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**PARASITIC INFECTIONS IN SEA TURTLES AND CETACEANS IN MEDITERRANEAN
SEA WATERS, WITH A FOCUS ON THE ADRIATIC SEA**

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Abstract

Parasitic infections contribute to natural mortality in population of free ranging sea turtles and cetaceans worldwide. Notwithstanding all host-dependant factors influencing the outcomes of parasitic infections, some parasites, spanning from protozoan to helminthic taxa, have a high pathogenic potential and can cause fatal disease in these animals. Sea turtles blood flukes (Digenea: Spirorchiidae) are a main factor in mortality of populations of green and loggerhead turtles in Atlantic and Pacific Ocean. In order to explore presence and pathology of spirorchiidiasis in the Mediterranean area, data from 168 loggerhead turtles (*Caretta caretta*) stranded dead along North Western Adriatic coast were collected during a six-years period. Parasitological study and pathological findings revealed the presence of eggs and adult flukes of *Hapalotrema mistroides* and *Neospororchis* – Neogen 11, with prevalence of 15.5% and 6.0% respectively. Mild lesions were observed, including multifocal granulomatous enteritis and chronic focal vasculitis of the major vessels. Egg emboli were found widespread in the tissues, mostly attributable to *H. mistroides*. Sequencing of rDNA markers (28S and ITS-2) and phylogenetic analyses revealed the identity of the Mediterranean isolates with Floridian specimens. Genetic analysis on the hosts led to the conclusion that the life cycles of both genera can be entirely supported by the Mediterranean ecosystem. To estimate the infection intensity, a new rapid method was set up. Correlation between spleen egg burden and fecal egg output was studied by means of statistical tests applied to splenic tissue and fecal material, revealing the absence of correlation and the unreliability of fecal burden to assess severity of the disease *in vivo*. Gastrointestinal helminth community of loggerheads was also studied, revealing a species diversity typical of demersal stage turtles and similar to that observed in other neritic areas of the Mediterranean.

As for cetaceans, crassicaudosis is considered one of most severe helminthic infections in these animals. Nevertheless, knowledge on the epidemiology of *Crassicauda* species is scarce, due to constraints of sampling free-ranging wild host species and to the difficulty of obtaining entire specimens suitable for morphologic studies. Presence and pathology of *Crassicauda boopis* were investigated in fin whales (*Balaenoptera physalus*) stranded along Italian coasts. Severe lesions linked to the presence of adult nematodes in circulatory system and kidneys were detected in five rorquals (5/7), associated with renal function impairment and arteritis of the mesenteric vessels linked to spirurid larvae migrants. Morphological studies enabled the identification of the adult nematodes as *C. boopis*. Sequencing of molecular barcode regions was performed on adult and larval nematodes from the whales; additionally, specimens of *Crassicauda* spp. isolated from toothed whales were morphologically and molecularly studied. A multigene analysis made on the barcode regions of rDNA and on the mtDNA revealed the ITS-2 spacer to be the most efficient

marker in species differentiation inside the genus *Crassicauda*. This analysis opened new insights on the identity of the isolated *larvae migrans* and on the life cycles of these poorly known nematodes. Furthermore, during the parasitological examination, immature elements of the genus *Pennella* were recovered from one fin whale and described from a morphological point of view for the first time. Preliminary molecular data were obtained, with the aim of clarifying the taxonomy of the genus *Pennella*. The coccidian *Toxoplasma gondii* was detected in the tissues of one rorqual as well, likely associated with a chronic infection.

In conclusion, these parasitological surveys highlighted the importance of a continue monitoring of the health of these endangered species avoiding underestimating the potential impact of parasitic diseases in natural mortality.

Background

The Mediterranean Sea, including the Adriatic basin, are the home of several species of cetaceans and sea turtles. Due to the migratory nature of these animals, most of the populations within the Mediterranean cannot be considered completely isolated from the adjacent Atlantic populations; nevertheless, genetic tools have demonstrated that Mediterranean populations of several species of cetaceans and sea turtles actually diverged in different ages from the Atlantic ones (Berubé et al., 1998; Gaspari et al., 2007).

Loggerheads (*Caretta caretta*) and green turtles (*Chelonia mydas*) regularly nest along Mediterranean coasts, the second species being much more abundant in the Eastern region. Loggerheads are instead regularly present in all the basin, and are found in the Adriatic Sea all over the year, with seasonal variations in their abundance and distribution (Margaritoulis et al., 2003; Casale et al., 2004; Lazar et al., 2004; Casale and Margaritoulis, 2010; Casale and Simone, 2017). The opportunistic nature of this species and the richness and variety of preys inside this arm of the Mediterranean Sea make it an important foraging area for loggerheads at the neritic stage. The deeper waters of the Ionian Sea and Balearic Sea provide a suitable habitat for smaller turtles at the pelagic stage. Rookeries from Greece and Crete, with Cyprus and Turkey, provide the most important contribution to the Adriatic stock; other important nesting sites for the Mediterranean population are included in the North African coasts and Southern Italy, nevertheless, the large-scale migratory habits of these reptiles enables individuals originating in the Western Atlantic coasts to reach the Mediterranean waters through the Gibraltar strait. Their presence has been occasionally registered also in the Adriatic Sea (Clusa et al., 2014).

Common bottlenose dolphins (*Tursiops truncatus*, Montagu, 1821), striped dolphins (*Stenella ceruleoalba*, Meyen, 1833), Risso's dolphins (*Grampus griseus* [Cuvier, 1812]) and Cuvier's beaked whales (*Ziphius cavirostris*, Cuvier, 1823) are regularly present in the Adriatic Sea. With the exception of the bottlenose dolphin, which is adapted to live also on the continental shelf of the Northern region of the Adriatic, all other species of odontocetes prefer the deep waters of the Southernmost sub-region and are most frequently sighted in other areas of the Mediterranean, such as in the Pélagos Sanctuary and in Western Mediterranean until the Alboran Sea. The fin whale (*Balaenoptera physalus*), the only mysticete regularly present in the Mediterranean, is considered also a seasonally regular species in the central Southern Adriatic. Recent surveys, carried out in the framework of the European Project “Network for the conservation of Cetaceans and Sea Turtles” (NETCET), confirmed the presence of fin whale specimens in the central Adriatic, likely

related to the presence of masses of krill in a limited part of the year (Fortuna et al., 2015). In the Mediterranean, it is more abundant in highly productive pelagic regions, such as the Ligurian-Corsical-Provençal Basin, where high whale densities, comparable to those of rich oceanic habitats, are described (Notarbartolo di Sciara et al. 2007). Relying on genetic analyses, the Mediterranean fin whales have been defined as a sub-population, the animals being mainly resident inside the basin. Total estimated abundance of this species in the Mediterranean reaches no more than 5,000 individuals (Fortuna et al., 2015).

Human-induced mortality is a well-known threat to the conservation of sea turtles and cetaceans worldwide; bycatch, ship strikes and pollution by both chemicals and marine debris are considered causes of major concern, and mitigation strategies are under researchers' attention to ensure conservation of marine species. Environmental stressors, here including immunotoxicity of chemical substances, direct and indirect fisheries interactions, traumatic injuries from vessel collisions and ingestion of plastic debris are considered to enhance the susceptibility of the animals to infectious diseases (Aguilar and Borrell, 1994; Aguirre et al., 1995; Jepson et al., 2005, Van Bresseem et al., 2009), and animals living inshore seem more at risk than the pelagic species (Herbst, 1994; Van Bresseem et al., 2009).

Acquisition of parasitic infections, their severity and outcome are known to be strongly connected on innate and acquired host immunity, both humoral and cell mediated. Among others, clinical disease by *Toxoplasma gondii* is surely associated to decreased immunity in mammals, with severity varying among host species and genotype involved (Hill et al., 2005). In marine mammals, acute clinical manifestations of toxoplasmosis have been associated to compromised immune system (Domingo et al., 1992), by both natural and anthropogenic factors (Mazzariol et al., 2012). Increased burden of ectoparasitic copepods of the genus *Pennella* has been considered as an easily visible indicator of general health status in odontocetes (Vecchione and Aznar, 2014). Besides, high burdens of leeches and barnacles are easily associated with impaired movement and diving activity in unhealthy sea turtles, and are often observed in animals injured by ship strikes or carrying gastrointestinal foreign bodies such as hooks and lines, coming from past sub-lethal contacts with human activities (Stamper et al., 2005; Flint et al., 2009).

The aim of this project was to focus on parasitic infections compromising sea turtles and cetaceans health, addressing the animals spanning over the Adriatic and Mediterranean Sea. Some of the most pathogenic parasites are investigated, trying to determine their impact on general health status of the hosts. Due to the limitations in sampling these wild, marine animals and their status of endangered, protected species, data on their parasitic fauna are still unavoidably constrained, if

compared with data available on domestic terrestrial species. Considering parasites as a precious part of the biodiversity as well as biological markers for studying the ecology of host populations (i.e., movements, stock composition), the project was also aimed at enhancing our knowledge on the ecology and biology of these parasites.

SECTION I – SEA TURTLES

1. Blood flukes (Spirorchiidae, Stunkard 1921)

Etiology

Parasites of the family Spirorchiidae Stunkard, 1921 are responsible for cardiovascular infection in both freshwater and marine turtles globally (Platt, 2002). Marine turtles' ancestors are considered to come from a freshwater turtle (Shaffer et al., 1997), and morphological and molecular studies revealed that marine spirorchiids derive from freshwater parasites as well, as a result of co-evolution or host-capture phenomenon (Snyder, 2004). The relationship between the families Spirorchiidae and Sanguinicolidae, included in the superfamily Schistosomatoidea, is still to be completely clarified, and, though considered for a long time as sister groups, most recent studies revealed the former to be basal to the second one. The successful colonisation of a marine bird by a marine turtle blood fluke hypothetically represented the starting point for the evolution of avian and mammalian schistosomatids (Snyder, 2004).

Revisions and re-description of species of marine spirorchiids, brought to the recognition of six genera, namely *Amphiorchis* Price, 1934, *Hapalotrema* Looss, 1899, *Learedius* Price, 1934, *Carettacola* Manter and Larson 1950, *Neospirorchis* Price 1934 and *Monticellius* 1939. Nevertheless, questions upon the validity of the genus *Learedius* have been raised by the most recent phylogenetic studies, as well the existence of at least two different genera inside the taxon *Neospirorchis* (Chapman et al., 2015; Stacy et al., 2017). Cryptic nature of *Neospirorchis* sp. flukes make it very difficult to collect specimens suitable for morphological studies; thus molecular tools revealed to be helpful, where not necessary, to clear these taxonomic issues (Stacy et al., 2010a and 2017; Chapman et al., 2016).

Extensive literature is available for freshwater spirorchiid life cycles, all of which involve pulmonate gastropods (heterobranch) as intermediate hosts (Wall, 1941; Pieper, 1953; Goodchild and Kirk 1960; Hollimann and Fisher, 1968). On the counterpart, only two intermediate hosts have been so far identified for marine species, including a fissurellid limpet, *Fissurella nodosa* (Vetigastropoda) for *Learedius learedi* and the vermetid *Thylaedus rugulosus* for *Amphiorchis* sp. (Caenogastropoda) (Stacy et al., 2010b, Cribb et al., 2017). The existence of such deeply phylogenetically distant intermediate hosts between marine spirorchiids makes the range of possible intermediate hosts very wide, and thus challenging to investigate for all spirorchiid species (Stacy et al., 2010b; Cribb et al., 2017). Nevertheless, knowledge on life cycles of these parasites is essential to epidemiological studies. For example, the higher proximity between the intermediate and definitive hosts in neritic areas is evoked as the reason for the higher prevalence

of the infection in turtles at neritic life stage and for the absence of these parasites in oceanic juveniles (Aguirre et al., 1998; Work et al., 2005; Orós et al., 2005 and 2016; Flint et al., 2010). Similarly, the increase of severity of spirorchiidiasis during the warm season in Australia has been suggested to be related to the seasonal increase in the availability of the intermediate hosts, as long as with higher cercarial emergence from them (Flint et al., 2010).

Pathology and epidemiology

Spirorchiidiasis in marine turtles is reported globally in tropical and subtropical areas, with most of the studies developed in the Western Atlantic (Florida - Wolke et al., 1982; Jacobson et al., 2006; Stacy et al., 2010a; Caribbean Islands - Greiner et al., 1980; Dyer et al., 1995; Santoro et al., 2007a), and in the Pacific Ocean off Australian coasts (Glazebrook et al., 1989; Gordon et al., 1998; Flint et al., 2010; Flint et al., 2015; Chapman et al., 2016; Chapman et al., 2017) and Hawaii (Dailey et al., 1992; Dailey and Morris, 1995; Graczyk et al., 1995; Aguirre et al., 1998; Work et al., 2005). High prevalence is registered in these areas – up to 79% in Queensland (Flint et al., 2010) and seroprevalence of 100% in green turtles from Hawaii (Graczyk et al., 1995), with spirorchiidiasis representing one of primary causes of concern in sea turtles' health.

Pathological effects induced by spirorchiids infection arise both from the localization of adult flukes in the blood vessels and from the embolization of their eggs in the tissues. The existence of a microhabitat selectivity is relevant to potential pathogenic effects of the different species and *Neosporichis* genotypes (Stacy et al., 2010a). A univocal identification of the infecting species would be necessary to study this specialization and molecular tools have been recently developed for this aim (Chapman et al., 2016 and 2017; Stacy et al., 2017). Host species also accounts for different pathogenic effects and frequency of occurrence of parasitic species (Stacy et al., 2010a and 2017), though most of spirorchiids have demonstrated to be not selective, infecting more than one host species. The available studies mostly focus on loggerheads (*Caretta caretta*) and green turtles (*Chelonia mydas*), but all sea turtle species, with the exception of the leatherback (*Dermochelys coriacea*), have been reported to be positive or possibly susceptible to spirorchiidiasis (*Eretmochelys imbricata* - Glazebrook et al., 1989; Dyer et al., 1995; Werneck et al., 2008; *Lepidochelys olivacea* - Santoro et al., 2007b). Differences in the diet of these species and their pelagic *versus* neritic habits probably account for different susceptibility of the hosts.

Granulomas associated with spirorchiid eggs have been reported in almost all body districts; the detection of such lesions by histopathology permits the diagnosis in the *post-mortem*, when adult flukes escape gross and histological detection. Severity of eggs-associated lesions varies from microscopic accumulation of small groups of elements to grossly visible, miliary granulomas

surrounding masses of eggs in the tissues, up to several millimetres in size. A layer of multinucleate giant cells is typically observed walling off the eggs in the granuloma; a fibrous layer, eosinophils and lymphocytes occasionally surround the reaction peripherally (Gordon et al., 1998; Flint et al., 2010; Stacy et al., 2010a). Vascular and perivascular deposition of eggs disrupt microcirculation and lead to thrombotic lesions, perivascular edema, hypertrophy of arterial muscular layer and infarcts (Flint et al., 2009; Flint et al., 2010). Arteritis and papillary proliferations inside the lumen of vessels and mural endocarditis are associated to chronic adult flukes adhesion to blood vessels' wall (Aguirre et al., 1998; Gordon et al., 1998; Stacy et al., 2010a). Secondary entry of Gram negative bacteria from intestinal lesions, bacterial infections in the central nervous system and septic thrombi adherent to arterial lesions can be responsible for systemic illness and death of the infected turtles (Wolke et al., 1982; Gordon et al., 1998; Raidal et al., 1998; Jacobson et al., 2006; Stacy et al., 2010a). Anemia, cachexia and listlessness to lethargia are clinically visible in critically ill animals (Gordon et al., 1998; Santoro et al., 2017); diminished reflexes up to paresis are also reported in neurospirorchiidiasis (Jacobson et al., 2006).

Grading disease severity is essential for assessing whether spirorchiids actually represent a primary, contributory or irrelevant element in the turtle death. Work et al. (2005), in the frame of an epidemiological study in the Hawaii, rated the intensity of infection by quantifying splenic egg burden by chemical digestion of portions of the organ, followed by count of the eggs trapped in the tissue. Stacy et al. (2010a) proposed an impact rating (from 1 to 5) depending on the severity of lesions, which included the proportion of organs involved, the distribution (focal to multisystem embolization) and eventually presence of secondary bacterial infections in the animals. Flint et al. (2010) gave a severity score based on histopathology, by counting the number of granulomas per high power field in the different organs, combining this number with that of grossly visible granulomas in the intestinal serosa and heart. Co-existence of other natural diseases such as fibropapillomatosis (FP) and infection by the systemic coccidian parasite *Caryospora cheloniae* enhance mortality in sea turtles' populations, in which they are geographically overlapped to spirorchiidiasis (Work et al., 2005; Flint et al., 2009; Chapman et al., 2017), and complicate the identification of the primary cause of death. Notwithstanding the difficulty in ranking spirorchiidiasis severity and defining their role in the single cases of death, spirorchiids are still considered as primary pathogens in free ranging sea turtles.

Spirorchiidiasis in the Mediterranean Sea

Spirorchiidiasis has been neglected for a long time in sea turtles' population of the Mediterranean Sea, as only historical, punctual reports existed until recently (Monticelli, 1986; Looss, 1899;

Gohar, 1934 and 1935). No other mention on its presence inside the Mediterranean basin was published until a case of fatal infection, sustained by the species *Hapalotrema mistroides*, was published recently (Santoro et al., 2017). The origin of the infected turtle was not clarified in that study, and since co-existence of loggerheads from Mediterranean and Western Atlantic rookeries is documented in the central Mediterranean (Clusa et al. 2014; Shamblin et al., 2014), the suspect still remained that the turtle could have acquired the infection in Atlantic waters. Comparative genetic analyses on ITS-2 and 28S fragments also revealed the identity of *H. mistroides* from this Italian study with the Floridian isolate (Santoro et al., 2017). Cribb et al. (2017) similarly isolated *Amphiorchis* sp. from captive reared loggerheads in the Oceanographic Aquarium in Valencia. The source of infection was identified in a turtle rescued from regional waters, in which the presence of turtles entering the Gibraltar strait from the Ocean is still more probable (Cribb et al., 2017). Studies on Central-Eastern Mediterranean have not reported the presence of spirorchiids in loggerheads (Manfredi et al., 1998; Santoro et al., 2010a; Gračan et al., 2012) and systematic studies on green turtles parasitofauna in the Mediterranean still lack.

2. Gastrointestinal helminth community of *Caretta caretta*

Several studies on gastrointestinal helminth fauna of loggerheads have been conducted in the Mediterranean (Aznar et al., 1998; Manfredi et al., 1998; Piccolo and Manfredi, 2001; Santoro et al., 2010a,b; Gračan et al., 2012). Different geographical areas have been sampled, including waters of the central and Western Mediterranean Sea (Aznar et al., 1998; Santoro et al., 2010a,b) and the Adriatic Sea (Manfredi et al., 1998; Piccolo and Manfredi, 2001; Gračan et al., 2012).

Comparing these studies with those conducted in Western Atlantic (Greiner et al., 2013), the parasitic fauna of *C. caretta* in the Mediterranean is similar in composition, with digenetic trematodes and nematodes accounting for the majority of helminth diversity (George, 1997; Aznar et al. 1998). The passage of juvenile turtles from Atlantic Ocean through Gibraltar strait to the Mediterranean Sea has been evoked as responsible for the partial similarity in community composition (Valente et al. 2008); phylogeographic studies on the epibiont *Chelonibia testudinaria* have already demonstrated the importance of sea turtles migration from Western Atlantic to Mediterranean Sea for the expansion of passively transported organisms in the latter basin through the host movement (Rawson et al., 2003). Moreover, molecular studies on the gastrointestinal nematode *Sulcascaris sulcata* from *C. caretta* from these two different basins evidenced the existence of a single panmictic population between the two areas (Santoro and Mattiucci, 2009). Nevertheless, a higher diversity and intensity of infection is found in Florida turtles (Greiner et al., 2013). The isolation of Mediterranean *C. caretta* from other turtle species, which share most of parasitic species, partly accounts for this lower richness (Aznar et al., 1998). The gastrointestinal helminths communities of loggerheads are anyway considered as depauperate and predictable, due to phylogenetic barriers with other marine organisms (Aznar et al., 1998). The high physiological diversity from the other marine vertebrates prevents turtles from sharing parasitic species, with the exception of occasional and accidental infections by immature or larval forms of fishes and cetaceans' parasites (Piccolo and Manfredi, 2001; Santoro et al., 2010a; Gračan et al., 2012; Greiner, 2013). *Anisakis* spp. in particular is encountered in loggerheads with relatively high frequency in the Northern Adriatic, in which it is the dominant species with prevalence 34.3% (Gračan et al. 2012), and has been reported in sea turtles around the world, including other host species - *Chelonia mydas* in Australia (Glazebrook and Campbell, 1990), *Lepidochelys kempii* in Western Atlantic (Innis et al., 2009). *Anisakis* spp. has also been recovered in *C. caretta* in other Mediterranean regions (Piccolo and Manfredi, 2001; Badillo, 2007; Orós et al., 2005; Santoro et al., 2010b;) and in Eastern Atlantic Ocean (Valente et al., 2008). Higher prevalence has been associated to great availability of *Anisakis*' definitive hosts, i.e. marine

mammals, both in the waters off Madeira Archipelago (Valente et al., 2008) and in the Adriatic Sea (Gračan et al., 2012). Sea turtles act in the life cycle of *Anisakis* spp. as paratenic or accidental hosts, harbouring the third stage larvae and probably representing a dead end host (Santoro et al., 2010b). Pathogenic effects of *Anisakis* spp. in sea turtles are due to their migration through the gastric walls and visceral tissues, which cause mucosal ulceration and granulomatous reaction (Orós et al. 2005; Innis et al., 2009; Santoro et al., 2010b). Molecular analyses on nematode larvae, essential for species identification, could be used as geographical markers in the study of turtles migrations, as they have already proved to be useful to reconstruct the origin and migratory routes of their definitive and intermediate hosts (Mattiucci et al., 2007).

The importance of the ontogeny of loggerheads in helminth community structure has been proven to account for communities dissimilarities among different geographical areas inside the Mediterranean (Santoro et al., 2010a). Juveniles, feeding in pelagic preys over oceanic habitats harbour a lower number of parasitic species than bigger juveniles, subadults and adults which feed in neritic grounds on benthic preys (Santoro et al., 2010a, Gračan et al., 2012). Lower overall prevalence and intensity of infection are found in the smaller turtles, and have been related to the dilution effect of oceanic conditions – i.e. dispersion of intermediate hosts - (Santoro et al., 2010a) and to the oligotrophic conditions of this ecosystem when compared to the neritic grounds (Valente et al., 2008). The digeneans *Enodiotrema megachondrus* (Looss, 1901) (Digenea: Plagiorchiidae) and *Calycodes anthos* (Braun, 1899) (Digenea: Calycofidae) are typically encountered in smaller, pelagic turtles and in those undergoing the ontogenic shift (Valente et al., 2009; Santoro et al., 2010a, Gračan et al., 2012), thus mostly in Balearic waters (Aznar et al., 1998) and Ionian waters off Calabria (Santoro et al., 2010a), as well as in juvenile turtles undergoing habitat shift in the North Eastern Adriatic (Gračan et al., 2012).

The largest turtles host the higher diversity of nematodes and trematodes. Higher prevalence for gastrointestinal helminthiasis is registered in neritic areas in the Mediterranean Sea and digenean trematodes are the most important in terms of species richness of gastrointestinal communities (Santoro et al., 2010a; Gračan et al., 2012). Molluscan intermediate hosts are supposed to be involved in their life cycles, as typical for flukes (Esch et al., 2001). The broader diet of turtles foraging in neritic areas, where these intermediate hosts are supposed to be more abundant, accounts then for this broader diversity, together with the life history of the host (Gračan et al., 2012). Nevertheless, specific intermediate hosts have been identified only for the nematode *S. sulcata* (Nematoda: Anisakidae). Its life cycle is modulated between benthic gastropods and bivalves (Berry and Cannon, 1981). The higher prevalence of *S. sulcata* in larger turtles feeding

in inshore waters is thus explained, since these sessile and slow moving intermediate hosts are abundant in shallow waters and are thus included in the diet of the turtles feeding on benthic organisms. The presence of *S. sulcata* is typical in loggerheads from the neritic grounds such as the Adriatic (Manfredi et al., 1998; Piccolo and Manfredi, 2001; Scaravelli et al., 2005; Gračan et al., 2012), the African shelf (Sey, 1977) or coastal waters off Campania Region (Santoro et al., 2010a). *S. sulcata* lives in the stomach or pyloric region of the host, and attaches to the gastric mucosa, thus showing a potential to cause gastritis and ulcers (Greiner, 2013; Mettee, 2014).

Helminthic infections of the gut have rarely been associated to pathogenic effects on their hosts, with the exception of Anisakidae larvae. Helminth populations in loggerheads from the Eastern Adriatic showed a typical aggregate distribution, mostly causing light infections (Gračan et al. 2012). The dynamics of parasite-host relationship anyway deserve continue attention, as they act as indicators of immunocompetence and health of the host. Moreover, helminth communities of marine turtles themselves can act as indicators of the health of marine ecosystem, since the impairment of the transmission of such organisms, which is mediated by multiple hosts, could reflect perturbations of the food web structure and biodiversity (Marcogliese, 2005).

3. Aims of the project and outputs

Presence and pathology of spirorchiidiasis in the Central-Eastern Mediterranean Sea have been investigated, collecting data from loggerhead turtles (*C. caretta*) stranded in North Western Adriatic coast from 2009 to 2015. Identification of the species present, their morphological and molecular characterization, prevalence, pathology associated to both adult and eggs presence have been studied. In order to verify whether the life cycle of the spirorchiids implicated could be entirely supported by the Mediterranean ecosystem, the origin of the infected turtles was investigated through use of mitochondrial DNA analyses. Identification of the natal rookery and literature on migratory routes of Mediterranean turtles permitted to trace the origin of the infection to Mediterranean foraging grounds.

The results of these studies were collected in the following papers and scientific communications in national and international conferences:

- Marchiori E., Negrisolo E., Cassini R., Garofalo L., Poppi L., Tessarin C., Marcer F., 2017. Cardiovascular flukes (Trematoda: Spirorchiidae) in *Caretta caretta* from the Mediterranean Sea. *Parasites and Vectors*, 10:467 – PAPER 1
- Tolve L., Casale P., Formia A., Garofalo L., Lazar B., Natali C., Novelletto A., Vallini C., Bužan E., Chelazzi G., Gaspari S., Fortuna C., Kocijan I., Marchiori E., Novarini N., Poppi L., Salvemini P., Ciofi C., 2018. A comprehensive mitochondrial DNA mixed-stock analysis clarifies the composition of loggerhead turtle aggregates in the Adriatic Sea, *Marine Biology*, 165:68 – PAPER 2
- Marchiori E., Bertuzzo F., Garofalo L., Negrisolo E., Poppi L., Tessarin C., Marcer F., 2016. First report of genus *Neosporichis* in sea turtles from Mediterranean Sea. *XXIX Congress SoIPa, Bari, 21-24 June. Atti, p. 40*.
Presentazione orale - Scientific communication 1
- Marchiori E., Poppi L., Centelleghes C., Menandro M. L., Pasotto D., Novarini N., Palmisano G., Marcer F., 2017. Pathological findings in sea turtles stranded along the Northwestern coast of the Adriatic Sea. *37th Annual Symposium on Sea Turtle*

Biology and Conservation, Las Vegas NV (USA), 15-20 April. Presentazione orale
- Scientific communication 2

- Marcer F., Negrisolo E., Poppi L., Tessarin C., Garofalo L., Centelleghes C., Marchiori E., 2017. Prevalence and host pathology of spirorchidiasis in stranded loggerheads (*Caretta caretta*) in North Western Adriatic Sea. *37th Annual Symposium on Sea Turtle Biology and Conservation, Las Vegas NV (USA), 15-20 April. Poster - Scientific communication 3*

Diffusion and pathology of spirorchidiasis in the different sub-regions of the Mediterranean Sea have not been clarified yet, and risk factors for the development and the severity of infection are as well ignored. An objective tool for the evaluation of the intensity of infection is essential to compare data from different geographical areas. A new technique has been set up, starting from the study by Work et al. (2005), simplifying the process for the quantification of tissue egg burden and therefore reducing costs and time of implementation.

Serology and copromicroscopic exams are, as far, the only diagnostic methods applicable *in vivo* for spirorchidiasis. We investigated the correlation between positivity at copromicroscopic exam and presence of spirorchids in the tissues, selecting the spleen as target organ for the comparison (Flint et al. 2009), in order to assess the reliability of the fecal exam to reveal infection by *Haplotrema mistroides* and *Neosporchis* sp. Moreover, we compared egg counts from feces and splenic tissue to evaluate whether a correlation exists between fecal egg burden and intensity of infection.

The results of these studies are collected in the following paper and scientific communication in national conference:

- Marchiori E., Cassini R., Ricci I., Marcer F., 2018. Qualitative and quantitative methods for estimating Spirorchidiasis burden in sea turtles. *International Journal for Parasitology: Parasites and Wildlife*, 7(3):409-414 – PAPER 3
- Marchiori E., Cassini R., Ricci I., Nardo M., Marcer F., 2018. Comparison between two quantitative methods and two different matrixes for the evaluation of parasitic burden in *Haplotrema mistroides* infection. *XXX Congresso SoIPA, Milano 23-26 Giugno. Poster - Scientific communication 5*

The study of the interactions between host and parasites is a useful tool for the assessment of the general health of the host; large number of parasites in the digestive system can indeed impact on the absorption of nutrients affecting the health, growth and reproductive outputs of the host. The gastrointestinal helminth community of loggerheads stranded along Western Adriatic coast has been studied on a wide sample of animals (n=113) to study host-parasites interactions and evaluate the helminth community diversity . The indexes of prevalence, intensity and abundance have been thus estimated for each parasitic species, and a comparison has been carried out with other surveys from the Mediterranean area.

Sulcascaris sulcata is one of most abundant species in Adriatic turtles' gut, and furthermore it is the species with the higher pathogenic potential, due to the attachment of fourth and fifth stage larvae to the gastric mucosa. Its life cycle has been elucidated and extensively studied by Berry and Cannon (1981) and several species of scallops have been found to be intermediate host for the third stage larvae of this nematode in different geographical areas.

Prevalence and pathology of this species has been studied in the sea turtles *Caretta caretta* stranded along North - Western Adriatic coasts, and the discovery of an intermediate host in the same geographical area opens new insights in the study of the epidemiology of this helminthiasis in the Mediterranean Sea.

The following scientific communications have been prepared and presented on these topics at national scientific conferences:

- Marchiori E., Cassini R., Centelleghes C., Poppi L., Zanetti E., Marcer F., 2017. Gastrointestinal helminth community of *Caretta caretta* in the Northwestern Adriatic Sea: preliminary results. *IV Congresso Nazionale di Ecopatologia della Fauna, Domodossola (VB), 11-13 Ottobre*. Poster - Scientific communication 4
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4. Paper 1

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Cardiovascular flukes (Trematoda: Spirorchiidae) in *Caretta caretta* Linnaeus, 1758 from the Mediterranean Sea

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Abstract

Background: The northern Adriatic Sea represents one of the most important neritic foraging grounds for the loggerhead sea turtle *Caretta caretta* L. in the Mediterranean Sea. Four genera of blood flukes with variable prevalence and pathogenic impact have been reported worldwide in this species. *Hapalotrema* Looss, 1899 and *Amphiorchis* Price, 1934 are the only two genera reported in Mediterranean waters; however, updated data describing spirorchiidiasis in the central and eastern Mediterranean and infection prevalence are still lacking. This work aimed to investigate the presence and pathology of spirorchiidiasis in *C. caretta* in the Mediterranean Sea.

Methods: One hundred sixty-eight animals stranded along the northwestern Adriatic coast between 2009 and 2015 were submitted to necropsy and subsequent analyses for the detection of adult flukes, detection of eggs in the faeces and spleen and histopathology. Molecular analyses were carried out on hosts (mitochondrial D-loop) and parasites (28S gene and ITS2 spacer) to trace the turtle origins and identify the fluke phylogenetic relationships.

Results: Spirorchiidiasis was detected in 16.7% of the animals. *Hapalotrema mistroides* (Monticelli, 1899) and *Neospororchis* sp. were found in twenty-six and ten cases, respectively. Adult flukes were found in six cases, while eggs were detectable through copromicroscopic examination for all infected turtles, and the results for the detection of eggs in the spleen agreed with the copromicroscopic analysis. Only mild lesions were observed. Eggs of types 1 and 3 were grossly visible in the gastrointestinal mucosa, vasculitis was rarely observed in the heart and great vessels, and multifocal granulomas were widespread in the tissues. Molecular identification unambiguously assigned the spirorchiid samples to *H. mistroides* and *Neospororchis* sp. Genetic characterization of loggerhead mtDNA pointed to a Mediterranean origin of the turtle hosts.

Conclusion: This survey provides new data on the spread of spirorchiidiasis in the Mediterranean loggerhead sea turtle population and reports for the first time the presence of *Neospororchis* spp. in this basin. The infections did not have a causal effect on the death nor a strong impact on the general health status of the animals.

Keywords: Sea turtles, *Caretta caretta*, Cardiovascular flukes, Spirorchiidae, Mediterranean Sea, Phylogeny

Background

Infection by blood flukes of the family Spirorchiidae Stunkard, 1921 causes morbidity and mortality in marine turtle populations worldwide. The presence of adult flukes in the circulatory system of infected animals and the embolization of eggs through host vessels leads to lesions of

varying severity, including arteritis, thrombosis, aneurysms of the great vessels and disseminated granulomas in all body districts [1-3].

Six genera of spirorchiids are currently recognized as parasites of marine turtles worldwide: *Hapalotrema* Looss, 1899; *Neospororchis* Price, 1934; *Carettacola* Manter & Larson, 1950; *Amphiorchis* Price, 1934; *Learedius* Price, 1934; and *Monticellius* Mehra, 1939. Most of the reports of spirorchiidiasis in the marine environment concern the green turtle (*Chelonia mydas* Linnaeus, 1758); however, the infection is also well documented in the loggerhead sea turtle (*Caretta caretta* Linnaeus, 1758) in which species of the genera *Hapalotrema*, *Neospororchis*, *Carettacola* and *Amphiorchis* have been reported.

Studies on the prevalence of infection in *C. mydas* report high percentages of positivity worldwide, ranging from 80 to 100% from Australia to Florida (USA) [3–9]. Similar surveys carried out on *C. caretta* from the north-western Atlantic region describe the prevalence as ranging between 33–96% [1, 4].

The genus *Hapalotrema* is globally distributed and is described in Florida and Australia [1, 4, 10–13]. Three species, i.e. *H. synorchis* Luhman, 1935, *H. pambanensis* Gupta & Mehrotra, 1981 and *H. mistroides* (Monticelli, 1989), have been reported in loggerhead sea turtles from both Florida and western Australia. Species of *Neospororchis* have been isolated from *C. caretta* in the north-western Atlantic region [1, 11, 14, 15]. *Neospororchis pricei* Manter & Larson, 1950 was morphologically identified by Manter & Larson [14] and by Stacy [11]; however, in the latter study molecular and phylogenetic analyses revealed the presence of other unidentified species among the collected specimens with different localization in the definitive hosts [11]. Species of the genus *Carettacola* have also been reported from the western Atlantic coast [14, 4], whereas there is only one report of *Amphiorchis* in *C. caretta* from the Mediterranean Sea [16].

Few descriptions of spirorchiids in loggerhead sea turtles and green turtles exist for the Mediterranean area, and information on infection prevalence is not available. In the late 19th century, Monticelli described the blood fluke *Mesogonimus constrictus* (Leared, 1862) (syn. *Hapalotrema mistroides*) in a specimen of *C. caretta* (syn. *Thalassochelys caretta* Bonaparte, 1838) from the Gulf of Naples, off Italy [17], and the same species was reported by other authors in both loggerhead and green turtles from the Egyptian coast a few years later [18-21]. Recently, Santoro et al. [22] described severe lesions due to *H. mistroides* infection in a loggerhead sea turtle stranded along the Italian Tyrrhenian coastline. *Amphiorchis* spp. associated with severe disease were recently reported by Cribb et al. [16] in neonate loggerhead sea turtles kept in a facility in Valencia, Spain. No reports of the genera *Neospororchis* and *Carettacola* are available for the Mediterranean Basin.

Methods for postmortem diagnosis include gross and microscopic observations of adult flukes, egg masses, and related lesions in the organs, tissues and vessels [2]. Other laborious techniques can be used to detect adult flukes in blood and organs [23] and to search for spirorchiid eggs in the spleen [7]; copromicroscopic examination has also been used to detect the presence of eggs in faecal material [1].

Parasite identification is achieved through observations of morphological features. However, intact adult parasites are often difficult to obtain from stranded animals, and success also depends on the preservation status of the carcasses [4]. The eggs represent the most resistant stage and have been classified into three types [1]: eggs with bipolar filaments (type 1) are attributed to different genera, i.e. *Hapalotrema*, *Learedius*, *Monticellius* and *Amphiorchis*; eggs with monopolar filament (type 2) are described for *Carettacola*; and round eggs (type 3) are attributed to *Neosporichis* [1, 24]. Thus, egg morphology alone is a helpful but limited tool for parasite identification [4]. Molecular approaches are therefore important for overcoming the limits of morphological identification and are the basis for analyses of phylogenetic relationships within the family Spirorchiidae [11, 16, 25, 26]. Considering these factors, the aims of this work were to (i) obtain prevalence data on spirorchiidiasis in the central Mediterranean population of *C. caretta* using different diagnostic approaches; (ii) evaluate their pathogenic impacts on the host; and (iii) increase our knowledge for the identification of blood flukes by morphological and molecular methods.

Methods

Between June 2009 and November 2015, 168 loggerhead sea turtles were found stranded and dead along the north-western Adriatic coast of Italy from Grado (Udine Province; 45°41'N 13°24'E) to Riccione (Rimini Province; 44°00'N 12°39'E). Necropsy and parasitological examination were performed on the carcasses at the Department of Comparative Biomedicine and Food Sciences and the Department of Animal Medicine, Productions and Health of Padova University (Italy), respectively. Permission for the execution of necropsies on stranded sea turtles was endorsed by the local health authority to the University of Padova.

