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Environmental Presence of *Mycobacterium tuberculosis* Complex in Aggregation Points at the Wildlife/Livestock Interface

J. A. Barasona[1], J. Vicente[1], I. Díez-Delgado[1,2], J. Aznar[3,4], C. Gortázar[1] and M. J. Torres[3]

[1] SaBio IREC, National Wildlife Research Institute (CSIC-UCLM-JCCM), Ciudad Real, Spain
[2] Departamento de Sanidad Animal, Facultad de Veterinaria, Universidad Complutense de Madrid, Madrid, Spain
[3] Departament of Microbiology, Universidad de Sevilla, Seville, Spain
[4] Biomedical Institute of Sevilla (IBiS), University Hospital Virgen del Rocío/CSIC, Seville, Spain

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Correspondence:
J. A. Barasona. SaBio-IREC (CSIC-UCLM-JCCM), Ronda de Toledo s/n, 13071 Ciudad Real, Spain.
Tel.:+34 926295450; Fax: +34 926295451;
E-mail: joseangel.barasona@uclm.es

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Summary

The members of the *Mycobacterium tuberculosis* complex (MTC) cause tuberculosis (TB). Infection is transmitted within and between livestock and wildlife populations, thus hampering TB control. Indirect transmission might be facilitated if MTC bacteria persist in the environment long enough to represent a risk of exposure to different species sharing the same habitat. We have, for the first time, addressed the relationship between environmental MTC persistence and the use of water resources in two TB endemic areas in southern Spain with the objective of identifying the presence of environmental MTC and its driving factors at ungulates' water aggregation points. Camera-trap monitoring and MTC diagnosis (using a new MTC complex-specific PCR technique) were carried out at watering sites. Overall, 55.8% of the water points tested positive for MTC in mud samples on the shore, while 8.9% of them were positive in the case of water samples. A higher percentage of MTC-positive samples was found at those waterholes where cachectic animals were identified using camera-trap monitoring, and at the smallest waterholes. Our results help to understand the role of indirect routes of cross-species TB transmission and highlight the importance of certain environmental features in maintaining infection in multihost systems. This will help to better target actions and implement control strategies for TB at the wildlife/livestock interface.

Introduction

Understanding infection dynamics and the transmission of pathogens shared by human beings, livestock and wildlife is the key element as regards implementing multihost pathogen control measures (Daszak, 2000; Cleaveland et al., 2001). Animal tuberculosis (TB) is one of these shared diseases and affects a wide range of hosts (Alexander et al., 2002; Michel et al., 2010). It is caused by members of the *Mycobacterium tuberculosis* complex (MTC) such as *M. bovis* among others. *M. bovis* causes chronic TB in cattle and is considered to be a public health and economic concern worldwide (Morris et al., 1994; Schiller et al., 2011). In developed countries, the eradication of TB in cattle has been attempted by employing test and slaughter campaigns, which have succeeded to a varying degree. Some of them have achieved a significant reduction in prevalence, but the disease has not been eradicated, probably owing to the existence of wildlife reservoirs (see Haydon et al., 2002) which may have hampered the success of this strategy (Corner, 2006; Gortázar et al., 2008).

In south-central Spain (SCS) and Portugal, TB eradication in cattle has not been accomplished and MTC infection has been found to be endemic in wild ungulates (Aranaz et al., 1996; Gortazar et al., 2011). Many studies have documented the relationship between TB in cattle and

wild ungulates in this region (Parra et al., 2005; Rodríguez-Prieto et al., 2012; Martínez-López et al., 2013; Barasona et al., 2014a), the existence of wild maintenance hosts of MTC (Eurasian wild boar, *Sus scrofa*, and red deer, *Cervus elaphus*; Gortázar et al., 2008; Vicente et al., 2006) and the danger that this disease poses to emblematic endangered species such as the Iberian lynx (*Lynx pardinus*; López et al., 2014). In summary, TB in SCS and Portugal is a multihost system, and it is probably the largest region with the highest prevalence of TB in wildlife reported in international literature (Vicente et al., 2006, 2013; Gortázar et al., 2008). Under these complex circumstances, it is difficult to understand the epidemiology of TB in host communities and the environment (the MTC reservoir as a whole) because a wide range of factors of a different nature may be involved.

