Control mediante vacunación de la tuberculosis en su principal reservorio silvestre en España, el jabalí.

Tuberculosis control by vaccination in its main wild reservoir in Spain, the wild boar.

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Los abajo firmantes, como directores de esta Tesis Doctoral, hacemos constar que la Tesis titulada "**Control mediante vacunación de la tuberculosis en su principal reservorio silvestre en España, el jabalí**", y realizada por **Iratxe Díez-Delgado**, licenciada en Veterinaria, reúne los requisitos necesarios para optar al grado de **DOCTOR CON MENCIÓN INTERNACIONAL**.

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A mis padres y mi hermana,

mis anclas e inspiración.

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El control de las enfermedades compartidas con la fauna silvestre requiere desarrollar estrategias que reduzcan la transmisión del patógeno entre esta y el ganado y/o los seres humanos. Esta revisión describe y analiza las opciones de control aplicadas en la actualidad, e intenta predecir las opciones de control en las próximas décadas. Antes de tomar decisiones de intervención (o no) es prioritario establecer un adecuado sistema de vigilancia y control (poblacional y sanitario). El control de enfermedades en fauna silvestre puede lograrse a través de diferentes medidas como: (1) acciones preventivas, (2) control de artrópodos vectores, (3) control poblacional de hospedadores mediante eliminación aleatoria o selectiva, gestión del hábitat o control de la reproducción, y (4) vacunación. Otras opciones como la zonificación o la no intervención también deben ser consideradas en función de la relación coste-beneficio. Idealmente, las múltiples opciones de control deben combinarse y aplicarse de forma integrada. El éxito de estas medidas en fauna silvestre depende de numerosos factores, incluyendo la ecología de la enfermedad, la historia natural y las características del patógeno así como la disponibilidad de métodos diagnósticos apropiados, las características de los hospedadores domésticos, silvestres y vectores, la extensión geográfica del problema, la escala de la intervención y las actitudes de las partes implicadas.

Abstract

The control of diseases shared with wildlife requires the development of strategies that will reduce pathogen transmission between wildlife and both domestic animals and human beings. This review describes and criticizes the options currently applied and attempts to forecast wildlife disease control in the coming decades. Establishing a proper surveillance and monitoring scheme (disease and population wise) is the absolute priority before even making the decision as to whether or not to intervene. Disease control can be achieved by different means, including: (1) preventive actions, (2) arthropod vector control, (3) host population control through random or selective culling, habitat management or reproductive control, and (4) vaccination. The alternative options of zoning or no-action should also be considered, particularly in view of a cost/benefit assessment. Ideally, tools from several fields should be combined in an integrated control strategy. The success of disease control in wildlife depends on many factors, including disease ecology, natural history and the characteristics of the pathogen, the availability of suitable diagnostic tools, the characteristics of the domestic and wildlife host(s) and vectors, the geographical spread of the problem, the scale of the control effort and stakeholders' attitudes.

Introduction

Diseases shared with wildlife are multi-host infections with an impact on human health, economy and wildlife management or conservation were wildlife itself plays a significant role on infection maintenance. Shared diseases represent a significant burden that affects public health, global economies and the conservation of biodiversity (Daszak et al. 2000; Gortázar et al. 2006; Caron et al. 2013). It has been suggested that 80% of the relevant animal pathogens present in the United States of America have a potential wildlife component (Miller et al. 2013). Furthermore, the number of emerging infectious disease (EID) events caused by pathogens originating in wildlife has increased significantly over time, suggesting that EIDs represent an increasing and highly significant risk to global health (Jones et al. 2008). Moreover, changes in wildlife management such as changes in harvesting/culling, conservation measures and translocations, feeding and fencing of natural habitat are among the drivers of zoonotic pathogen emergence (Gortázar et al. 2014a). A collaborative effort of multiple disciplines in a One Health context is crucial if the health of human beings, livestock, wildlife and the environment is to be improved (FAO/OIE/WHO/UNSIC/UNICEF/ WorldBank 2008). It is also widely accepted that the total eradication of a shared infectious agent is almost impossible if wildlife hosts, which serve as a natural reservoir of the pathogen are ignored (O'Reilly and Daborn 1995; Gortázar et al. 2007; Martin et al. 2011).

Disease emergence in wildlife (e.g. chronic wasting disease, CWD), and difficulties in the eradication of endemic shared diseases such as classical swine fever (CSF) and tuberculosis (TB), have, over the last few decades, prompted a growing interest in disease control in wildlife reservoirs (Artois et al. 2001 and 2011; Wobeser 2007; Delahay et al. 2009; Miller et al. 2013). The control of diseases shared by wildlife

requires the development of strategies to reduce pathogen transmission between wildlife and domestic animals or human beings. The control of wildlife disease often consists of an intervention in natural ecosystems and is, as such, often controversial (Artois et al. 2011). This review describes the options that are available for disease control at the wildlife-livestock-human interface, from preventive measures to population control and vaccination. This includes a critical review of the options currently applied and an attempt to forecast wildlife disease control in the coming decades. This review does not include those disease control efforts that are directed solely towards wildlife for conservation or game management purposes. Modelling (if not accompanied by actual intervention) is also beyond the scope of this paper. An outline if the steps and options that could be used to achieve disease control are shown in Figure 1 and some examples can be seen in Figure 2.

Disease monitoring in wildlife

The key requisite for any disease control in wildlife is that of establishing a proper surveillance and monitoring scheme. Surveillance and monitoring build on the steady collection, collation, and analysis of data related to animal health but differ at the aim and target population. Surveillance targets wildlife populations classified as healthy to demonstrate the absence of infection (OIE 2011). Conversely, monitoring focuses on known infected wildlife populations aiming to detect spatial and temporal trends (Artois et al. 2009). Disease control measures are only undertaken when disease is present; therefore, from now on this paper will focus on monitoring (since surveillance is applied when infection is absent). After disease discovery, descriptive studies are needed in order to assess whether the disease and the role of wildlife is relevant for

public or animal health or for wildlife conservation and management. If this is the case, then wildlife diseases must be monitored by defining the key wildlife hosts, host population background data and samples; choosing the appropriate methods for diagnosis and for space-time trend analysis, and establishing a reasonable sampling effort with suitable sample stratification (Boadella et al. 2011a). Each situation must be analyzed independently since being a "reservoir" or "spillover host" depends not only on the pathogen and wildlife species but other factors e.g. wild boar in the Iberian Peninsula are considered reservoir hosts for *Mycobacterium bovis* (*M. bovis*) but feral hogs in Australia are considered spill over hosts (see more examples at Palmer 2013). If properly performed, monitoring will allow changes in disease occurrence to be identified and the impact of any intervention to be critically assessed (e.g. Robinson et al. 2012). One example of the current trend as regards improved wildlife disease monitoring is the European research consortium APHAEA, whose goal is to harmonize approaches in order to develop a health surveillance network for wildlife at a European level by improving both population and disease monitoring (APHAEA 2013).

Disease control options

The primary means to control diseases shared by wildlife include (1) preventive actions, (2) arthropod vector control (if vector-borne), (3) host population control through random or selective culling, habitat management or reproductive control, and (4) vaccination. Ideally, tools from several fields should be combined in an integrated control strategy. Targeted and effective methods aiming to maintain natural environments will receive most support despite being potentially controversial (Dandy et al. 2012). Alternative options such as zoning (sensu Artois et al. 2011) or no-action

should also be considered, particularly in view of a cost/benefit assessment (Figure 1), but disease and population monitoring are always required.
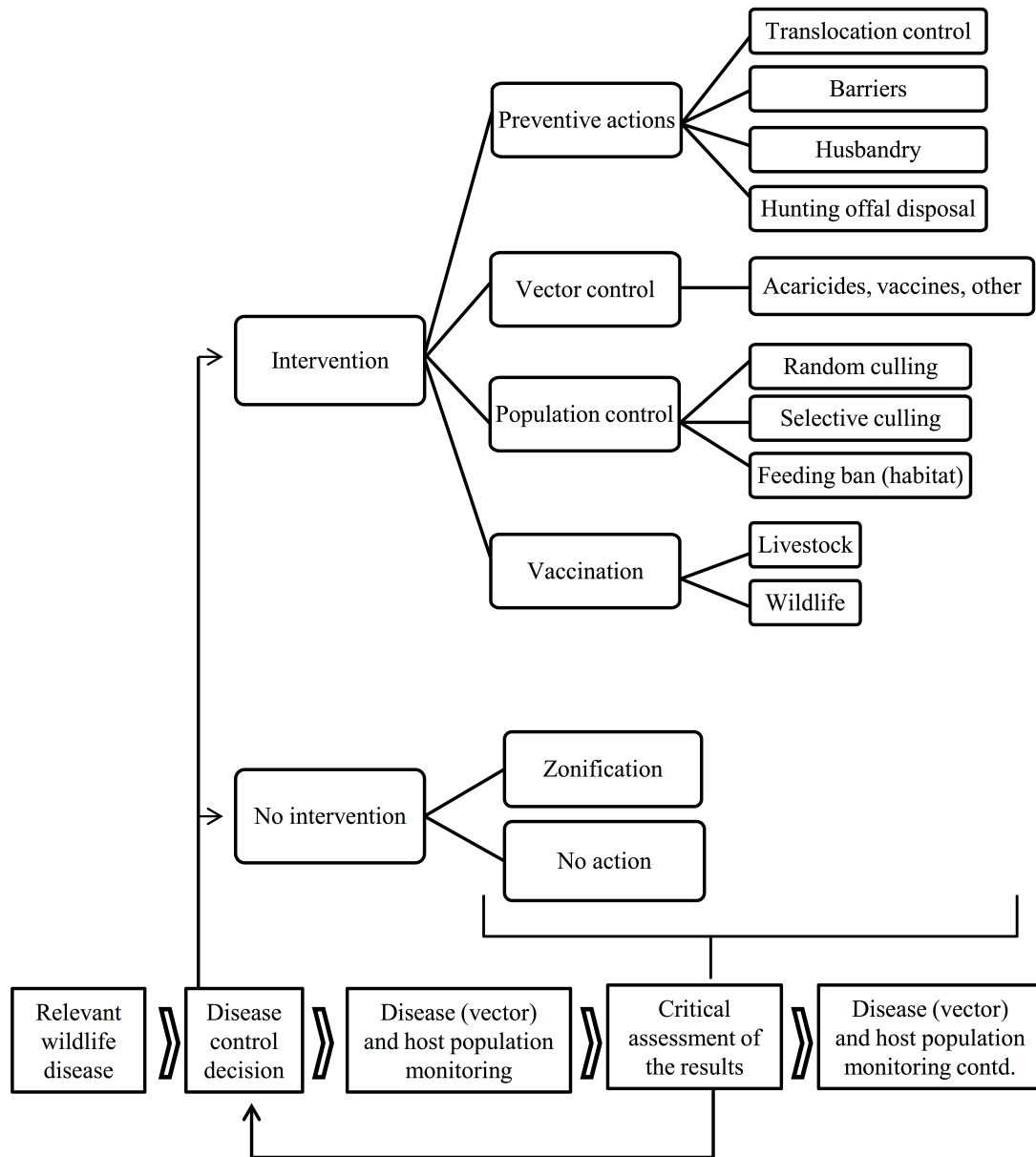
Figure 1. Flowchart of the available disease control options and result assessment in diseases shared with wildlife.
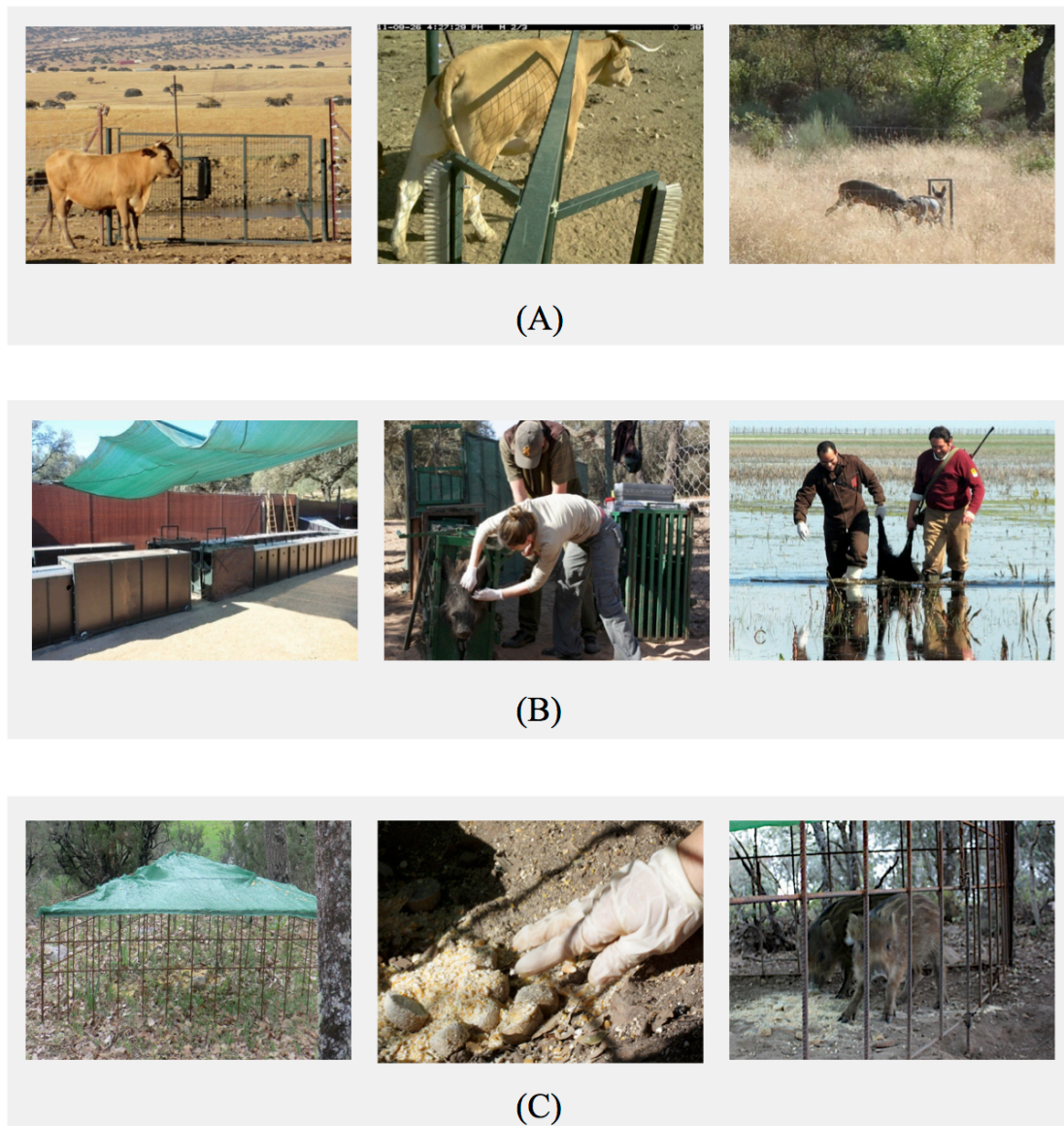
Figure 2. Examples of some disease control options currently applied: (A) Farm biosecurity by segregating wildlife and cattle using fences; (B) Selective culling by testing using animal side lateral flow ELISA (Chembio DPP test, New York, USA); (C) Vaccination against TB in wild boar using oral baits.

Preventive actions to control diseases

Disease prevention at the wildlife-livestock-human interface is a broad field that includes control methods such as translocation, fencing, feed and water management, farm biosecurity and hygienic hunting offal disposal, among others.

Translocation control

Movement control, known as translocation control in wildlife, is one of the most fundamental preventive actions in disease control for both domestic animals and wildlife (Gilbert et al. 2005; Smith et al. 2009; Carstensen et al. 2011). Translocation control is meant to prevent the introduction or re-introduction of pathogens via the release of infected free-living or captive wildlife. Global wildlife trade affects millions of individuals annually, with severe implications for disease emergence (Karesh et al. 2005). Several recent reviews discuss the importance of translocation control for disease prevention (e.g. Kock et al. 2010; Sainsbury and Vaughan-Higgins 2012), and new regulations have been enforced in some countries (e.g. OIE regulations for chytrid fungus control in amphibians, Royal Decree 1082/2009 in Spain).

Barriers

This concept includes the use of large or small scale fencing and any other barrier: physical, dogs, deterrents, barriers to vectors, etc. to prevent the transmission of diseases between animal populations by decreasing contact among them.

Large-scale fencing:

Certain livestock diseases, such as foot-and-mouth disease (FMD), are difficult to control due to the large numbers of infected wildlife hosts. This limits the ability to trade livestock products in international markets. Fencing has been used on very large scales to segregate wildlife from cattle. One successful example is from southern Africa where livestock and game-proof fences lengthier than 500 km where set up to prevent the spread of rinderpest and FMD (Sutmoller 2002; Schneider 2012). However, fences are vulnerable to certain animal species (e.g. suids may slip under them, or elephants may destroy them; Jori et al. 2011) being difficult and expensive to maintain. Expenses

and doubts on efficacy are some of the reasons why EU Commisioners did not back up proposal of Lithuania Minister on building a fence along Belarus´ border to prevent wild boar movement in order to control African Swine Fever (ASF) spread (PROMED 2014a and 2014b). Moreover, fencing may be an important impediment to conservation as such large barriers seriously interfere with animal migration (Owens and Owens 1980).

Farm-biosecurity, small-scale fencing and deterrents:

Although on a far smaller scale, fencing is a key tool in farm biosecurity. Farm biosecurity is becoming a prominent method to prevent infectious disease transmission and reduce wildlife-livestock interactions (Engeman et al. 2011; Judge et al. 2011). For example, industrial pig and poultry farms maintain their low disease status partly because they are effectively separated from potentially infected wildlife by fencing and other physical barriers. Farm biosecurity continues to be improved, not only in intensive rearing facilities, but also in open air systems and in livestock production systems in which wildlife contact is likely in pastures, water points or feed-storage sites. On UK cattle farms, appropriately deployed simple exclusion measures (sheet metal gates and fencing, feed bins and electric fencing) were 100% effective in preventing the Eurasian badger (*Meles meles*) from entering farm buildings. These exclusion measures also reduced the level of badger visits to the rest of the farmyard, thus potentially decreasing the risk of *M. bovis* (i.e. tuberculosis, TB) transmission between badgers and cattle (Judge et al. 2011). Wild ungulates, including white-tailed deer (*Odocoileus virginianus*) and Eurasian wild boar (*Sus scrofa*), are among the most damage-causing wildlife species. Fencing has been demonstrated to reduce the use of potential contact sites by wildlife (e.g. VerCauteren et al. 2006; Honda et al. 2011; Vilardell et al. 2012). In Riding Mountain N.P. (Canada) local fencing was combined with the use of guard

dogs to further decrease the risk of *M. bovis* transmission to cattle in conjunction with the on-going test and cull and a deer feeding ban to reduce the risk of elk (*Cervus elaphus*) and white-tailed deer transmitting *M. bovis* to cattle (O'Brien et al. 2011). Segregating wildlife and livestock from common resources such as waterholes or feed by setting up selective enclosures or by training dogs to reduce wildlife visits to farms may prove beneficial (VerCauteren et al. 2008 and 2012). Actions to prevent disease transmission at water points and feeding sites may also include dispersing or modifying the available water points and replacing feeding sites on the ground with selective feeders which are less accessible to certain species. For instance, an apparent reduction of 66% in cattle TB skin reactors was achieved using fencing to segregate waterholes for either cattle or wildlife on a farm in Spain (Barasona et al. 2013a). Care must be taken to select the appropriate segregation method; if it is applied incorrectly it can cause the opposite effect. For example, the policy of massively feeding elk during the winter in the Yellowstone Ecosystem (WY) in order to limit transmission of *Brucella abortus* in pastures shared with cattle may actually contribute toward disease transmission and maintenance within elk herds (Cross et al. 2007a).

Husbandry

Changes in animal husbandry include infinite possibilities as regards dealing with specific biosecurity problems. These changes include timing and the use or certain pastures, feeding livestock inside, or changing disease susceptible livestock species to less risky ones (Ward et al. 2009). For instance, agencies can promote substituting horses for ruminants or sheep for cattle in TB endemic areas. The latter option is occasionally being recommended to cattle owners in highly prevalent regions with high wildlife densities in Spain (C. Gortázar, personal communication).

Carcass and hunting-offal disposal

Another important field in biosecurity and wildlife disease control is the proper removal of harvested animals (including viscera and other remains) in order to limit potential infection spread, principally by mammals (Vicente et al. 2011). One specific case is the obligatory pre-movement testing of hunter-harvested wild boar carcasses for CSF. Wild boar shot, in potentially endemic areas, must remain (refrigerated in appropriate set ups to enable carcass maintenance until clearance) at the hunting site until blood and spleen have been analyzed for CSF in the corresponding laboratory (e.g. Attila and Tamas 1995). In New Zealand similar discussions are occurring around the movement of potentially *M. bovis* infected feral pig heads collected by hunters as trophies (G. Nugent, personal communication).

The disposal of carcasses and hunting remains has significantly contributed to wildlife disease-related conflict between hunters, government agencies, the livestock industry and conservationists in Mediterranean Spain (Gortázar et al. 2010). Recent field tests have revealed that simple and inexpensive fence designs prevent non-target species, including wild boar, from accessing the food provided for endangered avian scavengers (*Gyps fulvus*, *Aegypius monachus*, *Corvus corax* and *Aquila adalberti;* Moreno-Opo et al. 2012*)*. More observational and experimental research is needed in all the aforementioned control methods, since only a few of these methods have been scientifically assessed for their actual contribution to disease control.

Arthropod vector control

The control of arthropod vector infestations for the control of diseases shared with wildlife has principally been described in relation to West Nile virus (WNV) and

tick-borne infections such as Lyme borreliosis and babesiosis. West Nile exemplifies the complex interactions between health and the environment (Tedesco et al. 2010) as new conflicts are surfacing around culicoid mosquitoes control and environmental health (Siptroth and Shanahan 2011). Since there are no efficient vaccines or treatments available for WNV, efforts are focused on vector control mainly by using insecticides and on new strategies based on symbionts, such as *Wolbachia* sp (Bourtzis et al. 2014). Nevertheless, there is an increased concern about the toxic effects of insecticides on non-target insect populations, on humans and the environment (e.g. Clean Water Act versus pesticide use and wetland management practices such as drainage in Sacramento -San Joaquin Bay- Delta estuary, CA, USA; Siptroth and Shanahan 2011).

Ixodes tick control (including habitat management through burning, the use of acaricides, and white-tailed deer elimination) has been shown to reduce *Ixodes scapularis* populations by up to 94%, and acaricide application to deer decreased nymphal *I. scapularis* populations by up to 83%. However, the effect of these strategies on the incidence of Lyme disease in humans remains unknown (Poland 2001; Stafford et al. 2009).

Control efforts for *Babesia* sp vectors rely on culling wild ungulates in infected and neighbor farms in conjunction with acaricide control of tick infestations in the area. The systematic culling of white-tailed deer as a tick eradication method is regarded as unfeasible due to its high cost, regulations preserving wildlife in American Indian reservations and the ethical considerations behind this approach (George 1990). Pasture rotation methods to reduce the tick burden initiated in the 1970s appear to have failed due to the abundance of white-tailed deer and other wild ungulate species (Pound et al. 2010; Lohmeyer et al. 2011).

Two other methods to control ticks on white-tailed deer exist: acaricides and vaccination. Acaricides include systemic treatments through the consumption of ivermectin-medicated corn and/or topical treatments using 4-poster deer treatment bait stations and/or 2-poster deer treatment feeder adapters, both of which passively apply acaricide topically to deer (Pound et al. 2010). Vaccines against cattle ticks became available in the early 1990s as a cost-effective alternative for tick control that reduced acaricide use as well as the associated problems such as the selection of acaricide-resistant ticks, environmental contamination and the contamination of milk and meat products with acaricide residues (de la Fuente and Kocan 2003; de la Fuente et al. 2010). Vaccination trials with commercial vaccines containing the *Rhipicephalus microplus* BM86 and BM95 gut antigens, Gavac® and TickGARD® (Heber Biotec S. A., Havana, Cuba and Hoeschst Animal Health, Australia), reduced the number of engorging female ticks, their weight and their reproductive capacity, thus resulting in the reduction of tick infestations and in the prevalence of some tick-borne pathogens (de la Fuente and Kocan 2003; de la Fuente et al. 2010). Other candidate protective antigens such as subolesin (SUB) have recently been proposed for the control of different tick species and other ectoparasites (de la Fuente et al. 2011). Vaccination with BM86 and SUB tick protective antigens have reduced tick infestations in red deer (*Cervus elaphus*) and white-tailed deer with an overall vaccine efficacy of approximately 80% for the control of *R. microplus* infestations in white-tailed deer (Carreón et al. 2012).

Wildlife population control

Many factors contribute to the natural regulation of wildlife abundance. Herbivores, which are likely to be particularly relevant for shared disease maintenance,

are probably limited by food availability and predation or hunting harvests (Ripple and Beschta 2012). Disease itself is a mechanism that may regulate wildlife populations. The problem of overabundant wildlife populations and thus, an increased reservoir population, may occasionally be addressed by using relatively simple management actions such as feeding bans or increased harvesting (O´Brien et al. 2011; Carstensen et al. 2011; Boadella et al. 2012).

It has been demonstrated that the supplementary feeding of red deer has a strong effect on the reproductive success of hinds, and hence on population productivity (Rodriguez-Hidalgo et al. 2010). However, feeding bans will have little to no effect on overabundant populations that are not provisioned, such as those in protected areas (e.g. Gortázar et al. 2008). Feeding bans have been known to generate conflict with hunters and landowners if baiting and feeding is perceived as a traditional and rewarding practice by which to increase the hunting harvest (Carstensen et al. 2011, Gortázar et al. 2011) or other perceived values (e.g. deer as a symbol of natural resources for Michiganders, O´Brien et al. 2006).

The total elimination of a reservoir species is impractical, expensive, and ethically and ecologically unacceptable unless it targets an introduced species (Rupprecht et al. 2001). Moreover, hunting has limitations in its ability to control wildlife populations, for example, in protected areas or urban habitats, and the effects of culling are only temporary if population control is not sustained over time. It is also known that eliminating or substantially reducing the number of abundant species can have indirect effects on other species. For instance, fox numbers increased after badger culling for TB control in the UK (Trewby et al. 2008); and deer and moose (*Alces alces*) numbers increased, as well as grazing pressure and habitat damage, when carnivore culling was conducted in Canada (Macdonald 1980). Culling also has effects over the

targeted species such as increased movement due to social disruption (dispersal and immigration; Woodroffe et al. 2006; Holmala and Kauhala 2006; Carter et al. 2007; Woodroffe et al. 2008) and compensatory reproduction (Hanson et al. 2009). The aforementioned reasons have led some authors to state that culling reservoir populations in order to mitigate or control the transmission of pathogens has proven disappointingly inefficient (Bolzoni et al. 2007; Lachish et al. 2010; Artois et al. 2011, Hallam and McCracken 2011) and EFSA to advise against the wild boar mass culling carried out to control ASF transmission in some EU member estates (EFSA 2014).

Random culling may be considered for overabundant populations of introduced species or game species if feeding bans and sustainable habitat management are not feasible. Random culling to control overabundance should be explored before testing other more costly means. As shown in Table 1, random culling can, under certain circumstances, contribute to wildlife disease control. Models suggest that in pathogens that depend on frequency-dependent transmission, culling or increased harvesting can eradicate the disease when birth or recruitment induces the compensatory growth of new, healthy individuals, which has the net effect of reducing disease prevalence by dilution (Potapov et al. 2012). Harrison et al. (2010) proposed that the use of wildlife culls for disease control should be proposed only when: (i) the pathogen transmission cycle is fully understood including all the host (vector) interactions; (ii) the response of wildlife populations to culling is known; and (iii) a cost-benefit analysis shows that increased revenue or benefit from reduced disease prevalence exceeds the cost of culling. In practice, random culling is seldom a stand-alone tool but rather one of several elements of an integrated disease control strategy, often based on vaccination.

A more socially acceptable alternative to random culling is selective (or targeted) culling, similar to test and cull schemes applied to domestic animals. Such

actions can be very expensive, and their feasibility depends on access to the animals, the availability of convenient, sensitive and specific tests, the prevalence of the infection, and the spatial distribution of the target population (Table 1).

Table 1. Attempts to control diseases shared with wildlife through population control.

