UNIVERSIDAD COMPLUTENSE DE MADRID

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Departamento de Sanidad Animal

TESIS DOCTORAL

Presence of parasites in pinnipeds from the Antarctic Peninsula

Presencia de parásitos en pinnípedos de la Península Antártica

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

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Madrid, 2013

COMPLUTENSE UNIVERSITY OF MADRID
VETERINARY FACULTY
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PRESENCE OF PARASITES IN PINNIPEDS FROM THE ANTARCTIC PENINSULA

CLAUDIA DEL CARMEN RENGIFO HERRERA

Doctoral Thesis. *Madrid, 2013*

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PRESENCIA DE PARÁSITOS EN PINNÍPEDOS DE LA PENÍNSULA ANTÁRTICA

TESIS DOCTORAL

Claudia del Carmen Rengifo Herrera

Madrid, 2013

Memoria presentada por Dña. Claudia del Carmen Rengifo Herrera para optar al grado de Doctor por la Universidad Complutense de Madrid.

Madrid, 14 de enero de 2013

Luis Miguel Ortega Mora, Doctor en Veterinaria y Catedrático de Universidad adscrito al Departamento de Sanidad Animal de la Facultad de Veterinaria de la Universidad Complutense de Madrid y **Susana Pedraza Díaz,** Doctora en Ciencias e Investigadora contratada en la Universidad Politécnica de Madrid,

CERTIFICAN:

Que la Tesis Doctoral titulada: **"Presencia de Parásitos en Pinnípedos de la Península Antártica"** que presenta la Licenciada en Veterinaria, **Dña. Claudia del Carmen Rengifo Herrera**, para optar al grado de Doctor por la Universidad Complutense de Madrid, ha sido realizada en las dependencias del Departamento de Sanidad Animal de la Facultad de Veterinaria de la Universidad Complutense de Madrid bajo su supervisión y cumple todas las condiciones exigidas para optar al grado de Doctor por la Universidad Complutense de Madrid.

De acuerdo con la normativa vigente, firmamos el presente certificado, autorizando su presentación como co-directores de la mencionada Tesis Doctoral.

En Madrid, a 14 de enero de dos mil trece.

Fdo. Prof. Dr. Luis Miguel Ortega Mora Fdo. Dra. Susana Pedraza Díaz

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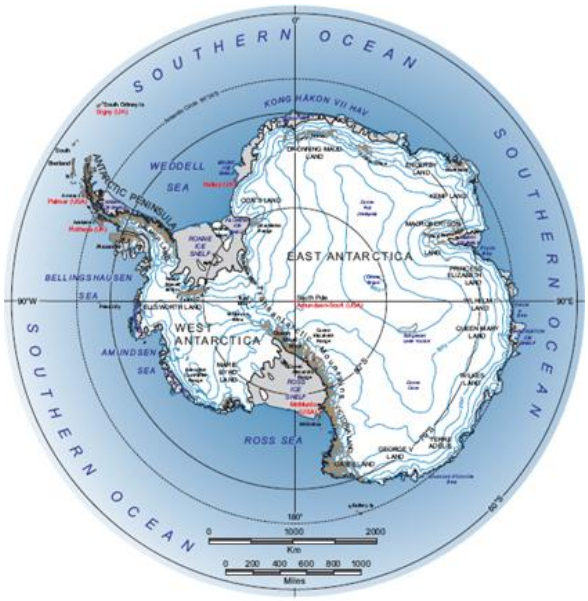
CHAPTER I

INTRODUCTION AND OBJECTIVES

I. INTRODUCTION

1. General characteristics of the Antarctic Environment

The Antarctic environment is the most isolated on Earth, possessing unique geographical and climatological characteristics (Kerry and Riddle, 2009). It comprises the south area of 60ºS, inclusive of the ice covered continent, isolated islands and a large part of the Southern Ocean. The continent is separated from South America by 1,000 km, Australia by 2,500 km and Africa by 4,000 km; along with three distinct morphological zones: East Antarctica, West Antarctica and the Antarctic Peninsula (Figure 1) (King and Turner, 1997; Shirihai, 2002). East Antarctica has the largest surface and is dominated by the high Antarctic plateau, which rises quickly inland of the coast with huge flat ice mass, with over 2 to 4 km in elevation. West Antarctica is more mountainous than East Antarctica, with an average elevation of 850 m and some areas reaching more than 2 km, with exposed peaks of buried mountains above 4 km. The Transantarctic Mountains separate East and West Antarctica and rise to a maximum elevation of 4,528 m (King and Turner, 1997).

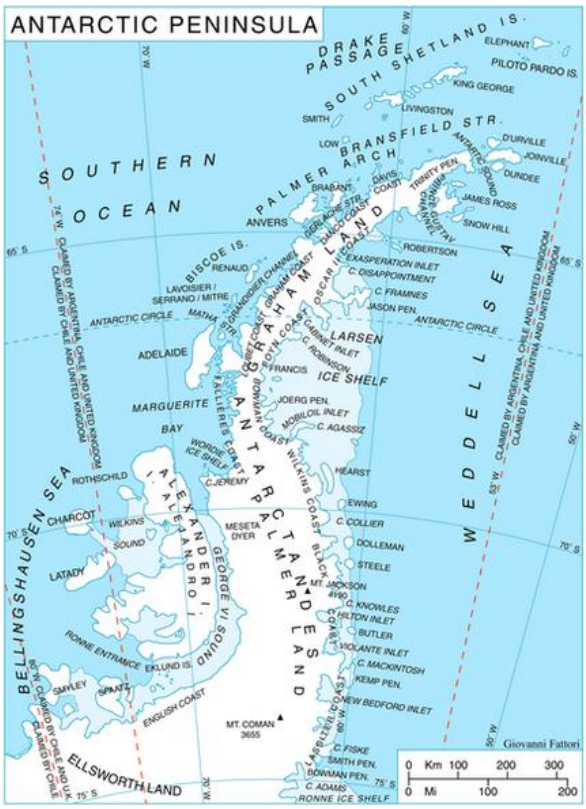


Source: Landsat Image Mosaic of Antarctica (LIMA)

Figure 1: Map of the Antarctic Region

The third noticeable area is the Antarctic Peninsula (Figure 2), which extends northwards from the main mass of the Antarctic continent. It is a narrow mountainous barrier of 70 km width and between 1,500 to over 3,000 m mean height, with

surrounding islands and archipelagos, mostly of volcanic origin. Within the archipelagos there are the South Shetland Islands, a chain of eleven major islands, several minor ones with islets, rocks; and volcanic matter. These are located 120 km north of the Antarctic Peninsula, in the Southern Ocean. The major islands are Elephant, Clarence, King George, Nelson, Robert, Greenwich, Livingston, Snow, Deception, Smith and Low (King and Turner, 1997). King George and Livingston are the largest islands of the group. The Peninsula with the Islands constitutes the warmest and certainly most accessible part of the Antarctic continent, yet it boasts a harsh climate (Shirihai, 2002).



Source: World Wildlife Fund

Figure 2: Map of Antarctic Peninsula

The Antarctic continent is ringed by a belt of sea ice, unlike the Arctic which is an ocean surrounded by continents. Most of the sea ice melts by late summer (February and March), and the edge retreats back, close to the shoreline of East Antarctica. In the depths of the continent´s winter (September), sea ice can reach almost 1,000 km offshore to north of the 60th parallel in open water (King and Turner, 1997; Shirihai, 2002).

The Southern Ocean surrounds the Antarctic continent and adds up to be a conglomeration of large parts of the South Pacific, South Atlantic and South Indian Oceans, covering about 9.6% of the surface area of the World Ocean (Shirihai, 2002; Aronson et al., 2011). Compared to the Arctic Ocean which has a considerable input of fresh water from continental runoff, the Southern Ocean has virtually no fresh water running off from the Antarctic continent (Aronson et al., 2011). It also has unique characteristics, like the physical barrier to water exchange created by the Antarctic Circumpolar Current and the related physiological barrier created by cold sea temperatures and salinity at high Southern latitudes. The Antarctic Circumpolar Current is one of the most powerful ocean currents on Earth. It has physically isolated the Southern Ocean from adjacent seas and protects it physiologically from some life thriving in warmer Sub Antarctic waters (Shirihai, 2002; Aronson et al., 2011).

Climate and weather are the defining characteristics of the Antarctic environment and to an extent the primary factors that set it apart from other regions of the world (Kerry and Riddle, 2009). The climate is strongly influenced by geography, the Earth´s orbital characteristics and the topography itself. The Earth´s rotation imposes limitations on the exposure to direct solar radiation ensuring one period of virtually continuous sunlight during summer, and subsequent polar night during the winter months. The summer in the Southern hemisphere occurs during the period when the distance between the Sun and Earth is at a minimum while the austral winter occurs when the Sun-Earth distance is at a maximum. For this reason, this region receives a greater share of solar emissions than the Arctic region during summer (Pook, 2009). The average air temperatures during summer range from near 0ºC around the coastal margin to about -40ºC on the plateau. In winter, mean coastal temperatures are generally within the range of -18 to -29ºC and mean temperatures on the plateau are around -68ºC. In contrast, monthly mean temperatures for the northern section of the Antarctic Peninsula exceed 0ºC in summer. In general, the combination of elevation and extreme cold in the Antarctic region results in one of the driest atmospheric conditions known on Earth (Pook, 2009).

All these characteristics provide isolation to the Antarctic environment, exerting an influence on Antarctic wildlife at different levels. Conditions such as temperature, humidity and solar radiation can influence the survival of hosts vectors and pathogens in the environment. It also affects every aspect of the living organisms that inhabit the

Antarctic region and defines the habitat in which the native species live, the range of conditions they must survive and the conditions that exotic wildlife, introduced pathogens and their vectors must withstand, if they are to establish and reproduce in this region (Pook, 2009).

2. Antarctic Human Activity

The earliest documented human history in the Antarctic region goes back to the seventeenth and eighteenth century when sailors gradually discovered the existence of the *Terra Australis.* Later, in the beginning of 1780´s, the exploitative industry characterised by sealing, whaling and fishing with a total lack of regulations were the major activity in the zone. The period after World War II (1945), may be considered the era of scientific activity in the Antarctic region, not only for the increment of research programmes, but also for the establishment of permanent stations.

The Antarctic tourism is a recent phenomenon, developed as an industry mainly from the 1950´s and 60´s. Today, several thousand tourists visit Antarctica annually. It is guided and controlled by the International Association of Antarctic Tour Operators (IAATO), created in 1991. This Association is a volunteer member organization that makes guidelines, procedures and regulations of touristic activities conducted in the Antarctic region, especially those related to environmental issues (Basberg, 2008). The tourism season runs from November to March, and is mainly shipbased. Passengers are taken ashore using small inflatable boats, generally for periods of 1-3 hours and they are generally focused on the Antarctic Peninsula, and in particular on a number of sites with wildlife, historical and scenic values (Mortimer and Prior, 2009). Until the early 1990´s some 60,000 people visited the Antarctic region as tourists. During the 1990´s and the following decades, touristic activities grew steadily. In the 2006/2007 season around 30,000 visitors made registered visits in tourist ships, from large cruise ships to small sailing yachts. However, some reduction has been observed the following years, probably due to saturation in market or reflecting the worldwide economic slow-down during these years (Figure 3) (Basberg, 2008; Mortimer and Prior, 2009). Still, the presence of tourists in the Antarctic region is quite marked.

In the present, scientific activity and tourism are the two main human activities allowed in Antarctica (Basberg, 2008). These frequent visits together with scientific activities in the region could potencially create a cumulative impact on the environment,

although there are policies and procedures established to minimize and mitigate environmental impacts (Mortimer and Prior, 2009).

3. Antarctic Treaty System

The Antarctic region is undoubtedly one of the least impacted sites of the planet. However, it may suffer from the human activities occurring both locally and elsewhere in the world. Since its discovery, natural resources have been exploited for commercial reasons, like fishing, sealing and whaling, including the introduction of alien species, and the long-term survival of a number of alien flora and fauna to the Sub Antarctic islands. Later, expeditions to and within the Antarctic region were driven for scientific endeavour (Jabour, 2009). Only recently, it has been formally recognised as a place worthy of very high standards of environmental protection (Riddle, 2009). The International Geophysical Year of 1957-58 (IGY) gave rise to the formulation of the Antarctic Treaty in 1959. The Treaty encouraged investigation in the Antarctic region and cooperation between nations to guarantee freedom to scientific research and exchange of data between members (Turner and Pendlebury, 2004). Initially, the Signatories of the Treaty included twelve countries: seven Antarctic territorial claimants (Argentina, Australia, Chile, France, New Zealand, Norway, United States and United Kingdom) and other countries, active during the IGY in the Antarctic region (Belgium, Japan, South Africa, and the former Union of Soviet Socialist Republics). They participated in the diplomatic conference in Washington (1959), where the Treaty was negotiated. There were twelve original parties who had the right to participate in the Consultative Meetings and are accordingly known as Consultative Parties. The Treaty provides for accession to any state which is a member of the United Nations, and any other state by invitation of all Consultative Parties. Nowadays the Antarctic Treaty has increased from 12 to 49 members (Austria, Belarus, Brazil, Bulgaria, Canada, China, Colombia, Cuba, Czech Republic, Denmark, Ecuador, Estonia, Finland, Germany, Greece, Guatemala, Hungary, India, Italy, Malaysia, Monaco, Netherlands, North Korea, Papua New Guinea, Peru, Poland, Portugal, Romania, Slovakia, South Korea, Spain, Sweden, Switzerland, Turkey, Ukraine, Uruguay, Venezuela, as well as the 12 initial parties), although only 26 countries are Consultative Parties (Jabour, 2009; Rothwell, 2009). The Antarctic Treaty is a very straight forward document comprising of only 14 articles. It combines some very basic measures dealing with the conduct of

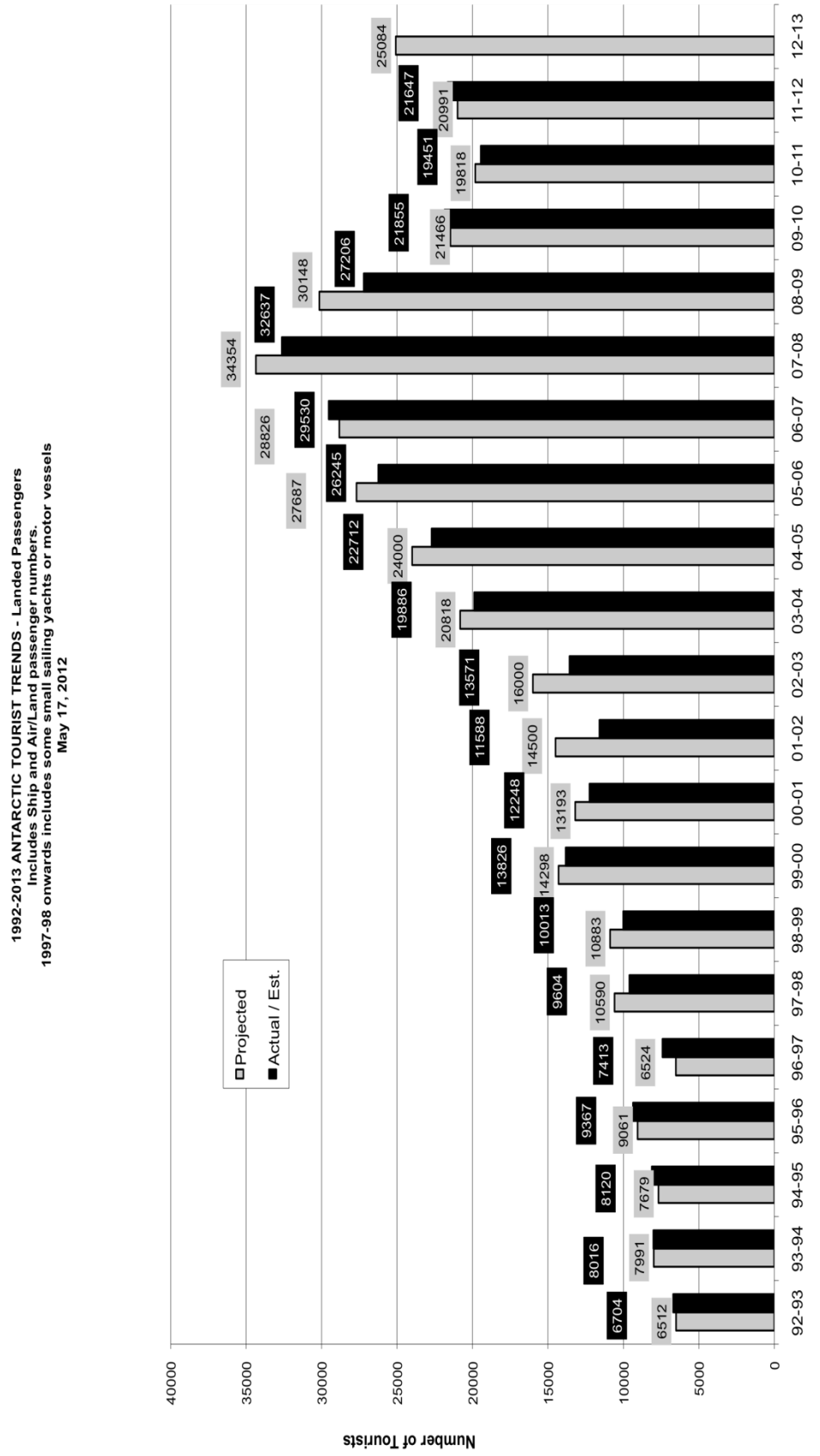
science, with some very sophisticated provisions with sovereignty and Treaty review (Rothwell, 2009).

The Antarctic Treaty System (ATS) is a whole complex of arrangements made for the purpose of coordinating relations among states with respect to the Antarctic region. Included are the Antarctic Treaty itself, Recommendations adopted at meetings of the Antarctic Treaty Parties, the protocol on Environmental Protection to the Antarctic Treaty (also referred to as the Madrid Protocol, 1991), and two separate Conventions for the Conservation of Antarctic Seals (CCAS, London 1972), and the Conservation of Antarctic Marine Living Resources (CCAMLR, Canberra 1980). It also includes the results of Meetings of Experts, the decisions of Special Consultative Meetings (ATCM´s) and, at a non-governmental level, reflects the work of the Scientific Committee on Antarctic Research (SCAR), established in 1958 on all aspects of the system. The ATS applies to the area south of 60º South Latitude, including all ice shelves and has drawn its attention to the protection of the Antarctic environment with relevance to the health of Antarctic wildlife (Rothwell, 2009).

As mentioned above, the Antarctic environment is considered the most pristine of the planet and one of the main focuses of the ATS has been to protect the Antarctic biota from the impact of increasing human activity. This is reflected in the XXIII Antarctic Treaty Consultative Meeting (1998), where the significant risk of the introduction of diseases into Antarctic wildlife species because of the increase in the numbers of people travelling to and within the Antarctic region was recognised. With regard to this topic, marine mammals such as pinnipeds have been described as prime sentinels of aquatic ecosystems because many species have long life spans, are long-term coastal residents and feed at a high trophic level. They provide an approach to evaluate the ecosystem health because they can be use as barometers for current or potential impacts on individuals and populations (Bossart, 2011).

4. Antarctic Pinnipeds

Pinnipeds have always been understood to represent a distinct group of aquatic mammals and the most conspicuous marine mammals in the Antarctic region (Shirihai, 2002; Berta, 2009).



Source: ATCM XXXV, 2012

Figure 3: Antarctic Tourist Trends 1992-2013

According to the taxonomic classification, they are divided in three families: Otariidae (eared seals), Phocidae (true seals) and Odobenidae (walruses). Currently, there are thirty-three living species distributed throughout the world; eighteen phocids (Table 1), fourteen otariids (Table 2) and one odobenid, represented by the walrus (*Odobenus rosmarus*), which is restricted to the Northern circumpolar waters (Riedman, 1990; Berta, 2009). They are marine mammals with unique anatomical and physiological adaptations to dive underwater, even though they spend considerable time on land and ice platforms.

Table 1: List of Pinnipeds of the Phocidae Family

Common Name	Scientific Name	Sub-species
	Sub-family Monachinae	
Lineage Monachini		
Hawaiian monk seal	*Monachus schauinslandi*	
Mediterranean monk seal	*Monachus monachus*	
Lineage Mirounginі		
Northern elephant seal	*Mirounga angustirostris*	
Southern elephant seal	*Mirounga leonina*	
Lineage Lobodontini		
Weddell seal	*Leptonychotes weddellii*	
Ross seal	*Ommatophoca rossii*	
Crabeater seal	*Lobodon carcinophagus*	
Leopard seal	*Hydrurga leptonyx*	
	Sub-family Phocinae	
Hooded seal	*Cystophora cristata*	
Bearded seal	*Erignathus barbatus*	
Grey seal	*Halichoerus grypus*	
Harp seal	*Phoca groenlandica (Pagophylus groenlandicus)*	
Ribbon seal	*Phoca fasciata*	
Largha (Spotted) seal	*Phoca largha*	
Caspian seal	*Phoca caspica*	
Baikal seal	*Phoca sibirica*	
Ringed seal	*Phoca hispida* (*Pusa hispida*)	*P.h. hispida* (Arctic Basin) *P.h. ocholensis* (Northern Japan) *P. h. botnica* (Baltic Sea) *P. h. ladogensis* (Lake Ladoga) *P. h. saimensis* (Lake Saimaa)
Harbour seal	*Phoca vitulina*	*P. v. vitulina* (eastern Atlantic) *P. v. richardsi* (eastern Pacific) *P. v. stejmegeri* (Kuril harbour seal) *P. v. concolor* (western Atlantic) *P. v. mellonae* (Seal Lake)

Source: Riedman, 1990

Table 2: List of Pinnipeds of the Otariidae Family

Common Name	Scientific Name	Sub-species
Sub-family Otariinae (Sea lions)		
Sea lion	*Zalophus californianus*	*Z. c. californianus* (California sea lion) *Z. c. wollerbaeki* (Galapagos sea lion) *Z. c. japonicus* (Japanese sea lion)
Northern or Steller sea lion	*Eumetopias jubatus*	
Southern sea lion (South American sea lion)	*Otaria byronia* (*O. flavescens*)	
Australian sea lion	*Neophoca cinerea*	
New Zealand sea lion	*Phocartos hookeri*	
Sub-family Arctocephalinae (Fur seals)		
Northern fur seal	*Callorhinus ursinus*	
Guadalupe fur seal	*Arctocephalus townsendi*	
Juan Fernandez fur seal	*Arctocephalus philippii*	
Galapagos fur seal	*Arctocephalus galapagoensis*	
South American fur seal	*Arctocephalus australis* (*A. gracilis*)	
New Zealand fur seal	*Arctocephalus forsteri*	
Antarctic fur seal	*Arctocephalus gazella*	
Sub Antarctic fur seal	*Arctocephalus tropicalis*	
	Arctocephalus pusillus	*A.p. pusillus* (South African /Brown fur seal) *A.p. doriferus* (Australian fur seal)

Source: Riedman, 1990

Phocids are characterised by their lack of visible ear pinnae and inability to turn the hindlimbs forward to support the body, resulting in a peculiar crawling locomotion on land but extremely efficiency on water. They inhabit both the Northern and Southern hemispheres, although they are largely restricted to polar and sub-polar regions (Berta, 2009). Comparing both polar populations, the Antarctic phocids generally have larger bodies, a characteristic that provides them with insulation against the cold above or under the frigid polar ice, and could be associated to the more plentiful food supply in the Southern Ocean (Riedman, 1990). Other phocids can survive in estuarine and freshwater habitats, like Caspian and Baikal seals. Molecular studies further support the division into two major sub-groups Monachinae and Phocinae. The Monachinae has three lineages: Monachini, Miroungini and Lobodontini. In contrast, the Phocinae includes differentiated species with extensive geographic distribution (Berta, 2009).

Otariids are characterised by the presence of external ear flaps or pinnae and ability to turn the hindflippers forward to walk on land. They are divided into two sub-families: Otariinae (sea lions) and Arctocephalinae (fur seals), although molecular studies have revealed that some species and sub-species of these two groups do not share

monophyletic lineage, but further taxonomic research are needed to elucidate this controversial subject (Berta and Churchill, 2011). They are called fur seals for the thick, dense fur, and except for the Northern and Guadalupe fur seals, they are located in the Southern hemisphere (Berta, 2009).

The global distribution patterns of pinnipeds reveal that certain species tend to be restricted to a particular region of the world, like those who live in the extreme Polar regions (Riedman, 1990). However, most of the Antarctic pinnipeds have been reported as vagrants from the Southern continents with certain predilection for the Antarctic region (McFarlane et al., 2009). In addition, fewer species inhabit the Antarctic than the Arctic, but the Antarctic populations are considerably larger (Riedman, 1990).

Antarctic phocids include the Crabeater seal (Figure 4), Ross seal (Figure 5), Leopard seal (Figure 6), Weddell seal (Figure 7) and the Southern elephant seal (Figure 8). The Crabeater seals are exclusively located on Antarctic pack ice south to 79ºS. They presumably migrate but movement patterns are still unknown. Occasionally, a select few reach the Sub Antarctic islands, New Zealand, Australia, South Africa, the Falklands and South America north to the Southeastern Brazilian coast. They feed mainly on krill, although they also forage opportunistically on mysids and fish (Shirihai, 2002; Kendall et al., 2003). The Ross seals are the least-known Antarctic pinniped. The distribution is circumscribed to ice packs in the Antarctic Ocean south to 78ºS, but they have also been recorded north to South Australia, Kerguelen and Heard Island. Evidence suggests Ross seals prefer heavy ice packs, providing substantiation of why so little is known about them. However, it is known they feed on krill, migrating squid and mid-water fishes (Shirihai, 2002). The Leopard seals are generally confined to the Antarctic pack, although they could be found widespread, yet uncommonly, in Antarctic and Sub Antarctic zones south to 78ºS, with some vagrancy occurring far north. Their broad diet includes krill, fish and seabirds especially penguins (Shirihai, 2002). The Weddell seals have a circumpolar distribution, breeding on both coastal pack and fast ice south to 78ºS, while making foraging trips up north with expanding ice pack during the winter. They can also reach South Orkney, South Shetland, South Georgia and South Sandwich, occasionally Sub Antarctic islands, with some wandering as far as New Zealand, South Australia and South America. Their diet is quite varied, mainly fish, cephalopods, krill and crustaceans. They feed occasionally in association with Crabeater seals, and there are records of penguins in their feeding habits (Shirihai,

2002). The Southern elephant seals are widely distributed in Southern hemisphere. They breed predominantly on Sub Antarctic islands but they are also found is the Antarctic Peninsula and Southern Argentina. Some bachelor males haul out to moult in summertime on the Antarctic continent. They have a varied diet, but mainly feed on fish and cephalopods (Shirihai, 2002).

Source: Saluvet Group

Figure 4: Crabeater seal (*Lobodon carcinophagus*)

Source: British Antarctic Survey

Figure 5: Ross seal (*Ommatophoca rossii*)

Source: Saluvet Group

Figure 6: Leopard seal (*Hydrurga leptonyx*)

Source: Saluvet Group

Figure 7: Weddell seal (*Leptonychotes weddellii*)

Source: Saluvet Group

Figure 8: Southern elephant seal (*Mirounga leonina*)

Antarctic otariids include the Antarctic fur seals (Figure 9), who breed from 61ºS to the Antarctic Convergence, forming colonies in Antarctic islands like South Georgia, South Orkney, South Shetland, South Sandwich, Bouvetoya, Marion, Kerguelen, Heard, McDonald and Macquarie; wandering in non-breeding season to Weddell Sea, Argentina coasts, and recorded recently in the south Pacific site of Chile (Acevedo et al., 2011). Females may migrate north of Antarctic Convergence. They feed on krill and fish, principally (Shirihai, 2002).

Source: Saluvet Group

Figure 9: Antarctic fur seal (*Arctocephalus gazella*)

The Antarctic Peninsula and the South Shetlands Islands have the most exciting wildlife on Earth and the most widespread are pinnipeds (Shirihai, 2002). Few time-series studies have been made in phocid and otariid populations from the Antarctic Peninsula. Some of them have gathered distribution and abundance data for Antarctic fur seals, Weddell seals and Crabeater seals throughout the South Shetland Islands and Antarctic Peninsula and some have focused on Deception Island in their surveys (Kendall et al., 2003). Antarctic fur seals and Southern elephant seals are both relatively ubiquitous in this area. Weddell seals are also frequently seen in many beaches and there are several areas where Crabeater seals can be encountered. Leopard seals are one of the least frequent phocid found in the Antarctic Peninsula and South Shetland Islands, but still they have several known sites. Ross seals are rare in dense pack ice in the Southern Peninsula region (Shirihai, 2002). In other studies, fur seals abundances and distributions have been documented at South Georgia Island, where the largest breeding population has been found (Kendall et al., 2003). In summary, Antarctic fur seals, Southern elephant seals, Weddell seals and Crabeater seals are all reasonably common in the South Shetland Islands (Shirihai, 2002).

5. Health status of Worldwide and Antarctic Pinnipeds

Research focused on marine mammals has resulted in a compilation of data, providing important information related to their health. In general, data have been collected from many sources including stranded animals, wild populations and animals in collection (Dailey, 2001). As a consequence of this fact, a large number of emerging

and remerging viral, bacterial, protozoal and fungal diseases have been reported. Additionally, complex diseases involving emerging infectious and neoplastic components have been reported, providing valuable data on aquatic ecosystem and public health (Bossart, 2011). They have also been widely used for neurotoxins and contaminant studies, especially anthropogenic and chemical pollutants in coastal areas that bio-accumulate in marine ecosystems, resulting in high tissue contaminant concentrations and decline of health in marine mammals, increasing the susceptibility to infectious diseases (Dailey, 2001; Bossart, 2011).

Toxins, neoplasia and viral, bacterial and parasitic diseases have been identified causing, or being associated with, significant morbidity and mortality in pinnipeds, especially in free-ranging populations. Additionally, mass mortality events have increasingly been observed and ascribed to infectious agents. In the Northern hemisphere, a mass mortality event was reported in harbour seals from the North Sea in 1988 (de Bruyn et al., 2008). Several vulnerable populations undergo high mortality, such as Caspian seals in the summer of 2000 (Kuiken et al., 2006) and Northern fur seals populations in St. Paul Island (Alaska, United States) from 1986 to 2006 (Spraker and Lander, 2010). In the Sub Antarctic region, mass mortality events affected New Zealand sea lions populations in 1998 (de Bruyn et al., 2008) and Sub Antarctic fur seals from Marion Island in 2007 (de Bruyn et al., 2008).

Within the infectious agents causing diseases, parasites have been known to cause health problems. They are also integral components of marine ecosystems, representing a valuable tool to explore the origins, distribution and maintenance of biodiversity (Dailey, 2001; Hoberg and Klassen, 2002). However, whereas the information on diseases caused by parasites in worldwide pinnipeds is substantial, in the Antarctic and the Sub Antarctic regions is sparse and limited.

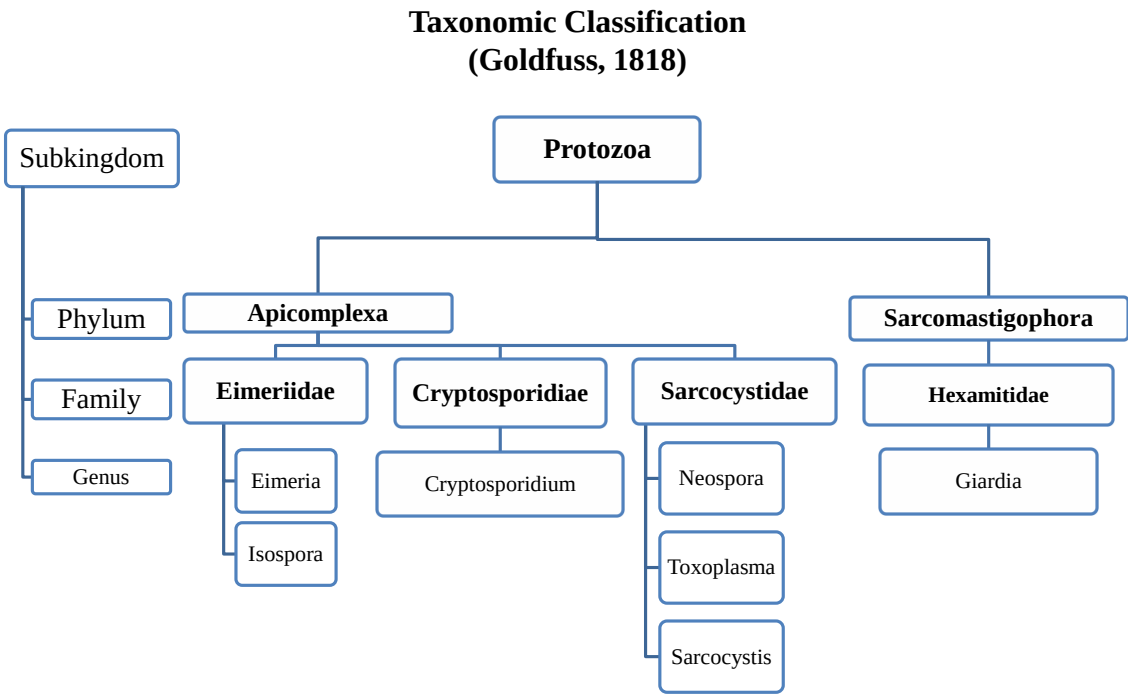
6. Presence of Parasites in Worldwide and Antarctic Pinnipeds

Parasites are organisms found in and on all animals of interest in Veterinary Medicine. They may or may not produce clinical disease, depending on a variety of environmental, ecological, immnunological and physiological factors that influence the host-parasite relationship. However, this relationship is constantly changing and different manifestations of disease are observed. For some hosts the presence of a parasite can cause illness and probably be lethal, while others are well adapted to the

parasite and no clinical signs are present (Foreyt, 2001). Large groups of parasites have been identified in captive and free ranging pinnipeds (Dailey, 2001). They have been found in incidental findings during necropsies, routine physical examination or causing major health problems and death in affected hosts.

6.1. Protozoan Parasites

Protozoa is a large group of primitive, unicellular, eukaryotic organisms with complex structures contained in a single cell. The classification is extremely complex but is intended to give an outline of the basic differences in the structure and life cycles of the main groups (Taylor et al., 2007). The most important genera recorded in pinnipeds are included in two Phyla, Apicomplexa and Sarcomastigophora (Figure 10) (McFarlane et al., 2009).



Source: Taylor et al., 2007

Figure 10: Taxonomic classification of Protozoans of relevance in pinniped populations

6.1.1. Apicomplexa

Apicomplexa is a group of parasites occurring intracellularly in host cells, having an apical complex at some stage of their development. Reproduction involves both asexual and sexual phases (Taylor et al., 2007). Some genera have complex life cycles requiring two different hosts for their development, like *Neospora*, *Toxoplasma* and

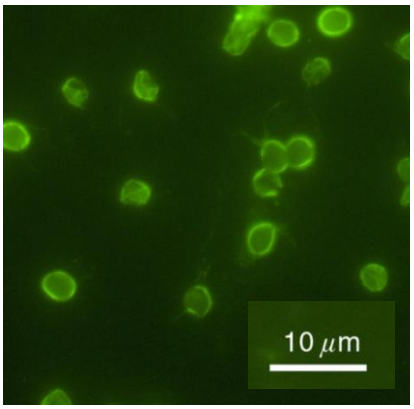
Sarcocystis. Others require only a single host to complete the life cycle, such as *Cryptosporidium*, *Eimeria* and *Isospora* (Yaeger, 1996). Some of these parasites, like *Cryptosporidium* and *Toxoplasma* are considered zoonotic agents with worldwide distribution, affecting a wide range of animals, including domestic and wild species and humans. They are also associated with outbreaks of infection resulting from drinking contaminated surface water or food (Fayer et al., 2004). In recent years, increasing interest has been carried out on marine mammals since they may act as indicator species for environmental contamination with these parasites (Appelbee et al., 2005).

6.1.1.1. Enteric Apicomplexan Parasites

6.1.1.1.1. *Cryptosporidium*

Cryptosporidium is a ubiquitous gastrointestinal parasite reported in a wide variety of hosts, including humans and vertebrate animals (Appelbee et al., 2005; Fayer, 2010). Currently, this genus contains up to 22 species and over 40 genotypes (Fayer, 2010; Fayer et al., 2010; Robinson et al., 2010; Ren et al., 2012). More than 150 mammalian hosts have been reported to be infected with this parasite (Fayer, 2004a). Most species appear to have some host specificity and surveys conducted in several groups of animals have shown that most hosts have been infected with only a few host-adapted *Cryptosporidium* species or genotypes, indicating that cross transmission is usually limited (Fayer, 2004a; Xiao and Fayer, 2008). However, cross-species transmission is possible when animals share a similar habitat and/or the parasite is biologically capable of infecting multiple hosts (Xiao and Fayer, 2008). *Cryptosporidium* completes its life cycle in the gastrointestinal tract of a single host and is generally associated with severe diarrheal disease (Deng et al., 2000; Fayer, 2004a). Oocysts (Figure 11) are transmitted by the faecal-oral route and remain infectious in cool wet conditions for 6 months or longer (Fayer et al., 2004; Xiao and Fayer, 2008). They can also survive in seawater for a long period of time (Fayer et al., 2004). After ingestion, sporozoites are released in the small intestine and invade epithelial cells. All subsequent endogenous stages are intracellular but extracytoplasmic, appearing to rest on the surface of villar epithelial cells. Two asexual cycles occur, each producing four to eight merozoites. Second stage merozoites develop into macrogametocyte and microgametocyte and fertilization results in oocyst formation. Sporulation takes place within the host, becoming immediately infective and might auto-infect but most oocysts are excreted in the faeces (Xiao and

Fayer, 2008). As mentioned above, watery diarrhea is the typical clinical sign of infection observed in affected individuals. However, asymptomatic infections have been also reported in animals and humans. Rarely, other clinical signs are present and severe cases can result in mortality. No specific virulence factors have been observed to cause direct or indirect damage to host tissue like the loss of absorptive epithelium including apoptosis and villus atrophy resulting in malabsorption; and the release of inflammatory cell mediators stimulating electrolyte secretion and diarrhea (Fayer, 2004a).



Source: Saluvet Group

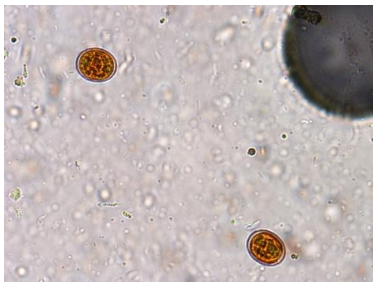
Figure 11: Immunofluorescence staining of *Cryptosporidium* oocysts (100X)

Cryptosporidium sp. has been described in worldwide pinnipeds, yet little is known about the prevalence or the role in the transmission in these hosts (Deng et al., 2000). It has been detected in California sea lions from Arctic Canada and ringed seals from both Arctic Canada and North Alaska, United States (Fayer et al., 2004; Hughes-Hanks et al., 2005). In ringed seals, prevalence observed were 20% (n=15) in Arctic Canada and 22.6% (n=31) in North Alaska (Hughes-Hanks et al., 2005). In California sea lions from Arctic Canada, prevalence observed was 50% (n=6) (Deng et al., 2000). It has been also described in 18.2% (n=55) of ringed seals from Canada (Appelbee et al., 2005; Santin et al., 2005; Dixon et al., 2008), 6.25% (n=176) of harbour seals and 100% (n=1) of hooded seals from the United States. Likewise, it has been found in 4.2% (n=24) of harp seals from the United States (Bass et al., 2012).