Direct oro-nasal transmission has historically been considered as the predominant infection route, while indirect transmission has been regarded as less significant (Morris et al., 1994). Pathology findings such as the presence of both thoracic and abdominal TB lesions in infected wildlife suggest that TB bacilli are transferred by means of a combination of direct oro-nasal transmission and indirect environmental transmission at shared watering and feeding sites (Martín-Hernando et al., 2007, 2010; Vicente et al., 2013). Moreover, the indirect route via shared resource use would appear to be important for cross-species transmission (livestock–wildlife interface; Palmer et al., 2004) in which direct interactions seem to be scarce (Böhm et al., 2009; Kukielka et al., 2013). Among others, waterholes are potential critical risk points for MTC transmission, particularly in semi-arid areas in which water is scarce, its availability is seasonal, and animals aggregate at waterholes daily (Barasona et al., 2014b). Cowie et al. (2014) recently indicated that TB is more frequent on cattle farms with a lower number of streams per hectare, probably owing to the fact that the reduced number of water sources forces more animals, both livestock and wildlife, to visit the same locations to obtain drinking water. In SCS, these shared resources which act as hazardous points for contagion have also been identified in previous TB risk analyses in wild ungulate populations (Vicente et al., 2007, 2013). The wallowing and rooting behaviour of wild boar in particular may contribute to contamination in the areas surrounding water points. Risk analyses provide a partial view of factors related to environmental transmission (Walter et al., 2012), and the presence of MTC in the environment may therefore be of epidemiological significance, although basic biological information such as distribution, quantity, viability and infectious potential continues to be largely unknown (Courtenay and Wellington, 2008). Such information is, however, crucial if the way in which these factors impact on MTC transmission and persistence is to be understood, in addition to its implications for management. This is particularly relevant at the wildlife/livestock interface.

Indirect transmission implies that, following excretion, the microorganism must endure environmental conditions during the time needed for it to become a potential source of infection for different hosts. According to experimental studies (spiked samples), MTC bacteria may persist in the environment for months (Maddock, 1933; Duffield and Young, 1985; Tanner and Michel, 1999; Fine et al., 2011a; reviewed and compared with the epidemiological context of our study area by Kukielka et al., 2013). Difficulties in diagnosis and failures in detection have often led to the role of the environment in the epidemiology of TB being underestimated (Jackson and Morris, 1995; Pillai et al., 2000; Witmer et al., 2010; and Fine et al., 2011b).

Here, we have focused on two areas (Doñana National Park and Ciudad Real province) that are characterized as complex epidemiological scenarios, with abundant and widely distributed cattle and wild ungulate populations, in which TB is endemic and highly prevalent. Our objectives were (i) to identify the presence of MTC at ungulates' aggregation points (water points) using a novel PCR technique and (ii) to determine the main factors behind the environmental presence of MTC at such aggregation points.

Materials and Methods

Study areas

Ciudad Real province

The trials were conducted in three open-air cattle breeding farms in the province of Ciudad Real (CR), Castilla-La Mancha, SCS (37°13′N, 6°40′W). Throughout the study area, there is a patchy distribution of farms in extensive regime (mainly in flat areas) and big game estates (mainly in hilly terrain). During the last decades, there has been an increasing tendency for private livestock farms to diversify into hunting estates. Furthermore, the management strategies applied to some wild ungulate populations (translocation, fencing, artificial feeding, etc.) have increased aggregation and shared space use between domestic and wild animals (Gortazar et al., 2006; Vicente et al., 2007), which could lead to indirect transmission of disease at the interface (Palmer et al., 2004) under the necessary conditions (Corner, 2006). A property is often divided into farm and game areas which are separated by livestock fences that are permeable to wildlife (Barasona et al., 2013).

This region is characterized by Mediterranean habitat, composed of forests and scrublands interspersed with 'dehesas' (savannah-like habitats). The annual rainfall in the region studied is variable (ranging from 300 to 700 mm), and the climate is Mediterranean with a continental

influence. The wet season typically starts in October/November and contributes most of the annual rainfall. Food and water resources become limited for ungulates during the dry season, from June to September. The artificially high densities of the managed populations and the provision of artificial food, along with the limited availability of water points in summer, cause wildlife to aggregate, probably increasing the risk of MTC transmission (Vicente et al., 2007).

Regarding cattle farms, the average stocking rate in CR is lower than in other European farming systems that are also considered to be extensive (Milan et al., 2006). The number of mixed-beef breed farms in Ciudad Real province was 660 during the study period, and the mean size was 467 ha (range 37–2040 ha) with an average of 114.2 heads per farm (maximum 1095). Most of the farms were located in the west of the province (the geographical distribution of extensive cattle farms during the study period can be seen in Rodríguez-Prieto et al., 2012).

The main big game species in the region studied are red deer and wild boar, which have locally high densities with approximately 20 deer/km2 and a wild boar hunting bag that doubled between 2000 and 2012 (Acevedo et al., 2008; Vicente et al., 2013). The TB prevalence figures reported in the literature are high for both species (up to 60% in wild boar and 12% in red deer; Rodríguez-Prieto et al., 2012; Vicente et al., 2013).

