies describing parasitic infections in sea turtles within the Mediterranean area are limited and often conducted on dead animals (Aznar et al., 1998; Santoro et al., 2010; Gračan et al., 2012; Marchiori et al., 2017; Santoro et al., 2020a,b; Gentile et al., 2021) or involved a small number of alive individuals (Pace et al., 2019; Marchiori et al., 2020). Furthermore, the effects of parasitic infections on the health status of sea turtles remain largely unexplored, although the anatomopathological characteristics, such as macroscopic and microscopic lesions, have been well documented (Flint et al., 2010; Santoro et al., 2017, 2020a,b). For example, to establish the effects of parasitism on hematological parameters in sea turtles it is essential to determine the alterations in the values of the blood parameters of interest and their correlation with parasitic infections (Aliko et al., 2018; Faggio et al., 2015). Sea turtles are known to be infected with parasites (Chapman et al., 2019; Flint et al., 2010; Gračan et al., 2012; Marchiori et al., 2020; Morick et al., 2023; Santoro et al., 2017, 2019, 2020a,b; Stacy et al., 2010) that may act as additional genotoxic factors in different scenarios of the marine environment. However, to the best of our knowledge, there is no information on the relationship between parasites and potential specific blood cell responses in free ranging sea turtles.

The purpose of this research study was twofold: first, to provide additional information on parasitic infections in free-ranging loggerhead and green sea turtles within the Mediterranean area, through a two-year parasitological investigation on sea turtles housed in a rescue center on the Adriatic coast, integrating copromicroscopy with molecular analysis. Second, to provide new information on the potential pathogenic effects of parasites, we simultaneously studied the effect of parasitism by comparing genotoxicity and immune system alterations in parasitized and non-parasitized sea turtles.

2. Material and methods

2.1. Study area

Between 2021 and 2023, 70 sea turtles (69 loggerheads and 1 green turtle) were hospitalized at the Sea Turtle Rescue Center of Manfredonia (Puglia, Italy), located along the Italian coast of the mid-lower Adriatic Sea (42°03′54.22″ N 14°47′16.45″ E - 41°13′50.14″ N 16°32′21.88″ E) (Fig. 1). This specific stretch of sea is located in the central Mediterranean Sea between Italy and the Balkan peninsula, covering an area of 132,000 km^2 and a depth of 1222 m. Due to the presence of shallow sandy beaches and the high density of edible fish species, many fisheries are widely practiced, such as trawling and small-scale fishing with longlines or gillnets (Lucchetti et al., 2017).

2.2. Animals

Out of 70 rescued sea turtles, 12 loggerheads were found stranded along the coast, still alive, while the green turtle and the remaining 57 loggerheads were accidentally caught by fishermen. In fact turtles recovered in this study area are not usually hospitalized for morbidity, but because they remain trapped in the fishing nets. Commonly after few days from the recovery, operators of the rescue center return the animals to the sea. Thirteen of them had a mean notch-to-tip Curved Carapace Length (CCL) $\geq$70 cm, 30 had a mean CCL $\geq$60–$\leq$69.9 cm, and 26 had a mean CCL $\leq$59.9 cm. In one case the mean CCL could not be determined. According to the classification of Casale et al. (2005), the rescued sea turtles were classified as adults, sub-adults, or juveniles, respectively. Following the Ministerial Guidelines of the Italian Institute for Environmental Protection and Research, ISPRA (Mo et al., 2013), once arriving at the Sea Turtle Rescue Center, each turtle underwent the first clinical examination, then received rehydration with fluidic therapy and vitamin administration if necessary and was finally kept in an individual basin with salt water. In addition to CCL, other biometric parameters such as tail length and total weight as well as health parameters (i.e., vitality, sensory evaluation, muscle tone or flaccidity, state of nutrition and appetite, absence or presence of injuries or trauma to hard tissues, fins and head, diving ability, and buoyancy position) were noted individually. Only the animals resulted clinically stable and healthy after the first examination were manually restrained out of the water and sampling procedures were initiated, in strict accordance with relevant national and international guidelines under CITES permits (CITES Nat. IT025IS, Int. CITES IT 007).

2.3. Fecal sample collection for parasitological survey

A fecal sample was collected from each turtle after the first spontaneous voiding as soon as possible (Marangi et al., 2020), to diagnose gastrointestinal infections and to detect parasite eggs/cysts/oocysts. After collection in a sterile falcon, the samples were labeled for turtle identification and stored in the refrigerator for future analysis.

2.4. Blood sample collection for molecular and biomarker analyses

Blood samples (2 mL) were collected from the cervical venous sinus of 70 sea turtles for molecular diagnosis of blood flukes by using a disposable heparinized syringe and transferred to solvent-rinsed glass vials with Teflon caps containing heparinized saline (heparin sodium). Of these, 57 randomly selected samples were used for biomarker analysis as follows. One drop of blood was used to make two blood smears for

