Universitat Autònoma de Barcelona

Effects of helminth co-infections on tuberculosis status in a wildlife reservoir

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SEFaS Servei d'Ecopatologia de Fauna Salvatge

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Los doctores Joaquim Segalés Coma, profesor del área de conocimiento de Sanidad y Anatomía Animal de la Universitat Autònoma de Barcelona, Pedro Fernández Llario, investigador y colaborador de la Universidad de Extremadura, y Emmanuel Serrano Ferron, investigador asociado al Servei d'Ecopatologia de Fauna Salvatge de la Universitat Autònoma de Barcelona y al departamento de biología y CESAM de la universidad de Aveiro (Portugal),

HACEN CONSTAR

Que la memoria titulada "Effects of helminth co-infections on tuberculosis status in a wildlife reservoir" presentada por Diana Gassó Garcia para la obtención del grado de Doctora en Veterinaria por la Universitat Autónoma de Barcelona, ha sido realizada bajo nuestra dirección y, considerándola satisfactoriamente finalizada, autorizamos su presentación para que sea juzgada por la comisión correspondiente.

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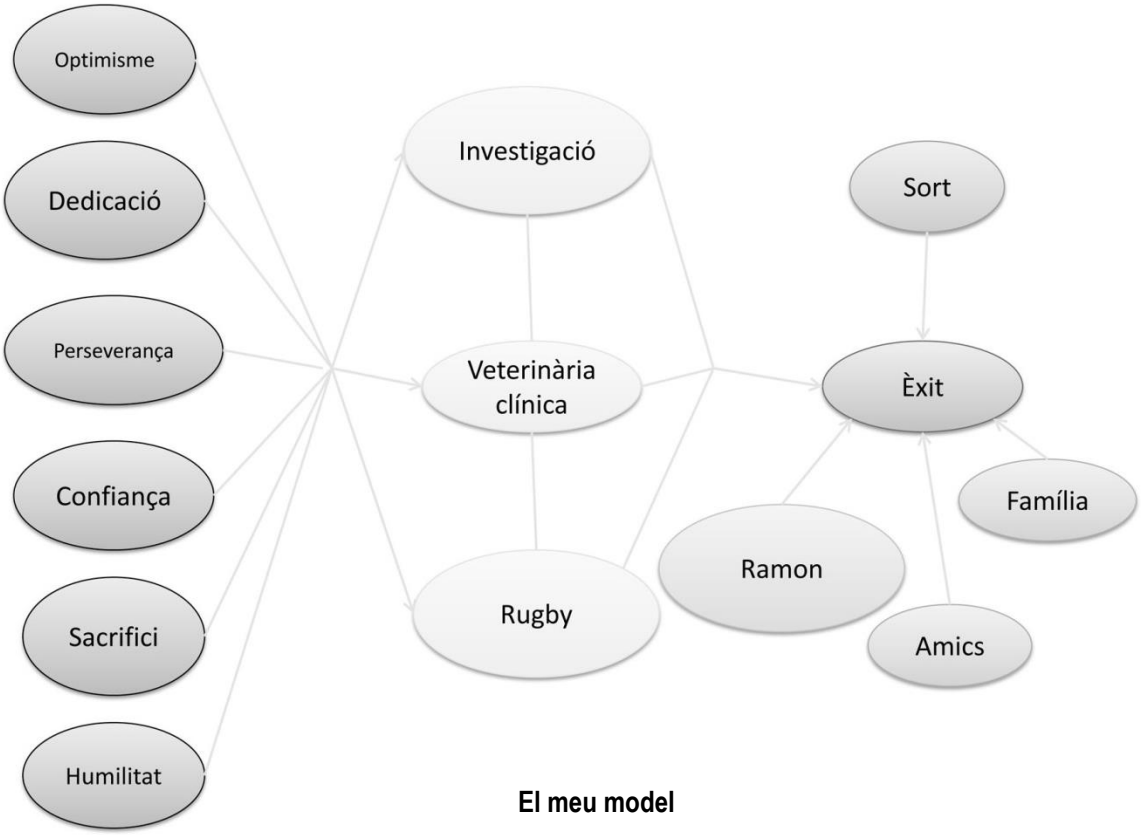

El meu model

Adaptat de Gastón Sanchez 2013

"No perseguiu la felicitat del futur,

sinó la pau i la tranquilitat en

aquest precís instant"

Thich Nhat Hantt

Il·lustracions I disseny de la portada originals de Mar H. Pongiluppi

Agraïments

Tot arriba… quan hi ha esforç, il·lusió i motivació, però sobretot quan tens l'ajuda de tots els que t'envolten.

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PREFACE

The three most devastating human diseases (HIV/AIDS, tuberculosis (TB) and malaria) overlap geographically with helminthiases, which are important neglected tropical diseases. Although is known that co-infections with helminthiasis adversely affects the naturally story and progression of the "big three" we are still in the early stages of appreciating the full extent of the comorbidity that occurs.

This doctoral thesis has two differentiated parts. The first one is more technical, built up to clarify some aspects of the wild boar parasitofauna: description, classification and quantification. Until now the same techniques used in wild herbivores were used without knowing with certainty if they work fine in this wild omnivorous. The second part, with a major ecological focus, studied indicators of health status in boars, how multiparasitism affects these indicators via direct or indirect effects and, finally the impact of deworming in the pathogen structure of a wild boar population naturally infected with *Mycobacterium tuberculosis complex* (MTC). The principal objective of this thesis was to proportionate management elements of the wild boar populations based on their parasitofauna and health status.

This thesis starts by introducing the concept of emerging and re-emerging diseases and the importance of wildlife research for this issue, forming part of the "one health" concept. This is followed up with a small revision of the co-infection concept ending up with the helminth-TB model. Finally the co-infection study system used in the works of this thesis (the host, the target disease and the worms). Next principal hypothesis and objectives of this doctoral work.are formulated. **Chapter 1** revises the five more common species of lung nematodes in the wild boar. Further the identification key to facilitate the differentiation of these common species with high prevalence in non-confined animals.is presented **Chapter 2** was designed to study *Macracanthorhynchus hirudinaceus* prevalence, specifically regardingin the limitation of flotation and sedimentation techniques for quantify this acanthocephalan. **Chapter 3**, reviews the uses and limitations of faecal egg count (FEC) for assessing worm burden in suids. This technique is a basic indirect tool used to assess worm burdens of wild hosts. **Chapter 4** shows the effects of TB on different oxidative stress (OS) biomarkers in wild boar experimentally challenged with *M. bovis*, and the role of body weight, sex, population and season are explored to explain the observed variability of OS indicators in two populations of free-ranging wild boar where TB is common. The following chapters studied changes of health biomarkers and pathogen structure of

a co-infected wild boar population naturally infected with MTC. **Chapther 5**, presents the investigations on direct and indirect impacts of helminth co-infections on selected physiological indicators of health status in wild boar with different TB status. Finally, **chapter 6** shows the impact of deworming in TB outcome and the pathogen community of a population of wild boars naturally co-infected with *M. bovis.*

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2. SUMMARY

1. SUMMARY

TITLE: Effects of helminth co-infections on tuberculosis status in a wildlife reservoir

This thesis uses basic parasitology studies of wild boar (*Sus scrofa*) to focus on a more ecological view of their pathogen populations, co-infections that arise and the effects on their health status. Specifically, six investigations were conducted being the first three aimed at reviewing aspects of the wild boar parasitofauna In particular, keys to identify the five most common species of lung nematodes (**Chapter 1**), the real prevalence of the Acanthocephala *Macracanthorhynchus hirudinaceus* denouncing the underestimation of this species (**Chapter 2**) and a review of the uses and limitations of faecal egg count for assessing worm burden in wild boars (**Chapter 3**) are presented. Furthermore, the levels of oxidative stress in a population of feral pigs infected naturally and experimentally by *M. bovis* (**Chapter 4**) and the effect of co-infection between helminths and tuberculosis in terms of physiological cost of health in a wild boar population naturally infected by MTC (**Chapter 5**) were addressed. Finally, the effect of an antiparasite drug treatment on the helminth community structure and on the outcome of disease infection in a wild boar population naturally infected by *M. bovis* (**Chapter 6**) was studied.

Keywords: Co-Infection, Faecal egg count, *Mycobacterium bovis*, Oxidative stress, Worms.

3. RESUM

2. RESUM

TÍTOL: Efectes de les co-infeccions per helmints sobre l'estat de la tuberculosis en un reservori de fauna salvatge

En aquesta tesi s'ha estudiat des d'aspectes bàsics de parasitologia del porc senglar (*Sus scrofa*), fins l'estudi més ecològic de les seves poblacions de patògens, les co-infeccions que en deriven i els efectes sobre el seu estatus sanitari. En concret s'ha portat a terme sis investigacions, on les tres primeres van ser orientades a revisar aspectes de la parasitofauna del senglar, que inclou una clau per identificar les 5 espècies de nematodes pulmonars més comuns (**Capítol 1**), les prevalences de l'acantocèfal *Macracanthorhynchus hirudinaceus* confirmant la seva subestimació (**Capítol 2**) i els usos i limitacions del recompte d'ous en femtes de senglar per avaluar la càrrega parasitària dels mateixos (**Capítol 3**). D'altra banda s'ha avaluat els nivells d'estrès oxidatiu en una població de porcs senglars infectats tant de forma natural com de forma experimental per *M. bovis* (**Capítol 4**) i s'ha abordat l'efecte de la co-infecció, entre els helmints i la tuberculosi, en termes de salut en una població de senglars naturalment infectada per *M. bovis* (**Capítol 5**). Finalment s'ha estudiat l'efecte de la desparasitació sobre l'estructura de la comunitat d'helmints i la tuberculosi (**Capítol 6**).

Paraules clau: Co-infecció, Estrès oxidatiu, Helmints, *Mycobacterium bovis*, Recompte d'ous en femta

4. RESUMEN

3. RESUMEN

TÍTULO: Efectos de las co-infecciones por helmintos sobre el estado de la tuberculosis en un reservorio de fauna salvaje

En esta tesis se ha estudiado desde aspectos básicos de parasitología del jabalí (Sus scrofa), hasta aspectos más ecológicos de sus poblaciones de patógenos, las co-infeciones que padecen y los efectos sobre su estatus sanitario. En concreto, se han llevado a cabo seis investigaciones, siendo las tres primeras orientadas a revisar aspectos de la parasitofauna del jabalí. En concreto, se ha desarrollado una clave para identificar las 5 especies de nematodos pulmonares más comunes (**Capítulo 1**), se han estudiado las prevalencias del acantocéfalo *Macracanthorhynchus hirudinaceus* confirmando su subestimación (**Capítulo 2**) y se ha revisado los usos y limitaciones del conteo de huevos en heces de jabalí para evaluar la carga parasitaria de los mismos (**Capítulo 3**). Por otro lado, se han evaluado los niveles de estrés oxidativo tanto en una población de jabalíes infectados de forma natural como experimentalmente por *M.bovis* (**Capítulo 4**) y se ha abordado el efecto sobre la salud de la co-infección, entre los helmintos y la tuberculosis, en una población de jabalíes naturalmente infectada por *MTC* (**Capítulo 5**). Finalmente, se ha estudiado el efecto de la desparasitación sobre la estructura de la comunidad de helmintos y la tuberculosis (**Capítulo 6**).

Palabras clave: Co-infección, Conteo de huevos en heces, Estrés oxidativo, Helmintos, *Mycobacterium tuberculosis*

5. INTRODUCTION

5.1. Emerging infectious diseases a key component of global change

Emerging infectious diseases (EIDs) are caused by newly identified species or strains that may have evolved from a known infection or spread to a new population or to an area undergoing ecologic transformation, or that may have existed previously but are rapidly increasing in incidence or geographic range (re-emerging infections). Domestic, wildlife and human rapid movements, urbanization, agricultural intensification, animal translocations, ecological manipulations, global climatic change are few examples of risk drivers for EIDs. Many wildlife species are reservoirs of pathogens that threaten domestic animal and human health. It is known from time that zoonotic pathogens are important and potentially source of EIDs for the human population (Morse 1995), but vice-versa humans also have been implicated in wildlife EIDs that cause severe consequences in wild populations (Daszak 2000) and on the conservation of global biodiversity. Then a multidisciplinary approach is required (Cook and Karesh 2008),the named "one health".

In short we can say that disease emergence most frequently results from a change in ecology of pathogen, host or both. The two pathogens of the wild boar studied in our work *Mycobasterium tuberculosis complex,* MTC (principally *M. bovis*) and some nematodes have a potential zoonotic paper, furthermore wild boar is a true reservoir to bovine TB, so is important to understand how their interact with the host and between them to pose good management practices and prevent disease emergence.

4.2. Beyond the one-pathogen one disease approach, the co-infection framework

As we have seen before the maintenance of established host-parasite relations would be important for the overall well-being of the host species. Disease control measures, climate change or novel parasite species introductions could break the equilibrium. Host-parasite interactions are rarely one-by-one. Instead, each host tends to have a well-established community of pathogens cohabiting within him. In these systems, co-infected pathogens could have synergistic or competitive effects favoring or disfavoring the host health, triggering epidemiological and clinical implications. The multihost-multipathogen interactions are complex to

study and understand; therefore, the study of pairwise co-infections may be a good first approximation. In a helminth-microparasite co-infection "bottom-up" (resource limitation) and "top-down" (immunity response, comparable to a predator "consume" of pathogen) ecological "rules" therefore detectable govern these microparasite populations (Graham 2008). For example helminths that caused anemia imposed resource limitation on RBC-dependent microparasites reducing its populations, but without resource limitation density of microparasites was positively affected by helminth co-infection because the reduction of INF-γ (Th1-Th2 trade-off).

Pedersen and Fenton 2007 proposed a hypothetical within-host parasite community interaction network (Fig 1) to understand parasite communities, where both direct and indirect interactions were considered in a three trophic levels: host resources, parasite community and host immune system. All these interactions would be present for apply the good control strategies in infectious diseases.

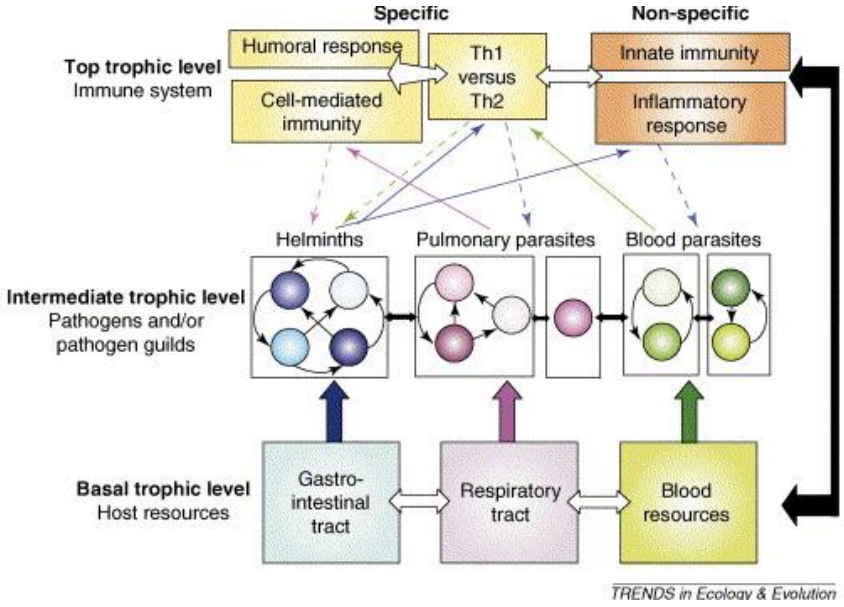

Figure 1. A hypothetical within-host parasite community interaction network. They define the within-host parasite interaction network with three levels of explicit trophic structure; given that parasites consume resources of the host for development, reproduction and transmission, and the immune system acts as a predator destroying the infecting pathogens. The basal level is defined by the host resources, analogous to the primary producers in a typical free-living food web. However, by contrast, host resources are inextricably linked to each other (white arrows) because the fitness and survival of the host depends on all resource components. The intermediate level comprises the parasites (colored circles) and parasite guilds that infect the host. Pathogens that consume similar resources, share a locality within the host and are attacked by the same components of the immune system can be considered

parasite guilds (boxes), in which direct interactions between parasites are most probable (unidirectional arrows). Parasite guilds can comprise a single species. The vertical arrows represent the flux of energy from host to pathogen. The top trophic level represents the diverse responses of the immune system that vary in their degree of specificity. Here, we highlight a few common components (boxes), and use solid colored arrows to represent the aspects of the immune system that target each parasite or parasite guild, whereas the dashed arrows represent the top-down indirect interactions of co-infection parasites, mediated by the immune system. Adapted from Pedersen and Fenton 2007.

4.3. What does co-infection mean? Types of co-infections

Despite the widespread acceptance of concurrent infections and its direct or indirect influence within the host and disease outcome seldom laboratory animals or wild animal works exist, probably because their complexity and difficulty to understand. As we say before parasites interact between them and between host via space, resource and immune system. Also the time when appear the new infection (at the beginning, its peak or during a chronic phase) is important for the course of the former infection or disease in general. Thus diseases could have unpredictable outcomes due to concurrent infections. To be able to understand complex interactions a first simple approach was necessary. A good started point was the review of pairs interactions in lab animals by Cox,(2001) where numerous examples of all combinations between virus, protozoa, helminthes and bacteria were reported and concluded that the immunodepression, Th1/Th2 dichotomy and worm immunomodulatory factors are three basic vertices indispensable to know about co-infection (Fig 2).

4.4. Impact of co-infections at individual, community and ecosystem scales

Infectious diseases remain a major threat to human health, economic and wildlife conservation. A common factor in the EIDs is the involvement of multiple hosts, vectors or parasites species in complex ecological communities. For these reasons a full ecological context is necessary to managing EIDs and prevent unexpected events before apply a treatment. For example in African buffaloes affected by bovine TB (bTB) deworming improved survival of individuals hosts infected by bTB but also enhanced pathogen fitness because infected animals not die (Ezenwa and Jolles 2015). Without reducing the infection prevalence of bTB in the population, individual and population effects of deworming were contraries. This and other disease examples show that infectious diseases need community ecology. Is necessary not only to understand and study

either within host or between host (spread) also between community (regional or biogeographical scales) see Fig 3, adapted from (Johnson et al. 2015). Thus both offensive and defensive strategies are necessary to minimize disease outcomes and reduce the emergence of diseases. Prevention of infection like limit reservoirs for parasites or vectors, and strategies for control ones a disease is in the area, limiting contacts between wild animals are some examples of possible actuations.

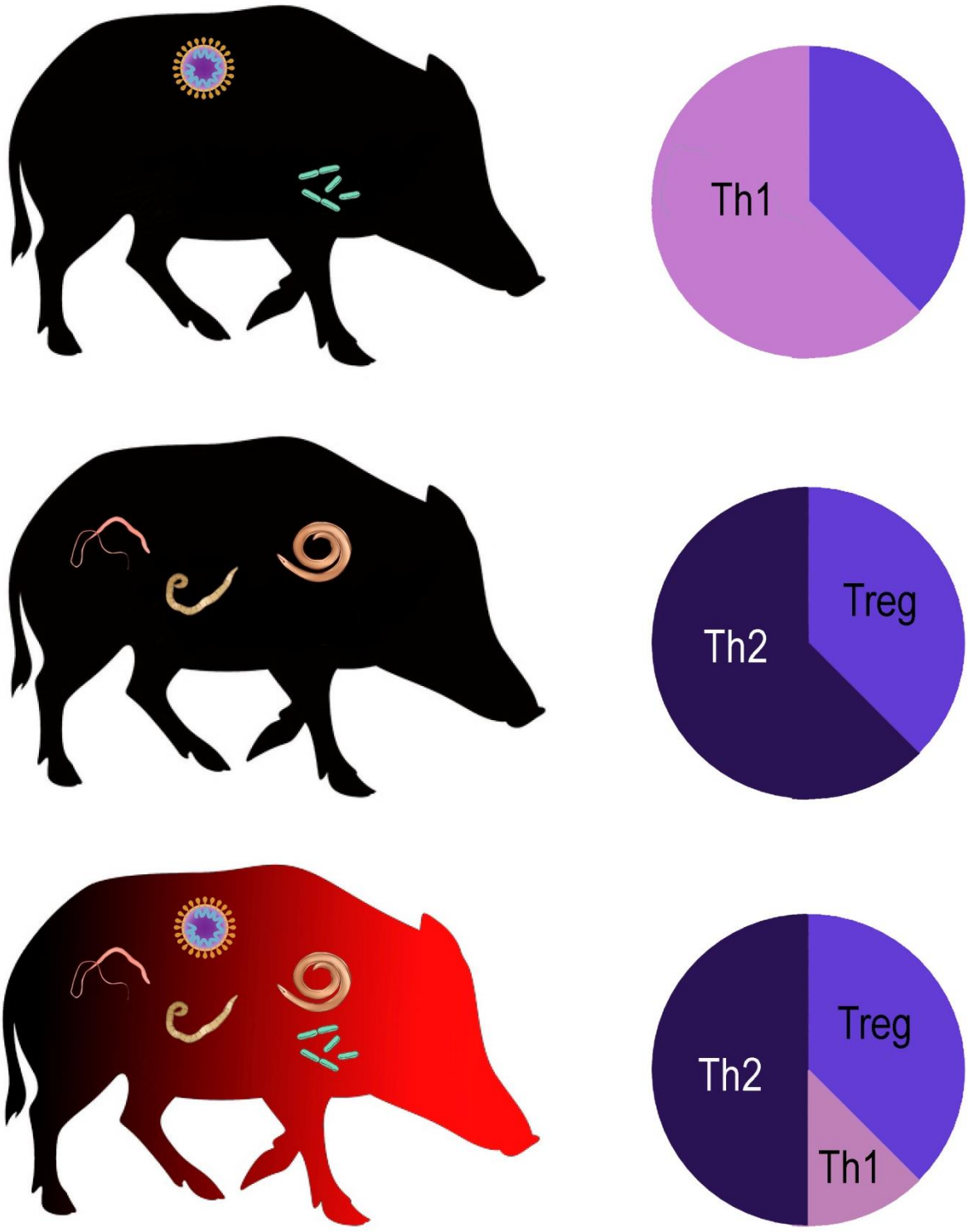

Figure 2.Th2/Th1 tradeoff in a co-infected wild boar. The first wild boar in black represents an animal infected with different types of microparasites, which response with a Th1 type cytokines to combat them. The second wild boar infested by macroparasites the principal immune response is a Th2 type cytokines. Finally the last wild boar, co-infected, is probably unable to combat with disease because the Th1-Th2 imbalance.

4.5. Helminth-TB, an exceptional co-infection model

Tuberculosis one of the major infectious diseases of humans is usually diagnosed in co-infection with other pathogens (Salgame et al. 2013). So is a good model for exploring the impact of multiple infections on host's health because its chronic nature, wide spread (affects one third of the human population (WHO 2012)), and commonly occur in co-infection with helminths (Hotez et al., 2008).

Helminths are ubiquitous concomitant pathogens in all vertebrate species (Cox 2001), they are worldwide distributed and almost always present in all wild animals but also in poor communities. These parasites play a well known immune regulation in the co-infected host (Maizels et al. 2004).

In a Helminth-TB co-infection, chronic helminth infections could induce T-helper type 2 cell (Th2), mast cells immunoglobulins and eosinophils response to create an anti-inflammatory environment which down regulate Type 1 inflammation necessary to deal with the bacteria infection (van Riet et al. 2007). So the outcome of TB disease would be influenced by worm species (Elias et al. 2006).

Wildlife is an excellent model to study the impact of co-infections because is almost always infected by multiple pathogens (Bordes and Morand 2011) and share major co-infections with humans. In wildlife the helminth-TB co-infection is wide distributed than in humans (Fig 4) with a broad range of possible hosts, so is a clear example of the before mentionedmultihost-multipathogen ecosystem. Also is a good example for the "one health" perspective, thus, when consider together studies of humans, lab animals, veterinary animals and wildlife might paint a more complete picture of worm-TB co-infection necessary for future research (Ezenwa 2016).

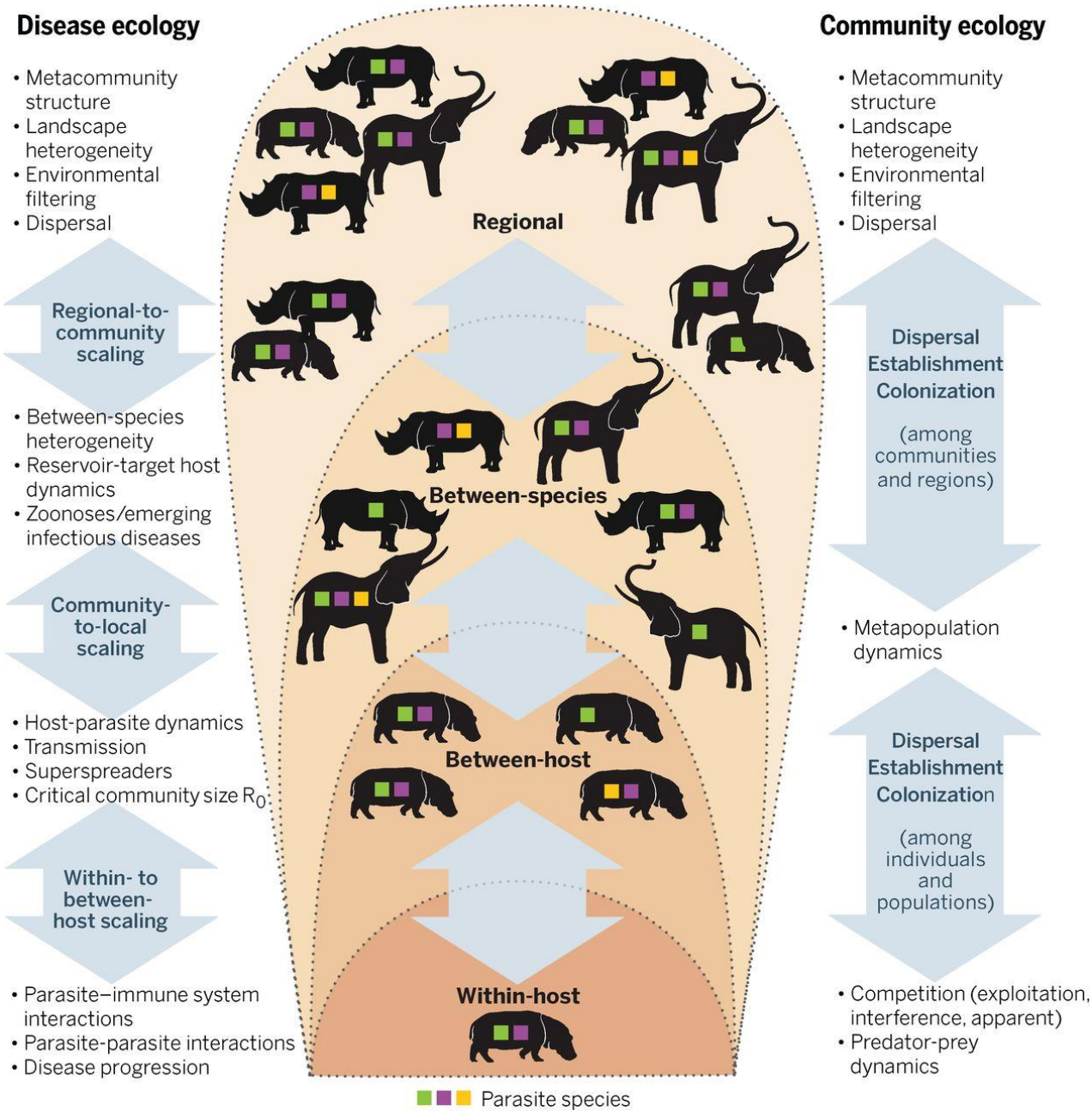

Figure 3 Ecological hierarchies applied to host-parasite interactions and analogous processes in community ecology. The range of scales includes within-host ("parasite infracommunity," often dominated by parasite-parasite and parasite-immune system interactions); between-host ("parasite component community," population biology); among species ("parasite supracommunity," community ecology); and across regions (macroecology and disease biogeography). The different colored squares represent different parasite species; the text at the right and left highlights the relevant processes from community ecology and disease ecology, respectively. The potential importance for interactions and feedback across these scales represents an essential research frontier in the field of disease community ecology. Adapted from Johnson et al. 2015.