Anatomopathological analyses

Necropsies were performed following guidelines given by Flint et al. [2] and Poppi et al. [27]. Before starting the dissections, morphometric data were collected that included SCL (straight carapace length) measurements. A body condition score (BCS) was assigned to the carcasses after evaluation of the adipose tissue covering the ventral muscles. In animals that were freshly dead (*n*

= 11) or in moderate decomposition ($n = 99$), all organs (adrenal gland, gastrointestinal tract, heart and major vessels, kidney, liver, lung, gonads, pancreas, salt gland, spleen, thyroid, thymus and urinary bladder) were separately observed to locate macroscopic vascular lesions or egg masses. The skull was opened for the examination of the brain only in fresh carcasses. In poorly preserved carcasses ($n = 58$), whenever possible, the heart, major vessels, gastrointestinal tract, and spleen were considered for gross examination.

Tissue samples (adrenal gland, $n = 16$; gastrointestinal tract, $n = 66$; heart and major vessels, $n = 37$; kidney, $n = 47$; liver, $n = 45$; lung, $n = 42$; gonads, $n = 18$; pancreas, $n = 15$; salt gland, $n = 3$; spleen, $n = 52$; thyroid, $n = 15$; thymus, $n = 44$; urinary bladder, $n = 8$) were collected, stored in 10% neutral buffered formalin, embedded in paraffin and sectioned at 4 μm for histological observation. The sections were stained with haematoxylin and eosin (HE) and observed under light microscopy (Olympus BX40F-3, Tokyo, Japan).

Parasitological analyses

Stool samples ($n = 168$) and organs (gastrointestinal tract, $n = 126$, spleen, $n = 64$, heart and major vessels, $n = 140$) were collected according to carcass conditions and thoroughly analysed for the presence of blood flukes and their eggs.

Faecal samples were collected from the terminal portion of the intestine in all animals. An aliquot of each sample was stored at $-20\text{ }^{\circ}\text{C}$ for molecular analysis, while another aliquot (2–5 g) was submitted to qualitative copromicroscopic analysis to search for eggs. A common centrifugal sedimentation/flotation technique was applied using a high-density solution (sodium nitrate, sodium thiosulphate and sucrose/1.450).

The gastrointestinal tract and serosal vessels were longitudinally opened and examined both grossly and under a dissecting microscope to detect eggs, adult worms, and related lesions.

A pre-weighed portion (2 g) of splenic tissue was diced and homogenized by a blender in tap water. The obtained fluid was centrifuged in a tube at $2000\times\text{ rpm}$ for 5 min. After removal of the supernatant, a high-density solution (sodium nitrate, sodium thiosulphate and sucrose/1.450) was added and mixed with the sediment until the tube was filled; a coverslip was left over the tube for a few minutes and finally observed under a light microscope ($10\times$) for the detection of spirorchiid eggs.

The heart chambers and major vessels (left and right aortas, brachiocephalic trunk and pulmonary arteries) were opened through longitudinal sectioning and rinsed with tap water. The washes were then submitted to sedimentation in conic flasks, and the sediment was observed under a stereomicroscope. Adult flukes were collected, counted and fixed in 70% ethanol for identification. The parasites were clarified in Amman's lactophenol or stained with Semichon's

acid carmine and mounted in Canada balsam. The morphometric characteristics were studied under a light microscope by a calibrated eyepiece micrometre (Olympus, ACH 40X-2) and compared with descriptions in the literature [13, 24]. Spirorchiid eggs detected in faeces and spleen were measured under a light microscope and classified as type 1, 2 or 3 depending on the presence and number of lateral processes according to Wolke et al. [1].

The concordance between the different parasitological methods used to diagnose spirorchiid infection (i.e. qualitative copromicroscopic analysis, search for eggs in the spleen and observation of adult flukes in the cardiovascular system) was calculated as the number of samples with identical results divided by the total number of samples commonly examined (% concordance). The data were also evaluated using kappa-type statistics [28], which express the proportion of agreement beyond chance and provide a value (parameter k) ranging from 0 (no agreement) to 1 (perfect agreement).

Genetic analysis of *Caretta caretta* hosts

To test the origin of the infected animals, a small amount (5 mm) of muscle tissues was collected from positive specimens. DNA was extracted using a QIAamp® DNA Mini and Blood Mini Kit (Qiagen GmbH, Hilden, Germany). A fragment of 815 bp of the mtDNA encompassing the control region (D-loop) was amplified by PCR using the primers LCM15382/H950 [29]. PCR conditions included an initial denaturing step at 94 °C for 3 min, followed by 36 cycles of 94 °C for 30 s, 52 °C for 30 s and 72 °C for 30 s, with a final step at 94 °C for 5 min. Amplification products were purified using a QIAquick PCR Purification Kit (Qiagen). A BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, California) and the same primers as those used in the first PCR were used in the sequencing reactions. Purification was carried out with an Agencourt CleanSEQ Dye Terminator Removal Kit (Beckman Coulter, Cassina De'Pecchi, Italy) and loaded onto an ABI Prism™ 3130 Genetic Analyzer (Applied Biosystems). Sequences were analysed, and base called using DNA Sequencing Analysis Software version 5.1 (Applied Biosystems). The multiple alignment programs included in the package Vector NTI version 9.1 (Invitrogen, Carlsbad, California) was used to align the sequences. D-loop haplotypes were classified by comparison to the mtDNA sequences available in GenBank and deposited in the Archie Carr Center for Sea Turtle Research database (ACCSTR; <http://accstr.ufl.edu/files/cclongmtdna.pdf>).

Molecular characterization of the parasites

Parasite DNA was extracted using a NucleoSpin® Tissue Kit (Macherey-Nagel, Duren, Germany) for adult flukes and using a PSP® Spin Stool DNA Kit (Invitex GmbH, Berlin, Germany) for stool samples that were positive in the copromicroscopic analysis.

The internal transcribed spacer 2 (ITS2) region of the rDNA was amplified using the primers described by Stacy et al. [30]. Amplification was performed by a standard PCR followed by a semi-nested PCR. The forward primer SPIR1 (5'-GAG GGT CGG CTT ATT ATC TAT CA-3') and the reverse primer SPIR2 (5'-TCA CAT CTG ATC CGA GGT CA-3') were used in the standard PCR. The forward primer SPIR1 and the reverse primer HLC4 (5'-TCA CAT CTG ATC CGA GGT CA-3') were used in the semi-nested PCR. The PCR reactions were performed with a 30 µl reaction volume composed of 1–3 µl DNA, 2 mM MgCl₂, 0.2 mM dNTPs (MBI Fermentas, Darmstadt, Germany), 1× PCR buffer, 0.5 µM each of the forward and reverse primer, and 1 U Platinum *Taq* DNA Polymerase (Invitrogen), with the remainder of the volume composed of sterile water. Cycling conditions included an initial activation step at 95 °C for 5 min., followed by 35 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s, with a final extension step of 72 °C for 10 min.

The 28S region of the rDNA was amplified using the primers described by Olson et al. [31]: LSU-5 (5'-TAG GTC GAC CCG CTG AAY TTA AGCA-3') and 1500R (5'-GCT ATC CTG AGG GAA ACT TCG-3'). The PCR reactions were performed in a 30 µl reaction volume composed 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 primer, 1 M Betaine solution, and 1.5 U Platinum *Taq* DNA Polymerase (Invitrogen), with the remainder of the volume composed of sterile water. Cycling conditions 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.

All positive samples from PCR were sequenced by the Macrogen company (Macrogen Europe, Amsterdam, the Netherlands). Single electropherograms were analysed with the software ChromasPro version 2.4.3 (Technelysium Pty Ltd, South Brisbane, Australia).

The consensus sequences were assembled with the SeqMan program (DNASTAR package, Lasergene). The new nucleotide sequence data reported in this paper are available in the GenBank, EMBL and DDBJ databases (accession numbers: LT617052, *H. mistroides* ITS2 and LT882715, *H. mistroides* 28S; LT617053, *Neospororchis* sp. ITS2 and LT882716 *Neospororchis* sp. 28S; see Additional file 1: Table S1).

Phylogenetic analysis

The nature of the newly generated sequences was confirmed by a BLAST search [32], performed against the non-redundant nucleotide database in GenBank. This search also allowed us to identify

the orthologous sequences obtained for spirorchiids and species of closely related fluke families [31] (Additional file 1: Table S1).

The family Spirorchiidae is a paraphyletic group with respect to the Schistosomatidae [33]. Thus, we created two data sets containing all available ITS2 and 28S sequences of the family Spirorchiidae (Additional file 1: Table S1). We also included sequences of members of the family Schistosomatidae and species belonging to the family Aporocotylidae. These latter species were used as outgroups in the phylogenetic analyses.

After downloading the sequences listed in Additional file 1: Table S1, they were aligned and successively trimmed while considering the coverage of different portions of the evaluated markers. The trimming served to minimize the amount of missing data. The ITS2 and 28S datasets were aligned with the MAFFT program [34], which is available at the EBI website [35]. The multiple alignments were then imported into the MEGA 6 program [36] for analyses. The ITS2 alignment was 451 nt positions long, while the 28S alignment encompassed 700 nt positions.

The phylogenetic analyses were performed according to the maximum likelihood (ML) and Bayesian inference (BI) methods [37]. The IqTree program (version 1.5.4) was used for ML analyses [38]. One hundred independent tree searches were performed to avoid entrapment in local optimal trees. The molecular evolution models selected by the IqTree program [39] were as follows: TVM + I + G4 for the 28S dataset and GTR + I + G4 for the ITS2 dataset. The statistical support for tree topologies was computed by performing 10,000 ultrafast bootstrap replicates [40]. The program MrBayes (version 3.2.6) was used for BI analyses [41]. Two simultaneous runs, each of four chains, were performed in all analyses. Each run consisted of 1,000,000 generations, and trees were sampled every 100 generations (trees generated = 2×10^4). Stationarity was considered reached when the average standard deviation of split frequencies was less than 0.005. 'Burn-in' was very stringent, and only the last 2000 generated trees were used to compute the majority-rule posterior consensus trees. The evolutionary model applied in Bayesian analyses was the GTR + I + G [37]. Pairwise distances among sequences were calculated with the MEGA 6 program [36].

Results

Parasitological analyses

Spirorchiidiasis was observed in 28 of the 168 examined animals (16.7%). The results of the analyses used to detect and identify adult flukes and spirorchiid eggs in the cardiovascular district, stool samples and spleen are reported in Table 1.

Adult flukes were found inside the heart and at the beginning of the major vessels in six animals. Because of the poor conditions of the specimens, the morphological approaches only

permitted identification of the parasites as members of *Hapalotrema* based on the presence of multiple testes anterior and posterior to the ovary and terminal genitalia.

Copromicroscopic examination proved positive results for spirorchiid eggs in 28 cases; type 1 and 3 eggs were found in 26 and 10 animals, respectively (15.5 and 6.0%), and there were 8 cases of mixed infection. The analyses carried out on spleen samples revealed the presence of spirorchiid eggs in 17 cases; type 1 and 3 eggs were observed in 16 and one sample(s), respectively. Of 28 animals found positive in the copromicroscopic exam, 14 specimens had eggs (i.e. type 1, type 3 or both) that were detected on the gastrointestinal walls. All animals with eggs in the spleen and adults in the cardiovascular system were also positive in the copromicroscopic analysis. Table 2 shows the comparisons among the performed techniques regarding the percentage of concordance and level of agreement (parameter k). The copromicroscopic analysis was in excellent agreement with the evaluation of eggs in the spleen ($n = 64$), whereas the correspondence with the evaluation of adults in cardiovascular system was only fair ($n = 140$).

Genetic analyses of *Caretta caretta* hosts

Twenty-one of 28 positive *C. caretta* were genetically analysed. Sequences of the same length (815 bp) were obtained for all the samples (Table 1). The comparison of these sequences with those registered in the databases for *C. caretta* led to the identification of three already known D-loop haplotypes. CC-A2.1 (GenBank: EU179445) was the most frequent haplotype and was detected in 19 host specimens (91%). One individual (4.5%) collected in 2013 exhibited the haplotype CC-A2.8 (FM200217), while the haplotype CC-A32.1 (JF837822) was recorded for one turtle (4.5%) collected in 2014.

Anatomopathological findings

Body condition score of the positive animals is shown in Table 1.

Spirorchiid eggs were detected in the intestinal mucosa and submucosa only in animals found to be positive by copromicroscopic exam. The distribution pattern of the eggs on the intestinal walls was macroscopically different for the two types. Type 1 eggs were arranged in small clusters or disseminated as single elements in the mucosal layer. Type 3 eggs were arranged in highly visible, serpiginous masses included in the mucosal layer or, less frequently, as large clusters with a cyst-like appearance and black (Fig. 1).

Egg clusters were surrounded by mild granulomatous inflammation with multinucleated giant cells, rare mixed inflammatory cells, and a thin fibrous capsule. Single elements and small groups of eggs ($n = 3-5$) were not associated with any inflammatory reaction. Granulomas were seen in the spleen, lung, thymus, and pancreas surrounding small, multifocal groups of eggs.

Isolated eggs were also observed in the gastric walls, liver, heart, kidney, adrenal gland, salt glands, and urinary bladder wall.

Mild to moderate arteritis was observed in four of six animals from which adult parasites were isolated. The beginning of the major vessels was the most affected section (Fig. 2). Small proliferative plaques were grossly visible on the intima with rough irregular surface. At histology, fibro-muscular proliferations were visible inside the intima of the inflamed vessels; isolated eggs were frequently seen more deeply in the vessel walls without any associated inflammatory processes. In one case, extensive sub-endothelial inflammatory infiltrate was observed in association with multiple degenerated eggs.

Molecular identification of parasites and phylogenetic analysis

The ITS2 sequences were obtained for twenty-three faecal samples positive for type 1 eggs and six adult flukes (one from each positive animal). All ITS2 sequences were identical. A BLAST search identified the ITS2 sequence from a specimen of *H. mistroides* (*H. mistroides* GU937893, Additional file 1: Table S1) as the closest relative to the ITS2 sequences in the current study. Pairwise comparisons among the new sequences and *H. mistroides* A ITS2 showed an identity of 99.63% (i.e. one base A vs G was different over 270 bases of the alignment). The different base is located near the 3' end, a portion not included in the phylogenetic analysis. Thus, the newly generated sequences were assigned to *H. mistroides* (see below). The ITS2 sequences obtained from eight faecal samples positive for type 3 eggs were found to be identical. These sequences were compared with data available in GenBank and were found to be identical to those obtained from *Neospororchis* sp. Neogen11 (KU601335) (Additional file 1: Table S1).

The 28S partial sequences obtained from four adults of *H. mistroides* were identical. Similarly, the 28S sequences obtained from five samples of *Neospororchis* sp. eggs were identical. We obtained a single haplotype for both taxa.

The ML tree obtained from the ITS2 alignment is presented in Fig. 3. The BI consensus tree exhibited a topology that was mostly congruent except for the arrangement of the *Neospororchis* clade (see below). The family Spirorchiidae was paraphyletic with respect to the Schistosomatidae. Two major clades occurred within Spirorchiidae. The first clade included exclusively taxa belonging to the genus *Neospororchis*. The second clade contained the other analysed spirorchiid taxa and was a sister group of the Schistosomatidae. The *Neospororchis* clade received strong bootstrap and BI posterior probability support. *Neospororchis* sp. Italy (LT617053) was placed within the *Neospororchis* clade as a sister taxon of *Neospororchis* sp. Neogen 11 (KU601335). In the ML tree, two clades (I and II) could be identified within the genus *Neospororchis*. Both clades received bootstrap corroboration. The phylogenetic relationships

among the different *Neospororchis* taxa were well resolved and received bootstrap corroboration in many cases. However, clade I was not found in the BI consensus tree. *Hapalotrema mistroides* Italy (LT617052) formed a clade with two other specimens assigned to the same species. This group received strong statistical corroboration by bootstrap and BI posterior probability values. Samples of *H. mistroides* were the sister group of *Hapalotrema postorchis* specimens, a phylogenetic relationship supported by both bootstrap and BI posterior probability values. *Hapalotrema pambanensis* and *Learedius learedi* were not monophyletic.

The maximum likelihood tree obtained from the 28S multiple alignment is presented in Fig. 4. The BI consensus tree exhibited a fully congruent topology (data not shown). Most of the nodes received support by bootstrap and BI posterior probability values. The family Spirorchidae was paraphyletic with respect to Schistosomatidae. Two main clades could be recognized within the spirorchid flukes. A clade containing mostly species of the genera *Carettacola*, *Amphiorchis*, *Hapalotrema* and *Learedius* was a sister group of the Schistosomatidae. Within this clade, *L. learedi* (represented by three sequences) and *H. pambanensis* (represented by two sequences) were monophyletic. Indeed, *Hapalotrema mehrai* Rao, 1976 is a junior synonym of *H. pambanensis* (Additional file 1: Table S1). The second major clade included various spirorchid taxa. *Neospororchis* sp. Italy (LT882716) grouped with the other taxa of the genus *Neospororchis*. Similarly, *H. mistroides* Italy (LT882715) was a sister taxon to *H. mistroides* (KU892016).

The average p-distance among the ITS2 sequences of the *Neospororchis* taxa was 0.128 ± 0.075 . The p-distances ranged from 0 [*Neospororchis* sp. Italy (LT617053) vs *Neospororchis* sp. Neogen11 (KU601335)] to the maximum value of 0.291 observed in *Neospororchis* sp. Neospirgen2 (KU600080) vs *Neospororchis* sp. Neogen15 (KU601339). The average p-distance among taxa belonging to the *Hapalotrema* + *Learedius* clade was 0.122 ± 0.064 . The range varied from 0 [*H. mistroides* Italy (LT617052) vs *H. mistroides* (GU937893)] to the maximum value of 0.195 observed in the pair *H. mistroides* (KY499798) vs *H. synorchis* (KM652618). Finally, within the genus *Schistosoma*, the average p-distance was 0.110 ± 0.072 , while values ranged from 0 [*Schistosoma japonicum* (FJ852567) vs *Schistosoma japonicum* (FJ852566)] to 0.188 [*Schistosoma japonicum* (FJ852566) vs *Schistosoma haematobium* (U22165)].

Discussion

Prevalence data and ecological considerations

Few studies on spirorchidiasis in loggerhead turtles have been carried out [1, 4, 15, 16, 42]; these studies involve a small number of animals and apply different methodologies for parasite detection. Only two surveys carried out in Florida can be considered for a comparison with the

current study due to having a similar diagnostic approach and sampling effort [1, 11]. The prevalence of infection observed in our study for both *Hapalotrema* sp. and *Neospororchis* spp. appears to be lower than that recorded in the two previous studies. In the most recent survey [11], the high percentage of loggerhead sea turtles infected by both *Hapalotrema* spp. and *Neospororchis* spp. (96 and 77%, respectively) demonstrated a wide diffusion of infection in the north-western Atlantic. In the present study, spirorchiidiasis does not appear to be widespread among loggerhead turtles feeding in the northern Adriatic grounds (prevalence of 15.5 and 6.0% for *Hapalotrema* and *Neospororchis*, respectively). There was one case of infection by *H. mistroides* observed in one loggerhead turtle stranded along the coast of central-western Italy [22]; however, other surveys performed in last few decades did not detect these parasites in *C. caretta* in the central or eastern Mediterranean areas [43–45]. These results may be partly due to different research methodologies or to minor sampling effort. Nevertheless, this finding supports the hypothesis that spirorchiidiasis did not raise the attention of researchers for frequent and serious patterns of infection in the region, which differs from the descriptions for North Atlantic waters [15]. The differences in the spread of infection may be due to environmental factors, including the presence and distribution of the intermediate hosts. Involvement of a gastropod intermediate host in the life-cycle of marine spirorchiids has been already demonstrated [16, 30].

The detection of adult flukes in a few cases may have been due to poor sample quality, the small body size of these trematodes and the tendency, especially for *Neospororchis* spp., to inhabit small vessels, as already shown by other authors [4, 3]. Therefore, it is commonly recognized that the diagnosis of spirorchiidiasis cannot rely only on the detection of adult specimens. Spirorchiid eggs have greater resistance to decomposition than adults both in carcasses and in the environment [46]. Faecal examination has been used to detect infections in sea turtles [1], although it is considered a diagnostic method lacking in specificity and sensitivity [25, 47]. In this study, copromicroscopic examination showed excellent agreement ($k = 0.886$) with the method used for searching for eggs in the spleen, a common site of egg deposition due to its role as a blood filtering organ [2]. The presence of eggs in the faeces is related to their migration through the intestinal walls and entrance into the gut lumen [12, 47], but not all species have the primary route of elimination *via* the gastrointestinal tract in all host species [4, 47]. Our positive results from the copromicroscopic exams were often associated with the presence of both *Hapalotrema mistroides* and *Neospororchis* sp. eggs in the intestinal mucosa; therefore, copromicroscopic examination remains a valid and noninvasive diagnostic method in the case of infection by *Hapalotrema mistroides* and *Neospororchis* spp. in *C. caretta* and is also feasible for use with live animals. Copromicroscopic analysis has additional limitations, such as recent infections (immature specimens) or low parasite burden, that could produce false negative results, and since different

spirorchiid genera have similar eggs, molecular approaches remain essential for certainty in identification.

Our genetic analyses were performed on positive hosts to test the hypothesis that spirorchiidiasis was acquired inside the Mediterranean Basin. Two recent reports [16, 22] demonstrated the presence of *H. mistroides* infection in loggerhead sea turtle specimens from the western Mediterranean and the Tyrrhenian Sea. However, no genetic analyses were done in those studies, and considering that both Atlantic and Mediterranean turtles can be found in the western part of the basin, uncertainty about their origins remains. The three D-loop haplotypes found in the present study are common in loggerhead sea turtles from the Mediterranean Basin [48]; haplotype CC-A2.1 is the most frequent in all Mediterranean rookeries [48] and is found at low frequencies in Atlantic colonies [49]. Haplotype CC-A2.8 was observed in loggerhead turtles nesting in Crete [48] and feeding in Ionian waters [50], while haplotype CC-A32.1 is private of Greek nesting colonies [48]. The presence of individuals from these two Mediterranean nesting colonies in Adriatic waters has been documented by genetic studies [50-52] as well as by satellite tracking studies [53, 54]. Therefore, the positive animals seem to belong to the stock usually encountered in the northern Adriatic waters. Loggerhead sea turtles of the Mediterranean, particularly those of Greek origin, show intra-Mediterranean migratory pathways and select neritic feeding areas inside the basin to which they show strong fidelity [55], sometimes sharing the same foraging area with turtles of Atlantic origin (i.e. the Gulf of Gabès, Tunisia [53]). These points enhance the probability that the turtles (at least those carrying Greek haplotypes) acquired the infection inside the Mediterranean Sea along their migratory routes.

Pathogenic impact

Spirorchiidiasis in sea turtles from the Atlantic and Pacific Oceans has been found to be causal or contributory to death in many cases [3, 11, 16, 56]. In the Mediterranean Sea, except for scarce historical data, there is only one case report of a free-ranging loggerhead turtle [22] in which severe lesions by spirorchiids likely contributed to the death of the animal. Nevertheless, in the present study spirorchiidiasis never affected the general health status of the hosts and represented an occasional finding during necropsy. Most of the positive turtles were in a good or excellent nutritional condition, and only mild lesions were observed.

Two patterns of lesions were observed for type 1 and type 3 eggs in the intestine and were similar to that described by Stacy et al. [4] in *C. caretta* for *Hapalotrema* spp. and *Neospororchis* spp., respectively. In *Hapalotrema*-infected hosts, the severity of the granulomatous reaction to egg masses was higher, with transmural and prominent lesions visible both on the intestinal

mucosa and on the subserosal vessels. In our study, the mucosal and (rarely) submucosal layers were affected, and no lesions were evident from the external surface of the intestinal walls.

Spirorchiids are known to produce serious pathological lesions in the circulatory system of the host. Arteritis, aneurysms and disseminated thrombi are described in various districts in *C. caretta* infected by *H. mistroides* and *Neospororchis* spp. [1, 4]. Some differences in the distribution of adult flukes and lesions are reported depending on the infecting species and genotypes [11]. In our study, most of the positive turtles had no lesions in the cardiovascular system except for a few cases with mild to moderate alterations at the emergence of the major vessels where adult specimens of *H. mistroides* were collected. Associations between *Hapalotrema* spp. (including *H. mistroides*) and endarteritis was already shown by Stacy et al. who also found parasites attached to the lesions [4].

Molecular and phylogenetic findings

The specimens assigned to the genus *Neospororchis* sequenced in our study shared the same ITS2 haplotype, *Neospororchis* sp. Italy (LT617053). The latter was identical to the *Neospororchis* sp. Neogen11 (KU601335) sequence obtained from a specimen previously isolated from the gastrointestinal tract of a *C. caretta* specimen in Florida [11]. Similarly, all the specimens assigned to *H. mistroides* Italy (LT617052) had identical ITS2 sequences that differed from *H. mistroides* (GU937893) by a single base (A vs G) located outside of the aligned portion.

The 28S sequences of *H. mistroides* Italy LT882715 and *H. mistroides* KU892016 were also identical (Fig. 4). The high levels of sequence similarity were mirrored by the phylogenetic results (Figs. 3, 4), where newly generated sequences grouped with the orthologous counterparts obtained from congeneric (*Neospororchis*) or conspecific specimens (*H. mistroides*). Multiple sequences were available for both *Neospororchis* spp. and *H. mistroides*. If our sequences do not belong to the genus *Neospororchis*/*H. mistroides*, these taxa become paraphyletic in the ITS2 analysis, a situation that is untenable. Thus, the phylogenetic analysis allowed the straightforward generic/specific assignment of the new sequences.

The level of molecular variation observed among the *Neospororchis* ITS2 sequences was higher than that observed among the species included in the *Hapalotrema* + *Learedius* clade or within the genus *Schistosoma*. These results support the view that the *Neospororchis* flukes parasitizing sea turtles belong to at least two distinct species.

The analysis performed on the ITS2 of *H. pambanensis* and *L. learedi* suggested a non-monophyly of these taxa. In our opinion, these results must be regarded as an artifact due to a mislabelling of the sequences submitted to GenBank. In favour of our hypothesis, there are the following considerations. First, Chapman et al. [25] generated and used this set of sequences in a

previous phylogenetic analysis where both species were monophyletic. Unfortunately, they did not present the accession numbers of the sequences in their phylogenetic tree. Secondly, in our 28S tree, both taxa are also monophyletic. Thirdly, it is unrealistic that two distinct species would present identical ITS2 sequences considering the level of variation also observed in the present study for these molecular markers.

Conclusions

In this study, eggs or adults from two species of spirorchiids, *H. mistroides* and *Neospororchis* sp., were observed in single or mixed infections and were identified by both morphological and molecular approaches. *Hapalotrema mistroides* was already described in loggerheads stranded along Egyptian [19, 18] and Italian coastlines [17, 22], whereas the genus *Neospororchis* is described in this basin for the first time. Our study noted the importance of molecular approaches and phylogenetic studies as a good complement for resolving problems related to poorly preserved specimens and to provide evidence of cryptic speciation within the currently recognized spirorchiid species, as previously suggested by other authors [11, 25]. Although spirorchiidiasis is one of the oldest observations in sea turtle literature [17], this study provides prevalence data with pathological features of the infection for the first time inside the Mediterranean Basin and updates our knowledge on the species present. Low prevalence and mild lesions were observed in the samples, leading to the conclusion that spirorchiidiasis does not represent a cause of severe morbidity or mortality for turtles in the region. Data on *C. mydas*, also a resident species in the Mediterranean, are completely lacking, and studies performed in the western Mediterranean included a limited number of loggerhead sea turtles. Therefore, further investigations are necessary for more thorough knowledge of the ecopathology and spread of spirorchiidiasis in the Mediterranean Sea. Considering the results of the genetic analysis carried out on positive turtles and the current knowledge of the migratory routes of Mediterranean turtles, the hypothesis that infection by both *Hapalotrema* and *Neospororchis* was acquired inside the Mediterranean Basin seems to be supported. The identity of the ITS2 sequences of *Neospororchis* sp. Italy and Neogen-11 from Florida suggest the possibility that the infection was transmitted by Atlantic turtles to Mediterranean turtles through an infected intermediate host in a shared feeding ground. If this hypothesis is confirmed, this would highlight the importance of studying the roles of these areas as ecological key points for the spill over of pathogens between the two populations.

Additional file

Additional file 1: Table S1. List of fluke taxa analysed in the present study with GenBank accession numbers, sources, hosts and localities.

Abbreviations

Not applicable.

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Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

The data supporting the conclusions of this article are included within the article. Sequences of *Hapalotrema mistroides* Italy and *Neospororchis* sp. Italy have been deposited in the GenBank database under the accession numbers LT617052 and LT617053 (ITS2), respectively and LT882715 and LT882716 (28S), respectively.

The D-loop haplotypes of the turtles were already described and deposited in the Archie Carr Center for Sea Turtle Research database (ACCSTR; <http://accstr.ufl.edu/files/cclongmtdna.pdf>).

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

EM contributed to the conceptualization of the study, performed necropsies, parasitological and histopathological analysis and was a major contributor in writing and editing the paper. EN contributed to conceptualization of molecular and phylogenetic studies, performed phylogenetic studies, provided molecular studies on parasites sequences and secondary structure of ITS2 and was a major contributor in writing and editing the paper. RC made statistical analyses and contributed in editing the manuscript. LG made genetic analysis on turtles mtDNA and drafted the corresponding part of the manuscript. LP provided the collaborations for the collection of turtles carcasses, performed necropsies, and histopathological analyses and contributed in editing the pathological findings. CT made molecular analysis on parasite samples and collaborated in editing the paper. FM contributed to the conceptualization of the study, performed parasitological analyses and the necropsies, was a major contributor in drafting and editing the manuscript, coordinated the project and provided funding for the study. All authors read and approved the final manuscript.

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Table 1 Biometric data and results of analyses for *Caretta caretta* positive for spirorchiid infection

ID	SCL (cm)	BCS	Sex	mtDNA haplotype	Examination for spirorchiid eggs			Examination for adult flukes ^a	
					Stool samples		Spleen samples	Detection and morphological identification	Molecular identification
				Egg type	Molecular identification ^b	Egg type			
23451	83	3	F	CC - A2.1	Type 1	<i>H. mistroides</i>	nd	nd	nd
27177	55	1	F	CC - A2.1	Type 1	<i>H. mistroides</i>	Type 1	Negative	nd
32010	55	2	M	CC - A2.1	Type 1	<i>H. mistroides</i>	nd	nd	nd
55775	40	2	M	CC - A2.1	Type 1	<i>H. mistroides</i>	nd	Negative	nd
56275	71	2	F	CC - A2.1	Type 1	Negative	nd	Negative	nd
56284	52	2	F	CC - A2.8	Type 1	<i>H. mistroides</i>	Type 1	Negative	nd
56300	61	2	F	CC - A2.1	Type 1	<i>H. mistroides</i>	Type 1	<i>Hapalotrema</i> sp.	<i>H. mistroides</i>
56301	49	2	F	CC - A2.1	Type 1	<i>H. mistroides</i>	Negative	Negative	nd
56365	53	2	M	CC - A2.1	Type 1	<i>H. mistroides</i>	Negative	Negative	nd
57391	N.D.	1	F	CC - A2.1	Type 3	<i>Neospororchis</i> sp.	Negative	Negative	nd
58955	75	2	F	CC - A2.1	Type 1	<i>H. mistroides</i>	Type 1	Negative	nd
61468	61	1	M	CC - A2.1	Type 1 + 3	Negative	Type 1	Negative	nd
61467	45	2	F	CC - A2.1	Type 1 + 3	<i>H. mistroides</i> and <i>Neospororchis</i> sp.	Type 1	<i>Hapalotrema</i> sp.	<i>H. mistroides</i>
61733	35	3	F	CC - A2.1	Type 1	Negative	Type 1	Negative	nd
61918	69	2	M	CC - A2.1	Type 3	<i>Neospororchis</i> sp.	Type 3	Negative	nd
62452	64.5	2	F	CC - A32.1	Type 1+3	<i>H. mistroides</i> and <i>Neospororchis</i> sp.	nd	Negative	nd
62955	68	2	F	CC - A2.1	Type 1	<i>H. mistroides</i>	Type 1	Negative	nd

ID	SCL (cm)	BCS	Sex	mtDNA haplotype	Examination for spirorchiid eggs			Examination for adult flukes ^a	
					Stool samples		Spleen samples	Detection and morphological identification	Molecular identification
					Egg type	Molecular identification ^b	Egg type		
63355	77	2	M	CC - A2.1	Type 1 + 3	<i>H. mistroides</i> and <i>Neospororchis</i> sp.	Type 1	<i>Hapalotrema</i> sp.	<i>H. mistroides</i>
62953	51.5	2	F	CC - A2.1	Type 1 + 3	<i>H. mistroides</i> and <i>Neospororchis</i> sp.	Type 1	<i>Hapalotrema</i> sp.	<i>H. mistroides</i>
63475	63	1	F	CC - A2.1	Type 1	<i>H. mistroides</i>	Type 1	<i>Hapalotrema</i> sp.	<i>H. mistroides</i>
63477	59	2	M	CC - A2.1	Type 1	<i>H. mistroides</i>	Type 1	Negative	nd
64893	43	2	M	nd	Type 1	<i>H. mistroides</i>	Type 1	Negative	nd
64985	51	2	M	nd	Type 1	<i>H. mistroides</i>	nd	<i>Hapalotrema</i> sp.	<i>H. mistroides</i>
65004	35	3	M	nd	Type 1	<i>H. mistroides</i>	Type 1	nd	nd
65734	58	2	F	nd	Type 1 + 3	<i>H. mistroides</i>	nd	Negative	nd
65735	73	1	F	nd	Type 1 + 3	<i>H. mistroides</i> and <i>Neospororchis</i> sp.	Type 1	Negative	nd
65000	80	2	F	nd	Type 1	<i>H. mistroides</i>	Type 1	Negative	nd
65225	44	2	F	nd	Type 1 + 3	<i>H. mistroides</i> and <i>Neospororchis</i> sp.		Negative	nd

^aIn the cardiovascular system

^bMolecular analyses in mixed infections were performed on eggs isolated directly from the intestinal wall

Abbreviations: ID, host identification code; SCL, straight carapace length; BCS, body condition score; nd, not determined

Table 2 Comparison between copromicroscopic examination and evaluation of spirorchiids in the spleen and cardiovascular system

		Coproscopic examination (eggs)			% concordance	Parameter <i>k</i>
		Neg	Pos	Total		
Spleen examination (eggs)	Neg	44	3	47	95.3	0.886
	Pos	0	17	17		
	Total	44	20	64		
Cardiovascular system (adults)	Neg	116	18	134	87.1	0.356
	Pos	0	6	6		
	Total	116	24	140		

Abbreviations: Neg, negative sample; Pos, positive sample



Fig. 1 Intestinal lesions in *Caretta caretta* associated with *Haplotrema mistroides* and *Neospororchis* sp. Different pattern of distribution on the intestinal district are easily visible for the two genera. Type 1 eggs are scattered on the intestinal wall, grossly looking as small brownish spots (**a**) composed of a low number of fusiform eggs as revealed by stereomicroscopy (**b**). Big masses of type 3 eggs are visible on the intestinal mucosa as elongated black serpiginous stripes (**c**), formed by thousands of elements (**d**). Different patterns of distribution are easily visible in this case of mixed infection, in which type 3 eggs appear like grouped in a cyst like structure (**e**). Multifocal granulomatous enteritis (**f**; HE) with multinucleated giant cells (black arrow) surrounding a core of eggs and necrotic debris is detectable in intestinal sections with minimal fibrotic reaction (inset). *Abbreviation*: HE, haematoxylin and eosin. *Scale-bars*: **a**, 0.5 cm; **b**, 1 cm; **c**, 0.37 cm; **d**, 560 μ m; **e**, 350 μ m; **f**, 150 μ m (inset: 120 μ m)

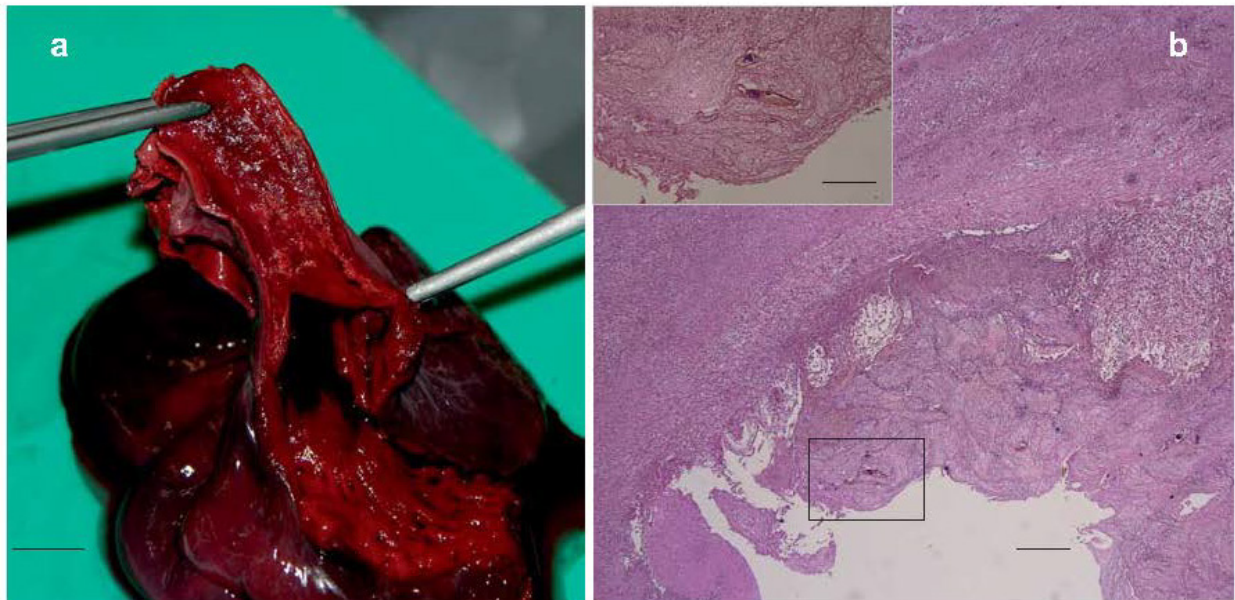


Fig. 2 a Heart and great vessels. Small proliferative plaques are visible on the supravulvular region on the intima of a great vessel in a specimen of *C. caretta* infected with *Hapalotrema mistroides*. **b** Heart, atrium (HE). Raised irregular plaques of severe chronic endocarditis and extensive fibroplasia with multifocal areas of undetermined mesenchymal cells surrounding some eggs (inset). *Abbreviation:* HE, Haematoxylin and eosin. *Scale-bars:* **a**, 1 cm; **b**, 290 μ m (inset: 135 μ m)