6.1.1.1.2. *Eimeria* and *Isospora*

Eimeria and *Isospora* are two gastrointestinal parasites, comprising a large number of species (1000 and 200, respectively), some of them capable of significant morbidity and mortality in infected hosts, like domestic mammals and birds. The life cycle is also

completed in a single host. The genera are differentiated on the basis of the number of sporocysts in each oocyst and the number of sporozoites in each sporocyst. *Eimeria* oocysts (Figure 12) contain four sporocysts, each with two sporozoites while *Isospora* oocysts contain two sporocysts each with four sporozoites. Host-specificity varies according to the species. The asexual stage starts when sporozoites are released from oocysts and sporocysts, invading the intestine cells and replicates, affecting the lamina propria of the small and large intestine by destruction of epithelial cells. The process repeats one to four times, depending upon the species involved. Eventually, the sporozoites become schizonts and later merozoites. Finally, merozoites develop into macrogametocyte and microgametocyte and the sexual stage begins when zygotes became oocysts. Oocysts are unsporulated when passed in the faeces and require a period of development in the environment to become infective (Taylor et al., 2007).



Source: Saluvet Group

Figure 12: *Eimeria*-like oocysts (40X)

Coccidiosis is the typical disease in animals and particularly affects immunologically naïve young animals exposed to a high level of infection. The clinical signs are mainly characterised by diarrhea, accompanied with mucus and blood. Some pathological changes are fibrohaemorrhagic enterocolitis, with large numbers of coccidial organisms affecting the superficial mucosa. Central nervous system signs have been associated with infections of *Eimeria* species in domestic cattle. In wild mammals, oocysts can be present in the faeces without showing obvious signs of illness, although some cases of morbidity and mortality from coccidial infection have been reported (Van Bolhuis et al., 2007).

Pathologies in marine mammals have been reported, such as fatal and self-limiting enterocolitis associated with *E. phocae* infection in captive harbour seals from the Netherlands, showing neurological signs and both sexual and asexual stages in affected intestines (Van Bolhuis et al., 2007; Colegrove et al., 2011). As well, *E. phocae* have

been described due to morphologic features in captive clinical ill western Atlantic harbour seals from the United States (Hsu et al., 1974a; Hsu et al., 1974b; Fowler and Miller, 2003). Coccidian oocysts not fully described have also been found in captured harbour seal pups with fatal diarrhea from Scotland and the United States (Dailey, 2001).

Molecular studies revealed three new enteric cyst-forming parasites named coccidian parasites A, B and C in California sea lions from the United States (Colegrove et al., 2011). Coccidian parasite C has also been described affecting a harbour seal (Colegrove et al., 2011; Gibson et al., 2011). Coccidians identified as Coccidia A and B have been detected in asymptomatic California sea lions stranded in the United States coasts (Carlson-Bremer et al., 2012). Both pinnipeds have been considered definitive hosts due to the observation of sexual and asexual stages in the gastrointestinal tract, causing enteritis (Colegrove et al., 2011; Gibson et al., 2011). In Antarctic pinnipeds, coccidian schizonts have been described in Antarctic fur seals (McFarlane et al., 2009). Also, in King George Island six morphologically different oocysts have been found with marked difference compared to *E. phocae* (Drozdz, 1987). Oocysts found in Southern elephant seals have been described as the new species *Isospora miroungae*. In Weddell seals, the new species *Eimeria weddelli* and *Eimeria arctowski* have been also described. As well, three species of *Eimeria* occurred rarely and have been described as *Eimeria* sp. 1, in Crabeater seals, *Eimeria* sp. 2 and *Eimeria* sp. 3 in Weddell seals (Drozdz, 1987; McFarlane et al., 2009). It was suggested that these parasites do not affect the pinniped´s health, unless the animal is stressed or immunocompromised (Dailey, 2001).

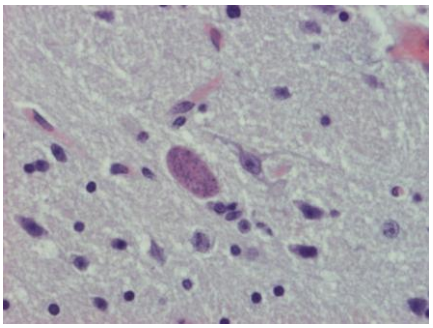
6.1.1.2. Systemic Apicomplexan Parasites

Toxoplasma, Neospora and *Sarcocystis* are three apicomplexa protozoans whose life cycle involves two hosts. The asexual stage occurs in an intermediate host and the sexual stage in the gastrointestinal tract of the final host. The significance is due to the asexual stage in intermediate hosts, affecting them systemically (Taylor et al., 2007).

6.1.1.2.1. *Toxoplasma*

The genus *Toxoplasma* has a single species, *Toxoplasma gondii*. Definitive hosts are domestic and wild felids and show a complete lack of species-specificity in the

intermediate host, being capable to infect any warm-blooded animal including humans and therefore are considered an important zoonosis (Jones and Dubey, 2010). In most intermediate hosts, infection is usually acquired via the digestive tract and disseminated by the lymphatic and portal systems with subsequent invasion of various organs and tissues. There are three stages of *T. gondii* infective to all hosts: tachyzoites, bradyzoites and sporozoites (Dubey, 1998). Tachyzoites enter the host cell of the intermediate and definitive hosts and multiply asexually until the host cell ruptures. Tachyzoites give then rise to tissue cyst (Figure 13) containing bradyzoites. Upon ingestion by definitive hosts, bradyzoites are released and multiply as tachyzoites, invading intestinal cells. Asexual and sexual stages occur in the lamina propria of the intestines. The sexual stage starts two days after ingestion of tissue cysts and finally oocysts form and mature (Jones and Dubey, 2010). Unsporulated oocysts are then passed in the faeces of the definitive host to the environment where sporulation occurs upon certain optimal conditions. (Taylor et al., 2007). Oocysts are very resistant in the environment, and the findings of *T. gondii* in marine mammals suggest the possibility of contamination with oocysts shed by felids and widespread by rainfall and wastewater outfalls to the marine environment (Jones and Dubey, 2010). *Toxoplasma* is also recognised as an important pathogen of marine mammals, causing morbidity and mortality (Lindsay and Dubey, 2009). Pathogenic effects are always related to the extra-intestinal phase of development in the central nervous system, although it may also affect other organs as well and in some cases can remain asymptomatic. *T. gondii* can also be transmitted transplacentally and by carnivorism (Jones and Dubey, 2010).



Source: Saluvet Group

Figure 13: *Toxoplasma gondii* cyst in brain tissue (100X)

T. gondii has been described in eastern Pacific harbour seals, associated to encephalitis in Northern fur seals and Northern elephant seals, and to disseminated

organ infection in California sea lions (Dailey, 2001; Dubey et al., 2004). Van Pelt and Dietrich (1973) reported how a hand-fed newborn harbour seal was affected with toxoplasmosis (Fayer et al., 2004). In this case report in particular, one of the probable routes of infection for this newborn seal was the transplacental transmission from the infected dam, exacerbated by the debilitating effect of other concurrent infections (Van Pelt and Dietrich, 1973).

Necropsy and histological findings have revealed pathological lesions in affected pinnipeds. Tachyzoites have been observed in a Hawaiian monk seal with visceral and cerebral lesions (Honnold et al., 2005). Colegrove et al. (2011) considered pinnipeds like Hawaiian monk seals, Northern elephant seals, Northern fur seals, harbour seals and California sea lions, intermediate hosts but still little is known about this subject. Experimentally, grey seals fed with *T. gondii* oocysts developed infection (Figure 11), but how marine mammals become infected in nature has not been well described (Fayer et al., 2004; Gajadhar et al., 2004).

T. gondii antibodies have been detected in several groups of marine mammals. In general terms, the presence of antibodies in a host does not necessarily mean that clinical signs will develop, but it does indicate that it may have been exposed to the agent in the recent past (Kerry and Riddle, 2009; McFarlane et al., 2009). Pinnipeds from the east coast of Canada, including hooded seals, grey seals and harbour seals have been tested, showing prevalence of 9% (n=122) in grey seals and harbour seals (n=34) and 1.7% (n=60) in hooded seals (Measures et al., 2004). In Hawaiian monk seals, prevalence has been 0.006% (n=332) in populations from Northwestern Hawaiian Islands (Aguirre et al., 2007). Several pinniped populations along the Pacific and Atlantic coasts of the United States have been also tested and prevalence observed was 16% (n=311) in harbour seals, 42% (n=45) in California sea lions, 16% (n=32) in ringed seals, 50% (n=8) in bearded seals and 11.1% (n=9) in spotted seals (Dubey et al., 2003). In the Northwest Pacific site of the United States, prevalence in harbour seals was 7.6% (n=380) (Lambourn et al., 2001). European pinnipeds have also been evaluated, showing prevalence of 23.4% (n=47) in grey seals and 5.4% (n=56) in eastern Atlantic harbour seals from the Atlantic coasts of United Kingdom and France (Cabezon et al., 2011). In Japan, prevalence observed was 4% (n=322) in Kuril harbour seals from different populations around Hokkaido (Fujii et al., 2007). All these descriptions reveal a worldwide distribution and natural exposure of the parasite in marine ecosystems.

6.1.1.2.2. *Sarcocystis*

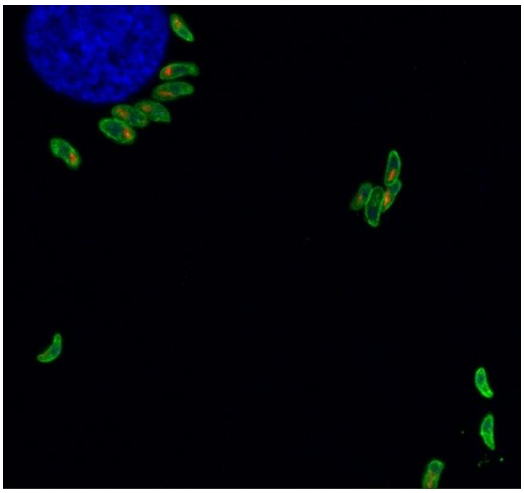
Sarcocystis sp. is one of the most prevalent livestock parasite, infecting mammals, birds, lower vertebrates and humans (Taylor et al., 2007). It generally has a two-host predator-prey life cycle with an asexual cycle in herbivores and omnivores and a sexual cycle in carnivores (Rohde, 2005). *Sarcocystis* derives the name from the intramuscular cyst stage (sarcocyst) present in the intermediate host and about 130 species have been described. Sporozoites excyst from sporocysts and invade the intestinal mucosa to reach endothelial cells in small arteries throughout the body. The asexual cycle begins with the initial reproduction form of schizonts. Schizonts with merozoites are released and go to muscle tissues, forming sarcocysts. They continue to divide until numerous mature infective bradyzoites are formed inside sarcocysts. When tissues containing sarcocysts with infective bradyzoites are consumed by the definitve host, bradyzoites are released and penetrate the cells of the intestinal lamina propria transforming into macrogametes and microgametocytes in the cell. The microgametocytes produce flagellated microgametes, which penetrate the macrogametes, forming oocysts containing sporocysts (Fayer, 2004b). When the newly created oocysts sporulate, often rupture releasing infective sporocysts. The definitive host sheds both sporocysts and sporulated oocysts in faeces. *Sarcocystis* infection in the intermediate host is usually asymptomatic and generally specific (Taylor et al., 2007). Sarcocysts have been found located in all strained muscles and, to a lesser extent, smooth muscle, neural tissue and spinal cord causing neurological disorders (Yantis et al., 2003; Fayer, 2004b).

Marine mammals are considered to be aberrant hosts because only schizonts have been described (Yantis et al., 2003). They have been reported to infect pinnipeds; yet no sexual stages have been described in these animals. Mense et al. (1992) reported the identification of *Sarcocystis* sp. in the liver and skeletal muscle of California sea lions. *Sarcocystis* spp. infections have also been reported in Pacific harbour seals, California sea lions, bearded seals, ringed seals, Northern fur seals and Leopard seals (Dailey, 2001; Colegrove et al., 2011). *Sarcocystis neurona* and *S. canis*-like are two related species reported in a captive Hawaiian monk seal (Yantis et al., 2003). *S. neurona* was found confined to the central nervous system and liver, producing fatal hepatitis, including random hepatic necrosis and cholestasis (Yantis et al., 2003). Similarly, encephalitis caused by *S. neurona* in captive and stranded Pacific harbour seals have been documented (Miller et al., 2001; Dubey et al., 2003; Mylniczenko et al., 2008).

Sarcocystis richardi have been reported encysted in a harbour seal diaphragm (Dailey, 2001). In Antarctic pinnipeds, *Sarcocystis richardi* and *S. hydrurgae* have been reported in Antarctic fur seals and Leopard seals, respectively (Dailey, 2001; McFarlane et al., 2009).

6.1.1.2.3. *Neospora*

Neospora caninum is a protozoan parasite morphologically similar to *T. gondii* although biologically different. Neosporosis is primarily a disease of cattle and dogs and is not considered zoonotic like Toxoplasmosis. Canids are considered the definitive host and include the domestic dog (*Canis domesticus*), coyotes (*Canis latrans*), Australian dingo (*Canis lupus dingo)*, and recently described gray wolf (*Canis lupus*); and ruminants are intermediate hosts (Dubey et al., 2007; Dubey et al., 2011). In general, the life cycle is very similar to *T. gondii*. It is typified by the three known infectious stages: tachyzoites (Figure 14), bradyzoites in tissue cysts, and sporozoites into the oocyst. Tachyzoites and tissue cysts occur intracellularly and are found primarily in the central nervous system. The environmentally resistant stage of the parasite, the oocyst, is excreted in the faeces of definitive hosts, sporulating in the environment. Nothing is known about the survival of *N. caninum* oocysts, but its close relationship with *T. gondii*, opens the opportunity to assume that environmental resistance can be similar (Dubey et al., 2007). Lesions have only been observed in the intermediate and definitive hosts, and these have been neuromuscular disease in canids and high rates of abortion in cattle (Gondim, 2006).



Source: Saluvet Group

Figure 14: Immunofluorescence staining of *Neospora caninum* taquizoites (100X)

Surveys indicate that a wide range of domestic and wild animals have been exposed to *N. caninum* (Dubey and Schares, 2011). However, the isolation of the parasite has not been described in marine mammals, although antibodies against *N. caninum* have been detected, indicating the exposure to this parasite. Dubey et al. (2003) detected antibodies in marine mammals along the coast of the United States. Prevalence observed in pinnipeds have been 3.5% (n=311) in harbour seals, 3.7% (n=27) in California sea lions, 12.5% (n=32) in ringed seals and 12.5% (n=8) in bearded seals. Also, serological detection have been reported in Kuril harbour seals and spotted seals from Japan with prevalence of 4.03% (n=322) and 4.34% (n=46), respectively (Fujii et al., 2007). These findings suggest that pinnipeds can be intermediate hosts for *N. caninum.* However, further studies are needed to confirm the role of marine mammals in the biology of the parasite. If marine mammals are confirmed to be intermediate hosts, many fundamental questions will have to be addressed regarding the transmission through the oceans (Gondim, 2006).

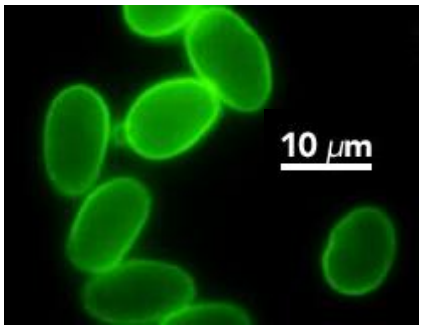
Polyparasitism commonly occur in human and wildlife populations and has been found to influence in the severity of diseases. Concurrent detection of *T. gondii, S. neurona* and *N. caninum* has been reported in 91% (n=161) of pinnipeds with clinical signs including Northern elephant seals, California sea lion, harbour seals, Guadalupe fur seals and Steller sea lions from California and Pacific Northwest sites of the United States and Canada. These three parasites share routes of transmission, like the faecal-oral and transplacental routes, and probably the transmission dynamics have been the same in these cases (Gibson et al., 2011).

6.1.2. Sarcomastigophora

6.1.2.1. *Giardia*

Within the phylum Sarcomastigophora, *Giardia* is the most relevant enteric protozoan in marine mammals. This parasite is unique in possessing a large adhesive disc on the flat ventral surface of the body, facilitating the attachment to the epithelial cells in the intestinal mucosa (Taylor et al., 2007). The life cycle starts with the infection by ingestion of cysts, followed by excystation and colonization of the small intestine by the trophozoite forms, which multiplies by vegetative growth in the intestine. Trophozoites undergo dramatic biological changes to differentiate into a resistant cyst, in a process called encystation. The cyst (Figure 15) is the resistant stage,

capable to survive outside the intestines (Bernander et al., 2001). Six species of *Giardia* are currently recognised and some have been found to infect several groups of animals but not humans (Xiao and Fayer, 2008). Of these species, *G. duodenalis* is the worldwide distributed parasite associated with outbreaks of infection resulting from drinking contaminated surface water or food (Fayer et al., 2004). It comprises a complex of genotypes or assemblages, based on host specificity and the analysis of conserved genetic loci. Currently, there are seven well defined assemblages, designated A through G (Feng and Xiao, 2011). In addition, an assemblage H has been recently described in pinnipeds (Lasek-Nesselquist et al., 2010). Assemblages A and B have the broadest host specificity and are considered zoonotics transmited by the faecal-oral route, causing cronic diarrhea in humans and other livestock and wildlife mammals (Xiao and Fayer, 2008).



Source: Saluvet Group

Figure 15: Immunofluorescence staining of *Giardia* cysts (100X)

Giardia cysts have been detected in ringed seals from western Arctic Canada, 20% (n=15), and North Alaska, 64% (n=31) (Olson et al., 1997; Fayer et al., 2004; Hughes-Hanks et al., 2005). In California sea lions from the United States, cysts were found in the faeces of 17% (n=6) of the pinnipeds sampled, (Deng et al., 2000). It has also been reported in 27% (n=74) of phocids, including grey, harp and harbour seals from Canada (Measures and Olson, 1999). Similarly, prevalence in grey seals, eastern Pacific harbour seals and eastern Atlantic harbour seals from the East and West coasts of the United States were 63% (n=27), 37.5% (n=8) and 4.5%, (n=112), respectively (Lasek-Nesselquist et al., 2010).

Molecular techniques have been used to describe Assamblage A of *G. duodenalis* in harp and hooded seals from Canada (Appelbee et al., 2005; Appelbee et al., 2010), as well as in eastern Atlantic harbour seals and grey seals from the East and West coast of

the United States (Lasek-Nesselquist et al., 2010). Similarly, Assemblage B has been described in ringed seals from Canada, eastern Pacific harbour seals, eastern Atlantic harbour seals and grey seals from the East and West coasts of the United States (Dixon et al., 2008). A novel *G. duodenalis* sequence (HS-1), and *G. duodenalis* canine genotype C and D have been identified in eastern Pacific harbour seals from Washington coast (Gaydos et al., 2008). This novel HS-1 genotype shared similarity with Assemblage H haplotype of grey seals from the East coast of the United States suggesting more genetic diversity, and perhaps a larger host range than previously believed (Lasek-Nesselquist et al., 2010).

6.2. Helminth Parasites

Helminths are multicellular eukaryotic organisms capable of causing a wide variety of infectious diseases in terrestrial and aquatic hosts. Taxonomically, the phylum has several groups of parasitic significance, including nematodes or roundworms, trematodes or flatworms and cestodes or tapeworms (Taylor et al., 2007). They are endoparasites and most of them use intermediate and paratenic hosts in immature and larval forms to complete the complex life cycle and ensure infection in the marine environment, involving several marine invertebrates, fishes and marine mammals (Mattiucci et al., 2005; McFarlane et al., 2009). Research in helminth taxonomy has illustrated the complexities of host specificity and revealed cryptic species infecting marine mammals (McFarlane et al., 2009).

6.2.1. Nematodes

Nematodes (Figure 16) are commonly called roundworms due to the appearance in cross-section (Taylor et al., 2007). The roundworms make up the largest number of parasites in marine mammals, serving as definitive hosts together with fish eating birds in the marine environment. In addition, crustaceans usually serve as first intermediate hosts and fish, squids and other invertebrates as intermediate or paratenic hosts. (Rohde, 2005; Mattiucci et al., 2008).

Source: Saluvet Group

Figure 16: Nematodes found in a faecal sample

6.2.1.1. Anisakids

Anisakids are parasites of the alimentary tract with cosmopolitan distribution and commonly found in marine mammals causing illness but rarely death (Mattiucci et al., 2008). Sometimes, despite the high intensity in the gastrointestinal tract, no apparent ill effects have been described (Rohde, 2005). In humans, clinical signs have been observed, becoming accidental hosts and therefore considered zoonotic agents (Hwang et al., 2012). They display an indirect life cycle in aquatic ecosystems and involve several hosts at different levels in food webs (Mattiucci et al., 2008). In general, the life cycle begins when eggs from female worms are released from the marine mammal host and passed out with the faeces into the seawater. Like all nematodes, Anisakids exist in four different larval stages before reaching maturity. The L1 larval form develops inside the egg. The L2 hatches from the egg in the ocean and becomes free swimming. The L2 molts and is then ingested by crustaceans (first intermediate host) where they mature to a L3 stage, tipically infective to hosts. Subsequently, the infected crustaceans are eaten by fishes and squids, becoming the paratenic hosts, and larvae migrate to the muscle tissues. After consumption by the definitive host, it matures to an adult (Klimpel et al., 2004; Rohde, 2005). These parasites are found free within the stomach or attached to the gastric mucosa, leading to ulcers, perforation into the abdominal cavity, and gastritis which may have an allergic component (Geraci and St Aubin, 1987). During necropsies,

mixed infections of Anisakids species have been found and findings include melena stool associated with anaemia (Banish and Gilmartin, 1992; Siebert et al., 2007; Byard et al., 2010; Papadopoulos et al., 2010). Findings also reveal that the intensity of Anisakids infections in the Antarctic and Sub Antarctic populations tend to be higher than the Arctic and Sub Arctic, presumably as the result of the lower degree of habitat disturbance in less stressed areas (Mattiucci and Nascetti, 2007).

6.2.1.1.1. *Anisakis*

Anisakis spp. have a worldwide distribution and infections are very common in marine mammals, although do not seem to affect their lifespan (Klimpel et al., 2004). They have been described in 0.3% (n=257) of ringed seals in Norway and New Zealand fur seals from South Australia. Eggs have been found in Hawaiian monk seals from Northwestern Hawaiian Islands (Byard et al., 2010). Larvae and adults stages have been identified in sub-adults and adults Northern fur seals and Steller sea lions from Alaska, and Northern elephant seals from California (Nadler et al., 2005; Spraker and Lander, 2010). *Anisakis pegreffii* (also named as *Stomachus similis*) has been identified in Mediterranean monk seals and Leopard seals (Dailey, 2001; Papadopoulos et al., 2010). In the Caspian sea, *Anisakis schupakovi* has been identified in Caspian seals (Kuiken et al., 2006). In Antarctic and Sub Antarctic pinnipeds, *Anisakis* infections have also been recorded in Weddell seals, where high gastrointestinal burdens have been considered normal (McFarlane et al., 2009). In addition, *Anisakis simplex C* has been described in Southern elephant seals (Mattiucci and Nascetti, 2007) and larval and adult stages of *Anisakis simplex* in Antarctic otariids (McFarlane et al., 2009).

6.2.1.1.2. *Contracaecum*

Contracaecum spp. are anisakids with mild morphological differences compared to *Anisakis* spp. They also have worldwide distribution and infections are very common in marine mammals and fish eating birds (Mattiucci et al., 2008). Adult stages have been described in California sea lions from the United States, Mediterranian monk seals from Greece and Southern elephant seals from the Argentinian coast (Mattiucci et al., 2003; Nadler et al., 2005; Papadopoulos et al., 2010). Within the *Contracaecum* species, *C. turgidum* has been identified in Hawaiian monk seals, showing a prevalence of 29.1% (n=282) in populations from Hawaiian Islands (Banish and Gilmartin, 1992; Reif et al.,

2006); and *C. margolisi* in California sea lions from Canada and Northeast Pacific Ocean (Nadler et al., 2005; Mattiucci and Nascetti, 2007). Other species described in Antarctic and Sub Antarctic pinnipeds include *C. mirounga* in Southern elephant seals from the Weddell sea, King George island and the Argentinian coast; and *C. radiatum* in Leopard seals and Weddell seals from the Weddell and the Ross seas (Mattiucci et al., 2003; Nadler et al., 2005; Mattiucci and Nascetti, 2007; McFarlane et al., 2009).

Contracaecum ogmorhini complex is a further group of *Contracaecum* species identified in otariids from Northern and Southern hemispheres, such as California sea lions from Canada and different otariid species from New Zealand, South Africa and Argentina (Mattiucci et al., 2003). Species found within this complex have been summarised in Table 3. In addition, a Southern elephant seal from the coast of Argentina hosted *C. ogmorhini* sensu stricto in mixed infection with *C. mirounga*, and a species not yet described, genetically related to *C. osculatum B* (Nadler et al., 2000b; Mattiucci et al., 2003; Nadler et al., 2005).

Table 3: List of *Contracaecum ogmorhini* complex in Northern and Southern Pinnipeds

Species	Host	Location
C. ogmorhini s. s.	Brown fur seal	New Zealand
	Brown fur seal	South Africa
	South American fur seal	Argentina
C. ogmorhini s. l.	South American fur seal	Canada
	South American fur seal	Argentina
	Australian fur seal	New Zealand
	South African fur seal	South Africa
	Leopard seal	Antarctic region
	Antarctic fur seal	Antarctic region

Source: Mattiucci et al., 2003; Nadler et al., 2005; Mattiucci and Nascetti, 2007

Contracaecum osculatum complex is another group of parasites which has undergone a subsequent redescription (Nascetti et al., 1993; Orecchia et al., 1994). It has been described in harbour seals from Germany and California sea lions from Mexico (Mawson, 1953; Claussen et al., 1991; Fauquier et al., 2004). In Antarctic and Sub Antarctic pinnipeds, larval and adult stages of *C. osculatum* have been reported in populations from Macquarie and Heard Islands (Mawson, 1953; McFarlane et al., 2009). A list of the different species included in the *C. osculatum* complex found in pinnipeds is provided below (Table 4).

Table 4: List of *Contracaecum osculatum* complex in Northern and Southern Pinnipeds

Species	Host	Location
C. osculatum s. s.	Grey seal	Baltic sea
	Northern fur seal	United States
C. osculatum A	Bearded seal	Canada
C. osculatum B	Harp seal	Barentsz sea
	Harbour seal	Canada
		Norway
C. osculatum D	Weddell seal	Antarctic region
C. osculatum E	Weddell seal	Antarctic region
C. osculatum baicalensis	Baikal seal	Russia

Source: Mawson, 1953; Orecchia et al., 1994; Fauquier et al., 2004; Nadler et al., 2005; Mattiucci and Nascetti, 2007; Mattiucci et al., 2008; Spraker and Lander, 2010

6.2.1.1.3. *Phocascaris*

Phocascaris spp. are anisakids occurring only in phocids, which have been reported as the only final host (Abollo and Pascual, 2002; Mattiucci et al., 2008). Molecularly, *Phocascaris* is closely related to the *C. osculatum* complex and some authors have even proposed that the *Contracaecum* species which have phocids as definitive hosts should be included in the genus *Phocascaris* although further data are necessary to confirm this fact (Mattiucci et al., 2008). Three different species have been described in the genus *Phocascaris: P. phocae* in harp seals from Canada and Norway and ringed seals from the High Arctic Archipelago of Norway, *P. netsiki* in ringed seals and *P. cystophorae* in hooded seals from Canada (Abollo and Pascual, 2002; Nadler et al., 2005; Mattiucci et al., 2008; Johansen et al., 2010).

6.2.1.2. *Pseudoterranova*

The genus *Pseudoterranova* is a parasite of the Anisakidae family with a wide distribution and pinnipeds are considered the definitive host (Paggi et al., 1991). The larvae have been called sealworms or codworms and the life cycle is similar to the rest of Anisakids with the important difference that larvae cannot be free in seawater and must be in an intermediate host for transmission (Palm, 1999). In the life cycle, partially embrionated eggs are released in the faeces of the pinniped and descend to the ocean floor before hatching. Water temperature must be between 0ºC and 25ºC for hatching and differentiation. The newly hatched larvae are most active at temperatures above 10ºC. They infect benthic crustaceans (copepods), exsheathe, penetrate to the

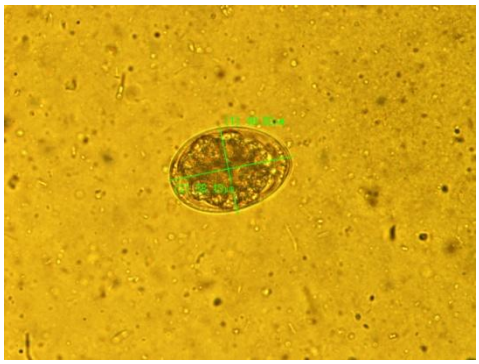
haemocoele and begin to grow. Later, they pass to fish and secondary fish where larval worm grows enough to become infective to the definitive host. Finally, the definitive host ingests the final fish host and after two moults, they mature and reproduce (McClelland, 2002; Rohde, 2005).

Pseudoterranova spp. have been described in the gastrointestinal tract of ringed seals from Norway (Johansen et al., 2010), California sea lions, Northern elephant seals, harbour seals and Steller sea lions from the United States (Nadler et al., 2005). *P. decipiens* (refered by some authors as *Terranova piscium*) has been identified in harbour seals from Germany and Leopard seals from the Antarctic region (Claussen et al., 1991; Dailey, 2001). Likewise, *P. decipiens* s.s. (also designated as *P. decipiens* B) has been described in harbour seals from Canada, Northeast and Northwest Atlantic Iceland, Norway and Sweeden; hooded seals from Canada; and grey seals from Norway and Iceland (Paggi et al., 1991; Nadler et al., 2005; Mattiucci and Nascetti, 2007). *P. krabbei* (also designated as *P. decipiens* A) has been reported in harbour seals from Norway; and grey seals from Northeast Atlantic Norway, Iceland and Canada (Paggi et al., 1991; Nadler et al., 2005; Mattiucci and Nascetti, 2007). *P. azarasi* (also designated as *P. decipiens* D) has been described in Steller sea lions from Northwest Pacific Ocean and Japan (Nadler et al., 2005; Mattiucci and Nascetti, 2007). *P. bulbosa* (also designated as *P. decipiens C)* has been identified in bearded seals from Northeast and Northwest Atlantic Oceans and Canada (Nadler et al., 2005; Mattiucci and Nascetti, 2007); and *P. cattani* in South American sea lions from Chile (Nadler et al., 2005). Finally in the Southern Ocean, *Pseudoterranova decipiens* E has been identified in Weddell seals (Mattiucci and Nascetti, 2007).

6.2.1.3. Ancylostomatids

Hookworms are haematophagus ancylostomatid nematodes, affecting a wide range of mammals, including humans and wild species, causing intestinal haemorrhage, severe anaemia and protein malnutrition (Acevedo-Whitehouse et al., 2009). They also have been described to infect the gastrointestinal tract of pinnipeds. The genus *Uncinaria* has been the cause of high mortality in young pinnipeds through haemorrhagic enteritis and anaemia (McFarlane et al., 2009). The life cycle has been modified to fit the seasonal at-sea habits of pinnipeds, demonstrating the adaptation that has been made in the transition from the terrestrial to the marine habitat. In terrestrial

hosts, infective third-stage larvae penetrate the skin of the host and grow to adults in the intestine. In pinnipeds, hookworm larvae penetrate the skin of pregnant cows and wait until the birth of the pup, being consumed with the milk during nursing (Rohde, 2005). Infective third stage larvae, possibly influenced by hormones in the pregnant cow, migrate from the belly blubber to the mammary glands. Newborn pups can consume as many as 1,500 larvae with the first meal of milk, and two weeks later develop potencially fatal hemorrhagic diarrhea and anaemia. The reproducing adult worms are found only in the pups where they feed on blood causing the clinical signs and subsequently the possible death of the animal (Rohde, 2005). The infection and associated intestinal lesions can be observed for the first time about three weeks after the majority of pups have been born, when parasites develop into mature adults, feeding on the intestinal mucosa, and are eliminated spontaneously three months post-infection (Nadler et al., 2000a; Castinel et al., 2006; Castinel et al., 2007). Eggs (Figure 17) shed in the soil through faeces can survive to free-living third-stage larvae (Geraci and St Aubin, 1987). The parasite in the neonatal host causes debilitation leading to trauma, malnutrition and hypothermia (Castinel et al., 2007).



Source: Saluvet Group

Figure 17: Ancylostomatid egg (40X)

Uncinaria spp. have been found in Juan Fernandez fur seal pups, Northern fur seal pups, California sea lions pups and Hawaiian monk seal neonates from the United States. Similarly, they have been found in adults and neonates of New Zealand sea lions from Auckland Islands, Australian fur seals and New Zealand fur seals, Australian sea lions and South American sea lions from the Southern region (Nadler et al., 2000a; Lyons et al., 2001; Castinel et al., 2007; McFarlane et al., 2009; Byard et al., 2010). The

presence of *U. lucasi* have been described in 6.25% (n=64) of Northern fur seals from the United States (Lyons et al., 2001; Ionita et al., 2008; Spraker and Lander, 2010); and *U. hamiltoni* in California sea lion pups from the United States and South American sea lions from the coast of Argentina (Nadler et al., 2000a; Beron-Vera et al., 2004). In Antarctic pinnipeds, *Uncinaria* sp. and *Uncinaria hamiltoni* have also been described in high numbers in Southern elephant seals adults and pups, although it is thought that they are not an important cause of mortality in the Southern hemisphere, based on the absence of lesions in the small intestines of affected animals (Beron-Vera et al., 2004; McFarlane et al., 2009).

6.2.1.4. Filarids

Filarids are nematode parasites transmitted by arthropod vectors to humans and other vertebrates, causing a disease called Filariosis. The disease can cause a serious and potentially fatal condition in hosts (Taylor et al., 2007). In general, the life cycle of Filarids is indirect and starts when the adult female release microfilariae into the definitive host bloodstream. The intermediate host (vector) becomes infected with microfilariae while taking blood meal from the infected animal. The microfilariae mature to the infective larval stage within the vector. The vector then bites another susceptible host, and the infective larvae enter through the bite wound. Inside the host, infective larvae extend to blood or tissue fluids, and mature into adult worms in deep connective tissue, membranes or visceral surfaces. Microfilariae cannot mature into adult worms without first passing through the intermediate host. Therefore the mode of transmission in marine mammals has yet to be proven. It is expected that pulmonary and cardiovascular complications associated with Filarids would seriously reduce the ability to dive and feed in infected animals (Geraci and St Aubin, 1987).

Filarids have been reported in harbor seals, ringed seals and hooded seals¸ but the precise identification has been uncertain (McFarlane et al., 2009). *Dipetalonema* and *Dirofilaria* are two genera of veterinary interest described in phocids and otariids worldwide (McFarlane et al., 2009). Some authors report the *Acanthocheilonema* sp. as a filarial worm. However, now is part of the genus *Dipetalonema* and the previous name is no longer in use (Fowler and Miller, 2003). Microfilariae have also been found in red pulp of spleen during histological examination in Northern fur seals and California sea lions from the United States, assuming they are *Dipetalonema odendhali* (Davis et al.,

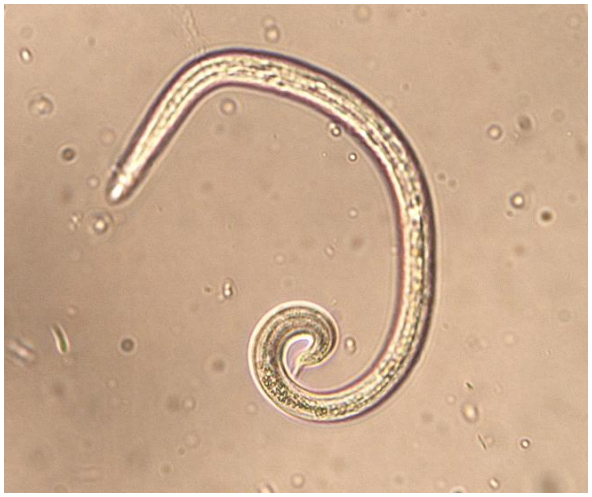
1971; Perry and Forrester, 1971). *Dipetalonema spirocauda* has been reported in several harbour seals populations from North America (Puget Sound, Southern California, New England and Atlantic Canada), western Atlantic harbour seals from the United States (MacDonald and Gilchrist, 1969; Dunn and Wolke, 1976; McDonald et al., 2006), harbour seals and Pacific harbour seals from Germany (Siebert et al., 2007) and Mediterranean monk seals from the Aagean and Ionian Islands along the coastline of Southern Greece (Papadopoulos et al., 2010). In all the reports, macroscopic and hystopathological lesions in heart atrium and ventricles, pulmonary arteries and lungs have been described (MacDonald and Gilchrist, 1969; Dunn and Wolke, 1976; Claussen et al., 1991). It has also been reported that *D. spirocauda* infection causes mortality mainly among juvenile seals, whereas adult seals generally tolerate the parasite burden although they can be adversely affected in the presence of secondary infections or immunocompromised conditions (Papadopoulos et al., 2010).

Antibodies against *Dirofilaria immitis,* the heartworm of canids have been detected in Hawaiian monk seals from Northwestern Hawaiian Islands, with a prevalence of 0.153% (n=332) (Aguirre et al., 2007). Geraci and St Aubin, (1987) also reported infection with *Dirofilaria immitis* in a captive harbour seal and sea lions. In the Antarctic region, filarial tissue and blood parasite have been reported in a Southern elephant seal from Heard Island. The description confirmed the presence of a fragment of a filarial nematode from a blood vessel, referred as *Filaria (sensu lato)* sp. (Mawson, 1953; McFarlane et al., 2009). Even if filarial nematodes can be occurring in Southern pinnipeds, their prevalence and significance remain uncertain (McFarlane et al., 2009).

