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Molecular analysis of endoparasites from marine mammals from southern Spain including the Campo de Gibraltar

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Abstract:

"Molecular analysis of endoparasites from marine mammals from southern Spain including the Campo de Gibraltar"

Marine mammals are susceptible to many parasitic infections that can have an influence on their health and ability to survive in their environments, especially nowadays with increasing threats and many cetacean species being endangered or even at risk of extinction.

This study presents the results of a comprehensive molecular analysis of endoparasites in marine mammals, including the Fin Whale (*Balaenoptera physalus*), the Common Bottlenose Dolphin (*Tursiops truncatus*), the Short-beaked Common Dolphin (*Delphinus delphis*), the Striped Dolphin (*Stenella coeruleoalba*), the Harbour porpoise (*Phocoena phocoena*), and the Long-finned Pilot Whale (*Globicephala melas*), found in southern Spain including the strait of Gibraltar. The aim of this study is to investigate parasitic infections in marine mammals and thereby contribute to the protection of these endangered species.

The fieldwork included the collection of biological samples from several cetacean species that were found dead and stranded in southern Spain. The necropsies were conducted in the Campo de Gibraltar, Algeciras where muscle, skin and internal organ tissue samples as well as multicellular parasites were collected as frozen or ethanol-soaked specimens. Tissue and parasite samples were analyzed with molecular methods including conventional PCR for the presence of acanthamoebae, trypanosomes, piroplasms, haemogregarines, cystogenic coccidia, rickettsiae, anaplasmas and bartonellae. PCR products of positive samples were sent for sequencing to the Biotechnology Research Institute in Gödöllő, Hungary. In all PCR-positive samples the presence of *T. gondii* was confirmed, with a 100% sequence match to GenBank data of this species in multiple organs (lungs, muscle, cerebellum, and encephalon) of a single *D. delphis*.

According to our best knowledge, these findings reveal and confirm, for the first time globally, the presence of *Toxoplasma gondii* DNA in the Short-beaked Common Dolphin *(D. delphis)* as previous studies only confirmed the presence of anti-*T. gondii* antibodies or *T. gondii* antigens with immunological methods. Furthermore, the study provides insights into the occurrence of other parasites in cetaceans found in southern Spain, in this way helping to understand ecological condition of ecosystems and cetacean populations in the strait of Gibraltar. The results contribute to our knowledge on the epidemiology of *T. gondii* and underline the importance of continuing research and monitoring of global and Mediterranean cetacean populations.

Összefoglalás:

Dél-Spanyolországból és a Gibraltári-szoros környékéről származó tengeri emlősök endoparazitáinak molekuláris vizsgálata

A tengeri emlősök számos parazitafertőzésre érzékenyek, amelyek nagyban befolyásolják a túlélőképességüket szabad környezetükben. Különösen igaz ez manapság, amikor a cetfélék túlélését fenyegető egyéb hatások száma folyamatosan növekszik. Mára már számos faj vált veszélyeztetté, sokuk a kihalás szélére sodródott. Ez a tanulmány tengeri emlősök: közönséges barázdásbálna (*Balaenoptera physalus*), palackorrú delfin (*Tursiops truncatus*), közönséges delfin (*Delphinus delphis*), a csíkos delfin (*Stenella coeruleoalba*), a barna delfin (*Phocoena phocoena*) és a hosszúszárnyú gömbölyfejű-delfin (*Globicephala melas*) endoparazitáinak molekuláris szűrővizsgálati eredményeit mutatja be. A minták Spanyolország déli részéről, illetve Gibraltári-szoros partjairól származtak. A tanulmány célja, hogy a tengeri emlősök parazitafertőzéseinek vizsgálata útján hozzájáruljon e veszélyeztetett fajok védelméhez.

A terepmunka során biológiai mintákat gyűjtöttünk több különböző cetfaj példányaiból, amelyeket elpusztulva találtak Spanyolország déli részén. A boncolásokat a Campo de Gibraltarban, Algecirasban végezték, ahol izom-, bőr- és belső szervi szövetmintákat, valamint többsejtű parazitákat gyűjtöttek. Ezeket fagyasztva vagy etanolban tárolták. A szövet- és parazitamintákat konvencionális PCR-rel elemeztük acanthamoebák, trypanosomák, piroplasmák, haemogregarinák, cystogén coccidiumok, rickettsiák, anaplasmák és bartonellák jelenlétére. A pozitív minták PCR-termékeit a Gödöllői Biotechnológiai Kutatóintézetbe küldtük szekvenálásra. Minden PCR-pozitív szervminta (tüdő, izom, kisagy és agyvelő) egyetlen *D. delphis* egyedből származott. Ezekben a szekvenálás megerősítette a *T. gondii* jelenlétét, 100%-os szekvencia egyezéssel e faj GénBankban hozzáférhető adataihoz. A legjobb tudomásunk szerint ez az első alkalom, hogy a *T. gondii* DNS-ének jelenlétét *D. delphis* mintákban igazolták, ugyanis korábbi kutatások eddig csak anti-*T. gondii* antitesteket, illetve *T. gondii* antigéneket írtak le immunológiai módszerekkel. Továbbá, ez a kutatás betekintést nyújt a dél-spanyolországi cetfélék egyéb parazitáinak előfordulásába is, ezáltal segít a Gibraltári-szoros vizeiben lévő ökoszisztémák és a cetpopulációk kapcsolatának mélyebb megértésében. Mindemellett hozzájárul a *T. gondii* epidemiológiájáról szerzett eddigi ismereteinkhez és rávilágít a cetpopulációk lokális, illetve globális monitoringjának fontosságára.

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List of abbreviations

CEGMA – Centros de Gestion del Medio Marino Andaluz
CITES – Convention on International Trade in Endangered Species
Cox – Cyclooxygenase
DNA – Deoxyribonucleic acid
EDTA – Ethylenediaminetetraacetic acid
EPS – Expanded polyesterene
gltA – Citrate synthase enzyme
NGO – Non-governmental organization
PCR – Polymerase chain reaction
RNA – Ribonucleic acid
ITS – Internal Transcribed Spacer
UVMB – University of Veterinary Medicine Budapest

Introduction

Marine mammals are fascinating animals including cetaceans that exhibit an extraordinary intelligence and social structure. The two largest animals that ever lived on earth are still swimming and traveling all over this world's oceans (1,2). Over centuries these remarkable creatures have been subject to fascination and threads. Several species went extinct and many populations were close to extinction (3,4). While regulations and conservation efforts have contributed to the recovery of some species, cetaceans now face an increasingly dangerous thread of parasitic infections, that are influenced by modern environmental challenges as ocean pollution and climate change (5–9). Parasites in general are intricate components of healthy ecosystems, serving essential roles in maintaining an ecological balance (10). However, the interplay between cetaceans and their parasitic counterparts is not well understood. This study seeks to contribute to understanding of endoparasites within marine mammals by providing comprehensive molecular analyses of endoparasites from various cetaceans found in southern Spain including the Strait of Gibraltar.

To this day many cetacean species are endangered or even at risk of extinction (11). Therefore, the necessity of investigating threatening parasitic infections becomes evident particularly given the current conservation status of various cetacean species. The research of this study aims to investigate the prevalence and distribution of certain endoparasites of

cetaceans, contributing to understand the significance of the epidemiology of specific parasitic infections and their potential thread and impact on certain cetacean populations. Underlining the importance of research and monitoring of global and Mediterranean cetacean populations, is crucial for future the health of complex ecosystems and environmental balance in a world where marine mammals face increasing threads that potentially disrupt their ecological niches and endanger their survival (5). Understanding the multifactorial relations between cetacean hosts, their parasitic counterparts and their shared habitat is fundamental for implementing effective conservation strategies redirecting trends of their decline of many marine mammal population especially with the inevitably growing impact of human activities that include habitat destruction, oceanic pollution and overfishing that potentiate the risk of transmission of parasitic infections, particularly zoonotic infections, for marine mammals and humans (6,7,12). It becomes evident that the preservation and monitoring of cetacean and marine mammals is not only important for their wellbeing but also for human health, by recognizing the interconnection of human actions and the environmental health (13). In the next section, provided description of the examined cetacean species, with a focus on key aspects such as morphology, distribution, migration patterns, population dynamics, feeding behavior and social behavior, this study will give a foundation for the broader context of this subject. Following this, the study presents the analyzed parasitic species, giving comprehensive insights on their relationship with the cetaceans and their impact on ecosystems. Furthermore follow, the objectives, materials and methods and the results along with their discussion.

Literature review

The Fin whale (*Balaenoptera physalus*)

Morphology:

The Fin whale is the second largest animal that has been living on Earth (2) and reaches lengths from 17.5 to 20.5 m (1), where females tend to surpass male individuals in size (14). The length of newborns varies around 5.2 meters (14,15). Only the Blue whale *(Balaenoptera musculus)* has a greater length with recorded sizes ranging from 20 to up to 28 m (1). Both species belong to the same family of Balaenopteridae and therefore share distinct morphological features like a V-shaped head with a central ridge. The Fin whale is

characterized by its dorsal fin, which is relatively small, pointed backwards and situated at two-thirds along the caudal back (1).

Distribution and migration:

Three subspecies are currently recognized: The northern Fin Whale *(B. p. physalus),* that is known to migrate to the Mediterranean Sea, the southern Fin Whale *(B. p. quoyi)* and the Pygmy Fin Whale *(B. p. patachonica)* (16). Alike other large cetaceans, B physalus is distributed from temperate to subpolar oceans throughout the world including the North Atlantic Ocean, the Mediterranean Sea (17) and the Sea of Cortez in the North Pacific Ocean (18). They tend to travel more than 8,000 km from high to low latitudes and form migratory patterns in order to reach feeding grounds or to reproduce (14,16,18). These migratory patterns may result in isolation of populations including resident populations in warm water regions such as the Mediterranean Sea (16). Thus beside migratory groups of Fin Whales passing the strait of Gibraltar, some populations from the Mediterranean Sea have significant genetic variations from north Atlantic populations and some genetic variations from populations of the Sea of Cortez (18). Furthermore, observations of newborn animals in the Ligurian Sea and sightings of whales all year round suggest that there is an occurrence of resident genetically distinct population in the Mediterranean Sea (14,17,18).

Population in the Mediterranean Sea:

It is only estimated that the population of Fin Whales in the North Atlantic Ocean as a whole is around 47.300 animals (1) including the population of the Mediterranean Sea that is around 2,000 to 3,500 Individuals (14) in summer (19) with a high concentration of around 900 Individuals in the western Ligurian Sea (17,19,20).

Feeding behaviour:

To reach its main prey, krill *(Meganyctiphanes norvegica)* (14,19), the Fin Whale exhibits a feeding behaviour as complex as its communication systems. The primary mechanisms for sound reception involve bone conduction and pressure mechanisms that allow it a capability for intelligent communication (15) over extensive distances (14). Different swimming-surfacing patterns were observed that are associated with traveling as an avoidance strategy of vessels and with feeding techniques (14,17) designed to follow krill aggregations. With a dozen dives as deep as 470 meters in a single feeding bout, it is presumed that the Fin Whale adapted its diving competences to adroitly pursue *M. norvegicas* vertical migration (19).

The Common Bottlenose Dolphin *(Tursiops truncatus)*

Morphology:

The Common Bottlenose Dolphin is the largest of the three dolphin species presented in this text and measures up to four meters in length (1). It is characterized by its robust head with a relatively short beak that is often white tipped on the lower jaw. Furthermore, they are identified by their colour scheme of a dark grey back, transitioning to a lighter grey on the lower flanks and a white belly. Their upper flanks have no significant markings. Other distinct features are their dorsal fins that are tall, centrally placed, sickle-shaped and often embellished with markings and patterns for individual identification (1).

Distribution and Migration:

T. truncatus is found in both tropical and temperate waters including the Mediterranean Sea (21). Seasonal movements have been observed in European waters from the Faroe Islands to the Cardigan Bay in Ireland, the coasts of England between Cornwall and Sussex (1) and migrations to the Mediterranean Sea through the strait of Gibraltar, along Spanish coasts, as well as populations in the western Mediterranean Sea near Sardinia and in the northern Adriatic Sea (22). They find their favourable environments and habitats in estuaries and bays to the continental shelf in coastal areas (1).