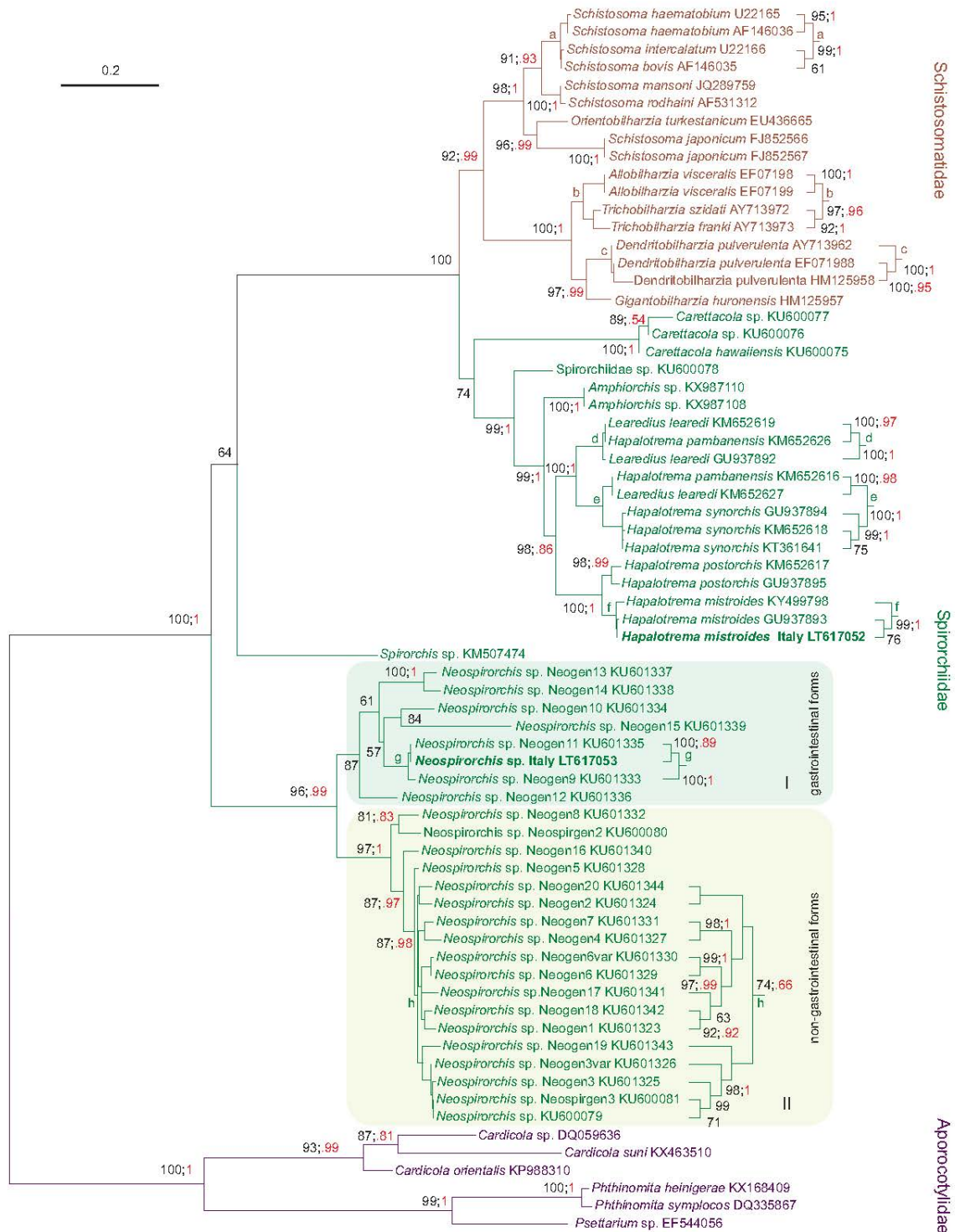


Fig. 3 Phylogenetic analysis of spirorchiid flukes based on ITS2 sequences. The ML tree ($-\ln = 6007.5517$) was computed with IQ-TREE program. The scale-bar represents 0.2 substitutions/state change per position. Numbers in black represent ultrafast bootstrap values ($> 50\%$) expressed in percent; numbers in red refer to Bayesian Inference posterior probabilities presented in a compressed way (e.g. 1 instead of 1.00; .95 instead of 0.95) to allow a better readability of the figure

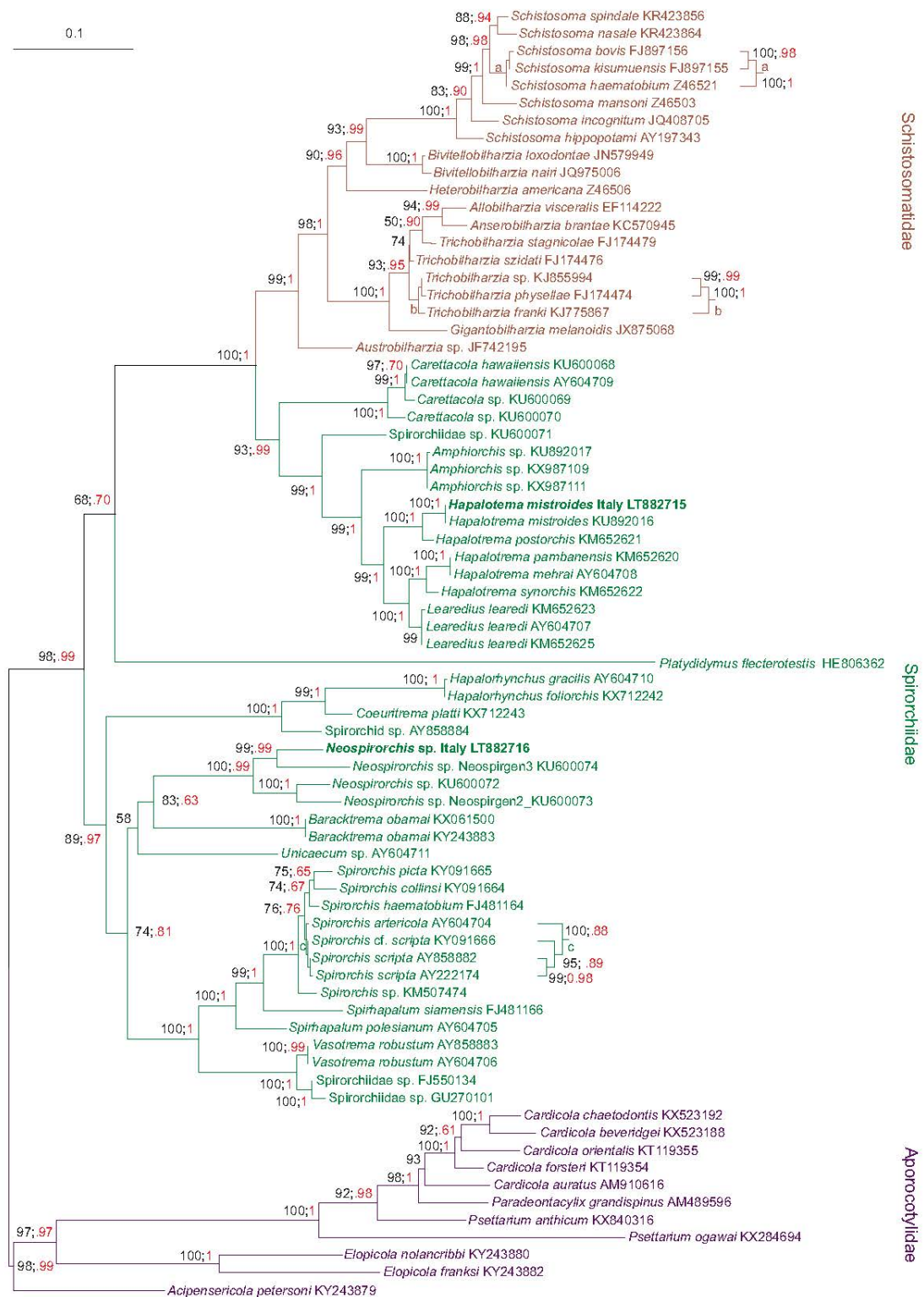


Fig. 4 Phylogenetic analysis of spirorchiid flukes based on 28S sequences. The ML tree ($-\ln = 10754.3072$) was computed with IQ-TREE program. The scale-bar represents 0.1 substitutions/state change per position. Numbers in black represent ultrafast bootstrap values ($> 50\%$) expressed in percent; numbers in red refer to Bayesian Inference posterior probabilities presented in a compressed way (e.g. 1 instead of 1.00; .95 instead of 0.95) to allow a better readability of the figure

5. Paper 2

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A comprehensive mitochondrial DNA mixed-stock analysis clarifies the composition of loggerhead turtle aggregates in the Adriatic Sea

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Abstract

Migration is one of several marine vertebrate traits increasingly affected by human encroachment. The Adriatic Sea is an important foraging and wintering site for loggerhead turtles (*Caretta caretta*), and one of the Mediterranean regions where they are most heavily impacted, particularly by fisheries bycatch. Conservation measures concern foraging and wintering areas and nesting grounds, and must, therefore, be informed by the natal origin of individuals in these mixed aggregates. Genetic diversity was investigated among 488 loggerheads found stranded or incidentally captured in fishing gear across the Adriatic and the origin of individuals was assessed through mixed-stock analysis based on comparison of 755 bp of mitochondrial DNA control region sequence. In addition, we highlight the importance of the Gulf of Manfredonia (Apulia, Italy), and of the northeastern Adriatic—a previously genetically undescribed foraging ground. No significant genetic divergence was recorded among sampling areas, between turtles sampled in cold and warm months or between adults and juveniles. The distribution of turtles across the Adriatic Sea appeared not to depend on individual origin. Rookeries in western Greece and Crete provided the most important contributions to the Adriatic mixed stock. In particular, the Greek populations were the most abundant locally in the Gulf of Manfredonia, so they are likely to experience an even greater impact than previously thought because of the severe fishery bycatch levels in this area. This study also provides an example of how substantial increases in sample sizes permit a relatively comprehensive testing of genetic structure across groupings in foraging aggregations.

Introduction

Patterns of dispersal and migration in marine vertebrates are known to embrace longdistance movements to breeding and nesting areas, foraging, and wintering grounds. Unfortunately, connectivity between habitats that play important roles in the life cycle of an organism can be disturbed or disrupted by a plethora of anthropogenic threats (Halpern et al. 2007). Bycatch, the incidental capture of non-target species in fisheries, is a prevalent cause of mortality particularly in the Mediterranean Sea, where marine species like sea turtles migrate on a regular basis from foraging to nesting grounds of great importance for population survival (Casale and Margaritoulis 2010; Fortuna et al. 2010; Coll et al. 2012; Luschi and Casale 2014).

The Adriatic Sea is one of the main foraging areas in the Mediterranean basin for marine wildlife, and in particular the loggerhead sea turtle *Caretta caretta*. The shallow (< 100 m) waters of the northern Adriatic are highly productive (Agostini and Bakun 2002) and a main neritic foraging and wintering ground for *C. caretta* (Margaritoulis et al. 2003; Casale et al. 2004; Lazar et al. 2004; Casale et al. 2010; Lazar et al. 2011; Luschi and Casale 2014). The southern Adriatic is mostly

oceanic, has a wide depression more than 1200 m deep (Cushman-Roisin et al. 2001), and is regarded as an important area for early life stage development, although all size classes are present throughout (Casale et al. 2010; Casale and Mariani 2014).

An additional, important neritic foraging area has recently been described in the Gulf of Manfredonia (Italy), off the Apulian coast, and directly west of the South Adriatic trench. This region is frequented by turtles of different size classes showing a certain degree of site fidelity (Casale et al. 2012; Casale and Simone 2017).

Describing the connectivity between natal areas and foraging grounds represents a major challenge in sea turtle research and conservation. A variety of different tools have been adopted to investigate the longrange migration of chelonians, including capture-mark-recapture, satellite tracking, and genetic analysis. Molecular tools, in particular, can provide a thorough understanding of the natal origin of foraging stocks and, among others, give insights into how anthropogenic threats affect wild populations occurring in specific marine areas and/or nesting grounds (Hamann et al. 2010; Rees et al. 2017). Furthermore, genetic structure was among the main biological parameters involved in the recent designation of regional management units (RMUs) as groups of nesting populations on possible, independent evolutionary trajectories (Wallace et al. 2010).

Flipper tagging and satellite tracking studies of loggerheads breeding in Greece have shown the importance of the Adriatic foraging grounds for the Greek nesting populations (Lazar et al. 2004; Zbinden et al. 2008), but comparable studies from other Mediterranean nesting sites are lacking. Similarly, population genetic studies conducted on *C. caretta* sampled in the Adriatic Sea have demonstrated a prevalence of individuals from Greece (Garofalo et al. 2013; Clusa et al. 2014). Molecular tracking of sea turtles is mainly based on interindividual variation at mitochondrial DNA (mtDNA) sequences, which occur at different frequencies in different rookeries—a result of population structuring due to natal homing (Bowen et al. 2005). Given this rookery baseline description, mixed-stock analysis (MSA) can then be used to estimate the relative contribution of different natal sites to foraging areas (e.g., Bass et al. 2004; Bowen et al. 2007; Bjorndal and Bolten 2008). However, loggerhead mtDNA population structure is rather weak in the Mediterranean, probably because of relatively recent colonization events from the Atlantic Ocean (Clusa et al. 2013). For this reason, MSA may have low power when using a relatively small sample size, which is less likely to include rare and more informative mtDNA haplotypes (Bolker et al. 2003). The previous attempts to characterize the genetic population structure of loggerhead turtles sampled in the Adriatic Sea were based on a relatively small sample size and did not include individuals from the northeastern Adriatic waters and the Gulf of Manfredonia as major loggerhead foraging sites (Casale et al. 2012).

A thorough assessment of the natal origin of loggerheads foraging in the Adriatic basin may have profound implications for our understanding of migratory patterns of turtles in the Mediterranean Sea. Different migration routes and displacement observed in tagged and satellite-tracked adult turtles may, in fact, depend on their rookeries of origin or on other factors such as ocean currents or different foraging strategies (Zbinden et al. 2011). Although juvenile movements and dispersal are mostly driven by marine currents, natal homing has been observed in juvenile sea turtles that show site fidelity to pelagic feeding areas near their rookery of origin or areas at similar latitudes (Avens et al. 2003; Bowen et al. 2004; Monzón-Argüello et al. 2009). If dispersal to foraging grounds depends on where individual turtles come from (i.e., location and/or size of rookery of origin), then genetic structuring is expected, for turtles from different rookeries would feed in different coastal areas. If, on the other hand, the migration of turtles from different rookeries to feeding sites is based on factors other than individual origin, then no genetic structure should be recorded across the Adriatic Sea. Moreover, there might be seasonal and/or body size differences when turtles of different origin congregate at specific foraging grounds.

In this study, we assessed the natal origin of the Adriatic foraging stock through MSA, using a comprehensive data set composed of loggerhead samples collected across the Adriatic Sea as a result of bycatch and stranding events off and along the coasts of Croatia, Slovenia, northern Italy, and the southern Italian region of the Gulf of Manfredonia. We conducted a population genetic analysis to characterize previously undescribed mtDNA haplotypes and investigate possible differences among turtles from the Adriatic foraging population. Using a relatively large sample size, we attempted an optimization of Bayesian MSA by (1) adopting a population abundance prior (Pella and Masuda 2001) to account for the relatively higher contribution of rookeries with a large number of nests, rather than relying on haplotype frequencies only; and (2) relaxing the assumption of equal possibility of reaching the foraging area using priors based on geographical distance between known Mediterranean rookeries and the Adriatic Sea.

Intensive human activity in the Adriatic Sea, including fisheries, shipping, and pollution, is considered a potential threat to sea turtle populations (Franzellitti et al. 2004; Casale 2011; Lazar and Gračan 2011). Comprehensive knowledge of the composition of the Adriatic foraging stock will help to inform decision-making processes aimed at identifying marine protected areas and mitigating the anthropogenic impact on sea turtle survival. An assessment of migratory routes from the main contributing rookeries can provide background data for revising regional policies on the use of fishing gear particularly harmful to loggerheads, such as set nets and bottom trawlers—responsible for most bycatch and likely one of the main factors affecting survival rates of *C. caretta* in the Adriatic Sea (Casale et al. 2004, 2012, 2015).

Materials and methods

Sample collection

A total of 488 *C. caretta* samples were collected between 1995 and 2015 in the Adriatic Sea off or along the coasts of Slovenia, Croatia, and northern and southern Italy (Fig. 1). Blood and skin biopsies were taken from live bycaught turtles, while muscle and skin were obtained from necropsies of stranded or floating individuals. Sea turtles sampled alive were tagged before release to avoid pseudoreplication. Our sample set included both juveniles and adults with the curved carapace length ranging from 8.5 to 86.0 cm (see Electronic Supplementary Material Fig. S1 for the body size distribution of turtles sampled in the Adriatic Sea). Blood samples were stored at $-20\text{ }^{\circ}\text{C}$, while tissue samples were preserved in 95% ethanol at $4\text{ }^{\circ}\text{C}$ or ambient temperature.

Genetic analysis

High-molecular-weight DNA was isolated using either a standard phenol/chloroform extraction protocol (Sambrook and Russel 2001), a NucleoSpin Tissue kit (Mach-erey–Nagel), or a QIAamp DNA Mini Kit (Qiagen). A fragment of the mtDNA was amplified by polymerase chain reaction (PCR) using the LTEi9 light-strand primer and H950 heavy-strand primer designed by Abreu-Grobois et al. (2006). The H950 primer anneals to the control region. The LTEi9 primer anneals between the threonine tRNA and proline tRNA genes, and in combination with H950 allows PCR amplification of an 887 bp mtDNA fragment. PCR was conducted in a total volume of 15 μl with 100 ng of total DNA, 1 \times PCR buffer, 1.5 mM MgCl_2 , 200 μM of each dNTP, 0.5 μM of each primer, and 0.5 units of *Taq* DNA polymerase. Thermal profiles consisted of an initial denaturation step of 5 min at $94\text{ }^{\circ}\text{C}$, followed by 35 cycles of 45 s at $94\text{ }^{\circ}\text{C}$, 45 s at $55\text{ }^{\circ}\text{C}$, and 1 min and 30 s at $72\text{ }^{\circ}\text{C}$, with a final extension step of 10 min at $72\text{ }^{\circ}\text{C}$. Amplicons were cycle-sequenced using BigDye Terminator v3.1 chemistry (Applied Biosystems) according to the manufacturer's protocol, and cycle sequencing reactions were resolved on Applied Biosystems 3100 Avant and 3130xl genetic analyzers.

Data analysis

Raw sequence chromatographs from both strands were edited and aligned to the loggerhead turtle mtDNA sequence using GENEIOUS 8.0.4 (Biomatters Ltd). The consensus sequence consisted of 755 bp of the mtDNA control region after trimming of tRNA gene sequences. We also trimmed sequences down to 380 bp to match the short mtDNA control region fragment described by Norman et al. (1994) and compared sequence diversity parameters to values reported for *C. caretta* in the previous studies. Mitochondrial haplotypes were classified by comparison to the mtDNA

sequences available on the Archie Carr Center for Sea Turtle Research database (ACCSTR; <http://accstr.ufl.edu/files/cclongmtdn a.pdf>). Relationships among haplotypes were inferred using an unrooted haplotype network based on the TCS inference approach of statistical parsimony implemented in POPART (Leigh and Bryant 2015). The method links haplotypes with the smaller number of differences as defined by a 95% confidence criterion (Templeton et al. 1992; Clement et al. 2000). Mitochondrial control region sequence diversity was inferred by the number of haplotypes (k), number of polymorphic sites (p), haplotype diversity (h), and nucleotide diversity (π) using ARLEQUIN 3.5.2.2 (Excoffier and Lischer 2010). The extent of mtDNA control region differentiation was investigated by principal coordinate analysis (PCoA) of a standardized pairwise haplotype distance matrix using GenAlEx 6.503 (Peakall and Smouse 2012). We then assessed the average number of pairwise differences in haplotype frequencies among turtles using the Φ -statistics of Excoffier et al. (1992) implemented in ARLEQUIN 3.5.2.2. The statistical significance of the Φ_{ST} values was obtained after 10,000 haplotype permutations. Following a preliminary pattern of haplotype clustering recovered by PCoA, Φ -statistics was performed by pooling samples according to sampling region, sampling season, and body size. The Adriatic Sea can be divided into a northern, central, and southern basin—each with distinct topographic, bathymetric, and dynamic features (Artegiani et al. 1997; Cushman-Roisin et al. 2001). Similarly, the eastern coast is generally high and rocky, whereas the western coast is low and mostly sandy. Such differences may be mirrored by latitudinal and longitudinal patterns of genetic divergence as recorded in several studies on marine vertebrates of the Adriatic (e.g., Garoia et al. 2004; Gaspari et al. 2015; Ruggeri et al. 2016). Geographical subdivision, therefore, considered a southern Adriatic region (southern Italy) and a northern group (Slovenia, Croatia, northern Italy), as well as a western Adriatic (Italy) and eastern Adriatic (Slovenia and Croatia) area. The seasonal partitioning divided samples collected during relatively colder months (November–May) from those obtained during warmer periods (June–October), the latter also being the nesting season for most Mediterranean populations. The differences between warmer and colder months in terms of loggerhead sea turtle distribution as well as the frequency of bycatch events were reported for the Adriatic Sea by Lazar et al. (2002). Moreover, sea temperature is known to affect the migratory strategies of *C. caretta* from nesting beaches to foraging grounds, as well as their seasonal habitat choice (e.g., Hawkes et al. 2007; Schofield et al. 2009). Cold months were defined as those showing average water temperatures lower than the average annual temperature recorded in the Adriatic Sea across 34 locations in Italy, Slovenia, and Croatia from 2013 to 2015 (available at <http://seatemperature.info/adriatic-sea-water-temperature.html>). Warm months were those with average temperatures equal to or higher than the annual average. Finally, sequences were analyzed by dividing our sample set into two sea

turtle body size classes defined according to the individual ability of swimming independently of water currents. Body size can affect the swimming ability of turtles and, therefore, their distribution under different sea-current speeds (Revelles et al. 2007). The mean speed of Adriatic currents, 0.30 m s^{-1} (Poulain 2001), was used to calculate the critical swimming speed (U_{crit}) which a turtle of a certain body size can sustain without enduring muscular fatigue as follows: $U_{\text{crit}} = 1.763 \text{ SCL} - 0.262$ (Revelles et al. 2007; Monzón-Argüello et al. 2009), where SCL is the straight carapace length (Bolten 1999). Turtles with an SCL of 32 cm or larger were defined as individuals capable of swimming independently of sea currents, while turtles with an SCL of less than 32 cm were defined as juveniles (Casale et al. 2005) whose movements were more likely to have been affected by currents. Conversion from curved to straight carapace length was conducted following the method described by Casale et al. (2017).

An MSA was performed using the Bayesian approach implemented in the MIXSTOCK package in R (Bolker and Okuyama 2014). The package compares mtDNA sequences from one or more mixed populations with baseline haplotypes from source populations (rookeries). We used the foraging ground centric approach to estimate the posterior distribution with 95% confidence intervals of unknown proportions of loggerhead turtle rookeries in the Adriatic foraging stock (i.e., the proportion of Adriatic sea turtle stock originating from distinct rookeries) (Pella and Masuda 2001; Bolker et al. 2003).

We assessed the composition of the Adriatic stock using a baseline of 10 rookeries from the Atlantic Ocean and 13 rookeries from the Mediterranean Sea (Fig. 1; Electronic Supplementary Material Table S1). Baseline sequences from the island of Zakynthos and the bays of Kyparissia and Lakonikos in the Peloponnese Peninsula were pooled into a single western Greece group according to Carreras et al. (2014) and non-significant pairwise Φ_{ST} values (ranging from -0.01 to -0.04 ; $P > 0.05$). Only short mtDNA control region sequences of less than 500 bp were available from the small rookeries of Lampedusa (Italy) and the Kuriat islands (Tunisia) (Laurent et al. 1998; Chaieb et al. 2010), so these rookeries were not included in the baseline. Haplotypes from single Mediterranean nests were also not part of our MSA for they could not be ascribed to a geographically defined rookery with a specific haplotype frequency. A few Mediterranean sites that receive sporadic nesting have the haplotype CC-A10.4 (Garofalo et al. 2016a; Maffucci et al. 2016), previously found with a 0.1% frequency in the Central East Florida rookery (Shamblin et al. 2012) and likely to occur in other Mediterranean rookeries. Samples carrying the CC-A10.4 haplotype were also excluded from the MSA in order not to overestimate the Atlantic contribution in a baseline where this haplotype is found in one Atlantic rookery only.

An MSA was also performed by pooling rookeries into Mediterranean, Northwest Atlantic, and

Northeast Atlantic RMUs (Wallace et al. 2010; Naro-Maciel et al. 2014). Subsequent analyses were conducted by (1) removing the contribution of the Atlantic nests from the baseline and (2) pooling 8 out of the 13 Mediterranean rookeries into four clusters, which included the two sites in Libya, Dalaman, and Dalayan in Turkey, middle, and eastern Turkey and the sites in Lebanon and Israel, respectively (Fig. 1; Electronic Supplementary Material Table S2). A similar subdivision of rookeries was proposed by Shamblin et al. (2014), whereby rookeries in Lebanon, Israel, Cyprus, eastern, and Middle Turkey were pooled into a single reference group. A Φ_{ST} test of pairwise differences in haplotype frequencies performed using ARLEQUIN 3.5.2.2 recorded no significant differences between rookeries pooled for MSA (Electronic Supplementary Material Table S3).

Priors were based on either of the following: (1) equal contribution from each rookery; (2) contribution weighted by population abundance; (3) contribution weighted by shortest swimming distance between rookeries and foraging ground; or (4) contribution weighted by both abundance and distance. The average number of nests per year was used as an estimate of population abundance (Electronic Supplementary Material Table S1). In the first MSA, where all 23 individual rookeries were considered, we used an equal contribution prior. On the other hand, all four different priors were used after pooling the eight rookeries from Libya, Lebanon, Israel, and Turkey into four distinct clusters. We also performed an MSA using the 380 bp mtDNA control region sequences to compare data obtained using mtDNA fragments of different length. As for the MSA based on the longer, 775 bp sequence fragment, the contribution of the Atlantic rookeries was close to zero (Electronic Supplementary Material Fig. S3). Subsequent analyses were, therefore, conducted following the same procedure used for the longer mtDNA control region sequence. We used a number of Markov chains equal to the number of source populations with 200,000 iterations and a 50% burnin. Convergence of Markov chains was assessed according to the Gelman–Rubin criterion whereby a value lower than 1.2 for all parameters is taken as a general rule of thumb to test for betweenchain variance being relatively smaller than withinchain variance, and for chains to converge (Gelman and Rubin 1992).

Results

Mitochondrial DNA control region sequence diversity

A total of 14 haplotypes were characterized in the *C. caretta* Adriatic foraging stock (Table 1). Four haplotypes (CC-A2.1, CC-A3.1, CC-A10.4, and CC-A20.1) were found in both Atlantic and Mediterranean rookeries and accounted for 91.6% of the Adriatic turtles. Among these, CC-A2.1 was the most abundant but the least informative as it is almost ubiquitous in Mediterranean

and Atlantic rookeries. Haplotype CC-A10.4 is found in occasional Tyrrhenian nesting sites in the Mediterranean Sea (Garofalo et al. 2016a; Maffucci et al. 2016; Fig. 1). Two out of three individuals carrying haplotype CC-A10.4 were sampled in the southern Adriatic, off the Apulian coast. Eight exclusively Mediterranean haplotypes (CC-A2.8, CC-A2.9, CC-A6.1, CC-A26.1, CC-A29.1, CC-A31.1, CC-A32.1, and CC-A53.1) accounted for 8.2% of the total Adriatic sequences. Eleven (2.3%) loggerheads carried haplotype CC-A2.8, which is specific to the Cretan rookery, while 10 (2.0%) individuals had haplotypes CC-A6.1 and CC-A32.1 endemic to western Greece. Two individuals sampled in Slovenia carried haplotype CC-A29.1, found only in the Israeli rookery. Haplotype CC-A26.1, exclusive to Sirte (Libya), was recorded in three individuals from the northern Adriatic and one individual from the Apulian waters. We found only one turtle with Turkish haplotype CC-A53.1, recovered in Slovenian waters. Similarly, only one haplotype CC-A31.1, assigned to Calabrian and Greek rookeries and recently found in occasional nesting sites in Sicily (Garofalo et al. 2016b), was found in the northwestern Adriatic. Only 0.2% of the foraging stock (one juvenile turtle sampled along the Apulian coast) carried the Atlantic haplotype CC-A1.1. One new haplotype was recorded in one individual sampled off the coast of Slovenia. The new haplotype differed from CC-A2.1 for one A-to-G transition at nucleotide site 15,648 of the published *C. caretta* mitochondrial genome sequence (Drosopoulou et al. 2012) (Fig. 2). The new sequence was named CC-A73.1 after submission to ACCSTR and deposited in GenBank under accession number MF182628. Haplotype CC-A73.1 was not considered in the MSA as its rookeries of origin have yet to be identified.

The number of haplotypes, number of polymorphic sites, and haplotype and nucleotide diversities were significantly higher in turtles sampled in the northern versus the southern Adriatic. Similarly, genetic diversity indices were higher in the eastern than in the western Adriatic (Table 2; Electronic Supplementary Material Table S4).

Eight polymorphic sites were recorded in turtles from the Apulian waters after removal of a single individual carrying haplotype CC-A1.1, which differed by 41 mutations from CC-A2.1 (see Electronic Supplementary Material Table S5 for genetic diversity measures obtained with the full data set). Haplotype and nucleotide diversities calculated for the short (380 bp) control region fragment were 0.22 ± 0.00 and 0.0006, respectively, for the northern Adriatic and 0.20 ± 0.00 and 0.0013, respectively, for the southern Adriatic. Molecular diversity indices were also significantly higher in turtles sampled during warmer months than in the colder season. A lower number of haplotypes and polymorphic sites were observed in adults versus juveniles, which accounted for 15% of our sample set. These values were recorded after removing the one individual carrying haplotype CC-A1.1 from the analysis.

The first and second coordinates of the PCoA explained 67.5 and 11.8% of the total variance, respectively. A pattern of haplotype differentiation similar to the one described by the statistical parsimony network was recovered, whereby CC-A26.1 and CC-A32.1 were the most divergent from the other haplotypes. However, the PCoA failed to recover a clear clustering between haplotypes exclusive of either the eastern (CC-A20.1, CC-A29.1, CC-A53.1, and CC-A73.1) or western (CC-A10.4, CC-A31.1, and CC-A32.1) Adriatic, and between haplotypes recorded in adults (CC-A31.1) or juveniles only (all others but CC-A2.1 and CC-A3.1). There were no haplotypes found only in warm or cold months or only in the northern or southern Adriatic (Electronic Supplementary Material Fig. S4). There was no significant differentiation in the average number of pairwise differences in haplotype frequencies as computed using Φ -statistics between the North and South Adriatic groups, West and East Adriatic groups, winter and summer groups, or body size clusters (Table 3). MSA was, therefore, conducted by pooling all samples in a single Adriatic stock.

Adriatic loggerhead turtle stock composition

The mixed-stock analysis performed using a baseline of 23 rookeries and equal contribution prior revealed a contribution of the Atlantic rookeries close to zero. However, the analysis had a shrink factor higher than 1.2 and reported relatively large confidence intervals for Mediterranean rookeries (Electronic Supplementary Material Table S6, Fig. S2). In contrast, the MSA of the relative contribution of three distinct RMUs to the Adriatic stock exhibited a shrink factor < 1.2 and very narrow confidence intervals. The analysis showed a remarkable discrepancy between contribution estimates of Atlantic rookeries, all very close to zero, and contribution proportions of Mediterranean rookeries (Fig. 3).

The MSA performed without Atlantic rookeries satisfied the Gelman–Rubin criteria, with shrink factors < 1.2 , and showed good chain convergence, albeit with wide confidence intervals recorded. According to the MSA with priors weighted by population abundance and distance from the rookeries, the western Greece rookery had the highest contribution to the Adriatic mixed stock ($P = 0.38$, 95% CI 0.14–0.67), followed by Crete ($P = 0.18$, 95% CI 1.65E-06 to 0.51) and western Turkey ($P = 0.18$, 95% CI 3.81E-20 to 0.47). Much lower contributions were recorded for rookeries from the remaining Turkish sites, Libya, Lebanon, and Israel (Fig. 4; Electronic Supplementary Material Table S7). A similar pattern was observed when using an equal prior, as well as a prior based on contribution weighted by either population abundance or distance only (Electronic Supplementary Material Figs. S5–S7). Western Greece was the most represented, while there was a slight increase in the contribution to the Adriatic stock from the rookeries of Israel and Lebanon when using an equal prior

(Electronic Supplementary Material Table S8).

Analysis of the shorter, 380 bp mtDNA control region fragment recovered a similar pattern of rookery contribution to the Adriatic stock except for Crete, which had a contribution approximately 10% lower than the one recorded using the longer control region sequence (Electronic Supplementary Material Figs. S8-S11). This is because the short sequence does not include a polymorphism of haplotype CC-A2.8, which is distinctive of Cretan rookeries.

DISCUSSION

The Adriatic Sea is one of the main Mediterranean European fishing grounds for both pelagic and demersal fisheries, in addition to being a very important feeding ground for *C. caretta*. This overlap highlights the urgent need for management actions to balance the exploitation of marine resources with the protection of vulnerable loggerhead turtles and their critical habitats across the Adriatic Sea. Describing the origin of loggerhead turtles frequenting the Adriatic and their genetic characterization can be particularly useful in the design of effective fishery management plans.

This study investigated the population genetic diversity of loggerhead turtles across the Adriatic Sea, using a comprehensive sample set from Slovenia, Croatia, and Italy. Stranded and bycaught individuals in Italian waters were obtained from the northern regions and from the southern area of the Gulf of Manfredonia—a particularly important foraging ground of conservation concern. Haplotype and nucleotide diversities were within the range of values recorded in the previous studies, despite such studies having been based on very small numbers of loggerheads sampled in the Adriatic Sea. For instance, Clusa et al. (2014) reported lower haplotype and nucleotide diversity values for juvenile *C. caretta* sampled in both the northern and southern Adriatic basins. Garofalo et al. (2013) recorded higher diversity values in the North Adriatic foraging ground, although the comparison was based on the short (380 bp) fragment of the mtDNA control region. The genetic diversity recovered in our study was, nevertheless, lower than the diversity values described for loggerheads sampled in other western and eastern Mediterranean foraging sites, probably because the Adriatic is geographically a semi-enclosed basin (Garofalo et al. 2013; Clusa et al. 2014).

Differences in genetic diversity measures were observed between the North and South Adriatic, West and East Adriatic, as well as between turtles of different body sizes or sampled in distinct seasons. While such differences may have been due to a discrepancy in sample size for the majority of comparisons, the eastern Adriatic had higher measures than the western Adriatic, despite the lower number of turtles sampled. Genetic divergence based on pairwise differences in haplotype frequencies was nil or negligible among sampling regions, between turtles sampled in winter and summer, and

between adults and juveniles. The absence of a clear genetic structure across the Adriatic basin strongly suggests the absence of a genetic distinctiveness of turtles foraging across an area extending from the northernmost part of the Adriatic Sea, genetically described here for the first time, to the southern Gulf of Manfredonia. The observed lack of genetic differentiation supports the hypothesis that loggerhead turtles do not disperse across the Adriatic foraging ground based specifically on their natal origin.

MSA based on RMUs recorded a negligible contribution from Atlantic rookeries to the Adriatic stock, corroborating the previous studies on loggerhead turtle distribution in the Mediterranean basin (Garofalo et al. 2013; Clusa et al. 2014). On the other hand, nesting sites in western Greece and Crete were the most important rookeries contributing to the Adriatic foraging population. Geographical proximity to the Adriatic basin, sea currents, and size of the Greek populations increase the likelihood of presence of adults, juveniles, and hatchlings from Greek rookeries in the Adriatic Sea (Casale et al. 2007; Casale and Mariani 2014). Moreover, eastern Mediterranean currents flow counterclockwise from North Africa to Turkey and the Balkan Peninsula (Fig. 1). Waters from the Aegean and Ionian Sea reach the northern Adriatic along the Croatian coastline and then flow back southwards to the Ionian Sea along the Italian peninsula (Millot and Taupier-Letage 2005; Cushman-Roisin et al. 2001). A likely migratory route to the Adriatic is, therefore, provided by sea currents flowing northwards from the Greek nesting grounds (Hays et al. 2010). Capture–mark–recapture and telemetry data from adult loggerhead turtles breeding in Mediterranean rookeries, and particularly in Greece, support genetic evidence of the extensive use of the Adriatic as a foraging ground for individuals from Greek nesting areas (Lazar et al. 2004; Luschi and Casale 2014). The fact that *C. caretta* from both the northern and the southern Adriatic foraging areas mainly originate from Greece is of particular conservation concern for the Greek population, which is likely to be affected by unmanaged Adriatic anthropogenic threats in greater proportion than previously estimated (Casale et al. 2004, 2010; Fortuna et al. 2010). In addition, increased numbers of nesting individuals or hatchlings from Greece would come under threat while feeding in the Adriatic, nullifying the conservation gains from the protection of nesting beaches.