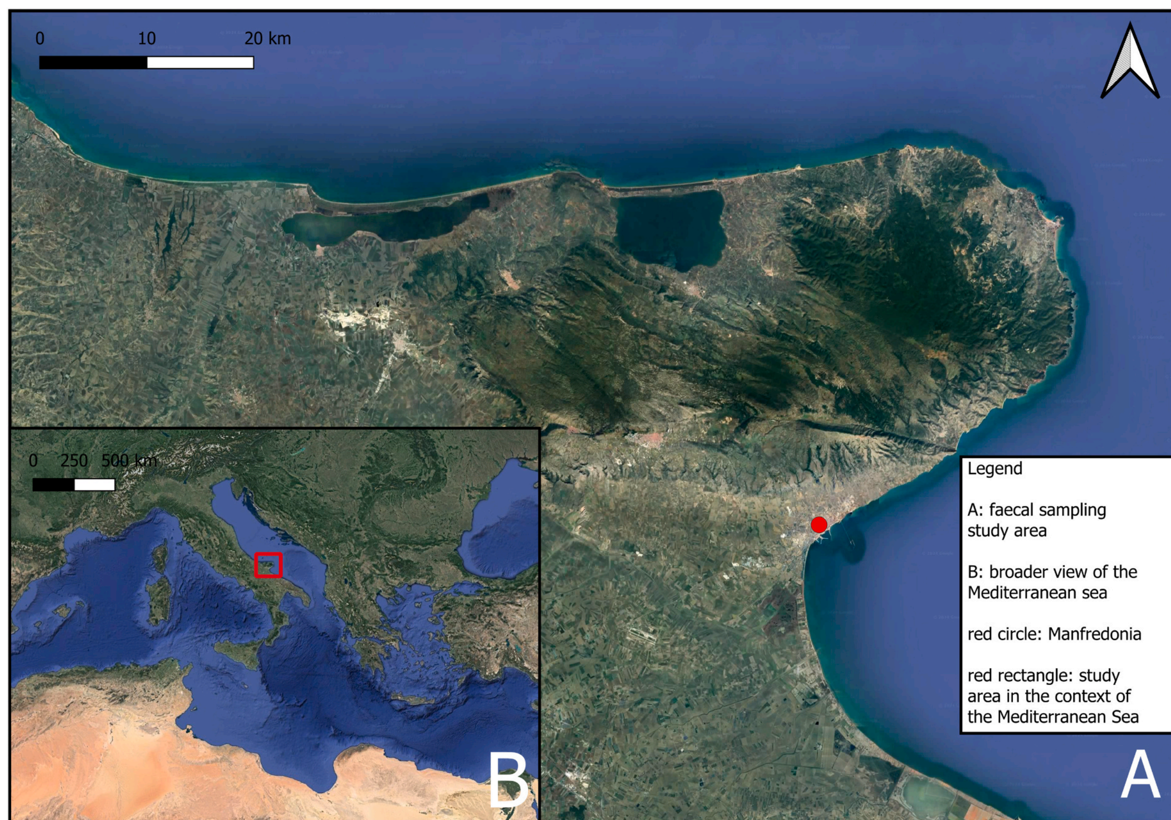

Fig. 1. Fecal sampling study area (A) in the context of the Mediterranean Basin (B): red circle (Manfredonia Sea Turtle Rescue Center, Southern Adriatic Coast). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

erythrocytic nuclear abnormalities (ENA) assay and differential white blood cell count (DWBCC); the rest of the blood was divided into two aliquots: one aliquot was used for biomarker analysis and the remaining part was stored and delivered within 24 h to the Microbiology Lab of the University of Foggia for molecular analysis. For biomarker analysis, a part of whole blood was used for respiratory burst, while the remaining was centrifuged to obtain plasma to evaluate lysozyme activity. Blood smears, whole blood and plasma were stored in dry ice and transported to the Laboratory Biomarkers at the University of Siena for biomarker analysis.

2.5. Coprological analysis

Each fecal sample was subjected to microscopic investigation by using the slightly modified Mini-FLOTAC® technique in combination with Fill-FLOTAC® and a ZnSO4 flotation solution (specific gravity 1.360). The method has an analytic sensitivity of 5 eggs per gram of feces, as originally reported by Cringoli et al. (2017). For this study, the technique was adapted to the small amount of fecal material available from turtles, as described by Pace et al. (2019) and Marangi et al. (2020).

Eggs were identified by their morphologic and morphometric characteristics as reported by Greiner (2013), Marangi et al. (2020) and Barnard and Upton (1994). For the purpose of our study, only eggs of the helminths belonging to Anisakidae and Spirorchiidae family have been taken into consideration.

2.6. Molecular analyses and sequencing

Genomic DNA was extracted from 200 μl of blood samples using the NucleoSpin® Kit (Macherey-Nagel, Duren, Germany) and the QIAamp DNA Blood kit (Qiagen, Milan. Italy), in order to recover the maximum DNA concentration. The 28S rDNA region within Spirorchidae family gene was amplified using the primers described by Olson et al. (2003): LSU-5 (5′-TAGGTC GAC CCG CTG AAY TTA AGCA-3′) and 1500R (5′-GCT ATC CTG AGG GAA ACT TCG -3′). PCR reactions were performed in a 30 μl reaction volume consisting of 3–5 μl DNA, 2.5 mM MgCl2, 0.2 mM dNTPs (MBI Fermentas, Germany), 1× PCR buffer, 0.8 μM each of forward and reverse primers, 1 M Betaine solution, and 1.5 U of Taq Platinum DNA Polymerase (Invitrogen), with the remainder of the volume composed of sterile water. The amplification protocol included an initial activation step at 95 °C for 4 min, followed by 35 cycles of 94 °C for 60 s, 56 °C for 60 s, and 72 °C for 120 s, with a final extension step at 72 °C for 4 min.

Purified amplicons were directly sequenced in both directions using the ABI PRIMS Big Dye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, California, USA) with the same primers as the respective PCR reaction, following the manufacturer's instructions. Obtained sequences were determined on the ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Waltham, Massachusetts, USA) and the chromatograms were inspected by eye using the FinchTV software v1.4.0 (Geospiza, Inc.; Seattle, WA, USA; http://www.geospiza.com). Primer regions plus bad-quality regions were trimmed. Once the sequences had been cleaned up, each sequence was compared with all the *H. mistroides* 28S and *Neospirorchis* sp. 28S homologous nucleotide sequences available in GenBank databases using the Blast tool (https://blast.ncbi.nlm.nih.gov). Then, the obtained sequences were gathered in a "fasta" file and aligned with reference *H. mistroides* 28S and Neospirorchis sp. 28S sequences using MAAFT software. Maximum Likelihood Phylogenetic Analysis was achieved according to partitions and optimal substitution models identified by the BIC and AIC metrics with 1000 bootstrap replicates as implemented in MEGA X v10.0.5.