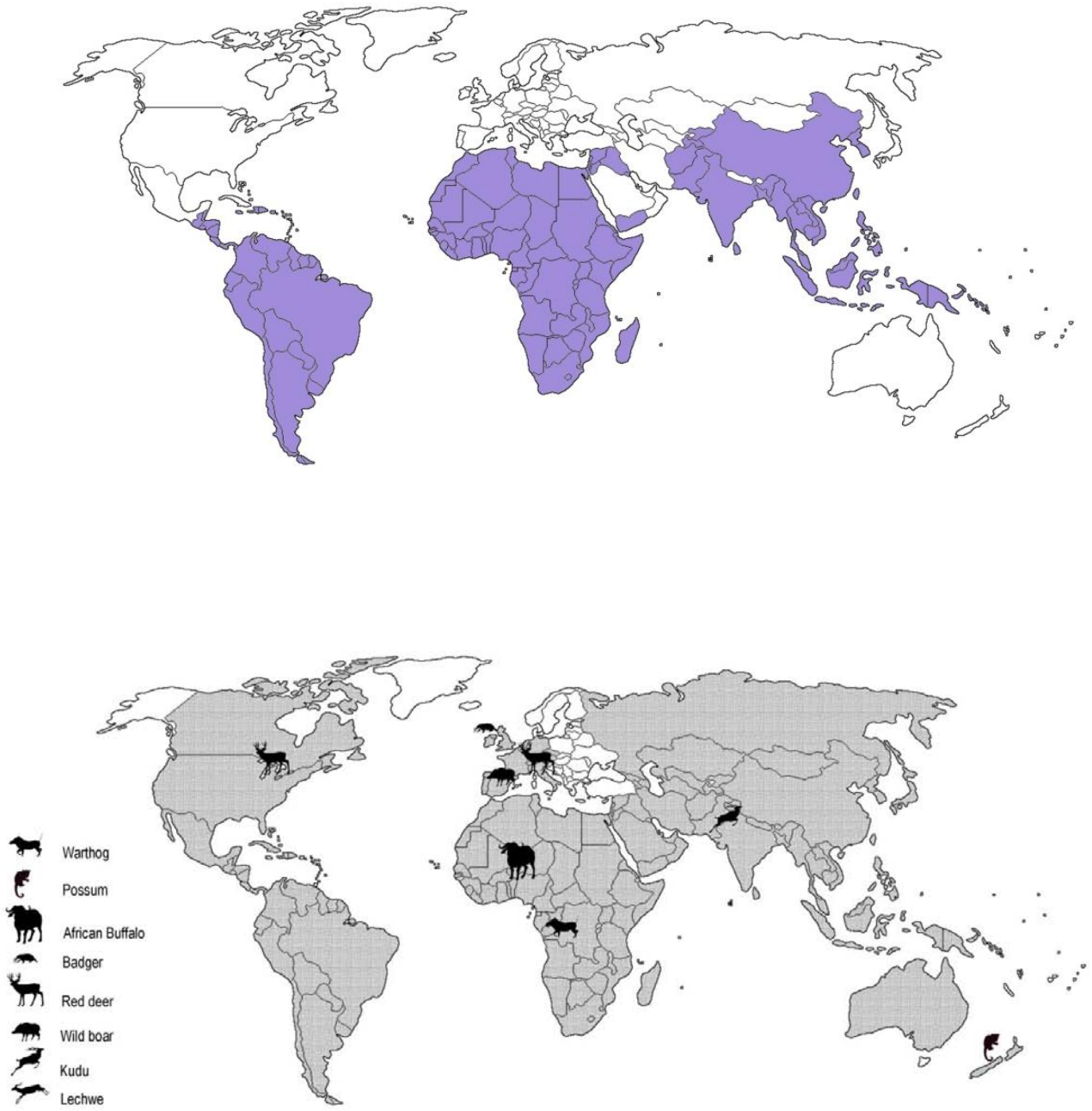

Figure 4. **World map showing the geographic distribution of co-infection between *Mycobacterium tuberculosis* complex (MTC) and helminths.** The first map painted in purple represents distribution in human beings and the second one in wildlife. Animals in black are known reservoirs of *Mycobacterium bovis*.

5. THE COINFECTION STUDY SYSTEM USED IN THIS THESIS

6.1. The host

The wild boar (*Sus scrofa*) or Eurasian wild pig (Fig 5) are among the most widely distributed large mammals in the world (Oliver and Leus 2008), well adapted to a large variety of ecosystems habiting in Europe, Asia and North Africa (Fig 6). Moreover this range has been greatly expanded by human introduction to the American continent, Australia, New Zealand and several islands in the Pacific Ocean (Rossell and Herrero 2007) then, this species currently occurs in all continents except Antarctica. In the Iberian Peninsula, two subspecies (*S. s. castilianus and S. s. baeticus*) have been reported, although this differentiation should be confirmed with molecular biology techniques (Rossell and Herrero 2007).

Figure 5. Wild boar (*Sus scrofa*). Adult female, male and piglets. Source UICN.

The wild boar have been an important resource of subsistence hunters since the earliest times, and it is nowadays one of the most important game species for recreational hunting.

In the last few decades populations have increased both in number and range throughout Europe (Massei et al. 2015), increasing human-wild boar conflicts. The principal causes of this increase are lack of large predations, reforestation, deliberate releases for sport hunting, supplementary feeding, habitat alteration due to human activities and mild winters which improved survival. In parallel there are an apparent decline in hunter numbers, so the main cause of wild boar mortality, will decrease.

Figure 6. Wild boar world distribution. Adapted from IUCN.In Yellow extant (resident) and in purple (reintroduced).

In Toledo, central Spain, where the studies of this thesis were carried, wild boars are in fenced areas mainly for the purpose of hunting. Boars shared the habitat with other wild ungulates such as red deer or fallow deer. This area has special ecological conditions, with Mediterranean forests mixed with farmland, an attractive habitat for wild boar. In this zone income from game hunting is a significant economic factor, and the sustainable management of wild boar is therefore an important issue (Fernández-Llario et al. 2003). Furthermore densities of boars tend to be high with an increased risk of diseases. The hunting method practiced in this area, called “montería” is a typical way of big game hunting in the southern regions of the Iberian Peninsula. It consists of a number of hunters waiting at fixed points while the animals are chased towards them by dog teams. Both male and female wild boars may be shot, and there is no limit to the number of animals that each hunter may shoot (Fig 7).

Figure 7.Hunted wild boar (*Sus scrofa*) after a "montería". Photo: X. Fernández-Aguilar.

These facts lead to a huge harvesting of game carcasses, which are a valuable protein source. However meat consumption continues to be very low in most regions (Paulsen et al. 2012), including Spanish ones.

Wild boar are susceptible to a variety of infectious diseases (Risco et al. 2014b), TB, salmonellosis, trichinellosis, metastrongylosis, Aujezsky's disease, brucellosis, porcine circovirus infection, classical swine fever and toxoplasmosis, just to name a few. Many diseases are shared with domestic pigs and humans, becoming a reservoir difficult to control. This can have negative impacts on the farming industry with high economic losses but also on public health. One well example of a zoonotic disease is TB, MTC in concrete *M. bovis* is the main pathogen of bTB. In human bTB although is not a high pathogenic disease, cause between 0.5-1.5% of the TB infections each year in industrialized countries. This numbers may be higher in developing countries but hardly quantifiable due the difficult differentiation between specimens in these areas (De La Rua-Domenech 2006). With a wide distribution, in Europe and other sites around the

world, bTB is difficult to control in domestic animals due to the wildlife reservoirs. The wild boar is one of these true reservoirs (Naranjo et al. 2008) which contribute to make hard TB control.

6.2. The target disease: MTC (*M. bovis*)

MTC, refers to a genetically related group of Mycobacterium species that can cause TB in a variety of organisms including humans. Are obligate pathogenic bacterial species in the family *Mycobacteriaceae*. This family of small bacillus is highly aerobic and requires high levels of oxygen to grow. It is divided extremely slow compared with other bacteria and can survive in a dry state for weeks. Primarily a pathogen of the mammalian respiratory system, it infects the lungs. The most frequently used diagnostic methods for TB are the tuberculin skin test, acid-fast stain, and chest radiographs. TB can only be spread through air droplets. In the lungs, the bacteria are taken up by alveolar macrophages, but they are unable to digest and eradicate then, they multiply unchecked within the macrophage. The bacteria also evade macrophage-killing by neutralizing reactive nitrogen intermediates (Flynn and Chan 2003).

Protective granulomas are formed due to the production of cytokines and upregulation of proteins involved in recruitment. Granulomatous lesions (Fig 8) are important in both regulating the immune response and minimizing tissue damage. Moreover, T cells help maintain Mycobacterium within the granulomas (Saunders and Cooper 2000).

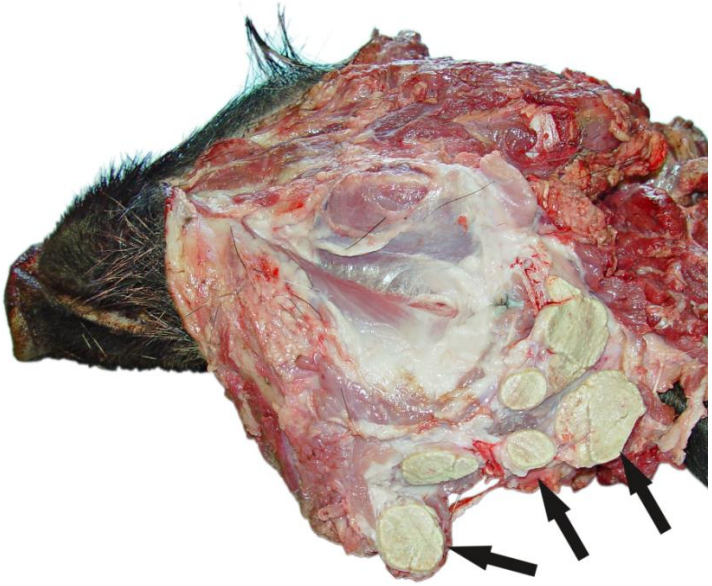

Figure 8. Granuloma in hunted wild boar (*Sus scrofa*) in National Game Reserve Ports de Tortosa i Beceit. Photo: G. Mentaberre.

Assuming that they all are derived from a common ancestor, it is intriguing that some are exclusively human (*M. tuberculosis, M. africanum, M. canettii*) or rodent pathogens (*M. microti*), whereas others have a wide host spectrum (*M. bovis*)

Bovine tuberculosis (bTB) is caused by ***Mycobacterium bovis*** and closely related mycobacteria of the MTC. They have an extensive host rang and may cause zoonotic TB. The European wild boar as we said before is a clear natural reservoir (Naranjo et al. 2008). So it would be necessary to control the infection in this and other wildlife hosts to bTB eradication in livestock.

6.3. The helminths

6.3.2. Gastrointestinal nematodes

Gastrointestinal nematodes can affect key stages of alimentation (food intake), digestion (motility) and absorption (epithelial structure) so, malabsorption and nutrient loss can result (Coop and Kyriazakis 1999). However, parasites might also reduce the probability of a host reproducing or surviving, although clinical signs are not present, and we refer to this as subclinical disease. Theoretical approaches have provided strong arguments to support the hypothesis that parasites can affect population dynamics (Anderson and May 1978) and experimental works with wild animals confirm this hypothesis (Pedersen and Greives 2008) demonstrating that removal of a group of intestinal parasites alleviates population crashes.

6.3.2.1. Gastric nematodes

Gongylonematidae,

Gongylonema pulchrum (Molin, 1857) “Gullet worm”. Easily distinguished microscopically by the presence of longitudinal rows of cuticular bosses in the anterior region of the body. The average length for male worms is 29.1 mm, while the average length for adult females is 58.7 mm. Eggs measure around 50 μm. *G pulchrum* is parasitic in the upper digestive tract (oesophagus and stomach) of various mammals throughout the world. It is most common in ruminants, which are suitable definitive hosts.

This parasite has an indirect biologic cycle (prepatent period: 8 weeks). Coprophagic beetles or cockroaches eat eggs of wild boars faeces and then the parasite develops just to L3. The wild boar infected by ingestion of the beetles and L3 is implanted in the oesophageal mucosa or submucosa.

Trichostrongylidae,

Hyostrongylus rubidus (Hassal and Stiles, 1892) "The red worm of the stomach". Slender reddish worms when fresh, males measure 4 to 7mm long by 0.86 width and females 5 to 11mm long by 1mm width. Strongylida order typical eggs, measure 60 to 82 µm large by 31 to 38µm width, in faeces have 16 to 32 blastomers.

This parasite has direct biologic cycle (prepatent period: 18 - 21 days). Since the expulsion via faeces, eggs hatches after 40 hours at 15 - 25ºC, then L1 past to L3 in 7 days. These infective larvae are ingested by wild boar, in the stomach penetrated to the fundus glands and turn about L4 and L5 (juvenile phase), finally return to the gastric lumen where copulate and start laying eggs. If the conditions (environmental or high infections) are not good it can occur hypobiosis during few months.

Spirocercidae,

Ascarops strongylina (Rudolphi, 1819) "The white and thick worm of the stomach". Small, slender worms, males measure 10 to 15 mm long with a characteristic rolled end and two unequal spicules. Females measure 15 to 22 mm long. They live on the stomach wall under a layer of mucus. Eggs measure 34 to 45 µm large by 18 to 26µm width and in faeces have a well-defined embryo.

This parasite has an indirect biologic cycle (prepatent period: 4 weeks). Coprophagic beetles eats eggs of wild boars faeces and then the parasite develops just to L3. The wild boar infected by ingestion of the beetles and L3 implanted in the gastric mucosa.

Physocephalus sexalatus (Molin, 1860) "The white worm of the stomach". Small, slender worms; the males measuring 6 to 13 mm long and also with a rolled end and two unequal

spicules and the females measure 10 to 22.5 mm long. Eggs, are identical in measure and shape than Ascarops ones, thick-shelled and are embryonated when passed.

This parasite has an indirect biologic cycle (prepatent period: 6 weeks). The cycle is the equal to *Ascarops sp*. one.

5.3.1.2. Intestinal nematodes

Ascarididae,

Ascaris suum (Goeze, 1782). "Large roundworm". Big, elongated, fusiform and yellowish pink colour, only could be confused by *Macracanthorhynchus sp.* Males measure 15 to 31 cm long and 2 to 4 mm width. Females measure 20 to 49 cm long by 3 to 6 mm width. Eggs measure 60 to 75 µm large by 50 to 55 µm width. Wild boar excrete no embryonated eggs, it will be infective after 3-5 weeks in the grass.

This parasite has a direct biologic cycle (prepatent period: 60 - 80 days). Embryonated eggs with L3 can be eaten by transport hosts (earthworms, beetles, etc.) or directly by de definitive host. In the intestine of the wild boar the egg hatches and L3 penetrates the intestinal mucosa and via linfa or blood do a "liver-pneumo-tachea-enteric" migration returning to the intestine where adults reproduce.

Ancylostomatidae,

Globocephalus urosubulatus (Alessandrini, 1909) "Pig hookworm". A very small, stout whitish worm, males measure 4.5 - 5.5 * 0.3 mm and females 5 - 5.7 * 0.3 - 0.4 mm. Eggs of these nematodes are thin shell, ovoid, slightly asymmetrical and measure 67 to 73 * 35 - 40 µm.

The live cycle is direct (prepatent period: 26 - 36 days), either by oral ingestion of L3 larvae or by percutaneus penetration. Larval migration through the heart, lungs, trachea, oesophagus and stomach occurs.

Trichuridae,

Trichuris suis (Schrank, 1788) “Whip worm” with a very thin filamentous anterior part (0.5 mm diameter) embedded in the mucosa where there is the oesophagus (2/3 of the worm) and continued by a thick back (0.65 mm diameter) where there is the digestive and the reproductive system. Females generally are bigger than males but the two genders range about 3 to 5 cm. Males ends rolled-up with a unique spicule. Wild boars excrete no embryonated eggs measuring 50 to 61 µm large and 21 to 35 µm width with a lemon like shape and brown orange colour.

This parasite has a direct biologic cycle (prepatent period: 6-8 weeks in optimal conditions). The infective stage is the egg with L1, which is eaten by the wild boar and hatches in the last portion of the small intestine. After three moults they reach the adult stage and finally go to the large intestine to fix in the distal ileum, caecal and colonic mucosa.

Strongyloidae,

Oesophagostotum dentatum (Rudophi, 1803) “Nodular worm”. Adult worms are white in colour 8 - 14 mm long. Males are 8 - 10 * 0.2 - 0.4 mm and females 11 - 14 * 0.4 - 0.5mm. Eggs are strongylida like shape, with a thin wall and about 75 * 40 µm and a consistent content with 8 to 16 blastomers.

Direct biologic cycle (prepatent period: 3 to 6 weeks), wild boar infection is by ingestion of L3, although there is limited evidence that skin penetration is possible in pigs. The L3 enter the mucosa of any part of the small or large intestine then emerge on to the mucosal surface, migrate to the colon and develop to the adult stage. In the grass eggs hatched and L1 develops to L2 and L3 (the infective stage).

6.3.3. Acanthocephalosis

Oligacanthorhynchidae,

Macracanthorhynchus hyrudinaceus (Pallas, 1781) “Thorny-headed worm”. Adults resembles *Ascaris suum*, but taper posteriorly. The anterior of the worm possesses a retractable proboscis, which is covered with recurved hooks. The males are up to 10 cm and the females

around 60 cm in length and slightly pinkish in colour when fresh. The worms are thick (5 - 10 mm in width) and the cuticle is transversely wrinkled.

This parasite has an indirect biologic cycle (prepatent period: 2-3 months). Adults, attached to the small intestinal mucosa, lay eggs which are passed in the faeces. These are produced in large numbers, are very resistant to extremes of climate and can survive for years in the environment. After ingestion by dung or water beetle larvae, the acanthor develops to the infective cystacanth stage in approximately 3 months. Infection of wild boar occurs after ingestion of either infected beetle grubs or adult beetles.

6.3.4. Lung nematodes

Metastrongylidae,

Metastrongylus apri (Gmelin 1790)**,** ***M. salmi*** (Gedoelst 1923)**,** ***M. pudendotectus*** (Vostokov 1905) and ***M. confuses*** (Jansen 1964)**.** "Pig lungworm". The adult male is up to 25 mm and the female up to 58 mm. To differentiate species microscopic details are necessaries (male bursa, spicules and hooks, female vulva and tail). Eggs are larval (L1) when expulsed by females and presented a little thick and rough wall. Their measure between 43 - 57 * 38 - 45 µm.

These parasites have an indirect biologic cycle (prepatent period: 24 days). In cold temperatures the eggs are very resistant and can survive for over a year in the soil. Normally, however, they hatch almost immediately, the intermediate host ingesting the L1. In the earthworm, development to L3 takes about 10 days. The longevity of the L3 in the earthworm is similar to the intermediate host itself and may be up to 7 years. The wild boar is infected by the ingestion of earthworms and the L3, released by digestion, travel to the mesenteric lymph nodes and moult. The L4 then reach the lung by the lymphatic-vascular route, the final moult occurring after arrival in the air passages.

M. bovis, *G. Pulchrum*, *A. Suum*, *M. hirudinaceus* and *M. apri* are potential zoonoses.

7. HYPOTHESIS AND OBJECTIVES

Co-infection is the rule rather than the exception both in wildlife and in people from underdeveloped countries. Therefore, an increased interest in this knowledge area has been gathered in the recent years, but impacts of multiparasitism in disease dynamics are only beginning to be recognized.

Helminths, ubiquitous in all vertebrates, worldwide distributed and masters of immune regulation are overlapped geographically with a significant number of diseases, including some of the most devastating ones malaria, AIDS and TB. Thus, interactions between macro and microparasites may exert effects on the outcome of these important diseases.

Wildlife research can generate new and valuable information about helminth-microparasite co-infection development in a natural context. In consequence, the main goal of the present thesis was clarify the specific interactions between worms and TB in a wild boar population naturally and/ or experimentally infected by MTC (especially *M. bovis*) as well as to assess basic parasitologic knowledge of the wild boar.

The hypotheses of the present thesis were:

1. Prevalence and identification of some wild boar worm species were confusing.
2. FEC, the most widely extended technique for quantify worm load in wildlife, might not be a reliable technique to assess wild boar worm burden.
3. MTC would increase OS in wild boar although the presence of other factors like environmental variation could mask TB effects under natural population conditions.
4. Co-infection with worms would be detrimental for wild boar health status in a population naturally infected by MTC.
5. Antihelminthic drug treatment may be a good tool to positively influence TB outcome.

To evaluate these hypotheses the followed **specific objectives** were proposed:

Objective 1: To revise the five more common species of lung nematodes of the wild boar and make an identification key to facilitate their recognition (**Chapter 1**) as well as to assess the limitation of flotation and sedimentation techniques to quantify *M. hirudinaceus* in wild boar (**Chapter 2**).

Objective 2: To establish the uses and limitations of FEC to assess worm burden in wild suids (**Chapter 3**).

Objective 3: To study the effects of TB on different OS biomarkers in wild boar experimentally and naturally infected with MTC (**Chapter 4**).

Objective 4: To investigate direct and indirect impacts of helminth co-infections on selected physiological indicators of a wild boar population naturally infected by MTC (**Chapter 5**).

Objective 5: To establish the impact of worm drug treatment in the pathogen community of a population of wild boar naturally co-infected MTC (**Chapter 6**).

7. CHAPTERS

Technical chapters

Chapter 1

An identification key for the five most common species of *Metastrongylus*

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Abstract

Species of the *Metastrongylus* genus, the lung nematodes of pigs that require an intermediate host (earthworm) to complete their cycle, pose a potential risk to both livestock and humans. This parasite can result in lung pathology and mixed infections with other pathogens (e.g. viruses) can be fatal to pigs. Although this genus distributed worldwide, there are no classification keys for identifying this common parasite species. In this work, we take advantage of parasitological surveys of wild boar (*Sus scrofa*) in northern and central Spain and southern Poland, to develop a morphological identification key for the five most common *Metastrongylus* species (*Metastrongylus apri, Metastrongylus pudendotectus, Metastrongylus salmi*, *Metastrongylus confusus* and *Metastrongylus asymetricus*). In addition, we provide the first record of *M. confusus* in Spain, probably unidentified until now due to the lack of appropriate identification keys. We hope that this user-friendly identification key will enable parasitologists and veterinary practitioners to avoid further misclassifications of *Metastrongylus* species.

Key words: Lung worms, *Metastrongyus confusus*, *Metastrongylus spp., Sus scrofa,* Wild boar

Introduction

Metastrongylus species are parasitic nematodes of the respiratory tract of swine with indirect life cycles in several earthworm species (Vanparijs and Thienpont 1982). These widely distributed helminths are particularly prevalent and abundant in young individuals, with acquired immunity usually developing in adults (Humbert 1992; Heise-Pavlov and Heise-Pavlov 2003). The *Metastrongylus* infection is ever-present in wild swine populations and in extensively raised domestic pigs (Adedokun et al. 2001; Carstensen et al. 2002).

Metastrongylus spp. causes dyspnea and progressive weight loss due to the destruction of interstitial tissues, obstruction and ultimately consolidation of the lungs (Alcaide et al. 2005), with those animals showing moderate or severe immunodepression more susceptible to negative health impacts. Concomitant infection, such aswith circovirus, can cause fatal bronchopneumonia (Marruchella et al. 2012).

Thus far, six *Metastrongylus* species have been described: *Metastrongylus apri* (alsocalled *Metastrongylus elongatus)*, *Metastrongylus salmi*, *Metastrongylus pudendotectus, Metastrongylus confusus, Metastrongylus asymetricus,* and *Metastrongylus madagascariensis* (the latter only in pigs of Madagascar). The first three species are, by far, the most commonly reported worldwide and usually present in mixed infections. In Florida (USA), for example, *M. apri*, *M. salmi* and *M. pudendotectus* prevalence in feral swine is as high as 94%, 76% and 64%, respectively (Forrester et al. 1982). In South America (Brasil), da Silva and Müller (2013) observed that *M. apri* was the most prevalent (52.5%) in feral pigs, followed by *M. salmi* (20%) and *M. pudendotectus* (7.5%). In Hungary (Europe), however, *M. salmi* was the most prevalent (90.2%), followed by *M. pudendotectus* 84.3%, *M. apri* 68.6%, *M. confusus* 66.7% and *M. asymmetricus* 43.1% in Wild boar (Nagy et al. 2013). Similar patterns of infection by four *Metastrongylus* species were observed in Belarus (East Europe, see Khrustalev 1981), and Japan (Morita et al. 2007a), with *M. apri, M. pudendotectus* and *M. salmi* present in both countries but *M. confusus* only in the former and *M. asymetricus* only in the latter. In France*, M. pudendotectus, M. salmi and M.confusus* (prevalences of the three species around 100%),and also *M. asymmetricus* and *M. elongatus* (prevalences of this two species around 25%), were highly common in wild boar populations (Humbert and Drouet 1990). In Poland, infections by five *Metastrongylus* species (76% *M. pudendotectus* and *M. confusus*, 72% *M. salmi*, 64% *M. apri* and 40% *M. asymetricus*) are also common (Nosal et al. 2010). In other countries, the three main species (i.e. *M. salmi, M. apri,*and *M. pudendotectus*) have exhibited varying prevalences: in Turkey (Senlik et al. 2011) the prevalence of the 3 species is between 50 and 60%;in Iran

(Solaymani-Mohammadi et al. 2003)*M. apri* 41.6%, *M. pudendotectus* 16% and *M. salmi* 8.3%; in Estonia (Järvis et al. 2007), *M. pudendotectus* 78%, *M. salmi* 77% and *M. apri* 41%; and in Spain (García-González et al. 2013)*M. apri* 71%, *M. pudendotectus* 28% and *M. salmi* only 0.6%.

Changes in the *Metastrongylus* genus distribution are expected given the global increase of wild boar (*Sus scrofa*) populations (Acevedo et al. 2014).Additionally, the increasing tendency toward outdoor pig production systems will also contribute to the spread of *Metastrongylus* species (Carstensen et al. 2002).

Concerning the identification of *Metastrongylus* species, the utility of morphological features for *Metastrongylus* has been supported by molecular analyses (Leignel et al. 1997; Conole et al. 1999). In fact, all *Metastrongylus* species can be relatively easily differentiated by observing their morphological features under a microscope. But curiously, there is no classification key for identifying such these species and misclassifications can be extremely common leading to the following:(1) spurious heterogeneities in the geographic ranges of species of *Metastrongylus* genus and (2) the failure to identify the role of particular *Metastrongylus* species in animal (e.g. *M. apri*, Yoshihara 2004; Alcaide et al. 2005) and human health (Moyle and Purdy 2012).

In the present work, taking advantage of *Metastrongyslus* samples from wild boars sampled in north eastern and central Spain and in southern Poland, we aimed to fill this gap developing a simplified identification key for the five most prevalent *Metastrongylus* species in the northern hemisphere. Moreover, *M. confusus* was recorded in Spain for the first time.

Material and Methods

Twenty-eight wild boar lung samples were obtained from north eastern (National Game Reserve Ports de Tortosa i Beseit, Tarragona, 40°48.11" N, 0°20.35"E, and the Campus of the Universitat Autònoma de Barcelona, Barcelona 41°30' 21"N, 2 °6' 27.86" E) and central Spain (39°55' 15.55"N, 5 °10' 32.33" E, Toledo). In addition, twenty-five additional wild boar lung samples were collected in the Małopolska Province, Poland (Nosal et al. 2010). In Spain, wild boars were box-trapped and euthanised with T61® at the Universitat Autònoma de Barcelona, and hunter-harvested in the other two Spanish areas and in Poland. The gender and age of animals were assessed by examining genitalia and dental eruption patterns (Boitani and Mattei 1992). The trachea and lungs were removed and collected in individual bags, maintained at 4ºC during transport and kept frozen (-18ºC) until the day of processing.