6.2.1.5. Metastrongyloids

Metastrongyloids (Figure 18) are also cosmopolitan parasites that inhabit the respiratory parenchyma of mammals (Taylor et al., 2007). In terrestrial hosts, the life cycle is indirect and the intermediate hosts are usually molluscs and earthworms (Rohde, 2005). However, the route of infection in aquatic mammals is unknown, though it is likely that infective larvae developed in the intermediate host switches to a fish infecting pinnipeds through the oral route, demonstrating one of the interesting transitions that the parasite has made in order to survive in the ocean environment (Geraci and St Aubin, 1987; Rohde, 2005; Rijks et al., 2008). *Parafilaroides decorus* is a parasite commonly found in California sea lions of which a life cycle is proposed,

using a vertebrate (a small tidal-pool fish, *Girella nigricans*) as a single intermediate host (Geraci and St Aubin, 1987). This fish lives at breeding rookeries where feeds on the pinniped excrement containing eggs with the first-stage larvae followed by the development into the infective L3 in the intestinal wall. Later, the California sea lion eats the fish containing the infective L3, which then matures and reproduces (Rohde, 2005). Metastrongyloids are poorly described in pinnipeds, and descriptions reported have been based on only a few specimens (Kennedy, 1986). Pathological findings associated include respiratory and cardiovascular system affections (Siebert et al., 2007). Sometimes the larvae form small, firm nodules in the lung parenchyma due to granuloma formation associated with degenerating parasites (Onderka, 1989).



Source: Saluvet Group

Figure 18: Metastrongyloid larva (40X)

Two species of *Parafilaroides*, *P. gymnurus* and *P. hispidus* have been reported in ringed seals, harp seals, harbour seals from Germany; grey seals, spotted seals, Steller sea lions and Baikal seals*;* primarily from eastern Canada and Europe. *Filaroides* (*Parafilaroides*) *hispidus* has been described in ringed seals from Canada (Kennedy, 1986). *Parafilaroides decorus* has also been reported in Steller sea lions from the eastern Central Pacific. *P. measuresae* has been redescribed in Northern elephant seals, while *P. gullandae* in eastern Pacific harbour seals from the United States. The structural identification indicates that prevalence gradient goes from common to uncommon in *P. decorus* for sea lions, *P. gullandae* for harbour seals and *P. measuresae* for Northern elephant seals (Carreno and Nadler, 2003; Dailey, 2006;

Siebert et al., 2007). *Parafilaroides* spp. have been reported in Australian sea lions and *Parafilaroides normani* in brown fur seals, Sub Antarctic fur seals and New Zealand fur seals from Southern waters (Dailey, 2009; McFarlane et al., 2009). In the Southern Ocean, *Parafilaroides hydrurgae* has been reported in Leopard seals (Mawson, 1953; Dailey, 2006; McFarlane et al., 2009). As mentioned above, it is well-known that lungworms are a common underlying cause of respiratory disease (verminous pneumonia), but the prevalence or significance in health of Sub Antarctic and Antarctic pinnipeds remain unclear (McFarlane et al., 2009). In addition, hypersensitivity reactions have been associated to *Parafilaroides decorus* infection in California sea lions, inducing acute bronchitis, bronchopneumonia, and stimulate mucous secretion, which in turn lead to asphyxiation (Geraci and St Aubin, 1987).

Otostrongylus circumlitus is a further Metastrongyloid considered the large crenosomatid nematode (Geraci and St Aubin, 1987). It is thought to interfere with the respiratory health and diving ability of pinnipeds and thus alter their ability to feed, grow and survive. It has been identified in Northern elephant seals and California sea lions from the United States; ringed seals from western Arctic Canada; harbour seals and eastern Pacific harbour seals from Germany and the United States (Geraci and St Aubin, 1987; Onderka, 1989; Elson-Riggins et al., 2001; Carreno and Nadler, 2003; Colegrove et al., 2005; Kelly et al., 2005).

6.2.1.6. *Trichinella*

Trichinella spp. are monoxenous parasites that mature in the intestine of carnivorous mammals, producing first stage (L1) larvae which migrate to the musculature. Marine mammals become infected by ingesting flesh with encapsulated L1. Direct transmission transplacentally and in breast milk has been proposed for cetaceans, but there is the speculation that small crustaceans may also be involved in the transmission. In addition, experimental evidence suggests that *Trichinella* may be transmitted by sarcophagous crustaceans and fish acting as paratenic hosts (Rohde, 2005). In terrestrial mammalian hosts, clinical signs are variable, non specific and depend on the host and the level of infection, including inappetance, weakness, diarrhea, fever, muscular pain, dyspnoea and peripheral eosinophilia (Taylor et al., 2007). In pinnipeds, the most relevant symptoms observed have been lethargy and anorexia (Kapel et al., 2003).

Freeze resistant *Trichinella nativa*, commonly found in Arctic wildlife, has been found in hooded seals and ringed seals from Greenland (Moller, 2007). In Finland, findings in grey seals, bearded seals, ringed seals and hooded seals have been also reported (Isomursu and Kunnasranta, 2011). In Greenland, the prevalence was 0.2% in ringed seals and 2.3% in hooded seals. In Finland, the prevalence in wild grey seals was 0.6% (n=171). In addition, grey seals have been successfully infected in laboratory conditions (Isomursu and Kunnasranta, 2011). *Trichinella*-specific IgG antibodies have been detected in 1.5% of ringed seals, 2.1% of harp seals and 0.3% of hooded seals (Moller, 2007). In the Southern hemisphere, incidental findings have been reported in Antarctic fur seals and Leopard seals (Dailey, 2001).

6.2.1.7. *Dioctophyme renale*

Dioctophyme renale, the giant kidney worm is the largest known nematode parasite infecting the kidneys of domestic and wild fish-eating carnivores, with occasional descriptions in humans and marine mammals (Hoffman et al., 2004; Duim-Ribeiro et al., 2007). The mink (*Mustela vison*) has been considered the principal definitive host, while humans and dogs are terminal or accidental hosts (Duim-Ribeiro et al., 2007). The life cycle is very complex and includes intermediate, paratenic and terminal hosts. Eggs are passed in the urine and must be ingested by the intermediate hosts (aquatic worms) where the parasite develops into the third larval stage. The process is temperature dependent. These aquatic worms can be then eaten by paratenic hosts (fishes). In the paratenic host, the infective larvae are encysted in tissues without further development. Definitive hosts are infected by ingestion of contaminated aquatic worms or paratenic hosts. In the definitive host the larva penetrates the duodenal wall, enters the abdominal cavity and migrates to the kidneys, where it remains until reaching the adult stage. Both sexes are necessary to parasitise, otherwise there is discontinuation of the life cycle in the definitive host (Duim-Ribeiro et al., 2007). Necropsy findings include alteration of the urinary bladder, with lesions and disfunctions like watery red fluid and a strong ammonia odor in abdominal cavity (Hoffman et al., 2004). In pinnipeds, *Dioctophyme renale* has only been reported in a stranded harbour seal from the United States and phocids from the Caspian Sea (Hoffman et al., 2004).

6.2.2. Cestoda

Cestodes are parasites with segmented and tape-like body. Each segment contains one or two sets of male and female reproductive organs (Taylor et al., 2007). In general, marine mammals harbour a rich tapeworm fauna, including several stages. The infection tends to be seasonal, reaching enormous intensities. Eggs are continuously discharged from the genital pores of the attached gravid segments of the strobila and pass to the environment in the faeces. The egg (Figure 19) must develop in water and finally hatches to liberate a motile ciliated coracidium, which if ingested by a copepod, develops into a first parasitic larval stage, a procercoid. When the copepod is ingested by a fish, the procercoid migrates to the muscles or viscera to form the second larval stage, the plerocercoid which possesses the characteristic scolex. The life cycle is completed when the infected fish is eaten by the final host. However, if the infected fish is eaten by a larger fish, the plerocercoid has the ability to establish itself in its new host (Taylor et al., 2007). Pathogenic effects in captive pinnipeds have been reported, although are varied and the effects may occur only when large numbers obstruct the intestinal lumen (Dailey, 2001). Infections with mature pseudophyllideans have been considered innocuous, but in extreme circumstances have been resulted in debilitation and death of the host. Parasites can encyst in the colonic wall or as a parasite mass obstructing the lumen of the gut (Geraci and St Aubin, 1987). Minimal inflammation has been found in the lamina propria throughout tissue sections with heavy tapeworm burden. High, moderate, and low burdens have also been found in the ileocecocolic junction of the intestinal tract (Spraker and Lander, 2010).

Source: Saluvet Group

Figure 19: Cestode eggs (40X)

Diphyllobothrium spp. are important parasites affecting humans and fish-eating mammals (Taylor et al., 2007). *Diphyllobothrium lanceolatum* and *D. terapterus* have been described in the gastrointestinal tract of captive pinnipeds (Dailey, 2001; Fowler and Miller, 2003). In the wild, *Diphyllobothrium* sp. has been identified in Northern fur seals, Hawaiian monk seals and ringed seals from the United States; and Mediterranean monk seals from Greek coasts (Fowler and Miller, 2003; Reif et al., 2006; Ionita et al., 2008; Papadopoulos et al., 2010). *Diphyllobothrium hians, D. elegans* and *D. cameroni* have also been described in Hawaiian monk seals from the United States (Banish and Gilmartin, 1992) and *Diphyllobothrium pacificum* in Northern fur seal pups from the United States (Spraker and Lander, 2010). Rausch et al. (2010) reported a redescription, according to morphological features, differentiating two different species: *Diphyllobothrium pacificum* and *D. arctocephalinum*. *D. pacificum* has been considered to be hosted by Juan Fernandez fur seals from the West coast of Chile and *D. arctocephalinum* in Australian fur seals from South Australia. In Antarctic and Sub Antarctic seals, the adult cestodes reported belong to several genera of the family Diphyllobothriidae (Order Pseudophylliidea). *Diphyllobothrium* spp. have been described in Sub Antarctic fur seals. In Weddell seals, two types of cestodes have been described, *Diphyllobothrium lashleyi* and *D. mobile* (McFarlane et al., 2009). The new species, *Diphyllobothrium lobodoni*, has also been described in the intestines of Crabeater seals (Iurkhano and Mal'tsev, 1994). *Glandicephalus antarticus* (also known as *Diphyllobothrium antarcticum* or *Dibothriocephalus antarticus*) has been reported in the stomach and intestines of the Ross seals, and *G. perfoliatus* in the bile-pancreatic duct of Weddell seals (Iurkhano and Mal'tsev, 1995; McFarlane et al., 2009). Heavy burden with adult forms have also been found in Leopard seals, whereas in Southern elephant seals and Antarctic fur seals only plerocercoids have been observed. These two species have been moved out of the family Diphyllobothriidae and are considered as the new family Glandocephalidae (superfamily Diphyllobothrioidea) (Iurkhano and Mal'tsev, 1995). The Southern elephant seal has been reported as definitive host of two species, *Baylisiella tecta* and *Flexobothrium microovatum* (Wojciechowska and Zdzitowiecki, 1995). In addition, *Baylisia baylisiis* has also been described in Crabeater seals (McFarlane et al., 2009). Necropsy findings associated with *Baylisiella tecta* and *Baylisia baylisiis* were nodular reaction at the site of scolex attachment in the wall of the rectum (McFarlane et al., 2009).

The rarer *Schistocephalus solidus* has been known to occur in ringed seals from the Northern Gulf of Bothnia (Nickol et al., 2002), and *Bothriocephalus* sp. in Hawaiian monk seals from the United States (Banish and Gilmartin, 1992). Similarly, the cysticerci larval stage of *Taenia solium* has been recorded in Cape fur seals, South African fur seals from United States and Mediterranian monk seals*;* named as *Cysticercus cellulosae* (De Graaf et al., 1980; McFarlane et al., 2009). The finding of *Taenia solium* is considered a clear exposure to human faecal material, perhaps indirectly by consumption of fish fed on gravid proglottids or infected by feeding with contaminated invertebrates or carrion (Geraci and St Aubin, 1987). *Phyllobothrium delphini* has been found in connective tissue of Arctic pinnipeds, although this finding has been incidental (Dailey, 2001). In the Antarctic region, larval cestodes have been often recovered from the blubber of pinnipeds. Cysticerci of *Phyllobothrium* spp. have been found in Weddell seals (McFarlane et al., 2009). Also, *P. delphini* has also been reported in Southern elephant seals, Antarctic fur seals and Leopard seals (Dailey, 2001; McFarlane et al., 2009).

Monorygma grimaldi, a parasite commonly found encysted in the mesentery of dolphins and thought to mature in sharks has been described encysted in the mesentery of a phocid and encysted in the testis of an Antarctic fur seal (McFarlane et al., 2009). The life cycle of *Monorygma grimaldi* suggests a crustacean (possibly a planktonic copepod) as the initial intermediate host with fishes and squids acting as the source of infection for sharks. The postulated life cycle in marine mammals is that they may be accidental accumulators of this metacestode due to the similar diet with sharks (MacColl and Obendof, 1982). In addition, the larval stage *Scolex pleuronectis* has been identified in the large intestine of Northern fur seals from Komandor Islands (Skrjabin and Yurakhno, 1987). The larval stage has also been identified in other marine mammals, primarily cetaceans, marine invertebrates and fishes but they have rarely been described in detail, and the ecological significance of infection is unclear (Agusti et al., 2005).

6.2.3. Trematodes

Trematodes are parasites with worldwide distribution and include species within the sub-class Digenea (Taylor et al., 2007). Digenea trematodes or flukes are considered of veterinary importance affecting bile ducts, alimentary tract and vascular system of

vertebrates. They are also found in marine mammals, specifically in the gastrointestinal tracts of pinnipeds (Rohde, 2005). Their life cycle is still not known completely, but it is considered complex, involving two or three hosts and several larval stages for their development. The first intermediate hosts of most digenetic flukes are gastropods, and the final host is almost always a vertebrate (Jousson et al., 1998; Taylor et al., 2007).

6.2.3.1. Gastrointestinal Trematodes

Within the genus *Galactosomum, G. stelleri* has been found in the small intestine of Northern sea lions from the United States, differing in morphology but closely related to *G. ubelakeri* and *G. humbargari,* both found in California sea lions and aquatic birds, respectively (Pearson, 1973; Dailey et al., 2002). Similarly, there are reports of *G. angeliae* in Australian sea lions (McFarlane et al., 2009). Trematode eggs described recently as *Heterophyopsis hawaiiensis* have been reported in Hawaiian monk seals from Northwestern and main Hawaiian Islands (Banish and Gilmartin, 1992; Reif et al., 2006). Other trematodes found in pinnipeds include the genera *Cryptocotyle*, *Rossicotrema* and *Phagicola,* (Dailey et al., 2002). *Cryptocotyle lingua* and *Mesorchis advena* have been described in Caspian seals (Kuiken et al., 2006). Within the genus *Strictodera, S. diplacantha* has been described in the intestines of Australian sea lions. The genus *Phocitrema* has been reported in all these pinnipeds as well (Dailey et al., 2002; McFarlane et al., 2009). Further trematodes found in pinnipeds include *Microphallus* sp., *Maritrema* sp. and *Ogmogaster* sp¸ described in the intestines of Australian sea lions (Dailey, 2001), and *O. antarticus* in Weddell seals (McFarlane et al., 2009). In addition, *Mesostephanus neophocae* has been described in the intestines of Australian sea lions (Dailey et al., 2002) and in a captive Southern elephant seal from South Australia (McFarlane et al., 2009). *Hadwenius* sp. has been observed in Australian sea lions guts; and *Orthosplanchnus* sp. in Weddell seals from Antarctic populations (McFarlane et al., 2009).

6.2.3.2. Hepatic Trematodes

Pricetrema spp. are the most common trematode occurring in massive numbers in pinnipeds (Dailey, 2001). They are not considered pathogenic, although colitis has been occasionally observed histologically in infected animals including thickened biliary ducts, probably as a consequence of infection (Dailey, 2001). Other liver trematodes

include the genera *Orthosplanchnus* and *Zalophotrema. Zalophotrema hepaticum* has been found in the biliary system of California sea lions, harbour seals and Northern elephant seals (Fowler and Miller, 2003). They normally inhabit the liver, gallbladder, and pancreas, causing portal fibrosis with bile duct proliferation and dilatation. Aberrant liver migration has also been described, causing meningoencephalitis, seizure activity, lethargy and ataxia. Histopathology has revealed the presence of eggs and larvae in organs of the central nervous system, although the cause and route of the aberrant migration into the brain is unknown (Fauquier et al., 2004). In addition, *Pseudamphistromum* sp., *Opistorchis* sp. and *Metorchis* sp. have also been found to affect the liver of pinnipeds (Fowler and Miller, 2003), such as *Pseudamphistomum truncatun* in Caspian seals (Kuiken et al., 2006).

6.2.3.3. Conjunctival Trematodes

Philophthalmus sp. is a cosmopolitan trematode, occurring only in various microhabitats in the orbits of birds. However, the eye fluke *Philophthalmus zalophi*, has been found in the conjunctiva lens of eye from Galapagos sea lions and Galapagos fur seals. This is considered the first description of eye flukes in naturally infected marine-mammal species (Dailey et al., 2005).

6.3. Acantocephalans

Acantocephalans or thorny-headed worms (Figure 20) are widespread in marine mammals and have worldwide distribution, but little is known about their effects on health in affected hosts (McFarlane et al., 2009). They appear as small, white-domed or coma shaped nodules 5-10 mm in diameter scattered over the surface of the mucosa of the infected intestines. Their life cycle is indirect, involving arthropods, usually amphipods of marine environment and fishes as the intermediate hosts. After ingestion by the intermediate host, eggs hatch and parasites migrate to the haemocoel or mesenteries of fish where they develop to become cystacanths. Infected tissues with encysted cystacanths are then ingested by pinnipeds where adult worms attach to the mucosa of the stomach or intestine with a retractable spinous proboscis. This provokes a localised inflammatory response, usually limited to the mucosa (Taylor et al., 2007; McFarlane et al., 2009). Occasional ulceration at the site of the attachment can be observed, even though they have little pathological significance despite the having been

found in high numbers in some hosts (Geraci and St Aubin, 1987). Morphometric and allozyme electrophoretic analyses have demonstrated very high levels of genetic diversity among acanthocephalans in marine mammals (Sardella et al., 2005). In Northern pinnipeds, there are descriptions of mortality and morbidity due to these parasites. However, in Southern pinnipeds there is lack of information and in view of the possibility of changes in the Antarctic and Sub Antarctic regions, systematic prospective studies in marine mammal species are urgently required (McFarlane et al., 2009).

Source: Saluvet Group

Figure 20: Acanthocephalan found in a faecal sample

Bolbosoma spp. and *Corynosoma* spp. are located in the gastrointestinal tract of pinnipeds (Fowler and Miller, 2003). *Corynosoma strumosum* has been described in grey seals, spotted seals, ringed seals, harbour seals from the United States and Baltic sea; in 21.7% (n=115) of Sub-Adults Northern fur seals from the United States (Garcia-Varela et al., 2005; Aznar et al., 2006; Siebert et al., 2007; Garcia-Varela and Perez-Ponce de Leon, 2008; Ionita et al., 2008; Byard et al., 2010), and in 100% (n=7) of Caspian seals (Kuiken et al., 2006). In Sub-Adult Northern fur seals, the presence of two species of *Corynosoma*, *C. obtuscens,* and *C. validum* have also been found (Aznar et al., 2006; Ionita et al., 2008). In addition, *C. villosum* has been described in Steller sea lions and *C. wegeneri* in ringed seals. These latter two parasites have also been commonly encountered in captive pinnipeds (Dailey, 2001; Garcia-Varela et al., 2005). *C. rauschi* has been described in Hawaiian monk seals from North Western Hawaiian Islands (Reif et al., 2006). Likewise, *C. capsicum* has been found in Caspian seals, *C. falcatum* in grey seals, *C. magdaleni* in ringed seals and grey seals from the Western

Baltic sea, and *C. reductum* in ringed seals from the United States (Nickol et al., 2002; Garcia-Varela et al., 2005; Aznar et al., 2006).

Some acanthocephalans have been identified in Antarctic and Sub Antarctic pinnipeds, with no evidence of any clear degree of host specificity. Thus, *C. arctocephali* has been reported in Crabeater seals, Leopard seals and Antarctic fur seal; *C. bullosum* in Crabeater seals, Weddell seals and Southern elephant seals; *C. hamanni* (syn. *C. antarticum*) in Crabeater seals, Leopard seals, Ross seals and Weddell seals; and *C. pseudohamanni* in Crabeater seals, Leopard seals, Ross seals, Weddell seals, Southern elephant seals and Antarctic fur seals (Garcia-Varela et al., 2005; McFarlane et al., 2009). *C. semerne* has been described in New Zealand sea lions, Southern elephant seals and South American fur seals (McFarlane et al., 2009). This parasite has also been associated with mild focal eosinophilic and granulomatous inflammation in 13.8% (n=355) of harbour seals from the coast of Germany (Siebert et al., 2007). *C. australe* has been identified in South American sea lions, South American fur seals and Southern elephant seals from different locations of the Argentinian and Uruguayan coasts; and New Zealand sea lions (Garcia-Varela et al., 2005; Sardella et al., 2005; McFarlane et al., 2009). Likewise, *C. evae* has been described in South American fur seals and Leopard seals (Nickol et al., 2002; Garcia-Varela et al., 2005; Aznar et al., 2006). Even though *C. cetaceum* (syn. *Polymorphus arctocephali*) has been generally found in cetaceans, it has been described in brown fur seals and South American fur seals (Garcia-Varela et al., 2005; Sardella et al., 2005).

Bolbosoma sp. has been recorded in spotted seals and Northern fur seals. Two species of *Bolbosoma*, *B. borbrovi* and *B. nipponicum*, have also been identified in Northern fur seals (Ionita et al., 2008). In this study, differences in density, maturity and reproductive stages at different levels of the intestines were observed, revealing there is still much to learn about the biology of these widespread and unusual parasites, and the potential effects on the health of the definitive hosts (McFarlane et al., 2009)

6.4. Arthropods

Arthropods are organisms generally associated with ectoparasitism in vertebrates (Taylor et al., 2007). However, some of these parasites are the cause of endoparasitic infestation in marine mammals. They parasitise the nasal passages, trachea, bronchi and occasionally in bronchioles and lungs of pinnipeds and cannot survive outside the moist

environment of their host (Geraci and St Aubin, 1987). Within the group of endoparasite arthropods, the lung mite *Halarachne* sp. has been described in Caspian seals (Kuiken et al., 2006) and *Halarachne laysanae* in Hawaiian monk seals (Banish and Gilmartin, 1992; Fowler and Miller, 2003). Similarly, the mites *Orthohalarachne attenuate* and *O. diminuata* have been found in the nasal turbinates, trachea and bronchi of Northern fur seal pups (Fowler and Miller, 2003; Kelly et al., 2005; Spraker and Lander, 2010). Histologic lesions associated with these parasites are mild hymphoplasmocytic rhinitis although they can cause enough damage to mucous membranes to affect the animal´s health adversely (Geraci and St Aubin, 1987). In the Southern region, *Halarachne* spp. have been reported in Weddell seals, *O. diminuata* in Sub Antarctic fur seals and *O. magellanica* in Southern sea lion (McFarlane et al., 2009).

In summary, although the current available information on the presence and distribution of parasites in Antarctic pinnipeds, as opposed to their worldwide counterparts, is limited, as mentioned above, several parasites have been described in Antarctic populations and they are summarised in tables 5 and 6.

Table 5: Parasites described in Antarctic fur seals (*Arctocephalus gazella*)

Parasite Agent	References
Apicomplexa	
Sarcocystis richardi	Dailey, 2001; McFarlane et al., 2009
Nematodes	
Contracaecum ogmorhini	Mattiucci et al., 2003; Nadler et al., 2005; Mattiucci and Nascetti, 2007
Trichinella nativa	Dailey, 2001
Cestodes	
Phyllobothrium delphini	Dailey, 2001; McFarlane et al., 2009
Monorygma grimaldi	McFarlane et al., 2009
Acanthocephala	
Corynosoma arctocephali	Garcia-Varela et al., 2005; McFarlane et al., 2009
Corynosoma pseudohamanni	Garcia-Varela et al., 2005; McFarlane et al., 2009

Table 6: Parasites described in Antarctic phocids

Parasite Agent	Hosts	References
Apicomplexa		
Isospora miroungae	Southern elephant seals	Drozdz, 1987
Eimeria weddellii	Weddell seals	McFarlane et al., 2009
Eimeria arctowkii		McFarlane et al., 2009
Eimeria sp. 2		Drozdz, 1987
Eimeria sp. 3		Drozdz, 1987
Eimeria sp. 1	Crabeater seals	Drozdz, 1987
Sarcocystis hydrurgae	Leopard seals	Dailey, 2001; McFarlane et al., 2009
Nematodes		
Anisakis pegreffii	Leopard seals	Dailey, 2001
Anisakis simplex C	Southern elephant seals	Mattiucci and Nascetti, 2007
Contracaecum mirounga	Southern elephant seals	Mattiucci et al., 2003; Nadler et al., 2005; Mattiucci and Nascetti, 2007; McFarlane et al., 2009
Contracaecum oghmorhini		
Contracaecum radiatum	Leopard seals Weddell seals	Mattiucci et al., 2003; Nadler et al., 2005; Mattiucci and Nascetti, 2007; McFarlane et al., 2009
Contracaecum osculatum	Southern elephant seals Leopard seals	Nadler et al., 2000b; Mattiucci et al., 2003; Nadler et al., 2005
Contracecum osculatum D	Weddell seals	Mawson, 1953; Orecchia et al., 1994; Fauquier et al., 2004; Nadler et al., 2005; Mattiucci and Nascetti, 2007; Mattiucci et al., 2008; Spraker and Lander, 2010
Contracecum osculatum E		
Pseudoterranova decipiens	Leopard seals	Dailey, 2001
Pseudoterranova decipiens E	Weddell seals	Mattiucci and Nascetti, 2007
Uncinaria sp.	Southern elephant seals	Beron-Vera et al., 2004; McFarlane et al., 2009
Uncinaria hamiltoni		
Filaria sp.	Southern elephant seals	Mawson, 1953; McFarlane et al., 2009
Parafilaroides hydrurgae	Leopard seals	Mawson, 1953, Dailey, 2006; McFarlane et al., 2009
Trichinella nativa	Leopard seals	Dailey, 2001
Cestodes		
Diphyllobothrium lashleyi	Weddell seals	McFarlane et al., 2009
Diphyllobothrium mobile		
Diphyllobothrium lobodoni	Crabeater seals	Iurakhno and Mal´tsev, 1994
Glandicephalus antarticus	Ross seals	Iurkhano and Mal'tsev, 1995; McFarlane et al., 2009
Glandicephalus perfoliatus	Weddell seals	Iurkhano and Mal'tsev, 1995; McFarlane et al., 2009
Baylisiella tecta	Southern elephant seals	Wojciechowska and Zdzitowiecki, 1995
Flexoborhrium microovatum		
Baylisia baylisiis	Crabeater seals	McFarlane et al., 2009
Phyllobothrium spp.	Weddell seals	McFarlane et al., 2009
Phyllobothrium delphini	Southern elephant seals Leopard seals	Dailey, 2001; McFarlane et al., 2009
Trematodes		
Ogmogaster antarticus	Weddell seals	McFarlane et al., 2009
Orthosplanchnus sp.	Weddell seals	McFarlane et al., 2009

continue Table 6

Parasite Agent	Hosts	References
Acanthocephala		
Corynosoma arctocephali	Crabeater seals Leopard seals	Garcia-Varela et al., 2005; McFarlane et al., 2009
Corynosoma bullosum	Crabeater seals Weddell seals Southern elephant seals	Garcia-Varela et al., 2005; McFarlane et al., 2009
Corynosoma hamanni	Crabeater seals Leopard seals Ross seals Weddell seals	Garcia-Varela et al., 2005; McFarlane et al., 2009
Corynosoma pseudohamanni	Crabeater seals Leopard seals Ross seals Weddell seals Southern elephant seals	Garcia-Varela et al., 2005; McFarlane et al., 2009
Corynosoma semerne	Southern elephant seals	McFarlane et al., 2009
Corynosoma australe	Southern elephant seals	Garcia-Varela et al., 2005; Sardella et al., 2005; McFarlane et al., 2009
Corynosoma evae	Leopard seals	Nickol et al., 2002; Garcia-Varela et al., 2005; Aznar et al., 2006
Arthropods		
Halarachne spp.	Weddell seals	McFarlane et al., 2009

II. OBJECTIVES

The Antarctic region is considered an isolated environment. Geographically, the Southern Ocean constitutes a closed ecosystem delimited by the Antarctic Convergence, acting as a biological barrier that hampered the crossing of birds and mammals. However, the risk of introduction and spread of diseases by wildlife and humans have been recognised as a concern by some specialists for decades (Shirihai, 2002). Disease-causing agents are present in Antarctic wildlife, some of which might be restricted to this region, while others may have a worldwide distribution. Until recent, no diseases have been demonstrated to be introduced or spread by human activities to Antarctic wildlife, although no systematic studies have been undertaken. In addition of mortality events in which diseases have been suspected and reported in Antarctic wildlife, only a few have been investigated and the causes of others are still not known, indicating a lack of information related to diseases affecting Antarctic populations (Kerry and Riddle, 2009).

In this aspect, the Antarctic Treaty indicates that science is to be conducted to "preservation and conservation of the living resources" (Article IX) (Rothwell, 2009). More recently, the report of the Committee for Environmental Protection (CEP IV) celebrated in Madrid, Spain (June 9-13, 2003) during the XXVI Antarctic Treaty Consultative Meeting indicate that "…Parties should continue to conduct research relevant to cumulative impacts, and in particular to study disturbed versus undisturbed areas…". It also indicates that "…marine species may be given special protection… Marine mammals are considered good sentinels of aquatic ecosystems. However, information about diseases in Antarctic marine mammals is scarce and fragmented, therefore it is important to monitor their health in order to identify potential sources of infection, routes of transmission of disease causing agents and dissemination of diseases.

To contribute increasing the information related to diseases affecting Antarctic pinnipeds and in response to the demands of the Antarctic Treaty System through the Consultative Parties, the main objective of this Doctoral Thesis has been to evaluate the presence and distribution of relevant parasites in Antarctic pinnipeds. In addition, to determine whether the presence of some of the parasites in the Antarctic fauna are endemic or could be influenced by the human impact in the region, some of the

parasites investigated are zoonotic agents closely related to antropogenic impact and environmental contamination worldwide. For these purposes, faecal and blood samples of pinnipeds have been analysed. Samples of the phocids Weddell seals (*Leptonychotes weddellii*), Crabeater seals (*Lobodon carcinophagus*), Leopard seals (*Hydrurga leptonyx*), Southern elephant seals (*Mirounga leonina*) and the otariid Antarctic fur seals (*Arctocephalus gazella*) were obtained in the years of 2006, 2007, 2010 and 2011 from different locations along the West coast of Antarctic Peninsula in a latitudinal gradient covering 5 degrees of latitude (ranging from 62º15'S; 58º37'W-67º46'S; 68º43'W), distances greater than 600 km, differences in mean annual temperatures of up to 2ºC and marked difference in human activity. This study was addressed by the following specific objectives.

Objective 1 (Chapter 2)

Detection of the systemic parasite *Toxoplasma gondii* in Antarctic pinnipeds

To fulfil the first objective aiming at the detection of systemic parasites commonly linked to environmental contamination possibly due to antropogenic activities in Antarctic pinnipeds, we investigated the presence of the zoonotic protozoan *Toxoplasma gondii.* The determination was based on the detection of specific antigen IgG against *T. gondii* in sera of samples collected from different pinniped species distributed along the South Shetland Islands and vicinities of the Antarctic Peninsula.

Objective 2 (Chapter 3, 4, 5 and 6)

Detection and characterisation of gastrointestinal parasites in Antarctic pinnipeds

To fulfil the second objective the following sub objectives were proposed:

Sub Objective 2.1 (Chapter 3 and 4)

Detection and characterisation of the zoonotic parasites *Cryptosporidium* and *Giardia* in faeces

The detection of the two zoonotic protozoans *Crypstosporidium* sp. and *Giardia* sp. in faecal samples of Antarctic pinnipeds was performed using two different diagnostic techniques, immnunofluorescence staining and PCR. In addition, to identify the

genotypes involved in infection and evaluate if could be related to environmental contamination in the Antarctic region, molecular characterisation was also performed.

Sub Objective 2.2 (Chapter 5 and 6)

Detection of helminth parasites in faecal samples

To complete the second objective, a survey was conducted to obtain information related to gastrointestinal parasites found in Antarctic pinnipeds. For this purpose, coprological techniques were used for detection of helminth parasites present in faecal samples. In addition, molecular techniques were also used in order to obtain more information and complete the identification of some of the parasites, previously identified morphologically.

CHAPTER II

DETECTION OF *Toxoplasma gondii* ANTIBODIES IN ANTARCTIC PINNIPEDS

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ABSTRACT

The presence of *Toxoplasma gondii* antibodies was investigated in Antarctic marine mammals. Two hundred and eleven sera from different species of pinnipeds collected in years 2007, 2010 and 2011 from different locations in the South Shetland Islands and Antarctic Peninsula were analysed using a commercially available agglutination test kit. The presence of antibodies (titres ≥ 1:25) against *T. gondii* was detected in a total of 28 animals (13.3%). Amongst animal species, percentages of detection were higher in Southern elephant seals (*Mirounga leonina*) (76.9%; 10/13) followed by Weddell seals (*Leptonychotes weddellii*) (41.9%; 13/31). Antibodies were also found in 4 of 165 (2.4%) Antarctic fur seals (*Arctocephalus gazella*) and 1 of 2 Crabeater seals (*Lobodon carcinophaga*). Highest titres (1:100–1:800) were also observed in Southern elephant seals and Weddell seals. To the best of our knowledge this is the first report on the detection of antibodies against *T. gondii* in Antarctic marine mammals.

1. Introduction

Toxoplasma gondii is an apicomplexan parasite with a worldwide distribution which affects a wide range of animals, including domestic and wild species and humans. In marine mammals, infections with *T. gondii* cause morbidity and mortality (Dubey, 2010).

In pinnipeds, clinical toxoplasmosis has been reported in a Northern elephant seal (*Mirounga angustirostris*) (Dubey et al., 2004), a Northern fur seal (*Callorhinus ursinus*) (Holshuh et al., 1985), a Pacific harbor seal (*Phoca vitulina richardsi*) (Van Pelt and Dietrich, 1973), a Hawaiian monk seal (*Monachus schauinslandi*) (Honnold et al., 2005) and California sea lions (*Zalophus californianus*) (Ratcliffe and Worth, 1951; Dubey et al., 2003). In addition, numerous serological studies have shown the presence of antibodies against *T. gondii* in true seals (Fam. Phocidae), eared seals (Fam. Otariidae) and walruses (Fam. Odobenidae) from different geographical areas which include USA, North-western Hawaiian islands, Japan, Svalvard, the Canadian Arctic, Mexico and the North-eastern Atlantic Ocean (Dubey, 2010; Jensen et al., 2010; Cabezon et al., 2011; Simon et al., 2011; Alvarado-Esquivel et al., 2012). The range of pinniped species in which *T. gondii* antibodies have been found include the Pacific harbor seal (*Phoca vitulina richardsi*) (Lambourn et al., 2001; Dubey et al., 2003), western Atlantic harbor seal (*Phoca vitulina concolor*) (Measures et al., 2004), Kuril harbour seal (*Phoca vitulina stejnegeri*) (Fujii et al., 2007), ringed seal (*Pusa hispida*), bearded seal (*Erignathus barbatus*), spotted seal (*Phoca largha*) (Dubey et al., 2003), grey seal (*Halichoerus grypus*) (Measures et al., 2004; Cabezon et al., 2011), hooded seal (*Cystophora cristata*) (Measures et al., 2004), Hawaiian monk seal (*M. schauinslandi*) (Aguirre et al., 2007), eastern-Atlantic harbor seal (*Phoca vitulina vitulina*) (Cabezon et al., 2011), California sea lion (*Z. californianus*), and the walrus (*Odobenus rosmarus*) (Dubey et al., 2003).

To the best of our knowledge, no investigations have been carried out in Antarctic pinnipeds. Marine mammals are regarded as good bio-indicators of environmental changes. However, the information available about the health status of the Antarctic marine mammals is very scarce and fragmented (Kerry et al., 2000). In addition, human derived activities in this pristine environment could be compromising these populations. In this sense recommendations have been made regarding the importance of health monitoring the Antarctic fauna (Anon, 2003). The purpose of this study was to

investigate the presence of *T. gondii* antibodies in pinnipeds from different regions in the Antarctic Peninsula.

2. Materials and Methods

2.1. Pinniped samples

Blood samples were collected during the month of February of years 2007, 2010 and 2011 from a total of 211 animals (Table 7): 31 Weddell seals (*L. weddellii*), 13 Southern elephant seals (*M. leonina*), 2 Crabeater seals (*L. carcinophagus*) and 165 Antarctic fur seals (*A. gazella*) from different locations along the west coast of the Antarctic Peninsula in a latitudinal gradient covering 5º of latitude (ranging from 62º15´S; 58º37´W-67º46´S; 68º43´W), distances greater than 600 km and differences in mean annual temperatures of up to 2ºC.

For the collection of samples, animals were randomly selected, captured and physically restrained. All captured animals were tagged with a coloured and numbered plastic tag for tracking purposes, ensuring that no animal was sampled more than once. Permissions for these activities were granted by the Spanish Polar Committee complying with the Antarctic Treaty System. Blood samples were centrifuged (700 × g for 10 min) and the sera stored at −20ºC until analysed.

2.2. Serological examination

Detection of antibodies against *T. gondii* was performed using a commercial kit based on detection of specific IgG from sera by direct agglutination (Toxo-Screen DA, BioMerieux®, France) according to the manufacturer's instructions. For initial screening, 1:25 and 1:100 final dilutions of sera were tested. Samples that showed agglutination at 1:25 were considered positive (see Section 4) and further tested for titre determination at two-fold serial dilutions from 1:25 to 1:6,400. All positive samples were retested to confirm the reliability of the results.

Table 7: Distribution of samples and *Toxoplasma gondii* antibody detection results in Antarctic pinnipeds

Animal species	Location*	Year	No. Samples	Positive	% Positive
Weddell seal (*Leptonychotes weddellii*)	Deception Island, South Shetland Islands	2007	8	0	0
		2010	14	9	64.3
		2011	6	2	33.3
	Ronge Island, Errera Channel	2010	1	0	0
	Avian Island, Marguerite Bay, Antarctic Peninsula	2010	2	2	100
	Total		**31**	**13**	**41.9**
Southern elephant seal (*Mirounga leonina*)	King George Island, South Shetland Islands	2007	6	5	83.3
	Deception Island, South Shetland Islands	2010	1	1	100
		2011	1	1	100
	Avian Island, Marguerite Bay, Antarctic Peninsula	2010	4	2	50.0
	Anvers Island, Palmer Archipelago, Antarctic Peninsula	2011	1	1	100
	Total		**13**	**10**	**76.9**
Crabeater seal (*Lobodon carcinophaga*)	Deception Island, South Shetland Islands	2007	1	0	0
		2011	1	1	100
	Total		**2**	**1**	**50.0**
Antarctic fur seal(*Arctocephalus gazella*)	Deception Island, South Shetland Islands	2007	40	0	0
		2010	44	1	22.7
		2011	48	2	41.7
	Avian Island, Marguerite Bay, Antarctic Peninsula	2010	14	0	0
		2011	15	1	66.7
	Barrientos Island, Aitcho Islands, South Shetland Islands	2011	4	0	0
	Total		**165**	**4**	**2.4**
Total			**211**	**28**	**13.3**

*Geographical coordinates: Deception Island 63º00´S; 60º40´W; Ronge Island 64º40’S; 62º40’W; Avian Island 67º46’S; 68°43’W; King George Island 62°15’S; 58°37’W; Anvers Island 64°48'S, 63°46'W; Barrientos Island 62˚24’S, 59˚44’W.