Population:

Since global population numbers are difficult to assess due to its migratory nature, some local scientifically valuable resident population estimates are available. Despite the fact that the common bottlenose dolphin is one of the most common sea mammals inhabiting the world's oceans, the following population numbers are limited to specific areas and well-studied groups and do not represent their numbers as a whole species. (1) In the Mediterranean Sea there are several areas with individual numbers of stable groups. For instance, in the Adriatic Sea at Croatia's coast, a population estimated at 184 individuals (1). As a prime example, on the Sardinian coast, there is a population of 121 photo-identified dolphins (22).

Feeding:

As other dolphin species, *T. truncatus* is an opportunistic feeder and their diet consists of a diverse range of prey animals. It consumes both benthic and pelagic species such as fish, cephalopods and shellfish. Some common prey animals are haddock, saithe, cod, hake and octopus (1). As apex predators, due to their diet array, ocean pollutants will accumulate in the dolphins over time. The exposure of various pollutants such as microplastic ingestion is a growing risk factor particularly in the Mediterranean Sea for the common bottlenose dolphin (7).

Behaviour:

Being able to form highly social structures in their groups of up to 25 individuals and to develop complex behaviours, the common bottlenose dolphin is known for intelligent hunting strategies and long-lasting associations between group members such as mothers and calves (1).

The Short- beaked Common Dolphin *(Delphinus delphis)*

Morphology:

It's a relatively small dolphin species that is characterized by a slender body , its pronounced beak, a light coloured hour-glass pattern on its lower flanks, a dark dorsal body and below its dorsal fin it has a remarkable dark triangle (1). Males of this species reach approximately 2.4 meters in length and surpass the females that grow to approximately 2.1 meters (1).

Distribution:

D. delphis have been observed in warm temperate and tropical waters around the globe (1). Although the species inhabits both the Atlantic Ocean and the Mediterranean Sea, there are differences in their morphology and habitat preferences (1,6,23). In the northeast Atlantic, relatively large specimens have been described that prefer deep-water habitats, however the smaller Mediterranean Short-beaked Common Dolphins are also found in bays like the Bay of Algeciras (1,6,7,23).

Migration:

In the Mediterranean Sea the presence of *D. delphis* is observed for example along the Spanish coast, with sightings increasing from the Bay of Almeria to the bay of Algeciras and the coast of Malaga (6,23). Animals passing through the Strait of Gibraltar have been documented with calf schools during July and August (23). The migration patterns of sea mammals including the Short-beaked Common Dolphin appear to be influenced by the disturbance of vessels (17) and the availability of suitable habitats (23).

Population:

Unlike in other species, there are scientifically significant population estimates for the Short-beaked Common Dolphin in the northeast Atlantic ranging around 120.000 individuals (1). Nevertheless, in the Mediterranean Sea, this species is not as common and their population is of particular concern as numbers appear to be decreasing as a result of possible reasons such as the exposure of pollutants (6,7). Another interesting circumstance in this context is the hybridisation of two different dolphin species in the Campo de Gibraltar (24). The Short-beaked Common Dolphin (*Delphinus delphis*) male and the Common Bottlenose Dolphin (*Tursiops truncatus*) female have hybridized in the Bay of Algeciras (24).

Feeding and behaviour:

The short-beaked dolphin feeds on small fish and squid, with pelagic schooling fishes being their primary prey in the northeast Atlantic ocean (1). Their coordination systems are complex and most probably influenced by the position of the sun with consistent directional movements depending on the time of the day (23).

The Striped Dolphin (*Stenella coeruleoalba*)

Morphology:

Stenella coeruleoalba exhibits a unique morphology described by a slender, streaming body that measures approximately from 2 meters to 2.4 meters in length (1). The black beak is relative to its body length proportionally medium-sized, and separated by a groove, a dark dorsal surface and light flanks. It is dorsal fin varies in shape from sickle shaped to nearly erect, it is positioned centrally and stands out eminently. In addition, they have a white to light grey blaze with distinctive black lines in the flanks (1).

Distribution:

Following the other two dolphin species described, the Striped Dolphin is just as common and known for its global distribution, populating in both the northern and the southern hemispheres, especially preferring the tropical and subtropical and warm tempered oceanic waters both shallow and deep (1,20,25–27).

Migration:

Striped Dolphins are known to live distributed in the open sea of the Mediterranean as well as close to shore lines (25,26). Especially the western Mediterranean Sea more exactly the Corso-Ligurian basin, is a region with remarkably high concentration of individuals that form resident populations that are well studied (20). These Mediterranean populations are genetically different from the populations found in the Atlantic Ocean as they do not interbreed (25). As an ecological factor the distribution of prey animals like fish and cephalopods impact the dolphin's migration patterns as they follow the abundance of their food. Furthermore, the geographical isolation of the Mediterranean Sea with its temperature, salinity and water current differences to the Atlantic Ocean is also a considerable factor for migration adjustment (20,25–27)

Population:

The estimated population of Striped Dolphins in the northeastern Atlantic Ocean is 73.843 individuals that inhabit waters from southwest Ireland to France (1). This habitat is rich in prey animals for many cetacean species and plays a critical role in the study of cetaceans. In

the Mediterranean Sea there is a total estimated population of 117.880 animals (25,26). Although this number may seem to suggest a sizable population, it's still considered as "vulnerable" as it encounters several threats as for example water pollution, habitat degradation, fishery bycatch, disturbance by boats and small vessels and its genetic isolation that makes it susceptible for environmental changes and diseases including parasitic infections (7,17,25,26,28,29).

Feeding:

Stenella coeruleoalba is an opportunistic feeder like other dolphin species and has a wide range diet that includes mostly pelagic teleosts as sprat, whiting, pout, hake and other fish (1,27). In their diet included are also cephalopods as squids and crustaceans such as shrimps (1).

Behaviour:

As this species is well studied, their highly social nature has been observed and documented. It reveals in their behaviour and group size, sometimes reaching more than 60 members (1). They use their group size to conceive intelligent hunting strategies and defence against predators. With whistles and clicks as much as physical contact they are able to have complex social interactions and behaviours. Not only their evolved intelligence is a factor for success but also their ability that enables them to be exceptionally agile and fast swimmers making them one of the most acrobatic species among dolphins (1).

The Harbour porpoise *(Phocoena phocoena)*

The harbour porpoise is a relatively small and the most numerous cetacean species in the north western European shelf waters including the northwest Atlantic Ocean, the North Sea and the Baltic Sea (1). Although its rich abundance globally, *P. phocoena* plays a small role in the Mediterranean ecosystems and faces increasing threats even in more populated waters where the species is more common (1,30).

Morphology:

As the cetacean that exhibits the lowest known size in its taxonomic group in the north-western European continental waters, the Harbour Porpoise female of the North Sea, measures about 160 cm in length, males reach around 145 cm and newborn calves approximately 70-80 cm (31). The size of the animals tends to vary depending on geographical location and age. Nevertheless, specimens of Iberian waters are generally larger than their North Sea cousins. The harbour porpoise is defined by a small, rotund body with a small triangular and a short blunt head that displays no beak (1,30,31).

Distribution and Migration:

Harbour Porpoises found in different geographical and ecological niches exhibit different genetics, behaviour and habitat preferences (1,30). *P. phocoena* prefers shallow coastal waters as much as estuaries (31) and forms a circumpolar distribution in the Northern Hemisphere (30). Its habitat reaches from the Barents Sea to the Atlantic coast of Great Britain, Ireland and the Bay of Biscay. Some Isolated populations have been observed in the Black Sea and in waters in South East Asia (30). Despite the wide distribution globally, in the Mediterranean Sea its activity has become of limited presence due to climatic changes since the last glacial maximum where water temperature has risen and its oligotroph conditions make the habitat not as favourable anymore for this species (30). Nowadays the few specimens found in the Mediterranean Sea are most often observed in the Strait of Gibraltar and are considered individuals from Atlantic or North Sea populations that have migrated to the Mediterranean Sea (31).

Population:

The North Sea population of the Harbour Porpoise is dense and well distributed with an estimated number of around 280,000 individuals (1). Nevertheless, the species is declining in number especially compared to their population before the Second World War (31). The population in the southern hemisphere exhibit a slightly larger body size but smaller population numbers and genetic isolation resulting in a lower genetic diversity that predisposes for being sensitive for ecological and environmental changes as well as for diseases (1,30,32).

Feeding and behaviour

The Harbour Porpoise is a species that is highly adapted to a life in shallow and turbulent waters (1,31). Their small body size and fins make them top predators and opportunistic feeders in their shallow environments (1,33). They are agile and equipped with a complex echolocation that they can use for hunting squid and small fish like herring and sprat (1,30), as much as for coordinating in their shoal water environment in such a way that it is unlikely for healthy individuals to strand on shores (31,33). Not much is known about their social behaviour and communication but they show active avoidance of trawl nets that could potentially drown them(31). There is also a high competition with other dolphin species like the Common Bottlenose Dolphins as their diet range overlaps and they compete about habitat and recourses (1). The niche of being adapted to shallow waters might be an avoidance strategy of competing with much larger species in the open deep sea. There have been reports of Common Bottlenose Dolphins attacking and killing Harbour Porpoises (32).

The Long-finned Pilot Whale *(Globicephala melas)*

Morphology:

As one of the largest of the delphinids, the Long-finned Pilot Whale males reach a length exceeding 6.25 meters while females are slightly smaller (1,34). They are characterized by distinct features such as the square bulbous head that tends to be particularly noticeable with the increasing age of the males (1). Their dorsal fins are positioned just ahead of the centre of their back with a black or dark grey in coloration with a white or light grey saddle shaped patch behind it (1,34). In young individuals it grows as a sickle shape but transforms into a flag shape as they age (1). They exhibit a black or dark grey body complemented by a greyish-whitish anchor-shaped patch situated on the chin region and notably elongated pectoral fins (1,34). Despite their distinctive characteristics, Long-finned Pilot Whales are frequently misidentified as their Short-finned counterparts (1).

Distribution and Migration:

Long-finned Pilot Whales predominantly inhabit continental shelves and offshore waters in temperate and sub arctic regions worldwide including the North Atlantic and southern oceans where they are well suited to deep water environments, favouring depths ranging from 200 to 300 meters (1,34,35). When it comes to the Mediterranean Sea, *G. melas* populations are concentrated mainly in the western basin specifically in the Strait of Gibraltar, Alborán Sea and the Gulf of Vera (34). Migration patterns in the Mediterranean Sea are formed by two different genetically isolated populations, one at the Strait of Gibraltar and the other resides at the Alboran Dorsal extending to the Ligurian Sea (1,34,35).

Population:

Their population number in the North Atlantic Ocean is estimated in the hundreds of thousands individuals, however exact numbers are difficult to determine (1). In the Mediterranean Sea, fewer than 250 individuals where observed with a population decline of 26,2% over 5 years following a morbillivirus outbreak that could lead to a possible extinction of the Mediterranean populations emphasizing the urgent need for conservation efforts (34).

Feeding and behaviour:

As deep water foragers these whales exhibit a diverse diet primary focuses on squid species such as *Todorodes sagittatus* and other cephalopods, fish and crustaceans (1,34). Their social structure is matrilineal with adult females in leadership roles, utilizing vocalisations like echolocation for communication and hunting in 600 to 800 meter deep waters as the Strait

of Gibraltar displaying a hierarchical social system organized into clans that engage in reproduction between unrelated pods and exhibit habitat segregation (34).

Toxoplasma gondii

Toxoplasma gondii is a worldwide distributed obligate intracellular protozoan parasite which undergoes its sexual cycle in feline species (36–38). Although felids are the only known definitive hosts of *T. gondii*, most warm-blooded animals can be infected, including humans implying zoonotic concerns (38). Rodents play an important role of the distribution of toxoplasmosis as they act as the main intermediate hosts (39). The parasite influences the rodents behavior according to the well-studied hypothesis of manipulation leading to decreased general anxiety, loss of avoidance to predators and less sensitivity to feline urine leading to an increased chance of being preyedon by felids (39,40). Those behavioral alterations are caused by a pathogenic mechanism that induces remodeling within neuronal circuits that are responsible for regulating reactional behaviors by processes such as hypomethylation in genes, located in the medial amygdala diminishing predator aversion (39–42).