Western Turkey had the third highest posterior probability of contributing to the Adriatic stock, along with the Libyan rookeries. The Libyan coastline is largely unsurveyed for the presence of nesting sites (Hamza 2010), meaning that the actual contribution to foraging areas might, in fact, be underestimated if a limited number of nests per year is used as the population abundance prior in the MSA. The Levantine coast (LBIS) nesting site had a surprisingly medium–high contribution, mainly as a result of two turtles recovered from the coast of Slovenia, in the northeastern Adriatic, carrying haplotype CC-A29.1, only recorded in Israeli rookeries to date. This haplotype might also occur in

other, larger rookeries that have not yet been sampled or are yet undiscovered, in which case the LBIS contribution to the Adriatic stock would be relatively lower. In fact, although rookeries from Cyprus and the southern and western Turkish coasts (pooled in the TKME and DYDL clusters, respectively) are closer to the Adriatic Sea than the Israeli nesting sites and noticeably more abundant (see Electronic Supplementary Material Table S1), they provided a similar or smaller contribution than the Levantine rookeries. The possibility that haplotype CC-A29.1 could originate from rookeries other than the Israeli ones implies that unknown or poorly sampled nesting areas might be important to loggerhead recruitment yet to be quantified.

Contribution from Calabrian nesting sites was close to zero despite the relatively short geographical distance from the Adriatic Sea. This is most probably due to the small rookery size of Calabria and the sea currents flowing southwards along the Ionian coast of Italy, hindering migration to the Adriatic basin (Millot and Taupier-Letage 2005). Moreover, the rookery from Calabria shares haplotype CC-A31.1 with both western Greece and an occasional nesting site recently described in Sicily (Garofalo et al. 2016b). This makes detection of a Calabrian contribution to the Adriatic stock even more difficult. The same is true for another rare haplotype, CC-A10.4, described only in occasional nesting sites along Tyrrhenian beaches (Garofalo et al. 2016a; Maffucci et al. 2016). The three individuals carrying CC-A10.4 are likely to originate from Mediterranean colonies where CC-A10.4 has yet to be described rather than from Tyrrhenian rookeries. The CC-A10.4 haplotype may be part of the CC-A10 haplogroup (based on sequencing of 380 bp of the mtDNA control region) recorded in Greece by Laurent et al. (1998) but never observed afterwards in the same rookery that was also the major source of our Adriatic mixed stock. In this study, we tried to optimize the MSA resolution primarily using a relatively large sample size. Although approximately 90% of the Adriatic stock included the most common CC-A2.1 and CC-A3.1 haplotypes, we found additional rare, rookery-specific sequences with respect to the previous studies based on much smaller sample sizes.

We recorded haplotypes CC-A26.1 and CC-A29.1 so far described in the Libyan rookery of Sirte and the Israeli nesting sites, respectively. Haplotype CC-A20.1, which occurs in several Atlantic rookeries and only in one Mediterranean nesting site (Calabria, southern Italy), was recorded in one turtle from the northeastern Adriatic. Moreover, one turtle sampled in the northern Adriatic, off the coast of Slovenia had haplotype CC-A53.1, which is specific to eastern and central Turkish rookeries and never previously reported in the Adriatic (Yilmaz et al. 2012; Garofalo et al. 2013; Clusa et al. 2014).

We further improved the MSA performance in detecting mixed stocks from the Mediterranean baseline by removing and pooling baseline rookeries. We found that using nine single and

clustered rookeries (namely the Libyan, Levantine, Cypriot, eastern, western and westernmost Turkish, Cretan, western Greek, and Calabrian) resulted in a relatively more informed baseline than using a set of reference sequences from 13 separate nesting grounds. Shamblin et al. (2014) pooled rookeries from Israel, Lebanon, Cyprus, and eastern and central Turkey into one single cluster despite significant Φ_{ST} values differentiating the Levantine and Cypriot nesting sites. Our scheme was similar to the one presented by Shamblin et al. (2014) except for retaining Cyprus, the two eastern Turkish sites (MTU and ETU) and the Levantine sites (LEB and ISR) as three distinct groups. The optimal grouping and/or splitting of rookery sites for baselines in MSA can, indeed, vary with geographical scale as well as conservation priorities. For instance, there may be compelling reasons to treat rookeries as demographically distinct for management purposes even in the absence of evidence of genetic differentiation. Formia et al. (2006) recommended recognition of Bioko and Ascension Island as demographically distinct rookeries despite lack of genetic divergence, given that they are distant by approximately 3000 km. A lack of marker resolution can make decisions on baseline structure even more complex, particularly at finer scale, such as in the eastern Mediterranean basin. Our pooling scheme considered geographically and ecologically close rookeries with similar genetic profiles, as recovered by Φ -statistics. This resulted in a relatively finer definition of some of the natal ground contributions to the Adriatic stock. For instance, the first MSA conducted with a baseline of 13 Mediterranean rookeries resulted in relatively wide confidence intervals, particularly for the contribution of the nesting sites in Misurata. Pooling of the Libyan rookeries drastically reduced the confidence intervals of the posterior probability of contribution to the Adriatic stock. Finally, priors weighted by population abundance (average number of nests per year) and length of migratory routes resulted in more realistic contributions from small, geographically distant rookeries. Approximately 20 and 10% of the Adriatic stock was found to include turtles from Lebanon and Israel, respectively, when using equal priors and priors weighted for population abundance. These values were reduced to less than 10% when using priors weighted for both abundance and geographical distance between nesting and foraging grounds.

MSA of Mediterranean foraging grounds is, nonetheless, hampered by two fundamental issues. First, the Mediterranean baseline is not a comprehensive one. For instance, frequencies of certain haplotypes were either large with respect to rookery size or inconsistent with the distance between sampling area and nesting sites. This suggests that haplotypes such as CC-A29.1 and CC-A10.4 might also originate at rookeries other than the ones described to date. Moreover, our finding of a novel mtDNA control region haplotype implies that more nesting areas need to be sampled (or sample sizes increased) to attain an inclusive baseline for sample assignment, particularly along the

largely unexplored coasts of Libya. Second, the resolution power of currently available mtDNA control region haplotypes seems inadequate to accurately describe unique rookeries and their fine-scale contributions to foraging grounds. Analysis of both mtDNA sequences and microsatellite allele frequencies can better recover patterns of genetic structure among nesting grounds (Carreras et al. 2007). For instance, an integrated approach proved to be a powerful tool for MSA and individual assignment of loggerhead turtle from western Mediterranean feeding grounds to either Atlantic or Mediterranean RMUs, whereby a 35% increase in robust assignment was recovered when using both markers rather than mtDNA alone (Carreras et al. 2011). Studies on interindividual variation using additional mtDNA and nuclear markers should, therefore, be explored, to better inform our understanding of rookery dispersal and distribution throughout the Mediterranean (Duchene et al. 2012; Novelletto et al. 2016).

Notwithstanding the need for improved genetic sampling at several Mediterranean rookeries, our results confirm a high degree of connectivity between the Adriatic and western Greece and Crete, and, therefore, the importance of collaborative management efforts throughout the range of these populations. While migratory routes to and from the Adriatic deserve concerted conservation attention, genetic results indicate that foraging loggerheads do not exhibit preferential habitat use within Adriatic sub-regions. However, further research is needed to assess whether they may be able to shift foraging zones in response to local threats or whether depletion in more threatened areas such as the northern Adriatic and the Gulf of Manfredonia may be compensated by shifts in distributions of the foraging population.

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6. Paper 3

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Qualitative and quantitative methods for estimating spirorchiidiasis burden in sea turtles

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Abstract

Infection by blood flukes *Hapalotrema mistroides* and *Neospororchis* sp. (Digenea: Spirorchiidae) has been recently reported in *Caretta caretta* (Linnaeus, 1758) in the Mediterranean Sea. Observations of post mortem lesions are generally used to assess disease severity, and few attempts have been made to standardize the evaluation of the parasitic burden from tissue egg counts. Faeces and spleen homogenates of 105 loggerheads from the northwestern Adriatic Sea were submitted to a sedimentation-flotation technique for the research of spirorchiid eggs; molecular techniques were used for unequivocal identification. Egg quantification for positive faeces and spleen samples was achieved using a modified McMaster method. Spleen samples were also submitted to quantification through the only method cited in the literature for similar purposes, which involves preventive chemical digestion. Correlations between splenic counts obtained from the two different methods and between faecal and splenic egg burdens were calculated using Spearman's rho test. Concordance between studies on eggs in faeces and spleen tissue was also calculated. Eggs of *H. mistroides* and Neogen-11 were found in spleen and faecal samples. Strong correlations were found between splenic egg burdens calculated from the two methods for *H. mistroides*, demonstrating that the modified McMaster method can be used for quantification. A multiplying factor must be used before drawing comparisons, as egg burdens are higher in value when measured after chemical digestion. High concordance was obtained from a qualitative examination of faeces and spleen tissue of *H. mistroides*, showing that copromicroscopic examination can be used for *in vivo* diagnosis. As weak correlations were found between faecal and splenic egg counts, faecal burden cannot be regarded as indicative of disease severity. For Neogen-11, low concordance was found between faeces and spleen tissue, likely reflecting lower levels of egg embolization in organs.

Keywords

Sea turtles; Spirorchiidae; McMaster; egg burden.

1. Introduction

Infections by blood flukes of the family Spirorchiidae (Digenea) have been recently re-described in loggerhead sea turtles from different regions of the Mediterranean Sea (Cribb et al., 2017; Santoro et al., 2017; Marchiori et al., 2017). The presence of *Hapalotrema mistroides* Monticelli, 1896 in the basin is historically recognized (Monticelli, 1896; Looss, 1899 and 1902; Gohar, 1934 and 1935) while *Neospororchis* sp. Neogen-11 and *Amphiorchis* sp. have been found in the region for the first time in recent years (Marchiori et al., 2017, Cribb et al., 2017). Epidemiological studies

characterizing distributions of different genera and effective impacts of the disease on sea turtle populations in the Mediterranean Sea are still lacking. Spirorchidiasis is globally found in sea turtles with varying degrees of severity: fatal disease is observed with asymptomatic infections, appearing as incidental findings during necropsy (Gordon et al., 1998; Flint et al., 2010; Stacy et al., 2010a; Santoro et al., 2017; Marchiori et al., 2017). Different risk factors for the acquisition of the infection and for disease severity are presented in the literature (e.g., geographical area, host species and parasites involved) (Work et al., 2005; Flint et al., 2010; Stacy et al., 2010a; Chapman et al., 2017). The severity of the disease is typically defined based on observations of gross and microscopic lesions during post mortem examination; few attempts to standardize the evaluation of parasitic burden have been made, generating an impact score based on one or more criteria: the number of granulomas per high power field (40X) (Flint et al., 2010; Santoro et al., 2017) or the number of adults and egg emboli and the presence and forms of organ injury (Stacy et al., 2010a), leaving room for subjectivity in operator expertise and in carcass preservation conditions. Complexities of such evaluations are compounded by incomplete knowledge of the site tropism of the different spirorchiid species. Quantifications of tissue egg burden are reported in only one work (Work et al., 2005) wherein the spleen is used as target tissue for such measurements while obtained values expressed as eggs per gram of spleen (epgs) are considered in an epidemiological analysis. Nevertheless, this method is limited by the cost of pepsin and by time-consuming procedures required for chemical digestion.

Copromicroscopic exams have also been used to detect spirorchiid infection (Wolke et al., 1982; Greiner, 2013) and are reported among methods used for ante mortem diagnosis, as this efficient and simple method can be easily applied *in vivo* in sea turtles rescue facilities. Nevertheless, faecal egg counts and infection sizes have been speculated to be unrelated as suggested by studies on schistosomiasis (De Bont et al., 2002) even though no specific studies on this matter have been carried out in relation to spirorchiiids.

The aim of this study is to propose and validate an efficient alternative means to quantify spirorchiid egg burdens of infested loggerhead spleen tissue and to compare derived results with those obtained with the only method published in the literature thus far (Work et al., 2005). To evaluate the feasibility of quantifying faecal egg output as an indicator, its correlations with egg burdens in spleen tissue are also investigated.

2. Materials and Methods

2.1 Sampling and laboratory analyses

In total, 105 dead loggerhead sea turtles found along northwestern Adriatic coast from 2013-2017 and were subjected to necropsy and successive analyses. During necropsy, stool samples taken from the rectum and spleen tissues were collected and stored frozen at -20°C at the Department of Animal Medicine, Productions and Health of Padova University for parasitological examination.

A qualitative analysis was performed on the spleens of all turtles for the detection of spirorchiid eggs through the use of a concentration-flotation technique described by Marchiori et al. (2017). Eggs were classified as type 1, 2 or 3 depending on their morphology according to Wolke et al. (1982).

Tissue egg burden quantification was applied to positive spleen samples using a new method based on mechanical homogenization (MH) and based on principles of the Mc Master technique, which is commonly used for faecal examination in different species of animals (Mc. Gordon and Whitlock, 1939). For this purpose, 2 grams of randomly selected splenic parenchyma were diced and homogenized in a blender in 6 ml of high specific gravity (s.g.) solution (sodium nitrate, sodium thiosulphate and sucrose, s.g. 1.450). Part of the obtained solution (0.30 ml) was then used to fill McMaster chambers. Egg counts were calculated as the number of eggs per gram of spleen (eggs) as follows: $\text{eggs} = \text{number of counted eggs} \times 10$.

To compare quantitative methods, a representative number of spleen samples (n=25) was selected and subjected to a second means of quantifying of tissue egg burdens through chemical homogenization (CH) as previously described by Work et al. (2005) with minimal modifications and as briefly described here. Two grams of randomly selected spleen were diced and left in PBS solution for 24 hours at 37°C. Homogenates were then digested in 2% (w/v) pepsin dissolved in 1% NaCl solution and in 0.03% HCl in a warm water bath (37°C) fitted to a shaker for 24 hours. The digested solutions were then filtered with coarse (100 µm) and fine (40 µm) mesh filters to recover spirorchiid eggs. The filters were then rinsed extensively with tap water to remove eggs from the meshes. The solutions were then centrifuged (3500 rpm x 5 min) and sediment was resuspended in 2000 µl of physiological solution. Egg counts were performed in an aliquot of 100µl of the obtained solution and their total volumes were calculated.

The filtered pepsin-solution was also centrifuged at 3500 rpm x 5 min and the sediment was observed to exclude the passage of type 3 eggs through filter meshes.

Faecal samples from the turtles were subjected to qualitative copromicroscopic examination by means of a common concentration-flotation technique using the same high s.g. solution applied to the spleen to identify spirorchiid eggs. Positive faecal samples were subjected to quantitative

analysis by use of the McMaster method using two grams of faeces in 6 ml of solution to provide an estimation of egg output in terms of eggs per gram (epg).

Molecular analyses were carried out on positive samples to identify spirorchiid species while targeting the ITS-2 spacer of rDNA as described by Stacy et al. (2010b).

2.2 Statistical analyses

The correlation between the two research methods (MH and CH methods) for the estimation of egg burdens in the spleen derived from quantitative estimation was investigated using Spearman's rho test. For both methods, a value of 5 epgs was arbitrarily assigned to samples deemed negative through quantitative estimation but positive through qualitative analysis while assuming a random distribution between the negative sample (=0 epgs) and threshold (=10 epgs). To evaluate the repeatability of the MH method, three samples with low, moderate and high egg counts were selected to repeat the same quantitative procedure on 8 different portions of the organ, including peripheral and central areas. The repeatability of the newly developed quantitative method on the spleen was assessed by investigating the CV (Coefficient of Variance expressed as a percentage).

To compare results derived using two matrixes (spleen and faeces), concordance between the copromicroscopic examination and the detection of eggs in spleen tissue from qualitative results (pos/neg) was calculated as the number of samples with same result of the total number of samples examined (% concordance). This was evaluated using kappa-type statistics (Landis and Koch, 1977), which express the proportion of agreement determined beyond chance with a value (parameter k) of 0 (no agreement) to 1 (perfect agreement).

Correlations found between quantitative estimations of the faeces and spleen samples (assessed via the MH method) were investigated by means of Spearman's rho test. In this case, the mean 5 epg value was assigned to positive samples under a threshold for quantitative analysis.

The overall level of statistical significance was set to $p < 0.05$. Statistical analyses were performed using the Excel[®] 14.7.1. and IBM SPSS Statistics 20 software programs.

3. Results

Only spirorchiid eggs of types 1 and 3 were identified in both faeces and spleen tissue (Fig. 1) with overall prevalence values of 27,6% (29/105) and 12,3% (13/105) for the two types, respectively.

Molecular analyses carried out on positive samples identified eggs of type 1 as *Hapalotrema mistroides* (GenBank accession number: LT617052) and eggs of type 3 as *Neospororchis* Neogen-

11 (GenBank accession number: LT617053) in all positive samples. We refer to eggs of type 1 as *H. mistroides* and to eggs of type 3 as Neogen-11 from here onward. The number of samples tested and the number of positive samples tested are reported in Table 1 for qualitative and quantitative analyses of both matrixes. Comparisons of quantitative results derived from individuals tested with both methodologies and of both matrixes are shown in Tables 2 and 3, respectively.

Qualitative and quantitative analyses of spleen tissues carried out with the MH method reveal tissue eggs of types 1 and 3 while the CH method did not detect any eggs of type 3. The correlation found between eggs values for spleen *H. mistroides* from the two quantitative methods was calculated from 25 samples (21 positive results from qualitative spleen testing; 2 samples with negative spleen results and positive results upon copromicroscopic examination; 2 samples with negative faeces and spleen results) with high Spearman's test results ($\rho=0.904$; $p<0.5$). Eggs values estimated from the CH method are generally higher with a factor of 2.5 as shown in Fig. 2. Correlations found for Neogen-11 were not calculated due to a lack of positive samples found from the CH analysis.

Regarding the repeatability of the MH method, average and standard deviation (SD) values of actual counts for the three series of 7/8 replications were respectively measured as 3,431.3 (SD=489.3) for sample 230/16, 525.0 (SD=148.6) for sample 192/16 and 58.6 (SD=20.4) for sample 194/17. It was thus estimated that samples with 100 eggs/gr would present a CV of roughly 33% (errors resulting from the methodology used allow for counts of 67 to 133 L1/g), that samples with 1,000 eggs/gr would present a CV of approximately 20%, and that samples with 10,000 eggs/gr would present a CV of approximately 12.5%.

Qualitative copromicroscopic exam results were found to be positive for 30 animals and mostly with *H. mistroides* (Tab. 1). Due to insufficient faecal material, a quantitative analysis was not performed on 1 faecal sample positive for *H. mistroides*. At the same time, two samples deemed negative for both types of eggs from qualitative faecal examination were subjected to quantitative analysis, as they belonged to animals with positive spleen results.

Concordance between qualitative copromicroscopic and qualitative exam results for spleen tissue (n=105) was observed at 95% (parameter $k=0.87$) and 92% (parameter $k=0.52$) for *Hapalotrema mistroides* and Neogen-11, respectively (Table 4 and 5). The correlation between the number of eggs of *H. mistroides* found in the same individuals (n=31) with modified McMaster levels in faeces and spleen tissue (MH method) was found to be low ($\rho=0.428$; $p=0.016$) as shown in Fig. 3. The same correlation was not investigated for Neogen-11 due to the limited number of positive samples observed.

4. Discussion

Attempts to standardize evaluations of the severity of spirorchiidiasis in sea turtles have been made based mostly on observations of gross and microscopic lesions (Stacy et al., 2010; Flint et al., 2010). The only quantitative method published (Work et al., 2005) evaluates the intensity of infection based on egg burdens expressed as eggs per gram of tissue while using the spleen as a target organ for such evaluation. The spleen indeed has been described as one of the most common sites of spirorchiid egg accumulation due to its role in blood filtering (Flint et al., 2009 and 2010). Nevertheless, the CH method (Work et al., 2005) takes considerable processing and mostly due to the duration of sample digestion with pepsin, which lasts up to 24 hours. The recovery of eggs from filters after digestion also necessitates a very careful and time-expensive procedure to guarantee the measurement of all eggs. Finally, economic costs of the procedure must take into account the cost of materials (pepsin solution, and filters) needed to carry it out. Through the present study we developed a new means of identifying spirorchiid eggs in spleen tissue that is as sensitive as the aforementioned method in detecting spirorchiid eggs in spleen tissue. Therefore, mechanical homogenization appears to be a sufficient means of releasing eggs from splenic parenchyma and is more efficient and cost effective because it exploits materials commonly used in laboratories. However, lower tissue egg burden values were detected through the mechanical process than through the chemical digestion of tissues when employing the CH method, suggesting that the proteolytic action of pepsin enhances the availability of eggs. Nevertheless, the strong correlation found between egg counts obtained from the two methods ($\rho=0,904$) shows that the two methods assess parasitic burdens of splenic tissue to similar degrees. However, a multiplying factor must be used to draw comparisons, as eggs values estimated using the CH method are nearly three times higher than those derived from the MH method.

According to our evaluation of the repeatability of the MH method, the CV method presented a decreasing trend with higher counts. The high percentage of CV found (roughly 30% in samples with roughly 100 eggs) may depend on variability intrinsic to the method in terms of a non-homogeneous distribution of eggs in the spleen with different parenchymal portions of the organ harbouring different egg burdens. In the future the precise definition of the specific part of the spleen to be sampled may improve the repeatability of the method. However, errors resulting from the methodology appear to be sufficiently limited when compared to the overall variability of eggs values that can be obtained from different turtles, which spans over thousands of units (in this study, from 0 to 11,300 eggs). As a consequence, this method appears to be reasonably repeatable for use in epidemiological studies.

Regarding *Neospiroorchis* Neogen-11, eggs of this genus were not found using the CH method in all samples deemed positive from the MH method. The filtered solution, after the digestion of the sample with pepsin, was also checked for the presence of *Neospiroorchis* eggs, which are very similar in size to filters with the smallest meshes (i.e., 40 µm), but this procedure never generated positive results, proving that eggs were not present in the sample after digestion. The absence of type 3 eggs observed when using the CH method is difficult to explain but may be due to the degeneration of such eggs during chemical digestion. Our results suggest that this method is not adapted to detect round eggs typical of genus *Neospiroorchis*.

Comparisons between different matrixes for the examination of spirorchiid eggs in sea turtles were drawn in this study to determine the reliability of copromicroscopic exams for detecting and quantifying of parasite burdens. Copromicroscopy has rarely been used for the detection of spirorchiid eggs during surveys of sea turtles (Wolke et al., 1982; Greiner, 2013; Santoro et al., 2017), as the observation of gross and microscopic lesions in post mortem exams in combination with the application of sensitive molecular tools (Chapman et al., 2016) allows for the detection of spirorchiidiasis without the need to repeat to such an exam. However, this approach can complement findings derived from other techniques to identify other stages of parasites or associated lesions. Copromicroscopy is another efficient and inexpensive diagnostic method that can be used *in vivo*, as ante mortem diagnosis remains difficult to achieve and as research on antibodies directed towards surface antigens is to date the only diagnostic tool available (Herbst et al., 1998, Graczyk et al., 1995, Work et al., 2005). Attempts to amplify DNA of spirorchiid flukes from blood samples have been recently made with negative results (Chapman, 2016).

Carcass decomposition and egg elimination through the decomposition of tissues have been hypothesized as potential means through which spirorchiids to emit their eggs into the environment and mostly for species with tropism for organs seemingly remote from access to the external environment (i.e., CNS and endocrine organs) (Stacy et al., 2010a; Stacy et al., 2017). Nevertheless, shedding by faecal route is very probable for all those species exhibiting a gastrointestinal localization of eggs and/or adults, including *H. mistroides* and the *Neospiroorchis* genotype examined here (Stacy et al., 2010a; Chapman et al., 2017; Marchiori et al., 2017). The presence of eggs of *H. mistroides* in the spleen with that in feces revealed high concordance values, demonstrating the potential usefulness of copromicroscopy for the detection of *H. mistroides* infections. The high likelihood of detecting *H. mistroides* eggs in spleen tissue may be explained by the localization of adult flukes, which are typically found in the left aorta or cardiac chamber and which likely release their eggs into the arterial system. Being the spleen a filter of arterial blood,

this could account for eggs emboli to be held in this organ (Flint et al., 2009). Further studies on egg distribution dynamics in the host's body could help confirm this hypothesis.

Quantitative estimations of parasitic burden in terms of eggs and egg are poorly correlated. This can be attributed to different dynamics of egg accumulation in the two matrixes. As suggested by Work et al. (2005), splenic egg burdens likely reflect chronic accumulation and infection by spirorchiids rather than adult spirorchiid burdens. The correlation observed between faecal counts and the number of adult worms is also difficult to assess and not necessarily existent as demonstrated in studies on schistosomiasis (Gryseels and De Vlas, 1996; De Bont et al., 2002). Difficulties associated with finding adult spirorchiids due to poor animal preservation conditions and to the well-known cryptic nature of these flukes prevented us from determining the value of this correlation in the examined animals by counting adult worms. Nevertheless, as pathological effects of spirorchiidiasis in turtles are known to be directly related to the presence of egg granulomas in tissues, splenic egg burdens appear to be more reliable than faecal egg counts in assessing disease severity in turtles; faecal egg burdens appear to have no utility in the assessment of disease effects on live sea turtles as speculated by Chapman (2016).

Regarding Neogen-11 we found low concordance between the two matrixes, and the limited number of positive samples observed prevented us from assessing this correlation through quantitative estimation. This finding may be justified by different migration dynamics observed within the definitive host of this parasite in comparison to those observed for *Hapalotrema*. In fact, adult flukes of genotype Neogen-11 deposit their eggs locally in the submucosa of the intestine, showing limited signs of embolization (Stacy et al., 2017) and thus explaining the bypassing of systemic circulation and of the spleen. This hypothesis is supported by low eggs values found for this species through spleen analysis compared to those derived via copromicroscopy.

Studies on the correlation between splenic and egg burdens of other organs are lacking in the literature, and the presence of preferential embolization sites in the host's body remains to be studied in relation to different spirorchiids species (Stacy et al., 2017). Studies of correlations between splenic egg burdens and other post-mortem data related to pathological effects of each spirorchiid species should be conducted to characterize the meaning of splenic egg burdens as a predictor of the impacts of infection on the host's health.

5. Conclusions

The proposed method, which is based on the mechanical homogenization of splenic parenchyma and a modified McMaster technique, is here demonstrated to serve as a good means of detecting

spirorchiids infection and calculating tissue egg burdens. The proposed method presents advantages over methods described in the literature (e.g., its efficient implementation and workability).

Research on *H. mistroides* eggs in the faeces of loggerhead turtles could be used to diagnose infections of this species *in vivo*. Nevertheless, faecal egg counts should not be regarded as indicative of the total parasitic burden and consequently of the severity of a disease affecting an animal.

The determination of parasitic burden expressed as eggs may be used as a starting point for future epidemiological studies assessing risk factors and disease impacts of different spirorchiid species found in the Mediterranean area.

Acknowledgments

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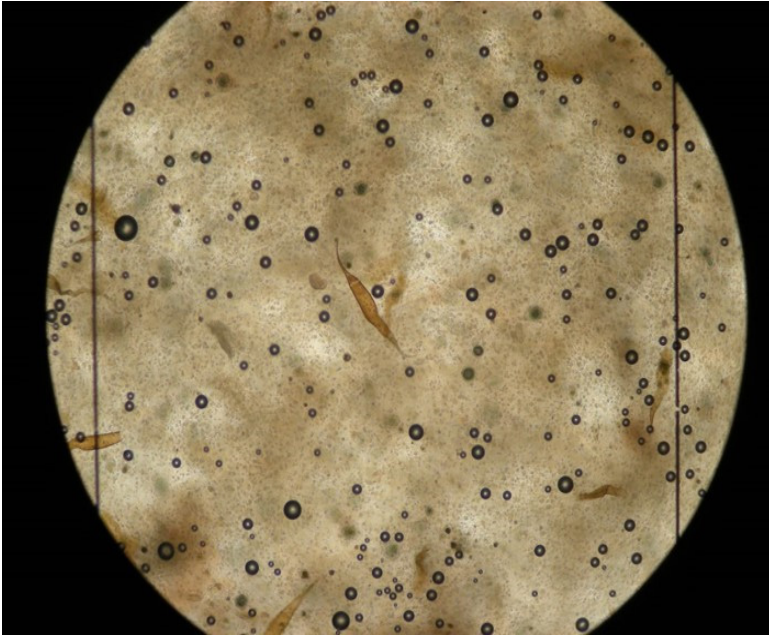


Figure 1
Eggs of *H. mistroides* in the McMaster chamber for quantification of splenic egg burden (Scale bar: 200µm)

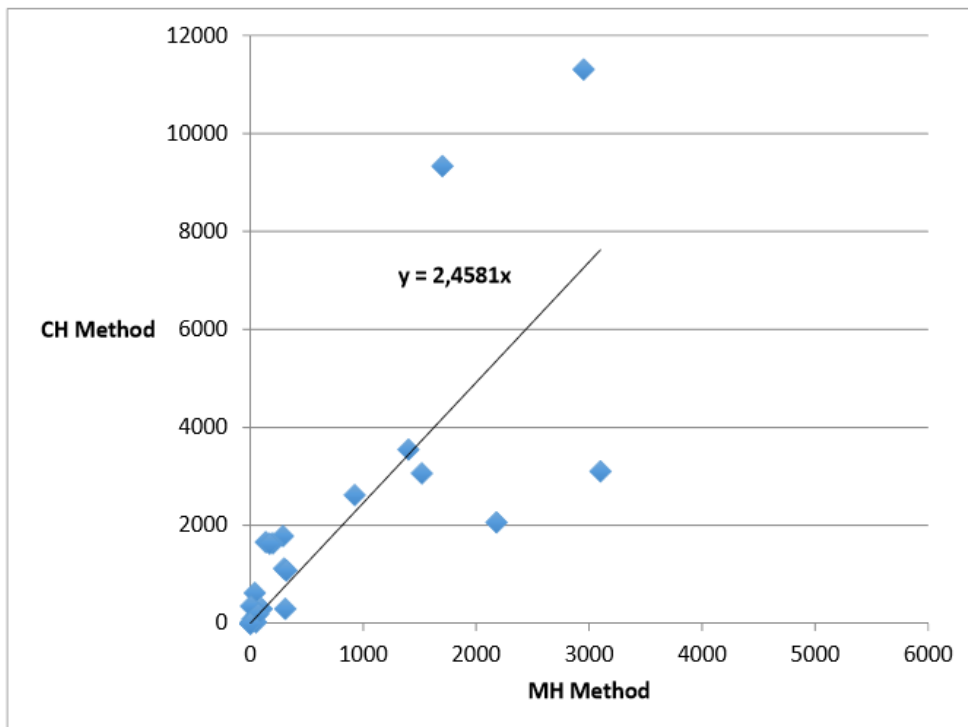


Figure 2
Scatter plot with trend line showing the correlation between eggs counts using the two quantitative methods in the spleen.

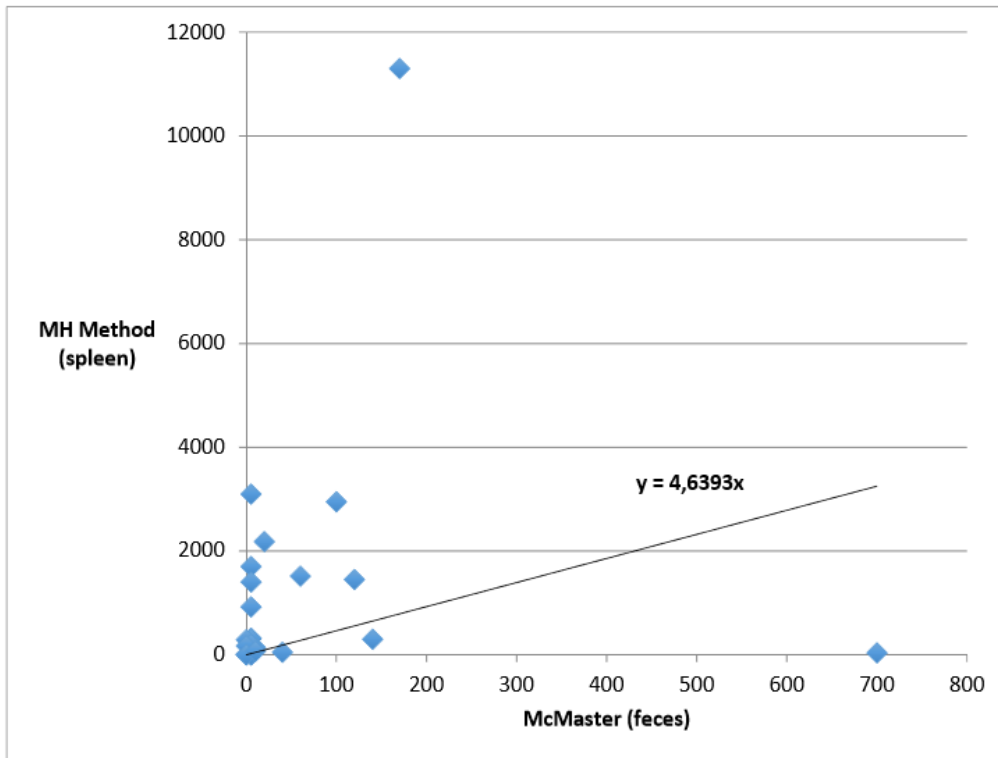


Figure 3

Scatter plot with trend line showing the correlation between fecal egg output and splenic egg burden calculated by Method MH.

Tables

Table 1 – Results of qualitative and quantitative analyses in spleen and feces samples

Tested individuals	Matrix	Analysis	Eggs type 1 (<i>Hapalotrema mistroides</i>)					Eggs type 3 (<i>Neospororchis</i> sp. Neogen-11)			
			Positive	Positive ^a	Range (epg/s)	Average	St. Dev.	Positive ^a	Range (epg/s)	Average	St. Dev.
All individuals (n=105)	Spleen	Qualitative	27	26	-	-	-	5	-	-	-
Individuals positive to qualitative analysis (n=27)	Spleen	Quantitative (MH)	-	25	0-11,300	1,065.4	2,242.2	3	0-20	2.2	5.6
All individuals (n=105)	Feces	Qualitative	30	27	-	-	-	13	-	-	-
Individuals positive to qualitative analysis (n=31)	Feces	Quantitative (McMaster)	-	10	0-700	46.8	129.3	9	0-533	30.4	100.3

^aSamples negative at quantitative analysis, but positive at qualitative are considered negative in these columns; St. Dev., Standard deviation.

Table 2 – Comparison between results of quantitative analyses in spleen using the two methodologies (MH and CH)

Tested individuals	Matrix	Analysis	Eggs type 1 (<i>Hapalotrema mistroides</i>)				Eggs type 3 (<i>Neospororchis</i> sp. Neogen-11)			
			Positive ^a	Range (epg/s)	Average	St. Dev.	Positive ^a	Range (epg/s)	Average	St. Dev.
Individuals tested by both quantitative methods (n=25)	Spleen	Quantitative (MH)	21	0-3,100	633.4	947.0	2	0-20	1.6	4.5
	Spleen	Quantitative (CH)	21	0-11,304	1,838.8	2,795.0	0	-	-	-

Table 3 – Comparison between results of quantitative analyses in spleen and feces

Tested individuals	Matrix	Analysis	Eggs type 1 (<i>Hapalotrema mistroides</i>)				Eggs type 3 (<i>Neospororchis</i> sp. Neogen-11)			
			Positive ^a	Range (epg/s)	Average	St. Dev.	Positive ^a	Range (epg/s)	Average	St. Dev.
Individuals tested by both quantitative methods (n=31)	Feces	Quantitative (McMaster)	10	0-700	46.8	129.3	9	0-533	30.4	100.3
	Spleen	Quantitative (MH)	25	0-11,300	927.9	2,118.7	3	0-20	1.9	5.3

		Feces		
		Negative	Positive	Total
Spleen	Negative	76	3	79
	Positive	2	24	26
	Total	78	27	105

Table 4 - Concordance matrix for Eggs type 1 (*H. mistroides*) detected by qualitative exam in feces and spleen

		Feces		
		Negative	Positive	Total
Spleen	Negative	92	8	100
	Positive	0	5	5
	Total	92	13	105

Table 5 – Concordance matrix for Eggs type 3 (*Neospororchis* Neogen-11) detected by qualitative exam in feces and spleen

7. Scientific communications

Scientific communication 1

XXIX Congress SolPa, Bari, 21-24 June 2016. Atti, p. 40. Presentazione orale.

FIRST REPORT OF GENUS *NEOSPIRORCHIS* IN SEA TURTLES FROM MEDITERRANEAN SEA

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Spirorchiid flukes (Digenea: Spirorchiidae) are parasites of circulatory system of freshwater and sea turtles. The presence of adults in heart and vessels and the spreading of eggs to various organs can lead to severe vasculitis, thrombosis and development of disseminated granulomas. *Neospororchis* sp. in particular has been associated to meningitis and mortality mass event because of its tropism for Central Nervous System (CNS) (Jacobson *et al.*, 2006).

One hundred forty-four carcasses of loggerhead turtle (*Caretta caretta*), stranded along North-Eastern coast of Adriatic sea in the period 2009-2015 were analyzed for spirorchidiasis. After necropsies, research of parasitic elements by stereomicroscopy in major vessels and organs was performed, followed by copromicroscopic analysis and histological examination of tissues. Internal transcribed spacer 2 region (ITS2) from parasitic elements was amplified and sequenced for comparison with data in literature.

Neospororchis eggs (Type 3) were identified in faecal samples of six turtles (4.16%); four out of these had a mixed infection with *Hapalotrema*. No adults of *Neospororchis* were found. Type 3 egg masses were grossly visible as black short stripes on the intestinal mucosa; big clusters of rounded eggs surrounded by granulomatous inflammation were histologically visible in mucosal and submucosal layers. Small granulomas surrounding isolated spirorchiid eggs were ubiquitous in several organs, but not in CNS. ITS2 sequences obtained from isolated eggs matched (100% identity) with those of *Neospororchis* (Neogen11) described by Stacy (2008). To identify the possible origin of the infected

turtles, sequences of mtDNA encompassing the D-loop region were analyzed. All turtles were carriers of Mediterranean haplotypes.

This represents the first report of genus *Neospirochis* in *C. caretta* living in the Mediterranean Sea. In this study spirorchidiasis seems not to have severely affected health status of the host, being lesions always mild in all districts.