The trachea and lungs were subsequently defrosted, dissected and washed to obtain adult worms. First, lungs were filled with pressurised water (with the aid of a hose), and the parenchyma was gently massaged. The obtained fluid was removed and passed through a 500 μm sieve. This process was repeated three times. The trachea and main bronchi were opened with scissors and carefully examined. Finally, the lungs were washed with running water and all pulmonary nematodes collected from the sieve in 70% ethanol for their identification and count. Obtained worms were clarified in a lactophenol solution and identified to the species level using both the classical (Holló 1965; Jansen 1964) and recent descriptions (Morita et al. 2007; Nosal et al. 2010). With the aid of a microscope (x10, x40 or x100 magnification), we used ten individuals from each species to describe and measure the principal morphological characteristics, summarised in Table 1. Photographs of both males and females are shown in Fig. 1.

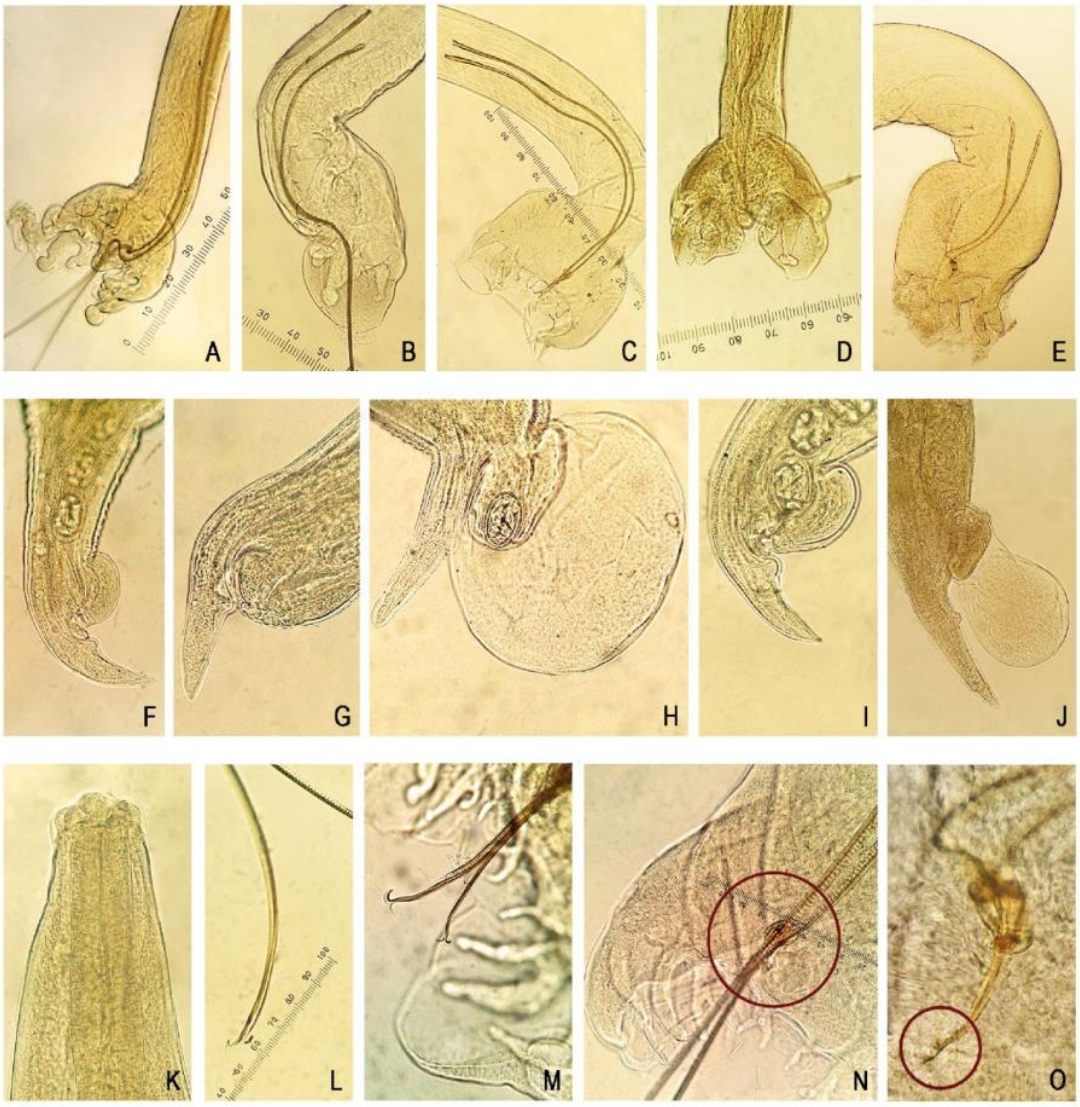

Figure 1. Morphological features of the caudal end of five *Metastrongylus* spp. collected. Males from **A** to **E**, females from **F** to **J**. **A** and **F** *M. confusus,* **B** and **G** *M. salmi,* **C** and **H**: *M. pudendotectus*, **D** and **I** *M. apri*, **E** and **G** *M. asymetricus*. **K** *Metastrongylus* head (rudimentary mouth capsule), **L** *M. confusus* single terminal barb of spicule, **M** *M. pudendotectus* double terminal barb of spicule, **N** *M. pudendotectus* gubernaculum, **O** *M. asymetricus* gubernaculum and double terminal barb of spicule inside the circle.

Characteristics	*M. pudendotectus*	*M. apri*	*M. confusus*	*M. salmi*	*M. asymetricus*
Females (n=10)					
Vulva	Near anus and posterodorsal to prevulvar swelling	Somewhat anterior to anus	and posterodorsal to prevulvar swelling	Somewhat anterior to anus and in	On ventral base of prevulvar swelling
Prevulvar cuticular dilatation	Present	Absent	Absent	)sent	Present
length*width (±SD)(µm)	262.75*254.5 (± 42.07*44.05) [200*200 - 350*330]	NA	NA	A	140*82.5 (± 28*19.3) [100*52.5 – 187.5*112.5]
Prevulvar swelling length (µm)	165.25±33.36 [87.5 - 200]	100.25±24.09 [77.5 - 160]	85.75 ± 17.53 [62.5 - 127.5]	12.92± 17.78 [75 - 125]	73.75 ± 6.27 [62.5 - 85]
width (µm)	88.75±22.21 [50 - 137.5]	50±11.67 [35 - 70]	48.26 ±14.71 [27.5 - 75]	.5±10.35 [50 - 82.5]*	30.94±7.06 [25 - 45]
Tail length 1 (µm)	119±14.99 [100 - 150]	71.75±9.59 [57.5 - 87.5]	71.75 ± 5.60 [60 - 77.5]	±9.59 [62.5 - 92.5]	102.97± 9.8 [87.5 – 117.5]
Eggs in vagina length (µm)	56±2.93 [50 - 60]	53±2.84 [47.5 - 57.5]	57.75 ± 6.84 [47.5 - 72.5]	.75±4.26 [47.5 - 60]	52±3.5 [47.5 – 57.5]
width (µm)	43.5±6.26 [37.5 - 55]	36.5±1.75 [35 - 40]	38.25 ± 2.51 [35 - 42.5]	.26±1.32 [35 - 37.5]	37.5±2.04 [35 - 40]
Males (n=10)					
Spicule length (mm)	1.38±0.07 [1.3 - 1.5]	4.20±0.21 [3.9 - 4.5]	2.92 ± 0.10 [2.8 - 3.1]	12±0.22 [1.6 - 2.4]	0.66±0.06 [0.6 – 0.75]
Barb of terminal	Double	Simple	Simple	mple	Double
Gubernaculum	Present	Absent	Absent	)sent	Present
length (µm)	34±5.55 [27.5 - 45]	NA	NA	A	NA
width (µm)	25.5±7.70 [12.5 - 35]	NA	NA	A	NA
Copulatory bursa length (µm)	458±49.62 [400 - 520]	365.7±39.10 [310 - 400]	351±26.85 [300 - 400]	6±50.60 [410 - 550]	592±29.5 [550 - 630]
width (µm)	509±56.66 [440 - 610]	360±25.82 [320 - 400]	306±46.71 [200 - 340]	7±39.45 [230 - 360]	460±42.43 [400-500]

Table 1. Measurements of five species of the genus *Metastrongylus*. Numerical values presented as average ± standard deviation and minimum, maximum range in brackets. Measurements of M. *asymetricus* males were done in only five individuals.* in some cases bit defined, no measurable limits.

Results and Discussion

The morphological features of the five most common *Metastrongylus* species are shown in Fig. 1 and described in Table 1. *M. pudendotectus* (mean prevalence 98%, 100- 94 max-min), *M. salmi* (75.5%, 31.3-100)and *M. confusus* (66.7%, 16.7-100) were found in Tarragona, Barcelona and Toledo, and*M. apri* (0-33.3%) was only found in Barcelona and Toledo. The presently reported finding of *M. confusus* constitutes the first record of this species in Spain. The prevalences of the *Metastrongylus* species in wild boar from Poland were the following: 48.8% for *M. pudendotectus*, 32.8% for *M. elongatus*, 14% for *M. salmi* and 4.4% for *M. asymetricus*(Nosal et al. 2010). A dichrotomic key to differentiate *M. pudendotectus, M. confusus, M. salmi, M. apri* and *M. asymetricus* on the basis of morphological criteria is provided in Table 2.

1	Distal extreme with copulatory bursa with spicules (male)	2
	Distal extrem with a vagina (female)	6
2	Metastrongylus male bursa copulatrix rays weakly developed (Fig. 1c, e), spicules ≈ 1mm with a barb of terminal double	3
	Metastrongylus male bursa copulatrix strongly developed (Fig. 1a), spicules >2mm with a barb of terminal simple	4
3	Spicule ≈ 1 mm	***M. pudendotectus***
	Spicule < 1 mm	***M. asymetricus***
4	Spicule > 3.5 mm, broad-brimmed, mushroom-like of the externo-lateral ribs (Fig. 1d)	***M. apri* (*elongatus*)**
	Spicule < 3.5mm	5
5	Spicule mostly > 2.5 mm, plus identation separating terminal spherical swelling (on the inner side of left medio-lateral ray) present	***M. confusus***
	Spicule generally < 2.5 mm, longer bursa and lack of identation on terminal spherical swelling	***M. salmi***
6	Metastrongylus female with prevulvar cuticular dilatation (Fig. 1f)	7
	Metastrongylus female without prevulvar cuticular dilatation	8
7	Vulva near to anus, posterodorsal to prevulvar swelling	***M. pudendotectus***
	Vulva on ventral base of prevulvar swelling	***M. asymetricus***
8	Vulva opens at half of the prevulvar swelling, away from the anal opening	***M. salmi***
	Vulava opens at the distal end of the prevulvar swelling, close to anal opening	9
9	Length of prevulvar swelling ≥ 90 µm	***M. apri* (*elongatus*)**
	Length of prevulvar swelling < 90 µm	***M. confusus***

Table 2. Identification key of the most common species of *Metastrongylus* genus

M. apri (Gmelin 1790) and *M. pudendotectus* (Vostokov 1905) were the first described species of the *Metastrongylus* genus and have been commonly reported as dominant species, or even the only species in several surveys (Pence et al. 1988; Takacs 1996; Rajković-Janje et al. 2002). They are easily distinguishable by their small copulatory bursa with strongly developed ribs, long spicules bearing a simple terminal barb in *M. apri* and a more conspicuous copulatory bursa with weakly developed rays and shorter spicules with double terminal barbs joined by a gubernaculum in *M. pudendotectus* (see Fig. 1 and, Holló 1965).

M. salmi was described nearly two decades later by Gedoelst (1923). Though this species bears some resemblance to *M. apri*, it can be easily differentiated from both *M. apri* and *M. pudendotectus:* in males, by the length of spicules (shorter than *M. apri* and longer than *M. pudendotectus*), their narrow and long copulatrix bursa and their ribs; in females, by the vulvar opening and by the poorly pronounced prevulvar swelling (see Fig. 1 and Table 2 and Holló 1965).

Jansen (1964) described *M. confusus* and noted their similarity to *M. salmi* and *M. apri*. Male *M. confusus* differs from the other *Metastrongylus* spp by the length of their spicules and by the shape of the copulatory bursa (Fig. 1, Table 2). In the case of females, the prevulvar swelling in *M. confusus* differs from that of *M. pudendotectus*, *M. asymetricus*, and *M. salmi* but not from *M. apri* females (Fig. 1). In this case, the length of the vagina can be used to distinguish between these two species (Jansen 1964).

Finally, Noda (1973) described *M. asymetricus,* with similarities to *M. pudendotectus* (males with weakly developed bursa copulatrix rays and spicules ≈ 1mm with a double terminal barb; females with prevulvar cuticular dilatation). The spicules of *M. asymetricus* males are shorter and females of *M. pudendotectus* have a vulva near the anus, posterodorsal to prevulvar dilatation while the *M. asymetricus* vulva is on the ventral base of prevulvar swelling. In spite of their easy identification, *M. asymetricus* has only been recorded in France (Humbert and Drouet 1990) and later in Japan (Morita et al. 2007; Sato et al. 2008).

Depending on the zone, country or continent, the prevalence of each *Metastrongylus* species varies. Most likely, *M. confusus* may have been identified as *M. apri* in most instances, but additionally, other species would have been completely overlooked due to the lack of an identification key. Indeed, today, parasitologists and veterinary practitioners continue to struggle with this lack of information that lends itself to the misclassification of *Metastrongylus* species.

To conclude, we would like to highlight the importance of considering both male and female adult individuals to achieve the proper identification of lung nematodes, but especially in the case of *M. confusus*, *M. apri* and *M. salmi*. We hope that this newly provided identification key will help to fill the gap in knowledge on this relevant genus.

Chapter 2

Coprological tests underestimate *Macracanthorhynchus hirudinaceus* burden in wild boar

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Abstract

The present study evaluated the limitations of the coprological sedimentation test to assess *Macracanthorhynchus hirudinaceus* infestation in 59 wild boar (*Sus scrofa*) from central Spain. Coprological sedimentation test appeared to be a poor predictor of both prevalence of infection and real parasite burden due to the high number of false negative results (prevalence was reduced from 61 to 16%). Because the potential increased risk of this zoonosis, it is suggested to use alternative techniques in wildlife surveillance programs.

Key words: *Sus scrofa*, McMaster, Sedimentation, Acanthocephalan, Faecal egg count

Introduction

The acanthocephalan *Macrachanthorhynchus hirudinaceus* is a worldwide gastrointestinal parasite of suids (e.g. domestic pigs, Gibbens et al. 1989; wild boar, de-la-Muela et al. 2001; or peccaries, Souza et al. 2006), canids (Alagaili et al. 2011) and also humans (Hamula et al. 2014a; Hamula et al. 2014b), representing a public health risk in most rural areas of Asia (Taraschewski 2002).

This acanthocephalan presents an indirect cycle with dung beetles as intermediate host (Pavlović et al. 2010). In the definitive host, this parasite causes serious damage to the intestinal mucosa by its proboscis, which penetrates deep into the intestinal wall. Heavy infestations may induce a catarrhal enteritis and, rarely, perforation of the intestinal wall, which can result in a fatal peritonitis. This zoonosis is not rare in countries that include beetles in their diet for gastronomic or medical purposes, such as China (Zhong et al. 1983).

In the last few decades, wild boar (*Sus scrofa*) populations have increased in both number and range throughout Europe (Massei et al. 2015). In parallel, some stages of pig production (mainly breeding stock) are changing from intensive systems to a loose housing (Haugegaard 2010), favouring the cycle of *M. hirudinaceus.*

Faecal egg count (FEC) is the reference coprological technique used to quantify parasitic burden in a broad range of domestic and wild vertebrate species (Torres et al. 2000; Taylor et al. 2007). Although the concentration method (McMM, (MAFF 1986) with 33% zinc sulphate (1.18sg)is the most widely used method for FEC, its limitations in estimating loads of worm that shed heavy eggs-like acanthocephalans, has recently been emphasised (Gassó et al. 2015). As other authors have noted previously, the sedimentation technique is necessary to quantify the presence of *M. hirudinaceus* in faecal samples (Dangjin 1996). Such limitations in FEC would result in the systematic underestimation of *Macrachanthorhynchus* parasitation, but little information currently exists.

In this work, taking advantage of a full parasitological examination in 59 wild boars harvested in central Spain, differences between prevalence and intensity of *M. hirudinaceus* infestation directly assessed by collection of adult worms from the gastrointestinal tracts, and indirectly by sedimentation FEC in faeces, were evaluated.

Materials and Methods

Fifty nine wild boars (20 males and 29 females, aged 7 to 36 month) were hunter-harvested in Toledo (central Spain; 39°55' 15.55"N, 5 °10' 32.33" E). The vegetation in this area is typical of a Mediterranean forest and hosts a density about more than 20 wild boar/ 100 ha. No

approvals were needed from any Ethics committee since wild boars were not sacrificed for research purposes. Wild boars were legally hunted in their own habitat by authorised hunters within the framework of an annual hunting plan approved by the regional authority in charge of livestock and wildlife management.

Digestive tracts (complete small intestine) were collected in individual bags, until they reached the laboratory and were then frozen at −20ºC until subsequent parasitological examination. Rectal faeces were also collected directly from the rectum and stored at 4ºC until coprological analysis. A formalin ethyl acetate sedimentation technique was used with 1 gram of faeces. The entire sediment was inspected under microscopy to quantify the egg burden. Finally, *M. hirudinaceus* parasites were directly collected from the small intestine at the necropsy room according to Gassó et al. (2015). Quantitative Parasitology program (QPweb) was used to analyse data (Reiczigel et al. 2013).

Results and discussion

The prevalence and intensity of *M. hirudinaceus* infestation based on FEC and adult worm species are shown in Table 1. Prevalence using direct estimates was four times higher than when using FEC. This decrease was observed previously by other authors (Fernandez-de-Mera et al. 2003; Mowlavi et al. 2006). The false negative results can be explained by infestations by single or few parasite individuals of the same sex (ten single and four same-sex infections in the present study) making reproduction of the worm impossible. This leads to the fact lack of eggs in faecal samples and the high number of false negative results obtained by the FEC procedure. Moreover, worms can be immature, further increasing the false negative rate. This limitation has previously been observed in other *Macrachanthorhynchus* species parasitizing foxes (Alagaili et al. 2011). Hence, previous surveys of *Macrachanthorhynchus* parasitation based on coprological techniques would have underestimated the true prevalence of this zoonotic parasite.

	Prevalence (CI 95%)	Mean abundance of infection (min-max)
Necropsy (Adults)	61.2 (46.2-74.8)	3.6 (1-19)
FEC eggs/g	16.3 (6.8-30.7)	8.21 (0-100)

Table 1. Prevalence and 95% confidence interval, mean, minimum and maximum abundance (involves the zero values of uninfected hosts) of *Macracanthorhychus hirudinaceus* infestation estimated by collection adult nematodes (necropsy) and indirect estimation by coprology in 59 wild boars harvested in central Spain.

The main limitation of assessing *Macrachanthorhynchus* load by direct collection of adult worms is the extremely time-consuming and laborious work required to perform a full parasitological examination (approx. 3 h/digestive tract). Consequently, fewer than 50 digestive tracts are typically evaluated in most studies (to include 95% of the population values with 95% of probability, 97 samples are required; see Table 1 in Walton 2001).

For poor communities in Asia, Africa and South America, insect harvesting is necessary for the local economy, contributing significantly to livelihoods in both rural and urban areas (Vantomme et al. 2004). Forest insects that are part of the human diet have generally been collected from their habitat, and currently, domestication has been limited to only a few species (Durst et al. 2010). Therefore, we want to highlight that both the increase of wild boar and the search for new sources of protein to combat world hunger and environmental impacts (FAO 2013), increase the risk of human infestation. Therefore, it is necessary to consider *M. hirudinaceus* in wildlife health surveillance programmes.

Conclusion

Although FEC is an easy cost-effective and non-invasive tool for animal health surveillance, it significantly underestimates *M. hirudinaceus* infestation in wild pigs. Thus, further improved methods for detection and parasite burden estimation of *M. hirudinaceus* should be implemented to address this limitation and the zoonotic risk of this parasitic disease.

Chapter 3

Uses and limitations of faecal egg count for assessing worm burden in wild boars

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Abstract

The most widely used technique to assess helminth infection in both domestic and wild mammals is the faecal egg count (FEC). Most efforts to test the reliability of FEC as a proxy for parasite load are in small ruminant studies and limited work has evaluated the use of FEC in pigs. The aim of this study was to explore whether FEC is a reliable indicator of helminth load, and to evaluate the effects of sample storage on FEC accuracy in 59 wild boars. Though FEC was useful for assessing most helminth infections (e.g., *Metastrongylus* spp., *Ascaris suum*, *Trichuris suis*), stomach nematodes were often missed. The accuracy of FEC decreased over time, and thus it is recommended that samples be processed within five days of collection.

Keywords: Coprological analysis, Helminth, McMaster egg counting, Sample storage, *Sus scrofa*

Introduction

Gastrointestinal nematodes can be an important cause of growth impairment, diarrhoea, dehydration and post-weaning death and they have recently highlighted as a neglected challenge to both indoor and outdoor pig production systems (Roepstorff et al. 2011). Faecal egg count (FEC) is a simple, non-invasive reference technique used to quantify parasitic burden in a broad range of domestic and wild vertebrate species, although some studies indicate that its accuracy may be affected by technical (Cringoli et al. 2004) and seasonal variations and host and helminth biological factors (Villanua et al. 2006). FEC relies on the relationship between adult worm burden and the number of eggs per gram of faeces. The McMaster Method (McMM) is the most widely used FEC technique to assess endoparasite burden in small and large ruminants among others.

Little is known about the limitations of FEC in pigs one of the most common livestock. This lack of information is probably due to the low infestation burden due to modern intensive production and the routine use of anthelmintics in extensive production. In fact, to date, only two studies have assessed the utility of FEC as a proxy for adult *Ascaris suum* (Pereckiene et al. 2007) or *Oesophagostomum spp.*(Christensen et al. 1995) burdens in domestic pigs.

In recent years, swine production in Europe has gone from tethered or single-stalled sows to loose housing, hence increasing the exposure of pigs to infective phases of parasites (Eijck and Borgsteede 2005; Roepstorff et al. 2011). Along the same lines, free-range pig production is gaining importance on the African continent (Kagira et al. 2012), making the assessment of worm burden by FEC indispensable. There is also an increasing interest in assessing the health status of wild boars (*Sus scrofa*), the wild counterpart of domestic pigs, due to the natural or artificial expansion of populations. The helminthofauna of wild boars is typically greater than in domestic pigs, especially for those parasites with indirect life cycles (de-la-Muela et al. 2001). Consequently, it is important to understand the uses and limitations of FEC as a proxy for endoparasite burden in wild boars, especially when veterinarians are working under field conditions and samples cannot be analysed as quickly as required.

In the present study, and taking the advantage of the total parasitological assessment of lung and gastrointestinal nematode load in 59 harvested wild boars, we: (I) evaluated the sensitivity of FEC as a diagnostic method and explored whether FEC is a reliable indicator of nematode load; and (II) assessed the effects of sample storage on the accuracy of FEC.

Material and Methods

Sampling procedure

Fifty-nine wild boar samples were obtained from three study areas in Spain: 30 from the National Game Reserve Ports de Tortosa i Beseit, (28,587.87 ha, 40°48.11" N, 0°20.35"E) and 4 from the campus of the Universitat Autònoma de Barcelona (41°30'1.21"N, 2 6' 27.86"E), both in Catalonia northeast Spain; and 25 from Oropesa de Toledo, Toledo (central Spain; 39°55' 15.55"N, 5 °10' 32.33" E).Animals were either captured by box trap and chemically euthanized or hunter-harvested (no approval was needed from any Ethics committee since the animals used in the present study were not sacrificed for research purposes, but we took advantage of the harvested animals for this aim. The harvested wild boar have been legally hunted (shot) or box-trapped in their own habitat by authorised gamekeepers and hunters within the framework of an annual hunting plan by the regional authority in charge of livestock and wildlife management).

Lungs and complete digestive tracts (stomach, small and large intestine) were collected in individual bags, until they reached the laboratory and then frozen at - 20 ºC for subsequent examination. Rectal faeces were then collected for later analysis. All samples were transported in cold boxes (4ºC) until laboratory analysis.

Coprological analysis

The first FEC of 20 wild boars was performed after a maximum of 48 h under refrigeration, day 0, and was subsequently repeated on days 5, 12 and 20 after collection. After the first coprology (day 0), faeces were kept in the laboratory at room temperature (ranging from 17 to 25ºC and 30 to 50% relative humidity). A concentration method (McMM, M.A.F.F. 1986) with 33% zinc sulfate (1.18 sg) was used for egg quantification, displaying a lower detection limit of 50 eggs per gram (e.p.g.) of faeces. To minimise false negatives, a test tubeflotation technique was also used (Roepstorff and Nansen 1998). Eggs were identified by their morphological characteristics with a microscope (Thienpont et al. 1986).

Adult worm identification

Trachea, lungs and digestive tract (stomach, small and large intestines) were dissected and washed to obtain adult worms (Roepstorff and Nansen 1998). These were collected in 5mm-500 µm sieves. Helminths were transferred in 70% ethanol solution for conservation. The worms were later immersed in lactophenol, observed under stereo microscope (×10, ×40 or ×100 magnification) and identified using Gassó et al. (2014) for lung nematodes and Frontera et al. (2009) for gastrointestinal helminths.

Statistical analysis

For exploring the accuracy of FECs, the intensity of infestation of different nematode species estimated after necropsy was correlated to the FEC made on the day of sampling (day 0). Specific linear regressions were carried out for those species identifiable by egg morphology (e.g. *Metastrongylus* spp., *Ascaris suum* and *Trichuris* sp.) and for those nematode species with unidentifiable eggs (called "strongyle eggs"): *Oesophagostomum- Hyostrongylus-Globocephalus*-type eggs and Spiruid-type eggs for *Physocephalus* sp. and *Ascarops* sp. (Straw et al. 2006).

Changes in FEC in the same sample over time were estimated using non-parametric Kruskal-Wallis ANOVA with FEC as the response variable and time (days 0, 5, 12 and 20) as a fixed factor. This analysis was performed for eggs of specific species e.g. *Ascaris suum*, *Trichuris suis* and *Metastrongylus spp*., and for those nematode species with unidentifiable eggs (e.g. spiruid and strongyle eggs). All statistical analyses were performed using R software version 3.2.1 (R Development Core Team 2015).

Results and Discussion

Prevalence and intensity of helminth infestation based on FEC and adult worm species are shown in Table 1.

The necropsy (adult worms found) revealed that all individuals were infested with 1-8 (mean = 4.5) helminth species: 13 helminth species, 12 nematodes and 1 acanthocephalan (*Macracanthorhynchus hirundinaceus*) were recorded in the studied wild boars. *Metastrongylus spp.*, *Ascaris suum* and *Trichuris spp*. were identified by coprological analysis. *Physocephalus*

sexalatus, *Ascarops strongylina, Hyostrongylus rubidus, Globocephalus urosubulatus, Oesophagostomum dentatum, M. hirundinaceus* and *Gonglyonema pulchrum* were identified by examining the respiratory and digestive tract. This last species was excluded from our analysis due to the lack of an oesophagus in some samples.