The life cycle:

Toxoplasma gondii has a complex life cycle that involves different stages in both intermediate and definite hosts. It begins when an infected felid that is typically a cat, sheds an oocyst in its feces (43,44). This oocyst is initially unsporulated non-infectious and must undergo the process of sporulation, that takes up to several weeks, to become infectious (43,45). The many sporulated oocysts that persist in the environment and survive for years, contain sporozoites that are the actual infectious form of *T. gondii* (43). The Intermediate host, typically rodents but occasionally birds and other mammals including humans and cetaceans ingest the sporulated oocyst from the environment through contaminated food, water and excretes (41,43). In the intermediate host, the sporozoites are released from the sporulated oocyst to invade the host cells focusing on myocytes and neurons where they differentiate into tachyzoites that are rapidly dividing causing acute infection by multiplying endodyogeny within the hosts cell (43,45). Some of the tachyzoites transform into a rather slow replicating form known as bradyzoites that encyst in tissues persisting in their protective structures for the hosts lifetime (38,43–45). The infection by *T. gondii* alters the behavior of the infected intermediate host in a way that chances increase to get preyed and consumed by predators as felids (39,40) When the intermediate host is consumed by the defined host, such as a cat, the tissue cysts are broken down triggering the infection of the

cat through the digestive tract (38,43). This release of the former encysted bradyzoites causes infection of epithelial cells of the small intestine where the bradyzoites develop oocysts that are shed in the environment with the cats feces starting the cycle anew (37,46,47). The felids only can act as the definite hosts that are required for *T. gondii´s* sexual phase (36–38). Other warm-blooded animals like humans and cetaceans can be infected, but most often serve as aberrant intermediate hosts (26,35). Nevertheless, the intermediate hosts can manifest clinical signs ranging from asymptomatic infections to severe disease depending on the immune status of the intermediate host and the pathogenicity of the *T. gondii* strain (40,41,46).

Human toxoplasmosis:

Since toxoplasmosis is a zoonotic disease, humans can under circumstances manifest clinical presentations with dramatically different outcomes depending on the immunological status of the particular human host (36,40,44,45). The infection route starts with the ingestion of oocysts from contaminated food, water, soil or feline feces but also through consumption of raw or undercooked infected meat particularly lamb, pork and venison (38,46,48). Furthermore, vertical infection during pregnancy from the infected mother to the fetus is also possible as it is, especially in humans and monitored animals, through blood transfusion and organ transplantation (40,46,49). The clinical manifestation is hardly accompanied by symptoms in healthy individuals (46,49). Most often humans are symptomless or complain about mild flu-like symptoms (40,49). Severe symptoms including fever, joint pain, swollen lymph modes, muscle aches and most importantly (46,49). As the immunological status of the infected individuals is essential for the clinical presentation of toxoplasmosis, humans with immunocompromising diseases or therapies including HIV/AIDS and chemotherapy can exhibit several clinical signs followed by potentially life threatening complications including encephalitis(36). There are some studies assuming the behavioral influence of the parasite on humans with psychiatric disorders as schizophrenia and bipolar disorders (40,50,51). *T. gondii* is present worldwide so are human infections (48). Seroprevalence rates indicate the human individuals that have been infected in different regions (38,44,46,48,49). Due to its potential impact on some human populations in different regions, toxoplasmosis is of public health where preventative measures are essential to prevent infection or to reduce the risk of it, especially in pregnant women and immunocompromised humans (40,46,48,49).

Pathomechanism of *T. gondii*:

Toxoplasma gondii infects a wide range of warm-blooded animals, as potential intermediate hosts, and causes disease (39). It has a complex pathomechanism that influences its hosts neural and immune systems changing the behavior towards threads in the environment (40–42). The infection begins when *T. gondii* oocysts are ingested by the host through contaminated matter (43). Inside the hosts body, the parasite goes through various stages of development including the formation of structural stable cysts containing the dormant form of *T. gondii*, bradyzoites, which can be found in several tissues including the brain where it actively invades neural tissues and causes structural changes in it (38,40,43). The presence of the cysts in infected intermediate hosts is associated with alterations in neural function leading to changes in behavior and cognition (40–42). Manipulation of neurotransmitter systems impacts among other the hosts dopamine production and utilization. Dopamine is a key neurotransmitter associated with reward, pleasure and motivation (40,44). These dopamine levels tend to be increased after modulation of neural systems by the parasite, leading to involuntary changes in behavior (40,45,50). Targeting specific neural circuits that are responsible for reactive behaviors, including those related to fear and aversion (42). The precise pathomechanisms are still being researched but it is well known that the structural changes and according functional changes induced within these circuits, influence the response to specific environmental stimuli leading to an increased risk of getting preyed in the everyday life of the infected intermediate hosts (40,42). Recent research suggests that toxoplasmosis induces epigenetic modifications changing gene expression without altering the DNA sequence of the host. In the case of *T. gondii* particularly, the process involving the reduction in addition of methyl groups to DNA, hypomethylation has been associated with genes influencing behavior such as those associated with arginine vasopressin, located in the amygdala, a brain region strongly associated with emotions such as fear and aversion (39–42). The evolutionary value and ultimate goal of the pathomechanism of *T. gondii* is to facilitate its transmission to its definitive feline host, illustrated by a broader example of parasite driven coevolution where traits that enhance an organisms reproductive success are favored (38,40–42,52). The manipulation of neurotransmitter expression leads to a diminished aversion to feline urine related odors and a generally decreased sense of fear and uncomfortability in open uncovered spaces in the environment (38,44,45). Those specific alterations in behavior increase the chance of the infected intermediate host, typically a rodent, being preyed and consumed by a feline, completing *T. gondiis* life cycle (40–42,50).

Cetacean Toxoplasmosis:

Although *T. gondii* is a well-studied widespread parasite that is known of being capable of infecting various warm-blooded terrestrial host species, recent studies have been discovering its presence in marine mammals including cetaceans (53–60). As the infective stage of *T. gondii* are oocysts shed by felids, the definite hosts, there is a high concentration of them in urban areas (38,43,53,54). Waterborne contamination of oocysts in marine environments near coastal cities is a probable transmission route to the world's oceans displaying risks for marine ecosystems and aquatic animal populations (53,54,56,57). Unlike for terrestrial intermediate hosts, the transmission and pathways of *T. gondii* to marine mammals remain unclear (53,57). A possible route of waterborne transmission mechanisms include biotic vectors as shrimps and fish that get preyed by marine mammals like cetaceans (53,54,57). Reported Infections of stranded cetaceans in different regions including the Atlantic Ocean, the Pacific Ocean and the Mediterranean Sea suggest that *T. gondii* infects cetacean in various marine ecosystems displaying a wider distribution of the parasite than previously acknowledged (53,54,56,57). The impact of toxoplasmosis on cetacean heath is still not well studied, but recent researches presented an association of *T. gondii* and pathological lesions of stranded dolphins including Striped Dolphins (*Stenella coeruleoalba*) (54,55,58,59) The most common pathological lesions associated with toxoplasmosis in cetaceans include meningo-encephalitis, lymphadenitis and abscesses in vital organs including the brain, often in combination with morbillivirus and herpesvirus infection (54,55,59). The circumstances and co-infections that could lead to a *T. gondii* infection becoming life threatening for cetaceans remain uncertain. Furthermore, there is little research on the potential clinical outcomes in association to different *T. gondii* genotypes and their pathogenicity and virulence in marine mammals (54,55,58,59). Common methods of confirming *T. gondii* are serological, immunological, histopathological, PCR and DNA analyses (54–56,60). In the Short-beaked Common Dolphin (Delphinus delphis) the presence of anti-*T. gondii* antibodies was confirmed to display the presence of *T. gondii* in this cetacean species (55). Understanding the transmission dynamics and how *T. gondii* affects marine mammals, particularly cetaceans, is essential for our comprehension of marine ecosystems. There is a need of further research regarding the complex interplay between different genotypes od *T. gondii*, their interactions with other pathogens and distribution of different geographical regions, and their pathological behavior to migration patterns. Genetic analysis would be helpful to display these multifactorial relationships, increasing the understanding of marine ecosystems and the health of cetacean and marine mammal populations.

Halocercus pingi

Halocercus pingi is a parasitic nematode that infects the lungs of certain cetaceans and has unique morphological characteristics and host preferences (61). It is commonly associated with the Narrow-ridged Finless Porpoise (*Neophocaena asiaeorientalis*) and the Indi-Pacific Finless Porpoise (*Neophocaena phocaenoides*) leading to its usual geographical occurrence from the Sea of Japan to the South China Sea (62–64). H. pingi exhibits distinct morphological characteristics that make it distinguishable from other Halocercus genus members (61). The adult male measures from 150 mm to 183 mm in length, while the adult females extend with 155 mm to 364 mm in length. It has a cylindrical body with a thickness of 0.45 mm to 0.68 mm (61). Specific features of *H. pingi*`s morphology include six papillae in the cephalic region, a smooth cuticle, a short oesophagus measuring 0.19 mm to 0.22 mm in length, unique male genitalia with long, slender spicules of 0.77 to 0.82 mm in length (61). The anterior end of the nematode contains two large unicellular glands that might play a role in its pathomechanism and the posterior end of the female differs with ventrally to the tail tip positioned anus from the male one (61). The uterine contains numerous larvae and seems viviparous (61). There is little research in the life cycle of *Halocercus pingi*, but potential transmission routes have been suggested after the discovery of the parasite in *Neophocaena asiaeorientalis* calves, where transplacental or lactational transmission would be plausible with a potential exposure to organochlorine pollutants and an immature neonatal immune system that may contribute to the calf's susceptibility to parasites particularly *H. pingi* (6,64). The geographical occurrence of *H. pingi* depends highly on its specific hosts` migration routes and has been documented in waters including the Sea of Japan and the South China Sea where studies have revealed the presence of the nematode in the lungs of various cetacean species (62–64). Halocercus pingi primary infects the Narrow-ridged Finless Porpoise (*Neophocaena asiaeorientalis*) and the Indi-Pacific Finless Porpoise (*Neophocaena phocaenoides*) in this area and is mainly found in the lungs (62,64). Another species of the same genus, *Halocercus delphini* was recorded with relatively high infection levels in Striped Dolphins (*Stenella coeruleoalba*) and Common Dolphins (*Delphinis delphis*) where phylogenetic relationships play a critical role in the parasites host specificity with a preference for hosts belonging to the taxonomic group of the delphinidae family(65). It is apparent that an overlap in the diet of different cetaceans contribute to the infection of the same lungworm species (65,66). There is are no records or confirmations of *H. pingi* found in the Mediterranean Sea (64–66).

Research

All activities and actions described in this study have been carried out by the Author, unless otherwise indicated.

Objectives:

The study aims to investigate the diversity, identification and molecular characteristics of endoparasites in certain marine mammals found on the coast of southern Spain around the area of the Campo de Gibraltar.

Therefore, specific objectives were then to be followed:

The leading objective of this research is to characterize and identify endoparasites and to determine the prevalence of endoparasites in marine mammals with a focus of certain cetacean species including the Fin Whale (*Balaenoptera physalus*), the Common Bottlenose Dolphin (*Tursiops truncatus*), the Short-beaked Common Dolphin (*Delphinus delphis*), the Striped Dolphin (*Stenella coeruleoalba*), the Harbour porpoise (*Phocoena phocoena*), and the Long-finned Pilot Whale (*Globicephala melas*), found in southern Spain. To fulfill this objective, tissue and parasite samples are to be collected from dead and stranded cetaceans during official necropsies regularly conducted in Algeciras, Spain. Advanced molecular techniques including conventional PCR is employed, designed to identify the presence of specific parasites such as acanthamoebae, trypanosomes, piroplasms, haemogregarines, cystogenic coccidia, rickettsiae, anaplasmas and bartonellae. A specific focus of the study is to confirm the presence of *Toxoplasma gondii* DNA in marine mammal tissue and to evaluate the prevalence of this parasite in the Short-beaked Common Dolphin (*Delphinus delphis).* By fulfilling these objectives, the study contributes to the understanding of diversity, prevalence and potential impact of endoparasites including *Toxoplasma gondii*, within the marine mammal population of southern Spain, thus providing foundations for the conservation and monitoring of these vulnerable species.