Scientific communication 2

37th Annual Symposium on Sea Turtle Biology and Conservation, Las Vegas NV (USA), 15-20 April 2017. Presentazione orale.

PATHOLOGICAL FINDINGS IN SEA TURTLES STRANDED ALONG THE NORTHWESTERN COAST OF THE ADRIATIC SEA

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The North Adriatic Sea represents one of the most important foraging grounds for loggerheads (*Caretta caretta*) in Mediterranean basin. Nevertheless, the area is also intensively exploited by anthropic activities; according to literature, bycatch and boat strikes represent most relevant causes of death for turtles in the area, though only sparse studies have been carried out on pathological issues on stranded animals.

Sea turtles strandings along Veneto coast (north western Adriatic) were monitored in the years 2013-2016, thanks to a collaboration with institutional Authorities, WWF Italy, Museum of Natural History of Venice and many others, in the framework of the European IPA Project NETCET. Dead animals were transferred to Padua University to perform necropsy and subsequent analyses.

A total of 252 carcasses were transported to the necropsy room; 172 out of these were in advanced decomposition state or mummified. When preservation conditions allowed it, life stage (N=233) and sex (N=176) were assigned according to biometric data and gonad inspection; gross lesions were investigated (N=138) and histological (N=59), bacteriological (cultures and molecular analysis) (N=68), parasitological (N=205) and virological (Transmission Electron Microscope -TEM) (N=4) analyses were performed. Whenever possible, a death cause was formulated. Badly preserved or mummified turtles were also included for evidence of human interaction (boat collisions, entanglement, hooks, lines).

Carcasses in the study period (N= 37 in 2013, N= 71 in 2014, N= 100 in 2015, N= 44 in 2016) were distributed in 3 age classes as follows: juveniles N=68 (38 females, 15 males, 15 undetermined),

subadults N=129 (60 females, 33 males, 36 undetermined), adults N=35 (19 females, 8 males, 8 undetermined).

The most frequent finding was multifocal hydrodynamic imbalance (N=93), showing as: mild to severe multifocal hemorrhagic edemas (especially perirenal, subcutaneous and on muscular masses; N=66), severe sero-hematic effusions in celomic and pericardial cavities (N=45), moderate internal organs congestion (N=25), often associated with diffuse, moderate to severe, acute to subacute enteritis (N=28).

Lesions connected to anthropic interaction were seen in 19 turtles, including acute, fatal (N=6) and chronic injuries (N=7) by boat impact, amputated limbs from line entanglement (N=4) and mortal ingestion of hooks and lines (N=2). Moreover, plastic debris was collected from gut content of 4 over 113 observed carcasses. Anemia and cachexia syndrome was detected in 6 turtles.

The most frequently isolated bacterial strain was *Photobacterium damsela damsela* (N=13), isolated from intestinal tract (N=11), heart (N=6) and central nervous system (N=1); in 4 animals the strain was isolated from more than one district. Research of virions from the intestinal tract by TEM were negative.

Ectoparasite leeches (*Ozobranchus margo*)(N=15/ 155), gastrointestinal helminth parasites belonging to 7 taxa (N=82/113) and spirorchidiasis infection (N=34/205) were also detected.

Unusual and still unexplained peaks of mortality were observed in autumn 2014 and summer 2015, when the number of dead turtles just in the period June-July 2015 clearly exceeded the total annual amount of the year 2013 and of 2016. Pathological and collateral analyses, such as microbiological and parasitological findings, are widely discussed to highlight possible hypothesis to explain the exceptional events.

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37th Annual Symposium on Sea Turtle Biology and Conservation, Las Vegas NV (USA), 15-20 April 2017. Poster.

PREVALENCE AND HOST PATHOLOGY OF SPIRORCHIIDIASIS IN STRANDED LOGGERHEADS (*CARETTA CARETTA*) IN NORTH WESTERN ADRIATIC SEA

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The Adriatic Sea represents an important foraging area for subadult and adult loggerhead turtles. Cardiovascular flukes (Digenea: Spirorchiidae) are considered worldwide an important cause of stranding and mortality in sea turtles, including loggerheads. The presence of adults in heart and vessels and the spreading of eggs to various organs can lead to severe vasculitis, thrombosis and development of disseminated granulomas. *Hapalotrema mistroides* is the only species historically reported in Mediterranean Sea, but updated data on species and prevalence still lack. The aim of this study was to investigate presence and pathological lesions of spirochiidiasis in *C. caretta* spanning over Mediterranean Sea.

Two hundred five carcasses of *C. caretta* stranded along the Italian coastline of the Northern Adriatic Sea between June 2013 and October 2016 were submitted to necropsies and parasitological analyses in order to detect spirorchiids infection (research of adult blood flukes in cardiovascular system, detection of Spirorchiid eggs in spleen and in faecal samples by sedimentation and flotation procedure). Tissue samples from different organs were formalin-fixed, paraffin-embedded and routinely processed for histological examination in order to detect Spirorchiid eggs and lesions. Adult flukes and eggs (type: 1, 2, 3) were morphologically identified according to the available literature references. Internal transcribed spacer 2 region (ITS2) from parasitic elements was amplified and sequenced for comparison with data in literature. In order to identify the possible origin of the infected turtles, sequences of mtDNA encompassing the D-loop region were analyzed.

Spirorchiidiasis was detected in thirtyfour animals (16.6%). *Hapalotrema* and *Neospororchis* infection were found in seventeen and two cases respectively, while mixed infections were observed

in additional fifteen animals. Adult parasites of the genus *Hapalotrema* were found in six animals; no adult flukes of genus *Neospororchis* were isolated. Spirorchiid eggs were observed in faecal samples of thirty-three infected turtles, while in one positive animal the eggs were detected only in the spleen. Molecular identification unambiguously assigned the spirorchiid samples to the taxa *Hapalotrema mistroides* and *Neospororchis* sp. In particular, ITS2 sequences obtained from eggs type 3 matched with those of *Neospororchis* sp. *Neogen* 11 sequence, obtained from a specimen previously isolated from gastrointestinal tract of *C. caretta* in Florida.

Only mild lesions were observed: eggs of type 1 and 3 were grossly visible in the gastrointestinal mucosa; focal vasculitis was rarely observed in heart and great vessels, while miliary granulomas were widespread in the tissues.

Genetic analyses, made on the D-loop region of mitochondrial DNA, pointed to a Mediterranean origin of the turtle hosts.

This survey provides new data on the spreading of spirorchiidiasis in Mediterranean loggerhead population, reporting for the first time *Neospororchis* genus in this basin and confirming the presence of *H. mistroides*. In this study spirorchiidiasis seems not to have severely affected health status of the host, being associated to mild lesions in all districts.

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Scientific communication 4

IV Congresso Nazionale di Ecopatologia della Fauna, Domodossola (VB), 11-13 Ottobre 2017.

Poster.

GASTROINTESTINAL HELMINTH COMMUNITY OF *CARETTA CARETTA* IN THE NORTH WESTERN ADRIATIC SEA: PRELIMINARY RESULTS

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The Adriatic Sea is one of the most important neritic feeding grounds for *Caretta caretta* inside the Mediterranean basin. The diet of this species at neritic ontogenic stage includes a variety of benthic preys, as molluscan and crustacean species, that are often involved as intermediate host in the indirect life cycles of digenetic trematodes and nematodes. The aim of this survey was to describe gastrointestinal helminthic fauna of loggerheads feeding in northern Adriatic grounds and to compare it with surveys made in other Mediterranean areas.

In the period 2009-2016, 113 loggerhead turtles, found stranded dead along Italian north Adriatic coasts, were investigated for the presence of gastrointestinal (GI) parasites. Content of stomach and gut were examined by mean of a filtration-sedimentation process. The parasites were isolated at stereomicroscopy, counted and stored in 70% alcohol. Identification of parasites was achieved by observation of morphometric features at stereo and light microscopy and comparison with literature. Prevalence (P), mean intensity (MI), mean abundance (MA) and relative abundance (RA) were estimated for each parasite species.

Totally 62.8% (71/113) of the turtles were positive for the presence of GI parasites, with total burden ranging from 0 to 626 individuals (mean abundance: 32.4 helminth parasites per turtle). Eight species of two different taxa were found: *Sulcascaris sulcata* (P=23.9%; MI=29.7; MA=7.1; RA=21.9%) and *Kathlania leptura* (P=5.3%; MI=78.5; MA=4.2; RA=12.9%) among Nematoda. *Rhytidodes gelatinosus* (P=38.9%; MI=23.2; MA=9; RA=27.9%), *Pleurogonius trigonocephalus* (P=15.9%; MI=30.3; MA=4.8; RA=14.9%), *Pachypsolus irroratus* (P=22.1%; MI=11.0; MA=2.4; RA=7.5%), *Orchidasma amphiorchis* (P=19.5%; MI=11.8; MA=2.3; RA=7.1%), *Enodiotrema megachondrus* (P=15.9%; MI=15.8; MA=2.5; RA=4.2%) and *Calycodes anthos* (P=1.8%; MI=2.0; MA=0.0; RA=0.1%) among Trematoda.

All the species encountered in our study were already observed in other loggerhead populations. The number of isolated taxa (richness index: 8) is similar to that observed in precedent surveys in the Adriatic basin (Gračan et al. 2009). However, these values are lower than number of parasite species reported in turtle populations of north Atlantic neritic areas, where up to 16 different species of GI parasites were recorded in single turtle populations (Greiner 2013).

R. gelatinosus and *S. sulcata* showed the highest values of prevalence and abundance. They were reported in all previous studies in the Adriatic Sea with similar prevalence. This finding copy with assumption that life cycles of these parasites involve benthic intermediate hosts, mostly present in the Eastern part of the Mediterranean basin and thus they are typically present among the helminthofauna of neritic stage turtles. On the contrary, *E. megachondrus* and *C. anthos* life cycles is supposed to involve pelagic intermediate hosts, and their abundance and prevalence are usually higher in oceanic habitats. Nevertheless, these two species were encountered in the investigated population and *E. megachondrus* in particular showed an unexpected high prevalence value. The significance of this finding has to be further addressed.

In conclusion, the investigated population showed a moderate prevalence of infection. Light infection levels (<100 parasite specimens per host) were present in most cases (63/71). The influence of biological and ecological parameters on infection levels and species prevalence should be evaluated, taking into account age and sex of the host and season.

COMPARISON BETWEEN TWO QUANTITATIVE METHODS AND TWO DIFFERENT MATRIXES FOR THE EVALUATION OF PARASITIC BURDEN IN *HAPALOTREMA MISTROIDES* INFECTIONS

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INTRODUCTION - Infection by the blood fluke *Hapalotrema mistroides* (Digenea: Spirorchiidae) has been recently reported in sea turtles *Caretta caretta* in different Mediterranean regions (Santoro et al., 2016, Dis Aquat Org 124:101-8, Marchiori et al., 2017, Parasites&Vectors 10: 467). Eggs of *H. mistroides* are commonly found in all host's organs, but release of eggs probably happens by fecal route (Stacy et al., 2010, Dis Aquat Org 89:237-259). Observation of post mortem gross and microscopic lesions is generally used to assess severity of the disease (Flint 2010), but few attempts have been done to standardize the evaluation of the parasitic burden by tissue egg counts (Work et al., 2005, J Parasitol 91(4):871-876).

MATERIALS AND METHODS - Feces and spleen homogenates of 105 loggerheads, stranded dead along North-western Adriatic Sea in the period 2013-2017, were submitted to a sedimentation-flotation technique for the research of spirorchiids eggs; quantification of eggs was then achieved in positive feces and spleen by a modified McMaster method (2 gr of feces/spleen homogenate in 6 ml of high density solution, s.g. 1450). Spleen homogenates were also submitted to a second quantification by the method previously proposed by Work et al. 2005 (J. Parasitol., 91(4):871-876), which includes a preventive chemical digestion of tissues with a 2% pepsin - 0,03% HCl solution. Concordance between research of eggs in feces and spleen was calculated and evaluated using the kappa-type statistics (Landis and Koch, 1977, Biometrics 33:159-174). Correlation between fecal and splenic egg counts and between splenic counts obtained with the 2 different methods were calculated using the test Rho of Spearman.

RESULTS AND CONCLUSIONS - High concordance was obtained between qualitative examination of feces and spleen (95%, $k=0,874$), revealing that copromicroscopic exam can be an appropriate method for the diagnosis of the infection *in vivo*. Low correlation ($Rho=0,418$) was found between fecal egg counts and splenic egg burden, thus fecal burden cannot be regarded as indicative of disease severity. High correlation was instead found between splenic egg burden calculated with the two methods ($Rho=0,904$). Thus modified McMaster method can represent a good alternative for counting tissue egg burden compared to that of Work et al. (2005), in consideration of the fact that it is faster and cheaper. However, counts result underestimated with modified McMaster method, therefore appropriate ranges must be calculated to compare results between the two methods and to correlate them with severity of the infection in the investigated geographic area.

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AN EPIDEMIOLOGICAL UPDATING ON THE NEMATODE *SULCASCARIS SULCATA* (ANISAKIDAE, NEMATODA) IN ADRIATIC SEA

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INTRODUCTION - The nematode *Sulcascaris sulcata* is frequently recovered in stomach and esophagus of loggerhead sea turtles *Caretta caretta* in neritic feeding grounds worldwide. The existence of an intermediate host in its life cycle, represented by benthic gastropods and bivalves (Berry and Cannon, 1981, Int. J. Parasitol. 11: 43-54), accounts for its presence in shallow coastal waters, such as those of the Eastern Mediterranean and the Adriatic Sea (Gracan et al. 2012; DAO 99:227-236). The adult parasite, harboring in the stomach of marine turtles is associated to cases of ulcerative gastritis (Santoro et al. 2010, Parassitologia 52:364). The aim of this work is to provide an updating on the epidemiology of *S. sulcata* in Adriatic Sea.

MATERIALS AND METHODS - During five years (2013-2017), the whole digestive tract of 134 loggerheads stranded along Northern Adriatic Sea was opened and examined to search for *S. sulcata* parasites. The isolated parasites were fixed in 70% ethanol, clarified in Amman's lactophenol and identified following the dichotomous keys. Copromicroscopic exam by sedimentation and flotation method with high-density solution (s.g.1.450) was carried out in all animals (n=134). Besides, in 2017, during routine sampling, 37 fresh scallops (*Pecten jacobaeus*) caught in Northern Adriatic Sea were submitted to parasitological examination. All the collected parasites were morphologically and molecularly identified by a PCR-RFLP specific for the family Anisakidae and a PCR targeting the Cox-2 mtDNA gene.

RESULTS AND CONCLUSIONS - Adult and/or larval stages or of *S. sulcata* were found in the digestive tract of 40 loggerheads and eggs were observed in the feces of other 12 animals (overall prevalence: 38.8%). Gastric ulcers were observed in fifteen animals. In eight animals, parasites were detected, with copromicroscopic exam being negative; most of these turtles had only immature

parasites or few specimens in the gastrointestinal tract. In all cases in which only eggs of *S. sulcata* were found, the bad conservation of the carcasses might have prevented the finding. Eighteen specimens (48.7%) of scallops were positive for larvae of *S. sulcata* in the muscle. The prevalence of *S. sulcata* in *C. caretta* in this study is higher than that previously reported by other Authors in Northern Adriatic Sea (Gracan et al. 2012; DAO 99:227-236). Although *S. sulcata* larvae have been found in several mollusc species in the world, *P. jacobaeus* is the only intermediate host detected in the Mediterranean basin. Further samplings will be required in order to define the real prevalence of *S. sulcata* in this intermediate host and to identify any other potential intermediate host of the parasite in this important foraging area for Mediterranean loggerhead population.

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XXX Congresso SoIPA, Milano 23-26 Giugno 2018. Poster

FIRST DETECTION OF *CUCULLANUS CARETTAE* BAYLIS, 1923 (NEMATODA: RHABDITIDA) IN LOGGERHEAD TURTLE (*CARETTA CARETTA*) FROM THE ADRIATIC SEA

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INTRODUCTION - *Cucullanus carettae* (Baylis 1923), is a nematode belonging to the subfamily Cucullaninae that has been described worldwide in loggerhead turtles (*Caretta caretta*). Regarding the Mediterranean, *C. carettae* has been just identified in Tyrrhenian and Ionian Sea (Santoro et al., 2010, Parasitol. Int., 59, 367-375). Conversely, until now a description of a unique specimen of *Cucullanus* sp. in loggerhead from the Adriatic sea is reported in literature (Piccolo and Manfredi, Proceedings, of First Mediterranean Conference on Marine Turtles. Rome, 2001, 207-2011).

MATERIALS AND METHODS - In a framework of a bio monitoring project of Abruzzo and Molise coasts, a parasitological survey was performed on stranded and accidentally caught sea turtles, at Istituto Zooprofilattico of Abruzzo and Molise “G.Caporale“. During necropsy, the gastrointestinal system of 73 stranded sea turtles (72 *C. caretta* and 1 *Chelonia mydas*) was inspected for the isolation and the collection of the parasites. At the same time intestine samples were collected for histology. Furthermore, 60 and 62 samples of feces were also collected from dead and alive animals respectively and were submitted to sedimentation and flotation technique, using a high density solution (s.g. 1300) for the detection of parasites eggs. Adult parasites of the genus *Cucullanus* were identified according to the specific literature.

RESULTS AND CONCLUSIONS - A massive infestation by *C. carettae* were found in the intestine of one loggerhead, (1.4% positive) associated with chronic lympho-plasmocytic enteritis, while the remaining 72 were negative for the presence of the parasite. To our knowledge this is the first identification of *C. carettae* in loggerhead turtles from Adriatic sea. In addition, three fecal samples from alive turtles and five stools (8/122; 6.6%), collected during necropsy, were positive for *C. carettae* eggs. Additional studies are needed to gain knowledge on the real prevalence and distribution of *C. carettae* in the Adriatic sea compared to other Mediterranean areas. Its presence among the helminthofauna of the animal could indeed contribute to track turtles migratory routes and also to assess the possible impact on this endangered sea turtle species.

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XXX Congresso SoIPA, Milano 23-26 Giugno 2018. Presentazione orale.

SEVERE CASE OF SPIRORCHIDIASIS IN A LOGGERHEAD SEA TURTLE (*CARETTA CARETTA*) FROM ADRIATIC SEA

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INTRODUCTION - Spirorchiid infections are considered the most important parasitic disease cause of stranding and mortality of sea turtles worldwide. According to Marchiori et al. (2017, *Parasites&Vectors* 10: 467), two species of spirorchiids are present in Adriatic Sea, nevertheless they seem to have not a causal effect on the death nor a strong impact on the general health status of sea turtle population in this area. This work reports a case of a severe spirorchiid infection in a specimen of loggerhead stranded along the Abruzzo coasts.

MATERIALS AND METHODS - In February 2018, an adult male of loggerhead (curved carapace length: 75 cm; weight: 44.7kg) accidentally caught was promptly hospitalized at Center Recovery treatment and rehabilitation Marine turtle (CRTM) “L. Cagnolaro”. The turtle showed lethargy, listlessness, neurological compromising and penile prolapse. Twenty-four hours later, the turtle died and it was necropsied at the Istituto Zooprofilattico Sperimentale dell’Abruzzo e del Molise “G. Caporale” (IZSAM). Histological and parasitological exams were carried out. Spirorchiid elements were morphologically and molecularly identified, by use of a PCR targeting the 28S gene and ITS2 spacer of rDNA.

RESULTS AND CONCLUSIONS – The turtle was thin with shells shrunken and with the central plastron area markedly depressed. The coloration of the coelomic organs was pale in respect of their normal coloration. Epididymism and vas deferents were bilaterally abnormal observed. Eggs of spirorchiids of both Type1 and Type 3 were observed by stereomicroscope in various organs.

Histological examination showed disseminated eggs in pancreas, spleen, kidney, lung, brain, intestine, adrenal gland and thymus. In the genital tract lesions of vas deferens and epididymis were also associated to spirorchiid eggs. also associated to spirorchiid eggs. Severe and diffuse multifocal granulomatous reactions surrounding numerous fluke eggs were observed. The eggs of type 1 and 3 were molecularly identified as *Hapalotrema mistroides* and *Neospororchis* sp. Neogen 11 respectively, confirming a co-infestation. This is the first case of severe spirorchiidiasis described in free-ranging loggerhead turtles in the Adriatic Sea, similar to that recently described from Tyrrhenian Sea (Santoro et al. 2017, Dis Aquat Org 124:101-8). Much is still left to know on the epidemiology of this parasitic disease in the Mediterranean basin, including genetics of hosts, identification of intermediate hosts, risk factors. Significant organ injury was associated to multisystemic embolization of eggs in this case. Impairment of circulatory system due to disseminated granulomatous lesions could have been contributory to death in this by-caught animal.

SECTION II – CETACEANS

1. Parasitofauna of cetaceans: metazoan parasites

The collection of data upon cetacean helminthofauna dates back to the start of commercial whaling; in last decades, stranded and bycaught animals have prompted the majority of scientific data on the ecology and biology of these cryptic species, which are unavoidably less explored than terrestrial mammals. Nowadays, 172 species of helminths have been reported in cetaceans, including both odontocetes and mysticetes, but a relatively high number of species are occasional or accidental finding of birds' or pinnipeds' parasites, infecting cetaceans (Raga et al., 2008). The phylum of the Nematoda accounts for the higher diversity, counting up to 62 species, followed by digeneans, cestodes and acantocephalans.

Spanning through the different groups of cetaceans' parasites, several species have shown to have a high pathogenic potential, affecting reproductive fitness or even leading to direct mortality (Delyamure, 1955; Raga et al., 1997; Raga et al., 2008). Given that hosts' health, parasitic burden and concurrence of other pathogens highly affect the severity and outcome of parasitic infections, the type of parasite itself can also be determinant (Raga et al., 2008).

Gastric ulcers have been found in association with different species of the family Anisakidae in toothed whales (Young and Lowe, 1969; Cowan et al., 1986; Abollo et al., 1998a). Nematodes of the genus *Anisakis* are generally reported within the stomach of both toothed and baleen whales, where they usually are found free in the lumen or attached to the mucosa (Geraci and St.Aubin, 1978; Jaber et al., 2006). The infection is rarely symptomatic, nevertheless, ulcers of the gastric walls, up to perforation of the stomach, have been observed in association with adults or larvae penetrating mucosal, submucosal or muscular layers (Fiscus et al., 1962; Abollo et al., 1998a).

Depending on parasitic burden, their species, co-existence of other secondary pathogens and immune response of the host, infection by bronchopulmonary nematodes of the family Pseudaliidae (Metastrongyloidea) can be potentially fatal, as a consequence of bronchitis and pneumonia (Moser and Rhinehart, 1993; Dailey, 2001; Measures, 2001). Neonatal animals are particularly at risk of developing heavy infection with fatal outcomes and a transplacental transmission has been suspected for *Halocercus* spp. (Dailey et al., 1991; Fauquier et al., 2009). The coiled cephalic ends of *Halocercus* spp. firmly fixed within bronchioli induce mucopurulent bronchitis and pneumonia (Dailey, 1985) which can impair diving activity (Geraci and St.Aubin, 1978). Massive infestations by *Skrjabinalius* spp., *Pseudalius inflexus*, *Stenurus ovatus* can lead to occlusion of the airways for the physical presence of plugs of worms (Measures et al., 2001; McColl and Obendorf, 1982). Suppurative bronchopneumonia, bronchitis, bronchiolitis either parasitic and bacterial for secondary infections are associated with these lungworms (Delyamure, 1955; McColl and Obendorf, 1982).

Besides, pseudaliids located in the cranial sinuses and middle ear of toothed whales can generate hemorrhages, non suppurative chronic inflammation, thickening and rarely purulent flogosis of the mucosal lining. Impairment of echolocation and diving ability have been suspected to be the consequence (Dailey and Perrin, 1973; Measures, 2001), and presence of pseudaliids in the auditory system is thought to be involved in individual strandings (Delyamure, 1955). Irreversible, perforating, lytic bone lesions of the pterygoid sinus are instead associated with *Crassicauda grampicola* (Crassicaudidae), which can co-occur with pseudaliids in the air sinuses and auditory system. Other species of the genus *Crassicauda* are responsible for severe, fatal infections, mainly in the kidneys and vascular system; for their importance in this project they will be treated separately.

Digenean parasites of cetaceans have been defined as “destructive” for the internal organs (Geraci and St. Aubin, 1978). *Nasitrema* sp. can be considered one of most pathogenic species of the family Brachycladiidae. This fluke can be found as well in pterygoid synuses and in the inner ear of odontocetes; lesions ranging from mild sinusitis to severe meningoencephalitis, even associated with abscesses have been observed (Neiland et al., 1970; Ridgway and Dailey, 1972); migration to the brain through the VIII cranial nerve has been reported in an aged bottlenose dolphin (Degollada et al., 2002). This fluke has been as well retained responsible for impairment of the echolocation and equilibrium, representing the primary cause for the stranding of infected odontocetes (Morimitsu et al., 1986, 1992; O’Shea et al., 1991; Degollada et al., 2002). Other members of the Brachycladiidae, the genera *Campula*, *Brachycladium* and *Oschmarinella* are found in pancreatic and bile ducts of odontocetes. Mild infestations by *Campula* spp. may be asymptomatic but heavy infestations may produce biliary hyperplasia, portal fibrosis and granulomatous hepatitis (Jaber et al., 2004); in most severe infections, diffuse organ damage can lead to impairment of the hepatic function (Dailey, 2001). *Campula oblonga* has been associated to pancreatic fibrosis in harbour porpoises, extensively compromising digestive and endocrine function and acting as an important factor in natural mortality (Geraci and St. Aubin, 1978). The trematode fluke *Pholeter gastrophilus* (Heterophyidae) is commonly associated to chronic gastritis and fibrosis of gastric walls in odontocetes. Hundreds of small flukes, eggs and parasite debris are observed within nodules included in the wall of the stomach; in order of frequency, fundus, pylorus and duodenal ampulla can be interested, depending on the host species and digestive physiology (Aznar et al., 2006). The granulomatous reaction surrounding parasitic nodules within muscular layer, gives rise to a subacute gastritis and fibrosis (Woodward, 1969; Jaber et al., 2006; Raga et al., 2008); in most severe infections, peritonitis, likely caused by perforation of gastric walls (Jaber et al., 2006) and pyloric obstruction have been also reported, as a consequence of severe diffuse granulomatous reaction (Kirkwood et al., 1997).

Ectoparasitic crustaceans *Pennella balaenopterae* (Copepoda: Pennellidae) act as primary pathogens and as vehicle of secondary infections in both toothed and baleen whales, and at the same time the intensity of infection by these copepods is regarded as a visual marker for impaired immune system in odontocetes (Vecchione, 1994). Adult female *Pennella* feeds on the host's blood through aspiration in a modified buccal complex, the siphonostoma; the cephalic portion of the parasite is deeply fixed in the skin and blubber of cetaceans thanks to two or three variably-developed cephalic horns, the rest of the body lying free in the water (Hogans et al., 1987). Inflammatory infiltrate, made up by neutrophils, macrophages and lymphocytes creates considerable masses around the parasite head. Massive infestations can lead to malnourishment, diffuse necrosis of tissues or entrance of secondary pathogens from the site of attachment (Vecchione, 1994; Gazzonis and Merella, 2012).

At least seventeen different species of cetaceans, including both toothed and baleen whales have been reported to be infected by *P. balaenopterae*. The higher prevalence in pelagic *versus* coastal species demonstrate that its life cycle is primarily oceanic (Fraija-Fernández et al., 2018). Subtle morphological differences in the first antennae of adult females have been used to discriminate morphologically this species from *P. filosa*, a species infecting fishes. Nevertheless, recent molecular data on the mtDNA of the two species revealed them to be conspecific (Fraija-Fernández et al., 2018). *Pennella balaenopterae* is a sexually dimorphic species, and only fertilized females parasitize the skin of cetaceans (Aznar et al., 2005); moreover, only the adult females and the first naupliar stage (i.e., the free-swimming larval stage) have been described to date. After the second naupliar stage, the preadult stage (chalmus 4) infects intermediate hosts, i.e. fishes or cephalopods, in which it attends sexual maturity and mate. The female then returns free into the water to search for the definitive host (Abaunza et al., 2001), while the entire life cycle of the male is not yet completely known (Arroyo et al., 2002).

Crassicauda spp.: taxonomy, life cycle and host pathology

The family Crassicaudidae belongs to the order Spirurida (Chitwood, 1933) (Nematoda: Secernentea). A typical bilaterally symmetric anterior extremity is observed in spirurid nematodes, with or without pseudolabia. The esophagus is divided into an anterior, muscular portion smaller than the posterior, glandular part, which can be not easily distinguishable. Caudal papillae in the males appear ventrally or ventrolaterally, spicules are absent or generally asymmetric; the caudal bursa is absent. Recent phylogenetic analyses on rDNA of *Crassicauda magna* placed this genus inside this superfamily Acuarioidea instead then Habronematoidea (Jabbar et al., 2014). The genera *Crassicauda* and *Placentonema*, belonging to the Crassicaudidae, are then distinguished between each other for the absence of caudal alae in the former genus (Delyamure, 1955). *Placentonema gigantissima*, the largest nematode known, infects the reproductive tract and placenta of the female sperm whales (Gubanov, 1951; Lambertsen, 1997), while nematodes of the genus *Crassicauda* (Nematoda: Spirurida) are specific to both toothed and baleen whales, infecting the urogenital system, air sinuses, mammary gland and subcutaneous tissues. Twelve species (*C. anthonyi*, *C. bennetti*, *C. boopis* [syn. *pacifica*], *C. carbonelli*, *C. costata*, *C. crassicauda*, *C. delamureana*, *C. fuelleborni*, *C. giliakiana*, *C. grampicola*, *C. magna* [syn. *duguay*], *C. tortilis*) are currently recognized but the poor characterization of the specimens makes the redefinition of species and taxonomic revisions unavoidable. The descriptions in literature, indeed, often concern only few details of the cephalic end or of the tails, and in some cases only one of two genders is characterized. The presence or absence of the spicules in the male allows a first species-distinction (Raga et al., 1987) (see Tab. 1 at the end of the chapter).

No descriptions of the larval stages are present, except for partial characterization of *larvae migrans* from histological sections of a Cuvier's beaked whale (Diaz-Delgado et al., 2016). The detection of larvae at histopathology from infected animals has anyway contributed to formulate some hypothesis upon their life cycle. Similar patterns of lesions have been reported in fin whales infected by *C. boopis* in the North Atlantic Ocean and in Cuvier's beaked whales infected by a *Crassicauda* sp. in the Canary Islands. In the latter case, the mesenteric arteries were the vessels most interested by larval migration phenomena, as visible from histology (Diaz-Delgado et al., 2016). This suggests that, after ingestion of the infecting stage, larvae migrate from the intestine to the kidneys through the wall of mesenteric arteries and aorta, creating severe flogosis and linear scars along their passage (Lambertsen, 1986, Diaz-Delgado et al., 2016). Entry of *C. boopis* into the kidney could involve movements of the larvae either remaining within aortic walls and renal arteries or through embolization in the arterial blood flow. Once in the kidneys, the larvae can grow to sexual maturity occupying the vena cava with their anterior extremity, the body staying in renal parenchyma and the

tails lying in the ureters (Lambertsen, 1986). In both cases described by Lambertsen (1986) and Diaz-Delgado et al. (2016), adult nematodes were present in the kidneys, but only the former achieved a species identification. The presence of larvated eggs and larvae in the urine of infected fin whales suggests as well the shedding of larvae of *C. boopis* in the environment with the urine (Lambertsen, 1986). Moreover, the presence of eggs in the pulmonary airspace, likely originated from embolization of parasitic thrombi, allowed Lambertsen to hypothesize that elimination of eggs through the whale's blow could also happen. Similar thrombotic lesions were also found recently by Lempereur et al. (2017) in the lungs of a young fin whale infected by *C. boopis*.

Nothing is known upon the extrasomatic life cycle of these species. The presence of an intermediate or paratenic host is highly probable, as typical for the marine Spirurida (Anderson, 2000), thus involving, in the case of *C. boopis*, a whale-krill-whale transmission (Lambertsen, 1986). Nevertheless, a direct transmission to the calf, through the ingestion of urine-contaminated milk, is also considered a reliable hypothesis, for the presence of adult parasites in the kidneys of weaning calves (Lambertsen, 1986; Lempereur et al., 2017). Though a transplacental and lactogenic transmission cannot be ruled out, no evidence of larvae either in the placenta or mammary gland have ever been found, therefore this remain as speculative.

Pathogenic impact of crassicaudosis on cetaceans

Several members of the genus *Crassicauda* (Nematoda: Spirurida) are considered primary cause of mortality of their hosts, or can contribute to the death through detrimental effects on general health. Their large size (up to 150 cm for *Crassicauda boopis*) coupled with their localization in the host account for extensive, severe and irreversible tissue damage (Lambertsen, 1986; Zucca et al., 2004; Van Bressemer et al., 2006; Fichi et al., 2013; Keenan-Bateman and McLellan, 2016).

Crassicauda grampicola and *Crassicauda* spp., are described in pterygoid air sinuses of small odontocetes; the purulent flogosis and osteolytic lesions have been associated with direct mortality in *Stenella* spp. (Perrin and Powers, 1980) and to sublethal effect in *Delphinus capensis* (van Bressemer et al., 2006); lesions to cranial nerves and to the meninges have been reported to be contributory to strandings events (Baker, 1992; Morimitsu et al., 1992; Raga et al., 1997; Zucca et al., 2005; Fichi et al., 2013). The same species has been also described in mammary glands in white sided dolphin *Lagenorhynchus acutus*; the subsequent pyogranulomatous mastitis and fibrosis of the parenchyma is hypothesized to reduce the reproductive success of the herds (Geraci et al., 1978).

The baleen whales *Balaenoptera physalus*, *B. musculus*, and *Megaptera novaeangliae* have been found to be affected by systemic disease caused by *C. boopis* adults (Lambertsen, 1985 and 1986;

Lempereur et al., 2017). Lambertsen (1992) estimated crassicaudosis to be contributory to a substantial fraction of natural mortality in Atlantic population of fin whales (up to 4.9%). The extensive tissue damage on renal and vascular systems is mediated by the immune-response, that, while trying to encapsulate the giant parasite body in the vessels and kidney, gives rise to severe thrombosis and phlebitis of the vena cava and renal vessels, finally leading to congestive renal failure. Moreover, extensive vasculitis of the mesenteric arteries related to larval migration is supposed to affect gut and vascular function (Lambertsen, 1986; Lempereur et al., 2017).

In *Ziphius cavirostris*, diffuse arteritis associated with *larvae migrans* has been supposed to have dramatically impacted normal circulation in these deep-diving animals, likely representing the case of death either directly or through enhancing the probability of ship strikes (Díaz-Delgado et al., 2016). Adult nematodes were found in the kidneys, causing pyogranulomatous pyelonephritis and extensive loss of nephrons, as described also in Stejneger's beaked whale infected by *C. giliakiana* (Tajima et al., 2014).

Crassicauda fuelleborni and *C. magna* are reported in the subcutaneous tissues and muscles of odontocetes, mainly in the thoracic-cervical region; their long body (up to 3 meters) is embedded by the reaction of the host and associated with myositis (Johnston and Mawson, 1939; Abollo et al., 1998b). A critical habitat for *C. magna* in kogiid whales is an exocrine gland located in the cervical region, which has been recently discovered to be likely involved in the emission of the eggs in the external environment (Keenan-Bateman et al., 2018).

Crassicauda crassicauda and *C. carbonelli* infect external genitalia of mysticetes and pilot whales respectively (Gibson, 1973; Lambertsen, 1986; Raga and Balbuena, 1990). Fibrotic reaction has been signalled in the urethra of the male rorquals induced by *C. crassicauda* (Lambertsen, 1986).

Molecular analyses on the genus Crassicauda

Molecular data upon the genus *Crassicauda* are very scarce, though the use of molecular tools could overcome problems of morphological identification due to the absence of important portions of the nematodes or to the presence of larval forms, or, finally, when only chronic lesions are observed but the nematodes bodies are no more recognizable. Recently, sequences of 18S gene from rDNA and of COX I from mtDNA have been obtained from few species. Sequences of the former marker have shown to be well conserved within the studied group, which includes *C. magna*, *C. boopis* (Jabbar et al., 2014, Lempereur et al., 2017) and *Crassicauda* sp. (Díaz-Delgado et al., 2016). As for the COX I, only one sequence, belonging to *C. giliakiana* is available in public databases. Regions inside COX I gene have been used efficiently for species identification inside the phylum Nematoda (Traversa

and Otranto, 2006), as well as the nuclear ribosomal ITS-2 fragment (Traversa et al., 2004, Chilton et al., 2004). A wider sampling inside the genus *Crassicauda* and a multigene analysis could help overcoming issues of morphologic species identification and help clearing taxonomic issues.