Type of population control	Wildlife species; Pathogen targeted; Site	% population reduction; % infection reduction in wildlife	Efficacy (in terms of reduced contact or infection in livestock or humans)	References
Culling and hazing (bison outside the park are hazed back or culled)	Bison; *Brucella abortus*; Yellowstone, Montana, USA	Negligible; n.a.	Cattle incidents continue	Plumb et al. 2009
Random culling	Wild boar; *Mycobacterium bovis*; Spain	50%; 21-48%	Wild boar abundance correlated with annual % of skin test reactor cattle; TB lesion prevalence declined in sympatric red deer	Boadella et al. 2012
Random culling	Wild boar; *M. bovis*; Spain	67%; Negligible	TB lesion prevalence declined in sympatric fallow deer	García-Jiménez et al. 2013
Random culling (local proactive culling)	Badger; *M. bovis*; RBCT, UK	69-73%; n.a.	Variable. Greater effects on cattle breakdowns during post-culling period	Jenkins et al. 2008; Woodroffe et al. 2008
Random culling (widespread proactive culling)	Badger; *M. bovis*; Ireland *(four areas)*	n.a.; 25%	52-82% less of confirmed cattle restrictions	Griffin et al. 2005
Random culling (reactive culling)	Badger; *M. bovis*; Laois Co., Ireland	n.a.; n.a.	Higher survival time to future bTB episodes in cattle herds	Olea-Popelka et al. 2009
Random culling (den gassing)	Badger; *M. bovis*; Avon, UK	n.a.; n.a.	Substantially reduced risk of infection for cattle and no new cases in 10 years	Clifton-Hadley et al. 1993; Corner et al. 2011
Random culling	Red deer and wild boar; *M. bovis*; Brotonne, France	Close to 100% in red deer and significant in wild boar; 86%, 82%	No new cattle breakdowns since 2006	Hars et al. 2010

Random culling	Possum; *M. bovis*; New Zealand	Locally close to 100%; n.a.	92% decline in number of infected herds	O'Brien et al. 2011
Random culling	Feral water buffalo; *M. bovis*; Australia	99%; 100%	100%	Radunz 2006
Random culling (restricted; + restricted feeding and baiting)	White-tailed deer; *M. bovis*; Michigan, USA	n.a.; 63% but still maintenance hosts	Herd breakdowns continue	Carstensen and DonCarlos 2011
Random culling (intense; +feeding and baiting ban)	White-tailed deer; *M. bovis*; Minnesota, USA	50%; 100%	Minnesota regained TB free status in 2010	Carstensen and DonCarlos 2011; Carstensen et al. 2011
Random culling	European starling; *Salmonella enterica*; Texas, USA (feedlots)	66%; n.a.	No apparent reduction in cattle, but disappeared from feed bunks and substantially declined within water troughs	Carlson et al. 2011
Random culling	White-tailed deer; Ticks (*Borrelia burgdorferi* vectors); Moneghan island, Maine, USA	100%; significant tick abundance reduction	n.a.	Rand et al. 2004
Random culling	Wild boar; CSF virus; French Vosges Forest, France	Hunting biased to piglets and juveniles; negligible	No measurable effect	Rossi et al. 2005 and references therein
Random culling	Wild boar; Suid Herpesvirus 1 - Aujeszky's disease virus; Spain	50%; 0%	n.a. (no pigs present on treatment sites)	Boadella et al. 2012
Random culling (several studies)	Fox and other carnivores; Rabies virus; Europe and North America	Variable; not sufficient	n.a.	Rupprecht et al. 2001 and references therein
Selective culling	Bison (fenced wood bison); *B. abortus*; Elk Island NP, Canada	n.a.; 100%	n.a. (no cattle present on treatment site)	Pybus and Shury 2012
Selective culling (+ vaccination of calves)	Elk and Bison (fenced plains bison); *B. abortus*; Elk Island NP, Canada	n.a.; 100%	n.a. (no cattle present on treatment site)	Pybus and Shury 2012

Selective culling	African buffalo; *M. bovis*; Kwazulu/Natal, South Africa	n.a.; 50%	n.a. (no cattle present on treatment site)	Michel et al. 2006
Selective culling	White-tailed deer; *M. bovis*; Michigan, USA	Negligible; 0%	n.a.	Cosgrove et al. 2012
Selective culling	White-tailed deer; Chronic Wasting Disease (prion); Colorado, USA	Negligible; estimated to take 5-10 years to reduce from 8% to <2%	Locally feasible, but not in large areas owing to costs ($300/animal plus personnel time)	Wolfe et al. 2004

n.a.: not available

Random and selective culling strategies are more likely to succeed in isolated populations than on large geographical scales, and the results will probably consist of a certain reduction of disease prevalence in the wildlife host and in the domestic host targeted, rather than in the total eradication of the infectious agent (Pybus and Shury 2012). The success of a culling scheme will also depend on the attributes of the specific infectious agent targeted (Boadella et al. 2012). Increased research into random and selective culling, with simultaneous alternative methods such as immunocontraception or feeding bans, is needed. Indeed, fertility control methods as immunocontraception are perceived by the general public as a more acceptable manner for limiting wildlife population than culling (Fagerstone et al. 2002; Rutberg and Naugle 2008). Immunocontraception may as well be a tool to control venereal and vertical transmitted diseases (Rhyan et al. 2013) and has several advantages over culling as no compensatory reproduction or behavior disturbances take place (Carter et al. 2009). However, long-term effectiveness and side effects have to be further investigated (Massei et al. 2012).

Vaccination and medication

In this context, wildlife vaccination to reduce infection prevalence emerges as a valuable alternative or complementary tool in disease control. Disease control through the vaccination of wildlife reservoirs may potentially have advantages over other approaches. As opposed to culling, vaccination may be more acceptable to the general public (Beltrán-Beck et al. 2012) since it is a non-destructive and sustainable (does not increase the susceptible animals in the population) method of controlling disease in wildlife.

The best vaccination method for wildlife populations spread over a wide geographical area is oral vaccination using baits. The oral vaccination of wildlife is the only disease management tool with proven efficacy on large spatial scales. This has been shown most clearly in the case of fox rabies control in Western Europe (Müller et al. 2005). Table 2 summarizes the most significant wildlife vaccination assays carried out in the field, and their outcomes. Many more host/pathogen binomia are currently being evaluated in the laboratory or are beginning to be investigated in preliminary field studies (e.g. Beltrán-Beck et al. 2012). Such ongoing studies are not included in this review.

However, wildlife disease control can eventually interfere with wildlife ecology. In diseases where vaccination significantly reduces target host mortality, effects on sympatric prey, predators or competitors may occur (Slate et al. 2009; Chauvenet et al. 2011) while this is unlikely for chronic and endemic diseases. In addition, some management tools commonly used to improve bait deployment, such as artificial feeding, are known drivers of reproductive success (Rodriguez-Hidalgo et al. 2010) and can increase wildlife spatial aggregation at feeding sites (Vicente et al. 2007). As

discussed previously, these methods can actually increase disease transmission if applied on a wide scale for prolonged periods of time. Vaccines must demonstrate biosafety for non-target species (vaccines against diseases, such as CSF, that affect only one species do not represent a risk for non-target species; Rossi et al. 2010) and physical stability to endure environmental temperature conditions, though inactivated vaccines circumvent this requirement (some effective oral inactivated vaccines are already being developed, Beltrán-Beck et al. 2014a). Approaches within natural ecosystems should therefore first be carefully tested in trials that are progressively extended to a larger scale (Artois et al. 2011).

Medication of wild animals can rarely be used to reduce the burden of disease in wild populations and very few examples exist in the literature of the medication of free-ranging wildlife in comparison to the plethora of reports on vaccination. Among these, the control of *Echinococcus multilocularis* in foxes is a prominent example. The adult fox tapeworm is sub-microscopic and infects foxes and, less efficiently, dogs. The larval form infects several wild rodents. In villages and small towns in central Europe, foxes are responsible for environmental *E. multilocularis* egg contamination in the vicinity of humans, leading to infection risk if humans accidentally ingest viable eggs (Janko et al. 2011). The knowledge developed for fox rabies vaccine delivery through oral baits has been built on to employ similar strategies by which to deploy the anthelminthic praziquantel (König et al. 2008).

An important concern when releasing drugs into the environment is biosafety (Boxall 2004; Horvat et al. 2012). Though, the presence of anthelmintic compounds in the environment is mainly derived from their massive use in the livestock industry.

Table 2. Attempts to control diseases shared with wildlife through vaccination.

Pathogen targeted; Wildlife species; Site	Vaccine deployment	% reduced infection in wildlife	References
Classical Swine Fever virus; Wild boar; France	Oral (preventive vaccination)	n.a Effective prevention of infection maintenance	Rossi et al. 2010
Foot and Mouth Disease virus; Buffalo and other wildlife; South Africa	Cattle vaccination in contact areas with infected wildlife	n.a Breakdowns linked with fence permeability, vaccination coverage, and efficiency of animal movement control measures	Jori et al. 2009
Mycobacterium bovis; Badger; UK	Parenteral	61-72% reduction in the incidence of positive test results	Chambers et al. 2011
M. bovis; Possum; New Zealand	Oral	95-96%	Tompkins et al. 2009
Rabies virus; Coyote; Texas, USA	Oral	100%	Sidwa et al. 2005
Rabies virus; Grey fox; Texas, USA	Oral	n.a.	Sidwa et al. 2005
Rabies virus; Raccoon; Ontario, Canada	Oral	n.a., contributed to geographical containment	Slate et al. 2009
Rabies virus; Raccoon; Wolfe Island, Ontario, Canada	Oral and parenteral (+ rabies-caused mortality)	100%	Rosatte et al. 2007
Rabies virus; Red fox; Germany	Oral	100%	Müller et al. 2005
Rabies virus; Red fox; Ontario, Canada	Oral	Close to 100%, but persists in skunks	MacInnes et al. 2001; Slate et al. 2009
Rabies virus; Red fox and raccoon dog Estonia	Oral	100%	Cliquet et al. 2012

Compartmentalization and zoning: knowing the problem and living with it

Both compartmentalization and zoning (or zonification) can and have been implemented by countries or states in order to define sub-populations of varying health statuses for disease control. This could become one of the best solutions for disease control at the wildlife-livestock interface in the future (see Artois et al. 2011 review). The idea of zoning consists of defining a geographical area in which an infection exists in order to differentiate its infection status from other zones. This has, for example, been proposed for Yellowstone bison (*Bison bison*), suggesting that the inherent cost of declaring a brucellosis-infected zone would be far lower than current management to avoid *Brucella abortus* spillback to cattle (Bienen and Tabor 2006). It is also carried out de facto as regards *M. bovis* and *B. abortus* infected wood bison in Wood Buffalo N.P. in Alberta, Canada (O'Brien et al. 2011; Pybus and Shury 2012) and for several wildlife species carrying FMD in Namibia and Zimbawue (Sutmoller 2002; Schneider 2012).

A related concept is compartmentalization, during which segregation is based on production-linked establishments and types of animal husbandry and biosecurity, rather than on geographical boundaries. Free-ranging domestic pigs could, for instance, belong to a different (and more at risk) compartment than industrial pigs, thus allowing a different status to be defined for each compartment.

Economic effects of no action

Inaction is a frequent decision in the control of wildlife diseases. This is due to the fact that, for most diseases, there is no strong justification for intervention (in terms of public or animal health conservation) or if justification exists there are no suitable and cost-efficient disease control tools available (Wobeser 2007). Regardless, the

decision to take no action should be accompanied by monitoring in order to assess the effect of this inaction on pathogen maintenance and on animal and human health. This would allow our strategy to be changed if monitoring proves that our decision should be reconsidered (Wobeser 2007).

Taking no action to control diseases can result in higher costs. One example is the dramatic increase in prevalence of TB in badgers after the suspension of TB cattle testing during the FMD epidemic in the UK in 2000-2001. This was ascribed to the high prevalence of cattle herd infection and cattle with advanced disease (Woodroffe et al. 2006). In New Zealand, the control of the invasive Australian brushtail possum (*Trichosurus vulpecula*) ceased during an economic crisis in the early 1980s. Almost immediately, cattle TB prevalence rose (P. Livingstone, personal communication). Modelling offers a useful alternative approach to the development of management criteria and facilitates the consideration of ecological-economic trade-offs, signifying that diseases are managed in a cost-effective manner (Fenichel et al. 2010; Alexander et al. 2012).

Wildlife disease control in the 21ˢᵗ century: towards integrated disease control schemes

Various general inferences can be made from the review given above. First, setting up a proper disease and population surveillance and monitoring scheme is an absolute priority; even before deciding whether or not to intervene (Figure 1). For example, the information provided by the European research consortia APHAEA and ANTIGONE constitutes valuable knowledge with which to start up a surveillance network (APHAEA 2013; ANTIGONE 2011). Second, all options for disease control at

the wildlife-livestock-human interface, including those of no intervention, need to be considered, either individually or combined. Third, combining several disease control tools in integrated strategies is likely to reduce the cost and effort required for disease control. Integrated strategies are also preferred since no single control measure is universally applicable (White et al. 2008). However, when more than one tool is used in a control strategy, the relative contribution of each one is confounded (Rosatte et al. 2007; Carstensen and DonCarlos 2011). Fourth, the success of disease control in wildlife depends on many factors, including a) the single or multi-host nature and other characteristics of the pathogen, b) the availability of suitable diagnostic tools, c) the characteristics of the wildlife host(s) and vectors, d) the geographical range of the pathogen/reservoir (improved control in isolated versus continuous populations) and the scale of the control effort (large scale longitudinal programs are better), e) the attitude of the stakeholders involved (highly dependent on their education and communication provided to them).

One particular field deserving increased attention is the One Health approach, meaning a need for better collaboration between public health, veterinary, and environment services in order to address shared diseases. For instance, game species depend on veterinary services while on the farm, on environment services after their release into the wild, and on public health services after being harvested for human consumption. Despite this fact, inter-agency information exchange and collaboration is often limited. To overcome this difficulty, governments should consider setting up "One Health working groups", aimed at improving inter-agency collaboration for instance through specific information exchange mechanisms and through joint risk assessment exercises considering not just one of the three compartments (e.g. Hartley et al. 2013). Also, the potential of wildlife rescue centers for the monitoring and early detection of

potentially zoonotic or economically relevant diseases is often neglected (Gourlay et al. 2014). In fact, disease in wildlife populations has been compared to an iceberg (with only the tip of the total mass being visible at any time; Wobeser 2007) because there were few people looking for it and other considerations related to the wilderness of wildlife (difficulties in detecting and measuring disease and individuals theirselves). Nowadays, several surveillance and monitoring schemes are operating in wildlife worldwide (Kuiken et al. 2011; CCWHC 2013; USGS-NWHC 2014; WHA 2014) and generating a considerable amount of valuable information. As mentioned earlier, the number of EID events caused by pathogens originating in wildlife and the risk they represent to global health evidences the necessity of engagement between these wildlife specialists and other agencies (WHO, OIE).

Most current monitoring and disease control efforts in wildlife are directed toward only a few relevant diseases, including rabies, ASF, CSF, FMD, CWD, brucellosis, TB, *E. multilocularis* and tick-borne diseases. In the future, it is likely that this list will become longer as new scenarios and disease control needs emerge. Future wildlife disease control efforts will probably rely on a better understanding and modelling of wildlife-pathogen interactions (Alexander et al. 2012), thus improving biosafety and prevention. Other fields expected to grow include immunocontraception for population control, selective culling and, most notably, vaccination. New vaccines will hopefully permit more cost-effective, biosafe and cheaper disease control in wildlife. Recent results with inactivated *M. bovis* vaccines (Garrido et al. 2011; Beltrán-Beck et al. 2014a) and recombinant arthropod vector vaccines for the control of both vector infestations and pathogen transmission (de la Fuente et al. 2011 and 2012) support this research direction. The development of effective vaccines for wildlife is still in its infancy, but the results reviewed here have demonstrated the possibilities and

advantages of integrated control strategies, and encourage support to expand research in this area in order to contribute to the eradication of wildlife-associated diseases.

Finally, from a global point of view, disease control schemes should be aimed at the accomplishment of a balance. Most of the above-mentioned examples of shared wildlife diseases are resultant of unbalanced situations in which, for instance, wildlife has increased in numbers, often as the result of anthropogenic factors (such as rural abandonment or land use changes, Gortázar et al. 2006). Any proposed control scheme that does not target re-establishing an ecological balance will probably be limited to a short-term success instead of long-term disease control.

Acknowledgements

Wildlife disease control case study: controlling tuberculosis in wild boar by vaccination

The present thesis approaches control options of shared diseases where wildlife plays a significant role in maintenance using the control of animal tuberculosis in wild boar (*Sus scrofa*) by vaccination as a case study.

Why animal tuberculosis?

Animal tuberculosis (TB, see Box 1) is a chronic disease that causes granulomatous lesions (with varying size and degrees of necrosis, calcification and encapsulation) affecting mainly lymph nodes and lungs (although lesion distribution depends on species affected and route of infection; Corner 2006). The etiological agents causing this disease are members of the *Mycobacterium tuberculosis complex* (MTC) such as *Mycobacterium bovis* (*M. bovis*) and *Mycobacterium caprae* (*M. caprae*).

TB is the perfect example of a shared disease as these pathogens are capable to infect and produce disease in multiple animal species, domestic and wild, including humans (Buddle et al. 2013; Langer and LoBue 2014). This multi-host situation allows for complex interactions (by direct contact, via the environment or by use of shared resources) at the wildlife-livestock-human interface (Kukielka et al. 2013; Cowie et al. 2016). This creates complex epidemiological scenarios that translate into an increased difficulty to control disease (Renwick et al. 2007; Michel et al. 2010; Nugent 2011).

Box 1. Animal tuberculosis, not just a cattle problem & not just *M. bovis*

For decades cattle have been recognized as the main reservoir and control target for *M. bovis* (de la Rua-Domenech et al. 2006), hence the disease was termed bovine TB (bTB).

Disease control of bTB is mainly conducted in developed countries through eradication programmes. These programmes are focused exclusively on cattle and aim to achieve an officially TB free status (OTBF, herd prevalence below 0.1 and 99.9% of herds officially free during 6 years) by means of compulsory testing, culling of positives and movement restrictions. This scheme has been successful reducing bTB incidence but was unable to achieve an OTBF status in all countries (Radunz 2006; Reviriego Gordejo and Vermeersch, 2006; Corner et al. 2007). This fact raised concerns about the existence of other reservoirs that could act as a source of infection hampering eradication efforts (Haydon et al. 2002; Gortázar et al. 2007; Pesciaroli et al. 2014).

Further studies have evidenced the existence of competent domestic hosts such as goats (Napp et al. 2013), sheep (Muñoz-Mendoza et al. 2015), pigs (Parra et al. 2003; Di Marco et al., 2012); and wildlife reservoirs (Table 1). All these hosts, none of them subjected to mandatory control strategies (except for goats coexisting with cattle herds), contribute to explain why many countries have reached a plateau or even an increase in bTB figures recently (both in OTBF and non-OTBF countries; Abernethy et al. 2013, Marsot et al. 2016).

Also, works on genetics have prompted the acceptance of *M. caprae* as a new species (Aranaz et al. 2003). *M. caprae*, is considered a primary goat pathogen but it is also a common cause of TB in other livestock species, wildlife and humans (Prodinger 2005 and 2014; Rodriguez et al. 2011).

Considering this knowledge the term bTB can be deceiving, as the disease is neither restricted to cattle population nor only produced by *M. bovis*. Hence the term animal tuberculosis can reflect more accurately the disease and improve risk perception.

In particular, the role of wildlife reservoirs in TB maintenance is increasingly recognized worldwide (Corner 2006). Known TB scenarios where a wild reservoir is involved can be seen in Table 1.

Table 1. Known wildlife reservoirs worldwide.

Area	Acknowledged wildlife reservoirs	Reference
Africa	Buffalo (*Syncerus caffer*) Kudu (*Tragelaphus strepsiceros*) Lechwe antelope (*Kobus leche*) Warthog (*Phacochoerus africanus*)	Rodwell 2001 Michel et al. 2006 Hlokwe et al. 2014
Europe	Red deer (*Cervus elaphus*) Fallow deer (*Dama dama*)	Gortázar et al. 2012 Fink et al. 2015
Iberian Peninsula	Wild boar (*Sus scrofa*) Red deer (*Cervus elaphus*) Fallow deer (*Dama dama*)	Naranjo et al. 2008 Gortázar and Boadella 2014
UK Republic of Ireland	Badger (*Meles meles*)	Clifton-Hadley et al. 1993 Gormley and Corner 2013
North America		Miller and Sweeny 2013
Canada	Elk (*Cervus canadensis*) Bison (*Bison bison*)	Nishi et al. 2006
USA (mainland)	White tailed deer (*Odocoeilus virginianus*)	O´Brien et al. 2002
USA (Hawaii)	Feral pig (*Sus scrofa*)	Bany and Freier 2000
New Zealand	Possum (*Trichosurus vulpecula*)	Nugent et al. 2014

Moreover, TB is a suitable example for disease control in wildlife as it fulfills the criteria to be considered relevant enough to intervene, i.e. it has an impact over Public Health, economy and wildlife management and conservation (Cousins 2001; Gortázar et al. 2015a).

According to the World Health Organization (WHO) human tuberculosis (hTB) represents the major cause of human deaths worldwide and is the leading cause of death due to an infectious disease (WHO 2017). While hTB primary etiological agent is

Mycobacterium tuberculosis the contribution of *M. bovis* and *M. caprae* (zoonotic TB, Figure 1) to hTB figures makes them a concern for Public Health services worldwide although is likely to be underrated (Müller et al. 2013; Langer and LoBue 2014; Prodinger et al. 2014). Nevertheless, the main impact of zoonotic TB occurs in developing countries where livestock sanitary status is less controlled, food safety measures as inspection and hygienization of animal products are largely absent and inmunosupression triggered by HIV/AIDS highly prevalent (Cosivi et al. 1998; Michel et al. 2010). Since the human burden of disease cannot be reduced without managing the animal TB reservoir, the "End TB strategy" set up by WHO includes zoonotic TB engaging all relevant sectors in line with a One Health approach. The aim of this strategy is to significantly reduce TB incidence and deaths in humans by 2030 in a context of increased immunosuppressive co-infections, co-morbidities and the emergence of drug resistant strains (WHO 2017).

Figure 1. Information about the impact of zoonotic TB and the transmission chain considering the role of wildlife (Source: Joint WHO- FAO-OIE initiative).

TB also has a severe economic impact over livestock industry, wildlife-related business and to national economies (Zingstagg et al. 2006). Firstly, it affects the livestock industry through decreased production due to the chronic nature of the disease, through animal loss (mandatory slaughter of reactors), carcass condemnation and movement restrictions due to the eradication scheme set up for cattle (Reviriego Gordejo and Vermeersch 2006; Zingstagg et al. 2006). Secondly, in wildlife-related business (hunting, commercial game farming and ecotourism) the profitability could be reduced due to the death of highly valuable individuals and /or through poorer trophy and sighting prospects (e.g. lions, *Panthera leo*, and African buffalo two of the "Big five", Michel et al. 2010; wild boar, Barasona et al. 2016). Thirdly, it also incurs in additional costs for the public administrations derived from eradication scheme associated costs (testing and compensation), loss of export revenues (due to trade barriers imposed to non-OTBF countries), medical costs (in public healthcare systems), and loss of human economic productivity due to sickness (Zingstagg et al. 2006; Smith et al. 2009; Kyu et al. 2018).

Regarding its relevance over conservation, due to the wide range of susceptible hosts TB can affect protected or even endangered species that might be involved in disease maintenance (e.g. badgers, Gormley and Corner 2013) or not (incidental or spill-over hosts, e.g. lions, Viljoen et al 2015) threatening conservation efforts. This impact is more drastic in endangered and small isolated populations such as the Iberian lynx (*Lynx pardinus*) where infectious diseases including TB are the main cause of mortality (López et al. 2014).

Finally, it is important to note that intervention over wildlife is intended as part of an integrated strategy and does not preclude from continuing control strategies over

domestic reservoirs especially, but not exclusively, in cattle, which is the main reservoir of *M. bovis* and major source of herd infection (Guta et al. 2014; Hardstaff et al. 2014).
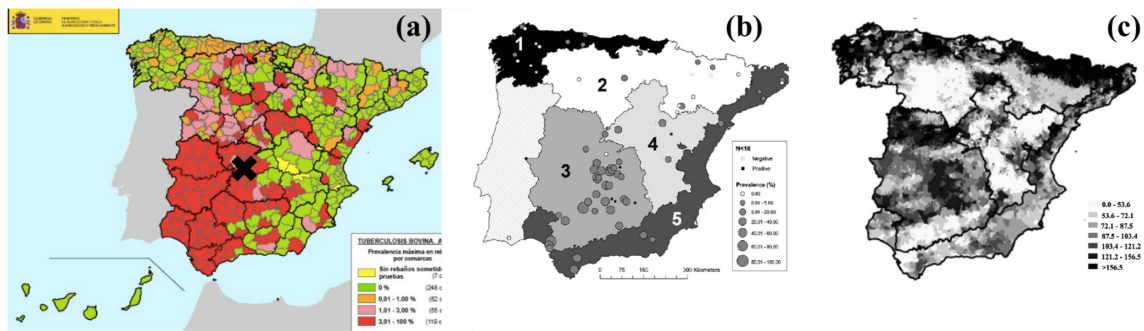
Why wild boar?

The variety of wild reservoirs and their relative importance among settings makes necessary to consider local specificities to identify the target of disease control tools (Corner 2006; Gortázar et al. 2015a).

In Spain, disease persists in cattle at levels above international standards of TB freedom but herd prevalence has decreased from the 11% to less than the 3% in the last 30 years although the situation is not homogeneous among regions (Figure 2a, Allepuz et al. 2011; MAPAMA 2018). In this context, the importance of reservoirs increases as disease control over cattle progresses (Gortázar et al. 2012; MAPAMA 2017). TB is widely distributed in the ecosystem and endemic in wild ungulates (Aranaz et al. 2004; Vicente et al. 2006 and 2013). Among them, wild boar is recognized as the main reservoir (Naranjo et al. 2008), role supported by cumulative evidence proving its ability to maintain and to transmit disease to sympatric species.

Wild boar is highly susceptible to TB and the recorded prevalence is among the highest reported for any species (mean prevalence 63 %; Vicente el al. 2013, that can be locally higher e.g. 92% in Doñana National Park; Gortázar et al. 2008). Lesion pattern suggests natural infection is acquired via direct oro-nasal transmission or via indirect food/waterborne transmission at shared watering holes and feeding sites (Martín-Hernando et al. 2007). The transmission potential harbored by wild boar is confirmed by de detection of *M. bovis* excretion by oral, nasal, urinary and fecal routes (Santos et

al. 2015; Barasona et al. 2017). Bacterial load and shedding pattern are associated with disseminated disease affecting various organs, thus generalized individuals are believed to be the major drivers of infection maintenance and key targets for disease control (super-spreaders, Kramer-Schadt et al. 2009; Santos et al. 2015; Barasona et al. 2017).

Figure 2. Map of Spain displaying (a) cattle TB prevalence (Source: MAPAMA 2018), (b) wild boar seroprevalence (dot size is proportional to prevalence, Source: Boadella et al. 2011b) and (c) wild boar relative abundances (Source: Acevedo et al. 2014). These maps evidence the contrasting situation of South Central Spain and the northern part of the country and the spatial overlap of cattle TB prevalence with wild boar abundance and TB prevalence. ✖ Montes de Toledo.

Regarding transmission, wild boar are capable of sufficient intra-species transmission being able to maintain TB in absence of other wild or domestic hosts (Gortázar et al. 2005; Vicente et al. 2007; Mentaberre et al. 2014). The high infection pressure faced by wild boar is evidenced by the high antibody prevalence in piglets (44%) and the high seroconversion rate (80%) experienced before becoming subadults (Che'Amat et al. 2016). Wild boar may spread disease to other species as well, as suggested by the existence of shared MTC genotypes among livestock and wildlife (Aranaz et al. 2004, Gortázar et al. 2005; Hermoso de Mendoza et al. 2006). In addition, the overlap in spatial TB trends in cattle and wild boar supports transmission among cattle and wild boar (Figures 2a and 2b; Rodriguez-Prieto et al. 2012; LaHue et al.

2016). Data also supports a relationship between wild boar abundances and TB incidence in sympatric hosts (Figure 2a and 2c; Acevedo et al. 2007; Rodríguez-Prieto et al. 2012; LaHue et al. 2016). Moreover, segregating wild ungulates from cattle reduces incidence in the later (Barasona et al. 2013) and reducing wild boar densities (culling) reduced TB in sympatric ungulates (Boadella et al. 2012; Garcia-Jimenez et al. 2013) and eventually in wild boar itself (Boadella et al., 2012).

The role of wild boar is not only linked to these features but also to wild boar characteristics as a species (see Box 2) and to risk factors. Risk factors associated with disease in wild boar and with potentially enhanced transmission are abundance, aggregation, intensive management, increasing age and environmental factors (Vicente et al. 2007 and 2013). This native species is increasing its abundance and expanding its geographical range in Spain and Europe in the last decades (Acevedo et al. 2014; Massei et al 2015). Locally higher abundances and aggregation might be the result of intensive management, typically occurring in South Central Spain (SCS), that includes supplementary feeding and watering, fencing and translocation (Acevedo et al. 2007; Vicente et al. 2007 and 2013). Nonetheless, TB may occur in the absence of management, either in overabundant populations (e.g. National Parks, Gortázar et al. 2008) or even in low-density populations (Mentaberre et al. 2014). Accumulated risk of infection occurs with increasing age (higher prevalence detected in adult age class) although juveniles are most likely to be generalized and suffer from TB-induced mortality (Martín-Hernando et al. 2007, Naranjo et al. 2008; Vicente et al. 2013). Additionally, environmental drivers as low rainfall and summer droughts that occur frequently in Mediterranean habitats promote aggregation around limited resources (Vicente et al. 2013). Once these factors are identified, management actions to reduce risk are key strategies in disease control schemes.

These studies provide compelling evidence of the main role of wild boar in TB epidemiology and make timely to consider wild boar a key target for TB control in Spain.