2.3. Statistical analysis

Seropositivity data with respect to animal species and year were analysed by pairs using the Chi-square or Fisher’s exact test using the Statgraphic Centurion XVI version 16.1.11, statistical software (©StatPoint Technologies, Inc., 1922–2010, Warrenton, VA, USA). Differences were considered significant at a probability level of $P < 0.05$.

3. Results

Antibodies against *T. gondii* were detected in 28 (13.3%) of the 211 Antarctic pinniped samples collected (Table 7). Percentages of detection, with titres ≥1:25, were significantly higher in Southern elephant seals (76.9%, 13/10), than in Weddell seals

(41.9%, 13/31) ($P < 0.05$), and than in Antarctic fur seals (2.4%, 4/165) ($P < 0.001$). In Crabeater seals antibodies were found in 1 of the 2 animals tested. Titres ranged from 1:25 to 1:800, most animals showing titres of 1:25 (10/28) and 1:50 (8/28). End-point titres of 1:100 (n=2) and 1:400 (n=1) were found in Southern elephant seals; and of 1:100 (n=5), 1:200 (n=1) and 1:800 (n=1) in Weddell seals.

Seropositive animals were recorded each year of the study, not finding any statistical differences, and in four of the six locations from which samples were screened: Avian Island (5/35), Deception Island (17/165), King George Island (5/6), and Biscoe Point (1/1).

4. Discussion

To the best of knowledge, the study presented here constitutes the first report on the presence of *T. gondii* antibodies in Antarctic pinnipeds. Our serological data using agglutination suggest an unexpected high level of exposure in these populations, especially in Southern elephant seals and in Weddell seals where *T. gondii* antibodies were found in 76.9% and 41.9% of the samples analysed, respectively. In Crabeater seals, one of the two animals (50%) analysed also showed antibodies against *T. gondii*. Direct agglutination has been widely used to detect *T. gondii* antibodies in a variety of marine mammals (Mikaelian et al., 2000; Dubey et al., 2003; Thoisy et al., 2003; Measures et al., 2004; Dubey et al., 2005; Aguirre et al., 2007; Dubey et al., 2008). It has been reported that amongst different serological tests available, the agglutination test is most useful because it is species independent (does not require species specific conjugates), sensitive, and specific (Desmonts and Remington, 1980; Dubey, 2002). In particular, the commercial kit used in the present study has proven its usefulness at detecting *T. gondii* antibodies in experimentally infected seals (Gajadhar et al., 2004) and in Arctic seals (Jensen et al., 2010; Simon et al., 2011). Most authors have considered titres of 1:25 as positive, although as low as 1:2 or 1:5 have also been reported in other hosts (Dubey and Jones, 2008). In addition, an agglutination titre of 1:25 was found in a beluga whale (*Delphinapterus leucas*) with confirmed toxoplasmosis, which led Mikaelian et al. (2000) to suggest that a low titre might be indicative of infection. Therefore in this study evidence of exposure was considered at titres ≥1:25. We found that most titres were low (1:25, 1:50 and 1:100). This is consistent with previous studies using the direct agglutination test in which low titres

have been reported in pinnipeds (Dubey, 2010; Jensen et al., 2010; Cabezon et al., 2011; Simon et al., 2011; Alvarado-Esquivel et al., 2012).

Seropositive animals were recorded in most of the locations included in the study. However, Palacios et al. (2010) did not find antibodies against *T. gondii* in penguins in these locations. The Antarctic pinnipeds analysed here, particularly Weddell seals and Southern elephant seals, have a widespread and circumpolar distribution around Antarctica, as well as occurring on Sub Antarctic islands. Occasional wandering individuals have also been recorded as far as Australia, New Zealand, Africa, and South America but seasonal movements are poorly known (Shirihai, 2002).

The route of *T. gondii* infection for marine mammals is not known. Felids are the only known definitive host for this parasite, playing a crucial role contaminating the environment with oocysts excreted in their faeces (Dubey, 2010). It has been suggested that contamination of sea water by freshwater run-off and sewer discharge carrying *T. gondii* oocysts from the terrestrial environment may result in infection in marine mammals (Miller et al., 2002; Conrad et al., 2005; Dabritz et al., 2007). Furthermore it has been experimentally demonstrated that *T. gondii* oocysts can sporulate in sea water and remain infectious for mice for up to 24 months (Lindsay and Dubey, 2009). There is no wild felid fauna in Antarctica and in 1991 the Madrid Protocol on Environmental Protection to the Antarctic banned all introduced species from the Antarctic to protect the native wildlife from introduced diseases, including cats. However, felids are present in the Sub Antarctic regions, areas within the normal distribution range of the animal species analysed here. Recently, Afonso et al. (2007) reported high seroprevalence values (51.09%) in feral cats in the Kerguelen archipelago in the Sub Antarctic region. Therefore exposure to *Toxoplasma* might have occurred outside Antarctica and is in agreement with the higher detection rates in Southern elephant seals and Weddell seals found here, which show wider distribution and migratory ranges. In addition, the differences observed here between the animal species analysed could be due to their different feeding habits. While Antarctic fur seals and Crabeater seals feed primarily on krill taking occasionally fish and cephalopods, the diet of the Weddell seal consists mainly on fish, eating also cephalopods and crustaceans and Southern elephant seals eat mainly cephalopods and fish consuming occasionally shellfish (Shirihai, 2002). It has been shown that *T. gondii* oocysts may be concentrated by marine filter-feeding invertebrates, bivalve molluscs, both under laboratory conditions (Lindsay et al., 2001;

Arkush et al., 2003; Lindsay et al., 2004) and in the wild (Miller et al., 2008) which may act as a source of infection for marine wildlife. In our study, only Southern elephant seals might sporadically consume shellfish, not representing therefore a likely route of transmission for Antarctic pinnipeds. However, recent studies performing experimental exposure of filter feeder fish to *T. gondii* oocysts have indicated that migratory fish may play a role in the transmission of *T. gondii* in the marine environment (Massie et al., 2010).

Further investigations are needed to elucidate the likely transmission pathways of *T. gondii* in marine mammals as well as the presence of *T. gondii* in the Antarctic marine ecosystem.

Acknowledgements

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CHAPTER III

DETECTION AND CHARACTERISATION OF A *Cryptosporidium* ISOLATE FROM A SOUTHERN ELEPHANT SEAL (*Mirounga leonina*) FROM THE ANTARCTIC PENINSULA

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ABSTRACT

The presence of *Cryptosporidium* and *Giardia* in 221 faecal samples from different species of Antarctic pinnipeds was investigated by immunofluorescence microscopy and PCR. *Cryptosporidium*, a skunk-like genotype, was detected only in a Southern elephant seal. *Giardia* was not detected. This is the first report of a *Cryptosporidium* sp. in Antarctic marine mammals.

Cryptosporidium and *Giardia* are ubiquitous protozoan parasites which infect a wide variety of hosts, including humans and domesticated and wild animals (Xiao and Fayer, 2008). In recent years, increasing research has been carried out in marine mammals since they may act as indicator species for environmental contamination with these waterborne parasites (Appelbee et al., 2005). *Cryptosporidium* oocysts and/or *Giardia* cysts have been identified in faeces or intestinal contents of various animal species, including an Australian dugong (*Dugong dugon*), California sea lions (*Zalophus californianus*), ringed seals (*Phoca hispida*), harp seals (*Phoca groenlandica*), grey seals (*Halichoerus grypus*), hooded seals (*Cystophora cristata*), bearded seals (*Erignathus barbatus*), and harbor seals (*Phoca vitulina*), as well as right whales (*Eubalaena glacialis*) and bowhead whales (*Balaena mysticetus*) from different locations worldwide (reviewed in references Appelbee et al., 2005; Hughes-Hanks et al., 2005; Dixon et al., 2008). However, no studies have been conducted on Antarctic marine mammals. Regarding the species or genotypes involved, the presence of zoonotic assemblages A and B of *Giardia duodenalis* has been commonly reported (Appelbee et al., 2005; Bogomolni et al., 2008, Dixon et al., 2008; Lasek-Nesselquist et al., 2008), as have assemblages F (Bogomolni et al., 2008) and D and novel genotypes related to the canine assemblages C and D (Gaydos et al., 2008). *Cryptosporidium hominis*, a species thought to be infective exclusively for humans, nonhuman primates, and gnotobiotic pigs (Morgan et al., 2000), has been identified only in a dugong (Hill et al., 1997). Other species reported include *Cryptosporidium muris* and two novel genotypes, designated *Cryptosporidium* sp. seal 1 and 2 (Santin et al., 2005; Bogomolni et al., 2008; Dixon et al., 2008). These studies indicate that marine mammals could represent potential zoonotic reservoirs for *Cryptosporidium* and *Giardia*, but they also reflect that human activities may have an impact on the health of marine mammals and the environment. It is therefore important to monitor the health status of wildlife in general and identify potential sources of infection and routes of transmission or dissemination, particularly in unspoiled areas.

In the present study, we investigated the presence of the zoonotic parasites *Cryptosporidium* and *Giardia* in Antarctic pinnipeds in order to determine the occurrence of these parasites, to identify the species or genotypes involved in infection, and to evaluate whether they might be linked to anthropogenic activities.

A total of 221 fresh faecal samples from different pinnipeds populations from different locations along the west coast of the Antarctic Peninsula (ranging from 62°15´S to 58°37´W-67°46´S and 68°43´W) (Fig. 21) were collected from the ground during the month of February in 2006 and 2007. These included samples from 31 Weddell seals (*Leptonychotes weddellii*), 2 Crabeater seals (*Lobodon carcinophagus*), 4 leopard seals (*Hydrurga leptonyx*), 53 Southern elephant seals (*Mirounga leonina*), and 131 Antarctic fur seals (*Arctocephalus gazella*).

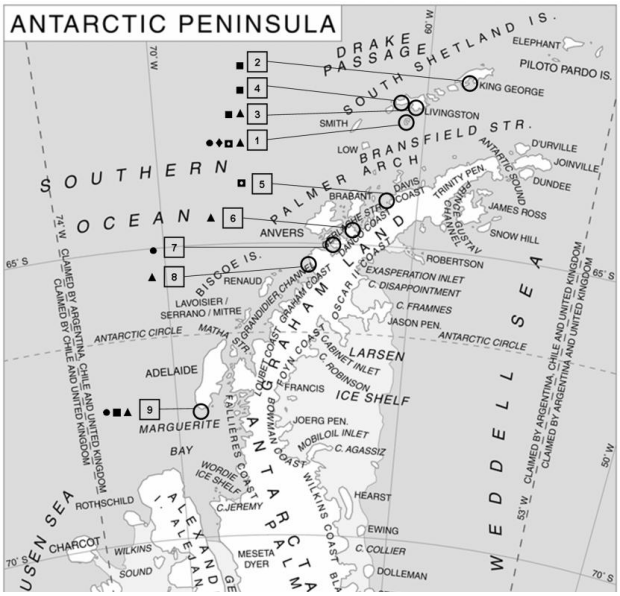

Figure 21: Locations of sampling areas and animal distribution (adapted from Wikimedia Commons [Giovanni Fattori]). 1, Deception Island, South Shetland Islands; 2, King George Island, South Shetland Islands; 3, Hannah Point, Livingston Island, South Shetland Islands; 4, Byers Peninsula, Livingston Island, South Shetland Islands; 5, Cape Primavera, Antarctic Peninsula; 6, Ronge Island, Errera Channel; 7, Paradise Bay, Antarctic Peninsula; 8, Galindez Island, Argentine Islands; 9, Avian Island, Marguerite Bay, Antarctic Peninsula. ● Weddell seal (*Leptonychotes weddellii*); ⬣ Crabeater seal (*Lobodon carcinophagus*); ◆ Leopard seal (*Hydrurga leptonyx*); ■ Southern elephant seal (*Mirounga leonina*); ▲ Antarctic fur seal (*Arctocephalus gazella*).

Faecal slides were prepared on the same day of sample collection by spreading in triplicate approximately 40 µl of homogenized sample onto a microscope glass slide and fixing in methanol and were stored at -20°C. Faecal samples were kept at -4°C without preservatives for periods of up to 2 months until they were analysed.

1. Detection of *Cryptosporidium* and *Giardia*

Immunofluorescence staining was performed using the *Crypto*/*Giardia* Cel IF test (Cellabs Pty. Ltd., Brookvale, Australia) on faecal slides. The numbers of oocysts/cysts on slides were determined at magnification x400, and the means for 20 fields were calculated. If no oocysts/cysts were seen in 20 fields, the entire slide was examined. To approximately calculate the number of oocysts, the following categories were established: no oocysts; <1 oocyst per field; 1 to 10 oocysts per field; 11 to 100 oocysts per field; and >100 oocysts per field, which corresponded to approximately 0, $<10^3$, 10^3 to 10^4, 10^4 to 10^5, and $>10^5$ oocysts per g (or per ml) of faeces, respectively, performing spiking trials with control *C. parvum* oocysts in negative seal faecal samples. Faecal slides were prepared as described above.

DNA purification was performed using 200 to 300 µl of homogenized faeces and comprised oocyst/cyst disruption with zirconia beads in the presence of guanidinium thiocyanate, followed by purification with activated silica as previously described (McLauchlin et al., 1999). Positive (both positive faecal samples, bovine and canine, and control oocysts/cysts of *C. parvum* and *G. duodenalis* assemblage D) and negative controls were included in each batch.

For *Cryptosporidium*, a nested PCR procedure was performed for amplification of an 827 to 840 bp polymorphic fragment of the 18S ribosomal DNA (rDNA) (Xiao et al., 1999). In addition, a 446 bp fragment of the HSP70 gene was amplified using the primers HSPF4 and HSPR4 (Morgan et al., 2001). For *Giardia*, a nested procedure was performed to amplify a 511 bp fragment of the beta-giardin gene (Lalle et al., 2005). Positive and negative controls were included for all PCRs.

The presence of *Cryptosporidium* oocysts was detected by immunofluorescence and PCR only in one sample (0.45%) from a Southern elephant seal collected in the southernmost sampling area, Avian Island, in 2006. The presence of *Giardia* was not detected by either method in any of the samples analysed. These results suggest that the presence of these parasites in these regions is rare. The detection methods used in this study are widely applied and have proven very sensitive. However, we did not perform concentration of the faecal material or purification of oocysts/cysts, and therefore samples with very low numbers of oocysts/cysts might not have been detected. Nevertheless, we consider the application of both immunofluorescence microscopy and PCR to enhance the detection power. To our knowledge, our study constitutes the first

report of the presence of *Cryptosporidium* in Antarctic marine mammals. Few studies have been conducted in this respect; Fayer (2008) has indicated that Antarctica was the only continent in which the presence of *Cryptosporidium* had not been reported. However, recently the presence of *Cryptosporidium* oocysts in Antarctic adelie (*Pygoscelis adeliae*) and gentoo penguins (*Pygoscelis papua*) from Ardley Island, South Shetlands (62°13´S, 58°54´W) has been described (Fredes et al., 2007b; Fredes et al., 2008), although other studies in different locations have reported the absence of *Cryptosporidium* and/or *Giardia* in gentoo and adelie penguins and in chinstrap penguins (*Pygoscelis antarctica*) (Fredes et al., 2007a; Palacios et al., 2010). In contrast to the results presented here, prevalence rates of *Cryptosporidium* in pinnipeds from other less-preserved areas range from 16 to 24% (Hill et al., 1997; Deng et al., 2000; Hughes-Hanks et al., 2005; Santin et al., 2005; Bogomolni et al., 2008), whereas for *Giardia*, they range from 12 to 64.5% (Olson et al., 1997; Measures and Olson, 1999; Hughes-Hanks et al., 2005; Bogomolni et al., 2008). This indicates that the Antarctic fauna has suffered from a lower level of exposure to these agents, which is in agreement with the relative geographical and biological isolation of the Antarctic continent. However, further studies are needed to investigate their potential sources of infection and to monitor their possible introduction and dissemination in this singular environment.

The number of oocysts observed per field was 5, which approximately corresponded to 10^3 to 10^4 oocysts per g of faeces, suggesting infection in this animal rather than passive transfer. In contrast to other animal species analysed in this study, whose migratory and foraging ranges seem to be confined to the Antarctic region, the Southern elephant seal is widely distributed in the Southern hemisphere. Therefore, infection in this animal might have been acquired outside Antarctica and introduced into the area. Nevertheless, this might have important implications for the Antarctic fauna, since these animals can act as reservoirs of the disease to those in close vicinity and also disseminate these pathogens to different geographic locations in the marine and terrestrial environments.

2. Molecular characterisation of the *Cryptosporidium* isolate

18S rDNA and HSP70 positive amplicons were directly sequenced in both directions at the Unidad Genómica del Parque Científico de Madrid. Sequences were

analysed using the BioEdit Sequence Alignment Editor software program, v.7.0.1 (Fredes et al., 2008; Hall, 1999). Multiple alignments were performed using the ClustalW software program, and neighbor-joining trees were constructed from the aligned sequences using the MEGA software program, version 4 (Tamura et al., 2007). Analysis of the 828 bp 18S rDNA fragment revealed a 99.5% to 99.6% similarity to the sequences of the *Cryptosporidium* skunk genotype published in GenBank, isolated from a skunk (accession no. AY120903), from environmental samples (AY737559 and EU825736), and from a human patient (EU437415). The sequence obtained for this isolate showed the deletion of a T base at position 285 with respect to the sequence under accession no. AY120903 and the insertion of a T base at positions 456, 457, and 508 with respect to all four sequences. The neighbor-joining analysis of the multiple alignment performed with *Cryptosporidium* sequences retrieved from the GenBank database (Fig. 22) showed that this genotype clusters closely with other intestinal *Cryptosporidium* species, such as *C. parvum*, *C. hominis*, *C. wrairi*, *C. meleagridis*, and *C. suis*, but constitutes a separate, distinct group.

Sequence and phylogenetic analysis of the HSP70 gene confirmed these results. The highest similarities, 99.8%, were observed with the *Cryptosporidium* skunk genotype isolated from a skunk (accession no. AY120917) and from a human patient (EU437414). The sequence obtained in this study varied by a T/C substitution at position 75 and an A/G substitution at position 240 with respect to the sequence under accession no. AY120917 and EU437414, respectively. Previously, the *Cryptosporidium* skunk genotype had been isolated from skunk, raccoon, eastern squirrel, opossum, river otter (Xiao and Fayer, 2008), environmental samples (Perz and Le Blancq, 2001; Jellison et al., 2009), and, also recently, from humans (Robinson et al., 2008; Davies et al., 2009). It was initially suggested that this genotype might be a fur-bearing wild mammal host-adapted type with no significance for public health (Xiao and Fayer, 2008). However, the identification of this genotype in a human patient who had suffered from diarrhea (Robinson et al., 2008) demonstrates that it is capable of causing infection in other hosts and could disseminate through different routes of transmission. More molecular data identifying the species and genotypes present in marine mammals are needed to compare with new and existing data from humans and other terrestrial animals in order to evaluate the potential impact of human activities on these populations.

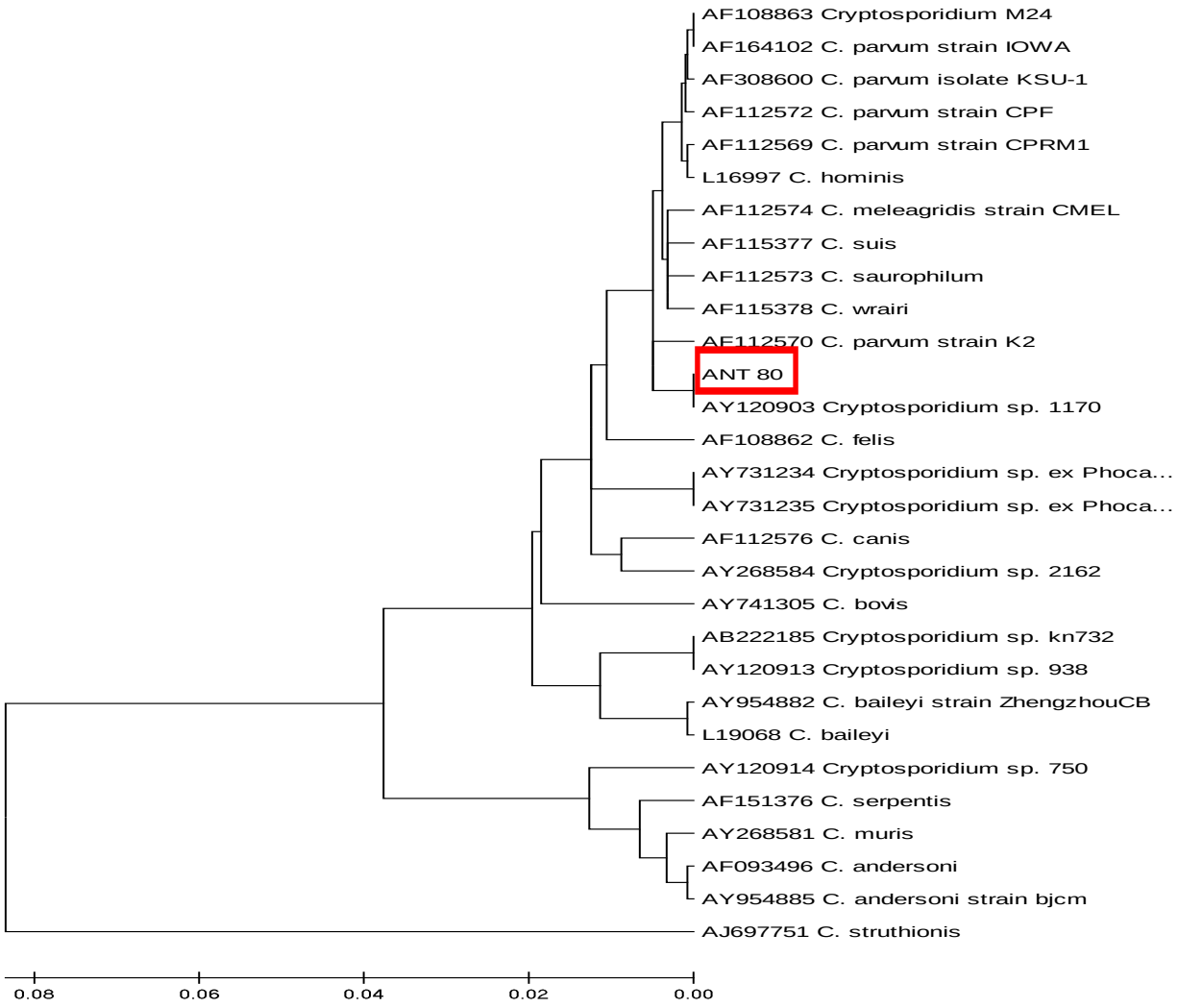

Figure 22: Phylogenetic relationships between the Southern elephant seal isolate ANT 80 (in box) and published *Cryptosporidium* species or genotypes, inferred by neighbor-joining analysis of the 18S rDNA fragment. Evolutionary distances were calculated by the Kimura-2 parameter model using *Eimeria tenella* as an outgroup.

3. Nucleotide sequence accession numbers

The nucleotide sequences generated in this study have been deposited in the GenBank database under accession numbers GQ421425 and GQ421426.

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CHAPTER IV

DETECTION OF A NOVEL GENOTYPE OF *Cryptosporidium* IN ANTARCTIC PINNIPEDS

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ABSTRACT

A study was conducted to investigate the presence of *Cryptosporidium* and *Giardia* in Antarctic marine mammals. A total of 270 faecal samples from different species of pinnipeds from different locations in the South Shetland Islands and Antarctic Peninsula were analysed by immunofluorescence microscopy and PCR. *Cryptosporidium* was detected by PCR in three samples from Southern elephant seals (*Mirounga leonina*) and 2 Weddell seals (*Leptonychotes weddellii*). However, no oocysts were observed in any of the samples by immunofluorescence microscopy. Molecular characterisation of the isolates, using the 18S rDNA, the HSP70 and the COWP loci, revealed the presence of a *Cryptosporidium* sp., previously reported from an Antarctic Southern elephant seal, in the elephant seals and a novel genotype in Weddell seals. *Giardia* could not be detected in any of the samples analysed.

1. Introduction

Cryptosporidium spp. and *Giardia duodenalis* (syn. *G. intestinalis*, *G. lamblia*) are protozoan parasites which infect a wide variety of hosts including humans and domesticated and wild animals worldwide (Xiao and Fayer, 2008). Currently, the genus *Cryptosporidium* contains up to 22 species and over 40 genotypes, while *Giardia duodenalis* includes 7 assemblages or genotypes, designated A through G (Fayer, 2010; Fayer et al., 2010; Robinson et al., 2010; Feng and Xiao, 2011; Ren et al., 2012). In addition, an assemblage H has been recently described in seals (Lasek-Nesselquist et al., 2010). Proper identification and characterisation of the species and genotypes involved in infection are needed to elucidate the routes of transmission. Traditionally, species were primarily differentiated according to host specificity, oocyst or cyst morphology and site of infection (Fayer, 2010). However, taxonomy based on these criteria has proven inadequate. Furthermore, genetic analysis has shown that these genera are complex. The advent of molecular characterisation tools has greatly contributed to establishing a correct taxonomy for both parasites setting the basis for a better understanding of the diseases they cause and their epidemiology.

In the last years increasing research has been carried out on marine mammals since they may act as indicator species for environmental contamination with these waterborne parasites (Appelbee et al., 2005). *Cryptosporidium* spp. oocysts and *Giardia* spp. cysts have been identified in different pinniped species which include California sea lions (*Zalophus californianus*), bearded seals (*Erignathus barbatus*), ringed seals (*Phoca hispida* syn. *Pusa hispida*), harp seals (*Pagophilus groenlandica*), grey seals (*Halichoerus grypus*), hooded seals (*Cystophora cristata*), harbour seals (*Phoca vitulina*), mainly from different locations in North America and an Antarctic Southern elephant seal (*Mirounga leonina*) (reviewed in *Chapter III*). Molecular analyses identified *Cryptosporidium muris* and two *Cryptosporidium* seal genotypes, seal genotypes 1 and 2, in ringed seals in Canada (Santin et al., 2005). Recently, two additional novel *Cryptosporidium* genotypes have been described in an Antarctic Southern elephant seal (*M. leonina*) and in a harp seal (*P. groenlandicus*) from the Gulf of Maine (*Chapter III*; Bass et al., 2012). *Giardia duodenalis* Assemblage A was identified in harp and hooded seals from Canada (Appelbee et al., 2005), Assemblage B in a harbour seal in the USA as well as in ringed seals in Canada, both Assemblages A and B in a harp seal and Assemblage F-like in mixed grey/harbour seal populations from beaches in the USA (Bogomolni et al., 2008; Dixon et al., 2008;

Lasek-Nesselquist et al., 2008). A further study has identified the canine genotype D and a novel genotype related to Assemblages C and D in faeces of harbour seals from Washington State´s marine waters (Gaydos et al., 2008). These studies highlight the need for more research that can provide additional information on the diversity and host range of these groups of parasites.

The purpose of this study was to further investigate the presence of *Cryptosporidium* and *Giardia* in pinnipeds from different regions in the Antarctic Peninsula.

2. Materials and Methods

2.1. Faecal samples

A total of 270 faecal samples from different pinniped populations from Deception Island, and other areas in the South Shetland Islands and Antarctic Peninsula were collected during the month of February in both 2010 and 2011 (Table 8). These included samples from Weddell seals (*Leptonychotes weddellii*), Southern elephant seals (*M. leonina*), and Antarctic fur seals (*Arctocephalus gazella*). Fresh samples were collected from the ground.

After sample collection, faecal slides were prepared, fixed in methanol, and stored at −20 ºC until analysed. Faecal samples were kept at +4 ◦C without preservatives for periods up to 2 months when they were received and analysed in the laboratory.

2.2. *Cryptosporidium* and *Giardia* detection and characterisation

Immunofluorescence staining was performed using the *Crypto*/*Giardia* Cel IF Test (Cellabs Pty Ltd., Brookvale, Australia) according to the manufacturer's instructions. Oocyst/cyst disruption and DNA purification from faecal samples were performed as described previously (McLauchlin et al., 1999).

For *Cryptosporidium* detection and characterisation, a nested PCR procedure was performed for amplification of an 827–840 bp polymorphic fragment of the 18 rDNA (Xiao et al., 1999; Xiao et al., 2000). For further characterisation, a 446 bp fragment of the HSP70 and a 550 bp fragment of the COWP genes were amplified according to the protocols described by Morgan et al. (2001) and Pedraza-Díaz et al. (2001), respectively.

Table 8: Distribution of samples and results obtained

Name	No. Samples	Year	Location	Positive	Cryptosporidium species/genotype		
					18S rDNA	HSP70	COWP
Weddell seal (*Leptonychotes weddelli*)	9	2010	Deception Island, South Shetland Islands	1	*Cryptosporidium* sp. Weddell seal genotype	*Cryptosporidium* sp. Weddell seal genotype	*Cryptosporidium* sp. Weddell seal genotype
	1		Rongé Island, Errera Channel				
	3	2011	Deception Island, South Shetland Islands	1	*Cryptosporidium* sp. Weddell seal genotype	nd	nd
	1		Byers Peninsula, Livingston Island, South Shetland Islands				
Total	**14**			**2**			
Southern elephant seal (*Mirounga leonina*)	16	2010	King George Island, South Shetland Islands				
	16		Hannah Point, Livingston Island, South Shetland Islands				
	18		Byers Peninsula, Livingston Island, South Shetland Islands				
	18		Avian Island, Marguerite Bay, Antarctic Peninsula				
	3	2011	King George Island, South Shetland Islands				
	3		Hannah Point, Livingston Island, South Shetland Islands				
	15		Byers Peninsula, Livingston Island, South Shetland Islands	1	*Cryptosporidium* sp. Southern elephant seal genotype	nd	nd
	13		Avian Island, Marguerite Bay, Antarctic Peninsula	1	*Cryptosporidium* sp. Southern elephant seal genotype	*Cryptosporidium* sp. Southern elephant seal genotype	*Cryptosporidium* sp. Southern elephant seal genotype
	7		Biscoe Point	1	*Cryptosporidium* sp. Southern elephant seal genotype	nd	nd
	2		Barrientos Island				
Total	**111**			**3**			

continue Table 8

Name	No. Samples	Year	Location	Positive	Cryptosporidium species/genotype		
					18S rDNA	HSP70	COWP
	53	2010	Deception Island, South Shetland Islands				
	8		Avian Island, Marguerite Bay, Antarctic Peninsula				
	1		Rongé Island, Errera Channel				
	3		King George Island, South Shetland Islands				
	39	2011	Deception Island, South Shetland Islands				
	4		Avian Island, Marguerite Bay, Antarctic Peninsula				
	16		King George Island, South Shetland Islands				
	2		Byers Peninsula, Livingston Island, South Shetland Islands				
	3		Hannah Point, Livingston Island, South Shetland Islands				
	3		Barrientos Island				
	13		Penguin Island				
Total	**145**			**0**			

Antarctic fur seal (*Arctocephalus gazella*)

nd: not done

For *Giardia*, a nested procedure was performed to amplify a 511 bp fragment of the beta-giardin gene (Lalle et al., 2005).

Positive (*C. parvum* and *G. duodenalis* assemblage D) and negative controls were included for all PCRs. A 5 µl aliquot of the PCR products was examined following electrophoresis in 1% agarose/ethidium bromide gels.

Positive amplicons were purified using the GENECLEAN Turbo kit (QBiogene, CA, USA) according the manufacturer's instructions and then directly sequenced in both directions using the Big Dye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA) and a 3730 DNA analyser (Applied Biosystems, CA, USA) at the Unidad Genómica del Parque Científico de Madrid. Sequences were analysed using BioEdit Sequence Alignment Editor v.7.0.1 (7) (Hall, 1999). Multiple alignments were performed using the ClustalW program and neighbour-joining trees were constructed from the aligned sequences using the MEGA5 software (Tamura et al., 2011). Accession numbers of Genbank *Cryptosporidium* 18S rDNA sequences used in the analysis: *C. andersoni* (AF093496), *C. baileyi* (L19068), *C. bovis* (AY120911), *C. canis* (AB210854), *C. cuniculus* (EU437413), *C. fayeri* (AF112570), *C. felis* (AF108862), *C. fragile* (EU162751), *C. galli* (HM116388), *C. hominis* (AB369994), *C. macropodum* (AF513227), *C. meleagridis* (AF112574), *C. molnari* (HM243548), *C. muris* (AB089284), *C. parvum* (L16996), *C. ryanae* (AY587166), *C. serpentis* (AF151376), *C. suis* (AF115377), *C. ubiquitum* (AF442484), *C. varanii* (AF112573), *C. wrairi* (AF115378), *C. xiaoi* (FJ896050), *Cryptosporidium* sp. 80ANT (GQ421425), *Cryptosporidium* sp. Cc444 (JN858905), *Cryptosporidium* sp. ferret genotype (GQ121022), *Cryptosporidium* sp. mink genotype (EF641015), *Cryptosporidium* sp. Pg453 (JN858909), *Cryptosporidium* sp. Pv140 (JN858906), *Cryptosporidium* sp. Pv245 (JN858907), *Cryptosporidium* sp. Pv270 (JN858908), *Cryptosporidium* sp. seal genotype 1 (AY731234), *Cryptosporidium* sp. seal genotype 2 (AY731235), *Cryptosporidium* sp. skunk genotype (AY120903).

Accession numbers of Genbank *Cryptosporidium* HSP70 sequences used in the analysis: *C. andersoni* (AJ567390), *C. baileyi* (AF221539), *C. bovis* (AY741306), *C. canis* (AY120920), *C. cuniculus* (GU967462), *C. fayeri* (AF221531), *C. felis* (AF221538), *C. galli* (AY168849), *C. hominis* (EF591788), *C. meleagridis* (AF221537), *C. muris* (AF221543), *C. parvum* (EF576953), *C. ryanae* (EU410346), *C. serpentis* (AF221541), *C. suis* (DQ833281), *C. ubiquitum* (EF362483), *C. varanii* (FJ429602), *C. wrairi* (AF221536), *C. xiaoi* (FJ896041), *Cryptosporidium* Pg453 (JN860884), *Cryptosporidium*

Pv140 (JN860883), *Cryptosporidium* Pv270 (JN860882), *Cryptosporidium* sp. ferret (AF221532), *Cryptosporidium* sp. hedgehog (GQ259143), *Cryptosporidium* sp. mink (EF428201), *Cryptosporidium* sp. seal 1 (AY731236), *Cryptosporidium* sp. seal 2 (AY731237), *Cryptosporidium* sp. seal 2 (AY731238), *Cryptosporidium* sp. skunk (AY120917), *Cryptosporidium* sp. 80ANT (GQ421426).

Accession numbers of Genbank *Cryptosporidium* COWP sequences used in the analysis: *C. andersoni* (DQ989570, AY282693), *C. baileyi* (AY282698, AF266276), *C. canis* (AF266274), *C. cuniculus* (EU437411), *C. fayeri* (AY237633), *C. felis* (AY282700), *C. hominis* (AF148741, AF481960), *C. meleagridis* (AF248742, AY282694, DQ116568), *C. muris* (AF161579, AY643491), *C. parvum* (AY282696, AY282687, AY282686, AY282695, AF248743), *C. serpentis* (AF266275), *C. ubiquitum* (HM209389), *C. wrairi* (U35027), *Cryptosporidium* sp. ferret (AB469366), *Cryptosporidium* sp. mink (EU197215).

2.3. Nucleotide sequence accession numbers

The nucleotide sequences generated in this study have been deposited in the GenBank database under accession numbers JQ740100–JQ740108.

3. Results

3.1. Detection of *Cryptosporidium* and *Giardia*

Cryptosporidium was detected in samples collected from three Southern elephant seals (*M. leonina*) and two Weddell seals (*L. weddellii*) of the 111 and 14 faecal samples analysed, respectively, by PCR (Table 8). Cryptosporidial DNA was not detected in any of the 145 samples from Antarctic fur seals (*A. gazella*) analysed. No *Cryptosporidium* oocysts were observed in any of the samples by immunofluorescence microscopy.

The presence of *Giardia* could not be detected either by immunofluorescence or by PCR in any of the samples analysed.

3.2. Molecular characterisation of the *Cryptosporidium* isolates

Sequence analysis of the 840 bp 18S rDNA fragment amplified showed that the three isolates present in the Southern elephant seals were an exact match (100% similarity) to the *Cryptosporidium* isolate previously obtained from an Antarctic Southern elephant seal

(GQ421425) and closely related to the *Cryptosporidium* skunk genotype (AY120903) (Fig. 23A). The two sequences obtained from Weddell seals were identical to each other and showed the highest similarity (98.6%) with the *Cryptosporidium* ferret genotype (GQ121022), being also closely related to *Cryptosporidium* mink genotype (EF641015) and *Cryptosporidium wrairi* (AF115378) (similarities of 98.5% and 98.4%, respectively).