Materials and methods

Sample collection and necropsies:

Tissue samples and parasites were taken from dead and stranded cetaceans including the previously described species the Fin Whale (*Balaenoptera physalus*), the Common Bottlenose Dolphin (*Tursiops truncatus*), the Short-beaked Common Dolphin (*Delphinus*

delphis), the Striped Dolphin (*Stenella coeruleoalba*), the Harbour porpoise (*Phocoena phocoena*), and the Long-finned Pilot Whale (*Globicephala melas*) that are found in southern Spain. Necropsied were carried out in collaboration with the NGO Seashore Ambiental and the public institution from the administration of the regional government of Andalusia CEGMA (Centros de Gestion del Medio Marino Andaluz), which is a marine wildlife rehabilitation centre that is responsible for biodiversity, conservation and coastal environments in the region.

A sample collection protocol for marine mammals was established in collaboration with Prof. Dr. Sándor Hornok, head of the department of Parasitology and Zoology from the University of Veterinary Medicine Budapest (UVMB).

For a controlled and protocolled sampling, an organised and numbered tube system was developed in collaboration with Dr. Gergő Keve from the UVMB. The sampling protocol and the numbered tube sampling system were combined to a renewed sampling protocol that was logically implementable in the fieldwork during the necropsies.

100 numbered (NS1-NS100) Starstedt tubes of 2ml volume and a screw cap containing 1ml 96% ethanol are for usage for the collection of small parasites referring to individuals smaller than 5-6mm in diameter and 1cm in length. 50 numbered (NS101-NS150) Monovette tubes of 9ml volume and a screw cap containing 5-6ml 96% ethanol are for the collection of large parasites referring to individuals larger than 5-6mm in diameter and 1cm in length. A high ethanol concentration of 96% is crucial for an effectively preserved parasitic sample that has to be carefully collected to ensure that the parasites are preserved effectively. Another 100 numbered (NS151-NS250) empty Monovette tubes of 9ml volume and a screw cap are for the collection and freezing of tissue and faecal samples with the aim of sampling unicellular parasites that potentially infect the hosts tissue are to be stored at -20°C and no ethanol should be added to the tubes containing tissues or faeces. If there is the need of more tubes, 50 numbered (SN251-SN300) empty Monovette tubes of 9ml volume should be used for tissue samples and parasites as it is in need. If a parasite is collected, 5-6ml 96% alcohol should be filled into the tubes and if it is for tissue sampling or faeces, it should be frozen shortly after. The sampling method is described specifically to use a clean metal spoon applicator that must be cleaned, decontaminated and ethanol sterilized for the application of each different sample and tissue to minimize the risk of cross contamination.

With this protocol established, basic sampling principles are set. The collection of all multicellular visible parasites from the same individual host should be in one numbered ethanol containing tube or several tubes if too many parasites are collected. Those ethanol

filled tubes should be stored at room temperature. Initially all parasites from various locations within the host including mouth, stomachs, intestine and lungs into an ethanol containing tube or more tubes if necessary and again stored at room temperature. The collection of tissue samples, in numbered empty tubes, includes several organs such as e.g., spleen, liver, tail muscle, cerebrum and cerebellum. Separately faecal samples should be collected in numbered empty tubes. Those samples should be frozen shortly after their collection. When tissue samples are collected, an ethanol sterilized scalpel blade has to be used to obtain samples from the middle of each organ. The faecal samples should be collected from the large intestine if possible. All relevant data are to be recorded including place and date of finding and the name of the cetacean mammalian species and any observable information regarding age, sex and general condition of the individual. Photographic documentation of during necropsies should be recorded to evaluate pathological conditions and all data should be linked to the numbered tubes. As an addition single EDTA blood samples from living turtles should be taken for future research. The analysis of the samples is planned to be conducted in Budapest at the University of Veterinary Medicine Budapest in collaboration with Prof. Dr. Sándor Hornok and Dr. Gergő Keve of the Department of Parasitology and Zoology. In order to achieve the objectives modern molecular analysation methods are planned to be applied such as PCR and DNA extraction and DNA sequencing. For the transport from Algericas, Spain to Budapest, Hungary, a cooling storage system was developed to ensure the quality of frozen tubes that kept the tubes frozen for the time of transport via public airplane. An EPS (expanded polyesterene) box with cooling packs and a thermometer for monitoring and maintaining low temperature and frozen samples should be equipped with emergency disposable cooling packs that could be activated if the temperature increases above a certain degree.

Prof. Dr. Sándor Hornok created a certificate on march 28, 2022 phrasing that the samples in labelled tubes transported to Budapest, Hungary are non-infectious, non-living and only to serve scientific research purposes representing no health hazard or commercial value. The transport of the samples to Hungary was approved by Seashore and conducted by the Author. Furthermore, a contract on cooperation in science and veterinary medicine was designed for the responsible use of all samples including all tissues and parasites of this study singed by three parties:

Prof. Dr. Péter Sótonyi as the rector of the University of Veterinary Medicine Budapest, Carolina Fernández-Maldonado DVM, PhD as the founder and director of Seashore

Ambiental, and Nicolas R. Specht, student of the University of Veterinary Medicine Budapest and Author of this study.

This contract forces all parties that the samples can only be used for this and future research in the frame of this study and under coordination and publication of the Author of this study to ensure the responsible and legal handling of the rare cetacean tissue samples and parasites. Due to the fact that this study works with endangered species under the CITES convention, Dr. Gergő Keve organized a certificate from the Pest County Government Office which defines the legal right for import, possession, utilization and researching the samples: PE-06/KTF/33276-8/2023.

Sample collection

A total of 118 tissue and parasite samples from 13 individuals were gathered (As shown in Table 1). The supervising veterinarian, Alejandra Cerezo Caro from Seashore Ambiental, oversaw and participated in various necropsies. All sampling tubes have been coded as previously explained to ensure the methodical categorization and recognition of the samples during the entire analysis process. Each tissue and parasite sample is associated with the specific cetacean individual from which it was obtained. Every cetacean is identified by a provincial code that carries crucial data within itself. BPH231222CAM serves as the provincial code for a singular Fin Whale (Balaenoptera physalus), where the first three letters of the code (**BPH**231222CAM) implicate the taxonomic name, followed by the date of discovery of the cetacean (BPH**231222**CAM) in this instance, the 23th of December 2022. The subsequent segment mentions the Spanish province using its initial two letters (BPH231222**CA**M), indicating the province of Cádiz, where the individual was found. The final part indicates the vital status, whether the individual was deceased or alive, using the initial letter of the Spanish translation for 'dead' or 'alive' (**m**uerto or **v**ivo) (BPH231222CA**M**). In the event of two individuals being found on the same day, in the same province, and having the same vital status, each cetacean is assigned a specific investigation code specifically linked to the individual. This incident did not happen in this study thus an investigation code was not necessary to identify the cases and individuals.

Necropsy

For every individual an official necropsy protocol from the public institution from the administration of the regional government of Andalusia CEGMA (Centros de Gestion del

Table 1. Summary of all tissue and parasite samples of this study

NS Code	Parasite	Organ	Location	Species	Date of Necropsy	Provincial Code
NS154	0	1	Sceletal muscle	*D. delphis*	14.08.22	DDE120822CAM
NS196	0	1	Lung	*D. delphis*	14.08.22	DDE120822CAM
NS181	0	1	Liver	*D. delphis*	14.08.22	DDE120822CAM
NS176	0	1	Feces	*D. delphis*	14.08.22	DDE120822CAM
NS186	0	1	Kidney	*D. delphis*	14.08.22	DDE120822CAM
NS174	0	1	Encephalon	*D. delphis*	14.08.22	DDE120822CAM
NS153	0	1	Spleen	*D. delphis*	14.08.22	DDE120822CAM
NS188	0	1	Blood	*D. delphis*	14.08.22	DDE120822CAM
NS189	0	1	Cerebellum	*D. delphis*	14.08.22	DDE120822CAM
NS1	1	0	Parasite caudal fin	*D. delphis*	14.08.22	DDE120822CAM
NS198	0	1	Liver	*S. coeruleoalba*	16.08.22	SC0150822CAM
NS167	0	1	Lung	*S. coeruleoalba*	16.08.22	SC0150822CAM
NS159	0	1	Sceletal muscle	*S. coeruleoalba*	16.08.22	SC0150822CAM
NS176	0	1	Intestinal lumen	*S. coeruleoalba*	16.08.22	SC0150822CAM
NS175	0	1	Intestines	*S. coeruleoalba*	16.08.22	SC0150822CAM
NS180	0	1	Kidney	*S. coeruleoalba*	16.08.22	SC0150822CAM
NS162	0	1	Encephalon	*S. coeruleoalba*	16.08.22	SC0150822CAM
NS195	0	1	Cerebellum	*S. coeruleoalba*	16.08.22	SC0150822CAM
NS152	0	1	Spleen	*S. coeruleoalba*	16.08.22	SC0150822CAM
NS170	0	1	Blood	*S. coeruleoalba*	16.08.22	SC0150822CAM
NS151	0	1	Skin/Fat	*S. coeruleoalba*	16.08.22	SC0150822CAM
NS2	1	0	Caudal fin	*S. coeruleoalba*	16.08.22	SC0150822CAM
NS127	1	0	Caudal fin	*S. coeruleoalba*	16.08.22	SC0150822CAM
NS131	1	0	Caudal fin	*S. coeruleoalba*	16.08.22	SC0150822CAM
EDTA	0	1	Blood	*C. caretta*	16.08.22	CCA150822CAV
NS110	1	0	Fin	*C. caretta*	16.08.22	CCA150822CAV
NS177	0	1	Sceletal muscle	*P. phocoena*	21.08.22	PPH230822HUM
NS171	0	1	Lung	*P. phocoena*	21.08.22	PPH230822HUM
NS156	0	1	Liver	*P. phocoena*	21.08.22	PPH230822HUM
NS158	0	1	Intestine	*P. phocoena*	21.08.22	PPH230822HUM
NS183	0	1	Lumen intestine	*P. phocoena*	21.08.22	PPH230822HUM
NS169	0	1	Kidney	*P. phocoena*	21.08.22	PPH230822HUM
NS199	0	1	Spleen	*P. phocoena*	21.08.22	PPH230822HUM
NS160	0	1	Skin/Fat	*P. phocoena*	21.08.22	PPH230822HUM
NS163	0	1	Mammary gland	*P. phocoena*	21.08.22	PPH230822HUM
NS192	0	1	Uterus	*P. phocoena*	21.08.22	PPH230822HUM
NS3	1	0	Fin	*P. phocoena*	21.08.22	PPH230822HUM
NS4	1	0	Fin	*P. phocoena*	21.08.22	PPH230822HUM
NS112	1	0	Fin	*P. phocoena*	21.08.22	PPH230822HUM
NS222	0	1	Subcutaneous	*S. coeruleoalba*	16.11.22	SCO151122CAM
NS114	1	0	Subcutaneous	*S. coeruleoalba*	16.11.22	SCO151122CAM
NS201	0	1	Kidney	*S. coeruleoalba*	16.11.22	SCO151122CAM
NS141	1	0	Intestine (proximal)	*S. coeruleoalba*	16.11.22	SCO151122CAM
NS207	0	1	Intestine	*S. coeruleoalba*	16.11.22	SCO151122CAM
NS136	1	0	Skin	*S. coeruleoalba*	16.11.22	SCO151122CAM
NS132	1	0	Skin	*S. coeruleoalba*	16.11.22	SCO151122CAM
NS145	1	0	Stomach (caudal, piloric stomach)	*S. coeruleoalba*	16.11.22	SCO151122CAM
NS129	1	0	Gallbladder	*S. coeruleoalba*	16.11.22	SCO151122CAM
NS133	1	0	Lung	*S. coeruleoalba*	16.11.22	SCO151122CAM
NS147	0	1	Hepatic ducts	*S. coeruleoalba*	16.11.22	SCO151122CAM
NS236	0	1	Stomach (caudal, piloric stomach)	*S. coeruleoalba*	16.11.22	SCO151122CAM
NS216	0	1	Lung	*S. coeruleoalba*	16.11.22	SCO151122CAM
NS226	0	1	Liver	*S. coeruleoalba*	16.11.22	SCO151122CAM
NS146	1	0	Skin	*D. delphis*	28.11.22	DDE261122MAM
NS218	0	1	Skin	*D. delphis*	28.11.22	DDE261122MAM
NS143	1	0	Peritoneum	*D. delphis*	28.11.22	DDE261122MAM
NS231	0	1	Peritoneum	*D. delphis*	28.11.22	DDE261122MAM
NS205	0	1	Liver	*D. delphis*	28.11.22	DDE261122MAM
NS140	1	0	Liver	*D. delphis*	28.11.22	DDE261122MAM
NS150	1	0	Skin	*T. truncatus*	14.12.22	TTR131222CAM
NS215	0	1	Skin	*T. truncatus*	14.12.22	TTR131222CAM
NS206	0	1	Muscle	*T. truncatus*	14.12.22	TTR131222CAM
NS239	0	1	Intestine	*T. truncatus*	14.12.22	TTR131222CAM