Doñana National Park

Five cattle farms were studied in Doñana National Park (DNP, 54 252 ha), which is located in SCS (37°0′N, 6°30′W). DNP is one of the most important natural reserves in Europe in terms of biodiversity. Human access is restricted, and management is carried out by conservation authorities. Agriculture and hunting are prohibited, and artificial feeding practices are not conducted but are limited to the traditional exploitation of some natural resources, such as pinecone collection and cattle and horse breeding.

It is a flat region consisting of sandy soils that borders the Atlantic Ocean, with altitudes ranging from 0 to 47 m a.s.l. The habitat is composed of an important proportion of sand dune habitat, marshland and pine forest, along with patches of Mediterranean scrubland. The region has a dry subhumid Mediterranean climate with marked seasons, particularly with regard to the water level and its effects on the vegetation. In the wet season (winter and spring), most of the marshlands are flooded and wildlife and cattle are concentrated in the slightly more elevated scrublands. In summer, the ecotone between scrublands and marshes produces an aggregation of wild and domestic ungulates (Barasona et al., 2014b).

The Doñana cattle are evenly distributed in the study area. The number of cattle has decreased by about 50% from the first survey carried out in 1979 to the present, and 988 animals are currently kept together on five extensive farms of a similar size (an average of 5676 ha) that cover DNP. The cattle move freely within but not among enclosures, whereas wildlife can cross the fences without any difficulty. Despite compulsory testing and the culling of infected animals (Gortázar et al., 2008), cattle TB reactor rates are still high in DNP (up to 8%; Veterinary Authorities).

Wild ungulate populations are composed of wild boar, red deer and fallow deer (*Dama dama*), and there are high abundances (kilometric abundance indices: up to two animals/km for each species; http://www-rbd.ebd.csic.es/Seguimiento/mediobiologico.htm). Previous studies have revealed that *M. bovis* infection prevalence is spatially structured, leading to among the highest rates of TB reported in wildlife worldwide (52% in wild boar, 27% in red deer and 18% in fallow deer; Gortázar et al., 2008; Gortazar et al., 2011). The culling of ungulate populations by park rangers took place until 2012 as part of the park management scheme (Boadella et al., 2012). This culling was principally directed towards wild boar, of which several hundreds were eliminated each year (10–20% of the estimated population).

Study design and sampling

The survey was carried out during the dry season (July–September) in 2011, when water is scarce, signifying that livestock and wild ungulates aggregate around water sites (Vicente et al., 2007; Kukielka et al., 2013; Barasona et al., 2014b). Sample point selection was based on the premise of the site being a risk point. A total of 61 water points (Fig. 1) on eight farms with culture-confirmed *M. bovis* in cattle and free-ranging wild ungulates were therefore selected for environmental sampling and ungulate use (abundance and aggregation) monitoring. In CR, 12 water points were sampled (10 artificial waterholes, one lagoon and one drinking trough) on three cattle-extensive farms. In DNP, 49 of the 153 available water points were sampled (45 waterholes, two lagoons and two drinking troughs) on five farms.

The samples, consisting of 500 g of mud substrate from the shore used and one litre of water, were collected in labelled sterile plastic bottles and stored in an insulated cooler surrounded by cooling packs. Therefore, mud and water were transported at 4°C to the laboratory and immediately frozen at −18°C until laboratory diagnostics were performed. Point location (x- and y-coordinates) and water point characteristics (size, filling status) were also recorded for each point.

Laboratory analysis

Samples frozen at −18°C were thawed at room temperature for DNA extraction. Briefly, the water was centrifuged at