2.7. Biomarker analysis

2.7.1. ENA assay and DWBCC

Blood smears were air-dried overnight, fixed and stained with Diff-Quick (Bio-Optica), and the same blood smear from each animal was used for both ENA assay and DWBCC. The ENA assay was carried out according to the method of Casini et al. (2018). Nuclear abnormalities were scored in 1000 mature erythrocytes per sample using an immersion light microscope (Olympus BX41) and were attributed to one of the following categories: micronuclei, lobed nuclei, segmented nuclei, and kidney-shaped nuclei. The results were expressed as ENA frequency, mean value (‰) of each abnormality and the sum of all the lesions scored and the % values of each abnormalities were determined. Two hundred leukocytes were counted and identified as lymphocytes, monocytes, eosinophils, heterophils and basophils and the results expressed as mean value (%) of each leukocyte. In addition, thrombocytes in their activated form were also identified and counted. From each DWBCC, the ratio between the number of heterophils (H) and lymphocytes (L) was also measured to obtain the H/L ratio.

2.7.2. Lysozyme activity

The lysozyme activity was measured in plasma samples with a standard turbidity test as previously described by Caliani et al. (2019). Briefly, 1 mg/mL stock solution of hen egg white lysozyme (HEL, Sigma, St. Louis, MO) was prepared in 0.1 M phosphate buffer (pH 5.9) and serially diluted in phosphate buffer to produce the standard curve of 0, 0.3, 0.6, 1.25, 2.5, 5, 10, 20, 25 μg/mL. A fresh solution of *Micrococcus lysodeikticus* (Sigma, St. Louis, MO) was obtained by dissolving 50 mg of the lyophilized cells in a 0.1 M phosphate buffer. Each concentration of the standard curve (25 μL/well) was added to a 96-well plate in triplicate, and 25 μL of each sample was added in quadruplicate to the same plate. *M. lysodeikticus* solution (175 μL/well) was quickly added to three sample wells and each of the standard wells. The blank was the fourth well and contained only 25 μL of plasma and 175 μL of phosphate buffer. A microplate ELISA reader (Microplate Reader Model 550, Bio-Rad) was used to measure absorbance at 450 nm. The optical density (O.D.) was measured immediately (T0) and after 5 min (T5) and the activity expressed as HEL concentration (μg/mL) by linear regression of the standard curve.

2.7.3. Respiratory burst

Respiratory burst activity was evaluated as the presence of intracellular oxyradical produced by NADPH oxidase, and was measured with the NBT assay, following the method of Caliani et al. (2019). For each sample, 100 μL of whole blood was added in triplicate to a 96-well plate, and the plate was incubated at 30 °C for 2 h to allow cell adhesion. Then, the plate was washed off 3 times with L-15 medium to eliminate unattached cells. Subsequently, 100 μL of L-15 medium supplemented with NBT (1 mg/mL) were added to each well and the plate was incubated at room temperature for 1 h. After incubation, the plate was unwrapped and fixed with 100 % methanol for 10 min. The plate was washed several times with 70 % methanol and air-dried. To disrupt cell wall and dissolve formazan blue crystals resulting from the reduction of NBT by the oxyradicals, 120 μL of KOH and 140 μL of DMSO were added to each well. Optical density (OD) was measured at 630 nm using an ELISA microplate reader (Microplate Reader Model 680XR, Bio-Rad).

2.8. Statistical analysis

In this study, we first analyzed the presence/absence of parasites. The sea turtles examined were considered parasitized if they tested positive for at least one of the two diagnostic methods used. Prevalence values were determined as the number of animals found positive for parasitic infections/number of animals examined x 100 with the corresponding 95 % confidence intervals (Tables 1 and 2). We divided the animals in three classes of age according to the von Bertalanffy model that uses as parameter the Curved Carapace Length (CCL). The von Bertalanffy growth function is expressed in the form $Lt = L\infty - (L\infty - L0)\ e(-kt)$, where Lt is the carapace length at age t, L0 is the initial carapace length and it is used to estimate the time required by turtles to grow within the observed size range (Von Bertalanffy, 1938). L0 was set as 20 cm, L∞ was 95.63 cm CCL and k = 0,077/yr (Casini et al., 2018).

The statistical significance of differences in the prevalence of infections between the age groups (juveniles, sub-adults, adults) was investigated using Fisher exact test (Table 2). A P value ≤0.05 was considered statistically significant. We then focused on host-parasite interactions and examined intraspecific responses to parasitic infections. For DWBCC, the percentage frequency of each leukocyte type was calculated in the first 200 white blood cells. The H/L ratio was calculated as the number of heterophils divided by the number of lymphocytes counted in these 200 white blood cells. The thrombocyte/white blood cell ratio (TWBCR) was calculated as the number of thrombocytes counted per 200 leukocytes, and converted to a percentage. Mean, standard deviation, median, and range of hematological, genotoxic, and immune parameter values were calculated. Differences were compared between parasitized (n = 20) and non-parasitized (n = 37) loggerhead sea turtles as well as within the three related age groups. The Shapiro-Francia (n > 50) and Shapiro-Wilk (n < 50) tests were used to check whether the data were normally distributed (Shapiro and Francia, 1972; Shapiro and Wilk, 1965). These two statistical tools were chosen because the Shapiro-Francia test can be considered the most

Table 1
Prevalence (%) with corresponding 95 % confidence intervals (95 % CI) of parasites and their associations detected in samples of loggerhead turtles (*Caretta caretta*) and a green turtle (*Chelonia mydas*) undergoing rehabilitation at a sea turtle center rescue, after stranding or being accidentally caught in fishing nets along the Adriatic Sea, as determined by combining the results of copromicroscopy and molecular analysis of peripheral blood.