	Prevalence		Intensity of Infestation			
Helminth species	Adult worm	Eggs in faeces	Adult worm		Eggs in faeces (x/g)	
	% (CI)		Mean	Min-max	Mean	Min-max
Metastrongylus spp (L)	84.3 (71.99-91.83)	58.9 (45.88-70.83)	91.5	2 - 333	265.1	25 - 1100
Ascaris suum (SI, LI)	36.2 (25.05-49.07)	25 (15.52-37.69)	6.2	1 - 23	882.1	150 - 3450
Trichuris suis (LI)	43.1 (31.18-55.88)	33.9 (22.92-47.00)	7	1 - 23	225.2	25 - 750
Others	ND	58.9 (45.88-70.83)	--	--	404.5	50 - 2500
Ascarops strongylina (S, SI)	72.4 (59.80-82.25)	ND	55.2	1 - 591	--	--
Physocephalus sexalatus (S, SI)	15.5 (8.38-26.93)	ND	8.8	1 - 51	--	--
Hyostrongylus rubidus (S)	8.6 (3.73-18.64)	ND	11.4	5 - 19	--	--
Globocephalus urosubulatus (SI)	44.8 (32.75-57.55)	ND	124.4	1 - 726	--	--
*Macracanthorhynchus hyrundinaceu*s (SI)	12.1 (5.97-22.88)	ND	2	1 - 4	--	--
Oesophagostomum sp (LI)	13.8 (7.16-24.93)	ND	6.9	1 - 31	--	--

Table 1. Prevalence and 95% confidence interval, mean, minimum and maximum intensity of infestation estimated by counting adult nematodes and by coprology in 59 wild boars harvested in north-east and central Spain. Letters within brackets indicate where parasites were found: lung (L), stomach (S), small intestine (SI), caecum and colon (LI). The ND indicates that adults or eggs in faeces were not distinguished; the short dashed lines (--) indicate that intensity of infestation or prevalence was not estimated

Coprological analysis is a reliable indicator for diagnosis for some - but not all - helminthic infections. Although the number of false negative infestations with *T. suis* (24%), *Metastrongylus spp.* (28%), *and A. suum* (33%) diagnosed by coprology was moderate (Table 2), detection sensitivity for other helminthic infestations was lower (e.g. all *Macracanthorhynchus* infections in boars were undetected by coprological analyses). Several factors can explain this low sensitivity, with low parasite loads, the low reproductive rate of some species (e.g. gastric helminths, Poelvoorde and Berghen 1980), and the absence of one of the sexes (see Table 2) being the main factors. Additionally, low sensitivity may be explained by the presence of undistinguished eggs. There were no false positives in this study.

Our results demonstrate that FEC can be used to assess the parasite burden of nematode infestations with special relevance to pig health such as *T. suis, Metastrongylus* spp. and to a lesser extent *A. suum* (Fig. 1). The best correlations were observed for *T. suis* (β =0.21, SE = 0.01, R^2 = 0.86, p-value < 0.001, Fig. 1C), followed by *Metastrongylus* spp. (β = 0.67, SE = 0.062, R^2 = 0.76, p-value < 0.001, Fig. 1A) and *A. suum* (β =0.13, SE = 0.01, R^2 = 0.68, p-value < 0.001, Fig. 1B). In all cases, FEC decreased over time, but did so especially from 20 days after sampling (Table 3). This decrease was more evident for both *Metastrongylus* spp. (F = 9.36, p-value = 0.0035, Fig. 2A) and nematodes with indistinguishable eggs (F = 3.52, p-value = 0.0659, Fig. 2D) than for *Trichuris* or *Ascaris,* in which there was no change in FEC until day 12 (F value = 2.225, p-value = 0.139 *T. suis* and F value = 2.8, p-value = 0.1 for *A. suum*). After day 20, eggs from spirurids and strongyles were not detected (Fig. 2D). Similarly, FEC for *Metastrongylus* spp. decreased 9.5-fold 20 days after sampling.

Nematode species	False negatives	Boars with a single worm individual	Boars with worms belonging to the same sex
Macracanthorhinchus spp	7/7	4	3
Ascaris suum	7/21	6	1
Trichuris suis	11/39	3	4

Table 2. False negative coprology for *Macracanthorhinchus spp., Trichuris suis* and *Ascaris suum* infections.

Thus, a proper coprology assessment for *Metastrongylus*, *Trichuris* and *Ascaris* species in faecal samples stored at temperatures between 17 to 25ºC is feasible for at least five days after sampling. On the contrary, eggs from other nematodes, including the stomach worms and *G. urosubulatus* from the small intestine, are more susceptible to storage conditions. Our results suggest that desiccation is the principal cause of egg destruction and, despite the high resistance and the long survival of eggs in optimal conditions cited in the literature, in practice the degradation and/or hatching of these eggs is significant. In some areas, such as in south-central Spain, wild boar populations are managed to maintain artificially high densities for hunting purposes, which implies a risk of disease emergence (Gortázar et al. 2006). Thus, routine coprological analysis of harvested boars is recommended not only to assess the sanitary status of wild boar populations (e.g. in the case of boars coinfected with helminths and bovine tuberculosis, see Risco et al. 2014), but also to evaluate the impact of specific population management practices such as supplemental feeding (Navarro-Gonzalez et al. 2013).

	Days after collection			
Helminth species	0	5	12	20
Metastrongylus spp (L)	262.5 (0-1200)	220 (0-1000)	87 (0-600)	25 (0-200)
Ascaris suum (SI, LI)	387.5 (0-3450)	417.5 (0-3200)	287.5 (0-3500)	27.8 (0-250)
Trichuris suis (LI)	90.95 (0-650)	129.5 (0-900)	67.5 (0-650)	25 (0-100)
Others	80 (0-400)	22 (0-100)	15 (0-150)	0 (0-0)

Table 3. Mean, minimum and maximum egg counts in faeces on different days post-collection. Faeces were maintained without refrigeration under standard laboratory conditions (17-25ºC and 30-50% relative humidity). Letters within brackets indicate where parasites were found: lung (L), stomach (S), small intestine (SI), caecum and colon (LI). In the category "others", eggs from species *Globocephalus urosubulatus*, *Oesophagotomum sp*, *Hyostrongylus rubidus*, *Ascarops strongylina* and *Physocephalus sexalatus* were included

In conclusion, according to the results in this study, FEC is a useful tool for quantifying some (e.g. *Trichuris*, *Ascaris*) but not all gastrointestinal nematode infestations of wild boar. Lung nematode infestations can also be properly assessed by coprology in wild boar. Stomach nematodes often go undetected by coprology, thus further research is required to evaluate the effectiveness of other flotation solutions or McMaster modifications (e.g. FLOTAC, d'Ovidio et al. 2014) to assess parasite burden of such species. Moreover, ethyl acetate/formalin sedimentation procedure can be also useful to assess *Macracanthorhynchus* sp., infection. Nevertheless, it is recommended that samples be processed as soon as possible because egg count decreases over time.

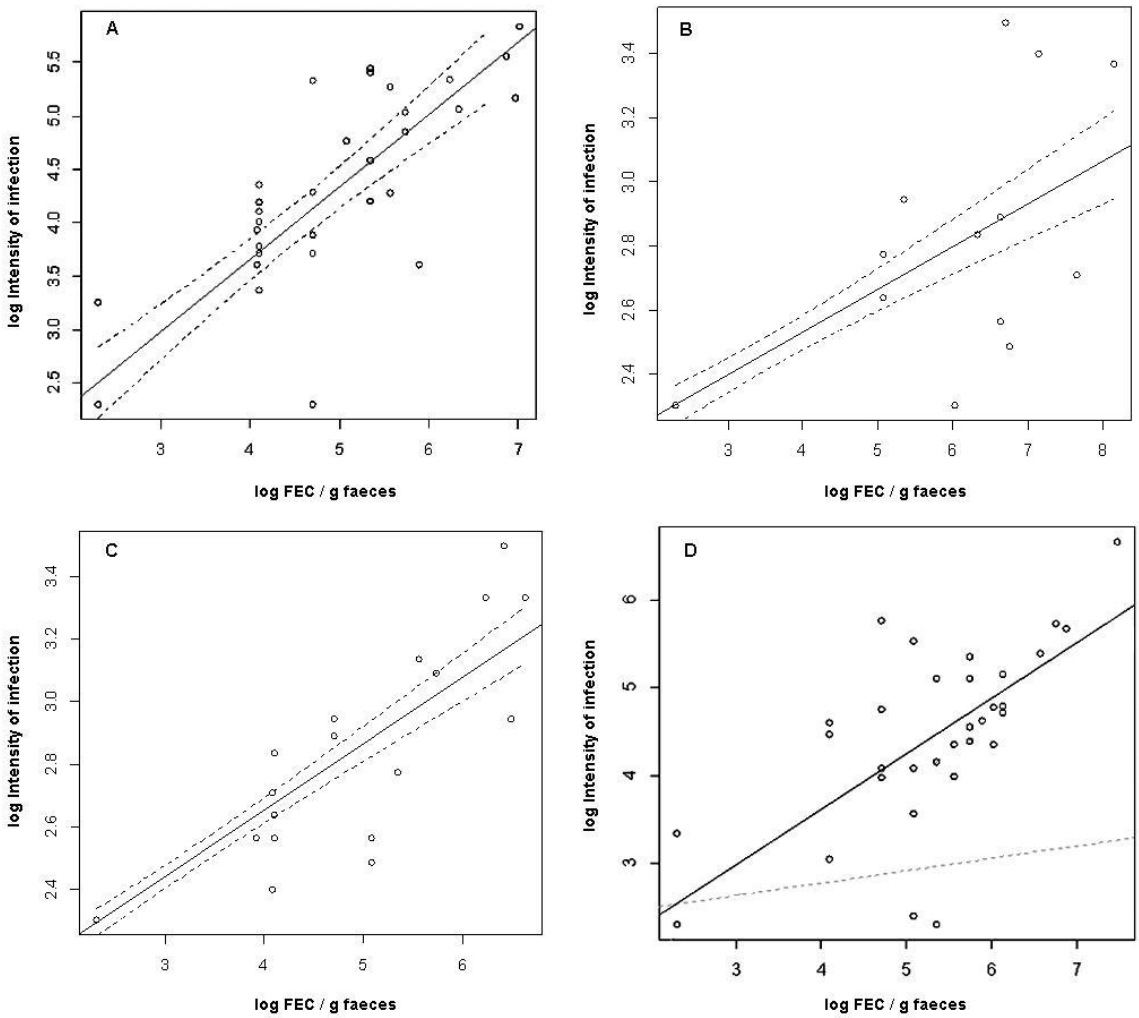

Figure 1. Relationship between intensity of infestation and eggs per gram of faeces (e.p.g.) of *Metastrongyus* spp. (A), *Ascaris suum* (B), *Trichuris suis* (C), and "strongyle eggs" nematode species (D) in 59 wild boars. In the latter group (D), *Oesophagostomum-Hyostrongylus-Globocephalus*-type eggs are represented by a solid line and the spiruid-type eggs with a dashed grey line. In A, B and C, dashed lines represent the confidence interval (95%) of the prediction. Both e.p.g., and intensity of infection have been log-transformed.

Integrative chapters

Chapter 4

Oxidative stress in wild boars naturally and experimentally infected with *Mycobacterium bovis*

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Abstract

Reactive oxygen and nitrogen species (ROS-RNS) are important defence substances involved in the immune response against pathogens. An excessive increase in ROS-RNS, however, can damage the organism causing oxidative stress (OS). The organism is able to neutralise OS by the production of antioxidant enzymes (AE); hence, tissue damage is the result of an imbalance between oxidant and antioxidant status. Though some work has been carried out in humans, there is a lack of information about the oxidant/antioxidant status in the presence of tuberculosis (TB) in wild reservoirs. In the Mediterranean Basin, wild boar (*Sus scrofa*) is the main reservoir of TB. Wild boar showing severe TB have an increased risk to *Mycobacterium* spp. shedding, leading to pathogen spreading and persistence. If OS is greater in these individuals, oxidant/antioxidant balance in TB-affected boars could be used as a biomarker of disease severity. The present work had a two-fold objective: i) to study the effects of bovine TB on different OS biomarkers (namely superoxide dismutase (SOD), catalasa (CAT), glutathione peroxidase (GPX), glutathione reductase (GR) and thiobarbituric acid reactive substances (TBARS)) in wild boar experimentally challenged with *Mycobacterium bovis*, and ii) to explore the role of body weight, sex, population and season in explaining the observed variability of OS indicators in two populations of free-ranging wild boar where TB is common. For the first objective, a partial least squares regression (PLSR) approach was used whereas, recursive partitioning with regression tree models (RTM) were applied for the second. A negative relationship between antioxidant enzymes and bovine TB (the more severe lesions, the lower the concentration of antioxidant biomarkers) was observed in experimentally infected animals. The final PLSR model retained the GPX, SOD and GR biomarkers and showed that 17.6% of the observed variability of antioxidant capacity was significantly correlated with the PLSR X's component represented by both disease status and the age of boars. In the samples from free-ranging wild boar, however, the environmental factors were more relevant to the observed variability of the OS biomarkers than the TB itself. For each OS biomarker, each RTM was defined as a maximum by one node due to the population effect. Along the same lines, the *ad hoc* tree regression on boars from the population with a higher prevalence of severe TB confirmed that disease status was not the main factor explaining the observed variability in OS biomarkers. It was concluded that oxidative damage caused by TB is significant, but can only be detected in the absence of environmental variation in wild boar.

Key words: Free radicals, *Mycobacterium bovis*, Oxygen stress, ROS-RNS, *Sus scrofa*

Introduction

Oxidative stress (OS) results from a disturbance of the balance between the production of reactive oxygen (ROS) or nitrogen (RNS) species and the organism's ability to compensate for their damaging effects (Valko et al. 2007). During an infectious process, macrophages and neutrophils produce large amounts of ROS and RNS for pathogen clearance (Dröge 2002). However, these biochemical products do not discriminate between pathogens and the host's own biological structures causing cell injury, triggering physiologic disorders and promoting the pathological process (Sorci and Faivre 2009). Since the OS response integrates both the activation of immune response and the ability of the organism to compensate for infection damage (Wiid et al. 2004), it is widely used as a biomarker for assessing the physiological cost of infection in human (Valko et al. 2007) and animal health (Lykkesfeldt and Svendsen 2007;Castillo Rodríguez et al. 2011).

A wide variety of studies have assessed oxidative damage in a broad range of pathologies such as tuberculosis (TB). Immunocompetent hosts infected with *Mycobacterium* spp. mount an immune response mainly based on T cell activation. Although a strong immune response is usually sufficient to control TB, infected hosts rarely clear the bacterium (Flynn and Chan 2003). One reason for its long persistence in an immunocompetent host is that *Mycobacterium* spp. is able to persist within macrophages through diverse evasion strategies, including the inhibition of macrophage RNS production (Bryk et al. 2002). In a murine model, these inhibitory effects result in the reactivation of persistent *Mycobacterium* spp. infection (Flynn et al. 1998).On the other hand, TB-affected individuals with poor antioxidant defences suffer from tissue damage due to OS (Lamsal et al. 2007; Palanisamy et al. 2011). In fact, inflammation caused by OS in TB-affected patients has been implicated in the pathogenesis of lung fibrosis and dysfunction (Kwiatkowska et al. 1999). In view of this, it seems clear that a proper oxidant/antioxidant balance is essential for the containment of both acute and chronic TB in mammals.

To date, no data have been reported regarding oxidant/antioxidant status in wild reservoirs of the *Mycobacterium tuberculosis* complex (MTC). In the Mediterranean Basin, wild boar (*Sus scrofa*) is the main reservoir of TB (Naranjo et al. 2008), one of the major diseases of domestic animals throughout the world. This wild pig can suffer from a severe form of the disease showing macroscopic lesions in cervical lymph nodes, lungs, liver, kidneys or testicles (Martín-Hernando et al. 2007). As described for other host models (Menin et al. 2013), wild boar suffering from severe TB are considered super-shedders (Barasona et al. 2015). Wild boar live at different

population densities in a wide range of environmental conditions, which may affect the values of OS biomarkers. However, only preliminary work has explored OS patterns in this mammal (Cánovas et al. 2015). Consequently, the wild boar is an excellent model for exploring how OS is affected by environmental and population sources of variation aside from disease occurrence. Furthermore, OS is interesting from an ecological perspective because of the link between OS and the fitness components of organisms (Alonso-Alvarez et al. 2008; Galván et al. 2012). In fact, there is clear evidence about the negative effects of oxidative stress on the reproduction (Costantini 2008), and survival rates (Freeman-Gallant et al. 2011) of wild vertebrates. For that reason, OS biomarkers can be excellent indicators not only to assess health status (Beaulieu and Costantini 2014)but also to measure the impact of environmental variation on individuals and populations.

In spite of the plethora of methods used to determine oxidative damage and antioxidant defences, little information exists about the reliability of these biomarkers to assess the impact of infectious diseases on free-ranging vertebrates. Moreover, the interpretation of OS biomarkers is not a trivial issue. In fact, the proper assessment of oxidative status must take into account not only the concentration of antioxidant defences (i.e., antioxidant vitamins and other substances, and/or up-regulation of endogenous antioxidants (enzymes)), but also oxidative damage (Beaulieu and Costantini 2014). For instance, an elevation of endogenous or exogenous antioxidants with stable oxidative damage concentrations suggests that individuals are successfully dealing with the new oxidative conditions. In contrast, the depletion of antioxidant substances and stable oxidative conditions suggest the physiological exhaustion to cope with the stressor and the risk of oxidative damage.

In addition, the pure effects of a given stressor on the OS biomarkers are hardly estimated because of both individual (e.g., growth (Sohal and Weindruch 1996), genetic diversity (Hybertson et al. 2011), reproductive status (Sharick et al. 2015) or immune response (Rada and Leto 2008; Costantini and Moller 2009)), and environmental variation (Rada and Leto 2008). To complicate matters, all of the previously mentioned factors can act in synergy (Marcogliese et al. 2005), making the comparison of OS biomarkers between populations and seasons (Isaksson 2010) difficult. Surprisingly, there is little information about these limitations in the use of OS biomarkers for most vertebrate species.

In the present work, we studied oxidative status and oxidative defences in 120 wild boar (*Sus scrofa*) living under contrasting environmental conditions. They ranged from dewormed,

supplementally fed animals experimentally challenged with *Mycobacterium bovis* to free-ranging individuals inhabiting areas affected by the disease. According to recent recommendations (Beaulieu and Costantini 2014) a broad panel of biomarkers of antioxidant and oxidative status were used. We assessed the concentration of four endogenous antioxidant enzymes (AE): superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) and glutathione reductase (GR). We also measured concentrations of a biomarker of lipid peroxidation (thiobarbituric acid reactive substances, TBARS). The study had a twofold objective: i) to assess the effect of *M. bovis* infection on the oxidant/antioxidant status of wild boar showing different degrees of disease severity, and ii) to evaluate the importance of individual (body weight and gender) and environmental (population and season) sources of OS variation when assessing oxidant/antioxidant status in free-ranging wild boar populations affected by TB.

Since TB is a chronic infection causing a host inflammatory response (Sasindran and Torrelles 2011),which in turn has been linked to both OS and pathogenesis of lung fibrosis in human (Kwiatkowska et al. 1999; Lamsal et al. 2007) and animal models (Palanisamy et al. 2011), we expected to find higher OS values in TB-infected wild boar, in particular in those individuals showing gross lesions. On the other hand, because of the effect of external sources of OS, we expected that the relationship between TB and OS would be more difficult to detect in free-ranging animals.

Material and methods

Study areas

Two different populations of free-ranging wild boars were studied, originating in Eastern (The National Game Reserve Ports de Tortosa i Beseit, NGRPTB) and Central (Ciudad Real, CR) Spain. The NGRPTB (40°48'N, 0°19'E; about 28% of the surface area is higher than 1000 m.a.s.l, with the highest peak being Mont Caro (1442 m) and the lower altitudes around 300 m.a.s.l.) is a rough limestone mountain massif of 28,587 ha, with a typical Mediterranean climate and annual mean temperatures (year 2014) of 10.6ºC (min = 0.0ºC in February, max = 23.4ºC in July) and a mean yearly cumulative rainfall (period 2010-2014) of 1084.4 mm (min = 0.1 mm in Setember-August, max = 512.5 mm in November, information kindly provided by *Servei Meteorològic de Catalunya,*www.meteocat.com). CR is located in central Spain (38º55'N, 0º36'E; 600–850 m.a.s.l.) and shows a continental Mediterranean climate with annual mean temperatures (year 2014) of 17.92ºC (min = -3.4ºC in December, max = 40.6ºC in July) and a mean yearly

cumulative rainfall (period 2009-2014) of 468.2 mm (min = 0 mm in July, max = 180.2 mm in March, information provided by www.meteociudadreal.com).

Sampling of free-ranging wild boar

Sixty-one hunter-harvested wild boar from 3 to 80 months of age were sampled in the NGRPTB (n = 24) and CR (n = 37), during the regular hunting season. After animal collapse, the gender of the boar was assigned visually by inspecting genitalia, and age was assessed by recording dental eruption patterns (Boitani and Mattei 1992). Additionally, body weight (with viscera) of animals was measured with a dynamometer (precision of 0.1 kg). A necropsy examination of animals was performed to assess the presence of TB-like gross lesions affecting lymph nodes (submandibular, retropharyngeal, mediastinal and mesenteric lymph nodes), and thoracic or abdominal organs (Martín-Hernando et al. 2007). Submandibular and/or retropharyngeal lymph nodes and blood (in separation serum tubes) were collected and stored at 4°C until processing within the following 24 hours.

Ethics statement

For hunter-harvested boars, no approval was needed from an Ethics Committee since the animals were not culled for research purposes. These animals were legally hunted in their own habitat by authorised gamekeepers and hunters within the framework of an annual hunting plan approved by the local environmental agencies: *Departament d'Agricultura, Ramaderia, Pesca, Alimentació i Medi Natural – Generalitat de Catalunya* (for NGRPTB), and the *Departamento de Medio Ambiente de Castilla la Mancha* (for CR). On the other hand, the approval for culling wild boars used for the experimental M. bovis infection was given by the Committee on Ethics of Animal Experiments of the Regional Agriculture Authority of CR, Permit number 2741-2009.

Experimental Mycobacterium bovis infection design

Wild boar (n = 59, 42 males and 17 females, ranging from six to 24 months of age) were purchased from a local TB-free commercial farm at 3-4 months of age and were used in experimental *M. bovis* infection trials (previously published in Ballesteros et al. 2009 and Garrido

et al. 2011). Farmed animals were negative for both *Mycobacterium* spp. by ELISA tests (Boadella et al. 2011), regularly performed by veterinary authorities, and to mycobacterial lesions at slaughter. Moreover, farmed wild boar were dewormed yearly and reared under regular veterinary inspection.

The animals were housed in biosecurity level 3 bio-containment facilities where they had *ad libitum* food and water (during three experimental periods October 2010, January 2011 and March 2011; for more information see (Garrido et al. 2011)), and 44 of them were infected with 5 ml of a suspension containing 10^6 colony forming units (CFU) of a *M. bovis* field strain (SBO339 described in (Ballesteros et al. 2009)). Non- inoculated boars were used as control animals (n = 15). The animals were handed four times during the experiment, where blood samples were collected (days 11, 46, 186 and 300 dpi). Only serums of the last day were used for this propose, the other samples were used for other studies. At the end of the experiment, animals were anaesthetised by intramuscular injection of Zoletil® (Tiletamine 50 mg; Zolacepam 50 mg), and euthanised by captive bolt. Necropsy and sampling were performed following (Garrido et al. 2011).

Handing procedures and sampling frequency were designed to reduce stress and health for subjects, according to European (86/609) and Spanish (R.D. 223/1988, R.D. 1021/2005). No adversal events occurred during the infection and no animals showed signs of illness prior to the study end point.

All experiments were carried out following European, National and Regional Law and Ethics Committee regulation

Sample processing

Lymph nodes were dissected and stored in sterile containers for further microbiological analyses. Peripheral blood was collected from the conjunctival sinus, cavernous sinus or cava vein of the wild boar using an 18-gauge needle (Arenas-Montes et al. 2013). The collected blood was placed into serum separator tubes and maintained at 4ºC in cold boxes until arrival at the laboratory. Serum was obtained by centrifugation at 3.500 rpm for 5 min and conserved at -80ºC until further analysis.

TB severity assessment

To examine the extent of bovine TB-like lesions, wild boar were classified into three groups: i) animals free from TB (TB free), ii) animals with localised gross lesions (mild TB) and iii) animals with disseminated gross lesions (severe TB). Animals showing a mild TB were those with TB lesions in only one location, mainly submandibular or retropharyngeal lymph nodes. Those wild boar with lesions in these lymph nodes or any other organ (e.g., lung, liver, mesenteric lymph nodes and/or spleen) were considered to have severe TB (Risco et al. 2014b).

Microbiological analysis

For MTC detection, samples of lymph nodes from the head and thorax were pooled, homogenised with sterile distilled water and decontaminated with 0.35% hexadecylpyridinium chloride for 30 minutes (Corner and Trajstman 1988), centrifuged at 3500 rpm (1068 g) for 30 min and cultured onto Coletsos and 0.2% (w/v) pyruvate-enriched Löwenstein-Jensen media (Biomedics, Madrid, Spain) at 37ºC. Isolates were identified by staining for acid alcohol fastness and PCR amplification of the Mycobacterium spp. genus-specific 16S rRNA fragment and the MPB70 sequence (Wilton and Cousins 1992).

Oxidative stress

To minimise the interpretation bias caused by the use of a single biomarker (Sharick et al. 2015), five different biomarkers were used to assess OS in serum samples of wild boar: SOD, CAT, GPX, GR and TBARS. The latter is considered an indicator of lipid peroxidation whereas SOD, CAT, GPX and GR are endogenous antioxidant enzymes (AE). SOD (U/mg of protein) provides an important antioxidant defence in nearly all cells exposed to ROS generated by cellular immune responses. SOD catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide measured by the inhibition degree of cytochrome C by this enzyme. The method followed for its estimation was that proposed by McCord and Fridovich (McCord and Fridovich 1969). CAT (U/mg of protein) catalyzesthe decomposition ofhydrogen peroxide produced in damaged tissues to water and oxygen and was estimated following the Cohen and Somerson method (Cohen and Somerson 1969). GPX (mU/mg of protein) is the general name of an enzyme family with peroxide activity; its biochemical function is to reduce lipid hydroperoxides to their corresponding alcohols and to reduce free hydrogen peroxide to water. Its concentration was determined by estimating NADPH oxidation by the method proposed by Carmagnol et al.

(Carmagnol et al. 1983). Finally, GR (U/mg of protein) catalyzes the reduction of glutathione disulfide (GSSG) to the sulfhydryl form glutathione (GSH), which is a critical molecule resisting OS and maintaining the redox environment of the cell. This last enzyme was measured following the method described by Cribb et al. (Cribb et al. 1989). Because ROS have an extremely short half-life, they are difficult to measure directly. Instead, several products of the damage produced by OS can be measured, such as TBARS (nmol MDA/ml), which is formed as a by-product of lipid peroxidation (i.e., as degradation products of fats). An assay of TBARS measures malondialdehyde (MDA), a low-molecular-weight molecule formed by the decomposition of primary and secondary lipid peroxidation products present in the sample. The Buege and Aust method (Buege and Aust 1978) was used to measure MDA. This technique minimises additional oxidation of the sample matrix that would overestimate lipid peroxidation (Monaghan et al. 2009). Biochemical analyses were performed at the Laboratory of Ecophysiology of the Estación Biológica de Doñana, Spain (EBD-CSIC) in a microplate, multilabel reader Victor 3 PerkinElmer.

Statistical analyses

To explore the effects of M. bovis infection and age on the OS biomarkers in the experimentally infected wild boar (n = 59), the Partial Least Squares Regression approach (PLSR) was used. This statistical tool is an extension of multiple regression analysis and combines features from principal component analysis. PLSR copes better with multicollinearity than generalised linear models (Geladi and Kowalski 1986) and is statistically more robust. The relative contribution of each variable to the derived factors was calculated by means of the square predictor weights. PLSR creates score vectors (also called latent vectors or components) by maximising the covariance between different sets of variables. In the present study, the response variables were the serum concentration of each biomarker of OS (i.e., SOD, CAT, GPX, GR and TBARS) and the explanatory variables were TB status and animal age. The use of this approach minimises the limitations of interpreting a single biomarker (Romeu et al. 2010). To the authors' knowledge, this technique has been rarely used in pathological studies; however, a revision of its use in the field of ecology can be found in Carrascal et al. 2009. In a second step, a Kruskal-Wallis post hoc comparison was performed to assess which markers of OS were the most affected by disease severity.