Box 2. Wild boar, species characterization

Wild boar is one of the 17 species within the Family Suidae. This species is native to Eurasia where is the most widespread ungulate and has propagated to every continent except Antarctica (Barrios-García and Ballari 2012).

Its success can be attributed to its high plasticity regarding diet, reproduction and behavior. Wild boar are opportunistic omnivores; their diet is mainly composed of vegetables but can also scavenge and predate on vertebrates (Ballari and Barrios-García 2014). Regarding reproduction wild boar are precocious and prolific (2-6 piglets/ litters and 1-2 farrows per year) and are able to adjust to changing habitat quality by eliciting compensatory breeding and reproductive responses (Bieber and Ruf 2005). Mortality is mainly caused by hunting since they mostly lack predators (Massei et al. 2015). The social structure depends on gender; females tend to gather in social groups, sounders, while males are more solitary and juveniles are the dispersing age group (Truvé and Lemel 2003). Wild boar are also intelligent, able to undercrosss fences and highly adaptable to different forms of management and multiple habitats even thriving in human-dominated landscapes like peri-urban settings (Podgórski et al. 2013).

Due to these characteristics, anthropogenic factors (changes in land use and farming practices, translocations etc) and climatic change wild boar are increasing in numbers and geographical range in the last decades (Bieber and Ruf 2005; Massei et al. 2015). Higher abundances can intensify wild boar impacts and human-wildlife conflicts. Conflicting views on wild boar already coexist as is perceived as an iconic game species by hunters and as an invasive pest by other stakeholders (Massei et al. 2015). Is important to note that the impact of wild boar is not just ascribed to sanitary issues but also to conservation (predation, habitat destruction, ecosystem damage), agriculture (crop damage) and traffic accidents (Barrios-García and Ballari 2012; Massei et al. 2015).

The field trials in this thesis have been conducted in Montes de Toledo (SCS, Figure 2) a mainly agricultural area and a TB hotspot. In this region, wild boar is a highly valued game species and an essential component of the local economy. The upsurge of associated economical activities has led to changes in wildlife management towards more intensive models (Gortázar et al. 2007). Therefore, wild boar may occur as unmanaged populations (open and truly free-ranging populations; e.g. protected areas, communal lands), as managed populations (through fencing, supplementary feeding and translocations, either as single species business or as a mixed practice with livestock) and as farmed stock; as shown in Figure 3. These scenarios complicate epidemiology and control of TB but provide a great opportunity to make the transition from laboratory to real life settings and to evaluate control tools under natural MTC transmission, in presence of other pathogens and hosts, under different management regimes and under the influence of environmental and climatic factors.

Unmanaged (free-ranging) wild boar → Managed (fenced) wild boar → Farmed wild boar
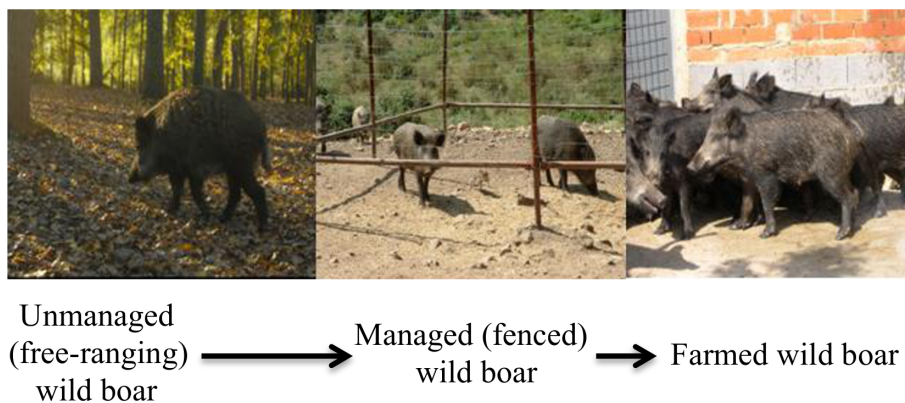
Figure 3. Gradient of wild boar management occurring in South Central Spain from extensive to intensive models (adapted from Gortázar et al. 2007).

Why vaccination?

As previously stated, the optimal approach is to combine tools from several fields in an integrated control strategy, however, is necessary to previously assess these tools individually to evaluate their performance. Since other options have already been evaluated in the TB- wild boar system (Table 2) and vaccination poses advantages over other methods (see Box 3) we proceeded to evaluate the potential of vaccination.

Vaccination, along with sanitation and hygiene, has been one of the most effective interventions to prevent and curb disease in humans and animals (Rappuoli et al. 2002; Woodland 2004) even achieving eradication of smallpox in humans (WHO 1980) and rinderpest in artiodactyls (Anderson et al. 2011). Vaccination is based on the delivery of a non-pathogenic but immunogenic antigen to stimulate a protective response against a later exposure to the pathogen.

Table 2. Summary of control strategies evaluated in the wild boar-TB system.

Control strategy	Results	References
Farm biosecurity Segregation of wild boar (and other wildlife) and cattle by fencing	66% reduction in cattle TB skin reactors.	Barasona et al. 2013a
Tailored biosafety schemes represent a great opportunity for the livestock sector to avoid disease transmission from wild reservoirs; nonetheless it has likely no benefit over wildlife sanitary status.		
Random culling Population control	21- 48% prevalence reduction in wild boar and decrease in sympatric wild ungulates.	Boadella et al. 2012.
	No effect over wild boar but observable decline in sympatric fallow deer prevalence.	García- Jiménez et al. 2013
	Transient reduction of wild boar TB prevalence.	Mentaberre et al. 2014 (coupled with cattle removal)
Selective culling Test and cull	No effect over wild boar prevalence.	Che´Amat et al. 2016
Culling through hunting, and occasionally by trapping, is the most used management tool to intervene in wild boar conflicts related to overabundance and disease. This low cost tool needs to target a sufficient proportion of the population (potentially entering in conflict with hunting business where selective culling might be more acceptable for owners) and is likely to achieve only transient effects since wild boar populations are capable to endure and compensate high removal rates.		

Box 3. Vaccination advantages

Vaccination provides a valuable alternative in settings where:

- Is not possible to remove etiologic agent or to interrupt transmission and treatment is not an option (Rupprecht et al. 2004; Gortázar et al. 2015a).

- Wildlife is valued for conservation or business purposes (Haydon et al. 2006; McDonald et al. 2008).

- Acceptability by stakeholders (e.g. owners, general public) is key (hunting business, farms or protected areas; Bengis et al. 2002; Cowie et al. 2015).

In wildlife, vaccination is perceived as a feasible disease control option for cost effective and long-term TB control (Cross et al., 2007b).

Nonetheless, wildlife vaccination presents particularities related to the ecological characteristics of the target population as the aim of the strategy and vaccine delivery. Wildlife vaccination goal is to control rather than eradicate disease and prioritizes population over individuals ultimately aiming to reduce disease burden and spread of infection at the population level in targeted and sympatric species (Cross et al. 2007b; Buddle et al. 2013; Gormley and Corner 2013). Regarding vaccine delivery is important to differentiate farmed from free-ranging wildlife. Vaccine delivery in farmed wildlife has similarities with vaccine administration in livestock, as individuals are accessible allowing parenteral regimes. Meanwhile vaccine delivery in free-ranging wildlife is challenging due to the technical and logistical difficulties to approach individuals, in this situation oral delivery via vaccine baits is the best option to reach populations over a wide geographical area (Cross et al. 2007b, capture and handling individuals is considered less cost-effective but still used in some species e.g. Chambers et al. 2011).

Thus, prior to the assessment of vaccination in wild boar against TB in real life settings is necessary to develop species-specific baits, to design appropriate baiting strategies and methods to estimate the proportion of vaccinated individuals as well as vaccines that are safe and effective (Box 4).

Vaccines against TB are an expanding field that needs and benefits from joint efforts of physicians and veterinarians since the discovery of Bacillus Calmette- Guérin (BCG) by Albert Calmette and Jean-Marie Camille Guérin (Vordermeier et al. 2014).

BCG is a live-attenuated *M. bovis* mutant obtained from the milk of an infected cow through serial passages in a medium containing glycerin, potato and ox bile. It was first administered in 1921 and remains the most used vaccine worldwide against TB in humans (Buddle et al. 2013; Fine 1995) and in animals (where is only licensed for badgers, Gormley et al. 2017, although experimental use is conducted in wildlife and livestock, Buddle et al. 2013). Despite being the only commercial option, BCG does not provide sterilizing immunity and has variable efficacy in humans and animals (Colditz et al. 1994; Buddle et al. 2013; Vordermeier et al 2014). Hence, the continued search of new vaccine candidates (more protective or suitable for field use) and alternative immunization strategies (such as heterologous protocols) to overcome BCG limitations. One of these candidates is heat-inactivated *M. bovis* (IV) obtained from an isolate of a naturally infected wild boar (Garrido et al. 2011). IV confers similar protection to BCG (no vaccine candidate has proven superior to BCG yet) and has added advantages for field use as being more stable (no refrigeration needed), cheaper (easier to produce, transport and store) and safer (no risk of reversion to a hazardous form and lack of multiplication) when compared to live BCG vaccines (Garrido et al. 2011; Beltrán-Beck at al. 2014a).

Another concern is the administration route, as not all formulations are suitable to be used by all routes (Aldwell et al., 2006; Garrido et al., 2011). As aforementioned oral formulations are preferred for mass vaccination of free-ranging wildlife and parenteral formulations are favored when vaccinating accessible and high valued or endangered animals. Moreover, differences in immunologic responses and protective immunity between routes are documented (Aldwell et al.1995; Garrido et al. 2011; Nol et al. 2008) which is interesting in terms of potential interference with diagnostic tests (Box 4).

Box 4. Wild boar-TB-vaccination system, state-of-the-art

The present thesis is based on previous knowledge generated in parallel research on:

- **Laboratory**, focused on fine-tuning vaccines and vaccine evaluation:

- Set up of an oropharingeal mycobacterial infection model and vaccination protocol for wild boar (Ballesteros et al. 2009a; Garrido et al. 2010).

- Development of IV vaccine (Garrido et al. 2011) and characterization of the protection mechanism (Beltrán-Beck et al. 2014b and 2014c; Juste et al. 2016).

- Evaluation of protection and response to immunization by BCG and IV, by oral and parenteral routes, in wild boar and pigs (Ballesteros et al. 2009a; de la Lastra et al. 2009; Garrido et al. 2011; Beltrán-Beck et al. 2014c).

- Test revaccination and high dose consumption effect (Gortázar et al. 2014b).

➜ BCG and IV confer similar protection in wild boar. Revaccination increases vaccine efficacy. Parenteral administration interferes with ELISA testing (generates antibodies) while oral route does not.

- **Field**, focused on developing techniques that enable wild boar oral immunization (parenteral delivery may adapt easily techniques and tools used in livestock as handling crushes and injection guns):

- Development and testing of species-specific bait to deliver pharmaceuticals orally to wild boar (Ballesteros et al. 2009b, Ballesteros et al. 2011).

- Design of delivery systems, selective piglet feeders, to target the preferred age-class (Ballesteros et al. 2009c).

- Optimization of the detection method and determination of serum persistence of the chemical marker added to baits (to estimate bait uptake rate and identify vaccinated individuals, Ballesteros et al. 2010).

- Biosafety evaluation of BCG and IV field deployment in terms of adverse reactions and excretion in the target host, vaccine strain survival and bait uptake by non-target species (Beltrán-Beck et al. 2014a).

➜ Baits coupled with selective feeders allow oral delivery of products to wild boar piglets with high specificity and uptake rates enabling to target 70% of the population with no adverse effects and minimal risks for non-target species.

A detailed review covering these issues is available in Beltrán-Beck et al. 2012.

According to Wobeser (2007) a last point to make clear before beginning any control program, including vaccination, is establishing how success will be measured. In the case of TB vaccines, the mechanism of protection remains unknown and no measurable correlate of protection has been elucidated (Vordermeier et al. 2014) complicating the development and evaluation of new candidates. Thus, TB vaccine efficacy in laboratory trials is assessed after challenge through clinical endpoints (e.g. infection, bacterial load, infectiousness, disease severity and survival) by means of culture, pathology, PCR, serology, gamma-interferon release assays (IGRA), intradermal tuberculin test (IDTB test) etc.

The most commonly used tool to assess the protective effects of TB vaccines is pathology scoring (e.g. Corner et al. 2008; Nol et al. 2008; Vordermeier et al. 2014). Pathology scoring evaluates disease severity by semi-quantitative estimation of TB lesions based on size, number and distribution in key organs. Advantages of the score over other methods are that it is easy to obtain, affordable, fast, repeatable and adds valuable information over binomial outcomes as lesion presence (Rodwell et. al 2001).

This reliable technique has been standardized for wild boar adapting inspection to previous knowledge on disease presentation (Martín-Hernando et al. 2007). In addition, scoring has been adapted to different scenarios being more detailed in laboratory trials (Ballesteros et al. 2009a) and simplified for large-scale field studies where the sample size is high and time and logistic constraints are faced (Díez-Delgado et al. 2014a). Increased accuracy and effectiveness of pathology scoring is achieved by subsequent confirmation by culture (recognized as the gold standard test but with limited sensitivity in wild boar, 82%, Santos et al. 2010) allowing the detection of individuals without visible lesions (Gavier-Widen et al. 2009).

OBJECTIVES

The present thesis aims to contribute to the field of disease control in wildlife addressing tuberculosis (TB) control in endemic areas by means of vaccination in wild boar. The **main objectives** are to evaluate the impact attained by vaccination and feasibility of this measure in real-life settings and to explore in the laboratory vaccine combinations that provide enhanced protection.

Four specific aims arise after reviewing current use of control tools in wildlife and the TB-wild boar-vaccination system. These aims are addressed by three separate experiments, each contributing one chapter to the thesis.

Specific aims:

1. To assess safety and efficacy of parenteral heat-inactivated *Mycobacterium bovis* candidate (IV) in a wild boar farm under natural TB transmission (Chapter I).

2. To compare the impact of two vaccines (traditional Bacille Calmette–Guerin, BCG, and IV) in free-ranging wild boar populations (managed and not) under natural TB transmission and the feasibility of vaccinating large areas (Chapter II).

3. To use mathematical models to answer questions that could not be tested in field such as exploring the influence of vaccination success gradients, the effect of long-term vaccination and vaccination cessation on population and disease dynamics under different initial prevalence/transmission scenarios (Chapter II).

4. To test BCG and IV combinations (heterologous vaccination) under controlled laboratory conditions in order to improve vaccine efficacy (Chapter III).

CHAPTER I

Parenteral vaccination with heat-inactivated *Mycobacterium bovis* reduces the prevalence of tuberculosis-compatible lesions in farmed wild boar

Resumen

En 2012, se implantó un programa de vacunación parenteral con *Mycobacterium bovis* (*M. bovis*) inactivado por calor (IV) con el objetivo de controlar la tuberculosis (TB) en una granja de jabalíes (*Sus scrofa*). Este trabajo pretende evaluar la seguridad y la eficacia de la administración intramuscular (IM) de IV en una granja con circulación natural de *M. bovis*. En base a resultados obtenidos previamente en condiciones de laboratorio, la hipótesis de trabajo era que los rayones vacunados mostrarían un score de lesiones compatibles con TB (LCTB) menor que los rayones control (no vacunados). No se detectó ninguna reacción adversa durante inspección visual ni en el examen postmortem (n=668 y n=97 respectivamente). Se obtuvieron datos de necropsia sobre LCTB para 97 jabalíes vacunados y 182 controles. La prevalencia observada de LCTB fue del 4.1% (95% IC= 0.2- 8%) en jabalíes vacunados y del 12.1% (95% IC= 7.1 -17.1 %) en jabalíes control (p <0.05). No se detectaron diferencias en el score medio de lesiones en los animales que presentaban LCTB (p>0.05). Los resultados muestran que la vacuna IV administrada IM a rayones es segura y protege eficazmente a los individuos vacunados (reduciendo un 66% la prevalencia de LCTB) frente a la exposición natural a *M. bovis* en un entorno de baja prevalencia. En el contexto actual de aumento de la prevalencia de TB en jabalí en hábitats mediterráneos, la vacunación permite reducir progresiva y paulatinamente la prevalencia desde el inicio de la estrategia vacunal, Así, la vacunación puede contribuir junto con otras herramientas en el control de la TB en jabalíes y cerdos.

Abstract

In 2012, a wild boar (*Sus scrofa*) tuberculosis (TB) control program was set up in a wild boar farm by means of intra-muscular (IM) vaccination with a heat-inactivated *Mycobacterium bovis* (*M. bovis*) vaccine (IV). The goal was to assess safety and efficacy of the parenterally administered IV in a large farm setting with natural *M. bovis* circulation. Based on preceding results under laboratory conditions, we hypothesized that vaccinated piglets would show smaller scores of TB-compatible lesions (TBCL) than unvaccinated controls. After vaccination, no adverse reactions were detected by visual inspection or at postmortem examination (n= 668 and 97 respectively). Postmortem data on TBCL were available for 97 vaccinated wild boar and 182 controls. The observed TBCL prevalence was 4.1 % (95% CI= 0.2- 8%) and 12.1% (95% CI= 7.1 -17.1 %) for vaccinated and control wild boar, respectively (p <0.05). Among those animals with TBCL, no difference in the mean lesion score was found (p>0.05). The results show that IV administered IM to wild boar piglets is safe and protects vaccinated individuals (66% reduction in TBCL prevalence) against natural challenge in a low prevalence setting. In a context of increasing TB prevalence in wild boar in Mediterranean habitats, vaccination achieved a progressive though slow decline in lesion prevalence since the onset of the vaccination scheme. Hence, vaccination might contribute, along with other tools, to TB control in wild boar and in pigs.

Introduction

Wildlife management has evolved towards a broad range of production schemes ranging from true natural populations to semi-intensive, farm-like settings maintaining high animal densities through supplementary feeding, fencing and translocations (Gortázar et al. 2007). The Eurasian wild boar (*Sus scrofa*), one of the most widespread and popular game species, is no exception. Artificial and natural high wild boar densities cause adverse effects on the environment, wild boar fitness and higher contact rates with transmissible pathogens (Massei et al. 2015).

Animal tuberculosis (TB), caused by *Mycobacterium bovis* and closely related members of the *M. tuberculosis* complex (MTC), is a zoonotic disease with implications for wildlife conservation, game production and for livestock production and trade (Cousins 2001). Wildlife reservoirs including the wild boar hamper the success of cattle TB eradication (Naranjo et al. 2008). *M. bovis* infection has been reported in feral and domestic pigs, too (Di Marco et al. 2012; Nugent et al. 2015). In farmed wild boar, TB is relevant because of legal restrictions on the sale and translocation of infected individuals (Spanish Royal Decree 1082/2009) and carcass condemnation.

In this context, wild boar farms can take advantage of the TB control tools developed for free-ranging wild boar such as vaccines (e.g. the newly developed inactivated *M. bovis* vaccine, IV; Garrido et al. 2011). In laboratory trials, oral and parenteral IV reduced disease progression and consequently infectiousness (Garrido et al. 2011; Beltrán-Beck et al. 2014b). However, only laboratory trial-derived information is available regarding the parenteral route (Garrido et al. 2011).

In 2012, a wild boar TB control program was set up in a wild boar farm by means of intra-muscular (IM) vaccination with IV. The goal of this experiment was

assessing safety and efficacy of parenterally administered IV vaccine under real conditions in a large farm setting with natural *M. bovis* circulation. We hypothesized that vaccinated piglets would show smaller scores of TB-compatible lesions (TBCL) than unvaccinated controls.

Material and Methods

Animal use and experimental vaccine permit

The experimental vaccine was deployed under permit of the Castilla – La Mancha regional government (D.G. Agricultura y Ganadería, Junta de Castilla - La Mancha; ref. 828493/2011). Handling was according to European (86/609) and Spanish legislation (R.D. 223/1988, R.D. 1021/2005). Postmortem inspection and sampling were performed on hunter-harvested wild boar. No animal was culled because of the experiment.

Study setting

The 1420 ha study site is located south of Toledo (Spain) and dedicated to recreational wild boar hunting with a breeding facility for re-stocking the hunting area. Wild boar proof fencing divides the site into (1) the farm area, that houses breeding stock and young offspring in a semi-intensive regime and (2) the hunting area, where almost free-ranging male offspring are relocated once they are 1.5-2 year old.

MTC infection was suspected in 2011 based on gross pathology and subsequently confirmed as *M. bovis* infection by the VISAVET laboratory, Universidad Complutense, Madrid. The detection of TB prompted the implementation of an experimental vaccination scheme in 2012.

Vaccination

Vaccine doses contained approximately 6 x10^6c.f.u. of heat-inactivated *M. bovis*. Vaccine strain derives from a field isolate obtained from a naturally infected wild boar (Neiker 1403, spoligotype SB0339) and prepared using Montanide ISA 50 V2 adjuvant (Seppic, Castres, France, further formulation details in Garrido et al. 2011). Piglets were vaccinated by injection of 1 ml of the IV vaccine into the longissimus dorsi at 3-4 months of age. All piglets underwent re-vaccination 1-2 months later and yearly re-vaccination thereafter until release into the hunting area or end of the productive life. A total of 306 and 362 piglets were vaccinated in 2012 and 2013, respectively (total n=668). One hundred eighty two individuals were not vaccinated and served as controls.

Post-mortem data

A careful post-mortem inspection enabling the record of macroscopic TBCL presence/absence and simple lesion scoring was conducted by the farm veterinarian (n=108 in 2013, n =169 in 2014 and 267 in 2015). The lesion score is based on lesion size as described in Díez-Delgado (2014a; 0 if no lesion is present, 1 for lesions < 1cm, and 2 for larger sized lesions) and simplified in terms of number of organs inspected (both mandibular lymph nodes, LN, both tracheobronchial LN, mediastinal LN and the 7 lung lobes separately). Hence, the total lesion score potentially ranged from 0 to 24.

Statistical analysis

Sterne's exact method was used to estimate 95% prevalence confidence intervals (95% CIs). A chi square test was used to compare the proportion of wild boar with TBCL between groups (control and vaccinated). Differences in lesion scores between groups were tested using the Wilcoxon rank-sum test. All analyses were undertaken in

the R statistical package (R Development Core Team 2015) using the gmodels library (Warnes et al. 2005). Significance was fixed at p <0.05.

Results

TBCL prevalence was 9.6% (95% CI= 6.1-13.1%; n= 274) in the farm area and 9.2% (95% CI= 6.1-12.3%; n=341) in the hunting area. Table 1 shows the apparent TBCL prevalence trend in wild boar from both sites independently of their membership in the vaccine study.

Table 1. Mean tuberculosis compatible lesions prevalence (TBCL prevalence) with associated 95% CIs and sample size (n) by area and year.

Area	2013		2014		2015	
	TBLC prevalence (95% CI)	n	TBLC prevalence (95% CI)	n	TBLC prevalence (95% CI)	n
Farm	16.7 % (9.6-23.7)	108	2.4 (-2.3-7.1)	41	6 (0.9-11.1)	83
Hunting area	-----------	----	10.9 (5.5-16.3)	128	8.2 (4.2-12.1)	184

After vaccination, neither adverse local or systemic reactions nor behavioral changes were detected by visual inspection in 668 vaccinated wild boar piglets. No vaccine-related lesions were noticed at postmortem examination (n= 97).

Postmortem data on TBCL were available for 97 vaccinated wild boar and 182 controls. The observed TBCL prevalence was 4.1 % (95% CI= 0.2- 8%) and 12.1% (7.3 -16.8 %) for vaccinated and control wild boar, respectively ($X^2 = 4.75$, 1 d.f., p <0.05). Thus, parenteral vaccination reduced TBCL prevalence by 65.9%. Among those

animals with TBCL, 4 vaccinated and 22 non-vaccinated controls, no difference in the mean lesion score was found (W= 40, p>0.05).

Discussion

The results show that IM administered heat-inactivated *M. bovis* (IV) is safe and protects wild boar piglets (66% reduction in TBCL prevalence) against natural challenge in a low prevalence setting. In a context of increasing TB prevalence in wild boar in Mediterranean habitats (Vicente et al. 2013), vaccination achieved a progressive though slow decline in lesion prevalence since the onset of the vaccination scheme.

Two aspects of experimental design need to be taken into account regarding this opportunistic experiment. First, vaccine efficacy assessment should ideally be based on several indicators, including mycobacterial culture. Funding constraints prevented its systematic use as a diagnostic tool in this case. Instead, TBCL was used as a proxy for disease as it provides a relatively accurate diagnostic tool for large-scale studies (Vicente et al. 2006). Second, this study was conducted on farmed wild boar, as opposed to "true" (free-ranging) wildlife. Our results suggest that vaccinated wild boar can endure low infection pressure during long periods without becoming infected. This knowledge could be useful for free-range pig production (Di Marco et al. 2012).

The live BCG vaccine has also been successfully applied to wildlife under field conditions (e.g. Tompkins et al. 2009). However, live vaccines have limited stability and may pose environmental risk, which limits their suitability for generalized field use. Inactivated vaccines such as IV overcome these limitations.

While oral formulations are favored when handling is not feasible, i.e. in "true" wildlife settings, a parenteral formulation was chosen for this farm because it ensures dosage and facilitates vaccination of large groups.

In contrast to our initial hypothesis, vaccine protection did not result in lower lesion scores but in lower TBCL prevalence. This suggests protection against infection rather than against disease progression. The challenge faced in low prevalence settings is probably lower in terms of dose than under laboratory conditions (10^6 c.f.u. of *M. bovis* in Garrido et al. 2011 and Beltrán-Beck et al. 2014b) and in terms of re-infection chance than in natural settings with a higher prevalence (Gortázar et al. 2008).

The increase of managed and farmed wildlife and the trend towards extensive production systems in livestock (Gortázar et al. 2007) will demand the application of new disease control tools including vaccination. Additional research on vaccination protocols, duration of vaccine-induced protection and vaccine performance under higher infection pressure and in complex (multiple host) scenarios is needed.

Acknowledgments

CHAPTER II

Impact of piglet oral vaccination against tuberculosis in endemic free-ranging wild boar populations
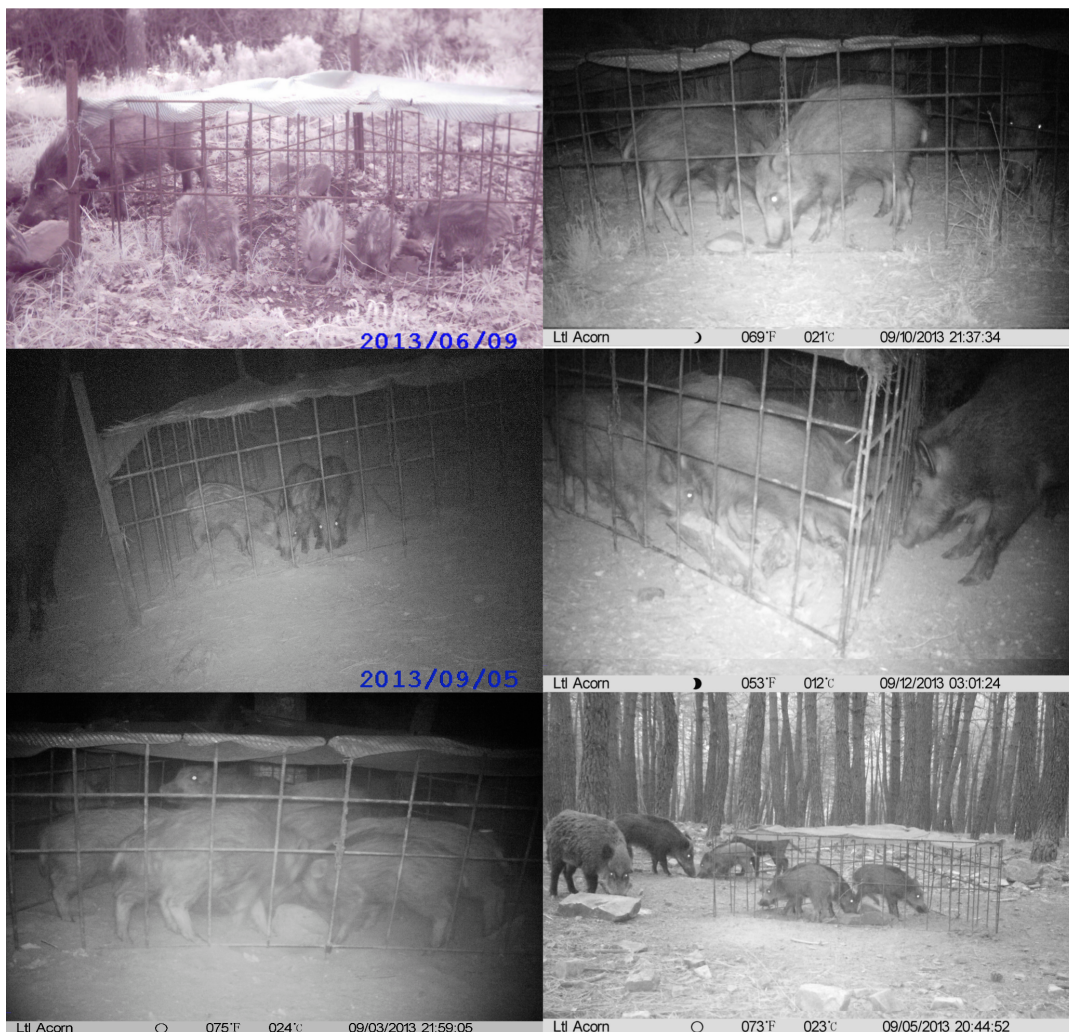
A version of this work has been published:

Resumen

El jabalí (*Sus scrofa*) es el principal reservorio de bacterias pertenecientes al complejo *Mycobacterium tuberculosis* en ecosistemas mediterráneos y un importante factor de riesgo para la aparición de tuberculosis (TB) en el ganado bovino. En este contexto, la vacunación en jabalíes puede ser una herramienta valiosa para el control de la TB. Este estudio evalúa dos candidatos vacunales administrados por vía oral, *Mycobacterium bovis* inactivado por calor (IV) y BCG, en cuatro fincas (una finca manejada y otra no manejada o natural por cada tipo de vacuna) durante cuatro años. Además de en las fincas vacunadas, se monitorizó la enfermedad en 15 fincas no vacunadas (controles espaciales) y en todos las fincas un año previo al inicio de la vacunación (control temporal). La hipótesis de partida es que la vacunación de rayones de 2-6 meses reducirá la prevalencia de la enfermedad a nivel poblacional durante el periodo de estudio. Esto es posible debido a la rápida renovación de las poblaciones de jabalí. La vacuna fue administrada mediante cebos vacunales colocados en comederos selectivos para rayones alcanzándose una tasa de consumo del 50 al 74% en las fincas naturales y del 89 al 92% en las fincas manejadas. Esto tiene importantes implicaciones para el potencial uso de esta herramienta en el control de otras enfermedades en esta especie. La prevalencia inicial de TB del área de estudio fue elevada oscilando entre el 50-100%. Durante el periodo de estudio la prevalencia se incrementó en las fincas no vacunadas (6%), mientras que descendió significativamente en la finca manejada vacunada con IV (34%). No se detectaron cambios significativos en el resto de fincas vacunadas. El impacto de la vacunación a largo plazo se estudió mediante modelos matemáticos representativos del sistema estudiado que mostraron que la vacunación de rayones reduce la prevalencia de la TB a nivel poblacional e incrementa la abundancia de jabalí. Por todo ello, la vacunación con IV puede ser una herramienta

complementaria útil en estrategias de control integrado de la TB, aunque su aplicación

ha de adaptarse a cada situación.