Table 1. Continued

NS Code	Parasite	Organ	Location	Species	Date of Necropsy	Provincial Code
NS219	0	1	Kidney	*T. truncatus*	14.12.22	TTR131222CAM
NS225	0	1	Liver	*T. truncatus*	14.12.22	TTR131222CAM
NS225	0	1	Lung	*T. truncatus*	14.12.22	TTR131222CAM
NS134	1	0	Lung	*T. truncatus*	14.12.22	TTR131222CAM
NS109	1	0	Lung	*T. truncatus*	14.12.22	TTR131222CAM
NS138	1	0	Skin around caudal fin	*G. melas*	20.12.22	GME191222MAV
NS113	1	0	Peritoneum	*G. melas*	20.12.22	GME191222MAV
NS101	0	1	Subcutaneous (parasitic cysts)	*G. melas*	20.12.22	GME191222MAV
NS148	1	0	Stomach (caudal, piloric stomach)	*G. melas*	20.12.22	GME191222MAV
NS126	1	0	Senos pterigoideos	*G. melas*	20.12.22	GME191222MAV
NS108	1	0	Intestine (proximal)	*G. melas*	20.12.22	GME191222MAV
NS237	0	1	Skin/Fat	*B. physalus*	24.12.22	BPH231222CAM
NS210	0	1	Muscle	*B. physalus*	24.12.22	BPH231222CAM
NS224	0	1	Tongue	*B. physalus*	24.12.22	BPH231222CAM
NS244	0	1	Traqueobron-chial LN	*B. physalus*	24.12.22	BPH231222CAM
NS238	0	1	Heart	*B. physalus*	24.12.22	BPH231222CAM
NS202	0	1	Thromb	*B. physalus*	24.12.22	BPH231222CAM
NS228	0	1	Blood	*B. physalus*	24.12.22	BPH231222CAM
NS240	0	1	Thymus	*B. physalus*	24.12.22	BPH231222CAM
NS229	0	1	Lung	*B. physalus*	24.12.22	BPH231222CAM
NS130	1	0	Nematodes location in lungs	*B. physalus*	24.12.22	BPH231222CAM
NS122	1	0	Skin (caudal fin)	*D. delphis*	28.12.22	DDE271222GRM
NS142	0	1	Skin (caudal fin)	*D. delphis*	28.12.22	DDE271222GRM
NS120	0	1	Skin (pectoral fin)	*D. delphis*	28.12.22	DDE271222GRM
NS117	1	0	Skin around pectoral fin. Unknown parasite.	*D. delphis*	28.12.22	DDE271222GRM
NS203	0	1	Muscle	*D. delphis*	28.12.22	DDE271222GRM
NS230	0	1	Intestine	*D. delphis*	28.12.22	DDE271222GRM
NS243	0	1	Heart	*D. delphis*	28.12.22	DDE271222GRM
NS249	0	1	Liver	*D. delphis*	28.12.22	DDE271222GRM
NS235	0	1	Thoracic aorta	*D. delphis*	28.12.22	DDE271222GRM
NS214	0	1	Lung	*D. delphis*	28.12.22	DDE271222GRM
NS242	0	1	Trachea	*D. delphis*	28.12.22	DDE271222GRM
NS248	0	1	Stomach	*D. delphis*	28.12.22	DDE271222GRM
NS233	0	1	Kidney	*D. delphis*	28.12.22	DDE271222GRM
NS250	0	1	Brain	*D. delphis*	28.12.22	DDE271222GRM
NS227	0	1	Cerebellum	*D. delphis*	28.12.22	DDE271222GRM
NS245	0	1	Medulla	*D. delphis*	28.12.22	DDE271222GRM
NS121	0	1	Unrecogniza-ble structure in atrium	*C. caretta*	30.12.22	CCA231222CAV
NS149	1	0	Subcutaneous (parasitic cysts)	*D. delphis*	09.01.23	DDE080123CAM
NS262	0	1	Subcutaneous (parasitic cysts)	*D. delphis*	09.01.23	DDE080123CAM
NS105	1	0	Mesentery	*D. delphis*	09.01.23	DDE080123CAM
NS263	0	1	Mesentery (cysts)	*D. delphis*	09.01.23	DDE080123CAM
NS115	1	0	Skin	*S. coeruleoalba*	26.01.23	SCO250123CAM
NS269	0	1	Skin	*S. coeruleoalba*	26.01.23	SCO250123CAM
NS102	1	0	Subcutaneous	*S. coeruleoalba*	26.01.23	SCO250123CAM
NS124	1	0	Muscle	*S. coeruleoalba*	26.01.23	SCO250123CAM
NS279	1	0	Lung	*S. coeruleoalba*	26.01.23	SCO250123CAM
NS173	0	1	Lung	*S. coeruleoalba*	26.01.23	SCO250123CAM
NS295	1	0	Piloric	*S. coeruleoalba*	26.01.23	SCO250123CAM
NS257	0	1	Piloro	*S. coeruleoalba*	26.01.23	SCO250123CAM
NS139	1	0	Stomach	*S. coeruleoalba*	26.01.23	SCO250123CAM
NS264	0	1	Stomach	*S. coeruleoalba*	26.01.23	SCO250123CAM
NS288	1	0	Intestine	*S. coeruleoalba*	26.01.23	SCO250123CAM
NS264	0	1	Stomach	*S. coeruleoalba*	26.01.23	SCO250123CAM

Medio Marino Andaluz) must be completed. Vital information for identification of the case and individual is recorded such as the provincial code, date of the necropsy, taxonomic name of the species, sex, weight, preservation method before the necropsy, investigation number, the location where the necropsy is conducted, the Spanish name of the species, approximate

age and total length of the carcass. Furthermore, specific measurements are conducted, such as the overall length, thoracic perimeter length at the axillary fold, beak center to dorsal fin tip length, beak center to blowhole center length, beak center to cranial melon start length, beak center to cranial pectoral fin insertion length, beak center to genital orifice center length, beak center to anal orifice center length, beak center to throat grooves end length, maximum caudal fin width, maximum dorsal fin width, central caudal fin notch depth, beak center to eye center length, right mammary fold length, left mammary fold length, beak center to dorsal fin base center length, pectoral fin length from tip to cranial insertion, pectoral fin length from tip to caudal insertion, genital slit length, anal slit length, maximum pectoral fin width, dorsal fin base length, number of teeth for the left and right maxilla, and the number of teeth for the left and right mandible.

Measures are noted in centimetre and are significant for many reasons. The measured numbers indicate the sex, age, development, species and health condition. Some of the measures are dedicated to a specific group of cetaceans such as the number of teeth that is only used for toothed whales and the measurements including the throat grooves are used for baleen whales.

Furthermore, the document notes additional data and information including the presence of any ectoparasites or epibionts found on the carcass. The taxonomic name and localisation of the parasites are documented, as well as the external lesions that should be well described. Specific pathological examination details are recorded for each following section.

The carcass is divided into different systems for the necropsy such as the integumentary system, musculoskeletal system, thoracic cavity, cardiovascular system including a specific tissue. Cetaceans have a distinctive anatomical structure referred to as the rete mirabile, which includes a network of blood vessels positioned near the brain. It facilitates the exchange of heat or substances between bloodstreams, regulating the body's temperature during cold-water swimming (67). Furthermore, examined is the respiratory tract. The health of the respiratory tract is particularly essential for the survival of marine mammals (68).

Moreover, the abdomen and the gastrointestinal tract including descriptions of the pancreas and liver are also recorded. The subsequent section addresses the urinary tract, genital tract, endocrine system, lymphatic system and a pathological section covers organs related to the senses, including the eyes and the acoustic system, incorporating acoustic fat, which surrounds the acoustic system for sound reflection and echolocation, and the pterygoid sinuses, influencing buoyancy and sound production. At last, the system examined

pathologically is the nervous system, which includes the cerebrum, cerebellum, brainstem, medulla oblongata, meninges, and peripheral nerves like the brachial plexus.

For potential future research specific organ samples are always collected. Each sample is divided into three parts for different potential purposes. One part is preserved in formaldehyde, another is intended to be frozen in a plastic bag, and the final part is to be frozen in aluminium foil to prevent any potential plastic contamination.

DNA extraction:

The DNA extractions took place at the UVMB in the Department of Parasitology and Zoology and were directed by Dr. Gergö Keve. The tissue and parasite samples of this study are to be selected by certain factors, as pathological findings and tissue probability of containing protozoan parasites. The QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) is being used for DNA extraction, following the manufacturer's instructions. This process includes an overnight digestion in tissue lysis buffer and Proteinase-K at 56 °C. Additionally, extraction controls (tissue lysis buffer) are being processed alongside the parasitic samples to monitor cross-contamination.

Molecular identification:

PCR analyses were conducted at the UVMB in the Department of Parasitology and Zoology and were directed by Nóra Takács. The cytochrome oxidase subunit I (cox1) gene is chosen as the general barcode for molecular analysis. The PCR amplifies an ~710-bp-long fragment of the gene. The primers HCO2198 (5′-TAA ACT TCA GGG TGA CCA AAA AAT CA-3′) and LCO1490 (5′-GGT CAA CAA ATC ATA AAG ATA TTG G-3′) are used in a reaction volume of 25 µl, containing 1 U (stock 5 U/µl) HotStarTaq Plus DNA Polymerase, 2.5 µl 10 × CoralLoad Reaction buffer (including 15 mM MgCl2), 0.5 µl PCR nucleotide Mix (stock 10 mM), 0.5 µl of each primer (stock 50 µM), 15.8 µl ddH2O, and 5 µl template DNA. For amplification, an initial denaturation step at 95°C for 5 min is followed by 40 cycles of denaturation at 94°C for 40 s, annealing at 48°C for 1 min, and extension at 72°C for 1 min. Final extension is performed at 72°C for 10 min. Several target groups were chosen for the parasite and tissue samples with their specific target gene such as the vertebrate barcode with cox1, flukes with cox1 and ITS2, *Sarcocystis sp.*with SUU, *Toxoplasma sp.* with repeat region, *Trypanosoma sp.* with ssu, piroplasms with 18SrDNS, *Hepatozoon spp.* With 18SrDNS, *Acanthamoeba sp.* 18SrRNA, anaplasmataceae with 16SrRNA, *Rickettsia sp.* with gltA and *Bartonella sp.* with 16S-23S ITS target gene. For each target group the specific target gene along with a primer,