Table 1. Summary of hosts and sites of infection by *Crassicauda* spp. and related references

Taxon	Morphological description	Spicules	Host	Site	References
<i>C. boopis</i> , Baylis, 1920 (Syn. <i>C. pacifica</i>)	Baylis, 1920 Margolis and Pike, 1955 Lambertsen, 1985	Absent	<i>Balaenoptera physalus</i> <i>Megaptera nodosa</i> <i>Megaptera novaeangliae</i> <i>Balaenoptera musculus</i>	Kidney	Baylis, 1920 Hamilton 1914,1915 Cockrill, 1960 Delyamure, 1955 Lambertsen, 1985, 1992 Lempereur, 2017
<i>C. tortilis</i> , Skrjabin, 1966	Skrjabin, 1959	Absent	<i>Balaenoptera musculus</i>	Kidney	Skrjabin, 1959
<i>C. costata</i>	Skrjabin 1969	asymmetric	<i>Eubalaena australis</i>	Kidney	Skrjabin 1969
<i>C. bennetti</i> (Spaul, 1926)	Spaul, 1926	Absent	<i>Hyperoodon planifrons</i> <i>Hyperoodon</i> sp.	Kidney	Spaul, 1926 Flower, 1882
<i>C. fuelleborni</i> (Hoepli and Hsü, 1929)	Hoepli and Hsü, 1929	Absent	<i>Neophocaena phocaenoides</i>	Muscles around the vagina	Hoepli and Hsü, 1929
<i>C. grampicola</i> (Johnston e Mawson, 1941)	Johnston e Mawson, 1941 Raga, 1987	Absent	<i>Delphinus delphi</i> <i>Grampus griseus</i> <i>Delphinus capensis</i> <i>Lagenorhynchus acutus</i>	Pterygoid sinuses, Mammary gland ¹⁴	Johnston and Mawson, 1941 Delyamure, 1955; Raga et al., 1982; Troncone et al., 1994; Scaravelli et al., 2006; Zucca et al., 2005; Geraci et al., 1978
<i>C. giliakiana</i> (Skrjabin and Andreewa, 1934)	Skrjabin and Andreewa, 1934 Kikuchi et al., 1995	asymmetric	<i>Delphinapterus leucas</i> <i>Ziphius cavirostris</i>	Kidney	Skrjabin and Andreewa, 1934 López-Neyra, 1958
<i>C. anthonyi</i> (Chabaud, 1962)	Chabaud, 1962	Symmetric	<i>Mesoplodon mirus</i> <i>Ziphius cavirostris</i>	Kidney	Chabaud, 1962 Piccolo et al., 1998 Fognani, 2005
<i>C. delamureana</i> (Skrjabin, 1966)	Skrjabin, 1966	Symmetric	<i>Balaenoptera borealis</i> ¹⁸	Kidney	Skrjabin, 1966
<i>C. magna</i> (Johnston and Mawson, 1939)[syn. <i>C. duguyi</i>]	Johnston and Mawson, 1939 Dollfus, 1966 Jabbar et al., 2014 Keenan-Bateman et al., 2018	asymmetric	<i>Kogia breviceps</i>	Neck region Subcutaneous tissues	Dollfus, 1966 Mignucci-Giannoni et al., 1998 Johnston and Mawson, 1939 Abollo et al., 1998b Jabbar et al., 2014 Keenan-Bateman et al., 2018
<i>C. carbonelli</i> Raga and Balbuena, 1992	Raga and Balbuena, 1990	asymmetric	<i>Globicephala melas</i>	Penis	Raga and Balbuena, 1990
<i>C. crassicauda</i> Creplin, 1929	Baylis 1916, 1922 Creplin, 1929	asymmetric	<i>Balaenoptera physalus</i> <i>Balaenoptera musculus</i>	Exernal genitalia and urethra	Baylis, 1920 Creplin, 1929 Gibson, 1973 Skrjabin, 1973 Lambertsen, 1986

2. Protozoan parasites of cetaceans: *Toxoplasma gondii*

Toxoplasma gondii (Apicomplexa) is a coccidian parasite with felids as definitive hosts, and an unusually wide range of intermediate hosts, which spans over all warm-blooded vertebrates, man included. As other coccidian parasites, it has a complex life cycle, which includes intestinal and extra-intestinal stages. After ingestion of the mature oocysts or cysts in the preys tissues, *Toxoplasma* multiplies sexually and asexually within the enterocytes of domestic or wild felids (Tenter et al., 2000), and is then shed in the environment with feces as unsporulated oocysts. Oocysts of *T. gondii* become infective in one to five days, depending on environmental conditions. The extra-intestinal phase of the cycle takes place in intermediate hosts (Jones and Dubey, 2010); after ingestion of the infective stage of parasite, they enter the cells of reticuloendothelial system and multiply rapidly generating the tachyzoites. Once spread into the organs, a chronic phase starts, with generation of bradyzoites, elements slowly multiplying inside terminal cysts, which may develop in visceral organs but are more prevalent in muscular and neural tissue, including the brain, eye, skeletal and cardiac muscle (Pietrobelli et al., 2003; Hill et al., 2005). These elements can persist with no harm for all the life of the host. Immunity does not eradicate infection; nevertheless, in immunosuppressed host, rupture of a tissue cyst can result in transformation of bradyzoites into tachyzoites and renewed multiplication, which can result in fatal disease (Hill et al., 2005).

Thanks to the high resistance of the infective oocysts, *T. gondii* is also widespread in the marine environment, both in coastal and pelagic waters. In experimental conditions, oocysts have been demonstrated to persist in marine water for months in the infective form (Lindsay and Dubey, 2009) and also to maintain infecting capability inside filter feeders mollusks (Lindsay et al., 2001, 2004; Arkush et al., 2003). The route of contamination of marine environment has to be conducted likely to the pouring of contaminated wastewaters into the sea. Studies of seroprevalence have demonstrated that marine otters which live closer to rivers mouths have a higher risk of getting the infection than those living in coastal areas where there is less spill of freshwater from the mainland (Miller et al., 2002). A study carried out among Mediterranean cetaceans found higher antibodies titres towards *T. gondii* in coastal species than in the pelagic ones, confirming that the risk of getting the infection depends on the habitat (Cabezón et al., 2004). How cetaceans living offshore can get the infection is still an open question. Food sources of cetaceans, which include fishes, cephalopods and other warm-blooded organisms, are not intermediate hosts for *Toxoplasma*, but in experimental conditions filter-feeding fishes have proved to maintain infective oocysts in their guts for some hours, possibly carrying the pathogen from neritic to pelagic waters (Massie et al., 2010). The role of these “biotic”

vectors should be further investigated to explain the spread of the infection in such a wide range of cetacean hosts. Direct evidence of *T. gondii* has been detected indeed in several species of toothed whales, including the bottlenose dolphin (*Tursiops* spp.) (Cruickshank et al., 1990; Inskoop et al., 1990; Di Guardo et al., 1995a,b; Schulman et al., 1997; Jardine and Dubey, 2002; Dubey et al., 2003, 2008, 2009; Marcer et al., 2010; Pretti et al., 2010), striped dolphins (*Stenella* spp.) (Migaki et al., 1990; Domingo et al., 1992; Di Guardo et al., 1995a,b, 2010, 2011; Dubey et al., 2007; Sierra et al., 2008; Pretti et al., 2010), Risso's dolphin (*Grampus griseus*) (Di Guardo et al., 1995a,b; Resendes et al., 2002), Beluga whale (*Delphinapterus leucas*) (De Guise et al., 1995; Mikaelian et al., 2000), Chinese white dolphin (*Sousa chinensis*) (Bowater et al., 2003) and sperm whales (*Physeter macrocephalus*) (Mazzariol et al., 2011). As for mysticetes, only one report of indirect diagnosis exists in a humpback whale (Forman et al., 2009). Several reports of acute infection by *T. gondii* exist also in toothed whales inside the Mediterranean Sea (Domingo et al., 1992, Di Guardo et al., 1995; Mazzariol et al., 2011).

In the acute phase of the disease in infected cetaceans, non-suppurative meningoencephalitis is the most frequent finding, associated with gliosis and perivascular cuffings; in necrotic foci, tachyzoites and tissue cysts are observed (Migaki et al., 1990; Resendes et al., 2002; Bowater et al., 2003; Dubey et al., 2003; Kreuder et al., 2003; Miller et al., 2004; Dubey et al., 2007, 2009). Tachyzoites can be also detected in other tissues, and lymphadenitis, myocarditis, interstitial pneumonia, adrenalitis, hepatitis have been described as well (Inskoop et al., 1990; van Bressemer et al., 2009). Correlation with immunosuppressive conditions has been demonstrated, confirming the opportunistic nature of this parasite, which has been often indicated as pathogenic when co-present with viral agent (Dolphin Morbilli Virus, DMV) or high levels of PCBs in tissues (Siebert et al., 1999; Bennett et al., 2001; Jepson et al., 2005; Hall et al., 2006). Nevertheless, it is not clear whether a role as a primary pathogen can be ascribed to *T. gondii* in dolphins, since severe encephalitis associated to the presence of *Toxoplasma* cysts and tachyzoites in the brain have been described without clear evidence of immunosuppressive conditions (Di Guardo et al., 2010).

Genotyping of *Toxoplasma gondii*

Only one species of *Toxoplasma* exists; historically, *T. gondii* was considered to have low genetic diversity and grouped only into three types (I, II, III) (Su et al., 2012). Recent molecular techniques, investigating polymorphisms in several loci of the genome and wider geographical sampling revealed a greater genetic diversity, and more than a hundred isolates, with different distributions, have been discovered (Ajzenberg et al., 2004). Genotype I to III are the most prevalent in Europe and in domestic cycles (human, cats, domestic and meat producing animals); atypical and mixed strains

prevail in areas where wild cycles occur more easily, such as in Africa and America among wild felids and several different wild intermediate hosts, and are far less frequent in Europe (Ajzenberg et al., 2004; Miller et al., 2004). Such “atypical” strains actually possess identical alleles to type I, II or III but have unique polymorphisms and “novel” alleles (Dardé, 2008).

The consequences of the infection may depend on the parasite genotypes and host species (Grigg et al., 2001; Dardé, 2004): in men, type I and type I variants are more frequently associated with severe retinochoroiditis and the atypical strains with disseminated toxoplasmosis (Bossi and Bricaire, 2004), while types II and III are generally less pathogenic (Miller et al., 2004).

As for the marine environment, genotype II has been isolated from marine otters (Miller et al., 2004), striped dolphin (Dubey et al., 2007), bottlenose dolphin and walrus (Dubey et al., 2008, 2009); in addition, genotype X and A (now “haplogroup 12”) have been found in marine otters and pinnipeds of Pacific and Atlantic American coasts (Conrad et al., 2005; Sundar et al., 2008). Genotype II and atypical strains are reported in marine mammals from the Mediterranean Sea (Marcer et al., 2010; Di Guardo et al., 2011).

Most used DNA-based diagnostic methods for toxoplasmosis include the PCR and Real Time PCR; several multi-copy genes are targeted for the detection in biological samples, including the B1, the 529 bp repeat element and the rDNA fragments ITS-1 or 18S; in rtPCR, the product is measured during each cycle using probes or intercalating dyes, thus the sequence must not be verified as in PCR to provide adequate specificity (Liu et al., 2015). For the identification of the genotypes, some molecular technologies have been developed, including microsatellite analysis and High Resolution Melting (HRM). The HRM analysis characterizes Single Nucleotide Polimorphisms (SNPs) located in the multicopy gene B1 by the analysis of the melting temperature. This method can correctly classify the three main lineages (Aksoy et al., 2014; Costa et al., 2013). Microsatellite analysis instead is based on the analysis of motif repeats inside the genome, which vary in their number inside the population creating multiple alleles in each given locus. Fifteen locus (markers) have been studied to genotype *T. gondii*, and Ajzenberg et al. (2010) developed a method for genotyping *Toxoplasma* in a single multiplex-PCR assay. Fifteen microsatellite markers (MS) are at the same time taken into account, differentiating the three main lineages (I, II, III) from all the atypical strains by use of 8 of these MS, and then typing closely related isolates by using the remnants 7 markers. Genotyping with such a degree of differentiation is of both clinical and epidemiological importance, since different strains can be associated to different outcomes in the host, and this highly-resolute method can help identifying common sources of infections through comparison of the strains (Ajzenberg et al., 2010).

This kind of typing could help studying epidemiology of toxoplasmosis in cetaceans, shedding light also on the route of spreading of the protozoan in pelagic species.

3. Aims of the project and outputs

Parasitological data on fin whales stranded along Mediterranean coasts are very scarce to date, and reports on the presence and pathology of crassicaudosis in this subpopulation are only punctual. In the frame of a collaboration with the Cetacean Emergency Response Team and the Mediterranean Marine Mammals Tissue Bank, necropsies were performed on seven fin whales stranded along Italian coasts and a complete parasitological examination was carried out on the field and later in the laboratory, in order to explore presence and pathology of parasitic infections, including metazoan and protozoan parasites.

The data obtained from seven animals stranded and analyzed in the period 2006-2015 were collected and presented in the following paper and scientific communications at national and international conferences:

- Marcer F., Marchiori E., Centelleghè C., Ajzenberg D., Gustinelli A., Meroni V., Mazzariol S., 2018. Parasitological and pathological findings in Fin Whales (*Balaenoptera physalus*) stranded along Italian coastlines. *Accepted in Diseases of Aquatic Organisms – PAPER 4*
- Marcer F., Danesi P., Mazzariol S., Negrisolo E., Pagiario M., Tessarin C., Marchiori E., 2016. Crassicaudosis in fin whales (*Balenoptera physalus*) stranded along Italian coasts. *XXIX Congress SoIPa, Bari, 21-24 June. Atti, p. 41*. Presentazione orale - Scientific communication 9
- Marcer F., Mazzariol S., Pagiario M., Mignone W., Tessarin C., Marchiori E., 2017. Parasitological findings in fin whales (*Balenoptera physalus*) stranded along Italian coastlines. *31st European Cetacean Society Annual Conference, Hindsgavl Castle Middelfart (Denmark), 1-3 May. Abstract book, p. 230*. Poster - Scientific communication 10

Immature stages of nematodes were detected during the aforementioned survey in two fin whales, likely referable to *larvae migrans* of *Crassicauda* sp. Since no description of such immature stages is present in literature and morphological data alone could not bring to species identification, genetic barcodes were created to confirm their identification. Genomic data are very scarce in public databases for the genus *Crassicauda*, thus we generated sequences from four further different species of the genus, carrying out a previous thorough morphological identification on adult nematodes

isolated from toothed whales, in order to compare sequences from larvae and identified adults. A multigene analysis was performed on all sequences, to study the utility of different nuclear and mitochondrial genomic markers in species identification, and to help clearing taxonomic issues within the genus. The data were collected in the following draft and presented at international scientific conference:

- Marcer F., Negrisolo E., Pietrobelli M., Tessarin C., Marchiori E., Morphological and molecular characterization of adults and larvae of *Crassicauda* spp. (Nematoda: Spirurida) from Mediterranean fin whales *Balaenoptera physalus* (Linnaeus, 1758). *In preparation* (draft) – PAPER 5
- Marchiori E., Negrisolo E., Tessarin C., Centelleghè C., Marcer F., 2017. Morphological and molecular characterization of *Crassicauda boopis* (Nematoda: Spirurida) from Mediterranean fin whales: preliminary results. *31st European Cetacean Society Annual Conference, Hindsgavl Castle Middelfart (Denmark), 1-3 May. Abstract book, p. 70.* Oral presentation - Scientific communication 11

Similarly, pre-adult stages of copepods were isolated from the skin of one fin whale; morphological features pointed to the genus *Pennella* spp. Since there are no descriptions of immature stages of *Pennella balaenopterae* from the skin of cetaceans in literature, we generated sequences of *mtcox1* gene from different congeneric species isolated from fishes and different cetaceans species and compared our data with those in public databases. Hypothesis upon the existence of a unique species of *Pennella* adapted to infect both fishes and marine mammals is formulated, as hypothesized by Fraija-Fernández et al. 2018 in odontocetes. A scientific communication was prepared on this topic and presented in a national scientific conference:

- Marcer F., Marchiori E., Tessarin C., Dotto G., Paoletti M., Mazzariol S., Mattiucci S., 2018. First molecular detection of *Pennella* sp. (Siphonostomatoidea: Pennellidae) in cetaceans from the Mediterranean Sea. *XXX Congresso SoIPA, Milano 23-26 Giugno.* Poster - Scientific communication 12

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4. Paper 4

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Parasitological and pathological findings in fin whales (*Balaenoptera physalus*) stranded along Italian coastlines

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Abstract

Mediterranean fin whales (*Balaenoptera physalus*) face many threats to their conservation, including both anthropogenic and natural issues. There are few records of the parasitic fauna of this species in this geographical area. To partially fill in this gap of knowledge, we investigated the presence and potential impact of parasitic diseases in Mediterranean fin whales. Seven animals stranded along Italian coastlines between 2006 and 2015 were submitted for necropsy and parasitological examination. The protozoan parasite, *Toxoplasma gondii*, was detected in one fin whale and, for the first time in mysticetes, it was successfully genotyped as a type II strain with 15 microsatellite markers. One crustacean (*Pennella* spp.) and four helminth taxa (*Crassicauda boopis*, *Ogmogaster antarcticus*, *Tetrabothrius ruudi* and *Bolbosoma* sp.) were detected and morphologically identified. Different degrees of ectoparasitism by adult *Pennella balaenoptera* were recorded. Immature stages of *Pennella* sp. were also detected in two animals and are here described for the first time in cetaceans. Infestation by *C. boopis* was confirmed or suspected in five cases. Parasitic thrombi, involving renal veins and caudal vena cava, and fibrosis of renal parenchyma were associated with *C. boopis* and likely resulted in some degree of renal dysfunction. Larval nematodes were found within foci of mesenteric endarteritis. Further research to evaluate the prevalence of this potentially fatal endoparasitosis in Mediterranean fin whales is warranted.

Keywords: *Balaenoptera physalus*, helminth parasites, crustacean parasites, *Toxoplasma gondii*, genotyping, Mediterranean Sea

1. Introduction

The fin whale *Balaenoptera physalus* (Linnaeus, 1758) is the most abundant mysticete in the Mediterranean Sea (Panigada & Notarbartolo di Sciara, 2012). Genetic studies confirm the existence of a resident Mediterranean population (Bérubé et al. 1998), which is currently listed as vulnerable by the International Union for Conservation of Nature (IUCN) Red List as it is progressively decreasing. The main threats for their conservation are linked to anthropogenic activities, with ship strikes overrepresented (Panigada & Notarbartolo Di Sciara 2012). However, natural disease processes, such as cetacean morbillivirus infections (CeMV), may pose a threat to fin whales (Mazzariol et al. 2016). Occasionally, CeMV infection is associated with opportunistic pathogens, such as *Toxoplasma gondii*. Most cases of cetacean toxoplasmosis involve odontocetes and can

present with abortion, encephalitis and systemic lethal disease (Migaki et al. 1990, Di Guardo et al. 2010). By contrast, knowledge of toxoplasmosis in mysticetes is limited to a young fin whale stranded in Italy (Mazzariol et al. 2012) and a seropositive humpback whale *Megaptera novaeangliae* stranded in England (Forman et al. 2009).

As for *T. gondii*, limited data on other parasites in Mediterranean fin whales are available (Tamino 1953, Malatesta et al. 1998, Çiçek et al. 2007, Giorda et al. 2017). The large copepod *Pennella balaenoptera* (Copepoda, Pennelliidae) Koren and Danielssen, 1877 is described in the ‘Pélagos Sanctuary’ and is regularly seen attached to the whales’ skin (Tamino 1953, Notarbartolo Di Sciara et al. 2003). This copepod is easily detectable since the posterior part of its body emerges from the host’s skin and trails freely in the water, while the head penetrates the skin and blubber occasionally reaching the muscular fascia (Arroyo et al. 2002). In striped dolphins *Stenella coeruleoalba* the intensity of infestation has possible association with immunosuppressive conditions, such as morbillivirus infection, environmental contaminants and malnutrition (Vecchione & Aznar 2014). The trematode *Ogmogaster antarcticus* Johnston, 1931 (Digenea, Notocotyliidae) was described in a fin whale stranded on the north-eastern coast of the Tyrrhenian Sea (Malatesta et al. 1998). As for nematodes, infestation by *Crassicauda* sp. was recently recorded in a fin whale, associated with a severe nephropathy and mesenteric lesions (Giorda et al. 2017). Infestation by *C. boopis* can cause serious illness in baleen whales, since the localization of adult worms in the vascular and urinary system can lead to severe renal dysfunction and death. This species has been recorded in fin whales from the Atlantic and Pacific Ocean, but not yet identified to species in the Mediterranean (Lambertsen 1986, 1992, Lempereur et al. 2017).

Herein, we evaluate the presence and potential negative effects and conservation implications of parasites in fin whales stranded along the Italian coastline between 2006 and 2015.

2. Material and Methods

Between October 2006 and January 2015 seven fin whales stranded along the Italian coastlines (Tyrrhenian and Ligurian Sea) and were necropsied in situ following a standard protocol (McLellan et al. 2004) by the Cetacean stranding Emergency Response Team (C.E.R.T.), established at Padova University by the Ministry for Environment, Territory and Sea, in collaboration with the local Units of Health Institutions (Local Health Units and Istituti zooprofilattici). Biometric data, carcass condition code, body condition score (Geraci & Lounsbury 2005), age estimation (Aguilar & Lockyer 1987, Aguilar, 2009) and sex of the stranded animals are reported in Table 1. Partial pathological data for some of the animals included in this study have already been reported in Mazzariol et al. 2012 (fin whale #3) and Mazzariol et al. 2016 (fin whale #3, #4, #5 and #6).

Parasitological and pathological analyses for metazoan parasites

Parasitological analyses were performed on the integument, including blubber, and respiratory, gastrointestinal, cardiovascular, and urogenital systems. Portions of skin and blubber with embedded copepods were collected. Portions of bronchial tree (primary, secondary and distal bronchi) were longitudinally opened and the pulmonary parenchyma was randomly sectioned and inspected for parasites. Main hepatic vessels and liver ducts were dissected and examined for lesions and parasites. A portion of the liver close to the hilum and from the periphery were sliced, washed with tap water and the sediment examined under a stereomicroscope. Stomach chambers and portions of intestine (small and large intestine at least 10m each) were sampled and the contents were filtered with 1.0 and 0.5 mm mesh sieves and the filtered material then observed with a stereomicroscope. As previously described by Lambertsen (1992), intestinal mucosa and mesenteric arteries were examined for lesions due to the migration of *Crassicauda* nematode larvae. The lumina of renal arteries and veins and the caudal vena cava were also examined for adult specimens of *C. boopis* or related lesions. The ureters were opened and examined to isolate the tails of this nematode. When possible, urine sediment, obtained by centrifugation at 2,000 rpm for 5 min (626 RCF), was analyzed for the detection of eggs or larvae of *Crassicauda*. All parasites recovered during post mortem examination were washed in physiological saline, counted and fixed in 70% ethanol.

Morphologic characteristics of genus *Pennella* were studied with a stereomicroscope. The nematodes and immature stages of copepods were examined as wet-mounts in clearing agents (glycerin or Amman's lactophenol), whereas flatworms were stained with Semichon's Acid Carmine or Borax carmine, dehydrated through a graded series of alcohol and mounted in Canada balsam (Kennedy 1979, Pritchard & Kruse 1982). All parasites were measured under a light microscope (Nis Elements D software, Nikon). Copepod parasites were compared with descriptions by Thompson (1905), Hogans (1987) and Abaunza et al. (2001). Dichotomous keys and literature data were used to identify the helminths (Delyamure 1955, Margolis & Pike 1955, Lambertsen 1985, Raga et al. 1986, Bray et al. 2008). Specimens of *C. boopis* were deposited at the Natural History Museum of London (NHMUK) (accession number: *Crassicauda boopis* 2015.10.11.1-4).

Tissue with gross lesions were fixed in 10% neutral buffered formalin, embedded in paraffin, cut at 4 µm thick and routinely stained with hematoxylin and eosin for microscopic examination. Further histochemical techniques, i.e., periodic-acid Schiff (PAS) and Masson's Trichrome were used on selected sections to better characterize the pathogens and tissue changes.

Analyses for protozoan parasites

Tissue samples of brain, spinal cord, heart, lymph nodes (mesenteric and mediastinal), skeletal muscle, lung, spleen, liver and kidney were collected; a portion of each tissue was processed for routine histological examination and a portion was stored at -20°C and subsequently analyzed by molecular methods to detect parasites of the subphylum Apicomplexa.

DNA extraction and PCR assay for detection of *Toxoplasma gondii*, *Neospora* and *Sarcocystis*

DNA extraction was performed on all the aforementioned tissues using NucleoSpin® Tissue kit (Macherey-Nagel, Germany). The PCR reaction was carried out in 30µL volume containing 1X PCR buffer, 2 mM MgCl₂, 200 µM each of the dNTPs, 2 U Platinum® Taq DNA Polymerase (Invitrogen, UK), 1µM of each primer, as described previously (Ho et al. 1996), and 1-3 µL of DNA extract. This PCR assay allows a conserved region of the *nss-rRNA* gene (300 bp in size) of *Neospora* spp. and other Apicomplexa coccidian as *Sarcocystis* and *Toxoplasma* to be amplified. The reaction mixture was first treated at 95°C for 5 min, followed by 12 cycles at 94°C for 30 sec and 58°C for 30 sec; 23 cycles at 94°C for 30 sec, 52°C for 30 sec and 72°C for 30 sec and a final extension step at 72°C for 7 min. DNA extracted from *T. gondii* oocysts, isolated from a domestic cat, were used as positive control. PCR products were analyzed by electrophoresis in SYBR Safe stained (Invitrogen, UK) 2% agarose gel, visualized with Geldoc XR (Bio-Rad Laboratories, USA) under UV light, purified and sequenced at BMR-Genomics (Padova, Italy). The sequences were analyzed using ChromasPro (version 1.42, Technelysium Pty Ltd., Australia) and compared with sequences available in the GenBank™ database using BLASTn program (<http://www.ncbi.nlm.nih.gov/>).

Extracted DNA was also tested by a commercial real time PCR assay (*Toxoplasma* Q-PCR Alert Kit, Nanogen Advanced Diagnostics S.p.a., Buttigliera Alta, Italy) on an ABIPRISM 7300 (Applied Biosystem, Carlsbad, USA) following the manufacturer's instructions. The real-time PCR assay targeted the 529 bp repeat region (*REP529*, GenBank accession no. AF146527) of *T. gondii* DNA (Homan et al. 2000). All DNA samples were tested in triplicate and each assay was considered positive if at least one test of the triplicate was positive. Each PCR run included a negative control without DNA and, to check the absence of PCR inhibitors, each sample was coamplified with an internal control consisting of beta-globin gene.

Genotyping of *T. gondii* strains

DNA samples extracted from tissues that tested positive for *T. gondii* DNA (C_t value < 32) were submitted to a genotyping analysis using 15 microsatellite markers distributed on 10 of 14 chromosomes, as described previously (Ajzenberg et al. 2010). Briefly, for each primer pair, the forward one was 5'-end labeled with fluorescein to allow sizing of PCR products electrophoresed in an automatic sequencer. PCR was carried out in a 25-µL reaction mixture consisting of 12.5 µL of

2X QIAGEN Multiplex PCR Master Mix (Qiagen, France), 5 pmol of each primer and 5 μ L of DNA. Cycling conditions were 15 min at 95°C; 30 s at 94°C, 3 min at 61°C, and 30 s at 72°C (35 cycles); and 30 min at 60°C. One microliter of the PCR product was mixed with 0.5 μ L of a dye-labeled size standard (ROX 500, Applied Biosystems) and 23.5 μ L of deionized formamide (Applied Biosystems). This mixture was denatured at 95°C for 5 min and then electrophoresed using an automatic sequencer (ABI PRISM 3130xl, Applied Biosystems). The sizes of the alleles in bp were estimated using GeneMapper analysis software (version 4.0, Applied Biosystems).

3. Results

Six out of seven fin whales had one or more parasitic species. Overall, 1,164 parasites were collected, including one crustacean and four helminth taxa (Table 2).

Metazoan parasites and histopathological findings

Adult females of the mesoparasitic copepod *Pennella balaenoptera* were detected in five whales. Mild infestation was observed in four animals, in which the parasites were anchored mainly on the back and on the abdominal region of the hosts. A severe infestation (>200 parasites) involving the entire body surface was observed in whale #3. The heads of the parasites were embedded into the skin and blubber without reaching the muscular fascia, and were associated with edema and hemorrhage in the subcutaneous tissues. The parasite trunk and abdomen protruded from the skin of the host. Microscopically, there was severe, focally extensive, chronic eosinophilic inflammatory infiltrates with edema and hemorrhage around the head of the parasites. Immature parasitic copepods of the family Pennellidae Burmeister, 1835 (Fig. 1) were collected from two animals. These specimens (n= 6) were small (mean length: 33.9 mm), showed a distinct cephalothorax dorsoventrally flattened, and flecked with black pigment (Fig. 1B). The second antennae were hamate and projected beyond the border of the cephalothorax, while the first pair of antennae was delicate and setose. On the ventral side of the cephalothorax, a prominent rostrum was also present and the sturdy maxillipeds were situated posteriorly (Fig. 1C); ventrally, in the anterior third of the cephalothorax, the cuticle folds form an hourglass design with a pair of spines in the central part. A pair of cuticular pointed structures was laterally located at the end of the cephalothorax. The filiform thorax extended to a length ten times that of the cephalothorax; the four pairs of thoracic limbs appeared well developed with bristles on the last two segments (Fig. 1D); the first and second limbs bifurcated in two extremities. The abdomen was filiform; eighteen pairs of bulges or short unbranched lateral appendages (with different degrees of development in the specimens) were visible along the abdomen, which terminated with a deep notch (Fig. 1E). On either side of this was a small bisetose

appendage. The morphometric data of the immature specimens of *Pennella* sp. Oken, 1815 are reported in Table 3.

Specimens of cestodes were collected from the small intestine of one adult animal (whale #5) (Fig. 2A). Morphometric characteristics of these parasites and the morphology of the scoleces (presence of clearly visible ear-like appendages and well-developed median lobes on the apical organ) (Fig. 2B) permitted the identification of these specimens as *Tetrabothrius ruudi* Nybelin, 1928 (Eucestoda, Tetrabothriidae). One specimen of acanthocephalan *Bolbosoma* sp. Porta, 1908 (Acanthocephala, Polymorphidae) was detected in the small intestine of one fin whale (whale #5), without any grossly evident change. The digenean *Ogmogaster antarcticus* Johnston, 1931 (Trematoda, Notocotyliidae) was found in the rectum of three fin whales (Fig. 2C, D).

Crassicaudosis was suspected and/or confirmed in five out of seven examined whales. Adult worms of the nematode *Crassicauda boopis* were identified in four cases (Table 2). A total of 95 tails of *C. boopis* (range: 7-40 parasites/host) were found inside the ureters of the infected animals (Fig. 3B). In three cases both kidneys were parasitized, while in one case a unilateral infestation was observed. The anterior part of the tails penetrated the wall of the ureters and extended into the renal parenchyma (Fig. 4A). Histopathological examination of the kidney revealed multifocal chronic nephritis with massive fibrosis, glomerular sclerosis and tubular atrophy in two animals (#4 and #5). In whale #4, larvated ova were histologically observed within the renal pelvis (Fig. 4B), in the lumen of large and medium caliber renal arteries and in adrenal gland vessels (small caliber arteries) (Fig. 4C). Additionally, larvated eggs of *Crassicauda* sp. were found in urinary sediment in whale #5.

Cephalic ends of *C. boopis* protruded freely into the lumen of the caudal vena cava in two animals (#4 and #5) (Fig. 3A). Large pendulous masses (Fig. 3C) and fragments of mineralized parasites were grossly observed within the lumen of renal arteries of two other whales (#2 and #3 respectively). Adult nematodes, pedunculated, botryoid and mineralized proliferations partially or totally obstructed the lumen of these vessels with severe thickening of the vessel wall. Microscopic examination revealed parasitic elements inside these vascular proliferations, containing large numbers of ova (Fig. 4D), completely embedded in concentric connective tissue layers with multifocal chronic inflammatory infiltrate (Fig. 5A,C). Nematodes were histologically observed in the renal parenchyma (Fig. 4A) without any tissue reaction. These parasites were associated with eggs in the renal pelvis (Fig. 4B) and in the lumen of the renal and adrenal arteries (Fig. 4C) and were also partially embedded by a mixed chronic inflammatory response mainly composed of eosinophils and marked fibroplasia. This inflammation with severe edema and congestion multifocally expanded and disrupted artery walls.

In whale #5, the mesenteric arteries were thickened and variably obliterated (Fig. 2E, inset) by intimal/subintimal hemorrhage, multifocal necrosis with cavitation and mineralization (Fig 5B). Microscopic examination confirmed chronic fibrosing endarteritis with severe eosinophilic infiltration and dystrophic mineralization were occasionally associated with nematode larva migrans (Fig. 5B, D). This animal also presented with several nodules in the intestine mucosa (Fig. 2E) (n >90; 6-7 mm-diameter) that contained amorphous material and occasionally associated with a single nematode larva (Fig. 2F). Skeletal muscles were grossly characterized by a diffuse and moderate pale discoloration and histological examination revealed a generalized muscular degeneration and edema with many fibers characterized by hyaline cytoplasm, mild atrophy and rare myocytolysis with scattered cells with central nuclei suggesting an ongoing regeneration process. Eosinophils were noted within the lumina of multiple arterioles and in several regional lymph nodes, there were numerous eosinophilic infiltrates with profound lymphoid depletion of germinal centers. Epicardial and endocardial granulomas were detected in fin whales #1 and #6 with PAS-positive translucent remains compatible with degenerated parasitic fragments digested by inflammatory reactions, and no intact parasites were identified. Additionally, nematode larvae were found free inside the lumen of the intestine of the calf whale #6; some of these featured pre and post-cloacal papillae, indicating a preadult stage.

Protozoan parasites

Whale #3 was PCR positive for *T. gondii* in heart, skeletal muscle, mesenteric lymph node and kidney samples (Table 4); the sequences showed 100% homology with *T. gondii* (GenBankTM accession no. AY 663792). The DNA sample that was genotyped was extracted from the skeletal muscle sample, successfully amplified at 3 microsatellite markers, and identified as *T. gondii* Type II. No lesions related to toxoplasmosis were observed in the histological sections of the examined tissues, even if *T. gondii* cysts were seen in sections of the kidney and heart (Mazzariol et al. 2012).

4. Discussion

Due to the technical complexities encountered during necropsy, the large size of these animals and the occasional difficulty of reaching the stranding site, complete parasitological surveys of fin whales in the Mediterranean Sea are lacking. Moreover, the preservation status of the carcasses affects the analyses that can be carried out.

The presence of ectoparasitic copepods *Pennella* sp. on Mediterranean fin whales has been known for a long time (Anthony & Calvet 1905). *Pennella* spp. are large mesoparasites parasitizing both teleost fishes and marine mammals. The high variability of morphological features of species

and stages of parasite development has generated much debate amongst taxonomists (Kabata 1979). Although numerous species of *Pennella* have been described, *P. balaenoptera* is the only recorded copepod species that parasitizes marine mammals (Hogans 1987, Abaunza et al. 2001). Recently, Fraija-Fernandez et al. (2018) provided evidence that *P. balaenoptera* should be considered a synonym of *P. filosa*, by comparing both morphological and molecular data for these two species. This finding demonstrates that the same species likely parasitizes both fishes and cetaceans, sharing the oceanic ecosystem.

The life cycle of *P. balaenoptera* is poorly understood and only the adult female and at present, only the first naupliar stage have been identified (Abaunza et al. 2001, Arroyo et al. 2002). Intensity of infestation by *P. balaenoptera* is considered an indicator of health status in cetaceans. Long-term, cumulative tendencies of *P. balaenoptera* burdens may be associated with a challenged immune system in striped dolphins, debilitating viral infection and high levels of polychlorinated biphenyls (Vecchione & Aznar 2014). Two cases of severe infestation by *Pennella* spp. were described in fin whales stranded alive along Italian and Turkish coastlines (Benvenuti et al. 1991, Cıçek et al. 2007). In our study, the degree of infestation was mild in most cases, and comparable to that reported by other authors in fin whales from Antarctic (Nishiwaki & Hayashi 1950, Mizue & Murata 1952) and Atlantic waters (Raga & Sanpera 1986). In one animal, positive for *Dolphin Morbillivirus* (DMV) and *T. gondii* infection, a severe infestation was reported (Mazzariol et al. 2012). Viral infection in this case suggested immunologic impairment which could also have favored the higher parasitic burden, as described in the literature for other species (Vecchione & Aznar 2014).

Two other DMV infected whales (Mazzariol et al. 2016) had immature stages of crustacean parasites belonging to the family Pennellidae. The morphological features of these parasites appear similar to those of the youngest individual of *Pennella filosa* (Linnaeus, 1758) described by Thompson (1905) in fish. This is the only report in the literature for immature stages of *Pennella* genus from a definitive host. Molecular analyses could be useful to ascribe these specimens to the species *P. balaenoptera*. The finding of young individuals of *Pennella* sp. in these two sick young whales suggests that the animals might had decreased their mobility shortly before death, allowing colonization by the parasites, as proposed by Aznar et al. (1994) in dolphins affected by a viral epizootic disease.

Adult tapeworms of the families Tetrabothriidae and Diphylobothriidae are described in cetaceans (Fraija-Fernández et al. 2016). Life cycles of these species involve zooplankton crustaceans as first intermediate hosts and marine mammals as their definitive hosts (Raga et al. 2008). Only the genera *Priapocephalus* Nybelin, 1922 and *Tetrabothrius* (Eucestoda: Tetrabothriidae) have been described in mysticetes, and members of the genus *Tetrabothrius* have been found in fin whales

worldwide. *Tetrabothrius affinis* (Lönnerberg 1891) (syn. *Tetrabothrius wilsoni*) is reported by Delyamure (1955) in Norway, South Africa, New Zealand and Antarctica (South Shetland islands); the same author reported *T. ruudi* in West Norway, France and Russian Pacific coast and Antarctica, but this genus had never been reported in the Mediterranean.

Acanthocephalans of the genus *Bolbosoma* are intestinal parasites of cetaceans and pinnipeds (Raga et al. 2008). Most of the species are typical of baleen whales, which are most likely infected by ingestion of the cystacanth larvae contained in an intermediate or paratenic host (Gazzonis & Merella 2012). Euphasiids and copepods have been shown to carry *Bolbosoma* larvae (Shimazu 1975, Tsimbalyuk 1980, Gregori et al. 2012). Five species are described in genus *Balaenoptera*, i.e., *B. brevicolle* (Malm, 1867), *B. nipponicum* Yamaguti, 1939, *B. turbinella* (Diesing, 1851), *B. balaenae* (Gmelin, 1790) and *B. hamiltoni* Baylis, 1929 (Delyamure 1955). The pathogenicity is linked to the anchorage of the proboscis to the intestinal wall, which can result in ulceration and even perforation (Gibson et al. 1998). No gross lesions were observed in the intestine of this animal, probably due to the presence of a single specimen.

The genus *Ogmogaster* Jägerskiöld, 1891 includes six species that are identified by the number of longitudinal ridges on the ventral surface, the presence or absence of spines on the tegument and body size (Raga et al. 1986). *Ogmogaster antarticus* was reported in fin whales from the Spanish Atlantic coasts and from the Mediterranean basin (Raga et al. 1986, Malatesta et al. 1998). The species shows wide diffusion and low host specificity being reported in both cetaceans and pinnipeds.