Parasites and their associations	No. positive		% (95 % CI)
	Caretta caretta (n = 69)	*Chelonia mydas* (n = 1)	
Hapalotrema mistroides	10	0	14.29 (6.09–22.48)
Sulcascaris sulcata	5	0	7.14 (1.11–13.18)
Neospirorchis sp.	5	0	7.14 (1.11–13.18)
Sulcascaris sulcata/ Neospirorchis sp.	3	0	4.29 (0.00–9.03)
Sulcascaris sulcata/ Hapalotrema mistroides	1	0	1.43 (0.00–4.21)
Total (n = 70)	24	0	34.29 (23.17–45.41)

Table 2
Prevalence (%) with corresponding 95 % confidence intervals (95 % CI) of parasitic infections in sea turtles (69 *Caretta caretta*, 1 *Chelonia mydas*) undergoing rehabilitation at a sea turtle rescue center, after having stranded or been accidentally trapped in fishing nets along the Adriatic Sea, based on age class, determined by combining the results of copromicroscopy and molecular analysis of peripheral blood.

CCL (Curved Carapace Length)	Class of age	Estimated age range	No. positive/ no. examined	% (95 % CI)
≤59.9 cm	Juveniles	7–15 years	7/26	26.92 (9.87–43.97)
≥60–≤69.9 cm	Sub-adults	16–19 years	10/30	33.33 (16.46–50.2)
≥70 cm	Adults	20–25 years	6/13	46.15 (19.05–73.25)
Undetermined	Unknown	Unknown	1/1	100 (100–100)
Total			24/70*	34.29 (23.17–45.41)

* All positive samples were from *C. caretta*.

powerful for determining whether the data follow a normal distribution (Mbah and Paothong, 2015) while the Shapiro-Wilk test has proven to be the best method for evaluating the normality of small samples (Ahad et al., 2011). The results of the Shapiro-Francia and Shapiro-Wilk tests showed significant ($P \leq 0.05$) departures from normality. Therefore, data values did not follow a normal distribution and were compared using an appropriate nonparametric test. For this purpose, the Mann-Whitney *U* test was used and differences were considered statistically significant if *P* values were $\leq$0.05.

3. Results

3.1. Coprological examination and molecular analyses

By coprological examination, 22/70 (31.43 %, 95 % CI: 20.55–42.3) sea turtles were found positive for helminths parasites. In particular, trematode eggs belonging to the Spirorchiidae family and eggs of *S. sulcata* were identified in 13/70 (18.57 %, 9.46–27.68 %) and 8/70 (11.43 %, 3.98–18.88 %) fecal samples, respectively. One (1.43 %, 0–4.21 %) turtle presented a mixed infection (Spirorchiidae/ *S. sulcascaris*). Eggs/cysts/oocysts referred to other parasites were reported in Supplementary Table 3.

By molecular analyses, 19/70 (27.14 %, 16.73–37.56 %) turtles were found positive for trematodes belonging to the Spirorchiidae family. All positive samples were successfully sequenced; after sequencing *H. mistroides* was identified in 11/70 samples (15.71 %, 7.19–24.24 %) and *Neospirorchis* sp. in 8/70 samples (11.43 %, 3.98–18.88 %). The sequences obtained showed a 98 % identity with *H. mistroides* 28S sequence (Accession Number LT617053) and 99 % identity with *Neospirorchis* sp. 28S sequence (Accession Number: LT882716).

Overall, combining the results of coprological examination and molecular analyses of peripheral blood samples, 24/70 (34.29 %, 23.17–45.41 %) rescued sea turtles were found to be infected with parasites. Specifically, 17 loggerhead sea turtles tested positive in both coprological and molecular analyses, five tested positive through coprological examination alone, and two through molecular analyses. In contrast, 46 sea turtles including the only *C. mydas* examined, tested negative for parasitic infections using both techniques. The detected parasites with their associations and prevalence values are presented in Table 1. According to age classes, parasitic infections were more prevalent in adults than in juveniles and sub-adults (Table 2). However, these differences did not reach statistically significant levels. Therefore, this suggests that loggerhead sea turtles can be infected with endoparasites at any developmental stage throughout their life, regardless of the estimated class of age.

3.2. Biomarker analysis

After the parasitological analysis, to investigate hematological, genotoxic and immune system alterations, blood analyses were carried out in two randomly selected groups of turtles, i.e. infected (n = 20) and non-infected (n = 37). Overall, the mean percentage frequency of heterophils (76.42 % vs 59.01 %) and the mean value of H/L ratio (4.99 vs 2.14) were significantly higher in parasitized turtles. In contrast, the mean percentage frequency of lymphocytes (20.35 % vs 32.1 %), the mean percentage value of TWBCR (9.35 % vs 14.36 %), and the mean percentage frequency of kidney-shaped nuclei (0.09 % vs 0.2 %) were significantly ($P \leq 0.05$) lower. The mean percentage frequencies of eosinophils, monocytes, lobed nuclei, micronuclei and total abnormalities tended to be lower in parasitized animals but did not show statistically significant differences. No segmented nuclei were identified in the examined samples. The difference in the measurement of lysozyme activity was negligible, and the measurement of respiratory burst activity was slightly higher in the parasitized subjects (Table 3, Table 1S). Similar results were observed across the three age groups. Briefly, the average percentage frequencies of heterophils (74.58 % vs 55.92 % and 77.56 % vs 59.47 %) and the mean values of H/L ratio (3.49 vs 2.13 and 5.34 vs 2.05) were statistically higher in juvenile and sub-adult turtles affected by parasitic infections. Conversely, the average percentage of lymphocyte frequency (17.87 % vs 35.52 %) was statistically lower in parasitized sub-adults (Table 4, Table 2S).

4. Discussion

In this study, parasitic infections and multiple health parameters related to genotoxic and immune system responses were evaluated in free-ranging sixty-nine loggerheads and one green sea turtle from the Mediterranean Sea.

Three helminth taxa, *H. mistroides*, *S. sulcata* and an unidentified Neospirorchis sp., were identified in loggerheads through a combination of coprological and molecular analysis and took into consideration. The only green sea turtle examined was found negative for these parasites.