Abstract

The Eurasian wild boar (*Sus scrofa*) is the main wild reservoir of the *Mycobacterium tuberculosis* complex in Mediterranean woodlands and a key risk factor for cattle tuberculosis (TB) breakdowns. Wild boar vaccination therefore has the potential to be a valuable tool for TB control. We tested two orally delivered vaccines, heat-inactivated *Mycobacterium bovis* (IV) and BCG, in four sites (two per vaccine type: one Managed and one Natural or unmanaged) during four years. TB was also monitored in 15 unvaccinated sites (spatial control), as well as in all sites from one year prior to intervention (temporal control). The rationale is that by vaccinating 2-6 month old wild boar piglets we can reduce disease at the population level during the study period. This is achievable due to the fast turnover of wild boar populations. Vaccine baits were deployed using selective piglet feeders and this method proved highly successful with uptake rates of 50 to 74% in Natural sites and 89 to 92% in Managed sites. This is relevant for the potential delivery of vaccines to control other diseases, too. Local wild boar TB prevalence at the beginning of the study was already high ranging from 50 to 100%. TB prevalence increased in unvaccinated sites (6%), while a significant decline occurred in the Managed IV site (34%). Changes recorded in the remaining sites were not significant. The short-term impact of vaccination observed in the field was complemented by mathematical modelling, representative of the field system, which examined the long-term impact and showed that vaccination of piglets reduced prevalence and increased abundance at the population level. We conclude that IV could become part of integrated TB control schemes, although its application must be tailored for each specific site.

Introduction

Vaccination is an effective tool to prevent, control and eradicate infectious diseases (Rappuoli et al. 2002). However, technical and logistical difficulties, coupled with the high cost of vaccinating free-ranging wildlife have limited its application to diseases that have a significant impact on public health, economy or conservation (Cross et al. 2007b). The turning point in wildlife vaccination was the successful use of an oral vaccine to control fox (*Vulpes vulpes*) rabies in Europe (Freuling et al. 2013). This success prompted research into field vaccination strategies to control other relevant diseases in wildlife, including animal tuberculosis (TB). TB is a chronic infection caused by *Mycobacterium bovis* (*M. bovis*), My*cobacterium caprae* (*M. caprae*) and other members of the *Mycobacterium tuberculosis* complex (MTC). It is a zoonosis, although the number of human cases is now low in industrialised countries (Langer and LoBue 2014) and therefore the impact of animal TB is mainly socio-economical, derived from eradication campaign costs, associated movement restrictions, and indirect losses to both the livestock (Zinsstag et al. 2006) and regional hunting industries (Barasona et al. 2016). Additionally, animal TB causes concern for the conservation of endangered species, e.g. the Iberian lynx (*Lynx pardinus*; Gortázar and Boadella 2014).

The majority of TB-control efforts focus on cattle (Reviriego Gordejo and Vermeersch 2006). However, TB is a well-recognized example of multi-host infection and is unlikely to be eradicated without targeting all relevant hosts (Gortázar et al. 2015b). The MTC host network in Mediterranean woodland habitats of the Iberian Peninsula is complex and includes several relevant domestic and wild host species (Gortázar and Boadella 2014). The native Eurasian wild boar (*Sus scrofa*) is considered the main wild reservoir for MTC in this region (Naranjo et al. 2008). This suid is also regarded as a key risk for cattle TB breakdowns (Hardstaff et al. 2014), mostly through

indirect contact (Kukielka et al. 2013; Cowie et al. 2016). Wild boar are consequently an additional target species for TB control. Evidence suggests that wild boar management interventions, such as biosafety measures that reduce wildlife–cattle contact rates (Barasona et al. 2013) or culling of wild boar (Boadella et al. 2012), may reduce TB prevalence in sympatric ruminants such as cattle and red deer (*Cervus elaphus*). In this context, wild boar vaccination might represent a valuable additional tool for TB control in Mediterranean Iberia.

Proof of principle of TB disease reduction by vaccination with the live attenuated *M. bovis* Bacillus Calmette-Guérin (BCG) has been demonstrated for several wild reservoirs in controlled experiments (Buddle et al. 2006; Lesellier et al. 2006; Nol et al. 2008). Further field experiments have been conducted in brush-tailed possums (*Trichosurus vulpecula*; Corner et al. 2002; Tompkins et al. 2009; Nugent et al. 2016) and Eurasian badgers (*Meles meles*; Chambers et al. 2011; Gormley et al. 2017) with promising results regarding protection (see summary in Supplementary Material SM1).

Two orally administered vaccine candidates have been tested in laboratory trials for their effectiveness at controlling TB in wild boar: BCG (Ballesteros et al. 2009a; Garrido et al. 2011; Gortázar et al. 2014b) and heat-inactivated *M. bovis* (IV; Garrido et al. 2011; Beltrán-Beck et al. 2014b). Both vaccines decrease disease severity, reducing lesion and culture scores, when compared to unvaccinated controls. Additionally, an injectable version of the IV vaccine successfully reduced TB lesion prevalence on a wild boar farm (66% reduction; Díez-Delgado et al. 2017). Vaccine safety and field species-specific delivery have been assessed in additional trials (Beltrán-Beck et al. 2014a).

Both BCG and IV vaccines are prophylactic and non-sterilising. Thus, as in other TB vaccines, their protective effect is expected to reduce the severity of the disease and subsequent transmission, rather than curing or completely preventing infection. The vaccines are formulated for oral delivery, as oral administration via baits is the most practical means for wildlife vaccination on large scales (Cross et al. 2007b). This coupled with complementary tools such as species-specific baits (Ballesteros et al. 2009b) marked with chemical compounds (Ballesteros et al. 2011) and selective baiting stations (Ballesteros et al. 2009c), enables a targeted vaccine delivery and the assessment of bait uptake.

Extensive field trials are needed to assess vaccine performance in free-ranging populations using oral delivery. This study reports the results from a large-scale (ca. 460 km^2) four-year wild boar oral vaccination experiment that began in 2012 and was implemented in a high prevalence area of Montes de Toledo, Spain. The field trial targeted vaccination of 2-6 month wild boar piglets with the rationale that disease prevalence could be reduced within the four-year trial period. Piglets were chosen as the target age class (Ballesteros et al. 2009c) as they are more likely to be uninfected and thus suitable for vaccination (age is a risk factor for this chronic disease, O´Brien et al. 2002; Vicente et al. 2013). Moreover, given the fast population turnover of wild boar in the study area (where wild boar are extensively hunted), most subadult and adult wild boar will have been vaccinated by the end of the fourth year. This will enable the population effect of the vaccine to be assessed.

To underpin the field studies and to assess the impact of piglet vaccination on the epidemiological dynamics we also developed a mathematical model of wild boar TB interactions. Mathematical models are crucial tools for understanding how disease management strategies modify host and pathogen dynamics and have a long history of

contributing to the understanding of the effectiveness of vaccination programmes (Scherer and McLean 2002; Keeling and Rohani 2008). Moreover, while the field study considered the short-term impact (after four years) of vaccination, the model can assess the long-term impact on prevalence and population abundance and test the consequences of vaccination success and of vaccine cessation on the resultant epidemiological dynamics.

This study therefore combines field trials of a four-year wild boar vaccination experiment with a mathematical modelling study of the field system. Our aims were to: first assess bait uptake rates under field conditions; second assess vaccine impact measured as changes in TB prevalence in the wild boar population based on pathology; and third mathematically model field vaccination in order to gather additional long-term insights into the influence of different levels of vaccination on disease prevalence and population density. Our hypothesis was that wild boar piglets would be efficiently targeted and that both IV and BCG would lead to measurable reductions in TB prevalence.

Material and Methods

Permits and ethics statement

The experiment was conducted under a research license (828493/2011) issued by D.G. Agricultura y Ganadería, Junta de Castilla- La Mancha. Post-mortem inspection and sampling were performed on hunter-harvested wild boar. No animals were culled for the experiment.
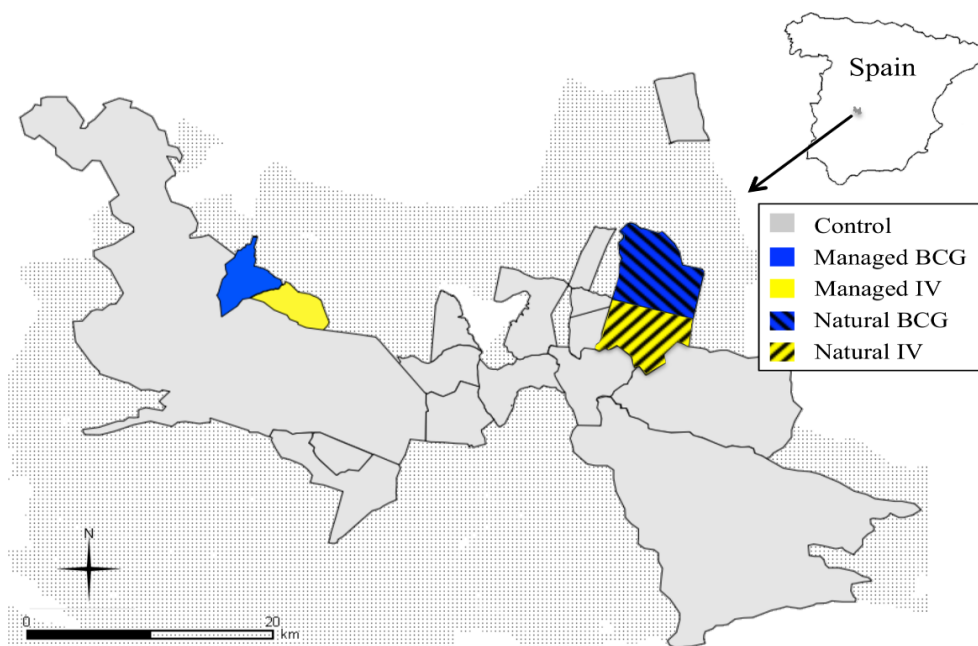
Study area

The study was conducted in Montes de Toledo, a mountain chain located in Central Spain (39º 25' to 39° 16'N, 4° 05' to 4° 23'W). This region has a Mediterranean wood and scrubland habitat dominated by evergreen oaks (*Quercus* sp.). The climate is typically Mediterranean, with mild to cold winters, hot summers and rainfall mostly limited to spring and autumn.

The study area is composed of an array of privately owned hunting estates, communal lands and natural areas representing a gradient of wildlife management levels. Natural or unmanaged populations are those in which free-living individuals live on open lands where no supplementary feeding takes place, while managed populations generally maintain high densities through supplementary feeding and fencing. Both of them, natural and managed populations are hunted (with differences in economical profitability). Land use changes have favoured the upsurge of a commercial hunting industry that is economically relevant for the area, in which the main big game species are red deer and wild boar (Vicente et al. 2013).

In this TB endemic area, wild boar TB-compatible lesion (TBCL) prevalence ranges from 52% to 70% and has increased over time (Vicente et al. 2013). Lower (12%) and relatively stable TBCL prevalence has been described for red deer (Vicente et al. 2013).

A total of 19 sites were selected for TB monitoring, of which two privately owned hunting estates and a natural park were devoted to vaccination (96 km^2) and the remaining sites, representative of the whole management spectrum, were pooled and used as control (n=15, ca. 360 km^2; further site characterization is provided in Table 1 Supplementary Material SM2).

BCG was deployed on one of the privately owned estates (Managed BCG) and IV on the other (Managed IV). The natural park was divided into two areas (accounting for two sites) delimited by a topographical barrier. BCG was deployed in the north area (Natural BCG) while IV was deployed in the south area (Natural IV; Figure 1). Thus, Natural BCG and Natural IV sites where separated by the main road that separates the north and south mountain chains and an exposed flat area of open grassland.

Figure 1. Study area, Montes de Toledo, central Spain.

Rationale

Given the high wild boar TB prevalence in the study area (63%) and the well-documented increasing trend (Vicente et al. 2013), measures to control TB in this species are needed. We propose that vaccination represents a chance to reduce TB in wild boar and can be readily implemented and assessed in the context of the existing monitoring scheme.

Piglets as target of vaccination

TB is a chronic infection that progresses slowly until it eventually kills the animal (Barasona et al. 2016). TB vaccines are preventive, thus in order to protect, the animal must be uninfected. Since increasing age is a well-known TB risk factor (O´Brien et al. 2002; Vicente et al. 2013), 2-6 month old piglets are the vaccination target as they are less likely to be infected. Moreover, vaccinating in early life could prevent the generalization of the disease. Generalized individuals are those with disseminated lesions that excrete large concentrations of mycobacteria (Santos et al. 2015; Barasona et al. 2017). These are known as super-shedders and are believed to be the major drivers of infection maintenance within populations, and thus are key targets for disease control (Kramer-Schadt et al. 2009).

Assessing disease at population level

In the study area wild boar are regularly hunted, i.e. hunting is an inherent feature of the study sites. This provides a suitable framework for data and sample collection that is, indeed, used for the national wildlife monitoring programmes (MAPAMA 2017). Thus, an effective control strategy should integrate in this set-up monitoring framework that also enables the assessment of the impact of the intervention. Monitoring based on sampling hunter-harvested wild boar provides a solid means for wildlife TB assessment, although it has some limitations. One of them is that hunters do not target piglets as they lack trophy value, so this age class is under represented. Also, assessing protection is difficult when piglets are vaccinated in summer and sampled 2-6 months later. Moreover, the effects of vaccination need to take place at a population level, i.e. cause a decrease in prevalence in the overall population. Since hunting leads to a fast population turnover, most of the population will belong to

a vaccinated cohort by the end of the experiment (four year vaccination). In summary, vaccine assessment is performed over the whole population and results are expected by the fourth year.

Vaccination program

Vaccines

The live attenuated BCG vaccine was derived from Danish *M. bovis* (CCUG strain 27863) and was prepared as described elsewhere (Ballesteros et al. 2009a; Garrido et al. 2011; Gortázar et al. 2014b). Vaccine doses consist of 0.15 ml of a suspension containing 10^6 c.f.u. (the dose tested in Ballesteros et al. 2009a; Garrido et al. 2011; Gortázar et al. 2014b). Vaccine doses were placed in sterile airtight polypropylene 0.2 ml vials (VWR®, Radnor, Pennsylvania, USA). BCG was freshly prepared for each vaccination cycle and stored at 4ºC until deployment (24 to 72 hours).

The IV vaccine was derived from a heat-inactivated field isolate obtained from naturally infected wild boar (Neiker1403, spoligotype SB0339) and was prepared as described in Garrido (2011). Each IV vial contained the equivalent of 10^7 c.f.u. in 0.2 ml of PBS.

Vaccine delivery

(i) Baits

BCG and IV vaccine vials were deployed in specific baits for wild boar piglets (Ballesteros et al. 2009b). The baits have a hemispherical shape (3.4 x 1.6 cm) and are made with piglet feed, wheat flour, paraffin, sucrose, and cinnamon-truffle powder attractant (Ballesteros et al. 2009b). These baits have proved stable, safe and effective as regards reaching the target species and age class in the field (Ballesteros et al. 2011). A

chemical marker, iophenoxic acid (IPA; PR EuroCHEM Ltd., Cork, Ireland), was added to the baits (as described in Ballesteros et al. 2011) to determine the proportion of wild boar piglets consuming baits (bait uptake). Two IPA derivatives, each associated with a vaccine type, were employed. Propyl-IPA was associated with BCG baits and ethyl-IPA with IV baits.

(ii) Selective piglet feeders spatial distribution

Baits were placed in selective piglet feeders (Ballesteros et al. 2009c). Experimental areas were divided into a 2 km^2 grid by means of GIS analysis (QGIS version 1.8.0 Lisboa). Two piglet feeders were distributed in each grid and were separated by approximately 100 meters to avoid monopolisation by any dominant family group. They were placed in the vicinity of a permanent waterhole (to ensure wild boar passed by) in a spot where they received afternoon shade (to avoid extreme heat). Managed sites (BCG and IV) had 10 pairs of piglet feeders each and Natural sites 14 pairs each (total piglet feeders =96). A detailed map of piglet-feeder distribution is provided in Figure 1 Supplementary Material SM2.

Vaccination schedule

Vaccination took place in summer to target the main peak of 2-6 month old wild boar after weaning and thus, able to consume baits and immunologically mature. Moreover, in summer natural food resources are at their lowest in Mediterranean habitats, which potentially enhances bait consumption (Ballesteros et al. 2009a). To increase the use of feeders by wild boar and to limit bait uptake by non-target species maize was pre-baited 2-5 times a week for 8 weeks prior to vaccine deployment (Kaden et al. 2000; Ballesteros et al. 2011). Also, sham baits (without vaccine or markers) were placed to habituate wild boar piglets to baits.

The vaccination campaign included three cycles that consisted of three nights each. Two consecutive cycles took place in early summer (end of June-July) and one in late summer (end of August-September). Twenty baits per piglet feeder were deployed each day at dusk, leading to a total of 17280 vaccine baits per year (180 baits/km^2 and year) during four consecutive years. Non-consumed baits were retrieved the next morning and fresh vaccine baits were newly placed each day (the vaccine spent a maximum of 12 hours in the environment).

Vaccine impact assessment

Hunter-harvested wild boar (n=1158) were sampled during the normal hunting season (October to February) from 2011-12 to 2015-16. Samples obtained prior to vaccination (hunting season 2011-12, "control year" hereafter) served as pre-intervention background, providing baseline data on infection and disease. A representative sample stratified by the age and sex of the hunted animals was randomly selected at each hunting event. Each specimen was subjected to sex and age determination, blood collection from the cavernous sinus (Arenas-Montes et al. 2013) and a general inspection of the whole carcass. Age was assessed on the basis of tooth eruption patterns (Saenz de Buruaga et al. 1991) and coat, establishing four categories: wild boar under 6 months were classified as very young piglets (n=24) which are the vaccination target, those from 6 to12 months were classified as piglets (n=227) and were sampled to assess bait uptake and vaccine impact, those between 12 and 24 months as yearlings (n=309), and those over 2 years as adults (n=598).

Organ samples taken in the field include the mandibular lymph nodes (LNs), tonsils, lung with tracheobronchial LNs and mediastinal LN, spleen, and mesenteric

LNs (Martín-Hernando et al., 2007). TBCL presence and lesion scoring were recorded by carrying out detailed inspections in the laboratory.

Prevalence was used to estimate vaccine impact (which is the common approach in wildlife TB studies e.g. Nugent et al. 2016; Díez-Delgado et al. 2017), as incidence is difficult to estimate in free-ranging wildlife (Delahay et al. 2013). Lesion presence is a recognised monitoring system to assess wildlife TB, since it is more practical and cost effective compared to culture, especially when working at a population level (Rodwell et al. 2001; Vicente et al. 2013). Lesion scoring is useful to determine the degree of vaccine-induced protection in laboratory trials, thus a simplified lesion scoring method was developed to report on lesion severity in field trials (Díez-Delgado et al. 2014a). Also, recording affected organs and cavities provides valuable information to determine disease severity (generalization) and infer infectiousness (Barasona et al. 2017). Briefly, the lesion score is based on lesion size (0 if no lesion is present, 1 for lesions <1 cm and 2 for larger lesions) and inspection of the routine target organs (considering each lung lobe separately and excluding the tonsils). An individual's total lesion score ranges from 0 to 26. Individuals with lesion scores >0 are defined as TBCL positive.

Processed tissues were stored at -20ºC. In order to confirm *M. bovis* or *M. caprae* presence, mandibular LN and tonsil pool plus a thoracic LN pool were cultured following the procedures described in Garrido (2011) and all isolates were spoligotyped (Kamerbeek et al. 1997).

Bait uptake assessment

Free-ranging wildlife does not allow for individual identification or individual assignation to a vaccine status unless marked and captured several times. Therefore, bait

uptake was used as a proxy for vaccine coverage (proportion of individuals that have received a vaccine; Ballesteros et al. 2010; Beasley et al. 2015).

Bait uptake is determined by the presence of a chemical marker in serum (IPA derivatives). The IPA derivatives analysis was carried out following the extraction method and LC/ESI-MS analysis described in Ballesteros (2010). Markers are detectable in serum for at least 18 months after bait ingestion (Ballesteros et al. 2010). Marker presence is, therefore, used to estimate bait uptake by individual wild boar piglets in the vaccination campaign prior to the hunting season. Discriminating whether older (>12months) wild boar consumed bait as piglets or as older individuals is not possible when the marker is used over several consecutive years. Results of marker presence in older wild boar can therefore not be used to relate individual vaccine status to individual outcome.

Statistics

Descriptive analysis, predictors, and logistic regression

Changes in temporal trends within the same site were analysed using a Chi square test or Fisher exact test (two tailed) when required.

In order to assess vaccine impact (defined as the combined probability of bait uptake and protection) for each site as compared to control sites, a logistic regression model was fitted using lesion presence as a dependent variable. Predictors tested in the model were known drivers of TB (Vicente et al. 2013): age (<12 months, 12 to 24 months and >24 months old), rainfall (m), relative wild boar abundance, years (1 to 4); and initial TB prevalence (proportion), to account for the situation prior to intervention.

Data on study area rainfall were obtained from the National Agency of Meteorology, Station 4184. The cumulative annual rainfall was calculated from

September to August to match sampling years rather than natural years. Wild boar populations were monitored by obtaining annual relative wild boar abundance estimates based on a dropping frequency index (FBII; Acevedo et al. 2007) for the vaccinated sites (n=4) and the majority of control sites (n=11).

All analyses and data visualisation were undertaken with the R statistical package (R Development Core Team 2015) and the ggplot2 package (Wickham 2009). Significance was fixed at $p < 0.05$. The 95% confidence intervals (CI) were calculated using bootstrapping.

Modelling

A mathematical model representing the key processes in the field system was developed to answer questions that could not be tested in the experimental trial and to gain insight into the mechanisms that govern the dynamics of vaccinating against TB in wild boar. The model reflects a single geographical estate containing a homogeneously mixed population with parameters that are representative of the field-trial sites. The model is deterministic and compartmental and uses a system of ordinary differential equations to represent the dynamics of susceptible, infected (which have TBCL but are not infectious) and generalized (which have lesions in more than one anatomical region and are considered to be infected, infectious and suffer high disease-induced mortality) individuals for piglet, yearling and adult age-classes. Piglets that are successfully vaccinated (those that receive the vaccine and are receptive to immunisation) have a reduced chance of infection and if infected a reduced rate of progression to the generalized class.

Two different scenarios representing our vaccination sites were modelled: (a) a site with medium initial prevalence where piglets have a low chance of infection prior to

vaccination and (b) a site with higher initial prevalence and greater rates of transmission combined with a greater proportion of piglets infected prior to vaccine delivery (through pseudo-vertical transmission from parent to offspring; piglets not receptive to immunization). Three situations were addressed: (i) the influence of different levels of vaccination success (which combines the effects of both coverage and efficacy by representing the proportion of effective immunisations) on disease prevalence, (ii) the influence of continued vaccination (25 years) and eventual cessation on population density, and on (iii) disease prevalence. The model framework, parameterisation and interpretation are further explained in Supplementary Material SM3.

Results

Bait uptake

The proportion of wild boar with chemical marker presence in serum by site and age class is displayed in Figure 2. Piglets from Natural sites had lower uptake rates (50 to 74%) than those from Managed sites (89 to 92%). The chemical marker was detected as well in older (>12 months) wild boar (42-59%).

The topographical barrier separating different vaccine types on the Natural sites was not fully effective: consumption of both vaccine types (presence of both markers) was detected in 22-39% of vaccinated wild boar from the Natural sites.
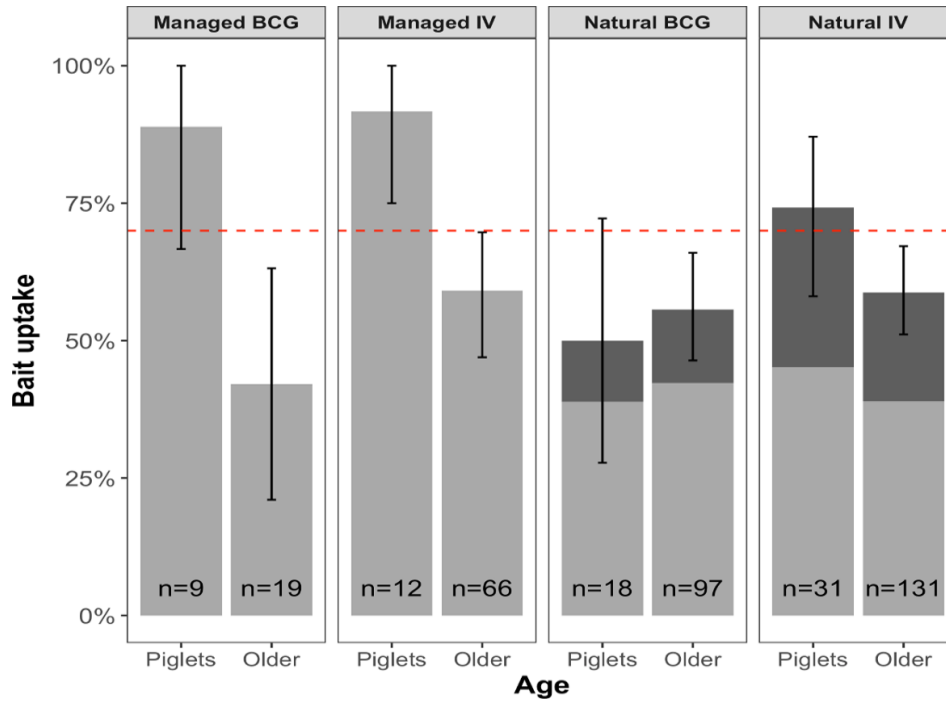
Figure 2. Bait uptake. Proportion of wild boar individuals positive to chemical marker detection by site and age class (piglets = wild boar <12 months; older = wild boar >12 months). Bars are the percentage of individuals positive to detection of chemical marker, light grey bar represents single chemical marker detection and dark grey the presence of both markers. Error bars are bootstrap 95% confidence intervals (CI). Horizontal dashed line stands for the minimum theoretical 70% uptake threshold required to achieve an effective intervention (Anderson et al. 2013).

Vaccine impact

Figure 3 presents the observed temporal trend of TBCL prevalence in the overall population and in the piglet age class. The agreement between TBCL and culture had a kappa value of 0.56 (raw data on TBCL, lesion score and culture are listed in Supplementary Material SM4).

TBCL prevalence increased steadily but not significantly when compared with the control year in the Control sites (6% increase, $X^2 = 0.922$, 1 d.f., $p>0.05$) during the study period. No individual control site had a consistently declining trend in TBCL

prevalence (Table 1 Supplementary Material SM2). With regard to the vaccinated sites, a significant decline occurred on the Managed IV site (34% reduction since control year; $X^2 = 7.665$, 1 d.f., $p<0.01$). Vaccination on this site appeared to prevent infection and reduce disease severity (see culture and score data in Supplementary Material SM4). No significant changes were recorded on the remaining sites ($p>0.05$). The inter-annual variability in TBCL prevalence was marked on the Natural sites (Figure 3). No significant trend was recorded for any site in the piglet age class ($p>0.05$).