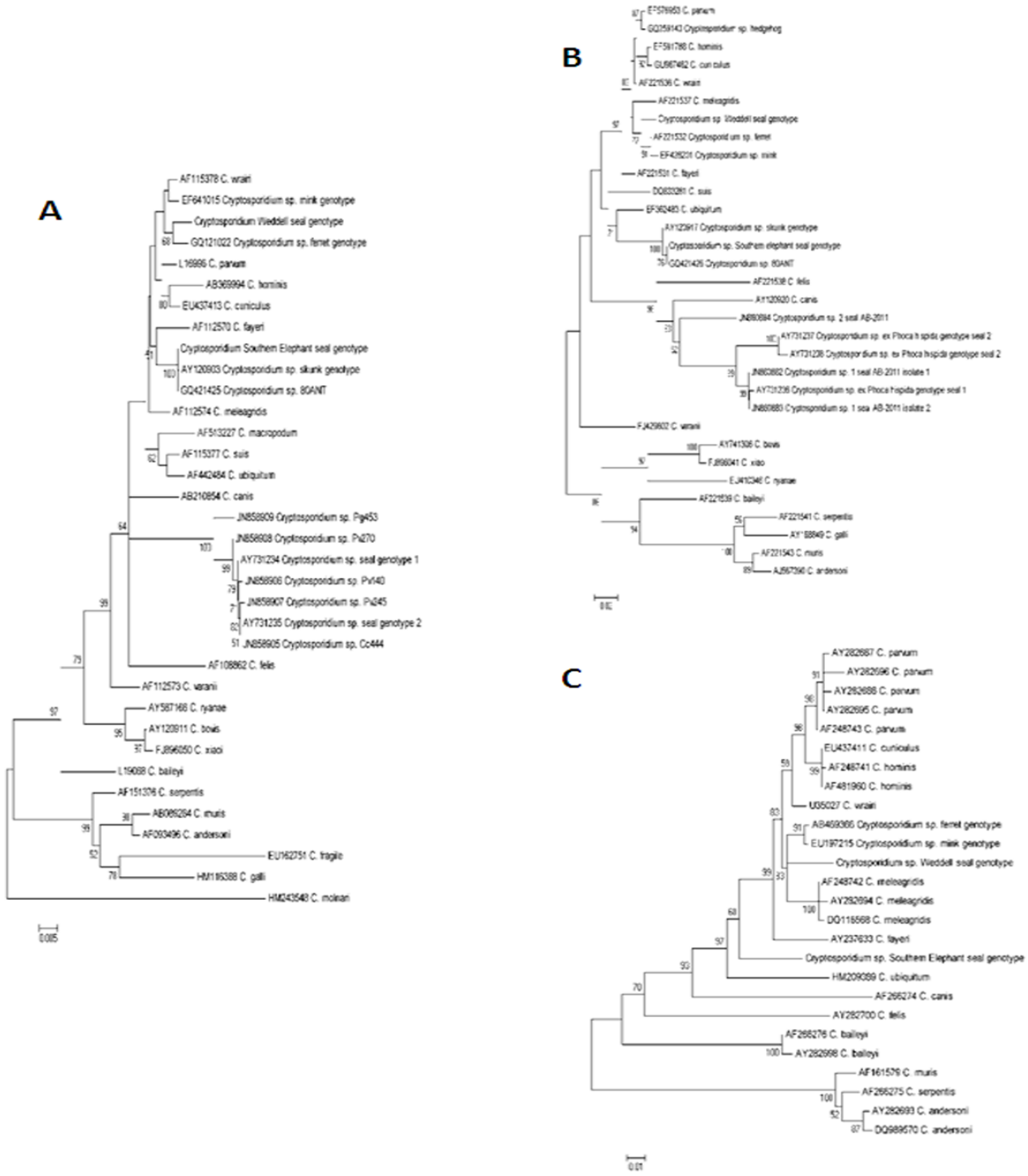

Figure 23: Phylogenetic relationships of the Southern elephant seal and Weddell seal genotypes and published *Cryptosporidium* species or genotypes inferred by neighbour-joining analysis of the 18S rDNA (A), the HSP70 (B), and the COWP (C) gene fragments. Evolutionary distances were computed using the Kimura-2 parameter method. Bootstrap values greater than 50% from 1000 replicates are shown.

For further characterisation, fragments of the HSP70 and COWP genes were amplified and sequenced from one sample of each of the 18S rDNA genotypes found (Table 8). Sequence and phylogenetic analysis of these markers confirmed these results. The neighbour-joining analyses of the multiple alignments performed with *Cryptosporidium* sequences retrieved from the GenBank database showed the genetic uniqueness of these genotypes, which cluster closely with other intestinal *Cryptosporidium* species (Fig. 23 A–C).

4. Discussion

Marine mammals are regarded as prime sentinel species for environmental changes (Bossart, 2011). However, the information available about the health status of the Antarctic marine mammals is very scarce and fragmented (Kerry et al., 2000). In addition, human derived activities in this pristine environment such as tourism and other causes like global warming could be compromising these populations. In this sense recommendations have been made regarding the importance of monitoring the health of the Antarctic fauna (Anon, 2003).

Recently, the detection of a *Cryptosporidium* genotype in an Antarctic Southern elephant seal was reported (*Chapter III*). In the present study further monitoring of the presence of the potentially zoonotic parasites *Cryptosporidium* and *Giardia* in Antarctic pinnipeds was carried out. Samples from 8 different locations along the west coast of Antarctic Peninsula in a latitudinal gradient covering 5 degrees of latitude (ranging from 62º15´S; 58º37´W–67º46´S; 68º43´W), distances greater than 600 km and differences in mean annual temperatures of up to 2º C were analysed. The results presented here confirm previous findings in that the presence of these parasites in the Antarctic region is not widespread (*Chapter III*): *Cryptosporidium* was only detected in 5 of the 270 animals sampled (1.8%) from 4 of the sampling areas included in the study, and *Giardia* was not detected in any of the animals analysed. However, the presence of *Cryptosporidium* seems to be constant in this region, since it has been detected it in three different years (2006, 2010 and 2011) (*Chapter III*; this study). The low percentages of detection found in these studies contrast with the results reported in pinnipeds from other areas in which prevalence rates of *Cryptosporidium* range from 6.5 to 24% (Hill et al., 1997; Deng et al., 2000; Hughes-Hanks et al., 2005; Santin et al., 2005; Bogomolni et al., 2008; Bass et al., 2012) whereas for *Giardia*, they range from 12 to 80% (Olson et al., 1997; Measures and Olson,

1999; Hughes-Hanks et al., 2005; Bogomolni et al., 2008; Dixon et al., 2008; Appelbee et al., 2010). It has been previously suggested that this indicates that the Antarctic fauna might experience a lower level of exposure to these agents, which is in agreement with the relative geographical and biological isolation of the Antarctic continent (*Chapter III*).

Detection of *Cryptosporidium* could be achieved by PCR but no oocysts were detected by immunofluorescence microscopy. Low numbers of *Cryptosporidium* oocysts in faecal samples could account for these results. The DNA extraction method used here utilises whole faeces, hence it is possible that target DNA is derived not only from oocysts, but also from other stages in the life cycle of this parasite as well as 'free' in the faeces (Pedraza-Díaz et al., 2001). The usefulness of PCR as diagnostic tool in the detection of *Cryptosporidium* and *Giardia* infections with intermittent shedding or low numbers of oocysts or cysts in faecal samples of different origin have been shown in previous studies (McGlade et al., 2003; Amar et al., 2004; Appelbee et al., 2010). In addition, the use of molecular methods allows the identification of the species or genotypes involved in infection and may contribute to understanding the routes of transmission. This has led to the description or redescription in the past few years of several novel *Cryptosporidium* species or genotypes, such as *C. ubiquitum* (Fayer et al., 2010), *C. ducismarci* (Traversa, 2010), *C. cuniculus* (Robinson et al., 2010), *C. tyzzeri* (Ren et al., 2012), or *C. viatorum* (Elwin et al., 2012) amongst others, or the *Giardia duodenalis* assemblage H (Lasek-Nesselquist et al., 2010).

Although the knowledge regarding the presence of *Cryptosporidium* and *Giardia* in marine mammals is increasing, few studies have identified the species and genotypes involved in infection. *Cryptosporidium hominis*, a species thought to be infective exclusively to humans, non-human primates and gnotobiotic pigs (Morgan et al., 2000) has only been identified in a dugong (Hill et al., 1997). Other species reported include *C. muris*, two seal genotypes, designated *Cryptosporidium* sp. seal 1 and 2 in ringed seals (Santin et al., 2005; Dixon et al., 2008); and a novel genotype from a harp seal (Bogomolni et al., 2008; Bass et al., 2012). Our studies have led to the description of a further two novel *Cryptosporidium* genotypes in Antarctic pinnipeds. The multilocus analysis performed, which included three of the most commonly used markers, 18S rDNA, HSP70 and COWP genes, has shown that these genotypes are more closely related to previously described *Cryptosporidium* genotypes in ferrets and mink and other intestinal *Cryptosporidium* species than to those reported from seals. Therefore the findings reported

here further widen the range of both *Cryptosporidium* host species and the parasite's species or genotypes and highlight the need for further studies to contribute to the understanding of the taxonomy and epidemiology of cryptosporidiosis.

The Antarctic pinnipeds analysed in this study, particularly Weddell seals and Southern elephant seals, have a widespread and circumpolar distribution around Antarctica, as well as occurring on Sub Antarctic islands. Occasional wandering individuals have also been recorded as far north as Australia, New Zealand, Africa, and South America but seasonal movements are poorly known (Shirihai, 2002). Therefore exposure to *Cryptosporidium* might have occurred outside Antarctica. This is in agreement with the higher detection rates in Southern elephant seals and Weddell seals found here, which show wider distribution and migratory ranges than Antarctic fur seals. In addition, the differences observed here between the animal species analysed could be due to their different feeding habits. While Antarctic fur seals feed primarily on krill taking occasionally fish and cephalopods, the diet of Weddell seal consists mainly of fish, although they also consume cephalopods and crustaceans. Southern elephant seals eat mainly cephalopods and fish, and occasionally shellfish (Shirihai, 2002). It has been shown that *Cryptosporidium* oocysts (and *Giardia* cysts) may be concentrated by marine bivalve shellfish (reviewed in Robertson, 2007) which may act as a source of infection for marine wildlife. In the present study, only Southern elephant seals might sporadically consume shellfish, not representing therefore a frequent route of transmission for Antarctic pinnipeds. Furthermore, the presence of *Cryptosporidium* oocysts in Antarctic adelie (*Pygoscelis adeliae*) and gentoo penguins (*Pygoscelis papua*) from Ardley Island, South Shetlands (62º13´S 58º54´W) has been recently described (Fredes et al., 2007b; Fredes et al., 2008) although there is no information available on the *Cryptosporidium* species or genotypes involved. In contrast, other studies in different locations have reported the absence of *Cryptosporidium* and/or *Giardia* in gentoo and adelie penguins as well as in chinstrap penguins (*Pygoscelis antarctica*) (Fredes et al., 2007a; Palacios et al., 2010). Nevertheless these findings might have important implications for the Antarctic fauna since these animals can act as vectors not just spreading the disease to those in close vicinity but also disseminating these pathogens to different geographic locations in the marine and terrestrial environments. Therefore, further studies are needed to expand our current knowledge of *Giardia* and *Cryptosporidium* in the marine environment.

Acknowledgements

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CHAPTER V

HELMINTH PARASITES FOUND IN FAECAL SAMPLES OF PHOCIDS FROM THE ANTARCTIC PENINSULA

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ABSTRACT

Information on helminth parasites in Antarctic phocids is scarce and fragmented. Anisakidae nematodes and Diphyllobothriidae cestodes have been reported in Antarctic and Sub Antarctic phocids, although the prevalence and health significance remain unclear. In the present study the presence of helminth parasites in faeces of *Leptonychotes weddellii*, *Hydrurga leptonyx* and *Mirounga leonina* have been investigated. Faecal samples were collected from different locations of the Antarctic Peninsula. Macroscopical inspection and standard flotation and migration techniques have been used for faecal examination. Eggs, larvae and adult parasites were found in 76.9% of samples analysed. Positive samples were from all locations surveyed and species investigated. The prevalence rate was 71.3% for *Mirounga leonina*, 95.4% for *Leptonychotes weddellii*, and 100% for *Hydrurga leptonyx.* Anisakidae (eggs and worms), Metastrongyloidea (larvae) and Diphyllobothriidae (eggs) were identified in *Mirounga leonina* and *Leptonychotes weddellii*. Metastrongyloidea (larvae) and Diphyllobothriidae (eggs) were found in *Hydrurga leptonyx*. Molecular characterisation of adult parasites found were *Contracaecum mirounga, Anisakis simplex C* and *Pseudoterranova* sp. in *Mirounga leonina;* and *Contracaecum* sp., *Contracaecum osculatum* and *Pseudoterranova* sp. in *Leptonychotes weddellii*. This study provides basic information related to the health status of phocids from the Antarctic Peninsula.

1. Introduction

The phocid population in the Antarctic environment is large, but with low variety of species including *Lobodon carcinophagus*, *Ommatophoca rossii*, *Hydrurga leptonyx*, *Leptonychotes weddellii* and *Mirounga leonina* (Riedman, 1990). Currently, information of helminth parasites in Antarctic phocids is scarce and fragmented, although some reports from Antarctic and Sub Antarctic regions are available and summarised in Table 9. Other reports using molecular tools identified mixed infections with sibling species of *Contracaecum*. In addition, a new taxon, genetically related to *Contracaecum osculatum B* has also been described in *M. leonina* from the Argentinian coast (Mattiucci et al., 2003). Likewise, Diphyllobothriidae cestodes have been frequently identified in phocid populations. In general terms, pathologies related to gastrointestinal nematodes and cestodes have been reported in Antarctic phocids. Moreover, it has been also indicated that heavy infections with gastrointestinal helminths are very common in Southern pinnipeds. Similarly, lungworms like *Parafilaroides* (Metastrongyloidea) have been described as a common underlying cause of respiratory disease in phocids. However, the prevalence and health significance in Antarctic and Sub Antarctic populations remain unclear (McFarlane et al., 2009). The aim of the present study was to investigate the presence of helminth parasites in faeces of phocids from different locations along the west coast of the Antarctic Peninsula.

2. Materials and Methods

2.1. Sample collection

A total of 212 faecal samples of *L. weddellii*, *H. leptonyx* and *M. leonina* were collected during the month of February of years 2006, 2007, 2010 and 2011 in seven different locations along the west coast of the Antarctic Peninsula. Sampled areas ranged from 62°15'S, 58°37'W to 67°46'S, 68°43'W, in a latitudinal gradient covering 5 degrees, distances greater than 600 km and differences in mean annual temperature of up to 2ºC. Sampling sites were located in some of the South Shetland Islands (Deception Island, King George Island, Barrientos Island and Livingston Island), Anvers Island, Ronge Island and Avian Island (Figure 24). In Livingston Island, two main locations were sampled, Hannah Point and Byers Peninsula.

Table 9: Helminth parasites identified in phocids from Antarctic and Sub Antarctic regions

Parasite Species	Host	Location	References
Cestoda			
Diphyllobothrium lashleyi	*L. weddellii*	N/D	Beverley-Burton, 1971
Diphyllobothrium mobile	*L. weddellii*	N/D	Beverley-Burton, 1971
Diphyllobothrium lobodoni	*L. carcinophagus*	Antarctic Pacific region	Iurkhano and Mal'tsev, 1994
Nematoda			
Anisakis. pegreffii (syn. *Stomachus similis*)	*M. leonina* *H. leptonyx*	Heard and Macquarie islands	Davey, 1971
Anisakis simplex C	*M. leonina*	Antarctic Ocean	Mattiucci and Nascetti, 2007
Contracaecum spp.	*M. leonina*	Península Valdes, Argentina	Dailey, 2001
Contracaecum ogmorhini	*H. leptonyx* *M. leonina*	South Australia Argentinian coast	Johnston and Mawson, 1945; Mattiucci et al., 2003
Contracaecum mirounga	*M. leonina*	Balleny island, King George islands, Weddell sea and Argentinian coast	Nadler et al., 2000b; Mattiucci et al., 2003; Mattiucci and Nascetti, 2007
Contracaecum radiatum	*L. weddellii* *H. leptonyx* *M. leonina*	Weddell sea and Ross seas	Nadler et al., 2000b; Mattiucci and Nascetti, 2007
Contracaecum osculatum	*H. leptonyx*	N/D	Dailey, 2001
Contracaecum osculatum D	*L. weddellii*	Weddell sea Ross sea	Orecchia et al., 1994; Mattiucci and Nascetti, 2007
Contracaecum osculatum E	*L. weddellii*	Weddell sea Ross sea	Orecchia et al., 1994; Mattiucci and Nascetti, 2007
Pseudoterranova decipiens E	*L. weddellii*	Antarctic Ocean	Mattiucci and Nascetti, 2007
Parafilaroides hydrurgae	*H. leptonyx*	South Australia and Heard Island	Mawson, 1953

Two hundred and seven faecal samples were obtained directly from the ground very close to animals. Only the top of droppings were recovered to avoid any contamination with free-living non-parasitic helminths. Thirty-nine samples were from *L. weddellii*, 164 from *M. leonina* and 4 from *H. leptonyx.* In addition, five faecal samples were collected directly from the rectum of *L. weddellii*, which were randomly selected, captured and physically restrained (permissions were granted by the Spanish Polar Committee CPE-EIA-2006-2 and CPE-EIA-2008-9, complying with the Antarctic Treaty System). The number of faecal samples per phocid species and sampling location are summarised in Table 10. All samples were fresh and animals showed no signs of

illness at the time of collection. Assessment of health status was based on body condition and normal behaviour.

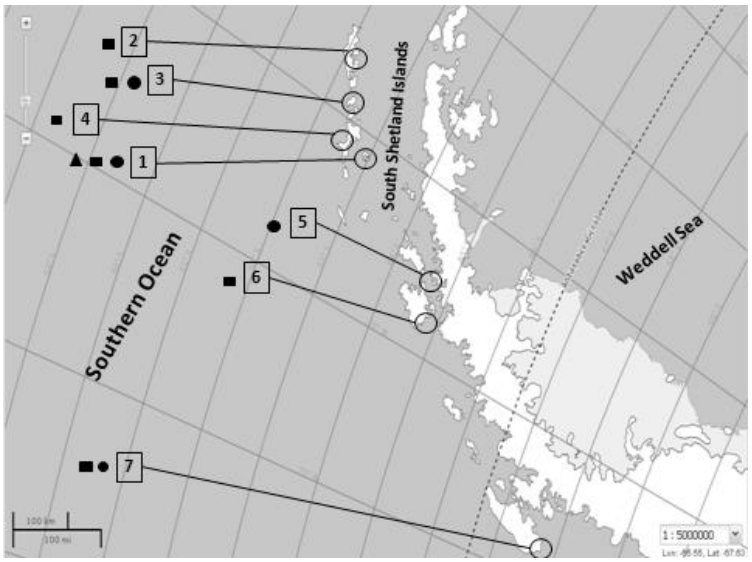

Figure 24: Locations of sampled areas and animal distribution. Image adapted from the SCAR Antarctic Digital Database (ADD). 1: Deception Island, South Shetland Islands, 2: King George Island, South Shetland Islands; 3: Barrientos Island, Aitcho Islands, South Shetland Islands; 4: Livingston Island, South Shetland Islands; 5: Ronge Island, Errera Channel; 6: Anvers Island, Palmer Archipelago, Antarctic Peninsula; 7: Avian Island, Marguerite Bay, Antarctic Peninsula. ● Weddell seal (*Leptonychotes weddellii*), ■ Southern elephant seal (*Mirounga leonina*), ▲ Leopard seal (*Hydrurga leptonyx*).

After collection, faecal samples were kept at 4°C without preservatives for further faecal analysis including macroscopic and microscopic examination. Faecal analysis was performed in the Gabriel de Castilla Military Station (Deception Island, South Shetland Islands). Afterwards, refrigerated and frozen samples were forwarded to the SALUVET Group laboratories in the Veterinary Faculty of the Complutense University of Madrid (Spain) for further morphological and molecular characterisation of parasites collected. Some parasites found in faeces collected in 2006 and 2010 were used for molecular characterisation.

2.2. Macroscopic examination

Faecal samples were examined macroscopically with a spatula. Visible parasites in stools were separated and repeatedly washed in a Petri dish containing physiological saline (pH 7.3) and stored individually in Ethanol 70% for morphological and molecular characterisation.

Table 10: Number of faecal samples collected per phocid species, date and location

Host Species	No. Samples	Year	Location
Weddell seal (*L. weddellii*)	17	2006	Deception Island, South Shetland Islands
	3		Avian Island, Marguerite Bay, Antarctic Peninsula
	10	2007	Deception Island, South Shetland Islands
	1	2010	Ronge Island, Antarctic Peninsula
	9		Deception Island, South Shetland Islands
	2	2011	Hannah Point, Livingston Island, South Shetland Islands
	1		Byers Peninsula, Livingston Island, South Shetland Islands
	1		Deception Island, South Shetland Islands
Leopard seal (*H. leptonyx*)	2	2006	Deception Island, South Shetland Islands
	2	2007	Deception Island, South Shetland Islands
Southern elephant seal (*M. leonina*)	24	2006	King George Island, South Shetland Islands
	3		Hannah Point, Livingston Island, South Shetland Islands
	7		Avian Island, Marguerite Bay, Antarctic Peninsula
	8	2007	Hannah Point, Livingston Island, South Shetland Islands
	11		Byers Peninsula, Livingston Island, South Shetland Islands
	16	2010	Hannah Point, Livingston Island, South Shetland Islands
	18		Byers Peninsula, Livingston Island, South Shetland Islands
	18		Avian Island, Marguerite Bay, Antarctic Peninsula
	16		King George Island, South Shetland Islands
	15	2011	Avian Island, Marguerite Bay, Antarctic Peninsula
	7		Anvers Island, Palmer Archipelago, Antarctic Peninsula
	3		Hannah Point, Livingston Island, South Shetland Islands
	13		Byers Peninsula, Livingston Island, South Shetland Islands
	2		Barrientos Island, Antarctic Peninsula
	3		King George Island, South Shetland Islands

2.3. Microscopic examination

For detection of parasite eggs in faecal samples, a flotation technique was performed using a commercial kit (Ovatec®Plus, Synbiotics Corporation, USA) according to the manufacturer´s instructions. In addition, approximately ten grams of faeces were examined by means of larvae migration technique using a Baermann apparatus (Kaufmann, 1996). Positive samples were preserved in Ethanol 70% for subsequent molecular characterisation.

2.4. Morphological characterisation

Of each worm, the anterior and posterior tips were preserved and cleared in lactic acid-phenol (1:1) for morphological identification. The remaining part was preserved in Ethanol 70% for molecular characterisation.

Parasites were identified according to Foreyt (2001) for eggs; Fagerholm (1988), Kloser and Plotz (1992), Paggi et al. (2000) and Mattiucci et al. (2005) for adult worms; and Dailey (2009) for larvae.

2.5. Molecular characterisation

DNA was extracted from excised midbody (approximately 0.5 cm) of individual adult worms or complete larvae suspensions obtained after being washed twice by centrifugation at 1,500 g for 5 min. DNA extraction was performed using a commercial kit (Durviz, Valencia, Spain) according to the manufacturer´s instructions. The large subunit of the ribosomal RNA gene (LSU rDNA) of larvae and adult parasites were amplified as described by Nadler et al. (2000b and 2005). PCR products were purified using the GENECLEAN Turbo kit (Qbiogene, CA, USA) according to the manufacturer´s instructions and directly sequenced in both directions using the Big Dye Terminator v.3.1 Cycle Sequencing kit (Applied Biosystems) and a 3730 DNA analyser (Applied Biosystems) at the Unidad Genómica del Parque Científico de Madrid. Sequences were analysed using BioEdit Sequence Alignment Editor v.7.0.1 (7) (Hall, 1999) (Copyright© 1997-2004 Tom Hall, Ibis Therapeutics, Carlsbad, CA 92008, USA). Basic Local Alignment Search (BLAST) from the National Center for Biotechnology Information (NCBI) was performed and relevant sequences were retrieved from GenBank.

2.6. Nucleotide sequence accession numbers

The nucleotide sequences generated in this study have been deposited in the GenBank database under accession numbers KC013593 - KC013600.

3. Results

3.1. Morphological characterisation

A total of 163 (76.9%) faecal samples showed the presence of eggs, larvae and/or adult worms. A 71.3% of *M. leonina* samples were positive showing Anisakidae eggs and adults (53.7%), Metastrongyloidea larvae (23.8%) and Diphyllobothriidae eggs (3%). In *L. weddellii* samples examined, 95.4% were positive for Anisakidae eggs and adults (90.9%), Metastrongyloidea larvae (9.1%) and Diphyllobothriidae eggs (63.6%). The presence and distribution of *M. leonina* and *L. weddellii* parasites along the different locations sampled are summarised in Tables 11 and 12, respectively. Faecal samples of *H. leptonyx* (n=4) from Deception Island were positive for Metastrongyloidea larvae (25%) and Diphyllobothriidae eggs (100%). Adult worms were identified morphologically as *Anisakis* sp., *Pseudoterranova* sp., and *Contracaecum* sp.

Table 11: Helminths present in faeces of *M. leonina* sampled in different Antarctic Peninsula sites. Parasite groups are expressed as % (number of positives)

Sites	Examined (positives)	Anisakidae	Metastrongyloidea	Diphyllobothriidae
Avian Island	40 (33)	62.5 (25)	15 (6)	5 (2)
Biscoe Point, Anvers Island	7 (4)	57.1 (4)	0 (0)	14.3 (1)
Byers Peninsula, Livingston Island	42 (30)	57.1 (24)	19 (8)	4.8 (2)
Hannah Point, Livingston Island	30 (19)	43.3 (13)	20 (6)	0 (0)
Barrientos Island	2 (2)	100 (2)	50 (1)	0 (0)
Potter Cove, King George Island	43 (29)	46.5 (20)	41.9 (18)	0 (0)
Total	164 (117)	53.7 (88)	23.8 (39)	3.0 (5)

3.2. Molecular characterisation

In the molecular characterisation of adults and larvae, the sequences obtained confirmed the presence of *Contracaecum mirounga*, *Anisakis simplex C* and *Pseudoterranova* sp. in *M. leonina* from Livingston Island. In Avian Island and King George Island, nematodes characterised were *A. simplex C* and *C. mirounga*. In *L.*

weddellii from Deception Island, nematodes characterised were *Contracaecum* sp., *Contracaecum osculatum* and *Pseudoterranova* sp. In Ronge Island, adult nematodes were identified as *Pseudoterranova* sp. Metastrongyloidea larvae collected from a faecal sample of *M. leonina* during 2006 were characterised as *Parafilaroides* sp.

Table 12: Helminths present in faeces of *L. weddellii* sampled in different Antarctic Peninsula sites. Parasite groups are expressed as % (number of positives)

Sites	Examined (positives)	Anisakidae	Metastrongyloidea	Diphyllobothriidae
Avian Island	3 (2)	66.7 (2)	0 (0)	66.7 (2)
Ronge Island	1 (1)	100 (1)	0 (0)	100 (1)
Byers Peninsula, Livingston Island	1 (1)	100 (1)	0 (0)	0 (0)
Hannah Point, Livingston Island	2 (2)	50 (1)	0 (0)	100 (2)
Deception Island	37 (36)	94.6 (35)	10.8 (4)	62.2 (23)
Total	44 (42)	90.9 (40)	9.1 (4)	63.6 (28)

Sequences analysis of the LSU rDNA fragments amplified showed for *Anisakis*, an exact match (100% similarity) to the *A. simplex C* sequence of the GenBank database AY821755, obtained from a *Mirounga angustirostris* from California, United States. Likewise, the sequence of *Pseudoterranova* from *L. weddellii* and *M. leonina* were identical to each other and showed 99% of similarity to the *P. decipiens* sequence of the GenBank database AY821760, obtained from a *M. angustirostris* from California, United States. For *Contracaecum* two different sequences were obtained in *L. weddellii* samples, one showed a 100% similarity to *C. osculatum baicalensis* sequence of the GenBank database AF226589, obtained from a *Phoca sibirica* from Lake Baikal, Russia. The other sequence showed 99% similarity to *C. osculatum* strain A sequence of the GenBank database AF226583, obtained from a *Erignatus barbatus* from Newfoundland, Canada. Finally, two more sequences were obtained for *Contracaecum.* The sequence from *M. leonina* was an exact match to *C. mirounga* sequence of the GenBank database AF226581, obtained from a *M. leonina* from Southern hemisphere (King George Island, Antarctica), while the one from *L. weddellii* showed the 99% similarity with the same sequence. Metastrongyloidea larvae sequence of *M. leonina* showed the highest similarity (94%) with the *Parafilaroides decorus* sequence AY292802, obtained from a *Zalophus californianus* from California, United States, and 93% to the sequence AM039757, obtained from an *Arctocephalus pusillus doriferus.* In addition, the sequence obtained has also been closely related to other parasites within

Metastrongyloidea, *Filaroides martis* (AY292795) and *Pseudalius inflexus* (AY292804), obtained from a *Mustela vison* from Canada (93% similarity) and a *Phocoena phocoena* from United States (92% similarity).

4. Discussion

The information available related to the health status of the Antarctic marine mammals is very limited (Kerry et al., 2000), particularly regarding the presence and effects of parasites. In this sense, there is a reduced understanding of the role of parasite diseases in wildlife population in this region (McFarlane et al., 2009). However, the rate of epidemics and disease in marine species in oceans are increasing and the Antarctic region is unlikely to be isolated from this event despite its apparently protected status (Harvell et al., 1999; McFarlane et al., 2009). The understanding of the continuum extending between ecological associations of Antarctic phocids and the normal commensal and/or parasitic fauna is pivotal in designing surveillance and investigative programmes (McFarlane et al., 2009). In this sense, recommendations have been made regarding the importance of health monitoring in the Antarctic fauna (Anon, 2003). The study presented here constitutes a report of the presence of gastrointestinal parasites in phocids from the Antarctic region, providing baseline data which is currently lacking for most of these species.

The analysis of faecal samples collected from phocids along the west coast of the Antarctic Peninsula showed a high number of positive samples (76.9%). Previous studies have indicated a high prevalence and intensity of nematodes and cestodes parasites in some Antarctic seals, like *H. leptonyx* and *L. weddellii* (McFarlane et al., 2009). In the study, *M. leonina, L. weddellii* and *H. leptonyx* have been infected in 71.3%, 95.4% and 100%, respectively. The parasites found in *M. leonina* and *L. weddellii* during macroscopic and microscopic examination were Anisakidae eggs and worms, Metastrongyloidea larvae and Diphyllobothriidae eggs; whereas in *H. leptonyx* the parasites found were Diphyllobothriidae eggs and Metastrongyloidea larvae. None of *H. leptonyx* samples had Anisakidae eggs or worms, differing from previous reports from the Antarctic and Sub Antarctic regions, where larval and adult stages of Anisakidae species like *Anisakis pegreffii, Contracaecum ogmorhini, Contracaecum radiatum* and *C. osculatum* have been identified (Johnston and Mawson, 1945; Mawson, 1953; Nadler et al., 2000b; Dailey, 2001). In *M. leonina, L. weddellii* and *H.*

leptonyx, Diphyllobothriidae eggs were found in low to high percentages (3%, 63.6% and 100% respectively). Mass infestations of cestodes in the gastrointestinal tract have been commonly described in Antarctic seals. It has also been described in *M. leonina*, although in lower percentage despite their more gregarious natures compared to the rest of Antarctic phocids. Probably both, diet and behavioural ecology can be relevant and presumably some Antarctic pinnipeds share parasite species (McFarlane et al., 2009), although further investigations related to this subject need to be performed.

Furthermore, with regard to locations where samples have been obtained, Livingston Island was the site with the highest number of collected samples (n=75), originating from two main areas within the Island, Hannah Point and Byers Peninsula. The percentage of positive samples was slightly higher in Byers Peninsula (72.1%) than in Hannah Point (65.6%), although there were no differences related to the presence of the different groups of parasites in the faeces. Deception Island and Avian Island were two other locations where a high number of positive samples have been observed (97.6% and 81.4%, respectively). Finally, King George Island and Anvers Island were the locations with the lowest number of positive samples (67.4% and 57.1%, respectively), although it might be considered equally high or moderate with respect to the rest of locations. In view to the currently available information, the results obtained in the study can be considered the first report on helminth parasites in phocids from these particular locations of the Antarctic Peninsula.

The presence of parasites in phocid populations does not necessarily mean that clinical signs will develop succumbing to disease (Kerry and Riddle, 2009). Dearborn (1965) reported that severe nematodes and cestodes burdens are common for *L. weddellii* in the Antarctic environment. In addition, pathologies related to these infections, especially on *L. weddellii*, have been also reported (McFarlane et al., 2009). In the present study, there was no evidence of illness or pathologies related to the presence of these parasites in any of the sampled animals, although parasite burden was not determined.

Some helminth species reported in the literature have undergone subsequent taxonomic revision, and research using molecular techniques has turned into a valuable tool illustrating the complexities of host specificity and revealed cryptic species among parasites infecting marine mammals (McFarlane et al., 2009). In the study the presence of *A. simplex C* and *C. mirounga* in *M. leonina* from Livingston Island, Avian Island

and King George Island was confirmed. Beside these two Anisakidae nematodes, *Pseudoterranova* sp. was also found in samples collected from Livingston Island. In *L. weddellii*, *Pseudoterranova* sp. was found in two sites, Deception and Ronge Islands. However, in Deception Island, additional nematodes identified were *Contracaecum* sp. and *C. osculatum.* Some of these parasites have been found in the same host, and these findings confirmed that mixed infections are common in Antarctic phocids, not only in *M. leonina* (Mattiucci et al., 2003), but also in *L. weddellii.*

Previous reports from Antarctic phocids indicated the presence of *Anisakis simplex C* and *C. mirounga* in *M. leonina* (Nadler et al., 2000b; Mattiucci and Nascetti, 2007); and *Pseudoterranova* sp. in *L. weddellii* (Mattiucci and Nascetti, 2007). In the study, the first description of two more Anisakidae nematodes has been completed in *L. weddellii*, *Contracaecum* sp. and *C. osculatum.* In addition, *Pseudoterranova* sp. has also been reported for the first time in *M. leonina*.

Some of the sequences obtained were an exact match to sequences previously reported to the GenBank database while others showed the highest similarity of 99% compared to published sequences of *P. decipiens* (AY821760), *C. osculatum* strain A genotype (AF226583) and *C. mirounga* (AF226581). The first two published sequences were from North America, while the last one was from the Antarctic region. Although molecular methods have proven to be more accurate for identification of sibling species than the morphological approach, there is an evident lack of genetic information for these parasites in the Antarctic environment. Therefore, more studies are needed to generate accurate data related to different group of parasites affecting Antarctic fauna.

Parafilaroides (Metastrongyloidea) are parasites found in the respiratory tract of pinnipeds from Antarctic environment, such as *Parafilaroides hydrurgae* in *H. leptonyx* (Mawson, 1953). In this study, one sample of *M. leonina* collected in 2006 from Deception Island was used for molecular characterisation, and *Parafilaroides* sp. was identified by sequence BLAST with similarity of 94% to *P. decorus*. The analysis retrieved other closely related parasites within Metastrongyloidea, *Filaroides martis* and *Pseudalius inflexus*. *Parafilaroides decorus* has been previously reported in *Z. californianus* from the Northern hemisphere (Dailey, 2009) and further data need to be collected to confirm the description of *P. decorus* in phocids from the Antarctic region.

The study presented here provides basic information related to the health status of Antarctic phocids and a key to identify and evaluate future changes in this pristine

environment. Furthermore, this information may also be useful to improve prevention and response measures related to wildlife conservation and environmental protection in the Antarctic region.

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CHAPTER VI

PRESENCE OF HELMINTH PARASITES IN ANTARCTIC FUR SEALS (*Arctocephalus gazella* PETERS, 1875) FROM THE ANTARCTIC PENINSULA

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ABSTRACT

In the present study the presence of helminth parasites in faecal samples of Antarctic fur seals (*Arctocephalus gazella*) from different locations along the west coast of the Antarctic Peninsula was investigated. Faecal samples were collected during the month of February of years 2006, 2007, 2010 and 2011. Standard flotation and migration techniques were used for faecal examination. Positive samples were found in all the locations sampled: Avian Island, Barrientos Island, Penguin Island, Ronge Island, Deception Island, Livingston Island and King George Island. Eggs, larvae and adults were found in 31.2% of samples collected (12% Anisakidae, 23.6% Metastrongyloidea and 1.1% Diphyllobothriidae). Helminth eggs identified belonged to the Anisakidae and Diphyllobothriidae families. Adult worms were identified morphologically as *Anisakis* sp., *Pseudoterranova* sp., and *Contracaecum* sp. Molecular characterisation of a subset of adult worms collected in Avian Island and Deception Island in 2010 confirmed the presence of *Contracaecum* sp. in Avian Island; and *Anisakis simplex C* and *Pseudoterranova* sp. in Deception Island. Metastrongyloidea larvae collected from Deception Island were characterised as *Parafilaroides* sp. This study provides basic information related to the presence and distribution of helminth parasites in Antarctic fur seals, which is currently lacking.

1. Introduction

The *Arctocephalus gazella* Peters, 1875 is an otariid species breeding from 61°S to the Antarctic Convergence, forming colonies in the Antarctic islands of South Georgia, South Orkney, South Shetlands, South Sandwich, Bouvetoya, Marion, Kerguelen, Heard, McDonald and Macquarie (Shirihai, 2002). They usually wander in the non-breeding season to the Weddell Sea, the Argentinian coast and some groups have been reported in Juan Fernandez Island and Southern Chile (Acevedo et al., 2011). Some Antarctic fur seals may also migrate northern than the Antarctic Convergence (Shirihai, 2002; Acevedo et al., 2011). The information on parasites in Antarctic fur seals is very scarce. Adult stage of gastrointestinal nematodes of the genera *Anisakis* Dujardin, 1845, *Contracaecum* Railliet & Henry, 1912 and *Pseudoterranova* Mozgovoy, 1953 have been found parasitising Antarctic marine mammals (Rocka, 2004). Larval and adult stages of *Anisakis simplex* Rudolphi, 1809 and species of the *Contracaecum ogmorhini* complex Johnston and Mawson, 1941 have been reported in otariid populations from Antarctic coast (Mattiucci et al., 2003). Several *Contracaecum* species, *Contracaecum osculatum* Rudolphi, 1802, *Contracaecum radiatum* Linstow, 1907 and *Conctracaecum mirounga* Nikolskij, 1974 have also been described in pinnipeds from various locations of the Antarctic and Sub Antarctic regions. In addition, the species *Anisakis similis* Caylis, 1920, *Anisakis physeteris* Baylis, 1923 and a species of the *Pseudoterranova decipiens* complex Krabble, 1878, *P. decipiens* E have also been found in the gastrointestinal tract of Antarctic pinnipeds (McFarlane et al., 2009). The aim of the present study was to investigate the presence of helminth parasites in faecal samples of Antarctic fur seals from different locations along the west coast of the Antarctic Peninsula by means of morphological and molecular techniques.

2. Materials and Methods

2.1. Sample collection

A total of 276 faecal samples of Antarctic fur seals were collected in different locations during the month of February of years 2006, 2007, 2010 and 2011. The colonies sampled were located along the west coast of the Antarctic Peninsula ranging from 62°15’S; 58°37’W to 67°46’S, 68°43’W (Figure 25). These locations were mainly in South Shetland Islands; including Deception Island, Penguin Island, King George

Island, Barrientos Island and Livingston Island. In addition, other locations such as Ronge Island and Avian Island were included in the study. In Livingston Island, two locations were sampled, Hannah Point and Byers Peninsula. These locations are distributed in a latitudinal gradient covering five degrees, distances greater than 600 km and differences in mean annual temperatures of up to 2°C. The numbers of faecal samples collected in each location are summarised in Table 13.

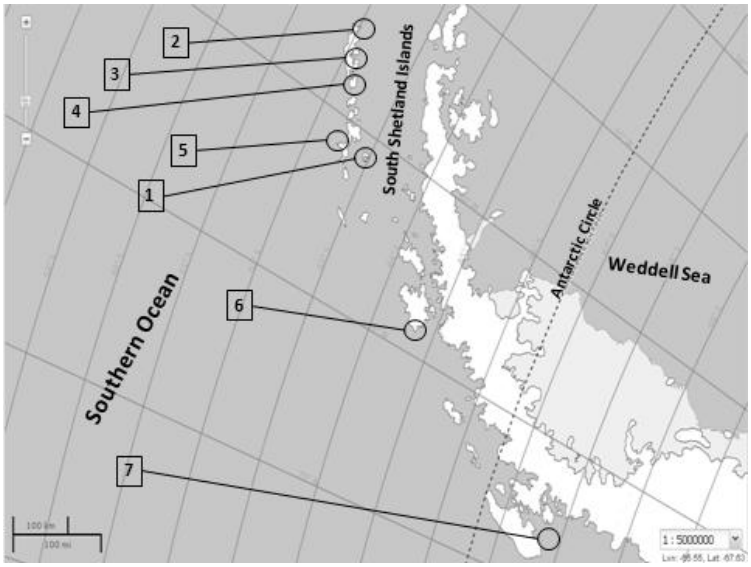

Figure 25: Sampling locations and distribution of *A. gazella* populations. Image adapted from the Antarctic Digital Database (ADD). 1: Deception Island, South Shetland Islands, 2. Penguin Island, Eastern King George Island, South Shetland islands 3: King George Island, South Shetland Islands; 4: Barrientos Island, Aitcho Islands, South Shetland Islands; 5: Livingston Island, South Shetland Islands; 6. Rongé Island, Errera Channel; 7. Avian Island, Marguerite Bay, Antarctic Peninsula.