In particular, *H. mistroides* was the most common parasite infecting the loggerhead sea turtles (14.29 %), followed by *S. sulcata*, *Neospirorchis* sp. (7.14 % each), and mixed infections (5.71 %). These helminths parasites have already been previously detected in Mediterranean dead loggerhead sea turtles with a prevalence of 13–16 %, 7.5 %, and 30 % respectively (Gentile et al., 2021; Marchiori et al., 2020; Santoro et al., 2019, 2020a,b), as they belong to the parasitic fauna characteristic of this host species (Gračan et al., 2012; Stacy et al., 2010). Interestingly, although 34.3 % of them were later found

Table 3
Mean, standard deviation (SD), median, and range (minimum-maximum) of hematological, genotoxicity and immune system parameters in a Mediterranean population of parasitized and non-parasitized loggerhead sea turtles (*Caretta caretta*) undergoing rehabilitation at a sea turtle rescue center, after stranding or being accidentally caught in fishing nets along the Adriatic Sea. The examined *C. caretta* population (n = 57) included juveniles (n = 19), sub-adults (n = 25), and adults (n = 13).

Parameters	Parasitized sea turtles (n = 20)		Non-parasitized sea turtles (n = 37)		*P* values
	Mean ± SD	Median (range)	Mean ± SD	Median (range)	
Heterophils (%)	76.42 ± 12.62	80.75 (35.5–89.75)	59.01 ± 13.8	61.5 (25–80)	0.00001[a]
Eosinophils (%)	3.22 ± 3.32	1.75 (0–12)	5.82 ± 6.81	3.5 (0–35)	0.25648
Lymphocytes (%)	20.35 ± 11.54	17.75 (7–60)	32.1 ± 11.58	30 (12.5–63)	0.00001[a]
Monocytes (%)	0 ± 0	0 (0–0)	0.06 ± 0.29	0 (0–1,5)	0.7414
TWBCR (%)	9.35 ± 9.31	4.5 (0–30)	14.36 ± 8.59	14.5 (1.5–38)	0.0251[a]
H:L ratio	4.99 ± 2.89	4.55 (0.59–12.14)	2.14 ± 1.13	2.13 (0.58–6.16)	0.00001[a,c]
Lobed nuclei (%)	13.55 ± 4.88	12 (6.6–23.5)	14.93 ± 5.98	14.9 (3.5–32.2)	0.20372
Kidney-shaped nuclei (%)	0.09 ± 0.12	0 (0–0.3)	0.2 ± 0.16	0.2 (0–0.5)	0.01174[a]
Micronuclei (%)	0.04 ± 0.06	0 (0–0.2)	0.05 ± 0.07	0 (0–0.2)	0.52218
Total ENA (%)	13.68 ± 4.96	12.2 (6.6–23.8)	15.19 ± 6.07	15 (3.7–32.7)	0.28014
Lysozyme (μg/mL)[b]	11.33 ± 5.47	11.26 (3.98–23.05)	10.90 ± 7.32	8.66 (1.77–34.24)	0.56868
Respiratory burst (OD)[c]	0.85 ± 1.33	0.21 (0.00–3.81)	0.51 ± 0.81	0.07 (0–2.44)	0.13622

[a] Statistically significant differences.
[b,c] For these parameters, the numbers of sampled *C. caretta* were 18 vs [b] 26 and [c] 27, respectively.

Table 4

Mean, standard deviation (SD), median, and range (minimum-maximum) of hematological, genotoxicity and immune system parameters in parasitized and non-parasitized juveniles, sub-adults, and adults of 57 loggerhead sea turtles (*Caretta caretta*) undergoing rehabilitation at a sea turtle rescue center, after stranding or being accidentally caught in fishing nets along the Adriatic Sea.