No significant differences in lesion scores were detected among vaccinated and control groups, probably due to heterogeneities in lesion evolution and challenge and to the simple scoring system used on field.
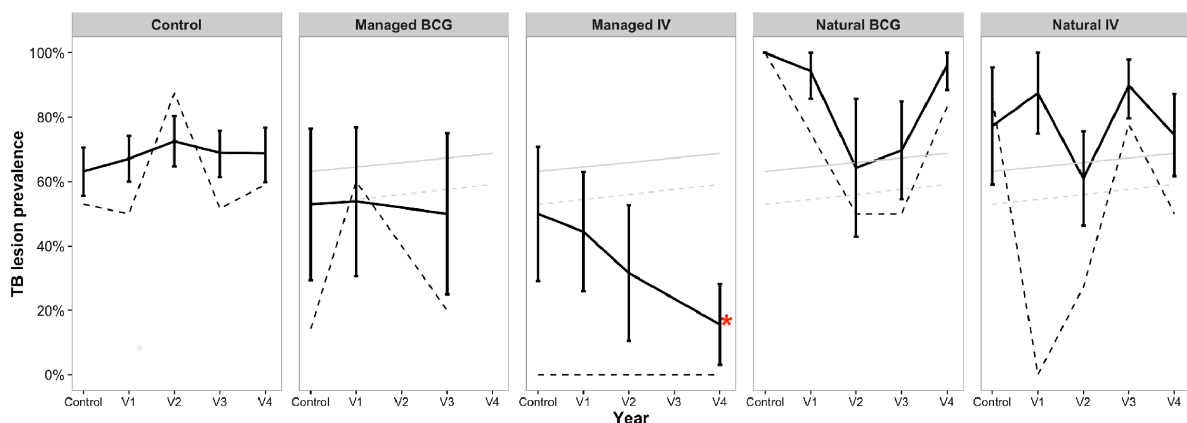
Figure 3. Temporal trend of tuberculosis (TB) lesion prevalence of piglets and total population by site. The dashed line represents piglet age class and the solid line the total population. Background information: the average trend for total population (solid line) and piglets (dashed line) found on the control site appears in light grey in the vaccine site figures. Error bars are bootstrap 95% confidence intervals (CI). Asterisk indicates a significant at p <0.01 decline in prevalence as compared to pre-vaccination levels.

Table 1 displays the results of the logistic regression model. Vaccination had a significant effect when IV was used on the Managed site ($p<0.001$). However, its effect was negligible for the sites on which BCG was deployed ($p>0.05$) and for the Natural IV site ($p>0.05$). Other significant variables explaining TBCL presence in our model were increasing age, low rainfall and initial prevalence.

Table 1. Results of the logistic regression model of tuberculosis compatible lesion presence. Estimates (B), estimate associated standard error (SE) and p-value are shown. Reference values for age class and site variables are "<12 month old" and "control" respectively.

Predictor		B (SE)		
(Intercept)		-0.542	(0.543)	
Age	Yearlings	1.044	(0.233)	***
	Adults	1.424	(0.216)	***
Rainfall		-1.394	(0.537)	**
FBII		-0.396	(0.361)	
Site	Managed BCG[a]	-0.500	(0.419)	
	Managed IV	-1.490	(0.296)	***
	Natural BCG	-0.058	(0.384)	
	Natural IV	0.262	(0.259)	
Initial prevalence		2.043	(0.541)	***
Year		-0.067	(0.079)	

[a] Only results of three vaccination years available

*** $p<0.001$ ** $p<0.01$ * $p<0.05$

Modelling

Two scenarios representing our vaccination sites were investigated: (a) similar to Managed sites (medium initial prevalence where piglets have a low chance of infection prior to vaccination) and (b) similar to Natural sites (high initial prevalence and greater rates of transmission combined with a greater proportion of piglets infected prior to vaccine delivery).

*Effects of vaccination success on disease prevalence*

Figure 4 a(i) & b(i) (see also Supplementary Material SM3) shows that as the proportion of successfully vaccinated piglets (those effectively immunised) increases, TBCL prevalence decreases (38% and 30% decrease, respectively, when vaccination success is 100%). This decrease in total prevalence is driven by a reduction in the density of generalized individuals and is greatest when piglets have a lower risk of infection prior to vaccination (Figure 4a(i)).

Effects of continued vaccination (25 years) and eventual cessation on population density and disease prevalence

Figure 4 a(ii) & b(ii) shows the epidemiological dynamics for a 25-year vaccination programme, with a vaccine success-rate of 75%. By the end of the vaccination period the proportion of the population belonging to the piglet age class is 26% in Figure 4a and 34% in Figure 4b and therefore vaccination of piglets against TB is an effective method of TB control (see also Supplementary Material SM3). It indicates that there is an initial reduction in the level of infected and generalized individuals, which lowers disease transmission and consequently leads to a decrease in prevalence. The impact of vaccination is largest when there is a reduced chance of piglet infection prior to vaccination and a lower initial prevalence. In the set-up that is most similar to Managed site IV the model predicts a 35% decrease in TB prevalence after 4 years (Figure 4a(iii)). This is comparable with the 34% decrease reported in the field study. A consequence of the vaccine-induced reduction in prevalence is a reduction in population mortality due to a decrease in disease-induced death. This drives an increase in total population density, which in the long-term allows the density of infected and generalized individuals to return to their pre-vaccination levels. Therefore, the long-

term reduction in disease prevalence shown in Figure 4 a(iii) & b(iii) is a consequence of an increase in total population density rather than a decrease in the density of infected and generalized individuals. These model results highlight how observations from the early years of a vaccination programme may not provide a clear picture of the effectiveness of a long-term vaccination strategy, since the benefits of vaccination on reducing the level of infection in the early years are countered by the subsequent increase in total population density.

The model results also indicate that when the vaccination programme is stopped there is an initial increase in disease prevalence and density of infected and generalized wild boar before levels return to those prior to vaccination. This is a consequence of the elevated population density resulting from vaccination and of the temporary nature of vaccine-derived immunity (see also Supplementary Material SM3).
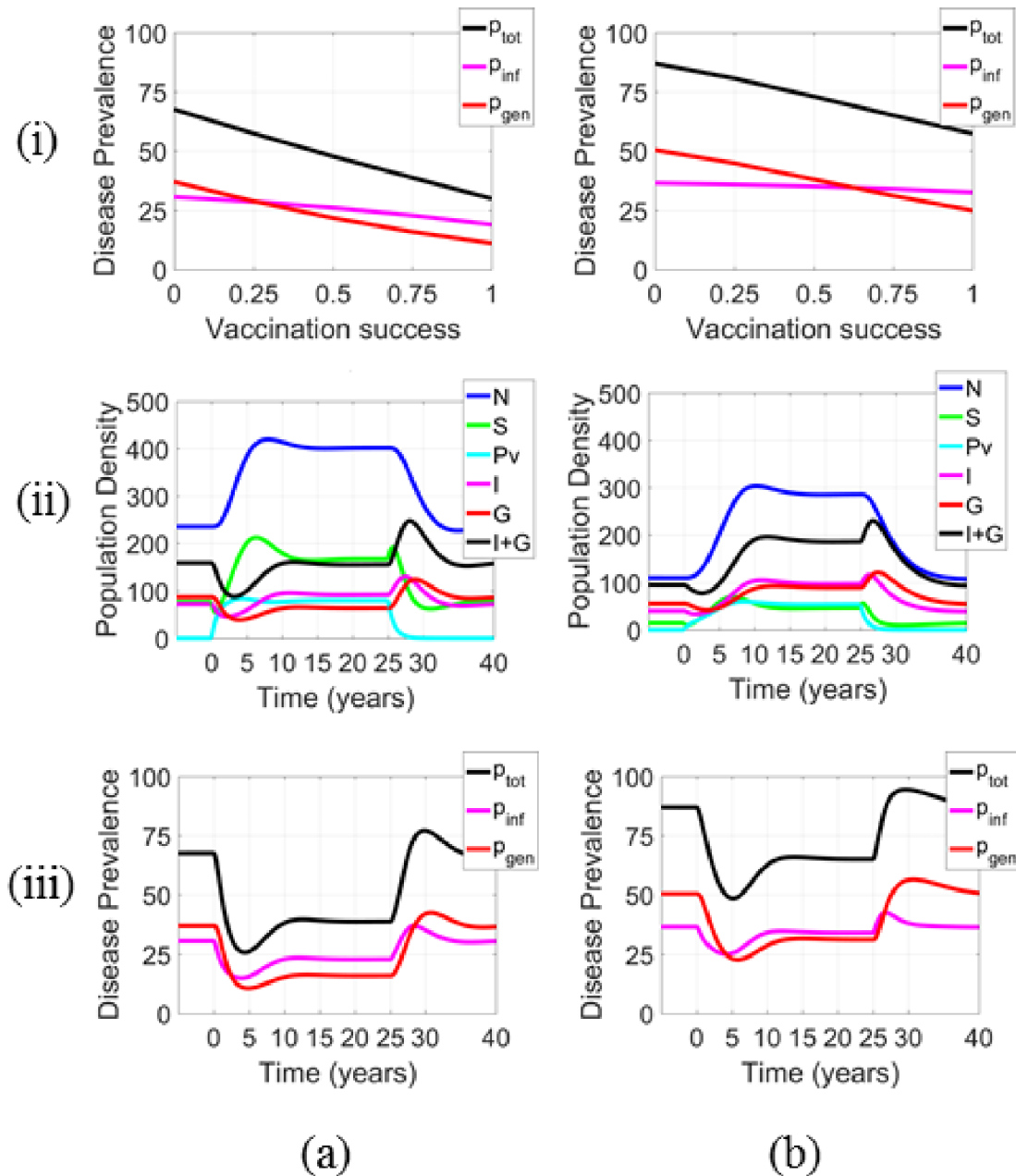
Figure 4. Modelling results for wild boar vaccination against tuberculosis. Column (a) represents a site with medium disease prevalence on which piglets have a low chance of infection prior to vaccination (default disease transmission rate and no pseudo-vertical transmission) and so is similar to a Managed site. Column (b) represents a site with higher initial prevalence and greater rates of transmission combined with a greater proportion of piglets infected prior to vaccine delivery (double transmission rate and 100% pseudo-vertical transmission) and so is similar to a Natural site. Row (i) shows disease prevalence against proportional vaccination success, vp, with results determined at the stable endemic steady state when the specified level of vaccination is included;

(ii) shows changes in population density against time (years) for a vaccination level of 75% (*vp* = 0.75) during 25 years of continued vaccination and eventual cessation; and (iii) shows changes in disease prevalence against time (years) for a vaccination level of 75% (*vp* = 0.75) during 25 years of continued vaccination and eventual cessation. N (blue) represents the total population density, I (magenta) represents the total density of infected but not generalized, G (red) the total density of generalized, S (green) the total density of susceptibles and Pv (cyan) the total density of vaccinated piglets. *ptot* (black) is the proportion of the total population infected with TB (*ptot* = (I+G)/N), *pinf* (magenta) is the prevalence of infected but not generalized (*pinf* = I/N); and *pgen* (red) is the prevalence of generalized infection (*pgen* = G/N).

Discussion

Contrary to our expectations we found no consistent reduction in prevalence after vaccination, the exception being IV vaccination on the Managed site. Here, under conditions of 90% bait uptake and 50% initial disease prevalence, IV appeared to prevent infection and reduce disease severity, lowering TBCL prevalence in the population by 34% after four years in a context of increasing prevalence in control sites. Model results confirmed that successful vaccination of piglets could lead to the observed reduction in prevalence but also predicted an increase in the overall host population density due to vaccine-derived reductions in disease-induced mortality in the long-term.

Achieving an adequate level of bait uptake is as important to the success of the strategy as vaccine efficacy (Massei et al. 2010). However, this goal is difficult to achieve and assess in free-ranging populations. Bait uptake by piglets is commonly a limiting factor in oral vaccination via baits (Kaden et al. 2000), but this trial was able to reach more than 70% (which is a reported threshold to achieve effective intervention;

Anderson et al. 2013) of this age class on three of the four sites. This is relevant as regards the potential of vaccination for controlling other diseases, e.g. classical swine fever, in the case of its eventual emergence in Mediterranean regions. In this study, higher uptake was achieved in populations used to being fed and to human presence, i.e. managed hunting estates. Therefore species management, a reported risk factor for TB (Vicente et al. 2013), can be helpful in vaccine delivery whilst naïve populations might take longer to get used to new food sources (Delahay et al. 2003). While the bait uptake in piglets was high the presence of the marker was also recorded in a proportion of older individuals (42-59%). This could be due to marker persistence for greater than 18 months or indicate that, despite using piglet feeders, some older wild boar gained access to baits. In previous studies that tested the viability of oral bait delivery (Ballesteros et al. 2011), older individuals gained access to baits in a proportion ranging from 8 to 43%. While the effect of vaccination on adults is unknown we speculate that it could act as a protective vaccination or revaccination, prolonging the individuals' immunity (as long as they are uninfected). It will nevertheless decrease bait availability for piglets.

There has been a dramatic increase in the prevalence of TBCL in wild boar over the last 20 years. For example, populations in Mediterranean Spain have shown a 26% increase in prevalence between 2000 and 2012 (Vicente et al. 2013). A similar increasing trend was observed in the control sites in this study, with a 10% increase in prevalence during the five-year study period. Our vaccination results should be interpreted in this context. Such findings make it increasingly important to assess new methods that can be used to reduce TB in wild boar. In this study we assess the potential of piglet vaccination as a tool for managing TB in wild boar.

Piglet vaccination using IV was successful in achieving a significant 34% reduction in TBCL prevalence at the population scale in the Managed site (90% piglet

bait uptake and moderate, 50%, initial TBCL prevalence). The model results confirmed this (Figure 4) showing that piglet vaccination could lead to the rapid reduction of prevalence. The model also highlighted that a similar (albeit reduced) decrease in prevalence would occur even if there was a high chance of piglets becoming infected prior to vaccination. This demonstrates that the vaccination of piglets using IV can be a valid and effective TB control tool in wild boar.

In contrast, the impact of IV in the Natural site was negligible. This suggests that the effect of vaccination may be context dependent. Vaccine performance can be affected by initial prevalence (which affects exposure to infection), pre-existing infection (since the vaccine is not curative), population dynamics, and may vary over time and space (Halloran et al. 1997; Kaden et al. 2000; Gormley and Corner 2011). Potential mechanisms for the different IV vaccine impact in the two sites in our study are: heterogeneous exposure to MTC, different levels of vaccination success at the two sites and inter-population mixing at the natural site.

First, exposure heterogeneity (in terms of infective dose and number of reinfections) could explain the different results obtained for IV as vaccines are believed to offer better protection against a light challenge of infection (Clemens et al. 2011). In our study, the Managed IV site was characterised by a moderate initial prevalence (50%), no generalization (lesions restricted to mandibular LNs) and low infection pressure for piglets, whereas the Natural IV site was characterised by a high initial prevalence (77%), a moderate proportion of generalized individuals (36%) and a high proportion of diseased 12-month-old wild boar (86%). Although the challenge dose is unknown in field trials, in the latter setting the potential exposure might have been sufficiently intense to resemble the challenge in laboratory trials, in which all individuals develop the disease despite receiving the vaccine. While the mathematical

model does not explicitly include the intensity of exposure to infection the model results suggest that both increased transmission and the proportion of infected piglets prior to vaccination reduce the impact attainable through vaccination (Figure 4).

Secondly, bait uptake achieved on both IV sites was not significantly different (92 and 74%), but nevertheless the proportion of successfully vaccinated individuals (those that received vaccine and were receptive to immunization) might have been. The likely higher level of infected piglets at the time of vaccination and the consumption of both vaccines (with possible non-protective outcomes; Díez-Delgado et al. 2014b) may have decreased the proportion of effectively immunised individuals and act as a confounder in the interpretation of vaccine impact based on vaccine type in Natural sites.

Thirdly, permeable fences in the Natural IV site allowed inter-population mixing (immigration/ emigration). These movements complicate the assessment of vaccine efficacy (dilution effect) and may act as a source of infection. Therefore we predict that enclosed and well-delimited (wild boar-proof fenced) populations will benefit most from vaccination.

We found no evidence of reduction of prevalence in any of the sites where BCG was deployed, as prevalence remained stable. BCG is known to confer variable protection in humans and cattle (Fine 1995; Buddle et al. 2013) and field trials in which BCG failed to provide any protection have been reported (in humans e.g. Colditz et al. 1994 and wildlife e.g. de Klerk et al. 2010). Field trials deploying BCG in other wildlife hosts have demonstrated protective effects on vaccinated individuals (Supplementary Material SM1). While this study confirmed BCG viability (Beltrán-Beck et al. 2014a),

we cannot rule out interference owing to non-tuberculous mycobacteria priming, genetic differences, nutritional status or co-infections (Fine 1995; Buddle et al. 2013).

A limitation of this study is that despite having four treatment sites we lack replication as they are divided by type of management and vaccine. Differences among sites beyond the tested confounders might have influenced the outcome.

The results from the mathematical modelling study indicate that the long-term use of piglet vaccination could reduce TB prevalence and thereby control TB in wild boar but would not be sufficient to achieve eradication. The model provides important insight on how the epidemiological dynamics respond to vaccination. Disease prevalence reaches its minimum value around 5 years after the start of the vaccination campaign (roughly the time frame of this field experiment). Thereafter, the reduction in disease prevalence, and associated reduction in disease-induced mortality at the population level, leads to an increase in population abundance. This finding could be tested in future wildlife vaccination programmes against virulent pathogens. This increase in population abundance also implies that increased hunting or population control may be required in order to balance the consequences of vaccination on population dynamics.

Furthermore, a consequence of the elevated population abundance is that disease prevalence can temporarily increase, beyond the pre-vaccinated level, if vaccination is stopped. The implication is therefore that disease management through vaccination requires a long-term commitment to maintain the reduction in disease prevalence. The oral bait method applied in this study provides an effective method for such long-term vaccine deployment.

Conclusions

Our efforts to deploy bait in free-ranging wild boar populations provided practical insights into the logistics of oral vaccination in Mediterranean ecosystems. Oral IV can contribute to TB control in its main Iberian reservoir, the wild boar. However, this study showed that IV performance could be context dependent. The study also showed that vaccination can have complex consequences on the population and epidemiological dynamics and this suggests that long-term disease control strategies need to be integrated with other wildlife management tools.

Council, Heriot-Watt University and the University of Edinburgh. I. Díez-Delgado was supported by a predoctoral grant from MINECO.

SM1 Table 1. Summary of the results and characteristics of vaccination field trials against tuberculosis conducted under natural exposure conditions in wild species.

| Species	Vaccine			Vaccine assessment		Reference
	Type	Dose	Route (delivery)	Endpoint	Efficacy	
Possum	BCG	10^6 cfu	Intranasal and conjunctival (trapping)	Clinical signs, lesion presence and cultured tissues	69%*	Corner et al. 2002
Possum	BCG	10^7 cfu	Oral (trapping)	Transition probability from susceptible to infected (estimated by modelling)	95%*	Tompkins et al. 2009
Possum	BCG	10^8 cfu	Oral (baiting)	Lesion presence and cultured tissues	81%	Nugent et al. 2016
Badger	BCG	10^6 cfu	Intramuscular (trapping)	Stat Pack serology INFg Culture	74%* 20% 27%	Chambers et al. 2011
Badger	BCG	10^8 cfu	Oral (trapping)	Stat Pack serology (hazard rate ratios)	36%-84%*	Gormley et al. 2017

* p<0.05

SM2 Table 1. Characterization of study sites. FBII is the relative wild boar abundance estimation based on a dropping frequency index and TBCL is the prevalence of tuberculosis-compatible lesions. Bold values represent significant p-values (p<0.05).

Site ID.	Status	Type of vaccine	Surface (km^2)	Type of site	Mean FBII	Fencing (permeability)	Supplementary feeding	Initial TBCL (%)	Final TBCL (%)
1	Control	None	8	Private hunting estate	0.1	Yes (wild boar permeable)	Yes	53	n.a.
2	Control	None	9	Private hunting estate	n.a.	Yes (wild boar permeable)	No	75	100
3	Control	None	103	National Park	0.3	Yes (wild boar permeable)	No	67	73
4	Control	None	27	Communal land	n.a.	No	No	50	29
5	Control	None	30	Communal land	0.1	No	No	36	33
6	Control	None	14	Private hunting estate	0.5	Yes (wild boar proof)	Yes	92	n.a.
7	Control	None	22	Private hunting estate		Yes (wild boar proof)	Yes	82	95
8	Control	None	8	Private hunting estate	n.a.	No	No	64	67
9	Control	None	22	Communal land	0.1	No	No	40	63
10	Control	None	9	Private hunting estate	1.2	Yes (wild boar proof)	Yes	56	n.a.
11	Control	None	26	Private hunting estate	0.2	Yes (wild boar proof)	Yes	87	83
12	Control	None	20	Private hunting estate	0.4	Yes (wild boar proof)	Yes	77	33
13	Control	None	21	Communal land	0.3	No	No	30	75
14	Control	None	23	Private hunting estate	0.3	Yes (wild boar proof)	Yes	88	69
15	Control	None	19	Private hunting estate	0.3	Yes (wild boar proof)	Yes	79	57
16[a]	Vaccine	BCG	19	Private hunting estate	0.4	Yes (wild boar proof)	Yes	53	50
17[b]	Vaccine	BCG	27	Natural Park (leased hunting land)	0.2	Yes (wild boar permeable)	No	100	96
18[c]	Vaccine	IV	29	Natural Park (leased hunting land)	0.2	Yes (wild boar permeable)	No	77	74
19[d]	Vaccine	IV	21	Private hunting estate	0.5	Yes (wild boar proof)	Yes	**50**	**16**

[a]Managed BCG [b]Natural BCG [c]Natural IV [d]Managed I

SM2 Figure 1. Distribution of piglet feeders throughout the vaccination sites: (a) Managed (BCG and heat-inactivated *M. bovis*; IV) and (b) Natural (BCG and IV).
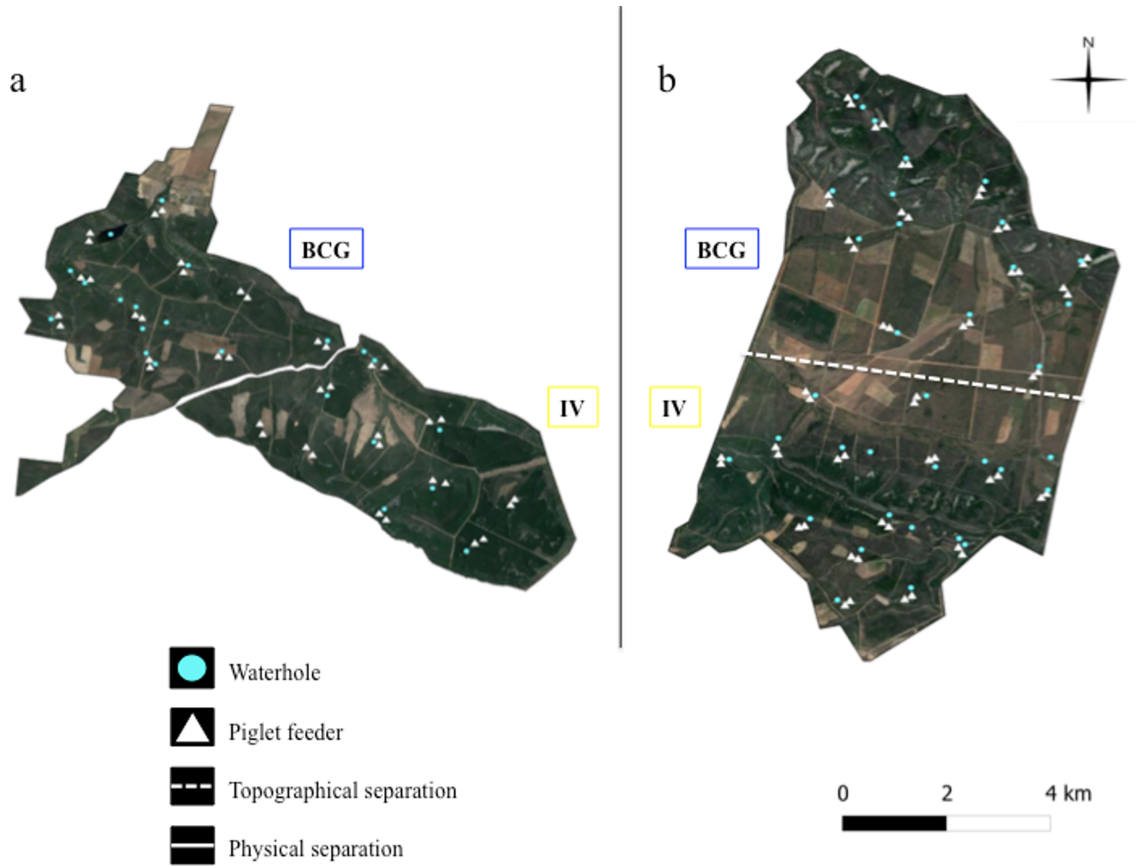
SM3. Information for the TB model.

1 Using mathematical modelling to examine the dynamics of the wild boar TB vaccination system

The model reflects a single geographical managed estate containing a homogeneously mixed population covering an area representative of a hunting estate. The population density of wild boar is separated into different age classes to capture distinct disease and reproductive characteristics for piglets (aged 0-1 year) P, yearlings (aged 1-2 years) Y, and adults (aged 2 years+) A. Further, the age-classes are split into susceptible, infected and generalized classes (subscripts S, I, G, respectively) to reflect the disease status of the population. The population dynamics of the wild boar TB system are represented by the following set of non-linear differential equations (which is an extension of classical disease modelling frameworks (see Anderson and May 1979; Keeling and Rohani 2008) and a schematic representation is shown in Figure S1:
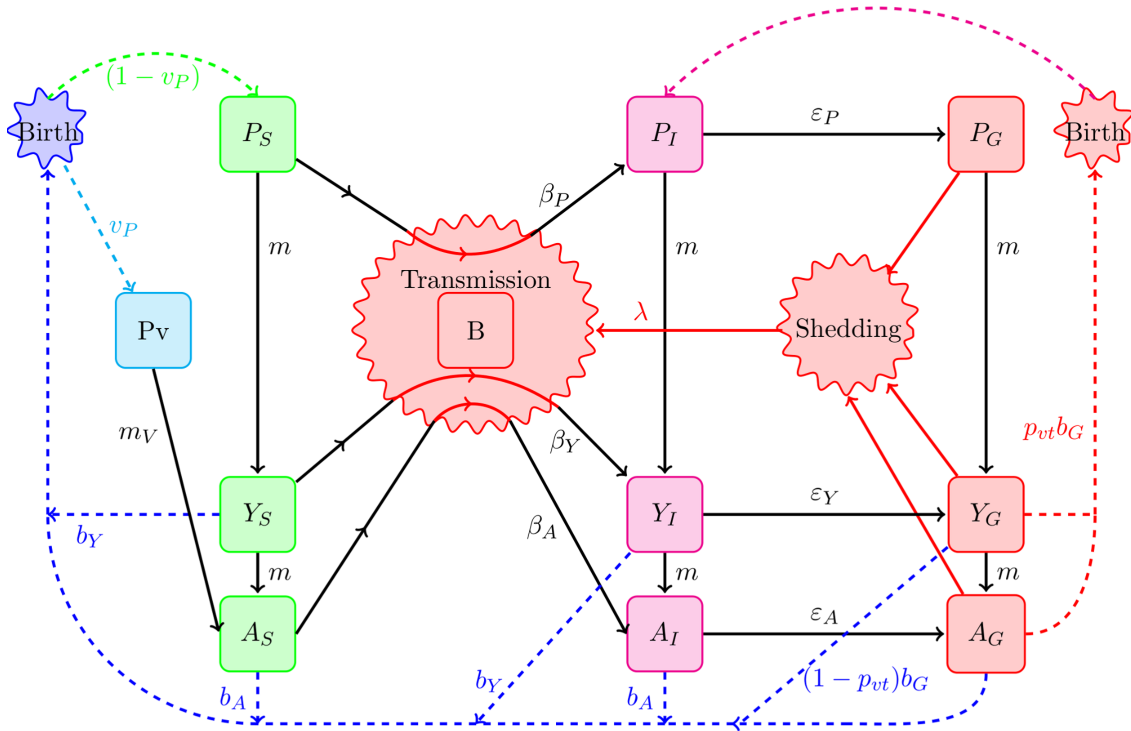
Figure S1: A schematic representation of the wild boar TB vaccination model represented by Equations 1. The model represents the density of piglets P, yearlings Y, and adults A with age-classes split into susceptible, infected and generalized classes (subscripts S, I, G, respectively). The class P_V represents vaccinated piglets and B represents the density of free-living TB particles. The parameters are detailed in Section 2.

$$\frac{dP_S}{dt} = (1 - v_P)(b_Y(Y_S + Y_I) + b_A(A_S + A_I) + (1 - p_{vt})b_G(Y_G + A_G))(1 - qN)$$
$$- mP_S - d_P P_S - \beta_P P_S B \tag{1a}$$

$$\frac{dP_I}{dt} = p_{vt}b_G(Y_G + A_G)(1 - qN) + \beta_P P_S B - mP_I - d_P P_I - \varepsilon_P P_I \tag{1b}$$

$$\frac{dP_G}{dt} = \varepsilon_P P_I - mP_G - \alpha P_G - d_P P_G \tag{1c}$$

$$\frac{dP_V}{dt} = v_P(b_Y(Y_S + Y_I) + b_A(A_S + A_I) + (1 - p_{vt})b_G(Y_G + A_G))(1 - qN) - m_V P_V - d_P P_V \tag{1d}$$

$$\frac{dY_S}{dt} = mP_S - mY_S - d_Y Y_S - \beta_Y Y_S B \tag{1e}$$

$$\frac{dY_I}{dt} = \beta_Y Y_S B + mP_I - mY_I - d_Y Y_I - \varepsilon_Y Y_I \tag{1f}$$

$$\frac{dY_G}{dt} = \varepsilon_Y Y_I + mP_G - mY_G - \alpha Y_G - d_Y Y_G \tag{1g}$$

$$\frac{dA_S}{dt} = mY_S + m_V P_V - d_A A_S - \beta_A A_S B \tag{1h}$$

$$\frac{dA_I}{dt} = \beta_A A_S B + mY_I - d_A A_I - \varepsilon_A A_I \tag{1i}$$

$$\frac{dA_G}{dt} = \varepsilon_A A_I + mY_G - \alpha A_G - d_A A_G \tag{1j}$$

$$\frac{dB}{dt} = \lambda(P_G + Y_G + A_G) - \mu B \tag{1k}$$

Here, N represents the total wild boar population. Susceptible and infected yearlings and adults give birth to susceptible piglets at rates b_Y and b_A respectively. Generalized yearlings and adults give birth to piglets at rate b_G with a proportion p_{vt} assumed infected (through pseudo-vertical transmission from parent to offspring) and the remainder, $(1 - p_{vt})$, assumed susceptible. In this study we assume that $b_Y = b_A = b_G$. The total population is regulated through a crowding parameter, q, that acts to stabilise the total population to a carrying capacity, $N = K$, in the absence of disease. Maturity from piglets to yearlings and yearlings to adults occurs at rate m and piglets, yearlings and adults may die of natural causes at rates d_P, d_Y, d_A respectively. Here we assume $d_P = d_Y = d_A$.