Of the total of faecal samples, 259 were freshly obtained directly from the ground very close to animals. Only the top of drops were recovered to avoid any contamination with free-living non-parasitic helminths. In addition, 17 animals were randomly selected, captured, physically restrained and faecal samples were collected directly from the rectum. Permissions were granted by the Spanish Polar Committee CPE-EIA-2006-2 and CPE-EIA-2008-9, complying with the Antarctic Treaty System. Animals sampled showed no signs of illness at the moment of collection. Assessment of health status was based on body condition and normal behaviour.

Table 13: Distribution of faecal samples collected in the study

Year	Total	Location	Sub-location	Nº of samples collected
2006	51	Avian Island		15
		Ronge Island		5
		Livingston Island	Hannah Point	1
		Deception Island		30
2007	80	Deception Island (80)	Whalers Bay	1
			Collins Point	5
			Fumarole Bay	5
			Penfold Point	2
			Lobera Beach	67
2010	65	Avian Island		8
		Ronge Island		1
		King George Island	Potter Cove	3
		Deception Island (53)	Fumarole Bay	12
			Penfold Point	4
			Lobera Beach	37
2011	80	Avian Island		4
		Livingston Island (5)	Byers Peninsula	2
			Hannah Point	3
		King George Island	Potter Cove	16
		Barrientos Island		3
		Penguin Island		13
		Deception Island	Lobera Beach	39

After collection, faecal samples were kept at 4°C without preservatives for further diagnostic procedures, including macroscopic and microscopic examination. Faecal analysis was performed in the Gabriel de Castilla Military facilities in Deception Island, South Shetland Islands. Afterwards, refrigerated and frozen samples were forwarded to the SALUVET Group laboratories in the Veterinary Faculty of the Complutense University of Madrid (Spain) for further morphological and molecular characterisation of parasites collected. Only samples from Avian Island and Deception Island collected in 2010 were used for molecular characterisation.

2.2. Macroscopic examination

Faecal samples were examined macroscopically with a spatula. Visible parasites present in stools were separated and repeatedly washed in a Petri dish containing physiological saline (pH 7.3) and stored individually in Ethanol 70% for morphological and molecular characterisation.

2.3. Microscopic examination

For detection of parasite eggs in faecal samples, a flotation technique was performed using a commercial kit (Ovatec®Plus, Synbiotics Corporation, USA) according to the manufacturer´s instructions. In addition, approximately ten grams of faeces were examined by means of larvae migration technique using a Baermann apparatus (Kaufmann, 1996). Positive samples were preserved in Ethanol 70% for subsequent molecular characterisation.

2.4. Morphological characterisation

Of each worm, the anterior and posterior tips were preserved and cleared in lactic acid-phenol (1:1) for morphological identification. The remaining part was preserved in Ethanol 70% and used for molecular characterisation.

Parasites were identified according to Foreyt (2001) for eggs; Fagerholm (1988), Kloser and Plotz (1992), Paggi et al. (2000) and Mattiucci et al. (2005) for adult worms; and Dailey (2009) for larvae.

2.5. Molecular characterisation

DNA was extracted from excised midbody (approximately 0.5 cm) of individual adult nematodes or complete larvae suspensions obtained after being washed twice by centrifugation at 1,500 g for 5 min. DNA extraction was performed using a commercial kit (Durviz, Valencia, Spain) according to the manufacturer´s instructions. The large subunit of the ribosomal RNA gene (LSU rDNA) of larvae and adults were amplified as described by Nadler et al. (2000a and 2005). PCR products were purified using the GENECLEAN Turbo kit (Qbiogene, CA, USA) according to the manufacturer´s instructions and directly sequenced in both directions using the Big Dye Terminator v.3.1 Cycle Sequencing kit (Applied Biosystems) and a 3730 DNA analyser (Applied

Biosystems) at the Unidad Genómica del Parque Científico de Madrid. Sequences were analysed using BioEdit Sequence Alignment Editor v.7.0.1 (7) (Hall, 1999) (Copyright© 1997-2004 Tom Hall, Ibis Therapeutics, Carlsbad, CA 92008, USA). Standard Nucleotide Basic Local Alignment Search (BLAST) from the National Center for Biotechnology Information (NCBI) was performed and relevant sequences were retrieved from GenBank.

2.6. Nucleotide sequence accession numbers

The nucleotide sequences generated in this study have been deposited in the GenBank database under accession numbers KC013601 - KC013604.

3. Results

3.1. Morphological characterisation

A total of 86 (31.2%) faecal samples were positive for eggs, larvae and/or adult worms. Helminths identified were Anisakidae (12%), Metastrongyloidea (23.6%) and Diphyllobothriidae (1.1%). The presence and distribution are shown in Table 14. Helminth eggs identified were mainly Anisakidae nematodes and Diphyllobothridae cestodes and different types were identified in morphometric-basis in both parasite groups. On the other hand, Metastrongyloidea larvae collected presented the same morphology in all samples. Adult worms were identified morphologically as *Anisakis* sp., *Contracaecum* sp. and *Pseudoterranova* sp.

Table 14: Presence of helminth parasites in faeces of Antarctic fur seals from seven Antarctic Peninsula sites. Results expressed as % (number of positives)

Sites	Examined (positives)	Anisakidae	Metastrongyloidea	Diphyllobothriidae
Avian Island	27 (10)	25.9 (7)	22.2 (6)	3.7 (1)
Barrientos Island	3 (1)	0 (0)	33.3 (1)	0 (0)
Penguin Island	13 (5)	7.7 (1)	38.5 (5)	0 (0)
Ronge Island	6 (2)	16.7 (1)	16.7 (1)	0 (0)
Deception Island	202 (57)	9.4 (19)	22.3 (45)	0.5 (1)
Hannah Point (Livingston Island)	4 (1)	0 (0)	25 (1)	0 (0)
Byers Peninsula (Livingston Island)	2 (1)	50 (1)	0 (0)	0 (0)
King George Island	19 (9)	21.1 (4)	31.6 (6)	5.3 (1)
Total	276 (86)	12 (33)	23.6 (65)	1.1 (3)

3.2. Molecular characterisation

In the molecular characterisation of adults and larvae, the sequences obtained confirmed the presence of *Contracaecum* sp. in Avian Island. In populations from Deception Island, nematodes characterised were *Anisakis simplex C* and *Pseudoterranova* sp. Metastrongyloidea larvae were characterised as *Parafilaroides* sp.

Sequence analysis of the LSU rDNA fragment amplified showed for *Contracaecum* sp., a 99% similarity to the *C. osculatum* strain A sequence of the GenBank database AF226583, obtained from *Erignatus barbatus* Erxleben, 1777 from Newfoundland, Canada. For *Pseudoterranova* sp., the fragment amplified showed a 99% similarity to the *P. decipiens* sequence of the GenBank database AY821760, obtained from *Mirounga angustirostris* Gill, 1866 from California, United States. For *Anisakis*, the fragment amplified was an exact match (100% similarity) to the *A. simplex C* sequence of the GenBank database AY821755, obtained from *M. angustirostris* from California, United States. Metastrongyloidea larvae obtained in a sample from Deception Island, showed a 99% similarity to *Parafilaroides decorus* sequence of the GenBank database AM039757, obtained from *Arctocephalus pusillus doriferus* Wood Jones, 1925, which are generally confined to Southeast Australian region.

4. Discussion

Current information available regarding the health status of Antarctic fur seals is very fragmented (Kerry et al., 2000). However, some descriptions regarding the presence of *Anisakis* sp. and *Pseudoterranova* sp. in Antarctic fur seals populations from Cape Shirreff (Livingston Island) have been reported due to morphometric-basis (Diedrichs-Alvarez, 2007). To the best of our knowledge, the present study is the first report of parasitological findings performed systematically using morphological and molecular approaches in apparently healthy Antarctic fur seals from several locations along the west coast of the Antarctic Peninsula.

In the study, faecal examination revealed a moderate (31.2%) prevalence rate in populations examined over several years, providing important baseline data so far unknown. Parasitised animals were recorded in all locations included in the study with differences in parasite diversity. Helminth parasites found belonged to Anisakidae, Metastrongyloidea and Diphyllobothriidae. The King George Island´s population presented the highest level of prevalence rate (47.4%) and diversity (21.1% Anisakidae,

31.6% Metastrongyloidea and 5.3% Diphyllobothriidae). In the rest of the Antarctic locations, parasites found were Anisakidae and Metastrongyloidea, except for Avian and Deception Islands where Diphyllobothriidae cestodes were also recorded although in lower rate (3.7% and 0.5%, respectively) than the populations from King George Island.

Diedrichs-Alvarez (2007) indicated that Anisakidae were the only group of parasites found in Antarctic fur seals from Cape Shirreff. In addition, they were the most prevalent group of parasites identified in Antarctic pinnipeds (McFarlane et al., 2009). In the present study, Anisakidae nematodes where also a prevalent parasite group found in most of the examined populations, except for Barrientos Island, although prevalence rates were low to moderate (7.7% up to 50%), only representing 12% of the total of infected animals.

Currently, there are no reports on the presence of Metastrongyloidea and Diphyllobothriidae in Antarctic fur seals. These results represent the first description of Metastrongyloidea and Diphyllobothriidae parasites in the west coast of the Antarctic Peninsula, based on larvae and eggs morphology, respectively. Metastrongyloidea larvae were found in all seven locations (Avian Island, Barrientos Island, Penguin Island, Ronge Island, Deception Island, Livingston Island and King George Island), with a 23.6% of the total of infected animals. The highest prevalence rate was found in Penguin Island (38.5%) and the lowest in Ronge Island (16.7%). *Parafilaroides* infection is considered the most common underlying cause of respiratory disease in pinnipeds (Measures, 2001). Several species, except *Parafilaroides decorus,* have been described in other otariids from the Southern hemisphere, such as *Neophoca cinerea* Peron, 1816. In addition, *Parafilaroides normani* Dailey, 2009 has been described in *Arctocephalus pusillus* Schreber, 1775, *Arctocephalus australis* Zimmermann, 1783 and *Arctocephalus forsteri* Lesson, 1828 (McFarlane et al., 2009). The identification of *Parafilaroides* sp. closely related to *P. decorus* (99% similarity), in a sample from Deception Island confirmed the presence of *Parafilaroides* in Antarctic otariids. However, further studies are needed to elucidate the presence and distribution of lungworms species affecting Antarctic fur seals.

Diphyllobothriidae eggs were identified in samples from three locations (Avian Island, Deception Island and King George Island) with low prevalence rate (ranging from 0.5% to 5.3%). Several genera of Diphyllobothriidae cestodes have been described

in other Southern fur seals, like *Adenocephalus pacificus* Nybelin, 1931 in *A. australis* and *Diphyllobothrium arctocephalinum* Johnston, 1937 in *A. pusillus* (Bray et al., 1994, McFarlane et al., 2009, Rausch et al., 2010*).* Likewise, descriptions related to host-parasite associations and infection rates have been reported in Antarctic phocids. However, the information in Antarctic fur seal populations is still devoid (McFarlane et al., 2009). In Livingston Island, sample collection was distributed in two locations, Hannah Point and Byers Peninsula. No Diphyllobothriidae parasites have been detected in any of these two locations. However, Anisakidae have been identified only in samples collected in Byers Peninsula coinciding with previous reports from Cape Shirreff´s populations (Diedrichs-Alvarez, 2007). In constrast, only Metastrongyloidea larvae were identified in Hannah Point (25%) suggesting that there are influential elements differing in these two locations. Probably, diet and behavioural ecology can be relevant on this subject (McFarlane et al., 2009). However, as mentioned above, further studies are needed to expand our current knowledge related to the ecology of the populations inhabit these two locations within Livingston Island.

Currently classical approaches based on morphological characteristics of eggs, larvae and adult parasites are considered insufficient for differentiating parasites to a species level. In this sense, molecular techniques have proven to be a valuable tool for answering questions related to parasite systematics (Andrews and Chilton, 1999). The application of simple standard molecular methods for identification of parasites have demonstrated to be successful for single parasites and homogeneous samples (Logan et al., 2004; Brabec et al., 2006; Aznar et al., 2007; Nakao et al., 2007; Trachsel et al., 2007; Wicht et al., 2010). However for heterogeneous infections the use of modified molecular techniques is recommended (Trachsel et al., 2007). Therefore in the present study, only nematodes were used for molecular characterisation. *Contracaecum* sp. and *Pseudoterranova* sp. were identified, however no exact match was found in the Genbank sequence database. In both cases the highest similarity found was 99% with previously published sequences of *C. osculatum* (AF226583) and *P. decipiens* (AY821760), respectively, from the Northern hemisphere. Likewise, *Parafilaroides* sp. showed 99% similarity with *P. decorus* (AM039757) from the Sub Antarctic zone. Although molecular methods have been proven to be more accurate for identification of sibling species than the morphological approach, there is an evident lack of genetic data of parasites from the Antarctic region. More molecular data are therefore needed in

order to generate accurate information related to the groups of parasites present in the Southern hemisphere.

Mixed infections with sibling species of Anisakidae nematodes, such as *A. simplex* and *C. ogmorhini* have been reported in pinnipeds (Mawson, 1953; Mattiucci et al., 2003; McFarlane et al., 2009). In the present study, no evidence of mixed infections was found. The presence of two Anisakidae species, *A. simplex C* and *Pseudoterranova* sp., was confirmed in Deception Island, although in different hosts.

Previous observations have indicated that heavy parasitic infections are not necessarily related to ill-effects in hosts (Dearborn, 1965; McFarlane et al., 2009). However, reports dealing with mortality due to some of these parasites in marine mammals have been described causing debilitation, anaemia, obstruction in gastrointestinal tract, inflammation and ulcers of the stomach wall, leading to peritonitis and death (Geraci and St Aubin, 1987; McFarlane et al., 2009). In the study, Antarctic fur seals showed low to moderate prevalence rate of infection with no apparent effect on health, although parasite burden was not determined and no necropsies were performed and therefore, no pathological lesions were studied. However, the animal condition may be altered under stress situations and immunocompromised conditions which might be influenced by a variety of environmental, ecological, immnunological and physiological factors, causing manifestations of disease and could become lethal (Foreyt, 2001).

Recommendations have been made regarding the importance of health monitoring in the Antarctic fauna (Anon, 2003) and the present study is a contribution providing baseline data. The information collected may be used as a key to identify or even evaluate future changes in the Antarctic environment. Likewise, it also may contribute to improve prevention and response measures related to wildlife conservation and environment protection.

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CHAPTER VII

GENERAL DISCUSSION

Since the adoption of the Antarctic Treaty in 1959 a direct attention to the protection of the Antarctic environment began, and nowadays is still remaining (Rothwell, 2009). The Antarctic region is the most pristine environment with unique and extreme conditions that set it apart from other regions of the world, and therefore is considered isolated and one of the least impacted ecosystems (King and Turner, 1997; Kerry and Riddle, 2009). However, the Antarctic isolation is relative due to the migration of marine mammals and birds, which travel to and from the Antarctic region and could be carrying potential pathogen agents. Also, the constant intromission of humans, currently represented by scientific and touristic activities, has been widely recognised to constitute a serious risk for the introduction and spread of diseases, exerting an influence on the health of the Antarctic wildlife (ATCM III, 1964; Anon, 2003; Frenot et al., 2005; Pook, 2009; Riddle, 2009). In this sense, the first SCAR Biology Symposium emphasized "There is an urgent need to find out what pathogenic organisms occur naturally in Antarctic populations before man introduce new ones" (SCAR VIII, 1964). On the other hand, the effects of climate change and worldwide environmental degradation is another important issue that draws the world´s attention. The polar climates such as the Antarctic region have been changing faster than others as a consequence of global atmospheric changes, characterised by the increase of mean annual temperatures especially in the Antarctic Peninsula, with a subsequent direct impact on the Antarctic fauna (Clarke et al., 2007).

In all ecosystems, health and disease have been part of a continuum (Kerry and Riddle, 2009). Disease is understood as any impairment of normal functions of organisms, interfering, modifying and meaning deleterious effects in hosts caused by pathological agents (Delahay et al., 2009; Kerry and Riddle, 2009). There is an enormous variety of pathological agents currently known such as parasites, capable of cause infectious diseases in hosts. They also have an important role in the ecosystems, sometimes with downstream effects in wild populations by reducing growth rates and fecundity, increasing metabolic requirements, changing patterns of behavior and ultimately causing the death of individuals (Delahay et al., 2009; Davidson et al., 2011). Parasites are therefore natural members of ecosystems and integral components of marine ecosystems, representing a useful tool to explore the origins, distribution and maintenance of biodiversity (Hoberg and Klassen, 2002; Delahay et al., 2009).

Marine mammals have been regarded as prime sentinels for environmental changes, and pinnipeds are the most conspicuous group in the Antarctic region. They spend considerable time on land and ice platforms although they range widely in the Southern Ocean and frequently travel beyond their normal range with some vagrancy occurring far north where human activity and the consequent environmental contamination are patent. This might cause the introduction of diseases into their own populations and increase the potential of interspecies diseases transmission into the Antarctic fauna (Shirihai, 2002; Geraci and Lounsbury, 2009). In this sense, animal health has been in the list of priorities of the SCAR, acknowledging that "A survey of diseases already present in Antarctica´s isolated or semi-isolated fauna is required…" (SCAR III, 1959) and marine mammals are of particular value as sensitive barometers, serving as key species under the premise that their protection will safeguard the ecosystem´s health (Delahay et al., 2009).

The Antarctic pinnipeds surely have experienced a variety of disease-causing agents, some of which may be restricted to Antarctic species while others may be more widespread worldwide although the environmental and biological conditions are believed to be different (Kerry et al., 2000). As mentioned above, parasitic diseases can influence the health and sustainability of wildlife populations, with downstream effects in individuals and therefore on populations and ecosystems (Davidson et al., 2011). Many parasites have a worldwide distribution and some of them are closely related to environmental contamination due to human activities. Similarly, some parasites have also been related to health affection in humans and are regarded as zoonotic agents such as the protozoans *Toxoplasma gondii*, *Cryptosporidium* sp. and *Giardia* sp. (Dierauf and Gulland, 2001; Appelbee et al., 2010). These three protozoans have been described in worldwide pinnipeds, and there are several reports on pathologies related to their presence (Van Pelt and Dietrich, 1973; Olson et al., 1997; Measures and Olson, 1999; Deng et al., 2000; Dailey, 2001; Lambourn et al., 2001; Dubey et al., 2003; Dubey et al., 2004; Fayer, 2004a; Measures et al., 2004; Appelbee et al., 2005; Honnold et al., 2005; Hughes-Hanks et al., 2005; Santin et al., 2005; Aguirre et al., 2007; Fujii et al., 2007; Dixon et al., 2008; Gaydos et al., 2008; Appelbee et al., 2010; Lasek-Nesselquist et al., 2010; Cabezon et al., 2011; Bass et al., 2012). Therefore two of the objectives of our study were the detection of *Toxoplasma,* and of *Cryptosporidium* and *Giardia* in Antarctic pinnipeds, respectively. There was no evidence of *Giardia* in any of the

studied populations. However, for *Cryptosporidium* and *Toxoplasma gondii*, our study constitutes the first report of their presence in Antarctic pinnipeds from the South Shetland Islands and the Antarctic Peninsula. The presence of these parasites in the Antarctic fauna has been confirmed by recent studies in which *Cryptosporidium* was detected in Antarctic penguin species from Ardley Island (South Shetlands) (Fredes et al., 2007a; Fredes et al., 2007b; Fredes et al., 2008), and antibodies against *Toxoplasma gondii* were detected in Antarctic pinnipeds from Bird Island, Hutton Cliffs (East Antarctica), Macquarie Island and South Georgia (South Atlantic) (Jensen et al., 2012). Other studies however did not detect the presence of *Toxoplasma* in Antarctic pinnipeds from Bouvetoya and Queen Maud Land (Tryland et al., 2012), nor the presence of *Toxoplasma, Cryptosporidium* and *Giardia* in penguins from the same locations along the Antarctic Peninsula and the South Shetland islands included in our study (Palacios et al., 2010), suggesting these parasites are not widespread in the Antarctic populations. In addition, we have identified and described two novel *Cryptosporidium* genotypes in two phocid species, Southern elephant seals and Weddell seals, highlighting the contributions of our work to the general study of the taxonomy and the molecular epidemiology of *Cryptosporidium.* All these findings warrant further investigations in order to elucidate whether there might be wild strains endemic in the Antarctic fauna or they have been introduced in these populations by vagrant individuals.

The intimate relationships between hosts and parasites have evolved over time into subtle and potentially complex interactions (Delahay et al., 2009). The presence of disease agents in hosts does not necessarily mean that clinical signs will develop (Kerry and Riddle, 2009). Some parasites have little or no detrimental effect on hosts, only causing pathological damage if the delicate balance of the organism is corrupted, when the parasites become too numerous or the immunological capability of the host is impaired, probably influenced by factors including nutrition, concomitant infections or the presence of a variety of physiological stressors (Delahay et al., 2009). Therefore, the description of a pathogen in wildlife does not necessarily mean that it is the underlying cause of disease. In the case of *Toxoplasma gondii,* we investigated the presence of antibodies against this parasite in Antarctic pinnipeds. Serological approaches reveal the exposure to a specific pathological agent in the past (Kerry and Riddle, 2009), but do not provide information related to the effects on health condition in hosts although mortality and morbidity events with severe pathology lesions have been observed in

marine mammals elsewhere (Dubey et al., 2003; Dubey et al., 2004; Fayer, et al., 2004a; Dubey and Jones, 2008). However, no outbreaks or clinical toxoplasmosis have been reported and the parasite has not been isolated in Antarctic wildlife. In the case of *Cryptosporidium*, the patent period of the disease can range from several days to months or years, demonstrating the potential of this infection to persist in hosts (Ramirez et al., 2004). During the acute phase of infection, high numbers of oocysts are shed in the faeces, although intermittent shedding with low numbers or no oocysts and no clinical signs can also be observed becoming asymptomatic individuals, which can act as reservoirs for the disease (Ramirez et al., 2004; Appelbee et al., 2010). In our studies, only one sample was positive with the two diagnostic methods employed and the rest of samples were positive only by PCR, suggesting that at the time of sampling animals may have been shedding low numbers of oocysts. These works are prospective studies focused on the detection of *Cryptosporidium* in Antarctic populations and is not possible to determine if infected animals developed clinical signs in the past or will develop them in the future. To be able to gain a deeper knowledge on this subject, further studies are required. However, the detection of *Toxoplasma gondii* antibodies (our study; Jensen et al., 2012) and the description of *Cryptosporidium* in faecal samples (our study) provide very important information on the occurrence of potentially harmful organisms in apparent healthy animals from the Antarctic environment.

In general, the introduction of exotic agents in communities may spread and impact native species (Rohde, 2005). However, to be able to identify new pathogens in any population, it is necessary to have a historic evidence of native organisms to infer the origin of emerging diseases and cryptogenic species. As part of our work, surveys of gastrointestinal helminths in Antarctic pinnipeds have also been performed, indicating a high prevalence of parasites in phocids (76.9%) and moderate in otariids (31.2%). These results agree with previous reports where high prevalence and intensity of nematodes and cestodes parasites have been described in some phocids like Leopard seals and Weddell seals and moderate in sibling otariid species (McFarlane et al., 2009). Helminth parasites described were Anisakidae, Metastrongyloidea and Diphyllobothriidae. Anisakidae parasites are the most prevalent group of helminth in Antarctic populations and were found in most of pinniped species, except in Leopard seals, differing from previous reports where several stages of Anisakidae species were identified (Johnston and Mawson, 1945; Mawson, 1953; Nadler et al., 2000b; Dailey,

2001). Regarding the percentages of parasitation, phocids were the group of pinnipeds with the highest parasitation (90.9% in Weddell seals and 53.7% in Southern elephant seals), contrary to the otariid Antarctic fur seals which only a 12% of parasitation was described. Diphyllobothriidae parasites have been described in high intensity in some pinnipeds such as Leopard seals and Weddell seals. Southern elephant seals have also been described but in lower percentage (McFarlane et al., 2009). In our study, similar findings have been observed, with highest percentages in Leopard seals (100%), followed by the Weddell seals (63.6%) and finally Southern elephant seals (3%). In Antarctic fur seals, our study constitutes the first description of Diphyllobothriidae eggs with a prevalence of 1.1%, differing from other otariid species from the Antarctic and Sub Antarctic regions, where prevalences observed were higher (up to 16%) (McFarlane et al., 2009). Metastrongyloidea larvae were found in phocids and otariids with differences in the percentages observed, 25% in Leopard seals followed by a 23.8% in Southern elephant seals and 23.6% in Antarctic fur seals, and finally a 9.1% in Weddell seals. These are parasites also found in the respiratory tract of pinniped species from the Antarctic and Sub Antarctic region (Mawson, 1953; McFarlane et al., 2009). However, for Antarctic fur seals, this is the first description of the presence of Metastrongyloidea parasites, which were found in all the locations sampled. In addition, we have described, for the first time, the presence of various species of nematodes in Antarctic pinnipeds, such as the Anisakids *Contracaecum* sp. and *C. osculatum* in Weddell seals, *Pseudoterranova* sp. in Southern elephant seals and the Metastrongyloid *Parafilaroides* sp., in Antarctic fur seals. Other parasites described in our study have also been reported by other authors in the same hosts, like *Anisakis simplex C* and *Contracaecum mirounga* in Southern elephant seals (Nadler et al., 2000b; Mattiucci and Nascetti, 2007), *Pseudoterranova* species in Weddell seals (Mattiucci and Nascetti, 2007) and *Anisakis simplex, Pseudoterranova* and *Contracaecum* species in fur seal species from the Antarctic and Sub Antarctic regions (Diedrichs-Alvarez, 2007; McFarlane et al., 2009). The differences observed in the studied populations suggest that there might be influential elements affecting the distribution of parasites, presumably diet and behavioural ecology, among others still unidentified (McFarlane et al., 2009).

Very little is known about the role of these parasites in the health and dynamics of pinnipeds although some of them have been reported causing severe affections in worldwide phocids and otariids, especially gastrointestinal pathologies associated with

mechanical damage and lesions in gastrointestinal tissues (Geraci and St Aubin, 1987; Banish and Gilmartin, 1992; Nadler et al., 2000a; Dailey, 2001; Spraker et al., 2004; McFarlane et al., 2009; Byard et al., 2010; Papadopoulos et al., 2010; Spraker and Lander, 2010), and lesions in the cardiac and respiratory systems (MacDonald and Gilchrist, 1969; Geraci and St Aubin, 1987; Onderka, 1989; Claussen et al., 1991; Elson-Riggins et al., 2001; Fowler and Miller, 2003; Kelly et al., 2005; Siebert et al., 2007; McFarlane et al., 2009). These affections may have repercussions in pinnipeds health (Geraci and St Aubin, 1987), but how helminth parasites affect the health status of Antarctic wildlife is still unknown. It has been reported that heavy infections with nematodes and cestodes are normal for some Antarctic and Sub Antarctic pinniped species, appearing to cause little pathology and in some cases no direct evidence of ill-effects were observed (McFarlane et al., 2009). However, the presence and impact of parasites on their hosts can be influenced by environmental stressors, such as pollution and habitat alteration (Papadopoulos et al., 2010). Both empirical and theoretical evidence suggest that parasites can reduce density and potentially control populations. They also have the most linkages to life-history parameters either directly or through indirect routes via other biotic interactions such as predation or competition or both. This feature contributes to their role in the stabilization of population dynamics, a role that is increasingly recognised (Torchin et al., 2002).

It is known that parasites can also be used as bio-indicators of environmental changes, with positive or negative effects: either increasing parasitism or be fatal for certain parasite species, leading to a decrease in parasitism (Rohde, 2005). The general tendency observed in affected environments has been a decrease of parasitism with increasing levels of contamination (Rohde, 2005; Siebert et al., 2007). On this subject, environmental pollutants are very closely related to human activities and although improved environmental controls in the Antarctic ecosystem have stopped many pollutant practices particularly arising from the Madrid Protocol on Environmental Protection; activities developed in the past as well as in the present may have a legacy in Antarctic and Sub Antarctic regions (Byard et al., 2010). In this sense, the Antarctic Treaty System (ATS) has indicated that "Parties involved should conduct research relevant to cumulative impacts, and in particular to study disturbed versus undisturbed areas" (Clarke et al., 2007). Furthermore, it is a fact that the Antarctic Peninsula is also experiencing one of the fastest rates of regional warming on Earth, probably having an

influence on wildlife and the ecosystem (Clarke et al., 2007). However, no significant differences were observed amongst the studied populations suggesting that the ecological balance and the host-parasite relationship might not have been affected yet in these populations, although there is an evident lack of baseline data in this sense for the Antarctic fauna. Our study is an approach to collect information related to the presence and distribution of relevant gastrointestinal and systemic parasites in Antarctic pinnipeds, contributing to provide further information on the health status of Antarctic marine mammals. This also creates useful baseline data that can be used to monitoring and evaluate any changes occurring at any level, affecting these populations, since alterations resulting from climate variation and human activities have been recognised to affect marine mammals in other ecosystems (Davidson et al., 2011). Furthermore, information on the health of the Antarctic fauna will also contribute to the improvement of environmental policies and animal management programs in ways to protect and sustain viable populations in the Antarctic environment (Geraci and Lounsbury, 2009; Kerry and Riddle, 2009). Our contributions are just the beginning and further research is required, not only related to these parasites and others, but also to other pathological agents affecting the Antarctic fauna.

CHAPTER VIII

CONCLUSIONS

Objective 1: Detection of the systemic parasite *Toxoplasma gondii* in Antarctic pinnipeds

First: The detection of *Toxoplasma gondii* antibodies in all Antarctic pinniped species surveyed constitutes the first documented report on the presence of this parasite in Antarctic marine mammals.

Second: The differences observed in the percentages of antibody detection and titres among pinniped species, which were significantly higher in Weddell seals and Southern elephant seals may have a direct correlation with the distribution patterns, migratory ranges and feeding habits of each species. However, further investigations are needed to elucidate the likely transmission pathways of *T. gondii* in marine mammals as well as the presence of *T. gondii* in the Antarctic marine ecosystem.

Objective 2: Detection and characterisation of gastrointestinal parasites in Antarctic pinnipeds

Sub Objective 2.1: Detection and characterisation of the zoonotic parasites *Cryptosporidium* and *Giardia* in faeces

First: The detection of *Cryptosporidium* in Southern elephant seals and Weddell seals represents the first description on the presence of this parasite in Antarctic marine mammals.

Second: The low percentages of detection, although constant throughout the study, of *Cryptosporidium* and the absence of *Giardia*, in contrast with the results reported in pinnipeds from other less preserved areas, indicate that the Antarctic fauna might experience a lower level of exposure to these agents, which is in agreement with the relative geographical and biological isolation of the Antarctic continent.

Third: Two novel *Cryptosporidium* genotypes, designated *Cryptosporidium* sp. Weddell seal genotype and *Cryptosporidium* sp. Southern elephant seal genotype have been described and characterised in Antarctic pinnipeds. These genotypes have shown to be more closely related to previously described *Cryptosporidium* genotypes in fur-bearing animals and other intestinal *Cryptosporidium* species than to those reported from seals. Therefore the findings reported here further widen the range of both *Cryptosporidium* host species and the parasite's species or genotypes.

Sub Objective 2.2: Detection of helminth parasites in faecal samples

First: The Antarctic phocids Southern elephant seals and Weddell seals have shown a high level of infection with different stages of nematodes belonging to the family Anisakidae and superfamily Metastrongyloidea, as well as cestodes of the family Diphyllobothriidae, while Leopard seals were only infected with Metastrongyloidea nematodes and Diphyllobothriidae cestodes, although the levels of parasitation were similarly high.

Second: The Antarctic fur seals have shown a moderate level of infection with the same stages of helminth parasites found in Antarctic phocids, which contrast with previous reports where only Anisakidae nematodes have been described. Therefore, this is the first report on the presence of Metastrongyloidea nematodes and Diphyllobothriidae cestodes in Antarctic otariids.

Third: The presence of the different helminth parasites in both phocids and otariid populations, provides further evidence on their extensive distribution in Antarctic pinniped species, although there are differences in the level of parasitation between host species and locations surveyed, probably influenced by diet and behavioural ecology, among other as yet unknown factors.

Fourth: Molecular methods have proven a useful tool, more accurate than morphology, for the identification of cryptic species of parasites infecting marine mammals, which has enabled the report on the presence of new genotypes closely related to previously described nematodes, along with others identified in these pinniped hosts, contributing to widen the genetic information for these parasites in the Antarctic environment, which is currently lacking.

SUMMARY

The Antarctic region is the most isolated on earth. Recently, it has been recognised as a place worthy of very high standards of environmental protection. However, the constant human intromission and the worldwide environmental degradation have been identified as serious risks in the introduction and spread of diseases, exerting an influence on health of Antarctic wildlife. With regard to this topic, marine mammals have been described as prime sentinels of aquatic ecosystems. Pinnipeds are the most conspicuous marine mammal group in the Antarctic region and they might therefore provide an approach to evaluate the health of this singular ecosystem. Research in pinnipeds has resulted in a compilation of data of emerging and reemerging diseases, some of them caused by parasites. However, whereas the information is substantial in worldwide populations, in the Antarctic and the Sub Antarctic regions is currently limited.

To contribute with further information and in response to the demands of the Antarctic Treaty System, the main objective of this Doctoral Thesis has been to evaluate health status of Antarctic pinnipeds through the investigation of the presence of relevant parasites in marine mammal populations. For this purpose, faecal and blood samples of the phocids Weddell seals, Crabeater seals, Leopard seals, Southern elephant seals and the otariid Antarctic fur seals from different locations along the Antarctic Peninsula and South Shetland Islands have been analysed. In the first part of the study, the presence of the systemic zoonotic protozoan parasite *Toxoplasma gondii* was investigated (Chapter 2). In marine mammals, infections with *T. gondii* cause morbidity and mortality and although the route of infection for these animals is not known, environmental contamination plays a crucial role. Exposure to *T. gondii* in different Antarctic pinnipeds was evaluated by means of a serological test. Antibodies were detected in all populations analysed although higher percentages and highest titres were found in Southern elephant seals and Weddell seals (76.9%, 1:400; and 41.9%, 1:800, respectively). The differences observed between animal species could be due to their different distribution and migratory ranges as well as their feeding habits. This is the first report on the detection of *T. gondii* in Antarctic marine mammals.

Other zoonotic parasites surveyed were the gastrointestinal protozoan parasites *Giardia* and *Cryptosporidium* (Chapters 3 and 4). These parasites are closely related to environmental contamination and anthropogenic impact, therefore they have been widely used to evaluate ecosystems health. *Cryptosporidium* spp. oocysts and *Giardia*

spp. cysts have been detected in different pinniped species worldwide, and potential host specific genotypes of these parasites involved in infection in seals have been identified. In Antarctic pinnipeds, the presence of these two protozoans was evaluated using immunofluorescence microscopy and PCR. Overall the presence of *Cryptosporidium* was detected in 2.4% of Southern elephant seals (4/164) and 4.4% of Weddell seals (2/45), representing a 1.2% of the pinniped populations analysed, whereas *Giardia* was not detected. These findings suggest that these two potentially zoonotic parasites are not widespread in the Antarctic region although the presence of *Cryptosporidium* may have important implications in the Antarctic fauna since infected animals can act as vector not just spreading the disease to those in close vicinity but also disseminating these pathogens to different geographic locations in the marine and terrestrial environment. In addition, the molecular characterisation performed, using three different molecular markers, led to the description of two novel genotypes of *Cryptosporidium*, which were designated *Cryptosporidium* sp. Southern elephant seal genotype and *Cryptosporidium* sp. Weddell seal genotype,. These results constitute the first report on the presence of *Cryptosporidium* spp. in Antarctic marine mammals and further widen the range of both *Cryptosporidium* host species and genotypes.

Helminths are the largest group of parasites present in marine mammals. The few studies carried out in Antarctic pinnipeds have indicated that heavy infections with helminths are very common in these populations, although their prevalence and health significance remain unclear. Therefore, in order to provide further information, a survey was performed to investigate the presence and distribution of helminth parasites in Antarctic phocids (Chapter 5) and otariids (Chapter 6) using coprological examination and molecular characterisation of selected specimens. In total, eggs, larvae and adult forms of helminth parasites were found in 71.3% of Southern elephant seals, 95.4% of Weddell seals and 100% in leopard seals, whereas otariid populations presented a lower prevalence (31.2%). Helminth parasites found were nematodes belonging to the family Anisakidae and the superfamily Metastrongyloidea and cestodes within the family Diphyllobothriidae. Anisakidae parasites were the most prevalent group of helminth in Antarctic populations and were found in most pinniped species, except in Leopard seals, with percentages of parasitation ranging from 12% in Antarctic fur seals to 90.9% in Weddell seals. Metastrongyloidea larvae were found in phocids and otariids with differences in the percentages observed, 25% in leopard seals followed by a 23.8% in

Southern elephant seals and 23.6% in Antarctic fur seals, and finally a 9.1% in Weddell seals. Diphyllobothriidae eggs were detected in all pinniped species analysed. The highest percentages of parasitation were observed in Leopard seals (100%), and Weddell seals (63.6%). However, in Southern elephant seals and Antarctic fur seals eggs were only observed in 3% and 1.1% of the samples, respectively. These findings constitute the first report on the presence of Diphyllobothriidae eggs and Metastrongyloidea larvae in Antarctic fur seals. In addition, the use of molecular techniques led to the identification, for the first time, of various species of nematodes in Antarctic pinnipeds. These include *Contracaecum* sp. and *Contracaecum osculatum* in Weddell seals, *Pseudoterranova* sp. in Southern elephant seals and *Parafilaroides* sp. in Antarctic fur seals. Other parasites identified in this study include *Anisakis simplex C* and *Contracaecum mirounga* in Southern elephant seals, *Pseudoterranova* sp. in Weddell and *Anisakis simplex* and *Contracaecum* sp. in Antarctic fur seal species, which have been previously reported in the same hosts. The differences observed in the studied populations and sites surveyed suggest that there might be influential elements affecting the presence and distribution of helminth parasites, presumably diet and behavioural ecology, among others still unidentified.

The results obtained in this Doctoral Thesis provide further information on the presence and distribution of parasites in Antarctic pinnipeds, highlighting the need for further surveys to elucidate the taxonomy and epidemiology of different pathogens and diseases. This information also provides useful background to evaluate and monitor any future changes that may occur in these Antarctic populations. These aspects will also contribute to create better measures to improve environmental policies and animal management programs to protect and sustain viable populations in the Antarctic environment.