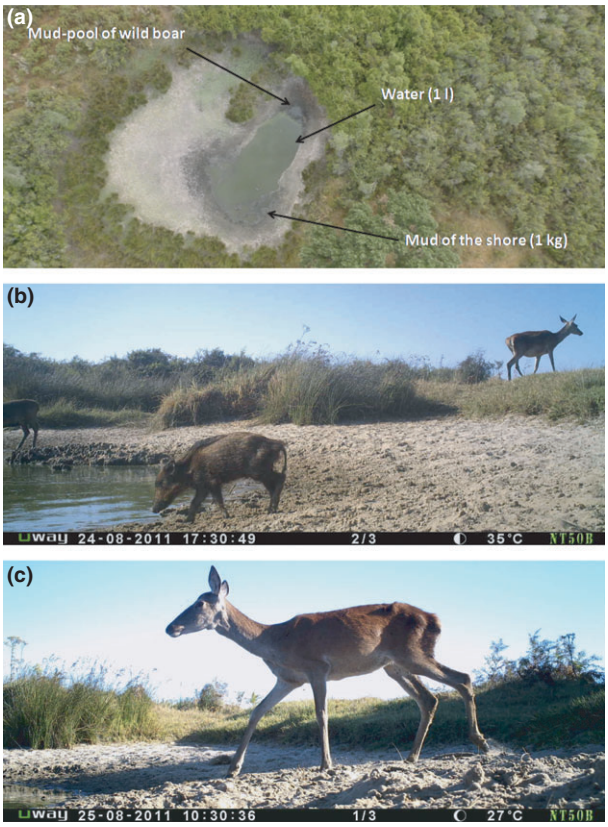

Fig. 1. (a) Detailed image of a sampled waterhole in Doñana National Park, Spain. High-resolution image obtained from Unmanned Aircraft System camera (Mulero-Pázmány M.). (b) Camera-trap image of a young wild boar with obvious cachexia sharing a sampled waterhole with two red deer (recorded as direct inter-specific interaction) and (c) a cachectic red deer with evident inflammation of head lymph nodes.

3500 ***g*** for 10 min, decanted and the pellet resuspended in sterile water. Prior to centrifuging, turbid samples with high levels of suspended solids were stored in a decanter to allow suspended solids to settle down. For samples where settling did not occur, a method involving stacking filters with large pore sizes (e.g. Whatman Grade 1 Filter Paper Standard Grade: 11-µm pore size) was used. This layering filtered out large debris. DNA extraction from de sediment was carried out using the automatic GenoXtract system (Hain Lifescience).

Microbial DNA was extracted directly from 250 mg portions of the composite soil samples using the Powersoil DNA Isolation kit (MoBio Laboratories, Mobio Inc., Solana Beach, CA, USA.) according to the manufacturer's protocol, which resulted in 100 µl soil DNA solutions.

MTC DNA extracted from water and soil was amplified via PCR using the FluoroType MTB test, which is performed on a FluoroCycler (HAIN Lifesciences GmbH, Nehren, Germany) and combines DNA amplification using specific primers and a subsequent melting curve analysis in one instrument. Recently, the efficacy of the FluoroType MTB assay based on HyBeacon fluorescence technology has been evaluated in human samples of respiratory tract and compared with culture, microscopy and molecular diagnosis (Eigner et al., 2013; Hofmann-Thiel and Hofmann, 2014; González Mediero et al., 2015). First, PCR mixes were freshly prepared by combining a 3 µl amplification mix A (AM-A) with a 7 µl amplification mix B (AM-B) in pre-amplification chamber I. Subsequently, 6 µl of isolated DNA was added in pre-amplification chamber II. Controls included 6 µl of PCR-grade water (negative control) and 6 µl of control DNA C + FT MTB (positive control). The PCR mixes were immediately loaded into the FluoroCycler. Fluorescence-labelled probes were bound to single-stranded amplicons, and the decrease in fluorescence was then measured and displayed as a melting curve. The evaluation was carried out using test-specific software (Fluoro-Software®, HAIN Lifesciences GmbH, Nehren, Germany), which automatically analysed the melting curves for the amplification control (AC) (70.5°C ± 3°C) and for MTB (melting point 60.0°C ± 3°C) and computed the results as 'no MTB complex DNA detected' (MTB negative, AC positive), 'MTB complex DNA detected' (MTB positive, AC positive or negative), 'not interpretable' in the case of unspecific peaks, or 'invalid' in the case of the failure of positive/negative controls or AC. The target for amplification in this test is IS 6110 which is an element found exclusively within the members of the MTC (Coros et al., 2008). This test, using mycobacterial culture as a reference, has shown 95.1% and 96.4% sensitivity and specificity, respectively, to detect MTC (Eigner et al., 2013). Further identification of MTC species or detection of other mycobacterial species not belonging to the MTC was not performed.

Camera-trap survey

Infrared camera trapping was performed at all the sampled water points during the dry season (July–September). Heat and motion infrared-triggered camera traps (NightTrakker NT50B 8.0 MP, Uway Outdoor Products, Norcross, GA, USA) were placed with the objective of covering natural and artificial water sources. The trapping stations consisted of a single camera placed 20 cm above the ground (detection distance average: 6.5 m, detection arc: 70°). The camera traps were set to take pictures during 24 h per day, with a delay of 1 min between exposures (the camera takes a second or third picture in the same pause time if additional motion or heat is detected). Passive systems use heat and motion detectors to take the photographs. The infrared source is connected to the movement sensor and has a maximum scope of 15 m. The date and time of each exposure were recorded by the camera and printed on each

image. A total of 5307 camera trap days were studied, and an index of relative abundance was then calculated for cattle, wild boar, fallow deer and red deer per study point. We therefore quantified the number of days during which a given species was present in relation to the total number of days monitored at each trapping station. The presence of individuals with obvious cachexia, probably owing to generalized TB (see images in Fig. 1; Martín-Hernando et al., 2007, 2010; Vicente et al., 2013), was also recorded. In addition, the number of direct inter-species interactions, that is the number of pictures showing the simultaneous presence of two or more MTC host species, was quantified (Fig. 1).