The prime driver for infection in the wild boar TB system is through environmental contact with free-living TB particles, with density B. We assume that free-living particles are shed from generalized wild boar at rate λ and decay at rate μ. Susceptibles may become infected through contact with free-living TB particles with transmission coefficients β_P, β_Y and β_A and infecteds can progress to the generalized class at rates ε_P, ε_Y and ε_A for the different age classes respectively. We assume that individuals in the generalized class suffer an additional disease induced mortality at rate α. We assume piglets and yearlings are more susceptible to TB infection than adults and so set $\beta_P = \beta_Y$, which we assume to be three times greater than transmission for adults, $\beta_A = 3\beta_Y$. Similarly we set the rate of progression to generalized infection for piglets and

yearlings to be the same, $\varepsilon_P = \varepsilon_Y$, and three times the rate for adults, $\varepsilon_A = \frac{1}{3}\varepsilon_Y$. In this way we have set the model so that the yearling class is the same as the piglet class in terms of disease characteristics, but the yearling class is the same as the adult class in terms of reproductive processes.

We represent vaccination in the model by assuming a proportion, v_P, of susceptible births enter the immune piglet class P_V. The vaccinated piglets lose their immunity at rate m_V maturing into the susceptible adult class. This implicitly assumes that when immunity is lost individuals have reached maturity and are able to reproduce but also have a reduced susceptibility to infection. Note, our vaccination coefficient combines the effects of both coverage and efficacy by representing the proportion of successful inoculations. In the model the vaccination process is represented as a continuous process whereas in the field vaccination is applied to piglets aged 3-6 months. Therefore, there is a chance of infection prior to vaccination and we approximate this with the inclusion of pseudo-vertical transmission from generalized individuals.

2   TB vaccination model parameters

We set values to approximate the observed prevalence and to be representative of the wild boar TB system in Central Spain. The parameters are as follows:

$b_Y = b_A = b_G = log(4)$ The population birth rate in a disease-free population when resources are unlimited. This constant rate means that for each reproductive member of the population, 3 piglets will be born, averaged over the population over a year. (This has been derived by assuming that there is a 50% sex ratio and that each female produces an average of 6 offspring per year when resources are not limited.)

$K = 500$ The carrying capacity for the total population in the target area in the absence of disease.

$q = \frac{1}{K}\left(1 - \frac{d_A(d_P+m)(d_Y+m)}{m(b_A m+b_Y d_A)}\right)$ This parameter limits the total population to the carrying capacity K in the populated disease-free steady state, and is derived from steady-state analysis of the model without infection.

$m = 1$ The rate that piglets mature to yearlings and yearlings mature to adults. These rates assume that it takes on average 1 year to enter the next age class.

$d_P = d_Y = d_A = \frac{1}{7}$ The natural death rate of all classes which implies an average life expectancy of 7 years.

$\beta_P = \beta_Y = c_\beta\beta_A = \frac{20}{K}$ The infection rates are fitted to give prevalence levels observed in the wild boar TB system in central Spain. We assume that $c_\beta = 3$ and so disease transmission to piglets and yearlings is three times that of the adult rate under the assumption that transmission is higher for piglets and yearlings than it is for adults.

$\varepsilon_P = \varepsilon_Y = 2$ The rate that infected piglets and yearlings become generalized. This assumes that it takes on average 6 months for an infected piglet or yearling to progress to the generalized class.

$\varepsilon_A = 2/3$ This is the rate that infectious adults become generalized. This assumes that it takes on average 18 months for an infected adult to progress to the generalized class.

$\alpha = 1$ This is the additional disease induced death rate of the generalized class and assumes that on average individuals spend 1 year in the generalized class before death.

$\lambda = 1$ The rate of shedding of infectious particles by generalized classes. We normalise this value to 1. This is valid as we have explored a range of values for β_P, β_Y and β_A which scale with the size of λ and the density of free-particles, B.

$\mu = 6$ This is the decay rate for free-living particles, indicating that they have an average life expectancy of 2 months.

p_{vt} The proportion of generalized births that result in pseudo-vertical transmission. In this study we assume $p_{vt} = 0$ or 1.

v_P The proportion of susceptible births successfully vaccinated. We explore the full range of possible values of v_P in this study.

$m_V = 1$ The rate that vaccinated piglets mature into the susceptible adult class. This assumes that when immunity is lost individuals are able to reproduce but also have the same reduced susceptibility to infection as adults.

3   TB vaccination model results

In the results that follow we refer to the total density of susceptibles as S where $S = P_S + Y_S + A_S$; the total density of infected but not generalized as I where $I = P_I + Y_I + A_I$; and the total density of generalized as G where $G = P_G + Y_G + A_G$. The total population density, N, can therefore be defined as $N = S + P_V + I + G$, which is at steady state, $N = P_S + Y_S + A_S = K$, in the absence of disease. All the densities are expressed in terms of population per geographical area. We define the total prevalence, $p_{tot} = \frac{I+G}{N}$, as the proportion of the total population infected with TB; the prevalence of infected but not generalized $p_{inf} = \frac{I}{N}$; and the prevalence of generalized $p_{gen} = \frac{G}{N}$; such that $p_{tot} = p_{inf} + p_{gen}$. We use MATLAB to obtain numerical results for the model as the proportion of successfully vaccinated piglets v_P is varied. We use the default parameter set detailed in Section 2 under conditions of 0 or 100% pseudo-vertical transmission, $p_{vt} = 0$ or 1. We consider results for both the default transmission coefficient, which results in a medium disease prevalence at steady state, and twice the default transmission value to reflect a greater risk of TB infection associated with increased aggregation of groups of wild boar at water holes during periods of drought or at feeding stations when extra food is made available resulting in a higher disease prevalence at the endemic steady state. We run the model until it has reached a stable endemic steady state then include vaccination for a period of 25 years to achieve a stable vaccinated steady state. We examine how vaccination affects the disease prevalence statistics p_{tot}, p_{inf} and p_{gen} and the epidemiological dynamics.

We examine results for the model described by Equations 1a-1k in different combinations of disease transmissions rates and pseudo-vertical transmission. Figure S2 (a) shows results for the default parameter set from Section 2 and 0% pseudo-vertical transmission; Figure S2 (b) shows results for default parameters with 100% pseudo-vertical transmission; Figure S2 (c) shows results for twice the default rate of disease transmission and 0% pseudo-vertical transmission; and Figure S2 (d) shows results for twice the default rate of disease transmission with 100% pseudo-vertical transmission. Note, Figure S2 (a) and (d) also appear in the main text, denoted Figure 4(a) & (b) and represent a site with medium initial prevalence where piglets have a low chance of infection prior to vaccination and a site with higher initial prevalence and greater rates of transmission combined with a greater proportion of piglets infected prior to vaccine delivery respectively.

Figure S2 (i) shows the change in disease prevalence for different levels of vaccination success (see also Table S1). Figures S2 (ii) and S2 (iii) show the epidemiological dynamics and changes in disease prevalence over time when we assume a 75% level of vaccination success. Figure S2 shows that the impact of vaccination on reducing TB prevalence is reduced if the transmission rate, initial prevalence and level of pseudo-vertical transmission is increased. These results support the findings in the main text that suggest that an increase in transmission intensity and proportion of infected piglets prior to vaccination reduces the impact attainable through vaccination.

v_P	Default Transmission						Doubled Transmission					
	$p_{vt} = 0\%$			$p_{vt} = 100\%$			$p_{vt} = 0\%$			$p_{vt} = 100\%$		
	p_{tot}	p_{inf}	p_{gen}	p_{tot}	p_{inf}	p_{gen}	p_{tot}	p_{inf}	p_{gen}	p_{tot}	p_{inf}	p_{gen}
0	68	31	37	79	34	45	79	34	44	87	36	50
0.25	57	29	29	70	32	38	70	34	36	81	36	45
0.5	48	26	22	61	30	31	61	33	28	73	35	38
0.75	39	23	16	51	27	24	53	31	22	65	34	31
1	30	19	11	41	24	17	46	29	17	57	33	25
	(a)			(b)			(c)			(d)		

Table S1: Table showing changes in p_{tot}, p_{inf} and p_{gen} for different levels of vaccination when pseudo-vertical transmission is 0% or 100%. Other details are as in Figure S2.
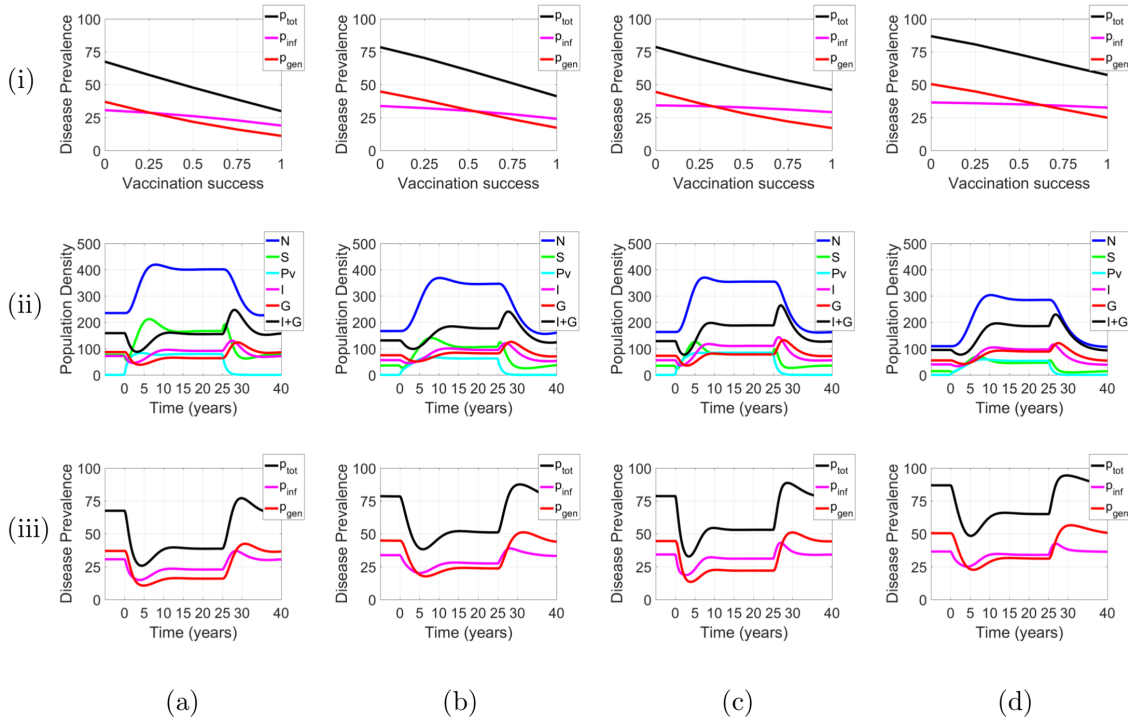
Figure S2: Using default parameter values described in Section 2 with (a) 0% pseudo-vertical transmission; (b) 100% pseudo-vertical transmission; (c) 0% pseudo-vertical transmission with disease transmission twice the default value; and (d) 100% pseudo-vertical transmission with disease transmission twice the default value. Row (i) shows disease prevalence against proportional vaccination success, v_p, with results determined at the stable endemic steady state when the specified level of vaccination is included; (ii) shows changes in density against time for the wild boar TB vaccination model for a vaccination level of 75%, ($v_p = 0.75$); and (iii) shows changes in disease prevalence against time for the wild boar TB vaccination model for a vaccination level of 75%, ($v_p = 0.75$). Here p_{tot}(black) is the proportion of the total population infected with TB; p_{inf}(magenta) the prevalence of infected but not generalized; p_{gen}(red) the prevalence of generalized; N(blue) is the total population density, I(magenta) the total density of infected but not generalized; G(red) the total density of generalized; S(green) is the total density of susceptibles; and P_V(cyan) is the total density of vaccinated piglets.

SM4 Table 1. Data on presence of tuberculosis-compatible lesions (TBCL, disease presence) of the total population and piglet age class by site and year. Sample size (n), absolute number of positives (+) and prevalence with associated 95%CI.

TBCL	Control year n	+	Prevalence (95%CI)	Vaccination year 1 n	+	Prevalence (95%CI)	Vaccination year 2 n	+	Prevalence (95%CI)	Vaccination year 3 n	+	Prevalence (95%CI)	Vaccination year 4 n	+	Prevalence (95%CI)
Control															
Total population	160	101	63.13 (56.25-70)	155	104	67.10 (59.38-74.84)	102	74	72.55 (63.73-80.39)	161	111	68.94 (61.49-75.79)	112	77	68.75 (59.82-76.81)
Piglets	34	18	52.94 (34.94-70.59)	36	18	50 (33.33-66.66)	16	14	87.5 (68.75-100)	31	16	51.61 (35.48-67.74)	22	13	59.10 (40.90-77.27)
Managed BCG															
Total population	17	9	52.94 (29.41-76.47)	13	7	53.85 (30.58-84.62)	16	8	50 (25-75)						
Piglets	7	1	14.29 (0-42.86)	5	3	60 (20-100)	5	1	20 (0-60)						
Managed IV															
Total population	24	12	50 (29.71-70.83)	27	12	44.44 (25.93-62.96)	19	6	31.58 (10.52-52.63)	32	5	15.63 (3.13-28.13)			
Piglets	1	0	0	2	0	0 (0-0)	6	0	0 (0-0)	4	0	0 (0-0)			
Natural BCG															
Total population	11	11	100 (100-100)	35	33	94.29 (85.71-100)	14	9	64.29 (35.71-85.71)	33	23	69.70 (51.51-84.85)	26	25	96.15 (88.46-100)
Piglets	1	1	100	2	1	50 (0-0)	6	3	50 (16.67-83.33)	6	5	83.33 (50-100)			
Natural IV															
Total population	22	17	77.27 (59.09-95.45)	24	21	87.50 (74.89-100)	41	25	60.98 (46.34-75.61)	49	44	89.80 (81.63-97.96)	47	35	74.47 (61.70-87.23)
Piglets	7	6	85.71 (57.14-100)	2	0	0 (0-0)	11	3	27.27 (0-54.54)	9	7	77.78 (44.44-100)	8	4	50 (12.50-85.50)

SM4 Table 2. Data on culture positivity of the total population and piglet age class by site and year. Sample size (n), absolute number of positives (+) and prevalence with associated 95%CI.

| TB CULTURE	Control year			Vaccination year 1			Vaccination year 2			Vaccination year 3			Vaccination year 4		
	n	+	Prevalence (95%CI)	n	+	Prevalence (95%CI)	n	+	Prevalence (95%CI)	n	+	Prevalence (95%CI)	n	+	Prevalence (95%CI)
Control															
Total population	43	25	58.14 (44.19-72.09)	84	30	35.71 (26.19-45.24)	46	26	56.52 (41.30-69.57)	152	130	85.53 (79.61-90.79)	110	56	50.91 (40.91-60.02)
Piglets	15	11	73.33 (53.33-93.33)	36	12	33.33 (19.44-50.00)	16	14	87.5 (68.75-100)	30	24	80 (66.58-93.33)	22	10	45.45 (22.73-68.18)
Managed BCG															
Total population	12	6	50 (25-75)	12	8	66.67 (41.67-91.67)				16	13	81.25 (62.50-100)			
Piglets	7	1	14.29 (0-42.86)	5	3	60 (20-100)				5	3	60 (20-100)			
Managed IV															
Total population	12	5	41.67 (16.67-66.67)	27	4	14.81 (3.70-29.63)	18	6	33.33 (11.11-55.56)				32	5	15.63 (3.13-28.13)
Piglets	1	0	0	2	0	0 (0-0)	6	0	0 (0-0)				4	0	0 (0-0)
Natural BCG															
Total population	7	5	71.43 (42.86-100)	35	25	71.43 (57.14-85.71)	14	11	78.57 (57.14-100)	33	26	78.79 (63.64-90.91)	26	23	88.46 (76.92-100)
Piglets	1	0	0	2	0	0 (0-0)	2	1	50 (0-100)	6	4	66.67 (33.33-100)	6	5	83.33 (50-100)
Natural IV															
Total population	16	13	81.25 (62.34-100)	24	18	75 (58.33-91.67)	41	25	60.98 (46.34-75.61)	49	46	93.88 (85.71-100)	47	35	74.47 (61.70-85.11)
Piglets	4	4	100 (100-100)	2	0	0 (0-0)	11	2	18.18 (0-45.45)	9	7	77.78 (55.56-100)	8	7	87.5 (62.50-100)

SM4 Table 3. Data on lesion score (disease severity) of the total population and piglet age class by site and year. Sample size (n), absolute number of positives (+), absolute number of generalized individuals out of the positives (G; lesions in more than one anatomical region, anatomical regions considered are head, thorax and abdominal cavity) and mean score (Mean).

TB LESION SCORE	Control year			Vaccination year 1			Vaccination year 2			Vaccination year 3			Vaccination year 4		
	n	+ (G)	Mean	n	+ (G)	Mean	n	+ (G)	Mean	n	+ (G)	Mean	n	+ (G)	Mean
Control															
Total population	160	101 (44)	2.81	155	104 (54)	4.30	102	74 (41)	4.59	161	111 (53)	4.27	112	77 (36)	4.71
Piglets	34	18 (3)	1.62	36	18 (6)	3.53	16	14 (9)	6.50	31	16 (10)	5.32	22	13 (5)	3.23
Managed BCG															
Total population	17	9 (3)	1.76	13	7 (7)	4.62	16	8 (1)	1.38						
Piglets	7	1 (1)	0.57	5	3 (3)	8.20				5	1 (0)	0.80			
Managed IV															
Total population	24	12 (0)	1.08	27	12 (4)	2.30	19	6 (3)	1.79	32	5 (3)	0.56			
Piglets	1	0 (0)	0	2	0 (0)	0	6	0 (0)	0	4	0 (0)	0			
Natural BCG															
Total population	11	11 (4)	5	35	33 (25)	6.43	14	9 (5)	7.29	33	23 (17)	6.73	26	25 (15)	7.15
Piglets	1	1 (0)	2		1 (0)	2	2	1 (0)	2	6	3 (1)	2	6	5 (3)	7
Natural IV															
Total population	22	17 (8)	4.64	24	21 (16)	5.67	41	25 (12)	3.68	49	44 (20)	6.27	47	35 (17)	4.43
Piglets	7	6 (2)	3	2	0 (0)	0	11	3 (2)	2.27	9	7 (4)	7.89	8	4 (2)	4.75

CHAPTER III

Tuberculosis vaccination sequence effect on protection in wild boar.
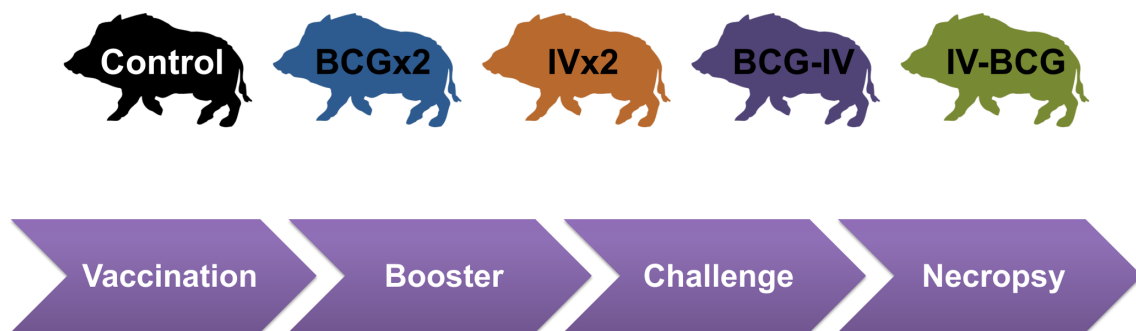
A version of this work has been submitted:

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Resumen

El jabalí (*Sus scrofa*) es un reservorio silvestre para las bacterias del complejo *Mycobacterium tuberculosis* causantes de la tuberculosis animal (TB). En este hospedador la vacunación representa una herramienta valiosa para el control de dicha enfermedad por lo que se están desarrollando nuevos candidatos vacunales así como nuevas estrategias de vacunación. En este sentido, los protocolos heterólogos de vacunación pretenden reforzar la protección vacunal o prolongar la duración de la misma. En este experimento se evaluó la protección y la respuesta inmune conferida por protocolos heterólogos y protocolos homólogos basados en las vacunas viva atenuada BCG y derivada de *Mycobacterium bovis* inactivada por calor (IV). Veintiún rayones fueron asignados aleatoriamente a uno de los siguientes grupos: Control, BCGx2, IVx2, BCG-IV e IV-BCG. Los rayones fueron vacunados por vía oral, retados con una cepa de campo de *M.bovis* por vía orofaríngea y necropsiados 4 meses después del reto. Se observaron reducciones significativas en el score total del lesiones respecto al grupo Control en los grupos IVx2 e IV-BCG (del 67% y el 66% respectivamente; $F_{4,16}= 6.393$, p=0.003; Tukey $_{\text{Control vs IVx2}}$ p=0.019, Tukey $_{\text{Control vs IV-BCG}}$ p=0.015). Sin embargo no se encontraron diferencias significativas para los grupos BCGx2 (pese a que se registró una reducción en el score total de lesiones del 48%) y BCG-IV (reducción del 3%). Los protocolos heterólogos con BCG e IV no mejoran la protección obtenida por los protocolos homólogos en jabalí y ofrecieron resultados dispares (desde no proporcionar protección a lograr resultados similares a protocolos homólogos). Por ello, los protocolos de vacunación homólogos siguen siendo la mejor opción en jabalíes (y potencialmente cerdos) frente a TB. Además, la influencia de la secuencia de administración de las vacunas evidencia la necesidad de estudiar la interferencia de la sensibilización previa sobre los resultados de la vacunación.

Abstract

The Eurasian wild boar (*Sus scrofa*) is a wild reservoir for members of the *Mycobacterium tuberculosis* complex causing animal tuberculosis (TB). In this host vaccination represents a valuable tool for TB control and new vaccine candidates and protocols are being studied. In this regard, heterologous prime-boost vaccination regimes aim to strengthen vaccine-induced protection or to expand duration of immunity. Thus, we evaluated the protection and immune response achieved by heterologous and homologous regimes administering live attenuated BCG and heat-inactivated *Mycobacterium bovis* (IV). Twenty-one wild boar piglets were randomly allocated in five groups: control, homologous BCG, homologous IV, heterologous IV-BCG, heterologous BCG-IV. Piglets were vaccinated orally, challenged by the oropharyngeal route with a field strain of *M. bovis*, and necropsied 4 months after challenge. Significant 67% and 66% total lesion score reductions were detected in homologous IV (IVx2) and heterologous IV-BCG groups when compared with Control group ($F_{4,16}= 6.393$, $p=0.003$; Tukey $_{Control\ vs\ IVx2}$ $p=0.019$, Tukey $_{Control\ vs\ IV\text{-}BCG}$ $p=0.015$). No significant differences were found for homologous BCG (although a 48% reduction in total lesion score was recorded) and BCG-IV (3% reduction). Heterologous regimes involving BCG and IV do not provide improved protection in the wild boar model and showed mixed results (from no protection to similar protection as homologous regimes). Therefore, homologous regimes remain the best option to vaccinate wild boar (and pigs) against TB. Moreover, vaccine sequence dramatically influenced the outcome underlining the relevance of studying the effects of prior sensitization in the outcome of vaccination.

Introduction

Heterologous prime-boost vaccination consists of priming the immune system against target antigen/s with one formulation and subsequently boosting with the same antigen/s using a different type of vaccine (Woodland et al. 2004; Lu 2009). This strategy aims to achieve an additive or synergistic effect that strengthens vaccine-induced protection or expands duration of immunity and is perceived as a potential strategy to control tuberculosis (TB) by immunization. In fact, the cellular response generated is stated to be stronger, broader and more durable than the one achieved by homologous vaccination regimes (Vordermeier et al. 2004; McShane and Hill 2005). This approach can be useful to control intracellular pathogens, as those causing TB, that require powerful T cell responses (Woodland et al. 2004).

TB is a major concern to both human and animal health worldwide. Animal TB produces economic losses in the livestock industry, has zoonotic potential and is an issue in wildlife conservation (Gortazar and Boadella 2014). Furthermore, the scenario of animal TB is complex because, although cattle are the key domestic host and the main TB control target, other livestock and several wild hosts do contribute to *Mycobacterium tuberculosis* complex (MTC) maintenance (Gortazar et al. 2015b).

In wildlife, vaccination is perceived as a feasible disease control option for cost effective and long-term TB control (Cross et al. 2007b). Thus, vaccination is studied worldwide in several reservoir host models in laboratory and field trials (Nol et al. 2008; Nugent et al. 2016; Gormley et al. 2017) using the live attenuated Bacille Calmette Guerin (BCG). BCG does not provide sterilizing immunity and has shown variable efficacy in cattle and wildlife (Buddle et al. 2013; de Klerk et al. 2010; Díez-Delgado et al. 2018); therefore, improved vaccines or alternative immunization

strategies such as prime-boost regimes that provide better protection are being investigated (Vordermeier et al. 2014).

In Spain, the native Eurasian wild boar (*Sus scrofa*) is considered the main wild reservoir for MTC (Naranjo et al. 2008), displaying a high TB prevalence (63%) and an increasing prevalence trend (Vicente et al. 2013), posing an evident risk to cattle (LaHue et al. 2016). In this context, wild boar vaccination represents a valuable tool that could be implemented in an integrated strategy for TB control in Mediterranean Iberia.

Consequently, laboratory trials with BCG (Ballesteros et al. 2009a) and a recently developed heat-inactivated *Mycobacterium bovis* vaccine (*M. bovis* inactivated vaccine, IV) have been conducted showing comparable levels of protection of both vaccines in wild boar (Garrido et al. 2011). Homologous re-vaccination (administering more than one dose of the same vaccine) with both BCG (Gortázar et al. 2014b) and IV (Beltrán-Beck et al. 2014b) resulted in increased protection against *M. bovis* challenge as compared to single dose strategies (Garrido et al. 2011) i.e. homologous vaccination works in the wild boar model. Moreover, recent field trials evidenced a positive effect of homologous prime-boost strategies for parenteral IV and oral IV, but not for oral BCG (Díez-Delgado et al. 2017 and 2018).

The aim of this study is to evaluate the protection and the immune response achieved by heterologous regimes compared to homologous regimes administering BCG and IV by the oral route in wild boar under the hypothesis that heterologous prime-boost strategies will be more immunogenic than homologous prime-boost ones (Lu 2009).

Material and Methods

Ethics statement

The protocol was approved by the Committee on the Ethics of Animal Experiments of the Regional Agriculture Authority (Diputación Foral de Bizkaia, Permit Number: JAVACON-2013/6329-BFA). Handling procedures and sampling frequency were designed to minimize stress and health risks according to European (Directive 63/2010) and Spanish legislation (Royal Decree 53/2013 and Act 6/2013).

Animals and experimental design

Twenty-one 3 to 4 month-old male wild boar piglets were purchased from a commercial farm known to be free of mycobacterial infections and tested on arrival by bPPD ELISA (Boadella et al. 2011c) to confirm absence of antibodies against *M. bovis*, yielding a fully negative result.