Parameters	Juveniles (n = 19)				Sub-adults (n = 25)				Adults (n = 13)			
	Parasitized (n = 6)		Non-parasitized (n = 13)		Parasitized (n = 8)		Non-parasitized (n = 17)		Parasitized (n = 6)		Non-parasitized (n = 7)	
	Mean ± SD	Median (range)	Mean ± SD	Median (range)	Mean ± SD	Median (range)	Mean ± SD	Median (range)	Mean ± SD	Median (range)	Mean ± SD	Median (range)
Heterophils (%)	74.58[a] ± 5.06	72 (69.5–81)	55.92[a] ± 15.57	61 (25–75)	77.56[b] ± 10.23	82.75 (56–85)	59.47[b] ± 12.96	62.5 (35.5–77)	76.75 ± 20.65	83.25 (35.5–85.5)	63.64 ± 12.8	59 (51–80)
Eosinophils (%)	2.58 ± 3.07	2 (0–8.5)	7.34 ± 9.6	2.5 (0.5–35)	4.56 ± 4.31	2.5 (0.5–12)	4.85 ± 4.41	3.5 (0–14.5)	2.08 ± 1.28	1.5 (1–4.5)	5.35 ± 5.85	5 (0–15.5)
Lymphocytes (%)	22.83 ± 5.78	22.5 (16.5–30.5)	29 ± 10.28	25 (17–46.5)	17.87[c] ± 7.33	16.25 (7–32)	35.52[c] ± 12.49	31.6 (12.5–63)	21.16 ± 19.49	14.75 (9–60)	29.57 ± 10.79	55 (20–43)
Monocytes (%)	0 ± 0	0 (0–0)	0.07 ± 0.27	0 (0–1)	0 ± 0	0 (0–0)	0.08 ± 0.36	0 (0–1.5)	0 ± 0	0 (0–0)	0 ± 0	0 (0–0)
TWBCR (%)	12.66 ± 11.34	10.75 (0–30)	15.92 ± 11.25	20.5 (1.5–38)	10.06 ± 10.18	4 (1.5–26)	14.44 ± 6.54	14.5 (3–26)	5.08 ± 4.45	3.75 (0–11)	11.28 ± 7.69	10 (3–21)
H:L ratio	3.49 ± 1	3.26[d] (2.28–4.91)	2.13 ± 0.87	2.13[d] (1.04–3.83)	5.34 ± 2.92	5.1[e] (1.75–12.14)	2.05 ± 1.27	2[e] (0.58–6.16)	6 ± 3.27	5.97 (0.59–9.94)	2.35 ± 1.1	2.31 (1.2–4)
Lobed nuclei (%)	12.61 ± 5.54	11.3 (6.6–22.5)	14.83 ± 7	15 (4.2–26.8)	14.22 ± 4.09	13.65 (10.1–19.8)	15.27 ± 6.14	15.5 (3.5–32.2)	13.58 ± 5.88	13.5 (6.7–23.5)	14.32 ± 3.84	13.4 (9.9–19.9)
Kidney-shaped nuclei (%)	0.06 ± 0.12	0 (0–0.3)	0.17 ± 0.18	0.1 (0–0.5)	0.1 ± 0.14	0 (0–0.3)	0.2 ± 0.14	0.2 (0–0.5)	0.11 ± 0.13	0.1 (0–0.3)	0.24 ± 0.17	0.2 (0–0.5)
Micronuclei (%)	0.06 ± 0.08	0.05 (0–0.2)	0.03 ± 0.06	0 (0–0.2)	0.01 ± 0.03	0 (0–0.1)	0.07 ± 0.07	0.1 (0–0.2)	0.05 ± 0.08	0 (0–0.2)	0.04 ± 0.07	0 (0–0.2)
Total ENA (%)	12.75 ± 5.66	11.5 (6.6–22.9)	15.04 ± 7.11	15 (4.2–27.3)	14.33 ± 4.13	13.8 (10.1–20.1)	15.54 ± 6.22	16 (3.7–32.7)	13.75 ± 5.99	13.7 (6.7–23.8)	14.61 ± 4	13.5 (9.9–20.4)
Lysozyme (μg/mL)[f]	13.1 ± 3.72	13.23 (7.7–18.72)	9.65 ± 6.45	7.61 (2.43–23.21)	12.43 ± 6	11.74 (4.29–23.05)	12.99 ± 8.26	11.84 (1.77–34.24)	7.81[h] ± 3.55	6.29 (3.98–12.98)	8.59[h] ± 3.35	7.74 (5.03–13.85)
Respiratory burst (OD)[g]	1.04 ± 1.38	0.15 (0.08–3.67)	0.63 ± 0.84	0.22 (0–2.36)	0.18 ± 0.13	0.17 (0–0.36)	0.38 ± 0.7	0.07 (0–2.4)	1.73[h] ± 1.6	1.19 (0.03–3.81)	0.57[h] ± 0.87	0.07 (0.06–2.08)

[a,b,c,d,e] Statistically significant differences: *P* values = [a] 0.00736, [b] 0.00244, [c] 0.00244, [d] 0.04338, [e] 0.0027.
[f,g] for these parameters, the numbers of examined samples were [f] 5, 8, 5 vs 11, 11, 4 and [g] 5, 8, 5 vs 11, 12, 4, respectively.
[h] Data between these subgroups could not be compared by Mann-Whitney *U* test as one sample was smaller than five.

parasitized, the sea turtles examined in this study showed a good body condition, were alert and responsive, and none of them presented signs of disease at clinical examination performed after they had been transferred to the rescue center. This is not an unexpected finding, as parasitic infections are often chronic and may not present obvious clinical signs, particularly in turtles that are well-nourished and maintained in optimal habitat conditions. Therefore, determining the health impact of parasitism can be difficult, as clinical signs are usually related to various factors, such as age, nutritional status, availability of intermediate hosts, parasitic load, concurrent diseases, hygiene conditions, environmental temperature, and stress. These factors can suppress the immune system defense mechanisms, causing clinical forms of parasitic infections (Diaz-Figueroa, 2005; Rataj et al., 2011). In particular, it has been shown that *H. mistroides*, *Neospirorchis*, and *S. sulcata* are capable of causing serious damages to the health status of loggerhead sea turtles, leading to great morbidity and being frequently implicated with cases of stranding and mortality in sea turtles worldwide (Chapman et al., 2019; Santoro et al., 2019).

Commonly, the diagnosis of parasitic infections in sea turtles is based on necropsy and histopathological findings in dead animals (Marchiori et al., 2017; Santoro et al., 2019) or coprological examination in live specimens (Marangi et al., 2020; Marchiori et al., 2020). In cases of Spirorchiidae infections, in vivo coprological diagnosis may not be sufficiently accurate for the proper management of sea turtles in rescue centers since fecal samples cannot always be quickly obtained and it is possible to assign eggs to a genus or several possible genera based on morphology. Furthermore, the frequency at which eggs are expelled through feces is unknown, and many eggs do not reach the intestinal contents, as they remain embolized in internal organs, leading to false-negative coprological results (Marchiori et al., 2020). For these reasons, an accurate diagnosis is important, based on coprological and molecular analysis of fecal and blood samples. In this study, coprological analysis (mini-FLOTAC® technique) and molecular analysis with sequencing were used for the detection and identification of helminths eggs in feces and circulating trematode DNA in blood samples, respectively. As already reported, the Mini-FLOTAC® can be considered one of the most sensitive methods for the coprological diagnosis of endoparasitic infections available today in veterinary medicine (Bosco et al., 2014) and has been successfully used in a previous investigation for the detection of gastrointestinal parasites in free-ranging loggerhead sea turtles (Marangi et al., 2020). In addition, molecular analysis can detect very low quantities ($<$10 pg in a blood volume of $<$400 μL) of free blood fluke DNA as reported according the manufacture's protocol (QIAamp DNA Blood kit). Therefore, the parasitological results of the present investigation can be considered accurate and reliable. However, an important consideration should be dedicated to the size of the parasitic burden, as the magnitude of changes in blood parameters may partly depend on the number of parasites to which the immune system is responding. It is possible that the presence of a small number of turtles with high parasitic burdens could skew the results. Although this could be a limitation of the study, it should be considered that is difficult to estimate in live turtles, given standard PCR is not quantitative and helminths egg numbers in feces do not necessary accurately reflect burden size.