Data analysis

Preliminary descriptive and exploratory analyses were conducted to investigate the characteristics of the data sets and the potential relations among (i) MTC presence (binary), (ii) the indices of relative host abundance – separately for livestock, wild boar, fallow deer and red deer – and (iii) the direct inter-specific interactions, using R software version 2.15.2 (R Development Core Team, 2013). A combination of descriptive statistics, graphical tools and correlation analyses was used to examine data patterns. *Chi*-square tests were used to compare prevalence (estimated using 95% confidence intervals; C.I.) between sample types and study areas, while Mann–Whitney *U*-tests were used to compare the indices of relative host abundance between study areas.

The first data exploration mainly consisted in checking the distribution of variables, and collinearity diagnostics among the explanatory variables, as well as the identification of potential interactions between them (Zuur et al., 2010). Collinear variables were excluded using a variance inflation factor (VIF) coefficient >2.5 threshold cut-off value (Zuur et al., 2010). Generalized linear mixed models (GLMMs) were used to investigate which factors influenced the presence of MTC DNA at water points. This statistical approach was chosen based on the nature of the data and type of inferences under consideration. The surveyed management estates were assumed to be a random sample of a larger number of mixed game-farm sites found across SCS. Therefore, the 'management estate' variable was included as a random factor in the tested models. Moreover, we also incorporated the 'area' variable as a fixed effect factor, instead of a random effect due to the reduced number of levels available (only two, CR and DNP; Zuur et al., 2010). Binomial distribution and logit link GLMMs were conducted to estimate risk factors, in which the detected environmental absence (coded as 0) or presence (coded as 1) of MTC at each water point studied was used as response variable. Contaminated samples or those whose PCR was inhibited were not included in the models. This approach included the main effects of explanatory variables that could potentially affect the response variables, as the indices of relative abundance for cattle, wild boar, fallow deer and red deer, and water point characteristics. Model selection based on an information-theoretic framework was used to test four possible combinations of these explanatory variables with biological and/or ecological significance based on our predictions (Table S1). Comparisons based on Akaike's information criterion (AIC; Akaike, 1974) were run among different models to identify the most important predictors. The model with the lowest AIC score was considered the most parsimonious, that is, as showing the most favourable trade-off between the number of parameters and model fit (Burnham and Anderson, 2002). Standard model checks were produced for all models with 'lme4' R package (Bates et al., 2014). Model residuals were examined and tested for spatial autocorrelation using the Moran's I to detect spatial structures (Diniz-Filho et al., 2003).

Results

Environmental detection of MTC using PCR

The PCR results for MTC DNA presence in the samples collected on the 8 TB-positive cattle farms investigated are shown in Table 1. In general, 24 water points (55.8%, CI 95% = 40.7–70.9) tested positive for MTC in mud samples, while a significantly lower proportion of positive samples (8.9%, CI 95% = 1.5–16.3) was found in water (Chi^2 = 25.81, 1 d.f., $P < 0.001$). However, all the positive water samples except one (PCR-inhibited mud sample) were also positive for mud (significant concordance; $|r| = 0.38$, $P < 0.05$). The proportion of PCR test inhibitions was higher in mud samples than in water samples (Table 1; Chi^2 = 9.05, 1 d.f., $P < 0.01$). Regarding comparison between study areas, no significant differences in the prevalence were observed in mud (Chi^2 = 0.79, 1 d.f., $P > 0.05$) or water samples (Chi^2 = 0.25, 1 d.f., $P > 0.05$), respectively. The proportion of PCR inhibitions was higher in DNP than in CR (Chi^2 = 4.56, 1 d.f., $P < 0.05$).