During the experiment wild boar were housed in the Biosafety Level 3 containment of the Basque Institute for Agricultural Research and Development (NEIKER-Tecnalia) with *ad libitum* food and water and monitored daily. The piglets were randomly allocated in five groups: control (n= 4), homologous BCG (n= 4), homologous IV (n= 4), heterologous IV-BCG (n= 5), heterologous BCG-IV (n= 4).

The wild boar vaccination schedule applied was the same used in previous experiments (Ballesteros et al. 2009a; Garrido et al. 2011; Gortazar et al. 2014b). Briefly, piglets were vaccinated orally with the first dose at T_0, revaccinated one month later on T_1 (day 31), challenged 4 months later on T_2 (day 125), sampled on T_3 (day 183) and necropsied on T_4 (day 238). During these five handlings restraint was not longer than 10 minutes and anaesthesia was not required. Vaccines were delivered

orally by means of a syringe. Challenge involved the oropharyngeal administration of 2 ml of a suspension containing 10^5 colony-forming units (CFU) of a *M. bovis* field strain (spoligotype SB0339, www.mbovis,org). At the end of the experiment, animals were anesthetized with the protocol described by Barasona (2013; intramuscular injection of tiletamine-zolazepam, 3 mg/kg, and medetomidine, 0.05 mg/kg) and euthanized by the use of the captive bolt method.

Vaccines

The two vaccines involved on the heterologous prime-boost strategy were BCG (*M.bovis* BCG Danish reference strain, CCUG 27863) and IV (obtained from a *M. bovis* field isolate spoligotype SB0339, Neiker 1403). The vaccines were prepared as described for previous experiments (Ballesteros et al. 2009a and Garrido et al. 2011). Single BCG and IV doses contained 10^6 and 10^7 CFU, respectively, in 2ml. The control group received 2 ml PBS.

Sample Collection and Necropsy

Blood samples were collected without anticoagulant for serum preparation to conduct bPPD ELISA, Complement component C3 ELISA and the cytokine array; in lithium heparin to conduct interferon-gamma (IFN-γ) release assays (IGRA) and in EDTA to perform the and the peripheral blood mononuclear cells (PBMC) RNA PCR.

A thorough post-mortem examination was conducted to assess visible TB-compatible lesions by carefully inspecting selected tissues after slicing them into 1–2 mm thick pieces. Organs inspected included lymph nodes (mandibular, parotid, retropharyngeal, tracheobronchial, mediastinal, hepatic, mesenteric, ileocecal and inguinal), oropharyngeal tonsils and visceral organs (lung considering each lobe separately, spleen, liver and kidneys). Scoring of TB-compatible lesions was based on

lesion distribution in the inspected organs and lesion severity (categorized from 0 to 4 according to the presence, size and number of lesions) following the method described in Ballesteros (2009a).

Microbiology

Samples for culture were immediately processed. Tonsils, main lymph nodes, lung, and ileocecal valve were cultured and isolates were spoligotyped in order to confirm the strain and calculate the culture score (number of samples yielding a positive result of the total number of cultured samples, score range 0-8) as previously described (Garrido et al. 2011).

Serology

Antibody response to bPPD. Serum samples (T_0 to T_4) were tested for immunoglobulin G (IgG) against purified protein derivative (PPD) derived from *M. bovis* (bPPD; CZ Veterinaria, Porriño, Spain) using IgG antibodies (Bethyl, Inc., Montgomery, TX) as a conjugate by means of an in-house ELISA (protocol described by Boadella et al. 2011c). Sample results were expressed as an ELISA percentage (E%) that was calculated using the formula: Sample E% = [(mean sample OD/ 2 x mean negative control OD) x100]. Samples with E% values ≥100 were considered positive.

Complement component C3 determination. For the quantitative determination of pig C3 protein concentration (μg/ml) in serum samples at T_0, T_2 and T_4, a sandwich ELISA was used (Pig Complement C3 ELISA kit, CUSABIO, Wuhan, China). Serum samples and standards were analysed following the manufacturer's instructions. Data was linearized by a standard curve and regression analysis was used to determine sample C3 concentrations.

Cytokine determination. The cytokine concentration in pooled sera was determined at T_0, T_2 and T_4 using the Quantibody porcine cytokine array (RayBiotech Inc, Norcross, GA, USA), an array-based multiplex ELISA system for the simultaneous quantitative measurement of multiple cytokines. Using this system, standard cytokines and samples were assayed in each array simultaneously through a sandwich ELISA, following the recommendations of the manufacturer. The signals were visualized using a Gene Pix 4100A laser scanner (Molecular Devices, Sunnyvale, CA, USA) and data were extracted by GenePix Pro 6 software (Molecular Devices). Finally, the quantitative data analysis was performed using the Quantibody Q-Analyzer software (RayBiotech Inc). Cytokine concentration was expressed in pg/ml.

IGRA

At T_2, T_3 and T_4, blood samples were collected into tubes with lithium-heparin. Within 8 h of collection, stimulation of whole blood with PBS (nil control) and the avian and bovine purified protein derivative (PDD) (CZ Veterinaria, Porriño, Spain) was performed as described for other species (Gormley et al. 2006). The detection of IFN-γ in the supernatant was performed using a quantitative ELISA (Pierce Endogen, Rockford, IL, USA), according to the manufacturer's recommendations.

RNA isolation and real time RT-PCR

Total RNA was extracted from wild boar PBMC using ARNzol spin kit (Real, Durviz, Spain) following manufacturer's recommendations. RNA was used for quantitative real-time RT-PCR analysis of mRNA levels of selected genes in individual samples. Selected genes were complement component 3 (C3), interleukin-1beta (IL-1B) and methylmalonyl CoA mutase (MUT). Real-time RT-PCR was performed with gene-specific primers (C3, SsC3-L: acaaattgacccagcgtagg and SsC3-R: gcacgtccttgctgtactga;

IL-1B, SsIL1beta-L: ccaaagagggacatg gagaa and SsIL1beta-R: ttatatcttggcggcctttg; MUT, Ss MUT-L: gtttgccaacggtgaaaagt and SsMUT-R: aatgagcttcaaggcagcat) using the Quantitech SYBR Green RT PCR kit and the Rotor GENE-Q (Qiagen Inc. Valencia, CA, USA) following manufacturer's recommendations. A dissociation curve was run at the end of RT-PCR reaction to ensure that only one amplicon was formed and that the amplicon denatured consistently at the same temperature range for every sample (Ririe et al. 1997). All reactions were performed in duplicate. The mRNA values were normalized against *Sus scrofa* cyclophilin (SsCyclophilin-L: agcactggggagaaaggatt and SsCyclophilin-R: cttggcagtgcaaatgaaaa), using the genNorm ddCT method (Pérez de la Lastra et al. 2009). The normalized expression was calculated at each time point and the mean of replicate values was used to compare data between vaccinated and control groups.

Statistical analyses

Differences in score (total lesion, thorax lesion and culture) between vaccinated groups and control group were tested using the Wilcoxon rank-sum test. Correlations among serology (E%), IFN-γ (optical densities, OD) values and total lesion score values were performed using Spearman´s rank test. Comparisons among groups regarding serology values (%E) and IFN-γ OD were conducted using ANOVA. Significance was fixed at $p < 0.05$. All analyses were carried out in the R statistical package (R Development Core Team) using the ggplot2 library to obtain the figures (Wickham 2009).

Results

Clinical signs

No clinical signs were observed in any of the wild boar after vaccination or challenge.

Pathology and M. bovis isolation

All wild boar developed TB-compatible lesions, and infection by challenge strain was confirmed by re-isolation from tissues and spoligotyping (SB0339). Figure 1 presents individual lesion score values and median by group for total lesion score and thorax lesion score. Figure 2 presents individual culture score values and median by group.
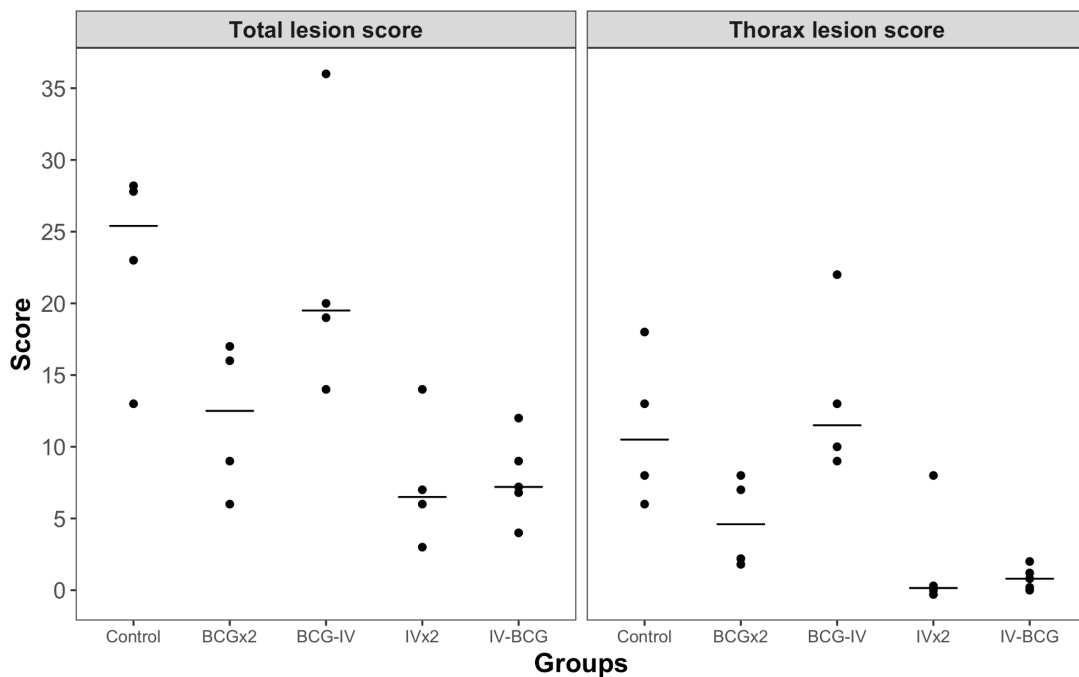
Figure 1. Individual total (left panel) and thorax lesion score (right panel) values by group. Solid lines represent median values for each group.
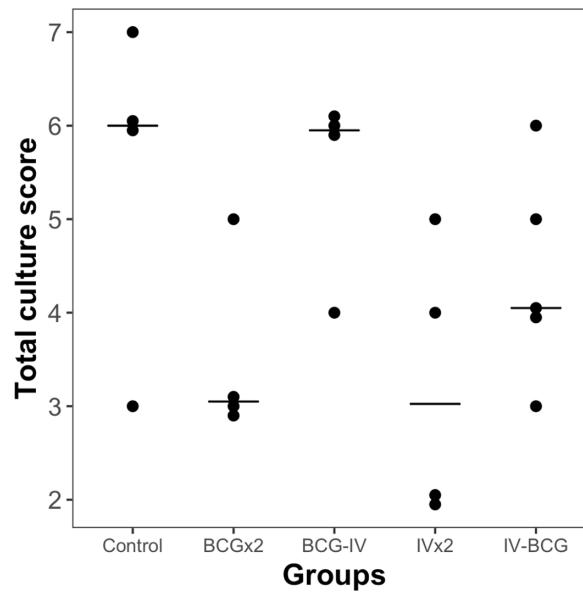
Figure 2. Individual total culture score values by group. Solid lines represent median values by group.

Considering the homologous vaccinates, wild boar of the IVx2 and BCGx2 groups showed total lesion score reductions of 67% and 48% and thorax lesion score reductions of 82% and 58%, respectively, as compared to controls. Heterologous vaccinates showed mixed results. Wild boar of the IV-BCG group showed reduced total and thorax lesion score (66% and 93% respectively) when compared to controls while BCG-IV vaccinates had negligible reduction in total lesion score and worse performance than controls in thorax lesion score (3% reduction and 20% increase, respectively).

This was confirmed by the detection of significant differences among groups in the total lesion score ($F_{4,16}= 6.393$, $p=0.003$). Post hoc tests revealed differences only among Control group and IVx2 and IV-BCG (Tukey $p=0.019$ and $p=0.015$ respectively) and between BCG-IV group and IVx2 and IV-BCG (Tukey $p=0.027$ and $p=0.022$ respectively).

Differences among groups in thorax lesion score were also significant ($F_{4,16}$= 7.902, p=0.001) and followed the same pattern affecting Control group versus IVx2 and IV-BCG (Tukey p=0.041 and p=0.013 respectively) and BCG-IV versus IVx2 and IV-BCG (Tukey p=0.009 and p=0.002 respectively).

Regarding lesion distribution, four individuals presented localized lesions circumscribed to the head and neck region (no thoracic or abdominal lesions, thus no generalization). These individuals belonged to groups IVx2 (3/4) and IV-BCG (1/5). Subsequent culture confirmed contained infection (negative thoracic cultures) in the three individuals belonging to the IVx2 group. Differences of total and thorax culture score with controls were not significant ($F_{4,16}$= 2.7, p>0.05).

Serological analysis

Antibody response to bPPD. Antibody levels against bPPD are represented in Figure 3. Antibody levels remained negative until challenge (T_0-T_2), thus oral vaccination did not generate antibody response. Positive responses started to be detected at T_3 and increased at T_4. Response to challenge generating detectable antibodies did not occur in all animals (at T_3 one individual of each group and at T_4 one individual of the BCGx2 group had not seroconverted, respectively). Differences between groups were not significant at any time point (p>0.05), further information on mean E% and standard deviation values by group for each time point is available in SM Table 1. However, individuals of the control group and the IV-BCG group had the highest responses, while those of the BCGx2 group had the lowest responses. No correlation between antibody response at T_4 and lesion or culture scores was found (p>0.05).
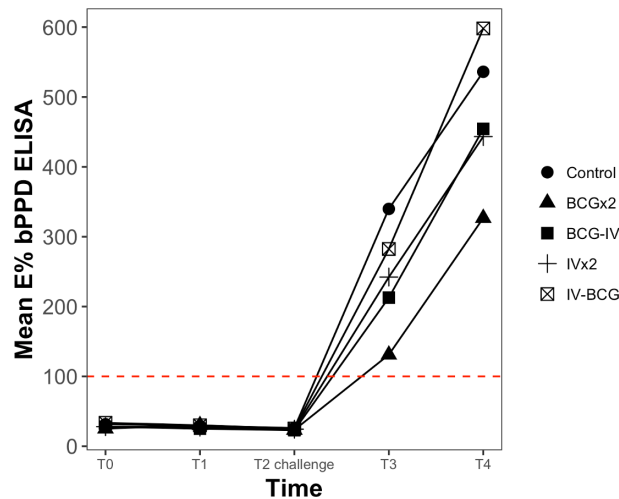
Figure 3. Mean antibody response (mean E%) of the bPPD ELISA by group for each sampling time point. Horizontal dashed line stands for cut off level (negative< 100 E%≤ positive).

Complement component C3. Serum C3 protein levels are shown in Figure 4. No significant differences were detected among groups for any time point (p>0.05), due to high individual variability. Serum C3 protein levels were significantly higher at T_2 and T_4 than in T_0 ($F_{2,60}$=4.531, p=0.015; Tukey p=0.032 and p= 0.029 respectively). No significant correlation between C3 in serum and lesion or culture scores was detected (p>0.05).
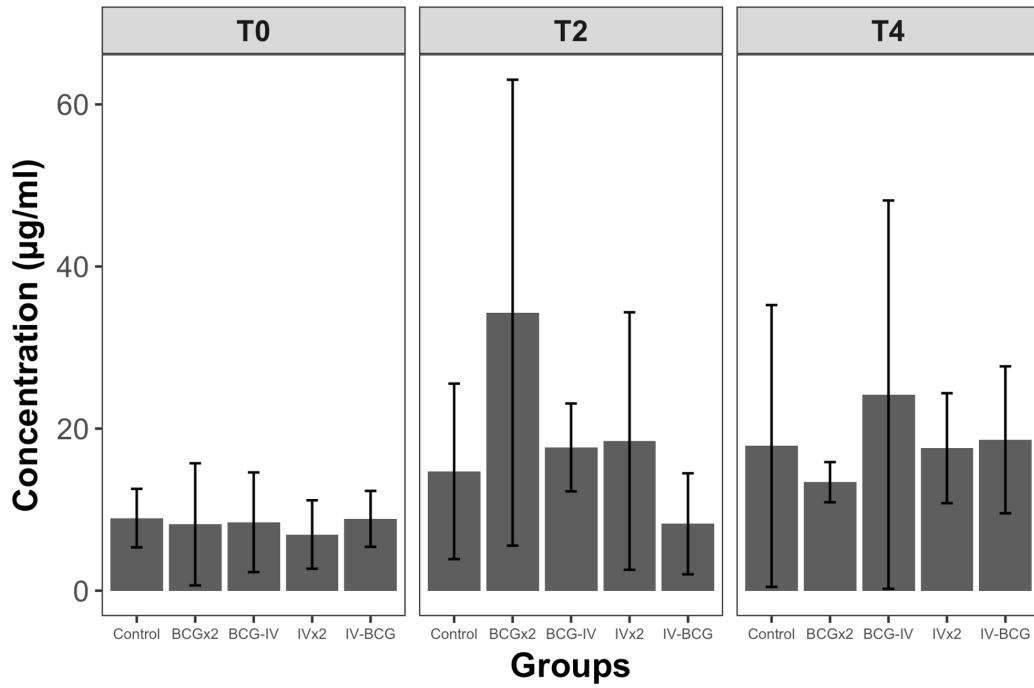
Figure 4. Serum C3 concentration (µg/ml) by group at T_0 (basal), T_2 (after completing vaccination regime) and T_4 (at necropsy, ca. 4 months after challenge).

Cytokines in serum. As Figure 5 show for IL-1B (left panel) and IL-6 (right panel) the levels of these cytokines at T_0 were similar among groups, rose at T_2 in vaccinated animals and decreased post-infection, at T_4, except for the control group for both cytokines and BCGx2 for IL-1B.
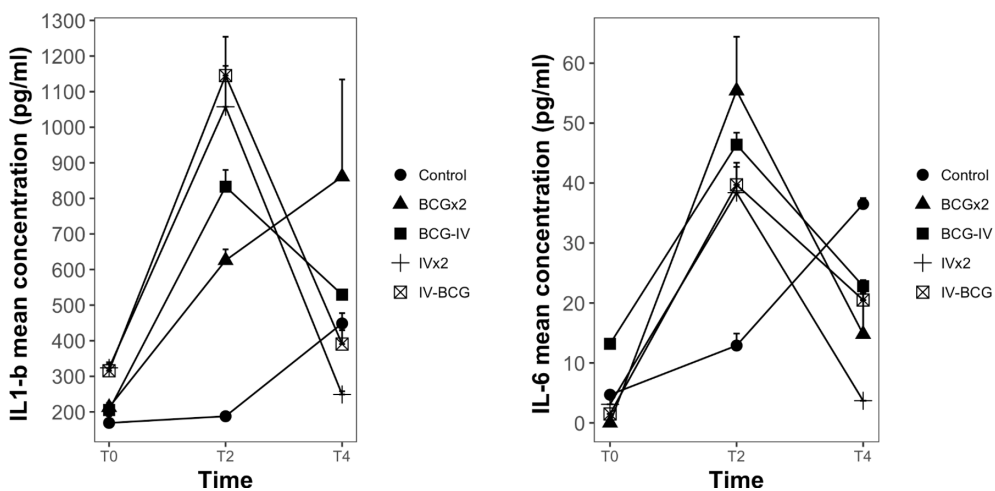

Figure 5. Mean IL-1B (left panel) and mean IL-6 (right panel) concentration in serum

(pg/ml) before vaccination (T_0), after full vaccination regime (T_2) and by end of the experiment (T_4) for each group. Error bars represent standard deviation of spot values for each cytokine in each well (sample).

IGRA

No response to bPPD stimulation was detected at T_2. An IFN-γ peak appeared at T_3 in all groups (mean OD ± SE ranging from 18.7 ± 4.5 in IV-IV group to 10.7±1.8 in controls) and a sharp decline (mean) at necropsy time (T_4) as shown in Figure 6. All PBS controls yielded consistently low results (mean OD ± SE, 0.12 ± 0.01). Mean OD and standard deviation values by group for each time point are shown on SM Table 2. Differences among groups were not significant at any time (p>0.05). No correlation with lesion or culture score was evidenced (p >0.05).
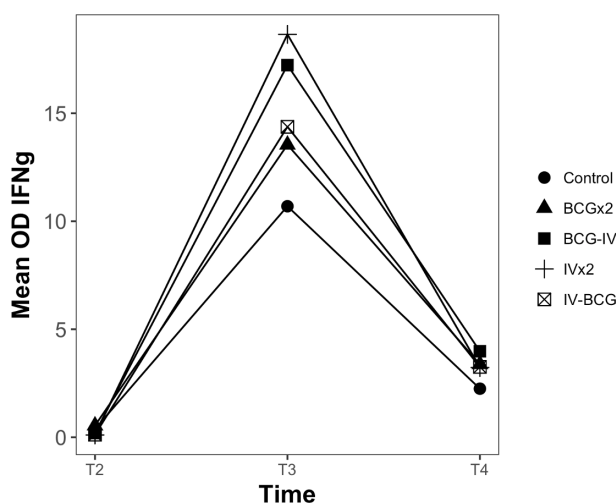
Figure 6. Mean optical density (OD) readings of the interferon-gamma (IFN-γ) release assay at T_2 (after full vaccination regime), T_3 (2 months post challenge) and T_4 (ca. 4 months post challenge) by group.

Gene expression.

The C3, MUT and IL-1B mRNA levels were analysed in PBMC at T_0 (before vaccination), T_2 (after full vaccination regime), and T_4 (at the end of the experiment ca. 4 moths after challenge). Figure 7 shows normalized mRNA values for C3 (left panel), MUT (middle panel) and IL-1B (right panel) by group for each time point. Mean normalized values (arbitrary units) and standard deviation for C3, MUT, IL-1B genes by group for each time point are displayed in SM Table3.
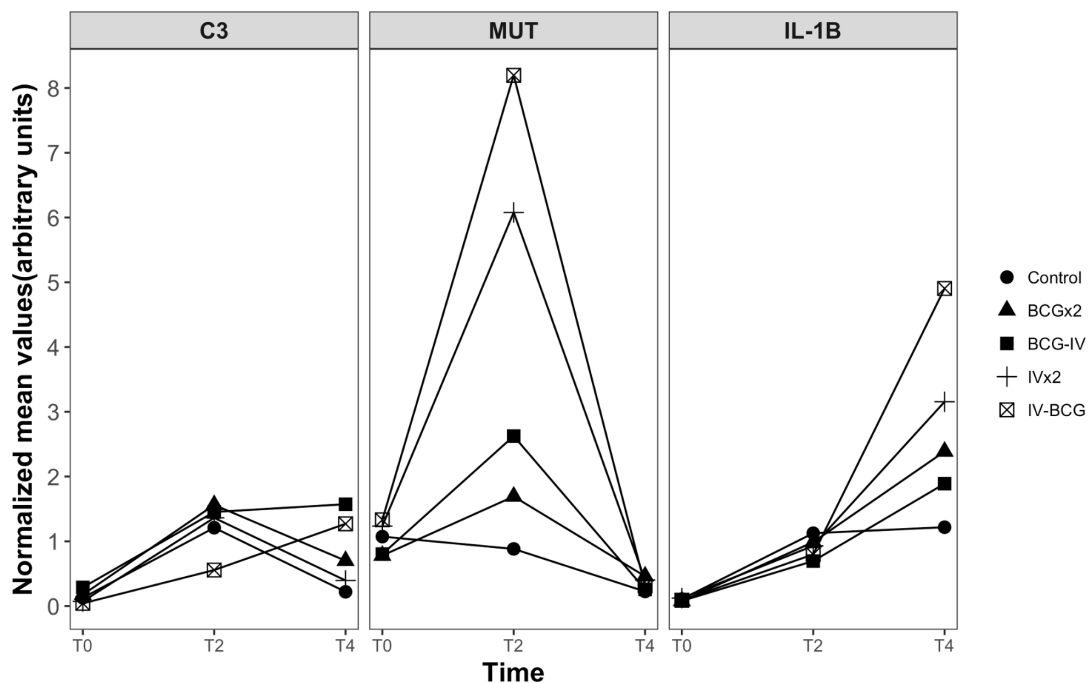
Figure 7. Quantitative C3, MUT and IL-1B gene expression analysis in PMBC using qRT-PCR. Mean normalized mRNA levels of C3 (left panel), MUT (middle panel) and IL-1B (right panel) before vaccination (T_0), after full vaccination regime (T_2) and by end of the experiment (T_4) for each group.

C3 expression levels significantly increased at T_2 and at T_4 (mostly driven by heterologous vaccination groups) compared to T_0 ($F_{2,60}$= 10.037, p=0.00, Tukey

p=0.000 and p= 0.012 respectively). Significant difference among groups was detected at T_4 ($F_{4,16}$= 3.059, p=0.047) but the Tukey test was not significant for any pair comparison (only marginal significance among control and BCG-IV groups, p=0.074). No correlation with lesion or culture score was evidenced (p>0.05).

MUT expression rose significantly after vaccination (at T_2; $F_{2,60}$= 15.471, p=0.000, Tukey p=0.000) and was of similar magnitude among BCG primed groups and IV primed groups, respectively. Significant differences among groups were found at T_2 ($F_{4,16}$= 4.704, p=0.011, Tukey p=0.000). Tukey posthoc analysis revealed significantly higher MUT expression levels in the IV-BCG group as compared to controls (p= 0.018) and to the BCGx2 group (p=0.039). MUT gene expression levels at T_2 negatively correlated with total lesion score (r_s= -0.499, p=0.021).

Regarding IL-1B, expression levels increased significantly at the end of the experiment (T_4) compared to T_0 and T_2 ($F_{2,60}$= 19.259, p=0.000, Tukey p=0.00 for both T_0 and T_2). No significant differences among groups were detected (p>0.05) although gene expression levels rose at T_4 in vaccinated groups and more intensely in IV primed groups. No significant correlations were found with lesion or culture score (p>0.05).

Discussion

Contrary to our expectations, heterologous prime-boost strategies were not more protective than homologous prime-boost ones, at least with this specific experimental design. The results suggest that IV effectively primes the protective immune response boosted by IV or BCG. By contrast, we did not found a significant effect in BCG followed by BCG (despite the substantial reduction in total lesion score, ca. 50%, and the cumulative evidence of the protection conferred by BCG in the wild boar model,

Ballesteros et al. 2009; Gortázar et al. 2014) or IV (no evidence of protection at all). The significant reduction in lung lesions and overall dissemination seen in the IVx2 and IV- BCG groups (Figure 1) was further highlighted by culture results (Figure 2) and is likely to translate into decreased *M. bovis* shedding and transmission. However, the only 3 wild boar without lesion generalization and thoracic infection belonged to the homologous IVx2 group, suggesting that homologous prime-boosting with this heat-inactivated vaccine is a good choice for TB control in wild boar (Beltrán-Beck et al. 2014b). Oral delivery of IV and BCG and their combinations did not induce antibodies or IFN-γ response (Figures 3 and 6) in agreement with results of previous studies (Ballesteros et al. 2009a; Garrido et al. 2011; Beltrán Beck et al. 2014b; Gortázar et al. 2014b). Thus, both oral BCG and oral IV vaccination, either with homologous or heterologous regimes, are compatible with TB bPPD ELISA and IGRA diagnostics.

The results of this study support the model for the protective mechanism elicited by IV through the activation of innate immune responses (Beltrán-Beck et al. 2014b; López et al. 2018; Risalde et al. 2018). The upregulation of C3 and proinflammatory cytokines IL-1B and IL-6 in response to vaccination, in agreement with previous studies with the IV in wild boar, deer and zebrafish (Garrido et al. 2011; Beltrán-Beck et al. 2014b; Thomas et al. 2017; López et al. 2018; Risalde et al. 2018), support a role for this molecule in the immune response to this vaccine. Moreover, complement components and proinflammatory cytokines have been identified to be involved in innate immune response to limit mycobacterial infection in vertebrate hosts including humans (Velasco-Velázquez et al. 2003; Garrido et al. 2011; O'Gara et al. 2013; Beltrán-Beck et al. 2014b; Mayer-Barber and Sher 2015; López et al. 2018).

These results also provide an additional explanation of the lack of detectable vaccine impact in a recent field trial in sites where 20-40% of wild boar accessed both

vaccines (Díez-Delgado et al. 2018). It was not possible to assess the prime-boost sequence in the field trial but unintended priming with BCG or with other mycobacteria may explain the low efficacy of vaccination in those sites (Díez-Delgado et al. 2018, labelled in the study as "Natural sites").

A limitation of this study is the low sample size (due to compliance to animal testing ethic requirements) that may have limited the statistical significance of the results. We found differences in protection due to sensitization sequence in our study, however the overall influence of the order of prime–boost administration remains unclear. Some authors have found that is not critical in terms of achieved protection (Vordermeier et al. 2004; Skinner et al. 2005) while others found that depending on the administration order some combinations fail to confer protection at all (Romano et al. 2006; Baldwin et al. 2013) as in the BCG-IV group of this experiment. These differences are probably due to the heterogeneity in vaccine types, challenge strains (*M. bovis* or *M. tuberculosis*), doses, administration and challenge routes, readouts and animal models used. .