The role of TB, individual, population and environmental sources of OS variation were explored in 61 free-ranging wild boar (24 from NGRPTB, and 37 from CR), using a recursive

partitioning approach and regression tree models (RTM). This statistical tool is ideally suited for the analysis of complex ecological data and provides several advantages over other regression techniques, but mainly a better ability to handle missing values in both explanatory and response variables (see De'ath and Fabricius 2000). In the present case, the response variable was each biomarker of OS whereas the explanatory variables were TB status (free, mild and severe infection), population (NGRPTB or CR), season (using seasonal solstices: autumn, spring, summer or winter), body weight and sex. By these variables, several sources of spatial (e.g., population), temporal (e.g., season) and individual variation (e.g., disease status, body weight and sex) potentially linked to OS biomarker variability were represented. Because boars showing severe TB were more abundant in CR that in NGRPTB, a post hoc tree model exploring the influence of season, sex, body weight and disease status on boars from this population was performed.

For the PLSR analysis the "plspm" package was used (Sanchez 2013)and for the RTM analysis the "rpart" statistical package was used (Therneau et al. 2015)(for more information see http://www.gastonsanchez.com and https://cran.r-project.org/web/packages/rpart/rpart). All statistical analyses were performed with the R software version 3. 2. 4 (R Development Core Team, December 2016).

Results

Assessment of TB lesions and OS biomarkers in studied wild boar

Globally, 16 out of 61 (26.2%) free-ranging wild boar had TB lesions, with severe TB lesions more frequently found in boars from CR than in NGRPTB. We detected a total of 5 spoligotypes, two in the five animals of NGRPTB (SB0294 (n=4) and SB0415 (n=1) and three in the 11 of CR (SB0119 (n=1), SB0339 (n=2) and SB0295 (n=8)). A part of *M. bovis*, *M. caprae* was detected in one animal of NGRPTB, in CR all *Mycobacteria* were *M. bovis*. For more information see (Mentaberre et al. 2014) regarding NGRPTB animals and (Górtazar et al. 2011) regarding CR wild boar. TB lesions were generated in all experimentally infected wild boar. All cases of suspected TB by gross examination were confirmed by means of bacteriological analyses. No *M. bovis* was detected in animals with a lack of gross lesions compatible with TB.

Table 1 summarises age, sex and TB lesion extent in experimental and free-ranging wild boar. In experimentally infected animals, 16 juveniles and four yearlings were infected with severe TB, and 14 juveniles and 10 yearlings were infected with mild TB; control animals(two juveniles and 13 yearlings) were negative for *M. bovis*. In free-ranging wild boar from CR, two

adults and two yearlings had severe TB, two yearlings and five juveniles displayed mild TB lesions, and 13 juveniles and 13 yearlings were TB free. No wild boars from NGRPTB showed severe TB status, while one piglet, two yearlings and two adults had mild TB and one piglet, three juveniles, four yearlings and 11 adults were free from MTC infection.

		Age				Sex		TB status		
		Piglet	Juvenile	Yearling	Adult	Male	Female	TB free	Mild TB	Severe TB
Experimentally infected	(n = 59)	0	32	27	0	42	17	15*	20**	24**
Free-ranging	CR	0	18	17	2	22	15	26	7	4
	NGRPTB	2	3	6	13	13	11	19	5	0

Table 1. Summary of the epidemiological values of the wild boar experimentally and naturally infected with *Mycobacterium tuberculosis* complex. Data from NI animals came from two populations, Ciudad Real (CR, n = 37)) and The Natural Game Reserve Ports de Tortosa i Beseit (NGRPTB, n = 24). *Control wild boar; ***M. bovis* inoculated wild boar

		Mean	SE	Min - Max
SOD (U/mg)	TB free	2.92	0.32	0.97 - 5.82
	Mild TB	1.75	0.44	0.08 - 12.04
	Severe TB	1.34	0.23	0.30 - 4.50
CAT (U/mg)	TB free	43.15	14.44	5.45 - 180.00
	Mild TB	32.23	8.47	7.54 - 202.19
	Severe TB	13.78	1.19	5.38 - 27.81
GPX (mU/mg)	TB free	17.83	1.29	9.36 - 29.31
	Mild TB	14.3	2.26	2.82 - 46.26
	Severe TB	12.04	1.94	2.71 - 48.74
GR (U/mg)	TB free	0.11	0.02	0.039 - 0.393
	Mild TB	0.13	0.02	0.036 - 0.387
	Severe TB	0.12	0.01	0.052 - 0.286
TBARS (nmol MDA/ml)	TB free	10.25	1.16	5.11 - 22.88
	Mild TB	8.49	0.68	3.57 - 20.84
	Severe TB	8.9	1.02	3.60 - 23.23

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Table 2. Descriptive statistics for oxidative stress biomarkers. Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione reductase (GR) and thiobarbituric acid reactive substances (TBARS), in serum from wild boar experimentally infected with *M. bovis*. The SOD, CAT, GPX and GR enzymes were measured in units of activity per mg of protein (U/mg), and TBARS in nanomoles of malondialdehyde per ml (nmol MDA/ml). SE: standard error; TB free: TB negative animals; Mild TB: animals with localised lesions in lymph nodes; Severe TB: animals showing generalised lesions in lung, liver, mesenteric lymph nodes and/or spleen

Tables 2 and 3 summarise descriptive values for OS biomarkers of experimental and free-ranging wild boars. Experimental TB free animals had higher values of AE; in contrast, the highest differences for free-ranging boars were detected between populations (CR versus NGRPTB) and not between TB groups.

		Population	Mean	SE	Min - Max
SOD (U/mg)	TB free	NGRPTB	4.04	0.40	1.69 – 7.33
		CR	3.01	0.44	0.16 – 8.96
	TB	NGRPTB	2.64	0.41	1.26 – 3.52
		CR	2.54	0.48	0.99 – 5.83
CAT (U/mg)	TB free	NGRPTB	54.66	3.25	38.04 – 89.04
		CR	20.90	3.5	5.72 – 78.16
	TB	NGRPTB	46.87	2.62	40.50 – 54.77
		CR	11.71	2.08	3.34 – 22.21
GPX (mU/mg)	TB free	NGRPTB	10.5	1.2	3.6 – 23.2
		CR	15.63	1.16	4.17 – 30.42
	TB	NGRPTB	9.87	2.1	4.85 – 14.89
		CR	13.5	1.2	7 – 18.6
GR (U/mg)	TB free	NGRPTB	0.262	0.035	0.098 – 0.567
		CR	0.0950	0.0078	0.0504 – 0.2014
	TB	NGRPTB	0.1924	0.0395	0.113 – 0.34
		CR	0.0857	0.0097	0.0448 – 0.1465
TBARS (nmol MDA/ml)	TB free	NGRPTB	4.99	0.46	2.82 – 12.31
		CR	13.30	0.68	9.21 - 20.23
	TB	NGRPTB	3.54	0.32	2.96 – 4.62
		CR	11.63	1.08	5.71 – 15.53

Table 3. Descriptive statistics for superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione reductase (GR) and thiobarbituric acid reactive substances (TBARS), in serum from wild boar naturally affected by TB. The SOD, CAT, GPX and GR enzymes were measured in units of activity per mg of protein (U/mg), and TBARS in nanomoles of malondialdehyde per ml (nmol MDA/ml). SE: standard error; TB free: TB negative animals; TB: animals with localised lesions in lymph nodes or generalised lesions in lung, liver, mesenteric lymph nodes and/or spleen. CR: Ciudad Real; NGRPTB: National Game Reserve Ports Tortosa i Beceit.

Oxidant/antioxidant status in experimentally M. bovis-infected wild boar

Since the initial PLSR model consisted of a PLSR Y's component including all of the OS biomarkers, only GPX, SOD and GR were retained in the final model. This model showed that

17.6% of the observed variability of wild boar antioxidant capacity was significantly (Q^2 = 0.132, p-value < 0.05) correlated with the PLSR X's component represented by both disease status and the age of animals (Fig 1). Both the age and TB status contributed similarly to the PLSR X's component (Table 4). In the Y's component, however, SOD, GPX and GR contributed in decreasing order of importance (Table 4 and Fig 1). In general, all the antioxidant biomarkers were negatively related to the TB status indicating low serum concentrations of these enzymes in individuals with severe TB (Table 2).

PLSR component	Predictors	Loads	Weights	VIP	Corr. Xu	Corr. Xt
X	Age	0.71	0.71	1.01	0.6	0.91
	TB status	-0.71	-0.71	0.99	-0.6	-0.91
					Corr. Yt	**Corr. Yu**
Y	GPX	0.21	--	--	0.27	0.65
	SOD	0.49	--	--	0.63	0.92
	GR	-0.18	--	--	-0.24	0.2

Table 4. Summary of the partial least squares regression between oxidative stress and tuberculosis status and age. The PLSR Y component representing antioxidant status by the enzymes superoxide dismutase (SOD), glutathione reductase (GR) and glutathione peroxidase (GPX) and the PLSR X component represented by the age and the tuberculosis status (i.e., TB free, Mild TB and Severe TB) of experimental wild boars. VIP: variable importance in projection; Corr. Xu: correlation between each explanatory variable in the X's component and the Y's component; Corr. Yt: correlation between each response variable in the Y's component and the X's component; Corr. Xt: correlation between explanatory variables and X's component; Corr. Yu: correlation between response variables and the Y's component.

SOD concentrations were the most affected by TB (chi-squared = 17.58, df = 2, p-value = 0.0001), with TB-free animals between 1.6 to 2.19 times higher than in boars showing mild or severe TB (Table 3 and Fig 2).To a lesser degree, GPX concentrations also varied according to TB status (chi-squared = 7.16, df = 2, p-value = 0.028), being 1.25 to 1.48 times higher in TB-free boars compared to mild or severely TB-affected individuals. Despite the fact that CAT followed

the same pattern, the post hoc Kruskall-Wallis test was not significant (chi-squared = 1.85, df = 2, p-value = 0.3969), probably because of the large standard error (SE = 14.44) and the wide rank of CAT values both in TB-free and affected animals (Table 3). Along the same lines, no differences in GR concentrations were observed between groups (chi-square = 3.09, df = 2, p-value = 0.213).

Oxidant/antioxidant status in free-ranging wild boar

The best RTM of the role of environmental and individual drivers of OS in both healthy and TB-affected wild boars were built with population as a single split factor (Table 5).In fact, mean concentrations of the five OS biomarkers differed mainly between populations, with the other factors (i.e., season and body weight) being irrelevant. As an example, for TBARS, which in turn built the model with the best fit (lower relative error and higher R^2, Table 5 and Fig 3), the mean concentration in boars from CR was 2.7 times higher than in NGRPTB animals. Along the same lines, mean GR concentration in boars from CR was 14.97 and 10.38 mU/mg of protein for animals from NGRPTB. For CAT, mean concentration in CR boars was 24.31 and 53.04 U/mg of protein in NGRPTB animals. Finally, although SOD variability was explained by the effects of the season, the high relative error and the low fit did not allow for model interpretation.

The post hoc regression tree analysis performed in CR, where severe TB was common among boars, showed that body weight was more important than disease severity for explaining the observed variability of OS biomarkers. The model fit, however, was so poor that conclusions could not be drawn (Table 5).

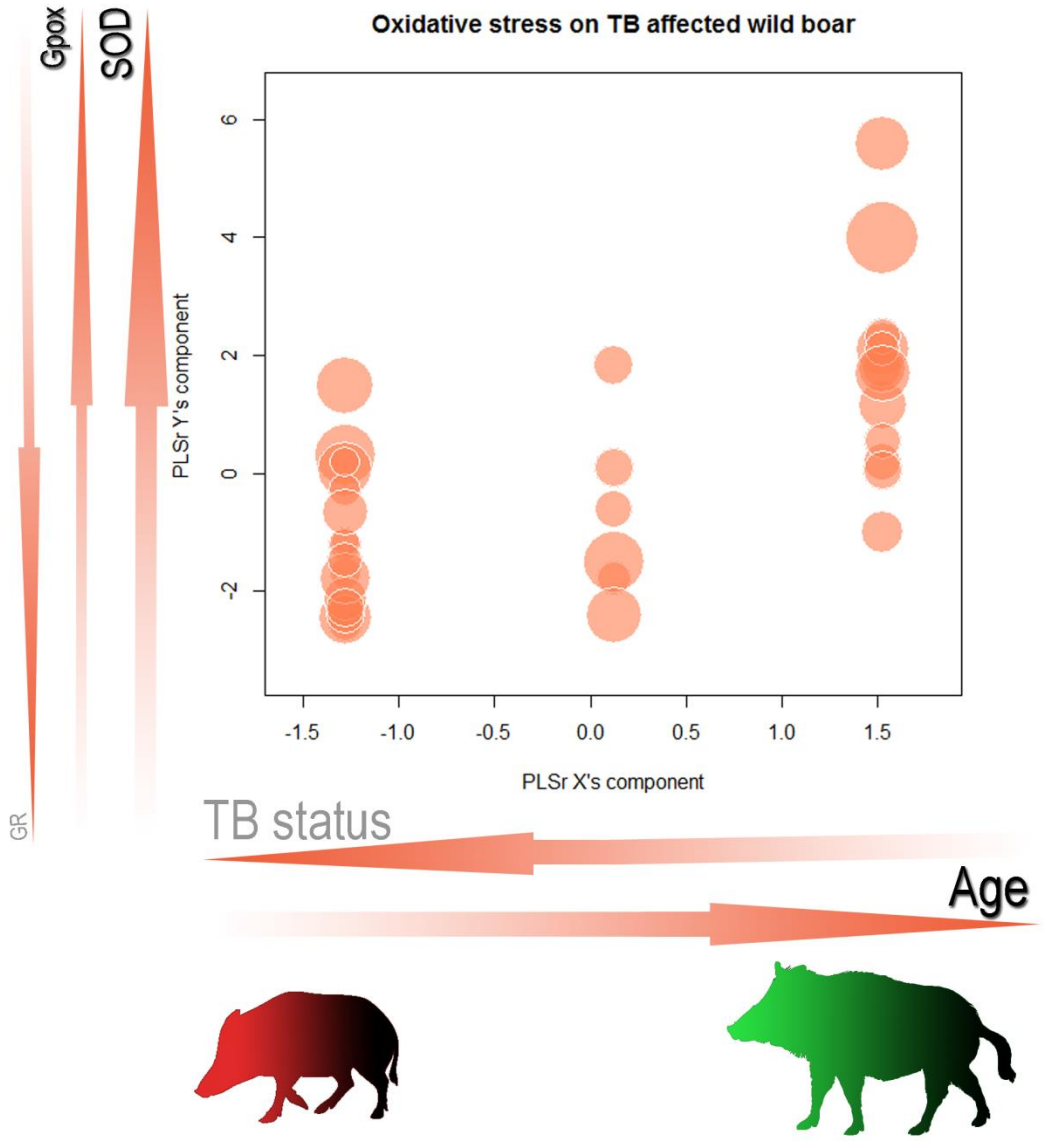

Figure 1. Bubble plot representing the relationships between a PLSR X component describing age and tuberculosis status and the PLSR Y component describing antioxidant capacity of 59 wild boar (*Sus scrofa*) experimentally infected with *M. bovis*. Font and arrow size indicate the weight of each variable whereas font colour indicates either the increase (black) or the decrease (grey) of the score components. Bubble diameter represents TBARS concentration (nmol MDA/ml), an oxidation biomarker. Thus, for proper interpretation both the bubble size and its position should be taken into account. Large bubbles at low PLSR Y scores suggest boars with oxidative damage whereas the same bubbles at high PLSR Y scores indicate that individuals are able to compensate for the increase in oxidation (high antioxidant /oxidant value). Each bubble represents individuals having the same X and Y score values. The red wild boar shape at the bottom represents a young wild boar suffering from oxidation due to *M. bovis*, whereas the green shape represents a TB-free adult wild boar in good antioxidant status.

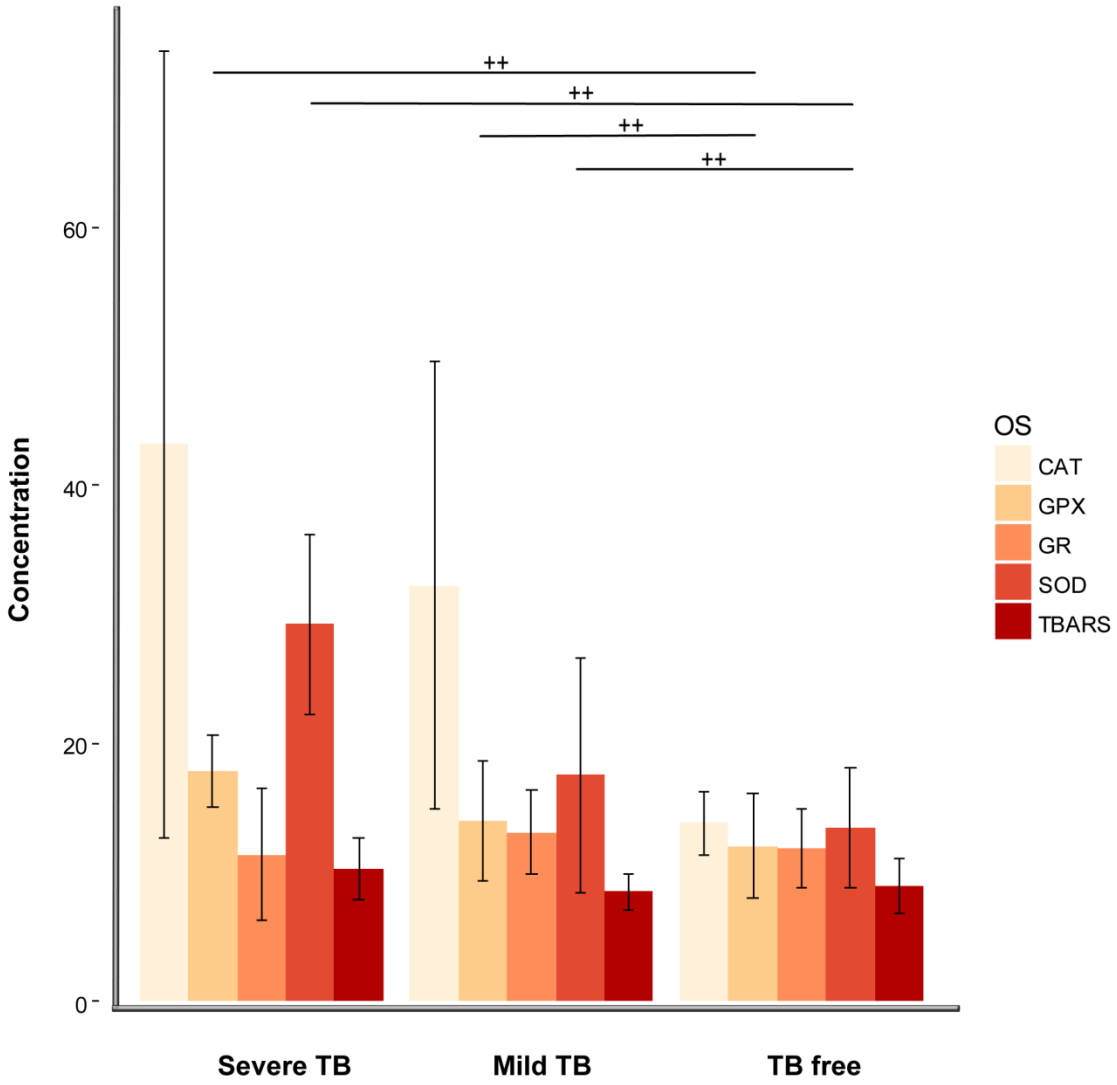

Figure 2.Mean concentration and associated standard error (SE) of lipid peroxidation (TBARS) and endogenous antioxidant enzymes (CAT, SOD, GPX and GR), in serum from wild boar experimentally infected with *M. bovis*. Concentrations of SOD, CAT and GR enzymes were measured in units of activity per mg of protein (U/mg), GPX in mU/mg, and TBARS in nanomoles of malondialdehyde per ml (nmol MDA/ml). Results of both SOD and GR were multiplied by ten for graphic representation. Wild boar were divided into three groups: TB free n=15, Mild TB (localised lesions in lymph nodes) n=20 and Severe TB (generalised lesions in lung, liver, mesenteric lymph nodes and/or spleen) n=24. Wiskers represent 95% confidence intervals and the horizontal lines the results of a post hoc Kruskall wallis test. Statistically significant differences, at $\alpha = 0.05$ are indicated by crosses.

Discussion

Based on our results, a positive oxidant/antioxidant balance (i.e., low antioxidant enzyme production for a given concentration of oxidant substances) in *M. bovis*-infected boars can be detected under experimental conditions but not in free-ranging animals exposed to environmental variation. The results obtained in our experimental challenge are in line with the basic pathophysiological mechanism proposed in other vertebrate models. In mice and humans, for example, an increase in OS is the main mechanism limiting *Mycobacteria* spp. multiplication, thus preventing the appearance of TB-like gross lesions (Yu et al. 1999;Chan et al. 1995). At the same time, the bacterium limits the exposure to free radicals by increasing the expression of antioxidant enzymes (Cumming et al. 2014), which slows down macrophage ROS and RNS production (Flynn et al. 1998; Bryk et al. 2002). These "attack and counterattack" interactions are key drivers for maintaining the TB granuloma in a latent form that can be reversed in case of immune depression (Sasindran and Torrelles 2011).

Our challenged boars may have experienced the same pathophysiological process since individuals with TB gross lesions showed low SOD and GPX concentrations for a given degree of oxidation (TBARS). In agreement with observations made in other vertebrate models (Wiid et al. 2004), in which AE mobilisation was needed to maintain OS homeostasis, our wild boars may show high SOD and GPX depletion to protect tissues from the intermediate production of macrophage reactive nitrogen during the severe form of the disease (Flynn et al. 1998). Since oxidative damage depends on whether AE production is sufficient to compensate for oxidative damage (Beaulieu and Costantini 2014), we can assume that our challenged wild boars developing TB-like gross lesions are suffering from oxidative stress.

This antioxidant capacity depression has also been observed in TB (Madebo et al. 2003) confirming the value of OS biomarkers as indirect indicators of bacteria proliferation during TB infection. Though it was ultimately not retained in our PLSR model, CAT concentration was also lower in individuals showing TB gross lesions. Previous research has also observed few variations in CAT concentrations between TB-affected and healthy patients (Mohod and Kumar 2012).

Age was also linked to changes in the oxidant/antioxidant balance in our experimental animals. In the last few decades, several works have shown that aging *per se* is a consequence of oxidative damage (Sohal and Weindruch 1996;Calabrese et al. 2015). In our case, the PLSR

model exemplifies that SOD and GPX were positively but poorly correlated with age. In contrast, GR was negatively and highly correlated with age, suggesting that yearling boar had lower levels of GR in serum than juveniles. In wild boar, glutathione levels were not sensitive to senescence and TBARS had a curvilinear pattern in relation to age (Galván et al. 2012). Similar inconclusive results have been obtained in domestic pigs infected by porcine reproductive and respiratory syndrome virus (Stukelj et al. 2013). In this study, the age group (weaners, fatteners or finishers) influenced concentration of AE in infected animals. The relationship between OS biomarkers and the aging process in wild boar, as well as pigs,is still unclear.

	Population	Cross-validation error	Relative error	Complexity parameter	R^2	VI
TBARS	NGRPTB + CR	0.30	0.28	0.02	0.72	P(46) > S(34)> BW(19)> TB(1)
	CR	1.06	1	0.11	0	BW(48) > A(40) > TBL(12)
SOD	NGRPTB + CR	0.96	0.86	0.05	0.14	S(68) > BW(24) > P(3) > TB(3) > G(2)
	CR	1.03	1	0.08	0	BW(47) > A(44) > TBL(8)
CAT	NGRPTB + CR	0.86	0.77	0.02	0.23	P(41) > S(29) > BW(27) > TB(3)
	CR	1.02	0.92	0.001	0.08	BW(47) > A(44) > TBL(8)
GPX	NGRPTB + CR	0.88	0.84	0.07	0.16	P(37) > BW(30) > S(26) > TB(4) > G(3)
	CR	0.99	0.73	0.001	0.27	BW(62) > A(25) > TBL(6) > G(5) > S(1)
GR	NGRPTB + CR	0.54	0.44	0.01	0.56	P(43) > S(32) > BW(20) > TB(3) > G(1)
	CR	1.13	1	0.14	0	BW(87) > TBL(9) > S(5)

Table 5. Summary of five regression tree models for evaluate the importance of individual and environmental sources of oxidative stress variation. We explore the relationships between population (P), season (S), body weight (BW), tuberculosis status (TB), gender (G) and age (A) and biomarkers of oxidative stress: thiobarbituric acid reactive species (TBARS), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) and glutathione reductase (GR) serum enzymes of wild boars naturally infected with *M. bovis*. Animals come from two populations: the National Game Reserve of Ports de Tortosa i Beseit (NGRPTB), and from Ciudad Real (CR). In CR, severe TB is more frequent than in NGRPTB, hence we performed a post hoc tree model using only boars from this population (shadow lines). The R^2 is the proportion of observed variability explained by a given tree model. Cross-

validation error: medium error of 10 cross validations; Relative error: is the part of the variance not explained by the tree model; Complexity parameter: minimum complexity benefit that must be gained at each step in order to make a split worthwhile. The default is 0.001; Variable importance (VI): sum of the goodness of fit measures for each split for which it was the primary variable, plus goodness * (adjusted agreement) for all splits in which it was a surrogate variable.

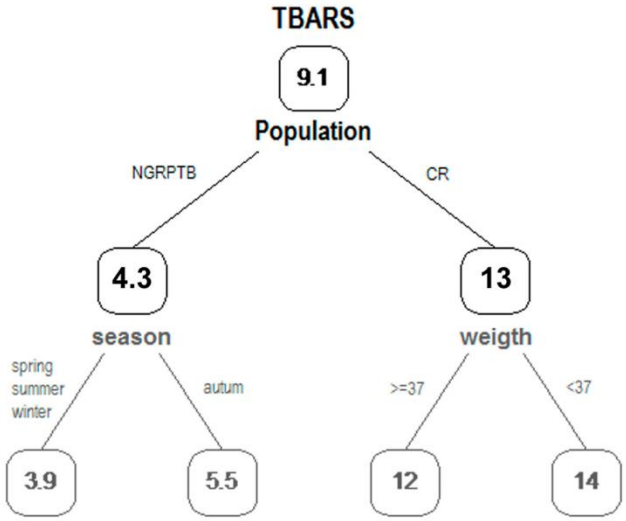

Figure 3.Regression tree model of lipid peroxidation (TBARS). The regression tree model used the parameter anova. The chosen partition was that which minimizes the sum of squared residuals. In this case the complexity parameter (CP) was 0.0001 (when de gain of the coefficient of determination was less than CP no additional partitions will be formed). The pruned tree was included (with only one node and with the lower cross-validation error) in black and the complete tree with the best response variable in gray. The values inside the boxes were TBARS serum values of wild boars (nmol MDA/ml). The explicative variable "population" divides the TBARS serum values with a mean of 12.82 nmol MDA/ml for CR animals and 4.29 for NGRPTB wild boars.

On the other hand, CR free-ranging wild boar had lower AE and higher TBARS levels compared to the NGRPTB boar. This would imply a higher oxidative imbalance and damage because the mobilisation of AE was lower than their utilization and maybe lower than their need (Beaulieu and Costantini 2014). However, TB status did not appear to be an important factor in explaining the observed variability in OS biomarkers; the fact that TB prevalence was lower (16 positive out of 61 wild boar) and mostly with localised lesions (12), could be an explanation. In

fact, no wild boar from NGRPTB and four from CR had severe TB lesions. The main variable to explain TBARS, GPX, CAT and GR variability was population (CR or NGRPTB) and not TB status. For SOD, however, the main explanatory variable was the season. Intrinsic (genetic (Ballerini et al. 2003), life-history traits (Alonso-Alvarez et al. 2008) or different TB granuloma phases (Sasindran and Torrelles 2011)) as well as extrinsic factors (such as other pathogens (Deblanc et al. 2013), population density, environmental contaminants (Rodríguez-Estival et al. 2012), season (Koziorowka-Gilun et al. 2011) and diet (Gabás-Rivera et al. 2014), among others) may hide TB effects (Beaulieu and Costantini 2014). Since all of these factors are related, it is difficult to recognize individual effects on OS. The fact that TB status did not appear to be a significant factor in the *ad hoc* model of CR animals, where extrinsic factors were reduced, suggests that intrinsic factors may also play an important role in OS levels.