Camera-trapping monitoring at sampled points

The camera-trapping survey yielded a total of 218 803 images from the sampled water points to be recorded and identified. The relative abundance indices by species and study area calculated by monitoring camera-trap stations are shown in Table 2. There was a sharp contrast between areas for the estimated abundance indices of domestic and wild ungulates. Cattle abundance was significantly higher in CR than in DNP (Table 1; Mann–Whitney *U*-test, $U = 442.5$, $P = 0.002$), while wild ungulate abundance was consistently higher in DNP (Table 1, Mann–Whitney

Table 1. Proportion of positive and inhibited samples to MTC DNA detection by PCR according to sample type and study area. Sample size is shown (*n*)

	Mud sample PCR		Water sample PCR		All sample PCR	
Study areas	Positive	Inhibited	Positive	Inhibited	Positive	Inhibited
CR	66.7% (8/12)	0% (0/12)	0% (0/10)	0% (0/10)	36.4% (8/22)	0% (0/22)
DNP	51.6% (16/31)	36.7% (18/49)	10.9% (5/46)	6.1% (3/49)	27.3% (21/77)	21.4% (21/98)
Total	55.8% (24/43)	29.5% (18/61)	8.9% (5/56)	5.1% (3/59)	29.3% (29/99)	19.2% (21/120)

Table 2. Mean and standard error (SE) of the relative abundance index for each ungulate species calculated by means of camera-trapping survey at sampled points. The relative abundance index was calculated as the number of days on which a given species was present in relation to the total number of days monitored at each trapping station

	Index of relative abundance of ungulates (Mean ± SE)			
Study areas	Cattle	Wild boar	Red deer	Fallow deer
CR	62.32 ± 10.12	10.79 ± 5.86	14.00 ± 6.89	0
DNP	21.03 ± 4.37	66.24 ± 5.08	60.34 ± 4.25	20.31 ± 4.28
Total	29.43 ± 4.56	54.96 ± 5.12	50.91 ± 4.38	16.18 ± 3.57

U-test; $U = 48.5$, $P < 0.001$, for wild boar; $U = 61.0$, $P < 0.001$, for red deer; and $U = 84.0$, $P < 0.001$, for fallow deer). A total of 134 direct interactions between species were recorded by the camera traps. There was a higher proportion of direct interactions between species in DNP than in the CR areas ($Chi^2 = 57.9$, 1 d.f., $P < 0.001$; shown by pairs of species in Fig. 2). In addition, 61 visits of cachectic individuals including 54 (88%) red deers, 6 wild boars and 1 fallow deer were identified in DNP, while only 1 cachectic wild boar was recorded in CR.

Factors affecting MTC presence at aggregation points

A significantly higher rate of MTC-positive samples was found at those water points at which cachectic animals had been identified by camera-trap monitoring ($Chi^2 = 4.30$, 1 d.f., $P < 0.05$). The most parsimonious GLMM describing the variation in the presence of MTC DNA included the effects of water point size and relative abundance indices for monitored ungulate species by camera trapping (Table S1). The models testing the statistical relationship among factors affecting MTC environmental presence revealed that the risk of MTC presence was significantly dependent on water point size ($\beta = -0.10$, SE = 0.04, $P < 0.01$; Table 3), with the proportion of positives increasing at the smallest waterholes (see Fig. 3). The residuals of this model were not spatially structured according to Moran's I index.

Discussion

The presence of MTC DNA in water points and its driving factors at a natural domestic/wild ungulates interface has been determined with the objective of better understanding the role of indirect routes of cross-species disease transmission. The analysis at *a priori* risk points for wildlife–cattle interactions showed a high environmental presence of MTC, particularly in mud samples. Water points are sites

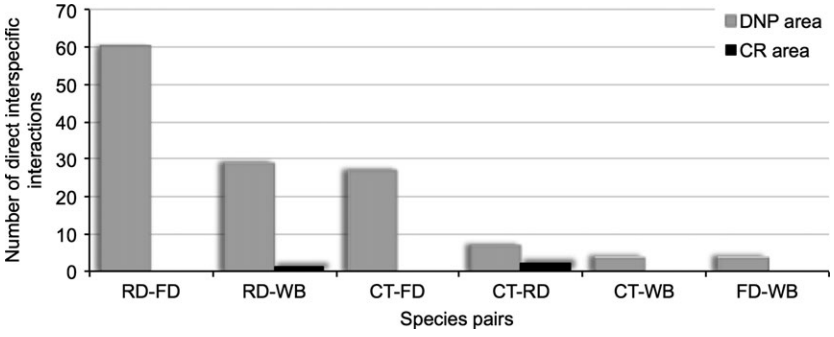

Fig. 2. Direct inter-species interactions (simultaneous presence of two species in the same image) measured by means of camera trapping at water points in Doñana National Park (DNP) and on Ciudad Real (CR) cattle farms. Results are shown for each pair of species coded as: red deer (RD), fallow deer (FD), wild boar (WB) and cattle (CT).

of aggregation where at least some viable MTC bacteria are likely to persist, and so inter-species transmission likely occurs there. Our results will help to better target actions and implement control strategies for TB at the wildlife/livestock interface in complex epidemiological scenarios (Barasona et al., 2013).