To our knowledge, no results of a similar investigation on free ranging loggerhead sea turtles are currently available in the literature, including the only one green turtle.

The evaluation of blood parameters and the subsequent ability to predict health status are important tools for monitoring the health of sea turtles, at both individual and population levels (Campbell, 1998; Oliveira-Júnior et al., 2009; Whiting et al., 2007). Specifically, the assessment of DWBC can be used as an important indicator of the health status of turtles when suffering from any adverse condition and can be a useful tool to evaluate possible immune system alterations. Our results showed some statistically significant differences in DWBC between parasitized and non-parasitized loggerhead sea turtles. Various studies have established that the innate immune system of sea turtles is broad and robust, and heterophils and lymphocytes are its primary components (Pellizzon and Lunardi, 2000; Zimmerman et al., 2010). The average percentage frequencies of heterophils was higher in parasitized sea turtles, reaching statistically significant differences in the total population examined, in juveniles and in sub-adults. Heterophils are specialized cells and are the most numerous leukocytes in reptile blood. Cytochemical and ultrastructural studies suggest they are analogous to mammalian neutrophils and serve similar functions. They respond to similar microbial stimuli, exhibit chemotaxis, phagocytize pathogens, and produce cytokines and metabolites to maintain immune homeostasis. Heterophils mediate various inflammatory responses (Alleman et al., 1999; Christopher et al., 1999; Mateo et al., 1984; Montali, 1988; Stacy et al., 2011), ranging from acute inflammation and defense against pathogens to wound healing and tissue remodeling. As the "first responders" to tissue injuries and infections, heterophils rapidly migrate from the peripheral blood to the site of injury/infection and then, as "effector cells", initiate the acute inflammatory response. Since heterophils are the key phagocytes in the first line of immune defense and heterophilia primarily indicates an increased demand for phagocytosis, results of this study show that significant increases in the number of heterophils in loggerhead sea turtles are associated with parasitic infections. Additionally, heterophilia is frequently associated with other inflammatory conditions, including necrosis, tissue injuries, and bacterial infections (Mader, 2006). Elevated heterophil count can also arise from stress or exposure to toxic compounds. Other causes include excess exogenous or endogenous glucocorticoids, gravidity, neoplasia and, rarely, granulocytic leukemia (Rosskopf Jr and Woerperl, 1982; Stacy et al., 2011). In agreement with observation from other authors (Caliani et al., 2019; Casal et al., 2009), our results indicate that heterophils were the most abundant leukocyte class, followed by lymphocytes. Our results show that parasitized animals had significantly lower mean lymphocyte percentage frequencies than non-parasitized animals. While lymphocytosis typically occurs in inflammatory states, some studies have documented a similar decrease in lymphocyte counts in malnourished and debilitated loggerhead sea turtles linked to the presence of parasites (Marchiori et al., 2023).

The innate immune response, in addition to macrophages, heterophils, eosinophils, and natural killer cells, includes cells such as thrombocytes. All of these cells play a key role in the early stages of a pathogenic invasion, producing and releasing a wide range of inflammatory, antimicrobial, and immune-modulating proteins (Ferdous and Scott, 2023). Thrombocytes have phagocytic capabilities; if activated, they can phagocytose melanin, hemosiderin, erythrocytes, nucleoprotein debris, and bacteria (Stacy et al., 2011). Our results also showed a significantly lower mean TWBCR percentage value in the total parasitized sea turtle population compared to non-parasitized ones. This finding is unexpected and warrants further investigations. Reptilian thrombocytes, like those of other vertebrates such as birds, amphibians and fish, are similar in shape and function to the mammalian platelets. In most vertebrates circulation, thrombocytes, after erythrocytes, are the most abundant blood cells. Some authors reported that thrombocytes were the predominant cell type in adult sea turtles undergoing rehabilitation (Camacho et al., 2014; Casal et al., 2009). This work, in agreement with Caliani et al. (2019) shows that thrombocytes are not the most abundant class.

The H/L ratio seems to be conserved across all vertebrates (Cabagna et al., 2005; Chen et al., 2007; Davis and Maerz, 2008; Moreno et al., 2002; Pfaff et al., 2007; Witeska, 2005) and it is an alternative and rapid approach to evaluate stress responses (Caliani et al., 2019; Davis et al., 2008). In DWBCCs, higher average values of H/L ratio were detected in parasitized turtles, reaching statistically significant differences in the total population examined, in juveniles and in sub-adults. In general, an elevated H/L ratio can be detected in the DWBCC in all cases where an increase in the number of heterophils is induced, like in this case. Therefore, this may be related to bacterial infections but may also be observed in other cases of increased phagocytosis such as in wound healing or tissue damage caused by toxic, metabolic, neoplastic and

infectious agents other than bacteria, as mentioned above (Mader, 2006; Rosskopf Jr and Woerperl, 1982; Stacy et al., 2011). Our results showed that an elevated H/L ratio could also be linked to parasitic infections. Parasites can affect the host's immune response in various ways, leading to immunosuppression and inhibiting the processes that facilitate the destruction of the parasites through humoral and cell-mediated immune response in mammals (Ferreira et al., 1995; Midttun et al., 2018; Molinari et al., 1998). Likewise, since different trematodes and nematodes parasites tend to establish a chronic infection in the host, it is possible that similar effects could also occur in parasitized loggerhead sea turtles, causing a particular impact on the WBCs profile of turtles.