Conclusions

Although TB causes the oxidant/antioxidant imbalance in experimental conditions, oxidative stress goes unnoticed in free-ranging *M. bovis*-infected animals. Accordingly, other environmental factors causing OS, such as parasites and/or food availability, should have a greater impact on OS than TB itself. Despite the growing interest in biomarkers of oxidative status for assessing the health status of individuals in conservation programmes, the regular use of these biomarkers to assess the impact of wildlife diseases is in its infancy. Biomarkers of oxidative status can help to quantify fitness costs of diseased animals but much work remains to be done to understand the natural sources of variation affecting these promising physiological indicators.

Chapter 5

Physiological cost of helminth co-infections in a wild host reservoir of tuberculosis

Abstract

Co-infection is now recognized as the norm rather than the exception in any animal species. The three major infectious diseases of humans, e.g., tuberculosis (TB), HIV and malaria, are usually diagnosed in co-infection with other pathogens. Among them, TB is probably the best disease for exploring the impact of multiple infections on host's health because it is widespread in wild and domestic animals, has a chronic nature and co-exist with other, ubiquitous concomitant pathogens like helminths. Wild boar (*Sus scrofa*), the main wild reservoir of bovine TB in Europe, is an excellent model to study the co-infection between TB and helminths. In this work, a full parasitological inspection (i.e., gastrointestinal and lung nematodes) was performed in 57 wild boar naturally infected with *Mycobacterium bovis.* Partial least square path models were used to understand direct and indirect relationships between helminth-mycobacteria co-infections and selected health biomarkers of boars namely (body condition (BC), acute phase proteins (APP), and oxidative stress (OS)). The age and the innate immunity of animals were also considered inthe analysis. Path models showed that helminths had direct and indirect, through TB effects, impact health status of boars deteriorating BC, and increasing inflammation and OS of boars. Worms were correlated positively with TB and between them depending on their biologic life cycle, sustaining positive interactions between them. It was concluded that co-infection with helminths increases the physiological cost of TB in terms of BC, APP and OS, in wild boars and thus may be involved in TB transmission and maintenance in this wild reservoir.

Key words: Body condition, Immune response, *Mycobacterium bovis*, Oxidative stress, Wild boar

Introduction

Despite the fast growing interest in studying the impact of infectious diseases from a community ecology perspective (Pedersen and Fenton 2007), research on the impact of multiple infections on host's health is still in its infancy. The broad spectrum of direct and indirect interactions between the host and the pathogen communities hamper co-infection research (Johnson et al. 2015), and hence new approaches to understand these complex systems are needed (Lello and Hussell 2008).

Co-infection is the norm rather than the exception in all living beings. In humans, for example, prevalence of co-infection exceeds one sixth of the global population, being associated with health impairment, reduced treatment efficacy and high treatment costs (Pullan and Brooker, 2008; Griffiths et al., 2011). The three major infectious diseases of humans (human immunodeficiency virus infection, HIV, malaria and tuberculosis, TB) are usually diagnosed in co-infection with other pathogens (Salgame et al. 2013). Among them, TB is probably the best model for exploring the impact of multiple infections on host's health because its chronic, and wide-spread distribution (affects one third of the human population (WHO 2012)) and frequent concomitance with helminths (Hotez et al., 2008 .

Helminths are ubiquitous parasites of vertebrates (Bordes and Morand 2011) and entail a well known immune modulation in the host (Maizels et al. 2004), especially when it is co-infected with microparasites as protozoa, bacteria or viruses (Cox 2001).

Chronic helminth infections induce proliferation of T-helper type 2 cells (Th2), mast cells, eosinophils and B-cell activation (increasing immunoglobulins), which create an anti-inflammatory environment that down regulates Th1 immune response necessary to deal with viral, protozoal or bacterial infections (van Riet et al. 2007). However, controversial data exits regarding the effect of helminth infection on the impact of TB lesions. As some authors did not detect any change in susceptibility to TB infection (Frantz et al. (2007), while others showed that helminths can aggravate TB (Li and Zhou, 2013). It was suggested that the outcome of helminth-TB co-infection is influenced by worm species (Elias et al. 2006), nematode burden and timing of TB infection (Garza-Cuartero et al. 2014).Thus, because of the huge number of helminth species in wild reservoirs, the outcome from helminth-TB co-infections is poorly understood.

To understand how immune system works in an ecological context is a big issue (Boughton et al 2011). The innate immunity is the responsible of the first-line response, which is

continued by the adaptive immune responses in case the organism survives the most acute phase of the infection (Kapetanovic and Cavaillon 2007). Both responses are necessary for pathogen clearance but can result in physiological damage for the host, in particular because the production of acute phase proteins (APP) (Mortensen 2001) and oxidative stress (OS) mediators (Sorci and Faivre 2009). Hence, regulatory cells, typically secreted during chronic parasitoses, are important to avoid an aberrant immune response that would provoke more severe tissue damage (Blok et al. 2015). Changes in these two biomarker families can be interpreted as the establishment of immune (innate and adaptive) responses and inflammatory reaction, and for this they can be used as indicators of physiological costs of the health (El-Deeb et al. 2014). Recently, an OS imbalance in wild boar experimentally infected with *M. bovis* has been demonstrated, although some intrinsic and extrinsic factors were implicated in the dynamics of the OS (Gassó et al. 2016). The present study deals with the assessment of OS in co-infection. The determination of haemaglutination (HA) and haemolysis (HL) has been recently proposed to be used as surrogates for innate humoral immunity in different ungulates species (Gilot-Fromont et al., 2012; Rossi et al., 2013). Contrary to APP and OS, HA and HL are not involved in inflammatory reaction.

Body condition (BC) was suggested to be another marker for costs of co-infection quantification (Serrano and Millán 2014). It is expected that more parasitized animals would have poorer BC and hosts in poorer BC would be predisposed to infections, which would further reduce BC and so on (Beldomenico et al. 2008). For example, in a concomitant infection with several helminth species, wild rabbits harbouring more than three helminth species had a worse BC than animals infected by one or two (Lello et al. 2005). However, host resistance and tolerance could change this *a priori* easy relationship (Råberg et al. 2009), in which case a high pathogen load would not necessarily be related to poor BC.

Wildlife is a relevant model to study the impact of co-infections because wild-living animals are almost always infected by multiple pathogens (Bordes and Morand 2011) and share some major co-infections with humans (e.g., *M. bovis*-helminths co-infection). Co-infections between *M. bovis* and other pathogens occur in a vast range of environmental conditions, thus have high potential to express potential interactions between resource limitations and co-infection outcomes. Wild boar (*Sus scrofa*) is the main wild reservoir of TB due to *Mycobacterium tuberculosis complex* in the Mediterranean basin (Naranjo et al. 2008), and also is a known reservoir of viruses, bacteria and parasites that are transmissible to domestic animals and humans (Ruiz-Fons et al., 2008, Meng et al., 2009). In the last few decades, wild boar

populations have increased in both number and range throughout Europe (Massei et al. 2015), increasing the possibility of contacts with domestic pigs and humans. This wild pig is often maintained in overcrowded conditions for commercial purposes, increasing the chance of disease transmission (Gortázar et al., 2006).

Some preliminary works have underlined the impact of co-infection on *M. bovis* infection in wild boar. Risco et al. (2013) observed higher TB prevalence in wild boar populations with porcine circovirus type 2 (PCV2) infection. Along the same lines, wild boar in contact with PCV2, Aujeszky's disease virus (ADV) and *Metastrongylus* spp, were more prone to suffer from severe TB (Risco et al. 2014b).

Disease maintenance in wild reservoirs is driven by intensity and frequency of pathogen shedding, the main responsible being high excretory or "super-spreaders" (Stein 2011). "Super-spreaders" are typically suffering from the acute form of the disease characterised for a generalized TB lesions and high bacteria loads (Martín-Hernando et al. 2007). Co-infection have also resulted in high bacterial loads in other helminth-bacteria models (Lass et al. 2013). Therefore, it will be expected that helminth-*M. bovis* co-infected wild boar would present poorer BC, higher OS and inflammation levels than non-co-infected individuals and may be super-shedders of helminths and *Mycobacteria* spp.

The aim of the present work was to assess the effects of multiparasitism on the health status of wild boars. For this, direct and indirect impacts of helminth co-infections were studied on selected physiological indicators in 57 wild boar with different TB status issued from a population were ADV and PCV2 circulated (Risco et al. 2014b). Gastrointestinal and lung nematode burden of all individuals was inspected in detail, and performance of a wide panel of health biomarkers was used to assess BC, OS and innate immunity. It was hypothesized that co-infection would increase the physiological cost of TB on health status. Taking into account that TB-affected wild boar have an increased OS, co-infected animals would have highest oxidative imbalance. Moreover, it was expected to find an increase of inflammation indicators and a decrease of fat reserves.

Materials and Methods

Study area

Sixty-three wild boar (13 males and 50 females, aged from five to 60 months) were hunter-harvested in a private hunting state of 2000 ha placed in Toledo (central Spain; 39°55' 15.55"N, 5 °10' 32.33" E). The vegetation in this area is typical of Mediterranean forest dominated by scattered holm oaks (*Quercus ilex*) and Mediterranean shrubs led by *Cistus ladanifer*, *Erica* spp or *Genista anglica*. Wild boar density was about 40 individuals per 100 ha. The game estate is fenced and supplementary food (special wild boar fodder) was provided in 5 feeders. Supplementary food is provided from June to September coinciding with the dry season and the decrease of natural food resources. During these four months the wild boar population receive supplemental food in specific feeders. On average, the boar population consume 42 tonnes (2100 Kg/month and feeder) of Jabalí Familia fodder (Mercoguadiana SL. Spain). Animals were legally hunted in their own habitat by authorised hunters within the framework of an annual hunting plan approved by the regional authority in charge of livestock and wildlife management. No approvals were needed from any Ethics committee since the wild boars were not sacrificed for research purposes.

Sampling procedure and processing

After the animal collapse, gender of boar was assigned visually by inspecting the genitalia, whereas age was assessed by simple dental biometry (Gonçalves et al. 2015). Body weight (with viscera) of animals was determined with a dynamometer (precision of 0.1 kg), and tarsus length and chest fat width were measured. Later, a necropsy examination of animals was performed to assess the presence of TB-like gross lesions affecting lymph nodes (submandibular, retropharyngeal, mediastinal and mesenteric lymph nodes), and thoracic or abdominal organs (Martín-Hernando et al. 2007). Respiratory (trachea and lungs) and gastrointestinal (stomach, small and large intestine) tracts were removed, placed in individual plastic bags and frozen at -20ºC for their later examination. Submandibular and/or retropharyngeal lymph nodes were dissected and stored in sterile containers for further microbiological analyses. Peripheral blood was collected from the cavernous sinus of the wild boar using an 18-gauge needle (Arenas-Montes et al. 2013) and placed into serum separator tubes. All samples were stored and maintained in cold boxes at 4°C until they were processed within the following 24 hours at the

laboratory. Later, serum was obtained after centrifugation at 1.200 G for 5 min and conserved at -80ºC or -20ºC until further analysis. All haemolytic sera were discarded.

Mycobacterium bovis, PCV2 and ADV infection assessment

Diagnosis of TB was based on the isolation of *Mycobacterium tuberculosis* complex (MTC) bacteria as well as on the presence of microscopic granulomatous TB lesions. To detect the presence of *MTC*, microbiological cultures from submandibular or retropharyngeal lymph nodes and from a piece of caudal lung lobes (both with TB-like lesions when possible) of each animal were carried out. For wild boar in which TB-like lesions were not found, a piece of submandibular lymph node and caudal lung lobe were identically processed for histopathology (Risco et al. 2014b).

Concerning viruses, a serologic survey was made to assess antibodies against PCV2 and ADV using commercial ELISA kits for swine and also following the manufacturer's recommendations (Risco et al. 2014b).

Parasite assessment

Trachea and one of the lungs (left or right) were dissected and washed to obtain adult pulmonary worms; the other lung was used for TB assessment. Adult helminths were collected in a 500 µm sieve. First, lungs were filled with pressure water, then parenchyma was massaged, and the process was repeated 3 times. Second, trachea and main bronchi were opened with scissors and carefully examined, in particular affected areas. Finally, lungs were washed with running water and all collected pulmonary nematodes placed in ethanol 70% solution for conservation. Later on, the number of nematodes was counted and clarified by lactophenol for their specific identification (Morita et al., 2007, Gassó et al., 2014). A post hoc Wilcoxon test analysis confirmed the lack of statistical differences between right and left lungs in 20 wild boar ($W = 158.5$ p-value = 0.27), hence parasite load estimated in one lung was multiplied by two.

For gastrointestinal nematodes, stomach, small and large intestines of boars were dissected, longitudinally opened and washed with running water, collecting them with insoluble food debris in different diameter sieves (5 mm to 500 µm). Finally, helminths were separated from food debris and conserved in ethanol 70% for their posterior classification. Adult worms were

cleared in lactophenol and finally identified using a 20x-60x stereoscope (Nikon eclipse 50i) and morphological keys (García-González et al., 2013, Frontera Carrión et al., 2009, Taylor et al., 2007).

Six animals were discarded of the analysis because the shot destroyed the lung or a part of the digestive tract, preventing the correct parasite assessment, then 57 animals were included in the final model, 46 females and 11 males.

Biomarkers of health and immunity status

Oxidant-antioxidant assessment

Serum paraoxonase/arylesterase-1 (PON-1) activity was determined using p-nitrophenyl acetate as substrate (Tvarijonaviciute et al. 2012). Serum glutathione peroxidase (Gpox) was measured according to a previously describe method (Paglia and Valentine 1967) and serum total oxidative status (TOS) was measured as previously described (Erel 2005), with some modifications (Franco et al. 2016). All the three analyses were measured in serum with an automated biochemistry analyzer (Olympus AU600; Olympus Europe GmbH, Hamburg, Germany). To calculate OS imbalance, TOS was divided by antioxidant enzymes (Gpox and Pon-1) (Abuelo et al. 2013).

Acute phase proteins

Serum concentration of haptoglobin (Hp) was quantified using a commercial automated spectrophotometric assay (Tridelta Development Ltd, Maynooth, Ireland). Serum amyloid-A (SAA) concentration was determined by using commercial solid phase sandwich Enzyme linked immunoabsorbent assay (Phase SAA assay, Tridelta Development Limited, Maynooth, Ireland). Analyses were performed according to the manufacturer's instructions, and the final absorbance of both techniques was measured in a microtiter plate reader (PowerWave XS, Bio.Tek Instruments Inc., Vermont, USA) at 630 nm.

Innate Immunity: haemoagglutination-haemolysis assay (HA-HL)

Serum samples (stored at -20ºC) were used for measuring the concentration of natural antibody (Nab) and complement (Cp) activity using a HA-HL assay (Rossi et al. 2013). HA level was interpreted as an indicator of Nab concentration and HL was interpreted as an indicator of Cp activity.

Statistical analyses

The relationships between the parasitism and physiological costs of wild boar were analyzed by means of Partial Least Squares Regression Path Model (PLSR-PM). This statistical technique was used to assess cause-effect relationships from observational data (Grace et al. 2010). The PLSR is advantageous when sample size is low and the number of variables is high with some degree of collinearity (Carrascal et al. 2009). The measured variables were considered either as explanatory or as response blocks and then either as latent (containing one or more related variables) or manifest variables (the measurable variables). For example regarding the response variables, three blocks were created, one for each of our physiological indicators: BC, OS and APP. BC was obtained by ordinary least square residuals (OLS) from the linear regression between age (in months) and chest fat (a proxy for body reserves in wild suids (Stribling et al. 1984). OS status was obtained dividing TOS by Gpox and Pon1. Finally, although APP status was initially defined by both SAA and Hp, only Hp was finally retained as proxy for chronic inflammation. The lack of relationships between SAA and the rest of variables justified our decision. The OS status and APP models only included no haemolytic sera (n=38).

On the other hand, the set of predictor variables was constituted by four blocks of latent variables: innate immunity (measured by HA and HL), TB status (free or infected with *MTC*), age of individuals in months, and gastrointestinal and lung different helminths species (in total nine species, five gastrointestinal and four pulmonary) and quantities. Specific PLSR-PMs were built for BC, OS and APP and were boostrapped to better adjust the models and improve the precision of estimators.

Conceptual model specification

The conceptual model, including, direct and indirect hypothetical relationships among explanatory and response variables, summarised in Fig. 2. To draw Fig 2, all physiological indicators of health have been shown in a single block. For the final analysis, however, the effects of explanatory blocks on BC, APP or OS were explored independently.

The conceptual model behind the relation among latent and manifest variables was drawn as a path diagram (Fig 2) in which ellipses represent latent variables and rectangles refer to manifest variables. Arrows show causations among blocks (latent variables), the direction of the array defines the direction of the relationship and, in the final model, arrows could be blue (positive relationship) or red (negative relationship). The first conceptual model suggests that health status of boars could be directly affected by TB and helminth load. TB status, on the other hand, would be influenced by age, innate immunity and helminth burden of boars. Worms were separated by their biologic cycle (direct and indirect) after exploring the parasites block with a principal component analysis (Fig 3A) and saw oppositely correlations with age (Fig 3B), depending of their life cycle (F-static = 8.07, p-value < 0.000). Then parasites with indirect biologic cycles (PIBC) affect directly parasites with direct biologic cycle (PDBC). So, these two blocks may also be influenced by innate immunity and age. ADV and PCV2 were excluded from the model because, due their high seroprevalence (100% and 90% respectively), these variables could not serve to explain individual-level changes or differences (Fig 2). Finally, three structural equation models were constructed (A, B and C, Figure 4) for each final latent variable representing physiological status (BC, OS status and APP) and analysed to facilitate and simplify direct and indirect effects of the model. Parasites were divided in two blocks (PDBC versus PIBC). Initial structural models with the entire manifest variables were not shown; the final model without the variables that caused background in the model (manifest variables with weights under 0.2 or more related with other latent variables than its own.) is represented.

Analyses were performed with the “plspm” package 0.4.7 version (Sanchez 2013) of the R software, version 3.3.1 (R Development Core Team, June 2016).

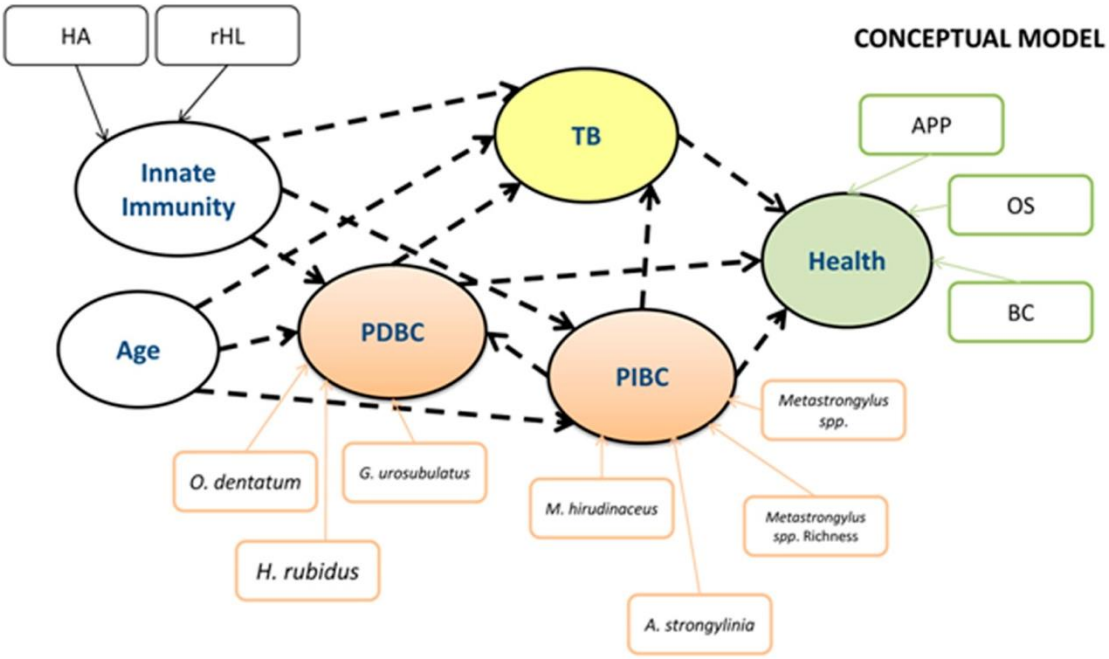

Figure 2 Two proposed path models showing the causal relations among variables. In 2A the variables are: TB (Tuberculosis), BC (body condition), OS (oxidative stress balance), APP (acute phase proteins), HA (haemoglutination), rHL (haemolysis residues) and Parasites (all the species in the same latent variable block). In 2B all variables are the same than in 2A less Parasites, divided in two latent variables blocks: PDBC (parasites with direct biologic life cycle) and PIBC (parasites with indirect biologic life cycle).

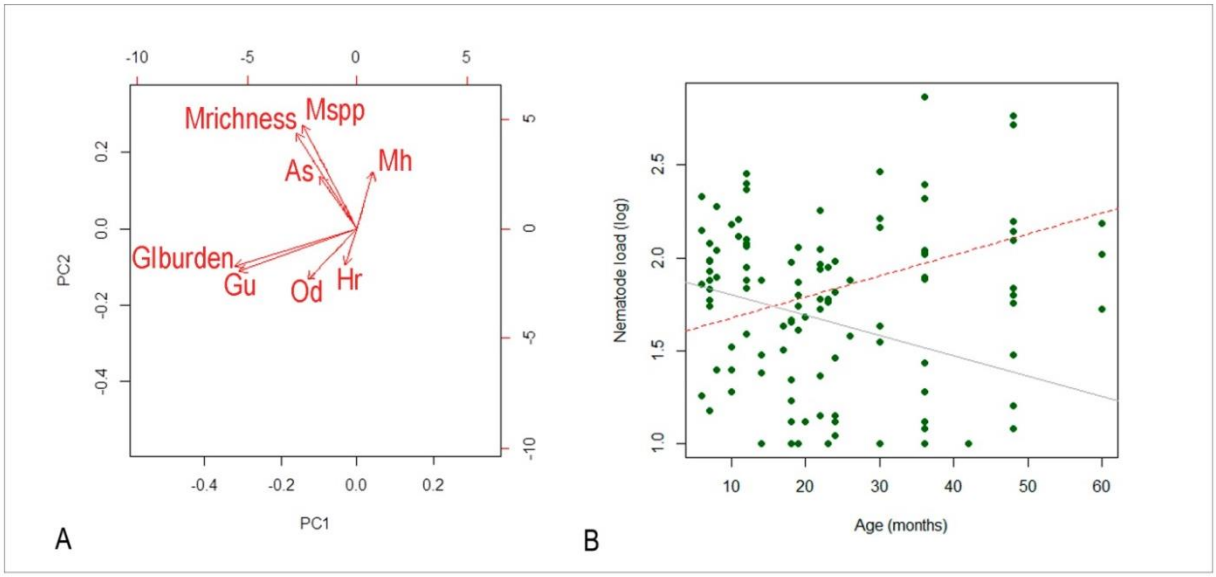

Figure 3. Analysis of the parasite block. 3A: principal component analysis graph of all worms: Mh (*Macracanthorhynchus hirudinaceus*), Mspp (*Metastrongylus spp*), Mrichness (*Metastrongylus richness*), As (*Ascarops strongylina*), GIburden (gastrointestinal worm burden), Gu (*Globocephalus urosubulatus*), Od (*Oesophagostotum dentatum*) and Hr (*Hyostrongylus rubidus*). 3B: correlation between age and worm loads depending on their biologic cycle: direct (red dotted line) and indirect (grey line).

Results

Simple descriptive statistics for both categorical and numeric variables included in the PLSR-PM are shown in Table 1. Correlations between latent variables and manifest variables and path coefficients results of the three PLSR-PM are shown in Tables 2 A, B, C and 3.

In general terms, parasite burden were highly correlated with the age of wild boars. This relationship was positive for PDBC (without intermediate host) but negative with the ones with indirect cycles. The probability to be infected with TB was positively correlated with worm loads. Parasite (both with direct and indirect biologic cycle) infection had two fold negative effects on BC, one direct and another indirect through TB (Table A). Besides, worm infection had a direct positive effect on OS and APP; the higher the parasite burden, the higher values of OS and APP. TB status, however, had no significant direct effects on physiological indicators of health. Regarding innate immunity, no significant relationships with the choosen variables were observed on *M. bovis*-helminth co-infection.

Body condition

Twenty one per cent of the observed BC variability of boar (Fig 4A) was explained by the direct and indirect effects of PDBC and indirect effects of PIBC, age and TB (Table 3A). PIBC had two fold negative effects on BC (β = -0.29, p-value = 0.02) direct and indirect through TB. Inetestingly, TB did not affect BC directly. Indirect negative effects of PIBC on BC enhanced total negative effects of parasites, being around β = -0.3 for both (PIBC and PDBC). Age also had indirect negative effects on BC trough PDBC. For the relationships between explanatory blocks, 37% of the observed PDBC variability was explained by age and PIBC. As expected, age was directly and positively correlated (β = 0.54, p-value = 0.00) with PDBC, but tended to be negatively correlated with their PIBC counterparts (not statistically significant). Also PIBC were directly and positively correlated with PDBC (β = 0.24, p-value = 0.02). Finally, PIBC were directly

and positively correlated with TB (β = 0.22, p-value = 0.04), explaining 23% of the observed TB variability. The goodness of fit (GOF) for the final BC model was 0.27.

Oxidative stress

Twenty six per cent of the observed OS variability of wild boar was explained by direct effects of TB status and PIBC, and indirect effects of age, parasites and innate immunity (Table 3B and Fig 4B). While PIBC were directly and positively correlated with OS (β = 0.28, p-value = 0.15), TB had a direct negative effect on OS imbalance (β = 0.18, p-value = 0.18). Besides, age had an indirect negative effect on OS through parasites. For the relations between explanatory blocks, 58% of the observed variability of PDBC and 25% of PIBC were mainly explained by age. As expected, similar to the BC model, age was directly and positively correlated (β = 0.71, p-value = 0.00) with PDBC, but negatively with their PIBC counterparts (β = 0.34, p-value = 0.01). Here, macroparasites were directly and positively (but not significantly) related with TB and the total variability of TB explained was the lowest (17%), compared with BC and APP models. The GOF for the final OS model was 0.34.

Acute phase proteins

Eighteen per cent of the observed APP variability of wild boar was explained mainly by direct effects of PDBC and indirect of age (Table 3C and Fig. 4C). PDBC were directly and positively correlated with APP (β = 0.24, p-value = 0.07). PIBC had also directly and positively effect, but it was not significant. On the contrary, TB status had a direct and negative effect, but also not statistically significant. Indirect effects of innate immunity, PIBC and PDBC were very low. For the relationship between explanatory blocks, 40% of the observed variability of PDBC and 18% of PIBC were mainly explained by age. As expected, similar to BC and OS models, age was directly and positively correlated (β = 0.50, p-value = 0.00) with PDBC, but negatively with their PIBC counterparts (β = 0.29, p-value = 0.03). Finally, the 28% of the TB status observed variability was explained mainly by PIBC and indirectly by age. Macroparasites had direct and positive effects on TB status, but only PDBC were statistically significant (β = 0.35, p-value = 0.03). Indirect effects in this case were minimal and non-significant. The goodness of fit (GOF) for the final APP model was 0.34.