High rates of MTC DNA presence were mainly found in mud of the sampled water points from two TB endemic areas, even when a reduced volume of material was analysed. Adams et al. (2013) determined that the PCR assay is a suitable method to detect environmental presence of MTC and that it is able to detect low levels of MTC DNA (even in the presence of interfering contaminating organisms). For instance, PCR enabled *M. bovis* detection in naturally infected soils and faeces (Courtenay et al., 2006; Sweeney et al., 2007) and up to 7 months after inoculation in spiked substrates (Adams et al., 2013). Although detection by this means does not guarantee that viable cells are present, several approaches have determined that detectable MTC DNA does not persist for more than 10 days after bacterial death (Young et al., 2005). Evidencing mycobacterial presence in the environment using cultures (the gold standard) is challenging, as several factors depending on the bacteria's characteristics (slow growth, dormant state acquisition), sample characteristics (contamination with other microorganisms) and sample treatments prior to culturing (harsh decontamination procedures) decrease sensitivity. Molecular techniques overcome the aforementioned limitations and are now commonly used in diagnostics (Sweeney et al., 2007). However, PCR also has technical constraints, namely cell lysis efficiency (when direct extraction is not performed), bacterial adherence to soil particles and the presence of inhibitors in the sample (Tebbe and Vahjen, 1993; Miller et al., 1999; Roose-Amsaleg et al., 2001). We therefore encourage the optimization and development of better techniques with which to assess both MTC presence in the environment (Fine et al., 2011a,b; Kukielka et al., 2013) and other biological features such as quantity, viability and infectious potential. This statement could also be generalized to other mycobacteria that do not belong to the MTC, such as non-tuberculous mycobacteria (NTM) including members of the *M. avium* complex. NTM are widely distributed in natural and man-made environments, and there is an increasing

Table 3. Results of risk factor analysis. Parameter estimates for the best generalized linear mixed models (binomial distribution and logit link) explaining the variation in the presence of *Mycobacterium tuberculosis* complex DNA at Mediterranean environments. The models were fitted using 'management state' as random effect. Significant results are highlighted in bold

Predictive variable	Parameter estimate	Standard error	*Z* value	*P*-value
Intercept	3.725	1.687	2.208	**0.027**
Area (DNP)*	−1.339	1.426	−0.939	0.347
Water point size	**−0.107**	**0.044**	**−2.396**	**0.016**
Cattle abundance	−0.006	0.015	−0.427	0.669
Red deer abundance	0.026	0.024	1.095	0.273
Fallow deer abundance	0.038	0.022	1.716	0.086
Wild boar abundance	−0.033	0.022	−1.520	0.128

*Parameter estimate for the levels of fixed factor was computed by considering a reference value of 0 for level 'CR' (Ciudad Real province).

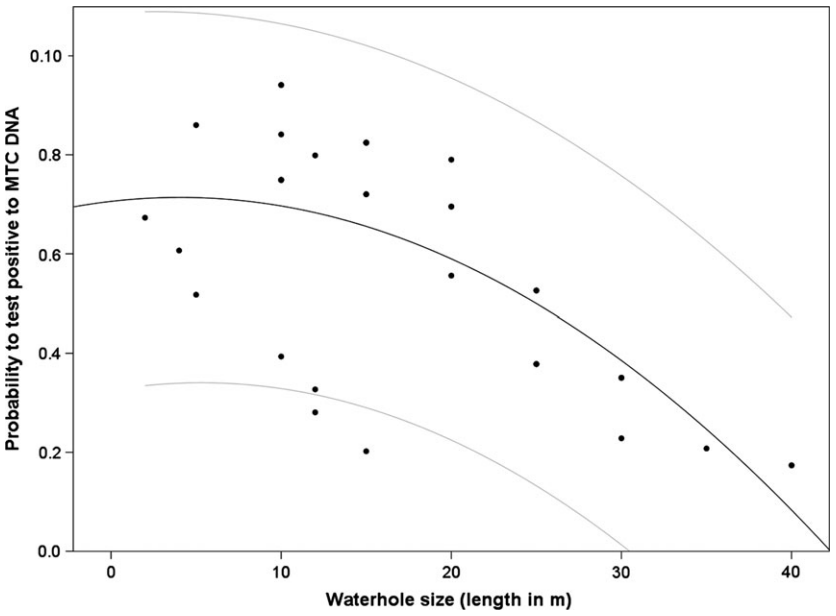

Fig. 3. Relationship between the probability of testing positive to MTC using PCR and the size (maximum diameter length in metres) of the sampled waterholes.

awareness of their pathogenic potential in humans and animals (Torkko et al., 2002).