One hypothesis for the differences in protection found in our study is that the interval between prime and boost must be long enough to allow the primer to induce a response before the booster is administered (McShane and Hill 2005). Since BCG is a slow replicating bacteria it would take longer to achieve a prime effect than IV and thus it would require a longer interval before administering the booster. The influence of timing between prime and boost has already been demonstrated in a homologous BCG vaccination study in the deer model (Griffin et al. 2006) and, perhaps, the optimization of protocols is an issue to address in future experiments.

Another hypothesis for this particular vaccine failure is the interference of protection mechanisms. Empirical evidence in human and animal trials (Mangtani et al. 2014 and Buddle et al.2002) suggests that pre-existing responses to mycobacterial antigens can interfere with protection of subsequent vaccination by blocking and/or masking mechanisms (Andersen and Doherty 2005; McShane 2014). This sensitization is attributed to contact with environmental bacteria, but environments with high circulation of *M.tuberculosis* or *M.bovis* or even BCG (if later boosting is intended) could have a sensitizing effect as well (Marinova et al. 2017). While is often assumed that this interference affects live vaccines due to restricted persistence or replication and not to non-replicating vaccines (McShane 2014), we observe that BCG priming interferes with the heat-inactivated vaccine IV. It is proposed that IV induces a protective immune response triggered by dendritic cells (DCs) mimicking phagocyte response to pathogen-associated molecular patterns (PAMPs) with a central role for C3 (Beltrán-Beck et al. 2014b). Thus, higher C3 levels may increase opsonophagocytosis and effective bacterial clearance (Juste et al. 2016), while interfering with CR3-mediated opsonic and nonopsonic phagocytosis of mycobacteria, a process that could be enhanced by specific antibodies against mycobacterial proteins and/or lipids induced by vaccination with the IV and the activation of IFN-γ producing CD8+ T cells by MHC I antigen presenting DCs. For BCG, the IFN-γ production by CD4+ T cells has been shown to play a major role in protection against TB after vaccination. Although both vaccines induce C3 and IL-1B production (Figure 4 and Figure 5a), these results suggest that prime vaccination with BCG may interfere with the protective mechanisms induced by IV vaccine, which in turn decreases the efficacy of BCG vaccination. The mechanism is unknown but may be connected with the activation of DCs and the

production of antibodies against mycobacterial proteins and/or lipids involved in the interference with CR3-mediated phagocytosis of mycobacteria.

This interference has important implications for mycobacterial vaccination and may explain cases where no protection is obtained underlining the need to consider very early vaccination and, if possible, testing for mycobacterial sensitization before vaccinating. In this regard, is important to consider mounting evidence on non-detectable sensitization by traditional techniques when contact occurs by the oral route (in the case of *M.bovis* in cattle, Serrano et al. 2018; and in the case of BCG and IV in several species, Ballesteros et al. 2009a; Jones et al. 2016; Roy et al. 2017; Thomas et al. 2017).

Conclusion

Heterologous regimes involving BCG and IV do not provide improved protection in the wild boar model as compared to homologous regimes. Moreover, our results indicate that when different vaccine products are administered vaccine sequence dramatically influences the outcome with differences in protection ranging from no protection at all to consistent significant reductions in scores and organic dissemination (similar to homologous regimes). Hence, in practical terms, homologous regimes are the best option for vaccination of wild boar (and pigs) against TB. These results also underline the relevance of studying the effects of sensitization in the outcome of vaccination.

Acknowledgments

Acknowledgments

SM Table 1. Mean E% and standard deviation (s.d.) values for bPPD ELISA by group for each time point.

Time / Group	Mean E% ± s.d.				
	T_0	T_1	T_2	T_3	T_4
Control	31.57 ± 12.38	29.23 ± 2.71	26.12 ± 5.33	339.79 ± 225.10	536.07 ± 223.77
BCGx2	25.26 ± 3.61	30.19 ± 9.24	23.44 ± 1.58	131.05 ± 72.70	326.73 ± 197.69
BCG-IV	28.03 ± 8.39	25.17 ± 2.10	23.01 ± 2.63	121.71 ± 117.66	454.32 ± 251.68
IVx2	28.02 ± 7.59	27.33 ± 5.73	24.48 ± 3.74	242.21 ± 151.81	443.34 ± 245.84
IV-BCG	33.56 ± 5.49	30.04 ± 4.39	25.40 ± 4.40	282.42 ± 163.64	598.13 ± 300.82

SM Table 2. Mean OD and standard deviation (s.d.) values for IGRA by group for each time point.

Time / Group	Mean OD ± s.d.		
	T_2	T_3	T_4
Control	0.38 ± 0.77	10.70 ± 3.61	2.25 ± 1.87
BCGx2	0.54 ± 0.99	13.54 ± 13. 57	3.38 ± 2.49
BCG-IV	0.19 ± 0.35	17.23 ± 15.50	3.98 ± 4.38
IVx2	0.11 ± 0.54	18.65 ± 8.96	3.23 ± 2.69
IV-BCG	0.12 ± 0.17	14.37 ± 9.31	3.26 ± 2.22

SM Table 3. Mean normalized values (arbitrary units) and standard deviation (s.d.) for C3, MUT and IL-1B gene expression by group for each time point.

Time / Group	Mean C3 normalized values (arbitrary units) ± s.d.		
	T_0	T_2	T_4
Control	0.13 ± 0.17	1.21 ± 1.07	0.22 ± 0.18
BCGx2	0.17 ± 0.19	1.56 ± 1.51	0.70 ± 0.063
BCG-IV	0.29 ± 0.36	1.45 ± 1.31	1.57 ± 1.20
IVx2	0.74 ± 0.34	1.36 ± 1.02	0.39 ± 0.20
IV-BCG	0.04 ± 0.02	0.56 ± 0.67	1.27 ± 0.60

Time / Group	Mean MUT normalized values (arbitrary units) ± s.d.		
	T_0	T_2	T_4
Control	1.07 ± 0.45	0.88 ± 0.59	0.22 ± 0.23
BCGx2	0.78 ± 0.28	1.69 ± 1.24	0.46 ± 0.30
BCG-IV	0.80 ± 0.20	2.62 ± 1.56	0.26 ± 0.37
IVx2	1.23 ± 0.39	6.08 ± 3.56	0.40 ± 0.25
IV-BCG	1.33 ± 0.62	8.19 ± 4.90	0.31 ± 0.33

Time / Group	Mean IL-1B normalized values (arbitrary units) ± s.d.		
	T_0	T_2	T_4
Control	0.11 ± 0.04	1.24 ± 1.27	1.22 ± 1.20
BCGx2	0.08 ± 0.03	0.99 ± 0.51	2.39 ± 2.00
BCG-IV	0.08 ± 0.02	0.69 ± 0.51	1.89 ± 1.42
IVx2	0.12 ± 0.04	0.93 ± 0.80	3.15 ± 1.17
IV-BCG	0.09 ± 0.05	0.80 ± 0.50	4.90 ± 3.66

DISCUSSION
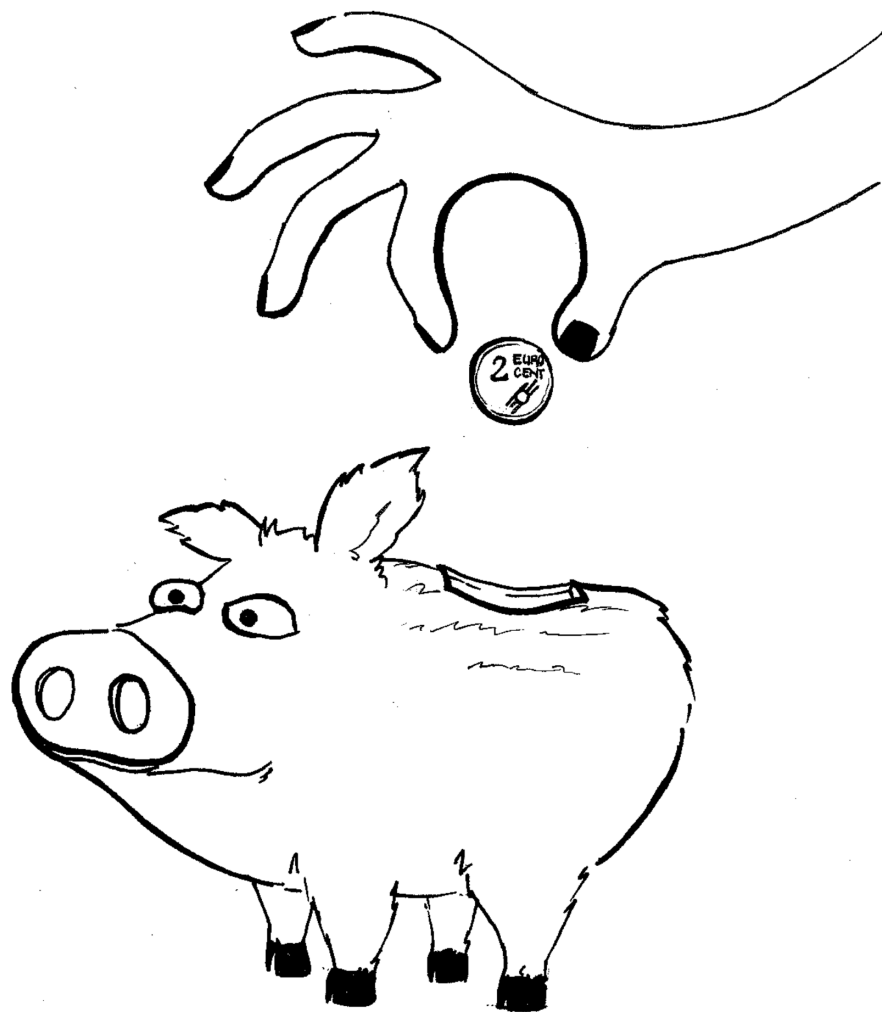
In the preceding chapters, we explore the use of vaccination against tuberculosis (TB) in wild boar under real life conditions and new vaccine combinations for this species in order to contribute to disease control in wildlife. In this section we synthesize the results obtained, discuss their global implications and outline future research prospects.

The emergence of new diseases, the comeback of diseases thought to be under control and the dissemination of antibiotic resistant pathogens mediated by wildlife become more frequent as pressure at the wildlife-livestock-human interface increases (Daszak et al. 2000; Jones et al. 2008; Arnold et al. 2016). Thus, disease control tools readily applicable over wildlife are needed to avert disease spread to humans and livestock as well as sanitary threats to wild species themselves (Artois et al. 2001 and 2011; Wobeser 2007; Delahay et al. 2009). However, the development of control tools in wildlife is challenging due to the difficulties to gather information pertaining the disease and the population and to the actual obstacles in implementing the tools (Artois 2001). Therefore, it requires interdisciplinary teams that generate knowledge and develop sustainable, feasible and cost-effective strategies that improve wildlife health (Artois et al. 2001 and 2011; McDonald et al. 2008). Joint efforts have led to the evaluation of different tools in several wild host-pathogen systems as reviewed in the **Introduction**, which also forecasts that wildlife disease control in the future will be based on integrated control strategies. Integrated control combines several tools, targets all the epidemiologically relevant hosts and involves all stakeholders to control disease in a rapid, cost-effective and balanced manner.

In this context, the results obtained in the present thesis are pertinent as wildlife TB, a complex and multi-host disease that has evaded control by conventional methods, is gaining international consideration. This recognition produces a corresponding need

for disease management strategies in real life settings (**Chapter 1** and **Chapter 2**) and for improving already existing tools (**Chapter 3**).

In **Chapter 1** we assessed the safety and efficacy of parenterally administered heat-inactivated *Mycobacterium bovis* vaccine (IV) in a large farm setting under low natural TB circulation. In this scenario IV use was safe and protected wild boar piglets from disease, achieving a significant 66% decline in TB prevalence in 3 years.

In **Chapter 2** we addressed the impact of short-term vaccination (4 years) in free-ranging wild boar belonging to managed and unmanaged sites in an endemic area with moderate to high natural TB circulation where wild boar is considered the main wild reservoir. Bacillus Calmette-Guérin (BCG) and IV vaccines were orally delivered via baits deployed in selective piglet-feeders; this approach enabled us to target a high proportion of wild boar piglets surpassing the theoretical threshold for effective intervention in most sites. No evidence of significant vaccine impact was detected in the sites where BCG was deployed; this result falls within the reported variability in BCG protection (Colditz et al. 1994; de Klerk et al. 2010; Buddle et al. 2013; Vordermeier et al 2014) that has led to the investigation of new vaccine candidates (as IV) and new vaccine protocols (as essayed in **Chapter 3**). Regarding IV only in the Managed site a significant decrease in TB prevalence was recorded (34%), suggesting that IV performance could be context dependent. While in the **Introduction** modelling was excluded from the review, in **Chapter 2** the use of a mathematical model representing the field system allowed us to overcome resource and time constraints of the field trial and to examine the long-term impacts of the proposed vaccination strategy. Model results evidenced that continuing vaccination (25 years) would reduce but not eradicate TB in wild boar and would have side effects over population dynamics (increased abundance).

In **Chapter 3**, we evaluated in the laboratory if heterologous regimes, involving combinations of BCG and IV, improved protection over homologous regimes in the wild boar model. Vaccination sequence dramatically influenced the outcome of this experiment but, as no significant increase in protection was observed, homologous regimes remain the best option for vaccinating wild boar against TB.

In summary, the evidence produced suggests that vaccine performance is greater if applied over well-defined populations (farmed or managed), in low to moderate (10 to 50%) prevalence settings and targeting a high proportion of the population (over 70%). Regarding the vaccines, IV is a suitable candidate for wild boar in real-life settings, as it reduces disease burden (despite not providing sterile immunity), is stable and safe under environmental conditions and does not interfere with diagnostics when administered orally overcoming limitations of live vaccines as BCG. Moreover, IV is an effective primer on homologous and heterologous regimes, although the former are more practical (simpler logistics and similar protection conferred). We advocate vaccination is a valuable contribution to reduce TB in wild boar and pigs although not as a stand-alone tool but as part of an integrated and adaptive (tailored to local risk and under continuous evaluation and adjustment of efforts) management strategy.

The results obtained in **Chapter 1** offer solutions for TB in farmed wild boar and the domestic pig sector. Wild boar farming for meat production and hunting (Piasentier et al. 2005; Hälli et al. 2012, Michel et al. 2017) is becoming very popular and requires the application of sanitary control measures as well (Gortázar et al. 2007). In addition, concerns regarding TB have been extended to outdoor raised pigs in a number of countries (Spain, Parra et al. 2003 and Cano-Terriza et al. 2018; Corsica,

Richomme et al. 2010; Sicily, Di Marco et al. 2012; UK, Bailey et al. 2013; and Argentina, Barandiaran et al 2015). Including vaccination as part of the sanitary management of both sectors would help to achieve an adequate TB status avoiding negative consequences over the profitability of farms, the quality of animal products, the sanitary status of co-existing extensive livestock, trade and national economies. The fact that animals are accessible and individually identified (well-defined and more controlled than their free-ranging counterparts) makes possible to ensure 100% vaccine coverage and avoid the limitations and handicaps of remote delivery. Moreover, it would also facilitate the implementation of an integrated strategy that combines vaccination with a selective culling approach (taking advantage of in vivo diagnostic techniques, Jaroso et al. 2010; Boadella et al. 2011c; Pesciaroli et al. 2012) and farm biosecurity (movement control, fencing, habitat management). This strategy could control TB without posing an excessive economic burden.

The application of the results obtained in **Chapter 2** can be direct in situations where feral pigs or wild boar are already recognized as reservoirs for TB (e.g. Spain, Naranjo et al. 2008; Portugal, Santos et al. 2009; Hawaii, Bany and Freier 2000) and eventually, over new scenarios. These may arise as knowledge gaps are filled with the detection of TB in new wildlife species and the description of new maintenance wild hosts worldwide (Renwick et al., 2007). In fact, there are still regions where information on wildlife TB is scarce (Asia, South America and northern Africa; Gortázar et al. 2015b) although awareness and data on the role of wildlife is increasing as disease prevalence in livestock is reduced (e.g. Canada, Wobeser 2009), as public health and biodiversity concerns arise (e.g. South East Asia, Thapa et al. 2017; Cantlay et al. 2017) and as non-typical scenarios are explored (low prevalence settings, Mentaberre et al. 2014; officially TB free countries, Malmsten et al. 2018; Meier and Ryser-Degiorgis

2018). In this regard, recent research has detected TB cases in wild boar in Morocco, South Korea, Poland and Brazil (El Mirni et al. 2016; Jang et al. 2016; Witkowski et al. 2017; Maciel et al. 2018). This new evidence adds up to the cases reported worldwide in wild boar and feral pig (Serraino et al. 1999; Bany and Freier 2000, Pavlik et al. 2002, Parra et al. 2003; Corner 2006; Zanella et al. 2008, Santos et al. 2009; Foyle et al. 2010; Meikle et al. 2011; Nugent 2011), to reports in other members of the Suidae family (as warthogs, *Phacochoerus* spp.; bushpig, *Potamochoerus spp.;* and giant forest hogs, *Hylochoerus meinertzhageni*; Michel et al. 2006; Tschopp et al. 2010) and to reports in the highly related Tayassuidae family (Mayer et al. 2012). While in most of these reports a role in TB maintenance (reservoir) is not attributed is important to bear in mind that disease is a dynamic process and, eventually, spill over hosts may become true reservoirs if changes in density, habitat and management occur (Naranjo et al. 2008; Nugent 2011).

While the high variability in TB dynamics among the aforementioned settings precludes from elaborating a universal protocol we advocate that the proposed vaccination strategy has straightforward application as part of a national wildlife TB framework in Mediterranean ecosystems (e.g. PATUBES in Spain, MAPAMA 2017) and that our experience could provide a basic guideline to control and prevent the establishment of reservoir in suid hosts in non-Mediterranean settings. We also acknowledge the limitations of this study as the nature of demanding field experiments has limited the aspects that could be assessed and the number of sites tested. The former have been partially addressed by mathematical models that have evidenced the consequences of long-term vaccination on disease and population dynamics. According to modeling vaccination will control but no eradicate TB, in this regard synergies with culling (conducted prior vaccination) and biosafety can achieve a lower initial

prevalence and lower population densities that translate in less baits needed and lesser transmission making vaccination more cost-effective. The increase in population density driven by long-term vaccination can be perceived as positive in hunting business, as it will increase yield and profitability, or as negative in peri-urban areas or natural areas, where increased impacts over conservation or conflicts with humans may require using culling to compensate the decrease in TB-induced mortality.

Additionally, the operational logistics of effective TB vaccine delivery to wild boar in a species-specific manner and the possibility of targeting a young age-class at large scales (ca. 100 km^2) described in **Chapter 2** can be applied to other products (other vaccines, toxicants, immunocontraceptives etc.). Oral delivery of other vaccines is a strategy to consider as wild boar and feral pigs are able to disseminate several pathogens and to serve as potential reservoirs of important infectious diseases for humans, livestock and other wildlife (Meng and Lindsay 2009; Ruiz-Fons 2015; Brown et al. 2018). In fact, the administration of an oral vaccine has already contributed to the control of classical swine fever in the wild boar reservoir in France (Rossi et al. 2010). An advantage of the present work is that it effectively targets young age classes, a population segment often key in disease dynamics (Rossi et al. 2005; Kramer-Schadt et al. 2009) and reported to be difficult to access (Kaden et al. 2000; Rossi et al. 2010). Baits could also vehiculate immunocontraceptives (Fagerstone et al. 2002; Massei et al. 2012) or toxicants (Shapiro et al. 2016; Snow et al. 2017) aiding in the control of growing wild boar and feral pig populations and in the mitigation of subsequent damages to ecosystems, agriculture and vehicle collisions (pest control tool) as well as health risks (disease control tool). Since population dynamics influence disease transmission (Acevedo et al. 2007; Vicente et al. 2013) and in turn disease is a natural phenomenon that shapes populations (modeling results of **Chapter 2;** Lachish et al.

2010; López et al. 2014; Barasona et al. 2016) harmonized knowledge on population structure and density is a vital component of disease management and remarkable efforts are being conducted in this regard (APHAEA 2013; Sonneburg et al 2017; Keuling et al. 2018).

A current example of the potential value of the delivery logistics presented in this thesis would be its use to contain African Swine Fever (ASF). ASF is rapidly spreading throughout European and Asian countries posing a severe socio-economic impact over the pig industry, food security and rural livelihoods. In the ongoing epizootic, wild boar has a key role on spread and maintenance complicating disease dynamics and control (Torre et al. 2013; Nurmoja et al. 2017). Oral delivery logistics could be applied either to break disease transmission by the administration of a vaccine (once it is available) or to reach wild boar densities that do not sustain ASF circulation by the administration of immunocontraceptives (once an oral formulation is developed, Massei et al. 2012) or toxicants (if an exception to EU biodiversity conservation legislation is made). These strategies would reinforce and complement the measures currently in place (biosecurity, fencing, movement control, culling and destruction of infected; EFSA 2014).

In **Chapter 3**, the empirical knowledge on heterologous vaccination regimes obtained may contribute to elucidate the immunological basis involved in BCG and IV-mediated protection and to fine-tune vaccination protocols. Fully understanding of TB infection, immune response and vaccine mediated protection mechanisms as well as determining markers of protection would help to explain the variable results observed in field trials (in animals and humans) and benefit the rational design of TB vaccine candidates (Vordermeier et al. 2014; Gonzalo-Asensio et al. 2017; Martín et al. 2018). In this sense, laboratory and field trials are complementary and the iteration between

them is necessary and enriching. On one hand laboratory trials are a first basic step to settle proof-of-concept, although they represent an oversimplified system; on the other hand field trials provide realistic conditions but have practical limitations and may result in unexpected outcomes that require further hypothesis testing in the laboratory. In this case, the experiment conducted could not find a more protective combination to apply in real-life settings but it provided an additional hypothesis on the lack of detectable vaccine impact in Natural sites where a high percentage of wild boar accessed both vaccines (Díez-Delgado et al. 2018).

This thesis provides science-based evidence on wild boar vaccination against TB; nevertheless, it leaves a number of questions surrounding the impact of wild boar TB vaccination open.

Further research on the impact of wild boar vaccination over sympatric species, including cattle, would add valuable information on the ability of wild boar vaccination to break inter-species transmission and would allow a more accurate cost-effectiveness analysis. Conducting a cost-effectiveness analysis is key as budgets are often limited and the effective allocation of resources is a must. Regarding the economic feasibility of the proposed strategy, costs could probably be optimized further (e.g. bait densities) and synergies with other tools could maximize benefits and reduce expenses.

The unintended effects of vaccination (decreased mortality) and its associated logistics (baiting) over wild boar populations (Kramer-Schadt et al. 2009; Chauvenet et al. 2011) need to be pondered considering the present context of growing populations and their associated impacts (Massei et al. 2015).

The evidence generated also prompts further research of the potential of heat-inactivated mycobacteria as immunogens in other species, particularly in ruminants. In

fact, an ongoing project exploring the administration logistics, host response and diagnosis is making great progress on deer (Thomas et al. 2017), livestock species not subjected to compulsory eradication (e.g. sheep and goat; Balseiro et al. 2017 and Roy et al. 2018) and even in cattle (demonstrating it does not interfere with test and cull schemes, Jones et al. 2016).

Finally, this research aims to bring solutions to the hunting and farming sector and to society as a whole by transferring field vaccination results into a marketable product, EMDIAR$^©$ a commercial IV intended for use in wild boar and extensively raised pigs.

CONCLUSIONS

1. Complex and multi-host diseases that evade control by conventional methods, such as animal tuberculosis (TB), would benefit from an integrated approach to achieve control in a rapid, cost-effective and balanced manner. Integrated approaches combine several tools, target all the epidemiologically relevant hosts and involve all the interested stakeholders. These strategies are informed by population and disease monitoring and need to be flexible and tailored to the scenario.

Las enfermedades complejas, con múltiples hospedadores, son difíciles de controlar ya que con frecuencia evaden los métodos convencionales de control. Este tipo de enfermedades, entre las que se incluye la tuberculosis animal (TB), se beneficiarían de un enfoque integrado que combine varias herramientas de control, actúe sobre todos los hospedadores relevantes desde punto de vista epidemiológico e implique a las partes interesadas para lograr el control de la misma de modo rápido rentable y equilibrado. Además, estas estrategias requieren de datos obtenidos mediante el seguimiento poblacional y sanitario y han de adaptarse a cada escenario y a la evolución del mismo.

2. Vaccination is a realistic option to contain TB in wild boar, the main wild reservoir in Mediterranean Iberia. The individual evaluation of vaccination by field studies, prior to its incorporation into an integrated strategy, is essential because experimental systems are oversimplified. Nevertheless, field studies have limitations (time-wise, resource-wise) that have to be acknowledged and can be partially overcome by modeling.

La vacunación es una alternativa realista para lograr contener la TB en el jabalí, el principal reservorio silvestre en los ecosistemas mediterráneos de la Península

Ibérica. La evaluación de esta herramienta mediante estudios en campo, previa a su incorporación en estrategias integradas, es esencial para complementar la información obtenida en laboratorio. No obstante, los estudios en campo poseen ciertas limitaciones (en lo referente a tiempo y costes) que han de ser consideradas y pueden ser minimizadas gracias al uso de modelos matemáticos.

3. Parenteral vaccination with heat-inactivated *Mycobacterium bovis* (IV) is safe and protects farmed wild boar, and eventually pigs, from disease (66% TB reduction).

La vacunación parenteral con Mycobacterium bovis inactivado por calor (IV) es segura y protege frente a la TB a jabalíes en condiciones de granja y potencialmente a cerdos (reduciendo la enfermedad en un 66%).

4. The use of baits and selective piglet-feeders over a large scale (ca.100 km^2) allows the oral administration of TB vaccines and other products in a species-specific and age-specific manner to free-ranging wild boar piglets. This delivery logistics resulted in the vaccination of more than 70% of wild boar piglets in most sites, especially in managed sites (fenced, administration of supplementary feeding).

El uso de cebos y comederos selectivos para rayones permite administrar vacunas frente a TB y otros productos por vía oral a rayones de jabalí de modo selectivo para esta especie y clase de edad en grandes áreas (aprox. 100km^2). Esta estrategia de administración logró vacunar más del 70% de la población diana en la mayoría de fincas ensayadas y especialmente en las fincas manejadas (valladas, que suministran alimentación suplementaria).

5. Oral vaccination with Bacillus Calmette-Guérin (BCG) under field conditions did not lead to significant reductions in TB disease prevalence at a population scale after four years of vaccine deployment.

La vacunación oral con el Bacilo Calmette-Guérin (BCG) en condiciones de campo no redujo significativamente la prevalencia de TB a escala poblacional tras cuatro años de liberación de cebos vacunales.

6. Oral vaccination with IV resulted in a significant reduction of TB prevalence after four years of vaccine deployment only in the population belonging to the managed site (34% decrease).

La vacunación oral con IV durante 4 años redujo significativamente la prevalencia de TB en la población perteneciente a la finca manejada (reduciendo la enfermedad en un 34%).

7. Long-term vaccination (25 years) would be able to control but not to eradicate disease and will have an impact over population dynamics (increased abundance via decreased mortality) according to modeling results.

La vacunación durante largos periodos (25 años) puede controlar pero no erradicar la TB y tiene impacto sobre las poblaciones de jabalí (aumentando su abundancia a través de la disminución de la mortalidad) según los resultados proporcionados por los modelos matemáticos.

8. The application of vaccination in real-life settings suggests that vaccine success is greater if applied over well-defined populations (farmed or managed), with low to moderate prevalence (10 to 50%) and targeting a large proportion of the population.

It also suggests that IV is a suitable candidate for wildlife in real-life settings, as it reduces disease burden (despite not providing sterile immunity), is stable and safe under environmental conditions and does not interfere with diagnostics.

La aplicación de la vacunación en entornos realistas sugiere que el impacto de la vacunación es mayor si se aplica sobre poblaciones bien definidas (en granja o delimitadas mediante vallado), en condiciones de baja a moderada prevalencia de TB (10-50%) y actuando sobre una alta proporción de la población. También indica que la vacuna IV es eficaz y adecuada para su aplicación en condiciones de campo debido a la protección registrada, a su estabilidad y seguridad en condiciones naturales y a que no interfiere en el diagnóstico.

9. Heterologous vaccination regimes involving BCG and IV do not provide improved protection in the wild boar model as compared to homologous regimes. Hence, homologous regimes are the best option for vaccination of wild boar and pigs against TB. Moreover, vaccine sequence dramatically influenced the outcome underlining the relevance of studying the effects of sensitization in the outcome of vaccination

   Los regímenes heterólogos de vacunación que combinan BCG e IV no confieren protección mayor que los regímenes homólogos en jabalí. Así, los regímenes homólogos son la opción de elección para la vacunación frente a TB en jabalíes y cerdos. Además, \a secuencia de la inmunización afectó notablemente el grado de protección evidenciando la necesidad de estudiar la interferencia de la sensibilización previa sobre los resultados de la vacunación.

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Sometimes a scream is better than a thesis

Raph Waldo Emerson

(ahí van los dos por si acaso)