Table 2. Primers and details for conventional PCR methods used in this study

Target group	Target gene	Primer name	Primer sequence (5'-3')	Amplicon length (bp)	Thermocycling profile	Reference
General barcode	cox1	LCO1490 HCO2198	GGT CAA CAA ATC ATA AAG ATA TTG G TAA ACT TCA GGG TGA CCA AAA AAT CA	~710	95 °C for 5 min; 40× (94 °C for 40 s; 48 °C for 1 min; 72 °C for 1 min); 72 °C for 10 min	Folmer et al., 1994 (69)
Vertebrate barcode	cox1	SFF_145f SFF_351r	GTH ACH GCY CAY GCH TTY GTA ATA AT CTC CWG CRT GDG CWA GRT TTC C	~250	95 °C for 5 min; 40× (94 °C for 40 s; 56 °C for 1 min; 72 °C for 1 min); 72 °C for 7 min	Walker et al., 2016 (70)
Flukes	cox1	JB3 JB4.5	TTT TTT GGG CAT CCT GAG GTT TAT TAA AGA AAG AAC ATA ATG AAA ATG	~450	95 °C for 5 min; 40× (95 °C for 40 s; 55 °C for 1 min; 72 °C for 1,5 min); 72 °C for 10 min	Bowles et al., 1993 (71); Wang et al., 2012 (72)
Flukes	ITS2	3S-fw A28S-rev	GGT ACC GGT GGA TCA CTC GGC TCG TG GGG ATC CTG GTT AGT TTC TTT TCC TCC GC	~522	95 °C for 5 min; 40× (95 °C for 40 s; 55 °C for 1 min; 72 °C for 1,5 min); 72 °C for 10 min	Prasad et al., 2007 (73); Sahu et al., 2016 (74)
Sarcocystis sp.	SSU	COC1* COC2*	AAG TAT AAG CTT TTA TAC GGC T CAC TGC CAC GGT AGT CCA ATA C	~350	95 °C for 10 min; 40× (94 °C for 30 s; 54 °C for 30 s; 72 °C for 30 s); 72 °C for 10 min	Ho et al., 1996 (75)
Toxoplasma sp.	repeat region	TOX-8 (fw) TOX5 (rev)	CCC AGC TGC GTC TGT CGG GAT CGC TGC AGA CAC AGT GCA TCT GGA TT	~480	95 °C for 5 min; 35× (95 °C for 40 s; 60 °C for 1 min; 72 °C for 1 min); 72 °C for 10 min	Homan et al., 2000 (76); Reischl et al., 2003 (77); Schares et al., 2008 (78)
Trypanosoma sp.	ssu	609F 706R	CAC CCG CGG TAA TTC CAG C CTG AGA CTG TAA CCT CAA	~800-1000	95 °C for 5 min; 40× (94 °C for 40 s; 49 °C for 1,5 min; 72 °C for 1 min); 72 °C for 5 min	Da Silva et al. 2004 (79); Ramírez et al. 2012 (80)
Piroplasms	18S rDNS	BJ1 BN2	GTC TTG TAA TTG GAA TGA TGG TAG TTT ATG GTT AGG ACT ACG	~500	95 °C for 10 min; 40× (95 °C for 30 s; 54 °C for 30 s; 72 °C for 40 s); 72 °C for 5 min	Casati et al., 2006 (81)
Hepatozoon spp.	18S rDNS	HepF HepR	ATA CAT GAG CAA AAT CTC AAC CTT ATT ATT CCA TGC TGC AG	~650	95 °C for 5 min; 35× (95 °C for 40 s; 57 °C for 40 s; 72 °C for 60 s); 72 °C for 7 min	Inokuma et al., 2002 (82)
Acanthamoeba sp.	18S rRNA	JDP1 JDP2	GGC CCA GAT CGT TTA CCG TGA A TCT CAC AAG CTG CTA GGG GAG TCA	~480	95 °C for 5 min; 35× (95 °C for 35 s; 56 °C for 45 s; 72 °C for 1 min); 72 °C for 7 min	Niyyati et al., 2016 (83)
Anaplasmataceae	16S rRNA	EHR16sD EHR16sR	GGT ACC YAC AGA AGA AGT CC TAG CAC TCA TCG TTT ACA GC	~350	95 °C for 10 min; 40× (95 °C for 30 s; 55 °C for 30 s; 72 °C for 45 s); 72 °C for 5 min	Brown et al., 2001 (84)
Rickettsia sp.	gltA	RpCs.877p RpCs.1258n	GGG GGC CTG CTC ACG GCG G ATT GCA AAA AGT ACA GTG AAC A	~380	95 °C for 5 min; 40× (95 °C for 20 s; 48 °C for 30 s; 72 °C for 1 min); 72 °C for 5 min	Regnery et al. 1991 (85)
Bartonella sp.	16S-23S ITS	BA325s BA1100as	CTT CAG ATG ATG ATC CCA AGC CTT CTG GCG GAA CCG ACG ACC CCC TGC TTG CAA AGC A	~600	95 °C for 5 min; 40× (94 °C for 30 s; 65 °C for 30 s; 72 °C for 50 s); 72 °C for 5 min	Maggi et al., 2006 (86); Maia et al., 2016 (87)
Ixodidae COI	16S rDNA	16S+1 16S-1	CTG CTC AAT GAT TTT TTA AAT TGC TGT GG CCG GTC TGA ACT CAG ATC AAG T	~460	95 °C for 5 min; 40× (94 °C for 40 s; 51 °C for 1 min; 72 °C for 1 min); 72 °C for 10 min	Beati et al., 2001 (88)

primer sequence (5'-3'), amplication length in bp and a specific thermocycling profile is used as seen in Table 2.

Sequencing:

In all PCRs, the non-template reaction mixture serves as the negative control. Extraction controls and negative controls should remain PCR negative in all tests. Purification and sequencing of the PCR products are planned to done by Biomi Ltd. (Gödöllő, Hungary). Quality control and trimming of sequences are to be performed with the BioEdit program, then alignment with GenBank sequences by the nucleotide BLASTN program (https://blast.ncbi.nlm.nih.gov). New sequences are to be submitted to GenBank.

Results

For each case and individual, the necropsy was conducted according to the protocol of the regional government of Andalusia CEGMA (Centers for the Management of the Andalusian Marine Environment). The sampling protocol of this study was intentionally designed to be easily implementable and seamlessly align with the governmental protocol, ensuring that there were no issues impacting the data quality due to sampling complexities. The most promising samples of specific individuals were selected to be further examined based pathological findings and the expertise of Prof Dr. Sándor Hornok and Dr. Gergő Keve and include DDE120822CAM, SCO150822CAM, PPH230822HUM, SCO151122CAM, DDE261122MAM, TTR131222CAM, BPH231222CAM, DDE2712222GRM. The pathological protocol and diagnosis of the individuals of the chosen tissue and parasitic samples is presented in the following section.

BPH231222CAM:

This deceased Fin Whale neonate (*Balaenoptera physalus*) female of approximately 1000kg bodyweight with a total length of 520cm, appeared floating in the Bay of Algeciras on the 13.12.2022 where it was collected and transported to the Los Barrios, Spain waste landfill, where the necropsy was performed on the 24.12.2022.

Morphological diagnosis: Severe haemorrhage in the latissimus dorsi muscle and craniodorsal area of the animal. Moderate to severe congestion of the central nervous system. Severe emphysema and alveolar oedema, along with areas of pulmonary atelectasis, and presence of nematodes in the bronchi and lung parenchyma. Associated reaction in lymph

nodes. Moderate serosanguineous pericarditis and presence of serosanguineous fluid in the thoracic and abdominal cavities.

SCO151122CAM:

This Individual is described as a deceased juvenile Striped Dolphin (*Stenella coeruleoalba*) female of 20.6kg bodyweight with a total length of 131 cm that appeared stranded on Levante Beach in La Línea de la Concepción on the 15.11.2022, and was collected by CEGMA personnel for its necropsy the following day. The necropsy was conducted as planned in Algeciras, Spain on the 16.11.2022.

Morphological diagnosis: Severe, chronic nematodosis in the respiratory system, causing collapse of the airways, resulting in areas of haemorrhages, alveolar oedema, pulmonary emphysema, and atelectasis. Associated reaction in lymph nodes. Additionally, high parasitosis in the digestive system, hindering proper feeding and exacerbating the complications.

DDE261122MAM

This cetacean identified as a Short-beaked Common Dolphin (*Delphinus delphis*) female and a body length of 183cm stranded on the 26.22.2022 on the beach of Nerja, with milk present in the mammary glands and linear lesions on the skin, and was collected by CEGMA personnel for its necropsy conducted in Algeciras on the 28.11.2022.

The animal can be considered a juvenile based on the total body length and the lack of teeth in the mandible.

The amount of subcutaneous fat and the presence of whitish content in the digestive system (consistent with milk) suggest that the animal was able to feed. The presence of various skin lesions, different lymph nodes, and the thymus with lymphadenomegaly, along with the appearance of the lungs and meninges, are indicators of infections.

DDE271222GRM

This individual identified as a Short-beaked Common Dolphin (*Delphinus delphis*) adult female with a bodyweight of 79kg and a length of 101cm stranded on the beach of Granada on the 27.12.2022 and was collected by CEGMA personnel for its necropsy in Algeciras on the 28.12.2022.

Morphological diagnosis: Moderate to severe congestion of the central nervous system. Severe alveolar emphysema and oedema, along with areas of pulmonary atelectasis. Associated reaction in lymph nodes. Severe and extensively focal haemorrhagic enteritis in the middle and distal regions. Moderately severe epithelial and subcutaneous, granulomatous

lesions caused by ectoparasites and likely viral lesions. Moderate serosanguineous pericarditis and presence of serosanguineous fluid in the thoracic and abdominal cavities.

PPH230822HUM

This Harbour porpoise (*Phocoena phocoena*) female with a bodyweight of 43,8kg was found stranded on a sandy beach in the province of Huelva on the 23.08.2022. It was transported to Algeciras and stored in a refrigerator until the following day the 24.08.2022 when the necropsy was performed.

Diagnosis: The animal under analysis is a pregnant female that did not give birth. The state of the animal, reproductive system, and foetus likely do not contribute to the diagnosis, but the discrepancy in size between the foetus and the placenta, along with their appearance, suggests that there may have been an issue at this level. The issues could stem from various origins, but regardless, they would have affected the mother's condition. Due to the preservation state of this animal, it is not possible to assess the body condition, but it was likely found weak and in an active stranding. This hypothesis could be supported by numerous findings, such as the large quantity of sand in the digestive system and the presence of subcutaneous hematomas.

SCO150822CAM

This Striped Dolphin (*Stenella coeruleoalba*) female neonate of 9.6kg bodyweight was stranded, deceased on the beach in the province of Cádiz on the 15.08.2022.

It was transported to CEGMA in Algeciras on the same day and refrigerated for the necropsy to be conducted the following day the 16.08.2022. The lesions in the liver related to circulation raise suspicion of antemortem cardiovascular shock. The lung lesions indicate a chronic infection, but they are not severe enough to have caused the animal's death.

The investigated animal is most probably a juvenile considering its length, the presence of vestigial hair, and the existence of milk-like fluid in the digestive system. Histological and microbiological analysis could help identify the cause of stranding and death. It is essential to consider the possibility of maternal separation due to the presence of a small amount of milk in the digestive system.

TTR131222CAM

This Common Bottlenose Dolphin (*Tursiops truncatus*) juvenile male with a length of 289cm stranded on the beach of Chipiona on the 13.12.2022 and was collected by CEGMA personnel for its necropsy the following day the 14.12.22.