RESUMEN

INTRODUCCIÓN

La Antártida está considerada actualmente como el entorno más aislado que existe, con características geográficas y climatológicas únicas que la diferencian del resto del mundo. Sin embargo, este aislamiento es relativo, observándose una migración constante de mamíferos y aves marinas, los cuales viajan hacia y desde la Antártida, pudiendo transportar agentes potencialmente patógenos. Igualmente, a través de la historia, se ha producido un incremento de la actividad humana en la Antártida, que hoy en día se debe fundamentalmente a actividades científicas y turísticas. Por otro lado, se considera que los cambios medioambientales que se están produciendo globalmente, pueden constituir también un riesgo inminente de introducción y diseminación de enfermedades, lo que puede ejercer una gran influencia sobre la fauna autóctona.

Recientemente, el ecosistema Antártico ha sido formalmente reconocido a nivel mundial como un lugar digno de un nivel muy alto de protección medioambiental. Por este motivo, a raíz del año Geofísico Internacional de 1957-58, se inició la formulación de lo que se denominó el Tratado Antártico, creado en 1959. Este tratado es un documento muy sencillo, en el que se establecen unas medidas básicas para la realización de actividades científicas en la Antártida con especial atención a la protección del medio ambiente Antártico y la salud de la fauna antártica.

El Tratado Antártico, a través del Comité Científico de Investigaciones Antárticas (SCAR) ha indicado que "es escasa la información que se tiene sobre las enfermedades propias de los animales antárticos". Además, "...hay una gran necesidad de realizar estudios que permitan conocer la situación sanitaria de las diferentes especies y poblaciones de animales que habitan el ecosistema antártico, con la finalidad de contribuir al conocimiento científico y orientar la creación de medidas de administración de los recursos vivos marinos de la Antártida", insistiendo además en que "...es fundamental destinar esfuerzos para estimar la situación sanitaria de las poblaciones naturales de vertebrados superiores de la Antártida, mediante su seguimiento periódico, como también, dimensionar su efecto sobre la dinámica poblacional de las especies afectadas y realizar estudios epidemiológicos de las enfermedades que los afectan. Tales estudios podrán servir como un indicador de la acción humana en ese ecosistema y mejorar las medidas de prevención de la potencial contaminación biológica, desde o hacia la Antártida, que surgen a partir de las actividades domésticas del hombre en la Antártida...".

En este sentido, los mamíferos marinos son considerados buenos centinelas de los ecosistemas marinos por encontrarse en la cúspide de la cadena trófica, por lo que muchas investigaciones se han centrado en estas especies como bio-indicadores de contaminación medioambiental en diferentes ecosistemas. Dentro de los mamíferos marinos, los pinnípedos son el grupo más numeroso y sobresaliente de la fauna Antártica, por lo que podrían ser de utilidad para evaluar este ecosistema en particular. Estos animales poseen características anatómicas y fisiológicas adaptadas a la vida marina, aunque pasan mucho tiempo en tierras costeras y plataformas de hielo.

Las investigaciones realizadas en mamíferos marinos a nivel mundial han generado valiosa información sobre el estado sanitario de estas especies en el mundo, habiéndose descrito un gran número de enfermedades, muchas de ellas causadas por parásitos. Se considera que los parásitos son elementos naturales con un papel importante en los ecosistemas, teniendo distintos efectos en las poblaciones silvestres, como la reducción de las tasas de crecimiento y fecundidad, el aumento de la exigencia metabólica, el cambio de los patrones de comportamiento y en última instancia, causando la muerte de los individuos. Por este motivo, los parásitos son también componentes integrales de los ecosistemas marinos, representando una herramienta muy útil para explorar los orígenes, distribución y mantenimiento de la biodiversidad.

OBJETIVOS

Se han identificado distintos parásitos en pinnípedos Antárticos y Sub-Antárticos. Sin embargo, el conocimiento sobre la presencia y distribución de parásitos en estas especies es escaso y fragmentado. Por lo tanto, la realización de estudios en pinnípedos Antárticos servirá para proporcionar un mayor conocimiento sobre la salud de estas especies y su entorno. Con el fin de contribuir a la información relacionada sobre agentes patógenos y enfermedades que afectan a la fauna antártica, y en respuesta a las demandas del Sistema del Tratado Antártico, el **objetivo principal** de la presente Tesis Doctoral ha sido el de **evaluar la presencia y distribución de parásitos sistémicos y gastrointestinales relevantes en las poblaciones de pinnípedos**. De igual manera, algunos de estos parásitos son también organismos estrechamente asociados al impacto humano en diferentes regiones, además de ser elementos que permiten la evaluación de la calidad medioambiental afectada por la contaminación a nivel mundial.

Por lo tanto, para realizar este estudio, se abordaron los siguientes **objetivos específicos**:

Objetivo 1: Detección del parásito sistémico *Toxoplasma gondii* en pinnípedos antárticos

En primer lugar se investigó la presencia del parásito protozoo sistémico *Toxoplasma gondii*. Este parásito, considerado de carácter zoonósico, ha sido reportado como causante de graves infecciones en diversas especies de mamíferos marinos y, aunque su epidemiología no es totalmente conocida en los ecosistemas marinos, está estrechamente vinculado a la contaminación ambiental.

Objetivo 2: Detección y caracterización de parásitos gastrointestinales en pinnípedos antárticos

Para la consecución de este objetivo, se propusieron los dos siguientes sub objectivos:

Sub Objetivo 2.1: Detección y caracterización de los parásitos zoonósicos *Cryptosporidium* y *Giardia* en muestras fecales

La determinación de la presencia de los protozoos gastrointestinales *Cryptosporidium* y *Giardia* resulta de especial interés debido a su carácter zoonósico, además de ser considerados buenos bio-indicadores de contaminación medioambiental e impacto antropogénico, por lo que han sido ampliamente utilizados en estudios de evaluación de calidad ambiental. Se han detectado ooquistes de *Cryptosporidium* y quistes de *Giardia* en varias especies de pinnípedos a nivel mundial, habiéndose descrito también en estos animales genotipos de estos parásitos que podrían presentar cierta especificidad de hospedador.

Sub Objetivo 2.2: Detección de parásitos helmintos en muestras fecales

Los helmintos son el grupo más amplio de parásitos presentes en los mamíferos marinos. Estudios realizados en pinnípedos antárticos indican que un alto grado de parasitación por helmintos es normal en estas poblaciones, aunque su prevalencia y significado en la salud de estos animales no está definido.

METODOLOGÍA

Para la consecución de los objetivos planteados en esta Tesis Doctoral, se tomaron muestras de heces y sangre de pinnípedos de las familias Phocidae y Otariidae. Las especies de la familia Phocidae muestreadas fueron foca de Weddell (*Leptonychotes weddellii*), foca leopardo (*Hydrurga leptonyx*), foca cangrejera (*Lobodon carcinophagus*) y elefante marino (*Mirounga leonina*). La especie de la familia Otariidae muestreada fue el lobo fino Antártico *(Arctocephalus gazella)*.

Las muestras fueron recogidas durante los años 2006, 2007, 2010 y 2011, en diferentes localizaciones a lo largo de la costa oeste de la Península Antártica, cubriendo un gradiente latitudinal desde el archipiélago de Shetland del Sur (islas Rey Jorge, Livingston, Barrientos, Pingüino y Decepción) hasta las islas Anvers, Rongé y Avian, abarcando un total de 5 grados de latitud hacia el sur de la Península Antártica. Este gradiente latitudinal se corresponde asimismo con distancias de más de 600 km entre los puntos más alejados y diferencias de temperatura medias anuales de hasta 2 ºC entre el norte y el sur. Estas zonas se caracterizan por concentrar la mayor variedad y número de especies de pinnípedos, además de presentar distintos niveles de actividad humana.

Las muestras de sangre se obtuvieron de animales capturados, mientras que las de heces se obtuvieron tanto de animales capturados como de muestras recogidas directamente del suelo. Los animales capturados fueron escogidos al azar e inmovilizados físicamente el tiempo mínimo necesario, de acuerdo con los procedimientos estándar C1 y C2 de la Comisión para la Conservación de los Recursos Vivos Marinos Antárticos (CCAMLR). Todos los animales capturados fueron identificados con crotales numerados, garantizando que ningún animal fuera muestreado más de una vez. Las heces se recogieron directamente del suelo, cerca del animal, tomando solamente la parte superior del excremento para evitar contaminación con parásitos de vida libre. Las heces recogidas fueron de 45 focas de Weddell, 164 elefantes marinos, 4 focas leopardo y 276 lobos finos Antárticos. Las muestras de sangre obtenidas fueron de 31 focas de Weddell, 13 elefantes marinos, 2 focas cangrejeras y 165 lobos finos Antárticos.

Objetivo 1: Detección del parásito sistémico *Toxoplasma gondii* en pinnípedos antárticos

La exposición a este parásito en pinnípedos antárticos fue analizada mediante la utilización de un kit de aglutinación directa comercial (Toxo-Screen DA, BioMerieux®, Francia), capaz de detectar antígenos específicos IgG contra *T. gondii* en suero.

Objetivo 2: Detección y caracterización de parásitos gastrointestinales en pinnípedos antárticos

Sub Objetivo 2.1: Detección y caracterización de los parásitos zoonósicos *Cryptosporidium* y *Giardia* en muestras fecales

La detección de *Cryptosporidium* y *Giardia* se realizó mediante inmunofluorescencia directa (*Crypto*/*Giardia* Cel IF test, Cellabs Pty. Ltd., Australia) y PCR utilizando como marcadores las regiones del 18S rDNA, en el caso de *Cryptosporidium,* y del gen de la beta-giardina para *Giardia*. La caracterización molecular de los aislados de *Cryptosporidium* se realizó mediante secuenciación de los fragmentos amplificados de las regiones del 18S rDNA, COWP y HSP70.

Sub Objetivo 2.2: Detección de parásitos helmintos en muestras fecales

Se utilizaron técnicas coprológicas para la detección de los helmintos presentes en muestras fecales, que incluyeron un examen macroscópico y microscópico de las muestras. En el examen macroscópico, las muestras se examinaron separando los parásitos visibles y lavándolos repetidamente en placas de Petri con solución salina fisiológica (pH 7.3), para finalmente almacenarlos individualmente en etanol al 70%. En el examen microscópico, se realizó una técnica de flotación (Ovatec®Plus, Synbiotics Corporation, USA), para la detección de huevos. Igualmente, aproximadamente 10 gramos de heces fueron examinados mediante la técnica de migración larvaria utilizando el aparato de Baermann. Adicionalmente, se utilizaron técnicas moleculares, amplificando los fragmentos de la region LSU rDNA con el fin de completar la identificación de los parásitos encontrados.

RESULTADOS

Objetivo 1: Detección del parásito sistémico *Toxoplasma gondii* en pinnípedos antárticos

La presencia de anticuerpos frente a *Toxoplasma gondii* en mamíferos marinos antárticos fue detectada (títulos ≥1:25) en 28 de los 211 animales analizados, representando un 13.3% de la población muestreada. Este estudio constituye la primera descripción de la presencia de anticuerpos frente a *T. gondii* en mamíferos marinos antárticos. Dentro de las especies analizadas el más alto porcentaje de detección se observó en elefantes marinos (76.9%) seguido de focas de Weddell (41.9%). Igualmente se detectaron anticuerpos en el 2.4% de los lobos finos Antárticos muestreados y el 50% de las focas cangrejeras. Los títulos más altos fueron detectados en elefantes marinos (1:400) y en focas de Weddell (1:800). Las diferencias observadas entre las especies estudiadas podrían deberse a su distinta distribución y características migratorias, ya que las especies con títulos de anticuerpos y porcentajes más altos se encuentran ampliamente distribuidas por toda la Antártida, trasladándose incluso fuera del ecosistema antártico, atravesando la corriente Circumpolar para llegar a zonas Sub Antárticas y otras aún más alejadas. De igual manera, las diferencias observadas entre las especies analizadas podrían deberse a los diferentes hábitos alimentarios, ya que estas especies también presentan un rango más amplio de especies marinas de las cuales se alimentan, siendo alguna de ellas capaces de concentrar el parásito en condiciones experimentales, en concreto, los moluscos filtradores.

Objetivo 2: Detección y caracterización de parásitos gastrointestinales en pinnípedos antárticos

Sub Objetivo 2.1: Detección y caracterización de los parásitos zoonósicos *Cryptosporidium* y *Giardia* en muestras fecales

No se observó la presencia de *Giardia duodenalis* en ninguna de las muestras analizadas en este estudio. Sin embargo, se detectó la presencia de *Cryptosporidium*, en elefantes marinos (2.4%, 4/164) y en focas de Weddell (4.4%, 2/45), lo que representa un 1.2% del total de las poblaciones analizadas (491 muestras). Estos resultados indican que la presencia de *Cryptosporidium* es constante, ya que se encontraron muestras positivas a lo largo de los 4 años, aunque en bajos porcentajes. Sin embargo, estos hallazgos tienen grandes implicaciones para la fauna antártica, ya que estos

hospedadores pueden actuar como vectores, esparciendo el parásito a zonas cercanas, además de diseminarlo en diferentes localizaciones geográficas dentro de los entornos terrestres y marinos.

La caracterización molecular realizada, utilizando tres de los marcadores moleculares más comúnmente utilizados, permitió la descripción de dos nuevos genotipos de *Cryptosporidium*, el genotipo encontrado en elefante marino se denominó *Cryptosporidium* sp. Southern elephant seal genotype y el genotipo encontrado en foca de Weddell se denominó *Cryptosporidium* sp. Weddell seal genotype. El análisis molecular realizado permitió comprobar que estos genotipos se encuentran más próximos genéticamente a especies o genotipos intestinales de *Cryptosporidium* hallados en animales mustélidos, como la mofeta, el hurón o el visón, que a aquellos hallados en pinnípedos. Este trabajo constituye, por tanto, la primera descripción sobre la presencia de *Cryptosporidium* en mamíferos marinos antárticos, ampliando además, tanto el espectro de hospedadores como de genotipos de este parásito.

Sub Objetivo 2.2: Detección de parásitos helmintos en muestras fecales

Se observaron distintas formas parasitarias de helmintos (huevos, larvas y adultos) en un 76,9% de las muestras de fócidos (163/212), encontrándose porcentajes de detección del 71.3% en elefantes marinos (117/164), 95.4% en focas de Weddell (42/44) y del 100% en las focas leopardo (4/4), mientras que en las poblaciones de lobo fino antártico, las prevalencias halladas fueron más bajas (31.2%, 86/276). Los parásitos encontrados fueron nematodos pertenecientes a la familia Anisakidae y a la superfamilia Metastrongyloidea, y cestodos de la familia Diphyllobothriidae. Los parásitos pertenecientes a la familia Anisakidae fueron el grupo de helmintos más prevalente en todas las poblaciones antárticas y fueron encontrados en la mayoría de las especies estudiadas (90.9% en focas de Weddell, 53.7% en elefantes marinos y 12% en lobos finos antárticos), excepto en foca leopardo. Se observaron larvas de Metastrongyloidea en fócidos y otáridos con diferencias en los porcentajes observados, 25% en focas leopardo, seguido de un 23.8% en elefantes marinos, 23.6% en lobos finos antárticos, y finalmente un 9.1% en focas de Weddell. Se detectaron huevos de Diphyllobothriidae en todas las especies analizadas. Los mayores porcentajes de parasitación fueron observados en focas leopardo (100%) y focas de Weddell (63.6%). Sin embargo, en elefantes marinos y lobos finos antárticos, solo se observaron en un 3% y 1.1% de las muestras analizadas, respectivamente. La presencia de huevos pertenecientes a la

familia Diphyllobothriidae y larvas de la superfamilia Metastrongyloidea constituye la primera descripción de estos dos grupos de parásitos en lobos finos antárticos. La utilización de técnicas moleculares ha contribuido a la identificación, por primera vez, de algunas especies de nematodos en pinnípedos antárticos. Estas especies fueron *Contracaecum* sp. y *Contracaecum osculatum* en focas de Weddell, *Pseudoterranova* sp. en elefantes marinos y *Parafilaroides* sp. en lobos finos antárticos. Otros parásitos identificados en este estudio, han sido previamente identificados en las especies animales de estudio, como *Anisakis simplex C* y *Contracaecum mirounga* en elefantes marinos, *Pseudoterranova* sp. en focas de Weddell, y finalmente *Anisakis simplex* y *Contracaecum* sp. en lobos marinos. Las diferencias observadas en estas poblaciones estudiadas sugieren que podrían existir elementos que influyen en la presencia y distribución de estos parásitos, presumiblemente la dieta y el comportamiento, aunque no se descarta la presencia de otros elementos aún desconocidos.

CONCLUSIONES

Objetivo 1: Detección del parásito sistémico *Toxoplasma gondii* en pinnípedos antárticos

Primera: La detección de anticuerpos frente a *Toxoplasma gondii* en todas las especies de pinnípedos antárticos estudiados constituye la primera descripción de la presencia de este parásito en mamíferos marinos Antárticos.

Segunda: Las diferencias observadas en los porcentajes de detección y los títulos de anticuerpos obtenidos entre las especies de pinnípedos estudiados, significativamente más altos en focas de Weddell y elefantes marinos, podrían estar correlacionados directamente con la distribución, características migratorias y diferentes hábitos alimentarios de las distintas especies de pinnípedos antárticos. Sin embargo, son necesarios más estudios que eluciden la presencia de *T. gondii* en el ecosistema Antártico, así como las posibles rutas de transmisión en los mamíferos marinos.

Objetivo 2: Detección y caracterización de parásitos gastrointestinales en pinnípedos antárticos

Sub Objetivo 2.1: Detección y caracterización de los parásitos zoonósicos *Cryptosporidium* y *Giardia* en muestras fecales

Primera: La detección de *Cryptosporidium* en elefantes marinos y focas de Weddell constituye la primera descripción de este parásito en mamíferos marinos Antárticos.

Segunda: Los bajos porcentajes de detección de *Cryptosprodium,* aunque constantes a lo largo del estudio, y la ausencia de *Giardia,* en contraste con los resultados referidos en pinnípedos procedentes de zonas menos protegidas ambientalmente, indican que la fauna Antártica puede experimentar un bajo nivel de exposición a estos agentes, lo que concuerda con el relativo aislamiento geográfico y biológico del entorno Antártico.

Tercera: Se han descrito y caracterizado dos nuevos genotipos de *Cryptosprodium,* denominados *Cryptosporidium* sp. Weddell seal genotype y *Cryptosporidium* sp. Southern elephant seal genotype en pinnípedos Antárticos. Estos genotipos están más próximos genéticamente a genotipos descritos en mustélidos, así como a otras especies intestinales de *Cryptosporidium* que a aquellos encontrados en pinnípedos de otras latitudes. Por lo tanto, estos hallazgos amplían el espectro de especies hospedadoras para *Cryptosporidium,* así como sus especies y genotipos.

Sub Objetivo 2.2: Detección de parásitos helmintos en muestras fecales

Primera: Los fócidos antárticos, elefantes marinos y focas de Weddell, mostraron un alto nivel de infección con diferentes estadios de nematodos pertenecientes a la familia Anisakidae y superfamilia Metastrongyloidea, así como cestodos de la familia Diphyllobothriidae, mientras que las focas leopardo solamente estuvieron parasitadas por nematodos de la superfamilia Metastrongyloidea y cestodos de la familia Diphyllobothriidae, aunque los niveles de parasitación fueron igualmente elevados.

Segunda: Los lobos finos Antárticos mostraron un moderado nivel de infección con los mismos grupos de helmintos encontrados en los fócidos antárticos, contrastando este hallazgo con estudios previos donde solo se había descrito la presencia de nematodos de la familia Anisakidae. Por lo tanto, esta es la primera descripción de la

presencia de parásitos de la superfamilia Metastrongyloidea y familia Diphyllobothriidae en otáridos Antárticos.

Tercera: La presencia de los diferentes grupos de helmintos, tanto en poblaciones de fócidos como en otáridos, es una prueba de su extensa distribución en pinnípedos Antárticos, aunque existen diferencias en los porcentajes de parasitación entre las especies hospedadoras y las zonas estudiadas, probablemente influenciadas por la dieta y el comportamiento, entre otros factores aún desconocidos.

Cuarta: Los métodos moleculares han demostrado ser una herramienta muy útil y más precisa que la caracterización morfológica para la identificación de especies desconocidas de parásitos que infectan a los mamíferos marinos, lo que ha permitido describir la presencia de nuevos genotipos de nematodos en pinnípedos antárticos, junto con otros identificados con anterioridad en estos hospedadores, incrementando la información disponible de estos parásitos en el entorno Antártico, la cual es actualmente escasa.

APORTACIONES FUNDAMENTALES DE LA PRESENTE TESIS DOCTORAL

Las contribuciones de esta Tesis Doctoral incrementan la información existente sobre la presencia y distribución de parásitos en pinnípedos antárticos, habiéndose publicado los resultados obtenidos en los objetivos 1 (Capítulo 2) y 2.1 (Capítulos 3 y 4) y habiendo sido los resultados obtenidos en el objetivo 2.2 (Capítulo 5 y 6) enviados para su publicación en las siguientes revistas recogidas en el SCI (Science Citation Index):

- **Capítulo 2:** Detección de anticuerpos frente a *Toxoplasma gondii* en pinnípedos Antárticos:
  Rengifo-Herrera, C., Ortega-Mora, L.M., Alvarez-García, G., Gómez-Bautista, M., García-Párraga, D., García-Peña, F.J., Pedraza-Díaz, S. Detection of Toxoplasma gondii antibodies in Antarctic pinnipeds. Vet. Parasitol. 190 (2012): 259-262.
- **Capítulo 3:** Detección y caracterización de un aislado de *Cryptosporidium* procedente de un elefante marino (*Mirounga leonina*) de la Península Antártica:

  Rengifo-Herrera, C., Ortega-Mora, L.M., Gómez-Bautista, M., García-Moreno, F.T., García-Párraga, D., Castro-Urda, J., Pedraza-Díaz, S. Detection and

characterisation of a Cryptosporidium isolate from a Southern Elephant Seal (Mirounga leonina) from the Antarctic Peninsula. Appl. Environ. Microbiol. 77 (2011): 1524-1527.

- **Capítulo 4:** Detección de un nuevo genotipo de *Cryptosporidium* en pinnípedos Antárticos.

  Rengifo-Herrera, C., Ortega-Mora, L.M., Gómez-Bautista, M., García-Peña, F.J., García-Párraga, D., Pedraza-Díaz, S. Detection of a novel genotype of Cryptosporidium in Antarctic pinnipeds. Vet. Parasitol. 191 (2013): 112-118.

- **Capítulo 5:** Presencia de helmintos encontrados en heces de fócidos de la Península Antártica.

  Rengifo-Herrera, C., Ferre, I., Ortega-Mora, L.M., García-Moreno, F.T., García-Párraga, D., García-Peña, F.J., Pereira-Bueno, J., Pedraza-Díaz, S. Helminth parasites found in faecal samples of phocids from the Antarctic Peninsula. Polar Biol.

- **Capítulo 6:** Presencia de parásitos helmintos en lobos finos antárticos (*Arctocephalus gazella* Peters, 1875) de la Península Antártica.

  Rengifo-Herrera, C., Ferre, I., Ortega-Mora, L.M., García-Moreno, F.T., García-Párraga, D., García-Peña, F.J., Pereira-Bueno, J., Pedraza-Díaz, S. Presence of helminth parasites in Antarctic fur seals (Arctocephalus gazella Peters, 1875) from the Antarctic Peninsula. Antarct. Sci.

Al mismo tiempo, estos estudios han puesto de manifiesto la necesidad de continuar realizando investigaciones encaminadas a generar mayor información sobre la epidemiología de los diferentes agentes patógenos y las enfermedades que estos causan en la fauna antártica. Además, estos estudios proporcionan datos base muy útiles para poder evaluar y monitorizar los posibles cambios futuros que puedan ocurrir dentro del entorno Antártico y pueden contribuir a desarrollar medidas más eficientes para optimizar las políticas medioambientales vigentes, así como también los programas de gestión existentes, creados para proteger y mantener viables las diferentes especies antárticas.

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APPENDIX

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Short communication

Detection of *Toxoplasma gondii* antibodies in Antarctic pinnipeds

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ABSTRACT

The presence of *Toxoplasma gondii* antibodies was investigated in Antarctic marine mammals. Two hundred and eleven sera from different species of pinnipeds collected in years 2007, 2010 and 2011 from different locations in the South Shetland Islands and Antarctic Peninsula were analysed using a commercially available agglutination test kit. The presence of antibodies (titres ≥ 1:25) against *T. gondii* was detected in a total of 28 animals (13.3%). Amongst animal species, percentages of detection were higher in Southern elephant seals (*Mirounga leonina*) (76.9%; 10/13) followed by Weddell seals (*Leptonychotes weddellii*) (41.9%; 13/31). Antibodies were also found in 4 of 165 (2.4%) Antarctic fur seals (*Arctocephalus gazella*) and 1 of 2 Crabeater seals (*Lobodon carcinophaga*). Highest titres (1:100–1:800) were also observed in Southern elephant seals and Weddell seals. To the best of our knowledge this is the first report on the detection of antibodies against *T. gondii* in Antarctic marine mammals.

1. Introduction

Toxoplasma gondii is an apicomplexan parasite with a worldwide distribution which affects a wide range of animals, including domestic and wild species and humans. In marine mammals, infections with *T. gondii* cause morbidity and mortality (Dubey, 2010).

In pinnipeds, clinical toxoplasmosis has been reported in a Northern Elephant seal (*Mirounga angustirostris*) (Dubey et al., 2004), a Northern fur seal (*Callorhinus ursinus*) (Holshuh et al., 1985), a Pacific harbor seal (*Phoca vitulina richardsi*) (Van Pelt and Dietrich, 1973), a Hawaiian monk seal (*Monachus schauinslandi*) (Honnold et al., 2005) and California Sea lions (*Zalophus californianus*) (Ratcliffe and Worth, 1951; Dubey et al., 2003). In addition, numerous serological studies have shown the presence of antibodies against *T. gondii* in true seals (Fam. Phocidae), eared seals (Fam. Otariidae) and walruses (Fam. Odobenidae) from different geographical areas which include USA, North-western Hawaiian islands, Japan, Svalvard, the Canadian Arctic, Mexico and the North-eastern Atlantic Ocean (Dubey, 2010; Jensen et al., 2010; Alvarado-Esquivel et al., 2012; Cabezon et al., 2011; Simon et al., 2011). The range of pinniped species in which *T. gondii* antibodies have been found include the Pacific harbor seal (*Phoca vitulina richardsi*) (Lambourn et al., 2001; Dubey et al., 2003), western Atlantic harbor seal (*Phoca vitulina concolor*) (Measures et al., 2004), kuril harbor seal (*Phoca vitulina stejnegeri*) (Fujii et al., 2007), ringed seal (*Pusa hispida*), bearded seal (*Erignathus barbatus*), spotted seal (*Phoca largha*) (Dubey et al., 2003), grey seal (*Halichoerus grypus*) (Measures et al., 2004; Cabezon et al., 2011), hooded seal (*Cystophora cristata*) (Measures et al., 2004), Hawaiian monk seal (*M. schauinslandi*) (Aguirre et al., 2007), eastern-Atlantic

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harbor seal (*Phoca vitulina vitulina*) (Cabezon et al., 2011), California sea lion (*Z. californianus*), and the walrus (*Odobenus rosmarus*) (Dubey et al., 2003).

To the best of our knowledge, no investigations have been carried out in Antarctic pinnipeds. Marine mammals are regarded as good bio-indicators of environmental changes. However, the information available about the health status of the Antarctic marine mammals is very scarce and fragmented (Kerry et al., 2000). In addition, human derived activities in this pristine environment could be compromising these populations. In this sense recommendations have been made regarding the importance of health monitoring the Antarctic fauna (Annon, 2003). The purpose of this study was to investigate the presence of *T. gondii* antibodies in pinnipeds from different regions in the Antarctic Peninsula.

2. Materials and methods

2.1. Pinniped samples

Blood samples were collected during the month of February of years 2007, 2010 and 2011 from a total of 211 animals (Table 1): 31 Weddell seals (*L. weddellii*), 13 Southern elephant seals (*M. leonina*), 2 Crabeater seals (*L. carcinophaga*) and 165 Antarctic fur seals (*A. gazella*) from different locations along the west coast of the Antarctic Peninsula in a latitudinal gradient covering 5° of latitude (ranging from 62°15′S; 58°37′W-67°46′S; 68°43′W), distances greater than 600 km and differences in mean annual temperatures of up to 2 °C.

For the collection of samples, animals were randomly selected, captured and physically restrained. All captured animals were tagged with a coloured and numbered plastic tag for tracking purposes, ensuring that no animal was sampled more than once. Permissions for these activities were granted by the Spanish Polar Committee complying with the Antarctic Treaty System. Blood samples were centrifuged (700 × *g* for 10 min) and the sera stored at −20 °C until analysed.

2.2. Serological examination

Detection of antibodies against *T. gondii* was performed using a commercial kit based on detection of specific IgG from sera by direct agglutination (Toxo-Screen DA, BioMerieux®, France) according to the manufacturer's instructions. For initial screening, 1:25 and 1:100 final dilutions of sera were tested. Samples that showed agglutination at 1:25 were considered positive (see Section 4) and further tested for titre determination at two-fold serial dilutions from 1:25 to 1:6400. All positive samples were retested to confirm the reliability of the results.

2.3. Statistical analysis

Seropositivity data with respect to animal species and year were analysed by pairs using the Chi-square or Fisher's exact test using the Statgraphic Centurion XVI version 16.1.11, statistical software (©StatPoint Technologies, Inc., 1922–2010, Warrenton, VA, USA). Differences were considered significant at a probability level of $P < 0.05$.

Table 1
Distribution of samples and *Toxoplasma gondii* antibody detection results in Antarctic pinnipeds.

Animal species	Location[a]	Year	No. Samples	Positive	% Positive
Weddell seal (*Leptonychotes weddellii*)	Deception Island, South Shetland Islands	2007	8	0	0
		2010	14	9	64.3
		2011	6	2	33.3
	Rongé Island, Errera Channel	2010	1	0	0
	Avian Island, Marguerite Bay, Antarctic Peninsula	2010	2	2	100
	Total		31	13	41.9
Southern elephant seal (*Mirounga leonina*)	King George Island, South Shetland Islands	2007	6	5	83.3
	Deception Island, South Shetland Islands	2010	1	1	100
		2011	1	1	100
	Avian Island, Marguerite Bay, Antarctic Peninsula	2010	4	2	50.0
	Anvers Island, Palmer Archipelago, Antarctic Peninsula	2011	1	1	100
	Total		13	10	76.9
Crabeater seal (*Lobodon carcinophaga*)	Deception Island, South Shetland Islands	2007	1	0	0
		2011	1	1	100
	Total		2	1	50.0
Antarctic fur seal (*Arctocephalus gazella*)	Deception Island, South Shetland Islands	2007	40	0	0
		2010	44	1	22.7
		2011	48	2	41.7
	Avian Island, Marguerite Bay, Antarctic Peninsula	2010	14	0	0
		2011	15	1	66.7
	Barrientos Island, Aitcho Islands, South Shetland Islands	2011	4	0	0
	Total		165	4	2.4
	Total		211	28	13.3

[a] Geographical coordinates: Deception Island 63°00′S; 60°40′W; Rongé Island 64°40′S; 62°40′W; Avian Island 67°46′S; 68°43′W; King George Island 62°15′S; 58°37′W; Anvers Island 64°48′S, 63°46′W; Barrientos Island 62°24′S, 59°44′W.

3. Results

Antibodies against *T. gondii* were detected in 28 (13.3%) of the 211 Antarctic pinniped samples collected (Table 1). Percentages of detection, with titres ≥1:25, were significantly higher in Southern elephant seals (76.9%, 13/10), than in Weddell seals (41.9%, 13/31) ($P < 0.05$), and than in Antarctic fur seals (2.4%, 4/165) ($P < 0.001$). In Crabeater seals antibodies were found in 1 of the 2 animals tested. Titres ranged from 1:25 to 1:800, most animals showing titres of 1:25 (10/28) and 1:50 (8/28). End-point titres of 1:100 ($n = 2$) and 1:400 ($n = 1$) were found in Southern elephant seals; and of 1:100 ($n = 5$), 1:200 ($n = 1$) and 1:800 ($n = 1$) in Weddell seals.

Seropositive animals were recorded each year of the study, not finding any statistical differences, and in four of the six locations from which samples were screened: Avian Island (5/35), Deception Island (17/165), King George Island (5/6), and Biscoe Point (1/1).

4. Discussion

To the best of knowledge, the study presented here constitutes the first report on the presence of *T. gondii* antibodies in Antarctic pinnipeds. Our serological data using agglutination suggest an unexpected high level of exposure in these populations, especially in Southern elephant seals and in Weddell seals where *T. gondii* antibodies were found in 76.9% and 41.9% of the samples analysed, respectively. In crabeater seals, one of the two animals (50%) analysed also showed antibodies against *T. gondii*. Direct agglutination has been widely used to detect *T. gondii* antibodies in a variety of marine mammals (Mikaelian et al., 2000; Dubey et al., 2003, 2005, 2008; Thoisy et al., 2003; Measures et al., 2004; Aguirre et al., 2007). It has been reported that amongst different serological tests available, the agglutination test is most useful because it is species independent (does not require species specific conjugates), sensitive, and specific (Desmonts and Remington, 1980; Dubey, 2002). In particular, the commercial kit used in the present study has proven its usefulness at detecting *T. gondii* antibodies in experimentally infected seals (Gajadhar et al., 2004) and in Arctic seals (Jensen et al., 2010; Simon et al., 2011). Most authors have considered titres of 1:25 as positive, although as low as 1:2 or 1:5 have also been reported in other hosts (Dubey and Jones, 2008). In addition, an agglutination titre of 1:25 was found in a beluga whale (*Delphinapterus leucas*) with confirmed toxoplasmosis, which led Mikaelian et al. (2000) to suggest that a low titre might be indicative of infection. Therefore in this study evidence of exposure was considered at titres ≥1:25. We found that most titres were low (1:25, 1:50 and 1:100). This is consistent with previous studies using the direct agglutination test in which low titres have been reported in pinnipeds (Dubey, 2010; Jensen et al., 2010; Cabezon et al., 2011; Alvarado-Esquivel et al., 2012; Simon et al., 2011).

Seropositive animals were recorded in most of the locations included in the study. However, Palacios et al. (2010) did not find antibodies against *T. gondii* in penguins in these locations. The Antarctic pinnipeds analysed here, particularly Weddell seals and Southern elephant seals, have a widespread and circumpolar distribution around Antarctica, as well as occurring on sub-Antarctic islands. Occasional wandering individuals have also been recorded as far as Australia, New Zealand, Africa, and South America but seasonal movements are poorly known (Shirihai, 2002).

The route of *T. gondii* infection for marine mammals is not known. Felids are the only known definitive host for this parasite, playing a crucial role contaminating the environment with oocysts excreted in their faeces (Dubey, 2010). It has been suggested that contamination of sea water by freshwater run-off and sewer discharge carrying *T. gondii* oocysts from the terrestrial environment may result in infection in marine mammals (Miller et al., 2002; Conrad et al., 2005; Dabritz et al., 2007). Furthermore it has been experimentally demonstrated that *T. gondii* oocysts can sporulate in sea water and remain infectious for mice for up to 24 months (Lindsay and Dubey, 2009). There is no wild felid fauna in Antarctica and in 1991 the Madrid Protocol on Environmental Protection to the Antarctic banned all introduced species from the Antarctic to protect the native wildlife from introduced diseases, including cats. However, felids are present in the sub-Antarctic regions, areas within the normal distribution range of the animal species analysed here. Recently, Afonso et al. (2007) reported high seroprevalence values (51.09%) in feral cats in the Kerguelen archipelago in the Sub-Antarctic region. Therefore exposure to *Toxoplasma* might have occurred outside Antarctica and is in agreement with the higher detection rates in Southern elephant seals and Weddell seals found here, which show wider distribution and migratory ranges. In addition, the differences observed here between the animal species analysed could be due to their different feeding habits. While Antarctic fur seals and crabeater seals feed primarily on krill taking occasionally fish and cephalopods, the diet of the Weddell seal consists mainly on fish, eating also cephalopods and crustaceans and Southern elephant seals eat mainly cephalopods and fish consuming occasionally shellfish (Shirihai, 2002). It has been shown that *T. gondii* oocysts may be concentrated by marine filter-feeding invertebrates, bivalve molluscs, both under laboratory conditions (Lindsay et al., 2001, 2004; Arkush et al., 2003) and in the wild (Miller et al., 2008) which may act as a source of infection for marine wildlife. In our study, only Southern elephant seals might sporadically consume shellfish, not representing therefore a likely route of transmission for Antarctic pinnipeds. However, recent studies performing experimental exposure of filter feeder fish to *T. gondii* oocysts have indicated that migratory fish may play a role in the transmission of *T. gondii* in the marine environment (Massie et al., 2010).

Further investigations are needed to elucidate the likely transmission pathways of *T. gondii* in marine mammals as well as the presence of *T. gondii* in the Antarctic marine ecosystem.

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Detection and Characterization of a *Cryptosporidium* Isolate from a Southern Elephant Seal (*Mirounga leonina*) from the Antarctic Peninsula▽

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The presence of *Cryptosporidium* and *Giardia* in 221 fecal samples from different species of Antarctic pinnipeds was investigated by immunofluorescence microscopy and PCR. *Cryptosporidium*, a skunk-like genotype, was detected only in a southern elephant seal. *Giardia* was not detected. This is the first report of a *Cryptosporidium* sp. in Antarctic marine mammals.

Cryptosporidium and *Giardia* are ubiquitous protozoan parasites which infect a wide variety of hosts, including humans and domesticated and wild animals (27). In recent years, increasing research has been carried out in marine mammals since they may act as indicator species for environmental contamination with these waterborne parasites (1). *Cryptosporidium* oocysts and/or *Giardia* cysts have been identified in feces or intestinal contents of various animal species, including an Australian dugong (*Dugong dugon*), California sea lions (*Zalophus californianus*), ringed seals (*Phoca hispida*), harp seals (*Phoca groenlandica*), gray seals (*Halichoerus grypus*), hooded seals (*Cyptophora cristatai*), bearded seals (*Erignathus barbatus*), and harbor seals (*Phoca vitulina*), as well as right whales (*Eubalaena glacialis*) and bowhead whales (*Balaena mysticetus*) from different locations worldwide (reviewed in references 1, 5, and 13). However, no studies have been conducted on Antarctic marine mammals. Regarding the species or genotypes involved, the presence of zoonotic assemblages A and B of *Giardia duodenalis* has been commonly reported (1, 2, 5, 16), as have assemblages F (2) and D and novel genotypes related to the canine assemblages C and D (10). *Cryptosporidium hominis*, a species thought to be infective exclusively for humans, nonhuman primates, and gnotobiotic pigs (19), has been identified only in a dugong (12). Other species reported include *Cryptosporidium muris* and two novel genotypes, designated *Cryptosporidium* sp. seal 1 and 2 (2, 5, 25). These studies indicate that marine mammals could represent potential zoonotic reservoirs for *Cryptosporidium* and *Giardia*, but they also reflect that human activities may have an impact on the health of marine mammals and the environment. It is therefore important to monitor the health status of wildlife in general and identify potential sources of infection and routes of transmission or dissemination, particularly in unspoiled areas.