Overall, younger turtles appeared more susceptible to changes in DWBCC compared to adults. Indeed, we observed statistically significant differences in the average percentage values of heterophils, lymphocytes and mean values of H/L ratio between parasitized and non-parasitized juveniles and/or sub-adults. These hematological effects of parasitic infections, as revealed by significant differences in DWBCC, may be reduced once the sub-adult life stage has been concluded and after the turtle has been exposed continuously for long periods (Barton, 1997). Thus, certain hematological effects of parasites may be attenuated in adult sea turtles once they have progressively adapted to the presence of parasites. Similar findings were previously documented in studies exploring tolerance to parasitic infections in wild rodents (Jackson et al., 2014), the correlation of immune response with fitness-associated factors in male black grouses (Soulsbury et al., 2018), and the effects of ectoparasite load in free-ranging Bahamian rock iguanas (Knapp et al., 2018). The results of these studies suggest that animal hosts can develop tolerance to parasites (Jackson et al., 2014) by triggering an immune response differently depending on classes of age (Soulsbury et al., 2018), and also that across life stages impacts of parasitism can be modulated by host size, sex, and co-infections (Knapp et al., 2018), as reviewed by Råberg (2014).

In the present investigation, we assessed the presence of nuclear abnormalities to explore potential impacts of parasitic infections on DNA integrity. The detection of micronuclei in the cytoplasm of erythrocytes is one of the most sensitive and widely method used for assessing DNA damage and chromosomal instability (Casini et al., 2018). Since any morphological changes in the nuclear membrane are considered a marker of mutagenic damage (Carrasco et al., 1990; Çavaş and Ergene-Gözükara, 2005; Guevara-Meléndez et al., 2023), and are considered as precursors for micronuclei formation (Carrasco et al., 1990; Fenech and Crott, 2002; Shimizu et al., 1998), the ENA assay in the peripheral blood can be considered as a marker of exposure to genotoxic agents and genomic damage. Since we did not observe significant differences in mean values of ENA frequency, no evidence supports a possible relationship between the presence of parasites and genotoxic effects. However, the mean percentage frequency of kidney-shaped nuclei was significantly lower in the total parasitized turtle population. Therefore, a possible genotoxic impact of helminths infections in sea turtles cannot be completely overlooked.

Respiratory burst is an essential component of innate immunity of sea turtles, enabling phagocytic cells to effectively eliminate pathogens (Caliani et al., 2019). Although not statistically significant, higher levels of respiratory burst activity observed in parasitized animals suggest that, in the presence of parasites, the organism may implemented certain immunity defense mechanisms.

Lysozyme is an enzyme that, along with the complement system and antimicrobial peptides, plays a primary role in the innate immune defense of sea turtles (Zhang et al., 2011). Upon release from phagocytes into the plasma, lysozyme primarily catalyzes the hydrolysis of the bacterial cell walls (Caliani et al., 2019). In our study, lysozyme activity did not exhibit significant variations between parasitized and non-parasitized animals. Other authors found a decrease in lysozyme activity in turtles exposed to plastic pollution (Adamovicz et al., 2020), heavy metals (Work et al., 2000), fluctuating temperature (Work et al., 2015), and organochlorine contaminants (Walsh et al., 2010). To date, no information is available on lysozyme levels in parasitized turtles, but our results could support the hypothesis that plasma lysozyme can be used as an innate immune response marker and give more information on turtles' health status.

These results suggest that the presence of parasitic infections by Spirorchiidae and *S. sulcata* does not play an important role in genotoxic damage, lysozyme and respiratory burst responses in loggerhead sea turtles. The presence of these parasites does not necessarily imply an overt form of disease; other causes, such as the presence of marine contamination, could also affect the animals' immune system, weakening it and making the animals more vulnerable to parasite infection.

5. Conclusion

This study aimed to investigate the physiological responses to parasitic infections and the effects of parasitism, furthering our understanding of the relationship between parasitic infections and biomarkers in rescued free-ranging sea turtles during rehabilitation. To the best of our knowledge, this is the first study on hematological, genotoxic responses and innate immune responses in sea turtles affected by parasitic infections in the Mediterranean Sea. Two parasite species (*H. mistroides* and *S. sulcata*) and one genus (*Neospirorchis* sp.) of helminths were identified by the combination of coprological and molecular findings in loggerhead sea turtles. The only green sea turtle examined was negative. Parasitized juvenile and sub-adult loggerhead sea turtles showed significantly higher values of heterophils and H/L ratio. Slightly higher lysozyme and respiratory burst values were measured in parasitized animals. Moreover, no relationship between the presence of parasites and genotoxic effects was found. We can assume that the parasite load could induce inflammation in the animals by a possible mechanism of regulation and downplaying the immune response. However, other factors should be taken into account for immune system alterations. Although our work present some limitations mainly due to not chance to calculating the size of the parasitic burden (the PCR is not quantitative and it is difficult to estimate in live sea turtles), this study suggests the importance of knowing possible immune system alterations in free-ranging sea turtles for the assessment of health status in infections with important pathogens such as blood flukes and gastrointestinal parasites. This is important because sea turtles are examined clinically and extensively through laboratory testing during their captivity in rescue centers. Therefore, these parameters can be successfully used to monitor the health status of parasitized loggerhead sea turtles and should be checked as part of the necessary examinations in free-ranging sea turtles rescued within the Mediterranean area. In conclusion, although some findings were unexpected and the significance of some findings remains unclear, this study suggests that the presence or absence of parasites, as well as other pathogens, should be taken into consideration when performing biomarker investigations in sea turtles since major pathogens, such as parasites, can influence differences in the values of some parameters between infected and uninfected turtles.

Consent for publication

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CRediT authorship contribution statement

Marianna Marangi: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Roberto Amerigo Papini:** Writing – review & editing, Writing – original draft,

Formal analysis. **Giovanni Furii:** Formal analysis. **Lorenzo Gordigiani:** Writing – review & editing, Methodology. **Agata Di Noi:** Writing – review & editing. **Maria Cristina Fossi:** Visualization, Validation. **Silvia Casini:** Writing – review & editing, Visualization, Validation. **Ilaria Caliani:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no conflict of interest and that they have no actual or potential competing financial interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marpolbul.2024.117274.

Data availability

No data was used for the research described in the article.

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