Morphological diagnosis: Severe, chronic nematodosis in the respiratory system, completely collapsing the airways, resulting in the appearance of haemorrhagic areas, alveolar oedema, emphysema, and pulmonary atelectasis as seen in figure 1-4. Associated reaction in lymph

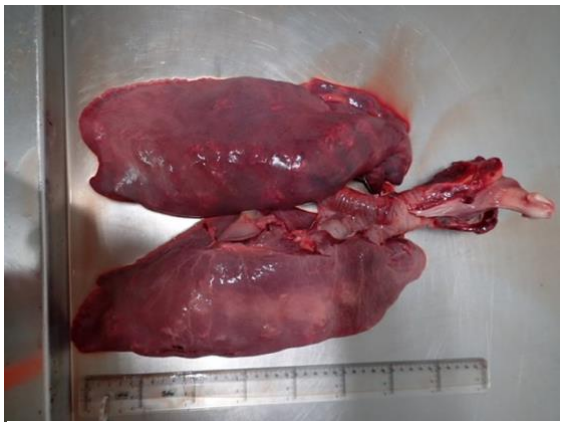

Figure 1: Photography of Lungs of TTR131222CAM

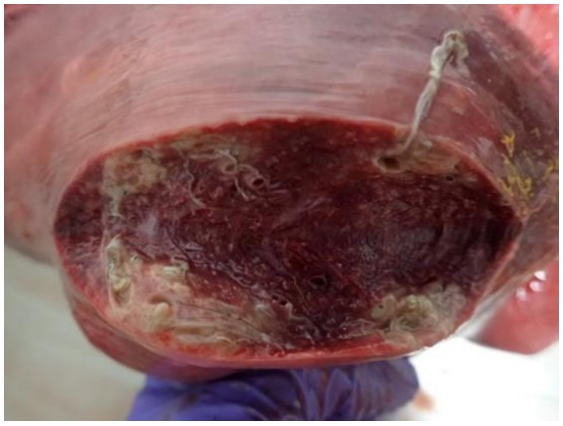

Figure 2: Photography of Lung of TTR131222CAM with nematidosis in tertiary bronchi

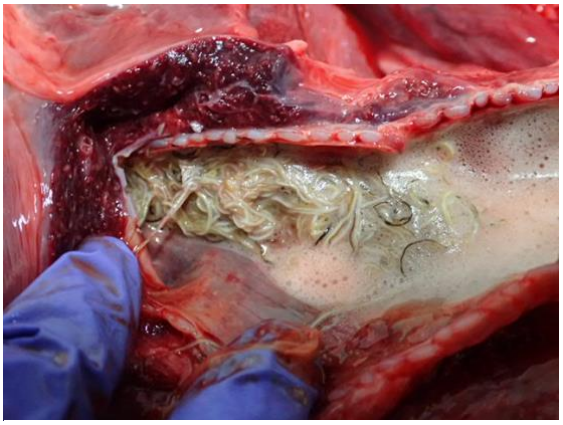

Figure 3: Photography of Lung of TTR131222CAM with nematidosis in secondary bronchi

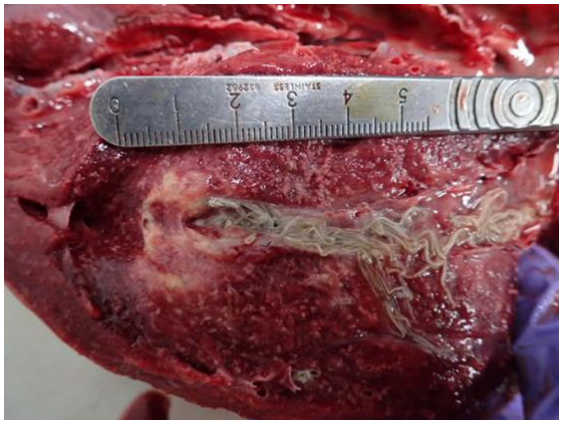

Figure 4: Photography of Lung of TTR131222CAM with longitudinal insertion of tertiary bronchi with nematidosis

nodes and severe hepatitis and ascites.

Presumptive diagnosis: This is a juvenile animal that is sexually mature, confirmed by its size and the development of its genital organs. This period is delicate for the animal's immune system as it is a developmental stage, making the animal more susceptible to infectious diseases, as evidenced by its poor body condition, high parasitosis, and associated systemic lesions. Additionally, the animal has severe lung damage, moderate to severe central nervous system congestion, severe hepatitis, and ascites, indicating clear systemic lesions (signs that suggest cardiopulmonary shock).

The molecular analysis targeting lungworm nematodes with the specific target PCR Ixodidae COI with the target gene 16S rDNA and the primers and its sequences 16S+1 3- CTG CTC AAT GAT TTT TTA AAT TGC TGT GG -5 and 16S-1 3- CCG GTC TGA ACT CAG ATC

AAG T-5 revealed a significant 94.16% positivity for Halocercus pingi DNA of the nematodes in the respiratory system of the stranded Common Bottlenose Dolphin (Tursiops truncatus) as seen in Table 3.

DDE120822CAM:

This Short-beaked Common Dolphin (*Delphinus delphis*) male juvenile with a bodyweight of approximately 17kg was found on a sandy beach in the province of Cádiz on the 12.08.2022 and was transported to the centre the same day. Two days later the 14.08.2022, the necropsy was performed in Algeciras, Spain, and meanwhile, the animal was kept refrigerated.

Presumptive diagnosis:

The animal can be considered a juvenile based on the total body length and the lack of teeth in the mandible. The amount of subcutaneous fat and the presence of whitish content in the digestive system (consistent with milk) suggest that the animal was able to feed. The presence of various skin lesions, different lymph nodes, and the thymus with

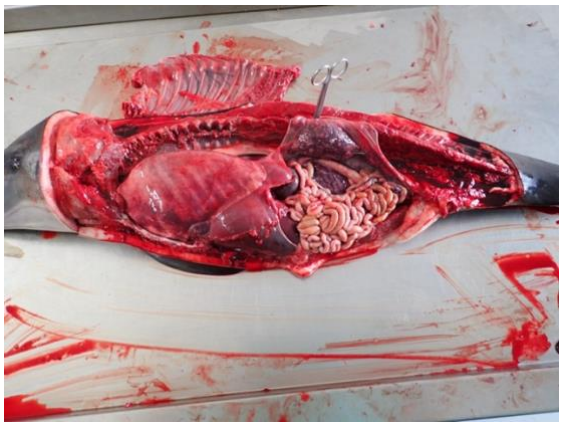

Figure 5: Photography of DDE120822CAM after preparation of thoracic and abdominal cavity

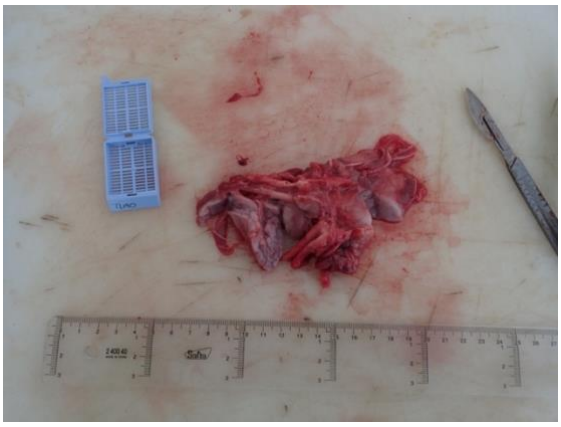

Figure 6: Photography of Thymus of DDE120822CAM

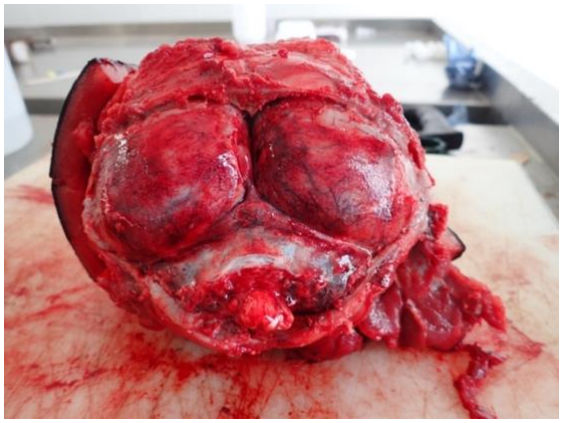

Figure 7: Photography of DDE120822CAM opened cranium with encephalon

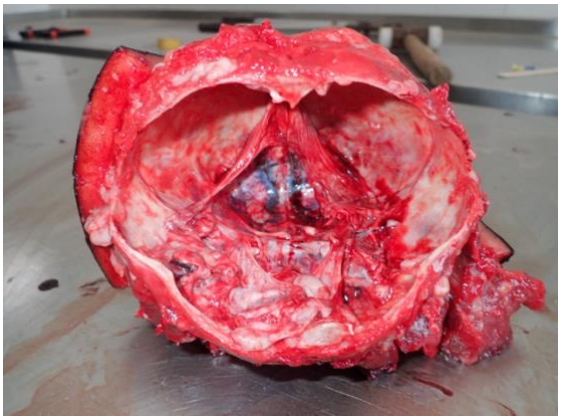

Figure 8: Photography of opened cranium and brain cavity of DDE120822CAM

lymphadenomegaly, along with the appearance of the lungs and meninges that displayed a moderately increased thickness and congestion, are indicative of a probable infection seen in figures 5-8. Some infections can cause abnormal behaviour in the animal or loss of strength. These conditions could have led to the live stranding, indicated by the facial and joint lesions, and consequently the death of the animal. Further microbiological and histopathological tests would be necessary to confirm and determine the cause of stranding and death. The molecular analysis utilizing specific primers TOX-8(fw) with a sequence of 3-CCC AGC TGC GTC TGT CGG GAT-5, TOX5(rev) with the sequence of 3-CGC TGC AGA CAC AGT GCA TCT GGA TT-5) and Sarcocystis PCR (as shown in table 2) confirmed the presence of *Toxoplasma gondii* DNA in various organ samples of this juvenile Short-beaked Common Dolphin. Specifically, the encephalon (sample NS174), cerebellum (sample SN189), and skeletal muscle (sample SN154) exhibited 100% positivity for *T. gondii* DNA as seen in Table 3 and Table 4. The other analyzed tissue samples were negative or amplified the hosts DNA.

Discussion:

***Toxoplasma gondii* in DDE120822CAM:**

The pathological findings observed in the necropsied Short-beaked Common Dolphin (*Delphinus delphis*) juvenile are suggestive of a interplay between various physiological and environmental factors leading to the animal's stranding and eventual demise. The presence of multiple skin lesions, lymphadenomegaly in the thymus, and congested lungs and meninges indicated a probable systemic infection. Such infections can significantly impact an animal's behavior and physical strength, potentially contributing to live stranding events, as evidenced by the facial and joint lesions identified in this case.

Moreover, the confirmed presence of *Toxoplasma gondii* DNA in various organ samples, including the encephalon, cerebellum, and skeletal muscle, further displays the impact of parasitic infections on the health of cetaceans, especially in vulnerable juvenile individuals. The 100% positivity for *T. gondii* DNA in these specific organs underscores the potential severity of the infection and its implications for the central nervous system and musculature of the heart. It is likely that the systemic effects *of T. gondii* contributed to the observed pathological changes, including the congestion and increased thickness of the meninges.

The molecular analysis utilizing specific primers TOX-8(fw) TOX5(rev) and Sarcocystis PCR confirmed the presence of *Toxoplasma gondii* DNA in various organ samples of the

Table 3. PCR tested organs + nematodes

Species	Provincial code	Muscle	Lungs	Liver	Spleen	Blood	Medulla	Cerebellum	Encephalon	Nematode
Short-baked common dolphin (D. delphis)	DDE120822CAM	1	1	1	1	1		1	1	
Striped dolphin (S. coeruleoalba)	SC0150822CAM	1	1	1	1	1		1	1	
Harbor porpoise (P. phoconea)	PPH230822HUM	1	1	1	1					
Striped dolphin (S. coeruleoalba)	SCO151122CAM		1	1						
Short-baked common dolphin (D. delphis)	DDE261122MAM			1						
Common bottlenose dolphin (T. truncatus)	TTR131222CAM	1		1						1
Fin whale (B. physalus)	BPH231222CAM	1	1			1				
Short-baked common dolphin (D. delphis)	DDE271222GRM		1	1			1	1	1	
Loggerhead sea turtle (C. caretta)	CCA150822CAM					1				

Table 4. Results of tested samples

Target and primer	Piroplasma sp. 18S BJ1+BN2	Hepatozoon sp. 18S HepF+HepR	Trypanosoma sp. Ssu 609F+706Rnew	Trypanosoma sp. VSG NTE1+NTE2	Trypanosoma sp. VSG NTE3+NTE4	Acanthamoeba sp. 18S JDP1+JDP2	Sarcocystis sp. Ssu COC1*+COC2*	Anaplasmataceae 16S EHR16sD+ EHR16sR	Rickettsia sp. gltA RpCS877p+ Rpcs1258n	Bartonella sp. 16S-23S ITS BA325s+ BA1100as	Ixodidae COI 16S rDNA 16S+1+16S-1
Positive results after sequencing (+ provincial code)							100% Tocoplasma gondii: NS174, NS189, NS154 (DDE120822CAM)				94,16% Halocerus pingi: NS109 (TTR131222CAM)

examined juvenile Short-beaked Common Dolphin. This study´s use of advanced molecular analysis insights

beyond the scope of previous research and investigations that primarily relied on the serological techniques for the detection of anti-*T. gondii* antibodies or *T. gondii* antigens using immunological techniques. Thus, these new findings serve to provide the first confirmation of the presence of *Toxoplasma gondii* DNA in the Short-beaked Common Dolphin (*Delphinus delphis*), that presents a notable advancement in the understanding of the prevalence and impact of this protozoan parasite, contribution to a more accurate assessment of parasitic infections in marine mammals in southern Spain and the region of the Campo de Gibraltar.