Our literature review reveals little knowledge of comparable reports, particularly in hot and dry conditions with high levels of solar radiation such as those of DNP and CR (Kukielka et al., 2013). The survival times of *M. bovis* under a range of environmental conditions have been examined by Kukielka et al. (2013). Temperatures and precipitation levels at most of the cited research sites differ greatly when compared to those of SCS (Kukielka et al., 2013). Nevertheless, two of the studies were carried out in Mediterranean-like climates where *M. bovis* could survive in spiked faeces for up to 28 days (Tanner and Michel, 1999) or 1 week to 28 days in spiked soil and faeces depending on temperature and exposure to sunlight (Duffield and Young, 1985). These results indicate that temperature, humidity and sunlight influence bacteria survival times in the environment. This may explain the high rates of MTC DNA presence found in shore mud, in which appropriate conditions – such as moisture, organic material and protection from direct sunlight – drive the survival of bacteria and their concentration by decantation (Duffield and Young, 1985). In this sense, mud would appear to be the sample of choice, because it is more reliable than water, although a significantly higher proportion of PCR test inhibitions occur when analysing this substrate. The climatological conditions in SCS signify that MTC will probably have lower survival rates than those previously reported and that contamination rates will probably be higher than in other epidemiological scenarios (Gortázar et al., 2008). In this respect, differences between areas could be associated with an increased availability of forests in DNP where wood and scrub areas may provide shady and moist conditions for the persistence of bacteria (Tanner and Michel, 1999). A higher environmental presence of MTC in DNP may also be associated with the higher abundances and prevalence of TB in the host community and hence in the environment.

Our observations at *a priori* risk points for cattle–wildlife interactions have evidenced that the use of extensive farm water resources by wild ungulates in the summer is frequent and widespread. This confirms the findings of other descriptive studies carried out in SCS (Barasona et al., 2013, 2014b; Kukielka et al., 2013). Overall, the relative abundance measured using the camera-trap monitoring of wild ungulates was higher in DNP than in CR, which is an area with numerous private cattle farms in which hunting is a secondary activity (Carrasco-Garcia et al., 2015). Although we did not find relationship between contamination and abundance, it is probably due to high abundance of ungulates in all the study sites, and therefore, the environmental presence of MTC is not sensitive to abundance in our study context. Future research must cover a wider range of abundance situations. In addition, the interface between wild and domestic ungulates consists mainly of indirect contact (Kukielka et al., 2013). This concords with our evidence from CR, in which direct inter-specific interactions were very limited. However, camera trapping permitted a higher proportion of direct inter-specific interactions to be observed at DNP water points in the present study. This could help to explain why DNP is the highest TB prevalent area recorded worldwide (Gortázar et al., 2008; Gortazar et al., 2011).

Regarding risk factors, we have detected higher rate of MTC-positive samples when cachectic animals were identified by camera-trap monitoring. In our study areas, visibly cachectic deer and wild boar most probably represent MTC-infected individuals with advanced generalized TB (Martín-Hernando et al., 2007, 2010; Vicente et al., 2013). These individuals might make a disproportionate contribution to environmental contamination with MTC (supershedders, Gortázar et al., 2013). In fact, *M. bovis* detection has been linked to the absolute number of excretors and the frequency of excretion by infected individuals (Courtenay et al., 2006).

One important determinant associated with the environmental presence of MTC in water and mud samples from DNP was waterhole size, because we found a clear relationship between MTC positivity and smaller waterhole size. A higher concentration of organic material in water and/or increased contact rates in terms of the number of ungulates per metre of shore length may be, respectively, responsible for the exposure, persistence and contamination of these waterholes. Knowing the effect of this risk factor permits us to suggest feasible applications (Gortazar et al., 2015) – such as removing the smallest waterholes or enlarging them – in order to reduce the environmental presence of MTC. In addition, practical measures may be complementarily addressed to minimize the risks associated to inter-specific aggregation at watering sites (Cowie et al., 2015). We specifically propose (i) dispersing or modifying the available water points that can potentially be shared between domestic and wild species, (ii) zoning or segregating wildlife and cattle from common waterholes by setting up selective barriers (Barasona et al., 2013) and (iii) implementing strategic animal husbandry, timing and the use of certain pastures (where animals also have to drink) or changing disease susceptible livestock species to less risky areas (Ward et al., 2010). Further research on specific biosecurity programmes should focus on managing these water source features in Mediterranean areas to reduce the opportunities of inter/intraspecies MTC transmission.

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Conflict of Interest

The authors declare that they have no competing interests.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1 Measures for model support