Crassicauda boopis has been reported in fin whales from the Atlantic and Pacific Oceans (Lambertsen 1986) and is considered endemic in the Atlantic population (Lambertsen 1992). Lambertsen described the dispersal and the mechanisms of this parasitic disease in Atlantic fin whales (1986, 1992). Infestation by *C. boopis* was already suspected in fin whales stranded in the Mediterranean, due to the presence of nematodes associated with the typical lesions in the urinary and vascular system (Fernández-Maldonado 2017, Giorda et al. 2017). Nevertheless, identification at species level was not achieved in those cases. The presence of intact terminal fragments of the nematodes allowed for such identification in our survey, confirming *C. boopis* in the animals covering the Mediterranean basin. The severity of the infestation ranged from moderate to severe in our survey. The anatomical locations of the parasites agreed with previous reports, with the cephalic portion of the female specimens in the lumen of the caudal vena cava and the male's head trapped in the renal veins. The localization of the head inside the lumen of vessels may allow the worms to feed on the host's blood (Lambertsen 1986). Reduced packed red cell volume in infected whales, potentially due to chronic consumption of blood by the worms, was reported by Lambertsen but due to autolysis, we

could not perform a complete blood count to confirm anemia. The massive inflammatory reaction of the host's tissues to the parasite was broadly observed, causing severe lesions that appeared very similar to those described in the literature (Lambertsen 1986). Wide thrombotic masses occupying the lumen of renal vasculature and diffuse inflammatory reaction and fibrosis of renal parenchyma were observed in all infested animals, with varying degrees of severity. Multidigitate masses within renal veins were also reported and attributed to the host's attempts to capsule the parasite. Such lesions are made up of fibrocellular tissue originating from the tunica media of the vessels. In chronic cases, a mineralized sheath can be observed completely around the worm. Occlusion of the renal vein lumen can occur as a consequence of the tissue reaction around the parasite. The lesions observed in this survey matched these features, with varying degrees of severity; they were particularly severe in the animal with a higher parasitic burden (n= 40). Multiple renal thromboses in this animal likely led to some degree of renal impairment by reduced blood flow. The severity of these lesions correlated with high serum concentration of creatinine (6.95mg/dL), urea (157.8 mg/dl) and phosphate (29.5mEq/L) in whale #5 (Mignone, personal communication). These results were significantly higher than those reported in the literature in infested whales (Lambertsen 1992). In this individual, eosinophils were also observed in several mesenteric lymph nodes and skeletal muscular vessels (*longissimus dorsi*) with no parasites evident. We surmise this condition could have played a role in the stranding together with the renal function impairment suggested by blood chemistry. Other potentially relevant parasitic lesion in this animal involved chronic verminous mesenteric arteritis. These findings could support the hypothesis of larval migration of *C. boopis* from intestine to renal vessels within the wall of the mesenteric arteries, as speculated by Lambertsen (1992) and similarly to crassicaudosis in Cuvier's beaked whales *Ziphius cavirostris* (Díaz-Delgado et al. 2016). Crassicaudosis could be a cause for concern in the Mediterranean fin whales.

Four types of Apicomplexa coccidians (Sarcocystiidae) are reported in odontocetes: *Cystoisospora delphini* in bottlenose dolphins *Tursiops truncatus*, *Sarcocystis* spp. in toothed whales and in a striped dolphin, *Neospora caninum* in bottlenose dolphins, and *T. gondii* in eight dolphin species, in one harbor porpoise (*Phocoena phocena* [Linnaeus, 1758]) and recently in one sperm whale (*Physeter microcephalus* Linnaeus, 1758) (Cruickshank et al. 1990, Inskoop et al. 1990, Migaki et al. 1990, Domingo et al. 1992, Di Guardo et al. 1995, Mikaelian et al. 2000, Jardine & Dubay 2002, Bowater et al. 2003, Cabezòn et al. 2004, Raga et al. 2008, Mazzariol et al. 2011, Gonzales-Viera et al. 2013). As for Mysticetes, the reports concerning Sarcocystiidae are limited. Akao (1970) described *Sarcocystis balaenopteralis* in muscle tissues of a sei whale *Balaenoptera borealis* (Lesson, 1828) and Forman et al. (2009) reported the presence of *T. gondii*-specific antibodies in a humpback whale

Megaptera novaeangliae) (Borowsky, 1781). Coinfection by *T. gondii* and DMV in one fin whale included in this study was reported by Mazzariol et al. (2012).

Genotyping of *T. gondii* from whale #3 identified type II genotype. To the authors' knowledge, this is the first genotyping attempt of a *T. gondii* strain from Mysticetes. Genotype II predominates not only in terrestrial mammals and birds of North America and Europe, but also in marine mammals of different geographic locations: in the California sea otter (*Enhydra lutris*), striped dolphin, bottlenose dolphin and walrus (*Odobenus rosmarus*) (Cole et al. 2000, Miller et al. 2004, Sundar et al. 2008, Di Guardo et al. 2011). Non-type II genotypes were reported in marine mammals such as those belonging to the haplogroup 12 in North America (formerly Type X and A) in the California sea otter (Khan et al. 2011), harbor seal (*Phoca vitulina*) and California sea lion (*Zalophus californianus*) (Cole et al. 2000, Miller et al. 2004, Sundar et al. 2008, Van Bressemer et al. 2009). In this animal, which had morbilliviral infection and high levels of organochlorine pollutants (Mazzariol et al. 2012), *T. gondii* cysts were not associated with a local inflammatory response. These findings could indicate an asymptomatic chronic *T. gondii* infection. In delphinids, such as striped dolphins, toxoplasmosis can cause severe brain lesions, especially in immunosuppressed animals (Di Guardo et al. 2010). However, this possibility could not be further investigated in this case due to lack of central nervous tissue samples.

In conclusion, our results increased knowledge of some parasitic diseases that may affect the fitness for survival of Mediterranean fin whales. Although anthropogenic threats are still the major concern for conservation of this species in the area, parasitic disease processes should be considered potential causes of morbidity and mortality and may be used as indicators in further health assessments.

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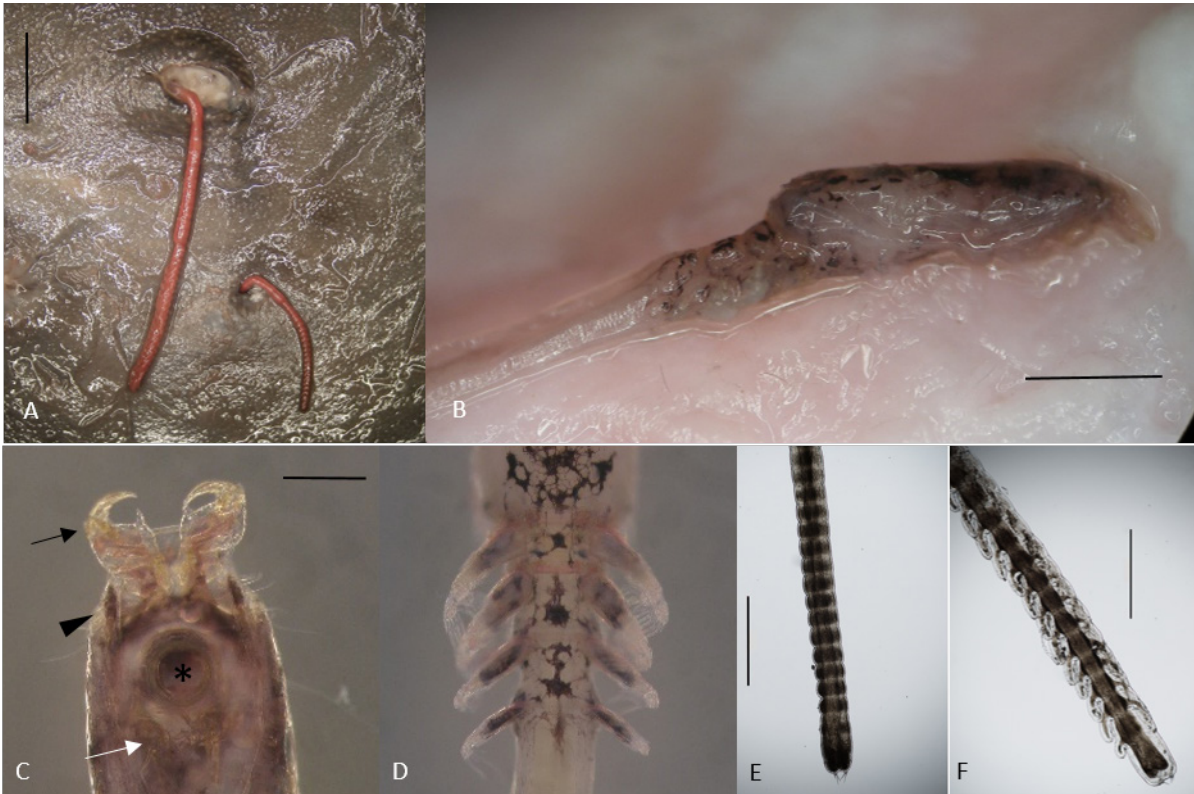


Fig.1: Immature stages of *Pennella* sp. (A). Abdominal portion of the parasite protruding from the host's skin (bar=0.5 cm); (B) Lateral view of cephalothorax after removing the surrounding tissues (bar=0.5 cm); (C) Ventral view of the head with first (black arrowhead) and second (black arrow) antennae, rostrum (*) and maxillipeds (white arrow) (bar=300 μ m); (D) Cephalothorax, details of the limbs (ventral view) (bar=500 μ m); (E, F) Abdomen of the parasite at two different developmental stages of the appendages (bars=1000 μ m).

Fig.2 (next page): Parasitological findings. (A). *Tetrabothrius ruudi* (arrow) collected from the intestine of whale #5; (B) Close-up view of the scolex of *T. ruudi* (bar=0.5mm); (C) filtration of the intestinal content by sieve and isolation of *Ogmogaster antarcticus* specimens in Petri dish; (D) specimen of *O. antarcticus* showing typical longitudinal ridges (bar=1mm); (E) Multifocal, well-defined nodules (arrows) on the intestine mucosa/submucosa; severe thickening of the wall of the mesenteric arteries (inset) (F) Detail of the nematode larvae (arrow) extracted from an intestinal nodule (bar=1mm)



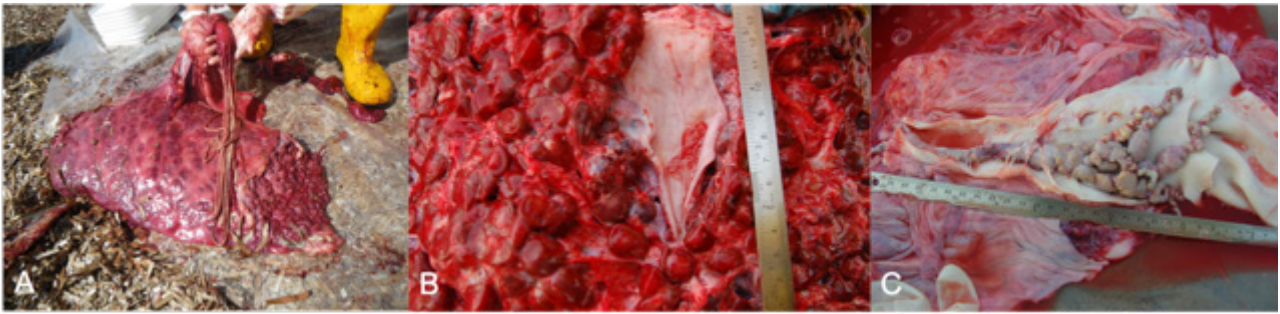


Fig.3: Gross findings. (A) Cephalic ends of *C. boopis* protruded freely into the lumen of the caudal vena cava in the whale #4; (B) Nematode tails in the proximal ureteral lumen in the whale #4; (C) Knobby and mineralized formation partially obliterating the vascular lumen of a renal artery of whale #2.

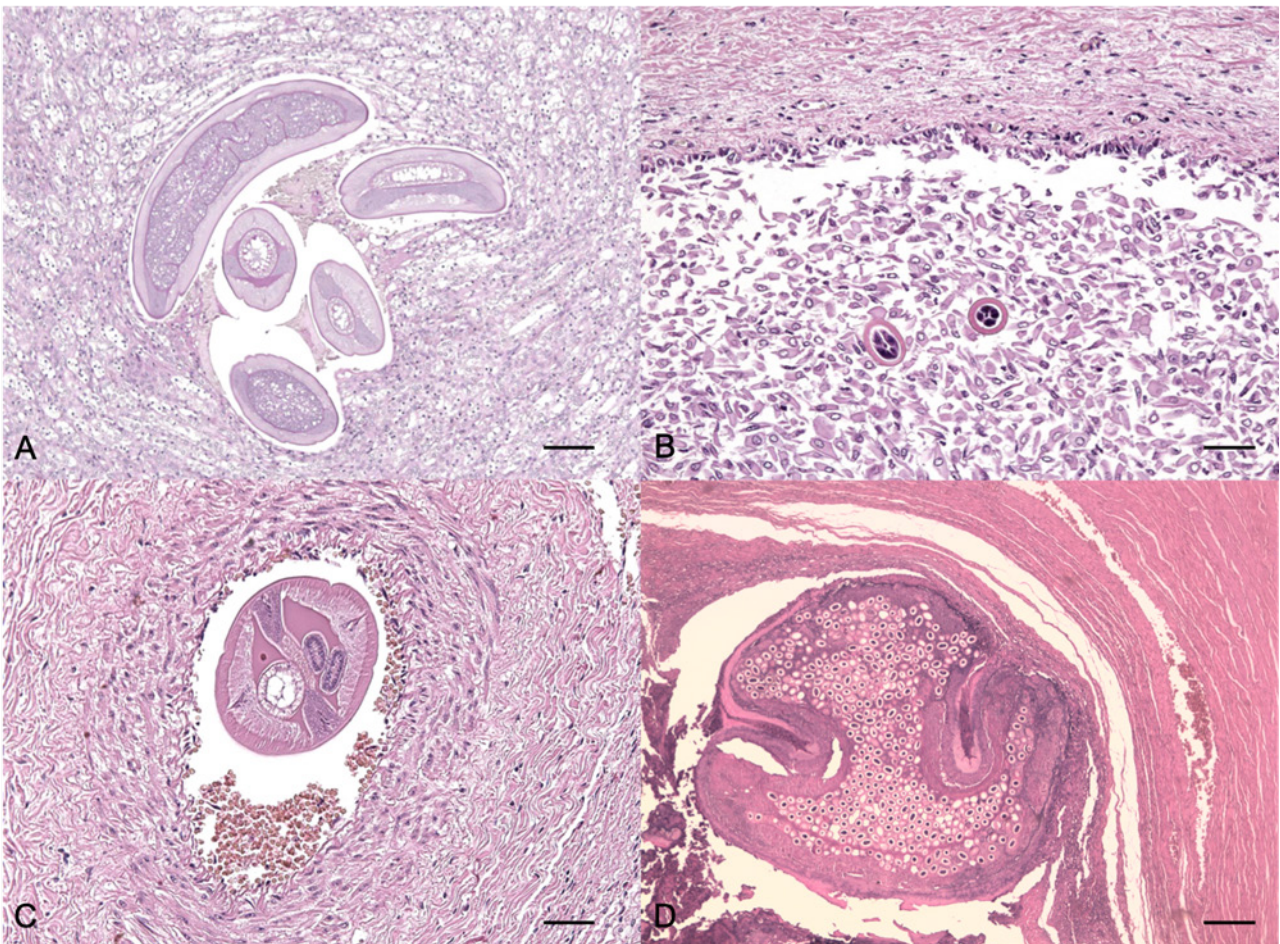


Fig. 4: Pathological findings. (A) *Crassicauda* adult nematodes within the renal medulla in whale #4 (10X magnification, Hematoxylin and Eosin (HE), bar=100µm); (B) *C. boopis* larvated eggs free in the renal pelvis of whale #4 (20X magnification, HE, bar=50µm); (C) Whale #4. *C. boopis* in the lumen of an artery in the adrenal parenchyma in whale #4 (10X magnification, HE, bar=100µm);

(D) In whale #2, presence of a parasitic thrombus formed by an adult *C. boopis* (4X magnification, HE, bar=200 μ m).

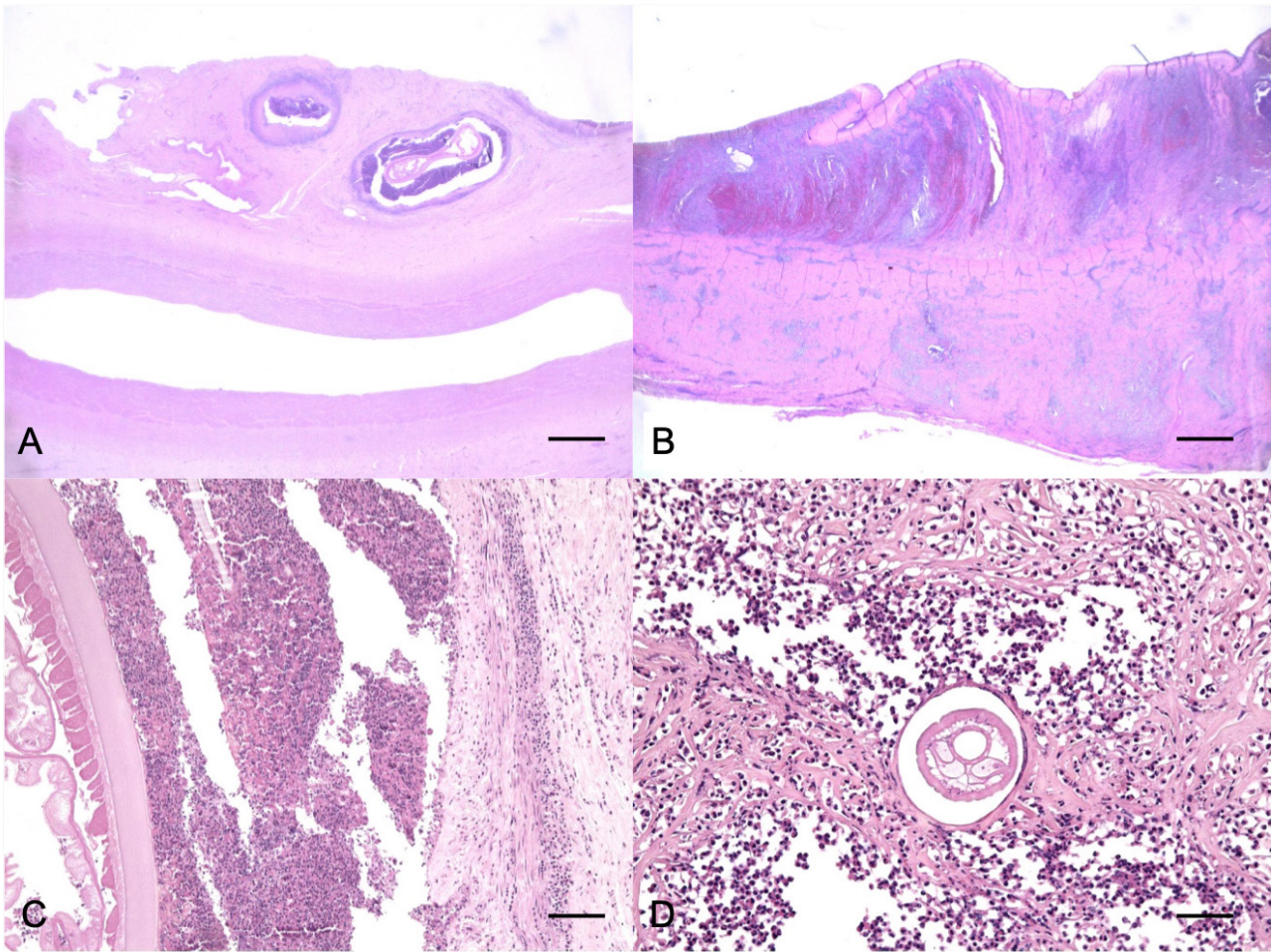


Fig. 5: Pathological findings. (A) and (C) Whale #4 renal artery with severe mural thickening (A) due to chronic inflammation and fibroplasia centered on nematode remnants (HE - A: 1X magnification, bar=1mm and C: 20X magnification, bar=50 μ m); (B) and (D) In whale #5 chronic mesenteric endarteritis with with subintimal hemorrhage, necrosis, cavitation and mineralization (B) and the presence of parasitic larva in the lumen of vasa vasorum (D) (HE - B: 1X magnification, bar=1mm and D: 20X magnification, bar=50 μ m).

Table 1. Fin whale specimens analyzed in this study. ID = Identification code; CCC = Carcass Condition Code, according to Geraci and Lounsbury, 2005; BCS = Body Condition Score; Age according to Aguilar and Lockyer, 1987 and Aguilar, 2009; F = Female; M = Male; DMV=Dolphin morbillivirus; RT-PCR = reverse transcription PCR; VN = virus neutralization; IHC = immunohistochemical analysis; * = data published in Mazzariol et al. 2016

Fin whale	ID	CCC	BCS	Age	Sex	Body length (m)	Year	Stranding site	DMV*
#1	109	3	Poor	Newborn	M	5.57	2006	Alassio (SV)	-
#2	134	2	Moderate	Juvenile	M	13.40	2008	Giannella (GR)	-
#3	194	3	Poor	Adult	M	16.7	2011	San Rossore (PI)	RT-PCR + (liver, spleen, lung)
#4	208	2	Poor	Juvenile	F	10.78	2011	Capo Testa (OT)	RT-PCR + (liver, spleen, lymph node, muscle)
#5	211	2	Poor	Juvenile	M	10	2011	Savona (SV)	VN +
#6	297	2	Poor	Newborn	F	5	2013	Marciana (LI)	RT-PCR + (brain, spleen, lung, thymus), IHC + (brain, thymus)
#7	342	3	Moderate	Adult	M	17	2015	Camaiore (LU)	-

Table 2. Metazoan parasites collected and identified from the examined fin whales. Neg = negative; Pos = positive for the presence of *C. boopis*, but not quantitatively determined; *”suspected” positive to crassicaudosis for presence of lesions (see in the text) F = female; M = male

Organs	Parasite	Fin whale #1	Fin whale #2	Fin whale #3*	Fin whale #4	Fin whale #5	Fin whale #6	Fin whale #7
Skin-blubber	<i>Pennella balaenoptera</i> (Copepoda; Pennellidae)	Neg	10	24	3	2	Neg	16
	Immature stage of <i>Pennella</i> sp. (Copepoda; Pennellidae)	Neg	Neg	Neg	15	Neg	3	Neg
Intestine	<i>Ogmogaster antarcticus</i> (Trematoda; Notocotylidae)	Neg	712	254	Neg	Neg	Neg	1
	<i>Tetrabothius ruudi</i> . (Eucestoda; Tetrabothriidae)	Neg	Neg	Neg	Neg	8	Neg	Neg
	<i>Bolbosoma</i> sp. (Acanthocephala; Polymorphidae)	Neg	Neg	Neg	Neg	1	Neg	Neg
	Nematoda larvae (inside nodules)	Neg	Neg	Neg	Neg	7	Neg	Neg
	Nematoda larvae (free into the lumen)	Neg	Neg	Neg	Neg	Neg	9	Neg
Kidney /ureters	<i>Crassicauda boopis</i> tails	Neg	40 (36 M; 4 F)	Neg	38 (24 M; 14 F)	10 (8M; 2 F)	Neg	7
Vena cava	<i>Crassicauda boopis</i> heads	Neg	Neg	Neg	11	Pos	Neg	Neg
Mesenteric arteries	Nematoda larvae (into the vessels wall)	Neg	Neg	Neg	Neg	4	Neg	Neg.

Table 3. Mean and individual measurements (in μm) of six immature specimens of *Pennella* sp. Oken, 1815 in different developmental stages.

		Mean	Specimen 1	Specimen 2	Specimen 3	Specimen 4	Specimen 5	Specimen 6
	Total length	33899	21582	26641	27245	32302	43525	52097
Cephalothorax	Total length including limbs	2573	2404	2516	2420	2644	2694	2763
	Head length	1910	1726	1967	1847	1908	1978	2034
	Head width	781	665	747	797	817	824	837
Thoracic region	Diameter in the middle region	444	392	344	402	472	494	559
	Total length	25257	14655	18260	18475	23192	34271	42689
Abdomen	Total length	6068	4522	5865	6351	6467	6560	6645
	Width in the middle region	364	340	365	370	310	324	476
	Width including appendages	631				630	668	594
	Appendages		Poorly developed	Poorly developed	Poorly developed	Present	Present	Present

Table 4. Results of *T. gondii*, *Neospora*, and *Sarcocystis* detection by PCR and sequencing.

Tissues	Fin whale #1	Fin whale #2	Fin whale #3 °	Fin whale #4	Fin whale #5	Fin whale #6	Fin whale #7
Brain	Negative	Negative	NOT DONE	Negative	Negative	Negative	Negative
Spinal cord	Negative	Negative	NOT DONE	Negative	NOT DONE	Negative	Negative
Heart	NOT DONE	Negative	<i>T. gondii</i> *	Negative	Negative	Negative	NOT DONE
Skeletal muscle	Negative	Negative	<i>T. gondii</i> *	Negative	Negative	Negative	Negative
Lymph nodes	Negative	NOT DONE	<i>T. gondii</i> *	Negative	Negative	Negative	Negative
Spleen	NOT DONE	NOT DONE	Negative	NOT DONE	NOT DONE	NOT DONE	NOT DONE
Liver	NOT DONE	Negative	Negative	Negative	Negative	Negative	Negative
Lung	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Kidney	NOT DONE	Negative	<i>T. gondii</i> *	Negative	Negative	Negative	Negative

* *T. gondii*, GenBank accession number AY663792; ° Mazzariol et al. 2012

5. Paper 5

In preparation (draft)

Morphological and molecular characterization of adults and larvae of *Crassicauda* spp. (Nematoda: Spirurida) from Mediterranean fin whales *Balaenoptera physalus* (Linnaeus, 1758)

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1. Introduction

Nematodes of the genus *Crassicauda* (Spirurida: Tetrameridae) are parasites of different species of cetaceans, both toothed and baleen whales. Different site tropism and consequent pathogenic impact are described among the members of this genus, whose localizations span over subcutaneous tissues, cranial sinuses, and urogenital system (Geraci and St. Aubin 1987; Lambertsen 1986, Jabbar et al. 2015). *Crassicauda boopis* (Baylis, 1920), with its large size and the localization in the renal and circulatory district, is considered to be one with the highest pathogenic potential in whales, similar to that of *Crassicauda anthonyi* in Cuvier's beaked whales (Diaz-Delgado et al. 2016). Fin whales (*Balaenoptera physalus*), humpback (*Megaptera novaeangliae*) and blue whales (*Balaenoptera musculus*) have been reported to be susceptible to *C. boopis* infections. Reports of this species exist from North and South Pacific Ocean (Baylis 1920, Margolis & Pike 1955, Rees 1953, Cockrill 1960, Delyamure 1955) and North Atlantic Ocean (Hamilton 1914, 1915, Lambertsen 1985, 1986, Lempereur et al. 2017). Recently, *C. boopis* has been recorded in fin whales stranded along the coast of the Mediterranean Sea (Marcer et al. *unpublished data*) and nematodes likely referable to the same species have been observed in a fin whale stranded along Spanish coasts (Fernandez-Maldonado, 2018) demonstrating that the infection is not rare also in this geographical area. Renal impairment is described in infected fin whales as a consequence of the severe damage caused by parasites and host's immune response in renal glomeruli and vessels. Thrombosis of the renal veins and of the vena cava, abscessation and fibrosis of the kidneys are lesions commonly associated to the presence of cephalic portions of female nematodes within the lumen of the vena cava and presence of the central body of the parasite in renal parenchyma. The impairment of blood supply to the kidney can finally result in congestive renal failure (Lambertsen 1992).

Few hypotheses on the life cycle of *Crassicauda* spp. have been formulated (Lambertsen 1992, Diaz-Delgado et al. 2016). Based on the presence of vasculitis, sclerosis, and linear scars along mesenteric arteries and aortic walls, a larval migration from the intestine to the kidneys through vascular walls, has been hypothesized for larvae of *C. boopis* (Lambertsen 1992), similarly to *Crassicauda* sp. in Cuvier's beaked whales (CBW) (Diaz-Delgado 2016). These migrating immature elements have never been isolated nor morphologically described in literature.

Identification of *Crassicauda* spp. relies on the observation of the morphometric features of cephalic and terminal fragment of the adult specimens, and comparison with literature. The great length of the body of some species and the massive host's reaction surrounding parasites' body often prevent the

collection of intact specimens, making molecular methods a useful alternative for species identification. Current molecular characterization of the *Crassicauda* nematodes is very limited both in term of studied species and analyzed genes. Thus, it is essential to increase the taxon sampling as well as the gene sampling for testing the utility of studied markers for species identification as well as to assess the phylogenetic relationships among different taxa.

The aim of this work is to characterize morphologically and molecularly adult and larval stage nematodes isolated from Mediterranean fin whales. Furthermore, we wanted to perform a multigene analysis, including ribosomal and mitochondrial markers, to test if these molecular markers allow identifying the different species of *Crassicauda*.

2. Materials and methods

2.1 Morphological characterization

Fragments and intact specimens of adult nematodes (N=95 tails, 11 cephalic portions) were isolated from vena cava, ureters and kidneys of three fin whales stranded along Italian Tyrrhenian coasts in the period 2008-2013. Two cephalic portions, 3 male tails and 3 female tails of these adult nematodes, were observed to achieve morphological identification.

Larvae of nematodes were isolated from two fin whales (*B. physalus*), stranded in the same geographical area. In particular, seven larvae were collected from nodules included in the intestinal wall and four included in mesenteric arteries' walls of one fin whale (whale#334); nine free larvae were collected from the intestinal lumen of whale #270. All these larvae were morphologically studied.

Additional comparative material

Specimens of adult nematodes isolated from the kidney of a Cuvier's beaked whale (*Ziphius cavirostris*), and from the pterygoid sinuses of a Risso's dolphin (*Grampus griseus*), were morphologically identified as *C. anthonyi* (4 cephalic portions, 1 male tail and 1 female tail) and *C. grampicola* (2 cephalic portions, 2 male tail and 2 female tail), respectively. One cephalic portion, one male tail and one female tail of both species were deposited at the Natural History Museum of London (NHMUK) (*C. anthonyi*: 2018.4.25.3-4; *C. grampicola*: 2018.4.25.1-2). Finally, fragments of *Crassicauda* sp. isolated from subcutaneous tissues of two bottlenose dolphins (*Tursiops*

truncatus) and of one striped dolphin (*Stenella ceruleoalba*) were included in the study. Data of the hosts and nematodes analyzed in this study are listed in Table 1.

All the collected nematodes were preserved in 70% ethanol; cephalic end and tails of the parasites selected for morphological identification were clarified in Amman's lactophenol solution and subsequently observed at light microscope (Olympus, ACH 40X-2) by NIS-Elements D software (Nikon).

2.2 Molecular analyses

DNA was isolated from the following specimens: 12 adult nematodes from fin whales; 4 larvae from mesenteric arteries and intestinal nodules and 3 larvae from the intestinal lumen of fin whales; 4 specimens of *C. anthonyi*; 4 specimens of *C. grampicola*; 6 fragments of *Crassicauda* sp. (Table 1 and 2). Extraction was performed using NucleoSpin® Tissue Kit (Macherey-Nagel, Germany), according to manufacturer's instructions. The small subunit (SSU; 18S) and the ITS-2 fragment of the rDNA, and the mt-*cox 1* gene region were amplified by polymerase chain reaction. Primers G18S4-F (5'-GCTT GTCTCAAAGATTAAGCC-3') and reverse 136-R (5'-TGATCCTTCTGCAGGTTACCTAC-3') (Nadler et al. 2007) were used for 18S amplification, while forward primer D (5'-GAGTCGATGAAGAACGCAG-3') and reverse B1 (5'-GAATCCTGGTTAGTTTCTTTTCCT-3') were used for the ITS-2 region (Traversa et al. 2004). Finally, primers JB3 (5'-TTTTTTGGGCATCCTGAGGTTTAT-3') (forward) and JB4.5 (5'-TAAAGAAAGAACATAATGAAAATG-3') (reverse) were used for *cox 1* (Bowles et al. 1992).

The PCR for 18S region was performed in a 30 µl reaction volume, comprising 1-3 µl DNA, 2mM MgCl₂, 0.25 mM dNTPs (MBI Fermentas, Germany), 1X PCR buffer, 0.5 µM of each forward and reverse primer, 1U Platinum Taq DNA Polymerase (Invitrogen), with the remainder of the volume made of sterile water. Cycling conditions comprised an initial activation step at 94°C for 2 min., followed by 35 cycles of 94° for 30 s, 58,7° for 30 s, 72°C for 80 s, with a final extension step of 72° for 7 min.

The PCR for ITS-2 region was performed in a 30 µl reaction, comprising 1-3 µl DNA, 2,5mM MgCl₂, 0.25 mM dNTPs (MBI Fermentas, Germany), 1X PCR buffer, 0.5 µM of each forward and reverse primer, 1U Platinum Taq DNA Polymerase (Invitrogen), with the remainder of the volume made of sterile water. Cycling conditions comprised an initial activation step at 94°C for 2 min., followed by 35 cycles of 94° for 30 s, 58° for 30 s, 72°C for 30 s, with a final extension step of 72° for 5 min.

The PCR for *cox 1* region was performed in a 30 µl reaction volume, comprising 1-3 µl DNA, 2,5mM MgCl₂, 0.5 mM dNTPs (MBI Fermentas, Germany), 1X PCR buffer, 1.25 µM of each forward and

reverse primer, 1U Platinum Taq DNA Polymerase (Invitrogen), with the remainder of the volume made of sterile water. Cycling conditions comprised an initial activation step at 95°C for 2 min., followed by 35 cycles of 94° for 40 s, ° for 30 s, 72°C for 30 s, with a final extension step of 72° for 5 min.

The PCR products were resolved in 2% agarose gel with SYBR® Safe DNA gel stain (Invitrogen™, USA). The amplicons of PCR (fragments of expected size 1700 bp for 18S, 510 bp and 710 bp for ITS-2 and *cox I* respectively) were directly sequenced by Macrogen (Macrogen Europe, the Netherland). A couple of internal primers were designed specifically with software Primer3 to facilitate sequencing the 18S fragment, i.e. forward primer 437F (5'-AACTAAGAACGGCCATGCAC-3') and reverse 1279R (CTCTCGGCATGAGGAGGTAG-3').

The chromatograms were corrected using the software ChromasPro version 2.4.3 (Technelysium Pty Ltd, Australia). The consensus sequences were assembled with the program SeqMan available in the DNASTar package. The consensus sequences were compared with the non-redundant data base available in the GeneBank® database using the software BLAST (Altschul et al. 1990).

2.3 Phylogenetic analysis

Data set including the *cox1* and *ITS2* new sequences as well as orthologous counterparts available in GenBank, obtained for taxa related in *Crassicauda* genus, were created (see results). The multiple alignments of these sets were performed with the program Muscle implemented in MEGA7 software. Phylogenetic trees were computed with the IqTree software according to the Maximum likelihood method by applying the best fitting evolutionary model selected by the program itself. To assess the statistical support to the tree topologies 10.000 of ultrafast bootstrap replicates were calculated.

3. Results

3.1 Morphological description of nematodes collected in *B. physalus*

3.1.1 Adult nematodes

Morphometric data of adult nematodes isolated from the kidneys of fin whales fitted with the description of *Crassicauda boopis* in literature (Baylis 1920, Delyamure 1955, Lambertsen 1985).

Female. Body elongate, maximum length of cephalic fragment 60.5 cm. Anterior end (lying free in the lumen of vena cava) tapering to head, with an evident constriction at 52 µm from buccal opening.

Buccal cavity compressed (width 23x21 μm); small lateral lips and 8 cephalic papillae are visible on the anterior end (Fig. 1A.). Esophagus is encircled by a nerve ring at 0.27 mm from anterior extremity. Paired ovaries and uteri. The posterior end, found in the ureter, bears a genital constriction, at 4.33 mm from the tip of tail, where the vulva opens ventrally (Fig. 1B). A pineal appendage contains last part of intestine and a terminal anus.

Male. Cephalic extremity fixed in renal parenchyma. The posterior end (found in ureters) is attenuate, with a coiled tail (2 turns); the cloaca opens at 1.056 mm from tip of tail, with no spicules. Eighteen caudal papillae are visible (Fig. 1C).

Specimens of *C. boopis* (1 head, 2 male tails and 2 female tails) were deposited at the NHMUK (*Crassicauda boopis* 2015.10.11.1-4).

3.1.2 Larvae

Intestinal nodules. Larval elements contained in the intestinal mucosa of one of the adult whales showed the following morphometric features: Total length of the body 9.99 mm (range 7.8-12.0), width 103.75 μm (range 95.49-113.62). Anterior end sharp, two pseudolabia and 6 prominent lateral (externo-labial) papillae. Width of anterior end's wall 5.86 μm (range 4.94-7.41). Genital pore (or cloaca) 65.30 μm from tip of tail, posterior end with a terminal button, starting from the cloacal opening and terminating with a rounded tip; width of posterior end's wall 4.01 μm (Fig. 2A, B).

Mesenteric arteries' wall. These elements were included in the thickness of the vessels' intimal layer. Morphological features were generally similar to those of the larvae included in the intestinal nodules. Total length of the body 10,6 mm (range 9,8-11,4), width 10,7 mm (range 9,2–13,0). Triangular shape of the head, bulging at the base. Intestinal tube evident in central and terminal segment of the body (Fig. 2C, D).

Intestinal lumen. Free larvae inside intestinal lumen: length 33500 μm , width 212.50 μm . Anterior end sharp, with two lips and six cephalic papillae. Buccal cavity 62.7 μm x 15.8 μm , continuing in an esophagus 26,8 μm long. Cuticle thickness of 3.07 μm . Posterior portion bearing a terminal restriction in which a vestigial genital pore and pre and post cloacal papillae (9-10 pairs) are occasionally visible (Fig. 2E, F).