This confirmation highlights the susceptibility of certain cetacean species to zoonotic pathogens emphasizing the potential for cross species transmission from different environments. As this diagnosed systemic infection is likely to be a leading factor of the associated pathological findings, a need for comprehensive monitoring and management strategies is well suggested.

***Halocercus pingi* in TTR131222CAM:**

The pathological findings from the necropsied Common Bottlenose Dolphin (Tursiops truncatus) juvenile male unveil a distressing presentation of severe and chronic nematodosis within the respiratory system, leading to the collapse of the airways and the manifestation of extensive haemorrhagic areas, alveolar edema, emphysema, and pulmonary atelectasis. The concurrent presence of severe hepatitis and ascites indicates the widespread impact an infection on multiple physiological systems. Notably, the unexpected molecular detection of a substantial 94.16% positivity for *Halocercus pingi* DNA within nematodes found in the respiratory system of the affected dolphin raises significant questions regarding the prevalence and clinical implications of this particular lungworm nematode in this cetacean species. The surprising identification of Halocercus pingi underscores the need for further investigations into the distribution, pathogenesis, and potential impact of this *T. truncatus*. The systemic lesions observed, including severe lung damage, central nervous system congestion, and indications of cardiopulmonary shock, underscore the debilitating effects of the parasitic infection and its potential implications for the overall health and survival of affected cetaceans. The susceptibility of juvenile marine mammals to such infections, compounded by their delicate immune systems during developmental stages, shows the vulnerability of these populations to the adverse effects of parasitic infestations, urging the

implementation of comprehensive monitoring and management strategies to mitigate the impact of such infections on marine mammal health and conservation efforts. The unexpected detection of *Halocercus pingi* DNA, a parasitic nematode primarily associated with the Narrow-ridged Finless Porpoise (*Neophocaena asiaeorientalis*) and the Indo-Pacific Finless Porpoise (*Neophocaena phocaenoides*)(62–64), within the respiratory system of the Common Bottlenose Dolphin (*Tursiops truncatus*), marks a notable departure from its typical geographical occurrence, which spans from the Sea of Japan to the South China Sea. Notably, this discovery represents the first instance of *H. pingi* being identified in the species *T. truncatus* and, more significantly, the first documented occurrence in the Mediterranean Sea region.

The DNA we examined showed a 94.16% match with the *H. pingi* isolate available in the GenBank dataset (https://www.ncbi.nlm.nih.gov/genbank/), which was the closest match. It did not match with the DNA sequences of *H. delphini* (65,66), which species is much more common in the Mediterranean region. It is important to mention that the 94.16% match could even mean the appearance of a new genotype of *H. pingi* or even a new species, as our phylogenetic tree suggests (figure 9). However, further studies are needed to confirm this. In the Mediterranean Sea, *Halocercus delphini* was recorded with relatively high infection

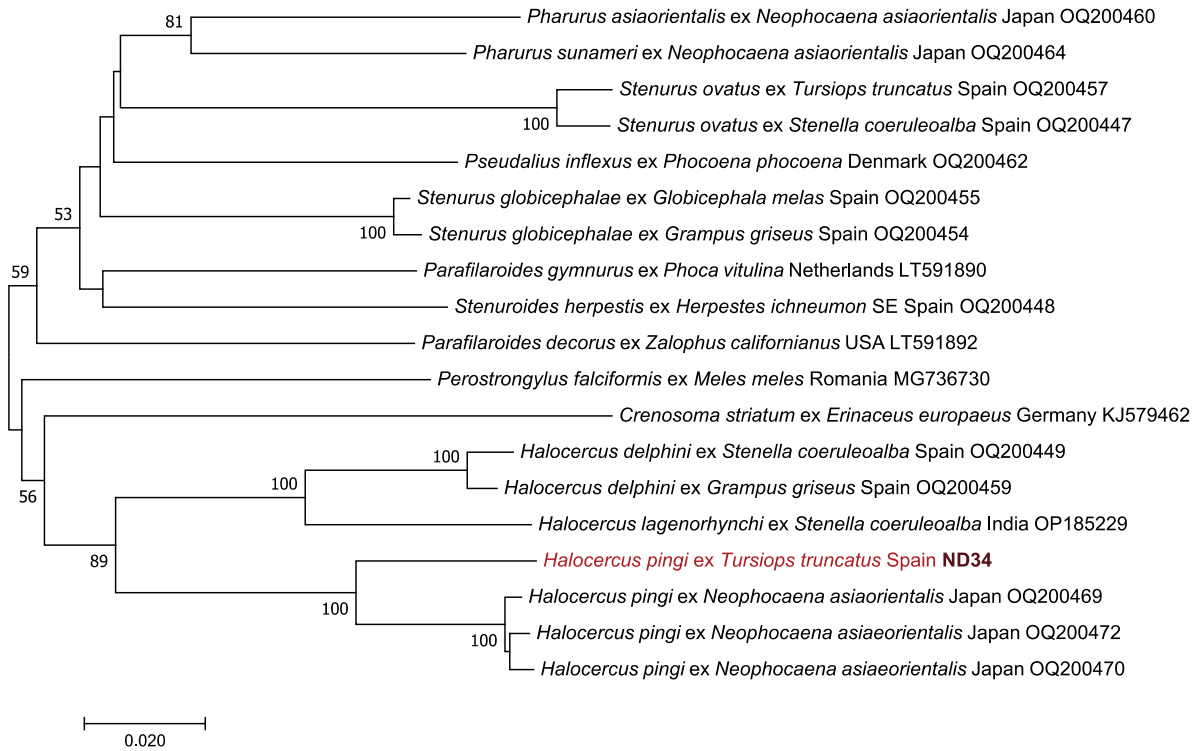

Figure 9: Phylogenetic tree including the *Halocercus pingi* sequence from this study

levels in Striped Dolphins (*Stenella coeruleoalba*) and Common Dolphins (*Delphinus delphis*)(65,66).

This discovery not only show the need for continued research of *Halocercus species* in Mediterranean cetaceans but also the need for further surveillance and monitoring of parasitic infections in *T. truncatus*. The need for taxonomic and molecular investigations is emphasized to clarify the evolutionary implications and potential ecological impact of the identified *H. pingi* strain on the cetacean populations in southern Spain including the Campo de Gibraltar.

Conclusion:

This comprehensive investigation into the endoparasites of marine mammals in southern Spain, particularly in the region of Campo de Gibraltar, has provided valuable insights into the prevalence and impact of parasitic infections, underscoring the vulnerability of cetacean populations to a range of pathogens. The identification of 100% Toxoplasma gondii DNA in the Short-beaked Common Dolphin (*D. delphis*) and the surprising detection of 94,16% *H. pingi* DNA in the respiratory system of the Common Bottlenose Dolphin (*T. truncatus*) display milestones in the understanding of parasitic dynamics in these marine mammal species.

These findings highlight the importance of employing advanced molecular techniques in parasite detection, shedding light on previously unknown occurrences of specific pathogens in these certain cetacean populations and species.

The implications of the identified infections of the researched parasite species extend to broader ecological knowledge of the complex relationships between parasitic prevalence and geographic distribution of cetacean species.

Moving forward, it is important to establish long-term monitoring programs and conduct further research to elucidate the epidemiology and ecological impact of these parasites on the health and conservation of cetaceans in the Mediterranean Sea. Additionally, the limitations of this study implicate the necessity of further updating sampling strategies and the inclusion of more specific molecular markers to provide accurate understanding of the dynamics of parasitic infections of *H. pingi* in marine mammal populations. By addressing

these challenges, future research can contribute significantly to the development of effective conservation and management strategies aimed to preserve the balance of marine ecosystems.

Limitations of the study:

The study's reliance on samples from stranded and deceased cetaceans might introduce potential biases, limiting the generalizability of the findings to broader, living populations. The use of a specific set of molecular markers and primers might have influenced the detection and identification of certain parasitic species, potentially leading to an underestimation of overall parasitic diversity in the examined marine mammal species. The absence of longitudinal data and a limited sample size could constrain the comprehensive understanding of the seasonal and geographical dynamics of parasitic infections in cetacean populations, calling for further research efforts to address these limitations and provide a broader perspective on the epidemiology of marine mammal parasites.

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- *facilitate professional relations and collaboration;*
- *support open access.*

Thesis statement for TDK thesis

I, the undersigned dr. Gergő Keve as the supervisor, declare that I have read and approved the thesis "*Molecular analysis of endoparasites from marine mammals from southern Spain including the Campo de Gibraltar*" of the student **Nicolas Specht** (Vth year student) and support his/her participation in the Scientific Student Conference of the University of Veterinary Medicine in 2023. Furthermore, I declare that the uploaded TDK thesis has been successfully checked for plagiarism and that any matches found comply with the University guidelines/rules.

Budapest, 2023. October 17th

.....

Supervisor

Appendix 5. Declaration regarding TDK research paper-thesis equivalence

DECLARATION

I hereby declare that the thesis entitled "**Molecular analysis of endoparasites from marine mammals from southern Spain including the Campo de Gibraltar**" is identical in terms of content and formal requirements to the TDK research paper submitted in 2023.

Date: Budapest, 2023. November 10th

Nicolas Specht

Thesis progress report for veterinary students

Name of student: NICOLAS SPECHT

Neptun code of the student: FW1950

Name and title of the supervisor: DR. GERGŐ KEVE ; PhD student, Veterinary scientist

Department: Dpt. of Parasitology and Zoology

Thesis title: Parasites of Marine Mammals and Turtles
MOLECULAR ANALYSIS OF ENDOPARASITES FROM MARINE MAMMALS FROM SOUTHERN SPAIN INCLUDING THE CAMPO DE GIBRALTAR

Consultation – 1st semester

	Timing			Topic / Remarks of the supervisor	Signature of the supervisor
	year	month	day		
1.	2022.	09.	20	Creation of a sampling protocol	
2.	2022.	09.	27	Basic methods of Thesis writing	
3.	2022.	10.	11.	Creation of sampling tubes + protozoology	
4.	2022.	10.	25.	Using Zotero	
5.	2022.	11.	13.	Protozoology	

Grade achieved at the end of the first semester: 5 (five)

Consultation – 2nd semester

	Timing			Topic / Remarks of the supervisor	Signature of the supervisor
	year	month	day		
1.	2023	03.	10	Online discussion	
2.	2023.	03.	22.	Online discussion	
3.	2023.	04.	22	Methodology discussion	
4.	2023.	05.	02.	Methodology discussion	

5.	2023	09.	13	TDK-dokumentum	

Grade achieved at the end of the second semester: 5 (jeles!)

The thesis meets the requirements of the Study and Examination Rules of the University and the Guide to Thesis Writing.

I accept the thesis and found suitable to defence,

.....
signature of the supervisor

Signature of the student: N. Spodh 15.11.2023

Signature of the secretary of the department: .....

Date of handing the thesis in..... .....