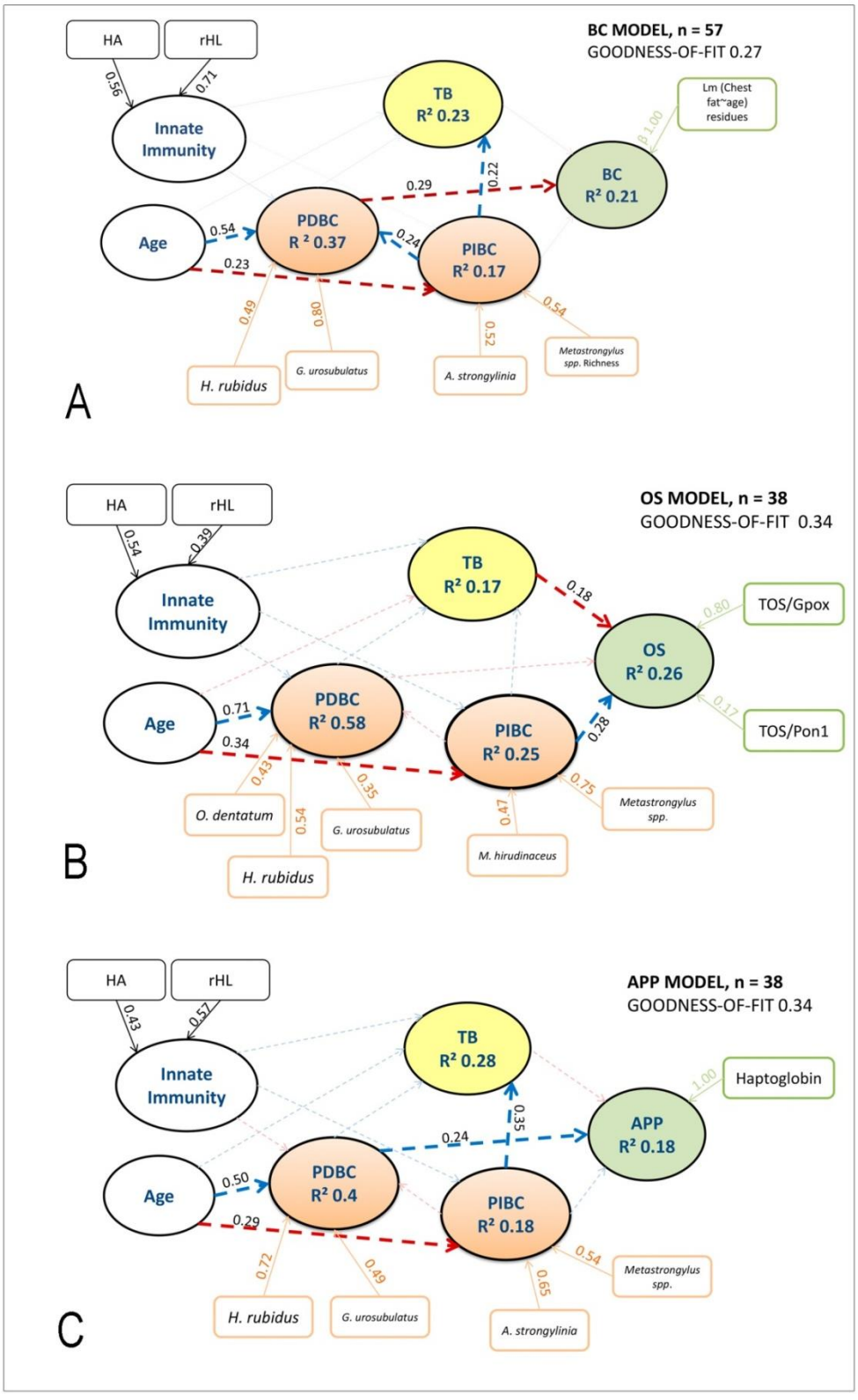

Figure 4.Path model with estimated weights of manifest variables and path coefficients of latent variables. Significant coefficients in black, not significant in grey (p value < 0.1). TB (Tuberculosis), BC (body condition), OS (oxidative stress balance), APP (acute phase proteins), HA (haemoglutination), rHL (haemolysis residues), PDBC (parasites with direct biologic life cycle) and PIBC (parasites with indirect biologic life cycle)

Latent variable		**Manifest Variable**	**bTB infectious status**	
Name	Label		Free	Infected
Individual characteristics	Age	Age in months	23.22 ± 2.5 (6 - 60)	25.52 ± 3.05 (6 - 48)
	Innate Immune response	HA (Haemagglutation) is a continuous variable ranging from 0 to 7 titers	4.25 ± 0.31 (1 - 6.5)	4.17 ± 0.3 (2.5 - 6.5)
		rHL (residuals of the correlations between Haemolysis and HA) is a continuous variable	-0.02 ± 0.05 (-0.51 - 0.35)	0.02 ± 0.05 (-0.34 - 0.36)
	Body condition	OLS residuals (Chest Fat Age)	0.09 ± 0.08 (-0.63 - 0.83)	-0.12 ± 0.08 (-0.72 - 0.38)
Health status	Antioxidant status	Serum TOS / Pon1 ratio (µmol/l / IU/ml)	2.44 ± 0.54 (0.33 - 9.02)	1.8 ± 0.43 (0.25 - 6.44)
		Serum TOS / Serum Gpox (µmol/l / H^2O*dilution factor)	0.086 ± 0.030 (0.0057 - 0.67)	0.047 ± 0.008 (0.0058 - 0.106)
	Acute phase proteins	Haptoglobin (g/L)	1.03 ± 0.12 (0.017 - 2.19)	1.01 ± 0.10 (0.37 - 1.63)
Tuberculosis	TB	0 = TB free 1 = TB affected	36 wild boars	21 wild boars
Helminths	PIBC	*Metastrongylus spp* (worms/WB)	43.97 ± 8.53 (0 - 216)	49.3 ± 14.14 (0 - 236)
		Metastrongylus spp. richness (species/WB)	1.83 ± 0.23 (0 - 4)	1.81 ± 0.33 (0 - 4)
	PDBC	*M. hirudinaceus* (worms/WB)	1.83 ± 0.44 (0 - 10)	1 ± 0.37 (0 - 7)
		A. strongylina (worms/WB)	2 ± 1.6 (0 - 56)	18.43 ± 5.31 (0 - 105)
		H. rubidus (worms/WB)	3.64 ± 1.94 (0 - 39)	3.19 ± 1.74 (0 -19)
		G. urosubulatus (worms/WB)	70.42 ± 11.36 (0 - 276)	153.52 ± 43.25 (8 - 726)
		O. dentatum (worms/WB)	1.53 ± 0.95 (0 - 31)	1.52 ± 1.05 (0 - 21)

Table 1Description of both latent and manifest variables used for fitting the causal model for body condition, oxidative and inflammatory status in wild boar. Descriptive statistics of manifest variables (mean ± standard error (minim – maxim)) are attached. Immune response, oxidative status and acute phase protein calculations were made without haemolytic data (free TB = 22 and TB-affected = 16). PIBC means parasites whose biologic cycle is indirect and PDBC means parasites whose biologic cycle is direct.

2A

	BC PM correlation with LVs					
Latent and measurable variables	Innate Immunity	Age	PIBC	PDBC	TB	BC
Innate Immunity						
HA	0.22	0.05	-0.06	0.01	-0.04	-0.05
rHL	0.94	0.29	-0.27	0.15	-0.07	-0.1
Age						
Months	0.3	1	-0.22	0.5	0.08	-0.05
PIBC						
Metastrongylus spp.	0.01	-0.38	0.48	0.01	0.05	-0.15
Metas. Richness	-0.19	-0.33	0.69	0.09	-0.01	-0.24
A. strongylina	-0.19	-0.2	0.67	0.03	0.38	-0.03
M. hirudinaceus	0.01	-0.19	-0.23	-0.13	-0.17	0.16
PDBC						
H. rubidus	0.09	0.45	-0.01	0.49	-0.04	-0.2
G. urosubulatus	0.13	0.33	0.18	0.89	0.3	-0.3
TB						
M. bovis	-0.08	0.08	0.32	0.24	1	-0.19
BC						
Chest fat corrected by age	-0.11	-0.05	0.32	0.24	1	-0.19

2B

		OS PM correlation with LV				
Latent and measurable variables	Innate Immunity	Age	PIBC	PDBC	TB	OS
Innate Immunity						
HA	-0.31	0.1	-0.19	0.04	-0.03	-0.26
rHL	0.97	0.19	0.26	0.21	0.09	0.12
Age						
Months	0.16	1	-0.38	0.75	0.01	-0.09
PIBC						
Metastrongylus spp.	0.24	-0.33	0.85	-0.15	0.16	0.25
Metas. Richness	0.09	-0.17	0.33	0.06	0.08	0.16
M. hirudinaceus	0.09	-0.2	0.32	-0.16	-0.12	0.1
PDBC						
H. rubidus	0.26	0.54	-0.16	0.74	-0.03	-0.18
G. urosubulatus	0.04	0.33	-0.19	0.56	0.29	0.01
O. dentatum	0.03	0.6	-0.25	0.69	-0.04	-0.19
TB						
M. bovis	0.09	0.01	0.12	0.07	1	-0.2
OS						
TOS/Pon1	0.37	-0.05	0.27	-0.14	-0.14	0.85
TOS/Gpox	-0.16	-0.1	0.12	-0.18	-0.18	0.74

2C

	APP PM correlation with LV					
Latent and measurable variables	Innate Immunity	Age	PIBC	PDBC	TB	APP
Innate Immunity						
rHL	1	0.19	0.14	0.25	0.09	0.38
Age						
Months	0.119	1	-0.32	0.61	0.01	0.01
PIBC						
Metas. Burden	0.2	-0.33	0.78	-0.12	0.16	-0.05
Ascarops	0.02	-0.17	0.79	-0.02	0.39	0.17
PDBC						
H. rubidus	0.27	0.54	-0.14	0.9	-0.03	0.28
G. urosubulatus	0.04	0.33	0.07	0.52	0.29	0.11
TB						
M. bovis	0.09	0.01	0.35	0.1	1	-0.02
APP						
Haptoglobin	0.38	0.1	0.08	0.28	-0.02	1

Table 2. Correlation between manifest variables (MVs) and latent variables (LVs) of the three partial least squares path modelling of wild boar health. 2A: body condition, 2B: oxidative status and 2C: acute phase proteins results.

3 A

BC PM Path coefficients

Paths	Direct effects	Indirect effects	Total	Bootstrapped mean Total effects	Confidence interval 0.025 to 0.975
Age ->PDBC	0.535	-0.041	**0.495**	0.495	0.328 – 0.701
PDBC -> BC	-0.308	-0.010	**-0.318**	-0.306	-0.527 – -0.146
PIBC -> BC	-0.185	-0.110	**-0.295**	-0.263	-0.542 – 0.217
PIBC -> TB	0.302	0.047	**0.349**	0.252	-0.455 – 0.610
PIBC -> PDBC	0.287	0.000	**0.287**	0.235	-0.264 – 0.644
Age ->PIBC	-0.142	0.000	-0.142	**-0.234**	-0.463 – 0.129
PDBC -> TB	0.163	0.000	0.163	0.164	-0.201 – 0.528
Innate Immunity->PIBC	-0.246	0.000	**-0.246**	-0.125	-0.449 – 0.403
Age -> BC	0.000	-0.133	-0.133	-0.118	-0.278 – 0.019
Age -> TB	0.074	0.038	0.112	0.103	-0.160 – 0.347
Innate Immunity -> TB	-0.044	-0.073	-0.117	-0.090	-0.415 – 0.234
TB-> BC	-0.061	0.000	-0.061	-0.082	-0.290 – 0.119
Innate Immunity -> BC	0.000	0.051	0.051	0.033	-0.156 – 0.210
Innate Immunity ->PDBC	0.076	-0.070	0.005	-0.009	-0.257 – 0.237
Innate Immunity -> Age	0.000	0.000	0.000	0.000	0.000 – 0.000

3 B

OS PM Path coefficients

Paths	Direct effects	Indirect effects	Total	Bootstrap mean Total effects	Confidence interval 0.025 to 0.975
Age ->PDBC	0.722	0.018	**0.741**	0.721	0.457 – 0.896
Age ->PIBC	-0.437	0.000	**-0.437**	-0.336	-0.623 – 0.220
PIBC -> OS	0.247	-0.023	**0.224**	0.276	-0.388 – 0.668
Age -> OS	0.000	-0.189	**-0.189**	-0.204	-0.467 – 0.234
TB-> OS	-0.220	0.000	**-0.220**	-0.181	-0.421 – 0.150
PDBC -> OS	-0.110	-0.030	-0.139	-0.113	-0.399 – 0.419
PDBC -> TB	0.135	0.000	0.135	0.066	-0.846 – 0.569
PIBC -> TB	0.132	-0.006	0.126	0.042	-0.397 – 0.447
Age -> TB	-0.043	0.042	-0.001	0.039	-0.290 – 0.338
Innate Immunity->PIBC	0.360	0.000	0.360	-.0.026	-0.565 – 0.457
Innate Immunity->PDBC	0.085	-0.015	0.069	0.015	-0.233 – 0.277
Innate Immunity -> TB	0.038	0.057	0.095	0.004	-0.413 – 0.397
PIBC ->PDBC	-0.042	0.000	-0.042	-0.004	-0.270 – 0.439
Innate Immunity -> OS	0.000	0.060	0.060	-0.002	-0.298 – 0.252
Innate Immunity -> Age	0.000	0.000	0.000	0.000	0.000 – 0.000

3 C

APP PM Path coefficients

Paths	Direct effects	Indirect effects	Total	Bootstrap mean Total effects	Confidence interval 0.025 to 0.975
Age ->PDBC	0.614	-0.030	**0.584**	0.467	-0.520 – 0.719
PIBC -> TB	0.389	0.007	**0.397**	0.374	-0.391 – 0.666
Age ->PIBC	-0.355	0.000	**-0.355**	-0.293	-0.517 – 0.146
PDBC -> Hp	0.309	-0.009	**0.300**	0.221	-0.390 – 0.578
TB-> Hp	-0.103	0.000	-0.103	0.177	-0.413 – 0.239
PIBC -> Hp	0.145	-0.015	0.130	0.145	-0.188 – 0.487
PIBC ->PDBC	0.084	0.000	0.084	0.129	-0.137 – 0.480
Age -> Hp	0.000	0.129	0.129	0.128	-0.075 – 0.396
PDBC -> TB	0.089	0.000	0.089	0.128	-0.453 – 0.459
Innate Immunity -> Hp	0.000	0.064	0.064	0.039	-0.161 – 0.21
Innate Immunity->PIBC	-0.235	0.000	-0.235	0.030	-0.533 – 0.494
Innate Immunity->PDBC	-0.123	-0.019	-0.142	0.025	-0.281 – 0.285
Innate Immunity -> TB	0.000	0.093	0.093	0.020	-0.335 – 0.400
Age -> TB	0.083	-0.086	-0.003	-0.009	-0.336 – 0.354
Innate Immunity -> Age	0.000	0.000	0.000	0.000	0.000 – 0.000

Table 3.**Path coefficients, direct, indirect and total effects of each latent variable relation**. Bootstrapped means and 95% confidence interval were added. Results were ordered from highest to lowest bootstrapped total effects. Statistically significant total effects were marked in bold.

Discussion

Multiparasitism has negative effects on physiological costs of health status of the wild boar, as it was observed in present study using TB-helminth co-infection model. Worm burden showed a direct and indirect (through TB) deleterious effect on host health, and was related with reduced BC, presence of OS, and increased APP in boars. Furthermore, a positive relationship between worm burden and probability to become infected by *MTC* was observed.

In recent works there are some examples about how helminths can aggravate the impact of TB lesions in humans (Li and Zhou, 2013). Pulmonary TB and parasitic diseases were shown to be risk factors for each other. Co-infection may significantly inhibit the host immune system, increase intolerance to antibacterial therapy and be detrimental to the prognosis of the disease. The present results, however, are similar to those observed by Ezenwa and collegues (2010), in which worms enhanced TB in African buffalo. In the present TB-helminth co-infection model, wild boars with high worm loads of PIBC were infected by bovine TB, suggesting that helminths are a risk factor to be infected with *MTC* or vice versa. This supports the existence of immune-mediated interactions between worms and *Mycobacteria* and the possibility that endemic helminths could drive to TB emergence (Ezenwa et al. 2010).

Chronic diseases imply a constant cost for the host in terms of immunity and this is expected to worsen BC. In line with this several works in wild animals demonstrate negative correlations between TB and BC (Caron et al., 2003; López-Olvera et al., 2013; Munyeme et al., 2010). However, other authors did not find evidence of poor BC in TB positive wild boar (Tschopp and collegues (2010) and unpublished data from Díez-delgado and collegues cited in their study (2014)) as occurred in present study. Noteworthy, the two former works did not consider the aggregated effects of worms that could potentiate the decrease of BC.

An increase of OS is one of the main mechanisms limiting *Mycobacteria spp.* multiplication (Chan et al. 1995), contrary to what was expected in the studied model, since TB positive wild boars had less OS values. This could be explained by the increasing expression of antioxidant enzymes due to the bacteria to limit exposure to free radicals (Cumming et al. 2014). It is important to have on mind that, under wild conditions, OS variations could be masked by environmental differences (Gassó et al. under review).

The three shown models indicated that innate immunity was not consistent results, relationships with co-infecting pathogens were not significant and changed between models. This

lack of relationships between HA-HL, TB status and macroparasites might reflect the difficulty of assessing immune system responses under natural conditions (Boughton et al. 2011). Innate immunity was previously related with antimicrobial first line defence (Kapetanovic and Cavaillon 2007) and Naranjo and colleagues (2006) demonstrate that wild boars with gene expression which potentiate innate immunity controls better TB. Recently, innate immunity was shown to be correlated to the risk and outcome of classical swine fever infected wild boar (Rossi et al. 2013). The lack of relationship of these two biomarkers in the present study with co-infections probably indicates the necessity of more explorations on this issue. However, the increase of APP and OS, two groups of known biomarkers indicators of the triad disease-immunity-pathology (Knowles et al., 2013, El-Deeb et al., 2014), in relation of high worm burden demonstrates the relation between parasites and host immunity.

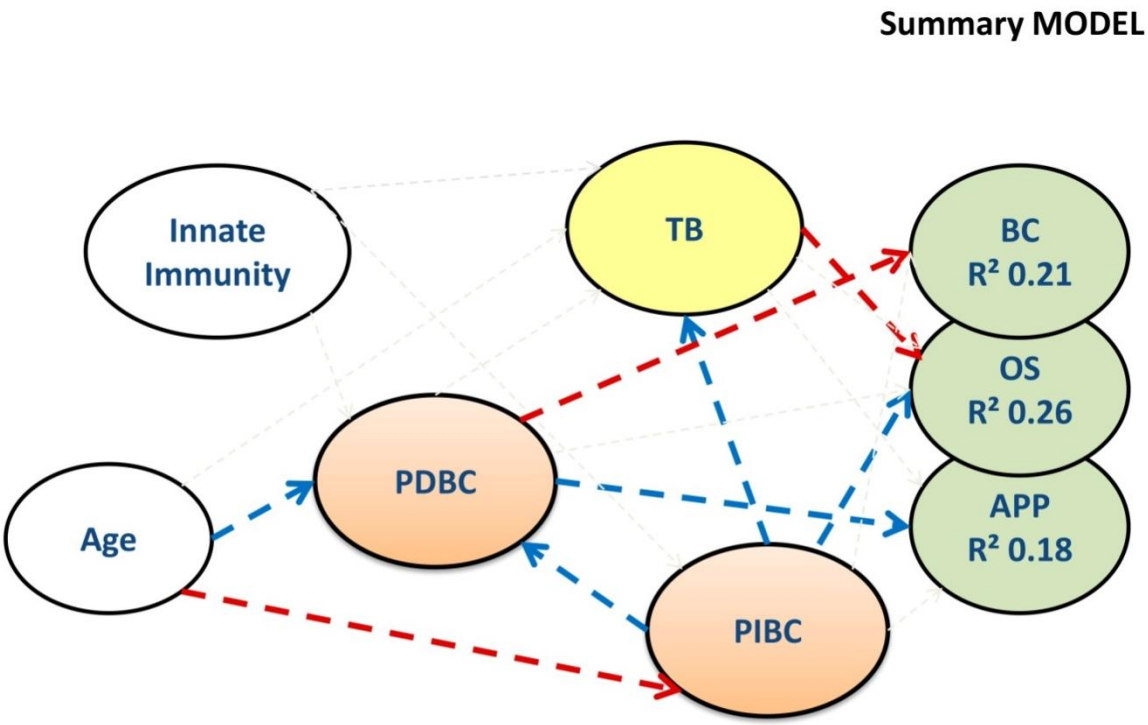

Figure 5. Path model summary. TB (Tuberculosis), BC (body condition), OS (oxidative stress balance), APP (acute phase proteins), HA (haemoglutination), rHL (haemolysis residues), PDBC (parasites with direct biologic life cycle) and PIBC (parasites with indirect biologic life cycle).

Although the rise of SSA has been described in domestic pigs infected by PRRSV, PCV2 and *Mycoplasma hyopneumoniae* under field conditions (Parra et al. 2006), the same variable was not included in the final model because previous exploratory statistics (data not shown) revealed no correlations between this variable and the rest of variables (helminth load or TB infection).

Concerning macroparasites, a clear relationship between the age of wild boar and the worm burden was found. PDBC were positively correlated and PIBC negatively correlated with age; in other words, the older the animal, the more PDBC and lesser PIBC worm load. This clear difference between these two groups of parasites could be explained by the two different types of host defence against parasites and pathogens: tolerance and resistance (Råberg et al. 2009). PDBC could be controlled by tolerance (limiting the harm caused by the pathogen) and PIBC could be controlled by resistance by acquired immunity (limiting parasite burden). Furthermore juvenile boar tends to eat more intermediate hosts (earthworms or beetles) than adults, increasing the risk of infection by PIBC.

The direct and positive relation observed between PIBC and PDBC indicates that the higher the worm burden of one group of parasites increased the worm load of the other group. Possible immune interactions caused by co-infections (Corrêa-Oliveira et al. 2002) of these groups of worms could explain these positive relations. Previous studies in mice demonstrated that immunological regulation of *Heligmosomoides polygyrus* depress mucosal inflammatory response to facilitate its own and other parasite survival (Behnke et al. 1993). On the contrary, in other combinations of parasites, immune cross-reactions reduced the concurrent infections (Yacob et al., 2002; Holmes, 2002). According to the helminth-helminth co-infection, wild boar with higher worm loads (via direct or indirect effects) had poorer BC. Similar effects were observed in wild rabbits harbouring more than three helminth species (Lello et al. 2005) and negative relationships between parasite abundance and BC were also observed in roe deer (Jégo et al. 2014).

TB and worms were probably not the unique pathogens in the studied wild boars, since it is very likely that other bacteria, protozoa, coccidian and viruses cohabit with them. Concerning the common viral pathogens in the area, PCV2 and ADV were very sero-prevalent and could not serve as good variables for the model. Although imperfectly understanding interactions, helminth-helminth and helminth-TB are considered good models to study co-infection. Furthermore

environmental factors were not controlled; in consequence, the present results and predictions would not serve to other zones with different ecological and environmental conditions.

Concluding remarks and perspectives

Direct and indirect interactions between co-infecting parasites within individual hosts have high effects on host health, fitness and disease outcome. In the present helminth-TB co-infection model in wild boar, worms worsened the overcome of disease by their positive relationship with the TB status and direct and indirect, negative or positive effects on physiological indicators of health status (increasing OS and inflammation and reducing BC). It is necessary to continue expanding the complex dynamics of multi-host-multi-parasite communities to make progress in controlling infectious diseases.

This study suggests that deworming could be a relevant tool to improve health status and maybe reduce the transmission rate of TB in a population of wild boars naturally infected by *MTC*. However, Ezenwa and Jolles (2015) observed contradictory effects of deworming African buffaloes, since the *M. bovis* survival increased.

Applied Chapter

Chapter 6

Effects of mass anthelmintic administration on TB dynamics and helminth community in a wildlife reservoir

Abstract

Helminths are ubiquitous parasites that impact human and animal health. Recent research suggests that most deleterious effects provoked by helminth infections are driven by their immunomodulatory effects. Such helminth-biased immune response results in a higher occurrence and persistence of bacterial, protozoa or virus infections. Though deworming is being considered as alternative method to deal with infectious diseases in co-infected hosts, few works have evaluated the pros and cons of this measure. This work was aimed at evaluating the effects of a two-year consecutive mass anthelmintic drug administration (oral ivermectin) on tuberculosis dynamics and parasite community structure in a wild boar (*Sus scrofa*) population naturally infected by *Mycobacterium bovis* in Central Spain. Ivermectin was provided in combination with a specific fodder and delivered in feeders during summer, when natural food is scarce. During the study period (2011-2015), 116 wild boar were hunter-harvested and inspected for TB infection status. Gastrointestinal and lung nematode burden was assessed in 67 wild boars of the above mentioned, harvested during the two consecutive years after the anthelmintic mass treatment. In the last five years TB prevalence remained stable after our anthelminthic treatment but TB severity decreased after deworming. Moreover, helminth community was structured, i.e., in a non-random assemblage, only in TB-free wild boar. We can conclude that punctual anthelmintic treatment may be beneficial to TB containment in wildlife reservoirs at host individual scale but not at population scale.

Key words: Co-infection, Community ecology, *Mycobacterium bovis*, *Sus scrofa,* Worms

Introduction

The negative effects of worms on human, livestock and wildlife health have been largely described in the scientific literature over the last half a century. The main treatment to deal with helminth infections in human and livestock have been mass drug administration. In fact, deworming is now being used to minimize the impact of the three major infectious diseases of humans (e.g., malaria, tuberculosis, TB, Human immunodeficiency virus, HIV (Hotez et al. 2006)).

In wildlife, however, deworming is rare and used only for scientific purposes. In fact, the impact of helminths on wildlife populations has hardly been demonstrated by observational studies and thus researchers have show a growing interest in using antiparasite drug treatments to infer the impact of parasites on the health and fitness of wildlife (Pedersen and Fenton 2015). Thought less experience has been gained in wildlife, there is an increasing interest in using anthelminthics to control emerging and zoonotic diseases (Ezenwa and Jolles 2015) in free ranging mammals.

Hosts can be considered as complex ecosystems (Pérez et al. 2006; Tompkins et al., 2011; Rynkiewicz et al., 2015), inhabited by symbionts and pathogens regulated by ecological rules (Graham 2008). As result, community ecology has recently provided a new perspective in the infectious diseases research and treatment. That new approach is based on understanding the role of co-infection in the success of treatments aimed at infectious diseases control (Johnson et al. 2015). The use of anthelmintic drugs to control infectious diseases caused by bacteria, virus or protozoa is among the most promising strategies (Pedersen and Fenton 2007). Immune response against helminths (Maizels et al. 2004) drives the ability of the host to clear microbial infections (Cox 2001). As result, it has been suggested that anthelmintics would improve the immune response of the host to deal with other infections such as malaria, tuberculosis and human immunodeficiency virus (Elias et al., 2006; Hotez et al., 2006; Fenton, 2013).

Nevertheless, the question was not as simple as it might seem. In fact, though the deleterious impact of helminth co-infections have been widely reported (Pullan and Brooker, 2008;Griffiths et al., 2011), other works have shown how nematodes have positive effects on the host (Nacher et al., 2000, Helmby, 2015). Consequently, the pros and cons of helminth co-infections on host populations are fare of being fully understood. In other words, there is not a clear picture on the impact of deworming on the dynamic of infectious diseases (see Walson and John-stewart, 2007 for a helminth-HIV systematic review and Nacher, 2011 for helminth-malaria review).

Detecting effects of pathogen communities on individual hosts or populations still remains a major challenge for disease ecology and conservation (Pedersen and Fenton 2015). Few works have attempted to assess the consequences of the removal of non-target pathogens (e.g., deworming) on the dynamics of infectious diseases with health significance (Knowles et al. 2013b); Pedersen and Antonovics, 2013). Furdermore, the scarce research peformed at that regard provide contrasting outcomes on the non-target pathogens. For example, the positive effects of an anthelmintic treatment at individual scale can increase the dissemination of a bacterial disease in the host population (Ezenwa and Jolles 2015). Overall, parasite communities are remarkably stable to the perturbations caused by punctual deworming since the effect of anthelmintic on nematodes is short-living (Knowles et al. 2013b). However, the effects of deworming have been traditionally assessed by indirect methods (e.g., coprology, (Pedersen and Fenton 2015) probably underestimating the role of anthelmintic to drive the colonising parasite community after perturbation.