In the present study, we investigated the presence of the zoonotic parasites *Cryptosporidium* and *Giardia* in Antarctic pinnipeds in order to determine the occurrence of these parasites, to identify the species or genotypes involved in infection, and to evaluate whether they might be linked to anthropogenic activities.

A total of 221 fresh fecal samples from different pinniped populations from different locations along the west coast of the Antarctic Peninsula (ranging from 62°15′S to 58°37′W-67°46′S and 68°43′W) (Fig. 1) were collected from the ground during the month of February in 2006 and 2007. These included samples from 31 Weddell seals (*Leptonychotes weddelli*), 2 crabeater seals (*Lobodon carcinophagus*), 4 leopard seals (*Hydrurga leptonyx*), 53 southern elephant seals (*Mirounga leonina*), and 131 Antarctic fur seals (*Arctocephalus gazella*).

Fecal slides were prepared on the same day of sample collection by spreading in triplicate approximately 40 μl of homogenized sample onto a microscope glass slide and fixing in methanol and were stored at −20°C. Fecal samples were kept at +4°C without preservatives for periods of up to 2 months until they were analyzed.

Detection of *Cryptosporidium* and *Giardia*. Immunofluorescence staining was performed using the *Crypto*/*Giardia* Cel IF test (Cellabs Pty. Ltd., Brookvale, Australia) on fecal slides. The numbers of oocysts/cysts on slides were determined at magnification ×400, and the means for 20 fields were calculated. If no oocysts/cysts were seen in 20 fields, the entire slide was examined. To approximately calculate the number of oocysts, the following categories were established: no oocysts; <1 oocyst per field; 1 to 10 oocysts per field; 11 to 100 oocysts per field; and >100 oocysts per field, which corresponded to approximately 0, $<10^3$, 10^3 to 10^4, 10^4 to 10^5, and $>10^5$ oocysts per g (or per ml) of feces, respectively, performing spiking

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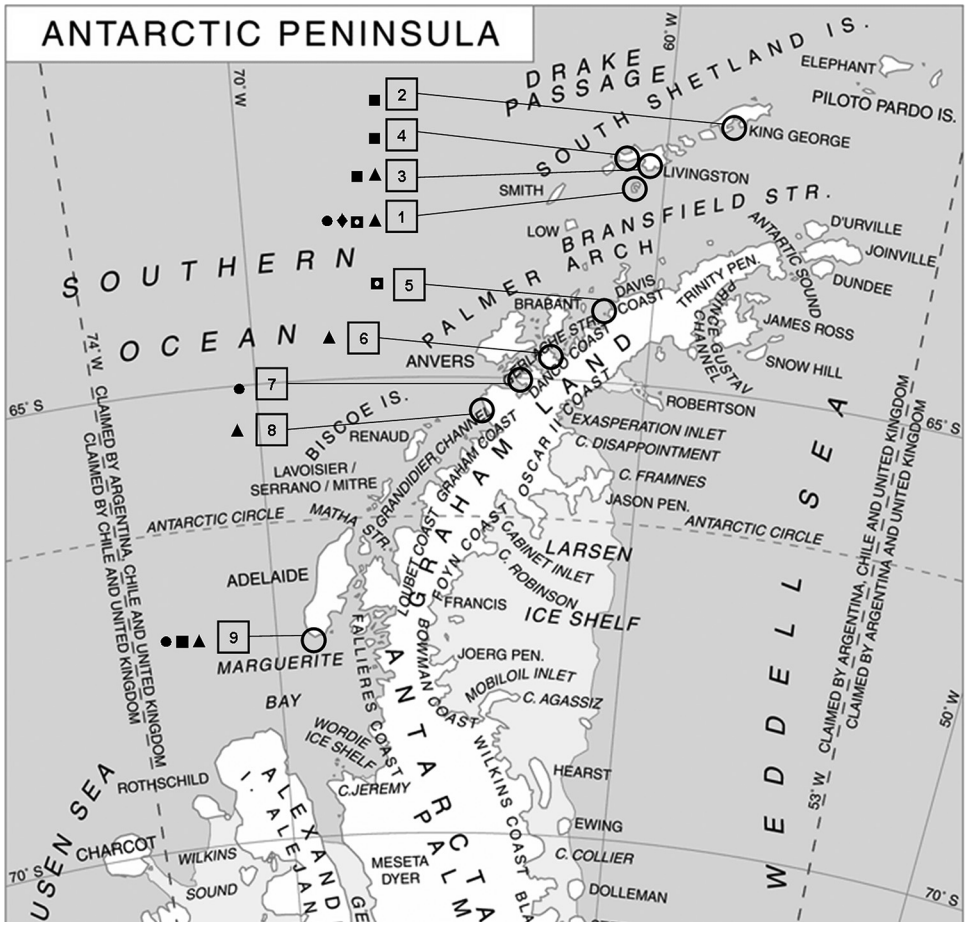

FIG. 1. Locations of sampling areas and animal distribution (adapted from Wikimedia Commons [Giovanni Fattori]). 1, Deception Island, South Shetland Islands; 2, King George Island, South Shetland Islands; 3, Hannah Point, Livingston Island, South Shetland Islands; 4, Byers Peninsula, Livingston Island, South Shetland Islands; 5, Cape Primavera, Antarctic Peninsula; 6, Rongé Island, Errera Channel; 7, Paradise Bay, Antarctic Peninsula; 8, Galindez Island, Argentine Islands; 9, Avian Island, Marguerite Bay, Antarctic Peninsula. ●, Weddell seal (*Leptonychotes weddelli*); ■, crabeater seal (*Lobodon carcinophagus*); ◆, leopard seal (*Hydrurga leptonyx*); ■, southern elephant seal (*Mirounga leonina*); ▲, Antarctic fur seal (*Arctocephalus gazella*).

trials with control *C. parvum* oocysts in negative seal fecal samples. Fecal slides were prepared as described above.

DNA purification was performed using 200 to 300 μl of homogenized feces and comprised oocyst/cyst disruption with zirconia beads in the presence of guanidinium thiocyanate, followed by purification with activated silica as previously described (17). Positive (both positive fecal samples, bovine and canine, and control oocysts/cysts of *C. parvum* and *G. duodenalis* assemblage D) and negative controls were included in each batch.

For *Cryptosporidium*, a nested PCR procedure was performed for amplification of an 827- to 840-bp polymorphic fragment of the 18S ribosomal DNA (rDNA) (28). In addition, a 446-bp fragment of the HSP70 gene was amplified using the primers HSPF4 and HSPR4 (20). For *Giardia*, a nested procedure was performed to amplify a 511-bp fragment of the beta-giardin gene (15). Positive and negative controls were included for all PCRs.

The presence of *Cryptosporidium* oocysts was detected by immunofluorescence and PCR only in one sample (0.45%) from a Southern elephant seal collected in the southernmost sampling area, Avian Island, in 2006. The presence of *Giardia* was not detected by either method in any of the samples analyzed. These results suggest that the presence of these parasites in these regions is rare. The detection methods used in this study are widely applied and have proven very sensitive. However, we did not perform concentration of the fecal material or purification of oocysts/cysts, and therefore samples with very low numbers of oocysts/cysts might not have been detected. Nevertheless, we consider the application of both immunofluorescence microscopy and PCR to enhance the detection power. To our knowledge, our study constitutes the first report of the presence of *Cryptosporidium* in Antarctic marine mammals. Few studies have been conducted in this respect; Fayer (6) has indicated that Antarctica was the only continent in which the presence of *Cryptosporidium* had not been reported. However, recently the presence of *Cryptosporidium* oocysts in Antarctic adelie (*Pygoscelis adeliae*) and gentoo penguins (*Pygoscelis papua*) from Ardley Island, South Shetlands (62°13′S, 58°54′W) has been described (7, 9), although other studies in different locations have reported the absence of *Cryptosporidium* and/or *Giardia* in gentoo and adelie penguins and in chinstrap penguins (*Pygoscelis antarctica*) (8, 22). In

contrast to the results presented here, prevalence rates of *Cryptosporidium* in pinnipeds from other less-preserved areas range from 16 to 24% (2, 4, 12, 13, 25), whereas for *Giardia*, they range from 12 to 64.5% (2, 13, 18, 21). This indicates that the Antarctic fauna has suffered from a lower level of exposure to these agents, which is in agreement with the relative geographical and biological isolation of the Antarctic continent. However, further studies are needed to investigate their potential sources of infection and to monitor their possible introduction and dissemination in this singular environment.

The number of oocysts observed per field was 5, which approximately corresponded to 10^3 to 10^4 oocysts per g of feces, suggesting infection in this animal rather than passive transfer. In contrast to other animal species analyzed in this study, whose migratory and foraging ranges seem to be confined to the Antarctic region, the southern elephant seal is widely distributed in the Southern hemisphere. Therefore, infection in this animal might have been acquired outside Antarctica and introduced into the area. Nevertheless, this might have important implications for the Antarctic fauna, since these animals can act as reservoirs of the disease to those in close vicinity and also disseminate these pathogens to different geographic locations in the marine and terrestrial environments.

Molecular characterization of the *Cryptosporidium* isolate. 18S rDNA and HSP70-positive amplicons were directly sequenced in both directions at the Unidad Genómica del Parque Científico de Madrid. Sequences were analyzed using the BioEdit Sequence Alignment Editor software program, v.7.0.1 (7, 11). Multiple alignments were performed using the ClustalW software program, and neighbor-joining trees were constructed from the aligned sequences using the MEGA software program, version 4 (26). Analysis of the 828-bp 18S rDNA fragment revealed a 99.5% to 99.6% similarity to the sequences of the *Cryptosporidium* skunk genotype published in GenBank, isolated from a skunk (accession no. AY120903), from environmental samples (AY737559 and EU825736), and from a human patient (EU437415). The sequence obtained for this isolate showed the deletion of a T base at position 285 with respect to the sequence under accession no. AY120903 and the insertion of a T base at positions 456, 457, and 508 with respect to all four sequences. The neighbor-joining analysis of the multiple alignment performed with *Cryptosporidium* sequences retrieved from the GenBank database (Fig. 2) showed that this genotype clusters closely with other intestinal *Cryptosporidium* species, such as *C. parvum*, *C. hominis*, *C. wrairi*, *C. meleagridis*, and *C. suis*, but constitutes a separate, distinct group.

Sequence and phylogenetic analysis of the HSP70 gene confirmed these results. The highest similarities, 99.8%, were observed with the *Cryptosporidium* skunk genotype isolated from a skunk (accession no. AY120917) and from a human patient (EU437414). The sequence obtained in this study varied by a T/C substitution at position 75 and an A/G substitution at position 240 with respect to the sequence under accession no. AY120917 and EU437414, respectively. Previously, the *Cryptosporidium* skunk genotype had been isolated from skunk, raccoon, eastern squirrel, opossum, river otter (27), environmental samples (14, 23), and, also recently, from humans (3, 24). It was initially suggested that this genotype might be a fur-bearing wild mammal host-adapted type with no significance for public health (27). However, the identification of this genotype in a human patient who had suffered from diarrhea (24) demonstrates that it is capable of causing infection in other hosts and could disseminate through different routes of transmission. More molecular data identifying the species and genotypes present in marine mammals are needed to compare with new and existing data from humans and other terrestrial animals in order to evaluate the potential impact of human activities on these populations.

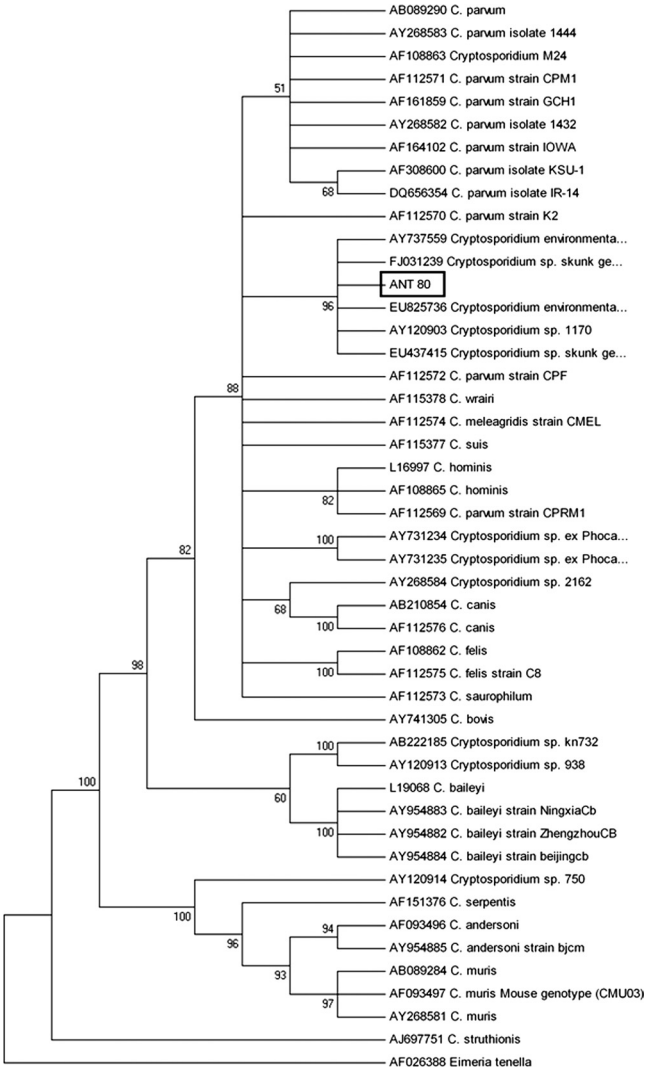

FIG. 2. Phylogenetic relationships between the southern elephant seal isolate ANT 80 (in box) and published *Cryptosporidium* species or genotypes, inferred by neighbor-joining analysis of the 18S rDNA fragment. Evolutionary distances were calculated by the Kimura-2 parameter model using *Eimeria tenella* as an outgroup.

Nucleotide sequence accession numbers. The nucleotide sequences generated in this study have been deposited in the GenBank database under accession numbers GQ421425 and GQ421426.

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Short communication

Detection of a novel genotype of *Cryptosporidium* in Antarctic pinnipeds

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ABSTRACT

A study was conducted to investigate the presence of *Cryptosporidium* and *Giardia* in Antarctic marine mammals. A total of 270 faecal samples from different species of pinnipeds from different locations in the South Shetland Islands and Antarctic Peninsula were analysed by immunofluorescence microscopy and PCR. *Cryptosporidium* was detected by PCR in three samples from Southern elephant seals (*Mirounga leonina*) and 2 Weddell seals (*Leptonychotes weddellii*). However, no oocysts were observed in any of the samples by immunofluorescence microscopy. Molecular characterisation of the isolates, using the 18S rDNA, the HSP70 and the COWP loci, revealed the presence of a *Cryptosporidium* sp., previously reported from an Antarctic Southern elephant seal, in the elephant seals and a novel genotype in Weddell seals. *Giardia* could not be detected in any of the samples analysed.

1. Introduction

Cryptosporidium spp. and *Giardia duodenalis* (syn. *G. intestinalis*, *G. lamblia*) are protozoan parasites which infect a wide variety of hosts including humans and domesticated and wild animals worldwide (Xiao and Fayer, 2008). Currently, the genus *Cryptosporidium* contains up to 22 species and over 40 genotypes, while *Giardia duodenalis* includes 7 assemblages or genotypes, designated A through G (Fayer, 2010; Fayer et al., 2010; Robinson et al., 2010; Feng and Xiao, 2011; Ren et al., 2012). In addition, an assemblage H has been recently described in seals (Lasek-Nesselquist et al., 2010). Proper identification and characterisation of the species and genotypes involved in infection are needed to elucidate the routes of transmission. Traditionally, species were primarily differentiated according to host specificity, oocyst or cyst morphology and site of infection (Fayer, 2010). However, taxonomy based on these criteria has proven inadequate. Furthermore, genetic analysis has shown that these genera are complex. The advent of molecular characterisation tools has greatly contributed to establishing a correct taxonomy for both parasites setting the basis for a better understanding of the diseases they cause and their epidemiology.

In the last years increasing research has been carried out on marine mammals since they may act as indicator species for environmental contamination with these waterborne parasites (Appelbee et al., 2005). *Cryptosporidium* spp. oocysts and *Giardia* spp. cysts have been identified in different pinniped species which include California sea lions (*Zalophus californianus*), bearded seals (*Erignathus barbatus*), ringed seals (*Phoca hispida* syn. *Pusa hispida*), harp seals (*Pagophilus groenlandica*), grey seals (*Halichoerus grypus*), hooded seals (*Cyptophora cristatai*), harbour seals (*Phoca vitulina*), mainly from different locations in North America and an Antarctic Southern elephant seal (*Mirounga leonina*) (reviewed in Rengifo-Herrera et al., 2011). Molecular analyses identified *Cryptosporidium muris* and two

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Cryptosporidium seal genotypes, seal genotypes 1 and 2, in ringed seals in Canada (Santin et al., 2005). Recently, two additional novel *Cryptosporidium* genotypes have been described in an Antarctic Southern elephant seal (*M. leonina*) and in a harp seal (*P. groenlandicus*) from the Gulf of Maine (Rengifo-Herrera et al., 2011; Bass et al., 2012). *Giardia duodenalis* Assemblage A was identified in harp and hooded seals from Canada (Appelbee et al., 2005), Assemblage B in a harbour seal in the USA as well as in ringed seals in Canada, both Assemblages A and B in a harp seal and Assemblage F-like in mixed grey/harbour seal populations from beaches in the USA (Bogomolni et al., 2008; Dixon et al., 2008; Lasek-Nesselquist et al., 2008). A further study has identified the canine genotype D and a novel genotype related to Assemblages C and D in faeces of harbour seals from Washington Stateĭs marine waters (Gaydos et al., 2008). These studies highlight the need for more research that can provide additional information on the diversity and host range of these groups of parasites.

The purpose of this study was to further investigate the presence of *Cryptosporidium* and *Giardia* in pinnipeds from different regions in the Antarctic Peninsula.

2. Materials and methods

2.1. Faecal samples

A total of 270 faecal samples from different pinniped populations from Deception Island, and other areas in the South Shetland Islands and Antarctic Peninsula were collected during the month of February in both 2010 and 2011 (Table 1). These included samples from Weddell seals (*Leptonychotes weddellii*), Southern elephant seals (*M. leonina*), and Antarctic fur seals (*Arctocephalus gazella*). Fresh samples were collected from the ground.

After sample collection, faecal slides were prepared, fixed in methanol, and stored at −20 °C until analysed. Faecal samples were kept at +4 °C without preservatives for periods up to 2 months when they were received and analysed in the laboratory.

2.2. Cryptosporidium and Giardia detection and characterisation

Immunofluorescence staining was performed using the *Crypto*/*Giardia* Cel IF Test (Cellabs Pty Ltd., Brookvale, Australia) according to the manufacturer's instructions.

Oocyst/cyst disruption and DNA purification from faecal samples were performed as described previously (McLauchlin et al., 1999).

For *Cryptosporidium* detection and characterisation, a nested PCR procedure was performed for amplification of an 827–840 bp polymorphic fragment of the 18 rDNA (Xiao et al., 1999, 2000). For further characterisation, a 446 bp fragment of the HSP70 and a 550 bp fragment of the COWP genes were amplified according to the protocols described by Morgan et al. (2001) and Pedraza-Díaz et al. (2001), respectively.

For *Giardia*, a nested procedure was performed to amplify a 511 bp fragment of the beta-giardin gene (Lalle et al., 2005).

Positive (*C. parvum* and *G. duodenalis* assemblage D) and negative controls were included for all PCRs. A 5 μl aliquot of the PCR products was examined following electrophoresis in 1% agarose/ethidium bromide gels.

Positive amplicons were purified using the GENECLEAN Turbo kit (QBiogene, CA, USA) according the manufacturer's instructions and then directly sequenced in both directions using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA) and a 3730 DNA analyser (Applied Biosystems, CA, USA) at the Unidad Genómica del Parque Científico de Madrid. Sequences were analysed using BioEdit Sequence Alignment Editor v.7.0.1 (7) (Hall, 1999). Multiple alignments were performed using the ClustalW program and neighbour-joining trees were constructed from the aligned sequences using the MEGA5 software (Tamura et al., 2011). Accession numbers of Genbank *Cryptosporidium* 18S rDNA sequences used in the analysis: *C. andersoni* (AF093496), *C. baileyi* (L19068), *C. bovis* (AY120911), *C. canis* (AB210854), *C. cuniculus* (EU437413), *C. fayeri* (AF112570), *C. felis* (AF108862), *C. fragile* (EU162751), *C. galli* (HM116388), *C. hominis* (AB369994), *C. macropodum* (AF513227), *C. meleagridis* (AF112574), *C. molnari* (HM243548), *C. muris* (AB089284), *C. parvum* (L16996), *C. ryanae* (AY587166), *C. serpentis* (AF151376), *C. suis* (AF115377), *C. ubiquitum* (AF442484), *C. varanii* (AF112573), *C. wrairi* (AF115378), *C. xiaoi* (FJ896050), *Cryptosporidium* sp. 80ANT (GQ421425), *Cryptosporidium* sp. Cc444 (JN858905), *Cryptosporidium* sp. ferret genotype (GQ121022), *Cryptosporidium* sp. mink genotype (EF641015), *Cryptosporidium* sp. Pg453 (JN858909), *Cryptosporidium* sp. Pv140 (JN858906), *Cryptosporidium* sp. Pv245 (JN858907), *Cryptosporidium* sp. Pv270 (JN858908), *Cryptosporidium* sp. seal genotype 1 (AY731234), *Cryptosporidium* sp. seal genotype 2 (AY731235), *Cryptosporidium* sp. skunk genotype (AY120903).

Accession numbers of Genbank *Cryptosporidium* HSP70 sequences used in the analysis: *C. andersoni* (AJ567390), *C. baileyi* (AF221539), *C. bovis* (AY741306), *C. canis* (AY120920), *C. cuniculus* (GU967462), *C. fayeri* (AF221531), *C. felis* (AF221538), *C. galli* (AY168849), *C. hominis* (EF591788), *C. meleagridis* (AF221537), *C. muris* (AF221543), *C. parvum* (EF576953), *C. ryanae* (EU410346), *C. serpentis* (AF221541), *C. suis* (DQ833281), *C. ubiquitum* (EF362483), *C. varanii* (FJ429602), *C. wrairi* (AF221536), *C. xiaoi* (FJ896041), *Cryptosporidium* Pg453 (JN860884), *Cryptosporidium* Pv140 (JN860883), *Cryptosporidium* Pv270 (JN860882), *Cryptosporidium* sp. ferret (AF221532), *Cryptosporidium* sp. hedgehog (GQ259143), *Cryptosporidium* sp. mink (EF428201), *Cryptosporidium* sp. seal 1 (AY731236), *Cryptosporidium* sp. seal 2 (AY731237), *Cryptosporidium* sp. seal 2 (AY731238), *Cryptosporidium* sp. skunk (AY120917), *Cryptosporidium* sp. 80ANT (GQ421426).

Accession numbers of Genbank *Cryptosporidium* COWP sequences used in the analysis: *C. andersoni* (DQ989570, AY282693), *C. baileyi* (AY282698, AF266276), *C. canis* (AF266274), *C. cuniculus* (EU437411), *C. fayeri* (AY237633), *C. felis* (AY282700), *C. hominis* (AF148741, AF481960), *C. meleagridis* (AF248742, AY282694, DQ116568), *C. muris* (AF161579, AY643491), *C. parvum* (AY282696, AY282687, AY282686, AY282695, AF248743), *C. serpentis* (AF266275), *C. ubiquitum* (HM209389), *C. wrairi* (U35027),

Table 1
Distribution of samples and results obtained.

Host species		No. samples	Year	Location	Positive	*Cryptosporidium* species/genotype		
						18S rDNA	HSP70	COWP
Weddell seal (*Leptonychotes weddelli*)		9	2010	Deception Island, South Shetland Islands	1	*Cryptosporidium* sp. Weddell seal genotype	*Cryptosporidium* sp. Weddell seal genotype	*Cryptosporidium* sp. Weddell seal genotype
		1		Rongé Island, Errera Channel				
		3	2011	Deception Island, South Shetland Islands	1	*Cryptosporidium* sp. Weddell seal genotype	nd	nd
		1		Byers Peninsula, Livingston Island, South Shetland Islands				
	Total	14			2			
Southern elephant seal (*Mirounga leonina*)		16	2010	King George Island, South Shetland Islands				
		16		Hannah Point, Livingston Island, South Shetland Islands				
		18		Byers Peninsula, Livingston Island, South Shetland Islands				
		18		Avian Island, Marguerite Bay, Antarctic Peninsula				
		3	2011	King George Island, South Shetland Islands				
		3		Hannah Point, Livingston Island, South Shetland Islands				
		15		Byers Peninsula, Livingston Island, South Shetland Islands	1	*Cryptosporidium* sp. Southern elephant seal genotype	nd	nd
		13		Avian Island, Marguerite Bay, Antarctic Peninsula	1	*Cryptosporidium* sp. Southern elephant seal genotype	*Cryptosporidium* sp. Southern elephant seal genotype	*Cryptosporidium* sp. Southern elephant seal genotype
		7		Biscoe Point	1	*Cryptosporidium* sp. Southern elephant seal genotype	nd	nd
		2		Barrientos Island				
	Total	111			3			
Antarctic fur seal (*Arctocephalus gazella*)		53	2010	Deception Island, South Shetland Islands				
		8		Avian Island, Marguerite Bay, Antarctic Peninsula				
		1		Rongé Island, Errera Channel				
		3		King George Island, South Shetland Islands				
		39	2011	Deception Island, South Shetland Islands				
		4		Avian Island, Marguerite Bay, Antarctic Peninsula				
		16		King George Island, South Shetland Islands				
		2		Byers Peninsula, Livingston Island, South Shetland Islands				
		3		Hannah Point, Livingston Island, South Shetland Islands				
		3		Barrientos Island				
		13		Penguin Island				
	Total	145			0			

nd: not done.

Cryptosporidium sp. ferret (AB469366), *Cryptosporidium* sp. mink (EU197215).

2.3. Nucleotide sequence accession numbers

The nucleotide sequences generated in this study have been deposited in the GenBank database under accession numbers JQ740100–JQ740108.

3. Results

3.1. Detection of Cryptosporidium and Giardia

Cryptosporidium was detected in samples collected from three Southern elephant seals (*M. leonina*) and two Weddell seals (*L. weddellii*) of the 111 and 14 faecal samples analysed, respectively, by PCR (Table 1). Cryptosporidial DNA was not detected in any of the 145 samples from Antarctic fur seals (*A. gazella*) analysed. No *Cryptosporidium* oocysts were observed in any of the samples by immunofluorescence microscopy.

The presence of *Giardia* could not be detected either by immunofluorescence or by PCR in any of the samples analysed.

3.2. Molecular characterisation of the Cryptosporidium isolates

Sequence analysis of the 840 bp 18S rDNA fragment amplified showed that the three isolates present in the Southern elephant seals were an exact match (100% similarity) to the *Cryptosporidium* isolate previously obtained from an Antarctic Southern elephant seal (GQ421425) and closely related to the *Cryptosporidium* skunk genotype (AY120903) (Fig. 1A). The two sequences obtained from Weddell seals were identical to each other and showed the highest similarity (98.6%) with the *Cryptosporidium* ferret genotype (GQ121022), being also closely related to *Cryptosporidium* mink genotype (EF641015) and *Cryptosporidium wrairi* (AF115378) (similarities of 98.5% and 98.4%, respectively).

For further characterisation, fragments of the HSP70 and COWP genes were amplified and sequenced from one sample of each of the 18S rDNA genotypes found (Table 1). Sequence and phylogenetic analysis of these markers confirmed these results. The neighbour-joining analyses of the multiple alignments performed with *Cryptosporidium* sequences retrieved from the GenBank database showed the genetic uniqueness of these genotypes, which cluster closely with other intestinal *Cryptosporidium* species (Fig. 1A–C).

4. Discussion

Marine mammals are regarded as prime sentinel species for environmental changes (Bossart, 2011). However, the information available about the health status of the Antarctic marine mammals is very scarce and fragmented (Kerry et al., 2000). In addition, human derived activities in this pristine environment such as tourism and other causes like global warming could be compromising these populations. In this sense recommendations have been made regarding the importance of monitoring the health of the Antarctic fauna (Anon, 2003).

Recently, the detection of a *Cryptosporidium* genotype in an Antarctic Southern elephant seal was reported (Rengifo-Herrera et al., 2011). In the present study further monitoring of the presence of the potentially zoonotic parasites *Cryptosporidium* and *Giardia* in Antarctic pinnipeds was carried out. Samples from 8 different locations along the west coast of Antarctic Peninsula in a latitudinal gradient covering 5 degrees of latitude (ranging from 62°15′S; 58°37′W–67°46′S; 68°43′W), distances greater than 600 km and differences in mean annual temperatures of up to 2 °C were analysed. The results presented here confirm previous findings in that the presence of these parasites in the Antarctic region is not widespread (Rengifo-Herrera et al., 2011): *Cryptosporidium* was only detected in 5 of the 270 animals sampled (1.8%) from 4 of the sampling areas included in the study, and *Giardia* was not detected in any of the animals analysed. However, the presence of *Cryptosporidium* seems to be constant in this region, since it has been detected it in three different years (2006, 2010 and 2011) (Rengifo-Herrera et al., 2011; this study). The low percentages of detection found in these studies contrast with the results reported in pinnipeds from other areas in which prevalence rates of *Cryptosporidium* range from 6.5 to 24% (Hill et al., 1997; Deng et al., 2000; Hughes-Hanks et al., 2005; Santin et al., 2005; Bogomolni et al., 2008; Bass et al., 2012) whereas for *Giardia*, they range from 12 to 80% (Olson et al., 1997; Measures and Olson, 1999; Hughes-Hanks et al., 2005; Bogomolni et al., 2008; Dixon et al., 2008; Appelbee et al., 2010). It has been previously suggested that this indicates that the Antarctic fauna might experience a lower level of exposure to these agents, which is in agreement with the relative geographical and biological isolation of the Antarctic continent (Rengifo-Herrera et al., 2011).

Detection of *Cryptosporidium* could be achieved by PCR but no oocysts were detected by immunofluorescence microscopy. Low numbers of *Cryptosporidium* oocysts in faecal samples could account for these results. The DNA extraction method used here utilises whole faeces, hence it is possible that target DNA is derived not only from oocysts, but also from other stages in the life cycle of this parasite as well as 'free' in the faeces (Pedraza-Díaz et al., 2001). The usefulness of PCR as diagnostic tool in the detection of *Cryptosporidium* and *Giardia* infections with intermittent shedding or low numbers of oocysts or cysts in faecal samples of different origin have been shown in previous studies (McGlade et al., 2003; Amar et al., 2004; Appelbee et al., 2010). In addition, the use of molecular methods allows the identification of the species or genotypes involved in infection and may contribute to understanding the routes of transmission. This has led to the description or re-description in the past few years of several novel *Cryptosporidium* species or genotypes, such as *C. ubiquitum* (Fayer et al., 2010), *C. ducismarci* (Traversa, 2010), *C. cuniculus* (Robinson et al., 2010), *C. tyzzeri* (Ren et al., 2012), or *C. viatorum* (Elwin et al., 2012) amongst others, or the *Giardia duodenalis* assemblage H (Lasek-Nesselquist et al., 2010).

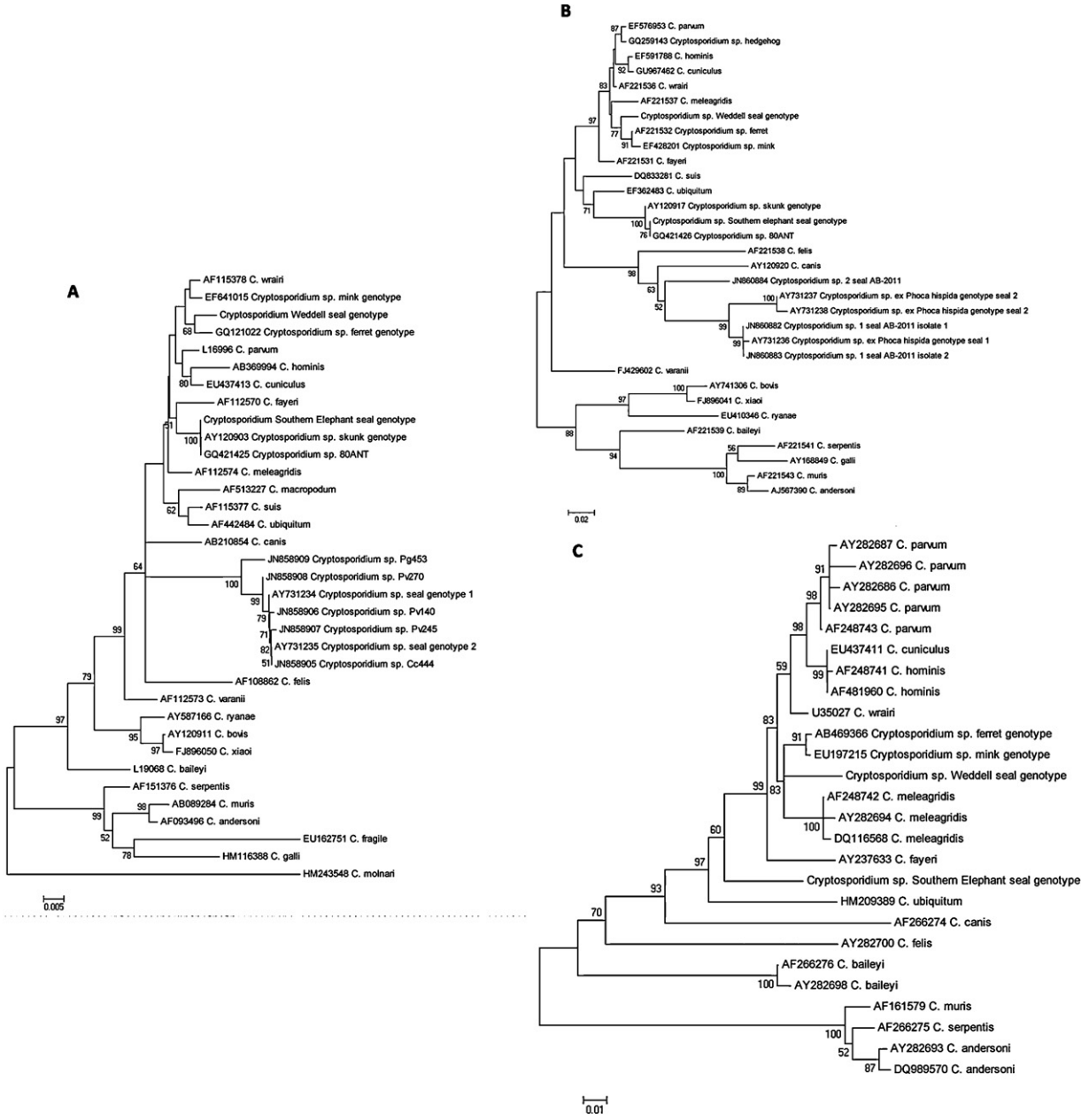

Fig. 1. Phylogenetic relationships of the Southern elephant seal and Weddell seal genotypes and published *Cryptosporidium* species or genotypes inferred by neighbour-joining analysis of the 18S rDNA (A), the HSP70 (B), and the COWP (C) gene fragments. Evolutionary distances were computed using the Kimura-2 parameter method. Bootstrap values greater than 50% from 1000 replicates are shown.

Although the knowledge regarding the presence of *Cryptosporidium* and *Giardia* in marine mammals is increasing, few studies have identified the species and genotypes involved in infection. *Cryptosporidium hominis*, a species thought to be infective exclusively to humans, non-human primates and gnotobiotic pigs (Morgan et al., 2000) has only been identified in a dugong (Hill et al., 1997). Other species reported include *C. muris*, two seal genotypes, designated *Cryptosporidium* sp. seal 1 and 2 in ringed seals (Santin et al., 2005; Dixon et al., 2008); and a novel genotype from a harp seal (Bogomolni et al., 2008; Bass et al., 2012). Our studies have led to the description of a further two novel *Cryptosporidium* genotypes in Antarctic pinnipeds. The multilocus analysis performed, which included three of the most commonly used markers, 18S rDNA, HSP70 and COWP genes, has shown that these genotypes are more closely related to previously described *Cryptosporidium* genotypes in ferrets and mink and other intestinal *Cryptosporidium* species than to those reported from seals. Therefore the findings reported here further widen the range of both *Cryptosporidium* host species and the parasite's species or genotypes and highlight the need for

further studies to contribute to the understanding of the taxonomy and epidemiology of cryptosporidiosis.

The Antarctic pinnipeds analysed in this study, particularly Weddell seals and Southern elephant seals, have a widespread and circumpolar distribution around Antarctica, as well as occurring on sub-Antarctic islands. Occasional wandering individuals have also been recorded as far north as Australia, New Zealand, Africa, and South America but seasonal movements are poorly known (Shirihai, 2002). Therefore exposure to *Cryptosporidium* might have occurred outside Antarctica. This is in agreement with the higher detection rates in Southern elephant seals and Weddell seals found here, which show wider distribution and migratory ranges than Antarctic fur seals. In addition, the differences observed here between the animal species analysed could be due to their different feeding habits. While Antarctic fur seals feed primarily on krill taking occasionally fish and cephalopods, the diet of Weddell seal consists mainly of fish, although they also consume cephalopods and crustaceans. Southern elephant seals eat mainly cephalopods and fish, and occasionally shellfish (Shirihai, 2002). It has been shown that *Cryptosporidium* oocysts (and *Giardia* cysts) may be concentrated by marine bivalve shellfish (reviewed in Robertson, 2007) which may act as a source of infection for marine wildlife. In the present study, only Southern elephant seals might sporadically consume shellfish, not representing therefore a frequent route of transmission for Antarctic pinnipeds. Furthermore, the presence of *Cryptosporidium* oocysts in Antarctic adelie (*Pygoscelis adeliae*) and gentoo penguins (*Pygoscelis papua*) from Ardley Island, South Shetlands (62°13′S 58°54′W) has been recently described (Fredes et al., 2007b, 2008) although there is no information available on the *Cryptosporidium* species or genotypes involved. In contrast, other studies in different locations have reported the absence of *Cryptosporidium* and/or *Giardia* in gentoo and adelie penguins as well as in chinstrap penguins (*Pygoscelis antarctica*) (Fredes et al., 2007a; Palacios et al., 2010). Nevertheless these findings might have important implications for the Antarctic fauna since these animals can act as vectors not just spreading the disease to those in close vicinity but also disseminating these pathogens to different geographic locations in the marine and terrestrial environments. Therefore, further studies are needed to expand our current knowledge of *Giardia* and *Cryptosporidium* in the marine environment.

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