Morphological features of larval specimens are here briefly described and the measurements are provided in Table 3.

3.2 Molecular and phylogenetic analysis

Amplification and sequencing of 18S gene resulted in 26 sequences of good and their alignment proved them to be identical for all species of the genus *Crassicauda* and for larval elements (ACC #1,#8,#11,#14,#18,#23). The BLAST research in GeneBank revealed a high identity (99%) with *C. magna* (accession number KM233410). Thus these sequences proved useless to discriminate among different *Crassicauda* species.

Amplification of ITS-2 fragment gave 24 sequences of good quality. The specimens, represented by fragments of adult parasites, belonging to *C. boopis* were split in two haplotypes (ACC #2,#3). All specimens belonging *C. grampicola* (ACC#19) exhibited an identical haplotype, and the same reasoning applied to specimens of *C. anthonyi* (ACC #15). Specimens, represented by fragments of adult parasites, assigned to *Crassicauda* sp. 1 (ACC #24) exhibited a single haplotype. As for larval nematodes, the ITS-2 sequence of the larvae found free in the intestinal lumen appeared to be identical to that of *C. boopis* (ACC #12). On the other side, larvae included in the intestinal nodules and in the mesenteric artery's wall exhibited a distinct haplotype of ITS-2 (*Crassicauda* sp. 2) (ACC #9). The phylogenetic analyses of all sequenced haplotypes is provided in Fig 3.

As for the *cox1*, the specimens belonging to *C. boopis* were split in four haplotypes (ACC#4,#5#6,#7) Specimens of *C. anthonyi* clustered in two haplotypes (ACC#16,#17) and the same applied to *Crassicauda* sp. 1 (ACC#25,#26). Conversely, the *cox1* sequences obtained from *C. grampicola* belonged to three distinct haplotypes (#20,#21,#22). No sequences were obtained from *Crassicauda* sp. 2 samples. The ML tree obtained for *cox1* sequences is provided in Fig 4. The analysis of the topology reveals that, with the exception of *Crassicauda* sp. 1, the *cox1* sequences obtained from other species of *Crassicauda* do not form monophyletic groups. This result strongly supports the view that ancestral polymorphisms characterize the mitochondrial genomes of *Crassicauda* species making the *cox1* marker useless for the molecular identification of this group of parasitic nematodes.

4. Discussion

Fourteen species have been described inside the genus *Crassicauda*, i.e. *C. crassicauda*, *C. giliakiana*, *C. anthonyi*, *C. bennetti*, *C. grampicola*, *C. boopis* (syn. *C. pacifica*), *C. magna* (syn. *C. duguyi*), *C. tortilis*, *C. delamureana*, *C. fuelleborni*, *C. costata*, *C. carbonelli*. These species occur in the kidney (*C. giliakiana*, *C. anthonyi*, *C. bennetti*, *C. boopis*, *C. tortilis*, *C. delamureana*, *C. costata*), reproductive system (*C. crassicauda*, *C. carbonelli*, *C. fuelleborni*), pterygoid sinuses (*C. grampicola*) and subcutaneous tissues and “gill slit” gland (*C. magna*) of the host (Keenan-Bateman et al., 2018). Among them, four species have been described in the kidneys of mysticetes, i.e. *C.*

boopis, *C. tortilis*, *C. delamureana*, *C. costata* showing low host-specificity. The morphological identification of adult specimens is primarily based upon presence of the spicules, which are absent in *C. boopis* and *C. tortilis* and present in *C. delamureana* and *C. costata* (Skrjabin, 1969). *C. boopis* has been isolated in fin whale, blue whale and humpback whale, whereas *C. tortilis* has been described in *Balaenoptera musculus* (Skrjabin 1973) (Skrjabin, 1959, Lambertsen, 1992). The distinction between the two species relies mainly upon measurements, that are yet similar. In the present study, the isolation of the intact tails and heads of males and females of *C. boopis* allowed the identification of the species in three hosts, by comparison with the description of the species made by Lambertsen (1985).

To date, complete knowledge on life cycle of *Crassicauda* spp. has not been reached, but hypothesis on transmission, migration and development of immature elements have been formulated for *C. boopis*, observing pathological findings in infected fin whales. A similar somatic migration has been recently evoked for larval *Crassicauda* sp. in CBW, as suggested by the very similar pattern of lesions in the hosts' circulatory system and the presence of adult nematodes in the kidneys (Díaz-Delgado et al. 2016). The presence of an intermediate or paratenic host is a reliable hypothesis, as marine spirurids have indirect cycles (Anderson, 2000). *Meganyctiphanes norvegica* or other crustaceans included in rorquals diet could instead play a role in a hypothetical indirect life cycle of *C. boopis*, in which infective larvae would get to the host during filter-feeding. Nevertheless, a direct transmission whale-to-calf has been hypothesized during lactation, for the presence of adult nematodes in the kidneys of waning calves (Lambertsen 1986, Lempereur et al. 2017); since no evidences of a nematodes in the placenta or in the mammary tissue have ever been detected, the passage of larvae through ingestion of urine-contaminated milk during lactation from mother to calf has been hypothesized. Successively, the pattern of lesions observed in infected whales suggested the passage of the immature stage parasites through the gut's mucosal and submucosal layers to reach mesenteric arteries' walls; always according to the evidence of lesions, they finally reach the aorta and renal arteries either by migrating within vessels' walls or by getting into the blood flow. Lambertsen (1986) indeed reported the presence of larval elements in the tunica media of the mesenteric arteries of fin whales infected at the same time by adult specimens of *C. boopis*. Larvae were observed in histological sections of the vessels, in association with severe flogosis and hemorrhages of the intima, but no morphological considerations were done on these elements. Such larvae were inferred to belong to the species *C. boopis* because of the absence of other parasitic nematodes in the examined animals, excluded *C. crassicauda* and anisakids.

In this study larval nematodes were isolated from mesenteric arteries, intestinal walls and intestinal lumen of fin whales, with morphological features of less and more advanced developmental stages for the different sites in this order. The phylogenetic analysis performed on 18S sequences permitted to ascribe all of them to the genus *Crassicauda*. The 18S marker presents highly conserved regions, which efficiently permit to distinguish deeply phylogenetically separated taxa inside nematode clade. This is confirmed by this study, in which only one sequence was obtained from all different species of *Crassicauda*, as already evidenced by Lempereur et al. (2017) and Díaz-Delgado et al. (2016).

Analysis of the ITS-2 sequences efficiently grouped together specimens morphologically identified as the same species (i.e. *C. grampicola* and *C. anthonyi*), as happens for other nematode families (Chilton et al. 2004, Powers et al. 1997) included the Spirurida (Traversa et al. 2004). The identity of ITS-2 sequences of the well-developed larvae isolated from intestinal lumen of the newborn calf with those from adult *C. boopis* may open new insights on migration routes of *C. boopis* in the host. We speculate the migration through the intestinal walls could occur very late in the developmental process of the nematode, which would get to the renal district in a late maturing stage; different migration routes could actually exist for *C. boopis* in newborn calves or aberrant migration could also occur in such cases.

Further studies are needed to ascertain the identity of the less developed larvae found in intestinal walls and mesenteric arteries of the adult whale #334. Unfortunately, no data are present in public databases on ITS-2 sequences from other *Crassicauda* species to help identifying these elements. We speculate that at least other two species should be molecularly characterized to perform comparison with the larval elements recovered, i.e. *C. crassicauda* and *C. tortilis*. The first one is the only other species of *Crassicauda* described in fin whales. In the study by Lambertsen (1992), adults of *C. crassicauda* were present simultaneously with adult *C. boopis* and with *larvae migrans* in the mesenteric arteries; no hypothesis on the life cycle of this species have been formulated. On the other side, *C. tortilis* is the only other species described in the kidneys of a mysticete (*Balaenoptera musculus*). Assuming that similar final localization could correspond to similar intra-somatic migration route - as observed for *Crassicauda* sp. in CBW's kidneys (Díaz-Delgado et al. 2016) and considering that the genus *Crassicauda* is characterized by a relatively low host specificity inside the suborders of the odontocetes and mysticetes, a wider molecular sampling inside the genus would be essential to confirm these hypotheses.

This work contributes both to enrich the genetic sequence data bank and the molecular taxonomy of this genus; it represents an important starting point for identification of ruined fragments or immature stages of *Crassicauda* spp.

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Table 1. List of specimens of *Crassicauda* spp. morphologically and molecularly studied in this work and their provenience.

	18S	ITS-2	COX I
<i>C. boopis</i> (n=12)	Accession number #1	Accession number #2	Accession number #4
Host: <i>B. physalus</i>		Accession number #3	Accession number #5
Localization: kidney			Accession number #6
			Accession number #7
<i>Crassicauda</i> sp. larvae (n=4)	Accession number #8	Accession number #9	Accession number #10
Host: <i>B. physalus</i>			
Localization: mesenteric arteries, intestinal nodules			
<i>Crassicauda</i> sp. larvae (n=3)	Accession number #11	Accession number #12	Accession number #13
Host: <i>B. physalus</i>			
Localization: intestinal lumen			
<i>C. anthonyi</i> (n=4)	Accession number #14	Accession number #15	Accession number #16
Host: <i>Z. cavirostris</i>			Accession number #17
Localization: kidney			
<i>C. grampicola</i> (n=4)	Accession number #18	Accession number #19	Accession number #20
Host: <i>G. griseus</i>			Accession number #21
Localization: cranial sinuses			Accession number #22
<i>Crassicauda</i> sp. (n=6)	Accession number #23	Accession number #24	Accession number #25
Host: <i>S. ceruleoalba</i> , <i>T. truncatus</i>			Accession number #26
Localization: subcutaneous tissue			

Table 2. Fin whales and specimens of nematode (larvae and adults) morphologically and molecularly (in brackets) analyzed in this study.

Fin whale	Site and year of stranding	Age	Sex	Adult nematodes				Nematode larvae		
				Female tails	Male tails	Heads	Internal fragments	Intestinal nodules	Mesenteric arteries	Intestinal lumen
#24-08 134	Giannella (GR), 2008	Juvenile	M	1 (1)	1	-	(1)	-		
#334- 11 211	Savona (SV), 2011	Juvenile	M	1 (1)	1 (1)	-	(1)	7 (2)	4 (2)	-
#308- 11 208	Capotesta (OT), 2011	Juvenile	F	1 (3)	1 (3)	2 (1)	-	-		
#270- 13 297	Marciana (LI), 2013	Newborn	F	-	-	-	-	-	-	9 (3)

	IN	MA	FL		IN	MA	FL
Total length	8356,81	10613,79	30417,45	Width of tail at cloacal pore opening	61,15	64,69	F : 154,00
Mid body width	81,53	107,33	151,08	Distance genital pore – terminal end	62,01	71,44	F: 544,11 M: 141,98
Head width	39,29	41,49	66,05	Cuticle width at mid body	5,63	5,48	7,8
Distance denticles – head base	25,69	21,73	36,08	Cuticle width in terminal end	4,22	4,43	4,8
Distance excretory pore- cephalic end	209,46	171,72	269,27	Length of buccal cavity	/	/	63,91
Denticles length	3,24	2,78	8,78	Width of buccal cavity	/	/	15,19
Head cuticle width	2,08	1,44	4,42				

Table 3. Main morphometric data on larval elements (μm). IN, Intestinal Nodules; MA, Mesenteric Arteries; FL, free larvae in intestinal lumen.

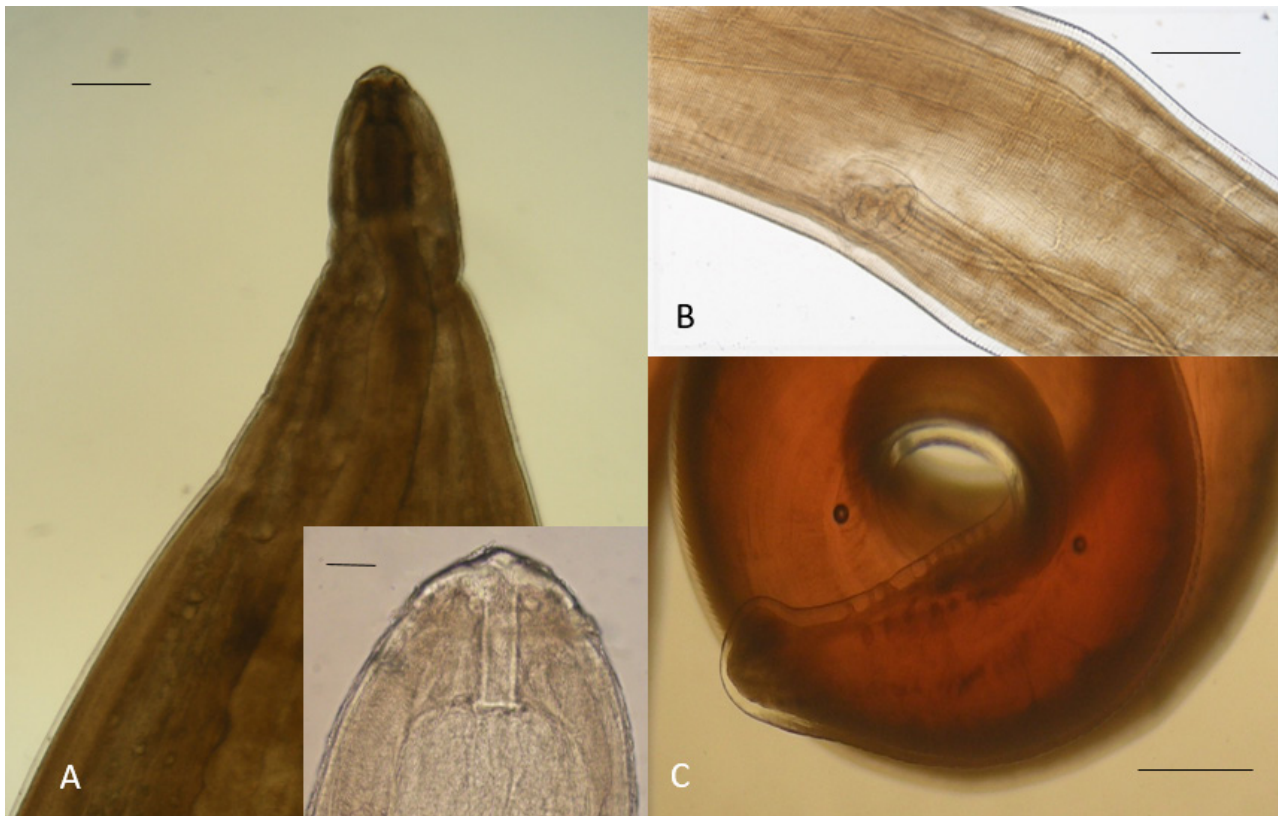


Figure 1. Adult *Crassicauda boopis*. A. Female. Cephalic end (bar=280 μm) with particular of the buccal cavity (inset; bar=70 μm). B. Female. Paired uteri opening in the vulva (bar=600 μm). C. Male. Coiled caudal extremity with caudal papillae (stereomicroscope) (bar=1 mm).

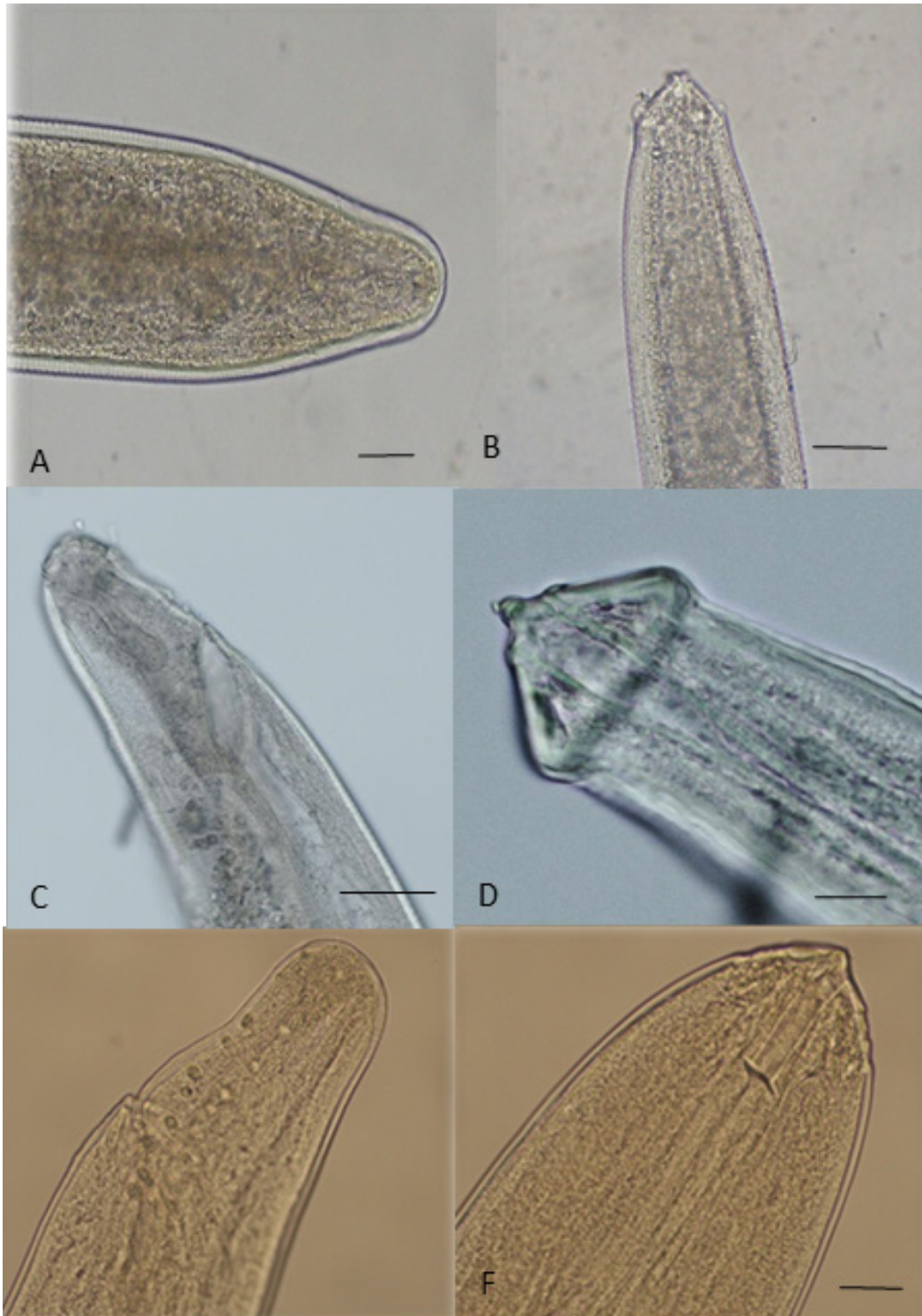


Figure 2. Larval *Crassicauda* spp. A, B. Caudal and cephalic end of larvae from intestinal nodules (A, bar=20 μ m; B, bar=40 μ m). C, D. Caudal and cephalic end of larvae isolated from mesenteric arteries wall (C, bar=50 μ m; D, bar=25 μ m). E, F. Caudal and cephalic end of larvae isolated from intestinal lumen, with caudal papillae visible in E (E, bar=50 μ m; F, bar=30 μ m).

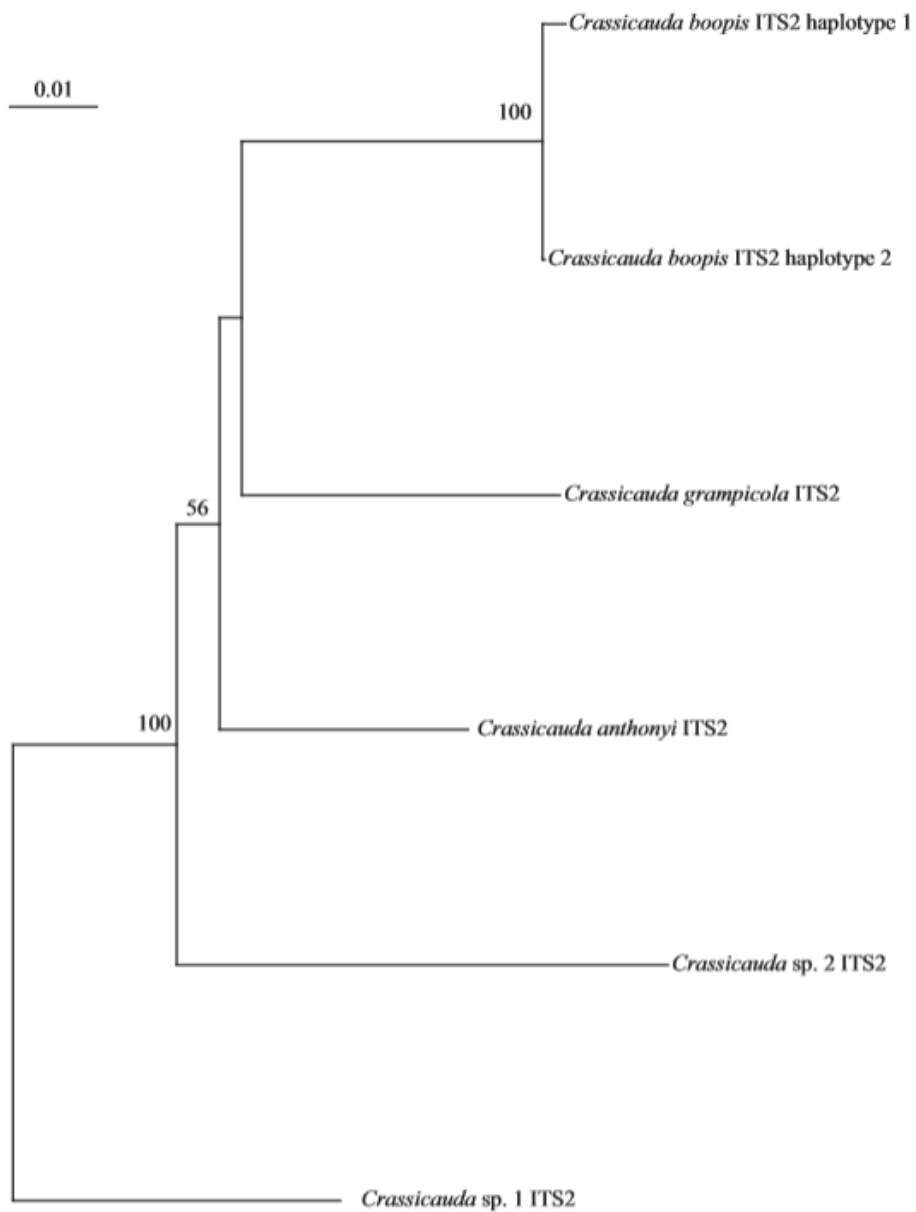


Figure 3. Maximum likelihood tree obtained from ITS2 alignment. The tree was arbitrarily rooted using *Crassicauda sp. 1*. Ultrafast bootstrap support values (>50%) are provided. Bar represent 0.01 substitution/site.

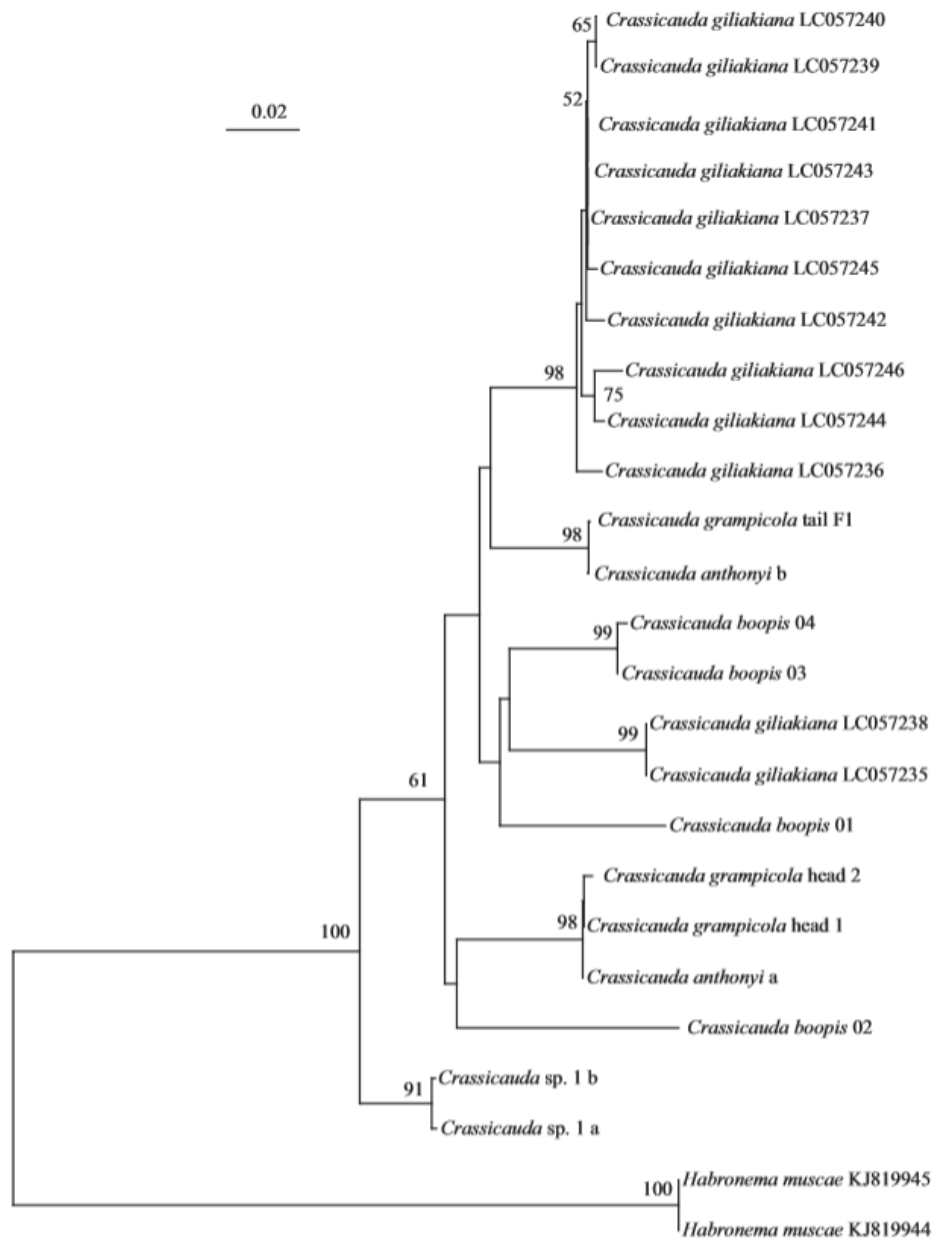


Figure 4. Maximum likelihood tree obtained from *coxI* alignment. Ultrafast bootstrap support values (>50%) are provided. Bar represent 0.02 substitution/site.

6. Scientific communications

Scientific communication 9

XXIX Congress SoIPa, Bari, 21-24 June 2016. Atti, p. 41. Presentazione orale.

CRASSICAUDOSIS IN FIN WHALES (*BALAENOPTERA PHYSALUS*) STRANDED ALONG ITALIAN COASTS

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Of several known crassicaudid infections, those caused by *Crassicauda boopis* (Nematoda, Spirurida) in whale are especially pathogenic. The giant adult nematode grows in the vascular and ureteral system of the kidney; it can cause complete vascular occlusion and kidney failure as described in Atlantic fin whales (*Balaenoptera physalus*) (Lambertsen, 1992); no data are still available in literature for this host species in Mediterranean basin.

Six fin whales, stranded dead along Italian coastline in the period 2006-2013, were analyzed for *Crassicauda* infection. The parasites were morphologically identified according to Lambertsen (1985); molecular analyses by amplification and sequencing of a portion of the 18S (Floyd et al., 2005; Jabbar et al., 2015) of the small subunit ribosomal and internal transcribed spacers 2 (ITS2) (Traversa *et al.*, 2004) of the rRNA were carried out. Formalin-fixed tissues were routinely processed for histology.

Crassicaudosis was observed in four out six examined animals. Adult *C. boopis* were found in three fin whales, one of which had also nematode larvae in intestinal nodules and mesenteric vessels' wall. Another animal showed vascular lesions with ruined fragments of the nematode inside. The sequences obtained from the parasitic elements (adults, larvae and lesions) showed a high identity with each other for ITS2 region; the 18S sequences had high identity with the unique *Crassicauda* sequence (*C. magna*) registered in GenBank (Accession number: KM233410.1).

Chronic vasculitis and/or thrombosis were observed in renal vessels, vena cava and mesenteric arteries, leading to almost complete occlusion of vessels lumen in three cases. Histology showed renal

fibrosis, perirenal granulomas and disseminated *Crassicauda* eggs in renal vessels, renal pelvis and adrenal glands.

This study provides data on the presence, pathology and biomolecular characterization of *C. boopis* in fin whale of the Mediterranean Sea.

Scientific communication 10

31st European Cetacean Society Annual Conference, Hindsøgl Castle Middelfart (Denmark), 1-3 May 2017. Abstract book, p. 230. Poster.

PARASITOLOGICAL FINDINGS IN FIN WHALES (*BALAENOPTERA PHYSALUS*) STRANDED ALONG ITALIAN COASTLINES

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The fin whale (*Balaenoptera physalus*) is the commonest large whale species in the Mediterranean Sea. These whales concentrate in some specific feeding areas of this basin, among which the International Pelagos Sanctuary. Few reports on parasitofauna of Mediterranean fin whale population are available in literature. In the period October 2006 - January 2015, post-mortem examinations were performed on seven fin whales, found stranded along Tyrrhenian and Ligurian coastlines. Biometric data, carcass conservation code, body condition score, age estimation and sex of stranded animals were collected during necropsy. A complete parasitological exam on skin, blubber and gastrointestinal, cardiovascular, respiratory and urogenital systems was carried out. The parasites were fixed in 70% ethanol for the subsequent morphometric identification. Tissue samples of the major organs were formalin fixed, paraffin embedded and routinely processed for histological examination. Six out of seven fin whales were positive for one or more parasite species; one crustacean (*Pennella balaenopterae*) and four helminth taxa (*Ogmogaster antarcticus*, *Crassicauda boopis*, *Tetrabothrium* sp., *Bolbosoma* sp.) were identified. Crassicaudosis infection was observed in five out of seven examined animals. Adult *C. boopis* were found in four fin whales. Another animal showed typical vascular lesions with ruined fragments of the nematode inside. Chronic severe vasculitis, associated with thrombosis, was observed in renal vessels and vena cava, leading to almost complete occlusion of vessels lumen in three cases. Nematoda larvae were found in the wall of the intestine and mesenteric arteries in one animal. Histology showed mild renal fibrosis, perirenal granulomas and disseminated *Crassicauda* eggs in renal vessels, renal pelvis and adrenal glands. This study provides data on the ecto and endoparasites of fin whales stranded along Italian coastlines and

particularly on presence and pathogenic effects of *C. boopis* in Mediterranean population of fin whales.

Scientific communication 11

31st European Cetacean Society Annual Conference, Hindsøglav Castle Middelfart (Denmark), 1-3 May 2017. Abstract book, p. 70. Oral presentation.

MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF *CRASSICAUDA BOOPIS* (NEMATODA; SPIRURIDA) FROM MEDITERRANEAN FIN WHALES: PRELIMINARY RESULTS

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Crassicauda boopis is considered one of most pathogenic nematode parasites in *Balaenoptera physalus* Atlantic population, but no data are available for Mediterranean one. The localization of the adults in the kidney can lead to organ impairment. Hypothesis on somatic migration of larvae through vascular walls have been made but no morphological description has been provided for larval elements.

To detect crassicaudosis infection, parasitological examination was carried out on seven carcasses of *B. physalus*, stranded along the Italian coastlines between 2006 and 2015.

During necropsy, specimens of adult nematodes (N=124) were found in renal vessels, vena cava, kidneys and ureters of four animals; larvae were also detected in the wall of the intestine (N=21) and inside mesenteric artery intima (N=4) of an adult whale.

After isolation at stereomicroscope, adults (10 female tails, 36 male tails, 2 cephalic fragments) and larvae (N=16) were observed using light microscope for morphometric characterization (NIS-D software). Amplification and sequencing of 18S and mt COX 1 genes was carried out on samples from adult and larvae.

Morphometric data of adult nematodes fit with the description of *C. boopis* in literature. Some morphologic differences were observed between larvae collected from the intestine and mesenteric arteries, including total body length, cephalic terminal fragment shape and definition of internal structures.

Sequences obtained from 18S gene of the examined samples showed high identity (99%) with *Crassicauda magna* (Accession number: KM233410.1); multiple alignment of sequences obtained from adults and larvae showed high identity with each other for mtCOX 1 gene.

This work provides data on crassicaudosis infection in Mediterranean population of fin whales, confirms the hypothesis of a somatic migration of larvae in the definitive host and gives the first morphological description of larval stages of *Crassicauda*; preliminary molecular data provide basis for further systematic studies on the *Crassicauda* genus.

FIRST MOLECULAR DETECTION OF *PENNELLA* SP. (SIPHONOSTOMATOIDEA: PENNELLIDAE) IN CETACEANS FROM THE MEDITERRANEAN SEA

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INTRODUCTION - Copepods of the genus *Pennella* (Oken, 1816) are large mesoparasites infecting teleost fishes and marine mammals. Two species are mainly described from fish (*P. filosa* and *P. instructa*); while, one (*P. balaenopterae*) is described in cetaceans. The reports in cetaceans concern specimens of adult females embedded into the skin of various host species and the description of first naupliar stage of parasite; the male is free-living (Arroyo et al. 2002, Sarsia, 87: 333-337). The aim of this report is to describe immature stages of *Pennella* sp. collected in a fin whale (*Balaenoptera physalus*) stranded along Italian coast.

MATERIALS AND METHODS - Specimens of immature stage copepods were collected from the skin-blubber of two fin whales (*B. physalus*) stranded along the Sardinian and Tuscan coast on 2011 and 2013, respectively. Six entire parasites were studied by light microscope (Nis Elements D software, Nikon) and were referred to *Pennella filosa*, according to the morphological features reported by Thompson (1905, Biol Bull 8 (5): 296-307). A portion of one specimen was submitted to molecular analyses by a PCR, amplifying the mtDNA *cox1* gene (using the primers LCO1490 and HCO2198 (Folmer et al., 1994; Mol Mar Biol Biotechnol. 3(5): 294–299)). The obtained sequence was compared with those previously obtained, at the same gene locus, from specimens of *P. balaenopterae* (from fin whale and sperm whale); *P. filosa* (from bluefin tuna) and *P. instructa* (from swordfish) Alignment of the sequences was performed using BioEdit and genetic analysis (MP, NJ) by MEGA6.0 was performed.

RESULTS AND CONCLUSIONS - The morphological features of the samples (size, characteristics of the cephalothorax and the abdomen) allowed to ascribe the specimens to the genus *Pennella*. The molecular analysis showed that sequence obtained clustered in the same clade with the specimens of *P. balaenopterae* *P. filosa* and *P. instructa*. The genetic similarity among all the different *Pennella* spp. suggests that other genetic/molecular markers should be used to clarify whether they are separated species, or morphotypes adapted to different hosts.

This work was financially supported by a Research Project of Padova University (Prot. CPDA149521/14)

Conclusions and perspectives

During this project, a first survey on the spirorchiidiasis in the Mediterranean Sea has been completed, including a wide sample of loggerhead turtles *C. caretta* and using a multidisciplinary approach to reach a more complete knowledge on ecology and pathology of the disease in the area. Genetic analyses on the hosts have revealed that loggerheads likely acquired the infection while following migratory routes within the Mediterranean Sea, demonstrating that life cycles of the involved cardiovascular flukes can develop entirely inside this basin; as a consequence, all the resident population of sea turtles is considerable at risk of infection. In our survey the infection never showed to be contributory to the death of the animals, causing only mild lesions. Nevertheless, fatal infections have been observed in other geographical regions along Italian coasts, enhancing the importance of continuing studying the epidemiology of this disease in the Mediterranean area (Santoro et al., 2017).

Diagnosis of spirorchiidiasis *in vivo* relies on serological tests or coprological examination. Nevertheless, both these methods are limited in specificity, and the presence of species in which fecal route is not the primary way for eggs shedding, may be excluded by coprologic diagnosis. Molecular tools show the higher specificity and in some cases the highest sensibility in the detection of spirorchiid eggs in turtles tissues, thus implementation of such tools for the diagnosis *in vivo* could be recommendable: a Real Time PCR has been already applied efficiently to detect the presence of *Cardicola* spp. (Trematoda: Aporocotylidae) in fishes blood samples (Polinski et al., 2013) and a similar tool could be implemented for detecting and identifying turtles blood flukes and managing the infection *in vivo*.

The parasitological survey carried out in fin whales *B. physalus* during this project is the first wide study on the parasitofauna of this species in the Mediterranean Sea. *Crassicauda boopis* was responsible for severe lesions and likely contributory to the death of the animals at least in one case. The importance of crassicaudosis in natural mortality rates should be further investigated among the Mediterranean population of fin whales.

At the light of molecular results obtained from this research from larvae of *Crassicauda*, the intra-somatic life cycle of *Crassicauda* spp. should be revised in baleen whales. Other species of *Crassicauda* should be included in the phylogenetic tree to clarify taxonomic issues due to limitations of morphologic descriptions.

Description of immature specimens of *Pennella* sp. from fin whales revealed the high morphological similarity with immature stage of *Pennella filosa* from fish. Preliminary molecular data further highlighted the genetic similarity of three different congeneric species, thus other genetic markers should be studied to clarify the taxonomy of this genus as proposed by Fraija-Fernández et al. (2018) in odontocetes.

Genotyping of *Toxoplasma gondii* was attended from the tissues of a fin whale infected by the protozoan, and this is the first direct detection of toxoplasmosis in a baleen whale. Genotyping of *T. gondii* strains isolated from other toothed whales stranded along Italian coasts might help studying host sensitivity, virulence and spread of the strains in marine mammals.

Parasitic infections potentially harmful for the health of both sea turtles and cetaceans have been detected during this project in the Mediterranean.

In order to get to the results of this project, a multidisciplinary approach was essential. Thanks to a classic and molecular parasitological approach, together with pathological and phylogenetic studies it was possible to get to a wider analysis of the host-parasite relationship and parasites diversity, addressing future researches.