In the present work we aimed at investigating the impact of two consecutive mass anthelmintic treatments on the parasite community and tuberculosis dynamics in a wild boar (*Sus scrofa*) population of central Spain. Then, gastrointestinal, lung nematode burden and tuberculosis severity status was assessed in detail. Wild boar is the main wildlife reservoir for TB in the Mediterranean basin (Naranjo et al. 2008) and is exploited for hunting commercial purposes. Hence, enclosed wild boar populations are common through the Iberian Peninsula (Gortázar et al. 2006). The study had a twofold objective: i) to study the effects of deworming on TB dynamics (i.e., probability and severity of *M. bovis* infection) and ii) to investigate the impact of deworming on the parasite community of boars in terms of composition and structure. Our main prediction is that alterations in the parasite community of boars after the anthelmintic treatment will drive TB dynamics at individual and population scales.

Material and Methods

Study area and studied population

Sampling was carried out in a private hunting estate of 2000 ha from Toledo, central Spain (39° 55' 15.55" N, 5 °10' 32.33" E). The natural vegetation in the area is typically Mediterranean dominated by scattered holm oaks (*Quercus ilex*) and shrubs of *Cistus ladanifer*, *Erica* spp and *Genista anglica*. Wild boar density is about 40 individuals per 100 ha. The hunting

estate is fenced and animals receive supplementary food in summer (June to September) when natural food is scarce. The supplement is provided in 5 feeders strategically disposed in the hunting state (Fig. 1). The boar population eat about 42 tonnes/year (2100 kg/month and feeder) of Jabalí Familia fodder (Mercoguadiana SL. Spain). In autumn and winter wild boar are legally hunted in by authorised hunters in a "Monteria" modality (Fernández-Llario et al. 2003). This activity is carried out within the framework of an annual hunting plan approved by the regional authority in charge of livestock and wildlife management. No approvals were needed from any Ethics committee since animals were not sacrificed for research purposes.

Experimental design

In June 2012 and 2013 the wild boar population was treated with a medicated fodder (Ivomec Premix, Merial) for 7 consecutive days. Since natural food is very scarce during summer, all animals are expected to feed on the supplement.

Sampling procedure and processing

From 2011 to 2015 TB infectious status was systematically evaluated in 138 young and adult wild boar (96 females and 42 males). After the animal's collapse, gender of boar was assigned visually by inspecting genitalia, whereas age was estimated by dental biometry (Gonçalves et al. 2015). Later, a necropsy examination of each boar was performed to assess the presence of TB-like gross lesions affecting lymph nodes (submandibular, retropharyngeal, mediastinal and mesenteric lymph nodes), and thoracic or abdominal organs (Martín-Hernando et al. 2007). Respiratory (trachea and lungs) and digestive (stomach, small and large intestine) tracts were removed and stored in individual plastic bags. Later, at the laboratory, organs were frozen to keep them for a further parasitological examination. Submandibular and/or retropharyngeal lymph nodes were dissected and stored in sterile containers for microbiological analyses.

Figure 1. A wild boar herd feeding on ivermectin medicated fodder aimed at controlling helminth infections. Animals are feeding at night to avoid the heat of summer (by Ingulados S.L.).

Bovine tuberculosis status

Diagnosis of TB was based on the isolation of *M. bovis* or other *Mycobacteria* spp. as well on the presence of microscopic granulomatosus TB lesions. To detect the presence of Mycobacterium tuberculosis complex (MTC) bacteria, microbiological cultures from intact submandibular or retropharyngeal lymph nodes and from a piece of caudal lung lobes (both with TB-like lesions when possible) of each animal were carried out. For wild boar in which TB-like lesions were not found, a piece of submandibular lymph node and caudal lung lobe were identically processed for histopathology see (Risco et al. 2014b) for more information and for TB severity assessment (TB free, mild TB or severe TB)

Parasite assessment

Trachea and lungs were dissected and washed to obtain adult pulmonary worms according to the protocol proposed by Gassó et al. (2014). A single lung was used for parasitological purposes whereas the other half part was sent for assessing TB severity. The lack of statistical differences on the median intensity of nematode infection between the right and left lungs was verified in 20 individuals by a non-parametric Mann-Witney test ($U = 49$, $Z = -0.03$, p-value > 0.05). Hence, lung parasite load was twice the parasite load assessed in a single lung. Adult gastrointestinal helminths, however, were collected in a 500 µm sieve and transferred in 70% ethanol solution. Finally, worms were immersed in lactophenol for identification using a stereomicroscope (10×, 40× or 100× magnification) and identification keys for lung (Gassó et al. 2014), and gastrointestinal (Frontera et al. 2009) helminths.

Statistical analyses

For assessing the effects of deworming on the dynamics of TB infection we used generalized linear models (GLM) with a binomial error structure and a Logit link function. TB infectious status was our binary response variable (0 for healthy and 1 for TB infected boars) whereas the anthelmintic treatment: pre- (2011-2012) and post-treatment (2013-2015), was the fixed explanatory factor. For assessing the effects of deworming on TB severity we used generalised additive models (GAM, (Wood 2006) and TB infected animals. In this case TB severity (0 for mild TB and 1 for severe TB) was the binary response variable and the treatment the fixed explanatory term. In both GLM and GAM analyses the age of animals in months was initially included as explanatory variable but finally excluded because the lack of relationship (non-significant effect) with our response variables. In both analyses overdispersion was checked following (Zuur et al. 2010). Because sample size was moderate and quite heterogeneous (Table 1), model coefficients were estimated by bootstrapping (Davison and Hinkley 1997) using the package "boot" 1.3-18 version (Canty and Ripley 2016). For GAM analyses we used "mgcv" package 1.8-15 version (Wood 2011) whereas the "ggplot2" package 2.1.0 version (Wickham 2009) for graphic representations. All analysis were conducted using the R software, version 3.3.1 (R Development Core Team, June 2016).

On the other hand, pathogen community structure was studied using null models (Gotelli and Graves 1996). Data were organized as presence-absence matrices in which each row

represented a pathogen species and each column represents an individual wild boar. In this matrix “1” indicates that species is present at a particular site or host, and “0” indicates that a species is absent (Gotelli and Entsminger 2001). Three matrices were created separately for TB free, mild TB and severe TB wild boar. The C-score index introduced by (Stone and Roberts 1990), quantifies the average amount of co-occurrence among all unique pairs of species in the assemblage, measures the tendency for species to not occur together. C-score index was used as for exploring co-occurrence patterns and the FE algorithm (fixed row-equiprobable column) chosen to analyze the results obtained. The C-score measures the average number of checkerboard units between all possible pairs of species. In a competitively structured community, the observed C-score should be significantly larger than expected by chance (O>E). Else, a C-score smaller than expected by chance (O<E) indicates a randomly assembled community. The observed C-score was calculated for each matrix and compared with the expected calculated for 5000 randomly assembled null matrices by Monte Carlo procedures. Moreover, to compare the degree of co-occurrence across data, a standardized effect size (SES) for each matrix was calculated. SES measures the number of standard deviations that the observed index (C-score) is above or below the mean index of the simulated communities. The analysis was carried out using the software EcoSim 7.0.(Gotelli and Entsminger 2001).

Results

TB dynamics

As we can see in Table 1, TB prevalence decreased throughout the study period ranging from 45.8% in 2015 to 58.3% in 2011. This slight decrease in TB prevalence, however, was undetectable by our statistical model ($\beta_{Treatment}$ = -0.123, Bias for the bootstrapped coefficient = -1.3e-05, E = 0.42, Z = - 0.309, p-value = 0.75, Fig. 2), indicating that TB prevalence in wild boar remained stable after deworming. On the contrary, TB severity changed over years ($\beta_{Treatment}$ = -1.4, Bias for the bootstrapped coefficient = - 0.08, SE = 0.63, Z = - 2.38, p-value = 0.02, Table 2, Fig. 2). Two years before the mass anthelmintic administration severe TB was 57.7% (2011 and 2012) but decreased to 32.5% over the following three years.

	Years				
TB status	2011	2012	2013	2014	2015
TB infected	58.3 (29.4 – 81.9) n = 7	47.8 (27.3-68.1) n = 11	46.5 (32.18-61.8) n = 20	54.1 (33.8-73.8) n = 13	45.8 (26.2-66.2) n = 11
Total	12	23	43	24	24

Table 1. Prevalence at 95% CI of bovine tuberculosis in 116 wild boars hunter-harvested in central Spain. This wild boar population received a mass anthelmintic treatment in summer 2012 and 2013 (cells with a grey background).

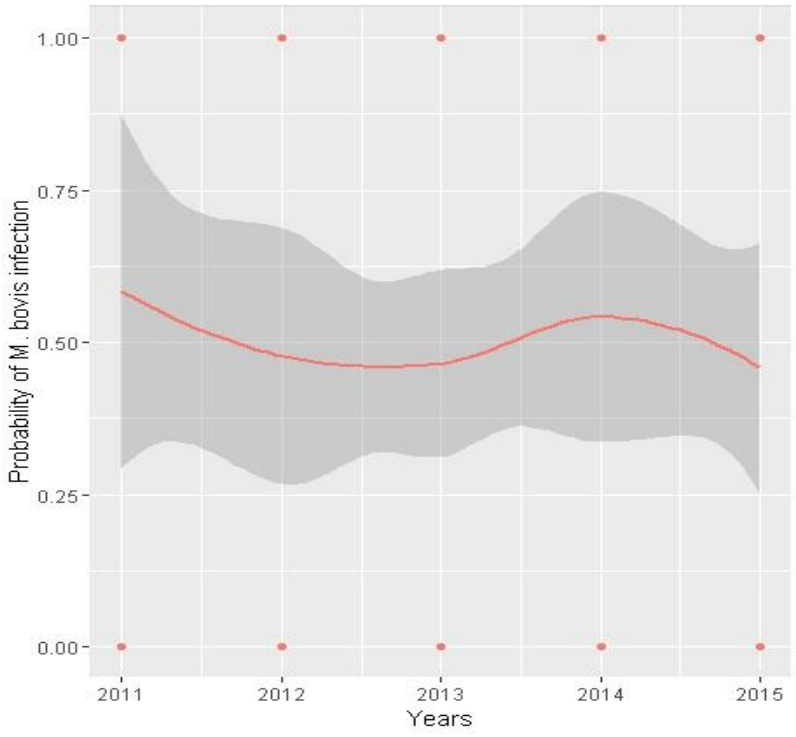

Figure 2. Trends in the probability of being infected with *Mycobacterium bovis* in a wild boar population from central Spain. The probability of *M. bovis* infection has been modelled by a generalised linear model

(GLM). The shaded area represents the 95% confidence interval for the prediction. Mass anthelmintic treatment was provided in summer 2012 and 2013.

	Years				
TB status	2011	2012	2013	2014	2015
Mild TB	57.1 (22.5 - 87.1) n = 4	27.3 (7.8-59.9) n = 3	85.1 (63.1-95.8) n = 17	53.8 (26.1-78.4) n = 7	45 (7.8-59.9) n = 7
Severe TB	42.8 (12.8 - 77.5 n = 3	72.7 (40.1-92.1) n = 8	15.1 (42.1-36.9) n = 3	46.1 (21.5-73.9) n = 6	36.3 (13.5-66.7) n = 4
Total	7	11	20	13	11

Table 2. Prevalence at 95% CI of mild and severe tuberculosis in 66 wild boars hunter-harvested in central Spain. This wild boar population received a mass anthelmintic treatment in summer 2012 and 2013 (cells with a grey background).

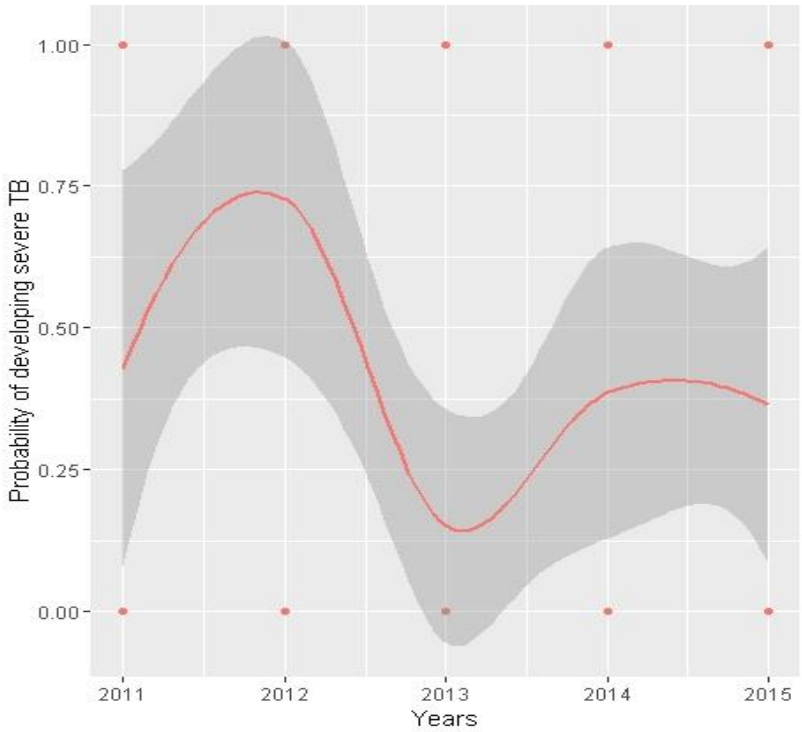

Figure 3. Trends in the probability of developing severe TB in a wild boar population of central Spain modelled by a generalised additive model (GAM). The shaded area represents the 95% confidence interval for the prediction. Mass anthelmintic treatment was provided in summer 2012 and 2013.

Changes in the parasite community

The prevalence and the mean intensity of helminth infection observed in our sample of wild boars can be seen in Table 3.

Helminths	**Prevalence** 95% CI	**Mean intensity of infection** (min - max)
NEMATODES		
Globocephalus urosubulatus	97 (89.9 - 99.46)	123 (16 – 726)
Ascarops strongylina	69 (57.01 - 79.61)	8 (1 – 39)
Metastrongylus spp.	67 (54.6 - 70.67)	35 (2 – 402)
Hyostrongylus rubidus	34 (23.03 - 46.84)	12 (1 - 39)
Oesophagostomum dentatum	18 (10.09 - 29.28)	8 (1 – 31)
Physiocephalus sexalatus	10 (4.09 - 18.80)	2 (1 – 3)
ACANTOCEPHALANS		
Macracanthorhynchus hirudinaceus	33 (17.51-39.26)	3 (1 – 7)

Table 3. Prevalence at 95 % CI and mean intensity of infection (min-max) of helminths collected in 67 hunter-harvested wild boar in a private hunting state from Toledo, central Spain.

Our null model analysis revealed that the observed C-score was greater and significant than expected by chance (O>E) only in TB-free boars (Table 4). This result suggests the existence of a competitively structured helminth community with non-random combinations. In animlas suffering from TB the observed C-score was also greater but not enough that that expected by chance indicating that helminth assemblages are random.

	C-score			
	O	E	P	SES
TB free	25.78	24.14	0.00 *	4.83
TB infected	17.8	17.5	0.16	0.98
Mild TB	9.97	9.7	0.2	0.82
Severe TB	1.3	1.4	0.24	-0.84

Table 4. Observed (O) and expected by chance (E) values of the C-score for presence/absence matrices of helminth communities on TB- free (n = 34), mild TB (n = 21) and severe TB (n = 9) wild boar from central Spain. Negative values of the standardized effect size (SES) indicates that O < E, whereas positive values indicate the contrary. The asterisk indicates significant P-value <0·05.

Discussion

These results suggest that a punctual mass anthelmintic treatment had a little effect on TB dynamics in our wild boar population. On the contrary, the anthelmintic treatment appeared to be beneficial for the host at individual scale, probably ameliorating the ability of the host to cope with the disease. On the other hand, the fact that we found high number of adult worms in the necropsied animals could be for two reasons, lack of oral ivermectin effect or more probably rapid re-infections, typical in wild animals (Thomas and Morgan, 2013; Knowles et al., 2013).

The lack of treatment effect on wild boar TB incidence could be explained by the rapid re-colonization of host by parasites or initial protection front TB infection involving several innate immune defences (van Crevel et al. 2002) not affected by the Th2/Th1 imbalance. Our hypothesis supports the second idea, because, although boars were re-infected rapidly, the drug treatment lasted the summer season. Then, when density and contacts between animals were elevated, and probability to be infected by *M. bovis* higher, effects of ivermectin were still present. Worm burdens would be low at the moment of TB infection. Furthermore in this estate wild boar had high natural diet resources almost all the year. Summer is the only season where natural resources availability are limited and then, were supplemented with animal feed. Thus we think that never had resource limitation. In these situations the trade-off between Th1-Th2 responses may be smaller (Ezenwa and Jolles 2011).

Our observation that worm treatment had different effects on TB prevalence and severity are in the same lines that results found by Ezenwa and Jolles (2015) where anthelmintic treatment in an African buffalo population affected by bovine TB had asymmetrical effects between infection probability and mortality. In the same lines other treatments (vitamin D3 in diet or oral vaccinations) in wild boar suggested for TB control, have reduced TB severity but not TB prevalence (see Risco et al. under review and Ballesteros et al., 2009).

Our results show that after an anthelminthic treatment a part of host re-infection the structure of helminth communities re-appears rapidly. Wild boar TB free had a structured helminth community four months after drug treatment, but in wild boar infected with *M. bovis*, being more significant in severe TB boars the helminth community was not structured. This can suggest that in the infection moment with *M. bovis* parasites communities were unstructured and then if the host was infected by the bacteria the parasite community hardly returns to be structured. Previous studies in European wild rabbits (Cattadori et al. 2007) and Mice (Carmo et al. 2009) demonstrated that microparasites also have the capacity to affect worms.

We would note that ivermectin, though is a wide-spectrum treatment front parasites not covers all the none-TB parasites. So non-target parasites like protozoa, virus or acanthocephalan among others cannot be forgotten or ignored because probably have effects in the co-infected hosts. Further research in the non-target parasites are needed to complete or knowledge in this issue.

Conclusions

In wild boar, a punctual anthelmintic treatment administrated orally, in supplemented feed, during scare resource season is beneficial for the host at individual level, decreasing severity of TB, but not enough to decrease prevalence and could see effect at the population level.

Then oral deworming could be a novel control measure to reduce wild boar TB severity in a resistant and natural reservoir of *M. bovis*. On the other hand, without minor importance, the Mycobacteria likewise affect the community of parasites. Further, non-target parasites also may have an effect in this co-infection model. To better understand these effects it will be necessary further research comprising different co-infection models and more large-time studies of target and non-target pathogens.

8. GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Infectious diseases remain a major threat to human health, economic sustainability and wildlife conservation. The multi-host-multipathogen approach is complex but necessary to understand and be able to control emerging diseases. Then recent advantages in community ecology may be applied in disease research (Seabloom et al. 2015) to look for the pathogen interactions within the host, and between hosts and species.

Interactions between helminths and microparasites have captured particular attention. The ubiquity of helminth infections in many host populations and their overlap with the most devastating diseases (malaria – HIV – TB), make necessary the research and knowledge of these co-infections (Salgame et al. 2013). Worm-TB co-infection is very suitable model for understanding why diseases need the "one health" approach with a multi-host-multipathogen study.

Wildlife research, where parasite interactions are in their natural context, can generate new and unique insights about helminth-microparasite. For this reason, increasing integration of wild studies with research in human, laboratory and veterinary animal populations will be necessary to understand the co-infection issue (Ezenwa 2016).

Wild boar, the main reservoir of bTB in Spain (Naranjo et al. 2008) can be consider an interesting model to study worm-TB interactions. However, before the study of these more complicated concepts, it is essential to have available good tools for assessing parasitofauna of wild boars.

8.1. Parasitological comments

There is an increase interest for assessing the health status of wild boar, the wild counter part of domestic pigs, due to the natural or artificial expansion of populations (Massei et al. 2015). The helminthofauna of wild boars is typically greater than in domestic pigs, especially for those parasites with indirect life cycles (de-la-Muela et al. 2001). Consequently, it is important to understand the uses and limitations of the tools used as a proxy for endoparasite burden in wild boar. According to the results in the three technical studies about suids parasitofauna, although FEC is an easy cost-effective and non-invasive tool for animal health surveillance, it significantly

underestimates some species. FEC using the McMaster method is a useful tool for quantifying some (e.g.*Trichuris*, *Ascaris*) but not all gastrointestinal nematode infestations of wild boar. This is mainly due to the low reproductive rate of some species and the absence of one of the two sexes. Stomach nematodes often go undetected by coprology. Nevertheless, it is recommended that samples be processed as soon as possible because egg count decreases over time. Furthermore, although the sedimentation method was used to assess acanthocephalans, it also significantly underestimated *M. hirudinaceus* infestation in wild pigs. Thus, further research is required to: I) evaluate the effectiveness of other flotation solutions or McMaster modifications (e.g. FLOTAC, d'Ovidio et al. 2014) to assess GI parasite burden and, II) improved methods for detection and burden estimation of acanthocephalan. On the other hand, concerning lung nematodes, their infestations can be properly assessed by coprology in wild boar. It is important to highlight the importance of considering both male and female adult individuals to achieve the proper species identification of these worms, especially in the case of *M. confusus*, *M. apri* and *M. salmi*.

In terms of co-infection, in wild boar, assessing parasitofauna by coprology could underestimate some species. This may, add new and not controlled factors of variability between hosts that may bias the final response variables. Consequently, although necropsy is hardest and time-consuming, always gives more consistent results.

8.2. Helminth-TB effects in physiological biomarkers of health status of wild boar

A wide variety of studies have assessed oxidative damage in a broad range of pathologies such as TB. Based on our results, a positive oxidant/antioxidant balance (i.e., low antioxidant enzyme production for a given concentration of oxidant substances) in *M. bovis*-infected boars can be detected under experimental conditions. The results obtained in our experimental challenge are in line with the basic pathophysiological mechanism proposed in other vertebrate models. In mice and humans, for example, an increase in OS is the main mechanism limiting Mycobacteria spp. multiplication, thus preventing the appearance of TB-like gross lesions (Yu et al. 1999; Chan et al. 1995). At the same time, the bacterium limits the exposure to free radicals by increasing the expression of antioxidant enzymes (Cumming et al. 2014), which slows down macrophage ROS and RNS production (Flynn et al. 1998). These "attack and counterattack" interactions are key drivers for maintaining the TB granuloma in a latent form that can be reversed in case of immune depression (Sasindran and Torrelles 2011).

Although TB causes the oxidant/antioxidant imbalance in experimental conditions, oxidative stress goes unnoticed in free-ranging *M bovis*-infected animals. Accordingly, other environmental factors causing OS, such as parasites and/or food availability, should have a greater impact on OS than TB itself. Despite the growing interest in biomarkers of oxidative status for assessing the health status of individuals in conservation programmes, the regular use of these biomarkers to assess the impact of wildlife diseases is in its infancy. Biomarkers of oxidative status can help to quantify fitness costs of diseased animals but much work remains to be done to understand the natural sources of variation affecting these promising physiological indicators.

In the TB-helminth co-infection model used in this thesis, apart from OS, body condition (BC) and inflammation with acute phase proteins (APP) were studied. Multiparasitism had negative effects on physiological biomarkers of health status of the wild boar, the augment of worm burden reduce BC and increase OS and APP. Direct and indirect interactions between co-infecting parasites within individual hosts have high effects on host health, fitness and disease outcome. In the used helminth-TB co-infection model, worms worsened the overcome of disease by their positive relationship with the TB status and direct and indirect, negative or positive effects on physiological indicators of health status (increasing OS and inflammation and reducing BC). It is necessary to continue expanding the complex dynamics of multi-host-multi-parasite communities to make progress in controlling infectious diseases.

Thus, confirming the idea that parasites have significant impacts on the dynamics of TB disease in wild boar, the parasite removal could be a good strategy to control TB overcome in this population. As parasites can have significant impacts on the dynamics of wildlife populations (Pedersen and Fenton 2015), it is important to remark that before practice a systemic deworming for disease management these types of works are necessaries. Moreover, in African buffalo Ezenwa and Jolles (2015) observed contradictory effects of deworming, increasing the pathogen survival. Thus, it would be important to assess what happens after a deworming treatment in controlled conditions in this host reservoir of MTC to predict consequences in host individual and population scales.

8.3. Worm treatment for TB control

To assess directly the consequences of helminth infections on the health and fitness in natural populations, antiparasite drug treatments were used (see Pedersen and Fenton 2015 for a review). But few works were focused in the effects of deworming for the concurrent diseases. Our results suggest that in this natural population of wild boar affected by bTB a punctual

treatment with oral anthelmintic drug during 7 days not affect the pathogen overcome, in this case TB prevalence. Contrary, results show that there was a correlation between the TB severity and the treatment, because once the treatment started the percentage of hunted wild boar with severe TB (generalized lesions) decreased. In this case we propose that anthelmintic treatment is beneficial for the host at individual level, decreasing severity of TB, but not enough to decrease prevalence and could see the effect at the population level.

The lack of treatment effect on bTB incidence could be explained by the rapid recolonization of host by parasites or initial protection front TB infection involving several innate immune defences (van Crevel et al. 2002; Blok et al. 2015) not affected by the Th2/Th1 imbalance. Natural antibodies and complement protect in the early stages of microbial pathogens (Kapetanovic and Cavaillon 2007). Although in the fifth chapter no consistent relations between pathogens and biomarkers of innate immunity, the formulated hypothesis supports the second idea (innate immunity is important in the early events of TB disease): although boars were re-infected rapidly, the drug treatment was in the start of the summer and its effect was lasted at the end of this season. Then, when density and contacts between animals were the highest, and also probability to be infected by *M. bovis* increase (Risco et al. 2014a), worm burdens were the lowest.

In summary, anthelmintic treatment administrated orally in supplemented feed during summer season in wild boar did not affect *M. bovis* prevalence but decrease disease severity. Then oral deworming could be a novel control measure to reduce TB severity in a resistant and natural reservoir of MTC (especially *M. bovis*). It cannot be ruled out that non-target parasites, like protozoa, other bacteria or virus, may also have an effect in this co-infection model. On the other hand, the Mycobacteria likewise affect the community of parasites, changing patterns of community structure which could affect both macro and microparasites relations and thus, host health at individual or population level in a near or distant time of period. To understand better these effects it will be necessary further research comprising different co-infection models and more large-time studies of target and non-target pathogens.

8.4. Closing remarks

Since co-infection is a rule in many parts of the world it is necessary to pay attention on the interactions between helminths and microparasites to better understand disease overcome. Good

parasitological tools are necessary to assess with precision worm burdens the key factors of many emerging diseases.

Direct and indirect interactions between co-infecting parasites within individual hosts have significant effects on host health, fitness and disease outcome. It is necessary to continue expanding the complex dynamics of multi-host-multi-parasite communities to make progress in controlling infectious diseases.

More field experimental studies are needed, because the majority of community ecology studies are often descriptive. Natural infection outcomes are more difficult to predict to compared to experimental ones, since other important factors such as nutritional challenges, seasonality, immunocompetence or extrinsic factors that alters intensity of exposure to parasitesmay modulate such outcome.

Parasite removal may be a good strategy to modulate the outcome of an infectious disease like TB. Before practicing a systemic deworming for disease management, these types of works are necessaries to predict consequences in host individual and population scales.

Co-infection can have adversely effects on public health, so integrating wild studies with ongoing human, laboratory animal and veterinary research is necessary.

9. CONCLUSIONS

9. CONCLUSIONS

1. *M. confusus* and other *Metastrongylus spp*. are usually overlooked due to the lack of an identification key for this genus. FEC sedimentation technique significantly underestimates *Macracnthorhynchus hirudinaceus* a high prevalent acanthocephalan in some areas of Spain.
2. FEC is a useful tool for quantifying some but not all nematode infestations. Samples should be processed as soon as possible because egg count decrease over time after faecal sample collection.
3. TB causes an oxidat/antioxidant imbalance under experimental conditions that goes unnoticed in free-ranging *Mycobactrium bovis*-infected wild boar. Environmental factors causing OS such as parasite and food availability seem to have greater impact on OS than TB itself.
4. Worms worsen physiological indicators of the health status by their direct and indirect effects through TB, increasing OS and inflammation and reducing BC).
5. Anthelmintic treatment administrated orally in supplemented feed during summer season in wild boar does not affect *M. bovis* prevalence but decrease disease severity.

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